HEMATOLOGY
Basic Principles and Practice

3rd Edition

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Churchill Livingstone
A Division of Harcourt Brace & Company

New York Edinburgh London Philadelphia San Francisco

CHURCHILL LIVINGSTONE
A Division of Harcourt Brace & Company
The Curtis Center
Independence Square West

Philadelphia, Pennsylvania 19106

Library of Congress Cataloging-in-Publication Data
Hematology : basic principles and practice / [edited by] Ronald Hoffman [et al.]. 3rd ed.
p. cm.
Includes bibliographical references and index.
ISBN 0443079544
1. Hematology. 2. BloodDiseases. I. Hoffman, Ronald.
[DNLM: 1. Hematologic Diseasesdiagnosis. 2. Hematologic Diseasestherapy. WH 120 H487 2000]
RC633.H434 2000
616.15dc21
DNLM/DLC 98-43923

HEMATOLOGY: BASIC PRINCIPLES AND PRACTICE ISBN0443079544

Printed in the United States of America

Last digit is the print number: 9 8 7 6 5 4 3 2 1

To my wife Nan, my daughter Judith, and my son Michael, for their encouragement and patience during this project. To my parents Sarah and Morris, who made great sacrifices in order for me to pursue my education. To my colleagues at the University of Illinois at Chicago who have participated in this project and supported my efforts. To Esmail D. Zanjani, Ralph Zalusky, Bernard G. Forget, and Lawrence Frohman who have each at different points in my career encouraged my commitment to academic medicine. To Ed Benz, Sandy Shattil, Bruce Furie, Harvey Cohen, Les Silberstein, and Phil McGlave who have been great colleagues and friends during the preparation of this third edition.

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To my wife Peggy, for your friendship, counsel, and patience, and for understanding why this book is important to me. To my children Tim and Jen. To my parents and my mentors, whose example and caring nurtured my interest in academic hematology: Bernard G. Forget, MD, David G. Nathan, MD, Arthur W. Nienhuis, MD, and Arthur B. Pardee, PhD. To my secretary Carol Dankelman, for her help during the preparation of the textbook.

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To my wife Gloria, my son Jason, my mother Helen, my sister Sherry, and my late father, Arthur. I would like to thank my mentors at the Harvard Medical Unit of the Boston City HospitalDrs. Richard Cooper, Neil Abramson, James H. Jandl, and the late William B. Castlewho instilled in me a profound respect for the importance of basic research in blood diseases and a deep appreciation for the privilege of contributing to this endeavor.

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To William J. Williams and the late Adrian J. Gottlieb, for introducing me to hematology and, specifically, the field of blood coagulation. To Alan N. Schechter and the late Christian B. Anfinsen, for their guidance into serious scientific research. To Robert S. Schwartz and Jane F. Desforges, for their nurturance and guidance. To my loving wife Barbara, my children Eric and Gregg, my mother Bernice, and in loving memory of my late father, J. Leon Furie.

Bruce Furie, MD

To my wife Ilene, who continues to sustain me, my children Philip, Jonathan, Nicole, and Renee, and my grandchildren Sara and Ethan, who always remind me of the importance and joy of being a father and a grandfather; and to my special friends who make my life so full and rewarding.

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To my friends and family, for their love and support. To my mentors Eugene M. Berkman and Robert S. Schwartz, who continue to serve as my mentors. To my colleagues at the University of Pennsylvania Medical Center, for helping me to develop an academic transfusion medicine program.

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Preface to the Third Edition

Over four years have elapsed since the second edition of this book was published. During this period, an enormous amount of new scientific information has been generated in the area of hematology. This knowledge has been the result of both laboratory-based research and clinical trials. Exciting new data have been reported in virtually all areas of preclinical and clinical hematology research. This new edition serves as a resource for students, residents, fellows, scientists, and practicing physicians who are eager to learn about the science and practice of hematology. This third edition of *Hematology: Basic Principles and Practice* should be viewed as a work in progress; since the appearance of the first edition of this textbook in 1991, the book has evolved as it relates to content, focus, and size. This evolution reflects, in large part, the new directions and growth of hematology as an academic and clinical discipline. In order to retain the quality and vitality displayed in the first two editions, over thirty new primary authors have been added to this book. This changing of the guard is healthy for a text in order to ensure its timeliness and freshness. A significant number of new chapters appear in this volume. These new chapters represent a commitment by the editorial group to constructive change in order to create an excellent book. The authors have continued to update their chapters several times during the 24 months it has taken to complete this volume. The editors would like to thank the authors for these additional efforts, which assure that the text is up to date and comprehensive.

The editors have recognized the increasing importance of transplantation to hematologists; the section on Stem Cell Transplantation in this volume has been expanded in size, almost threefold since the second edition. As many as 14 chapters in this edition are devoted to stem cell transplantation. This emphasis reflects the growth and importance of this area to the discipline of hematology. Phil McGlave from the University of Minnesota was added to our editorial board for the purposes of enhancing our expertise in transplantation. He has selected a wonderful group of authors who have made important contributions to the text. The area of stem cell transplantation has blossomed over the last decade. The indications for high-dose chemo-radiotherapy and stem cell transplantation have been extended and the management of transplant recipients markedly improved. The use of alternative donor grafts and the engineering of allogeneic and autologous grafts have been areas of particular focus. This information is summarized in this edition and is important to all practicing hematologists.

The editors have remained responsive to the readership. Before embarking on this edition, we analyzed reviews of the book that appeared in a variety of journals, as well as solicited and unsolicited criticisms, and corrected any deficiencies or weaknesses. This edition represents the culmination of such efforts and results in a more readable and informative text. In addition, areas of overlap have been eliminated. Many of the readers of the text have appreciated the inclusion of personal strategies provided by many authors for the diagnosis and treatment of hematologic disorders. The number of personal strategy boxes has been expanded to provide a text that is useful not only to academic scientists but also to practicing physicians.

Some mention should be made of the debt that the editors owe to the contributing authors. As we all realize, the world of academic medicine has changed over the last decade. Physician accountability has become the new buzz word in many institutions. Contributions to textbooks are not valued by some institutions since such efforts fail to contribute to an institutional balance sheet in a positive fashion. The contributors to this book continue, however, to adhere to the traditional values of academic scholarship. In the sometimes frenetic world of institutional mergers and institutional mission statements this return to core values is refreshing and greatly appreciated. Only with the aid of such altruistic contributors can *Hematology: Basic Principles and Practice* remain the creative, dynamic, scholarly book that our readership has come to expect. We as a group remain committed to these ideals and standards. We are hopeful that our readership enjoys this text and continues to provide us with the constructive criticism needed to generate the creative energy required for future editions.

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Preface to the Second Edition

The first edition of *Hematology: Basic Principles and Practice* appeared in 1991. This work was intended to be a comprehensive and up-to-date textbook developed to serve a diverse group of individuals ranging from practicing general hematologists, students of the field, basic scientists involved in hematologic research, as well as practicing internists and pediatricians. During the past four years, the science and practice of hematology have continued to evolve. The rapid advancement of this field has necessitated the creation of a second edition, in order that the book remain current and useful to our readers as the field of hematology progresses.

We retained the features of the first edition that were regarded by our audience as most effective, but altered the second edition as required to further enhance the quality of the book. In response to the constructive criticisms offered in numerous positive reviews of the first edition, we have added an entirely new section titled *Immunology.* It is organized in a manner resembling the section titled *Molecular and Cellular Basis of Hematology,* which provides an overview of the basic science needed to gain insight into the field. Some chapters have been eliminated in the interest of reducing redundancy and balancing the content. The editors met twice for extended work sessions during the inception of this edition in order to organize the information to be included. These meetings led to the development of close relationships between the editors and staff which has, we believe, led to the generation of a more cohesive and comprehensive text.

The growing importance of transfusion medicine has been recognized by the appointment of Leslie E. Silberstein, MD to the editorial group. The second edition has 16 chapters that deal exclusively with transfusion medicine. This section provides a concise review of an increasingly significant and complex area. An understanding of transfusion medicine is required of all practicing hematologists. We hope this primer will serve as a framework by which students of hematology can gain mastery of this rapidly evolving area.

In the first edition we asked authors to contribute their personal strategies for managing difficult clinical problems in sections set apart from the main text. These boxed paragraphs were highlighted by a shaded tint so the reader could easily identify these clinical points. Such contributions now appear in an expanded form in most of the chapters dealing with clinical hematology.

The remarkably rapid progress of both experimental and clinical hematology, coupled with the accelerated entry of molecular and cellular concepts and techniques into clinical practice, has resulted in an entirely new set of terminologies relevant to the clinical hematologist. Oncogenes, chromosomal translocations, cell-surface antigens, growth factors, and specialized cellular receptors comprise but a few examples of entities that were identified only by the arcane jargon of research laboratories a few years ago, but are now important for the classification, diagnosis, and treatment of hematologic diseases. The translation of knowledge from research laboratory to the bedside has been so rapid that the names of these substances and phenomena have often remained in the shorthand of the basic research community. A formidable challenge facing the editors and publisher of this volume has been the development of a coherent approach to the terms used to identify these materials. In some cases, loose rules of usage have been developed. For example, the abbreviations used to designate human and mouse proto-oncogenes, their oncogene derivatives, and the proteins resulting from their expression are distinguished by differential use of lower case and upper case letters, italics, and so forth. However, these rules are not universally honored in either the primary research literature, or in reviews or textbooks. We have tried to conform to recommended usage in every situation in which a recommended terminology has been developed. However, we have deferred to the best judgment and preference of the authors of individual chapters whenever they thought the conventional terminology was inappropriate for the subjects they discuss. We have attempted to alert the reader to the use of alternative terminologies, and to define potentially confusing terms.

The contributors to this edition have done an outstanding job, and the editors are indebted to them for their diligence, perseverance, and scholarly presentations.

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Preface to the First Edition

The past several decades have seen an exponential increase in new medical knowledge related to hematology. New disease entities, new diagnostic methods, and novel therapeutic modalities have been introduced into the discipline. These clinical advances have largely been based on insights into the pathophysiology of hematologic disorders and have been gained by applying the tools of cell biology, biochemistry, immunology, and molecular genetics to this discipline. Inevitably, this explosion of knowledge has also led to further subspecialization within hematology, with the appearance of experts in transfusion medicine, red cell disorders, platelet and coagulation disorders, neoplastic diseases, and immunohematology.

The practicing general hematologists, whether operating at a university or in private practice, faces the challenge of caring for patients who have a variety of hematologic disorders. It has become increasingly difficult for one individual to remain current in all aspects of this expanding discipline, yet clinical care requires intimate familiarity with the enlarging knowledge base. In addition, basic scientists without clinical training have been attracted to hematology research. To put their work in a clinical context, these scientists require a broad background in the fundamentals of hematology and comprehension of clinical presentations, diagnostics, and therapy of specific diseases. Furthermore, students of the field, including medical residents, hematology fellows, and internists with special needs for understanding hematologic diseases have a requirement for an up-to-date source of information. *Hematology: Basic Principles and Practice* is a comprehensive textbook developed to serve these diverse groups.

The structure of the book reflects the diversity of its readership. *Hematology: Basic Principles and Practice* was designed during a meeting of the editors with Bob Hurley and Beth Barry of Churchill Livingstone in Woods Hole, Massachusetts, during the summer of 1988. As our starting point, all of the Editors stressed the importance of emphasizing the scientific underpinnings of modern hematology. Part I, *Molecular and Cellular Basis of Hematology,* is the foundation of the book and provides the overview of basic science needed to gain insight into the practice of hematology during the 1990s and into the next century. The next six parts are devoted to the major subspecialty areas, *Hematopoiesis, Red Blood Cells, White Blood Cells, Hematologic Malignancies, Hemostasis and Thrombosis,* and *Transfusion Medicine.* Each part contains an up-to-date review of the scientific fundamentals, followed by the description of specific hematologic diseases and their diagnosis and treatment. To further extend the books clinical usefulness, Part VIII deals with *Consultative Hematology.* With nine chapters covering general medicine and surgery, we hope this section will be especially valuable to the clinician performing consultations on patients who do not have a primary hematologic disorder but whose primary disorder results in hematologic abnormalities. The book closes with a section on *Special Tests and Procedures* that are integral to the practice of hematology.

To strike a balance between the scientific and clinical, we believe the text should be academic and scholarly, yet still offer a practical approach to diagnosis and patient management. To reconcile these objectives and provide a forum for sharing clinical approaches, we asked authors to contribute their personal strategies for managing difficult problems in nonreferenced editorial sections set off from the main text. The boxed paragraphs are highlighted by a shaded tint so the reader can easily identify these clinical pearls.

We also asked the contributors to seek out and create helpful illustrations to make difficult concepts easier to understand. We have redrawn much of the artwork to render primary visual material in a uniform style throughout the book, and have added color and tints to emphasize the more important points in the illustrations. We hope this makes our overall presentation more comprehensible and greatly enhances the heuristic value of these illustrations.

Ultimately, the quality of this project is closely related to the quality of the contributions received from the many participants in this multi-authored textbook. The preparation of each chapter has taken considerable effort and time, and we are grateful that the experts in the field agreed to participate in this undertaking. The contributors have done an outstanding job in writing comprehensive and timely treatises on specialized subjects, and we have responded by minimizing the delay between the preparation of each chapter and publication. We want to especially thank Les Silberstein at the University of Pennsylvania School of Medicine and Ed Snyder at the Yale University School of Medicine, who organized the section on blood banking. Their knowledge and guidance have been indispensable in editing this major section of the book.

Each of the editors brings to the book different interests and perspectives. We all worked together as equal partners to create a shared vision of a hematology text for a new generation of scientists and clinicians. We hope that we have accomplished this lofty goal, and that this will be the initial edition of a book that will continue to serve its varied readership for many editions to come.

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Color Plates

Plate 4-1 Arrangement of integral and peripheral membrane proteins in the red cell. Note the asymmetric distribution of lipid, the anastomosing lattice formed by spectrin-spectrin and spectrin-actin junctions.

Plate 7-1 (A) Lymph node. Several lymphatic follicles with well-developed germinal centers are visible. (H&E × 200). **(B)** Lymph node. Lymphatic follicle with prominent germinal center pushing away the follicular mantle. (H&E × 350.) **(C)** Lymph node. B cells are stained (brown color) with a peroxidase-labeled monoclonal antibody against CD20, a pan-B-cell marker. (× 250.) **(D)** Lymph node. B cells are stained (brown color) with a peroxidase-labeled monoclonal antibody against light chains. (× 300.) **(E)** Lymph node. Stain for lysozyme. Macrophages containing the enzyme are scattered throughout the germinal center and mantle zone. (× 300.) **(F)** Lymph node. T cells stained (brown color) by a peroxidase-labeled monoclonal antibody (UCHL-1) against the pan-T-cell antigen CD45RO. (× 300.) **(G)** Thymus. Thymic lobule with densely packed cellular cortex and less cellular medulla in which large, pale Hassals corpuscles are visible. (H&E × 250.)

Plate 12-1 Colony-forming unit-mixed erythroid, myeloid, megakaryocyte (CFU-Mix). (See also Fig. 123.)

Plate 12-2 Proerythroblast (upper left corner), promyelocyte (middle) and myelocyte (below, right), and metamyelocyte (lower right).

Plate 12-3 Erythrocyte maturation. Basophilic (four in middle, middle right), polychromatic (three middle right), and orthochromatic erythroblast (upper left).

Plate 12-4 Erythrocyte maturation. Basophilic (four in middle, middle right), polychromatic (three middle right), and orthochromatic erythroblast (upper left).

Plate 19-1 Bone marrow morphology in severe aplastic anemia. **(A)** Bone marrow biopsy specimen showing severe hypocellularity. **(B)** Aspirate smear showing residual stromal and lymphoid elements.

Plate 19-2 Clinical presentations of aplastic anemia. **(A)** Ecchymosis in pancytopenic women. **(B)** Submucosal hematomas. **(C)** Petechial eruptions in a thrombocytopenic patient.

Plate 19-3 Peculiar morphologic features occasionally observed in aplastic anemia. **(A)** Marrow necrosis with eosinophilic ground substance (arrowhead) on biopsy. **(B)** Scanty marrow aspirate in severe disease showing only blood elements. **(C)** Megaloblastoid erythroid dysplasia in a patient with moderate aplastic anemia. **(D)** Hemophagocytosis (small arrows) in a young man with posthepatitis aplasia. **(E&F)** Megaloblastoid erythropoiesis in the marrow aspirates of two young women recovering from aplastic anemia.

Plate 19-4 Morphology of other diseases that may present with pancytopenia. **(A)** Biopsy in myelofibrosis. **(B)** Touch preparation of biopsy specimen showing syncytium of malignant cells in metastatic breast cancer. **(C)** Biopsy specimen and **(D)** aspirate smear of lymphoma in bone marrow. A monoclonal population of B lymphocytes was determined by flow cytometry.

Plate 19-5 Cutaneous eruptions due to serum sickness.

Plate 35-1 Fragmentation hemolysis. Note the irregular, distorted forms and small fragments.

Plate 35-2 Peripheral smear from a patient with -thalassemia intermedia shows the extensive tailed forms that largely disappear following splenectomy. Similar observations have been made on tailed RBCs in patients with agnogenic myeloid metaplasia and myelofibrosis.

Plate 35-3 Peripheral smear from a patient with severe alcoholic liver disease. Note the acanthocytes, spur cells, and transitional forms. Macrocytes are also present.

Plate 35-4 Bite cells from a patient with oxidative hemolysis. Note the thin veil of membrane across a clear zone, giving the appearance of a blister.

Plate 36-1 -Carbon backbone and overall surface shape of the intact Dob IgG structure. The antigen combining sites are located at the ends of the two horizontal Fab arms formed by the association of the light chains (-carbon backbone as red lines and surface as light blue dots) and heavy chains (-carbon backbone as yellow lines and surface as blue dots). On the basis of amino acid sequence studies, Dob has a substantial deletion in the hinge region, and this probably limits its segmental flexibility. The molecular surface represents the area accessible to a probe sphere the size of a water molecule (1.4-A radius). In this representation the surface of the IgG is composed of convex regions, formed by the solvent-accessible van der Waals surface of individual atoms and concave regions. Small gaps and crevices inaccessible to the probe sphere are smoothed over. *(From Getzoff et al.,[5] with permission.)*

Plate 40-1 Peripheral blood smear stained with Wrights stain, containing a single eosinophil (right) and neutrophil (left). Note the characteristic bilobed nucleus and eosin staining of the cytoplasmic specific (secondary) granules filling the eosinophils cytoplasm.

Plate 47-1 (A) High power (× 156) Giemsa stain of a mast cell lesion in a plastic embedded bone marrow. **(B)** High power (× 250) toluidine blue stain of a mast cell lesion in a plastic embedded bone marrow. Note that on Giemsa stains, mast cell granules stain dark blue, whereas eosinophils stain bright orange. *(From Parker RI: Hematologic aspects of mastocytosis. 1. Bone marrow pathology in adult and pediatric systemic mast cell disease. J Invest Dermatol 96:47S, 1991, with permission.)***(C)** Giemsa plastic embedded bone marrow biopsy demonstrating metachromatic granules in mast cells. (× 1,000.) *(From Travis WD, Li C-Y, Bergstralh EJ et al: Systemic mast cell disease: analysis of 58 cases and literature review. Medicine 67:345, 1988, with permission.)*

Plate 53-1 Leukemia cutis in a patient with monoblastic leukemia.

Plate 53-2 Sweet syndrome in a patient with AML. Tender, pseudovesicular, erythematous plaques of Sweet syndrome. *(From Cohen,[159] with permission.)*

Plate 53-3 Gingival infiltration in a patient with myelomonocytic leukemia.

Plate 53-4 M0: Acute myeloid leukemia.

Plate 53-5 M1: Acute myeloid leukemia. Cells demonstrate no evidence of maturation or differentiation. The blasts often have large irregular nuclei with varying amounts of eccentrically placed cytoplasm. There is no definite granulation, and Auer rods are absent.

Plate 53-6 M2: Myeloblastic leukemia with differentiation. The leukemic cells demonstrate maturation through the promyelocytic stage. Azurophilic granules are seen with occasional Auer rods. *(Courtesy of Guido J.K. Tricot, MD, PhD.)*

Plate 53-7 M3: Acute promyelocytic leukemia. The leukemic cells contain multiple abnormal, coarse azurophilic granules, and Auer rods are prominent. *(Courtesy of Guido J.K. Tricot, MD, PhD.)*

Plate 53-8 M3v.

Plate 53-9 M4: Acute myelomonocytic leukemia. Both myeloid and monocytic elements are found, and myeloblasts differentiate through the promyelocyte stage. Monocytes and monoblasts make up >20% of the total number of nucleated cells. Monoblasts demonstrate a pale cytoplasm, with occasional vacuoles and granules with folded or rounded nuclei.

Plate 53-10 M4Eo: Acute myelomonocytic leukemia with eosinophils. Similar to the M4 variant but with increased eosinophils; eosinophils are atypical in appearance, with prominent basophilic granules.

Plate 53-11 M5a: Acute monocytic leukemia, undifferentiated. The predominant cell is an undifferentiated monoblast. The cytoplasm is pale, with occasional vacuoles. The nuclei are round and lack

the usual folded monocytoid appearance.

Plate 53-12 M5b: Acute monocytic leukemia, differentiated. Most of the cells are mature-looking monocytes with centrally placed folded nuclei. Rare monoblasts are found. *(Courtesy of Dr. Lorrie F. Odom.)*

Plate 53-13 M6: Erythroleukemia. Abnormal erythroid precursors with megaloblastic features are prominent. All stages of the erythroid series are found, typically with many bizarre and dysplastic features. Typical myeloblasts are also noted. *(Courtesy of the Blood and Bone Marrow Cell Recognition and Interpretation audio-visual seminars. American Society of Clinical Pathologists, Chicago, 1987.)*

Plate 53-14 M7: Acute megakaryocytic leukemia. Increased marrow fibrosis is frequent. Note the cytoplasmic budding, which is typical of megakaryocytic leukemias.

Plate 55-1 (A) Monoblastic leukemia cutis at diagnosis; lesion on forehead preceded disseminated rash by 68 weeks. **(B)** Gingival infiltration with M5a leukemia in a 21-month-old girl. **(C)** Retinal infiltration at presentation of M4 disease in a 5-year-old boy.

Plate 55-2 Cerebrospinal fluid double concentrate cytocentrifuge preparation of M5a leukemia cells.

Plate 57-1 French-American-British Cooperative Working Group classification of acute lymphocytic leukemia. **(A)** L1 lymphoblasts are small, with scanty cytoplasm and inconspicuous nucleoli. *(Courtesy of Guido J.K. Tricot, MD, PhD.)* **(B)** L2 lymphoblasts are generally larger and demonstrate considerably more variation in size and have prominent nucleoli and abundant cytoplasm. **(C)** L3 lymphoblasts are characterized by cytoplasmic basophilia and prominent cytoplasmic vacuolation. (Plates B and C courtesy of the Blood and Bone Marrow Cell Recognition and Interpretation audiovisual seminars, American Society of Clinical Pathologists, Chicago, 1987.)

Plate 67-1 Nodular sclerosis: Reed-Sternberg cell (left) and lacunar variants.

Plate 67-2 Nodular sclerosis: cohesive tumor cells in syncytial variant.

Plate 67-3 Lymphocyte predominance, diffuse: rare diagnostic Reed-Sternberg cell with mirror-image nuclei in center of field.

Plate 67-4 Lymphocyte predominance, nodular: L&H or popcorn variants of Reed-Sternberg cells.

Plate 67-5 Lymphocyte predominance, nodular: progressively transformed germinal centers.

Plate 67-6 Lymphocyte depletion: two mitoses (left), classic Reed-Sternberg cell (right), and numerous Reed-Sternberg pleomorphic variants.

Plate 67-7 Mixed cellularity: multinucleated Reed-Sternberg cell and numerous inflammatory cells.

Plate 67-8 Diagnostic Reed-Sternberg cell: note huge nucleoli and peripheral condensation of nuclear chromatin creating halo around nucleoli.

Plate 67-9 Spleen: expanded periarterial sheath with evidence of early Hodgkin disease.

Plate 67-10 Bone marrow biopsy: two focal nodules of Hodgkin disease.

Plate 67-11 Bone marrow biopsy: fibrosis and mononuclear variants of Reed-Sternberg cells.

Plate 67-12 Immunoperoxidase (brown) stain of Ki-1 (CD30) antigen on Reed-Sternberg cell membranes in paraformaldehyde-fixed frozen tissue of Hodgkin disease.

Plate 67-13 Bone marrow biopsy: isolated perivascular granuloma.

Plate 67-14 Immunoperoxidase stain of Leu-M1 (CD15) antigen on membrane and in perinuclear Golgi zone of Reed-Sternberg cells in paraffin section.

Plate 67-15 Immunoperoxidase stain of small lymphocytes but *not* Reed-Sternberg cell for leukocyte common antigen in paraffin section of Hodgkin disease.

Plate 67-16 Ki-1^+ anaplastic large cell lymphoma: Reed-Sternberg-like cell among histiocyte-like tumor cells.

Plate 67-17 Post-thymic or peripheral T-cell lymphoma: Reed-Sternberg-like cells, eosinophils, and medium-size lymphocytes with convoluted nuclei.

Plate 67-18 Mediastinal B-cell lymphoma with sclerosis containing Reed-Sternberg-like cell in center.

Plate 70-1 Peripheral T-cell lymphoma involving the skin. Note multiple nodular lesions on the chest wall due to a systemic lymphoma with cutaneous involvement.

Plate 70-2 Primary diffuse large B-cell lymphoma of the skin with multiple lesions on the trunk, some of which are ulcerating. There was no involvement of any other organs.

Plate 70-3 Appearance of the brain in a patient with leptomeningeal recurrence of large cell lymphoma. Note multiple small areas of tumor on the surface.

Plate 71-1 Histologic and clinical features of NHL in children. The upper panels show the histologic appearance of SNCC (Burkitt) lymphoma **(A)**, lymphoblastic lymphoma **(B)**, and the anaplastic subtype of large cell lymphoma **(C)**. The inserts in panels A and B show the characteristic L3 blasts of Burkitt lymphoma and the characteristic L1 blasts of lymphoblastic lymphoma, respectively. The lower panels show common clinical presentations of the three histologic subtypes of lymphoma: encasement of the bowel lumen by Burkitt lymphoma on abdominal computed tomography **(D)**, airway compression by lymphoblastic lymphoma on computed tomography of the anterior mediastinum **(E)**, and bony destruction of the tibia by large-cell lymphoma on magnetic resonance imaging **(F)**. *(From Sandlund JT, Downing JR, Crist WM: Non-Hodgkin lymphoma in childhood. N Engl J Med 334:1238, 1996, with permission.)*

Plate 72-1 Peripheral blood smear in CLL. Wright stain. Increased numbers of mature-appearing lymphocytes; the nucleus filling almost the entire cell; dense clumped nuclear chromatin, with no visible nucleolus. *(Courtesy of Rabia Mir, MD.)*

Plate 72-2 Nodular pattern of lymphocytic infiltration in the bone marrow biopsy specimen in CLL. (H&E × 35.) *(Courtesy of Rabia Mir, MD.)*

Plate 72-3 Interstitial pattern of lymphocytic infiltration in the bone marrow biopsy specimen in CLL. (H&E × 35.) *(Courtesy of Rabia Mir, MD.)*

Plate 72-4 Diffuse pattern of lymphocytic infiltration in the bone marrow specimen in CLL. (H&E × 35.) *(Courtesy of Rabia Mir, MD.)*

Plate 72-5 Smudge cells in the peripheral blood smear in CLL. (H&E × 35.) *(Courtesy of Rabia Mir, MD.)*

Plate 78-1 (A) Florid fatal infectious mononucleosis involving a lymph node (A) shows a polymorphous, immunoblastic infiltrate. (H&E × 400.) **(B)** Burkitt-type non-Hodgkin lymphoma (B), by contrast, shows a monomorphous infiltrate. (H&E × 400.)

Plate 78-2 Cytomegalic inclusion disease. **(A)** A characteristic cytomegalovirus nuclear inclusion and cytoplasmic inclusions are evident. (H&E × 1,000.) **(B)** Immunoperoxidase with anti-CMV confirms the presence of cytomegalovirus (dilute hematoxylin counterstain × 400).

Plate 78-3 Virus associated hemophagocytic syndrome. Extensive histiocytic erythrophagocytosis is evident in sinusoids of the lymph node. Histiocyte cytoplasm is distended by numerous red blood cell ghosts. (H&E × 400.)

Plate 78-4 Castleman disease. **(A)** The hyaline-vascular subtype is characterized by small, sclerotic reaction centers, penetrated by a radial artery and surrounded by a cuff of lymphoid cells with

onion skinning. (H&S × 400.) **(B)** The plasma cell subtype shows larger reaction centers, with sheets of plasma cells in the interfollicular areas. (H&E × 400.)

Plate 78-5 Angioimmunoblastic lymphadenopathy, a marked vascular proliferation with endothelial hyperplasia, small and intermediate sized immunoblasts, mature background lymphocytes, and reactive-appearing plasma cells are evident. (H&E × 400.)

Plate 105-1 Valsalva petechiae.

Plate 105-2 Stasis purpura (palpable).

Plate 105-3 Senile purpura.

Plate 105-4 Scurvy.

Plate 105-5 Ecthyma gangrenosum.

Plate 105-6 Meningococcemia.

Plate 105-7 Acute bacterial endocarditis.

Plate 105-8 Rocky Mountain spotted fever.

Plate 105-9 Schambergs pigmented purpuric eruption.

Plate 105-10 Majocchis pigmented purpuric eruption.

Plate 105-11 Lichen aureus.

Plate 105-12 Letterer-Siwe disease.

Plate 105-13 Histiocytosis X.

Plate 105-14 Disseminated intravascular coagulation.

Plate 105-15 Coumarin necrosis.

Plate 105-16 Coumarin necrosis.

Plate 105-17 Purpura fulminans status post herpes zoster.

Plate 105-18 Psychogenic purpura.

Plate 105-19 Subungual hemorrhage due to cryoglobulins.

Plate 105-20 Hyperglobulinemic purpura.

Plate 105-21 Rheumatoid vasculitis.

Plate 105-22 Wegeners granulomatosis.

Plate 105-23 Henoch-Schönlein purpura.

Plate 105-24 Leukocytoclastic vasculitis.

Plate 105-25 Hereditary hemorrhagic telangiectasia.

Plate 105-26 Telangiectatic mats.

Plate 105-27 CREST.

Plate 105-28 Kaposi sarcoma (AIDS).

Plate 105-29 Kaposi sarcoma (AIDS).

Plate 105-30 Kaposi sarcoma (AIDS).

Plate 105-31 Kaposi sarcoma (AIDS).

Plate 105-32 Angiosarcoma.

Plate 105-33 Fabry disease.

Plate 105-34 Fabry disease.

Plate 155-1 Platelet satellitism.

Plate 155-2 Agglutinated red cells in cold hemagglutinin disease.

Plate 155-3 Rouleaux in multiple myeloma.

Plate 155-4 Fibrin strands (inadequately anticoagulated sample).

Plate 155-5 Plasmodium vivax trophozoites showing ameboid shape.

Plate 155-6 New methylene blue stain showing reticulocytes.

Plate 155-7 Basophilic stippling in thalassemia minor.

Plate 155-8 Howell-Jolly bodies; ancanthocytes (spiculated cells) in postsplenectomy patient.

Plate 155-9 Siderotic granules in hereditary sideroblastic anemia.

Plate 155-10 Toxic changes in neutrophils secondary to inflammatory stimulus (toxic granulation vacuolation).

Plate 155-11 Chediak-Higashi disease; note abnormal granules in neutrophil and lymphocyte.

Plate 155-12 Hypersegmented neutrophils in pernicious anemia.

Plate 155-13 Normal red cell morphology.

Plate 155-14 Microcytes in iron deficiency anemia.

Plate 155-15 Megalocytes in pernicious anemia.

Plate 155-16 Hereditary elliptocytosis.

Plate 155-17 Spherocytosis.

Plate 155-18 Stomatocytosis.

Plate 155-19 Hereditary pyropoikilocytosis.

Plate 155-20 Target cells (codocytes) in liver disease.

Plate 155-21 Teardrops (dacryocytes) in myelofibrosis.

Plate 155-22 Fragmented red cells (schistocytes) in hemolyticuremic syndrome (arrow shows typical helmet cell).

Plate 155-23 Sickle cells.

Plate 155-24 Misshapen red cells with hemoglobin crystals in hemoglobin SC disease.

Plate 155-25 Hemoglobin CC disease in splenectomized patient showing hemoglobin crystals.

Plate 155-26 Polychromatophilic red cells.

Plate 155-27 Giant platelets in myeloproliferative disorder.

Plate 155-28 Drying artifact.

Plate 155-29 Cryoglobulin aggregates; may interfere with white cell count.

Plate 155-30 Normal bone marrow cellularity (marrow biopsy); note dark colored erythroid islands (arrow) and large multinucleated megakaryocyte (center).

Plate 155-31 Hypocellular bone marrow (marrow biopsy).

Plate 155-32 Normal bone marrow cellularity (marrow aspirate direct smear).

Plate 155-33 Metastatic tumor in bone marrow biopsy.

Plate 155-34 Clump of neuroblastoma cells in bone marrow aspirate forming rosette-like structure.

Plate 155-35 Clump of damaged normal hematopoietic cells in bone marrow concentrate.

Plate 155-36 Individual neuroblastoma cells in bone marrow aspirate smear.

Plate 155-37 Lymphoma nodule.

Plate 155-38 Normal lymphoid aggregate.

Plate 155-39 Increased bone marrow reticulin in myeloproliferative syndrome.

Plate 155-40 Abnormal macrophage in Gaucher disease.

Plate 155-41 Abnormal macrophage in Niemann-Pick Disease.

Plate 155-42 Bone marrow granuloma.

Plate 155-43 Leishmania organisms in patient with HIV and kala-azar.

Plate 155-44 Histoplasma capsulatum in bone marrow macrophage.

Plate 155-45 Erythrophagocytosis in patient with overwhelming EBV infection.

Plate 155-46 Psuedo Pelger-Huet anomaly in MDS (bilobed neutrophil).

Plate 155-47 Megaloblastoid red cell (binucleate, enlarged nucleus) in MDS.

Plate 155-48 Ring sideroblast in MDS (refractory anemia with ring sideroblasts)iron stain.

Plate 155-49 Micro-megakaryocyte in MDS.

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Part I - Molecular and Cellular Basis of Hematology

Chapter 1 - Anatomy and Physiology of the Gene

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Normal blood cells have limited life spans; they must be replenished in precise numbers by a continuously renewing population of progenitor cells. Homeostasis of the blood requires that proliferation of these cells be efficient, yet strictly constrained. Many distinctive types of mature blood cells must arise from these progenitors by a controlled process of commitment to, and execution of, complex programs of differentiation. Thus, developing red cells must produce large quantities of hemoglobin, but not the myeloperoxidase characteristic of granulocytes, the immunoglobulins characteristic of lymphocytes, or the fibrinogen receptors characteristic of platelets. Similarly, the maintenance of normal amounts of coagulant and anticoagulant proteins in the circulation requires exquisitely regulated production, destruction, and interaction of the components. Understanding the basic biologic principles underlying cell growth, differentiation, and protein biosynthesis requires a thorough knowledge of the structure and regulated expression of genes, because the gene is now known to be the fundamental unit by which biologic information is stored, transmitted, and expressed in a regulated fashion.

Genes were originally characterized as mathematical units of inheritance. They are now known to consist of molecules of deoxyribonucleic acid (DNA). By virtue of their ability to store information in the form of nucleotide sequences, to transmit it by means of semiconservative replication to daughter cells during mitosis and meiosis, and to express it by directing the incorporation of amino acids into proteins, DNA molecules are the chemical transducers of genetic information flow. Efforts to understand the biochemical means by which this transduction is accomplished have given rise to the discipline of molecular genetics.

Our ability to study the molecular genetics of hematologic problems has been greatly advanced by the development of recombinant DNA technology, which permits us to isolate, characterize, synthesize, and manipulate individual genes controlling known proteins or biologic phenomena. A brief overview of the basic methods and terminology of recombinant DNA technology is included in this chapter.

THE GENETIC VIEW OF THE BIOSPHERE THE CENTRAL DOGMA OF MOLECULAR BIOLOGY

The fundamental premise of the molecular biologist is that the magnificent diversity encountered in nature is ultimately governed by genes. The capacity of genes to exert this control is in turn determined by relatively simple stereochemical rules, first appreciated by Watson and Crick in the 1950s. These rules constrain the types of interactions that can occur between two molecules of DNA or ribonucleic acid (RNA).

DNA and RNA are linear polymers consisting of four types of nucleotide subunits. Proteins are linear unbranched polymers consisting of 21 types of amino acid subunits. Each amino acid is distinguished from the others by the chemical nature of its side chain, the moiety not involved in forming the peptide bond links of the chain. The properties of cells, tissues, and organisms depend largely on the aggregate structures and properties of their proteins. The central dogma of molecular biology states that genes control these properties by controlling the structures of proteins, the timing and amount of their production, and the coordination of their synthesis with that of other proteins. The information needed to achieve these ends is transmitted by a class of nucleic acid molecules called RNA. Genetic information is stored in the form of DNA nucleic acid sequences and expressed in the form of protein synthesis through the mediation of RNA. Genetic information thus flows in the direction DNA RNA protein. This central dogma provides, in principle, a universal approach for investigating the biologic properties and behavior of any given cell, tissue, or organism by study of the controlling genes. Methods permitting direct manipulation of DNA sequences should then be universally applicable to the study of all living entities. Indeed, the power of the molecular genetic approach lies in the universality of its utility.

One exception to the central dogma of molecular biology that is especially relevant to hematologists is the storage of genetic information in RNA molecules in certain viruses, notably the retroviruses associated with T-cell leukemia/lymphoma and the human immunodeficiency virus. When retroviruses enter the cell, the RNA genome is copied into a DNA replica by an enzyme called reverse transcriptase. This DNA representation of the viral genome is then expressed according to the rules of the central dogma. Retroviruses thus represent a variation on the theme, rather than a true exception to or violation of the rules.

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ANATOMY AND PHYSIOLOGY OF GENES

DNA Structure

DNA molecules are extremely long, unbranched polymers of nucleotide subunits. Each nucleotide contains a sugar moiety called deoxyribose, a phosphate group attached to the 5 carbon position, and a purine or pyrimidine base attached to the 1 position (Fig. 1-1). The linkages in the chain are formed by phosphodiester bonds between the 5 position of each sugar residue and the 3 position of the adjacent residue in the chain (Fig. 1-1). The sugar phosphate links form the backbone of the polymer, from which the purine or pyrimidine bases project perpendicularly.

The haploid human genome consists of 23 long, double-stranded DNA molecules tightly complexed with histones and other nuclear proteins to form compact linear structures called chromosomes. The genome contains 3 billion nucleotides; each chromosome is thus 50 to 200 million bases in length. The individual genes are aligned along each chromosome. Blood cells, like most somatic cells, are diploid. Each chromosome is present in two copies, so that there are 46 chromosomes consisting of approximately 6 billion base pairs (bp) of DNA. The length of a DNA molecule is often described in terms of the length of the number of nucleotide bases it contains. Because each nucleotide contains one such base, it is equivalent to say a DNA molecule is 1,000 bases (1 kilobase [kb]) or 1,000 nucleotides in length. As noted later, DNA usually exists as a double-stranded structure, held together by hydrogen bonds between the bases at equivalent positions of each strand (base pairing); the length of these double-stranded molecules is described in base pairs (e.g., 1,000 bp = 1 kilobase pair [1 kbp]).

The four nucleotide bases in DNA are the purines (adenosine and guanosine) and the pyrimidines (thymine and cytosine). The basic chemical configuration of the other nucleic acid found in cells, RNA, is quite similar, except that the sugar is ribose (having a hydroxyl group attached to the 2 carbon, rather than

Figure 1-1 Structure, base pairing, polarity, and template properties of DNA. **(A)** Structures of the four nitrogenous bases projecting from sugar phosphate backbones. The hydrogen bonds between them form base pairs holding complementary strands of DNA together. Note that A-T and T-A base pairs have only two hydrogen bonds, whereas C-G and G-C pairs have three. **(B)** The double helical structure of DNA results from base pairing of strands to form a double-stranded molecule with the backbones on the outside and the hydrogen-bonded bases stacked in the middle. Also shown schematically is the separation (unwinding) of a region of the helix by mRNA polymerase, which is shown using one of the strands as a template for the synthesis of an mRNA precursor molecule. Note that new bases added to the growing RNA strand obey the rules of Watson-Crick base pairing (see text). Uracil (U) in RNA replaces T in DNA and, like T, forms base pairs with A. **(C)** Diagram of the antiparallel nature of the strands, based on the stereochemical 3 5 polarity of the strands. The chemical differences between reading along the backbone in the 5 to 3 and 3 to 5 directions can be appreciated by reference to part **(A)**.

the hydrogen found in deoxyribose) and the pyrimidine base uracil is used in place of thymine. The bases are commonly referred to by a short-hand notation: the letters A, C, T, G, and U are used to refer to adenosine, cytosine, thymine, guanosine, and uracil, respectively.

The ends of DNA and RNA strands are chemically distinct, because of the 3 5 phosphodiester bond linkage that ties adjacent bases together (Fig. 1-1). One end of the strand (the 3 end) has an unlinked (free at the 3 carbon) sugar position, and the other (the 5 end) a free 5 position. There is thus a polarity to the sequence of bases in a DNA strand: the same sequence of bases read in a 3 5 direction carries a different meaning than if read in a 5 3 direction. Cellular enzymes can thus distinguish one end of a nucleic acid from the other; most enzymes that read the DNA sequence tend to do so only in one direction (3 5 or 5 3, but not both). Most nucleic acid-synthesizing enzymes, for instance, add new bases to the strand in a 5 3 direction.

The ability of DNA molecules to store information resides in the *sequence* of nucleotide bases arrayed along the polymer chain. Under the physiologic conditions in living cells, DNA is thermodynamically most stable when two strands coil around each other to form a double-stranded helix. The strands are aligned in an antiparallel direction, having opposite 3 5 polarity (Fig. 1-1). The sugar phosphate backbones are arrayed on the outside of the helix with the nucleotide bases stacked in the center (Fig. 1-1).

The DNA strands are held together by hydrogen bonds between the bases on one strand and the bases on the opposite (complementary) strand. The stereochemistry of these interactions allows bonds to form between the two strands only when adenine on one strand pairs with thymine at the same position of the opposite strand, or guanine with cytosine. These are the Watson-Crick rules of base pairing: only A-T and G-C base pairs can form. Two strands joined together in compliance with these rules are said to have complementary base sequences.

These thermodynamic rules imply that the sequence of bases along one DNA strand immediately dictates the sequence of bases that must be present along the complementary strand in the double helix. For example, whenever an A occurs along one strand, a T must be present at that exact position on the opposite strand; a G must always be paired with a C, a T with an A, and a C with a G. In RNA-RNA or RNA-DNA double-stranded molecules, U-A base pairs replace T-A pairs.

Storage and Transmission of Genetic Information

The rules of Watson-Crick base pairing apply to DNA-RNA, RNA-RNA, and DNA-DNA double-stranded molecules. Enzymes that replicate or polymerize DNA and RNA molecules obey the base pairing rules. By using an existing strand of DNA or RNA as the template, a new (daughter) strand is copied (transcribed) by reading processively along the base sequence of the template strand, adding to the growing strand at each position only that base that is complementary to the corresponding base in the template according to the Watson-Crick rules. Thus, a DNA strand having the base sequence 5-GCTATG-3 could be copied by DNA polymerase only into a daughter strand having the sequence 3-CGATAC-5. Note that the sequence of the template strand provides all the information needed to predict the nucleotide sequence of the complementary daughter strand. Genetic information is thus stored in the form of base-paired nucleotide sequences.

If a double-stranded DNA molecule is separated into its two component strands, and each strand is then used as a template to synthesize a new daughter strand, the product will be two double-stranded daughter DNA molecules, each identical to the original parent molecule. This semiconservative replication process is exactly what occurs during mitosis and meiosis as cell division proceeds (Fig. 1-2). The rules of Watson-Crick base pairing thus provide for the faithful transmission of exact copies of the cellular genome to subsequent generations.

Expression of Genetic Information Through the Genetic Code and Protein Synthesis

The information stored in the DNA base sequence achieves its impact on the structure, function, and behavior of organisms by governing the structures and amounts of protein synthesized in the cells. The primary structure (i.e., the amino acid sequence) of each protein determines its three-dimensional conformation and therefore properties (e.g., shape, enzymatic activity, ability to interact with other molecules, stability). In the aggregate, these proteins control cell structure and metabolism. Genes determine the structures of proteins that are synthesized, the timing of their production during development or differentiation, and the amounts produced in different cells or tissues. The process by which DNA achieves its control of cells through protein synthesis is called gene expression.

An outline of the basic pathway of gene expression in eukaryotic cells is shown in Figure 1-3 . The DNA base sequence

Figure 1-2 Semiconservative replication of DNA. **(A)** The process by which the DNA molecule on the left is replicated into two daughter molecules, as occurs during cell division. Replication occurs by separation of the parent molecule into the single-stranded form at one end, reading of each of the daughter strands in the 3 5 direction by DNA polymerase, and addition of new bases to growing daughter strands in the 5 3 direction. **(B)** The replicated portions of the daughter molecules are identical to each other (red). Each carries one of the two strands of the parent molecule, accounting for the term *semiconservative replication.* Note the presence of the replication fork, the point at which the parent DNA is being unwound. **(C)** The antiparallel nature of the DNA strands demands that replication proceed toward the fork in one direction and away from the fork in the other (red). This means that replication is actually accomplished by reading of short stretches of DNA, followed by ligation of the short daughter strand regions to form an intact daughter strand.

Figure 1-3 Synthesis of mRNA and proteinthe pathway of gene expression. The diagram of the DNA gene shows the alternating array of exons (red) and introns (shaded color) typical of most eukaryotic genes. Transcription of the mRNA precursor, addition of the 5-CAP and 3-poly (A) tail, splicing and excision of introns, transport to the cytoplasm through the nuclear pores, translation into the amino acid sequence of the apoprotein, and post-translational processing of the protein are described in the text. Translation proceeds from the initiator methionine codon near the 5 end of the mRNA, with incorporation of the amino terminal end of the protein. As the mRNA is read in a 5 3 direction, the nascent polypeptide is assembled in an amino carboxyl terminal direction.

is first copied into an RNA molecule, called premessenger RNA, by messenger RNA (mRNA) polymerase. Pre-mRNA has a base sequence identical to the DNA coding strand. Genes in eukaryotic species consist of tandem arrays of sequences encoding mRNA (exons); these sequences alternate with sequences (introns) present in the initial mRNA transcript (pre-mRNA) but absent from the mature mRNA. The entire gene is transcribed into the large precursor, which is then further processed (spliced) in the nucleus. The introns are excised from the final mature mRNA molecule, which is then exported to the cytoplasm to be decoded (translated) into the amino acid sequence of the protein, by association with a biochemically complex group of ribonucleoprotein structures called ribosomes. Ribosomes contain two subunits; the 60S subunit contains a single, large (28S) ribosomal RNA molecule complexed with multiple proteins, whereas the RNA component of the 40S subunit is a smaller (18S) rRNA molecule.

Ribosomes read mRNA sequence in a ticker tape fashion *three bases at a time*, inserting the appropriate amino acid encoded by each three-base code word or codon into the appropriate position of the growing protein chain. This process is called mRNA translation. The glossary used by cells to know which amino acids are encoded by each DNA codon is called the genetic code (Table 1-1). Each amino acid is encoded by a sequence of three successive bases. Because there are four code letters (A, C, G, and U), and because sequences read in the 5 3 direction have a different biologic meaning than sequences read in the 3 5 direction, there are 4^3 , or 64, possible codons consisting of three bases.

There are 21 naturally occurring amino acids found in proteins. Thus, more codons are available than amino acids to be encoded. As noted in Table 1-1 , a consequence of this redundancy is that some amino acids are encoded by more than one codon. For example, six distinct codons can specify incorporation of arginine into a growing amino acid chain, four codons can specify valine, two can specify glutamic acid, and only one each, methionine or tryptophan. In no case does a single codon encode more than one amino acid. Codons thus predict unambiguously the amino acid sequence they encode. However, one cannot easily read backward from the amino acid sequence to decipher the *exact* encoding DNA sequence. These facts are summarized by saying that the code is degenerate but not ambiguous.

Some specialized codons serve as punctuation points during translation. The methionine codon (AUG), when surrounded by a consensus sequence (the Kozak box) near the beginning (5 end) of the mRNA, serves as the initiator codon signaling the first amino acid to be incorporated. All proteins thus begin with a methionine residue, but this is often removed later in the translational process. Three codons, UAG, UAA, and UGA, serve as translation terminators, signaling the end of translation.

The adaptor molecules mediating individual decoding events during mRNA translation are small (40 bases long) RNA molecules called transfer RNAs (tRNAs). When bound into a ribosome, each tRNA exposes a three-base segment within its sequence called the anticodon. These three bases attempt to pair with the three-base codon exposed on the mRNA. If the anticodon is complementary in sequence to the codon, a stable interaction among the mRNA, the ribosome, and the tRNA molecule results. Each tRNA also contains a separate region that is adapted for covalent binding to an amino acid. The enzymes that catalyze the binding of each amino acid are constrained in

TABLE 1-1 -- The Genetic Code[a] Messenger RNA Codons for the Amino Acids

Alanine	**Arginine**	**Asparagine**	**Aspartic acid**	**Cysteine**
5-GCU-3	CGU	AAU	GAU	UGU
GCC	CGC	AAC	GAC	UGC
GCA	CGA			
GCG	AGA			
	AGG			
Glutamic acid	**Glutamine**	**Glycine**	**Histidine**	**Isoleucine**
GAA	CAA	GGU	CAU	AUU
GAG	CAG	GGC	CAC	AUC
		GGA		AUA
		GGG		
Leucine	**Lysine**	**Methionine**	**Phenylalanine**	**Proline**[c]
UUA	AAA	AUG[b]	UUU	CCU
UUG	AAG		UUC	CCC
CUU				CCA
CUC				CCG
CUA				

CUG				
Serine	**Threonine**	**Tryptophan**	**Tyrosine**	**Valine**
UCU	ACU	UGG	UAU	GUU
UCC	ACC		UAC	GUC
UCA	ACA			GUA
UCG	ACG			GUG
AGU				
AGC				
	Chain termination codons[d]			
	UAA			
	UAG			
	UGA			

[a] Note that most of the degeneracy in the code is in the third base position (e.g., lysine = AA [G or C], asparagine = AAC/U, valine = GUN [where N is any base]).
[c]Hydroxyproline, the 21st amino acid, is generated by post-translational modification of proline. It is almost exclusively confined to collagen subunits.
[b]AUG is also used as the chain initiation codon when surrounded by the Kozak consensus sequence.
[d] The codons that signal the end of translation, also called nonsense or termination codons, are described by their nicknames *amber* (UAG), *ochre* (UAA), and *opal* (UGA).

such a way that each tRNA species can bind only to a single amino acid. For example, tRNA molecules containing the anticodon 3-AAA-5, which is complementary to a 5-UUU-3 (phenylalanine) codon in mRNA, can only be bound to or charged with phenylalanine; tRNA containing the anticodon 3-UAG-5 can only be charged with isoleucine, and so forth.

Transfer RNAs and their amino acyl tRNA synthetases provide for the coupling of nucleic acid information to protein information needed to convert the genetic code to an amino acid sequence. Ribosomes provide the structural matrix on which tRNA anticodons and mRNA codons become properly exposed and aligned in an orderly, linear, and sequential fashion. As each new codon is exposed, the appropriate charged tRNA species is bound. A peptide bond is then formed between the amino acid carried by this tRNA and the C-terminal residue on the existing nascent protein chain. The growing chain is transferred to the new tRNA in the process, so that it is held in place as the next tRNA is brought in. This cycle is repeated until completion of translation. The completed polypeptide chain is then transferred to other organelles for further processing (e.g., to the endoplasmic reticulum and the Golgi apparatus) or released into cytosol for association of the newly completed chain with other subunits to form complex multimeric proteins (e.g., hemoglobin), and so forth, as discussed in Chapter 2 .

mRNA Metabolism

Eukaryotic and prokaryotic cells differ in the way that the initial mRNA transcript is structurally related to the mature mRNA species that is ultimately translated on ribosomes. In prokaryotes, the initial transcript and the translated transcript are essentially the same. In eukaryotes, the situation is far more complex.

In eukaryotic cells, mRNA is initially synthesized in the nucleus (Figs. 1-3 and 1-4). Before the initial transcript becomes suitable for translation in the cytoplasm, several complex events, mRNA processing and transport, must occur: excision of the portions of the mRNA corresponding to the introns of the gene (mRNA splicing), modification of the 5 and 3 ends of the mRNA to render them more stable and translatable, and transport to the cytoplasm. Moreover, the amount of any particular mRNA moiety in both prokaryotic and eukaryotic cells is governed not only by the composite rate of mRNA synthesis (transcription, processing, and transport), but by its degradation by cytoplasmic ribonucleases (RNA degradation). Many mRNA species of special importance in hematology (e.g., mRNAs for growth factors and their receptors, proto-oncogene mRNAs, acute-phase reactants) are exquisitely regulated by control of their stability (half-life) in the cytoplasm.

Post-transcriptional mRNA metabolism is complex. Only a few relevant details are considered in this section.

mRNA Splicing

The initial transcript of eukaryotic genes contains several subregions (Fig. 1-4). Most striking is the tandem alignment of exons and introns. Precise excision of intron sequences and ligation of exons is critical for production of mature mRNA. This process is called mRNA splicing, and it occurs on complexes of small nuclear RNAs and proteins called snRNPs; the term *spliceosome* is also used to describe the intranuclear organelle that mediates mRNA splicing reactions. The biochemical mechanism for splicing is complex. A consensus sequence, which includes the dinucleotide GU, is recognized as the donor site at the 5 end of the intron (5 end refers to the polarity of the mRNA strand coding for protein); a second consensus sequence ending in the dinucleotide AG is recognized as the acceptor site, which marks the distal end of the intron (Figs. 1-4 and 1-5). The spliceosome recognizes the donor and acceptor and forms an intermediate lariat structure that provides for both excision of the intron and proper alignment of the cut ends of the two exons for ligation in precise register.

Messenger RNA splicing has proved to be an important mechanism for greatly increasing the versatility and diversity of expression of a single gene. For example, some genes contain an array of more exons than are actually found in any mature mRNA species encoded by the gene. Several different mRNA protein products can arise from a single gene by selective inclusion or exclusion of individual exons from the mature mRNA products. This phenomenon is called alternative mRNA splicing. It permits a single gene to code for multiple mRNA and protein products with related, but distinct structures and functions. The mechanisms by which individual exons are selected

Figure 1-4 Anatomy of the products of the structural gene (mRNA precursor and mRNA). This schematic shows the configuration of the critical anatomic elements of an mRNA precursor, which represents the primary copy of the structural portion of the gene. The sequences GU and AG indicate, respectively, the invariant dinucleotides present in the donor and acceptor sites at which introns are spliced out of the precursor. Not shown are the less stringently conserved consensus sequences that must precede and succeed each of these sites for a short distance.

Figure 1-5 Regulatory elements flanking the structural gene.

or rejected remain totally obscure. For present purposes, it is sufficient to note that important physiologic changes in cells can be regulated by altering the patterns of mRNA splicing products arising from single genes.

Many inherited hematologic diseases arise from mutations, which derange mRNA splicing. For example, some of the most common forms of the thalassemia syndromes and hemophilia arise by mutations that alter normal splicing signals or create splicing signals where they normally do not exist (activation of cryptic splice sites).

Modification of the Ends of the mRNA Molecule

Most eukaryotic mRNA species are polyadenylated at their 3 ends. mRNA precursors are initially synthesized as large molecules that extend further downstream from the 3 end of the mature mRNA molecule. Polyadenylation results in the addition of stretches of 100150 A residues at the 3 end. Such an addition is often called the poly-A tail, and is of variable length. Polyadenylation facilitates rapid early cleavage of the unwanted 3 sequences from the transcript and is also important for stability or transport of the mRNA out of the nucleus. Signals near the 3 extremity of the mature mRNA mark positions at which polyadenylation occurs. The consensus signal is AUAAA (Fig. 1-4). Mutations in the poly-A signal sequence have been shown to cause thalassemia.

At the 5 end of the mRNA, a complex oligonucleotide having unusual phosphodiester bonds is added. This structure contains the nucleotide 7-methyl-guanosine, and is called CAP (Fig. 1-4). The 5-CAP enhances both mRNA stability and the ability of the mRNA to interact with protein translation factors and ribosomes.

5 and 3 Untranslated Sequences

The 5 and 3 extremities of mRNA extend beyond the initiator and terminator codons that mark the beginning and the end of the sequences actually translated into proteins (Figs. 1-4 and 1-5). The functions of these so-called 5 and 3 untranslated regions (5 UTR, 3 UTR) remain poorly understood. It appears that UTRs are involved in determining mRNA stability and, possibly, the efficiency with which mRNA species can be translated. For example, if the 3 UTR of a very stable mRNA (e.g., globin mRNA) is swapped with the 3 UTR of a highly unstable mRNA (e.g., the c-*myc* proto-oncogene), the c-*myc* mRNA becomes more stable. Conversely, attachment of the 3 UTR of c-*myc* to a globin molecule renders it unstable. Instability is often associated with repeated sequences rich in A and U in the 3 UTR (Fig. 1-4).

Transport of mRNA from Nucleus to Cytoplasm: mRNP Particles

An additional potential step for regulation or disruption of mRNA metabolism occurs during the transport from nucleus to cytoplasm. mRNA transport is an active, energy-consuming process. Moreover, at least some mRNAs appear to enter the cytoplasm in the form of complexes bound to proteins (mRNPs). mRNPs may regulate stability of the mRNAs and their access to translational apparatus. There is some evidence that certain mRNPs are present in the cytoplasm but are not translated (masked message) until proper physiologic signals are received.

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GENE REGULATION

Virtually all cells of an organism receive a complete copy of the DNA genome inherited at the time of conception. The panoply of distinct cell types and tissues found in any complex organism is possible only because different portions of the genome are selectively expressed or repressed in each cell type. Each cell must know which genes to express, how actively to express them, and when to express them. This biologic necessity has come to be known as gene regulation or regulated gene expression. Understanding gene regulation provides insight into how pluripotent stem cells determine that they will express the proper sets of genes in daughter progenitor cells that differentiate along each lineage. Major hematologic disorders (such as the leukemias and lymphomas), immunodeficiency states, and myeloproliferative syndromes result from derangements in the system of gene regulation. An understanding of the ways that genes are selected for expression thus remains one of the major frontiers of biology and medicine.

Active and Inactive Configurations of Genomic DNA in Chromatin

Most of the DNA in living cells is inactivated by formation of a nucleoprotein complex called chromatin. The histone and

nonhistone proteins in chromatin effectively sequester genes from enzymes needed for expression. The most tightly compacted chromatin regions are called euchromatin. Heterochromatin, less tightly packed, contains actively transcribed genes. Activation of a gene for expression (i.e., transcription) requires that it become less compacted and more accessible to the transcription apparatus. Little is known about these processes, but it is clear that both *cis*-acting and *trans*-acting factors are involved. *Cis*-acting elements are regulatory DNA sequences, within or flanking the genes. They are recognized by *trans*-acting factors, which are nuclear DNA binding proteins needed for transcriptional regulation.

DNA sequence regions flanking genes are called *cis*-acting because they influence expression of nearby genes only on the same chromosome. These sequences do not usually encode mRNA or protein molecules. They alter the conformation of the gene within chromatin in such a way as to facilitate or inhibit access to the factors that facilitate transcription. These interactions may twist or kink the DNA in such a way as to increase exposure to other molecules. When exogenous nucleases are added in small amounts to nuclei, these exposed sequence regions are especially sensitive to the DNA cutting action of the nucleases. Thus, nuclease-hypersensitive sites in DNA have come to be appreciated as markers for regions in or near genes that are interacting with regulatory nuclear proteins.

Methylation is another structural feature that can be used to recognize differences between actively transcribed and inactive genes. Most eukaryotic DNA is heavily methylated, that is, the DNA is modified by the addition of a methyl group to the 5 position of cytosines (5-methyl-C). In general, heavily methylated genes are inactive, whereas active genes are relatively hypomethylated, especially in the 5 flanking regions containing the promoter and other regulatory elements (see later). These flanking regions frequently include DNA sequences with a high content of Gs and Cs (G-C-rich islands). Hypomethylated G-C-rich islands (detectable by methylation-sensitive restriction endonucleases) serve as markers of actively transcribed genes. For example, a search for undermethylated G-C-rich islands on chromosome 7 facilitated the search for the gene for cystic fibrosis.

Several other structural configurations of DNA and their interaction with chromatin have been implicated as important for gene regulation. These include the B or Z conformation of the DNA helix, the degree of supercoiling or torsion of DNA superstructures, acetylation, phosphorylation, or ribosylation of nuclear proteins, and so forth. However, none of these mechanisms is understood in sufficient detail to merit further discussion.

Enhancers, Promoters, and Silencers

Several types of *cis*-active DNA sequence elements have been defined according to the presumed consequences of their interaction with nuclear proteins (Fig. 1-5). *Promoters* are found just upstream (to the 5 side) of the start of mRNA transcription (the CAP). mRNA polymerases appear to bind first to the promoter region and thereby gain access to the structural gene sequences downstream. Promoters thus serve a dual function of being binding sites for mRNA polymerase and marking for the polymerase the downstream point at which transcription should start. *Enhancers* are more complicated and less well understood DNA sequence elements. Enhancers can lie on either side of a gene, or even within the gene in introns. Enhancers appear to bind transcription factors and thereby stimulate expression of genes nearby. The domain of influence of enhancers (i.e., the number of genes to either side whose expression is stimulated) varies. Some enhancers influence only the adjacent gene; others seem to mark the boundaries of large multigene clusters (gene domains) whose coordinated expression is appropriate to a particular tissue type or a particular time. For example, the very high levels of globin gene expression in erythroid cells depend on the function of an enhancer that seems to activate the entire gene cluster and is thus called a locus-activating region (LAR; Fig. 1-5). The nuclear factors interacting with enhancers are probably induced into synthesis or activation as part of the process of differentiation.

Silencer sequences serve a function that is the obverse of enhancers. When bound by the appropriate nuclear proteins, silencer sequences cause repression of gene expression. There is some evidence that the same sequence elements can act as enhancers or silencers under different conditions, presumably by being bound by different sets of proteins having opposite effects on transcription.

Transcription Factors

Assays for detecting nuclear proteins that exhibit gene-specific DNA binding are now achieving widespread utility. Considerable information is now available about these nuclear proteins and their biochemical properties, but their physiologic behavior is incompletely understood. Proteins involved in the regulation of several gene systems have been isolated and their genes cloned. Common structural features have become apparent. Most transcription factors have DNA-binding domains sharing homologous structural motifs (cytosine-rich regions called zinc fingers, leucine-rich regions called leucine zippers, and so forth), but other regions appear to be unique. Many factors implicated in the regulation of growth, differentiation, and development (e.g., homeobox genes, proto-oncogenes, antioncogenes) appear to be DNA-binding proteins and may be involved in the steps needed for activation of a gene within chromatin. Others seem to bind to, or modify, DNA-binding proteins. These factors are discussed in more detail in Chapter 6 .

Regulation of mRNA Splicing, Stability, and Translation (Post-transcriptional Regulation)

It has become increasingly apparent that *post-transcriptional* and *translational* mechanisms are important strategies used by cells to govern the amounts of mRNA and protein accumulating when a particular gene is expressed. The major modes of *post-transcriptional* regulation are regulated alternative mRNA splicing, control of

mRNA *stability*, and control of *translational* efficiency.

A cell can regulate the relative amounts of different protein isoforms arising from a given gene by altering the relative amounts of an mRNA precursor that are spliced along one pathway or another (alternative mRNA splicing). Several striking examples of this type of regulation are described, including the ability of B lymphocytes to make both IgM and IgD at the same developmental stage, changes in the particular isoforms of cytoskeletal proteins produced during red cell differentiation, and a switch from one isoform of the *c-myb* proto-oncogene product to another during red cell differentiation. The effect of controlling the pathway of mRNA processing used in a cell is to include or exclude portions of the mRNA sequence. These portions encode peptide sequences that influence the ultimate physiologic behavior of the protein, or the RNA sequences that alter stability or translatability.

The importance of the control of mRNA stability for gene regulation is being increasingly appreciated. The steady-state level of any given mRNA species ultimately depends on the balance between the rate of its production (transcription and mRNA processing) and its destruction. One means by which stability is regulated is the inherent structure of the mRNA sequence, especially the 3 and 5 UTRs. As already noted, these

sequences appear to affect mRNA secondary structure or recognition by nucleases, or both. Different mRNAs thus have inherently longer or shorter half-lives, almost regardless of the cell type in which they are expressed. Some mRNAs tend to be highly unstable. In response to appropriate physiologic needs, they can thus be produced quickly and removed from the cell quickly when a need for them no longer exists. Globin mRNA, on the other hand, is inherently quite stable, with a half-life measured in the range of 50 hours. This is appropriate for the need of reticulocytes to continue to synthesize globin for 2448 hours after the ability to synthesize new mRNA has been eliminated from the terminally mature erythroblasts.

Messenger RNA stability can also be altered in response to changes in the intracellular milieu. This phenomenon usually involves nucleases capable of destroying one or more broad classes of mRNA defined on the basis of their 3 or 5 UTR sequences. Thus, for example, histone mRNAs are destabilized after the S phase of the cell cycle is complete. Presumably this occurs because histone synthesis is no longer needed. Induction of cell activation, mitogenesis, or terminal differentiation events often results in the induction of nucleases that destabilize specific subsets of mRNAs. Selective stabilization of mRNAs probably also occurs, but specific examples are less well documented.

The amount of a given protein accumulating in a cell depends on the amount of the mRNA present, the rate at which it is translated into the protein, and the stability of the protein. Translational efficiency depends on a number of variables, including polyadenylation and presence of the 5 CAP. The amounts and state of activation of protein factors needed for translation are also crucial. The secondary structure of the mRNA, particularly in the 5 UTR, greatly influences the intrinsic translatability of an mRNA molecule by constraining the access of translation factors and ribosomes to the translation initiation signal in the mRNA. Secondary structure along the coding sequence of the mRNA may also have some impact on the rate of elongation of the peptide.

Changes in capping, polyadenylation, and translation factor efficiency affect the overall rate of protein synthesis within each cell. These effects tend to be global, rather than specific to a particular gene product. However, these effects influence the relative amounts of different proteins made. mRNAs whose structures inherently lend themselves to more efficient translation tend to compete better for rate-limiting components of the translational apparatus, whereas those mRNAs that are inherently less translatable tend to be translated less efficiently in the face of limited access to other translational components. For example, the translation factor eIF-4 tends to be produced in higher amounts when cells encounter transforming or mitogenic events. This causes an increase in overall rates of protein synthesis, but also leads to a selective increase in the synthesis of some proteins that were underproduced before mitogenesis.

Translational regulation of individual mRNA species is critical for some events important to blood cell homeostasis. For example, as discussed in Chapter 26 , the amount of iron entering a cell is an exquisite regulator of the rate of ferritin mRNA translation. An mRNA sequence called the iron response element is recognized by a specific mRNA-binding protein, but only when the protein lacks iron. mRNA bound to the protein is translationally inactive. As iron accumulates in the cell, the protein becomes iron bound and loses its affinity for the mRNA, resulting in production of apoferritin molecules available to bind the iron.

Tubulin synthesis involves coordinated regulation of translation and mRNA stability. Tubulin regulates the stability of its own mRNA by a feedback loop. As tubulin concentrations rise in the cell, it interacts with its own mRNA through the intermediary of an mRNA-binding protein. This results in the formation of an mRNAprotein complex and nucleolytic cleavage of the mRNA. The mRNA is destroyed, and further tubulin production is halted.

These examples of post-transcriptional regulation emphasize that cells tend to use every step in the complex pathway of gene expression as points at which exquisite control over the amounts of a particular protein can be regulated. In other chapters, additional levels of regulation are described (e.g., regulation of the stability, activity, localization, and access to other cellular components of the proteins that are present in a cell).

Additional Structural Features of Genomic DNA

Most DNA does not code for RNA or protein molecules. The vast majority of nucleotides present in the human genome reside outside structural genes. Structural genes are separated from one another by as few as 15 kb, or as many as several thousand kilobases of DNA. Almost nothing is known about the reason for the erratic clustering and spacing of genes along chromosomes. It is clear that *intergenic* DNA contains a variegated landscape of structural features that provide useful tools to localize genes, identify individual human beings as unique from every other human being (DNA fingerprinting), and diagnose human diseases by linkage. A more detailed discussion of these techniques is included in Chapter 160 . Only a brief introduction is provided here.

The rate of mutation in DNA under normal circumstances is approximately $1/10^6$. In other words, 1 of 1 million bases of DNA will be mutated during each round of DNA replication. A set of enzymes called DNA proofreading enzymes connect many of these mutations. When these enzymes are altered by mutation, the rate of mutation (and, therefore, the odds of neoplastic transformation) increase considerably. If these mutations occur in bases critical to the structure or function of a protein or gene, altered function, disease, or a lethal condition often results. Most pathologic mutations tend not to be preserved throughout many generations because of their unfavorable phenotypes. Exceptions, such as the hemoglobinopathies, occur when the heterozygous state for these mutations confers selective advantage in the face of unusual environmental conditions, such as malaria epidemics. These adaptive mutations drive the dynamic change in the genome with time (evolution).

Most of the mutations that accumulate in the DNA of *Homo sapiens* occur in either intergenic DNA or the silent bases of DNA, such as the degenerate third bases of codons. They do not pathologically alter the function of the gene or its products. These clinically harmless mutations are called DNA polymorphisms. DNA polymorphisms can be regarded in exactly the same way as other types of polymorphisms that have been widely recognized for years (e.g., eye and hair color, blood groups). They are variations in the population that occur without apparent clinical impact. Each of us differs from other humans in the precise number and type of DNA polymorphisms that we possess.

Like other types of polymorphisms, DNA polymorphisms breed true. In other words, if an individual's DNA contains a G 1,200 bases upstream from the -globin gene, instead of the C most commonly found in the population, that G will be transmitted to that individual's offspring. Note that if one had a means for distinguishing the G at that position from a C, one would have a linked marker for that individual's -globin gene.

Occasionally, a DNA polymorphism falls within a restriction endonuclease site. (Restriction enzymes cut DNA molecules into smaller pieces, but only at limited sites, defined by short base sequences recognized by each enzyme.) The change could abolish the site or create a site where one did not exist before. These polymorphisms change the array of fragments generated when the genome is digested by that restriction endonuclease. This permits detection of the polymorphism by use of the appropriate

restriction enzyme. This specific class of polymorphisms is thus called restriction fragment length polymorphisms (RFLPs).

Restriction fragment length polymorphisms are useful because the length of a restriction endonuclease fragment on which a gene of interest resides provides a linked

marker for that gene. The exploitation of this fact for diagnosis of genetic diseases and detection of specific genes is discussed in Chapter 160 ; Figure 1-6 shows a simple example.

Restriction fragment length polymorphisms have proved to be extraordinarily useful for the diagnosis of genetic diseases, especially when the precise mutation is not known. Recall that DNA polymorphisms breed true in the population. For example, as discussed in Chapter 110 , a mutation that causes hemophilia will, when it occurs on the X chromosome, be transmitted to subsequent generations attached to the pattern (often called a framework or haplotype) of RFLPs that was present on that same X chromosome. If the pattern of RFLPs in the parents is known, the presence of the abnormal chromosome can be detected in the offspring.

An important feature of the DNA landscape is the high degree of repeated DNA sequence. A DNA sequence is said to be repeated if it or a sequence very similar (homologous) to it occurs more than once in a genome. Some multicopy genes, such as the histone genes and the ribosomal RNA genes, are repeated DNA sequences. Most repeated DNA occurs outside genes, or within introns. Indeed, 3045% of the human genome appears to consist of repeated DNA sequences.

The function of repeated sequences remains unknown, but their presence has inspired useful strategies for detecting and characterizing individual genomes. For example, a pattern of short repeated DNA sequences, characterized by the presence of flanking sites recognized by the restriction endonuclease Alu-1 (called Alu-repeats), occurs approximately 300,000 times in a human genome. These sequences are not present in the mouse genome. If one wishes to infect mouse cells with human DNA and then identify the human DNA sequences in the infected mouse cells, one simply probes for the presence of Alu-repeats. The Alu-repeat thus serves as a signature of human DNA.

Classes of highly repeated DNA sequences (tandem repeats) have proved to be useful for distinguishing genomes of each human individual. These short DNA sequences, usually less than a few hundred bases long, tend to occur in clusters, with the number of repeats varying between individuals (Fig. 1-6). Alleles of a given gene can therefore be associated with a variable number of tandem repeats (VNTR) in different individuals or populations. For example, there is a VNTR near the insulin gene. In some individuals or populations, it is present in only a few tandem copies, whereas in others it is present in many more. When the population as a whole is examined, there is a wide degree of variability from individual to individual as to the number of these repeats residing near the insulin gene. It can readily be imagined that, if probes were available to detect a dozen or so distinct VNTR regions, each human individual would differ from virtually all others with respect to the aggregate pattern of these VNTRs. Indeed, it can be shown mathematically that the probability of any two human beings sharing exactly the same pattern of VNTRs is exceedingly small if approximately 1012 different VNTR elements are mapped for each person. A technique called DNA fingerprinting that is based on VNTR analysis has become widely publicized because of its forensic applications.

Variable-number tandem repeats can be regarded as normal sequence variations in DNA that are similar to, but far more useful than, singlebase-change RFLP polymorphisms. Note that the odds of a single base change altering a convenient

Figure 1-6 Two useful forms of sequence variation among the genomes of normal individuals. **(A)** Presence of a DNA sequence polymorphism that falls within a restriction endonuclease site, thus altering the pattern of restriction endonuclease digests obtained from this region of DNA on Southern blot analysis. (Readers not familiar with Southern blot analysis should return to examine this figure after reading later sections of this chapter.) **(B)** A variable-number tandem repeat (VNTR) region (defined and discussed in the text). Note that individuals can vary from one to another in many ways according to how many repeated units of the VNTR are located on their genomes, whereas restriction fragment length polymorphism differences are in effect all-or-none differences, allowing for only two variables (restriction site presence or absence).

restriction endonuclease site are relatively small, so that RFLPs occur relatively infrequently in a useful region of the genome. Moreover, there is only one state or variable that can be examinedthat is, the presence or absence of the restriction site. By contrast, many VNTRs are scattered throughout the human genome. Most of these can be distinguished from one another quite readily by standard blotting and hybridization technology (see Chap. 160). Most important, the amount of variability from individual to individual at each site of a VNTR is considerably greater than for RFLPs. Rather than the mere presence or absence of a site, there is a whole array of banding patterns possible, depending on how many individual repeats are present at that site (Fig. 1-6). This reasoning can readily be extended to appreciate that those VNTRs occurring near genes of hematologic interest can provide highly useful markers for localizing that gene, or for distinguishing the normal allele from an allele carrying a pathologic mutation.

There are many other classes of repeated sequences in human DNA. For example, human DNA has been invaded many times in its history by retroviruses. Retroviruses tend to integrate into human DNA and then jump out of the genome when they are reactivated, to complete their life cycle. The proviral genomes often carry with them nearby bits of the genomic DNA in which they sat. If the retrovirus infects DNA of another individual at another site, it will insert this genomic bit. Through many cycles of infection, the virus will act as a transposon, scattering its attached sequence throughout the genome. These types of sequences are called long interspersed elements. They represent footprints of ancient viral infections.

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BASIC TENETS OF RECOMBINANT DNA TECHNOLOGY

The informational content of DNA molecules resides in the nucleotide sequence rather than in the sugarphosphate backbone. Unfortunately, traditional methods of biochemical fractionation do not provide straightforward means for distinguishing nucleic acid molecules from one another on the basis of their nucleotide sequences. Even if such methods were available, the quantity of bulk genomic DNA necessary to isolate a gene of typical size (a few thousand or tens of thousands of nucleotides long) from a complex genome such as the human (3 billion bp long) renders these methods impractical. In addition, genes do not exist in cells as discrete DNA molecules; rather, genes are linked together in tandem with very long stretches of intergenic DNA to form chromosomes. For example, in the human genome, each chromosome is approximately 100 million bp long. These facts render DNA an unworkable substance for direct physical purification of genes.

Recombinant DNA technology circumvents the biochemical problems inherent in the properties of DNA by combining enzymologic, microbiologic, and genetic approaches.

Restriction Endonucleases

Restriction endonucleases recognize short DNA base sequences and cleave DNA within or near these recognition sequences (Table 12). For example, *Eco*RI, a restriction endonuclease isolated from *Escherichia coli*, cuts DNA at the sequence 5-GAATTC-3, but nowhere else. Thus, each DNA sample will be reduced reproducibly to an array of smaller-sized fragments whose size ranges depend on the distribution with which 5-GAATTC-3 is encountered in that particular genome. However, the DNA will not be degraded in any other way by the enzyme. Restriction endonucleases differ from other nucleases by the specificity and limited manner with which they degrade DNA.

Restriction enzymes are usually named after the bacteria from which they were isolated. Thus, the first restriction endonuclease activity purified from *Serratia marcescens* is called *Sma*I, the second from *Hemophilus parainfluenzae* is called *Hpa*II, and so forth. Each of the nearly 500 restriction endonucleases that have been described recognizes a unique oligonucleotide sequence and cleaves the DNA only at those points. Table 1-2 shows the names and recognition sites of some typical restriction endonucleases.

In some cases, two or more different restriction enzymes recognize exactly the same recognition sequence. Such restriction enzymes are called isoschizomers. A useful type of isoschizomer is a pair of restriction enzymes that recognize the same sequence

TABLE 1-2 -- Some Common Restriction Endonuclease Enzymes and Their Recognition Sequences

Name of Enzyme	Microorganism from Which Derived	Recognition and Cleavage Site
*Eco*RI	*Escherichia coli*	5-GAATTC3
		3-CTTAAG5
*Bam*HI	*Bacillus amyloliquefaciens*	5-GGATCC3
		3CCTAGG5
*Hind*III	*Hemophilus influenzae*	5AAGCTT3
		3TTGCAA5
*Sau*BA, Pst	*Providencia stuartii*	5CTGCAG3
		3GACGTC5
*Sma*I	*Serratia marcescens*	5-GGGCCC3
		3CCCGGG5

Types of Cuts Made by Restriction Enzymes

5 overhang (e.g., *Eco*RI):		
5------GAATTC----3	5-------G-3	5-AATTC------3
3------CTTAAG----5	3-------CTTAA-5	3-G------5
3 overhang (e.g., *Pst*I):		
5------CTGCAG----3	5-----CTGCA-3	5G------3
3------GACGTC----5	3-----G-5	3-ACGTC------5
Blunt end (e.g., *Sma*I):		
5---GGGCCC---3	5----GGG--3	5CCC--------3
3---CCCGGG---3	3----CCC--5	3GGG--------5

but cut or fail to cut according to modifications of the DNA bases, notably methylation. For example, both *Hpa*II and *Msp*I recognize the sequence 5-GCCG-3. *Msp*I cuts regardless of whether the C residues are methylated, but *Hpa*II will cut only if the C residues are not methylated. These paired enzymes are useful for identifying positions in mammalian genomes that are methylated.

Even though their physiologic function remains unknown, restriction enzymes have proved to be extraordinarily useful in the laboratory. They reduce the sizes of DNA fragments in a controlled and reproducible manner from several hundred million base pairs long to fragment arrays ranging from a few dozen to a few tens of thousands of bases long. These ranges are far more manageable experimentally. Moreover, by digesting a DNA sample with combinations of restriction enzymes, maps or fingerprints of the restriction endonuclease sites in a genome can be constructed. Restriction endonuclease digestion is as useful an approach for

characterizing the fine structure of genomes as partial proteolytic digestion (peptide fingerprinting) has been for protein chemists.

Many restriction endonucleases cut the DNA so as to leave short, single-stranded overhanging regions or sticky ends at the 5 or 3 end of the cutting site, whereas other enzymes leave blunt or flush double-stranded ends. Because many restriction endonuclease sites are palindromes (reading exactly the same on each strand, provided one reads in the same direction (e.g., 5 3) on each strand, these overhanging ends are particularly useful. For example, if DNA from two different sources (such as a bacteriophage preparation and human genomic preparation) is digested with *Eco*RI, the sticky ends will be complementary by Watson-Crick base pairing and can thus be annealed at the single-stranded overhangs (Table 1-2). This is the most popular method for generating recombinant DNA molecules.

Enzymes Useful for Modifying and Synthesizing DNA

Several other nucleic acid-modifying enzymes have been critical to the development of recombinant DNA technology. Notable among these are reverse transcriptase (RNA-dependent DNA polymerase) and DNA ligase. Reverse transcriptase is the enzyme packaged inside retroviruses that have an RNA genome. For retroviruses to reproduce themselves within their cellular hosts, their RNA genomes must be transcribed into DNA molecules (RNA DNA) that can then be replicated (DNA DNA) and expressed by host cell machinery (DNA RNA).

Reverse transcriptase has a very useful property. If provided with an appropriate primer DNA sequence complementary to a small region of an mRNA molecule, it can read the mRNA strand in a 3 5 direction and transcribe a single-stranded DNA copy (copy DNA, complementary DNA [cDNA]) of the RNA molecule. Using an oligonucleotide consisting of 1218 dT residues (oligo dT) (recall that nearly all mRNAs in eukaryotes have a 3 poly[A] tail, complementary to the oligo dT), mRNA isolated from a cell or tissue of interest can be incubated with reverse transcriptase, generating a population of single-stranded DNA molecules representing the entire array of mRNAs expressed in that cell or tissue. For example, using additional enzymes that have been characterized and purified, a DNA-dependent DNA polymerase can synthesize a complementary second strand of DNA from the single-stranded cDNA template. This creates double-stranded DNA molecules containing the sequence information originally expressed in the form of the mRNAs in the specimen. These DNA molecules can then be manipulated in the same ways that native genomic DNA molecules can, by restriction endonuclease digestion, radioactive labeling, or insertion into microbial host vectors for cloning.

DNA ligase is an enzyme that can join the ends of two DNA molecules together to form a single DNA molecule. For example, double-stranded cDNA molecules can be joined with bacteriophage DNA molecules by incubating DNA from both sources together in the presence of DNA ligase. This ability to generate artificially recombined, or recombinant, DNA molecules has given rise to the term *recombinant DNA technology*.

Many other important enzymes have also been useful for the development of recombinant DNA technology. These include a variety of polymerases, kinases, endonucleases, and exonucleases that are used to introduce radioactive residues into DNA molecules, to phosphorylate or dephosphorylate their termini, to synthesize new strands, to elongate the ends of DNA molecules by adding single-stranded overhanging sequences, to truncate or trim single-stranded overhangs in order to generate blunt-ended molecules, and so forth. A vast array of elegant methods have been developed to exploit these enzymes to synthesize, modify, and combine DNA molecules with exquisite precision, thus re-engineering DNA.

Microbial Hosts and Infectious DNA Molecules

The development of methods to fragment DNA in a controlled fashion, polymerize it, modify it, or ligate two DNA molecules from dissimilar sources represents an impressive advance. However, these tools would have been of limited value except for the discovery of certain small DNA molecules that possess remarkable biologic properties. Microbial geneticists found that many bacteria harbored DNA molecules that were not part of the single major bacterial chromosome. These novel DNA molecules are small (a few thousand to approximately 100 thousand bases long), have circular structures, can replicate independently in host cells, and, most remarkably, are infectious in the form of naked DNA. These DNA molecules can be thought of as elemental commensal organisms, residing in the cell and capable of infecting other host bacteria. They have come to be called extrachromosomal elements or episomes.

The most relevant episome types are plasmids and bacteriophages. Plasmids (Fig. 1-7) useful in recombinant DNA technology usually carry one or more antibiotic resistance genes, an origin of DNA replication, and a limited but useful array of restriction endonuclease sites. Many useful plasmid vectors have been engineered for customized applications. Such vectors are 3,00010,000 bases long, carry one or two genes for antibiotic resistance, and include a short DNA sequence (polylinker) containing several tightly clustered restriction endonuclease sites. The polylinker sequence is in a noncritical region of the plasmid genome, so a restriction site in the linker can be cut without damaging the plasmid's genes. Cells infected with these plasmids can be detected and purified by their ability to grow in media containing the relevant antibiotic.

The most useful plasmids for recombinant DNA work are those in which the plasmid or its polylinker include several restriction endonuclease sites that occur only once in the plasmid genome. A single cut in the circle causes opening or linearization, while leaving all the biologically critical sequences intact. A DNA molecule can then be inserted into the opening and the loop resealed with DNA ligase, thereby generating a recombinant DNA molecule that retains all the useful biologic activities of the original plasmid.

Bacteriophages are viruses infective in certain species of bacteria. Their genomes are somewhat larger than plasmids (5,000100,000 bp), and the DNA is covered during the extracellular part of the viral life span by a protein coat. However, bacterial genomes relevant to this discussion can also exist in the cell as

Figure 1-7 Plasmidsstructure and properties. The upper portions of the diagram outline the basic structure of bacterial plasmids in terms of two hypothetical examples (Plasmid 1 and Plasmid 2). The three major functional attributes of plasmids are shown at the top of the figure: origin of DNA (O), one or more phenotypic marker genes such as tetracycline resistance (Tet^R [shown in red]) or ampicillin resistance (Amp^R), and restriction endonuclease sites suitable in their location and number for a particular application, such as the *Eco*RI sites shown for Plasmids 1 and 2. The remainder of the figure outlines a rudimentary recombinant DNA experiment, which results in formation of a novel plasmid carrying the gene for tetracycline resistance in addition to the ampicillin resistance gene. Note how digestion with *Eco*RI linearizes Plasmid 2 because it has only a single site, leaving the basic structure of the plasmid intact; in contrast, the two *Eco*RI sites in Plasmid 1 result in fragmentation into two pieces, one of which carries the Tet^R gene. Ligation of the two digestion mixtures results in several possible ligation products, including those formed by self-ligation of the individual plasmids or plasmid fragments by means of their own sticky ends as well as all possible combinations formed by one fragment annealing to another. However, out of this complex mixture the desired recombinant can be clearly selected, because only it will possess both ampicillin and tetracycline resistance. Thus, microbial genetics can be used to identify and purify a DNA fragment that was created chemically or enzymatically. This illustrates the fundamental strategy of recombinant DNA technology, whereby genetic selection of biologically active macromolecules can be used to identify, isolate, purify, and amplify particular products of enzymatic reactions.

episomes. The most useful phages for molecular genetics have been bacteriophage-, which can be used as a gene cloning vehicle, and the single-stranded bacteriophage M13, which is useful for DNA sequencing. Many bacteriophage genomes have been engineered to provide useful vectors.

The essential aspect of episomes important for this discussion is that they are biologically active even when they exist as naked DNA molecules. By combining the ability to attach episomal DNA to DNA from mammalian sources (using restriction enzymes and ligase) with the capacity of the episomes for infection and phenotypic alteration of host cells, these molecules can be used to introduce foreign DNA into host bacteria. Then all the useful properties of the vast array of microbial strains available become accessible for the study of genes from other species. An individual strain of bacteria can be readily isolated as a single-cell clone, inexpensively grown in large quantities, and used as factories for the production of the foreign DNA sequence contained within it, as well as any protein product encoded by the foreign DNA. Moreover, the recombinant episomal DNA can be readily isolated free of the host bacterial chromosome; this provides a simple way to retrieve large quantities of the mammalian DNA that rides as a passenger in the episomal DNA.

Advances in Nucleic Acid Chemistry

The development of automated anhydrous methods for the synthesis of DNA molecules in vitro has provided a means of synthesizing short DNA molecules without benefit of a template or DNA polymerase. For example, the polylinker sequences used to introduce restriction endonuclease sites into plasmids can be readily synthesized by automated instrumentation and ligated into a plasmid to alter its restriction endonuclease map. Synthetic oligonucleotides can also be radiolabeled and used as customized molecular hybridization probes, or used as primers for synthesis of DNA strands complementary to any desired region of a DNA template.

The tendency of DNA and RNA molecules to form double-stranded hybrids in physiologic solution has been exploited by nucleic acid chemists for the development of molecular hybridization assays. If DNA or RNA molecules are heated or exposed to certain denaturants, such as formamide, the hydrogen bonds

holding two strands together are disrupted, and the molecule is denatured into the single-stranded form. Temperature, salt, and denaturing conditions that favor reannealing into the double-stranded form can then be restored. This reannealing process is called *molecular hybridization:* reannealing rates under a given set of conditions of temperature, salinity, and denaturant are a function of the time of incubation and the initial concentration of the annealing strands.

Denatured DNA or RNA strands reanneal only with strands having a complementary sequence by the rules of Watson-Crick base pairing. This specificity forms the basis for the use of molecular hybridization as a means for detecting or quantifying (or both) specific DNA or RNA moieties in a complex mixture. A specimen of denatured DNA or RNA (e.g., mRNA from human bone marrow) can be incubated with a radioactively labeled, defined DNA or RNA sequence (e.g., a cloned human myeloperoxidase gene). The labeled denatured DNA probe will hybridize only to those mRNA molecules that are complementary by Watson-Crick base pairing (i.e., myeloperoxidase mRNA molecules). Any one of several available techniques can then be used to detect the fraction of radioactively labeled DNA probe molecules that have been bound into a double-stranded form. (For example, the enzyme S_1 nuclease degrades single-stranded DNA molecules, leaving only the double-stranded hybridized molecules intact.) The result is a highly sensitive and specific assay for identifying (in our example) myeloperoxidase mRNA in the complex mix of mRNA species present in the bone marrow mRNA. By extension of this reasoning, molecular hybridization strategies can be used to detect, quantitate, and map specific DNA or RNA sequences, provided that a complementary defined DNA probe is available.

Many hybridization assays have been devised. The range of applications, theoretic rationale, and utility of many of these assays can be appreciated by their analogy to the use of antigenantibody reactions in immunochemistry. The DNA probe is used by the molecular geneticist in much the same way as a defined antibody probe is used by the immunologist. The principles underlying the various molecular hybridization techniques are similar to those of immunochemical assays.

Polymerase Chain Reaction

The development of the polymerase chain reaction (PCR) was a major breakthrough that has revolutionized the utility of a DNA-based strategy for diagnosis and treatment. It permits the laboratorian to detect, synthesize, and isolate specific genes and to distinguish among alleles of a gene differing by as little as one base. It does not require sophisticated equipment or unusual technical skills. A clinical specimen consisting of only minute amounts of tissue will suffice; in most circumstances, no special preparation of the tissue is necessary. PCR thus makes recombinant DNA techniques accessible to clinical laboratories. This single advance has produced a quantum increase in the use of direct gene analysis for diagnosis of human diseases.

Polymerase chain reaction is based on the prerequisites for copying an existing DNA strand by DNA polymerase: an existing denatured strand of DNA to be used as the template, and a primer. Primers are short oligonucleotides, 12100 bases in length, having a base sequence complementary to the desired region of the existing DNA strand. The enzyme requires the primer in order to know where to begin copying. If the base sequence of the DNA of the gene under study is known, two synthetic oligonucleotides complementary to sequences flanking the region of interest can be prepared (see Chap. 160). If these are the only oligonucleotides present in the reaction mixture, then the DNA polymerase can only copy daughter strands of DNA downstream from those oligonucleotides. Recall that DNA is double stranded, that the strands are held together by the rules of Watson-Crick base pairing, and that they are aligned in antiparallel fashion. This implies that the effect of incorporation of both oligonucleotides into the reaction mix will be to synthesize two daughter strands of DNA, one originating upstream of the gene and the other originating downstream. The net effect is synthesis of only the DNA between the two primers, thus doubling only the DNA containing the region of interest. If the DNA is now heat denatured, allowing hybridization of the daughter strands to the primers, and the polymerization is repeated, then the region of DNA through the gene of interest is doubled again. Thus, two cycles of denaturation, annealing, and elongation result in a selective quadrupling of the gene of interest.

The cycle can be repeated 3050 times, resulting in a selective and geometric amplification of the sequence of interest to the order of 2^{30} 2^{50} times. The result is a millionfold or higher selective amplification of the gene of interest, yielding microgram quantities of that DNA sequence.

The PCR reaction achieved practical utility when DNA polymerases from thermophilic bacteria were discovered, when synthetic oligonucleotides of any desired sequence could be produced efficiently, reproducibly, and cheaply by automated instrumentation, and when DNA thermocycling machines were developed. Thermophilic bacteria live in hot springs and other exceedingly warm environments. Thermophilic DNA polymerases can tolerate 100°C incubations without substantial loss of activity. Their advantage is that they retain activity in a reaction mix that is repeatedly heated to the high temperature needed to denature the DNA strands into the single-stranded form. Microprocessor-driven DNA thermocycler machines can be programmed to increase temperatures to 95100°C (denaturation), to cool the mix to 50°C rapidly (a temperature that favors oligonucleotide annealing), and then to raise the temperature to 7075°C (the temperature for optimal activity of the thermophilic DNA polymerases). The rapidity of these changes (3060 seconds for each phase of the cycle) allows the laboratorian to include the test specimen, the thermophilic polymerase, the primers, and the chemical components (e.g., nucleotide subunits) of the reaction mix in a single tube, place it in the thermocycler, and conduct many cycles of denaturation, annealing, and polymerization in a completely automated fashion. The gene of interest can thus be amplified over one millionfold in a matter of a few hours. The DNA product is readily identified and isolated by routine agarose gel electrophoresis. The DNA can then be analyzed by restriction endonuclease, digestion, hybridization to specific probes, sequencing, further amplification by cloning, and so forth.

Producing and Isolating Recombinant DNA Molecules

Most recombinant DNA methods require defined, purified DNA molecules encompassing all or part of the gene of interest. One basic algorithm for isolating genes by molecular cloning is presented (Fig. 1-8). Genomic DNA is isolated from nuclei and digested with restriction endonucleases to generate overhanging sticky ends. (Alternatively, a restricted subset of DNA sequences that represents the genes expressed in a given cell can be created by first isolating messenger RNA, converting it into cDNA by incubation with reverse transcriptase, and then converting it to double-stranded cDNA using other enzymes.) The plasmid or bacteriophage DNA molecule to be used as a vector to carry the DNA into microbes is cut with the same restriction enzyme. The DNA molecules from the two sources thus have complementary sticky ends. They are annealed together by means of their sticky ends under conditions of a slight excess of the microbial DNA vector. This procedure ensures that most of the vector molecules ligate to only one molecule from the mammalian source. The recombinant plasmids are then sealed with DNA ligase. Each recombinant molecule is thus an

Figure 1-8 Isolation of a genomic gene copy by molecular cloning. Illustrated here is a prototypical experiment whereby the chromosomal or genomic copy of the -globin gene (shown in red) can be isolated by molecular cloning, using globin cDNA as a molecular hybridization probe. A suitable bacteriophage- cloning vector is digested with *Hind* III, which in the example cuts the phage only once, leaving two arms with sticky ends. Genomic DNA is similarly digested. A vast array of fragments (only a few of which are shown) results, their sizes depending on the location of the *Hind* III recognition sites. Ligation of the two digests to each other results in an array of bacteriophage DNA molecules, each containing a single fragment from the human genomic digest. The genomes are packaged into bacteriophage coats so that they become infectious viruses. These infect a culture of bacterial cells; each bacteriophage yields a plaque on a Petri plate as the result of subsequent rounds of reinfection and lysis of host cells on the plate. A replica of the plate is made by laying a filter over the plate, allowing it to absorb the colonies in situ, and hybridizing the filter to the radioactive globin cDNA probe. Only phage carrying the globin gene in the inserted genomic fragment will yield a positive autoradiography signal. This plaque is then isolated and used as starting material for isolation of the phage and the phage DNA. The globin gene can then readily be isolated by routine methods of DNA chemistry. The result is a highly purified representation of the gene and its surrounding sequences as it was configured in the original human genome.

infectious DNA species carrying a single DNA fragment from the mammalian source as a passenger.

The plasmids are then used to infect an excess of host bacteria; the excess cell number ensures that each bacterium, on the average, acquires only a single recombinant DNA molecule. The host bacteria chosen lack some phenotypic property conferred by the infecting molecule, such as antibiotic resistance. The infected cells are then plated onto antibiotic-containing Petri plates at a density allowing detection of individual colonies or bacteriophage plaques. Each colony or phage plaque represents the progeny of a single cell or bacteriophage and is thus a clone of a single cell or phage carrying a single DNA fragment from the mammalian source. Therefore, that DNA fragment, or gene, has been physically and genetically isolated in the colony or plaque, separated from all other mammalian DNA

fragments by the cloning process.

What remains is the need to identify the DNA fragment representing the specific gene targeted for purification. Those cells or phages carrying the DNA sequence of interest must be identified within the array of plaques or colonies (called a recombinant DNA library). Numerous stratagems have been devised for screening these libraries for the presence of the occasional clone bearing the gene of interest. The approaches that are suitable depend on what information is available about the particular gene or its protein product. In some cases, it is possible to use molecular hybridization to a DNA probe synthesized to contain a sequence encoding a known partial amino acid sequence (determined by sequencing a peptide or protein fragment of interest). In other cases, the microbial vector has the capacity to express part or all of the protein encoded by the DNA clone. The library can then be screened with antibodies raised against the protein of interest by conventional means.

Once the colony or bacteriophage plaque containing the recombinant molecule of interest has been identified, that colony or plaque can be purified free of the remainder of the library and amplified by growth in bacterial culture. In this manner, substantial quantities of recombinant DNA molecules can be produced from the cloned host cell. With respect to other DNA molecules derived from the original tissue source, the cloned gene will be pure. The purified gene can then be used as a hybridization probe, as the substrate for obtaining its DNA sequence, or as a template for controlled expression and production of its mRNA and protein products.

DNA and RNA Blotting

There are many ways that a cloned DNA sequence can be exploited to characterize the behavior of normal or pathologic genes relevant to hematology. Blotting methods deserve special mention because of their widespread use in clinical and experimental hematology. A cloned DNA fragment can be easily purified and tagged with a radioactive or nonradioactive label. The fragment provides a pure and highly specific molecular hybridization probe for the detection of complementary DNA or RNA

Figure 1-9 Southern gene blotting. Detection of a genomic gene (red) that resides on a 14-kb Bam HI fragment. To identify the presence of a gene in the genome and the size of the restriction fragment on which it resides, genomic DNA is digested with a restriction enzyme, and the fragments are separated by agarose gel electrophoresis. Human genomes contain from several hundred thousand to 1 million sites for any particular restriction enzyme, which results in a vast array of fragments and creates a blur or streak on the gel; one fragment cannot be distinguished from another readily. If the DNA in the gel is transferred to nitrocellulose by capillary blotting, however, it can be further analyzed by molecular hybridization to a radioactive cDNA probe for the gene. Only the band containing the gene yields a positive autoradiography signal, as shown. If a disease state were to result in loss of the gene, alteration of its structure, or mutation (altering recognition sites for one or more restriction enzymes), the banding pattern would be changed.

molecules in any specimen of DNA or RNA. One set of assays that has proved particularly useful involves Southern gene blotting, named after Dr. E. Southern, who invented the method. Southern blotting allows detection of a specific gene, or region in or near a gene, in a DNA preparation (Fig. 1-9). The DNA is isolated and digested with one or more restriction endonucleases, and the resulting fragments are separated according to their molecular size by electrophoresis on agarose gels. Under conditions routinely used, the largest fragments migrate most slowly and the smallest fragments most rapidly. Unfortunately, the size of the fragment containing the gene of interest often is not known. Moreover, a human genomic DNA preparation digested with most restriction enzymes yields many hundreds or thousands of fragments, producing a blur or streak on the gel. A final impediment to detection of the individual gene in this massive array of fragments is the unsuitability of agarose and acrylamide for molecular hybridization conditions.

Gene blotting circumvents these problems. The agarose gel is placed on top of a pad or sponge saturated in a high-salt buffer. A sheet of nitrocellulose, nylon, or a similar permeable membrane is laid on top of the gel. Large numbers of dry towels are laid on top of the filter, and a weight is placed on top of the entire stack to ensure air- and watertight contact among all layers. By capillary action, fluid is drawn from the saturated sponge or pad through the porous gel and the nitrocellulose membrane and into the dry pad of towels. The DNA is also drawn out of the gel, but, if care is taken to denature the DNA to the single-stranded form before initiating the blotting, it will not pass through the semipermeable membrane. Single-stranded DNA and RNA molecules stick noncovalently but tightly to the membrane. In this fashion, the membrane becomes a replica or blot of the gel. These membranes are much more amenable to subsequent manipulation and can be used in molecular hybridization assays.

After the blotting procedure is complete, the membrane is incubated in a hybridization buffer containing the radioactively labeled probe. The probe hybridizes only to the gene of interest and renders radioactive only one or a few bands containing complementary sequences. After appropriate washing and drying, the bands can be seen by autoradiography.

Digestion of a DNA preparation with several different restriction enzymes allows a restriction endonuclease map of a gene in the human genome to be constructed. Southern blotting has thus become a standard way of characterizing the configuration of genes in the genome.

Northern blotting represents an analogous blotting procedure used to detect RNA. RNA cannot be digested with restriction enzymes (they cut only DNA); rather, the RNA molecules can be run intact (mRNAs are 0.512 kb in length) through the gel, blotted onto membranes, and probed with a DNA probe. In this fashion, the laboratorian can detect the presence, absence, molecular size, number of individual species, and so forth of a particular mRNA species. Western blotting is an analogous procedure used to detect proteins with antibody probes.

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USE OF TRANSGENIC AND KNOCKOUT MICE TO DEFINE GENE FUNCTION

Recombinant DNA technology has resulted in the identification of many disease-related genes. To advance the understanding of the disease related to a previously unknown gene, the function of the protein encoded by that gene must be verified or identified, and the way changes in the gene's expression influence the disease phenotype must be characterized. Analysis of the role of these genes and their encoded proteins has been made possible by the development of recombinant DNA technology that allows the production of mice that are genetically altered at the cloned locus. Mice can be produced that express an exogenous gene and thereby provide an in vivo model of its function. Linearized DNA is injected into a fertilized mouse oocyte pronucleus and reimplanted in a pseudopregnant mouse. The resultant *transgenic mice* can then be analyzed for the phenotype induced by the injected transgene. Placing the gene under the control of

a strong promoter that stimulates expression of the exogenous gene in all tissues allows the assessment of the effect of widespread overexpression of the gene. Alternatively, placing the gene under the control of a promoter that can function only in certain tissues (a tissue-specific promoter) elucidates the function of that gene in a particular tissue or cell type. A third approach is to study control elements of the gene by testing their capacity to drive expression of a marker gene that can be detected by chemical, immunologic, or functional means. For example, the promoter region of a gene of interest can be joined to the cDNA encoding green jellyfish protein, and activity of the gene assessed in various tissues of the resultant transgenic mouse by fluorescence microscopy. Use of such a reporter gene demonstrates the normal distribution and timing of expression of the gene from which the promoter elements are derived.

Transgenic mice contain exogenous genes that insert randomly into the genome of the recipient. Expression can thus depend as much on the location of the insertion as it does on the properties of the injected DNA. In contrast, any defined genetic locus can be specifically altered by targeted recombination between the locus and a plasmid carrying an altered version of that gene. If a plasmid contains that altered gene with enough flanking DNA identical to that of the normal gene locus, homologous recombination can occur, and the altered gene in the plasmid will replace the gene in the recipient cell. Using a mutation that inactivates the gene allows the production of a null mutation, in which the function of that gene is completely lost. To induce such a mutation, the plasmid is introduced into an embryonic stem cell, and the rare cells that undergo homologous recombination selected. The knockout embryonic stem cell is then introduced into the blastocyst of a developing embryo. The resultant animals are chimeric; only a fraction of the cells in the animal contain the targeted gene. If the new gene is introduced into some of the germline cells of the chimeric mouse, then some of the offspring of that mouse will carry the mutation as a gene in all of their cells. These heterozygous mice can be further bred to produce mice homozygous for the null allele. Such knockout mice reveal the function of the targeted gene by the phenotype induced by its absence. Genetically altered mice have been essential for discerning the biologic and pathologic roles of large numbers of genes implicated in the pathogenesis of human disease.

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DNA-BASED THERAPIES

Gene Therapy

The application of gene therapy to genetic hematologic disorders is an appealing idea. In most cases, this would involve isolating hematopoietic stem cells from patients with diseases with defined genetic lesions, inserting normal genes into those cells, and reintroducing the genetically engineered stem cells back into the patient. Candidate diseases for such therapy include sickle cell disease, thalassemia, hemophilia, and adenosine deaminase (ADA)-deficient severe combined immunodeficiency. The technology for separating hematopoietic stem cells and for performing gene transfer into those cells has advanced rapidly, and clinical trials have begun to test the applicability of these techniques. However, despite the fact that gene therapy has progressed to the enrollment of patients in clinical protocols, major technical problems still need to be solved, and there are no proven therapeutic successes from gene therapy. However, progress in this field continues rapidly. The scientific basis for gene therapy and the clinical issues surrounding this approach are discussed in Chapter 95 .

Antisense Therapy

The recognition that abnormal expression of oncogenes plays a role in malignancy has stimulated attempts to suppress oncogene expression to reverse the neoplastic phenotype. One way of blocking mRNA expression is with antisense oligonucleotides. These are single-stranded DNA sequences, 1720 bases long, having a sequence complementary to the transcription or translation start of the mRNA. These relatively small molecules freely enter the cell and complex to the mRNA by their complementary DNA sequence. This often results in a decrease in gene expression. The binding of the oligonucleotide may directly block translation, and clearly enhances the rate of mRNA degradation. This technique has been shown to be promising in suppressing expression of *bcr-abl* and to suppress cell growth in chronic myelogenous leukemia. The technique is being tried as a therapeutic modality for the purging of tumor cells before autologous transplantation in patients with chronic myelogenous leukemia.

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SUMMARY

The elegance of recombinant DNA technology resides in the capacity it confers on investigators to examine each gene as a discrete physical entity that can be purified, reduced to its basic building blocks for decoding of its primary structure, analyzed for its patterns of expression, and perturbed by alterations in sequence or molecular environment so that the effects of changes in each region of the gene can be assessed. Purified genes can be deliberately modified or mutated to create novel genes not available in nature. These provide the potential to generate useful new biologic entities, such as modified live virus or purified peptide vaccines, modified proteins customized for specific therapeutic purposes, and altered combinations of regulatory and structural genes that allow for the assumption of new functions by specific gene systems.

Purified genes facilitate the study of gene regulation in many ways. First, a cloned gene provides characterized DNA probes for molecular hybridization assays. Second, cloned genes provide the homogenous DNA moieties needed to determine the exact nucleotide sequence. Sequencing techniques have become so reliable and efficient that it is often easier to clone the gene encoding a protein of interest and determine its DNA sequence than it is to purify the protein and determine its amino acid sequence. The DNA sequence predicts exactly the amino acid sequence of its protein product. By comparing normal sequences with the sequences of alleles cloned from patients known to be abnormal, such as the globin genes in the thalassemia or sickle cell syndromes, the normal and pathologic anatomy of genes critical to major hematologic diseases can be established. In this manner, it has been possible to identify many mutations responsible for various forms of thalassemia, hemophilia, thrombasthenia, red cell enzymopathies, porphyrias, and so forth. Similarly, single base changes have been shown to be the difference between normally functioning proto-oncogenes and their cancer-promoting oncogene derivatives.

Third, cloned genes can be manipulated for studies of gene expression. Many vectors allowing efficient transfer of genes into eukaryotic cells have been perfected. Gene transfer technologies allow the gene to be placed into the desired cellular environment and the expression of that gene or the behavior of its products to be analyzed. These surrogate or reverse genetics systems allow analysis of the normal physiology of expression of a particular gene, as well as the pathophysiology of abnormal gene expression resulting from mutations.

Fourth, cloned genes enhance study of their protein products. By expressing fragments of the gene in microorganisms or eukaryotic cells, customized regions of a protein can be produced for use as an immunogen, thereby allowing preparation of a variety of useful and powerful antibody probes. Alternatively, synthetic peptides deduced from the DNA sequence can be prepared as the immunogen. Controlled production of large

amounts of the protein also allows direct analysis of specific functions attributable to regions in that protein.

Finally, all of the aforementioned techniques can be extended by mutating the gene and examining the effects of those mutations on the expression of or the properties of the encoded mRNAs and proteins. By combining portions of one gene with another (chimeric genes), or abutting structural regions of one gene with regulatory sequences of another, the researcher can investigate in previously inconceivable ways the complexities of gene regulation. These activist approaches to modifying gene structure or expression create the opportunity to generate new RNA and protein products whose applications are limited only by the collective imagination of the investigators.

The most important impact of the genetic approach to the analysis of biologic phenomena is the most indirect. Diligent and repeated application of the methods outlined in this chapter to the study of many genes from diverse groups of organisms is beginning to reveal the basic strategies used by nature for the regulation of cell and tissue behavior. As our knowledge of these rules of regulation grows, our ability to understand, detect, and correct pathologic phenomena will increase substantially.

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Chapter 2 - Protein Synthesis and Intracellular Sorting

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INTRODUCTION

The previous chapter dealt with early events in gene expression, including transcription of messenger RNA (mRNA) molecules and their translation into polypeptides. This chapter considers what happens to polypeptides after synthesis, detailing their folding, their assembly into oligomeric complexes, and their delivery to the appropriate intracellular destination. The accuracy of the delivery process is essential, especially in eukaryotic cells, which are subdivided into many membrane-bound compartments termed organelles. Each organelle serves a particular purpose. For example, oxidative phosphorylation, the process responsible for generating the majority of cellular energy (adenosine triphosphate [ATP]), occurs on mitochondrial inner membranes. The capacity of any organelle to fulfill its role in cellular physiology depends in turn on that organelle possessing a characteristic set of protein components. An elaborate targeting process ensures that the requisite set of protein components is incorporated into each organelle. Understanding the machinations that underlie protein folding, assembly, and targeting is important to the study of hematology as it provides the necessary foundation for understanding how malfunctions in these processes cause blood disorders.

Background

Proteins are polymers created by the templated joining of amino acid monomers (see Table 2-1 for single-letter designations), each of which has unique properties. The overall character of a protein is influenced both by its amino acid content and by the order in which those amino acids were incorporated. Both are dictated by the mRNA template. The mRNA is read by tRNA molecules, which link particular amino acids to the corresponding triplet of nucleotides found in the mRNA. The whole process is orchestrated by a ribonucleoprotein complex called a ribosome that contains over 80 distinct proteins and four different RNA molecules. These proteins and RNAs are

TABLE 2-1 -- Examples of Import Signals

Post-translational uptake	
Nucleus	P**KKKRK**V (SV40 large T antigen)
Mitochondria	MLGI**R**SSV**K**TCF**K**PMSLTS**KR**L (iron-sulfur protein of complex III)
Peroxisomes	S**K**L (C-terminus of firefly luciferase)
Co-translational uptake	
ER	MMSFVSLLLVGILFWAT***E***A***E***QLT**K**C***E***VFQ (bovine lactalbumin)

Abbreviations of amino acids: A, alanine; C, cysteine; ***D, aspartic acid; E, glutamic acid;*** F, phenylalanine; G, glycine; **H, histidine**; I, isoleucine; **K, lysine;** L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; **R, arginine;** S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Note: Acidic residues (negatively charged) are in ***bold italic*** type; basic residues (positively charged) are in **bold** type.

TRANSLATIONAL REGULATION

Because of structural peculiarities, not all mRNA molecules are translated with equal efficiency. Moreover, the efficiency of translation can be modulated by cellular factors that bind mRNA at specific sites. A well-characterized example of this mode of regulation is the control of iron metabolism. When cytosolic iron levels are low, the regulatory protein aconitase binds to mRNA molecules encoding ferritin (cytosolic iron storage protein) and represses ferritin synthesis. Also, under low iron conditions, aconitase binds to the mRNA of transferrin receptor (Tf-R; surface receptor responsible for iron uptake) and stabilizes it, thus increasing Tf-R synthesis. When cytosolic iron levels are increased, iron complexes with aconitase and prevents its binding to both of the mRNA species, which promotes cytosolic iron storage by allowing ferritin synthesis and decreasing Tf-R mRNA levels. (See Chapter 26.)

incorporated into two macromolecular complexes, one of 60S (S refers to the sedimentation rate in an ultracentrifuge and is a measure of size) and the other of 40S. Translation begins when the 40S ribosomal subunit binds to the 5 cap (methyl guanine) of the mRNA molecule and then scans toward the 3 end until the translation start site is encountered (usually the first AUG). At that time the 60S subunit assembles with the 40S subunit to produce an 80S ribosome. Transcription RNA molecules ferry amino acids to the ribosome, upon which they are sewn together in sequence as the ribosome moves toward the 3 end of the mRNA template. Translation is terminated when a stop codon (UAA, UAG, or UGA) is encountered, which releases the nascent polypeptide from the ribosome.

Although the synthesis of all cellular proteins begins on free ribosomes in the cytosol, the transport of nascent polypeptides to their eventual destination requires navigation through several sorting branch points (Fig. 2-1). These sorting events are governed by short linear sequences of particular amino acids (sorting motifs) and their cognate receptors. The first sorting decision encountered occurs after 30 amino acids of the nascent polypeptide have been extruded from the ribosome. If

the nascent polypeptide lacks a signal sequence, most often found near the protein's amino (N)-terminus (Table 2-1),

Figure 2-1 Flow diagram describing intracellular protein transport. Synthesis of all cellular proteins begins in the cytosol, from which they can be diverted into the indicated compartments. The sorting events responsible for directing the nascent protein to its intracellular location are governed by amino acid sequence motifs recognized by cognate receptors.

then translation of the polypeptide is completed in the cytosol. After synthesis is complete, the protein can either stay in the cytosol or be post-translationally incorporated into one of the indicated organelles (Fig. 2-1). Alternatively, if the protein does contain an N-terminal signal sequence, the polyribosome complex is docked on the membrane of the endoplasmic reticulum (ER) and the emerging polypeptide is extruded co-translationally (during synthesis) into the ER lumen. The ER is a tubular network of membranes from which all of the more distal membrane structures of the secretory apparatus (ER, Golgi complex, endosomes, lysosomes, and plasma membrane) are derived. Protein components synthesized in the ER are delivered to those compartments in transport vesicles.

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PROTEIN FOLDING IN THE CYTOSOL

In general, proteins are not functional in the extended linear conformation in which they emerge from the ribosome. Rather, the polypeptide strand must be folded back on itself into a conformation that is dictated by the primary amino acid sequence. Although this process can occur spontaneously in a test tube at very low protein concentrations, it occurs very inefficiently and requires an extended time period. In the very densely packed cytosol of eukaryotic cells (protein concentrations of over 100 mg/mL) emerging polypeptides require assistance to fold, and that assistance comes in the form of a specialized set of proteins called molecular chaperones. The population of chaperones that assists folding in the cytosol is distinct from the population operating within the ER or mitochondria. Most molecular chaperones are members of the heat shock protein (Hsp) family, so called because their expression is induced by briefly heating cells to 42°C. Chaperones attach to nascent polypeptide strands and start the folding process even before translation is completed. Indeed, as the nascent polypeptide is being extruded from the ribosome, it is bound by chaperones that recognize short sequence motifs containing hydrophobic amino acids. By undergoing cycles of binding and release (linked to ATP hydrolysis), these chaperones help the nascent polypeptide find its native conformation, one aspect of which is the tucking away of hydrophobic sequence motifs in the protein interior so that they no longer contact the hydrophilic environment of the cytosol. Some properly folded protein monomers are assembled with other proteins to form multisubunit complexes. This is also facilitated by chaperones. Proteins can contain mutations that prevent them from folding properly. Such proteins are marked for destruction by modification with a small (8,000 daltons) polypeptide appendage called ubiquitin. Ubiquitin serves as an identification marker for a cylindric multisubunit proteolysis machine called the proteasome. Ubiquitin-tagged molecules are fed into the lumen of the proteasome cylinder, where they are degraded. The end products, i.e., amino acid monomers, are then reused in the translation of new proteins.

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SORTING FROM THE CYTOSOL INTO OTHER COMPARTMENTS

Some proteins synthesized on free polyribosomes remain in the cytosol. These include enzymes involved in glycolysis, signal transduction (soluble kinases and phosphatases, etc.), and components of the cytoskeleton. However, other proteins are post-translationally (after synthesis) translocated out of the cytosol and into organelles that are not part of the secretory apparatus. These organelles include the nucleus, mitochondria, and peroxisomes (Fig. 2-2).

Nuclear Proteins

The nucleus contains the cellular genome and is bounded by two concentric membranes that form the nuclear envelope (Fig. 2-2).

Figure 2-2 Intracellular protein trafficking. From the cytosol, proteins can be imported post-translationally into mitochondria, peroxisomes, or the nucleus. Alternatively, proteins can be imported co-translationally into the ER, from which selective packaging into transport vesicles can result in their delivery to any of the other membrane-bound compartments of the secretory apparatus.

The outer membrane of the nuclear envelope is continuous with the ER and has a polypeptide composition distinct from that of the inner membrane. The inner membrane contains binding sites for a two-dimensional protein lattice, termed the nuclear lamina, that serves both as a scaffold that provides shape to the nucleus and as a link between the DNA and the nuclear envelope. The nuclear envelope is perforated with nuclear pore complexes, the number of which is a general indicator of the overall level of transcriptional activity in the nucleus. Nuclear pore complexes (NPCs) are approximately 30 times larger than a ribosome and comprise more than 100 different proteins, which are arranged into an envelope-piercing channel with fibrils extending from the cytoplasmic face and a cagelike structure extending beneath the inner membrane of the nuclear envelope. NPCs are selective gates that allow passive bidirectional movement of molecules less massive than 60,000 daltons and active bidirectional passage of selected molecules as large as several million daltons (e.g., nuclei must export newly assembled ribosomal subunits). Candidate proteins for nuclear import, such as transcription factors, DNA repair enzymes, etc., most often bear a nuclear localization signal (NLS) consisting of one or more short clusters of positively charged amino acids (lysine and arginine; Table 2-1). A protein containing an NLS is bound by a heterodimeric receptor complex (/-importins) that attaches to the cytosolic fibrils of the NPC and then is translocated into the nucleus through the pore complex. Upon arrival in the intranuclear space, the /-importins release the NLS-containing proteins in response to guanosine triphosphate (GTP) hydrolysis. The importins are then shuttled back to the cytosol, where they can facilitate another round of transport. Many aspects of the regulation of nuclear import are currently unclear, but the key to its control is thought to be a GTP-binding protein, Ran. Unlike import into mitochondria or the ER, proteins to be imported into the nucleus are not unfolded prior to entry, nor is the NLS proteolytically cleaved from the imported protein. Presumably, NLS are not proteolytically removed following nuclear import because nuclear proteins must exit the nucleus and then re-enter it each time the nucleus is dismantled and reassembled during mitosis.

Mitochondrial Proteins

Although mitochondria do contain DNA and have the capability to make protein, most mitochondrial proteins are taken up after they are synthesized in the cytosol. Like nuclei, mitochondria have two membranes: an outer one that contacts the cytosol and an inner one in which the respiratory chain resides. The space enclosed by the inner mitochondrial membrane is called the matrix. Cytosolic proteins are targeted to mitochondria by a sorting motif referred to as a matrix targeting signal (MTS) or presequence that is generally between 20 and 60 residues in length and is rich in amino acids that are positively charged or

CONDITIONAL NUCLEAR LOCALIZATION

Modulation of nuclear import is an important means of regulating the activity of nuclear proteins that are relevant to the study of hematology. In particular, nuclear factor B (NF-B) is a transcription factor that is important in alterations in gene expression induced by signaling through the antigen receptors of B and T lymphocytes, the interleukin-1 receptor on macrophages, as well as the cytokine/growth factor receptors on other cell types. NF-B has a nuclear localization signal (NLS) but remains in the cytosol because its NLS is masked by association with the inhibitor of NF-B (IB). Upon exogenous stimulation through one of the receptors mentioned above, IB is phosphorylated, resulting in release of NF-B, exposure of its NLS, and import into the nucleus. Thus, the activity of NF-B is regulated by conditionally granting it access to the nucleus, and consequently the genes whose transcription it regulates.

hydroxylated (Table 2-1). After synthesis, MTS-containing proteins (also called preproteins) are kept in an unfolded state by interacting with cytoplasmic chaperones of the heat shock family and are thought to be guided to the mitochondria by a targeting factor called mitochondrial import stimulation factor (MSF). Mitochondrial import of preproteins occurs at contact sitessites where the outer and inner membranes appear to join. Both the outer and inner membranes contain complexes of proteins that act as translocators. The translocator on the outer membrane is referred to as Tom, while that on the inner membrane is termed Tim. These translocator complexes appear to have outward-facing proteins that bind the MTS as well as membrane-buried proteins (or domains) that form a transmembrane (TM) channel. Transfer of the preprotein from cytosolic chaperones to MTS-binding components of the Tom complex is facilitated by ATP hydrolysis. After transfer, the presequence binds sequentially to sites of increasing affinity, and this moves the elongated preprotein toward the Tim complex on the inner membrane. Opening and closing of the (Tim) translocation channel is regulated by MTS binding as well as by the proton gradient that exists across the inner membrane. As the preprotein emerges on the matrix side of the inner membrane channel it is met by a mitochondrially encoded Hsp70 chaperone that is thought to provide the motive force for protein import by reversibly binding incoming proteins in response to both ATP hydrolysis and nucleotide exchange. After arrival in the matrix, the preprotein is folded by another

mitochondrial chaperone, Hsp60, after which the MTS of the preprotein is removed by a matrix peptidase. In addition to the soluble proteins that reside in the mitochondrial matrix, there are membrane proteins that reside in the inner and outer membranes as well as within the intermembrane space. There is no uniform route taken by such proteins, and discussion of the various options falls outside the scope of this chapter.

Peroxisomal Proteins

Peroxisomes, which are essential for human survival, are so named because they are the site of many oxidative reactions that generate H_2O_2. H_2O_2 is used (particularly in the liver) by the enzyme catalase to oxidize toxic substances such as ethanol. Peroxisomes are also the site where long chain fatty acids are catabolized by -oxidation. The protein components that make up peroxisomes are, like those of mitochondria, synthesized in the cytosol and then imported post-translationally. Although the details of how peroxisome protein import occurs are currently unclear, most imported proteins contain a short carboxy (C)-terminal peroxisomal targeting signal, or PTS (Table 2-1). Peroxisomal import is blocked by mutations in the gene *PEX5*, whose protein product is thought to be a PTS receptor. Defects in *PEX5* also underlie the disease termed Zellweger syndrome. Zellweger syndrome is characterized by the presence of empty peroxisomes. Affected individuals are born with brain, liver, and kidney abnormalities that cause death shortly after birth.

PROTEIN TRAFFICKING WITHIN THE SECRETORY APPARATUS

Nascent proteins intended for residence within the cellular transit system termed the secretory apparatus are first diverted to the on ramp of the secretory apparatus, the ER. From the ER, the proteins can be exported in vesicles to more distal organelles (Golgi complex, plasma membrane, etc.; Fig. 2-2). The vesicles undergo successive rounds of budding and fusion, and by selective incorporation of protein cargo into those vesicles (dictated by sorting motifs), cargo proteins are delivered to their intended destination. Each of the compartments of the secretory apparatus comprises a unique collection of resident proteins and lipids. A major question in cell biology today is how the unique composition of these compartments can be maintained while at the same time allowing unimpeded passage of other, nonresident proteins.

Co-translational Import and Processing of Proteins in the ER

Overview of ER Structure and Function

The ER is an extensive membrane network that serves as the cellular site for synthesis of the massive amounts of lipid and protein used to construct the membranes of most cellular organelles. The ER is divided into three different domains according to morphologic characteristics visible with the electron microscope. Those domains are the rough ER, smooth ER, and the transitional elements. The rough ER is so called because it is studded with bound ribosomes that are actively synthesizing proteins. Smooth ER lacks ribosomes and is not very abundant in most cells (except hepatocytes). Smooth ER is thought to be the domain where lipid biosynthesis occurs and where cytochrome P-450 family proteins detoxify compounds such as phenobarbital. Finally, transitional elements are a tubulovesicular network of smooth membranes also called the intermediate compartment (IC) because it lies between the ER and Golgi complex. Protein mixtures leaving the ER are sorted in the IC, ensuring that escaped ER proteins are retrieved while allowing continued forward progress of other proteins.

Co-translational Import into the ER

Incorporation of proteins into the ER occurs co-translationally, and this distinguishes import into the ER from that into any other organelle (Fig. 2-3). Nascent secretory proteins are earmarked for import early during their synthesis on cytosolic polyribosomes by the presence of an N-terminal signal sequence (Table 2-1). Immediately after its extrusion from the ribosome, the signal sequence is bound by a ribonucleoprotein complex termed the signal recognition particle, or SRP. The bound SRP particle targets the polysome (synthesizing the secretory protein) to the ER membrane by interacting with a specific SRP receptor (SRP-R) or docking protein. Both the SRP and its receptor can bind GTP, which is a critical regulator of SRP-SRP-R interaction. When the SRP polysome complex contacts the SRP-R on the ER membrane, GTP hydrolysis is thought to

Figure 2-3 Co-translational translocation into the ER. During synthesis of nascent secretory proteins in the cytosol, an N-terminal signal sequence is extruded from the ribosome. The signal sequence is bound by the signal recognition particle (SRP), which delivers the polysome complex to the translocation machinery in the ER membrane. Ongoing translation then allows the protein to enter the ER through an aqueous channel, where it is met and folded by molecular chaperones such as the immunoglobulin heavy chain binding protein, BiP.

induce a conformational change that causes release of SRP into the cytosol, where it can participate in subsequent rounds of targeting. The timing of GTP hydrolysis is such that it occurs only after the ribosome has been productively engaged with the translocation apparatus. The translocation apparatus comprises multiple proteins, of which Sec61p (so named for the yeast mutant from which it was cloned) is a major component. Once the ribosome is firmly attached to the translocation complex, ongoing protein synthesis is thought to provide some of the motive force involved in translocating the nascent secretory protein (in an unfolded state) into the ER lumen. The growing polypeptide chain passes into the ER lumen at an approximate rate of 45 amino acids per second through a dynamic aqueous channel, which is open during passage of the unfolded protein and then closes when synthesis is completed. During passage of the nascent protein through the pore, the signal sequence is cleaved off by a signal peptidase associated with the luminal side of the translocator. After removal of the signal sequence, soluble proteins are simply released into the ER lumen; however, establishing the correct orientation (topology) of integral membrane ER proteins is somewhat more complicated and involves motifs called stop transfer sequences. Stop transfer sequences contain a hydrophobic core followed by positively charged amino acids that are thought to cause the protein to be discharged from the translocator, thus embedding it in the ER membrane.

Co-translational Addition of N-Linked Glycans

During translocation of nascent proteins into the ER, proteins that bear the motif N-X-S/T (where X is any amino acid except P) have appended to them carbohydrate moieties termed N-linked glycans. N-linked oligosaccharides are so called because they are linked to the asparagine (N) residue of the motif. These large hydrophilic sugar complexes are thought to be important in maintaining protein solubility by preventing formation of nonproductive aggregates. The N-linked glycan is constructed as a lipid-linked precursor containing mannose, glucose, and N-acetylglucosamine molecules (Fig. 2-4), and is then transferred to nascent polypeptides as a preformed oligosaccharide. The transfer is facilitated by an oligosaccharide transferase complex consisting of ribophorins I and II, and ost48. After addition, the N-linked glycans begin to be processed in the ER, and this continues during their movement through subsequent compartments (Fig. 2-4 ; discussed in detail following). The processing of N-linked glycans serves many physiologically relevant purposes. Indeed, the trimming of terminal glucose residues in the ER can modulate interaction between nascent glycoproteins and chaperones.

Folding in the ER Lumen

Even as nascent polypeptides are arriving in the lumen of the ER, their folding is initiated by chaperones like the Hsp70 family member, immunoglobulin-binding protein (BiP), which is associated with the luminal face of the translocation apparatus. Along with BiP, many other chaperones are active in folding incoming polypeptides, including a set of calcium-binding animal lectins, calnexin and calreticulin. These different classes of chaperones are thought to act in sequence in chaperone relays to achieve complete protein folding and assembly. Some aspects of protein folding occur only in the unique environment of the ER lumen. Indeed, the ER lumen is an oxidizing environment (the cytosol is reducing) in which the sulfur-containing amino acid cysteine can be linked through a disulfide bond to another cysteine residue. These bonds can form either within a given protein or between protein subunits of a receptor complex. This reaction is facilitated by the enzyme protein disulfide isomerase and is a critical reaction for proper folding of proteins of the immunoglobulin (Ig) domain superfamily. For example, each fully folded and assembled IgM monomer requires the formation of at least 16 disulfide bonds. Folding of nascent proteins does not always go to completion, particularly for proteins bearing mutations, such as the mutant forms of the cystic fibrosis TM conductance regulator found in cystic fibrosis patients. Consequently, the ER must have a disposal system to eliminate such proteins. For many years it was thought that such proteins were proteolytically degraded in the ER lumen. However, it has

been determined that mutant proteins are not degraded in the ER but instead are dislocated back into the cytosol, where they are destroyed via a ubiquitin-dependent degradation pathway. Because the ER contains both mutant proteins that cannot fold properly and normal proteins that are in the process of folding, there must be some means whereby the two are distinguished; however, it is unclear how this is accomplished. Interestingly, the ER seems able to monitor the number of unfolded proteins it contains and, when that number exceeds a certain threshold, to trigger a transcriptional increase in chaperone expression. This unfolded protein signal is transduced across the ER membrane to the nucleus by an ER membrane-bound kinase called Ire1p. This unfolded protein response presumably exists to match the magnitude of the ER's chaperone activity with its workload.

Figure 2-4 Processing of N-linked glycans during trafficking. During import of nascent proteins into the lumen of the ER, their asparagine (N) residues are modified by the addition of oligosaccharides. These N-linked oligosaccharides are then processed as indicated during passage of the protein through the secretory apparatus. Prior to exit from the ER, the triplet of terminal glucose residues is removed and mannose trimming begins. Mannose trimming continues during movement through the cis and medial cisternae of the Golgi apparatus. In addition, N-acetylglucosamine residues are added that confer resistance to cleavage by endoglycosidase H (endo H), an enzyme used as a diagnostic tool to determine if a nascent glycoprotein has traversed the medial Golgi compartment. Subsequently, galactose and terminal sialic acid residues are added in the trans-Golgi/TGN. Sensitivity to neuraminidase digestion (which removes sialic acid) can also be used as a diagnostic tool to indicate that a glycoprotein has arrived in the trans-Golgi/TGN.

Achieving Transport Competence

Many of the proteins that are translocated into the ER lumen or inserted into its membrane are only temporary residents intended for a more distal compartment in the secretory pathway. How does the ER know when the temporary residents are ready to leave, and how are these proteins distinguished from those intended to remain in the ER? For many years it was thought that to exit the ER, proteins had to have motifs directing them to do so; currently, however, the consensus is that there is no absolute requirement for such exit signals; rather, proteins must achieve transport competence. Transport competence means simply that the protein must be fully folded before exit can occur. Although the molecular definition of transport competence is currently unclear, in many cases it correlates with the absence

of persistent chaperone association. Generally, chaperone association is transient with normal proteins, but that association persists with mutant polypeptides. This is presumably because the amino acid sequence of the mutant polypeptide cannot assume a conformation that allows its hydrophobic chaperone-binding sites to be hidden within the protein. Conversely, when a normal protein has folded, its chaperone recognition sequences are tucked inside the protein in question, causing release from the chaperone and allowing the protein to exit the ER. Exit from the ER occurs from tubulovesicular transitional elements, where secretory proteins are packaged into either tubules or vesicles (hereafter referred to as vesicles), which shuttle them to the Golgi complex. The packaging of proteins into vesicles is likely to involve factors in addition to release from chaperone association, as cargo proteins appear to be markedly concentrated in vesicles relative to their overall abundance within the ER. The encapsulation of cargo proteins into transport vesicles as well as the mechanics of their budding and movement will be described below. Interestingly, transport competence sometimes requires assembly of two independently folded subunits. For example, the IgM heavy chain, Ig, cannot exit the ER until it has paired with the Ig or light chain, thus ensuring that only functional IgM molecules reach the circulation. Moreover, transport competence can be conditionally defined. Before they are activated, B lymphocytes produce a secreted form of Ig (s) and a form that is membrane bound ($_m$). Although both of these assemble with Ig light chain, only $_m$ is competent to exit the ER. After stimulation by its cognate antigen, the B lymphocyte becomes activated and still produces both $_m$ and $_s$, but now only $_s$ is competent to exit the ER.

ER Retention

Given that resident ER proteins are also completely folded, why are they compartmentalized in the ER and not exported to the plasma membrane along with the secretory proteins? ER proteins localize in the ER because they possess retention signals. There are currently two consensus ER retention motifs at the C-termini of ER proteins: KDEL, for soluble ER proteins, and KKXX, for membrane-bound proteins. Curiously, however, when these ER retention motifs are grafted onto test proteins, those test proteins still acquire modifications conferred by resident Golgi enzymes. This suggests that the test proteins were permitted to exit the ER and reach the Golgi apparatus. Indeed, there is good evidence that the above ER retention signals are really retrieval signals. That is, proteins bearing retrieval signals can transiently escape from the ER into the IC/cis-Golgi, from which they are returned to the ER via the retrograde (reverse) transport pathway (Fig. 2-5). Although escaped proteins are probably most often retrieved before they move past the cis-Golgi, modification of these proteins by trans-Golgi enzymes has been observed. For the KDEL motif of luminal ER proteins, a specific retrieval receptor has been identified. However, for the KKXX motif it remains unclear whether there is a specific receptor or, alternatively, whether the retrieval motif interacts directly with the retrograde transport machinery. In support of

Figure 2-5 Retrieval of escaped ER proteins from the IC/cis-Golgi. Occasionally, a resident ER protein is packaged along with secretory proteins in anterograde vesicles leaving the ER. Upon arrival in the IC/cis-Golgi stack, protein sorting occurs. The secretory proteins are allowed to continue on to the plasma membrane, while the escaped ER proteins are bound by a retention receptor and returned to the ER.

IMPACT OF RETENTION SIGNALS ON RECEPTOR FUNCTION

In addition to their role in maintaining organelle integrity, retention/retrieval signals can facilitate assembly of multisubunit complexes. In particular, the T-cell antigen receptor (TCR) complex must be capable of both recognizing a specific antigen and transducing a signal to the cytoplasm, which can only occur if the TCR possesses all of its six subunits. The surface expression of only complete TCR complexes is facilitated by the presence of ER retention motifs on several TCR subunits. These subunits exit the ER only after assembly into a full complex because assembly serves to bury the ER retention motifs of the individual subunits, preventing their binding by putative retention receptors. In this way, partial receptor complexes that are only marginally functional are prevented from reaching the cell surface, where they might interfere with the function of complete TCR complexes.

the latter possibility, recent reports demonstrate that KKXX-containing proteins can interact with the coatomer complex, which coats certain transport vesicles. Retrograde transport, along with its role in the specific retrieval of escaped ER proteins, serves to replenish the vesicle components lost due to anterograde (forward) transport. The need for balance between the anterograde and retrograde pathways is clearly exhibited when cells are treated with the fungal metabolite Brefeldin A (BFA). BFA treatment of cells selectively blocks the anterograde pathway, causing all but the most distal cisternae of the Golgi complex to collapse into the ER (Fig. 2-5). Interestingly, most endogenous ER proteins are not modified by Golgi enzymes, which suggests that they do not escape the ER even transiently. The means whereby these proteins are sequestered in the ER is unclear, but may result from formation of oligomeric complexes.

Protein Transport Within the Golgi Complex

Subdivision of the Golgi Complex

The Golgi complex comprises a stack of flattened, membrane-bound cisternae that is highly dependent on microtubules for structural integrity. The stack of cisternae can be subdivided into three parts, referred to as *cis*, *medial*, and *trans.* Both the cis and trans faces of the Golgi complex are associated with tubulovesicular bundles of membranes. The bundle on the cis side of the Golgi stack is termed the transitional elements/IC (see above) and is the site where incoming proteins from the ER dock and are sortedthat is, where escaped ER proteins marked for retrieval by the retrograde transport pathway are separated from proteins bound for more distal compartments. Protein cargo is moved vectorially across the Golgi stack from the cis to the trans side, where a second tubulovesicular bundle is encountered. The bundle at the trans face of the Golgi stack is called the trans-Golgi network (TGN) and, like the IC, is a major site of sorting where proteins can be diverted into lysosomes or secretory granules or, alternatively, continue by default to the plasma membrane. During vectorial movement across the Golgi stack, the N-linked glycans on cargo proteins are modified by resident Golgi enzymes (Fig. 2-4). The processing events are ordered in time and separated in space because the processing enzymes have a characteristic distribution across the Golgi stack. Following entry and during movement into the medial Golgi stack, the N-linked glycans of incoming glycoproteins are trimmed by mannosidases I and II and then have N-acetylglucosamine (GlcNAc) appended to them by GlcNAc transferase. Addition of GlcNAc confers resistance to the enzyme endoglycosidase H, a widely used diagnostic test for passage of proteins through the medial Golgi. During movement from the medial to the trans-Golgi stack and TGN, the N-linked glycans are appended first with galactose by galactosyltransferase and then with sialic acid by sialyltransferase. Sialic acid is a negatively charged carbohydrate that, when expressed on the surface, helps to protect cells from mechanical damage. In addition, the loss of sialic acid from secreted glycoproteins is a means of marking aged proteins for removal from the circulation and degradation by hepatocytes.

Retention of Resident Golgi Proteins

The ordered processing of glycoproteins during their movement across the Golgi stack reflects the distinctive protein composition of each Golgi subdomain. For example, mannosidases I and II are found in the cis/medial cisternae, whereas sialyltransferase is found in the trans-Golgi stack and TGN. Consequently, there must be a subdomain-specific means of retaining resident Golgi proteins while at the same time allowing the passage of itinerant proteins. However, extensive mutational analysis of resident Golgi enzymes has failed to reveal a consensus retention motif. Sequences important for retention have been found in all domains of Golgi complex proteins (luminal, TM, and cytoplasmic). Furthermore, overexpression of certain Golgi proteins does not result in their release to the plasma membrane, which suggests that retention is not mediated by a saturable retention receptor. Based on these analyses, two hypotheses to explain the retention of Golgi proteins have been put forth. They are the bilayer thickness and the kin-recognition/oligomerization models. The bilayer thickness model proposes that Golgi complex proteins are not packaged into anterograde transport vesicles exiting the Golgi complex because their TM domains are too short to be stable within the thicker membrane bilayer found at the plasma membrane. Evidence supporting this model is as follows: (1) TM domains of Golgi proteins are on average about 5 amino acids shorter than those of plasma membrane proteins; (2) there is an increasing cholesterol gradient in the lipid bilayers from the ER to the plasma membrane, and cholesterol has been reported to increase bilayer thickness; and (3) lengthening the TM domains of certain Golgi enzymes results in their escape to the cell surface. The kin-recognition/oligomerization model proposes that proteins of a given subdomain can form large detergent-insoluble oligomers that prevent their entry into the vesicles and thus their traffic to more distal cisternae. Mechanistically, the graded distribution of oligomers across the Golgi stack would result from the selective triggering of oligomerization by the different lipid compositions in different cisternae. Evidence in support of the oligomerization model is as follows: (1) the TM domains of many of the Golgi glycosyltransferases have been implicated in retention; and (2) ER retention of one medial Golgi enzyme, GlcNAc-transferase, causes the ER retention of another medial Golgi enzyme, mannosidase II. Possibly a combination of both retention mechanisms is active in maintaining the protein composition of the Golgi complex.

Anterograde Movement of Proteins

Transport competent proteins exit the ER and enter the IC in vesicles covered with a protein coat called COPII (detailed later). COPII-coated vesicles then fuse with the membranes of the IC, releasing their cargo proteins. However, the means whereby cargo proteins move from the IC to the trans-Golgi stack remains controversial. Two explanations have been proposed. The first proposal is that anterograde transport through the Golgi apparatus occurs in transport vesicles covered with a second kind of protein coat, COPI or coatomer (described later).

COPI-coated vesicles are thought to shuttle proteins around the outer rims of Golgi cisternae. Although COPI-coated vesicles are found swarming around the rims of Golgi cisternae, they do not appear to contain anterograde cargo. The second proposal, the directed maturation or fractional distillation model, states that anterograde movement occurs because nascent Golgi cisternae, which are formed by vesicular fusion in the IC, drift forward (anterograde) like icebergs while resident Golgi proteins are selectively pinched off in vesicles and trafficked back to the cis side of the Golgi stack. The farther away from the cis side the cisternae have drifted, the more their contents have been refined by the extraction of proteins in retrograde transport vesicles. Although it is currently unclear which of these models is correct, the bulk of the experimental evidence supports the fractional distillation model.

Sorting Events at the TGN

The TGN is an extremely important site of intracellular sorting where proteins bound for lysosomes or regulated secretory vesicles are separated from those entering the constitutive transport pathway leading to the plasma membrane. The constitutive or default pathway is so called because it was previously thought that transport to the plasma membrane occurred by default (i.e., no specific structural features were required) unless the protein was actively diverted into a different compartment. Although we still lack a thorough understanding of the molecular criteria for entry of cargo proteins into constitutive secretory vesicles, the concentration of cargo proteins within those vesicles suggests that active recruitment of cargo proteins is required. Proteins not entering the default pathway can be diverted into either lysosomes or, alternatively, regulated secretory granules. In polarized epithelial cells, structures called tight junctions subdivide the plasma membrane into two domains: an apical domain, which faces the lumen or sinus, and a basolateral domain, which faces adjoining cells and underlying connective tissue. In such polarized cells, proteins in the TGN have an additional sorting option: to go to the apical or basolateral domain of the plasma membrane.

Sorting into Lysosomes

Lysosomes are membrane-bound organelles with an acidic lumen (pH 5.05.5) containing numerous hydrolytic enzymes designed to destroy proteins, carbohydrates, and lipids. The lysosomal membrane has a unique composition that includes a proton pump that maintains the low pH of this organelle and numerous heavily glycosylated proteins, which presumably protect the membrane from hydrolysis. Soluble hydrolases are marked for sorting into lysosomes by a post-translational modification acquired during transport from the ER to the cis-Golgi. Indeed, the hydrolases bear a three-dimensional signal patch that induces phosphorylation of their N-linked sugars, creating the mannose-6-phosphate (M6P) sorting signal. Upon arrival at the TGN, the modified hydrolase is bound by an M6P receptor (M6P-R), which delivers it to lysosomes. It is thought that delivery to lysosomes is not direct but instead involves passage through a late endosomal compartment (Fig. 2-2 and following) where the low pH releases the hydrolase from the M6P-R. Subsequently the hydrolase is delivered to the lysosome and the M6P-R returns to the TGN to capture another hydrolase molecule.

M6P marks hydrolases for delivery to lysosomes, but what targets the M6P-R itself? The motif that enables M6P-R to deliver cargo to lysosomes is YSKV, found in its cytosolic domain. YSKV is recognized by the machinery responsible for formation of clathrin-coated vesicles (see later), which in turn underlies proper routing of the M6PR. In addition to the soluble hydrolases that reside in the lumen of lysosomes, there is also a

LYSOSOMAL STORAGE DISEASES

Failure to accurately target lysosomal hydrolases underlies two well-known human diseases, Hurler's syndrome and I-cell disease. Hurler's syndrome is caused by a mutation in a hydrolase responsible for breakdown of glycosaminoglycans that prevents the hydrolase from acquiring the M6P modification, consequently preventing targeting to lysosomes. Similarly, in I-cell disease, undigested material accumulates in lysosomes because a mutation in the enzymes that create the M6P modification causes missorting of lysosomal hydrolases.

wide array of proteins residing in the lysosomal membrane. The targeting signals for many of these integral membrane proteins have been identified and comprise either of two consensus motifs: (1) GYXXZ, where X is any amino acid and Z can be I, F, V, L, or M (the Z position may determine the efficiency of sorting); and (2) a leucine-based motif (LL or LI). Although the motifs are well established, the exact route taken by lysosomal membrane proteins after leaving the TGN is a matter of debate. The clathrin-coated vesicles containing these hydrolases may be transported to late endosomes, as is thought to occur for the M6P-R, or, alternatively, may be transported first to the plasma membrane from which they are retrieved to lysosomes.

Sorting into Regulated Secretory Granules

Regulated secretion differs from constitutive secretion in that proteins are condensed into stored secretory granules that are released to the plasma membrane only after the cell has received an appropriate stimulus (Fig. 2-2). For example, the cross-linking of Fc-epsilon receptors on mast cells by IgE-antigen complexes induces the release of preformed histamine-containing secretory granules. The biogenesis of secretory granules is not well understood but is thought to occur in two phases. The first involves sorting proteins into immature secretory granules that bud from the TGN, and the second involves the maturation of the granules after budding has occurred. Clues to the basic mechanism(s) have been gathered by analysis of the granule proteins themselves. In general, the luminal components of granules tend to self-associate and appear to exist in both soluble and membrane-associated forms. Based on these observations, a model has been proposed. It suggests that in the TGN a fraction of the granule protein molecules associate with the inner leaflet of the TGN membrane, possibly through a receptor protein. The remainder of the granule protein population attaches to the membrane-bound form through self-association, resulting in packaging of the whole population into a vesicle. After budding, the granule proteins are concentrated (up to 200-fold in some cases) by selective removal of extraneous contents into clathrin-coated vesicles. At some point during budding or maturation, the granule proteins, which are often packaged as proforms, are proteolytically cleaved to generate the mature form(s). Mature granules are then thought to be stored in association with microtubules until the stimulation of a surface receptor triggers their exocytosis, e.g., when the TCR complex of a cytotoxic T lymphocyte encounters its antigen on the surface of a target cell. Upon conjugation with its target, the cytotoxic cell's microtubules and their associated granules are reoriented to face the target cell, after which the motor protein kinesin conveys the granules along microtubules until they fuse with the plasma membrane, releasing their hydrolytic contents on the target cell. It is unclear why the granule contents damage only the target cell, but it may be because the cytotoxic cell is protected by the granule inner membrane. After release of the granule

contents, the granule membrane components are internalized and transported back to the TGN, where the granule can be refilled with cargo proteins.

Sorting to Plasma Membrane Domains in Polarized Cells

The apical and basolateral plasma membrane domains of polarized cells (epithelium) have distinct protein compositions generated by sorting events occurring in the TGN. This routing is of obvious importance, as a violation of cellular polarity would have disastrous consequences. (Consider the effect of gut epithelium secreting digestive enzymes at the basolateral surface instead of into the gut cavity at the apical surface.) Currently, there are two models for establishing the distinct protein compositions of the apical and basolateral surfaces: the direct and indirect pathways. The direct transport model proposes that basolateral and apical proteins are packaged into distinct vesicles at the TGN and transported directly to the basolateral and apical domains, respectively. The indirect transport model proposes that all proteins go first to the basolateral surface, from which apical proteins are selectively retrieved. It is currently unclear which model is correct, and it is likely that the answer will depend on the protein in question. Sorting motifs underlying basolateral targeting are similar to those of lysosomal membrane proteins (Y or LL motifs) and are usually associated with a protein structure called a type I turn. The apical surfaces of cells are enriched for glycolipids (sphingomyelins) and proteins bearing a glycophosphatidylinositol (GPI; added in the ER) anchor. In fact, the lipid component of these molecules may underlie their targeting to apical surfaces. The glycolipids and lipoproteins are thought to self-associate to form subdomains or rafts that exclude dissimilar proteins, thereby achieving segregation from basolaterally targeted proteins; however, it is completely unclear how such rafts would be packaged into transport vesicles and trafficked to the apical surface. After the polarity of an epithelial cell is established, it can be exploited to move cargo from one side of the cell (basolateral) to the other (apical) by a process termed transcytosis. A pertinent example of transcytosis is important in the maintenance of mucosal immunity. The polymeric Ig receptor (pIgR) acquires dimeric IgA molecules at the basolateral surface of gut epithelium, which induces trafficking of the Ig molecules to the apical surface where a proteolytic cleavage event destroys the pIgR and releases its cargo into the gut. The means whereby Ig binding triggers transcytosis of the pIgR is unclear but appears to involve phosphorylation of an S residue in the pIgR cytoplasmic tail.

Endocytic Traffic

The previous sections described delivery of endogenous proteins from the intracellular site of synthesis to their intended destination. This section considers the means whereby substances are imported from the extracellular milieu by a process termed endocytosis. Endocytosis also serves to recover protein and lipid placed on the plasma membrane by ongoing secretory activity. There are two types of endocytosis: phagocytosis (cell eating) and pinocytosis (cell drinking). Defects in endocytosis can underlie human disease. In particular, the receptor for low-density lipoprotein (LDL) regulates serum cholesterol by endocytically removing LDL from the circulation and delivering it to lysosomes, where the cholesterol is catabolically released for reuse in the biosynthesis of cellular membranes. Individuals with familial hypercholesterolemia have elevated serum cholesterol levels because mutations in the LDL receptor prevent removal of LDL from the circulation.

Phagocytosis

Phagocytosis refers to the ingestion of large particles (>0.5 M) and is primarily executed by specialized cells such as macrophages and neutrophils. Phagocytosis serves not only to engulf and destroy invading bacteria, but also to clear cellular debris at wound sites and to dispose of aged erythrocytes. Estimates are that human macrophages destroy 10^{11} aged erythrocytes each day. Phagocytosis is triggered when specific receptors on the phagocytic cell contact structural triggers on the particle. These structural triggers include bound antibodies and complement components (opsins) as well as certain oligosaccharide structures. When a particle binds a cellular triggering receptor, the polymerization of actin (a cytoskeletal component) is stimulated, driving the extension of pseudopods, which surround the particle and engulf it in a vacuole. The engulfed material is destroyed when the vacuole fuses with a lysosome, exposing the contents to hydrolytic enzymes. In addition to being a means of destroying invading pathogens, phagocytosis is a means of presenting the pathogen's components to lymphocytes, thus eliciting an immune response to protect against future exposure.

Pinocytosis

Pinocytosis refers to the constitutive ingestion of fluid in small vesicles (<0.2 M), which in some cells can result in turnover of the entire plasma membrane in less than 1 hour. After invagination and budding, the vesicle is part of the endocytic compartment, a complex network of interrelated membranous vesicles and tubules that are in intimate contact with one another and in flux. The four types of endocytic vesicles are early endosomes (EE), late endosomes (LE), recycling vesicles, and lysosomes. Each will be described in the order in which it is encountered during endocytic trafficking (see Fig. 2-2).

The invagination of endocytic vesicles occurs in regions of the plasma membrane containing a coat on the cytoplasmic side called clathrin. Cargo molecules can be captured in these clathrin-coated pits either stochastically in the fluid phase or in association with a specific surface receptor. Receptor-mediated capture (endocytosis) is much more efficient, enriching cargo up to 1,000-fold in the resulting endocytic vesicle relative to its normal concentration in the extracellular fluid.

The enrichment of cargo is so extensive because the formation of clathrin-coated pits is linked by adaptors to the receptors themselves (detailed later). Receptors are recognized by the adaptor molecules via sequence motifs very similar to those involved in trafficking membrane proteins to lysosomes: (1) a YXXZ motif (Z, an amino acid with a large hydrophobic side chain) that will adopt a tight-turn configuration; and (2) a dileucine motif.

Clathrin-coated pits invaginate into coated vesicles, after which the clathrin coat is removed by a cytoplasmic ATPase of the Hsp70 family. Removal of the clathrin coat facilitates vesicle fusion with EE, a dynamic network of tubules and vesicles that is occupied by endocytic tracers after 1 to 5 minutes of incubation with the cell. The primary function of EE is sorting, distinguishing receptors that will rapidly recycle back to the plasma membrane (transferrin receptor) from those that will continue on to lysosomes and be destroyed (Ig-antigen complexes). EE are also the compartment where receptor and ligand are uncoupled. For example, the slightly acidic pH (6.36.8) of EE causes uncoupling of transferrin receptor (Tf-R) from its cargo. The vacant Tf-R molecules are moved into the tubular extensions of EE, from which they are recycled back to the plasma membrane by recycling vesicles; meanwhile, the dissociated cargo molecules accumulate in the large central cavity. Recycling vesicles appear to have a biochemical composition distinct from that of EE, which validates the idea that membrane components are specifically sorted into these vesicles; however, the basis for this discrimination is unclear.

The cargo contained in EE is then delivered into late endosomes (LE) in a microtubule-dependent manner. It is unclear whether EE mature into LE through acquisition of nascent protein

INFLUENCE OF MHC-I AND MHC-II BIOSYNTHESIS ON THE TYPE OF ANTIGEN PRESENTED

Peptide antigens are presented to T lymphocytes in association with either class I or class II major histocompatibility complex (MHC) molecules. Peptides presented by class I are derived largely from endogenously synthesized proteins (or viruses) whereas those presented by class II are derived from proteins found in the extracellular milieu. The mechanistic basis for the differences in antigens presented results from differences in the intracellular trafficking of class I and class II MHC molecules. Class I MHC molecules are held in the ER in association with molecular chaperones until they acquire cytosolic peptide that has been fed into the ER by a transmembrane transporter. After acquiring peptide, class I molecules dissociate from chaperones and transit directly to the cell surface, where they display their peptide to T lymphocytes. Like class I molecules, class II molecules are also assembled in the ER; however, they are prevented from acquiring peptide there by an associated protein called invariant chain, which plugs the peptide-binding pocket of the class molecule. In addition, invariant chain possesses sorting signals that divert class II molecules into the endosome/lysosome pathway, where proteolytic removal of invariant chain allows acquisition of peptides that were derived from degradation of endocytosed material. Thus, the respective roles of class I and class II molecules in presenting antigens to the immune system are dramatically influenced by their biosynthesis.

components or if the EE and LE are distinct compartments between which cargo is moved by vesicular intermediates. LE are defined as endocytic vesicles that label after 5 to 15 minutes of incubation with an endocytic tracer and are usually located near the Golgi apparatus or nucleus. LE have abundant internal membranes, contain lysosomal hydrolases, and are likely to be the site where degradation of endocytosed material begins. LE, sometimes called prelysosomes, are distinct from lysosomes in that they are less dense, contain M6P-R, and are not the end point where endocytosed material accumulates. Endocytosed material accumulates in the lysosome, which it probably receives by fusion with LE.

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MECHANICS AND SPECIFICITY OF VESICULAR TARGETING

The movement of protein cargo from one compartment of the secretory apparatus to another generally involves the selective packaging of cargo proteins into transport vesicles, which are themselves targeted to a particular intracellular destination. The previous sections described the motifs known to be involved in selective incorporation of cargo into vesicles. This section considers the mechanics of vesicle budding and the means whereby vesicles find and fuse with the correct target membrane.

Linking Cargo Acquisition with Vesiculation

Cargo proteins are concentrated in transport vesicles, which markedly increases the efficiency of vesicular transport. The concentration occurs because cargo selection is linked to vesicle formation. The key to this linkage is the complex of coat proteins that surrounds the vesicle exterior. The coat proteins bind to transmembrane cargo molecules, either directly or through intermediate adaptor proteins. Coat binding also provides some of the force that causes the membrane to deform into a bud, thereby linking cargo acquisition with vesiculation. There are three kinds of coats: (1) clathrin, (2) COPI or coatomer, and (3) COPII. Clathrin-coated vesicles mediate endocytosis and the diversion of proteins from the TGN to endosomes and lysosomes. COPI-coated vesicles mediate retrograde transport from the Golgi complex to the ER. COPII-coated vesicles mediate anterograde transport from the ER to the Golgi complex. At steady state, COPII is located on ER membranes, COPI on the Golgi complex and IC membranes, and clathrin on the TGN and plasma membrane.

Clathrin-Mediated Vesiculation

Clathrin molecules do not recognize cargo receptors directly, but instead do so through intermediate molecules called clathrin adaptor proteins (APs) (Fig. 2-6). APs are soluble cytoplasmic heterotetrameric complexes that bind both sorting motifs in the cytoplasmic tails of cargo receptors (the tyrosine-based and dileucine motifs described previously) and clathrin. Through the specificity of the AP (particularly the subunit), a unique set of cargo receptors is incorporated into the coated transport vesicle. Indeed, the APs that mediate coated vesicle formation at the plasma membrane (AP-2; comprising , , 2, and 2 subunits) are different from those that mediate budding of vesicles at the TGN (AP-1; comprising , , 1, and 1 subunits). How AP recruitment to the membrane is regulated is not clear; however, phosphorylation may play a role. Indeed, it is clear that phosphorylation of the Y residue in the tyrosine-based sorting motif can abrogate AP binding. Furthermore, recruitment of AP-1 proteins to the TGN requires the function of the cytosolic GTPase, ADP-ribosylating factor (ARF). After attachment of the AP complex to the membrane, the clathrin coat is recruited to the AP complex. The clathrin coat comprises a heavy chain (180,000 daltons) and three light chains (3035,000 daltons) that are arranged in a functional unit called a triskelion. Clathrin triskelions are able to self-assemble into a lattice comprising 36 triskelions in vitro in the absence of ATP. Consequently, lattice formation may provide some of the energy required for vesiculation. The final stages of vesiculation are dependent on the GTPase dynamin, which is proposed to be a pinchase. Dynamin is recruited to the clathrin lattice, forming rings around the constricted neck of the budding vesicle. GTP hydrolysis is then thought to induce a conformational change in dynamin that severs the neck, releasing the vesicle from the membrane. Soon after release of the clathrin-coated vesicle, its clathrin coat is removed by a cytosolic protein of the Hsp70 family.

COPI-Mediated Vesiculation

Unlike clathrin, the coatomer or COPI complex (which comprises -, -, -, -, -, epsilon-, and -COP) recognizes at least some cargo proteins directly. Indeed, COPI subunits are able to bind in vitro to KKXX retention motifs on ER proteins, which suggests that direct binding of the retention motifs of escaped ER proteins underlies their retrieval in COPI-coated vesicles. The regulation of COPI recruitment is not completely understood but requires the function of the GTPase ARF. ARF is a monomeric GTPase with a fatty acid tail that is found abundantly in the cytosol in a GDP-bound form. The donor membranes from which COPI vesicles bud contain guanine nucleotide exchange factors that activate ARF (by binding GTP), exposing its fatty acid tail, and inducing membrane association (Fig. 2-7). Membrane-bound ARF in turn recruits coatomer subunits that form the vesicle coat and induce budding. The COPI coat is maintained on the transport vesicle until arrival at the correct recipient membrane, at which time a GTPase in the recipient membrane triggers hydrolysis of ARF's bound GTP molecule,

Figure 2-6 Linking the packaging of cargo with vesicle budding. Surface receptors acquire ligands from the extracellular milieu; the ligands are delivered to the cell interior by endocytosis. The receptors have motifs in their cytoplasmic tails that are bound by adaptor molecules (AP). Adaptor proteins in turn are bound by clathrin coat proteins, which deform the membrane and start the process of vesiculation. After budding, the clathrin coat is removed by a cytoplasmic uncoating ATPase of the Hsp70 family. Uncoating promotes fusion of the endocytic vesicle with early endosomes.

Figure 2-7 Vesicle targeting. Donor membranes contain targeting receptors called SNAREs that are packaged into transport vesicles along with cargo proteins. As with clathrin, coatomer complexes also initiate vesicle budding; however, before coatomer can bind, the GTP-binding protein ARF must be activated and must bind to the membrane. Along with the coatomer, another GTP-binding protein, Rab, is recruited to the transport vesicle. Rab aids in the targeting reaction, possibly by improving the interaction between the SNARE on the vesicle (v-SNARE) and the SNARE on the target membrane (t-SNARE). If the v-SNARE and t-SNARE are compatible, the vesicle fuses with the target membrane, thereby delivering its cargo.

triggering ARF dislocation from the vesicle membrane and removal of the COPI coat.

COPII-Mediated Vesiculation

The structural features on cargo proteins that are required for packaging in COPII-coated vesicles have not been identified, but the process appears to require the function of a new class of adaptor proteins that are similar to the yeast protein Emp24p. Recruitment of the COPII coat complex (which comprises four proteins: Sec31p, Sec13p, Sec23p, and Sec24p) to the ER membrane requires the function of a GTPase, Sar1p. Much like ARF, in its GTP-bound form Sar1p becomes

membrane associated, providing binding sites for the COPII components.

Vesicular Targeting

Vesicular traffic proceeds from a specific donor membrane to a specific target membrane and does not go astray, suggesting that each vesicle must have an accurate targeting mechanism. Although we presently lack a detailed mechanistic explanation for the accuracy of vesicular trafficking, the SNARE hypothesis fits well with the available data. The SNARE hypothesis proposes that receptors called SNAREs on vesicles (v-SNAREs) fit precisely with a corresponding set of SNAREs on the target membranes (t-SNAREs), forming a lock-and-key interaction that is required to permit vesicle fusion (Fig. 2-7). Consequently, every fusion step in vesicular transport is controlled by compartment-specific SNAREs. Analysis of the subcellular locations of known members of the SNARE family supports this contention. Interestingly, the neurotoxic proteases in snake venom that block neurotransmission do so by cleaving SNARE proteins, which blocks synaptic vesicle fusion. It is thought that the SNAREs in donor membranes (v-SNAREs) are maintained in an inactive state. Recent evidence suggests that SNARE activity may be controlled by association with a repressor protein, which can be selectively dissociated after vesicle budding and prior to v-SNARE/t-SNARE interaction.

Prime candidates for controlling the association between SNAREs and their putative repressor proteins are the Rabs. Rabs are a family of small GTP-binding proteins (40 in mammalian cells), each of which is able to associate with a characteristic membrane when in the GTP-bound state, consequently controlling the activity of a particular subset of SNAREs. Upon arrival of a vesicle at the target membrane, the vesicle-associated Rab protein is thought to deprotect the t-SNARE by dissociating its repressor. The Rab-induced deprotection is thought to be transient because GTPase-activating proteins in the target membrane return Rab to the GDP-bound state, causing it to dissociate from the vesicle. If the SNAREs are compatible, fusion can proceed. If not, the vesicle dissociates and moves to a new target membrane to try again. Additional proteins that are required for membrane fusion are NSF (NEM-sensitive factor, a trimeric ATPase) and SNAP (soluble NSF attachment protein), which are thought to act in dissociating the SNARE complexes so they can be recycled for future targeting events. Although these proteins are necessary to prime vesicles for fusion, they do not appear to play a role in the specificity of targeting.

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SUMMARY

Substantial progress has been made in understanding the mechanistic basis for the fidelity of intracellular vesicular trafficking. Protein motifs and their cognate receptors have been identified for almost all intracellular transport steps imaginable. The challenge now is to find ways of exploiting that knowledge to intervene in the numerous disease processes resulting from errors in protein targeting.

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Chapter 3 - Protein Architecture: Relationship of Form and Function

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INTRODUCTION

The previous chapters have described how the information stored in the gene is transcribed into mRNA, how the mRNA template is translated to synthesize proteins, and how newly synthesized proteins are transported to their appropriate site of function. It is the selective expression of a constellation of proteins from the genomic firmament that provides different cell types with their distinct characters. Proteins are linear polymers made up of amino acids linked together by peptide bonds. Twenty different amino acids are incorporated into these polymers when protein is synthesized from mRNA. The features distinguishing one protein from another are determined not by the polypeptide backbone but by the different side chains of the amino acids incorporated into the protein and the sequence in which they occur. These long arrays of amino acids fold to form compact structures with a specific three-dimensional architecture. The three-dimensional structure of a protein is determined by its unique amino acid sequence. Folded proteins have the capacity to accomplish many different tasks: formation of larger structures (as in the assembly of fibrin monomers into polymers), transport of ligands (as in the binding of oxygen by hemoglobin), catalysis of chemical reactions (as in proteolytic zymogen activation during the propagation of blood coagulation), or the modulation of biologic processes (as in regulation of DNA function by DNA-binding proteins).

A mutation within the coding region of a gene can have varied consequences for synthesis of the gene product. Some mutations are silent, that is, the alteration in the gene product, usually the replacement of one single amino acid by another, has no effect on the ability of the protein to assume its normal three-dimensional structure or to perform its normal function. On the other hand, a single amino acid replacement, if it occurs at a critical position in a protein, can have profound consequences for function. The abnormal factor VIII or factor IX molecules that result in hemophilia A or B frequently arise as the result of a single point mutation (see Chaps. 107 and 108). Similarly, a single point mutation at residue 6 in the -chain of hemoglobin, a change from glutamic acid to valine, results in sickle cell disease (see Chap. 30). Introduction of a stop codon into the translated portion of a gene, insertion or deletion of codons, or addition or deletion of bases leading to a shift in reading frame can all result in production of nonfunctional proteins. The altered proteins may fail to function either because a critical functional residue has been lost or because alterations in amino acid sequence prevent the protein from achieving its proper three-dimensional structure. Proteins that are improperly folded or that are drastically altered in sequence may never escape from the site of synthesis to their proper location within the cell or into the secretory pathway.

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AMINO ACIDS: THE BUILDING BLOCKS OF PROTEINS

The structure of an amino acid is illustrated in Figure 3-1 . The central or -carbon atom bears four substituents: an amino group, a carboxyl group, a group of varying chemical structure (R, usually referred to as the side chain), and a hydrogen atom. Since, with the exception of glycine, which bears two hydrogen atoms, each of the substituents carried by the -carbon is chemically different, amino acids are chiral molecules that may exist in two different forms, the L form or the D form. These chiral forms differ from one another in the same sense as your left hand differs from your right. Proteins contain only L amino acids.

The side chains of the amino acids provide each one with its distinct chemical character. The amino acids can be divided into three general classes based on the properties of their side chains, as illustrated in Figure 3-2 . In one group are amino acids whose side chains are hydrophobic (i.e., they are most at home in a nonaqueous environment). Amino acids in this category include alanine, valine, leucine, isoleucine, proline, tryptophan, phenylalanine, and methionine. The side chains in a second group of amino acidsaspartic acid, glutamic acid, lysine, and argininеare charged at physiologic pH and thus are at home in an aqueous environment. The side chains of the third group of amino acidsserine, threonine, tyrosine, histidine, cysteine, asparagine, and glutamineare considered polar. The side chains of this group of amino acids may participate in hydrogen

Figure 3-1 Schematic representation of an amino acid, including an amino group, a carboxyl group, a side chain (R), and a hydrogen atom all linked to the -carbon (C). Each of the substituents on the -carbon is different, rendering the amino acid chiral. The enantiomeric forms of the amino acid, the D and L forms, are shown.

Figure 3-2 The 20 different amino acids that are incorporated into proteins are shown, with the side chain groups highlighted. The amino acids are grouped according to the chemical nature of the side chains. **(A)** Hydrophobic amino acids, those that prefer a nonaqueous environment. **(B)** Amino acids that bear side chains charged at physiologic pH. **(C)** Amino acids with polar side chains that can form hydrogen bonds. As described in Chapter 2 , amino acids other than these 20 can appear in proteins. These additional amino acids arise by post-translational chemical modification of 1 of the 20 amino acids incorporated into a growing polypeptide chain during translation of messenger RNA. Each amino acid has a three-letter abbreviation and is also referred to by a single-letter code.

Figure 3-3 In a hydrogen bond two electronegative atoms share a single proton. The proton is located at the normal covalent distance from the atom to which it is formally bound and at a somewhat shorter distance than the normal van der Waals contact from the other. Hydrogen bonds form in proteins between electronegative atoms in two polar side chains (shown between serine and glutamine side chains), between water and a polar amino acid side chain (shown between water and the glutamine side chain), between carbonyl oxygen atoms and amide nitrogen atoms of the protein backbone (shown between the carbonyl oxygen of a methionine residue and the amide nitrogen of a phenylalanine residue), and between polar side chains and the polypeptide backbone (not shown). Amino acid side chains are shaded.

bond formation. Hydrogen bonds may be formed between these polar side chains and water molecules, making them adaptable to the aqueous milieu. Hydrogen bonds may also form between two polar side chains (Fig. 3-3). Glycine, which

Figure 3-4 Formation of a peptide bond occurs when the carboxyl (C) group of one amino acid condenses with the amino (N) group of the next amino acid, with concomitant loss of a water molecule.

has as its side chain a second hydrogen atom, is sometimes considered to be the single member of a fourth class of amino acids and is sometimes included, as here, in the first class.

It is interesting to note that histidine is unique in that it is the only amino acid whose side chain may be protonated or unprotonated, and therefore charged or uncharged, at physiologic pH. This property of the histidine side chain is functionally significant. For example, the catalytic mechanism of serine proteases, such as the enzymes in the blood coagulation cascade or the complement pathways, is dependent on the ability of a histidine residue in the active site of the enzyme to act as a general base, accepting and then releasing a proton in sequential steps of the enzymatic reaction.

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NATURE OF THE PEPTIDE BOND

The amino acid building blocks become incorporated into proteins in a pattern determined by the encoding genes and are held together in this array by peptide bonds. A peptide bond is formed when the carboxyl group of one amino acid condenses with the amino group of a second, eliminating water and establishing the covalent link between the two amino acids (Fig. 3-4). The peptide bond is formed between the carbonyl carbon of one amino acid and the nitrogen of the next amino acid in the protein sequence. This process is repeated as the polypeptide chain elongates. The result is a structure in which the amino group of the first amino acid in the chain and the carboxyl group of the last are not modified: a protein's polypeptide chain, the main chain or backbone, is described as running from its N terminus to its C terminus. The peptide backbone is comprised of the repeating unit

. (The carbonyl carbon of the protein backbone is referred to as C for ease of identification.)

The fundamental properties of the peptide bond dictate the conformation of the main chain of a protein. The chemical nature of the peptide bond (partial double-bond) requires that each segment of the polypeptide chain between one C and the

Figure 3-5 In a peptide bond, the amide nitrogen shares its lone pair of electrons with the carbonyl oxygen, lending a considerable double-bond character to the CN bond. As a consequence, the main chain of a protein is planar from one C to the next C . Each planar unit has 2 degrees of freedom; it can rotate about the NC bond and about the C C bond. The peptide bonds of the polypeptide depicted are in the trans configuration; adjacent C carbons and the side chains they bear are on opposite sides of the planar CN bond. This is the preferred configuration for most amino acids, as it minimizes steric hindrance. For proline the trans configuration is not favored as much as for other amino acids, and the cis configuration occurs with significant frequency. The planar units of the polypeptide backbone are enclosed in gray boxes. The amino acid side chains are highlighted.

next one in the main chain be planar (Fig. 3-5). The conformation of the polypeptide backbone is thus dictated by the angles between these planar segments. The angles of rotation about the NC bond and the angles of rotation about the C C bond are restricted because some angles of rotation would result in steric interference between the main chain and amino acid side chains. The only exception to these restrictions is the angles of rotation permissible for glycine residues. Since the side chain of glycine is a single hydrogen atom, a much wider range of conformations is available about the glycine C . Conversely, the bulky side chain of a tryptophan residue would be most restrictive of these angles of rotation.

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DISULFIDE BRIDGES

In addition to the peptide bond, there is one other covalent bond between amino acid residues that frequently occurs in proteins. The sulfhydryl groups on proximal cysteine residues can be oxidized to form cystine, which contains a disulfide bond (Fig. 3-6). This reaction requires an oxidizing environment. Since the intracellular space is a reducing environment, disulfide bonds are not usually found in intracellular proteins. Disulfide bonds are frequently found in extracellular soluble and integral membrane proteins: they are formed in the lumen of the endoplasmic reticulum, the initial compartment of the secretory pathway. Disulfide bonds may be formed from cysteines that are members of the same polypeptide chain, in which case they are thought to stabilize an already folded polypeptide backbone. Alternatively, a disulfide bond may serve to join covalently two different polypeptide chains, for example, the heavy and light chains of immunoglobulins.

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ELEMENTS OF SECONDARY STRUCTURE

The amino acid sequence of a protein is termed its primary structure. This structure is coded in the gene. When a number of consecutive amino acid residues have similar angles of rotation about NC and C C, the main chain of the protein will assume a regular structure. The rotational constraints imposed on these bonds dictate that these regular structures are almost always one of two forms, -helices and -sheets. These structural elements of proteins are termed secondary structures.

Formation of these secondary structures resolves a dilemma posed by the folding of a polypeptide chain by permitting formation of hydrogen bonds between the NH groups and the C=O groups of the protein main chain. The major driving

Figure 3-6 Disulfide bonds are formed between neighboring cysteine residues in an oxidizing environment. The side chains of cysteine and cystine are shaded.

force for folding of proteins in the aqueous environment is to remove nonpolar amino acid side chains from water by sequestering them in the hydrophobic core of the protein. This is akin to the coalescence of oil droplets when oil is dropped into water. The formation of larger globules minimizes contact of hydrophobic moieties of the oil with water. The main chain of a protein with its NH and C=O groups is highly polar. To bring the nonpolar side chains into the hydrophobic interior of the protein the main chain must follow. The hydrophilic nature of the main chain is neutralized in regions of secondary structure by the formation of hydrogen bonds between its polar elements, as illustrated in Figure 3-3 .

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-HELIX

-Helices ranging from four or five residues to 40 residues are found in compact globular proteins such as hemoglobin, while long rodlike proteins like the tail of the cytoskeletal protein myosin are made up of long helices twisted around each other to form coiled coils. The membrane-spanning regions of integral membrane proteins, for example, those of the red cell membrane protein glycophorin and the platelet $_2$ -adrenergic (epinephrine) receptor, are frequently -helical regions of about 20 amino acids. An -helix is formed from a continuous sequence of amino acid residues in a protein. One turn of an -helix contains 3.6 amino acid residues, with hydrogen bonds forming between the carbonyl oxygen of residue n and the amide hydrogen of residue n + 4 (Fig. 3-7A). The distance between one turn of an -helix and the next, its pitch, is 5.4 A. With 3.6 residues per turn, the rise per residue along the vertical axis of the helix is 1.5 A. Theoretically, an -helix can be either right-handed or left-handed with regard to direction of screw. In proteins, left-handed helices are rarely seen as the side chains of L amino acids approach the C=O group too closely.

With the exception of proline, there are no strong predictors of whether a particular amino acid side chain is likely or not to be incorporated into an -helix. The last atom of the proline side chain is bonded to the main chain nitrogen atom, preventing the nitrogen atom from participating in hydrogen bond formation

Figure 3-7 The -helix is formed from a continuous sequence of amino acids. **(A)** A right-handed -helix is illustrated. The hydrogen bonds between residue n and residue n + 4, which stabilize the helix, are illustrated by the dotted lines. **(B)** Amino acids in the propeptide of factor IX are plotted on a helical wheel. The asymmetric distribution of hydrophilic amino acid side chains (color) and hydrophobic amino acid side chains (gray) about the helix is easy to visualize with this representation.

(Fig. 3-2A). In addition, the proline side chain sterically hinders the -helical conformation, producing a bend in the helix if it appears after the first turn. Therefore, proline is not found within -helices.

The distribution of residues on the surface of a helix can be visualized using a helical wheel. Residues are plotted every 100 degrees (360°/3.6) around a circle or spiral (Fig. 3-7B). The plot shows the projection of the position of side chains on a plane perpendicular to the helix axis with side chains on one side of the helix appearing on one side of the wheel. Given the periodicity of the residues in an -helix, charged and polar residues may be arrayed on one face of the helix, while hydrophobic residues appear on the opposite face. A region of the propeptide of factor IX is an amphipathic helix, having a hydrophilic face and a hydrophobic face (Fig. 3-7B).

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-SHEETS

The second important element of secondary structure is the -sheet. In contrast to the -helix, the -sheet is formed from several different regions of a polypeptide chain. The stretches of polypeptide, usually 510 residues long, that form the -sheet are called -strands. The -strands are aligned to form hydrogen bonds between the carbonyl oxygen atoms on one strand and the amide hydrogen atom on the opposite strand. If in successive -strands the amino acids are running in the same direction, N terminal to C terminal, then the -sheet is termed parallel (Fig. 3-8A); if successive strands alternate directions, the sheet is termed antiparallel (Fig. 3-8B). Mixed -sheets in which some of the -strands are parallel and some antiparallel are uncommon. The hydrogen-bonding pattern is distinct in the two forms of -sheet. In either case, however, the sheet appears pleated; alternate C groups appear above and below the plane of the -sheet. Likewise, the amino acid side chains on a

Figure 3-8 -Sheets are formed from several regions of a polypeptide chain. The strands of polypeptide assembled in a -sheet may be parallel, that is, aligned in the same direction from N to C terminus, or the strands may be antiparallel, that is, aligned in alternating direction from N to C terminus. **(A)** Schematic drawing of a parallel -sheet. **(B)** Schematic drawing of an antiparallel -sheet. The hydrogen bonds that stabilize these structures are highlighted.

-strand are alternately above and below the plane of the -sheet.

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PROTEIN TERTIARY STRUCTURE: ASSEMBLY OF SECONDARY STRUCTURES AND DOMAINS

Most proteins are made up of combinations of -helices and -sheets connected by regions of less regular structure usually termed *loops.* The -helices and -sheets pack together to form the hydrophobic core of a protein, while the loop regions tend to appear on the surface of the protein. Loops can be of three general types: (1) tight turns, in which the carbonyl oxygen of the first residue and the amide hydrogen of the fourth residue share a hydrogen bond; (2) loops that are stabilized primarily by side chain-to-main chain hydrogen bonds; and (3) extended structures that do not contain hydrogen bonds. Comparison of homologous proteins among species suggests greater mutability of loop regions than of regions of regular secondary structure. The structures of the folded cores of the proteins are preserved during evolution, while insertions or deletions of several amino acids occur primarily in loop regions.

The arrangement of secondary structures within a polypeptide defines its tertiary structure. The packing of secondary structural elements into a compact folded form brings distant parts of the polypeptide chain into close proximity. Proteins may be assigned to one of four classes, depending on the content and arrangement of secondary structure within the core of the protein. Proteins of the -class are made up primarily of -helices with connecting loop structures. Similarly, -proteins are made up of -sheets connected by loop regions. In -proteins the two elements of secondary structure alternate, again connected by loops. A fourth class of proteins, + , incorporates both -helices and -sheets, but these elements are not arranged in an easily identifiable pattern. Most proteins studied to date are of the -, -, or -classes. Figure 3-9 shows examples of -, -, and -proteins.

Figure 3-9 Models illustrating the three-dimensional structures of several protein domains. **(AC)** Ribbon diagrams, making it easy to discern the elements of secondary structure that make up a protein domain. **(A)** -Subunit of hemoglobin, composed almost entirely of -helices. **(B)** H-*ras* oncogene protein p21, composed of both -helices and -sheets. **(C)** Variable domain of an immunoglobulin heavy chain, composed almost entirely of -sheets. Although the ribbon diagrams, which omit amino acid side chains and trace the protein backbone, are useful for observing the secondary structures within a protein, they give the misleading impression that there is a large amount of empty space within the molecule. This is not the case. In fact, the core of globular proteins is densely packed with atoms. **(D)** -Subunit of hemoglobin in a space-filling model in which the atoms are represented by spheres with radii proportional to their van der Waals radii. It is clear from this representation that very little empty space is present within the protein core.

Figure 3-10 Structure of ovine prostaglandin H_2 synthase-1 is used to illustrate the hierarchical nature of protein folding. The organization of the secondary structures within a domain defines its tertiary structure; the orientation of protein domains and protein modules within a protein subunit defines its tertiary structure. When a protein is made up of more than one subunit, the spatial relationship of the subunits in the assembled protein defines the protein's quaternary structure. *(The coordinates for this structure were kindly provided by Dr. Michael Garavito, Department of Biochemistry and Molecular Biology, University of Chicago; the structure is described in Picot et al.)*

Many proteins are made up of multiple domains. A protein domain, frequently encoded in a single exon, is a region of a polypeptide chain that can fold autonomously into a stable tertiary structure. The spatial relationship of these independent domains within a protein is part of the description of its tertiary structure. The relationship of secondary structure, protein domains, tertiary structure, and quaternary structure is illustrated in Figure 3-10 .

During evolution a limited number of protein domains have been used repeatedly: related domains from different proteins share enough sequence homology to preserve the polypeptide fold but may have markedly different amino acid sequences

and functions. Such domains, which appear to be associated with exon shuffling and duplication, have been termed modules. Examples of proteins whose tertiary structures are built from such conserved modules abound in plasma and as components of cells within the vasculature. A demonstration of the assembly of discrete proteins from such modules is given in Chapter 103 for the proteins involved in hemostasis and fibrinolysis. These proteins incorporate epidermal growth factor-like domains, kringle domains, and type I and type II fibronectin domains, all of which are widely distributed in mammalian proteins. The DNA-binding zinc finger domain is an example of the importance of these conserved domains to intracellular proteins as well. It is hypothesized that the modules within a given class share a stable core structure with discrete functions determined by the nonconserved amino acids expressed on the module's surface.

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QUATERNARY STRUCTURE: ASSEMBLY OF POLYPEPTIDE CHAINS

For some proteins the functional unit is made up of more than one independently synthesized polypeptide chain. The orientation of the polypeptide chains to one another within the functional unit is termed the quaternary structure of a multimeric protein. A multimeric protein may be made up of identical subunits or polypeptide chains, as in glucosephosphate isomerase, a dimer of two identical polypeptide chains, or different subunits, as in hemoglobin, in which the functional protein contains two - and two -subunits. The interaction between the subunits of a multimeric protein may be stabilized by disulfide bonds between the polypeptide chains. The interaction of the light and heavy chains of immunoglobulin molecules and the A-chains, B-chains, and -chains of fibrinogen are examples of such proteins. The subunits of a multimeric protein may influence one another, as in the binding of oxygen to hemoglobin, in which the occupancy of one heme group with oxygen influences the affinity of the heme groups of the remaining three subunits for oxygen. Alternatively, the subunits of the assembled multimeric protein may provide a unique function, as in the formation of the antigen-binding site of an immunoglobulin, the complementarity-determining site being formed by the variable regions of both the heavy and light chains of the immunoglobulin molecule. Finally, there are multi-subunit proteins in which each subunit has a distinct function: RNA polymerase is an example of such a protein.

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Chapter 4 - Plasma Membrane Dynamics and Organization

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INTRODUCTION

The plasma membrane is so fundamental to the cellular basis of life that the concept of the membrane as a semipermeable fluid lipid bilayer is taught in elementary biology courses. However, the common depiction of the membrane as a soap bubble or as a fluid mosaic ignores the complexity of real cells and the diverse mechanisms by which cell membranes are stabilized and organized. Of particular interest is how different membrane components are assembled to provide unique material properties and form specialized receptor domains to achieve vectorial exchange of nutrients and information with other cells and their milieu. As a result of this complexity and the fundamental importance of the plasma membrane, it is difficult to conceive of a disease process that at the cellular level does not involve altered membrane function, often as the primary event.

The plasma membrane consists of a complex, ordered array of lipids and protein stretched over the outer surface of the cell in the form of a lipid bilayer punctuated by penetrating or attached proteins. The membrane thus forms the interface between each cell and its environment. Biologic membranes display numerous properties that may seem paradoxical. These features issue from the general properties of the lipids and proteins that compose the plasma membrane and from specialized interactions between specific membrane proteins or lipids, or both. Three features stand out: (1) Plasma membranes are noncovalent assemblies of billions of molecules, yet they tend to be self-assemblying, self-sealing, and stable. A typical cell plasma membrane contains a billion molecules of lipid and 10 million protein molecules. All these molecules remain indefinitely associated as an extraordinarily thin (610 nm), continuous sheetlike membrane enveloping the cell. (2) Plasma membranes are fluid structures, yet they are highly ordered with respect to the distribution of molecules both across the bilayer as well as within the plane of the bilayer. Thus, the distribution of both membrane lipids and proteins is highly asymmetric. Many factors,

both intrinsic and extrinsic, act to order the membrane. (3) Plasma membranes function as a barrier, yet they readily pass ions, nutrients, and information (signals) between the cytoplasm and the extracellular environment. They are more than simply a semipermeable membrane, since they can transport some molecules against a concentration gradient. In addition, transmembrane signaling (information transfer) often occurs without transmembrane molecular passage.

In this chapter the biochemical basis of the general properties of biologic membranes is discussed, emphasizing the common principles that underlie their assembly and function. The red cell membrane is chosen as the central focus of this discussion because it is the best characterized plasma membrane and because understanding the structural basis for its function has direct relevance to understanding a number of hematologic disorders. Factors that establish and maintain order within the membrane are outlined, because it is the extent of organization of the plasma membrane that distinguishes it from simple lipid bilayers.

While the red cell membrane serves as the best-defined model for understanding the organization and interactions that maintain cell shape and the integrity of the structures inside and outside the cell, the role of the membrane as a communications surface is universal among many cell types. Thus, a brief introduction to signal transduction is also included.

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STRUCTURAL REQUIREMENTS FOR RED CELL FUNCTION

The human red cell leaves the bone marrow in the form of the reticulocyte; thereafter, the mature erythrocyte spends its circulatory lifetime of approximately 120 days performing its function of oxygen delivery. To carry out this function optimally, it must possess a remarkable ability to undergo cellular deformation. The diameter of the human red cell (8 m) far exceeds that of the capillaries (23 m) through which it must pass in the process of delivering oxygen to the tissues. The ability of normal red blood cells (RBCs) to undergo marked deformation during passage through capillaries was originally documented in 1675 by Leeuwenhoek, who noted that the globules underwent deformation into an ellipsoidal shape during passage through the microvasculature but resumed their original shape when they returned to large vessels. After observing his own blood, Leeuwenhoek noticed, with remarkable prescience, that when he was greatly disordered, globules of his blood appeared hard and rigid, but grew softer and more pliable as his health returned to normal. Since these insightful original observations, a substantial body of evidence has been gathered that suggests the ability of the red cell to undergo extensive deformation is essential not only for its function, but also for its survival. [1] [2] [3] [4] [5]

The ability of the red cell to undergo the deformation necessary to negotiate splenic channels and capillaries (Fig. 41) while withstanding continuous circulatory stresses is programmed into its geometry, the viscosity of the intracellular milieu, and the material properties of its membrane. [6] [7] The biochemical composition and anatomic organization of various components of the red cell membrane determine these cellular characteristics. The red cell is unique among eukaryotic cells in that its principal physical structure is its membrane, which encloses a concentrated hemoglobin solution. In contrast to other cells, the RBC has no cytoplasmic structures or organelles and lacks a nucleus. Thus, all the structural elements of the red cell are in one way or other linked to the cell membrane. The mechanisms by which different membrane components and their interactions contribute to the unique features of the human red cell are summarized.

Figure 4-1 (AC) Scanning electron micrographs of red cells passing through splenic endothelial slits. Note the remarkable deformation the red cells exhibit during their transit through these structures. The cell with lobular and irregular surface in **(B)** is a reticulocyte in transit.

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RED CELL MEMBRANE STRUCTURAL ORGANIZATION

The red cell membrane is composed of a lipid bilayer to which is anchored a filamentous network of proteins that underlies the cytoplasmic surface of the membrane. This network, also referred to as the membrane skeleton, is important for maintaining red cell shape and regulating membrane properties of deformability and mechanical stability.[6] [7] The red cell membrane has been well characterized biochemically. The structural organization of the various lipid and protein components has been well delineated.[8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18] [19] [20] About 52% of the membrane mass is protein, 40% is lipid, and 8% is carbohydrate. The major lipid components are unesterified cholesterol and phospholipids, present in nearly equimolar quantities. Free fatty acid and glycolipids are present in small amounts. [8] The phospholipid composition of the membrane is phosphatidylcholine (PC), 30%; sphingomyelin (SM), 25%; phosphatidylethanolamine (PE), 28%; and phosphatidylserine (PS), 14%. Phospholipids such as phosphatidic acid, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-diphosphate make up about 23% of the total. These phospholipids are asymmetrically distributed in the membrane, with more than 75% of the choline-containing uncharged phospholipids PC and SM found in the outer monolayer of the lipid bilayer, while 80% of PE and all PS, the charged phospholipids, reside in the inner monolayer. [9]

The maintenance of asymmetric phospholipid distribution in the normal red cell membrane appears to be the result of (1) a slow and symmetric rate of passive diffusion of choline-containing phospholipids (PC and SM) across the two lipid monolayers (half-times on the order of hours), (2) a direct interaction

of the charged phospholipids and membrane skeletal proteins, and (3) active transport of the aminophospholipids (PS and PE) by an ATP-dependent aminophospholipid translocase or flippase from the outer to the inner monolayer. [10] In contrast to phospholipids, cholesterol diffuses across the membrane on a physiologic time scale (a half-time of seconds or less). [20] The mature red cell is unable to synthesize lipids de novo, but a number of lipid renewal pathways produce considerable turnover of the various phospholipids with no net change in lipid composition. In addition, the cholesterol content of the membrane is regulated by the exchange that takes place between plasma cholesterol and membrane cholesterol. The function of the phospholipid bilayer is to provide physical continuity to the membrane. This membrane continuity, in turn, is responsible for solute impermeability. The lipid bilayer also serves as the matrix in which transmembrane proteins reside. The fluidity of this matrix is regulated by lipid composition.

Analysis of proteins of the red cell membrane by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) in one dimension indicates the presence of about a dozen distinct species, most of which have been well characterized. By contrast, two-dimensional separation employing isoelectric focusing and SDS/PAGE shows the membrane to be composed of more than 100 different proteins, many of which have yet to be characterized. The proteins of the membrane are divided into two general groups, integral and peripheral proteins. The integral proteins are tightly bound to the membrane through hydrophobic interactions with the lipids in the bilayer. Band 3, aquaporin, and glycophorins belong to this class; these proteins span the membrane and have distinct structural and functional domains, both within the bilayer and on either side of the membrane (Fig. 42). The peripheral proteins are located on the cytoplasmic surface of the lipid bilayer and can be readily released from the membrane by manipulation of the ionic strength of the milieu or other protein perturbants. The peripheral proteins, which include spectrin, actin, ankyrin, adducin, tropomyosin, tropomodulin, and protein 4.1, constitute the membrane skeleton (Fig. 42).

The most abundant and best-studied integral proteins of the red cell membrane are band 3, aquaporin, and a family of sialoglycoproteins called glycophorins. Band 3, the anion exchanger, is the major integral protein, constituting about 25% of total membrane protein. Recent cloning studies have demonstrated that this protein is the product of a gene residing on chromosome 17 that encodes a 911-amino acid protein (101,791 Mr). [21] Each red cell contains approximately 1×10^6 copies of band 3. This protein is composed of three dissimilar and functionally distinct domains. The hydrophilic cytoplasmic domain (residues 1403) interacts with a variety of peripheral membrane and cytoplasmic proteins, including ankyrin, protein 4.1, protein 4.2, hemoglobin, and a variety of glycolytic enzymes. The hydrophobic transmembrane domain (residues 404882) contains multiple membrane-spanning domains and forms the anion transporter. The acidic C-terminal domain (residues 883911) has no known function, although recent evidence suggests that this domain can bind carbonic anhydrase. Band 3 contains a single N-glycosidically linked oligosaccharide that possesses the blood group antigen activities I and i. The two clearly established functions of band 3 in the membrane are (1) anion transport, resulting in one-for-one exchange of HCO_3 or Cl across the membrane; and (2) physical linkage of the lipid bilayer to the underlying membrane skeleton, primarily through its interaction with ankyrin and secondarily through binding to protein 4.1 or protein 4.2.

Four sialic acidrich glycoproteins (glycophorins A, B, C, and D) compose a class of integral proteins termed glycophorins, constituting approximately 2% of the total red cell membrane proteins.[22] Glycophorins A, B, and C are distinctly different polypeptides containing 131, 72, and 128 residues, respectively. Recent cloning and sequencing studies have shown that these proteins are the product of distinct genes. The genes coding for glycophorins A and B reside on chromosome 4, and glycophorin C is encoded by a gene residing on chromosome 2. Glycophorins consist of three domains: a cytoplasmic domain, containing a cluster of basic residues positioned near the plasma membrane; a hydrophobic domain, which forms a single -helix spanning the bilayer; and an extracellular domain, which is heavily glycosylated. The glycophorin A molecule carries the MN blood group specificity, glycophorin B the Ss specificity, and glycophorin C the Gerbich blood group specificity. The presence of carbohydrates imparts a strong net negative charge to the cell surface; this is functionally important in reducing the interaction of red cells with one another as well as with other cells, including vascular endothelium. Glycophorin C appears to interact with p55 and protein 4.1, and through this association it regulates the membrane content of p55 and protein 4.1.

A number of other integral proteins are present in the red cell membrane. Aquaporin-1, present in the red cell membrane, belongs to a family of membrane channel proteins that serve as selective pores for water transport.[23] Colton blood group antigens represent a polymorphism on the aquaporin-1 protein. Of the different human red cell surface antigens, the Rh blood group system is unique in that its antigens are carried by a family of nonglycosylated but palmitoylated membrane proteins consisting of Rh30 (RhD and RhCE) polypeptides and the Rh50 glycoprotein. [24] The function of this protein complex remains undefined. Other blood group-specific determinants on the red cell membrane include Duffy, Kell, Kidd, Lutheran, and Lewis. Although the function of these proteins remains to be defined, it is likely that some of these integral proteins possess transport

Figure 4-2 Schematic diagram of the red cell membrane organization based on our current understanding of various membrane components. The key protein interactions identified to be important in regulating red cell membrane mechanical properties include spectrin dimer-dimer interaction, spectrin-ankyrin-band 3 interaction, and spectrin-actin-protein 4.1 interaction.

functions while others are involved either in mediating adhesive interactions or in linking the bilayer to the membrane skeleton.

The filamentous network of peripheral proteins is anchored to the bilayer by interaction with several integral proteins. The network, generally referred to as the membrane skeleton, is composed of seven principal components: spectrin, actin, ankyrin, adducin, tropomyosin, tropomodulin, and protein 4.1 (Fig. 42 ; Plate 41). Spectrin is a flexible, rodlike molecule present in red cells at approximately 200,000 copies per cell. It is composed of two nonidentical subunits of 260,000 daltons (spectrin -subunit) and 246,000 daltons (spectrin -subunit) intertwined side to side, forming a heterodimer with a contour length of approximately 100 nm. The gene that codes for -spectrin resides on chromosome 1 and that for -spectrin on chromosome 14. Each polypeptide is organized into a number of independently folded domains, each containing 106 residues. The subunits are linked, like beads on a string, by flexible joining regions. Spectrin heterodimers associate head to head to form $()_2$ tetramers, which have a contour length of approximately 200 nm. Tetrameric species of spectrin appear to predominate in the membrane skeleton. Oligomers larger than the tetramers are also observed; they appear to be formed by spectrin dimers joined by head-to-head linkages into a central ring. [25] The tail ends of spectrin tetramers are associated with short oligomers of actin composed of 12 monomers. Red cell actin is unusual in being organized into short, highly uniform filaments of approximately 35 nm in length. The strict control of actin filament length may be modulated by the proteins tropomyosin and tropomodulin. [26] On average, six spectrin ends complex with each actin oligomer, creating an irregular network with an approximately hexagonal lattice. Each spectrin-actin junction is stabilized by the formation of a ternary complex with protein 4.1. The gene coding for protein 4.1 has been sequenced and localized to chromosome 1. The protein is composed of 622 amino acids; approximately 200,000 copies of this molecule are present in each red cell. Spectrin-actin interactions that are independently weak are greatly stabilized by protein 4.1. [26] This stabilization occurs through the direct interaction of protein 4.1 with both spectrin and actin. Adducin is yet another skeletal protein that stabilizes the interaction of spectrin with actin. However, in contrast to protein 4.1, adducin is much less abundant in the red cell, with only 30,000 molecules present in each cell. An interesting feature of adducin is that it is a target for the calcium-dependent regulating protein, calmodulin; as such, its ability to promote spectrin-actin interactions is regulated by intracellular calcium concentrations. The red cell membrane skeleton can be envisioned as an irregular network in which the basic unit is composed of a hexagonal lattice with six spectrin molecules. The structural model for organization of the membrane skeleton has been confirmed by high-resolution electron microscopy of isolated membranes. [27] The micrographs show a highly repeated and remarkably regular organization of the spectrin-actin-protein 4.1 complexes in which each complex is linked to adjacent complexes by multiple spectrin tetramers.

One physical linkage of the membrane skeleton to the lipid bilayer is accomplished by ankyrin (band 2.1), which simultaneously interacts with spectrin in the skeleton and band 3, localized in the bilayer. [14] [16] The gene coding for ankyrin has been sequenced and localized to chromosome 8. Ankyrin is 1,879 amino acids long. It contains distinct binding sites for both -spectrin and band 3 and is present in the red cell at 100,000 copies per cell. The ankyrin-binding site on -spectrin is located approximately 20 nm from the C-terminus. The second linkage between the skeleton and the bilayer is through the interaction of the integral protein glycophorin C with the skeletal component, protein 4.1. Other direct linkages also exist, but the nature of these in the red cell is less well understood. [26] [27] Through these linkages, the lipid bilayer is mechanically coupled to the membrane skeleton; this composite structure is responsible for the unique properties of the red cell membrane. [28] [29]

The discussion thus far may seem to imply that various protein-protein associations are fixed in space and time, but this is a simplistic view. During its time in circulation, the red cell constantly undergoes cycles of deformation and relaxation, requiring the membrane to accommodate extensive and dynamic changes in cell shape. In all likelihood this is accomplished by dynamic regulation of the protein interactions. Potential mechanisms for regulation of skeletal protein associations and skeletal protein-integral protein interactions include phosphorylation, variations in intracellular magnesium and 2,3-diphosphoglycerate (2,3-DPG) during the oxygenation-deoxygenation cycle, and calmodulin effects due to elevated intracellular calcium. [14] [28] Only limited information is available regarding these regulatory pathways, but most available evidence suggests that phosphorylation tends to lower the affinity of protein-protein interactions. For example, phosphorylation of protein 4.1 results in a fivefold decrease in its affinity for spectrin and in its ability to promote spectrin-actin association. Phosphorylation of -spectrin reduces membrane mechanical stability. Similarly, increased levels of intracellular 2,3-DPG and elevated cytoplasmic concentrations of calcium, in association with calmodulin, decrease membrane mechanical stability by destabilizing spectrin-actin-protein 4.1 and spectrin-actin-adducin interactions, respectively. Thus, the red cell membrane is a dynamic structure in which multiple protein-protein associations are subjected to regulation. The most important function of these protein assemblies is to provide the cell with a flexible yet mechanically resilient and stable membrane.

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RED CELL MEMBRANE MECHANICS AND SKELETAL PROTEINS

During its 120-day life span, the erythrocyte must undergo extensive passive deformation and resist fragmentation. These two essential qualities require a highly deformable yet remarkably stable membrane. The property of membrane deformability determines the extent of membrane deformation that can be induced by a defined level of applied force. The more deformable the membrane, the less applied force is necessary to allow the cell to pass through the capillaries. By contrast, membrane stability is defined as the maximum extent of deformation that a membrane can undergo, beyond which it cannot completely recover its initial shape, that is, the point at which it fails. Normal membrane stability allows red cells to circulate without fragmenting, while decreased stability leads to cell fragmentation under normal circulating stresses.

A model designed to conceptualize how these membrane properties are regulated by the membrane components is presented in Figure 43 . [28] This model takes into account the known structure, associations, and stoichiometry of the skeletal proteins. In the nondeformed state, spectrin molecules exist in a folded confirmation. Reversible deformation of the red cell membrane occurs with a change in geometric shape, but at constant surface area. During reversible deformation, a rearrangement of the skeletal network occurs in which some spectrin molecules become uncoiled and extended, while others become more compressed and folded. Direct evidence for such reversible structural changes in the spectrin network has recently been documented. [29] With increased deformation, the membrane becomes increasingly extended; some of the spectrin molecules attain their maximal linear extension. This point is the limit of reversible deformability. A continued application of force would necessitate an increase in surface area and the breaking of junctional complexes. When the red cells are exposed to fluid forces great enough to require an increase in surface area, the membrane fails. This failure will occur at the weakest of the lateral protein-protein associations (spectrin-spectrin

Figure 4-3 Conceptual depiction of rearrangements in membrane skeleton during reversible deformation of red cells. Reversible deformation occurs with a change in geometric shape, but at a constant surface area. **(A)** Organization of membrane skeleton in the nondeformed membrane. **(B & C)** With increased deformation, the membrane becomes increasingly extended. Further extension beyond stage C would result in an increase in surface area and the breaking of junction points. This is the stage at which membrane fragmentation occurs. (
) protein 4.1-actin-spectrin junction; () spectrin-spectrin junction. Linear coils are spectrin dimer.

junction or spectrin-actin-protein 4.1 junction), leading to membrane fragmentation. Another form of membrane failure involves the separation of the lipid bilayer from the underlying membrane skeleton as a result of decreased cohesion between the bilayer and the membrane skeleton. A decrease in spectrin density beneath the membrane skeleton, or decreased numbers of linkages between the bilayer and the membrane skeleton, as a result of deficiencies in band 3, protein 4.2, or ankyrin leads to this form of membrane failure. By contrast, for the membrane to deform normally, the skeletal network must be able to undergo rearrangement and the spectrin molecules must be able to fold and unfold. Thus, an increase in intermolecular or intramolecular associations of the skeletal proteins or an increased association of integral membrane proteins such as band 3 with the skeletal network will markedly decrease membrane deformability. [7] Deformability and mechanical stability measurements of pathologic red cells with different mutant forms of membrane proteins, as well as of biochemically perturbed normal red cells, strongly support this model and point unequivocally to a crucial role for membrane proteins and their associations in regulating these two membrane properties. [6] [7]

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ROLE OF THE MEMBRANE IN DETERMINING THE SHAPE AND DEFORMABILITY OF THE RED CELL

Cellular deformability is the term generally used to characterize the ability of the red cell to undergo deformation during flow. This property is influenced by three distinct cellular components: cell shape or cell geometry, which determines the ratio of cell surface area to cell volume; cytoplasmic viscosity, which is regulated by intracellular hemoglobin concentration; and membrane deformability. [6] [7] Membrane deformability, in and of itself, is regulated by multiple membrane properties, which include elastic shear modulus, bending modulus, and yield stress. Directly or indirectly, membrane components and their organization play an important role in regulating each factor that influences cellular deformability.

Cell Shape

The biconcave disk shape of the normal red cell creates an advantageous surface area-volume relationship, allowing the red cell to undergo marked deformation while maintaining a constant surface area. The normal human adult red cell has a volume of 90 fl and a surface area of 140 m^2 . If the red cell were a sphere of identical volume, it would have a surface area of only 98 m^2 . Therefore, the discoid shape provides half again as much surface area as would a sphere of equivalent volume, and it is this excess surface area of approximately 40 m^2 that allows the red cell to undergo extensive deformation. Most deformations occurring in vivo and in vitro involve no increase in surface area. The normal red cell can undergo large linear extensions of up to 230% of its original dimension, but an increase of even 34% in surface area results in cell lysis. Because the acquisition and maintenance of a favorable surface area-volume relationship is crucial to red cell function, it is useful to understand how the cell acquires a discoid shape during its development from a nucleated erythroid precursor cell. The immediate

Figure 4-4 Morphology of cells during reticulocyte maturation. Left column, phase-contrast microscopy; middle column, scanning electron microscopy; right column, transmission electron microscopy. The nuclear extrusion process is illustrated in the top row. The least mature reticulocytes, which are motile and multilobular, are shown in the second row. The more mature reticulocytes, which are cup-shaped and nonmotile, are depicted in the third row. Mature red cells are illustrated in the bottom row.

precursor of the mature discocytic red cell is the nonnucleated reticulocyte, produced when the normoblast extrudes its nucleus (Fig. 44). In contrast to red cells, these immature reticulocytes are multilobular and motile, contain mitochondria and ribosomes, and synthesize hemoglobin and certain membrane components, including protein 4.1 and glycophorin C. Reticulocytes exhibit much less cellular deformability and mechanical stability than are displayed by mature red cells. Both deformability and mechanical stability improve during maturation. [30] In 23 days, motile reticulocytes evolve further in the bone marrow, first to become deep cup-shaped nonmotile cells that still contain ribosomes, and finally to mature, fully hemoglobinized discocytic RBCs lacking organelles. Major reorganizations of membrane phospholipids, skeletal proteins, and integral proteins accompany the acquisition of discoid shape and enhanced deformability. These reorganizations include loss of membrane lipids, loss of integral proteins such as transferrin receptors, insulin receptors, and fibronectin receptors, and a major reorganization of the skeletal protein network.

Having acquired the discoid shape with its favorable geometric parameters during reticulocyte maturation, the red cell must maintain this favorable surface area-volume relationship during its circulating life span. Either membrane loss, leading to reduction in surface area, or an increase in cell water content, leading to an increase in cell volume, will create a more spherical shape with less redundant surface area. This loss of surface area redundancy leads to decreased cellular deformability, compromised red cell function, and reduced survival. Skeletal protein defects involving spectrin, ankyrin, band 3, or protein 4.1 lead to a mechanically unstable membrane, resulting in cell fragmentation and generation of cells with reduced surface area in congenital hemolytic anemias, such as hereditary spherocytosis and hereditary elliptocytosis. [31] [32] [33] Similarly, partial phagocytosis of the red cell by macrophages leads to reduced surface area and generation of spherocytes in certain immune hemolytic anemias. By contrast, in some forms of hereditary stomatocytosis, increased cell volume due to deranged volume regulation as a result of a membrane defect generates red cells with reduced redundant surface area. Increased osmotic fragility is a characteristic feature of all these red cell populations with less than normal surface redundancy. A different type of red cell abnormality is manifested in liver disease. In this instance, elevated membrane cholesterol content causes increased cell surface area; in the setting of normal cell volume, this results in higher than normal redundant surface area. This excess surface area, in turn, is manifested morphologically by the presence of target cells and functionally by increased osmotic resistance. Conversely, decreased cell volume in the setting of a normal membrane surface area (e.g., in thalassemic red cells as well as in hemoglobin CC- and hemoglobin EE-containing red cells) generates the same abnormality. Abnormal red cell shape and altered cell geometry in various red cell disorders are thus characterized by the inability of these cells to maintain their surface area and control their cell volume appropriately as a result of membrane defects involving lipids, integral and skeletal proteins, or abnormal interactions of mutant hemoglobins with the red cell membrane.

Cytoplasmic Viscosity

Cytoplasmic viscosity is regulated by cellular hemoglobin concentration, and consequently by cell water content. Viscosity is another regulatory component of cellular deformability.[6] As the hemoglobin concentration increases from 27 to 35 g/dl, the viscosity of the hemoglobin solution increases from 5 to 15 centipoise (cP) (the viscosity of water is 1 cP). The distribution of red cell hemoglobin concentrations in whole blood from normal persons is 2735 g/dl. In this range of hemoglobin concentrations, the contribution of cytoplasmic viscosity to cellular deformability is negligible. Concentrations of greater than 37 gdl, however, increase viscosity exponentially, reaching 45 cP at 40 g/dl, 170 cP at 45 g/dl, and 650 cP at 50 g/dl. At these levels, the contribution of cytoplasmic viscosity to cellular deformability begins to dominate. Thus, the failure of normal-volume homeostasis mechanisms, which results in cellular dehydration, can limit cellular deformability owing to increased cytoplasmic viscosity. Mutations in proteins involved in transport processes, alterations induced in these proteins as a result of membrane oxidation, or other changes due to mutant hemoglobin interacting with the membrane can lead to marked cellular dehydration. Reduced cellular deformability in hereditary xerocytosis, sickle red cells, and hemoglobin CC red cells is primarily due to increased cytoplasmic viscosity as a consequence of cellular dehydration. [4] [6] The lipid components and the integral proteins of the red cell membrane involved in transport function play a crucial role in volume homeostasis of the red cell in order to maintain the cellular hemoglobin concentration at levels that do not unduly influence the ability of the cell to deform.

Membrane Material Properties

The mechanical behavior of the red cell membrane is complex. Its responses depend on the duration and magnitude of applied stresses. [6] [7] [34] [35] At small values of applied force for short duration, the red cell membrane behaves as a viscoelastic solid; that is, it is capable of undergoing large elastic extensions and completely recovers its initial shape. A reversible unfolding and refolding

of spectrin networks is crucial for the elastic behavior of the membrane. By contrast, when small forces are applied over a long period, or when large forces are applied for a short period, the membrane yields and is unable to recover its initial shape. Under these circumstances, the membrane exhibits permanent plastic deformation owing to inability of the skeletal network to recover its prestressed configuration. Whereas normal red cells completely recover their shape following repeated cycles of deformation in the circulation, pathologic red cells with weakened junctions between skeletal proteins fail to recover their initial shape and undergo plastic deformation. This explains in part the generation of elliptocytic shapes in congenital hemolytic anemias with defective spectrin oligomerization and decreased spectrin-actin-protein 4.1 association. The generation of irreversibly sickled cells in sickle cell anemia is also the result of plastic deformation of the membrane due to skeletal protein reorganization during repeated cycles of sickling and unsickling in the circulation.

In addition to contributing significantly to the regulation of the elastic behavior of the membrane, the membrane skeleton has a crucial role in regulating membrane mechanical stability. The junctional complexes in the skeletal network are the key regulators of mechanical stability. Decreased membrane mechanical stability is a hallmark of hereditary elliptocytosis due to either weakened spectrin-spectrin association as a result of mutations in either - or -spectrin or a weakened spectrin-actin-protein 4.1 junction due to mutations in protein 4.1. [6] [7] A decrease in the number of linkages between the bilayer and the membrane skeleton also results in membrane surface area loss such as that seen in hereditary spherocytosis. By contrast, an increased number of linkages between the bilayer and the membrane skeleton results in decreased membrane deformability.[6] [7] Although substantial changes in lipid composition of the membrane have little effect on membrane mechanical properties, small changes in individual protein components can profoundly alter membrane behavior. The integral proteins in association with the skeletal protein network determine the complex mechanical behavior of the red cell membrane.

The complex and regulated assembly of lipids and proteins in the membrane lends the unique shape and deformability characteristics to the red cell, enabling it to survive its tortuous and at times turbulent journey through the vasculature. Derangement in the structural organization as a result of changes in any of the individual components of the membrane, either lipid or protein moieties, leads to altered cell shape or deformability characteristics, or both, the hallmarks of many red cell disorders.

Membranes of other cell types appear to be organized in a manner analogous to the red cell membrane. Clearly, however, these membranes need not exhibit the same adaptations to the mechanical, chemical, and osmotic forces of the circulation. Rather, membrane cytoskeletal interactions analogous to those just described seem to maintain the distinctive shape and organization of the cell surfaces that are crucial to the organization of tissues. Examples include the polarity of ion transport molecules in epithelial cells, the localization of tight junctions in epithelial cells and endothelial linings, and the dynamic reorganization (capping or clustering) of receptors or antigens that occurs during ligand or antibody binding. Unfortunately, the biochemical basis for the membrane interactions is insufficiently understood to be discussed in this chapter. For our purposes, it is sufficient to note that similar lipids, integral proteins and peripheral proteins, seem to participate, and that analogous interactions seem to mediate the nonrandom organization of distinct membrane domains on different regions of the cell surface. In other words, the membrane cytoskeleton appears not only to strengthen the membrane but also to limit its diffusional degrees of freedom, thereby promoting topological organization.

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SIGNAL TRANSDUCTION: RELIANCE ON ALTERED MEMBRANE ORDER

Many signals are propagated across the plasma membrane by the passage of effector molecules, such as steroid hormones, Ca^{2+} ions, Na^{+} or K^{+} ions, lipophilic drugs, and so forth. Usually such events involve specific membrane proteins that function as pores or transport molecules. Alternative pathways of signal transduction do not involve the passage of molecules across the membrane but instead rely on changes in the organization of the membrane to transmit information. Four such signal transduction mechanisms are depicted in Figure 45 . All involve rearrangements of either integral or peripheral membrane proteins, or of certain membrane lipids, as an essential step in the transduction process. None requires the net passage of molecules across the bilayer. Two involve the generation of second messengers (cAMP and IP_3) at the cytoplasmic face of the membrane.

One of the first signal transduction pathways to be clearly elucidated was that involving the -adrenergic receptor. Subsequently it was recognized that this receptor is just one of many hormone receptors coupled to their effector proteins by the intermediary of a guanyl nucleotide-binding protein (G protein). (Fig. 45A).[36] [37] Other receptors acting via heterotrimeric G proteins include the $_2$ -adrenergic receptor (a cyclase inhibitory receptor), the M_2 -muscarinic receptor, and all of the rhodopsins. These receptors are all integral membrane proteins with an approximately globular structure composed of seven transmembrane -helices (similar to bacteriorhodopsin). The nature of the G protein varies between tissues, as does the nature of the effector protein. For example, stimulation of the -adrenergic receptor releases a G protein that subsequently activates adenylate cyclase to produce cAMP, whereas stimulation of rhodopsin (by light) energizes a different G protein (called transducin), which then activates phosphodiesterase and leads to the conversion of cGMP to 5 GMP. The product of the *ras* oncogene (p21ras) is also a G protein of sorts, except that it appears to function as a monomer. In yeast (*Saccharomyces cerevisiae*), this protein stimulates adenylate cyclase. Its function in mammalian cells remains uncertain. However, regardless of the variability in the specific molecules participating in different G-protein receptor systems, all are activated by an allosteric rearrangement within an integral transmembrane protein, followed by a redistribution of specific peripheral membrane proteins (the G-protein complex).

A similar rearrangement of peripheral proteins may also accompany activation of the PIP_2 -IP_3 signal pathway. In this pathway, there is a rearrangement of membrane lipid as well, due to the hydrolysis of PIP_2 to DAG (Fig. 45B). As with the -adrenergic receptor, recent evidence[36] [38] suggests that rearrangement of a G protein is involved in the activation of phospholipase C. The activation of this enzyme has two consequences: (1) the release of IP_3 as a soluble second messenger and (2) the conversion of PIP_2 to DAG in the membrane. DAG enhances the activity of protein kinase C and its association with the cytoplasmic face of the membrane. [39] Thus, signaling via the PIP_2 pathway involves a transmembrane allosteric signal, followed by the rearrangement of phospholipid and peripheral membrane.

Another family of receptors includes those that recognize polypeptide growth factors and those that possess tyrosine kinase activity. [40] [41] Such receptors include epidermal growth factor receptor (EGF-R), platelet-derived growth factor, and colony-stimulating factor-1 receptors. They all share a tripartite domain structure similar to that of glycophorins, with a single -helix spanning the membrane. On binding of ligand to their extracellular domain, tyrosine kinase activity is elicited in the cytoplasmic domain. This remarkable effect is propagated across the membrane (using a single stretch of -helix) by the

Figure 4-5 Signal transduction pathways that require changes in membrane order. **(A)** Cascades utilizing cyclic nucleotides as second messengers rely on a redistribution of the peripheral G-protein unit (a heterotrimer composed of -, -, and -subunits) from the transmembrane receptor to adenylate cyclase (or other catalytic unit, such as transducin). The same event occurs with a different G-unit for inhibitory cascades. **(B)** Pathways based on the hydrolysis of PIP_2 probably require the redistribution of a G-protein-like unit from the transmembrane receptor to phospholipase C. The hydrolysis of PIP_2 leads to a redistribution of these lipids in the membrane, with DAG stimulating the binding of C-kinase to the membrane and the release of protein 4.1 from the membrane. **(C)** The binding of ligands to receptors of the epidermal growth factor type (EGF-R) promotes the self-association of the receptor. Self-association appears to be a necessary and probably sufficient signal to activate the latent tyr-kinase activity resident in the cytoplasmic domain of EGF-R. **(D)** Receptor clustering is induced by the binding of an external polyvalent ligand. In the case of B lymphocytes, if the cluster achieves a sufficient size (an immunon), cell activation will occur. [44] Note that in all of the pathways depicted here, no molecules pass across the membrane.

ligand-induced dimerization of the receptor in the plane of the membrane. Once two receptors are brought together, their cytoplasmic domains interact in a way that evokes their latent tyrosine kinase activity. It is uncertain whether ligand-induced allosteric changes in the external domain play a role in this process or whether the growth factor peptide itself may cross-link two receptors. Given that the transmembrane domain of glycophorin A may control its dimerization, it is even possible that ligand-induced changes in the transmembrane domain of the growth factor receptors may activate their self-association. In any event, it appears that the actual propagation of the signal across the membrane, as measured by the activation of tyrosine kinase activity, requires only a change in the organization of the receptors in the plane of the membrane.

Many other receptors also appear to transmit their message to the cell interior via rearrangements in the distribution of integral and peripheral membrane proteins (Fig. 45D). Examples include integrin-mediated signaling[42] or the homotypic binding of adhesion proteins such as those of the cadherin family. [43] Clustering of receptors with weak affinity for cytoplasmic proteins might activate events in the cell that result in a strong binding site for cytoplasmic proteins. A similar mechanism has been proposed for the T-cell-independent activation of B lymphocytes by polyvalent antigens. [44] The activation of B lymphocytes by such antigens appears to be a threshold phenomenon, since it was found that only certain polyvalent ligands (polyacrylamides with more than 20 DNP haptens per polymer unit) were capable of activating the lymphocytes. Presumably, the threshold effect arises from the need to form confluent patches of surface receptors of some minimal size, since occupancy of the same number of receptor sites by ligands of lesser valency does not activate the cells. The receptor clusters created by such activation-competent ligands were called immunons.[44] Very recent work with T cells also indicates that a similar process involving the ligand-specific oligomerization of T-cell receptor molecules (TCR + MHC) sends a biochemical signal to the cell. In these cells, the size of the oligomers involving two to six ternary complexes was sufficient to send a graded signal to the cell. [45]

Finally, the clustering of membrane proteins into functional signaling complexes need not be driven only by protein-protein interactions. Recent evidence indicates that the dynamic clustering of sphingolipids and cholesterol in the plane of the membrane, so as to form rafts of laterally separated lipid microdomains, is yet another mechanism by which order in the membrane is achieved. Such rafts form relatively stable microdomains in which certain membrane- and GPI-linked proteins are stably associated. It is believed that the concerted movement of such lipid islands through the secretory pathway (with their entrapped proteins) is one mechanism by which selective targeting of proteins to the apical pole of epithelial cells is achieved. [46] The clustering of such protein-laden lipid rafts, via direct lipid interactions (that

are cholesterol dependent), is another way that the various components of membrane-based signaling systems can be brought into proximity.

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Chapter 5 - Cell Adhesion

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INTRODUCTION

Cell adhesion is essential for the development and maintenance of multicellular organisms. Cell-cell and cell-matrix contacts facilitate intercellular communication and define the architecture of organs. The regulated nature of cell adhesion is particularly evident in the hematopoietic system, where cells routinely make transitions between nonadherent and adherent phenotypes during differentiation and in response to stimuli in the circulation or extravascular tissues.

In the bone marrow, proliferation and differentiation of hematopoietic stem cells is controlled not only by soluble growth factors, but also by adhesion to stromal cells and matrix molecules. Weakening of these adhesive bonds is required for mature blood cells to enter the circulation. Circulating erythrocytes normally remain nonadhesive until they are finally cleared by the reticuloendothelial system. Other circulating cells often participate in regulated adhesive events during their life span. For example, prothymocytes adhere to components of the thymus, where they undergo further maturation before reentering the circulation. Lymphocytes regularly stick to the specialized high endothelial venules of lymphoid tissues, migrate into these tissues for sampling of processed antigens, and then exit via the lymphatics. During inflammation, specific classes of leukocytes roll on the endothelium, then adhere more tightly, and finally emigrate between endothelial cells into the tissues. There, neutrophils and monocytes phagocytose invading pathogens, whereas lymphocytes adhere to antigen-presenting macrophages. During hemorrhage, platelets stick to exposed subendothelial matrix components, spread, and recruit additional platelets into large aggregates that serve as an efficient surface for thrombin and fibrin generation. Leukocytes also adhere to activated platelets, a mechanism for linking hemostatic and inflammatory responses. The endothelial cells express molecules that affect the adhesiveness of platelets or leukocytes. Tight contacts between adjacent endothelial cells also limit access of blood cells to the underlying tissues.

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ADHESION MOLECULES

Cells adhere through noncovalent bond formation between macromolecules on cell surfaces with macromolecules on other cell surfaces or in extracellular matrix. These interactions involve either protein-protein or protein-carbohydrate recognition. Although some adhesion molecules are expressed only by blood or endothelial cells, most are also synthesized by other cells. Many adhesion molecules can be grouped into families according to related structural and functional features.

Extracellular Matrix Proteins

The principal constituents of the extracellular matrix are adhesive proteins and proteoglycans. The major proteins are von Willebrand factor (vWF), thrombospondin, collagen, fibronectin, laminin, and vitronectin. These proteins are large, often highly extended, and consist of multiple domains with different binding functions. In some proteins such as fibronectin, alternative splicing can increase diversity by producing molecules with variable numbers of domains. The many binding domains allow adhesive proteins to interact with each other as well as with cell-surface receptors, resulting in multipoint contacts that stabilize matrix structure. One large adhesive protein, fibrinogen, is found predominantly in plasma but may also be deposited in exposed subendothelial matrix following vascular injury. Fibronectin, vitronectin, thrombospondin, and vWF are located predominantly in the extracellular matrix, but are also found in plasma. Several adhesive proteins are also stored in -granules of platelets, where they are secreted following platelet activation at sites of hemorrhage.

Proteoglycans contain protein cores to which are covalently attached many glycosoaminoglycans, long linear polymers of repeating disaccharides. Most proteoglycans are in the extracellular matrix, but some are anchored on cell surfaces through a core protein that contains a membrane-spanning domain. Hyaluronan is a unique glycosoaminoglycan that forms polymers with molecular masses up to several million that are not covalently attached to a protein. Hyaluronan forms noncovalent interactions with globular domains on the protein core of proteoglycans and with a small molecule called link protein. The resultant hyaluronan-proteoglycan complexes can become very large, contributing to the structural stability of matrix. Hyaluronan can also bind to cell-surface receptors (see following).

Integrins

Integrins are a broadly distributed group of cell-surface adhesion receptors that consist of noncovalently associated - and -subunits (Fig. 51 and Table 51). There are more than 15 - and 8 -chains that pair in many, but not all, of the possible combinations. All blood cells have several different integrins. The four $_{2}$ integrins, each paired with a unique -subunit, are expressed only by leukocytes, and the $_{IIb\ 3}$ integrin (also known as glycoprotein IIb/IIIa or GPIIb/IIIa) is expressed only by megakaryocytes and platelets. Multidomain adhesive proteins of the extracellular matrix are ligands for many integrins. Some integrins bind to specific domains of several different proteins, and some adhesive proteins bind to several different integrins. These interactions generally mediate cell-matrix adhesion. Cell-cell interactions result from integrin recognition of cell-surface members of the immunoglobulin superfamily. Binding of fibrinogen to $_{IIb\ 3}$ integrins on adjacent platelets serves as a molecular bridge that promotes platelet aggregation. Furthermore, fibrinogen simultaneously binds to the $_{M\ 2}$ integrin on leukocytes and to an immunoglobulin-like receptor on endothelial cells, promoting leukocyte adhesion to the endothelium.

Immunoglobulin-like Receptors

Immunoglobulin superfamily members contain a variable number of disulfide-stabilized -barrel motifs (also found in antibodies) that are linked to transmembrane and cytoplasmic domains (Fig. 51 and Table 52). The immunoglobulin-like motif provides a framework to which specific recognition structures for other proteins can be added. Some of these motifs also

Figure 5-1 Schematic diagrams of several types of cell-surface adhesion receptors. Integrins consist of noncovalently linked - and -subunits, both of which contribute to ligand binding. The platelet $_{IIb\ 3}$ integrin is illustrated. Immunoglobulin-like receptors contain a variable number of immunoglobulin homology domains, some of which bind ligands, while others extend the ligand-binding domains from the membrane. Shown is VCAM-1, which contains seven immunoglobulin domains. The platelet GPIb/IX/V complex consists of several leucine-rich protein subunits. CD44 contains an N-terminal domain that binds to hyaluronan. Each of the selectins contains an N-terminal carbohydrate-recognition domain that binds sialylated and fucosylated oligosaccharides on specific cell-surface glycoprotein ligands. Illustrated is P-selectin, the largest of the three selectins.

TABLE 5-1 -- Integrins on Blood Cells

Integrin Designation	Other Names	Expressed By	Ligand	Function
$_{1\ 1}$	VLA-1	Leukocytes, other cells	Collagens, LM	Adhesion to ECM
$_{2\ 1}$	VLA-2 GPIa/IIa	Leukocytes, platelets, other cells	Collagens, LM	Adhesion to ECM
$_{3\ 1}$	VLA-3	Leukocytes, other cells	Collagens, LM, FN	Adhesion to ECM
$_{4\ 1}$	VLA-4	Monocytes, lymphocytes, eosinophils	VCAM-1, FN	Adhesion to cells, ECM
$_{5\ 1}$	VLA-5 GPIc/IIa	Leukocytes, platelets, other cells	FN	Adhesion to ECM
$_{6\ 1}$	VLA-6 GPIc/IIa	Leukocytes, platelets, other cells	LM	Adhesion to ECM
$_{L\ 2}$	LFA-1 CD11a/CD18	Leukocytes	ICAM-1, -2, -3	Leukocyte aggregation and adhesion
$_{M\ 2}$	MAC-1, CR3 CD11b/CD18	Neutrophils, monocytes	ICAM-1, FIB	Neutrophil aggregation and adhesion to EC
$_{X\ 2}$	p150,95 CD11c/CD18	Neutrophils, monocytes	?	Adhesion to EC

D 2	CD11d/CD18	Monocytes, lymphocytes	ICAM-3	Leukocyte aggregation
IIb 3	GPIIb/IIIa	Platelets	FIB, FN, vWF, VN, TSP	Platelet adhesion and aggregation
V 3	VN receptor	Platelets, EC	FIB, FN, vWF, VN, TSP, collagens	Platelet adhesion, angiogenesis
4 7	LPAM-1	Lymphocytes	VCAM-1, MAdCAM-1, FN	Lymphocyte adhesion to EC and ECM

Abbreviations: CR, complement receptor; EC, endothelial cells; ECM, extracellular matrix; FIB, fibrinogen; FN, fibronectin; GP, glycoprotein; LFA-1, leukocyte functionassociated antigen-1; LM, laminin; LPAM-1, lymphocyte Peyers patch adhesion molecule; MAdCAM-1, mucosal addressin cell adhesion molecule-1; TSP, thrombospondin; VCAM-1, vascular cell adhesion molecule-1; VN, vitronectin; vWF, von Willebrand factor.

TABLE 5-2 -- Immunoglobulin-like Receptors

Name	Other Names	Expressed By	Ligand	Function
ICAM-1		Macrophages, EC, other cells	M 2 , L 2 , FIB	T-cell responses, leukocyte adhesion to EC
ICAM-2		EC	L 2	Leukocyte adhesion to EC
ICAM-3		Leukocytes	L 2 , D 2	T-cell responses, leukocyte aggregation
PECAM-1	CD31	Leukocytes, platelets, EC	PECAM-1, V 3 ?	EC junctions, leukocyte transmigration, platelet signaling?
VCAM-1	INCAM-110	Activated EC, smooth muscle cells	4 1 , 4 7	Mononuclear cell adhesion to EC
MAdCAM-1		EC of Peyers patches	4 7	Lymphocyte homing
CD2		T cells	LFA-3[a]	T-cell responses
CD4		T cells	Class II MHC[a]	T-cell responses
CD8		T cells	Class I MHC[a]	T-cell responses
CD3	T-cell receptor	T cells	Antigen on MHC[a]	T-cell responses
CD22		B cells	Sialylated glycoproteins	B-cell adhesion and signaling
Sialoadhesin		Macrophages in bone marrow and lymphoid tissue	Sialylated glycoproteins	Hematopoiesis?

Abbreviations: ICAM-1, -2, -3, intercellular adhesion molecules; PECAM-1, platelet and endothelial cell adhesion molecule-1. For EC, FIB, LFA, MAdCAM-1, and VCAM-1, see Table 51 footnote.

[a]LFA-3 and classes I and II MHC molecules are also immunoglobulin-like receptors.

recognize glycoconjugates (see following). The immunoglobulin-like molecules intercellular adhesion molecule (ICAM)-1, ICAM-2, and vascular cell adhesion molecule (VCAM)-1, expressed on endothelial cells, as well as ICAM-3, expressed on leukocytes, mediate cell-cell contact through recognition of specific integrins on leukocytes. Interactions between immunoglobulin-like molecules help to mediate adhesion between T cells and antigen-presenting cells. Thus, the immunoglobulin-like molecules CD8 and CD4 on T cells bind to the conserved membrane-proximal domains of class I and class II MHC proteins, respectively, whereas the T-cell receptor (CD3) binds to the polymorphic antigen-presenting domain. In addition, the immunoglobulin-like protein CD2 on T cells binds to the immunoglobulin-like protein leukocyte functionassociated antigen-3 (LFA-3) on antigen-presenting cells. In many tissues, the immunoglobulin-like neural cell adhesion molecule (NCAM) mediates cell-cell contact through homotypic interactions, that is, binding of an NCAM molecule on one cell to an NCAM molecule on another cell. Similarly, the immunoglobulin-like receptor platelet and endothelial cell adhesion molecule-1 (PECAM-1) (CD31) may use homotypic interactions to promote contacts between adjacent endothelial cells and mediate adhesion of leukocytes to platelets and endothelium.

Other Adhesion Receptors That Mediate Protein-Protein Interactions

Cadherins are cytoskeletally linked membrane proteins that mediate cell-cell contact in many organs through homotypic binding to cadherins on adjacent cells (Table 53). Cadherins have not been described on blood cells, but are found on endothelial cells, where, like PECAM-1, they help form cell junctions.

The GPIb/IX/V complex on platelets consists of leucine-rich protein subunits (Fig. 51). Under conditions of high shear like those found in the arterial circulation, this complex promotes the initial platelet adhesion to injured vessels by binding avidly to vWF exposed in the subendothelium. The GPIb complex is also expressed by endothelial cells exposed to inflammatory cytokines such as tumor necrosis factor-, but its function on the endothelium is less well understood.

TABLE 5-3 -- Other Adhesion Receptors

Name	Other Names	Expressed By	Ligand	Function
Cadherins		EC, many other cells	Homotypic binding	Formation of EC junctions
GPIb/IX/V		Platelets, activated endothelial cells	vWF	Platelet adhesion to ECM under shear
CD36	GPIV	Platelets, many other cells	Collagens, TSP	Platelet adhesion to ECM
CD44		Leukocytes, other cells	Hyaluronan, serglycin	Lymphopoiesis, lymphocyte activation?
Natural killer cell receptors		Natural killer cells	Carbohydrate?, MHC molecules	Recognition of virus-infected or other foreign cells

For abbreviations, see Table 51 footnote.

CD36 is a receptor with at least two membrane-spanning domains that is expressed on many cell types. On platelets, it has been implicated as a receptor for collagen and perhaps for thrombospondin; both interactions could facilitate adhesion to subendothelial matrix at sites of hemorrhage.

Lectin Adhesion Receptors

CD44 is an unusual transmembrane glycoprotein expressed to variable degrees on many subsets of leukocytes (Fig. 51). It has a membrane-distal domain that is structurally related to link protein of extracellular matrix and, like link protein, can bind to hyaluronan. CD44 also binds to serglycin, a proteoglycan secreted by hematopoietic cells. The hyaluronan-binding function of CD44 may modulate a number of leukocyte responses. The most clearly demonstrated function is in lymphopoiesis, where maturation of lymphocyte precursors requires contacts with bone marrow stromal cells bearing surface hyaluronan. CD44-hyaluronate interactions may also promote lymphocyte entry to and transit through organized lymphoid tissues. The membrane-proximal regions of CD44 are structurally diverse

because of the insertion of variable numbers of domains through alternative splicing. These insertions may regulate the ability of CD44 to bind hyaluronan and may mediate postbinding events that affect cell signaling.

The selectins are a group of three asymmetric receptors that terminate in a membrane-distal carbohydrate-recognition domain related to those in Ca^{2+} -dependent (C-type) animal lectins such as the hepatic asialoglycoprotein receptor (Fig. 51). L-selectin is expressed on leukocytes, E-selectin on cytokine-activated endothelium, and P-selectin on platelets and endothelial cells exposed to secretagogues such as thrombin (Table 54). The selectins mediate leukocyte adhesion to platelets, endothelium, or other leukocytes through Ca^{2+} -dependent interactions of the carbohydrate-recognition domains with cell-surface carbohydrates on apposing cells. High-affinity binding appears to require specific carbohydrate structures that are displayed on a limited number of membrane glycoproteins. The best characterized glycoprotein ligands for selectins are mucins that have large numbers of clustered, sialylated O-linked oligosaccharides. Site-specific construction of O-glycans with specific sialylated, fucosylated, and in some cases sulfated moieties is required for these mucins to bind optimally to selectins. In the case of one mucin, P-selectin glycoprotein ligand-1 (PSGL-1), sulfation of tyrosine residues near a specific O-glycan is required for binding to P- and L-selectin.

Natural killer cells express a group of membrane proteins that are distinct from the selectins but also have membrane-distal C-type carbohydrate-recognition domains. Although these receptors are important for interactions of natural killer cells with target cells, their carbohydrate-recognition functions have not been established.

I-type lectins are a subgroup of membrane proteins of the immunoglobulin superfamily that bind to carbohydrates instead of proteins (Table 52). The first two N-terminal domains appear to be necessary and sufficient for carbohydrate recognition. The N-terminal domain is a V-type structure that includes an unusual disulfide bond that is not found in the more common C-type immunoglobulin domains. I-type lectins bind well to sialylated glycans on some but not all glycoproteins. Depending on the lectin, recognition requires that the sialic acid be either 2,6- or 2,3-linked to an underlying galactose residue. The functions of I-type lectins have not been fully defined. CD22, expressed on B cells, may promote adhesion to other leukocytes. Sialoadhesin, expressed on bone marrow macrophages, may regulate hematopoietic cell differentiation.

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LIGAND BINDING VERSUS CELL ADHESION

Like all macromolecular interactions, adhesion molecules bind to each other with equilibrium affinities that are defined by their on and off rates. However, the efficiency of cell adhesion is not simply a function of the solution-phase equilibrium affinities of adhesion molecules for one another. Adhesion molecules in cell membranes and matrix are primarily limited to two dimensions, and even low-affinity molecular interactions may allow time for additional bonds to form along the plane of cell contact, stabilizing adhesion. The efficiency of cell attachment, and the ensuing strength of adhesion, may reflect multiple factors that dictate the probability of bonds forming between adhesion molecules on cell or matrix surfaces. The requirements for cell attachment are particularly stringent in the circulation, where

TABLE 5-4 -- **Selectins**

Name	Other Names	Expressed By	Ligand[a]	Ligands Expressed By	Function
P-selectin	CD62P, GMP-140, PADGEM	Thrombin-activated platelets and EC, cytokine-activated EC	PSGL-1	Leukocytes	Leukocyte adhesion to activated EC and platelets
E-selectin	CD62E, ELAM-1	Cytokine-activated EC	ESL-1, others	Leukocytes	Leukocyte adhesion to activated EC
L-selectin	CD62L, LECAM-1, LAM-1	Leukocytes	GlyCAM-1, CD34, PSGL-1, others	EC of lymph nodes, activated EC, other leukocytes	Leukocyte adhesion to activated EC and other leukocytes, lymphocyte homing

Abbreviations: EC, endothelial cells; ELAM-1, endothelial leukocyte adhesion molecule-1; ESL-1, E-selectin ligand-1; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; GMP-140, granule membrane protein-140; LAM-1, leukocyte adhesion molecule-1; LECAM-1, leukocyte endothelial cell adhesion molecule-1; PADGEM, platelet activationdependent granule external membrane protein; PSGL-1, P-selectin glycoprotein ligand-1.

[a] The selectins bind to sialylated, fucosylated, and (in some cases) sulfated oligosaccharides on specific glycoproteins, of which only some have been identified.

platelets and leukocytes must rapidly adhere to the blood vessel wall under shear conditions. Factors that affect bond formation include the number of adhesion molecules on a cell or matrix surface, the distance the binding domain of an adhesion receptor protrudes from the cell membrane, the lateral mobility of receptors, and the clustering of receptors on microvilli or other membrane domains. Cell adhesion can be further stabilized by events that occur after bond formation between adhesion molecules. For example, the cytoplasmic domains of the integrins, cadherins, CD44, L-selectin, and some immunoglobulin-like molecules interact with cytoskeletal components, allowing clustering of receptors into surface patches that strengthen adhesion and promote cell spreading or migration.

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REGULATION OF ADHESION RECEPTORS

To prevent inappropriate interactions of cells with each other or with extracellular matrix, the expression and function of adhesion receptors must be tightly controlled. Three primary control mechanisms are used: (1) the rate of synthesis of the receptor, (2) the time the receptor is displayed on the cell surface, and (3) the binding affinity/avidity of the receptor for ligands (Table 55). All these mechanisms are used to control interactions of blood and vascular cells, examples of which are given following.

Regulation of Synthesis

The synthesis of many adhesion receptors is regulated. Erythroid precursors synthesize integrins that mediate their interactions with stromal cells and with extracellular matrix in the bone marrow. As the precursors mature, synthesis ceases, resulting in loss of expression of cell-surface integrins by the time a mature erythrocyte enters the circulation. Lymphocyte precursors synthesize CD44 during differentiation in the bone marrow, stop synthesis prior to release, and resume synthesis during maturation in the thymus. On exposure to antigens, immunologically naive lymphocytes synthesize increased amounts of several adhesion receptors during their conversion to the memory phenotype; this process presumably allows these cells to become more adhesive in response to a subsequent antigenic challenge. When exposed to inflammatory cytokines such as tumor necrosis factor- and interleukin-1, endothelial cells transiently increase synthesis of E- and P-selectin, ICAM-1, and VCAM-1, resulting in an adhesive surface for leukocytes.

Regulation of Surface Expression

The surface expression of some adhesion receptors is tightly controlled. L-selectin is present on the plasma membrane of leukocytes, where it is available to bind to inducible ligands on the endothelial cell surface. Stimulation of the leukocyte causes L-selectin to be shed into the plasma by proteolytic cleavage, a mechanism for down-regulating adhesion. P-selectin is constitutively synthesized by megakaryocytes (where it is incorporated into platelets) and by endothelial cells. Rather than being directly delivered to the plasma membrane, however, it is sorted into secretory storage granules: the -granules of platelets and the Weibel-Palade bodies of endothelial cells. On stimulation of these cells by agonists such as thrombin or histamine, P-selectin is rapidly transported to the cell surface during fusion of granule membranes with the plasma membrane. Once on the surface of the endothelium, both E- and P-selectin are internalized and delivered to lysosomes for degradation. The cytoplasmic domain of P-selectin contains signals that direct sorting into secretory granules, internalization through coated pits of the plasma membrane, and movement from endosomes to lysosomes; the latter two signals are probably also present in the cytoplasmic domain of E-selectin. The net result of these events is to control the duration that E- and P-selectin are exposed on the endothelium, where they can mediate adhesion of leukocytes. Activation of leukocytes also mobilizes a pool of $_{2}$ integrins from storage compartments to the plasma membrane, although some of these molecules are also constitutively expressed on the cell surface. Finally, platelet activation redistributes a portion of the GPIb/IX/V complexes from ligand-accessible positions on the plasma membrane to sequestered, invaginated membrane domains known as the surface-connected canalicular system. This process, which requires interactions of the cytoplasmic domain of GPIb/IX/V with the cytoskeleton, may serve to down-regulate GPIb-mediated adhesion of platelets to immobilized vWF.

Regulation of Binding Affinity

Regulation of binding affinity is an important control mechanism for other adhesion receptors. Many integrins are constitutively present on the cell surface but interact poorly with their ligands. Cell activation by a number of agonists induces conformational changes in integrins so that they effectively recognize their ligands. An example is the $_{IIb\ 3}$ integrin, which requires platelet stimulation to bind fibrinogen. If this binding affinity were not regulated, circulating platelets would indiscriminately aggregate in the fibrinogen-rich plasma milieu. The mechanisms for activation-induced affinity increases in integrins are still being defined. The cytoplasmic domains of integrins can exert both positive and negative influences on binding affinity. Binding of specific cytoplasmic proteins to these domains may propagate structural changes to the extracellular ligand-binding regions of the integrins. Low-affinity ligand binding may stabilize the active conformation of integrins, which may explain why integrins on unactivated cells will sometimes bind to immobilized, multivalent adhesive proteins but not to the same proteins in solution. Cellular activation may also regulate the

TABLE 5-5 -- Regulation of Adhesion Receptors

Mechanism	Example
Synthesis	Erythroid precursor synthesis of $_{5\ 1}$ Lymphocyte synthesis of CD44 Cytokine-induced synthesis of E-selectin, P-selectin, ICAM-1, and VCAM-1 in endothelial cells
Surface expression	Proteolytic cleavage of L-selectin from leukocytes Redistribution of P-selectin from granule membranes to plasma membrane of platelets and endothelial cells Endocytosis of P- and E-selectin on endothelial cells
Ligand affinity	Activation-induced increased affinity of many integrins for their ligands Activation-induced increased avidity of CD44 for hyaluronan

For abbreviations, see Table 51 footnote.

binding avidities of CD44 and L-selectin through their cytoplasmic domains.

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CELL SIGNALING THROUGH ADHESION MOLECULES

In addition to their roles in cell-cell and cell-matrix contacts, adhesion molecules may affect cell signaling through indirect or direct mechanisms. Proteoglycans in the extracellular matrix can sequester growth factors that can be released to bind to surface receptors on nearby cells. Some chemoattractants bind to proteoglycan-like molecules on the surface of endothelial cells, where they can activate adherent leukocytes. Binding of adhesive ligands to cell-surface integrins, GPIb/IX/V, CD44, cadherins, CD36, PECAM-1, selectins, and perhaps other receptors can directly trigger intracellular events. The consequences of these signals include increases in affinity/avidity of other adhesion receptors for their ligands, shape change, secretion, proliferation, synthesis of cytokines and other molecules, and migration. In some cases, binding of a monovalent adhesive ligand to a receptor may induce a signal. More commonly, signaling requires cross-linking of several receptors through interactions with multivalent ligands in matrix or on apposing cells.

Many of the recent studies on adhesion receptor signaling have focused on integrins. Binding of the same ligand to different integrins can mediate different responses in the same cell. Furthermore, ligand binding to the same integrin expressed in different cells can result in different signals. These data suggest that very specific interactions occur between ligand-occupied integrins and intracellular components. The cytoplasmic domains of integrins are probably essential for initiating signaling. Phosphorylation of such domains, while an attractive candidate for regulation, has not been shown to have important effects on cellular responses in the integrins that have been studied. However, tyrosine kinases have been localized at the interaction zones between integrins and the cytoskeleton, and tyrosine phosphorylation of a number of proteins accompanies integrin-mediated cell signaling. The localization of tyrosine kinases near the cytoplasmic domains of integrins is analogous to the *lck*-encoded tyrosine kinase associated with the cytoplasmic domains of CD4 and CD8, which appears to function in T-cell signaling. Tyrosine phosphorylation can initiate a cascade of signaling events, including the activation of serine-threonine kinases that cause a variety of cellular responses. Ligand binding to integrins also results in generation of lipid second messengers, alkalinization of the cytoplasm, and influxes of Ca^{2+}. The latter event may be due to Ca^{2+} transport through an integrin-associated protein that has several membrane-spanning domains.

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COOPERATIVE INTERACTIONS BETWEEN SIGNALING AND ADHESION MOLECULES

Signaling and adhesion molecules frequently function cooperatively in sequential cascades to enhance the specificity of cell adhesion. Three examples of how these cooperative interactions facilitate blood cell responses are illustrated.

Platelet Adhesion and Aggregation.

At sites of hemorrhage in the arterial circuit, platelets rapidly adhere to the damaged vessel through interactions of GPIb/IX/V receptors with immobilized vWF exposed in the subendothelial matrix (Fig. 52). This interaction is actually facilitated by high shear rates, perhaps because of shear-induced conformational effects on GPIb or vWF, or both. An important feature of this initial adhesive event is that prior activation of the platelets is not required. After adhesion, however, the interaction of immobilized vWF with GPIb receptors triggers intracellular signals that lead to platelet activation. These signals may be synergistic with those produced by small amounts of locally produced thrombin to enhance platelet activation. Platelet activation, in turn, increases the affinity of platelet integrins for other matrix adhesive proteins such as collagen and fibronectin, which stabilize adhesion. Binding of these ligands transduces signals that propagate further activation responses such as spreading, secretion of granule contents, and recruitment of additional platelets through cell-cell contact mediated by binding of fibrinogen to activated $_{IIb\ 3}$ integrins. This adhesion cascade allows unstimulated platelets to home to the site of vascular injury and subsequently be activated by locally generated mediators.

Neutrophil Rolling, Spreading, and Migration.

Near sites of extravascular bacterial infections, neutrophils first tether to and roll on the endothelial surface of venules through the interactions of selectins with cell-surface carbohydrate ligands (Fig. 53). Neutrophil rolling on the endothelium must occur under shear forces, just as platelets must adhere to subendothelial matrix under shear forces, although the shear rates in postcapillary venules are lower than those in arteries. The selectins are capable of forming rapid, yet reversible, bonds with their ligands under these conditions. Just as the initial adhesion to vWF does not require prior activation of platelets, selectin-mediated rolling does not require prior activation of neutrophils. Instead, locally

Figure 5-2 Platelet adhesion and aggregation. In response to arterial injury under high shear forces, platelets rapidly adhere to the subendothelial matrix. The initial contacts are made between GPIb/IX/V on platelets and vWF in the matrix. These molecular interactions help activate platelets, thereby increasing the affinity of several platelet integrins for other adhesive matrix proteins such as fibronectin, laminin, and collagen. CD36 also interacts with both collagen and thrombospondin. Fibrinogen cross-links activated platelets into aggregates by binding to $_{IIb\ 3}$ integrins. The platelet plug then serves as an efficient surface for generation of thrombin and fibrin.

Figure 5-3 Neutrophil rolling, spreading, and emigration. At sites of tissue injury or infection, neutrophils first roll on the endothelial cells in postcapillary venules. These transient adhesive interactions are mediated by activation-induced expression of E- or P-selectin, or a ligand for L-selectin, on the endothelial cell surface. E- and P-selectin bind to carbohydrate ligands on the neutrophil, whereas L-selectin on the neutrophil binds to a carbohydrate on the endothelial cell. These molecular bonds can form under the shear forces in the venular circulation. The rolling neutrophils are then activated by locally generated inflammatory mediators that increase the affinity of $_{2}$ integrins for immunoglobulin-like receptors such as ICAM-1 on the endothelium. These bonds strengthen adhesion. Neutrophil migration between endothelial cells into tissues at the site of infection requires disengagement of old adhesive bonds and formation of new bonds between integrins, PECAM-1, and their respective ligands.

generated inflammatory mediators induce expression of E- or P-selectin, and probably a ligand(s) for L-selectin, on the endothelial cell surface. The requirement for activation of endothelial cells rather than leukocytes allows the latter to adhere to vessels only at the site of microbial invasion. Once situated on the vessel wall through selectin-mediated contacts, however, the neutrophils become exposed to activators such as platelet-activating factor, a phospholipid signaling molecule, and interleukin-8, a potent chemoattractant, both of which are presented on the surface of activated endothelial cells. Neutrophil activation increases the affinity of $_{2}$ integrins for immunoglobulin counter-receptors on the endothelial cell surface such as ICAM-1 and ICAM-2. Although these bonds will not form under shear conditions, they do form once neutrophils are transiently arrested on the endothelium by the selectins. The integrin-ICAM interactions strengthen adhesion, promote spreading, and ultimately favor migration, presumably because of disengagement of integrin-ICAM bonds and redistribution of integrins to the leading edge of the cell, where new bonds form. Subsequent interactions of leukocytes with PECAM-1 in interendothelial cell junctions facilitates transendothelial migration of the neutrophils into the underlying tissues. Adhesion of leukocytes to the endothelium disrupts cytoskeletal tethers to the endothelial cadherins; this leads to dissociation of homotypic cadherin interactions that normally prevent passage of leukocytes. Both the integrin- and PECAM-1-mediated adhesive events may signal cytoskeletal redistributions in leukocytes that enhance migration toward chemotactic molecules released in the vicinity of the infection. Once in the tissues, integrin recognition of extracellular matrix protein ligands may trigger secretion of proteolytic enzymes and production of superoxide anions, both required for optimal bactericidal function.

Adhesion of T Lymphocytes to Antigen-Presenting Cells.

The initial interaction of T lymphocytes with antigen-presenting cells requires that the T-cell receptor (CD3) recognize antigen

Figure 5-4 Adhesion between T lymphocytes and antigen-presenting cells. The initial contact is mediated by the T-cell receptor (CD3), which binds with low affinity but high specificity to a specific antigen presented by an MHC molecule. Additional contacts, also of low affinity, are between CD4 (on helper cells) or CD8 (on cytotoxic cells) and MHC, and between CD2 and LFA-3. These interactions signal the T cell to increase transiently the affinity of the $_{2}$ integrin, LFA-1, for the immunoglobulin-like molecules ICAM-1, -2, and -3 on the antigen-presenting cell. These bonds strengthen adhesion and transduce further signals to the T cell that cause proliferation and cytokine secretion. Additional signals result from binding of $_{1}$ integrins on the T cell to adhesive proteins in the extracellular matrix.

presented by the polymorphic domain of MHC molecules (Fig. 54). Subsequent interactions include the binding of CD8 or CD4 to MHC class I or II molecules, respectively, plus the binding of CD2 to LFA-3. These molecular contacts are all of low affinity but are highly specific because they first require specific antigen presentation to the appropriate T cell. The combination of these binding events triggers signals that increase the affinity of LFA-1 ($_{L\ 2}$), a $_{2}$ integrin on T cells, for its ligand, ICAM-1 on antigen-presenting cells, strengthening adhesion. After ICAM-1 binds to LFA-1, the T cell is further activated, resulting in cytokine secretion and proliferation. Additional signaling is mediated through binding of other integrins on T cells to protein ligands in the extracellular matrix.

Principles of Cooperative Interactions

The first key principle of the three responses just described is that the initial adhesive event, while relatively limited, is highly specific. Thus, platelets bind only to exposed subendothelial matrix, neutrophils bind only to endothelium near the site of infection, and T cells bind only to cells presenting specific antigen. The second key principle is that subsequent activation events strengthen cell adhesion and lead to further responses such as secretion, fibrin formation, migration, proliferation, or release of cytotoxic mediators. Activation often results from cooperative signaling by soluble agonists and by binding of ligands to adhesion receptors. Co-stimulation by multiple signals can amplify, and provide specificity to, cellular responses by mechanisms not always feasible for individual mediators. Thus, adhesion and cell signaling are highly interrelated processes.

The process of reversing cell adhesion, while less well understood, is equally important for the control of cell behavior. Some molecules such as the selectins can be proteolytically cleaved or internalized. The activation-induced increases in affinity of integrins and CD44 for their ligands are generally transient, but the mechanisms for return to the inactive conformation are obscure.

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ALTERED EXPRESSION OF ADHESION MOLECULES

The highly regulated nature of adhesive events by hematopoietic cells suggests that defects in, or excessive expression of, adhesion

TABLE 5-6 -- Genetic Deficiencies in Adhesion Molecules

Molecule	Disease	Laboratory Findings	Clinical Findings
IIb 3	Glanzmann thrombasthenia	Impaired platelet aggregation	Mucocutaneous bleeding
GPIb/IX/V	Bernard-Soulier syndrome	Impaired platelet adhesion to vWF	Mucocutaneous bleeding
2 integrins	Leukocyte adhesion deficiency-1	Impaired adhesion of activated leukocytes to EC	Frequent infections
Selectin ligands	Leukocyte adhesion deficiency-2	Impaired fucose metabolism resulting in defective carbohydrate ligands for selectins, impaired rolling of leukocytes on venules	Frequent infections

For abbreviations, see Table 51 footnote.

molecules could contribute to the pathogenesis of disease. A variety of clinical observations support this hypothesis.

Genetic Deficiencies in Adhesion Molecules

Genetic deficiencies in platelet adhesion receptors such as the GPIb complex (Bernard-Soulier syndrome) and the $_{IIb\ 3}$ integrin (Glanzmann thrombasthenia) result in hemorrhagic symptoms similar to those seen in patients with thrombocytopenia. Genetic deficiencies in the leukocyte $_{2}$ integrins (leukocyte adhesion deficiency-1) are associated with frequent severe bacterial infections and a failure of neutrophils to enter the infected tissues. Similar symptoms are seen in patients with a congenital defect in fucose metabolism that prevents synthesis of the carbohydrate ligands for selectins (leukocyte adhesion deficiency-2) (Table 56).

Dysregulated Expression of Adhesion Molecules

Inappropriate expression of adhesion molecules has been implicated in thrombotic and inflammatory disorders and in tumor metastasis. For example, erythrocytes from patients with sickle cell anemia adhere to each other and to the endothelium, contributing to vaso-occlusive crises. These adhesive events may reflect, in part, the expression of integrins and other adhesion molecules not normally found on mature erythrocytes. Inappropriate adhesion and activation of platelets on exposed atherosclerotic plaques may contribute to thrombosis. Dysregulated expression of selectins on the endothelium of ischemic blood vessels during myocardial infarction or shock may contribute to neutrophil-mediated tissue necrosis following reperfusion of the vessel. Mediators released while the neutrophils are adherent in the reperfused vessels may activate integrin function, strengthening adhesion and generating further signals that release destructive oxygen radicals and proteases within the vasculature. Finally, malignant cells appear to utilize molecules normally used for adhesion of blood cells to promote metastatic spread through interactions with platelets, endothelial cells, and extravascular matrix.

These examples underscore the importance of proper regulation of adhesion molecule expression in the physiology of blood cells. Further studies of the structure and function of these molecules may lead to effective treatments for disorders in which their expression becomes dysregulated.

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Chapter 6 - Control of Cell Growth and Differentiation

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INTRODUCTION

Somatic cells undergo one of three general fates: They (1) proliferate by mitotic cell division, (2) differentiate and acquire specialized functions, or (3) die and are eliminated from the body. Cell proliferation is necessary for growth of the organism and ensures repletion of cells lost to terminal differentiation, cell death, or cell loss; in the case of lymphocytes, it serves the additional function of amplifying immune responses to specific antigens. Differentiation provides the organism with a supply of cells to execute specific and specialized functions. In some cell types, such as muscle and nerve cells, differentiation and proliferation are mutually exclusive fates, and cells undergo terminal differentiation. In other cell types, such as those of the hematopoietic lineage, proliferation may continue after cells acquire differentiated characteristics. For example, erythroblasts, myeloblasts, and megakaryoblasts committed to particular differentiation pathways and possessing lineage-specific markers continue to proliferate, and T and B lymphocytes expressing antigen-specific receptors and immunoglobulins actively proliferate when appropriately stimulated. Cell death is an active process, initiated by the cell itself (apoptosis), and is physiologically as important as cell proliferation and differentiation. It allows tissue renewal and changes in cellular composition without undesirable cell accumulation. When the regulation of any of these three cellular processes goes awry and their balance becomes abnormal, the consequences to the organism are usually dire and result in either functional insufficiency or neoplasia. The relevance of these events to normal tissue function and neoplasia has led to investigations of their mechanisms and regulation at a molecular level.

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SIGNAL TRANSDUCTION AND CELL PROLIFERATION

Cells normally proliferate, differentiate, and die in response to signals from their environment. Of these, mitogenic signals and signaling mechanisms are the best studied and provide a paradigm for how cells respond to environmental signals in general. Cell proliferation is normally stimulated by extracellular growth factors interacting with specific receptors located at the cell surface, and signal transduction is the process by which information about growth factors at the cell surface is transmitted to the nucleus where ultimate control of most cellular events resides. Signal transduction pathways leading to differentiation and apoptosis are being unravelled and operate on similar principles. An overview of some of the biochemical events involved in mitogenic signal transduction is provided as an introduction to the discussion of cell cycle regulation. [1] [2] Chapter 5 provides a more detailed discussion of signal transduction.

Much of what is known about signal transduction is based on studies of the cellular biochemical response to platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). When these ligands bind to their cognate cell surface receptors (PDGF-R and EGF-R, respectively), the receptors dimerize, activate their intrinsic tyrosine kinase activity, and catalyze the transfer of phosphate groups from adenosine triphosphate (ATP) to tyrosine residues of specific cellular proteins, including the receptors themselves (Fig. 61). [3] Some other types of receptors, such as the T-cell antigen receptor, are not tyrosine kinases, and the tyrosine phosphorylation that they induce upon ligand binding is mediated by associated nonreceptor tyrosine kinases. The presence of phosphotyrosines in target proteins enables them to form noncovalent complexes with proteins containing SH2 domains (src-homology region 2; defined by homology to a region in the src retroviral oncoprotein), which are peptide domains that bind phosphotyrosine-containing peptides. [4] Thus, phosphorylation of the EGF-R and PDGF-R enables them to interact with SH2-containing proteins near or at the plasma membrane, which initiates downstream signaling events. Certain enzymes with SH2 domains, such as the 1 isoform of phospholipase C (PLC1), directly associate with phosphorylated EGF-R and PDGF-R and become tyrosine phosphorylated by them, which, in the case of PLC1, results in enhancement of enzymatic activity. Activation of PLC1 catalyzes the hydrolysis of phosphotidylinositol (PIP_3) into diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3), both of which act as second messengers that launch additional actions inside cells: DAG activates protein kinase C (PKC, a kinase that phosphorylates serine/threonine residues in substrate proteins) and IP_3 induces Ca^{2+} release from intracellular stores, which in turn activates Ca^{2+} /calmodulin-dependent serine/threonine protein kinases and other Ca^{2+} -dependent events.[5]

Another signaling molecule that is activated when receptors bind their ligand and become phosphorylated is Ras. This proto-oncoprotein is a member of the class of low-molecular-weight GTP-binding proteins that are inactive in their GDP-bound form and active in their GTP-bound form. The intrinsic GTPase activity of Ras, enhanced by the presence of a GTPase-activating protein (GAP), hydrolyzes bound GTP to GDP and maintains Ras in its inactive state. [6] [7] Following EGF binding by EGF-R, two cytoplasmic proteins, Grb2 and SOS, that exist as heterodimers in unstimulated cells physically link EGF-R with Ras in a quaternary complex through binding of phosphorylated EGF-R with the SH2 domain of Grb2 and the binding of SOS to Ras. Formation of this complex activates the function of SOS as a guanine nucleotide exchange factor (GEF), resulting in the conversion of Ras-GDP to Ras-GTP and Ras activation. Activation of Ras initiates a cascade of serine/threonine kinase activation, which may begin with the association of GTP-Ras with Raf-1, leading to activation of the latters serine/threonine kinase function. Raf-1 phosphorylates and activates the kinase (MAPK kinase, or MEK) that phosphorylates and activates MAP kinase (MAPK, or mitogen-activated protein kinase). The host of serine/threonine kinases activated following mitogen exposure phosphorylate diverse cellular proteins and modulate their activities. [8] [9] Prominent among these targets are transcription factors.[10] Phosphorylation may directly alter the ability of transcription factors to bind DNA or activate transcription. Alternatively, phosphorylation may indirectly activate a transcription factor by inactivating an antagonist. Mitogen stimulation may result in activation of protein phosphatases that dephosphorylate specific phosphorylated residues in certain transcription factors to alter function. [11] The end result is a rapid change in the transcriptional program of the cell independent of any new mRNA or protein synthesis. These initial post-translational

Figure 6-1 Mitogenic signal transduction. Shown are signal transduction pathways activated by the binding of mitogenic ligands (L), such as PDGF or EGF, to their cognate receptors (R) at the cell surface. Binding results in dimerization and autophosphorylation (P) of the receptors on tyrosine residues (Y). This enables them to associate with and activate specific SH2 domain-containing downstream components of the signaling pathway. In the case of phospholipase C1 (PLC1), association leads to tyrosine phosphorylation by the receptor kinase and an enhanced ability to hydrolyze phosphoinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3); in turn, DAG activates protein kinase C (PKC) and IP_3 mobilizes Ca^{2+} from intracellular stores. In the case of Grb2-SOS, association with phosphorylated receptors stimulates its ability to facilitate Ras GTP-GDP exchange; GTP-Ras activates the MAP kinase (MAPK) cascade, which eventually induces serine (S)/threonine (T) phosphorylation of nuclear proteins that modulate gene transcription. Note that MAPK is activated by serine/threonine and tyrosine phosphorylation and that both result from the activity of a single dual-function kinase, MAPK kinase.

changes lead to rapid transcriptional induction of certain genes (so-called immediate early response genes, e.g., c- *fos* and c-*myc*), which adds to the transcriptional reprogramming of the cell that eventually enables cells to undergo DNA synthesis and cell cycling. Not surprisingly, some of the transcription factors at the end of the mitogenic signaling pathway, such as c-Myc, c-Fos, and c-Jun, have oncogenic potential when inappropriately activated.

Ligands other than EGF and PDGF may use different schemes for signal transduction. Neuroactive and vasoactive peptides (e.g., epinephrine and thrombin) activate responsive cells through specific receptors that have seven membrane-spanning domains. These receptors are typically coupled to heterotrimeric G-proteins that resemble Ras in being regulated by GTP and GDP.[6] [12] These receptor-coupled G-proteins are linked to effector enzymes (e.g., adenylyl cyclases) that generate molecular signaling intermediates (e.g., cyclic AMP) upon ligand binding. Steroid and thyroid hormones and retinoids can enter cells by virtue of their lipophilic nature. Their receptors are intracellularly located and able to bind sequence-specific DNA and directly modulate the transcription of responsive genes. Thus, the receptors are transcription factors whose activities are influenced by binding of the cognate hormone. [13] Interferon signaling uses a different signal transduction paradigm. Tyrosine kinases of the Janus kinase (JAK) family associate with interferon receptor subunits. Upon ligand binding, association of the receptor subunits allows these JAKs to phosphorylate and activate each other and to phosphorylate the associated receptors. Specific members of the STAT (signal transducers and activators of transcription) family of latent cytoplasmic transcription factors, which have SH2 domains, dock to the receptor phosphotyrosines and become phosphorylated by JAK. Tyrosine phosphorylation allows STATs to dimerize and translocate to the nucleus, where they bind sequence-specific DNA and modulate transcription of interferon-responsive genes.[14] Signal transduction using JAK-STAT protein is used by many peptide ligands and cytokines of hematologic interest (e.g., erythropoietin, IL-2, IL-3, IL-4, IL-6, IL-12).

The signal transduction shemes outlined permit a single event, ligand-receptor interaction, to have several downstream consequences. Its multiplex, frequently cascading nature allows signal amplification and diversification but also permits their modulation and fine regulation. Signaling pathways can intersect and interact at different levels, which allows one ligand to modify the signals generated by another ligand. For example, STATs can be phosphorylated by receptor tyrosine kinases, such as PDGF-R and EGF-R, as well as by JAK and can undergo serine/threonine phosphorylation, which modulates their transcriptional activity. [14] This allows PDGF and EGF to initiate some events usually initiated by cytokines and interferons, and the phenotypic changes brought about by cytokines and interferons may be altered in the presence of PDGF and EGF.

Transcription factors are final participants in afferent signal transduction pathways and initiators of cellular responses to these signals. [10] In general, they are sequence-specific DNA-binding proteins that modulate the expression of genes to which they bind. When these factors bind their cognate DNA sequence, they interact with the basal transcription machinery either directly or via intermediary proteins (co-activators and co-repressors) to initiate, enhance, or inhibit transcription. Transcription factors have peptide domains with characteristic secondary structures that are responsible for their ability to bind DNA. Many bind DNA only as dimers, making the peptide domain responsible for dimerization essential for DNA binding. Transcription factors use one of a number of peptide motifs to dimerize and bind DNA, among them zinc finger, basic region-leucine zipper (bZip), basic region-helix-loop-helix (bHLH), or helix-turn-helix motifs. [15] Factors that activate gene transcription generally do so because they have a distinct transcriptional activation domain that is frequently acidic in nature, glutamine rich or proline rich. Transcriptional gene regulation is highly complex, not only because of the multitude of transcription factors present in cells but also because of the ability of many factors to heterodimerize and form combinatorial pairs that have DNA-binding, transactivation, and/or regulatory properties that differ from those of the parental homodimers. A striking example is provided by heterodimers containing the Id protein, which is an HLH protein that can dimerize with selected bHLH proteins, such as the myogenic transcription factor MyoD, but that does not possess a DNA-binding basic region (see following). Id-containing heterodimers are incapable of binding DNA, making Id a negative transcriptional regulator that inhibits the function of positive factors. Negative gene regulation also occurs by active repression of transcription, and certain transcriptional repressors have been shown to recruit factors that bind histone deacetylases. Histones are a family of nuclear proteins that interact with DNA and organize it into higher-order structures consisting of DNA wrapped around a histone core (nucleosomes). Acetylation of histones masks their basic residues, destabilizes their interaction with DNA, loosens nucleosome DNA, and facilitates transcription. Deacetylation of histones, in contrast, stabilizes their interaction with DNA, which tightens nucleosome DNA and inhibits transcription. Reversible, regional histone acetylation, through recruitment of co-activators with acetyltransferase activity or recruitment of co-repressors with deacetylase activity, may be a general mechanism by which transcription factors facilitate or repress expression of specific genes. [16]

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THE CELL CYCLE

A cell stimulated to divide passes through a cyclic series of states, defined by biochemical and morphologic criteria, collectively termed the cell cycle (Fig. 62). Passage through the cell cycle provides an ordered sequence to the complex series of events necessary for the production of two identical progeny cells. The normal cell cycle is divided into discrete and sequential phases, S, G_2 , M, and G_1 .

S Phase.

S phase is the period of wholesale DNA synthesis during which the cell replicates its genetic content; a normal diploid somatic cell with a 2N complement of DNA at the beginning of S phase acquires a 4N complement of DNA at its end. The duration of S phase may vary from only a few minutes in rapidly dividing, early embryo cells to a few hours in most somatic cells. Early embryo cells generally live off the accumulated stores of maternal RNA and proteins present in the egg and are transcriptionally silent, whereas cells in later development and mature organisms must actively transcribe subsets of their genes to survive and maintain specialized functions. The longer time required for the latter to complete S phase may be adaptive, allowing the cell to coordinate DNA replication with

Figure 6-2 The cell cycle. The somatic cell cycle is divided into phases of DNA replication (S), mitosis (M), and the gaps in between (G_1 between M and S; G_2 between S and M). G_0 is not shown for the sake of simplicity but would be a side loop exiting and entering G_1 . The point in late G_1 at which cells become committed to DNA replication is called the restriction point (R). The inner circle shows the pattern of Rb phosphorylation through the cell cycle, with the density of stippling indicating the degree of Rb phosphorylation. Places in the cell cycle where individual cyclins (A, B, D1, D2, E) appear are shown by the outer arcs.

transcription and to preserve higher-order structural information, influencing gene expression for transmission to progeny cells.

M Phase.

M phase or mitosis is the period of actual nuclear and cell division during which the duplicated chromosomes are divided equally between the two progeny cells. It is obvious microscopically as the period of chromosome condensation, nuclear envelope breakdown, chromosome segregation to opposite poles, reformation of nuclear envelopes (which completes nuclear division, or karyokinesis), and physical separation of the two daughter cells (which completes cell division, or cytokinesis). A cell entering M phase has a 4N DNA content and finishes as two cells, each with an identical 2N complement of DNA.

G_1 and G_2 Phases.

$G_1$and G_2phases were originally conceived of as gaps between the distinctive M and S phases of the cell cycle. G_2 is the period between S and M when cells have finished replicating their DNA, are preparing to divide, and have a 4N DNA content. For most cells entering S phase, passage through G_2 is automatic, and the duration of G_2 is fixed except under unusual circumstances. G_2 duration can be extremely short and is essentially undetectable in rapidly proliferating, early embryonic cells. G_1 , which occupies the period between M and S, is the interval between the completion of one round of cell division and initiation of the next. Its duration is the most variable, can be prolonged (depending on the cell type), and is subject to regulation by environmental factors such as the availability of growth factors and nutrients. It is the period of cell growth, and a certain increase in mass is usually required before the cell initiates the next S phase. As a first approximation, the amount of time a cell spends in G_1 is inversely related to its rate of proliferation. When conditions are unsuitable for proliferation, cells arrest in G_1 , and those that are already in S, G_2 , or M usually complete the round to which they

have been committed and arrest only when they reach G_1 again. On the other hand, when rapid cell proliferation is mandated, as in embryos shortly after fertilization, G_1 is virtually undetectable, and there is no cell enlargement. This results in the original mass of egg cytoplasm being partitioned among thousands of cells within a few hours without a noticeable increase in size. G_1 has been subdivided into segments and regulatory points largely based on the study of the proliferative response of cells to sequential application of different growth factors, nutrients, and metabolic inhibitors. From the standpoint of cell cycle regulation, a particularly important point in G_1 is the restriction point or R, which occurs near the G_1 -S boundary. This is the point at which cells become committed to entering S phase regardless of growth factor stimulation or the availability of essential amino acids, and is analogous to the commitment point in the yeast cell cycle called Start.

G_0 Phase.

Viable cells may remain for prolonged periods in a nonproliferative G_0 state. Such cells have a 2N DNA content and may be difficult to distinguish from cells in prolonged G_1 . The distinction, if not obvious morphologically, may be made biochemically since they have differences in their protein and RNA metabolism. Terminally differentiated cells, such as neutrophilic granulocytes, have irreversibly exited the cell cycle during the process of differentiation and are examples of cells in G_0 . However, other cells reversibly enter G_0 and may be induced to return to G_1 and begin cycling with appropriate stimuli. For example, hepatocytes are usually in G_0 unless partial hepatectomy induces them to proliferate to reconstitute the functional mass of the liver; resting, antigen-specific lymphocytes are in G_0 until antigen and cytokine stimulation induces them to proliferate.

The enforced sequence, G_1 -S-G_2 -M, during normal progression through the cell cycle means that a cell must duplicate its DNA before dividing and that it must divide before duplicating its DNA again. This sequence ensures euploidy, and its enforcement maintains genetic stability. The dependence of later events in the cell cycle on normal completion of earlier events is ensured by checkpoint control mechanisms that prevent a cell that has not successfully completed one phase of the cycle from entering the next.[17] [18] The existence and importance of checkpoints are illustrated by yeast mutants defective in the *RAD9* gene. Normally, yeast cannot enter M phase until their DNA is fully replicated. Yeast defective in the *RAD9* gene enter M phase even if they are prevented from completing DNA replication. They also die more rapidly, presumably because the progeny inherit incomplete or damaged genetic material. In mammalian cells, the activity of checkpoints is seen after exposure to DNA-damaging agents, such as ionizing radiation, that delay cell entry into S and M by inducing temporary G_1 or G_2 arrest. This delay allows cells time either to repair the damaged DNA or to execute a program of self-destruction or apoptosis if the damage is irreparable. Caffeine antagonizes the G_2 /M checkpoint mechanism, and cells that are exposed to caffeine fail to arrest in G_2 and are less viable after irradiation. The p53 tumor suppressor protein participates in the G_1 /S checkpoint

mechanism, and its loss predisposes to cancer (see following).

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CELL CYCLE REGULATION

The progression of cells through the cell cycle is driven by serine/threonine kinases of the Cdk (cyclin-dependent kinase) family. As their name implies, the catalytic activities of these kinases are under the stringent control of associated regulatory proteins called cyclins. The latter were so named because levels of the first to be described, cyclins A and B, were seen to fluctuate periodically with the cell cycle. Numerous Cdks and cyclins exist in the cell and form various combinatorial pairs with distinct activities. Control of cyclin/Cdk activity exists at many levels and occurs by the appearance and disappearance of the cyclins at specific phases of the cell cycle, by post-translational modification of Cdks, and by association with Cdk inhibitors. In turn, cyclin/Cdks regulate cell cycling by modulating the activity of critical transcription factors.

Cyclins and Cyclin-Dependent Kinases

Entry into M

The molecular mechanisms regulating cell entry into M [19] were first revealed by studies of a conditional cell cycle mutant of *Schizosaccharomyces pombe* (fission yeasts) called *cdc2* (cell division cycle 2). Grown under nonpermissive conditions, these mutants arrest in G_1 or G_2 and do not enter S or M. Cloning of the *cdc2* gene revealed that it encodes a 34-kd serine/threonine kinase. Human cells have a structurally and functionally similar protein, which underscores the evolutionary conservation and importance of this regulatory mechanism. p34 *cdc2* is the prototypic Cdk (Cdk1), and subsequently discovered members of this kinase family have been designated Cdk2, Cdk3, and so forth. An independent line of study examining the effect of cytoplasmic extracts from mature *Xenopus* frog eggs microinjected into immature frog oocytes showed that these extracts contain a material that induces oocytes to mature and undergo typical M-phase changes such as nuclear membrane breakdown. After purification, the maturation-promoting factor (MPF) in these extracts was found to contain two proteins: one is p34 *cdc2*, and the other is a B-type cyclin. Cyclin B has no known enzymatic function and plays a regulatory role in the MPF complex, evidenced by the fact that p34 *cdc2* exhibits kinase/MPF activity only in association with cyclin B.

Cyclin B levels increase during S and G_2 (Fig. 62), and levels of the cyclin B/p34 *cdc2* complex sufficient for the G_2 -M transition are reached well before the onset of M. Mitosis is not prematurely triggered because the complex accumulates in an inactive form (Fig. 63). During S and G_2 , the p34*cdc2* complexed with cyclin B accumulates as a multiply phosphorylated protein. In human p34 *cdc2*, phosphorylation of threonine (Thr) 161 stabilizes its association with cyclin B and is essential for activity. The kinase responsible for Thr 161 phosphorylation, CAK (Cdk-activating kinase), is itself a Cdk (designated Cdk7) that associates with a novel cyclin, cyclin H. On the other hand, phosphorylation of Thr 14 and tyrosine (Tyr) 15 in p34 *cdc2* suppresses its kinase activity and keeps the cyclin B/p34 *cdc2* complex inactive. Activation of p34*cdc2* just prior to entry into M requires dephosphorylation of both Thr 14 and Tyr 15, which is accomplished by a dual-specificity phosphatase, Cdc25. The kinase and phosphatase that regulate p34 *cdc2* activity and time cell entry into M are themselves regulated by phosphorylation. This inhibits the kinase responsible for Tyr 15 phosphorylation (homolog of the product of the *S. pombe wee1* gene) and enhances the phosphatase function of Cdc25. Once activated, cyclin B/p34 *cdc2* can phosphorylate Cdc25 and create a self-amplifying feedback loop that generates more oocyte MPF activity from a small initial amount of active MPF and the large preexisting stock of inactive MPF. What starts this sequence of events by initially phosphorylating Cdc25 is unclear, although cyclin A/Cdks are candidates because they are active prior to cyclin B/p34 *cdc2* activation, have MPF activity, and inhibition of cyclin A during S prevents entry into M.

Activated p34*cdc2*/cyclin B complex can phosphorylate serine/threonine residues in many cellular proteins. Discerning its physiologic substrates is difficult, however, because many other cyclin/Cdk complexes are also active. Candidates include the lamins and vimentin, which are respectively nuclear and cytoplasmic proteins important for the structural organization of their compartments. These proteins undergo M-phase phosphorylation and are substrates for p34 *cdc2*/cyclin B kinase activity in vitro. Phosphorylation of lamins is important for nuclear

Figure 6-3 Regulation of cell entry into M by p34 *cdc2*/cyclin B (maturation promoting factor [MPF]). p34 *cdc2* kinase activity controls cell entry into M and is regulated during the cell cycle. Association with cyclin B, which first appears during S phase, is necessary for its kinase activity, and formation of the p34 *cdc2*/cyclin B complex (MPF) is stabilized by phosphorylation of Thr (T) 161. Accumulating MPF is maintained in an inactive state by phosphorylation of Thr 14 and Tyr (Y) 15, which is catalyzed by the homolog of the *S. pombe wee1* gene product and another kinase. At the G_2 /M transition, MPF is activated by dephosphorylation of Thr 14 and Tyr 15 by the homolog of the *S. pombe cdc25* gene product. This may be a self-amplifying reaction because activated MPF can phosphorylate and activate more Cdc25. Activated MPF phosphorylates cellular substrates and brings about the biochemical changes needed for M phase. During progression through M, degradation of cyclin B generates inactive p34*cdc2* and permits cell exit from M.

lamina disassembly and envelope breakdown, and phosphorylation of vimentin may cause depolymerization of vimentin intermediate filaments. If these are physiologic substrates, p34 *cdc2*/cyclin B kinase activity may initiate the structural reorganization that is essential for mitosis. As M phase progresses, p34 *cdc2*/cyclin B is inactivated by degradation of the cyclin B component via the ubiquitin pathway. Inactivation is important for cells to exit M, because recombinant cyclin B that is resistant to proteolysis induces cell arrest in M.

Entry into S

The rate of cell proliferation in somatic cells is generally determined by events in G_1 , with the irreversible decision to undergo DNA replication made at R. The importance of the decisions made in G_1 and their relevance to neoplastic cell behavior have made the identification of regulatory factors involved in G_1 and the G_1 -S transition a prime objective. [20] In *S. pombe*, p34*cdc2* is responsible for both the G_1 -S and G_2 -M transitions. In higher organisms, p34*cdc2* has a clear role in G_2 and M, but appears unnecessary for G_1 - and S-phase events, which are conducted by other Cdks. The specific function of several Cdks has yet to be defined, but some, like Cdk2 and Cdk4, are known to be important in G_1 and S. Their activities are regulated by the cyclins with which they pair, and the permitted partnerships determine where and how in the cell cycle the individual cyclin/Cdk complexes function. The disappearance of cyclins A and B during M and their reappearance only in S (Fig. 62) preclude a role for them in G_1 . Other cyclins, therefore, must be important in G_1 and the G_1 -S transition, and human cyclins potentially fulfilling these roles (C, D, and E) have been identified by functional complementation of *S. cerevisiae* (budding yeast) mutants deficient in three G_1 cyclin genes (*CLN13*). D-type cyclin genes were independently identified during the search for an oncogene involved in parathyroid adenomas and for genes induced during mitogenic stimulation of macrophages. Studies indicate that the D cyclins (of which there are threeD1, D2, and D3) associate with Cdk2, 4, and 6 and that Cdk4 and 6 associate only with D cyclins. Cdk2, in contrast, associates with cyclins A and E as well, and cyclin A associates with p34 *cdc2* as well as Cdk2.

D-type cyclins are clearly important regulators of G_1 events. Cyclin D/Cdk4, 6 complexes phosphorylate Rb, the protein product of the retinoblastoma susceptibility (*Rb*) gene and an inhibitor of S-phase entry in its hypophosphorylated state (see following). Rb substrate specificity is conferred in part by the D cyclins, which bind Rb via a peptide motif that is shared with Rb-binding viral oncoproteins, SV40 T antigen and adenovirus E1A. With the observations that inhibition of cyclin D1 expression

prevents cell entry into S and that cyclins D1 and D2 can reverse Rb-induced G_1 growth arrest, these data suggest that D-type cyclins function as regulators of G_1 progression and entry into S phase. Cyclin E is thought to play an important role in the G_1-S transition. In mitogen-stimulated cells, it appears later in G_1, peaks near the G_1/S boundary, and declines in S (Fig. 62). Cyclin E associates primarily with Cdk2, and in cells in G_1 is found in a quaternary complex with Rb-related p107 protein, transcription factor E2F, and Cdk2. This complex disappears as cells enter S, just as a similar complex containing cyclin A instead of E makes its appearance. Cyclin E induces Rb phosphorylation and counters G_1 growth arrest induced by hypophosphorylated Rb. Cyclin A first appears at the beginning of S and declines in G_2 and M and has an expression pattern that parallels but precedes that of cyclin B (Fig. 62). It may associate with either $p32^{cdc2}$ or Cdk2 and, in S phase, is found in a quaternary complex with Cdk2, E2F, and p107. These data and the results of cyclin A inhibition and addition experiments have led to the view that cyclin A plays a major role in driving S-phase events. Cyclin A in complex with $p32^{cdc2}$ may trigger the G_2-M transition by phosphorylating Cdc25 and initiating activation of cyclin B/$p32^{cdc2}$.

Inhibitors of Cyclin-Dependent Kinases

More recently, another layer of cell cycle regulation complexity has been discovered with the identification of inhibitors of Cdk and cyclin/Cdk complexes. [21] The first inhibitor to be identified and cloned in mammalian cells was p21 (Waf1, Cip1, Sdi1), which binds several different cyclin/Cdk complexes and has been classified as a universal inhibitor. While addition of p21 inhibits the activity of many cyclin/Cdk complexes, p21 is also found in complexes with kinase activity, indicating that the stoichiometry of p21 association with cyclin/Cdk probably determines inhibition. p21 also binds PCNA (proliferating cell nuclear antigen, a processivity factor during DNA synthesis) and inhibits its ability to facilitate the function of DNA polymerase-, the polymerase responsible for DNA replication. While these properties of p21 readily account for its ability to induce cell cycle arrest, the regulation of p21 expression sheds even more light on its potential function. Expression is transcriptionally induced by p53, the tumor suppressor protein activated by DNA damage (see following), suggesting that p21 is responsible for halting cell proliferation after DNA damage to allow time for damage assessment and repair. However, p21 can also be abundantly expressed in cells lacking functional p53, indicating that p53-independent pathways of expression exist. These other pathways may account for increased p21 expression in other cellular circumstances, such as in senescent cells that have reduced proliferative capacity and in cells induced to terminally differentiate. Other Cdk inhibitors with structural and functional similarities to p21 exist. These include p27 (Kip1) and the more recently described p57 (Kip2), which, like p21, bind multiple cyclin/Cdk complexes. Up-regulation of p27 is thought to mediate the growth arrest of TGF--treated or contact-inhibited cells. Regulation of p27 expression differs from that of p21 and seems to occur post-transcriptionally, with levels of the protein increasing while mRNA levels remain constant. Studies to date have not revealed a significant role for defects in universal Cdk inhibitors in carcinogenesis.

p16 (INK4a, MTS1, Cdk4I) and p15 (INK4b, MTS2) are Cdk inhibitors that differ structurally from the universal inhibitors and have restricted Cdk-binding specificity. Both p16 and p15 bind Cdk4 and Cdk6 exclusively, and binding inhibits Cdk4 and Cdk6 association with cyclin D and kinase activity. Rb is believed to be a critical target of cyclin D/Cdk4,6 kinase activity, and Rb phosphorylation prevents it from arresting cells in G_1 (see following). Thus, p15 and p16 may inhibit cell proliferation by preventing Rb phosphorylation. This view is supported by the observation that p16 overexpression inhibits proliferation of cells expressing Rb but not of cells devoid of Rb. p16 is probably important for preventing neoplastic cell proliferation in vivo. Its importance was initially suggested by the finding that the *p16* and *p15* genes were, in the region of chromosome 9, mutated in the germline of patients with familial melanoma and in the genome of many human tumor cell lines. Questions arose when *p16* was found to be much less frequently mutated in freshly isolated human tumors. However, with the discovery that p16 knockout mice are cancer prone and that many human tumors carry *p16* that is unmutated but transcriptionally silent due to aberrant methylation of upstream regulatory CpG islands, there is no doubt of its importance in carcinogenesis.

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TRANSCRIPTIONAL REGULATION AND THE CELL CYCLE

Cell proliferation requires coordinate expression of the genes needed for the complex biochemical processes involved, and proliferating cells must orchestrate the timely expression of these genes beginning with their transcription. Since the necessary genes are many (those encoding cyclins, DNA polymerases, accessory factors for DNA replication, purine/pyrimidine biosynthetic enzymes, just to name the obvious), a mechanism for orchestration must exist. Studies of the Rb protein have provided insights into how this occurs.[22] Rb is the product of the retinoblastoma susceptibility gene, *Rb*, which predisposes individuals to retinoblastomas and other tumors when only one functional copy is present in the germline. *Rb* is a tumor suppressor gene, and certain somatic cells (e.g., retinoblasts) become transformed when both copies are functionally inactivated. *Rb* is ubiquitously expressed, and Rb is normally present throughout the cell cycle. The known antiproliferative effect of Rb is explained by the cyclic regulation of its activity by phosphorylation (Fig. 64). Rb is hypophosphorylated in early G_1 . As cells progress through G_1 and approach the G_1 -S transition, Rb is phosphorylated on serine and threonine residues, probably by cyclin D/Cdk4 or 6 complexes, and becomes hyperphosphorylated. This persists until M, at which time Rb is dephosphorylated and returned to its hypophosphorylated, early G_1 state. Hypophosphorylated Rb inhibits cell cycling, and inhibition can be relieved either by phosphorylation or by Rb binding to certain viral oncoproteins that enforce cell cycling, such as adenovirus E1A, SV40 large T antigen, or human papillomavirus (HPV) E7. Two other cellular proteins, p107 and p130, are related to Rb (p105) structurally and functionally and have similar but not identical properties.

The link between Rb proteins and transcription of genes needed for cell cycling is established by members of the E2F family of transcription factors. Originally described as a cellular factor necessary for adenovirus E2 gene transcription, E2F proteins heterodimerize with members of the DP family of proteins to activate transcription. E2F proteins complex with hypophosphorylated Rb, which renders them transcriptionally inactive. E2F is released and its transcriptional activity restored by Rb phosphorylation or Rb binding to E1A, large T, or E7 protein (Fig. 64). In normal cells, cyclic Rb phosphorylation results in cyclic E2F activation and expression of E2F-responsive genes, a process that links specific cell cycle events near the end of G_1 (accumulation of cyclin D/Cdk4 complexes) to specific gene activation and production of proteins necessary for DNA synthesis (e.g., dihydrofolate reductase, thymidine kinase). In cells where Rb is absent or inactive due to homozygous deletion or mutation (e.g., in retinoblastoma and many other types of tumor cells) and in virally transformed cells where hypophosphorylated Rb is inactivated by viral oncoproteins, loss of normal regulation of E2F transcriptional activity leads to deregulated expression of cell cycleregulated genes and cell proliferation.

Figure 6-4 Regulation of the retinoblastoma susceptibility gene product (Rb) through the cell cycle. Rb is regulated by serine/threonine phosphorylation (P) through the cell cycle. Non- or hypophosphorylated Rb present in early and mid-G_1 can bind transcription factor E2F and thereby alter or sequester its activity. In late G_1 , Rb becomes hyperphosphorylated (perhaps due to Cdk/cyclin D kinase activity), releasing E2F for transcriptional duties or formation of other complexes. Removal of phosphate groups in M restores Rb to its hypophosphorylated form. In cells transformed by adenovirus E1A, SV40 large T, or HPV E7, these oncoproteins can bind hypophosphorylated RB and displace E2F (dashed lines). The Rb-like p107 protein also binds E2F and is found in quaternary complexes with Cdk2-cyclin E in G_1 or with Cdk2-cyclin A in S phase.

The regulatory model outlined is consistent with Rb being an integral and necessary part of the cell cycle mechanism, helping to drive cells to enter S through phosphorylation-regulated interactions with E2F. Alternatively, Rb may be a regulatory element of the cycling mechanism, such that Rb phosphorylation is permissive for cell cycling, but maintaining Rb in a hypophosphorylated state is how differentiation and growth-inhibitory signals stop cells from cycling. The latter view is supported by the phenotype of *Rb* knockout mice. *Rb*$^{-/-}$ embryos arise in expected numbers up to day 13 of gestation but fail to develop much beyond that, with evidence of abnormal central nervous system (CNS) development and defective erythropoiesis. Their development more than halfway through gestation and grossly normal appearance indicate that Rb is not essential for cell proliferation, while the failure of CNS and erythroid development argues for a role for Rb in enforcing terminal differentiation in these tissues. Neither are the p107 and p130 members of the Rb family obligatory components of the cell cycle: knockout of *p107* and *p130* individually permits normal mouse development, and knockout of both in combination results in abnormal chondrocyte development and neonatal lethality. Although the developmental abnormalities in *Rb*$^{-/-}$ embryos suggest a tissue-restricted role for Rb in normal development, Rb is part of a more global regulatory pathway for controlling cell growth, evidenced by the fact that the p16-cyclin D/Cdk4-Rb pathway is corrupted in many types of cancers. Depending on the particular cancer, one or another element of this pathway is frequently found to be altered: growth-inhibitory Rb and p16 are inactivated by mutation or epigenetic silencing, while growth-promoting cyclin D or Cdk4 are activated by deregulation or overexpression.

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p53 AND DNA DAMAGE CHECKPOINTS

In multicellular organisms, host welfare after exposure to DNA-damaging agents demands that cells either repair their damaged DNA or eliminate themselves if the damage is severe. The *p53* tumor suppressor gene plays a central role in this protective mechanism, and its derangement predisposes to cancer. [23] Originally described as a 53-kd cellular protein that is overexpressed in a wide variety of transformed cells, p53 was believed to be an oncoprotein, because *p53* genes cloned from nontumorigenic cell lines could immortalize and help transform primary rodent cells. Subsequently, mutations were found in these *p53* genes, and normal *p53* was found to suppress rather than promote cell transformation. The results of many studies have contributed to the current view that normal *p53* is a tumor suppressor gene with a negative effect on cell proliferation, generally stopping cells at the G_1-S transition. Some mutant *p53* genes exert a dominant negative effect and transform cells by inhibiting the activities of the normal gene. The facts that *p53* is the most frequently deleted or mutated gene in human tumors and that certain kindreds prone to developing cancer (Li-Fraumeni syndrome) are heterozygous for mutated *p53* in their germline attest

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to its importance in preventing malignant transformation. At the biochemical level, p53 is a homotetrameric, sequence-specific DNA-binding protein with transcriptional activation properties. Mutant p53 from tumor cells loses this ability to bind DNA and fails to activate transcription. p53 associates with a variety of other cellular (TBP [TATA-binding protein], c-Abl, Mdm2, etc.) and viral (SV40 large T antigen, adenovirus E1B, and HPV E6) proteins, and these associations have functional significance. For example, the Mdm2 oncoprotein complexes with p53 and inhibits its ability to transactivate a responsive promoter, while the viral oncoproteins that complex with p53 either inactivate its function or accelerate its decay.

A gene that plays so prominent a role in oncogenesis might be expected to perform crucial cellular functions and be indispensible for normal development. It was surprising, therefore, to find that mice with homozygous deletions of *p53* developed normally and were abnormal only in being prone to developing spontaneous tumors. How *p53* prevents tumorigenesis but is not essential for normal growth and development is best explained by a conditional requirement for its activity. Cells without p53 function fail to arrest in G_1 after DNA damage, and the absence of this checkpoint mechanism predisposes to genomic instability and failure to eliminate genetically damaged cells by apoptosis. Under conditions that engender DNA damage (e.g., exposure to -irradiation and chemotherapy agents), p53 levels increase by post-transcriptional mechanisms, and the protein becomes functionally activated. p53 transcriptionally activates expression of several cellular genes, some of which, such as *p21* and *GADD45* (growth arrest and DNA damage-inducible gene), inhibit cell cycling and DNA replication and are responsible for p53s DNA damage G_1 checkpoint function. The ensuing pause presumably allows the cell time for repairs before the damaged DNA is replicated and transmitted to progeny cells and becomes an ongoing source of genetic misinformation. Other genes transcriptionally activated by p53, such as *Bax*, engender apoptosis rather than cell cycle arrest and presumably lead to elimination of cells that are genetically damaged beyond repair. The cell cycle arrest and apoptosis-inducing functions of p53 that protect genetic integrity following genotoxic insults must not be needed for developmental programs, given the normal development of *p53*$^{/}$ mice. However, the absence of p53 protective mechanisms in *p53*$^{/}$ mice and somatic loss of these mechanisms in Li-Fraumeni kindreds must allow DNA damage to accumulate, culminating in cell transformation. Although both the cell cycle arrest and apoptosis function of p53 would seem to help maintain genomic integrity, the latter may be more important for tumor suppression. That p21-mediated cell cycle arrest may not be essential for p53 tumor suppression is suggested by the observation that *p21*$^{/}$ mice develop normally and are not predisposed to developing malignancies. In contrast, p53-induced tumor cell apoptosis has been shown to retard tumorigenesis induced by SV40 large T antigen transgenes and to influence the survival of cells transformed by other oncogenes. With evidence suggesting that p53 may also influence tumor cell responses to therapy, p53-dependent apoptotic mechanisms may have impact beyond tumor development.

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DIFFERENTIATION

Cell proliferation stocks an organism with the necessary number of cells to allow growth and to compensate for cell loss, but differentiation is the process by which these cells become endowed with the specialized functions needed. Each year an average adult man or woman produces about three to four body weight equivalents (200300 kg) of blood cells to maintain the steady-state numbers of cells in the circulation. Such a high cellular turnover suggests that control of hematopoietic cell proliferation and differentiation must be tightly regulated; otherwise a slight increase in proliferation rate could result in leukemia or hyperviscosity. By contrast, a tip of the balance in the opposite direction could lead to severe cytopenias.

The lineage-specific pathways of hematopoiesis have provided a paradigm for the study of cell differentiation. However, the molecular switches that determine lineage-specific cell differentiation remain incompletely understood. Nevertheless, limited insights into the molecular basis of cell differentiation have been gained through the study of various lower eukaryotic genetic systems as well as model cell culture systems. For example, conceptual paradigms of the transcriptional regulation of cell differentiation have been generated from the study of myogenesis and gene regulatory sequences in *Drosophila*.

Organization of Genomic Regulatory Systems

The regulation of cell differentiation and development is encoded in DNA in the form of arrays of transcription factorbinding sites (cis-regulatory elements). Decoding the cis-regulatory elements requires multiple transcription factors. These bind together to form higher-order protein complexes that provide machineries with vast combinatorial possibilities capable of activating specific gene transcription. The hard-wiring of transcription factors to each other and to the cis-elements enables them to process genomic information in the differentiation gene regulatory networks consisting of cis-elements of all relevant genes (Fig. 65). To fully understand cell differentiation, the organization of genomic regulatory networks must be comprehended.

Some general patterns of genomic regulatory systems have emerged.[24] When a gene is expressed in a number of different circumstances in a differentiating cell, separate groups of cis-elements, termed modules, may carry out the overall regulatory function (Fig. 65). Many modular cis-regulatory organizations are now well studied, particularly in *Drosophila*, which is highly amenable to genetic and biochemical analyses. From comparing and studying the complexity of cis-regulatory modules, it appears that modules usually consist of four to eight different factors. That is, the number of transcription factors bound per module averages five. Cell-specific cis-acting modules may be defined by specific combinations of transcription factors, which together are able to tether to the cis-regulatory DNA sequences and regulate transcription of specific differentiation-related genes.

The tethering of transcription factors to their cognate sites is restricted by nucleosomes. [25] Regions of genomic DNA that are transcriptionally active are marked by DNase I hypersensitivity sites that lack a nucleosome. Studies of the chicken erythroid-specific (A)/epsilon-globin gene enhancer, consisting of both tissue-specific and ubiquitous transcription factorbinding sites, suggest that hypersensitivity is determined primarily by erythroid factors and that their binding additively increased accessibility. Accessibility of DNase I to hypersensitive sites is an all-or-none phenomenon, such that binding of erythroid factors additively increases the probability of switching a particular cis-acting module into a hypersensitive state free of a canonical nucleosome. [26] These and other observations contribute to the emerging concept (Fig. 65) that transcriptional regulation is stochastic and that binding of transcription factors to their target sites in cis-regulatory modules enhances the probability of switching a transcription unit into an active state. [27] The all-or-none phenomenon of transcription provides a basis for the stochastic nature of hematopoietic differentiation.

The Myogenic Differentiation Model

The cell culture myogenesis model has provided astounding insights into some molecular mechanisms of lineage-specific cell differentiation induction. [28] [29] Early studies of myogenesis

Figure 6-5 Wiring of extracellular stimuli to transcription of genes during differentiation. The upper diagram depicts growth factor- or extracellular matrix/integrin-mediated signal transduction to activate transcription factors shown as a square, circle, or triangle. Activation of a subset of transcription factors is shown to regulate a specific category of genes (X and Y). The lower diagram illustrates the stochastic nature of transcription. Each gene may be regarded as an all-or-none transcription unit. Transcription factors bound to their target DNA sites increase the probability of a unit being switched on. For example, the square transcription factor may impart a 0.1 probability, which on a population basis would cause 10% of cells expressing the square factor to express a specific gene. The transcription factors are shown to increase the probability of transcription additively.

generated the idea that a single master gene may control muscle cell differentiation. Brief treatment of C3H 10T 1/2 fibroblastic cells with 5-azacytidine induced the formation of many myogenic colonies and fewer numbers of chondrogenic and adipogenic colonies. Transfection experiments showed that genomic DNA from 5-azacytidine-induced myoblasts, but not DNA from parental cells, conferred the muscle phenotype to C3H 10T 1/2 cells. A cDNA for the gene termed *myo*D was cloned by subtractive hybridization to identify mRNAs that were specifically expressed in myoblasts but not in parental C3H 10T 1/2 cells. Transfection of *myo*D cDNA alone was sufficient for myogenic conversion of C3H 10T 1/2 cells. MyoD is a transcription factor that was subsequently shown to activate muscle-specific genes. As with the adipocyte differentiation model, overexpression of c-*myc* also inhibited myogenic differentiation, suggesting that signals for cell proliferation could override cues for differentiation.

MyoD, a member of the helix-loop-helix family of transcription factors, binds to consensus DNA sites that include a CATG sequence present in most muscle-specific enhancers. MyoD is also a member of a family of myogenic transcription regulators, such as myogenin, myf-5, and myf-6-mrf-4-herculin, that can confer muscle phenotype to C3H 10T 1/2 cells. The MyoD protein is comprised of a basic-helix-loop-helix (bHLH) DNA-binding domain and an N-terminal transcriptional activation domain. MyoD binds its target sites as homodimers but more tightly so as heterodimers with ubiquitous bHLH E2A transcription factors. The association of MyoD with E2A is regulated by a third factor that was sought because MyoD protein is present in proliferating undifferentiated myoblasts. Indeed, the *Id* (inhibitor of differentiation) gene was cloned as a cDNA whose protein product was homologous to helix 2 of MyoD. Id contains an HLH motif but lacks the DNA-binding basic region. It can form heterodimers with MyoD or E2A proteins, but these dimers are unable to bind DNA. Transfection experiments have demonstrated that Id could inhibit the ability of MyoD to trans-activate a muscle-specific gene and retard myogenic differentiation. The myogenic differentiation model led to the discoveries of MyoD and Id, both of which participate in a network of protein-protein interactions that alters muscle-specific gene expression. It will become apparent in the subsequent discussions that these general paradigms hold true for myeloid maturation as well.

Hematopoietic Differentiation

Hematopoiesis is a fascinating system in which pluripotent hematopoietic stem cells (PHSCs) differentiate into many highly specialized circulating blood cells. [30] [31] [32] The long-term repopulating PHSCs are capable of self-renewal as well as limited differentiation toward the common lymphoid stem cell or myeloid multipotent stem cell (Fig. 66). Most PHSCs are believed to be dormant and stochastically awakened to enter the cell cycle. The common lymphoid stem cell differentiates into either mature T cells or B cells. The myeloid multipotent stem cell differentiates into a variety of circulating blood cells, including basophils, eosinophils, neutrophils, monocytes, erythrocytes, and megakaryocytes (platelets). The developmental program for each type of mature circulating cell consists of networks of cellextracellular matrix, cellcell, and cellgrowth factor/lymphokine interactions.

It has been established experimentally that PHSCs can differentiate toward either a lymphoid or a common myeloid progenitor. Retroviral labeling of the genome to analyze the clonality of differentiated cells revealed that myeloid and lymphoid cells arise from pluripotent stem cells. The mechanisms determining the commitment of PHSCs to differentiate toward either lymphoid or myeloid progenitors remain incompletely understood; however, the process by which cells differentiate along specific lineages of defined phenotypes is now known to be a result of coordinated lineage specific gene expression. It has been proposed that commitment to differentiate is a stochastic process based on paired daughter cell analysis. In these studies, daughter cells derived from a single parent progenitor cell frequently displayed varied combinations of cell lineages. Such observations have been construed to suggest that random activation of a group of differentiation genes might be required for single-lineage expression. This suggestion has precedence in the known random shuffling of immunoglobulin gene fragments through V(D)J rearrangements to generate antibody diversity. Clones of B cells are then expanded by specific antigens that select for specific antibody variable region structures arising from random V(D)J rearrangements. A stochastic model for hematopoietic cell differentiation has been proposed based on notions similar to those for generation of antibody diversity.

The stochastic model for commitment suggests that dormant PHSCs are recruited into the cell cycle by many lymphokines, including combinations of IL-1, IL-3, IL-4, IL-6, IL-11, IL-12, G-CSF, GM-CSF, FLT, and stem cell factor. Although the molecular basis for synergisms between these factors remains unknown, certain functional and structural homologies among them are notable. For example, IL-6, IL-11, and leukemia inhibitory

Figure 6-6 Stochastic switches and transcription factors in hematopoiesis. The diagram depicts hematopoietic differentiation as a combinatorial switching of key signaling pathways or factors (squares) in hematopoietic cells. Homologous recombination allowing for the inactivation of specific transcription factor genes has revealed the level of participation of different factors in hematopoiesis. The points at which knockout of each factor results in developmental abnormalities are shown. For example, knockout of GATA-1 deranges erythroid development of FC3, osteoclast development, and so forth. *(Adapted from Shivdasani and Orkin,[33] with permission.)*

factor (LIF) require cooperation with a signal-transducing protein GP130 for function. Likewise, receptors for IL-3, IL-5, and GM-CSF share a common -subunit that associates with unique factor-specific chains. Once recruited into the cycling fraction of cells, PHSCs must divide, survive, and differentiate into a certain lineage of cells. It is hypothesized that the stochastic establishment of specific differentiation programs (perhaps by random expression of groups of differentiation genes) in PHSCs provides a population of partially committed cells that are poised to receive additional instructions. Intermediate stage-acting, lineage-nonspecific factors such as IL-3, GM-CSF, and IL-4 support the proliferation of the multipotential progenitors after they have been awakened from their dormant state.

Lineage-specific factors support the survival, proliferation, and maturation of progenitors that are committed through hypothetical stochastic expression of specific groups of differentiation genes. [33] [34] For example, M-CSF promotes the proliferation and differentiation of macrophage/monocytes. Erythropoietin promotes the survival, proliferation, and differentiation of erythrocytes. IL-5 and G-CSF are thought to be specific for eosinophils and granulocytes, respectively. A late-acting lineage-specific factor for megakaryocytopoiesis, termed MGDF or thrombopoietin, has been cloned and extensively studied in recent years. [35] These late-acting lineage-specific factors could be viewed as factors that induce specific transcription factors such as PU.1, GATA-1, and a variety of HOX proteins (Fig. 66). Activation of cis-regulatory modules by these transcription factors selects out and promotes the proliferation of subsets of committed cells whose differentiation programs have been randomly selected. The activation of groups of differentiation genes that are not supported by lineage-specific factors will hypothetically result in cell death.

Lymphoid Differentiation

The commitment of PHSCs to lymphohematopoietic progenitors is thought to be mediated in part by the lineage-nonspecific factors mentioned previously. The mechanisms of early lymphoid development remain poorly understood. B-cell differentiation occurs in the bone marrow of adults, where expression of the immunoglobulin (Ig) heavy chain precedes that of the light chain. Completed Ig chains expressed on the surface lead to the surface-Ig-positive B lymphocytes that migrate to the peripheral lymphoid organs such as the spleen. [36] [37]

Several molecular events that are critical for maturation of lymphoid cells have been identified. Study of Bruton X-linked agammaglobulinemia, which is a disorder of B-cell development, has localized the defect to a B-cell-specific cytoplasmic tyrosine kinase gene locus. This tyrosine kinase, termed BPK, has classic tyrosine kinase, SH2, and SH3 domains. Pre-B or B-cell lines from X-linked agammaglobulinemia patients displayed reduced or absent *BPK* mRNA. Study of genetically engineered (knockout) mice in which the recombination-mediating protein RAG1 or RAG2 (necessary for V[D]J recombination)

is absent revealed that RAG1 and RAG2 are required for the normal maturation of lymphocytes. The V(D)J reaction appears restricted to particular antigen receptor loci in a lineage-specific and stage-specific fashion. [38] Cis-regulatory elements, which regulate the transcription of the germline (unrearranged) antigen receptor loci, are involved in the specificity of the V(D)J reaction. In the T-cell lineage, the first gene rearranged is TCR-. In B cells, the IgH locus is rearranged first. The products of the rearranged antigen receptor genes themselves appear to generate signals that regulate the develpment of T and B lineages. For example, deletion of the transmembrane domain for IgM causes a block of differentiation from the pro-B to the pre-B stage. In addition to these internal cues for differentiation, a number of cytokines such as IL-2, IL-4, IL-7, IL-9 and IL-15 provide other signals that are required for lymphocytic differentiation and maturation.

Myelomonocytic Differentiation

The study of myeloid differentiation underscores our lack of understanding of early events in the commitment of PHSCs to the multipotent myeloid progenitor CFU-GEMM (colony-forming unit-granulocytes, erythrocytes, monocytes, and megakaryocytes). Colony-forming units specific for granulocytes (CFU-G) are thought to arise from a precursor common to granulocytes and monocytes (CFU-GM). The committed myeloid precursors (CFU-G) are presumably selected for and amplified by a myeloid-specific factor, G-CSF. The monocyte-macrophage lineage is selected by M-CSF (CSF-1), which stimulates growth and differentiation through the CSF-1 receptor (CSF-1R) encoded by the proto-oncogene c-*fms*.

CSF-1 exerts its effects by binding to a class of high-affinity receptor that is expressed predominantly on monocytes and macrophages. [39] The receptor CSF-1R is a member of growth factor receptors that display ligand-induced tyrosine-specific protein kinase activity. Activation of CSF-1R triggers a cascade of events, many of which are described for the activation of EGF-R and PDGF-R, that convey signals from the plasma membrane to the cell nucleus. Dissection of CSF-1R by mutagenesis has led to the paradigm that a single plasma membrane receptor can give rise to separate nuclear transcription programs. Specifically, a point mutation in CSF-1R has been identified that results in the activation of the *fos* and *jun* proto-oncogenes, but not *myc*, by CSF-1.[40] This mutant receptor cannot trigger mitogenesis in transfected cultured cells unless complemented by a constitutively expressing *myc* allele. In separate studies of cell lines with the potential for differentiation into neutrophils or macrophages, the zinc finger transcription factor Egr-1 has been shown to be essential for differentiation along the macrophage lineage. Egr-1 appears to restrict differentiation of HL-60 (promyelocytic leukemia) cells into macrophages. Farther downstream events leading to the monocyte-macrophage phenotype remain unestablished.

With the availability of recombinant G-CSF, several cell culture models for granulocytopoiesis have been studied at the molecular level. [41] [42] For example,

differentiation of the myelomonoblastic murine cell line 32D C13 induced by G-CSF is accompanied by differential expression of the C/EBP family of proteins. In contrast to fat cell differentiation, in which C/EBP- protein increases with differentiation, myeloid differentiation of the 32D C13 cells is accompanied by a marked decrease in C/EBP- protein levels. The role of C/EBP- in myeloid differentiation has been further substantiated by the observation that genetically engineered C/EBP- null mice lack mature neutrophils. Myeloid differentiation is also associated with the expression of the *myb*-responsive myeloid-specific *mim*-1 gene, which encodes a protein with an as yet unknown function. Full activation of *mim*-1 requires both myb and C/EBP- binding to their cognate sites in the *mim*-1 promoter. In fact, this combination of activators is sufficient to activate *mim*-1 in heterologous cells such as fibroblasts or erythroid cells. [43] These observations support the hypothesis that tissue-specific gene expression could arise from combinations of transcription factors that are not tissue specific.

Azurophilic cytoplasmic granules are formed with the differentiation of myeloblasts to promyelocytes. [44] Myeloperoxidase is a major protein of the azurophilic granules, and hence its gene is an ideal target to study cis-regulatory modules. Recent studies on the myeloperoxidase gene promoter suggest that myeloid-specific expression of myeloperoxidase might in part be dictated by a cell-type-restricted transcription factor MyNF1 (myeloid nuclear factor 1), which is not yet cloned. Other transcription factors that may form the neutrophil-specific cis-regulatory module include myb, PEBP2/CBF/AML1, and ETS. [42] Identification of relevant cis elements and trans-acting factors required for myeloid-specific gene expression will provide novel avenues to connect extracellular signals from G-CSF to nuclear events that lead to the expression of the myeloid phenotype.

Erythroid Differentiation

Cells committed to differentiate stochastically into the erythroid lineage are amplified by the erythroid-specific factor erythropoietin. Erythropoietin is thought not to determine lineage commitment but to function as a growth factor as well as a survival factor in cell culture studies of erythroid cell lines and normal bone marrow erythroid precursors. Thus, according to the stochastic lineage commitment model, erythropoietin serves to rescue those cells (CFU-E or BFU-E) that express the erythropoietin receptor. [45] When bound to its receptor, erythropoietin initiates a signal transduction pathway that involves the serine/threonine protein kinase raf, protein kinase C, STAT5, and a genetic program that activates the expression of the c- *myc* proto-oncogene. Expression of Bcl-X_L , a Bcl-2 family member, is highly elevated in late erythroid differentiation induced by erythropoietin. Without erythropoietin, the caspase CPP32/apopain is activated and committed erythroid cells undergo programmed cell death with the characteristic laddering of DNA due to nuclease-mediated fragmentation. In these models, hemoglobin synthesis and other manifestations of the erythroid phenotype follow; however, the molecular events responsible for the initiation of these events remain unknown.

Insights into the regulation of the globin gene have emerged with the identification of cis and trans factors that regulate erythroid-specific genes. Erythroid-specific cis-acting elements have been identified in several erythroid genes. The sequence (A/T)GATA(A/G) is found in the globin gene enhancer in several species and in the promoter of the erythropoietin receptor (EpR) gene. [33] This consensus sequence is recognized by a cell-restricted transcription factor, GATA-1, found in megakaryocytes, erythroid, and mast cells. GATA-1 is one of four related members of the GATA family of transcription factors. High expression of globin also requires a regulatory region, located about 20 kb upstream of the epsilon-globin gene, termed the locus-activating region (LAR), which also contains a GATA sequence. Genetic knockout of GATA-1 in mice results in a block in normal erythropoiesis, attesting to the importance of GATA-1 in erythroid differentiation. The block in maturation occurs at the proerythroblast stage. Myeloid cells, however, do arise from hematopoietic cells lacking GATA-1, indicating that GATA-1 acts at a later stage than PHSCs or their immediate descendants. Homozygous deletion of the Kruppel-like factor EKLF gene results in absent -globin gene transcription but persistent epsilon- and -globin gene expression. This observation suggests the EKLF may be important for the interaction between the LAR and the -globin gene. The accumulation of knowledge about these factors will contribute to a fuller understanding of erythroid differentiation.

Megakaryocytic Differentiation

Megakaryocytic differentiation is an intriguing biological phenomenon in which a cell undergoes several rounds of DNA replication without cytokinesis. The resultant mature polyploid megakaryocyte then sheds small, membrane-bound, metabolically active cytoplasmic fragments that become circulating platelets. The platelet cell participates in thrombosis through several signal transduction pathways, including activation of the thrombin receptor and fibrinogen receptor (GPIIb/IIIa). The activated platelet lets the cellular glue plug up small anatomic defects in the vasculature. It is clinically apparent that thrombocytopenia due to peripheral destruction is accompanied by recruitment of mature megakaryocytes from precursor cells. The factor involved in this feedback loop has been purified, cloned, and termed thrombopoietin, or MDGF. [35]

Some of the later megakaryocytic differentiation events are beginning to emerge from studies of megakaryotic cell lines and megakaryocyte-specific genes. Several cell lines can be induced to differentiate and express various megakaryocytic phenotypes, including the platelet-specific fibrinogen receptor GPIIb/IIIa. These cells also display repeated rounds of DNA replication that result in polyploidism. The molecular mechanisms that control polyploidism are likely to involve certain molecules that determine cell fate, such as the cyclins, molecules that control the mitotic checkpoint, [18] as well as genes that regulate the initiation of DNA replication such as *CDC6*. Future studies of these genes in megakaryocytic differentiation will shed more light on this fascinating phenomenon.

As the promoters of several platelet-specific genes are cloned and thrombopoietin is available as a specific megakaryoctyic differentiation and growth factor, the mechanism of regulation of platelet-specific genes is beginning to emerge. [46] Inspection of the promoters for platelet genes GPIb, GPIIb, and GPIIIa indicates that there is a common putative DNA-binding site, termed putative meg-specific element (PMS-E), that may be megakaryocyte specific. It is also apparent that unique combinations of many ubiquitous transcription factors, such as SP1, are likely to dictate tissue specificity. Indeed, various DNA-binding sites have been identified in promoters of megakaryocyte-specific genes. Among these sites is the GATA sequence that was previously thought to be erythroid specific. In fact, enforced expression of GATA-1, a GATA-binding protein, in a myeloid cell line induced the megakaryocyte phenotype. Conversely, selective loss of megakaryocyte GATA-1 expression resulted in mutant mice with markedly reduced platelet numbers, deregulated megakaryocyte proliferation, and impaired cytoplasmic maturation. Intriguingly, when mice lacking the p45 subunit of the heteromeric erythroid transcription factor NF-E2 were generated, the NF-E2 $^{/}$ mice lacked circulating platelets and died of hemorrhage. [33] Their megakaryocytes showed no cytoplasmic platelet formation and yet proliferated in vivo in response to thrombopoietin. Loss of NF-E2 affected the erythroid lineage only mildly. With the availability of thrombopoietin, it is expected that the thrombopoietin-responsive cis elements in megakaryocyte-specific genes, such as those encoding GPIIb and platelet factor 4, are likely to be rapidly identified. In the next few years, the challenge remains to link events triggered by thrombopoietin through its receptor, c-Mpl, to the activation of cis-regulatory modules found in megakaryocyte-restricted genes.

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PROGRAMMED CELL DEATH (APOPTOSIS) AND HEMATOPOIESIS

Apoptosis and Programmed Cell Death

Apoptosis is a morphologically unique process of cell death that is distinct from necrosis. [47] [48] [49] Necrosis denotes accidental cell death resulting from severe and sudden thermal, physical, or

Figure 6-7 Apoptosis is a morphologically and biochemically defined phenomenon in which cells die. The apoptotic cell undergoes blebbing of the plasma membrane and condensation of nuclear material with nuclease digestion, resulting in nicked DNA ends that are detectable by fluorescent (FL) polyuridyl (UUU) labeling using terminal deoxynucleotidyl transferase (TUNEL assay). Release of nucleosomal DNA is detected as a ladder of DNA fragments.

chemical trauma. Morphologically, there is early mitochondrial and cellular swelling with ensuing cytoskeletal disruption and ruptured plasma membrane and organelles. During necrosis, the nuclear structure remains intact. In contrast, apoptosis is a sequential process that starts with condensation of the chromatin and shrinkage of cell volume (Fig. 67). The plasma membrane becomes ruffled and blebbed. The nucleus and cytoplasm then become partitioned into membrane-bound apoptotic bodies that are shed from the dying cells. The term apoptosis was proposed by Kerr, Wyllie, and Currie to describe these cellular alterations and used in Greek () to describe the falling off of petals from flowers or leaves from trees. [50] Most cells in the last stages of apoptosis display DNA strand breaks, which are detectable by terminal deoxynucleotidyl transferase labeling, and a characteristic degradation of nuclear DNA into multimers of 180 base pairs (DNA laddering; see Fig. 67). Throughout apoptosis, the mitochondria remain morphologically normal. Apoptosis participates in many physiologic processes, including morphogenesis, death of short-lived neutrophils, elimination of self-reactive T cells, and perhaps death of B cells with nonproductive V(D)J gene rearrangements.

Genetic Basis of Programmed Cell Death

Although apoptosis is commonly equated with programmed cell death in vertebrate systems, a genetic program for cell death

has been unequivocally established for the death of individual cells in *Caenorhabtditis elegans* (a small worm) and *Drosophila*. [49] [51] In *C. elegans*, 131 out of 1,090 somatic cells are programmed to die during development. Studies of mutant *C. elegans* have revealed at least 14 genes (complementation groups) that are involved in cell death. Of these, *nuc-1* is a gene that encodes an endonuclease. Mutants of *ced-3* or *ced-4* genes that are required for cell death have extra somatic cells. Mutation of the locus *ced-9*, which antagonizes *ced-3* and *ced-4*, results in developmental arrest. The product of *ced-9* blocks apoptosis, and hence its loss presumably results in untimely cell death. The biochemical properties of *ced-3, ced-4,* and *ced-9* are now better understood and their mammalian homologs have all been identified.[52] The emerging picture of this highly conserved suicide program is that the *ced-3* and *ced-4* proteins directly bind each other (Fig. 68). Ced-3 is a member of an ever-expanding family of proteases, termed caspases, that cleave critical cellular substrates after (to the C-terminal side) aspartate residues leading to irreversible cell damage and hence death. [53] [54] Ced-4 was found to bind directly with Ced-3 or Ced-9, which is homologous to Bcl-2 and is able to block cell death.

An understanding of the genetic regulation of programmed cell death in vertebrates is only beginning to emerge. An intriguing clue to the genetics of vertebrate apoptosis came from the study of *bcl-2*, a gene that is characteristically translocated [t(14:18)] in most follicular lymphomas. [55] [56] The protein Bcl-2 has significant sequence homology to the product of the *C. elegans ced-9* gene.[52] Expression of the Bcl-2 protein is restricted to tissues characterized by apoptotic death. For example, Bcl-2 is found in the thymic medulla, where surviving T cells are located, but Bcl-2 is not found in cortical thymocytes destined to die. In transgenic mice experiments, targeted *bcl-2* expression in B cells resulted in memory cells with an extended lifetime. Targeted *bcl-2* expression in T cells resulted in immature thymocytes that were resistant to apoptosis induced by glucocorticoids, irradiation, or anti-CD3 antibody. Thus, follicular lymphomas, which are characterized by a low growth fraction and deregulated expression of *bcl-2*, proliferate predominantly by a decrease in the rate of cell death. The function of Bcl-2 at the molecular level still eludes complete characterization; however, the Bcl-2 protein is known to exist in mitochondrial, endoplasmic reticular, and perinuclear membranes. Recent studies suggest that Bcl-2 may form channels for small electrolytes in lipid bilayers. Other studies suggest that its antiapoptotic effect is mediated through the retention of cytochrome c, which is released from mitochondria to activate caspases and initiate the final apoptotic pathway.

Figure 6-8 Studies in the nematode *C. elegans* reveal genetically determined pathways that regulate cell death. The death genes (ced) are shown with their corresponding mammalian analogs in parentheses. The CED3/CED4 complex is shown to culminate in programmed cell death (PCD).

Insights into signal transduction and activation of caspases have come from the study of the membrane protein termed Fas/APO-1 (Fig. 69), which induces apoptosis when cross-linked by anti-Fas/APO-1 antibody. [57] [58] The Fas protein is homologous to receptors for tumor necrosis factor and nerve growth factor. A lymphoproliferative condition is found in mice bearing the *lpr* mutation that is responsible for a defect in Fas or the *gld* mouse which is defective in the Fas ligand (FasL). The interaction of Fas with its ligand is now known to be central for clonal deletion of lymphocytes. The signals transduced by Fas are rapidly emerging as a complex yet tangible system. Conversion of sphingomyelin to ceramide by sphingomyelinase may play an important role in this signal transduction pathway, although the role of ceramide as a second messenger of death is controversial and therefore requires additional studies. [59] The apoptotic signaling by Fas or a related tumor necrosis factor receptor (TNF-R1) requires a cytoplasmic death domain (DD). [60] Adaptor proteins, such as FADD or RAIDD, tether to these receptors through their own DD domains. A death effector domain (DED) of the adaptor FADD is required for the propagation of the death signal. In the case of FADD, the DED binds to the prodomain of caspase 8 (MACH/FLICE) and activates its proteolytic activity. Some of the targets of the caspases include poly(ADP-ribose) polymerase, DNA-dependent protein kinase, and actin. Presumably the cleavage of these and other proteins contributes to the demise and morphologic changes of apoptotic cells.

The tumor suppressor protein p53 has been shown to participate in the regulation of cell cycle G_1-S transition.[23] [61] It is thought to be involved in the monitoring of DNA breaks induced by various physical and chemical agents such as ionizing radiation. Through an unknown mechanism involving the ataxia-telangiectasia protein ATM, DNA breaks are detected and p53 protein levels are subsequently increased (by post-transcriptional events), resulting in cellular arrest in G_1. Overexpression of wild-type p53 protein in tissue culture cells has been associated with an increase in the rate of apoptosis. On the other hand, homologous genetic knockout of the *p53* gene resulted in thymocytes that did not undergo apoptosis on exposure to ionizing radiation but continued to die on exposure to glucocorticoid. These observations suggest that several pathways leading to apoptosis in thymocytes exist and that at least one of these pathways is p53 dependent.

The participation of c-Myc in cell proliferation and neoplastic transformation has been established by a variety of studies. [61] [62] A new twist on the function of the c-Myc

protein in cell fate, however, was unveiled when cells overexpressing Myc were deprived of nutrients and growth factors through serum deprivation. In contrast to parental fibroblastic cell lines that undergo growth arrest and withdraw into the G_0 phase with serum deprivation, the Myc-overexpressing cells undergo apoptosis. The apoptotic phenotype is dependent on domains of the c-Myc protein required for transcriptional activation and cellular transformation, suggesting that Myc actively participates in apoptosis. Hypothetically, the Myc-overexpressing cells appear to contend with conflicting signals for cell proliferation (Myc) and for growth arrest (serum withdrawal) by executing the cell death program. Although serum deprivation provides a convenient experimental maneuver, its physiologic significance is obscure unless one considers the growth of a tumor mass. In a mass that is sufficiently large, access to nutrients becomes diffusion limited toward the center of the mass as tumor size increases. Thus, cells at the center of the mass may undergo apoptosis unless additional genetic alterations are sustained. Such additional genetic alteration might be the expression of Bcl-2 that can block the apoptotic effects of Myc.

Eosinophils, monocytes, and neutrophils all participate in the initiation and control of inflammatory responses. [63] Their elimination from circulation may in part be mediated by

Figure 6-9 The Fas and related TNF-R pathway are depicted with the intermediate adaptor molecules (TRADD, FADD). The adaptors interact with and activate FLICE, which belongs to the family of caspase proteases. The caspases cleave cellular protein substrates to mediate the final pathway of cellular death.

apoptosis. Isolated eosinophils undergo apoptosis after about 80 hours in culture. IL-5 appears to extend eosinophil lifetime by delaying apoptosis. Mature neutrophils have also been shown to undergo apoptosis. Likewise, monocytes undergo programmed cell death that can be averted by exposure to IL-1b, TNF-, GM-CSF, and interferon-.

Programmed cell death appears to be an integral component of the stochastic hematopoietic cell lineage commitment and differentiation model. Apoptosis also appears to participate in the physiology of the more mature hematopoietic cells. For example, thymocytes undergo a stringent selection process that uses apoptosis to eliminate cells that recognize self antigen. In addition to the roles of apoptosis in normal hematopoiesis, its role in the pathophysiology of hematopoietic disorders is rapidly and extensively becoming recognized.

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Chapter 7 - Overview of the Immune System (Including Compartmentalization of the Immune Response)

Ellen Puré

The immune system must detect and respond to a broad range of antigenic challenges from foreign substances. Although the immune system has evolved to protect the host against pathogenic microorganisms, similar types of immune responses are elicited by noninfectious foreign antigens as well. Furthermore, many of the nonspecific mechanisms that are engaged as the result of antigenic stimulation are used in the inflammatory response and in the repair of tissue damaged as the result of noninfectious injury such as wounding, burns, and trauma.

To succeed in its protective function, the immune response must react to particular antigens quickly and efficiently, especially those pathogens that it encounters repeatedly. The two host defense systems that provide immunity against infectious agents are the innate, or natural immune system, and the acquired (adaptive) or specific immune system (Table 71). The innate immune system is the first to respond and, as a result, limits or cures infection before an adaptive immune response is generated. The innate immune response involves mainly nonlymphocytic cells, including macrophages and polymorphonuclear leukocytes, as well as complement and acute-phase proteins. The cells that mount the innate immune response are also important effectors in acquired or adaptive immunity. Innate immunity is present in all individuals all the time and does not change with repeated exposure to antigen. If the innate immune response cures rather than limits an infection, it can prevent development of adaptive immunity, in which case no long-lasting immunity is established.

In comparison, the central players in the adaptive, inducible response are antigen-specific lymphocytes. The main challenge of the adaptive immune system is the requirement to respond to the myriad of foreign antigens introduced at any site with only a very small number of lymphocytes specific for any one antigen. Several features of the cells and tissues of the immune system allow this challenge to be met successfully. One key feature is the anatomic organization of the immune system. The primary lymphoid organs (i.e., the thymus and bone marrow) produce, under antigen-independent conditions, millions of lymphocytes displaying a vast diversity of antigen-specific receptors that form the basis of the broad immune response. The lymphocytes generated in the primary lymphoid organs are exported to the peripheral blood, where they form a naive pool of circulating lymphocytes capable of recognizing most antigens (Fig. 71). Importantly, recirculating lymphocytes migrate and are exchanged between the circulation and the peripheral or secondary lymphoid organs (i.e., the spleen and lymph

TABLE 7-1 -- Innate and Adaptive Immunity

Innate	Adaptive
Early: First 45 days	Late: 5 days
Constitutive	Inducible by antigen
No memory	Memory/immunity
Nonlymphocytic/not clonal	Lymphocytic/clonal

Figure 7-1 Leukocyte circulation. Leukocytes are bone marrow derived. Most leukocytes differentiate in the bone marrow and migrate from the marrow directly to the peripheral circulation. However, T-cell precursors migrate from the marrow to the thymus where they undergo maturation before they are exported to the periphery. Naive lymphocytes migrate and are exchanged between the circulation (blood and lymph) and the peripheral lymphoid organs where antigen-dependent immune responses are initiated. Antigen-stimulated effector cells home to sites of antigen exposure or inflammation.

nodes), where antigen-dependent immune responses are initiated. The specialized architecture of the secondary lymphoid organs makes them optimal sites for acquiring and concentrating antigens and for antigen-induced lymphocyte growth and differentiation to effector cells. On antigenic stimulation, the relevant lymphocytes become activated, proliferate, differentiate to effector cells, home to sites of antigen exposure, and are retained at these sites and eventually dispense with the antigen. A second peripheral lymphocyte compartment is formed when some of the antigen-stimulated lymphocytes become diverted to a memory pool, composed of cells that respond to antigen more immediately than those in the naive pool. The memory cells differ from the naive cells in their surface phenotype, homing properties, signaling requirements, and cytokine secretion pattern. The establishment of memory cells ensures that recurrent exposure to the same antigen results in a more rapid response, and changes the quality and maximal level of the response. Interactions between antigen-specific lymphocytes and other cells that are involved in the cognitive and effector phases of immune responses serve to optimize these responses. Multiple amplification loops magnify the effects of stimulating the few antigen-specific lymphocytes for one antigen.

There are two branches of adaptive immunity; humoral adaptive immunity and cellular adaptive immunity (Fig. 72). An essential difference between the B lymphocytes, which are responsible for humoral immunity, and T lymphocytes, which are responsible for cellular immunity, is the form of antigens that they recognize. Each clone of B and T cells expresses distinct antigen receptors. However, B lymphocytes recognize conformations

Figure 7-2 The two branches of adaptive immunity: humoral adaptive immunity and cellular adaptive immunity. The main function of B lymphocytes is to produce the antibodies that mediate humoral immunity. Antibodies neutralize pathogens or toxic products, opsonize pathogens for phagocytosis, and activate complement to enhance phagocytosis and destroy pathogens. T cells are required for cellular immunity. T cells act to eliminate antigen directly by differentiating into cytolytic cells. T cells also mediate cellular immunity indirectly by activating macrophages. T cells are also typically required to stimulate B cells to produce antibodies.

of native antigen, whereas the receptors expressed by T lymphocytes recognize peptides derived from processed antigen that are bound to cell surface protein products of the major histocompatibility complex (MHC). The main function of B lymphocytes is to produce the antibodies that mediate humoral immunity by neutralizing pathogens or toxic products, opsonizing pathogens for phagocytosis, or activating complement to enhance phagocytosis and destroy pathogens. B cells can also serve as antigen-presenting cells (APCs) for T cells. T cells generate cell-mediated immunity directly by differentiating into cytolytic cells that are able to kill, for example, virus-infected cells. T cells also mediate cellular immunity indirectly by activating macrophages against intracellular pathogens like *Mycoplasma tuberculosis*. In addition, T cells are typically required to stimulate B cells to produce antibody.

The multiple components of the immune system, including innate immunity and humoral and cellular adaptive immunity, all must work as an integrated host defense mechanism to effectively eliminate any infectious agent and provide long-lasting protective immunity. In this chapter, we briefly review the various components of the innate and adaptive immune systems with an emphasis on the links between innate and adaptive immunity and on how and where antigens and lymphoid cells meet and cooperate at various stages of infection to effectively eliminate invading pathogens.

CELLS OF THE INNATE AND ADAPTIVE IMMUNE SYSTEMS

The nonlymphocytic leukocytes that mediate innate immunity and also serve as effector cells of adaptive cellular immunity are all bone marrow derived and develop from multipotential hematopoietic stem cells. Lineage commitment and differentiation of the various cell types depend on stromal cell contact and cytokines.

Dendritic cells are bone marrow derived, professional APCs that possess characteristic processes that accommodate contact with T cells. Dendritic cells can develop from CD34+ peripheral blood monocytic precursors, in the presence of granulocytemacrophage colony-stimulating factor and interleukin (IL)-4 or IL-13, into immature dendritic cells that effectively take up and process antigens. Activation by stimuli such as tumor necrosis factor (TNF), lipopolysaccharides, or IL-1 induces further differentiation to mature dendritic cells that effectively stimulate antigen-specific T cells. Dendritic cells are found in the interstitium of most organs and mainly in the T-cell areas of peripheral lymphoid organs. As is characteristic of APCs, dendritic cells express both MHC class I and class II molecules.

A second population of unrelated cells that are mainly localized to the follicular, B-cell areas of peripheral lymphoid organs are referred to as follicular dendritic cells. Follicular dendritic cells are *not* bone marrow derived and are not related to interdigitating dendritic cells. Follicular dendritic cells display antigens for recognition by B cells in the form of immune complexes that are retained long-term on the cell surface.

There are two major classes of phagocytic cells, polymorphonuclear leukocytes (PMNs) and macrophages. Granulocytes are inflammatory cells with abundant cytoplasmic granules that are essential for the elimination of microbes and dead tissue. Eosinophils are leukocytes that contain eosinophilic granules. They express Fc (crystalline fragment) receptors for IgE, which is prevalent in the response to parasitic infections and mediates activation of eosinophil killing mechanisms. Eosinophils also play a role in immediate hypersensitivity to allergens and cause tissue injury and inflammation. Eosinophils are recruited and activated by IL-5 made by T cells. Basophils in the circulation and mast cells in tissues also express Fc receptors for IgE, and IgE immune complexes induce their granular exocytosis, resulting in the release of the chemical mediators of immediate hypersensitivity.

Neutrophils are the major class of phagocytic granulocytes. They contain multilobed nuclei and neutrophilic granules. Neutrophils respond rapidly to chemotactic stimuli and phagocytose and destroy foreign particles. Neutrophils are activated by macrophage- and endothelial-derived cytokines and are the major population of cells involved in acute inflammatory responses. Neutrophils express Fc receptors for IgG and complement receptors and thus bind and phagocytose opsonized antigens, providing an important link between innate immunity and humoral immunity. Neutrophils are the major cellular component of the acute inflammatory response and are the primary effector cells in immune responses to pyogenic organisms such as extracellular bacteria.

By contrast, macrophages are the major cellular component in most chronic immunologic responses and act as key regulators of the specific acquired response. A well established dichotomy within the immune system is that between an acute and a delayed inflammatory cellular response. PMNs and macrophages are both professional phagocytes whose major effector function is killing microorganisms. However, PMNs respond within minutes to hours, appearing in large numbers in the tissue and being largely responsible for the acute phase of inflammation. Macrophages are fewer in number, with peak response in hours to days, and participate in both acute and chronic inflammation.

Polymorphonuclear cells can regulate the activation and recruitment of macrophages primarily by the release of a wide variety of cytokines. In addition, macrophages are the principal scavengers of PMNs undergoing apoptosis in the secondary phase of an acute inflammatory response. The most important microbial killing mechanisms in phagocytes are the activation of the respiratory burst and the production of cytotoxic reactive oxygen intermediates, and, through a different mechanism, the generation of reactive nitrogen intermediates such as nitrous oxide.

Tissue macrophages are the second major class of phagocytic cells. They provide innate cellular immunity in tissues and initiate host defense responses. A second function of macrophages is the release of inflammatory cytokines. Macrophages also play a key role in many other phases of host defense, including acting as APCs, and provide important links between innate immunity and adaptive humoral and cellular immunity.

Large granular lymphocytes or natural killer (NK) cells are important cellular effectors of innate immune responses and

TABLE 7-2 -- Modes of Antigen Recognition

B Cells	T Cells	Other
Membrane immunoglobin	T-cell receptor	Fc receptor; complement receptor
Direct	Presented by antigen-presenting cell	Indirect
Native antigen	Peptide/major histocompatibility	Immune complexes

as such are often regarded as a first line of defense. NK cells target virus-infected cells or tumor cells for killing. NK cells can also participate in antigen-specific immune responses by virtue of their expression of receptors for IgG that mediate their antibody-dependent cell cytotoxicity function.

An essential difference between the cells of the innate immune system and the lymphocytes that mediate adaptive immunity is the means by which they recognize microorganisms (Table 72). Cells of the innate immune system recognize microorganisms directly through receptors for components of microorganisms or their products. These receptors usually recognize carbohydrate structures. For example, macrophages express a mannose receptor with a broad carbohydrate specificity. Macrophages also express a receptor for lipopolysaccharides, which are a major component of the surface of gram-negative bacteria. Cells of the innate system also detect microorganisms indirectly through receptors for opsonins such as antibody and complement and receptors for products of the acute-phase reaction.

In contrast, B and T lymphocytes, which mediate the adaptive immune response, recognize antigen specifically and directly. Lymphopoiesis is governed by the tenets of clonal selection. Each clone of lymphocytes expresses antigen receptors with a single specificity. B and T lymphocytes somatically rearrange the V, D, and J elements of the immunoglobulin and T-cell receptor genes, respectively, to generate as many as 10^{11} different clones of B and T lymphocytes that express distinct antigen receptors. The basis of antigen recognition by B and T lymphocytes is, however, quite distinct. B lymphocytes recognize native antigens, whereas T lymphocytes recognize processed antigens in the context of macromolecular complexes with products of the major histocompatibility locus. In either case, antigen

binding to the receptor leads to lymphocyte activation and clonal expansion. The progeny and resulting effector cells or products of a lymphocyte clone all have the same specificity as the parental cell. Furthermore, self-reactive clones are deleted or inactivated.

Antigen-independent maturation of lymphocytes occurs in the primary lymphoid organs, the thymus and bone marrow, whereas antigen-dependent differentiation occurs in secondary lymphoid organs. B and T lymphocytes are indistinguishable morphologically but are phenotypically and functionally distinct. B cells generate antibody responses, whereas T cells mediate cellular immunity but are also required for the generation of the vast majority of antibody responses. B and T lymphocytes reside predominantly in architecturally distinct areas of peripheral lymphoid organs.

B lymphocytes are formed along with other white blood cells in several tissues, including the yolk sac and the fetal liver and spleen during development. The bone marrow is the major source of B cells postnatally and in the adult. There are two subsets of B cells recognized. B1 B cells develop earlier in the bone marrow, but in adults are renewed from peritoneal cells rather than bone marrow cells. They are a self-renewing population and long lived. B1 B cells in general produce antibodies to common bacterial polysaccharides. Conventional B cells, B2 cells, are bone marrow derived throughout life. The main function of B cells is to produce antibodies that neutralize pathogens or toxic products, opsonize pathogens for phagocytosis, or activate complement to enhance phagocytosis and destroy pathogens. They also serve as APCs for T cells. The activation of B cells and their differentiation into antibody-producing lymphoblasts and plasma cells usually depend on T-cell help. Plasma cells are end-stage cells that produce large quantities of antibody. They are found initially in the medullary cords of lymph nodes but then in large part home to bone marrow or sites of inflammation. Memory B cells are generated in the germinal centers of the secondary lymphoid organs, where somatic mutation and isotype switching to downstream immunoglobulin heavy chain genes occur. Memory cells respond rapidly on reexposure to an antigen. They are long lived cells and can reside in the bone marrow or recirculate. Memory cells exhibit decreased activation thresholds/requirements and produce antibodies of higher affinity for the eliciting antigen.

T cells generate cell-mediated immunity directly by differentiating into cytotoxic T cells that kill, for example, virus-infected cells. T cells also mediate cellular immunity indirectly by activating macrophages, such as in the case of infection with intracellular pathogens like *M. tuberculosis*. T lymphocytes are also intimately involved in humoral immunity by providing essential help for B lymphocytes.

T cells develop from bone marrow progenitors that migrate to the thymus, where they undergo a careful selection process to eliminate or inactivate self-reactive clones and promote the survival of cells expressing receptors that recognize self-MHC. There are two lineages of T cells that are defined by their utilization of distinct T-cell receptor genes, / and /. The latter represent a small proportion of the total T-cell population and exhibit somewhat more limited diversity and a distinct tissue distribution; they are enriched in underlying mucosal epithelium.

The two major subsets of / T cells, CD4 and CD8 T cells, are defined by the restriction of their clonal recognition of antigenic peptides in the context of MHC class II and class I molecules, respectively. Also, in general, CD4 T cells function mainly as helper cells that stimulate the immune response and inflammation, whereas CD8 cells mainly function as cytotoxic cells that recognize infected targets and lyse them. Activated mature T cells can differentiate further into functionally distinct type 1 and type 2 populations that are characterized by the profile of cytokines they produce. Type 1 T cells secrete IL-2 and interferon-, whereas IL-4, 5, and 10 are typically produced by type 2 cells.

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OVERALL ANATOMIC AND FUNCTIONAL ORGANIZATION OF THE IMMUNE SYSTEM

The anatomic organization of the cells of the immune system and their ability to circulate are critical for the generation of the immune response. They are present as circulating cells in blood and lymph, defined collections in lymphoid organs, and scattered cells in virtually all tissues (Table 73).

The central or primary lymphoid organs, the thymus and bone marrow, are the sites of hematopoiesis and lymphopoiesis. The secondary organs include the spleen, lymph nodes, and Peyer's patches. The secondary lymphoid organs are architecturally ordered collections of lymphoid cells that are intricately connected to the vascular and lymphatic systems to provide optimal

TABLE 7-3 -- Organization of the Immune System

Lymphoid Organ System	Primary Function	Sites
Primary	Lymphopoiesis	Bone marrow Thymus
Secondary	Immune sensitization	Lymph nodes Spleen Mucosal Cutaneous
Tertiary	Effector responses	
Circulation	Surveillance Dissemination	Lymphatics

sites for the concentration of free or cell-associated antigens and recirculating lymphocytes. Furthermore, the cells in secondary lymphoid organs are compartmentalized to optimize the generation of immune responses. Immune effector responses usually are manifested at tertiary sites of extralymphoid tissues that, under normal circumstances, contain few lymphoid components but that have the capability to rapidly import appropriate lymphoid and accessory populations after inflammatory provocation.

The peripheral or secondary lymphoid organs are the sites of initiation or the sensitization phase of immune responses and their anatomy is specialized for acquiring antigen and mediating cell-cell interactions. Lymph nodes are nodular aggregates of lymphoid tissue situated along lymphatic channels, epithelium, and the mucosa of the gastrointestinal and respiratory tracts. Compartmentalization of lymphoid organs optimizes the immune response by facilitating exposure of antigen-specific cells to free antigen or antigens in the context of professional APCs, and facilitates cell-cell interactions required for initiation and amplification of the immune response. Secondary lymphoid organs are in general compartmentalized into B- and T-cell areas, but the various cell types can access the different compartments at critical stages to interact with other appropriate cells.

Lymphocytes enter the peripheral lymph nodes through postcapillary venules that are lined by a specialized endothelium (referred to as high endothelial venules [HEV]) that facilitates the extravasation of circulating cells into the organ (Fig. 73). Afferent lymphatics drain from each tissue into the draining nodes, allowing for collection of free or cell-associated antigens in the lymph nodes where the circulating lymphocytes can sample them. APCs, such as tissue dendritic cells, can carry antigen from tissue to the draining nodes, or free antigen can access the draining node through the lymphatics, where it can then be taken up by APCs as it percolates through the cortex and medulla (Fig. 73). Antigen is then presented in the context of the MHC molecules expressed on the APC to T cells in close proximity. T cells that come into contact with APCs displaying their cognate antigen in the context of the appropriate MHC restricting element are activated and temporarily detained in the lymph node, where they interact with antigen-activated B cells and differentiate into effector cells.

B lymphocytes preside mainly in the primary follicles. B cells migrate through the T-cell area on their way to primary follicles (Fig. 73). B-cell blasts that result from early activation events move into follicles to expand in an oligoclonal fashion to give rise to secondary follicles containing germinal centers. Using multiparameter flow cytometry, it is possible to separate five B-cell populations of the germinal center (BMI-5) (Fig. 74 ; Plate 7-1). Germinal centers contain follicular dendritic cells, which display on their surfaces antigen in the form of immune complexes, and proliferating B cells. Proliferating B cells in germinal centers undergo somatic mutation of their antigen receptors, and selection of clones expressing new antigen receptors leads to affinity maturation of the humoral immune response. In addition, B cells in the germinal centers undergo isotype switching, leading to the production of isotypes of immunoglobulin such as IgG, IgA, and IgE that use various downstream heavy chain genes, and thus determine the nature of the humoral immune response. Plasma cells are found in the medullary cords, then circulate to other tissues, especially bone marrow. Newly generated memory cells reenter the circulation.

In spleen, where the immune response to blood-borne antigens is initiated, antigen is delivered into the marginal sinus. T lymphocytes are activated in the periarterial lymphatic sheaths and then move to the marginal zone, where they interact with B cells. Activated B lymphocytes then form germinal centers and plasma cells enter the red pulp.

The mucosal lymphoid system services the gastrointestinal, pulmonary, and genitourinary tracts. The structure of mucosal

Figure 7-3 Morphology of a lymph node. The peripheral or secondary lymphoid organs are the sites of initiation or the sensitization phase of the immune response. The architecture of these organs is specialized for acquiring antigen and mediating the cell-cell interactions involved in initiation of an immune response. Secondary lymphoid organs are compartmentalized into B-cellrich areas (follicles) and T-cellrich areas (parafollicular regions). Lymphocytes enter lymph nodes through postcapillary venules. Afferent lymphatics carry antigen from all tissues to the draining lymph nodes, where it can be recognized by circulating lymphocytes.

Figure 7-4 Activated B cells enter lymphoid follicles and generate germinal centers. Activated B cells in follicles undergoing rapid proliferation are referred to as centroblasts and collectively form the dark zone. B cells in the dark zone give rise to the centrocytes that localize to the light zone where they interact with the network of follicular dendritic cells. The mantle zone contains naive B cells that reside in the follicles that are not participating in the ongoing immune response.

lymphoid organs varies somewhat from site to site, but overall is similar and comparable to that of the lymph nodes. A major difference is how they are exposed to antigen. In mucosal secondary lymphoid tissues, antigen influx occurs across a mucosal epithelium, rather than through afferent lymph. They are usually situated within the lamina propria, the highly vascularized connective tissue immediately subjacent to the mucosal epithelium. The mucosal lymphoid tissues contain clear B- and T-cell areas. The overlying epithelium is specially modified for the uptake and transport of luminal antigens across the epithelium by cells designated M cells. Lymphatic capillaries surround the mucosal secondary lymphoid tissues and serve to transport lymphocytes and accessory cells, as well as antigen, to draining lymph nodes and ultimately to the thoracic duct.

Finally, the cutaneous immune system is associated with the epithelium and underlying vascularized connective tissue (dermis) of the skin.

The tertiary lymphoid tissues are sites of the effector phase of the immune response including in nonlymphoid tissue. Tertiary sites contain two compartments; the first is a vascularized connective tissue with fibroblasts, macrophages, mast cells, and blood and lymphatic vascular channels embedded in an extracellular matrix that provide the route of lymphocyte recruitment into the tissues and egress by lymphatics. This compartment is adjacent to a second compartment consisting of differentiated parenchymal cells that make up a given organ or tissue.

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INTEGRATED HOST RESPONSE TO INFECTION

Only occasionally do the microorganisms encountered daily in the life of a normal, healthy individual cause perceptible disease. Most organisms are detected and destroyed within hours by host defense mechanisms that are not antigen specific and do not require a prolonged period of induction; these are the mechanisms of innate (natural) immunity. Only if an infectious organism survives this early line of defense will an adaptive (acquired) immune response ensue. The adaptive response leads to the generation of antigen-specific effector cells that specifically target the pathogen, and the generation of memory cells that prevent subsequent infection with the same microorganisms.

Infection occurs in several stages, each of which can be blocked by different host defense mechanisms. In general, the first phase involves adherence of the pathogen to epithelium. The epithelial surfaces of the body serve as an efficient barrier to most microorganisms. Epithelium represents a mechanical, chemical, microbiologic, and immunologic barrier to infection. Inhibition of attachment or binding by organisms to the host, for example by neutralizing antibodies present in the secretions at epithelial surfaces, can prevent infection. Next, local infection is established as the pathogen penetrates the epithelium and infects the local tissue. The major host defense mechanisms at this stage include intraepithelial (/) T cells, wound-healing processes, the release of antibacterial peptides (e.g., defensins), and phagocytes. Antibodies against invasins also can inhibit at this stage. Most of the pathologic process associated with an infection occurs as the result of the third phase of infection, in which the organism spreads. Extracellular pathogens spread by direct extension of the infected foci through the lymphatics and, usually only if the lymphatic system is overwhelmed by the infection, through the bloodstream. Intracellular pathogens spread from cell to cell either by direct transfer from one cell to the next or by release into the extracellular fluid and reinfection of adjacent and distant cells. Protective mechanisms at this stage include activation of the alternative complement pathway, phagocytes, cytokines, and NK cells. Assuming that the infection persists in spite of the early innate immune response and other host defense mechanisms, the host then mounts an adaptive immune response. In addition to clearing the infectious agent, an effective adaptive immune response prevents reinfection. Mechanisms include specific antibody, T-cell dependent macrophage activation, and cytotoxic T cells.

Two key features make the immune system effective against infection by diverse pathogens. The first is the variety of mechanisms of host defense, which includes innate immunity, humoral adaptive immunity, and cellular adaptive immunity. The second is the diversity of antigen-specific receptors and the polymorphism of MHC molecules required for the recognition of foreign antigens by T cells. Immunity to intracellular pathogens that reside in the host cell cytoplasm requires the generation of cytotoxic T cells (cellular immunity) and possibly antibody-dependent cell-mediated cytotoxicity (humoral and cellular immunity). Immunity to intracellular pathogens that reside in vesicular compartments requires T-celldependent macrophage activation (cellular immunity). Extracellular pathogens in interstitial spaces, blood, and lymph require antibodies, complement activation, phagocytosis, or neutralization, whereas the major defense at epithelial surfaces consists of antibodies (mainly IgA).

The alternative pathway of complement activation provides a nonadaptive first line of defense against many microorganisms. The alternative complement pathway can proceed on some microbial surfaces in the absence of specific antibody and lead to lysis or opsonization (by C3b) of the organism.

Phagocytosis is the principal mechanism for destruction of extracellular bacterial pathogens as well as several viral and fungal organisms. The importance of phagocytosis is indicated by the increased susceptibility to infection of patients with disorders of phagocytic function (e.g., chronic granulomatous disease; Chediak-Higashi syndrome; glucose-6-phosphate dehydrogenase deficiency; and secondary disorders like side effects of immune suppression and C and Ig deficiencies. Phagocytic cells enter the site of infection in response to products of coagulation,

fibrinolytic, and kinin pathways (especially for PMNs); bacterial production of N-formylmethionine (PMNs and macrophages); products of the activation of the classic and alternative complement pathways (C5a, C3a, C3b); and cellular products (i.e., chemokines, histamine). Most pathogenic bacteria resist direct phagocyte attachment, but the host produces opsonins that make them susceptible to phagocytosis through receptors for immunoglobulins (Fc receptors) and complement receptors (C3b).

Once a microorganism has been ingested into phagocytic vacuoles, phagocytes kill the pathogen either by aerobic (oxygen-dependent) or anaerobic (oxygen-independent) mechanisms. Infection is associated with increased oxygen consumption. Activation of nicotinamide-adenine dinucleotide phosphate hydrogenase (NADPH) oxidase catalyzes the reduction of O_2 to superoxide (O_2). Superoxide dismutase catalyzes the production of hydrogen peroxide (H_2O_2) that results in microbial killing by myeloperoxidase-catalyzed oxidation of halide ions to halide radicals. H_2O_2 can also kill directly and can interact with superoxide to form toxic hydroxyl radicals. Anaerobic mechanisms include degradation of ingested material by the activity of multiple lysosomal enzymes, including lysozyme, phospholipase A_2 , serine esterases, lipases, and acid phosphatases, and basic proteins, the defensins.

Pathogens and their products also induce cytokine production by phagocytes, including IL-1, IL-6, IL-8, IL-12, and TNF, which induce subsequent host defense mechanisms. TNF initiates a local inflammatory response and induces the expression of adhesion molecules on local blood vessels that mediate leukocyte adhesion, infiltration, retention, and activation. The small vessels later clot, preventing spread of infection to the blood. However, once an infection spreads to the bloodstream, these same effects of TNF at a systemic level can lead to septic shock. Chemokines such as IL-8 produced locally by macrophages, endothelial cells, and connective tissue cells promote the migration of monocytes, neutrophils, and lymphocytes into the inflammatory lesion. Cytokines (IL-1 and IL-6) released by macrophages also activate the acute-phase response that leads to the production of molecules that bind to bacteria: C-reactive protein is an opsonin and activates complement; mannose-binding protein is an opsonin for monocytes. Macrophage-derived cytokines induce a leukocytosis (increase in circulating PMNs).

Adaptive immunity is triggered when an infection eludes the innate defense mechanisms and generates a threshold dose of antigen. It does not become effective until several days after infection. The lag represents the proliferation and differentiation processes required to generate effector cells from the 1 per 10^6 resting lymphocytes that can recognize the antigen.

B-cell responses develop in lymphoid tissues. The production of antibodies by B cells to most antigens depends on helper (CD4) T cells. Activated B cells proliferate, forming germinal centers where they undergo isotype switching and somatic hypermutation. The antibodies secreted initially provide protection but may also trap antigen in the form of antigenantibody (immune) complexes on the surface of follicular dendritic cells for a long time, thus contributing to B-cell selection in the germinal center. The antibody-secreting plasma cells generated in the lymphoid tissue remain in the medullary cords of lymph nodes and in the red pulp of the spleen. However, other B cells leave the germinal center and migrate to the bone marrow, where they complete their differentiation into plasma cells, and approximately 90% of all antibody is produced by these bone marrow plasma cells. Antibodies are the key defense against extracellular bacteria. Antibodies interact directly with the bacteria or bacterial products.

T cells are the central mediators of protection against intracellular bacterial infections. The T cells do not interact with the microbes directly, but instead interact with

the infected host cells. T-cell activation is initiated when recirculating T cells encounter specific antigen in draining lymphoid tissues. Antigens in tissues are trapped by APCs in draining lymph nodes, whereas blood-borne antigens are taken up by APCs in the spleen. The trapping of antigen by APCs in the lymphoid tissues and the continuous recirculation of T cells through the lymphoid tissues ensure that rare, antigen-specific T cells will encounter that antigen on a professional APC (e.g., dendritic cells). Activation of T cells results in increased expression and function of cell adhesion molecules, which bind to their ligands that are upregulated at sites of inflammation and thereby promote the migration of effector cells to sites of infection.

Activated CD4 T cells can differentiate into functionally distinct effector cells that are distinguished based on the cytokines they produce. TH_1 (inflammatory) cells selectively produce IL-1, TNF, and interferon- and promote macrophage activation. TH_2 (helper) cells selectively produce IL-4 and IL-5 and preferentially promote antibody production. Infections with intracellular pathogens are accompanied by delayed-type hypersensitivity. Thus, in an individual immune to an intracellular bacterium, a skin reaction develops at the site of local administration of soluble antigens from this agent. This reaction is mediated in large part by T cells and is characterized by monocyte infiltration 2472 hours post-exposure.

One of the most important consequences of an adaptive immune response to natural infection or vaccination is the establishment of a state of immunologic memory. Immunologic memory is defined as the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously because of increased numbers of antigen-specific cells (as the result of antigen-induced clonal expansion). Secondary and subsequent responses differ qualitatively from primary responses; for example, the affinity as well as the amount of antibody increase with repeated immunization, and memory T cells are increased in frequency and have distinct activation requirements and cell surface phenotypes.

In summary, a variety of innate and adaptive defense mechanisms act in a concerted fashion to protect the host against a myriad of antigenic challenges. Phagocytes, neutralizing antibodies, and antibody-dependent complement-mediated and cellular cytotoxicity are the major protective mechanisms involved in defense against extracellular pathogens. Extracellular pathogens typically cause acute disease, which develops soon after entry of the organism into the host and is terminated once the immune response has developed. Defense against intracellular pathogens results in attack against the infected cells (lymphocyte-mediated cytotoxicity) or provides a potentially lethal cellular environment (activated macrophages). In general, intracellular pathogens coexist with their cellular habitat for longer periods of time and result in chronic disease. Tissue reactions against intracellular pathogens are granulomatous. Granuloma formation is responsible for the protection of the host, but also causes the pathologic processes associated with these lesions.

Chapter 8 - B-Cell Development

Kenneth Dorshkind

B cells are the subset of lymphocytes specialized to synthesize and secrete immunoglobulin (Ig); their name derives from the fact that they were first characterized in the *B*ursa of Fabricius in birds. However, B lymphopoiesis in adult mammals occurs in the bone marrow. Production in that site, which is antigen-independent, is referred to as primary B-cell development.

B-cell production is a dynamic process in which immature hematopoietic precursors progress through a series of developmental stages that culminate in the generation of newly produced B lymphocytes that express Ig on their cell surface. However, transforming events can occur in B lineage cells at particular developmental stages and may result in various forms of B-cell leukemia. Additional abnormalities of primary B lymphopoiesis can result in failure of surface Ig-expressing cells to develop, thereby resulting in deficiencies of humoral immunity.

This chapter describes the cellular and molecular events that occur during normal, primary B-cell development, in order to provide a basis for understanding abnormalities of that process. Although human B lymphopoiesis is emphasized, murine studies have contributed to much of what is known about B-cell development and will be discussed in parallel. A detailed list of primary references on each topic cannot be cited due to space limitations. Therefore, key reviews on particular subjects will be noted and may be consulted for additional information.

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STAGES OF B-CELL DEVELOPMENT

B lymphocytes, like all blood cells, are the progeny of pluripotential hematopoietic stem cells (PHSC). [1] [2] [3] [4] PHSC in humans are CD34+ cells that lack expression of additional lineage-specific determinants (lin cells) present on the surface of cells committed to development of the myeloid, erythroid, and lymphoid lineages. PHSC in mice are also lin and express the Thy-1 antigen at low levels (Thy-1 ^{lo}) and stem cell antigen-1 (Sca-1).

As shown in Figure 81 , progeny of the PHSC ultimately become committed to the development of myeloid or lymphoid cells. The existence of a multipotential myeloid-restricted precursor that generates progenitor cells committed to the erythroid, granulocytic, monocytic, or megakaryocytic lineages is well established. Figure 81 depicts a lymphoid-committed precursor and there is recent evidence supporting its existence. Nevertheless, developmental relationships during early lymphocyte production are unresolved, and reports of precursors that can generate both B cells and macrophages, but not cells of other hematopoietic lineages, [5] indicate that lineage diagrams such as those shown in Figure 81 will continue to evolve.

There is less ambiguity in defining precursorprogeny relationships within the B-cell developmental pathway itself. Although a uniform terminology for naming stages of B-cell differentiation has not been formulated, in both humans and mice the earliest committed B-cell precursors are referred to as pro-B cells. Their immediate progeny, referred to as pre-B cells, express immunoglobulin heavy chain protein of the class in their cytoplasm. Subsequently, as light chain protein is expressed, it combines with heavy chain protein, and the newly assembled IgM molecule is expressed on the surface of immature B lymphocytes. Immature B cells generally do not proliferate or differentiate in response to antigen, and their encounter with self-antigens may lead to their tolerization or elimination. As these cells mature, they acquire IgD. Surface IgD expression is indicative of the mature B-cell stage of development. These newly produced B cells leave the bone marrow to seed secondary lymphoid tissues such as spleen and lymph nodes, and during this period they undergo further maturation. If antigen is not encountered, these B cells die within a few days, but upon antigen binding IgM+/IgD+ B cells proliferate and differentiate into short-lived immunoglobulin-secreting plasma cells and long-lived memory B cells. Figure 81 shows a scheme of human and murine B-cell differentiation from the pro-B cell to the surface IgM/IgD stage of development.

As described previously, the expression of immunoglobulin has traditionally been used to delineate different stages of B lymphopoiesis. However, the development of antibodies that recognize additional cell surface antigens on B lineage cells and the use of flow cytometry have permitted stages of B-cell development to be defined more precisely. These molecules are described below, followed by a detailed discussion of the steps involved in the rearrangement and expression of the immunoglobulin heavy and light chain genes.

Cell Surface Antigens Expressed on Developing Human B Lineage Cells

Figure 81 shows a scheme of B lymphopoiesis in which several cellular determinants used to stage developing human B lineage cells are depicted. [1] [6] [7] [8] As shown in the figure, CD34, the human PHSC marker, is also expressed on pro-B cells. However, pro-B cells express additional determinants that distinguish them from the lin PHSC population.

Several of these determinants, which include CD10, CD19, and CD45, are expressed on the earliest B lineage-committed pro-B cells and continue to be expressed at most subsequent stages of primary differentiation. CD10, also known as common acute lymphocytic leukemia antigen, or CALLA, is a 100,000 molecular weight (MW) member of a family of peptidases expressed on lymphoid precursors, myeloid cells, and most acute lymphocytic leukemias. CD10 expression is down-regulated by the time IgD appears. CD19 is a 95,000 MW Ig superfamily protein that associates with other cellular determinants to generate a signal transduction complex on B lineage cells. Cross-linking of CD19 results in calcium mobilization and activation of phospholipase C and protein tyrosine and serine kinases. There is a gradual increase in expression of CD19 with maturation in the B-cell lineage. CD45 encodes a family of protein tyrosine phosphatases involved in the regulation of signal transduction in leukocytes.

Other cell surface determinants have a somewhat more restricted pattern of expression. For example, CD20 is expressed at the pro-B to pre-B cell transition and is lost upon differentiation into plasma cells. It may be involved in B-cell activation and proliferation. CD21 is the receptor for the C3d component of complement and the Epstein-Barr virus. It is expressed by

Figure 8-1 The hematopoietic hierarchy with an emphasis on B-cell development. Stages of human and murine B-cell development, and the cell surface, cytoplasmic, and nuclear determinants expressed on those cells are indicated. When comparing this scheme to others, it is important to understand that a uniform terminology for stages of development has not been adopted. Placement of lineage markers and their intensity of expression at various stages of development, indicated by the thickness of lines, is an approximation. Letters next to cells refer to the Hardy stages of murine B-cell development.

most IgD^{+} B cells and is lost upon B-cell activation. CD21 and cell surface IgM can form an activation complex with CD19 and CD81 (not shown in Fig. 81), a 26 kd integral membrane protein expressed on various cell types. This complex amplifies signaling through the B-cell receptor. CD22 is first detected in the cytoplasm of pro-B and pre-B cells and is expressed on the surface of a subset of B cells. CD22 is an adhesion receptor that may be involved in B-cell activation. CD24 is a GPI-linked sialoglycoprotein expressed at low levels from the late pro-B cell stage until plasma cell differentiation. CD38 is a type II membrane glycoprotein expressed on pro-B cells. CD38 expression is then extinguished before its reappearance in activated B cells. It may function as an adhesion receptor. Finally, expression of CD40 initiates at the pro-B cell stage of development. It is involved in B-cell proliferation, somatic hypermutation, and isotype switching in germinal centers. Defects in the CD40 gene result in X-linked hyper-IgM syndrome.

Cell Surface Antigens Expressed on Developing Murine B Lineage Cells

Hardy and colleagues have developed a scheme of murine B lymphopoiesis based on expression of various cell surface determinants. [9] The most immature pro-B cells (Fraction A) express the CD45R tyrosine phosphatase and CD43, a molecule of unknown function that may be involved in activation of B lineage cells. As they mature into Fraction B cells, they continue to express CD45R, CD43 and acquire CD24, the Heat Stable Antigen (HSA). Fraction B cells have initiated Ig heavy chain gene rearrangements. As cells mature to the Fraction C stage, they continue to express CD45R and HSA and acquire BP-1, a cell surface metallopeptidase. Cells in Fraction D, which are CD43, CD45R+, and HSA+, have undergone a functional heavy chain gene rearrangement and express heavy chain in their cytoplasm. Finally, as light chain protein is expressed, the Ig molecule is assembled and IgM appears on the surface of immature B cells. These newly produced B cells are distinguished based on the differential expression of IgM (Fraction E) or IgD (Fraction F). Finer resolution of the above fractions can be achieved based on the level at which

particular determinants are expressed and through the introduction of additional markers. [10]

Cells at most of the previous stages also express CD19, a cell surface antigen that is also a reliable indicator of commitment to the B-cell lineage. Pro-B cells express CD19 at relatively high levels and there is a 2.5-fold increase in CD19 expression as cells mature. B-1 B cells, described below, express CD19 at levels 50% higher than conventional B lymphocytes.

Expression of the Surrogate Light Chain Proteins

Analysis of various pre-B-cell lines revealed that heavy chain protein could be detected on the cell surface in association with two noncovalently linked proteins referred to as Vpre-B and lambda()5. These proteins were termed surrogate light chains

because of their homology with conventional light chains and are encoded by the Vpre-B and 5 genes which are located on chromosome 16 in mice and chromosome 22 in humans.[2] [11] Their detection on the surface of murine pre-B cell lines led investigators to hypothesize that expression of a cell surface /surrogate light chain complex occurred during normal development and that its ligand was expressed on cells of the hematopoietic microenvironment.

In an attempt to define the stage at which surrogate light chains are expressed during normal B-cell differentiation, analysis of primary bone marrow B lineage cells with monoclonal antibodies to surrogate light chain proteins has been made. The results are not always consistent between laboratories, possibly due in part to the use of different antibodies for detection of surrogate light chains. Thus, this area of research is an evolving one.

One model that has resulted from these studies is that murine pro-B and early pre-B cells express surrogate light chain on their surface as a pro-B-cell receptor. These cells do not express heavy chain protein, and the surrogate light chains may form a complex with another as yet uncharacterized 130 kDa glycoprotein. At later stages of development, a subpopulation of early pre-B cells may express a cell surface surrogate light chain//Ig/Ig receptor (Ig and Ig are defined in a subsequent section). However, surrogate light chain expression is down-regulated as differentiation proceeds, and most pre-B cells that express heavy chain protein no longer express surrogate light chain. In humans, there have been suggestions that, analogous to mice, some pro-/pre-B cells may express cell surface surrogate light chains in the absence of protein. However, there is general agreement that a /surrogate light chain/Ig/Ig complex is expressed on the surface of normal human pre-B cells. [12]

Pre-B cell and B-cell numbers are drastically reduced in 5 knockout mice, [2] [11] indicating that surrogate light chains must play a crucial role during B-cell development. However, although /surrogate light chain complexes can transduce signals resulting in mobilization of intracellular Ca_2^+ and tyrosine phosphorylation of intracellular proteins, their ligand has not been identified and their precise role during primary B lymphopoiesis remains undetermined.

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REARRANGEMENT AND EXPRESSION OF IMMUNOGLOBULIN GENES

The Ig molecule consists of two heavy chains and two light chains (Fig. 82). The amino acid sequences at the antigen binding amino terminal ends of the molecule are highly variable, thus this area is referred to as the variable region. Highly divergent regions within the variable portion of the heavy and light chains are referred to as hypervariable or complementary-determining regions. The remaining part of the heavy and light chains is more conserved and is referred to as the constant region.

The expression of Ig is unique to B lineage cells and is dependent on the successful rearrangement of genes that encode the heavy and light chain proteins. [13] [14] [15] [16] [17] In general (and as described following), heavy chain gene rearrangement and expression precedes light chain recombination. However, there may be exceptions to this sequence of events during normal B lymphopoiesis, and light chain gene rearrangements with heavy chain genes in the germ-line configuration have been detected in some leukemias and lymphomas.

Heavy Chain Gene Rearrangements

The genes that encode the heavy chain protein are located on chromosome 14 in humans and chromosome 12 in mice. Both the human and murine heavy chain gene include variable (V), diversity (D), and joining (J) regions that encode the variable portion of the immunoglobulin molecule located at its amino terminus (Figs. 82 and 83). V region genes, which number around 100 in humans and up to 1,000 in mice, are located at the 5 end of the heavy chain locus. Each V region exon is approximately 300 base pairs in length and is separated from other V region genes by short intronic sequences. V genes are organized into families based on sequence homology with other members; seven humanV_H gene families have been described. 5 of each V gene is an exon that encodes a translation initiation signal and leader peptide involved in intracellular trafficking of the heavy chain protein. Sequences encoded by this exon are not part of the mature heavy chain protein. There are about 30 human and 12 murine D region genes located 3 to the V region. These may also be grouped into families; at least 10 families have been described in humans. There are 6 human J region genes and 4 murine ones and multiple C region genes arranged in tandem with a species-specific order. [18] Each C region gene includes multiple exons.

As noted above, the first Ig rearrangements in developing B-cell precursors most commonly occur in the heavy chain genes. The recombination process in humans, depicted in Figure 83 , is similar to that in mice. The initial event during heavy chain gene rearrangement in a pro-B cell juxtaposes one D region segment to a J_H segment. Although in theory any one D locus can join with equal frequency to any one J_H region gene, there may be preferential use of selected D and J_H region genes at various stages of development. Murine studies also indicate that rearrangement of a particular D region gene to a J heavy chain gene segment can result in recombination at any one of three possible reading frames. A strong bias for reading frame I is seen in conventional B cells, and there is counterselection against reading frame III; approximately 70% of such D rearrangements have a stop codon. Pre-B cells in which reading frame II is used may produce a DJC protein. The precise role played by such a molecule is not understood, but it is hypothesized that its expression may somehow signal a cessation of further development. Following successful D-J_H recombination, a V_H region gene rearranges to the D-J_H complex during the pro-B to pre-B-cell transition. There are indications that biased use of J_H proximal V_H genes occurs in the newly generated repertoire of neonatal mice and humans. [18]

The heavy chain constant (C) region remains separated from the rearranged $V_H DJ_H$ complex by an intron, and this entire sequence is transcribed. Subsequent RNA processing leads to deletion of the intron between the $V_H DJ_H$ complex and the most proximal C region genes. Following translation, heavy chain protein is expressed in the cytoplasm of pre-B cells (Fig. 81). The heavy chain is retained there by a heavy chain binding protein (Bip) until it binds to light chain proteins.

Each B-cell precursor has two sets of immunoglobulin heavy chain genes, and if initial rearrangements are successful and protein is produced, further heavy chain rearrangements at the second locus are inhibited. [19] This process is known as allelic exclusion and results in expression of only one Ig heavy chain allele in any given cell. However, if rearrangements are unsuccessful at one heavy chain locus during B-cell development, recombination will initiate at the second one.

Light Chain Gene Rearrangements

The Ig light chain protein can be encoded by the kappa () or lambda () genes (Fig. 84). Over 90% of murine B cells and 60% of human B cells express light chain protein, which is encoded by a gene located on human chromosome 2 and murine chromosome 6. The human locus consists of approximately 76 V region genes, clustered into 6 families; 5 functional J region genes; and 1 constant region C gene. The murine light chain gene consists of approximately 160 V exons, organized into 19

Figure 8-2 The immunoglobulin molecule and the B-cell antigen receptor complex. The Ig molecule consists of two heavy and two light chain proteins. Two additional transmembrane proteins, Ig- and Ig-, are noncovalently associated with the heavy chain, and linked to these molecules are various protein kinases. Following binding of antigen to the Ig molecule, signaling cascades are activated, resulting in events that can include cell proliferation, synthesis of additional Ig classes, and Ig secretion.

families; 5 J region genes; and a single C region gene. There may be skewed use of particular V genes during fetal and adult B-cell development. Alternatively, B cells can express light chain protein, encoded by a gene located on human chromosome 22 and murine chromosome 16. Approximately 52 human V genes, grouped into ten families, have been described. There are seven human C genes, three of which are pseudogenes. Each C gene is located 3 of a respective J gene. [18] D region segments are not present in light chain genes.

Light chain gene rearrangements initiate following expression of cytoplasmic heavy chain protein in most B lineage cells. Rearrangements occur first at the locus, where the initial event is the joining of a V segment to a J segment. The V -J complex remains separated from the light chain C region by an intron, this entire complex is transcribed, and further splicing of the intron between the J and C segment results in formation of a mature V -J -C transcript. If rearrangements at the first allele are unsuccessful, attempts are made to rearrange the second gene. If this fails, the locus is used. Lambda-producing murine B cells may have deletions of both loci or exhibit rearrangements to a recombination signal sequence that is 1020 kB 3 of the C exon. Light chain gene rearrangements may only be productive in approximately one-third of pre-B cells, because up to 60% of nondividing pre-B cells never progress to the surface IgM+ stage.

Ig Gene Recombination

Immunoglobulin gene recombination is dependent on an enzymatic machinery that deletes intronic sequences and joins coding segments of DNA. [13] [16] [17] The

enzymes that mediate these functions act through recognition of recombination signal sequences that are located 3' of each heavy and light chain V-region exon, 5 of each heavy and light chain J segment, and 5 and 3 of each heavy chain D region gene. Figure 83 shows the association of these recognition sequences with the various

Figure 8-3 Rearrangement and expression of the human Ig heavy chain gene. The figure shows the Ig heavy chain gene and the signal sequences 3 of each V region locus, 5 and 3 of each D region locus, and 5 of each J region locus. These consist of a heptamer and nonamer sequences separated by either 12 or 23 base pairs. During Ig recombination a signal sequence of 12 base pairs can only join to another of 23 base pairs (the so-called 12-23 rule). As shown in the figure, initial heavy chain gene rearrangements form coding joints between D and J regions as well as signal joints that are ultimately degraded. Subsequently, joining of the V region gene to the DJ complex occurs. Following a successful rearrangement, the VDJ complex, the intron, and portions of the constant regions are transcribed. RNA processing and differential splicing results in formation of an mRNA molecule that is then translated. In the example shown, the rearranged VDJ complex and the constant region, with the and C region genes, is transcribed. Following RNA processing and translation, a particular B cell could then express and/or protein.

Figure 8-4 Structure of the human Ig light chain genes and their chromosomal locations.

heavy chain exons. Each recombination signal sequence consists of conserved heptamer and nonamer sequences, separated by nonconserved DNA segments of 12 or 23 base pairs. During immunoglobulin gene recombination, these recognition sequences form loops of DNA which in turn bring the coding exons in apposition to one another. These noncoding loops are subsequently deleted and degraded.

The expression of two proteins, referred to as Recombinase Activating protein (RAG)-1 and RAG-2, is required for heavy and light chain gene recombination in developing B lineage cells. Results from cell free systems that measure V(D)J recombination indicate that RAG proteins are involved in cleavage of DNA at recombination signal sequences, thereby producing a blunt DNA end on each signal sequence and a hairpin end on adjacent coding gene segments. The RAG proteins, in addition to various molecules that ligate the free DNA ends, are also needed for subsequent efficient joining of coding sequences to one another. [20]

Immunoglobulin Gene Transcription

Following productive heavy and light chain gene rearrangements, transcription of the rearranged loci occurs. Transcription of Ig genes is dependent on the interaction of various DNA binding proteins with specific promoter sequences located 5 of each heavy and light chain V region and one or more heavy and light chain enhancer regions.[13] [14] [15] [16] Enhancer sequences have been identified in both human and murine heavy and and light chain genes. Heavy chain enhancers are located in the intron and downstream of the C_H region genes and light chain enhancers are located in the intron and downstream of the C gene (Figs. 83 and 84).

A number of transcription factors that bind to motifs in these enhancers and potentiate transcription have been identified. For example, the E12 and E47 transcription factors, which are basic helix-loop-helix (HLH) proteins encoded by the E2A gene, bind to specific sequences in the heavy chain enhancer. E2A proteins are expressed in pro-B cells, but they may be complexed to another HLH protein known as Id that can form heterodimeric complexes with these proteins and prevent their binding to DNA. It is thought that upon cessation of Id expression, E12 and E47 molecules are free to bind their enhancer and pro-B cell maturation can occur. Studies of two transgenic mouse strains provide support for this sequence of events. Mice expressing an Id transgene have a complete block in B-cell differentiation, and B-cell development is blocked at Fraction A in knockout mice in which the E2A gene has been inactivated.

DNA binding proteins such as NF-B are also involved in regulating light chain gene expression. Analogous to the situation in which the actions of E2A proteins are inhibited by Id, NF-B is complexed to an inhibitory molecule, I-B, in the cytoplasm of pre-B cells. During pre-B-cell differentiation I-B dissociates from NF-B and the latter molecule translocates to the nucleus where it can then bind DNA.

Studies of mice in which the genes encoding these transcription factors have been disrupted have demonstrated that many

TABLE 8-1 -- Selected Transcription Factors That Regulate B-Cell Development

Transcription Factor	Function/Targets	Effect of Loss of Expression
PU.1/Ets proteins	Ig H and L chain genes, RAG-1, Ig, Ig, Vpre-B, 5, bcl-2, TdT, CD19, J chain	B-cell development blocked. Few CD45R+ cells; no Ig gene rearrangements
Ikaros	Lymphoid-specific genes in early lymphoid precursors	Block in all lymphocyte development
BSAP/Pax-5	Lymphoid-specific genes in early lymphoid precursors	Block at pro-B-cell stage
E2A/Basic helix loop helix proteins	Heavy chain enhancer	Block at pro-B-cell stage
Id	Binds to and inhibits E2A binding to DNA	Block at pro-B-cell stage when overexpressed
Bmi-1	Hox genes	Reduction of CD45R+ cell number
Early B-cell factor (EBF)	Ig promoter	Block at pro-B-cell stage; no D-J_H rearrangements
NF-B	Kappa light chain enhancer	Modest effects on primary B-cell development

of these molecules play an obligate role during B-cell development. Selected examples of transcription factors which have been demonstrated to play a key role in B-cell development are given in Table 81 , and in-depth reviews on this topic can be consulted for additional details. [21]

Sterile Transcripts

It is clear that transcription of Ig genes occurs subsequent to the formation of productive heavy and light chain gene rearrangements, but transcription of unrearranged heavy chain genes also takes place. This results in the production of developmentally regulated transcripts of unrearranged Ig genes, referred to as germ line or sterile transcripts. Multiple species of heavy and light chain gene germ line transcripts have been described, and some could conceivably encode proteins. It has been hypothesized that there is a mechanistic link between transcription and Ig gene rearrangement. For example, transcription might make unrearranged Ig genes somehow accessible to both RNA polymerase and V(D)J recombinase; the germline transcripts could function in the rearrangement reaction; or transcription could alter structural characteristics of DNA, making the recombination signal sequences better targets for recombination. [13] [14] [15]

Class Switching

At the terminal stage of primary B-cell development, B cells can express both IgM and IgD. This occurs through alternative processing of a primary RNA transcript. As noted above, the rearranged V_H -D-J_H heavy chain, part of the constant region, and the intron separating these exons are transcribed following productive rearrangements in a cell. If the intron is spliced, resulting in association of the C region with the VDJ complex, the B cell expresses IgM. Alternatively, if the C exon is

spliced out along with the heavy chain intron, the VDJ complex and the C exon become contiguous and the B cell expresses IgD. The differential processing of heavy chain transcripts within a single cell explains why some newly produced B cells coexpress both IgM and IgD (Fig. 83).

Class switching resulting in expression of additional heavy chain constant regions other than IgM and IgD can also occur. Deletion of germ line DNA resulting in religation of the VDJ complex with a particular downstream heavy chain constant gene appears to be the mechanism by which this takes place. In this case, for example, deletions of and result in juxtaposition of the VDJ complex to the next downstream 3 CH segment. These DNA deletions are believed to occur at or near nucleotide sequences called switch regions, which are located in the intron 5 to each C_H exon. Class switching that results in expression of , , and epsilon is a highly regulated, secondary differentiation event potentiated by helper T cells and their products. [22] [23]

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GENERATION OF THE IMMUNOGLOBULIN REPERTOIRE

The murine B-cell repertoire has the potential to include up to 10^9 antigen-binding specificities. There are several means by which this repertoire diversity is generated during Ig gene recombination. First, heavy and light chain proteins are encoded by multiple germline V, J, and, in the case of heavy chain, D region genes; the combinatorial diversity among them is enormous. Second, DNA joints that form during recombination are often imprecise and can occur at any of several nucleotides in the germline. This junctional diversity has the potential to generate different amino acid sequences resulting in added diversity of the Ig repertoire. However, as noted previously, out-of-frame joints that cannot be transcribed may also result. Finally, nucleotides not encoded in the germline can be added to D-J_H and V_H -DJ_H junctions during postnatal (but not prenatal) life by a nuclear enzyme known as terminal deoxynucleotidyl transferase (TdT). It is reasonable to assume that similar processes are operative in humans.

Not all B lineage cells successfully rearrange their heavy and light chain genes. Heavy chain gene rearrangements are productive in approximately one-third of pre-B cells, and of these cells, only about one-third progress to make functional light chain gene rearrangements. Those cells with nonproductive rearrangements are eliminated from the marrow, possibly by a macrophage-mediated mechanism.[3] [24] In view of this, it is obvious that a considerable degree of cell loss must occur during B lymphopoiesis, and, as a result, the number of B-cell precursors exceeds the number of mature B cells produced. In this regard, murine bone marrow has been estimated to contain up to 10^8 B lineage cells, but only about 3×10^6 mature B cells are produced each day. Thus, the generation of millions of mature B lymphocytes, each of which expresses an Ig molecule with a unique antigen-binding specificity, is dependent upon a high daily rate of bone marrow B lymphopoiesis.

As noted, those mature B cells that are produced in the bone marrow emigrate to secondary lymphoid tissues, such as spleen and lymph nodes. If the newly produced B cell does not encounter antigen, it is a short-lived cell that soon dies. Thus, that particular Ig antigen-binding specificity is lost and must be regenerated de novo during primary B lymphopoiesis in the bone marrow. However, if the mature B cell binds antigen, it clonally expands and subsequently reverts to small resting memory cells

that are retained by the organism. Thus, that Ig antigen-binding specificity is retained and if the same antigen is encountered again, a rapid secondary immune response is generated. Within secondary lymphoid tissues, such as spleen and lymph nodes, somatic mutation of V region genes can occur during the immune response. These latter events involve point mutations in V region genes that can often result in an increase in the affinity of an antibody for antigen.

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B-CELL TOLERANCE

It is obviously critical that the mature B cells that exit the bone marrow not be autoreactive, but because the process of Ig recombination is random, it is possible for B cells that recognize self-antigens to be generated. Several mechanisms have been proposed to account for the fate of such autoreactive B cells. In some cases the presence of self-antigen may not activate the self-reactive B cells. This scenario may result if binding of autoantigen to the B cell is weak, if the B-cell affinity for the antigen is weak, or if the autoantigens are present at an extremely low concentration. In other instances interaction of antigen with the autoreactive B cell may result in an anergic state. The level of membrane Ig on such anergic B cells may be reduced up to twentyfold, the cell's ability to proliferate may be impaired, and differentiation into immunoglobulin secreting cells may be blocked. Finally, self-reactive B cells may be clonally deleted. Clonal deletion may result from cytolysis by other cells, such as bone marrow macrophages, or autoreactive B cells may undergo a physiologic change resulting in cell death following receptor engagement. The intracellular mechanisms that underlie these processes remain to be defined, but further understanding of signaling mechanisms in B lineage cells should reveal differences between activated and anergized populations. [25] [26]

Recognition of self-antigen by a B cell may not necessarily result in anergy or apoptosis, however, because receptor editing is thought to occur in some self-reactive B lymphocytes. In this process, rearranged chain alleles can be replaced by secondary rearrangements of upstream V genes to downstream, unrearranged J segments. These secondary rearrangements, which may delete the primary V J complex or separate it from C by inversion, are possible because of the continual presence of unrearranged V regions upstream of the joined V J coding segments. The detection of RAG-1 and RAG-2 in B lymphocytes suggests that the recombinatorial machinery necessary for further Ig gene recombination is present in mature cells. Although most data in support of receptor editing are derived from murine studies, it has been suggested that this process may also occur in human B cells. [27]

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B-CELL ANTIGEN RECEPTOR COMPLEX

The interaction of antigen with the B cell results in cross-linking of membrane Ig and the transduction of intracellular signals. However, although Ig is a transmembrane protein, its cytoplasmic carboxy tail is relatively short. For example, the carboxy terminus of IgM and IgD consists of only three amino acids, and signal transduction after antigen binding to B cells is dependent upon the noncovalent association of Ig with two additional transmembrane proteins termed Ig and Ig. The Ig Ig proteins, which are encoded by the mb-1 and B29 genes, respectively, are expressed at early stages of B-cell development before the production of heavy chain protein and link membrane Ig to cytoplasmic tyrosine kinases (Fig. 82). Following binding of antigen to the Ig molecule, Ig and Ig proteins are phosphorylated by these associated protein kinases, and multiple signaling cascades are generated within the B cell. The end result of these activation events is cell proliferation and Ig secretion. Details of B-cell signaling events following antigen binding can be found in several recent reviews. [28] [29] [30]

As noted previously, the Ig and Ig molecules may be part of the pre-B-cell receptor that also includes surrogate light chain proteins and heavy chain. Thus, it is not surprising that Ig, Ig, and other components of the B-cell antigen receptor and the associated signaling machinery also play a key role in B-cell development. [31] Pro-B-cell differentiation in mice lacking Ig is blocked and failure to express Ig results in a marked reduction in the number of peripheral B cells. Many of the protein kinases expressed in B lineage cells have also been demonstrated to transduce signals required for normal differentiation. For example, patients with X-linked agammaglobulinemia, a condition in which B-cell numbers are reduced by over 95%, resulting in a severe deficiency of circulating Ig, have a mutation in the gene encoding Bruton's tyrosine kinase (Btk). Btk is a member of a family of nonreceptor tyrosine kinases. Mutations in this gene also account for the X-linked immunodeficiency of xid mice. Other kinases that appear to play key developmental roles during B lymphopoiesis include JAK, Syk, and Lyn. JAK knockout mice have a severe lymphocyte deficiency, Syk knockout mice have a block in the development of CD43 cells from CD43+ precursors, and production of B-1 cells (see following) is defective in Lyn knockout mice. [31]

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FETAL B-CELL DEVELOPMENT AND B-1 (CD5) B CELLS

The yolk sac is a major site of erythropoiesis, and murine studies have demonstrated that precursors with lymphoid developmental potential are present in that site. It has traditionally been considered to be the site at which hematopoiesis initiates during embryogenesis. However, recent evidence that B-cell progenitors can be detected in murine embryos at the same time or just before their appearance in the yolk sac has challenged this view and suggested that hematopoietic stem cells originate in an intraembryonic site. [32] This issue aside, it is clear that during embryogenesis, hematopoiesis, and B-cell development in particular, occurs in several intraembryonic sites in both humans and mice.[1]

The human fetal liver is seeded by hematopoietic precursors at week five to six of gestation, and pre-B cells that express cytoplasmic are detectable in it at seven weeks of gestation. Surface Ig-expressing B cells are present in the liver two to four weeks thereafter. Liver hematopoiesis ceases at birth. The spleen is also a site of human embryonic hematopoiesis by week five of development, and blood cell production continues in that organ until late fetal life. Surface Ig+ cells can be detected in the human embryonic spleen by 12 weeks of gestation. After birth the spleen is a secondary lymphoid tissue in which mature B and T cells are present. Hematopoiesis in the bone marrow is evident by three months of gestation, and by the time of birth bone marrow hematopoiesis predominates. [33]

A similar scenario is observed in mice. By day 13 of murine development, fetal liver B-cell progenitors that express cytoplasmic heavy chain protein are easily detectable, and by day 17 surface IgM-expressing cells are present. B lineage cells in murine fetal liver express the AA4 antigen, which has been useful for their isolation from that tissue. Fetal spleen is also a site of lymphopoiesis. However, shortly after birth the spleen becomes a secondary lymphoid organ and the bone marrow, which has been seeded with bloodborne hematopoietic precursors during embryogenesis, becomes the definitive site of primary B-cell development.

In addition to the above organs, other embryonic tissues, including the omentum, placenta, and blood have been reported to contain B-cell progenitors.

It is thought that B cells that develop during fetal life, referred to as B-1 or CD5 B cells, are distinct from those, termed B-2 cells, that are produced after birth in the bone marrow. These B-1 (CD5) B cells are distinguished from conventional B-2

cells by their high levels of surface IgM, low levels of membrane IgD, expression of CD5, their localization in the peritoneal and pleural cavities, the preferential use of certain immunoglobulin heavy chain variable region family members, and their predominant secretion of IgM and IgA antibodies, which are thought to provide defense against environmental flora. B cells from patients with chronic lymphocytic leukemia commonly express CD5, as do many autoreactive B lymphocytes. [34]

Although the existence of B cells with these different phenotypic and functional properties is accepted, whether or not they represent distinct B-cell lineages has been the subject of considerable debate among some investigators. Evidence that B-1 (CD5) B cells are a distinct B-cell lineage derived from a fetal stem cell is supported by experimental evidence demonstrating that repopulation of CD5-expressing B-1 cells occurs following reconstitution of mice with fetal liver cells but not with adult bone marrow. In addition to the phenotypic characteristics noted above, adult bone marrow pre-B cells in the mouse express a novel myosin light chain that is not detected in fetal liver pre-B cells. Others argue that B-1 cells are conventional B cells whose characteristics result from selective pressures following antigen exposure.

REGULATION OF B LYMPHOPOIESIS BY STROMAL CELLS AND THEIR PRODUCTS

Hematopoiesis takes place in the intersinusoidal spaces of the medullary cavity in association with a fixed population of stromal cells. The latter cells are nondividing and form a three-dimensional framework with which developing blood cells associate. The stroma is a source of signals that regulate the growth and differentiation of B lineage cells through direct interactions with B-cell progenitors and through the secretion of soluble mediators. [7] [35] [36] [37]

CellCell Interactions

Direct contact between developing B lineage and stromal cells can be observed upon analysis of intact bone marrow or of B lymphopoiesis in long-term cultures, and the molecular bases for these associations are being defined in both humans and mice. For example, both murine and human pre-B cells express the VLA-4 integrin that interacts with a stromal cell ligand identified as vascular cell adhesion molecule-1 (VCAM-1). VLA-4 also promotes binding to fibronectin, an extracellular matrix protein. CD44 on developing B lineage cells has also been implicated in mediating stromal celllymphocyte interactions in the mouse through binding to stromal cell derived hyaluronate. These intercellular interactions would presumably allow B cells to receive proliferative and/or developmental signals from stromal cells. It is important to understand that the stromal cells may not be passive populations that constitutively provide these signals. Instead, the binding of the B lineage cell may stimulate the stromal cell to produce such differentiation or growth-potentiating activities.

Stromal Cell Derived Cytokines

Another means by which stromal cells regulate B-cell development is through the secretion of soluble mediators. [38] A selective list of factors that have been implicated in the growth and/or differentiation of B lineage cells is presented in Table 82 . Although a clear definition of all the factors that may affect B lymphopoiesis is still evolving, studies of knockout mice have demonstrated that many of the cytokines described in the table and below play an obligate role during B-cell development. Some of the cytokines involved in normal B-cell development include the following.

IL-7

Although there is recent evidence that Interleukin-7 (IL-7) acts as a differentiation factor that potentiates Ig heavy chain gene rearrangements, [39] it has generally been considered to be a B-cell growth factor. [40] In mice, generation of normal numbers of murine B lineage cells is dependent upon stromal cell-derived IL-7, and cells that have initiated Ig heavy chain D-J_H rearrangements are particularly IL-7 responsive. By the time cells have matured to the late pre-B cell stage of development responsiveness to IL-7 is lost, presumably as a result of failure of cells to express the IL-7 receptor. Studies in which mice are treated with antibodies to IL-7 or its receptor, and studies of IL-7- and IL-7-receptor knockout mice indicate that IL-7 is an obligate B lymphopoietic factor, because the number of B lineage cells is dramatically reduced in these animals. However, these findings may be species-specific, because the proliferative response of human B lineage cells to IL-7 is not as vigorous as in mice. That IL-7 may not be a critical human B lymphopoietic factor is supported by the finding that B cells are generated in humans whose hematopoietic cells lack IL-7-receptor expression.

IL-7 Cofactors

A number of stromal cell-derived proliferation cofactors have been described that synergize with IL-7, but when used alone these cofactors have little or no demonstrable effect on the growth of B lineage cells. These include c-kit ligand (KL), flk2/flt3 ligand, insulin-like growth factor-I (IGF-I), pre-B-cell colony enhancing factor, and pre-B-cell growth stimulatory factor/stromal cell derived factor-I (PBSF/SDF-1). However, PBSF/SDF-1 may play an additional role during B lymphopoiesis. Mice lacking PBSF/SDF-1, a member of the CxC group of chemokines, die perinatally as a result of targeted gene inactivation and exhibit a number of abnormalities that include a significant reduction of CD43+CD45R+ pro-B cells and pre-B cells in fetal liver. The precise role of PBSF/SDF-1 in B-cell development remains unclear.

Differentiation Factors

One of the first transitions that must occur during B lymphopoiesis is the maturation of pro-B cells into pre-B cells, and stromal cell derived cytokines that include insulin-like growth factor-I (IGF-I) and fltk2/flt3 ligand may be involved in this process. Upon culture of murine CD45R negative populations with either of these cytokines, maturation of cells into CD45R+, cytoplasmic expressing pre-B cells occurs. The combination of c-kit ligand (KL) and IL-11 has also been reported to support the B-cell potential of lymphomyeloid progenitors and potentiate their maturation. Studies of mice in which the IGF-I gene has been inactivated have failed to indicate an obligate role for that factor during B-cell development. On the other hand, the frequency of pro-B cells in fractions A-C of flk2/flt3 receptor knockout mice is significantly reduced, indicating a requirement for flk2/flt3 ligand during the early stages of primary B-cell development.

Little information is available about mediators that act at later stages of development, although it has been suggested that stromal cells secrete an IL-4 like molecule that potentiates the pre-B to B-cell transition.

TABLE 8-2 -- Selected Cytokines and Hormones That Regulate Primary B Lymphopoiesis

Cytokine/Hormone	Source	Reported Effects
Positive Regulators		
IL-7	Stromal cells	Proliferation of DJ_H rearranged B-cell progenitors; rearrangement of Ig heavy chain genes
c-kit ligand (KL)	Stromal cells	Synergizes with IL-7 to stimulate proliferation of B-cell progenitors
Pre-B cell stromal cell derived factor/stromal cell derived lymphopoietin (PBSF/SDF-1)	Stromal cells	Synergizes with IL-7 to stimulate proliferation of B-cell progenitors. CD43+CD45R+ cell numbers reduced perinatally in mice lacking PBSF/SDF-1
Insulin-like growth factor-I (IGF-I)	Stromal cells	Synergizes with IL-7 to stimulate proliferation of B-cell progenitors; differentiation of CD45R cells to CD45R+ and cytoplasmic +

Pre-B-cell enhancing factor (PBEF)	Activated peripheral blood lymphocytes	Stimulates pre-B-cell growth in the presence of IL-7 + KL
Flk2/flt3 ligand	Stromal cells	Differentiation of CD45R cells to CD45R+
Thyroid hormone	Thyroid gland	Required for normal B-cell production in murine bone marrow
IL-11 + KL	Stromal cells	Supports B-cell potential of lymphomyeloid progenitors; potentiates maturation of immature B-cell precursors
IL-4-like molecule	Stromal cells	Potentiates pre-B to B-cell transition
Thymic stromal cell derived lymphopoietin (TSLP)	Stromal cells	Stimulates growth of B lineage cells
Negative Regulators		
IL-1	Macrophages	Indirectly inhibits B-cell production through stimulation of CSF production by stromal cells; may also directly inhibit proliferation of B-cell progenitors
IL-4	Stromal cells	Inhibits pro-B to pre-B-cell transition
Transforming growth factor beta (TGF-)	Stromal cells; ubiquitous	Inhibits pre-B to B-cell transition; inhibition of proliferation/differentiation of earlier stages?
Interferon- (IGN-)	T cells	May directly inhibit proliferation of B-cell progenitors and may indirectly affect the ability of stromal cells to support B lymphopoiesis
Estrogens	Ovaries	Inhibition of B-cell development in vivo; may function through direct effects on B-cell progenitors and indirect effects on stroma
Colony stimulating factors	Stromal cells; T cells	Augments myelopoiesis and inhibits B-cell development in vivo and in vitro; may indirectly inhibit B-cell production due to myeloid overgrowth

Negative Regulators

Although the preceding discussion has focused on positive regulation of B-cell development, some agents may inhibit growth and/or differentiation of B lineage cells at various stages of development.[41] IL-1 and TNF- have been shown to inhibit the proliferation of human pro-B cells, and these cytokines, as well as transforming growth factor-, -interferon, and various colony stimulating factors, have also been shown to inhibit murine B-cell development in vitro or in vivo. Some of these cytokines may directly bind to B lineage cells and inhibit their growth and/or differentiation, whereas others may act indirectly. For example, IL-1 may function by stimulating stromal cells to secrete factors that in turn inhibit B-cell production. The study of negative regulators is further complicated by the fact that even within the B-cell lineage, the same factor may stimulate some stages of development while inhibiting others. In this regard, IL-4 has been proposed to potentiate the pre-B to B-cell transition while inhibiting pro-B-cell growth.

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REGULATION OF B-CELL DEVELOPMENT BY SYSTEMIC FACTORS

In addition to regulatory signals produced in the medullary cavity, increasing evidence indicates that mediators of systemic origin also influence B-cell production. In particular, many classical endocrine hormones can affect B-cell development. For example, B-cell development in mice appears to be dependent upon the integrity of the pituitary/thyroid axis. A growing literature also suggests that steroid hormones may act as negative regulators of B lymphopoiesis. Increased levels of estrogens occurring during pregnancy inhibit B-cell development. [42] As with stromal cell-derived factors, hormones could act directly on B

lineage cells or mediate their effects indirectly through actions on the stroma.

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Chapter 9 - T-Cell Immunity

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ANTIGEN RECOGNITION AND T-CELL DEVELOPMENT

Lymphocytes recognize and respond to antigenic challenge. They are divided into B lymphocytes, which produce antibodies, and T lymphocytes, which regulate cell-mediated immunity. T lymphocytes, so called because they are thymus derived, are further subdivided into helper and cytotoxic T lymphocytes. Antigen receptors of T cells are structurally related to antibodies but, instead of recognizing soluble proteins, recognize foreign antigens as protein fragments (peptides) bound to major histocompatibility complex (MHC) molecules and presented on the surface of antigen-presenting cells. During development, T-cell receptors rearrange, much as antibodies do, and also undergo both positive and negative selection so that mature T cells recognize a broad spectrum of foreign but not self antigens.

T-Cell Antigen Receptor

The antigen receptor on the majority of T cells is a heterodimer consisting of two polypeptides, and , that are covalently linked by disulfide bonds (Fig. 9-1).[1] A minority of T cells express

Figure 9-1 Schematic of the T-cell receptor. C and V are Ig-like constant and variable domains. The transmembrane regions of the two chains are indicated as helices interrupting the plasma membrane phospholipid bilayer. S-S, disulfide bonds; indicates the approximate location of carbohydrate moieties.

a related receptor composed of and chains.[2] The chain is a 4050-kd acidic glycoprotein and the chain is a 4045-kd uncharged or basic glycoprotein. There are a number of structural similarities between the T-cell receptor (TCR) and immunoglobulin (Ig). All TCR chains contain variable (V) and constant (C) regions (Fig. 9-2). The junction between the V and C regions is encoded by a joining (J) segment, and, for the and chains, one or two diversity (D) segments. In addition, there are three highly diverse regions in the TCR chains that correspond to the complementarity-determining regions (CDR) of Ig. These regions form the contact points between the TCR and peptide/MHC.

The TCR recognizes a complex of processed peptide, generated from proteolysis of foreign proteins, bound to self MHC. Although the TCR confers specificity for the particular antigen/MHC complex, it depends on associated proteins called the CD3 complex for signal transduction (Fig. 9-3).[3] The CD3 complex consists of five separate integral membrane proteins (, , epsilon, , and) that are noncovalently associated with each other and with the TCR. The CD3 , , and epsilon chains exist as monomers in the TCR/CD3 complex. In mice, the chain is a homodimer in 90% of T cells and a heterodimer with the chain in the remaining 10% of T cells. However, the chain has not been identified in humans. Antibodies specific for CD3 react with 100% of peripheral T cells.

The synthesis, assembly, and expression of the TCR/CD3 complex are tightly regulated and coordinated in the thymus during T-cell differentiation (see below). [4] The CD3 , , and epsilon genes are expressed in very immature thymocytes, before the and TCR genes. In mature T cells, the TCR- heterodimer associates with the CD3-epsilon complex in the endoplasmic reticulum

Figure 9-2 Linear representation of the polypeptide chains of the , , , and TCR. The relation of the variable (V), diversity (D), and joining (J) segments of the TCR chains is shown. The C (constant), H (hinge), T (transmembrane), and CY (cytoplasmic) regions are also shown. S-S, disulfide bonds; indicates the approximate location of carbohydrate moieties. The approximate positions of the hypervariable regions CDR1, CDR2, and CDR3 are depicted by *, , and , respectively. *(Modified from Davis and Bjorkman,[1] with permission. Copyright 1988 Macmillan Magazines, Ltd.)*

Figure 9-3 The TCR associates with the multimeric CD3 complex. The CD3 complex, composed of a epsilon heterodimer, a epsilon heterodimer, and a homodimer, noncovalently associates with the TCR in the plasma membrane. S-S, disulfide bonds.

(ER), and the complex is then transported to the Golgi complex for glycosylation of N-linked oligosaccharides. The CD3 chain homodimers must associate with the TCRepsilon CD3 complex prior to transport of the entire assembly to the cell surface.

Recently the three-dimensional structure of two different TCRs bound to their specific peptide/MHC complex was solved by x-ray crystallography. [5] [6] The most surprising finding of this work is that the TCR is diagonally oriented, with the CDR loops aligning over the strands that form the base of the peptide-binding groove (Fig. 9-4). This arrangement may lock the TCR and MHC structures, with the TCR- chain covering the amino-terminal half of the bound peptide and the TCR- chain covering the carboxy-terminal half. Thus, the antigenic peptide is completely buried at the TCR/MHC interface.

As noted above, approximately 10% of human peripheral T cells express an alternative TCR designated . [2] The and chains are glycoproteins ranging from 35 to 60 kd, depending on the size of the polypeptide backbone and the degree of glycosylation. Although the TCR also associates with the CD3 complex, cells expressing this alternative receptor do not appear to recognize peptide fragments bound to MHC molecules (see below).

Rearrangement and Expression of T-Cell Receptor Genes

During T-cell maturation, TCR genes undergo rearrangement analogous to that for immunoglobulin genes (Fig. 9-5). In the earliest precursors, TCR genes are in a nonfunctional germline configuration consisting of variable (V), joining (J), and constant (C) gene segments separated by intervening sequences of DNA. The chain also contains diversity (D) gene segments. In humans, the -chain locus is on chromosome 7 and the -chain locus is on chromosome 14. The -chain locus consists of two very similar C genes, associated with a cluster of J segments and one D segment. During development, somatic rearrangement occurs, allowing these gene segments to come together.

Somatic rearrangement of the TCR V, D, and J genes is directed by recombinases that recognize specific heptamer and nonamer sequences of nucleotides adjacent to each rearranging segment. The chain rearranges before the chain (see following), and diversity is generated in the joining process from the possible arrangement of VDJ, VJ, and VDDJ joins. D segments are used in all three reading frames, adding to the potential -chain diversity. The -chain diversity comes from the joining of V and J segments. Other mechanisms, such as N nucleotide insertion at the V/D/J joining sites, also increase diversity. Interestingly, the -chain V and J genes are encoded on chromosome 14 between the -chain V and J genes. Therefore, rearrangement of the chain inactivates the chain. The locus is similar to the -chain locus and is also located on chromosome 7. There are many fewer V gene segments for the and loci than for the and loci (Table 9-1), but increased junctional variability of the chain may compensate for this reduced number of possible variable regions. The TCR shares many features with the TCR but also resembles Ig in a number of ways. The CDR3 region shares more homology with Ig than with the TCR. Structural analysis of the CDR3 suggests that it accommodates many different types of antigenic ligands and may bind to antigen directly in the absence of antigen-presenting cells, similar to Ig.

Although somatic mutation is an important mechanism for generating diversity in immunoglobulin molecules, it does not occur in TCR genes. [7] This may prevent the development of autoreactive TCR once T cells exit the thymus. It has been estimated that approximately 4.5×10^{13} different forms of V and 6.5×10^{12} forms of V are possible.[8] If only 1% of the sequences coded for viable proteins, 3×10^{24} receptors are still possible. If 99% of these are deleted because of self-reactivity, low affinity, or structural defects, 3×10^{22} murine TCRs are possible. That fewer than 10^{9} thymocytes exit the thymus during a mouses lifetime raises the question as to how random the generation of TCR is.

Each immature T cell contains two genes for each chain of the TCR. However, only one of the two inherited genes for the chain and one of the two inherited genes for the chain are

Figure 9-4 (A) Complex of TCR (top) and HLA-A2/peptide (bottom) shown as an -carbon trace. The TCR C domain is not shown. *(From Garboczi et al.,[5] with permission. Copyright 1996 Macmillan Magazines, Ltd.)* **(B)** Interaction of the TCR with HLA-A2. Ribbon diagram of the 1 and 2 domains of the HLA-A2 molecule is shown. The regions of interaction of the TCR and chains are shown in dark lines, with the rough positions of the CDR loops shown in numerals. This view is perpendicular to that shown in **A.** *(From Parham P: Pictures of MHC restriction. Nature 384:109, 1996,with permission. Copyright 1996 Macmillan Magazines, Ltd.)*

functionally rearranged and expressed in a single T cell. [9] This phenomenon, called allelic exclusion, occurs because the productive rearrangement of the TCR locus on one chromosome prevents the rearrangement of the corresponding allelic gene on the other chromosome. If the rearrangement on the first chromosome is not functional, i.e., if it cannot be transcribed or its transcript cannot be translated, the corresponding locus on the other chromosome will rearrange. Therefore, nonfunctional TCR transcripts are often found in T cells. The -chain genes rearrange first, so if both rearrangements are unproductive, no -chain gene rearrangements occur and the T cell dies.

T-Cell Development

Blood cells are derived from multipotent hematopoietic stem cells (HSC) produced in the liver in fetal life and then in the bone marrow in adults. [10] These HSC must receive signals provided by thymic stromal cells in order to develop into mature T cells. [10] [11] That the thymus is critical for T-cell development was shown in the early 1960s in a series of experiments by Miller[12] and others. Researchers found that mice thymectomized within a few days of birth had a marked deficiency of lymphocytes, did not reject foreign skin grafts, and were susceptible to infections. HSC reach the thymus via the blood and migrate through the vessel walls to enter the stroma, where the earliest differentiation steps begin in the cortex.

Current understanding of T-cell differentiation is largely derived from studies in mice in which combinations of antibodies recognizing specific cell-surface markers were used to select subpopulations of thymocytes.[13] These cells were then reintroduced into lethally irradiated animals to determine their ability to differentiate into mature T cells. Four major developmental stages can be identified in the thymus prior to expression of a mature TCR/CD3 complex and the lineage-specific antigens CD4 and CD8 (Fig. 9-6).[14]

Thymic Lymphoid Progenitors

The precursor cells that have newly entered the thymus express high levels of CD44, a homing receptor that may be responsible for targeting these cells to the thymus. They also express CD117 (stem cell factor receptor) and the interleukin-7 (IL-7) receptor, which promote thymocyte growth and survival. Thymic lymphoid progenitors are not, however, committed to the T-cell lineage; intravenous injection of these cells can also give rise to B lymphocytes and dendritic cells.

Pro-T Cells

Within a day of thymic entry, thymic lymphoid progenitors up-regulate expression of CD25 (IL-2 receptor) and are now defined as pro-T cells. These cells begin to proliferate rapidly, although the TCR- and TCR- in pro-T cells remain in germline configuration and are not expressed. This suggests that the pro-T-cell stage involves a TCR-independent activation event, for activation of mature T cells requires stimulation through their

Figure 9-5 Schematic representation of TCR and TCR chain rearrangement. The leader (L), variable (V), diversity (D), joining (J), and constant (C) genes are shown. Each C gene consists of multiple exons that are not shown. (See Table 9-1 for the number of V, D, J, and C exons in each locus.) In this example, recombination of the TCR- locus uses the V_{1} exon with the J_{7} exon; following recombination, intervening V and J exons are deleted.

TABLE 9-1 -- Human T-Cell Receptor Gene Segments

Variable (V)	7080	52	12	4[a]
Diversity (D)	0	2	0	3
Joining (J)	61	13	5	3
Constant (C)	1	2	2	1

[a] The TCR locus is located within the TCR locus. The V genes are interspersed with the V genes, but there are at least four TCR V genes.

TCRs. Finally, commitment to a T-cell lineage occurs during the transition from thymic lymphoid precursors to pro-T cells and is dependent on cytokines produced by the thymic stroma. In particular, IL-1 and tumor necrosis factor- (TNF-) play pivotal roles in the expression of CD25 on pro-T cells.

Early Pre-T Cells

Early pre-T cells down-regulate expression of CD44 and CD117, continue to express high levels of CD25, and also up-regulate expression of Thy-1, heat-stable antigen (HSA), and Sca-1/2. Early pre-T cells constitute the majority (60%) of triple-negative (CD3/CD4/CD8) thymocytes, but nearly three quarters of them fail to progress to the late pre-T-cell stage. During the transition from pro-T to early pre-T cell, the rate of proliferation slows and TCR- rearrangement begins. TCR- rearrangement precedes that of TCR- and is required for further differentiation. Mice with severe combined immune deficiency (SCID) or recombinase-activating gene deficiency cannot carry out V(D)J recombination, and thymocyte maturation arrests at the early pre-T-cell stage. Reconstitution of SCID mice with a productively rearranged TCR- transgene is sufficient to initiate the differentiation of early pre-T cells into double-positive (CD4+/CD8+) thymocytes. In mice that have been genetically altered such that they cannot express TCR- protein (TCR- knockout mice), thymocyte differentiation is arrested at the early pre-T-cell stage, although TCR- rearrangement proceeds normally. These findings underscore the important regulatory role served by TCR- and indicate that TCR- partners with a molecule other than TCR- at the early pre-T-cell stage. That molecule was identified by von Boehmer and co-workers in 1993 and designated pre-T. [15]

Pre-T is a type I transmembrane protein and a member of the Ig superfamily (Fig. 9-7).[16] It contains a single extracellular Ig-like domain that exhibits little homology with any other antigen receptor molecule and does not contain a J-like sequence. Pre-T and rearranged TCR- form a disulfide-linked heterodimer called pre-TCR. The cytoplasmic tail of pre-T is about 30 amino acids long, but it is not necessary for signal transduction. The asymmetric structure of pre-TCR suggests that other, as yet unidentified small proteins may be part of this receptor, and some evidence for this exists. In addition, results from biochemical and gene targeting studies indicate that CD3-, CD3-epsilon, and CD3- are complexed with pre-TCR and are responsible for signal transduction following engagement of pre-TCR with ligand. This allows cells that have successfully rearranged their TCR- to escape from programmed cell death and to undergo TCR- rearrangement that is terminated only when the TCR binds to intrathymic ligands. Thus, a key function of pre-TCR is to select TCR-+ cells for expansion and further differentiation, thereby permitting a large number of rearranged TCR- chains to combine with a single TCR- chain.

Late Pre-T Cells

Late pre-T cells lose CD25 expression and once again begin to proliferate rapidly. TCR- rearrangement occurs at this stage, and the newly synthesized TCR chains displace the pre-T, forming a mature TCR complex. At this point, pre-T expression is turned off, and early, large CD4+/CD8+ thymocytes evolve into small resting CD4+/CD8+ thymocytes.

Figure 9-6 Maturation of hematopoietic stem cells (HSC) in the thymus.

Figure 9-7 Schematic of the pre-T/TCR- heterodimer. The transmembrane regions of the two chains are indicated as helices interrupting the plasma membrane phospholipid bilayer. S-S, disulfide bonds; indicates the approximate location of carbohydrate moieties.

CD4/CD8 Lineage Commitment

The interaction of TCR with peptide/MHC prompts the CD4+/CD8+ T cell to make a commitment to either the CD4 or CD8 lineage. CD4 and CD8 are called co-receptors because during TCR interaction with peptide/MHC, CD4 and CD8 simultaneously bind to invariant parts of the MHC molecule. CD4 binds to constant regions of MHC class II, while CD8 binds to constant regions of MHC class I. Selection of CD4 or CD8 lineage occurs by two distinct processes. [17] [18] In the instructional model, cells whose TCR binds to MHC class II molecules lose expression of CD8, while those cells whose TCR interacts with MHC class I molecules lose expression of CD4. In the stochastic model, CD4+/CD8+ thymocytes randomly lose expression of either CD4 or CD8. Cells whose co-receptor expression correlates with the MHC specificity of the TCR continue to develop, while the majority of cells whose TCR MHC specificity does not match co-receptor expression (e.g., TCR specificity for MHC class II on a CD8+ cell) are deleted. Evidence exists for both the instructional and stochastic mechanisms of lineage selection in thymocyte development.

Positive and Negative Selection of T Cells

The vast majority of thymocytes undergo apoptosis in the thymus, never reaching the periphery. To exit the thymus, T cells must pass two tests, called positive and negative selection.[19] [20] In positive selection, T cells are selected for binding to self MHC. [21] Presumably, interaction between the TCR and self MHC molecules on thymic stromal cells sends a positive signal to the immature T cell that blocks apoptosis and activates further developmental steps. Surprisingly, the particular peptide bound to thymic MHC molecules does not play a critical role in positive selection. [22] [23] [24] TCR transgenic mice, expressing a single TCR on all their T cells, develop normally in the thymus even in the absence of the peptide to which the mature transgenic T cells respond. In addition, most T cells selected by a single peptide/MHC complex will respond to that MHC molecule bound to a spectrum of peptides. Thus, it appears that there is some flexibility in the requirement for peptide/TCR interaction such that a single peptide/MHC complex can select a large variety of TCR. In contrast, if the affinity of the TCR interaction with the selecting peptide/MHC complex is too high, the T cell will undergo apoptosis. [25] This process, called negative selection, prevents autoreactive T cells from reaching the periphery. Therefore, T cells that emerge from the thymus are both self-tolerant and self-MHC restricted.

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CELLULAR BASIS OF THE IMMUNE RESPONSE

Antigen Presentation

T cells are both effectors and regulators of the immune response. To mediate these activities, T cells must first recognize specific antigenic peptide/MHC complexes. These complexes are formed by two distinct pathways (Fig. 9-8).[26] [27] Intracellular antigens, such as viral proteins, are synthesized in the cytoplasm of infected cells. A fraction of these newly synthesized proteins are broken down into peptides in specialized cytoplasmic structures called proteosomes. These peptides are then transported into the ER by the transporter of antigenic peptides (TAP). In the ER they associate with newly synthesized MHC class I molecules. In contrast, extracellular antigens, such as extracellular microbes and secreted molecules, are taken up into specialized antigen-presenting cells (dendritic cells and macrophages) by endocytosis. The proteins are broken down into peptides in lysosomes and associate with newly synthesized or recycled MHC class II molecules in postlysosomal vesicles before transport to the plasma membrane. Thus, class I and class II MHC molecules present largely nonoverlapping antigenic peptides, with class I molecules generally presenting endogenous (intracellular) antigens and class II molecules generally presenting exogenous (extracellular) antigens.

Accessory Molecules

Although T-cell specificity is dependent on interaction of the TCR with antigenic peptide/MHC, activation of T cells requires the binding of additional, or accessory, molecules on the T cell to their ligands on the antigen-presenting cells (macrophages, dendritic cells, and B cells). This may reflect both the low affinity (estimated at about 10^5 M) of the TCR for peptide/MHC and that only a very small fraction of MHC molecules on an antigen-presenting cell are bound with the specific peptide recognized by an individual TCR. [28] [29] Accessory molecules can increase the strength of the interaction between the T cell and can provide additional signals for T-cell activation.

T-Cell Subsets and Their Functions

CD4+ Helper T Cells

CD4+ T cells initiate specific immunity by recognizing antigenic peptides bound to MHC class II molecules on B cells or other antigen-presenting cells such as dendritic cells and macrophages. Upon activation, these cells secrete soluble proteins such as IL-2, IL-4, and IFN- that are collectively referred to as cytokines (Table 9-2). Cytokines provide the necessary help for the activation and differentiation of other lymphoid cells; hence the designation of CD4+ T cells as helper T cells.

Figure 9-8 MHC class I and MHC class II molecules acquire antigenic peptides from different compartments. MHC class II molecules bind exogenous antigen. Peptide-free MHC class II molecules are synthesized in the rough endoplasmic reticulum (rER), mature through the Golgi complex, and fuse with endosomes that contain peptides derived from antigens brought into the cell by endocytosis. MHC class I molecules bind endogenous antigens. Soon after completion of synthesis of MHC class I molecules in the rER, they bind antigenic peptides derived from intracellular antigens such as viruses. Viral proteins are broken down into peptides in the proteosome and moved into the rER by the transporter of antigenic peptides (TAP).

TABLE 9-2 -- Functions of T-Cell-Derived Cytokines

IL-2	Stimulates T-cell proliferation and differentiation
	Induces antibody production by B cells
	Increases lytic activity of NK and LAK cells
IL-3	Stimulates production and differentiation of macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytic cells, and erythroid cells
	Supports proliferation of HSC
IL-4	Induces differentiation of Th2 cells
	Induces proliferation and differentiation of B cells
IL-5	Stimulates growth and differentiation of eosinophils
IL-6	Induces growth and differentiation of T cells, B cells, hepatocytes, keratinocytes, and nerve cells
	Stimulates production of acute phase proteins
IL-8	Chemotactic for neutrophils, T cells, and basophils
	Causes adhesion of neutrophils to endothelial cells
IL-9	Synergizes with erythropoietin to support development of erythroid burst-forming units by bone marrow cells
	Promotes in vitro T-cell growth and survival
IL-10	Suppresses macrophage function
	Induces B-cell proliferation and Ig secretion
IL-12	Induces differentiation of Th1 cells
	Stimulates growth and function of NK and T cells
IL-13	Induces B-cell growth and differentiation
	Inhibits inflammatory cytokine production by macrophages
IL-14	Induces proliferation of activated B cells
IL-15	Stimulates growth of T cells and NK cells

IL-17	Induces fibroblast and endothelial cell cytokine production
G-CSF	Stimulates production of granulocytes and neutrophils
	Activates neutrophils
GM-CSF	Promotes differentiation and growth of HSC
IFN-	Antiviral activity
	Enhances expression of MHC molecules
	Activates macrophage activity
MIP-1	Chemoattracts T cells, monocytes, and eosinophils
MIP-1	Chemoattracts monocytes and T cells
RANTES	Chemoattracts monocytes, T cells, and eosinophils

In 1989 Mossman and Coffman reported that murine CD4+ helper T-cell clones could be divided into two mutually exclusive subsets. [30] We now know that these subsets can be distinguished not only by the cytokines they produce, but also by the lymphocytes they affect and the types of pathogen to which they respond (Table 9-3). [31] [32] The Th1 subset produces cytokines such as IL-2, IFN-, and tumor necrosis factor-/, while the Th2 subset produces IL-4, IL-5, IL-6, IL-10, and IL-13. Th1 cells promote secretion of IgG_2 antibody, whereas Th2 cells induce IgG_1 and IgE. Th1 cells mediate delayed-type hypersensitivity and cytotoxicity, and have limited effects on B cells; Th2 cells mediate B-cell help and also activate eosinophils and mast cells. Th1 cells are beneficial in immune response to leishmaniasis, leprosy, viruses, and allergy, while Th2 cells are beneficial in arthritis, autoimmunity, helminth infection, and pregnancy. In humans, a Th0 subset has also been described. These cells can produce various combinations of both Th1 and Th2 cytokines.

There is now overwhelming evidence that Th1 and Th2 cells are derived from a common precursor. The major factor that determines Th1 versus Th2 lineage is the predominance of a particular cytokine in the microenvironment of the developing cell. Cells that differentiate in the presence of IL-4 largely become Th2 cells, whereas those that differentiate in the presence of IL-12 and IFN develop into Th1 cells. The source of these cytokines is still under investigation, but in some cases, T cells and natural killer (NK) cells can provide IL-4 early in an immune response while phagocytes can produce IL-12 in response to certain pathogens.

CD4+ T cells also regulate immune responses by a mechanism that involves direct cell-cell contact rather than secreted factors. Activated T cells and some other cells express a cell surface molecule called Fas (CD95), a member of the TNF receptor family. [33] Engagement of Fas by another cell-surface molecule called Fas ligand triggers apoptosis in the Fas-expressing cell. Homeostasis of the immune system is in part maintained by activated CD4+ cells expressing Fas ligand on their surface. Once the number of activated lymphoid cells in a particular site exceeds a critical number, Fas ligandbearing cells induce apoptosis of Fas+ cells. Without such a regulatory mechanism,

TABLE 9-3 -- Characteristics of Th1 and Th2 Cells

	Th1	Th2
Cytokines	IL-2	IL-4
	IFN-	IL-5
	TNF-/	IL-6
		IL-10
		IL-13
Functions	DTH	B-cell help
	Cytotoxicity	Eosinophil activation
	Macrophage activation	Mast cell activation
Help for pre-dominant antibody isotype	IgG_2	IgG_1
		IgE
Beneficial in	Leishmaniasis	Arthritis
	Leprosy	Autoimmunity
	Viral infection	Helminths
	Allergy	Pregnancy
		Organ transplantation
Detrimental in	Arthritis	Allergy
	Autoimmunity	Viral infection
	Helminths	Leprosy
	Organ transplantation	

the immune compartment would continue to expand enormously and eventually overwhelm the animal. In fact, this idea is confirmed by *lpr* and *gld* mice, two mutant strains that lack functional Fas and Fas ligand, respectively. These animals exhibit severe lymphoproliferative disorders, and eventually die with lupus-like symptoms.

Other cells have adopted expression of Fas ligand as a way to protect against immune cells. [34] Tissues such as cornea and testis are said to enjoy immune privilege in that they are not infiltrated by lymphocytes. Recent studies have shown that these tissues consitutively express Fas ligand, so that infiltrating Fas+ lymphocytes undergo apoptosis. Unfortunately, some tumors also express Fas ligand, thereby allowing escape from immune surveillance by activated tumor-specific T cells.

CD8+ Cytotoxic T Cells

CD8+ T cells recognize antigenic peptides presented by MHC class I molecules. The majority of CD8+ T cells kill target cells that bear the peptide/MHC complex for which the CD8 T-cell receptor is specific. These cells, therefore, are designated cytotoxic T lymphocytes (CTL). CD8+ T cells that emerge from the thymus are not yet capable of lysing targets, but upon stimulation with antigen/MHC and cytokines such as IL-2 and IFN- produced by CD4+ helper cells, they differentiate from precursor CTL into mature effector CTL.[35] Acquisition of effector CTL function also involves the synthesis of lytic molecules and their sequestration into membrane-bound granules. The granules contain a number of different components, including perforin, a pore-forming protein with structural similarities to complement proteins, and granzymes, a family of serine esterases. Effector CTL also acquire the capability to produce and secrete cytokines and, as for CD4+ cells, can be divided into type 1 and type 2, depending on the pattern of cytokines produced. [32]

Lysis of targets by CTL is well studied (Fig. 9-9). Upon conjugation of the target and CTL, the CTL cell becomes activated and the cytoplasmic granules are polarized toward the target cell. A fraction of these granules are exocytosed into the junction between the two cells, and the granules deliver their lethal contents to

the target cell. At this point, the CTL can detach, and target lysis proceeds in the absence of the CTL. Video microscopy has shown that conjugation and delivery of the lethal hit takes less than 20 minutes and that a single CTL is capable of lysing many targets in a short period.

Target cell death is mediated by the synergistic activity of the granzymes and perforin (Fig. 9-10). Perforin polymerizes in the target cell membrane to form pores that allow water and ions to move into the cytosol, leading to osmotic swelling and lysis. Granzymes activate a cascade of enzymes called caspases that culminate in the degradation of DNA, similar to the apoptosis induced by engagement of Fas. Although CTL can express Fas ligand, the greater part of CTL-mediated lysis results from granule exocytosis and not engagement of Fas. Interestingly, most CTL are resistant to CTL-mediated lysis. This may result from directed release of lytic granules toward the target cell and/or intrinsic resistance of the CTL to perforin and granule-mediated lysis.

Much of our understanding of the mechanism of CTL comes from studies using CTL recognizing non-self MHC molecules, as is the case in organ allograft rejection. However, the primary function of CTL is to eradicate viral infection. This is accomplished by two mechanisms: (1) CTL lysis of host cells that are the source of replicating virus, and (2) secretion of the antiviral cytokine, IFN-.

T Cells

A minority of peripheral blood T cells (<10%) express the TCR. Although our understanding of the rules governing TCR recognition of antigen lags far behind that of the TCR, some features have recently been identified. [36] Although the TCR is associated with the CD3 complex, many of these cells do not express either CD4 or CD8 (double negative). This is consistent with the observation that, with a few exceptions, antigen recognition by T cells is not MHC restricted. Some T cells recognize intact proteins while others recognize nonpeptidic antigens from bacteria or other pathogens. [37] T cells also recognize heat shock proteins or phosphorylated alkyl, carbohydrate, or nucleotide residues. Thus, T cells appear to recognize an overall structure in the absence of antigen processing

Figure 9-9 Schematic of target recognition and lysis by CTL.

Figure 9-10 Apoptosis can be caused by either granule exocytosis or Fas/Fas ligand pathways. Following the interaction of the TCR with MHC class I/peptide, granules containing perforin/granzymes localize toward the target cells and are released. Perforin inserts into the plasma membrane, and granzymes move into the target cell. Perforin and granzyme act synergistically to activate the caspase cascade, resulting in apoptosis. Binding of Fas ligand (FasL) to Fas also activates the caspase cascade, resulting in apoptosis.

and MHC presentation, in contrast to the fine specificity for antigenic peptide and MHC restriction observed for T cells.

The response of T cells to antigen is often more rapid than that of T cells, perhaps because T-cell antigens can be presented earlier than T-cell antigens. T cells secrete a variety of cytokines, and many mediate antigen-unrestricted cytotoxicity. Finally, an increasing body of evidence supports the proposition that T cells play a unique protective role in microbial infections, such as tuberculosis. This has stimulated research into whether vaccines or other therapies directed toward T cells may be important therapeutics.

Delayed-Type Hypersensitivity Responses

Delayed-type hypersensitivity is a cell-mediated immune response in which the ultimate effector cell is an activated mononuclear phagocyte (macrophage). DTH is the primary immune defense against intracellular bacteria such as *Listeria monocytogenes* and mycobacteria. It can also be induced by sensitization to chemicals or environmental antigens or by intradermal injection of microbial antigens, e.g., purified protein derivative (PPD) from *Mycobacterium tuberculosis* in presensitized or vaccinated individuals. The response begins when CD4+ or CD8+ T cells respond to the presence of MHC-associated antigenic peptide and produce inflammatory cytokines such as IL-2, TNF, and IL-8. Within a few hours, neutrophils accumulate at postcapillary venules at the injection site, and by 12 hours, neutrophils are replaced by T cells and macrophages. The T cells produce additional inflammatory cytokines, including IFN-, which activate the macrophages. The endothelial cells lining the postcapillary venules swell and fibrinogen leaks from the blood vessels into the surrounding tissue, where it is cleaved to fibrin. This deposition of fibrin, as well as the infiltrating T cells and macrophages, causes the tissue to become swollen and hard. Induration is obvious by about 18 hours after injection with antigen and peaks by 2448 hours. The activated macrophages eliminate the antigen, leading to resolution of the response.

T-Cell Memory

In most cases, the immune responses to infectious agents have distinct short- and long-term phases. Immediately after the primary antigenic challenge there is a rapid expansion of specific effector T cells that destroy the pathogen. Most effector cells are relatively short-lived and are eliminated at the end of the primary immune response. However, some of these cells are spared, and a state of long-lived memory develops. Subsequent challenge with the same pathogen leads to a more rapid and effective response, reflecting both an increase in the precursor frequency of antigen-specific T cells and an increased sensitivity

to antigen. [38] Memory T cells are small resting cells that may be sequestered in areas such as lymph nodes, where they can be periodically stimulated by low levels of residual antigen. Although memory T cells do not express activation antigens such as CD25 (IL-2 receptor), they typically express a low-molecular-weight isoform of CD45 (CD45R0).

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SUMMARY

Although B cells use cell surface Ig to recognize foreign antigens in soluble form, T cells have evolved to discriminate self from nonself based on TCR recognition of foreign antigenic peptides bound to self MHC molecules. TCR resemble Ig in many ways and may use similar molecular mechanisms to generate the receptor diversity needed to recognize the variety of pathogens that confront the immune system. T cells produce soluble mediators called cytokines and can also directly damage infected cells by both secretory and nonsecretory pathways. In these ways, T lymphocytes both regulate and orchestrate the antigen-specific immune response.

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Chapter 10 - Regulation of Activation of B and T Lymphocytes

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OVERVIEW

As discussed in previous chapters in this section, the immune system is compartmentalized into different cell types that perform defined effector functions and have evolved to respond to specific types of pathogens. Lymphocytes comprise the effector cells of the adaptive antigen-specific immune response. They are the cells responsible for the characteristics that we often associate with the immune system, namely memory, specificity, and tolerance to self-antigens. Lymphocytes can be subdivided into two general subpopulations, B lymphocytes and T lymphocytes. The former is responsible for antibody production and derives its name from its hematopoietic origin, the bursa of Fabricius in birds and the bone marrow in mammals. T lymphocytes, although derived from hematopoietic precursors in the bone marrow, become committed to the T-cell lineage and undergo most of their maturation in the mammalian thymus, from whence they derive their name. T lymphocytes are the

mediators of cellular immunity: cytotoxic T-cell responses, delayed-type hypersensitivity, graft-versus-host reactions, and so forth.

Postulated as long ago as 1900 by Ehrlich [1] and confirmed by numerous studies since then, lymphocytes express on their surface clonally distributed receptors that confer specificity for antigen and serve as signaling molecules to initiate the activation of these cells. On an individual lymphocyte, all of the expressed receptors for antigen exhibit identical specificity. As discussed later and in Chapter 8 , the specificity of the B- and T-cell antigen receptors is determined at the DNA level. Because the antigen specificity of the lymphocyte is determined at the genetic level, the progeny and effector cells derived from a B or T lymphocyte after antigen activation maintain the identical antigen specificity of the original responding cell. This process maintains the clonality of the immune response. A specific exception to this rule is the induced somatic hypermutation of the B-cell antigen receptor that occurs during germinal center reactions. [2] [3]

B lymphocytes recognize and bind to antigen by a surface form of immunoglobulin. Likewise, the T-cell antigen receptor is a complex of antigen recognition and signal transducer proteins. Most T cells use a molecule composed of two disulfide-linked proteins called the and chains as their receptor for antigen recognition. Notably, some T cells use a different receptor for antigen recognition. In this case, these lymphocytes express / T-cell receptors (TCRs). In all cases, B and T cells generate their receptor structures by a complex genetic mechanism. The variable regions, which are the regions that bind to antigen, are assembled by combining multiple genetic segments that serve to generate enormous diversity in the antigen-combining regions of these receptors. As a consequence, the potential variability and therefore the limits on the theoretic number of antigenic epitopes that can be recognized has been calculated to exceed 10^{11} .

Besides recognizing and binding antigen, the B- and T-cell antigen receptors also generate signals necessary for initiation of an antigen-specific response. However, neither the surface immunoglobulin molecule nor the TCR / or / proteins possess intrinsic signaling capabilities. Moreover, each of these proteins has negligible cytoplasmic domains and therefore is likely incapable of direct interaction with intracellular signal transduction molecules. For each of these receptors, coupling to cytoplasmic signaling pathways is accomplished by other transmembrane proteins that interact noncovalently with the antigen recognition proteins. As such, the antigen receptors of B and T cells are in fact complexes of proteins, some serving as antigen binding proteins and others functioning to generate signals for the lymphocyte response. More detail on the structure and function of the various components of these signaling complexes is presented later.

Although theoretically sufficient for antigen binding and signal initiation, the B- and T-cell antigen receptors are aided by other surface proteins called co-receptors. These co-receptor molecules play a role in the types of antigens that are bound to the receptors and influence the strength of the signal that is generated as a consequence of antigen binding. CD4 and CD8 influence the ability of the TCR to interact productively with antigen-presenting cells expressing peptide in the context of major histocompatibility complex (MHC) class II or class I, respectively. On the other hand, B-cell receptor (BCR) recognition and binding is not influenced by the MHC because these receptors can bind native antigen. Nonetheless, CD22 and CD19/CD21 serve as co-receptor-like molecules on B cells. As is discussed in more detail later, these co-receptors probably play specific roles in modulating receptor sensitivity under defined conditions in the case of CD22 or to specific forms of antigen in the case of CD19/CD21.

Finally, although signals generated as a consequence of antigen binding through the BCRs or TCRs is important in initiating activation responses by these cells, they are by no means sufficient to generate complete functional responses. Both types of lymphocytes require signals generated through secondary receptors called co-stimulatory molecules to achieve a complete activation response. In the case of T cells, signaling through the CD28 co-receptor is required for growth factor interleukin (IL)-2 production and clonal expansion. Similarly, B cells require CD40 co-receptor signaling for optimal clonal expansion and antibody production. Finally, for both types of lymphocytes, soluble cytokines play specific roles in amplifying and modifying the activation response and altering the effector functions of these cells.

In this chapter, we discuss the current understanding of how antigen-induced lymphocyte responses are initiated and regulated. Our focus will be on (1) the mechanism and role of the antigen receptor-induced activation signals; (2) to what degree these signals are necessary for lymphocyte-mediated immune responses; and, (3) how they can be modified to adapt the response to different pathogens.

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B LYMPHOCYTES

B lymphocytes are the primary effector cells of the antibody-mediated immune response and are antigen-presenting cells for activated, and in some circumstances, resting T cells. Antigen recognition and triggering is accomplished by the BCR, expressed on the surface of mature, immunocompetent B cells. On binding antigen, the BCRantigen complex is endocytosed, and protein antigens processed and re-presented as peptides on the B-cell surface in association with class II MHC antigens. These peptideMHC class II complexes are recognized by antigen-specific T cells. This interaction serves not only to facilitate activation of the T cell (see section on T-Cell Activation), but the activated T cell in turn delivers activation and differentiation signals to the B cell. In this context, the antigen-specific B and T cells can be viewed as communicating with each other, each in turn providing cues to the other to increase levels of activation and effector cell commitment. This stepwise process provides multiple levels of regulation, providing the immune system with critical checkpoints to ensure the appropriate magnitude and effector function is achieved. In addition to its role as a recognition structure and as a mechanism for antigen capture, the BCR serves also as a signaling molecule. In this regard, it is responsible for generation of the initial antigen-induced activation signals for the B cell.

B-Lymphocyte Antigen Receptor

The antigen recognition structure of the BCR is a surface form of immunoglobulin (sIg). The basic monomeric structure of sIg is identical to that of the secreted form of this molecule. Each molecule possesses two light chains disulfide-coupled to two disulfide-linked heavy chain molecules. The surface or membrane form of immunoglobulin differs from the secreted form with respect to the carboxy end of the molecule. Surface immunoglobulin includes additional amino acid residues comprising a spacer, transmembrane, and cytoplasmic region. The carboxy-terminal end of the membrane form is encoded by two additional exons, which differ for each immunoglobulin isotype. For sIgM and sIgD, the cytoplasmic region is only three amino acids long, whereas IgG and IgA are slightly more extensive, 28 and 14 amino acids, respectively. Antigen binding is mediated by the amino-terminal end of the molecule. There is enormous variability in this region between individual B cells. The primary sequence of the variable region of these receptors determines the antigen specificity of a particular B cell clone, and the

diversity of the variable region sequences expressed determine the repertoire of the individual.

All of the immunoglobulin isotypes function as antigen receptors on B cells. For the most part, only one is expressed on an individual cell at any given time. The exceptions are IgM and IgD, which are co-expressed on the majority of resting mature B cells. Co-expression of other isotypes has been reported, but they likely represent cells in transition during isotype switching. For isotypes other than IgM and IgD (i.e., IgG, IgA, and IgE), genetic mechanisms similar to those involved in variable region gene rearrangement (see later) make it genetically impossible to co-express these other isotypes.

The amino acid sequence of the variable region is determined by a genetic process involving the rearrangement of the exons that encode heavy and light chain variable regions. Through a process involving stochastic selection of variable region segments, random nucleotide additions, and junctional diversity, receptor genes are generated that have only a 1 in 10^9 10^{12} chance of being identical to any other BCR. Because this genetic process occurs without antigen selection, the intrinsic antigen specificity of the B cells produced is random and highly varied, enhancing the likelihood of clones responsive to new foreign antigens. Although this potential diversity can never be realized in the immune system of a single individual, it provides a tremendous advantage to the species because it ensures that the potential exists in a group of individuals to recognize and mount a response to a nearly infinite spectrum of antigens. However, this survival advantage must be balanced against the likelihood that many of the BCRs produced will be reactive to self-antigens. Although it is not discussed here, the immune system has evolved mechanisms to identify and remove self-reactive B and T lymphocytes from the repertoire based in part on signals generated through their BCRs (the reader is directed to reviews by Klinman, Monroe, and Goodnow et al. [4] [5] [6]).

The BCR exists as a protein complex in the plasma membrane of the B cell. IgM and IgD are the isotypes expressed on resting B cells with no prior history of antigen-induced activation (naive or virgin B cells). However, the brief cytoplasmic domain of cell surface IgM or IgD is unable on its own to generate a signal in response to sIg cross-linking by antigen or to couple to intracellular signal transducers. To initiate signals for B-lymphocyte activation, sIg exists noncovalently associated with at least two other proteins, Ig (CD79a) and Ig (CD79b). [7] [8] These proteins are products of the *mb-1* and *B29* genes, respectively, and constitute the signaling components of the BCR. [9] [10] Furthermore, expression of Ig and Ig are necessary for surface expression of membrane immunoglobulin.

The structures of CD79a and CD79b are superficially very similar. Each is a transmembrane protein with a single immunoglobulin superfamily domain in the extracellular sequence. Importantly, each has extensive cytoplasmic domains. As first noted by Reth, [11] within each of these cytoplasmic domains are regions of high homology with other immunological signaling molecules such as the , , and chains of the CD3 complex (see later), CD5, and the high-affinity IgE receptor and chains. These regions exist as motifs containing two tyrosines in a pattern of Y X X L/I (X 6-8) Y X X L/I, where X represents any amino acid. In chains of the TCR (see later), it was shown that these tyrosine-containing motifs are necessary and sufficient for signal transduction. [12] This motif is referred to as an ITAM, for *i*mmunoreceptor *t*yrosine-based *a*ctivation *m*otif. These motifs serve to couple the receptor to cytoplasmic proteins. The tyrosines (Y) associated with this motif are significant because they represent substrates for tyrosine phosphorylation by specific kinases and subsequent docking sites for intracellular signaling molecules, which is discussed later.

The earliest detected BCR-induced event is the activation and phosphorylation of tyrosine-specific kinases, [13] [14] particularly those belonging to the src family of protein tyrosine kinases. [15] [16] The tyrosine phosphorylation of CD79a and CD79b after BCR cross-linking provided the first clue that these proteins were involved in transmembrane signaling. Phosphorylation of these proteins is believed to be accomplished by specific protein tyrosine kinases that exist in noncovalent association with the BCR in an inactive form in resting B cells. [17] Clustering of the BCR signaling complex as would occur after binding to multivalent antigen or antigen aggregates results in the activation of these receptor-associated tyrosine kinases. The initial tyrosine phosphorylation of CD79a and CD79b is restricted to the ITAM-associated tyrosines and facilitates recruitment of secondary intracellular signal transducers. [10] Figure 10-1 illustrates the basic composition of the BCR of resting B cells, including the known receptor-associated tyrosine kinases. The stoichiometry is inferred by theoretic considerations based on the transmembrane region sequences of the CD79a (Ig), CD79b (Ig), and Ig heavy chain proteins. [18]

B-cell receptorassociated protein tyrosine kinases so far identified are Lyn, Blk, Fyn, and Lck. [15] [16] All are members of the src family, containing a conserved arrangement of proteinprotein interaction domains, as well as a kinase domain and sites for autoregulation by phosphorylation. [19] Kinases of the src family each have a myristylation sequence at the N-terminal, which facilitates an association of the kinases with the plasma membrane. Adjacent to the myristylation sequence is a stretch of unique sequence, which differs for each member of the src family kinases. This region is thought responsible for interactions with the unphosphorylated Ig/Ig complex in resting B cells. [17] At the C-terminal of the unique region is a single SH3 domain, which is known to mediate proteinprotein interactions by recognition of sequences rich in proline residues. C-terminal to the SH3 domain in the kinase is the SH2 domain. SH2 domains are known to interact with phosphotyrosine residues. An emerging common theme for molecules intimately involved in signal transduction is their modular construction associating different discrete proteinprotein interaction domains with domains mediating effector function. These interaction domains not only facilitate recruitment of enzyme substrates, as in the case of

kinases, but facilitate the localization of these signal transducers with membrane receptors and cytoplasmic signaling molecules that lack intrinsic enzymatic activity. For the src family kinases, the enzymatically active kinase domain is at the C-terminal of the protein. Each of these family members also shares common regulatory mechanisms. One tyrosine residue, at the C-terminal of the protein, provides negative regulation. If this site is phosphorylated, the kinase is inactivated, thereby providing a mechanism to turn off the response. Conversely, initiation of the response requires dephosphorylation at this site. [19] Dephosphorylation is believed to be mediated by co-clustering of CD45 (see later) with the aggregated BCR complex. The mechanism by which phosphorylation and dephosphorylation are believed to regulate kinase activity for the src family protein tyrosine kinases is illustrated in Figure 10-2 .

Subsequent to the initial wave of protein tyrosine kinase activation mediated by the BCR-associated kinases discussed previously, there follows a second wave of protein tyrosine kinase activity. Two of the kinases thought to play unique and critical roles in B-cell activation are Brutons tyrosine kinase (Btk) and Syk. As the name implies, Btk activity is associated with Brutons immunodeficiency. Accordingly, mutations in the Btk kinase have been shown to be responsible for X-chromosomelinked B-cell immune defects in both mouse and man.[20] [21] [22]

The Syk kinase bears homology to the ZAP-70 (*z*eta-*a*ssociated *p*rotein with an Mr of *70*,000) tyrosine kinase found associated with the chain of the TCR complex [12] (see later). Both ZAP-70 and Syk contain tandem SH2 domains at the N-terminal end of the protein, and contain a kinase domain in the C-terminal region. [23] [24] Syk kinase is also activated and

Figure 10-1 Composition of the B-lymphocyte antigen receptor complex (BCR) on resting B cells. Shown are heterodimers of Ig and Ig in noncovalent association with the membrane form of IgM. Also illustrated are BCR-associated src family tyrosine kinases because they are believed to associate with the complex in the resting state. These kinases are tethered to the cytoplasmic side of the plasma membrane by a short myristylated sequence and in noncovalent association with Ig and Ig by the N-terminal portion of the kinase. The specific association of each kinase with either Ig or Ig is not known and is illustrated here only for convenience.

Figure 10-2 Regulation of the activity of the src family protein tyrosine kinases. Illustrated is the model accounting for decreased activity as a consequence of Csk-mediated phosphorylation of the C-terminal tyrosine, which then interacts with the SH2 domain (shaded) of the kinase, resulting in an inaccessible kinase domain (cross-hatched). Cross-linking of the B-cell or T-cell receptor is believed to cluster CD45 and the receptor-associated src family tyrosine kinases. CD45-associated phosphatase-catalyzed dephosphorylation of the C-terminal tyrosine results in an open configuration in which the kinase domain-associated tyrosine is phosphorylated by adjacent tyrosine kinase molecules. This phosphorylated tyrosine serves as a docking site to position substrate proteins near the catalytic domain of the kinase. P, phosphate group; Y, tyrosine.

tyrosine phosphorylated on BCR cross-linking, although with slower kinetics than the src family kinases and Btk. [25]

Although not considered a principal component of the signaling machinery leading to B-cell activation, another kinase expressed by B cells is the Csk tyrosine kinase. This kinase is structurally related to the src family kinases, containing single SH2 and SH3 domains near the N-terminal of the molecule. Csk is functionally unique, however, in its substrate specificity. The main function of Csk kinase is to phosphorylate src family kinases, at their C-terminal negative regulatory site. [26] As such, the principal function of Csk is to maintain the BCR in the inactive state in the absence of antigen and possibly to modulate signaling once antigen is no longer present.

B-Lymphocyte Intracellular Signaling

The previous discussion has centered on the initiation and transmembrane transduction of BCR signals. Now we focus on how these signals are amplified, propagated, and translated by the B cell into appropriate responses. Many of the issues are common to T cells as well. The linkage of antigen receptor-initiated signals to changes in gene expression necessary to alter the activation state and effector capabilities of the lymphocyte occurs through the generation of cytoplasmic second messengers. These second messenger molecules are produced as a consequence of initiation of second messenger pathways. For all defined second messenger pathways linked to the BCRs and TCRs, there is a requirement for tyrosine kinase activation as an initiating event. Interestingly, as will become evident, the initial and secondary waves of tyrosine kinase activity are followed by activation of either serine/threonine-directed protein kinases or phosphatases. The activity of these proteins appears generally directed at the activation of transcription factors that

preexist in the resting lymphocyte but in an inactive state (reviewed in Birkeland and Monroe [27]).

Thus far, three major second messenger pathways have been implicated in BCR-mediated signaling. These include the inositol phospholipid (PI) hydrolysis pathway, which is initiated by the activation of phospholipase C (PLC). In B cells, PLC is a substrate for the Syk protein tyrosine kinase. [28] Syk-mediated phosphorylation of PLC is necessary for initiation of this signaling pathway. The preferred substrate of PLC is a relatively rare plasma membrane phospholipid, phosphatidyl 4,5-bisphosphate (PIP2). PLC-mediated cleavage of PIP2 generates diacylglycerol and inositol 1,4,5-trisphosphate, which trigger two second messenger molecules, a serine/threonine protein kinase called protein kinase C (PKC) and cytoplasmic free Ca^{2+} . The rise in intracellular free Ca^{2+} probably triggers a number of changes in lymphocyte physiology; however, minimally it results in the activation of calcineurin. Calcineurin is a cytoplasmic serine/threonine phosphatase whose activity is required for the activation response of B and T lymphocytes through its ability to activate the transcription factor NFAT. [29] The immunosuppressive effects of cyclosporine A and FK506 are due to the ability of these molecules to inhibit calcineurin. The precise role of PKC in B-cell activation is not known, although there is some evidence that its activation may be required for BCR-induced proliferation [30] and responses to thymus-independent antigens. [31]

A second pathway linked to BCR signaling is the phosphatidyl inositol-3-kinase (PI3-K) pathway. This pathway is initiated by kinase-mediated tyrosine phosphorylation of the regulatory subunit of PI3-K. [32] [33] Activated PI3-K in turn catalyzes the phosphorylation of inositol phospholipids, in one case converting PIP2 to PIP3. Although activation of PI3-K has been shown to be important in growth factor-mediated signaling, its role in B-lymphocyte signaling is not completely clear, although it is known to be activated by BCR-mediated signaling. [34] [35] One recently described consequence of PI3-K activity is the ability of the PIP3 product to activate a unique isoform of protein kinase C, PKC .[36] PI3-K may also be important for the activation of another kinase, S6K, which has been shown to be important for the progression of stimulated cells through the cell cycle. [37] [38] [39] PI3-K in this case exhibits a different enzymatic activity and is able to trigger serine and threonine phosphorylation of S6K.[40] The role of PKC and S6K in BCR signaling is still unclear.

Another network of regulatory proteins activated by BCR signaling are the proteins of the *ras* pathway.[41] [42] This signaling pathway represents the third pathway demonstrated to be involved in BCR signal transduction. In its active state, $p21^{ras}$ binds the guanine nucleotide guanosine triphosphate (GTP), and can activate raf-1, a serine/threonine kinase. Activated raf-1 is able to phosphorylate and activate MAP kinase/ERK kinase (MEK), a tyrosine and serine/threonine protein kinase that can then activate mitogen activated protein kinase (MAPK). MAPKs can in turn phosphorylate and activate transcription factors, thereby causing their localization to the nucleus, ultimately leading to specific gene activation. The activity of *ras* is controlled by a number of modulator proteins, which can either negatively regulate *ras* by promoting GTP hydrolysis to guanosine diphosphate (GDP), or positively regulate *ras* by promoting the exchange of bound GDP for GTP, thereby activating *ras*. Proteins that promote *ras* activity (guanine nucleotide exchange factors, or GNEF) include Vav and SOS. Localization of these components of the *ras* pathway is achieved by a number of SH2 and SH3 domain-containing proteins. [43] One such protein, Grb-2, binds to SOS via its SH3 domain. [44] Grb-2 itself consists of two SH3 domains that flank a single SH2 domain. It has been shown in other systems that the Grb-2 SH2 domain binds phosphorylated tyrosines of activated receptors. [45] The Grb-2 SH2 domain can also bind to phosphorylated Shc, a protein that is phosphorylated after antigen receptor cross-linking in B cells. [42] [46] [47] Nagai et al.[46] showed that both src family kinases and the Syk kinase were important for Shc phosphorylation. The Vav protein is expressed only in cells of hematopoietic origin. Vav is phosphorylated on tyrosines, [48] and a GNEF activity is stimulated[49] on BCR cross-linking. Deficiencies of Vav in B cells are associated with reduction in the number of peripheral B cells and inability to proliferate in response to BCR signaling. [50] [51]

BCR Co-receptor Molecules

Although the BCR is capable of initiating those signaling pathways described previously, its ability to do so is influenced by the selective recruitment of co-receptor molecules by antigen. One of these co-receptors is the CD19 complex. CD19 is a protein expressed on all cells of the B-cell lineage. CD19 is a B-cellspecific, single

transmembrane cell surface glycoprotein. The protein has a large (238242-amino-acid) intracellular domain, which contains 9 tyrosine residues. On mature-stage B cells, co-ligation of CD19 with the BCR dramatically shifts the dose response of B cells to antigen, enhancing the signaling generated through the BCR when CD19 is co-ligated.[52] [53] The cytoplasmic tail of CD19 interacts with the PI3-K p85 regulatory subunit and the Fyn tyrosine kinase. [54] Other studies have suggested that the CD19 cytoplasmic domain can bind to the Lyn tyrosine kinase. [55] These interactions suggest that the CD19 cytoplasmic domain will be important for signaling. In this regard, its nine cytoplasmic region tyrosines are rapidly phosphorylated after BCR cross-linking. [54]

On mature B cells, CD19 exists on the surface of the cell as a ternary complex consisting of the complement receptor CD21 as well as Leu-13 and target of an antiproliferative antibody (TAPA-1) (CD81). [56] [57] [58] [59] To this complex each protein seems to bring a unique function: CD19, with its extensive intracellular domain, is a substrate for tyrosine kinases and can associate with a variety of intracellular proteins to facilitate transmembrane signal transduction. CD21 is able to link this complex with the BCR because it can bind antigens that are associated with components of the complement cascade. In so doing, CD21 drags CD19 and its cytoplasmic associated signaling molecules into the BCR complex. Thus, this co-receptor functions to enhance the signal generated through the BCR when antigencomplement complexes are bound by the B cell. The roles of TAPA-1 (CD81) and Leu-13 are less clear. Both of these proteins have been shown to mediate homotypic cell adhesion, so it may be that their role is to facilitate cellcell interaction during the immune response. Figure 10-3 is a diagram of the BCR/CD19/CD21 co-receptor interaction, illustrating its enhanced signaling capabilities when co-ligated by an antigencomplement bridge.

Another molecule that exhibits activities consistent with a BCR co-receptor is CD45. CD45 is an abundant, highly glycosylated, leukocyte-specific cell surface protein. It is estimated to comprise 10% of the total cell surface glycoprotein. [60] [61] CD45 proteins appear in various sizes on different lymphocyte cell types, and on the same cell type during development and differentiation. This variation in the size of CD45 is achieved by alternate splicing of three or four exons that encode the N-terminal of the protein. Differential exon usage is found on different lymphocyte types, but the highest-molecularweight isoform of the molecule, containing all of the alternately spliced exons, is found on the B-cell surface. The cytoplasmic domain of the CD45 protein contains an active phosphatase domain that appears to play an important function in BCR signaling. B-cell lines lacking CD45 show defects in early BCR-mediated signal transduction. [62]

B cells from mice lacking CD45 cannot proliferate in response to BCR cross-linking, although they are able to proliferate in response to the mitogen lipopolysaccharide (LPS).[63] Further characterization of the phenotype and responses of B cells from CD45-deficient mice showed that these cells had severe

Figure 10-3 Model for antigen-complement C3b,d-mediated co-aggregation of the B-cell receptor (BCR) and the CD19CD21 co-receptor complex. Co-clustering mediated by simultaneous binding of BCR and CD21 serves to recruit the CD19-associated signaling proteins to the BCR signaling complex. As discussed, this is believed to enhance the signal that is generated by BCR aggregation.

functional defects.[64] The population of high-density, IgM^{low} IgD^{high} B cells was greatly diminished in the knockout mice. The cells from these CD45-deficient mice were also defective in their ability to flux calcium from the extracellular space, although they could release calcium from intracellular stores in response to BCR cross-linking.

It can be concluded from these studies that CD45 expression is necessary for optimal antigen receptor signaling. Whether CD45 is a part of the actual signaling complex and therefore functions as a legitimate co-receptor is still unclear. Because of the abundance of this protein, it might be expected that its associated phosphatase activity would be in close physical proximity to any cell surface protein in need of dephosphorylation, obviating a need for co-ligation with the BCR for effects on BCR signaling. One argument for proximity being sufficient for CD45 action is that CD45-deficient cells were shown to have antigen receptor signaling restored when the cells were transfected with only the cytoplasmic domain of CD45. [65] [66]

Another B-cell surface molecule, CD22, may also play a co-receptor role in early BCR signal transduction. It has been observed that CD22 can be co-immunoprecipitated with sIg from B-cell lysates made in mild detergent, and it has also been shown that cross-linking the BCR with anti-Ig leads to phosphorylation of the CD22 cytoplasmic domain.[67] [68] [69] CD22 is a single transmembrane-spanning glycoprotein that contains seven immunoglobulin superfamily domains in its extracellular region.[70] A ligand for CD22 has not been definitively identified, although the CD22 extracellular domain is able to bind sialic acid residues on a variety of proteins.[71] The intracellular domain of CD22 contains six tyrosine residues, which can be substrates for tyrosine kinases. These two observations led to the inclusion of CD22 into some models of the BCR complex. It seems more likely, given that not all sIg has CD22 associated with it,[67] that CD22 can instead transiently associate with the BCR during an immune reaction, and can use its phosphorylated tail to bring phosphotyrosine binding proteins into the antigenreceptor complex, where they can then modify other receptor components. In this regard, it has been shown that the CD22 cytoplasmic domain is able to associate with a tyrosine-specific phosphatase, protein tyrosine phosphatase 1C SH2-containing tyrosine phosphatase (PTP1C[SHP-1]). [72] [73] PTP1C(SHP-1) is found physically associated with the BCR in resting B cells, where it probably functions to dephosphorylate tyrosine residues within the ITAM motifs of Ig and Ig. One paradigm explaining CD22 enhancement of BCR signaling is that in secondary lymphoid organs, sequestration of CD22 is accomplished by B-cell association with sialic acid-modified proteins on the surface of T cells or stroma. In antigen-stimulated B cells where CD22 is tyrosine phosphorylated, its putative co-receptor function would be to sequester PTP1C(SHP-1) away from the BCR, thereby enhancing the tyrosine phosphorylation of the Ig/Ig ITAMs. This process is diagrammed in Figure 10-4 .

Negative Modulators of BCR Signal Transduction

B-cell receptormediated signaling is also subject to negative modulation by potential co-receptor proteins. It was observed

Figure 10-4 One hypothetical model to account for the ability of CD22 clustering to enhance B-cell receptor (BCR) signal transduction. In the resting B cell, the cytoplasmic phosphatase SHP-1 is in proximity to the complex, where it functions to maintain dephosphorylation of the Ig- and Ig-associated immunoreceptor tyrosine-based activation motifs (ITAMs), thereby decreasing the ability of the receptor to generate signals. Simple cross-linking of the BCR by antigen activates src family kinases, leading to ITAM phosphorylation as well as tyrosine phosphorylation of the cytoplasmic domain of CD22. Phosphorylated CD22 recruits SHP-1. However, in this instance it is still in the vicinity of the BCR, so SHP-1 can still down-modulate BCR signal transduction through ITAM dephosphorylation. In contrast, in environments where T cells or stromal cells expressing sialylated proteins are present, CD22 and its associated SHP-1 is sequestered away from the BCR. As a consequence, antigen-induced signals through the BCR would be expected to be stronger when antigen is encountered in appropriate anatomic niches or in the presence of T-helper cells.

many years ago that treatment of B cells with anti-Ig antibodies that lack the crystalline fragment (Fc) portion of the molecule led to B-cell proliferation whereas anti-Ig antibodies with the Fc region intact did not. [74] Furthermore, intact anti-Ig antibodies fail to trigger the signaling pathways leading to PI hydrolysis that is observed when BCR is cross-linked with F(ab)2 anti-Ig that does not contain the Fc portion. [75] This effect was presumed to be due to the cocross-linking of the sIg receptor complex with Fc receptors (FcR) on B cells. This would provide a mechanism for down-modulating a B-cell response late in an immune reaction, if B cells encounter an antigen that has been coated with secreted antibody. As illustrated in Figure 10-5 , simultaneous stimulation of FcRs and the BCR would send a signal to the B cell to shut down production of specific antibody.

The FcR expressed by B cells is the FcRIIB1. The cytoplasmic domain sequences important for FcR negative signaling in B cells have been defined, and a 13amino-acid motif has been identified that is both necessary and sufficient for FcR-mediated inhibition of the intact anti-Ig response. A single tyrosine residue contained in this motif was shown to be critical for inhibition of BCR-induced Ca^{2+} flux. [76] The proteins that interact with this 13amino-acid motif were subsequently defined and the motif referred to as the ITIM, for *i*mmunoreceptor *t*yrosine-based *i*nhibition *m*otif.[77] This sequence has been established only in FcRII receptors thus far, although other molecules such as CD22 may have similar motifs. However, in these cases the function of these putative ITIMs has not been established. The FcR ITIM motif is responsible for the interaction of this protein with the SHP-1 and SHIP-1 cytoplasmic phosphatases. The tyrosine residue contained within the putative ITIM motif is inducibly phosphorylated, and can thereby interact with these phosphatases through the tandem SH2 domains of the latter. Recruitment of these enzymes into the BCR complex presumably allows dephosphorylation of key tyrosine residues present in the cytoplasmic domains of Ig and Ig, or in other signaling molecules that interact with them, and thus can inhibit antigen receptor signaling.

These examples of co-receptors, all of which are able to modulate signaling through the antigen receptor, indicate that BCR-mediated signaling is a tightly regulated process. More interestingly, it again points out similarities to antigen receptor signaling in T cells, in which the participation of co-receptors (e.g., CD4, CD8, CD2) for

optimal responses is well established. Definition of the roles of these co-receptors suggests that optimal BCR signaling is not mediated by cross-linking sIg molecules

Figure 10-5 Model illustrating modulation of B-cell receptor (BCR) signal transduction by Fc RII B1. Antigen (black)antibody complexes co-cluster the BCR and the FcR. The latter positions the cytoplasmic phosphatases SHP-1 and SHIP-1 in proximity of the Ig and Ig ITAMs thereby dephosphorylating them (note faded P) and down-regulating their ability to recruit SH2-containing signal transduction proteins.

alone, but can involve an accumulation of signaling receptors into a large complex, each element of which brings along associated proteins. When the minimum threshold for a response is reached, by accumulation of a critical concentration of enzymes and modulators at the cytoplasmic face in association with the BCR and its co-receptors, B-cell activation can ensue. However, when sIg is extensively cross-linked (i.e., by anti-Ig antibody, or by multivalent antigens), a requirement for co-receptor engagement may not be evident. Although ligands for some of the putative co-receptors are known (i.e., complement components and the Fc portion of Ig), specific ligands for others, such as CD22, CD45, or CD19, remain to be defined. Definition of ligands for these proteins will clarify the roles that they play, and the circumstances that require their presence in BCR-mediated signaling.

Secondary, Co-stimulatory Signals for B-Lymphocyte Activation

The preceding discussion focused on the process by which antigen initiates activation signals in mature, immunocompetent B cells. However, by themselves these signals are usually insufficient to cause clonal expansion and antibody secretion. For these processes, secondary signals provided by CD4+ antigen-specific T cells are necessary. In this way, a B-cell immune response requires the integration of T- as well as B-cell activation.

Secondary signals for B-cell activation come in two forms: (1) those requiring direct physical contact between B and T cells, and (2) those mediated by soluble cytokines secreted by antigen-activated T cells. CD40 on the surface of resting and stimulated B cells is the primary receptor for cell contact-mediated secondary signals. CD40 is a 48-kd transmembrane protein belonging to the tumor necrosis factor receptor family. [78] Gp39 (CD40 ligand) on the surface of antigen-activated CD4+ T cells binds to CD40 and generates signals that promote B-cell survival and maintain or facilitate B-cell proliferative responses. [78] [79] Agents that block gp39CD40 interactions greatly impair the ability of activated T cells to interact with and drive B-cell proliferation. In humans, lack of gp39 is the molecular defect causing X-linked hyper-IgM syndrome. These patients can make IgM responses to T-cellindependent antigens, but fail to generate any B-cell responses to T-celldependent protein antigens. Even to T-cellindependent antigens, switching to other Ig isotypes is absent. [80] [81] [82] [83] Thus, although BCR signals may initiate B-cell activation, co-stimulation through CD40 is necessary for effector-level B-cell responses.

It is generally held that BCR signaling in the absence of T-cellmediated co-stimulatory signals results in an abortive activation response that is followed by B-cell death and elimination from the immune cell repertoire. [6] [84] This may then provide a mechanism for deletion of self-reactive mature B cells because they would be expected to encounter antigen but the necessary co-stimulatory signals by antigen-specific T cells would be absent.

Resting B cells express CD40, and there is evidence that CD40 signals together with cytokine signaling can activate the B cell in the absence of BCR signaling. This might be expected to be problematic in an immune response because it would allow activation of B cells with antibody specificities irrelevant to the pathogen or, worse, autoreactive. However, in vivo it is likely that CD40 stimulation does not occur without prior BCR engagement on resting B cells. The ability of the B cell to interact productively with the T cell (see next section) depends on molecules such as class II and B7, each of which is up-regulated on BCR-stimulated B cells.

In addition to CD40gp39 interactions, other surface ligandreceptor pairs mediate physical interactions between the B and T cell. These are diagrammed in Figure 10-6 . Some of these, as

Figure 10-6 Receptorligand pairs contributing to association-dependent secondary signals for B- and T-cell activation. These interactions illustrate the tremendous level of communication between the B and T cells during a lymphocyte-mediated immune response. Expression of some of these proteins is enhanced or their signaling capabilities activated as the B- and T-cell activation response proceeds. In this way, there is continued reinforcement and checkpoints to ensure that the immune response continues to be appropriate.

has been and will be discussed later, function as signaling molecules; others, such as intercellular adhesion molecule-1 and lymphocyte function associated-1, are adhesion molecules that increase the strength of the B-cellT-cell interaction. Even these adhesion molecules may possess some signaling capabilities as well.

Besides co-stimulatory signals generated through direct B-cellT-cell contact, various T-cellderived cytokines facilitate and regulate the activation response of the B cell after BCR initiation of the activation response. Although their precise role in B-cell activation responses and effector function have not been as well defined as for CD40, they clearly play a role in response of B cells in certain instances. [86] For example, IL-4 facilitates isotype switching to IgG1 and IgE, whereas transforming growth factor- results in switching to IgA. In addition, IL-4 can augment the early stages of BCR-induced activation, whereas IL-5 has been suggested to play a role in maintaining clonal expansion. Because they do not require cellcell contact for their function, cytokine-mediated effects on B-cell activation are likely limited to previously antigenand CD40-activated clones. In this manner, they probably function to enhance and modify the response to specific pathogens.

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T LYMPHOCYTES

As mentioned previously, T cells are responsible for mediating the cellular arm of the immune response. Effector T cells are basically of two types, helper T cells (Th), which produce cytokines important for the regulation of both cellular and humoral responses, and cytotoxic T cells (CTL), which serve to eliminate virally infected, and in some cases, transformed cells. As with B cells, T lymphocytes recognize specific antigen by virtue of a structurally unique cell surface antigen receptor (TCR). However, unlike B cells which have evolved to protect us from extracellular pathogens and which are capable of recognizing foreign antigens in their native conformation, T cells have evolved to protect us from intracellular pathogens and typically recognize a peptide determinant (epitope) bound to a molecule encoded in the MHC, and presented on the surface of an antigen-presenting cell such as a dendritic cell or B cell. Although there are certainly exceptions, most Th cells express the CD4 co-receptor and recognize antigenic peptide displayed on MHC class II molecules, whereas CTLs express the CD8 co-receptor and recognize peptide bound to MHC class I molecules.

T-Lymphocyte Antigen Receptor

The TCR is a multisubunit complex composed of at least eight chains. The antigen-specific, ligand-binding capacity is provided by only two chains, the TCR and chain on most T cells and the TCR and chains on the remaining cells (the so-called / T cells). The ligand-binding TCR chains are members of the immunoglobulin gene superfamily,[86] and are therefore related to a large number of proteins of immunologic interest, including immunoglobulin, CD4, CD8, and MHC class I and class II molecules. The extracellular portion of each ligand-binding chain contains the NH_2 -terminal polymorphic region involved in antigen recognition. [87] Analysis of the TCR crystal structure shows considerable similarity to that of the immunoglobulin antigen-combining site in this region. [88] The heterodimeric, ligand-binding subunits are identical for each TCR on a given T cell and its progeny (a T-cell clone), but each independent T-cell clone expresses a unique TCR with a distinct primary amino acid sequence (clonal variation).

As with B cells, the ligand-binding chains of the TCR are assembled from individual gene segments, variable (V), diversity (D), joining (J), and constant (C). Clonally diverse TCRs are generated following unique patterns of rearrangement of distinct members of these gene families in individual maturing T cells, with each ligand-binding chain containing just one representative family member from each gene segment (V, J, and C for TCR- and - and V, D, J, and C for TCR- and -). Thus, the germline diversity contributed by the unique nucleotide sequence of multiple, germline-encoded V, D, and J family members, coupled with the combinatorial diversity achieved during independent, random rearrangement of V, D, and J segments, provides the ability to generate an enormous number of genes that encode proteins with different primary amino acid sequences. Additionally, junctional diversity provided by nucleotides that are not present in the original DNA gene sequence (so-called N-regions) can occur in all four of the polymorphic TCR chains, [89] further adding to the diversity of antigen recognition elements that can be generated. Finally, because both chains of the TCR ligand-binding complex contribute to antigen specificity, diversity is increased by the heterodimeric structure of the TCR ligand-binding chains. In contrast to the BCR, TCR ligand-binding chains are not thought to undergo somatic hypermutation in peripheral lymphoid organs, except in rare cases.[90]

As with the BCR, the COOH-terminal intracytoplasmic portions of the polymorphic chains are quite small, ranging from 5 to 11 amino acids, a strong indication that they have no intrinsic signaling capacity. [91] [92] The remaining polypeptides in the TCR complex are nonpolymorphic (invariant in their sequence) and appear to be critical for the proper assembly and cell surface expression of the TCR as well as for the transduction of activation signals (see later). The nonpolymorphic components include three 2026-kd structurally related polypeptides, CD3-, -, and -epsilon [93] [94] (not to be confused with the polymorphic, ligand-binding chains TCR- and -), the structurally distinct chain, [95] and, in the mouse, . However, the functional significance of the murine subunit is in question because of the lack of an obvious human analog, the poor conservation across species, and a failure to demonstrate a unique function for . [95] [96] Finally, stoichiometric analyses reveal that the TCR complex is actually an octamer composed of four dimers, a disulfide-bonded ligand-binding heterodimer (TCR- or -), noncovalently coupled CD3-epsilon and CD3-epsilon heterodimers, and a disulfide-linked homodimer (Fig. 10-7).

TCR Signal Transduction

T-cell receptormediated cellular activation is initiated after interaction with membrane-bound ligand. Interaction with antigenic

Figure 10-7 Composition of the T-lymphocyte antigen receptor (TCR) complex. Shown are the nonpolymorphic members (CD3-, -, -epsilon, and chains) in association with the ligand-binding polymorphic TCR- and - chains. Also illustrated are the immunoreceptor tyrosine-based activation motifs (ITAMs; unfilled ovals in the cytoplasmic domains of the CD3 complex and the chain) that are tyrosine phosphorylated upon receptor cross-linking and are responsible for the recruitment of other signaling molecules to the TCR complex.

peptides on the surface of antigen-presenting cells results in the aggregation of TCR complexes, a process that appears to be critical for signaling through the TCR. TCR cross-linking then induces a complex series of biochemical cascades that result in T-cell activation and initiation of effector functions (e.g., cytokine production, proliferation, cytotoxicity). Recognition of several MHCpeptide complexes by multiple TCRs on a given T cell also increases the apparent strength of the interaction because the actual affinity of individual TCRs for appropriate antigenMHC complexes appear to be quite low (Kd 10^{-5} 10^{-6} LM). In addition, co-receptors such as CD4 and CD8 increase the affinity of the antigenantigen receptor interaction as well as increasing the sensitivity of the TCR by recruiting other important signaling molecules into the activation complex (see later).

Cross-linking of the TCR results in the rapid phosphorylation of many proteins on tyrosine residues as well as on serine/threonine residues. For the main part, tyrosine kinases and serine/threonine kinases are nonoverlapping subsets of protein kinases. Activation of tyrosine kinases occurs rapidly on TCR cross-linking, resulting in tyrosine phosphorylation of a subset of molecules [97] [98] as well as the other CD3 components.[99] Tyrosine phosphorylation of and the CD3 components occurs on a common motif, the ITAM, which is also present in subunits of the BCR and the Fcepsilon receptor [11] (see earlier). Each of the three CD3 components has one ITAM motif, while each monomer contains three. Although the kinase(s) responsible for phosphorylation in mature T cells has not been identified with certainty, the most likely candidates include the src-like kinases $p56^{lck}$ (Lck) and $p59^{fyn}$ (Fyn), because co-transfection studies in non-T cells demonstrate that Fyn or Lck can phosphorylate the ITAMs of ,[100] [101] and Lck appears mainly responsible for phosphorylating in thymocytes. [102] Furthermore, the localization of Lck and Fyn to the plasma membrane by a myristylation signal and the association of Lck with CD4 and CD8 (see later) and Fyn with TCR components [103] may contribute to their ability to phosphorylate in response to TCR aggregation or co-engagement of antigenMHC complexes by the TCR and the CD4 or CD8 co-receptors. The importance of both Lck and Fyn in normal T-cell activation has been revealed in Lck-deficient mice, which have very few peripheral, mature T cells, all of which are deficient in their ability to transduce TCR-induced signals, [104] and in mice deficient in both Lck and Fyn, in which mature T-cell development is totally abrogated. [105]

After phosphorylation of by src kinase family members, a member of the Syk family of tyrosine kinases, ZAP-70, associates with phospho-. This interaction is thought to be mediated by the N-terminal tandem SH2 domains in ZAP-70 interacting with the phosphotyrosines in the ITAM motif of [106] (see earlier; Fig. 10-8). During transduction of an activation signal through the TCR, binding of ZAP-70 to phospho- appears to lead to an increase in its kinase activity, resulting in an increase in intracellular phosphorylation levels. [107] However, in cases where a full activation signal is not transduced through the TCR, inactive ZAP-70 may be found bound to phospho-,[108] a phenomenon that may be related to the induction of tolerance in these cells. [109] [110]

The ITAM motifs in the invariant subunits of the TCR are thought to be critical for TCR signaling. Chimeric molecules containing intracellular ITAM motifs and the transmembrane and extracellular domains of unrelated proteins can, on cross-linking, transduce signals that mimic both early and late signal transduction events characteristic of the intact TCR. [111] As described previously, phosphorylated ITAMs of the invariant subunits of the TCR appear to act as a scaffolding for recruiting other signaling molecules by virtue of phosphotyrosineSH2 domain interactions. When compared with the other CD3 components, the prominent role of in TCR-induced signaling could be due to its higher concentration of ITAM (three ITAMs per monomer) relative to those observed in the CD3 complex (one ITAM per monomer). In support of this, chimeric molecules containing multimers of a single ITAM from the chain transduce signals more efficiently than do chimeric proteins containing a single ITAM motif. [101] However, it is also possible that the individual TCR subunits may have unique roles in signal transduction. The latter possibility is supported by the

Figure 10-8 The T-cell receptor (TCR) complex and co-receptor molecules in both resting and antigen-activated T cells. In the resting T cell, the TCR complex, the CD4 co-receptor, and CD45 are not associated in the membrane. The tyrosine kinase Lck is found associated with CD4 and low levels of the Fyn kinase are thought to associate with the TCR complex. However, neither enzyme is active because of phosphorylation at a negative regulatory site. Upon receptor ligation, CD45 and CD4 are drawn into the receptor complex. CD45 is thought to be responsible for dephosphorylating the negative regulatory tyrosines on Lck and Fyn (notice the light-colored P). Activation of Lck or Fyn is thought to lead to phosphorylation of the CD3 complex and the chain, allowing for the association of ZAP-70 by its tandem SH2 domains and its subsequent activation.

observation that stimulation of cells through or CD3-epsilon chimeras results in different patterns of tyrosine phosphorylated substrates. [112]

There have been several reported cases of inherited immunodeficiencies associated with abnormalities in the expression of invariant TCR components; [113] for a more complete description, see Chapter 42 . In these patients, mature T cells are often found in peripheral lymphoid organs, but T-cell function is impaired. A severe combined immunodeficiency syndrome that is marked by an absence of CD8+ peripheral T cells and a deficiency in TCR signal transduction in peripheral CD4+ T cells was found to be associated with mutations in ZAP-70. [114] Interestingly, although peripheral CD4+ T cells from a ZAP-70deficient patient were not able to transduce signals through the TCR, thymocytes from this patient were TCRsignal-transductioncompetent. [115] It appears that in ZAP-deficient thymocytes, but not mature T cells, Syk kinase can compensate for ZAP-70 in TCR-induced signal transduction. The maturation of CD4+, but not CD8+ T cells in these patients, suggests that the stronger association of Lck with CD4 (see later) may be required to activate Syk in the absence of ZAP-70.

Intracellular Signaling

Amplification, propagation, and translation of early TCR-induced signal transduction events in T cells utilize many of the same pathways already described for B cells (see previous discussion), including: (1) increased intracellular calcium levels and activation of PKC as a consequence of PLC activation, (2) activation of PI3-K, and (3) activation of the *ras* pathway (Fig. 10-9). As in B cells, most of the secondarily induced signaling cascades depend initially on the activation of tyrosine kinases. Increased intracellular Ca^{2+} levels and PKC activation after the tyrosine phosphorylation of PLC has been causally linked to TCR-induced cellular responses such as the transcriptional activation of the IL-2 gene. [111] [116] The molecular consequences of increased intracellular Ca^{2+} levels have been revealed using the immunosuppressive drugs cyclosporine A and FK506. Cyclosporine A and FK506 bind to distinct

Figure 10-9 Second messenger pathways activated by activation through both the T-cell receptor (TCR) and co-stimulatory molecule, CD28. Upon TCR cross-linking, tyrosine phosphorylation of the chain is thought to lead to the association of several important signaling molecules such as the tyrosine kinase, ZAP-70 (*z*eta-*a*ssociated *p*rotein with an Mr of *70*,000), and adaptor molecules such as Shc, p36, and Crk that are involved in linking tyrosine-phosphorylated receptor chains to guanine nucleotide exchange factors important for activating small guanosine triphosphates (GTPases) such as Ras, Rac1, and Rap-1. Activation of these small GTPases activates a cascade of serine/threonine kinases (the MAPK pathway) that leads to the activation of Erk-1 and Erk-2 as well as Jun N-terminal kinase (JNK), kinases that are critical for up-regulating the activity of various transcription factors such as AP-1 and NFAT. ZAP-70 activation leads to the phosphorylation and activation of phospholipase C (PLC) and the breakdown of the inositol phospholipid PIP2 into IP3 and DAG. IP3 generation leads to a release of calcium from intracellular stores, resulting in the activation of calcium-dependent enzymes such as the phosphatase calcineurin. Calcineurin dephosphorylates a subunit of the NFAT transcription factor, which promotes its translocation to the nucleus. Upon binding of its ligand, CD80 or CD86, co-stimulatory signals transduced through CD28, activate PI-3K, which serves to up-regulate JNK fully and also activate another transcription factor, NF-B. AP-1, activating protein-1; NFAT, nuclear factor of activated T cells; IP3, inositol triphosphate; DAG, diacylglycerol; NF-B, nuclear factor-B.

molecules, collectively referred to as immunophilins, which form complexes to inhibit a calcium/calmodulin-dependent serine phosphatase, calcineurin. [117] Because cyclosporine A is an effective inhibitor of IL-2 transcription, it has been inferred that at least one critical Ca^{2+} -regulated pathway in T cells involves activation of calcineurin. Subsequent experiments showed that calcineurins role in TCR-initiated signal transduction is to dephosphorylate and promote nuclear localization of a subunit of the transcription factor, NFAT, which is involved in IL-2 transcription. [118] The exact role of PKC in TCR-induced activation events is not yet clear, in part because phorbol esters, pharmacological activators of PKC, also lead to *ras* activation in T cells,[119] so that many downstream effects originally ascribed to PKC activation may actually be mediated by the *ras* pathway.

Although PI3-K is thought to be activated after TCR engagement, the stimulatory effect is actually quite weak. In T cells, PI3-K is thought to be optimally stimulated after engagement of the co-stimulatory molecule, CD28[120] (see later). It is not yet clear what role PI3-K may have during TCR-mediated signal transduction or after engagement of CD28. It is possible that PI3-K is indirectly involved in the activation of the transcription factor nuclear factor (NF)-B through the regulation of PKC-. [121] In addition, the serine/threonine kinase Akt is thought to be activated by the phosphorylated product of PI3-K, phosphatidylinositol-3,4-bisphosphate. [122] Finally, as described later, activation of PI3-K after CD28 signaling may up-regulate the activity of Jun N-terminal kinase (JNK), a stress-activated protein kinase involved in the regulation of transcription factors such as activating protein (AP)-1 and NFAT that are required for the transcriptional activation of IL-2.

As in BCR signal transduction, TCR cross-linking is also linked to the activation of the *ras* pathway,[120] resulting in the downstream activation of the MAPK pathway (see earlier). Activation of *ras* in T cells is thought to occur, at least in part, by the activation of the nucleotide exchange factor, SOS (described previously). Although somewhat controversial, it appears that SOS is linked to the TCR by the adaptor protein Grb2 interacting with other adaptor proteins, such as p36 or perhaps Shc. [123] Activation of *ras* leads to the induction of a series of serine/threonine kinases, which culminates in the activation of two MAPKs, Erk-1 and Erk-2. These kinases are critical for the phosphorylation of transcription factors that up-regulate the expression and activity of AP-1, a transcription factor complex required for IL-2 gene expression.

To make matters more complicated, in addition to *ras* gene products, there are several other small GTP-binding proteins expressed in T cells that may contribute to TCR-induced signal transduction. For instance, the adaptor Crk is linked to the nucleotide exchange factor, C3G, which serves to activate the small GTPase, Rap-1. In addition, members of the Rho family (Rac and Cdc42) of GTPases may serve to regulate the activation of JNK in T cells. [123] It is likely that all of these pathways contribute to the functional responses induced by TCR signal transduction.

CD4 and CD8 Co-receptors

The CD4 and CD8 co-receptors act to enhance TCR signal transduction by (1) increasing the affinity of the TCR complex for its MHCpeptide ligand, and (2) by recruiting signaling molecules such as Lck to the signal complex (see Fig. 10-8). CD4 and CD8 increase the affinity of the TCR for MHCantigenic peptide complexes both by physically associating with the TCR and by binding MHC molecules, ligands that are also bound by the TCR. [124] Although the expression of CD4 and CD8 was originally thought to define specific functional T-cell subsets (Th cells being CD4+ and CTLs being CD8+), it has become increasingly clear that the expression of these molecules on a particular T cell closely correlates with the type of MHC molecule that is recognized by its TCR: CD4+ T cells recognize antigen in the context of class II molecules, CD8+ T cells recognize antigen presented by class I molecules.

CD4 and CD8 are quite distinct structurally; CD4 is a single-chain, type I transmembrane glycoprotein containing four immunoglobulin-like domains, whereas CD8 is composed of two disulfide-linked type I membrane-spanning glycoproteins, each with a single immunoglobulin-like extracellular domain. [125] Despite the structural dissimilarity, CD4 and CD8 serve analogous co-receptor functions by virtue of their ability to bind MHC class II and I molecules, respectively. The extracellular portion of CD4 binds to a monomorphic element on the 2 domain of MHC class II molecules, [126] a site that is structurally analogous to the CD8 binding domain on the MHC class I 3 membrane proximal domain. [127] [128] Importantly, co-receptor binding to monomorphic regions of the MHC molecule does not sterically inhibit TCR binding to the polymorphic, peptide-binding groove. Indeed, the ability of the co-receptor and TCR to co-engage the same MHCpeptide complex has been shown to be critical for the enhancement of TCR-mediated signal transduction. [129]

CD4 and CD8 enhance the signaling efficiency of the TCR complex by drawing the src family kinase, Lck, into the TCR complex. CD4 is in general considered a stronger co-receptor because it binds more Lck than does CD8. In addition, CD4 cross-linking results in a clear increase in the enzymatic activity of Lck, whereas similar increases are not observed after CD8 cross-linking. [130] CD4 and CD8 interact with a Cys-X-X-Cys motif in the NH_2 -terminal region of Lck through a Cys-X-Cys motif found in their cytoplasmic tails. [130] During antigen recognition by the TCR, co-engagement of the TCR and co-receptor molecules helps draw the co-receptor molecules into the TCR complex, thus bringing the associated Lck into the signaling complex and enhancing tyrosine phosphorylation of intracellular substrates. Once substrates such as the CD3 components are phosphorylated, the SH2 domain of Lck may interact with the phosphorylated ITAMs, anchoring the co-receptor to the activated TCR complex and further promoting co-receptor function. [131]

CD45

As described for B cells, CD45 is a highly abundant transmembrane glycoprotein that contains an active phosphatase domain in its cytoplasmic tail. Like in B cells, expression of CD45 appears to be required for efficient TCR signaling; T-cell lines deficient in CD45 expression are impaired in TCR signal transduction. [132] In CD45-deficient mice, T-cell development is impaired such that few mature T cells are found in peripheral lymphoid organs, and those that are produced have a diminished ability to proliferate in response to TCR cross-linking. [63] As described previously, CD45 is thought to mediate some of its effects on TCR signaling by dephosphorylating the C-terminal phosphotyrosine of Lck and Fyn; when phosphorylated, this residue down-regulates the kinase activity of these src family kinases. Consistent with this hypothesis, TCR-induced stimulation of CD45-deficient T-cell lines does not result in enhanced tyrosine kinase activation [133] (although these cells exhibit constitutive hyperphosphorylation, presumably because of the absence of CD45 phosphatase activity).

Although CD45 is known to bind to CD22 on B cells, [134] the lack of an obvious ligand on other types of antigen-presenting cells that would promote aggregation into the TCR complex precludes CD45 from being considered a legitimate TCR co-receptor. However, CD45 has been shown constitutively to associate with the TCR, [135] suggesting that CD45 may have an intrinsic ability to interact with the TCR, either by a specific interaction or by mass action due to its shear abundance in the membrane. Moreover, as mentioned previously, expression of the cytoplasmic domain alone in CD45-deficient cells is sufficient

to reconstitute efficient TCR signaling, suggesting that ligand binding by CD45 is not required for augmentation of TCR signaling, [136] [137] and there is some evidence to suggest that dimerization may actually have a negative effect on CD45 function. [138]

Secondary Signals for T-Cell Activation

Co-stimulatory Signals

Although ligation of the TCR results in the induction of many signal transduction pathways, TCR signaling alone is not sufficient to drive proliferation of naive T cells (T cells that have never been exposed to antigen). For naive T cells to proliferate, they need to receive a signal through both the TCR and the co-stimulatory molecule, CD28. In fact, in the absence of co-stimulatory signals, TCR stimulation of at least some naive T cells results in long-term functional inactivation or anergy. [139] Co-stimulation through CD28 is thought to lower the threshold of activation as well as increase the longevity of the TCR-induced response by stabilizing important transcripts such as those for IL-2 and IL-4. [140] Finally, although in some cases early stages of T-cell proliferation can be induced in the absence of obvious co-stimulation, such cells eventually undergo apoptotic death. [141] The increase in apoptotic cell death in the absence of CD28 co-stimulatory signals appears to be due to the inability to up-regulate an antiapoptotic molecule, Bcl-X_L . [142]

CD28 is a member of the immunoglobulin superfamily that is constitutively expressed on the T-cell surface as either a disulfide-linked homodimer or as a monomer. [141] For CD28 to transduce a signal, it must engage a ligand (CD80 or CD86) on the surface of an antigen-presenting cell. CD80 and CD86 are also members of the immunoglobulin supergene family and, although they share relatively little homology with each other, both are capable of binding CD28. In addition, both CD80 and CD86 bind CTLA-4, a molecule that is structurally analogous to CD28, with an affinity that is even higher than their affinity for CD28. Interestingly, whereas CD28 is thought to transduce a positive signal during TCR ligation, CTLA-4 is apparently involved in negative regulation of this response [140] (see following).

Although much is known about the functional requirements for CD28 during T-cell activation, less is known about the signaling events responsible for the co-stimulatory effect. After ligation, tyrosine phosphorylation of the CD28 cytoplasmic tail allows for the association of downstream effector molecules such as Grb-2, the tyrosine kinase Itk, and perhaps most important, PI3-K. [140] Although stimulation through the TCR has been found to result in an association of PI3-K with the TCR complex, the association of PI3-K with CD28 is far greater and presumably leads to a much stronger activation signal. The importance of PI3-K activation during co-stimulation appears to be due, at least in part, to the activation of JNK through an as-yet-unidentified mechanism that may involve the small GTPase, Rac1. [143] The co-stimulatory effects of CD28 appear to be tightly linked to the activation of JNK, which leads to the phosphorylation of cJun and the activation of AP-1containing transcription factors [144] (see Fig. 10-9).

The requirement for CD28 in T-cell activation has been supported by the diminished T-cell proliferative responses and IL-2 production observed in mice deficient in CD28 expression. [145] [146] Because interruption of this pathway leads to an inhibition of T-cell activation, novel immunosuppressive agents that target this pathway have been developed in an attempt to tolerize antigen-specific T cells. For example, based on the differential affinity of CTLA-4 and CD28 for ligand binding, a soluble chimeric molecule, CTLA-4Ig, which contains the secretory domains of immunoglobulin and the extracellular domains of CTLA-4, has been successfully used specifically to compete for binding of CD80 and CD86 by CD28. Administration of soluble CTLA-4Ig leads to the prolongation of graft survival and the diminution of autoimmune disease. [141]

In contrast to the positive co-stimulatory role played by CD28, CTLA-4 appears to play an important negative immunoregulatory role. Mice deficient in CTLA-4 expression are hyperresponsive to antigenic stimulation, and the proliferation of T cells (perhaps to self-antigens) leads to massive lymphadenopathy and autoimmune disease. [147] [148] Coupled with the ability of activated, but not resting, T cells to express CTLA-4, these data suggest that CTLA-4 plays an important role in the negative regulation of immune responses, either by directly inducing apoptosis of activated T cells [149] or by competing with CD28 for its ligands and preventing the up-regulation of Bcl-X_L . [141]

Cytokines

After TCR activation, Th lymphocytes are stimulated to secrete a wide variety of cytokines, including interleukins, interferons, and chemokines. These cytokines are critical for promoting lymphocyte proliferation and effector function and may act in either an autocrine (on the same cell that secretes it) or paracrine (on other cells) fashion. For instance, the proliferation of naive T cells in response to TCR cross-linking depends on the presence of IL-2. [150] IL-4 and -10 support the expansion of a subset of Th, Th_2 , which help regulate humoral immune responses, while at the same time impeding the development of cells that mediate cellular immune

responses, Th_1 cells.[151] Conversely, interferon- and IL-12 promote the expansion of Th_1 cells at the expense of Th_2 cells.[152] By regulating the extent of lymphocyte proliferation and differentiation into specific effector populations, cytokines provide a potent, late-acting mechanism for regulating T-cell activation and modifying effector function in a manner appropriate to a particular immune system insult.

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Chapter 11 - Tolerance and Autoimmunity

Mark J. Shlomchik

INTRODUCTION

The immune system must balance the capacity to respond to foreign antigens and the need not to respond to self-antigens. A complex and multilayered approach has evolved to successfully handle this problem. However, autoimmune diseases, in which this balance is upset, are remarkably common in the population. The diversity and variable severity of such diseases most likely reflects the various approaches the immune system takes to regulate antiself responses, and thereby, the various points at which this multilayered system can break down. The normal functions that may prevent autoimmune disease are collectively known as self-tolerance mechanisms.

Autoimmune diseases are relevant to hematology at several levels. Autoimmune hemolytic anemia (AIHA) and idiopathic thrombocytopenic purpura are syndromes in which spontaneous autoimmunity to formed blood components may require transfusion support that is rendered difficult due to the presence of autoantibodies. Some cases of aplastic anemia may also fall into this category. Autoantibodies to red blood cells, whether pathogenic or not, are often problematic in terms of typing and screening. Another class of diseases are those induced by transfusion, but which are nonetheless autoimmune in nature: these include post-transfusion purpura (PTP)[1] [2] [3] and possibly AIHA associated with transfused thallasemia.[4] [5] [6] Finally, graft-versus-host disease, a common complication of allogeneic stem cell transplantation, although not a classical autoimmune disease, shares many features of autoimmune syndromes.[7] [8]

An important principle in understanding the etiology of autoimmune diseases is that no special mechanisms, cells, antibody types, or reactions are specific to autoimmune diseases. Rather, the pathogenesis involves the inappropriate or dysregulated triggering of the normal mechanisms of immunity. Therefore, an understanding of autoimmune disease induction and pathogenesis requires a grounding in the basic immune cell functions and interactions, which can be found in the preceding chapters.

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SELF-REACTIVE LYMPHOCYTES: ORIGIN AND CONTROL

Origins

Inevitably, autoreactive lymphocytes are generated as a consequence of the fact that immunoglobulin (Ig) and T-cell receptor (TCR) genes are encoded in pieces that rearrange in the DNA of precursor lymphocytes to ultimately form a complete gene. This process allows for many possible gene segment combinations (e.g., 4,000 different ones for the human Ig heavy chain alone), and in addition, small deletions and random additions at the sites where the pieces are joined together create additional diversity. There are two implications of this process for self-tolerance. First, it is impossible to prevent the assembly of a self-reactive receptor by filtering these out of the germline gene repertoire. Second, a developing lymphocyte cannot be considered autoreactive until the assembly process is complete and the Ig or TCR is expressed. Thus, autoreactive lymphocytes are produced every day, and it is at this key developmental stagewhen Ig or TCR is first expressed by the cellthat the immune system can first eliminate these potentially harmful cells. In particular for B cells, this occurs in the bone marrow, the primary central lymphoid organ (Fig. 11-1). The process is thus termed central tolerance.

Regulation: Central Tolerance

Clonal Deletion

The classical experiments of Pike and Nossal were the first to demonstrate that developing autoreactive B cells can be eliminated in the bone marrow. [9] [10] [11] The details of this process remained murky until the Goodnow and Nemazee groups each developed an Ig transgenic mouse system for the study of self-tolerance. [12] [13] [14] These mice have been genetically altered to carry the preformed Ig variable (V) genes that encode a specific autoantibody. Mice that have undergone this genetic transfer are termed transgenic and the gene that is transferred is termed the transgene (Fig. 11-2). The presence of this preformed transgene short circuits and prevents the normal rearrangement process at the natural Ig gene loci. Thus each B cell in the animal expresses only the transgene and has the same specificity. By choosing a target antigen that is carried by only some strains of mice (such as the polymorphic major histocompatibility complex class I genes used by Nemazee), it is possible to render the transgenic B cells autoreactive when crossed onto one strain (Fig. 11-3) but not autoreactive in a different strain. The results of such systems were dramatic. A complete loss or deletion of the B cells was demonstrated in the strain of mice that had the autoantigen, but perfectly good expression of the B cells was observed when the autoantigen was absent. This provided clear proof of B-cell clonal deletion. Further, it was shown that this deletion occurred at the immature B-cell stage, just when the cells first express their Ig receptor. [15] [16] [17] The exact fate of deleted B cells is still controversial. [15] [17] [18] [19] It has been recognized, under some circumstances, that B cells which have completed H- and L-chain rearrangement and then recognize self-antigen while still immature in the bone marrow may actually undergo a second round of V gene rearrangement. This most likely occurs at the L-chain loci, which are particularly suited to secondary V to J rearrangements. This process has been termed receptor editing. As expected, the editing process is accompanied by reexpression of the key lymphoid-specific genes that are required to catalyze the V gene rearrangements, *rag*-1 and *rag*-2. These genes had been preciously down-regulated when the cell first succeeded in making productive or expressible H- and L-chain rearrangements. Evidently, a cell has a certain period of time in which to produce a second L-chain rearrangement that will inactivate the cells self-reactivity. If this does not occur, the cell fails to mature and is eventually eliminated. The physiologic role of the editing process is still unclear, but it could represent a way to maximize the efficiency of B-cell generation while still maintaining an effective filter against strongly self-reactive B cells.

Figure 11-1 Stages at which self-tolerance can block B-cell development. Arrows indicate the normal pathway of development. X indicates where these differentiation steps can be interrupted for self-reactive B cells as a consequence of encountering self-antigen. Each X is labeled with the type of self-tolerance it represents. The clonal disability steps are somewhat more hypothetical than the earlier steps. See text for details.

Clonal Anergy

Another type of self-tolerance mechanism was also revealed by these experiments. This form, clonal anergy, involves inactivation of the self-reactive cell, but not its elimination.[10] [14] Such B cells seem to remain in the peripheral lymphoid circulation, albeit with a shorter life span than normal B cells. In addition, these cells have a lower amount of surface Ig and, moreover, seem much less capable of sensing the presence of antigen when the sIg receptor is triggered. This second form of B-cell tolerance, demonstrated dramatically through the use of Ig transgenic mice, was also anticipated in the experiments of Pike and Nossal. [10] The physiologic advantage of maintaining these anergic cells is unclear. They can be activated by strong stimulation under certain conditions; thus it has been suggested that they are maintained as a secondary repertoire to maintain greater B-cell diversity and thus better protect against a broader spectrum of pathogens. However, the presence of these cells also raises a danger that they may be activated by self-antigens as well, which could represent a source of autoantibodies. Indeed, it has been suggested that autoantibody-secreting B cells can arise by the activation of anergic B cells.

Clonal deletion and clonal anergy are often referred to as central self-tolerance because they can occur in the central lymphopoietic organs and act on immature lymphocytes that have just expressed their antigen receptors. Central tolerance is probably most important in purging or controlling very high affinity antiself lymphocytes.

Tolerance of Memory B Cells

Figure 11-1 indicates that there are yet other stages of B-cell development at which one could imagine that self-tolerance should occur. The most important of these is development of memory B cells, the long-lasting cells that harbor the ability of the immune system to respond better and faster to antigens that have already been encountered once. An important and unique process occurs during memory cell developmentthe genes that encode the antibody receptor molecule undergo a process of random mutation.[20] [21] [22] This process is thought to provide mutants with an increased affinity for the immunizing antigen, and in fact, the secondary immune response is known to be of higher affinity. However, a side effect of any random process, just as in the receptor rearrangement itself, is the potential to create novel antiself specificities. [23] [24] Thus, many have postulated that there should be a screening of cells for self-reactivity during memory B-cell development. [25] [26] [27] In fact, some evidence suggests this process exists, but it is much more elusive than clonal deletion or clonal anergy.

T Cells

In many respects, self-tolerance for T cells is similar to that for B cells; both deletion and anergy exist. [28] [29] [30] [31] [32] The principle differences reflect the basic differences in B and T development. Deletion for T cells occurs in the thymus (where TCR gene rearrangement occurs), not in the bone marrow. In addition, the

self-antigens for T cells consist of self-peptides, just as the foreign antigens for T cells are foreign peptides. Recent data have shown that T cells also undergo somatic mutation in germinal centers, but whether there is any mechanism to eliminate self-reactive T cells at this stage is unknown. [33]

Limitations of Central Tolerance

Although these mechanisms to eliminate or inactivate self-reactive B cells as they first emerge are clearly critical for the viability of an animal, they only account for part of the overall system that protects against autoimmunity. There are many reasons to believe that central tolerance cannot and should not be perfectly efficient. One is that the ability to tolerate self must

Figure 11-2 Clonal diversity in a normal versus transgenic mouse. The great diversity of B-cell specificities found in a normal mouse is indicated by the different patterns in each type of B cell. In contrast, in a transgenic mouse, each B cell expresses the same specificity because each carries the genes for a preformed heavy and light chain Ig gene (the transgenes). This is indicated by the same pattern in each B cell. The one B cell with a different pattern signifies that occasional cells will express a unique specificity even in a transgenic because the system is imperfect.

be balanced against the ability to efficiently respond to a wide variety of foreign antigens. Each cell that is eliminated in the interests of self-tolerance is one that cannot respond to a potential foreign antigen. This concept is illustrated metaphorically in Figure 11-4 . Thus one must suppose that it might be advantageous to allow some (weakly) antiself cells to escape these purging mechanisms. This is indeed the case. A second way to view this same problem is that even if it were desirable to have complete elimination of antiself lymphocytes, it would be impossible. It is unlikely that during development each cell will be exposed to a sufficient quantity of each and every self-antigen in the body to be functionally tested for self-reactivity. Furthermore, some antigens are tissue specific, such as thyroglobulin, and are unlikely to be found in the circulation at appreciable quantities.

Persistence of Self-reactive Lymphocytes

Despite central tolerance, self-reactive cells nonetheless exist in peripheral lymphoid organs of normal animals. It has been observed for some time that many immune responses are accompanied by transient antiself antibody responses. [34] [35] [36] [37] [38] For example, rheumatoid factors with specificity for self-IgG often accompany strong secondary immune responses to foreign proteins

Figure 11-3 Mating strategy to generate transgenic mice with and without a polymorphic autoantigen. Two mice are crossed, each of which is heterozygous: one for the transgene and the other for a polymorphic autoantigen (much as people can be heterozygous for blood group antigens). Shown are the possible resulting progeny of such a cross, each of which would occur at 1/4 frequency. TG indicates transgenic and Ag indicates presence of the autoantigen. The first two types of mice, one with TG and Ag and the other control with TG and not the Ag are compared in experiments to determine how autoantigen affects the development of the autoreactive B cells.

or viruses. [34] [35] [36] The simplest explanation for such phenomena is that the B cells that make these autoantibodies already exist in the peripheral lymphoid compartment, but are quiescent until they receive the proper stimulus. (How such cells get activated and why in normal animals this does not pose a threat are discussed following.)

Transgenic mouse models similar to those described above have provided the most convincing evidence of the existence of such B cells. One of particular relevance to hematology was generated by Honjo and colleagues. [39] These workers isolated the V genes that came from an actual anti-red blood cell autoantibody originally obtained from an NZB mouse with AIHA. Much like Goodnow and Nemazee, they used the transgenic approach to express the anti-red blood cell antibody in a normal nonautoimmune mouse. Although central deletion was seen in most of the transgenic mice studied, many also had some residual autoreactive B cells in spleen and lymph nodes, and some otherwise normal mice even developed frank AIHA. These results were interpreted as follows: central tolerance is not completely efficient even in a nonautoimmune mouse and some autoreactive B cells can be stimulated to cause disease. Shlomchik and colleagues, also using a transgenic approach, demonstrated that a rheumatoid factor autoantibody that was isolated from a diseased mouse was not subject to self-tolerance when expressed in a normal BALB/c mouse. [40] These B cells generally remained quiescent in a normal animal suggesting that those B cells that are not usually regulated by self-tolerance (perhaps because they recognize the self-antigen only weakly) may be the precursors of pathogenic autoantibodies in disease.

Control of Self-reactive Lymphocytes: Preventing Activation

The recognition that potentially self-reactive lymphocytes exist in the peripheral lymphoid repertoire of normal individuals, [41] despite central tolerance, raises the question of why they do not usually cause disease. One reason is the second layer of immune tolerance that prevents activation of self-reactive lymphocytes that exist in the periphery. This layer consists of several facets, which are described in the following sections.

Figure 11-4 How elimination of autoreactive cells affects the repertoire of lymphocytes capable of responding to foreign antigens. A hypothetical population of diverse B cells representing the entire repertoire available to respond to foreign antigen is depicted. The population is arrayed according to increasing self-reactivity (left panel). Tolerizing only the high affinity antiself B cells (right panel) leaves most of the potential repertoire intact. However, as the affinity cutoff for self-reactivity increases, fewer B cells will be included. It can be readily seen (bottom panel) that a low threshold for inactivation or deletion of self-reactive cells will lead to a small number of competent residual cells available for responses to foreign antigens. Thus, a stringent tolerization of low affinity antiself cells will compromise the ability to respond to nonself.

Absence of Self-antigen

The simplest explanation for why a self-specific lymphocyte is not spontaneously activated in the peripheral lymphoid compartment is the absence of self-antigen. This may be the reason why it was not eliminated in the first place. This situation has been termed clonal ignorance. It is related to the scenario described for rheumatoid factor B cells above in that the cell does not seem to care about the concentration of its autoantigen. In the case of rheumatoid factor, though, this is because the cell has relatively low affinity for self-IgG; in the case of thyroglobulin, for example, this is because the antigen concentration is vanishingly small. However, a change in antigen concentration, such as after thyroid damage from a viral infection, might then precipitate activation of these heretofore ignorant cells, leading to autoimmunity.

This antigen sequestration concept only applies to a limited set of autoantigens. A more general reason that self-specific lymphocytes remain quiescent, despite the ubiquity of self-antigens, is that T cells and B cells are dependent on each other for activation [42] [43] [44] [45] (Chap. 10). It is evident that for this to occur, B cells and T cells specific for the same self-antigen must be in the same place at the same time. If such cells are rare, then the requisite coexistence of two such cells will happen very infrequently, minimizing the chance of starting an autoimmune reaction. A second consequence of T-B interdependence is that specific inefficiencies of central tolerance in one limb can be compensated for in the other. For example, T cells are probably very efficiently purged of cells that react with thymus-specific antigens, whereas B cells are probably not. However, antithymus B-cell responses are unlikely even though many thymus-specific B cells probably circulate; the cognate T cell with specificity for the same self-antigen simply does not exist.

Co-stimulation

Even when a B cell and a T cell that do recognize the same self-antigen encounter each other, the result may still not be activation. This is because a positive response by a lymphocyte to antigen encounter also requires a second signal, aside from the stimulus of antigen recognition itself. These signals are transmitted through a series of ligand-receptor molecular pairs known as co-stimulatory molecules (Chap. 10). The most important of these are: B7.1 and B7.2 [46] [47] [48] [49] [50] [51] (expressed on B cells, macrophages, and dendritic cells) and CD28 [50] [51] (expressed on T cells). Another important pair is CD40 [52] [53] (expressed on B cells,

macrophages, and dendritic cells) and CD40 ligand [54] [55] [56] [57] [58] [59] (CD40L, expressed on T cells and missing in patients with X-linked immunodeficiency/hyper-IgM syndrome[60] [61] [62] [63]). CD40 stimulation is especially important for B cells, as it is for other antigen-presenting cells as well. Also in this category are lymphokine signals. For B cells, interleukin-4 (IL-4) signaling is important, but other cytokines such as interleukins-5, -6, and -2 (IL-5, IL-6, IL-2) also play roles in growth and differentiation. As shown in Figure 11-5 , some of these molecules are constitutively expressed, whereas others are induced in activated cells. This pattern of expression and induction leads to a cascade of events that occur during immune activation.

Figure 11-5 Timing of expression of costimulatory molecules. The schematic shows the regulated expression of the CD40-CD40L family (light red) and the B7-CD28/CTLA4 family (dark red) of molecules. From left to right is depicted increasing cellular activation as time elapses after initial encounter with antigen. The expression level of each molecule over time is indicated by a polygonal shape. The vertical width of the shape at any time reflects the degree of expression at that time. For example, CTLA4 is expressed little at the start and the expression increases continuously over time. The shapes depicting expression of molecules that are thought to deliver signals are outlined in bold, whereas those of molecules thought to receive signals are in fine line. The top three molecules are chiefly expressed on B cells and the lower three on T cells.

In general, for proper transmission of this second signal, one or the other of the lymphocytes must have been previously activated. This concept generates a paradox in that if one lymphocyte must already be activated, how is it possible to start an immune response at all? This is resolved in several ways. First, it is indeed difficult to start immune responses, and this is one of the mechanisms by which nonresponsiveness to self is maintained. However, a strong first signal to a T or B cell may be sufficient for it to induce its co-stimulatory molecules. [46] [64] Second, inflammation of any type is a powerful nonspecific inducer of these same co-stimulatory molecules.[65] [66] [67] Thus, in the presence of ongoing inflammation, such as would occur with infection or trauma, immune responses are much easier to start. Third, certain professional antigen-presenting cells, such as dendritic cells, may constitutively express these co-stimulatory molecules at moderate levels and can start the cascade, for example, by activating T cells, which is then amplified by T-B interactions. [67] In summary, there are two main functions of co-stimulatory requirements: they focus the interactions between two antigen-specific T and B cells and limit nonspecific interactions; and they restrict immune responses in the absence of inflammation. Both of these features of co-stimulation tend to prevent the activation of self-reactive lymphocytes that exist in peripheral lymphoid organs. For B cells this means that tolerance in the T-cell compartment alone will prevent many self-reactive B cells from being activated.

Even with antigen sequestration and co-stimulatory regulation, mechanisms that prevent the activation of self-reactive lymphocytes are incomplete at best. For example, it seems likely during infection or trauma that antiself responses could initiate because co-stimulatory molecules will be nonspecifically induced. Indeed, this is the case. Further, during infection and tissue damage, self-proteins that ordinarily are sequestered can be released. This leads to activation of the ignorant cells circulating in the body. [68] [69] [70] [71] [72] [73] In fact, (usually) self-limited autoimmune responses after infection are well known, such as poststreptococcal glomerulonephritis or postmycoplasmal cold agglutinins. Although these syndromes can cause serious clinical problems, they are self-limited, unlike autoimmune diseases such as systemic lupus erythematosus (SLE).

Control of Self-reactive Lymphocytes: Down-regulation

The difference between transient autoimmune responses and chronic severe autoimmunity may lie in the third layer of protection against autoimmunity: down-regulation of ongoing responses. Again, this layer is a normal part of the immune system, functioning to regulate both normal and autoimmune responses. Initially, in a normal response to a viral pathogen, there is great proliferation of lymphocytes specific for viral antigens. This process leads ultimately to the elimination of the pathogen, which was traditionally thought of as the signal to stop an immune response. However, when the pathogen is eliminated, in the absence of any other regulatory mechanism, there would be many residual cells that had been responding to the pathogen. Although a few such cells could be retained to provide immunologic memory, most of these are no longer useful in the short term. In addition to unnecessarily filling the lymphoid compartment, these cells may be a risk for causing autoimmunity. This is because of the possibility of the generation of newly autoreactive B cells by virtue of random somatic mutation. [24] B cells responding to foreign antigens begin to mutate their antibody V region genes. Mutation is a random process, and thus a mutation could occur that converts a nonautoimmune B cell into a self-reactive B cell. [24] No clear mechanism exists by which the body can discriminate and specifically eliminate these newly self-reactive mutant B cells. However, at a minimum, elimination of most of the reactive B cells regardless of specificity would mitigate this problem.

Over the last several years, several pathways for the elimination of such postexpansion cells have been elucidated. One seems to be an inborn program that causes cells to apoptose after undergoing a certain amount of proliferation. [74] [75] [76] Certain signals that probably include CD40 signaling in concert with BCR and IL-4 signaling may rescue a few of these cells from this self-destructive fate, and it is believed that these cells become long-lived memory cells. [77] [78] [79] [80] There are also active mechanisms that signal cells to apoptose. One receptor-ligand pair called Fas and FasL is central in this process. Generally, when Fas is ligated by FasL, the cell expressing Fas is triggered to die by apoptosis. [81] [82] [83] [84] Fas and FasL are not expressed at high levels on unstimulated resting lymphocytes. On activation, T cells express both Fas and FasL, whereas B cells express Fas. [84] [85] Sensitivity to the Fas signal may also be regulated in the Fas-expressing cell as well. Thus, after a certain degree of activation and proliferation, a T cell (expressing FasL) encountering an activated B cell (expressing Fas) may actually kill that B cell. There are likely other ligand pairs, particularly those in the tumor necrosis factor family that may serve similar functions, both for B and T cells.

A particularly interesting and instructive receptor ligand pair that down-regulates ongoing responses has been elucidated. The receptor, CTLA4, is expressed on activated T cells, and when ligated, causes inactivation or death of the receptive T cell; it is said to therefore transduce a negative signal. [86] [87] [88] [89] The other ligand in this pair is none other than B7, the same ligand that gives a positive signal to naive T cells by ligating CD28. The same molecule can promote activation early on in the immune response while, through a change in the receptive T cell, it can inhibit activation at a later time. This underscores the careful means by which the immune system regulates and dampens activation presumably to prevent autoimmunity.

How does regulation of ongoing immune responses prevent autoimmunity? In the first place, these normal forms of down-regulation undoubtedly prevent common transient autoimmune responses from becoming chronic. More subtlely, elimination of cells after immune responses will prevent the accumulation of a large number of self-specific memory cells. As long as such cells are rare, it is unlikely for autoreactive T cells and B cells, each specific for the same self-antigen, to wind up in the same place at the same time. Thus, down-regulation and elimination of responding cells prevents a critical mass of self-reactive cells from ever forming.

Control of Self-reactive Lymphocytes: Channeling the Type of Effector Response

A final layer of protection against self-inflicted immune damage involves channeling of responses so they are not harmful. Depending on the context, only certain effector functions will effectively eliminate certain pathogens. For example, antibodies will not be effective against intracellular pathogens. By analogy, only certain effector functions may cause autoimmune disease, depending on the circumstances. It is clear that there are two major types of T-helper cell responses, Th1 and Th2, that in turn lead to very different effector functions. [90] [91] [92] The propensity to make these various types of responses depends on a number of ill-understood factors, but these include genetics, route of antigen exposure, and dose of antigen. [93] [94] Intriguingly, in certain murine models of autoimmunity such as the NOD diabetes model, experimental manipulations that shift responses away from Th1 and toward Th2 are highly protective against disease. [95] [96] [97] This is also relevant to B-cell autoimmunity per se because, through the use of different isotypes of Ig, different effector functions can occur. The cytokines secreted by Th1 and Th2 cells have profound effects on the isotypes of Igs that are

produced during a response. Thus, not only is the T-cell component of the response channeled in this way, but the humoral response is also influenced.

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BREAKDOWN OF SELF-TOLERANCE IN AUTOIMMUNE DISEASES

Presumably, for autoimmune diseases and autoantibody production to occur, one or more of the multilayered mechanisms to prevent autoimmunity must fail. Surprisingly, the precise nature of these failures is not well understood. The mechanism of failure will differ for different autoimmune diseases and perhaps even for different patients with similar syndromes. Moreover, it seems likely both from phenomenologic and genetic studies that failures at several levels are required to generate clinically significant autoimmunity. In the following section, examples of the current state of knowledge are given.

This chapter is not meant to review the nature of autoimmune diseases; however, before considering the likely points at which self-tolerance mechanisms break down it is useful to review some basic concepts about these diseases. Grossly, autoimmune diseases have often been divided into organ-specific and systemic autoimmune syndromes. This classification is useful, but as these diseases are becoming better understood, the dividing lines are blurring; pathogeneses of all these diseases are likely to have much in common. In particular, systemic autoimmune diseases are actually much more specific in their antigenic targets than is commonly realized. Table 11-1 shows the types of autoantibodies commonly found in several systemic autoimmune diseases. Certain autoantibodies are diagnostic for specific autoimmune diseases, such as anti-Sm in SLE. Thus Sm is a specific target in SLE, but other autoimmune patients, such as those with rheumatoid arthritis, do not respond to this autoantigen. In fact, only 30% of all patients with SLE make anti-Sm, meaning that the other 70% are tolerant of their own Sm, despite having a systemic autoimmune disease.[98] Another salient feature of most human autoimmune diseases is adult onset. Both the selective nature of disease and its late onset argue against gross defects in the basic central tolerance mechanisms as being the cause.

Instead, these considerations suggest that most clinical autoimmune diseases are likely to arise from defects in the later stages of self-tolerance, such as preventing the activation of autoreactive cells or down-regulating them once they are activated. Because in no case is the primary cause of an autoimmune disease known, it cannot be excluded that subtle defects in the earlier stages, including central tolerance, may also play a role. However, it does seem clear that a gross defect in central tolerance would lead to a severe syndrome of congenital autoimmunity.

Genetic and Environmental Factors

Genetic Factors

Both genetic and environmental factors play a role in the occurrence of autoimmunity in some individuals and not others. The most well-known genetic factor is the major histocompatibility complex, known as HLA in the human. Many different autoimmune diseases are more or less associated with specific genotypes at this polymorphic locus. Among these are ankylosing spondylitis (HLA-B27), insulin-dependent diabetes mellitus (HLA-DR3/4), rheumatoid arthritis (HLA-DR4), and to some degree SLE (HLA-DR2/3).[99] It should be emphasized that although individuals with these genotypes are relatively more prone, most will not develop the autoimmune disease. How certain HLA genes predispose to autoimmunity is not very clear. These genes could be involved in the efficiency or specificity of central tolerance in the thymus, but could also be involved in the activation of autoreactive T cells in the periphery.

Inheritance patterns of all systemic autoimmune diseases suggest that multiple genes, in addition to the HLA locus, contribute to susceptibility. Such genes are beginning to be identified in animal models. Recent work in murine SLE has used genomic scanning with polymerase chain reaction-based polymorphic short simple repeat sequences to identify and map genes that are associated with autoimmune phenotypes. In this type of analysis, an autoimmune strain is crossed with a non-autoimmune strain and then the progeny are back-crossed to the autoimmune strain. Genetic loci segregate in this cross, and for each individual mouse, with the help of the murine genome map, the origin of 100200 genetic locations all along the chromosomes are identified as either from the autoimmune parent or the nonautoimmune parent. Simultaneously, the autoimmune phenotype of each mouse is determined in a variety of assays, most commonly including autoantibody production and glomerulonephritis. By typing a few hundred mice in this way, correlations can be made that link certain genetic loci with certain phenotypic traits. Several genes have been mapped with this approach, and their phenotypes investigated in greater detail. [100] [101] [102] [103] Interestingly, most of them seem to have direct effects on B cell function or activity. In the next several years, some of these genes may be identified and cloned and the exact nature of the defects defined. This will in turn permit screening for defects in the homologous genes in human autoimmune disease patients.

TABLE 11-1 -- Patterns of Autoantibody Expression in Systemic Autoimmune Diseases

Autoantigen	Autoimmune Diseases (% of patients *with* autoantibody)			
	Systemic Lupus Erythematosus	Rheumatoid Arthritis	Scleroderma	Sjögren Syndrome
dsDNA	40			
ssDNA	70			
Histones	70			
Sm	30			
nRNP	30			
Ro (SS-A)	35			60
La (SS-B)	15			40
IgG (RF)	5	90		1020
Scl-70 (Topo I)			70	
Centromere			70	

Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; Sm, Smith ribonucleoprotein; nRNP, native ribonucleoprotein; Scl-70, scleroderma 70-kD antigen (topoisomerase I). Blank space indicates rarely or never detected.

Information derived from Tan.[98]

TABLE 11-2 -- Genes Involved in Regulation of Autoimmune Responses

Category	Types of Genes[a]	Known Examples[b]
Central and peripheral deletion and anergy	Receptor signaling, MHC genes, receptor V genes	CD45,[119] PTP-1C, [120] [121] [122] HLA (certain types),[99] CD3,[123] CD4, CD8, CD28/B7[32]
Initiation of response	Receptor signaling, co-stimulatory molecules, adhesion molecules	CD45, PTP-1C, FcRII[121]
Down-regulation of response	Apoptosis genes, interleukins, negative co-stimulatory molecules	Fas, [107] [105] TNF,[124] [125] CTLA4,[87] CD40, CD3,[126] CD28/B7[50] [127]
Channeling of response	Interleukins, interleukin receptors	IL-4, IL-10, IL-12, IFN-[96] [97]

Abbreviations: PTP-1C, phosphotyrosine phosphatase; IL, interleukin; FcRII, receptor for IgG-type II; Fas, see text; TNF, tumor necrosis factor, CTLA4, see text; IFN-, interferon gamma; MHC, major histocompatibility complex.

[a]Indicates some of the categories of genes that may be involved in regulating autoimmunity at the indicated step.
[b]Some genes in the Types of Genes category which have been shown to play a role in the process indicated in the left column. Some have also been directly shown to play a role in autoimmunity.

Although animal models suggest multigenic inheritance, there are certain instructive cases in which single gene defects play a major role. Table 11-2 is a list of categories of genes that are likely involved in genetic predisposition to autoimmune disease. Note that these include genes involved in the processes of antigen sequestration, T-B collaboration, and immune response down-regulation that were discussed above as key features of the self-tolerance mechanisms that normally prevent autoimmune disease.

The best studied example of mutations in these genes is the *lpr/lpr* mouse, a natural variant originally discovered at the Jackson Laboratories, which carries an inactivated murine Fas.[81] [104] [105] The *gld* mutation (another natural variant discovered at Jackson), which inactivates murine Fas ligand (FasL) [84] has a very similar phenotype to the *lpr.* Both of these mutations lead to an age-dependent autoimmune syndrome with auto antibody profiles that remarkably resemble human SLE. [105] These mice die prematurely of renal failure. They also have an accumulation of lymphocytes that leads to marked lymphadenopathy. [106] Presumably, this is the result of failure to eliminate postactivation T and B cells by the Fas-based mechanism. [107] [108] [109] Exactly how defects in the apoptotic Fas pathway lead to autoimmunity has yet to be elucidated. Interestingly, a rare syndrome in humans with incomplete penetrance, called autoimmune lymphoproliferation syndrome, has recently been traced to mutations in human Fas.[110] Often these patients are misdiagnosed with leukemia or lymphoma and some have even been treated for (and survived) these neoplasms. Clonality and chromosomal studies in autoimmune lymphoproliferation syndrome reveal polyclonal B and T cell proliferations with normal karyotypes, in distinction with true lymphoma or leukemia.

The phenotypes of these mutants in the Fas pathway, though more fulminant than most human autoimmune syndromes, illustrate two important points. They demonstrate the critical nature of the late down-regulatory controls in preventing autoimmune disease. They also point out pathways in which less severe mutations might be discovered that account for human disease.

Environmental Factors

Environment plays a role that is at least as important as genetics. This is illustrated by the fact that concordance rates among identical twins, even raised in the same household, are surprisingly low. Only 20% of twins of patients with rheumatoid arthritis also get rheumatoid arthritis. [99] There are many examples of environmental factors causing either chronic or transient autoimmune diseases. There are postinfectious syndromes such as postmycoplasmal cold agglutinin disease. The pattern of incidence of multiple sclerosis suggests a viral etiology, though no causative virus has ever been convincingly demonstrated. Another category of infectious associations includes postviral myocarditis, which follows certain coxsackievirus infections. [72] It is sometimes conceptually difficult to draw a line between viral damage and consequent immune system damage; however, if sensitization to self-antigens occurs as a consequence of viral infection, and these later are pathogenic targets independent of viral antigens, it seems reasonable to consider the syndrome as autoimmune.

Infections are not the only source of environmental stimuli for autoimmunity. Toxins, such as mercury, cause autoimmunity in animal models. [111] [112] Another form more familiar to those in hematology is drug-induced autoimmunity, as in AIHA. Drugs that cause lupus-like syndromes, such as procainamide, are particularly prominent examples.[113] [114] Despite these specific examples, the environmental factors that play a role in promoting common autoimmune diseases such as rheumatoid arthritis or SLE are unknown.

Immunodeficiency and Autoimmunity

Paradoxically, immunodeficiency is often accompanied by B-cell autoimmunity. A clear example that is particularly relevant to hematologic practice is advanced human immunodeficiency virus (HIV) infection. Autoantibodies, along with clinically significant idiopathic thrombocytopenic purpura and/or AIHA are not uncommon. Serum protein electrophoresis often reveals oligoclonal IgG bands. Indeed, the extent to which features of acquired immunodeficiency syndrome (AIDS) are related to B-cell autoimmunity is not completely defined. The mechanism for this paradoxical autoimmunity is not proven, but it almost certainly relates to the lack of regulation of T cells by B cells. T cells not only promote B-cell activation, but also can negatively regulate and even kill B cells. The Fas-FasL ligand pair is instrumental in this. The tumor necrosis factor-family cytokines also likely play this role, and transforming growth factor-, another T-cell-derived cytokine, may have a negative regulatory effect. Because B cells that have already been activated by T cells may not require much in the way of additional positive signals, the growth of such clones may actually be promoted by a lack of T cells that on balance would provide more negative than positive signaling. Indeed, the B-cell lymphoproliferations associated with T cell immunodeficiency, such as HIV and iatrogenic immunosuppression, may also have their route in this mechanism.

Examples in Hematology: Epitope Spreading in PTP

One potential way to break self-tolerance may be particularly relevant to syndromes found in hematology and is worthy of

elaboration. This is a form of environmental stimulation, albeit iatrogenic. In PTP, transfusion with allogeneic platelets that contain a platelet-specific antigen (such as HPA-1^a) lacking in the (HPA-1^b) recipient leads to rapid destruction of the transfused platelets and antibody formation to the foreign platelet antigen. [3] [115] However, several days later, the recipient becomes severely thrombocytopenic due to increased destruction of the recipients own platelets. Although how such destruction of self-platelets occurs secondary to destruction of allogeneic platelets may still be controversial, [3] [115] [116] the best explanation is an autoimmune response. [1] [2] [3] How does this response get stimulated? The probable pathway bears significant parallels to one demonstrated in mice several years ago by Mamula, Janeway, and colleagues.[117] [118] These workers immunized normal mice with human cytochrome c, which differed slightly from endogenous murine cytochrome c. The mice made both an antibody response and a T-cell response to the human cytochrome c; however, since the human and mouse cytochromes are so similar, the antibody response (but not the T-cell response) cross-reacted with murine cytochrome c. Presumably, this reflected activation of ignorant B cells with specificity for self-cytochrome c (and also human). However, several weeks later, if the mice were given a dose of self-cytochrome c, now both a vigorous B-cell and T-cell antiself response ensued. These authors suggested that priming with the cross-reactive antigen first induced self-reactive B cells, which in turn could then break tolerance in anergic or ignorant self-reactive T cells.

How does this relate to PTP? Figure 11-6 illustrates the authors hypothetical adaptation of this mechanism to the platelet transfusion situation. The foreign platelets actually share many common antigens with the host, as well as differ at the HPA-1^a locus. The foreign antigenic difference allows ignorant self-specific B cells (as well as HPA-1^a -specific B cells) to interact with helper T cells that are specific for the foreign HPA-1^a antigen and become activated. Moreover, these activated B cells can then present self-platelet antigens along with co-stimulatory signals to self-reactive T cells. When this happens, the immune

Figure 11-6 Epitope spreading as a possible autoimmune mechanism for PTP. Events are depicted as progressing from left to right. An HPA-1 ^{b} person is transfused with an HPA-1$^{a/b}$ platelet product. An alloantibody response ensues as an HPA-1^{a} -specific B cell recognizes the platelet, becomes activated to secrete antibody, and presents the HPA-1 ^{a} antigen to an anti-HPA-1^{a} T cell (step 1). In addition, the activated B cell may now activate a previously ignorant anti-HPA-1 ^{b} -specific T cell to initiate an autoimmune response (step 2). The activated B cell acquired the self-HPA-1 ^{b} antigen as a passenger on the HPA-1$^{a/b}$ allogeneic platelet. This autoreactive T cell can then activate an ignorant anti-HPA-1 ^{b} B cell to make an autoantibody response (step 3) in response to autologous platelets. Note that the sensitization involved in steps 1 and 2 may take place in a primary response during the first transfusion or exposure and that step 3 may take place in a clinically noticeable way only after a secondary exposure to homologous platelets. [, Ag Receptor; =, MHC Class II.

response can perpetuate even in the absence of the foreign platelets. This is exactly what is seen in PTP, where a delayed response continues to eliminate self-platelets for many days after the disappearance of the transfused platelets. Thus, a foreign platelet is analogous to foreign cytochrome c in having a few different antigens along with many shared antigens. In the same way as shown experimentally with cytochrome c, it is hypothesized that the few foreign antigens existing on the same particle (in the case of cytochrome c it is the same molecule) allow spreading of autoimmunity from a foreign antigen to self-antigens. The key events are the activation of ignorant B cells that cross-react with both self and foreign molecules, and then the activation by these B cells of T cells that are specific for self.

It is reasonable to question how such antiself responses are ever stopped once started. PTP, for example, is a self-limited syndrome. In fact, the answer is not known; however, both down-regulation of antigen as the platelet count falls to near zero as well as the natural mechanisms that cause apoptosis of responding lymphocytes probably play a role. In the absence of an autoimmune prone host who has mutations affecting the down-regulation of immune responses, these autoimmune reactions will remain transient. It is speculated that when similar events occur in people who do have genetically based problems in down-regulating such responses that a chronic autoimmune syndrome can be induced.

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IMPLICATIONS AND THERAPY

The significance of this issue to hematology ranges from syndromes such as AIHA and idiopathic thrombocytopenic purpura to iatrogenically induced autoimmunity as in PTP. In the latter case, a phenomenon known as epitope spreading, which is documented in murine models, but little discussed in terms of PTP, is speculated to be a relevant pathogenetic mechanism. A basic understanding of the mechanisms of self-tolerance and their breakdown in autoimmune disease raises the possibility of many types of specific therapeutic interventions. One of the clearest would be to identify initiating factors, such as infections, and to prevent or treat them. A second approach would be to reset tolerance. Some of the previous examples, such as in PTP, illustrate how an initiating event can be amplified leading to broken tolerance. If the system can be set back to the state before that event, the disease could be cured. At present, it is unclear how to do this; however, an autologous or even allogeneic hematopoietic stem cell transplant may have the desired effect. In fact, this sort of radical therapy has been tried in selected cases of severe SLE and seems to have some efficacy. Another promising area is in channeling the immune response, particularly as the steering mechanisms are becoming better understood at the molecular level. Work in this area is currently active. A third area is to design more specific modulators of inflammation, including interfering with co-stimulatory signals. These latter approaches have seemed promising in various animal models and may be ready for clinical trials soon.

Current therapy is much more crude, and typically involves general nonspecific immunosuppression either with steroids or cytotoxic drugs. Although these therapies can be effective, they have numerous undesirable side effects, not the least of which is increased susceptibility to infection due to immunosuppression. Therapies should ultimately be directed toward either prevention or else specific down-regulation of ongoing responses. Future work in this area will include continuing to define how self-tolerance is imposed and how it is broken in disease, what the critical triggers and autoantigens are, and how to use immunomodulation to treat autoimmune diseases based on a better understanding of the pathogenesis.

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Chapter 12 - Stem Cell Model of Hematopoiesis

David A. Williams

INTRODUCTION

Healthy individuals require adequate production of enormous numbers of differentiated blood cells daily. Mature blood cells are derived from undifferentiated stem and progenitor cells in a complex series of maturational and divisional steps not yet completely understood. The complexity of this system is enormous, since as many as 15×10^9 erythrocytes and 15×10^9 white blood cells are produced each hour each day during the lifetime of the individual. Additional complexities include the need for rapid responses to acute stress (blood loss or infection) and the need to maintain a pool of undifferentiated cells from which mature cells are derived. Finally, mature blood cells must function in anatomic locations widely separated from the bone marrow, where most cells arise. These general concepts are important for the understanding of blood formation. Clearly any perturbations, even small ones, in the enormous production of hematopoietic cells will cause large abnormalities in blood production. Abnormalities of this process are manifested by sometimes life-threatening diseases such as aplastic anemia, cytopenias, leukemias, and other myeloproliferative disorders.

Understanding of the complex relationships involved in blood cell production has increased through basic science research by experimental hematologists, radiobiologists, and immunologists. During hematopoietic cell production, hematopoietic stem and progenitor cells give rise to morphologically distinguishable daughter cells, called precursors. Careful staining and microscopic observation of bone marrow cells by pathologists and hematopathologists have led to a clear understanding of the maturational steps involved in precursor cell differentiation. In 1961 Till and McCullough [1] developed an in vivo colony assay that identified a murine multipotent stem cell, the colony-forming unit-spleen (CFU-S); this assay allowed initial characterization of a transplantable primitive bone marrow cell. Pluznik and Sachs[2] and Bradley and Metcalf[3] later described in vitro colony assays for hematopoietic progenitor cells, which led to new understanding of the role of growth-stimulatory proteins in hematopoietic cell survival and differentiation. The molecular cloning of a number of growth factors, including several now in clinical use, is in part due to these early accomplishments (see Chap. 51). The influence of the bone marrow environment on hematopoiesis was initially suggested by Wolf and Trentin.[4] Again, progress in our understanding of the role of environmental signals in hematopoiesis was brought about by the description by Dexter et al. [5] of an in vitro culture system that mimicked the medullary environment and allowed continued production of myeloid hematopoietic cells for months in culture flasks.

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HEMATOPOIETIC CELL HIERARCHY

The experimental systems described previously, as well as many other experimental approaches and observations, have led to some general concepts concerning hematopoiesis. Blood cell formation occurs as a result of a series of maturational cell divisions. The hematopoietic system, which gives rise to all circulating blood cells, can be envisioned as a series of overlapping functional compartments (Fig. 12-1). The stem cell compartment is made up of rare primitive cells that are multipotential (maintain the capacity to give rise to all lineages of blood cells) and have a high self-renewal capacity (give rise to identical daughter stem cells). [6] A characteristic of the stem cell compartment is that most stem cells are mitotically quiescent.

A process termed commitment involves the transition from pluripotent, self-renewing cells in the stem cell compartment to the progenitor cell compartment. This process is not completely understood, but is characterized by restriction in the stem cell

Figure 12-1 Schematic view of hematopoiesis. Primitive stem cells reside in close association with cells making up the hematopoietic microenvironment, represented in vitro by CFU-F/RF. Several in vitro and in vivo assays are represented below. Since the relationship of these cells and the reconstituting hematopoietic stem cell is unclear, these compartments are shown overlapping. Primitive cells are represented as long-term and short-term repopulating cells, LTC-IC and CAFC. Cells in the progenitor compartments are defined by in vitro colony assays, such as CFU-C, CFU-Mix and HPP-CFC. Precursor cells are morphologically recognizable cells in the bone marrow environment. See text for full details.

differentiative and proliferative capacity. Progenitor cell compartments are comprised mainly of cells with the capacity to differentiate along one lineage (unipotential progenitors), with lower frequencies of bipotential and multipotential primitive cells. Therefore, commitment involves the acquisition of some specific growth factor receptors and loss of others. Progenitor cells are generally defined functionally, that is, by the capacity of the cells to form colonies in in vitro assays; they demonstrate little self-renewal capacity. Mitotically active cells are far more frequent in progenitor compartments compared with stem cell compartments.

Most cells in the bone marrow make up the precursor compartment. These cells exhibit easily recognized nuclear and cytoplasmic morphologic characteristics that can be used to classify the lineage of commitment of the precursor cell. For example, a myeloblast has distinguishing morphologic characteristics that allow classification into the lineage of cells destined to become granulocytes. Little self-renewal capacity exists in this compartment, but because of the large number of precursor cells and the high mitotic activity of these less primitive cells, considerable amplification in absolute cell numbers occurs within these compartments. Therefore, early cells have the capacity to give rise to large numbers of progeny cells through cloning during the transition from stem cell to differentiated and functional cell.

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PURIFICATION AND FUNCTIONAL CHARACTERISTICS OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

Stem cells have been defined functionally by the ability to reconstitute both lymphoid and myeloid hematopoiesis when transplanted into a recipient. Initial studies used radioprotection as an assay for this rare cell, [7] whereas Till and McCulloch[1] developed the first quantitative assay for stem cells based on reinfusion of cells into an irradiated recipient (Fig. 12-2). Detailed understanding of the biology of the hematopoietic stem cell has been hindered by the low frequency of this cell in the bone marrow nucleated cell population, the lack of reagents to distinguish the stem cell from other immature cell types, and the lack of practical and quantitative assays for human hematopoietic stem cells. Recent advances have been made in all of these problematic areas. Hematopoietic stem cells have been reported to be purified to a high degree using density gradient centrifugation, labeling with antibodies, lectins, or dyes, and separation using fluorescence-activated cell sorters. Additional separation methods include immunomagnetic bead selection or the use of immunopanning. [8] Immunologic characteristics of murine hematopoietic cells include lack of antigens present on more lineage-restricted progenitors (termed Lin), expression of the antigens Sca-1 (Ly6), [9] Qa-m7, [10] and Thy-1.[9] In addition, several investigators have used the intensity of staining with rhodamine 123, which is effluxed by the multidrug-resistance gene product or P-glycoprotein. [11] Similarly, putative human hematopoietic stem cells have been purified using expression of the CD34 antigen, [12] [13] lack of HLA-DR expression,[14] and lack of antigens expressed on more lineage-restricted progenitors. [15] Recent studies have provided evidence that a population of primitive CD34-negative cells exists in multiple species with culture characteristics similar to stem cells. [16] These cells, isolated by flow cytometric analysis based on staining with the DNA-binding dye Hoechst 33342, have been shown to be lineage marker-negative and capable of initiating long-term hematopoiesis in mice in vivo. A complicating feature of the analysis of human stem cells is the inability to test purified populations routinely in in vivo reconstitution assays. New approaches include the use of xenografts for reconstitution of human cell populations after transplantation. [17] [18] These models include the classical C.B-17 scid/scid severe combined immunodeficiency (SCID) mouse, [19] the beige athymic nude X-linked (bnx) immunodeficient mouse,[20] the humanized SCID (SCID-hu) mouse, in which human (fetal) hematopoietic tissues such as liver, thymus, and bone fragments, are surgically transplanted, [21] and a transgenic SCID mouse that expresses the genes for IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF). [22] Recently, a new mouse strain (NOD/TtSz-scid/scid, called NOD/SCID) was developed by crossing SCID mice with nonobese diabetic (NOD/Lt) mice.[23] In all these models, human hematopoiesis can be established, repopulating frequency can be estimated, and some degree of proliferation and differentiation takes place. For a recent review see Wermann et al. [24] Table 12-1 summarizes characteristics of murine and putative human hematopoietic stem cells. Human stem cell purification is an area of intense research and may provide new therapeutic approaches to the treatment of certain diseases, such as chronic myeloid leukemia (CML), for which

Figure 12-2 CFU-S assay. Hematopoietic colonies arising from the CFU-S stem/progenitor cell 14 days after injection of murine bone marrow into lethally irradiated mice.

TABLE 12-1 -- Phenotypic Characterization of Murine and Human Hematopoietic Stem Cells

Mouse	Human
Ly6A (Sca-1)$^{+}$	CD34+
Lin	HLA-DR
thy 1+	c-kit+
c-kit+	CD15, CD77
Rhodamine^{dull}	thy 1^{+}
5-FU resistant	Rhodamine^{dull}
	5-FU resistant
	4-HC resistant

From Williams[32] with permission.

the goal of current work is to purify normal stem cells from cytogenetically abnormal CML stem cells.

Both murine and human primitive hematopoietic cells can form colonies in semisolid media and sustain hematopoiesis in specialized stromal-containing liquid cultures (Table 12-2). Several different assays have been established, but the relationship between the cells defined by these assays and a transplantable and reconstituting hematopoietic stem cell is not clear. Multipotent and unipotential progenitor cells can also be assayed by the ability to form colonies in semisolid medium in vitro (Fig. 12-3 and Plate 12-1). These colonies are stimulated by inclusion of appropriate growth regulatory proteins, termed growth factors, and are comprised of maturing granulocytes and macrophages: CFU-granulocyte/macrophage (CFU-GM), erythrocytes (burst-forming unit-erythroid [BFU-E], and CFU-erythroid [CFU-E]), megakaryocytes (BFU- and CFU-megakaryocyte [BFU-Mk and CFU-Mk]), and mixtures of all lineages (CFU-Mix or CFU-GEMM). Progenitor colonies are generally composed of 50 to 100,000 cells, most which exhibit a relatively mature phenotype. In addition, murine [5] and to a lesser extent human[25] hematopoietic stem and progenitor cells can be cultured for prolonged periods in vitro in a culture system, termed long-term marrow or Dexter culture, which allows direct contact between these hematopoietic cells and supporting cells derived from the medullary cavity. A recent modification of these long-term cultures, called the long-term culture-initiating cell (LTC-IC) assay, also allows precise measurement of the frequency of the cell responsible for sustained hematopoiesis in such cultures. [26]

Commitment

The factors that regulate hematopoietic cell proliferation and differentiation, including commitment, remain unknown. Theories of stem cell behavior include the stochastic model,[27] which considers stem cell renewal versus differentiation to be based on probability, and the hematopoietic inductive environment theory, [4] which links commitment to local environmental signals. If stem cell self-renewal capacity is unlimited and stem cells are truly immortal, they could likely function for an individual's entire lifetime. Stem cell immortality would confer stability on the clonal composition of an animal's hematopoietic system. On the other hand, stem cell self-renewal capacity could also be much more limited. Under these circumstances, only a

TABLE 12-2 -- Assays for Primitive Human Hematopoietic Cells

High proliferative potential-colony forming cell [90]

Colony-forming cell blast[91]
Long-term culture-initiating cell[92]
Xenograft models[24]

portion of the total stem cell population would be active at any given time, and new stem cells would be required to contribute to active blood cell production to replace dying cells. This constant turnover in the active stem cell populations would likely lead to changes in the clonal makeup of the different hematopoietic lineages over time. Hematopoiesis then would be maintained by a succession of short-lived clones (clonal succession model). The concept of short-lived stem cells is supported by the finding that the hematopoietic systems of irradiated, reconstituted animals undergo clonal changes with time. Equally convincing evidence, however, indicates that at least a subpopulation of stem cells are long-lived, able to function for prolonged periods, and able to expand clonally during the regeneration of a new hematopoietic system. Recent evidence suggests that stem cells derived from different stages of development function in distinct fashion, implying that factors intrinsic to the stem cell, itself, may regulate stem cell fate. In addition, these changes in stem cell function correlate with measurable changes in telomere length, suggesting that the replicative potential of some stem cells may be limited. [28] The survival and differentiation of hematopoietic stem cells can also be influenced by an ever-increasing number of glycoproteins, termed cytokines, which stimulate both cell division and survival [29] (see Chap. 14). Evidence suggests that the provision of appropriate cytokines in vitro can induce the expansion of primitive murine progenitor cells without the loss of reconstitution capacity. [30] Growth factors that appear to be important in the survival of stem cells and expansion of progenitor cells in vitro include steel factor (also called stem cell factor, kit ligand, and mast cell growth factor, and discussed later in this chapter), interleukin-1 (IL-1), [30] IL-6, and possibly IL-11,[31] granulocyte colony-stimulating factor (G-CSF), [32] leukemia inhibitory factor (LIF),[33] megakaryocyte growth and development factor (MGDF, also called thrombopoietin) [34] and FLT3 ligand.[31]

Based on in vitro data, Ogawa[35] has proposed that these factors may function by triggering cell cycle-dormant primitive cells into proliferation. Some of these factors (G-CSF, IL-3, and steel factor) may be required for the survival of primitive hematopoietic cells in G_0. Interestingly, IL-6, IL-11, and LIF appear to interact with distinct receptors, but may share common signal-transduction pathways, whereas IL-6 and G-CSF share some structural homology.

At the molecular level, little is known about the process of commitment. Although it is clear that differentiation is accompanied by increased expression of certain lineage-specific genes, the regulation of gene expression during hematopoietic cell differentiation is due to largely unknown transcriptional regulatory proteins. It has become increasingly understood that extracellular regulatory signals affect cell behavior by activation of intracellular targets, which ultimately determine lineage or cell-specific gene expression by a complex of transcriptional factors (reviewed by Shivdasani and Orkin; [36] see Chaps. 6 and 52). These regulatory proteins bind to DNA sequences that either up- or down-regulate the expression of a nearby gene. [37] The best-studied transcription regulators in hematopoietic cell differentiation are the GATA-1, PU.1, tal-1/SCL, and NFE-2. Both GATA-1 and NFE-2 proteins appear to be critical for expression of many erythroid-specific genes. DNA sequences that lie within the regulatory regions of erythroid-specific genes are recognized by these proteins. GATA-1-binding protein recognizes a specific DNA sequence [T/A(GATA)A/G] (hence the name GATA-1) that is present near the promoter, enhancer, or locus control region (LCR) of erythroid genes. [38] Protein binding to the cognate DNA stretch via domains resembling zinc fingers transactivates the neighboring promoter. [37] GATA-1 motifs appear to be required for full promoter activity of a wide variety of erythroid-specific genes, including - and -globin, erythropoietin-receptor gene, porphobilinogen deaminase, pyruvate kinase, and glycophorin. [38] Genetic disruption of

Figure 12-3 Progenitor assay. Colonies arising in vitro in semisolid medium after plating of human bone marrow cells. **(A)** Colony-forming unit-granulocyte/macrophage (CFU-GM). **(B)** Burst forming unit-erythroid (BFU-E).**(C)** Colony-forming unit-megakaryocyte (CFU-Mk), upper right and CFU-GM, lower left.(See also Plate 12-1)

the murine GATA-1-binding protein gene is incompatible with development of erythrocytes either in vitro or in vivo. [39] Other members of the GATA family, especially GATA-2, may play an important role in hematopoietic cell differentiation. Both GATA-1 and GATA-2 are expressed not only in erythroid cells, but also in megakaryocytes and mast cell lineages.[40] [41] Several promoters of lineage-specific genes in these lineages have been demonstrated to contain GATA-1 sites.

NF-E2 binds to an activator protein-1-like site upstream of several erythroid-specific promoters and in the LCR of both - and -globin. NF-E2 has the same tissue expression profile as GATA-1. However, NF-E2 affects transcriptional activation as a heterodimer with a ubiquitous protein, p18. [41] Thus NF-E2 and GATA-1 proteins function together and largely control the tissue-specific expression of a great number of genes expressed only in red blood cells. The transcription factor PU.1 is a member of the ets family of proteins, the expression of which is restricted to hematopoietic cells. Mice deficient of PU.1 expression created by gene knock-out studies die early after birth with a deficiency of monocytes, granulocytes, and T- and B lymphocytes. [42] Tal-1/SCL is a hematopoietic basic-loop-helix factor which binds to cis elements of genes containing the sequence CANNTG (e boxes). Mice homozygous for deletion of this gene die during development from a complete absence of blood formation, even in the yolk sac.[43] [44] In addition, there are virtually no myeloid colony forming cells demonstrating that this transcription factor is essential for early hematopoietic cell proliferation or differentiation. Although multiple transcription factors affecting hematopoietic cell proliferation and differentiation have been described, it remains unclear how the interaction of growth factors with growth factor receptors and other cell-cell interactions modulate transcription factor function, and thus ultimately control blood formation under physiologic conditions or in response to commonly encountered stress situations.

C-kit Proto-oncogene and Its Ligand

Two mouse mutants have contributed significantly to our understanding of the hematopoietic stem cell biology. The murine mutants of bone marrow failure syndromes, *dominant white spotting (W)* and *steel (Sl)* have now been fully characterized at the molecular level. Because of the key role of these mutations in our understanding of the basic biology of hematopoietic stem cells, a summary of their phenotypic abnormalities and molecular biology is included here.

Phenotypically the *Sl* and the better characterized *W* mutants are black-eyed white mice with reduced fertility and macrocytic anemia[45] (Fig. 12-4). All hematopoietic lineages are affected. [45] [46] [47] The hematopoietic abnormalities associated with the *W* mutation can be corrected by bone marrow transplantation, whereas the abnormalities of *Sl* mutants are manifested in supporting cells, such as stromal cells in the bone marrow environment. In recent years, the molecular bases of both mutations have been delineated. The *W* mutations affect the c-kit proto-oncogene, a growth factor receptor and member of the tyrosine kinase receptor family, which includes the receptor or CSF-1.[48] [49] This receptor is expressed on primitive hematopoietic stem and progenitor cells. Specific c-kit mutations and the severity of the phenotypic abnormalities have been correlated with functional impairment of the c-kit-associated tyrosine kinase activity. [50] Piebald syndrome in humans appears to be associated with genetic mutations of the c-kit receptor[51] (Fig. 12-5).

The molecular and biochemical nature of *Sl* mutations has also been identified.[52] [53] [54] The gene identified in these studies encodes a protein that is the ligand for the c-kit receptor mutated in W mice. [55] [56] [57] The gene maps to chromosome 10 in mice and is deleted in alleles associated with embryonic lethal phenotypes. [56] [57] [58] The cloned Sl cDNA predicts a membrane-associated and glycosylated protein with structural homology to CSF-1. Although the physiologic role(s) of these isoforms are unknown, Kapur et al.,[59] using transgenic mice crossed into Steel mutants, have demonstrated that membrane-associated protein appears critical for erythroid cell production. Recombinant growth factor expressed from the cloned cDNA, which has been called stem cell factor, kit ligand, mast cell growth factor, and steel factor, corrects the bone marrow manifestations of the mutation when administered to mice.[56] The protein has pleiotropic effects on hematopoietic stem and progenitor cells in vitro, which has been tested in human trials in vivo. [60]

Hematopoietic Microenvironment

Hematopoiesis occurs within a complex environment in the medullary cavity of adults[61] and the fetal liver[62] and yolk sac[63] [64] of the developing fetus. Many cells making up this hematopoietic microenvironment (HM) are not derived from hematopoietic

Figure 12-4 Sl and W mouse mutants. Photograph of homozygote **(left)** and heterozygote **(right)** mice showing coat color abnormalities of Sl and W mouse mutations. *(Photographs courtesy of Dr. Roger Fleischman.)*

Figure 12-5 Comparison of **(A)** mouse W heterozygote and **(B)** infant with piebald syndrome. *(From Fleischman et al.[51] with permission.)*

stem cells, whereas hematopoietic cells develop in nests termed cobblestone areas (Fig. 12-6). Adventitial reticular cells reside on the adluminal surface of venous endothelial cells, which branch through the medullary cavity. [65] These cells appear to provide a reticular network that supports developing blood cells. In addition, both adventitial reticular cells and adipocytic cells play an active role in hematopoiesis by producing both soluble and membrane-associated growth factors. [66] These cells also respond to hematopoietic stress by changing volume; impaired hematopoiesis is associated with increased accumulation of fat inclusions in both cell types, and accelerated hematopoiesis is associated with loss of fat vacuoles and the provision of increased space for hematopoietic cells. [67] Adipocytes may play an additional role in blood cell production as a reservoir for lipids needed in cell metabolism during proliferation. Macrophages and osteoclasts, cells derived from hematopoietic precursor cells and osteoblasts, may also play important roles in the HM. The organization of the HM in situ is detailed in Chapter 13 .

The HM provides more than structural support for hematopoietic cells during proliferation and differentiation. Studies by Wolf and Trentin [4] show the effects of specific microenvironments on stem cell differentiation. Femoral shafts placed in the spleen pulp of mice give rise to hematopoietic colonies located at the junction of the spleen and femur tissues. Differentiation of cells derived from a single stem cell at this junction occurred in a lineage-specific manner according to the geographic distribution of the colony. Myeloid cells developed in the part of the stem cell-derived colony arising in the medullary cavity, while erythroid cells developed in the part of the colony arising in the splenic environment. These and similar observations led to the theory that the HM in which stem and progenitor cells reside influences the commitment process, termed the hematopoietic inductive microenvironment.

Dexter et al.[5] described a culture system in which the HM is replicated in tissue culture flasks. These cultures (Dexter cultures or long-term marrow cultures) allow survival and proliferation of primitive cells for extended periods in vitro. Modifications of the cultures allow support of lineages restricted to myeloid, erythroid, [68] [69] or lymphoid (termed Whitlock-Witte cultures) [70] cells. Cells produced in such cultures can be collected and used for bone marrow transplantation. There is some evidence that CML and acute myeloid leukemia (AML) stem or progenitor cells survive less well in such cultures than phenotypically normal cells. [71] [72] These observations have led to the use of long-term marrow cultures for experimental autologous transplants in CML and AML patients. Some patients treated with these protocols have re-established normal hematopoiesis, although it appears unlikely that long-term sustained remissions can be obtained using this approach.

The HM provides support for hematopoietic stem cell proliferation and differentiation both in vivo and in vitro. At least two hematopoietic growth factors have been shown to be important in vivo in the normal function of the HM. Mice deficient in steel factor (homozygous Sl/Sl mice [see discussion earlier in this chapter]) and LIF (LIF-deficient mice generated by gene targeting) [33] have hematopoietic deficiencies as a result of the lack of presentation by the HM of each protein. Sl/Sl ^{d} mice (from a viable mutant allele of Sl) have deficient CFU-S stem cells and low peripheral blood counts. [56] [73] LIF-deficient mice have normal peripheral blood counts but are deficient in CFU-S and committed progenitor cells. The bone marrow of both animals

Figure 12-6 Hematopoietic nest arising in long-term marrow (see Dexter et al. [5]) culture. Round refractile cells in the middle represent area of active hematopoiesis within the stromal hematopoietic microenvironment of the adherent layer.

is capable of reconstituting normal hematopoiesis when transferred by bone marrow transplantation into a normal HM.

Several theories concerning the physiologic roles of HM in the regulation of hematopoiesis have been proposed and are under investigation (Table 12-3). The HM provides adhesive interactions important for co-localization of stem and progenitor cells and growth regulatory proteins within the medullary cavity. Long [74] has suggested the categories of cell-cell, cell-matrix, and cell-growth factor for these interactions. Important matrix adhesive molecules include fibronectin, thrombospondin, glycosaminoglycans, and proteoglycans. Integral membrane proteins important in these interactions include haemonectin and vascular cell adhesion molecule-1. Pluripotent hematopoietic stem cells (CFU-S_{12} , reconstituting murine hematopoietic stem cells, and CD34+ human bone marrow cells) have been demonstrated to adhere to the CS-1 sequence in the alternatively spliced IIICS region of the extracellular matrix protein fibronectin. [75] [76] Other investigators have demonstrated adhesion of primitive hematopoietic cells to the matrix protein thrombospondin and the high-affinity heparin binding site in the C-terminal sequence of fibronectin.[77] [78] The receptor for the fibronectin CS-1 sequence is the integrin VLA-4, which has been shown to be expressed on primitive hematopoietic cells. The thrombospondin receptor is expressed on multilineage human progenitor cells.

Adhesion of primitive cells to the HM via lectins has also

TABLE 12-3 -- Possible Roles of Hematopoietic Microenvironment in Hematopoiesis

Direct communication via tight cell-to-cell contact
Stabilization of growth factors via binding to extracellular matrix molecules or membrane proteins
Production of both positive and negative regulators of hematopoiesis
Co-localization of growth factors and hematopoietic cells in a local area network, allowing receptor modulation by small quantities of cytokines
Outside-in signaling through adhesion receptors

From Williams[32] with permission.

been demonstrated. Aizawa and Tavossoli,[79] using sugar-modified bovine serum albumin, have demonstrated inhibition of adhesion of CFU-S to stromal cells in vitro and inhibition of homing in vivo. The specificity of this interaction is via unknown core proteins, but appears to be related to galactose or mannose sugar residues (or both). CD44 expression has also been detected on early hematopoietic progenitor cells. [80]

Both myeloid and erythroid differentiation are accompanied by changes in adhesive interactions. Patel and colleagues [81] [82] have demonstrated that erythroid differentiation is accompanied by increased adhesion to the RGDS-containing central cell-binding domain of fibronectin via the VLA-5 receptor. Loss of adhesion to RGDS is temporally related to enucleation and terminal differentiation, and increased efficiency of enucleation of some erythroid cell lines has been demonstrated on fibronectin in vitro. Adhesion to the protein haemonectin by myeloid progenitor and precursor cells has been demonstrated. [83] Loss of adhesion to this protein has been postulated to be involved in maturing granulocyte egress from the marrow.

The HM may also provide binding sites for hematopoietic growth factors. Proteoglycans and glycosaminoglycans are produced by several cells in the HM. [84] Both GM-CSF and IL-3 have been shown to bind noncovalently to glycosaminoglycans in the extracellular matrix of bone marrow. [85] [86] Co-localization of hematopoietic cells with locally high concentrations of growth factors bound in this fashion may be one way in which local area networks are established throughout the medullary cavity.[87] Multiple other adhesive interactions have been studied and are important in lymphocyte homing and leukocyte trafficking in the periphery. [88] These interactions utilize receptors in the immunoglobulin superfamily, integrins, selectin/LEC-CAM, and CD44. Many hematopoietic cell/stromal cell adhesive interactions have been described with receptor/ligand pairs of unknown identity. [89]

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PRECURSOR CELL DIFFERENTIATION

Most cells present in the bone marrow of a healthy individual are recognizable precursor cells of the myeloid or erythroid lineage. The ratio of myeloid/erythroid precursors (M/E) is normally

3:1, with approximately 10% of nucleated cells recognizable as lymphocytes, plasma cells, macrophages and rare morphologically indistinct blasts, progenitor cells, and stem cells. Megakaryocytes are present at a frequency of 45/1,000 nucleated bone marrow cells. Alterations in the M/E can be determined by staining bone marrow aspirates and are often useful in evaluating peripheral cytopenias. However, absolute cellularity is difficult to determine by examination of aspirate samples and is best determined by bone marrow biopsy. A relative lymphocytosis and variation in the M/E ratio are normal developmental differences seen in the bone marrow of infants and young children and make the interpretation of these bone marrow samples more difficult.

As seen on standard Wright-Giemsa staining at the light microscope level, the normal differentiation of erythrocytes occurs in well-defined stages. The first identifiable and also largest precursor of the erythroid lineage is the erythroblast (Plate 12-2). The cell diameter is 1219 m, and the nucleus is large, oval and homogeneously staining (violet) with indistinct nucleoli. The cytoplasm is darkly basophilic, with lighter staining areas (hyaloplasm) usually near the nucleus that reflect the position of the Golgi apparatus and lipid-containing mitochondria. Maturation to the basophilic normoblast is accompanied by reduction in cell size and pronounced changes in the nuclear chromatin structure (Plate 12-3). The basophilic normoblast is 1217 m in diameter, with basophilic cytoplasm, and the nuclear chromatin shows a coarsening and prominent clumping leading to descriptions of a spoked wheel or cartwheel appearance. Nucleoli are generally not seen. The polychromatic normoblast is nearly the same size as the basophilic normoblast (Plate 12-4). The accumulation of hemoglobin is now seen by the presence of less basophilic and muddy gray-colored cytoplasm. The nucleus shows further condensation and is nearly black. The last nucleated red cell precursor is the orthochromatic normoblast (Plate 12-3). The cell now approaches the diameter of a reticulocyte (812 m), and the eosinophilic staining cytoplasm contains nearly a full amount of hemoglobin. The nucleus is now fully condensed and pyknotic.

Extrusion of the nucleus results in the reticulocyte, a cell slightly larger than a fully mature erythrocyte. The reticulocyte is characterized by the presence of a fine granular or reticular network of ribosomal RNA observed with supravital stains such as cresyl violet or methylene blue. Such cells are present at low frequency in the peripheral blood of normal individuals but are increased in response to stress on the erythroid lineage, such as hemolysis or blood loss. Stress reticulocytes are prematurely released into the peripheral blood, where further maturation is completed. Characteristics of stress reticulocytes include a larger cell diameter and more basophilic cytoplasm than reticulocytes.

Additional morphologic indications of stress erythropoiesis include polychromatophilia (violet tinting seen with Wright-Giemsa staining) and basophilia (blue or black stippling diffusely distributed throughout the cell). These staining characteristics are the result of RNA remnants that stain in the cytoplasm of the erythrocyte. The premature destruction of erythroid precursors in the medullary cavity, termed ineffective erythropoiesis, is accompanied by elevation of serum lactate dehydrogenase, decreased levels of serum haptoglobin, slight elevation of the reticulocyte count (1.54%), and the occasional appearance of nucleated red blood cells in the peripheral blood. The destruction of erythrocyte precursors in the medullary cavity normally occurs in <10% of developing erythrocytes. Increased destruction of erythrocyte precursors accompanies hemoglobinopathies, iron deficiency anemia, megaloblastic anemias, and other rarer congenital anemias, such as congenital dyserythropoietic anemias.

The final stage of erythroid maturation is the erythrocyte. The cell is a biconcave, relatively flat, non-nucleated disk 78 m in diameter. Normal erythrocyte survival is 100120 days in humans.

Leukocytes that circulate in the peripheral blood are divided into those of the myeloid, monocyte/macrophage, and lymphocytic lineages. The differentiation and function of the lymphocyte lineage is discussed elsewhere (see Chaps. 7 , 8 , and 9). In myeloid and monocyte lineages, four distinct granule populations are seen that distinguish these cells morphologically during differentiation. Azurophilic granules are present in cells of both lineages, stain pink with Romanowsky dyes, and contain myeloperoxidase, acid phosphatase, basic cationic protein, and other hydrolases. Eosinophilic granules are a conspicuous reddish orange, and basophilic granules are intensely blue. Specific neutrophilic granules do not stain intensely with standard Wright-Giemsa stains.

The earliest identifiable cell of the myeloid lineage is the myeloblast (Fig. 12-7). The cell is approximately 1214 m in diameter,

Figure 12-7 Myeloblast transition to promyelocyte. Neutrophil below.

Figure 12-8 Promyelocyte in the middle. Eosinophilic myelocyte on the left.

with a round or oval nucleus and basophilic cytoplasm that lacks granules. Nuclear chromatin is fine, and one to five nucleoli are easily visible. The nucleus often stains reddish. Promyelocytes are the largest and most frequent of the primitive myeloid precursors (Fig. 12-8). Promyelocytes are variable in nuclear shape, with less prominent nucleoli, and chromatin of medium density that is coarser than that of the myeloblast. The cytoplasm is deeply basophilic and contains variable numbers of peroxidase-positive granules. These granules can overlie the nucleus and can vary in color from deep red to blue; they distinguish the promyelocyte from the myeloblast.

Myelocytes are characterized by round-to-oval nuclei with characteristic nuclear indentations, indistinct nucleoli, and unevenly stained, coarse chromatin structure (Fig. 12-9). The cytoplasm stains pale gray-brown or pink-brown, with numerous specific granules covering the nucleus and throughout the cytoplasm except in a clear area near the nuclear indentation (centrosphere), which represents the Golgi apparatus. The myelocyte is also characterized by the first appearance of specific granules and is the last cell capable of cell division during myeloid differentiation. The metamyelocyte exhibits a characteristic bean-shaped nucleus, the band neutrophil a horseshoe- or S-shaped nuclear structure without recognizable nuclear constrictions, and the segmented neutrophil is characterized by the typical

nuclear segmentations for which it is named, which divide the nucleus into two to five lobes (Fig. 12-9). These myeloid forms exhibit cytoplasmic characteristics similar to the those of the myelocyte without the centrosphere and with progressive reduction in the overall cell size. An additional nuclear lobule, termed a drumstick, is seen in 1012% of neutrophils of females.

Maturation of the eosinophil and basophil follows the same morphologic steps as the neutrophil until the appearance of

Figure 12-9 Myeloid maturation. Myelocytes, band, and segmented neutrophils.

Figure 12-10 Eosinophilic myelocyte (left) and basophil (right).

specific granules (Fig. 12-10). The eosinophil exhibits a bilobed nucleus, and the basophilic cytoplasm is filled with prominent orange-red granules (Fig. 12-11). Basophils exhibit less segmentation of the nucleus and cytoplasm that is sparsely filled with deeply staining blue-to-purple metachromatic granules (Fig. 12-10). The granules often obscure the nucleus and cytoplasm, which stains pink to reddish pink and frequently exhibits vacuoles. Frequencies in the peripheral blood of normal individuals are 04% and 00.5%, respectively, for eosinophils and basophils.

The earliest cell of the monocyte/macrophage lineage is difficult to distinguish from the myeloblast. The cell is 1218 m in diameter, with a round or oval nucleus that is frequently convoluted. The chromatin is fine, and nucleoli are sometimes present. The cytoplasm is basophilic, with a grayish cast and, although devoid of granules, may contain vacuoles. Blunt pseudopodia are sometimes present. The naphthol-AS-acetate esterase reaction is positive and is inhibited by fluoride. The promonocyte is a large cell with an indented nucleus exhibiting fine chromatin and a single nucleolus (Fig. 12-12). The cytoplasm stains light blue with azurophilic granules and a small centrosphere. The monocyte is the largest cell present in the peripheral blood and measures 1320 m in diameter (Fig. 12-13). The nucleus is large, lobulated, or bean-shaped, with coarse nuclear chromatin; it lacks nucleoli and is often eccentric in location. The cytoplasm stains a characteristic smoke-blue with azurophilic granules. The chromatin structure of the monocyte is less clumped and the cytoplasm grayer than the lymphocyte, characteristics that (along with nuclear convolutions) help to distinguish these cells from moderate-size lymphocytes in the peripheral blood. Other helpful distinguishing characteristics are blunt pseudopodia. Monocytes usually make

Figure 12-11 Mature, bilobed eosinophils (middle and right).

Figure 12-12 Promonocyte in peripheral blood.

up <510% of the peripheral leukocytes. Egress of monocytes from the blood into tissues is associated with increased cell size and larger, more lightly staining nucleus. These cells, termed macrophages or histiocytes, are phagocytic and mobile, and intracellular debris is often present.

Morphologic differentiation of platelet-forming cells is distinctly different from either erythroid or leukocyte cell lineages. Distinct morphologic maturational steps and divisions do not take place; these processes are instead replaced by the process of polyploidization. In the process, successive nuclear divisions without concomitant cytoplasmic divisions lead to megakaryocytes with 132 nuclei representing diploid to 64N nuclear content. The first identifiable cell in this lineage is the megakaryoblast, a distinctly large cell with a high nuclear/cytoplasmic ratio. The nucleus shows variable chromatin coarseness and may contain nucleoli. The nuclear shape varies, but the nucleus frequently displays convolutions or deep furrows. The cytoplasm stains basophilic, does not contain granules, and may exhibit fraying at the cytoplasmic membrane. Some megakaryoblasts may contain multiple nuclei. The promegakaryocyte exhibits a lobulated nuclear structure without nucleoli and with a coarse chromatin structure. The cytoplasm is basophilic, with azurophilic granules adjacent to the nucleus, and formed platelets are seen on the cytoplasmic cell surface.

Occasionally the presence of other cells apparently within the cytoplasm (emperipolesis) of the promegakaryocyte is noted, although the mechanism leading to this occurrence is unknown. Mature megakaryocytes are the largest hematopoietic cell in the bone marrow (Fig. 12-14). The nucleus is lobulated and exhibits coarse and clumped chromatin. Nuclear number varies from eight diploid nuclei (65% of marrow megakaryocytes) to four nuclei (10% of marrow megakaryocytes). The cytoplasm is basophilic, with numerous azurophilic granules. Platelets can be seen at the periphery of the cytoplasm

Figure 12-13 Monocyte with band neutrophil (above).

Figure 12-14 Megakaryocytes.

and are attached to the cell membrane. Megakaryocytes constitute 0.10.5% of cells in the medullary cavity of normal individuals.

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SUMMARY

The formation of blood cells represents a complex interaction between stem cells, cells making up the HM, and growth regulatory proteins, which are presented in soluble and localized forms. These interactions give rise to an enormous number and diversity of cells that function in widely separated parts of the body to transport oxygen, defend against infectious agents, and provide a stimulus for clotting. Abnormalities that affect this process can lead to life-threatening illnesses such as aplastic anemia and myeloproliferative diseases. In addition to contributing to our understanding of the basic pathophysiology of these diseases, research in hematopoiesis is providing new therapeutic tools, such as growth factors, which are being used in treatment of both congenital and induced hypoplastic conditions of the bone marrow.

ACKNOWLEDGMENT

Special thanks to Atillio Orazi, MD, Department of Pathology, Indiana University School of Medicine, Indiana University Medical Center, Indianapolis, Indiana.

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Chapter 13 - Anatomy and Physiology of Hematopoiesis

Catherine M. Verfaillie

INTRODUCTION

Hematopoiesis is a complex process in which hematopoietic stem cells (HSC) can self-replicate but also differentiate into myeloid and lymphoid lineage committed hematopoietic progenitor cells (HPC). The committed HPC undergo further multiplications before they terminally differentiate into mature blood elements. The process of myelopoiesis and lymphopoiesis occurs in close proximity with a permissive microenvironment. In adult life the hematopoietic microenvironment is provided in the bone marrow (BM). Earlier in ontogeny, hematopoiesis takes place in the aorta-gonadal-mesonephros (AGM) region, yolk sac, and fetal liver. The hematopoietic microenvironment contains stromal cells of mesenchymal origin, such as endothelial cells, fibroblasts, adipocytes, and osteoblasts, and cells of nonmesenchymal origin, including macrophages. These stromal cells produce and deposit a complex extracellular matrix (ECM) and produce and concentrate locally hematopoietic cytokines that can induce or inhibit progenitor proliferation and differentiation. Hematopoietic cells interact through specific cell surface receptors with either immobilized or secreted cytokines and with adhesive ligands present on stromal cells or ECM components, interactions that underlie the normal hematopoietic process. Once HPC differentiate into mature blood elements, they egress from the BM in the blood or secondary hematopoietic organs, a process that is still not well understood. This chapter focuses on the ontogeny of hematopoietic tissues and the interactions between hematopoietic elements and the adult marrow microenvironment that underlie the normal hematopoietic process. Mechanisms that underlie egress of mature as well as more primitive progenitors from the BM, known as mobilization, and the mechanisms underlying the homing and engraftment of HPC and HSC into the BM will also be discussed.

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ONTOGENY OF HEMATOPOIETIC TISSUES

Until recently it was thought that hematopoiesis originated solely from the extra-embryonic mesoderm in the yolk sac (Fig. 13-1).[1] It was believed that HSC originating in the yolk sac colonize sequentially the fetal liver, thymus, and BM, providing definitive hematopoiesis in embryonic, fetal, and adult life. Data on mice indicate, however, that HSC can also be found in the AGM region and may arise from hemangioblastic cells that can differentiate into both HSC and endothelial cells.[2] [3] [4] [5] The analogous area in birds is responsible for the development of adult hematopoiesis. In the mouse, HSC can be detected around the dorsal aorta and in the urogenital ridges as early as days 9 and 10 of development, possibly before the development of HSC in the yolk. When serially transplanted in adult conditioned recipients, cells from the AGM region can restore long-term multilineage hematopoiesis. This has led to the notion that HSC arising in the AGM region are responsible for the colonization of fetal liver, thymus, and BM and should therefore be considered stem cells that give rise to definitive hematopoiesis in adult life. Some studies suggest that HSC found in the yolk sac may not contribute significantly to adult hematopoiesis. Other studies demonstrate, however, that transplantation of HSC recovered from the yolk sac on days 9 and 10 of development into newborn conditioned recipients can also reestablish hematopoiesis. [6] [7] If HSC from the AGM region colonize the yolk sac or vice versa, and are therefore derived from the same stem cell pool, is not known. Additional studies will therefore be needed to clarify the contribution of HSC found in the AGM region and the yolk sac to the establishment of embryonic, fetal, and adult hematopoiesis.

A similar population of $CD34^+$ cells has been identified in the AGM region in human embryos at 3037 days of gestation. [8] In humans, HSC colonize the fetal liver at 5 weeks of gestation.[1] Fetal liver hematopoiesis becomes active at 6 weeks and the liver remains the dominant hematopoietic organ throughout fetal life. From the fetal liver, HSC seed the BM at 8 weeks of gestation. In contrast to the fetal liver where hematopoiesis is predominantly erythroid, hematopoiesis occurring in the fetal BM is mainly myeloid in nature and contributes only minimally to the blood pool throughout fetal life. HSC colonize the spleen at 12 weeks and the thymic rudiment at 8 weeks. B-cell development first begins in the fetal liver at 7 weeks of gestation and shifts to the BM at 1214 weeks. Liver stem cells differentiate in the thymus into mature T lymphocytes which populate fetal lymph nodes, spleen, and gut by the 12th week of gestation and other peripheral lymphoid tissues by week 1415 of gestation. Finally, NK cell activity has been reported in human fetal liver cells at 812 weeks of gestation, with $CD56^+$ cells first seen in fetal liver at 6 weeks.

Figure 13-1 Ontogeny of human hemopoiesis. The hemopoietic stem cell is believed to arise within the aorto-gonadal-mesonephros region and/or extra-embryonic yolk sac. After 5 weeks of gestation, hemopoietic stem cells colonize the liver from which they migrate to the bone marrow at 8 weeks, thymus at 8 weeks, and spleen at 12 weeks. During fetal life, the liver is the chief organ responsible for production of myeloid and erythroid cells. B-lymphopoiesis initially takes place in the liver, but shifts to the bone marrow after 12 weeks of gestation. T-lymphopoiesis starts in the thymus around week 10. After birth, the main hemopoietic organ becomes the BM.

During fetal life and immediately after birth a large number of HPC and HSC can also be found in the circulation. [9] However, within 2448 hours after birth, circulating progenitors disappear from the circulation, presumably because of lodgment in the BM cavity. In the adult, hematopoiesis takes place chiefly in the BM.

The reasons for the migratory behavior or the mechanisms underlying the localization, migration, and homing in different organs of HSC throughout embryonic and fetal life are not well understood. Changes in sites of hematopoiesis may be due to differences in the adhesive behavior of HSC and HPC at different stages of ontogenic differentiation, enabling them to interact with a specific hematopoietic microenvironment. Alternatively, differences in the capacity of the different hematopoietic organs to support the development of progenitors of different ontogenic ages may underlie the sequential establishment of hematopoiesis in AGM and yolk sac, liver, and BM. In vivo and in vitro biological evidence supports both hypotheses. Transplantation of fetal liver HSC in fetal sheep results in homing to the fetal liver but not BM if the transplants are performed before day 60 of gestation when the liver is the only hematopoietic organ. [10] By contrast, when fetal liver HSC are transplanted beyond day 80 of gestation, at which stage endogenous HSC have colonized the BM, exclusive homing to the BM is observed rather than homing to the fetal liver. Interestingly, although fetal liver HSC engraft in the fetal BM, appearance of their mature progeny in the blood does not occur until the perinatal period when BM hematopoiesis becomes active. The nature of receptors expressed on stem cells at different stages of ontogeny that govern their ability to interact with, for instance, the liver but not the BM microenvironment, is still unknown. Studies done with HSC derived from 1-integrin-deficient embryonic stem cells have demonstrated that 1-deficient HSC cannot colonize the fetal liver hematopoiesis even though these cells can differentiate in vivo in erythroid, myeloid, and B-lymphoid progeny, suggesting an important role for 1-integrins in the migratory behavior of embryonic and fetal HSC. [11] Supporting the hypothesis that supportive capacity differs among various hematopoietic organs is the observation that transplantation of day 10 murine yolk sac HSC in conditioned adult recipients do not engraft, whereas the same yolk sac HSC can repopulate the hematopoietic system of newborn mice in which the liver and spleen, like the BM, function as a site for hematopoiesis.[7] In vitro studies have shown that factors secreted by adult BM stroma inhibit growth of fetal liver erythroid progenitors. [12] These studies strongly suggest that homing and development of HSC may not occur when they are transferred to an ontogenically more mature microenvironment. These observations have important implications for the use of fetal liver progenitors for transplantation into adult recipients.

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COMPONENTS OF THE BONE MARROW MICROENVIRONMENT

Stromal Cells

The BM microenvironment is a complex organ in which stromal cells are responsible for providing most, if not all, factors required for the orderly development of the HSC (Fig. 13-2). Stromal cells are both mesenchymal and hematopoietic in origin, and include osteoblasts, fibroblasts, adipocytes, myocytes, endothelial cells, dendritic cells, and macrophages.[13] [14] When hematopoietic BM is examined in vivo, hematopoietic cells of different stages of differentiation and lineage commitment can be found in distinct areas throughout the bone marrow space. For instance, cells with an immature morphology can be found lining the subendosteal region in close proximity with osteoblasts.[15] [16] More differentiated progenitors and precursors of the

Figure 13-2 Bone marrow microenvironment. The BM microenvironment is composed of stromal cells (1) that are of mesenchy-mal (adipocyte, fibroblast, and endothelial cell, and osteoblast) and hemopoietic (macrophages) origin. Stromal cells produce both soluble and membrane-bound cytokines (2) as well as ECM components (3). Adhesion receptors (5) on hemopoietic stem cell and progenitor cells (4) support interaction with stromal adhesive ligands and cytokine receptors allow interaction with bound or soluble cytokines. Interactions between hemopoietic stem and progenitor cells and stromal cells, ECM components, and cytokines are responsible for the ordered production of mature blood elements.

myeloid, erythroid, and megakaryocytic lineage are located throughout the marrow. The apparent association of lineage-specific progenitors and their more differentiated precursors in islands suggests that lineage-specific differentiation may depend on specialized progenitor-stromal cell interactions. This has led to the concept of stem cell/progenitor niches.[17] [18] Such niches would consist of specialized stromal cells that produce extracellular matrix components and hematopoietic supportive cytokines that are conducive for the commitment and/or differentiation of progenitor cells at a specific stage of differentiation (Fig. 13-3).

Characterization of the role of the BM microenvironment in the hematopoietic process became possible with the development of stroma-dependent cultures, initially developed by Dexter and coworkers, also termed long-term marrow cultures (LTC).[19] [20] In LTC, human or mouse BM is cultured in serum-containing medium in plastic culture vessels. The stromal cells establish an adherent feeder layer, and HSC and HPC in the inoculum proliferate and differentiate in the stromal feeder for weeks to months in the absence of exogenous factors.[21] More primitive progenitors, termed long-term culture initiating cells (LTC-IC) or cobblestone area forming cells (CAFC)[22] [23] can be found between or under the stromal cells and have an appearance of cobblestones when evaluated by phase contrast microscopy. Because LTC-IC persist for long periods of time in culture and can re-initiate secondary LTC, they are closely related to HSC. [24] [25] More differentiated progeny, including colony-forming cells (CFC) and more mature precursors, are present in or suspended above the stromal layer. In the murine system, long-term repopulating HSC survive weeks in such stroma-dependent LTC, indicating that examination of hematopoiesis in stroma-based cultures can provide insights on the role of stromal cells in hematopoiesis.

A number of investigators have cloned murine stromal cell

Figure 13-3 Stem cell and progenitor niches. Stromal cell lines that support growth of stem cells but not more mature progenitors have been described. [26] [27] Likewise, stromal cell lines that support myelopoiesis[26] [27] or B-lymphopoiesis,[30] but not more immature progenitors have been cloned. Progenitors at different stages of differentiation can interact with certain but not all ECM components and can be induced to proliferate or differentiate by some but not all cytokines. This has led to the concept of stem cell niches and progenitor niches which would consist of specialized stromal cells and their secreted cytokine and/or ECM product that interact with progenitors at specific stages of differentiation and of a specific hemopoietic lineage. These interactions would tightly regulate progenitor differentiation and proliferation. Depicted are two putative niches: for instance, primitive CD34 $^{+}$ cells adhere through 1-integrins to fibronectin or VCAM-1. Primitive progenitors can also interact with SCF, which is expressed by fibroblasts. The combined stem cellECMcytokine interactions regulate growth of hemopoietic stem cells. Alternatively, more mature myeloid progenitors and their precursors adhere, for instance, to hemonectin. These progenitor and precursors are stimulated to proliferate and differentiate by the single lineage cytokine, G-CSF. The combined action of myeloid progenitor specific ECM components and cytokines is thought to regulate proliferation and differentiation of this cell lineage.

lines from either fetal liver or adult BM. [26] [27] [28] [29] [30] [31] [32] [33] [34] Some of these cell lines support the ex vivo maintenance and expansion of multipotent HSC populations, while they support poorly more committed HPC.[26] [27] Other cell lines support myelopoiesis or B-cell development but are incapable of supporting survival and proliferation of more immature, multilineage progenitors.[27] The nature of the factors expressed or secreted by stromal cells capable of supporting multipotent or lineage restricted progenitors is still largely unknown. However, the availability of these cell lines provides a potent tool to begin characterizing factors required for self-renewal or lineage commitment of HSC, or factors necessary for the proliferation or differentiation of lineage-committed HPC.

Fibroblasts

The best-studied BM microenvironment stromal cell is the fibroblast. Numerous murine BM or fetal liver fibroblast cell lines have been cloned. [28] [29] [30] [31] In addition, SV-40 transformed fibroblast-like cell lines have been generated from murine or human BM or fetal liver. [26] [27] In general, primitive and to a lesser extent more committed HPC adhere to the fibroblast feeders.[35] [36] Progenitor adhesion occurs either to cell surface expressed ligands, such as vascular cell adhesion molecule (VCAM),[36] cell surface expressed growth factors, such as stem cell factor (SCF), [37] [38] or to ECM components secreted by the fibroblast. [35] Although the type and concentrations of cytokines produced by the different cloned fibroblast feeders does not differ significantly, [26] [27] [28] [29] [30] [31] [32] [33] [34] [39] [40] some but not all feeders support the primitive progenitor compartment whereas others support committed myeloid or lymphoid progenitors, and still others support hematopoiesis poorly or not at all. Further, fibroblasts cloned from nonhematopoietic organs express frequently the same types of cytokines as fibroblasts derived from hematopoietic organs. [39] [40] However, fibroblasts from nonhematopoietic tissues often do not support hematopoiesis. This indicates that aside from the known cytokines, other factors produced and/or secreted by BM- or fetal liver-derived fibroblasts are important for the support of hematopoiesis. As yet unidentified growth factors may underlie the differences in hematopoietic supportive ability of different fibroblast cell lines. Alternatively, differences in presentation or local concentration of cytokines, which are regulated by the ECM, may affect the ability of fibroblast cell lines to support hematopoiesis. As adhesive interactions per se may influence the growth and differentiation of HSC and HPC, differences in adhesive ligands on the fibroblasts themselves or in the cell ECM may be important determinants of their hematopoietic supportive capacity.

Endothelial Cells

In vivo, endothelial cells line the sinusoids of the bone marrow cavity. As in other tissues, entry to and exit from the BM requires that progenitors and mature blood cells pass through the endothelial barrier of the sinusoids. Because endothelial cells are an integral part of the hematopoietic microenvironment, they may also affect the growth of HSC and HPC. Purified BM endothelial cell (BMEC) feeders have been generated. [34] [41] [42] [43] Like fibroblasts, BMEC produce a number of cytokines known to support hematopoiesis. When CD34^{+} cells are co-cultured with BMEC, primitive LTC-IC are maintained and more committed HPC proliferate and

differentiate, indicating a role for BMEC in the regulation of progenitor proliferation and differentiation. [34] [42] $CD34^+$ cells adhere to endothelial cells. [41] Furthermore, $CD34^+$ cells can migrate through BMEC barriers, a process that depends on the interaction of 2-integrins on $CD34^+$ cells and ICAM-1 expressed on BMEC.[43] Endothelial cell feeders can also be generated from tissues other than BM, such as human umbilical vein endothelial cells (HUVEC). [41] $CD34^+$ cells adhere to and migrate through BMEC significantly better than to HUVEC.[44] HUVEC also support in vitro hematopoiesis significantly less than BMEC. Endothelial cells derived from hematopoietic organs, like fibroblasts, have as yet undefined unique characteristics that make them suitable for the support of hematopoiesis.

Osteoblasts

Until recently, little attention was given to the potential role of osteoblasts as stromal cells in the BM microenvironment. However, the simple fact that adult hematopoiesis is localized almost exclusively in the bone may well be because bone cells themselves provide specific signals for either homing of stem cells or for the development of adult HSC and HPC. In addition, the most primitive progenitors can be found in close proximity with the endosteal lining of the BM cavity. [15] [16] In vitro studies have shown that human $CD34^+$ cells adhere tightly to osteoblast feeders, even though the nature of the receptorligand pairs in this interaction is not yet known.[45] Osteoblasts produce cytokines, such as G-CSF, GM-CSF, and IL-6, known to support hematopoiesis. [45] [46] When human $CD34^+$ cells are cocultured with osteoblast feeders, primitive LTC-IC can be maintained and expanded to the same or greater extent than when cocultured with mixed BM stromal feeders. [45] [46] These studies thus suggest an important

role for the osteoblast component of the BM microenvironment in hematopoiesis.

Extracellular Matrix Components

Stromal cells produce cytokines and ECM components, both of which are required for the regulation of hematopoiesis. A number of ECM components can be found in BM-derived stromal feeders. The same ECM components can be detected in situ in BM biopsies. They include several types of collagen, laminin, fibronectin, thrombospondin, proteoglycans, and hemonectin. Although the exact role of ECM components in hematopoiesis is not known, they serve to localize HPC and HSC in the BM microenvironment. In addition, adhesion of HSC and HPC to the ECM may affect proliferation and differentiation of progenitors directly or may alter the response of progenitors to cytokines. Finally, ECM components, in particular proteoglycans, may play a role by concentrating, protecting, and presenting cytokines.

Fibronectin

Fibronectin is a large 450 kDa fibril-forming glycoprotein composed of two similar subunits joined by a pair of disulfide bonds at their carboxyl termini and folded in a series of globular domains separated by regions of flexible polypeptide chains. [47] Alternative splicing of the mRNA results in a variety of fibronectin isoforms in which certain cell adhesion promoting domains can be present or absent. Fibronectin is found in almost all tissues. Various domains of the fibronectin molecule that interact with different cell surface receptors have been identified. A 75120 kDa proteolytic fragment in the center of the fibronectin molecule contains the sequence arginyl-glycyl-aspartyl-serine (RGDS). [48] The 51 integrin mediates interaction of different cell types with this RGDS-containing fragment of fibronectin. Adhesion to fibronectin can also occur in an RGDS-independent fashion via the 33/66 kDa heparin-binding fragment at the COOH-terminal end of the fibronectin molecule. Several RGDS-independent cell-adhesion promoting peptides have been identified within the 33/66 kDa COOH-terminal fragment of fibronectin. These include CS1, to which the 41 integrin binds,[49] and a number of heparin-binding peptides to which cells adhere through cell surface heparin-like molecules. [50] It has long been established that adhesion of cells to fibronectin serves to localize cells in a specific microenvironment. It has also become clear that adhesion to fibronectin can influence the growth of cells. Aside from serving a permissive role in up-regulating the sensitivity of cells to soluble cytokines, the adhesive interaction itself between cells and fibronectin may affect cell survival and proliferation. [51] [52]

Fibronectin is abundantly present in normal adult BM [53] where it is produced by endothelial cells and fibroblasts. Fibronectin may play an important role in homing of HSC and affect proliferation and differentiation of HSC and HPC. In vivo and in vitro studies have shown the importance of the CS1-binding 41-integrin in mobilization and homing of HSC/HPC, which will be discussed further in the following sections. [36] [54] [55] Further, engagement of 41- and 51-integrins affects progenitor proliferation and survival[52] [56] [57] This demonstrates that adhesion of HPC to fibronectin may play an important role in the growth regulation of these progenitors. Interaction with fibronectin is also required for the terminal differentiation of erythroid progenitors [51] [58] and for the survival and differentiation of B-lymphoid progenitors. [59] Because fibronectin is universally present, specificity and selectivity for the adhesion of different cell types or the same cells at different stages of maturation to fibronectin is provided by tissue-specific expression of various isoforms of fibronectin, [47] [60] and by differential expression of the multiple fibronectin cell surface receptors. [48] [49] [50]

Thrombospondin

Thrombospondin is a 450 kDa glycoprotein produced by platelets, endothelial cells, and fibroblasts. [61] As for fibronectin, several thrombospondin domains have been identified that bind to other ECM components, such as glycosaminoglycans, fibrinogen, or cell-surface receptors. The molecular basis of the thrombospondin-cell interaction is complex. Cell-surface proteoglycans, 41-integrins, av3-integrins, and CD36 bind to different sites in thrombospondin. [62] [63] [64] Thrombospondin is abundantly present in the BM microenvironment localized in megakaryocytes, fibroblasts, and the ECM associated with active hematopoiesis. [65] Thrombospondin serves as a ligand for committed progenitors[66] and adhesion of HPC to thrombospondin may constitute a signal that modulates the response of progenitors to cytokines. Co-culture of CFC with immobilized SCF and thrombospondin amplifies the response of CFC to soluble cytokines such as IL3 and GM-CSF. [67]

Proteoglycans and Glycosaminoglycans

Like most tissues, the BM microenvironment is rich in proteoglycans. These include heparan, dermatan, and chondroitin sulfate proteoglycans and hyaluronic acid. [68] Proteoglycans consist of a core protein to which one or more glycosaminoglycans (GAGs) are attached. GAGs are long, negatively charged, unbranched polysaccharide chains composed of repeating sulfated disaccharide units. [69] The type of sugar residues, the type of linkage between these residues, and the number and location of sulfate groups are important for the interaction of GAGs with ECM components, cells, and growth factors, and provide specificity. Heparan-sulfate GAGs secreted by hematopoietic supportive stromal cell lines play an important role in the maintenance and expansion of LTC-IC. [40] [70] Characteristics associated with the hematopoietic supportive nature of heparan-sulfate GAGs include a high degree of sulfation, localization of the sulfate groups (6-O rather than N-sulfation), and length of the GAG-chains.[71] BM-specific heparan-sulfate GAGs allow adhesion of $CD34^+$ cells.[72] The receptors on human hematopoietic progenitors responsible for their interaction with BM-derived heparan sulfate GAGs have not yet been identified. In addition, BM-specific heparan-sulfate GAGs can concentrate hematopoietic growth factors, such as IL3, GM-CSF, b-FGF, and MIP-1.[71] [73] [74] [75] The ability of BM-specific heparan-sulfate GAGs to selectively colocalize certain growth factors and HPC at a specific stage of differentiation may be crucial for the regulation of proliferation and differentiation. Heparan-sulfate GAGs can therefore be seen as the orchestrators of the putative HPC or HSC niche (Fig. 13-4).

Besides heparan-sulfate GAGs, chondroitin-sulfate GAGs are important in the hematopoietic process. When -d-xyloside, an agent that uncouples chondroitin sulfate glycosaminoglycan synthesis from proteoglycan synthesis, is added to LTC, a significant increase in chondroitin/dermatan sulfate GAGs is seen in stromal supernatants.[76] Hematopoiesis in -d-xyloside treated LTC is significantly increased, suggesting a role for chondroitin sulfate in hematopoiesis. Finally, as will be discussed later $CD34^+$ cells can adhere to hyaluronic acid in BM ECM through the CD44 receptor. [77] [78] Engagement of the CD44 receptor by hyaluronic acid may result not only in adhesion of HPC but may also affect their growth. [79]

Collagens and Laminin

Collagens are fibrous proteins found in the extracellular space. [80] The most common types are collagens type I, II, III, and IV. Collagen types IIII are assembled in collagen fibers and constitute the structural backbone of the extracellular space, seen as reticulin fibers on BM biopsies. [81] [82] Collagen type IV, in contrast,

Figure 13-4 Glycosaminoglycans are orchestrators of progenitor niches. Proteoglycans are common to all tissues. The glycosaminoglycan (GAG) side chains of proteoglycans have multiple functions: They support adhesion of cells, they bind to other ECM components, and they allow binding of cytokines. The type of sugar residues, the type of linkage between these residues, and the number and location of sulfate groups provide specificity to these interactions. Heparan-sulfate GAGs secreted by hemopoietic supportive stromal cell lines play an important role in the maintenance and expansion of LTC-IC.[40] These heparan-sulfate GAGs allow adhesion of $CD34^+$ cells.[72] In addition, BM-specific heparan-sulfate GAGs can concentrate hemopoietic growth factors such as IL-3, GM-CSF, and MIP-1 [71] [73] [74] [75] and can bind to ECM components, including thrombospondin (TSP) and fibronectin. The ability of BM-specific heparan-sulfate GAGs to selectively colocalize certain growth factors, other ECM components, and progenitors at a specific stage of differentiation may be crucial for the regulation of proliferation and differentiation of these progenitors. Heparan-sulfate GAGs can therefore be seen as the orchestrators of the putative progenitor or stem cell niche.

is not assembled in large fibrils but into a sheetlike mesh that constitutes a major part of basal membranes. [83] *Laminin* is an 850 kDa complex of three very long polypeptide chains arranged in the shape of a cross held together by disulfide bonds. Like fibronectin, it consists of a number of functional domains that can bind collagen type IV (basal membranes), proteoglycans, and cell surface receptors. [83] A specific role for collagen or laminin in the process of localization, proliferation, or differentiation of human hematopoietic progenitors in the bone marrow microenvironment has, however, not been defined. Egress of mature (and sometimes immature) hematopoietic cells from the BM microenvironment into the bloodstream requires passage of these cells through the basal membranes of endothelium. [84] 21- and 61-integrins present on granulocytes allow them to bind to and migrate through basal membranes. [85] [86] 21- and 61-integrins are not present on normal HPC and HSC. Of note, certain leukemic $CD34^+$ cells, in contrast to their normal counterparts, express 21- and 61-integrins, [85] [86] which may enable them to egress from the BM at an undifferentiated stage.

Hemonectin

Hemonectin is a 60 kDa extracellular matrix protein found exclusively in the BM microenvironment. [87] [88] It is believed that hemonectin preferentially supports binding of myeloid progenitors. Hemonectin is detected in fetal liver and fetal BM immediately before the onset of granulocytopoiesis. [89] The molecular structure of hemonectin and the cell-surface receptors responsible for the interaction of granulocytic progenitors with hemonectin have not yet been identified. The exact role of hemonectin in hematopoiesis is therefore still unknown.

Cytokines

The role of cytokines in hematopoiesis is discussed at length in Chapter 14 . However, a few concepts are discussed here in order to describe the regulation of hematopoiesis by the BM microenvironment.

First, almost all known cytokines are produced by cells present in the BM microenvironment. Low concentrations of most cytokines can be detected in supernatants of stromal feeders.[39] [40] [90] In vitro and in the absence of stromal cells, nanogram concentrations of cytokines are required to induce progenitor proliferation. How then do the picogram concentrations of cytokines found in stromal cultures (and in serum in vivo) affect progenitor growth? One possibility is that the combination of several different growth factors in small concentrations may induce progenitor proliferation and differentiation. [90] [91] In addition, growth factors can be bound and concentrated by ECM components, such as glycosaminoglycans.[71] [73] [74] [75] Therefore, the concentration of cytokines in local microenvironments may actually be significantly higher than that found in the circulation or in culture supernatants. There is also evidence that interaction with these ECM components may present cytokines in a biologically more active form to hematopoietic progenitors. [67] [74] Aside from secreted cytokines, stromal cells also produce cytokines that are presented in a membrane-bound form[38] [92] which would not be measured in stromal supernatants. Further, certain membrane-bound cytokine isoforms are significantly more active than in their soluble form. [38] Finally, adhesive interactions between HPC and stromal cells or ECM components may enhance the responsiveness of progenitors to small concentrations of cytokines. [40] [52] [67]

Second, although more than 40 cytokines have been described that promote proliferation and/or differentiation of progenitors, and other cytokines are thought to inhibit these processes, it remains unclear how the normal hematopoietic process is regulated. For instance, except for erythroid cells [93] it is unknown how HSC and HPC are instructed that increased numbers of mature blood elements of a specific lineage are required. Alternatively, it is unclear how HSC/HPC learn that sufficient mature blood elements are present and that further proliferation is not necessary (Table 13-1). One possible mechanism is contact-mediated regulation of progenitor proliferation, similar to contact inhibition seen in other biological systems. [94] In the BM, HSC and HPC are located in close proximity with progenitors at the same stage of differentiation, more mature precursors and blood cells and stromal cells. [15] [19] [20] Progenitors therefore receive signals through adhesion to neighboring hematopoietic or nonhematopoietic cells. These adhesive interactions are at least in part responsible for instructing HPC to continue or stop proliferating because of the lack or excess of maturing and terminally differentiated blood elements. As is seen in vivo, [95] HPC cultured in vitro in the presence of a stromal feeder are generally quiescent. [96] [97] Quiescence is in part mediated by growth inhibitory cytokines, such as TGF- [97] and MIP-1 [98] which are concentrated in the stromal ECM. [75] [99] There is also evidence that adhesive interactions per se signal HPC under physiological conditions to remain quiescent, [56] which will be discussed at length in the next section. A second mechanism involved in the local regulation of HPC proliferation is that HPC themselves can instruct the BM microenvironment to increase or decrease production of cytokines. For instance, osteoblast production of G-CSF is up-regulated by soluble factors produced by $CD34^+$ cells.[100] Likewise, when $CD34^+$ cells are cocultured with human primary BM stromal layers, stromal cells increase production of IL6 and GCSF. [101] When more mature $CD15^+$ precursors are present, production of G-CSF and IL6 returns to basal levels. Presence of increased cytokine concentrations induced by the $CD34^+$ cell population can override the contact-mediated inhibitory signals and induce $CD34^+$ cell proliferation and differentiation. [97] [102] [103] Once sufficient mature cells have been generated, stromal cell production of cytokines returns to baseline and increased hematopoiesis ceases. Thus, contact interactions between progenitors and stromal elements may be responsible for inhibiting excessive proliferation of these progenitors, and these growth inhibitory signals can be overridden by cytokines produced likely in response to a declining number of mature blood elements.

TABLE 13-1 -- Growth Factors Regulating Hematopoietic Cell Production

Growth Factor	Depletion of Blood/Progenitor Compartment	Producing Organ	Trigger
Erythropoietin	Anemia	Kidney	Oxygen tension
G-CSF	Leukopenia	Marrow ?	Soluble factor produced by HPC?
MGDF	Thrombocytopenia	Marrow ?	?
Flt-3 L	Aplasia	Marrow ?	?

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ADHESIVE INTERACTIONS AND HEMATOPOIESIS

What are the receptors on hematopoietic cells that allow them to interact with cells and ECM in the BM microenvironment? As discussed in the previous section, HSC and HPC as well as more mature precursors express cytokine receptors that allow them to interact with cell-surface expressed cytokines or cytokines present in the ECM. In addition to cytokine receptors, HPC also express a number of adhesion receptors. [104] [105] It is well accepted that adhesion receptors serve to localize progenitors in the BM microenvironment. Adhesion receptors can also transmit signals that modulate the response of the cell to other extracellular factors or directly induce or inhibit cell proliferation and differentiation.

The role of 1-integrins on HSC, HPC, and their progeny has been most extensively studied. 1-integrins allow interaction with the ECM components, fibronectin, collagens, laminin, and thrombospondin, as well as with the cell-surface expressed vascular cell adhesion molecule (VCAM)-1. [106] [107] [108] In addition to 1-integrins, HPC also express a number of other adhesion receptors, whose function has not been as extensively studied, including 2-integrins, 3-integrins, CD44, selectins, members of the immunoglobulin family, sialomucins, and lectins (Table 13-2).

Integrins

Integrins are a family of divalent cation-dependent cell-surface glycoproteins, consisting of an - and -chain (Fig. 13-5). They are responsible for cell-ECM and cell-cell adhesion events. [106] [107] [108] Integrins have a large, heterodimeric extracellular domain, a small membrane-spanning domain, and a cytoplasmic domain. Ligand-binding specificity is dictated mostly by the extracellular domain of the -chain. The cytoplasmic domain of the subunit is responsible for mediating integrin-cytoskeletal interactions and for activating signal pathways that may affect the growth and survival of cells (outside-in signaling). The cytoplasmic tail of the subunit is also responsible for affinity modulation of the integrin in response to signals provided to the cells through cytokine or other adhesion receptors (inside-out signaling).

Presence of an integrin on the cell surface does not necessarily indicate that the receptor has functional significance. Integrins can be constitutively expressed in a nonfunctional or low affinity state. Different activation signals, including stimulation of cells through other adhesion receptors, through cytokines, with PMA, and incubation with activating antibodies can switch the integrin to a functional, high-affinity state. [109] [110] [111] The increased affinity state of the integrin following one of these stimuli, a process termed inside-out activation, enables more avid binding of the integrin to its adhesive ligands (Fig. 13-4). Integrins colocalize with -actinin, talin, vinculin, and F-actin in focal contacts or adhesions [112] (Fig. 13-6). Because integrins do not have intrinsic kinase activity, activation of signal pathways requires recruitment of nonreceptor kinases. Engagement of integrins results in recruitment and autophosphorylation of the focal adhesion kinase, Fak, [113] which can bind to the integrin itself or with talin. Phosphorylation of FAK creates a binding site for c-Src, which phosphorylates Fak further, creating binding sites for a number of SH2-containing proteins, such as paxillin, Crk, Grb-2, and the p85 subunit of PI3-kinase. Thus, much like stimulation of cytokine receptors, integrins can activate the Ras/MAPK pathway that leads to the increased expression of c-myc and c-fos, activate PI3K, which affects cell proliferation and survival, induce immediate-early inflammatory response genes and alter levels of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors (CKIs). [114] [115] [116] [117] How these observations made mainly in fibroblasts and cell lines of nonhematopoietic origin relate to the assembly of focal contacts that affect adhesion, migration, or integrin-dependent growth modulatory signaling in HPC is still unknown.

1-Integrins

41-integrins play an important role in the interaction of HPC and the BM microenvironment. Several in vivo studies demonstrate a dominant role for the 1-integrin family in the homing and retention of HSC in the BM microenvironment. Engraftment of murine HSC can be inhibited by anti-4 antibodies. [55] [118] Homing of human HSC to a xenogeneic ovine BM microenvironment can be inhibited by anti-human 4 antibodies. [119] Intravenous infusion of anti-4 antibodies in mice or baboons results in peripheralization of HPC and HSC [118] [120] (Fig. 13-7). Finally, 1-defective HSC cannot successfully compete with wild type stem cells in competitive engraftment experiments, due to their inability to migrate. [11] All these studies indicate that 41-dependent interactions are needed to localize HSC in the BM and to allow their migration and adhesion to the BM. In vitro, CFC and LTC-IC adhere to BM stroma [36] at least in part through 1-integrins. Further, LTC-IC and CFC adhere to fibronectin and VCAM. [36] [54] [85] Of interest, although more than 80% of $CD34^+$ cells express 41- and 51-integrins, only a fraction adheres to CS1 or GRGDSP, [54] [85] indicating that a proportion of these receptors is present in a nonfunctional state. As in other biological systems, the functional state of 1-integrins on HPC can be affected by cytokines that can increase or decrease their affinity. In addition, intrinsic cellular changes, such as the presence of the BCR/ABL gene rearrangement in CML, can decrease the affinity of 1-integrins. [85] [121] It is thought that the decreased ability of integrins present on malignant CML progenitors to adhere to stroma may at least in part be responsible for their premature release into the PB. [122] [123] However, one needs to keep in mind that 1-integrins are responsible for only 50% of the adhesion of CFC or LTC-IC to stroma. It is not known which receptors account for the remainder. [35] [36]

TABLE 13-2 -- Adhesion Receptors and Their Ligands

Receptor Family	Receptor	Cell	Ligand
Integrins:			
1-family	41	HSC, HPC, erythroid, myeloid, and lymphoid precursors and blood cells	Fibronectin, Thrombospondin, VCAM
	51	HSC?, HPC, erythroid, myeloid, and lymphoid precursors and blood cells	GRGDSP
	11, 31, 21, 61	Mature WBC and platelets	Collagen and laminin
2-family	CD11a/18	HSC?, HPC, myeloid, and lymphoid precursors and blood cells	ICAM-1, ICAM-2, ICAM-3
	CD11b/18	HPC, myeloid, and lymphoid precursors and blood cells	ICAM-1, fibrinogen, iC3b
	CD11c/18	Myeloid and lymphoid precursors and blood cells	Fibrinogen
3-family	av3	Megakaryocytes and platelets	Fibrinogen, thrombospondin
7-family	47	Lymphoid progenitors and cells, mature myeloid cells	MadCAM and VCAM
CD44		HSC?, HPC, erythroid, myeloid, and lymphoid precursors and blood cells	Hyaluronate, fibronectin, collagen

Lectins	L-Selectin	HSC?, HPC, erythroid, myeloid, and lymphoid precursors and blood cells	s-Lex glycoproteins
	P-Selectin	HSC?, HPC, fibroblast, endothelial cells	s-Lex glycoproteins
	E-Selectin	Endothelial cells	s-Lex glycoproteins
Sialomucins	CD34	HSC, HPC, endothelial cells	Selectins
	CD43	HSC?, HPC, myeloid, and lymphoid precursors and blood cells	?, ICAM-1
	CD164	HSC?, HPC, myeloid, and lymphoid precursors and blood cells	?
Immunoglobulin superfamily	VCAM	Fibroblasts, endothelial cells	41, 47
	ICAMs	HSC?, HPC, mature myeloid, and lymphoid cells, fibroblasts, endothelial cells	CD11a/CD18, CD11b/CD18
	NCAM	Lymphoid cells, fibroblasts	N-CAM
	PECAM	HSC?, HPC, platelets, endothelium	PECAM

Figure 13-5 Affinity modulation of integrins. Integrins are transmembrane receptors consisting of an alpha and beta chain which connect the cytoskeleton of the cell with the extracellular milieu. Presence of an integrin on the cell does not mean that the receptor has functional activity. Signals from cytokines, other adhesion receptors, or oncogenes can affect the affinity (ability of the integrin to bind to its ligand) of integrin receptors (*inside-out signaling*).

Figure 13-6 Integrin-dependent regulation of adhesion and cell growth. Following engagement of integrins by their ligand, they laterally associate and translocate to focal adhesions/contact. Engagement of an integrin leads to the recruitment and activation of signal and adaptor molecules as well as structural proteins to such focal contacts/adhesion, including F-actin, Fak, c-SRC, Tensin, Paxillin, GRB-2, CRK, CBL, and PI3-K. These are then responsible for establishing firm adhesions as well as for initiating signal cascades that can affect cell survival and proliferation.

Figure 13-7 41-integrins are responsible for localization and homing of HSC. A number of in vivo studies have demonstrated an important role of the 41-integrin in the retention of stem and progenitor cells in the BM and for the homing of these cells to the BM microenvironment. [50] [118] [119] [120] [214] Infusion of anti-4 antibodies in mice or baboons results in the mobilization of long-term repopulating stem cells in the blood. Likewise, preincubation of murine, baboon, or human HSC with anti-4 antibodies prevents their homing and eventual engraftment in the BM microenvironment.

1-integrin-mediated interactions between HPC and fibronectin or other stromal components may also directly influence their survival, proliferation, and differentiation. For instance, co-culture of $CD34^+$ cells with fibronectin or engagement of 1-integrins on $CD34^+$ cells with blocking monoclonal antibodies under physiological cytokine conditions inhibits CFC proliferation. [56] [124] In contrast, engagement of 1-integrins by its ligand in the presence of high concentrations of cytokines promotes progenitor proliferation [52] while engagement of 1-integrins of HPC maintained under serum- and cytokine-starved conditions may delay apoptotic cell death. [122] [125] Thus, aside from anchoring progenitors in the BM, 1-integrins are also responsible for transferring growth modulatory signals. As has been seen in other biological systems, the final result of integrin-mediated signals depends on other external signals to which the progenitors are subjected.

Once progenitors differentiate, the expression pattern and functional status of 51- and 41-integrins changes. For instance, CFU-E adhere significantly better to fibronectin than more differentiated proerythroblasts. [126] [127] Further differentiation into erythroblasts and normoblasts is accompanied with a complete loss of adhesion to fibronectin and a concomitant loss of expression of the 51-integrin receptor at the reticulocyte stage. In contrast to more primitive HPC, erythroid differentiation may require interaction between progenitors and fibronectin. For instance, suspensions of MEL-14 erythroleukemic cells, but not fibronectin-adherent MEL 14 cells, fail to differentiate to the reticulocyte stage after induction with DMSO. [51] [58] [127] Likewise, differentiation of CFU-E to erythroblasts and erythrocytes requires interaction with fibronectin. [58] [128]

Similar observations have been made for B-lymphocyte maturation. [129] [130] Upon differentiation, B-cell precursors and mature B cells lose their capacity to adhere to fibroblasts. This loss is due to a decreased 41- and 51-integrin expression. Generation of B cells in stroma-dependent cultures requires close interaction between the immature progenitors and the stromal layer, as survival and proliferation of B-cell precursors depends on 41/VCAM and possibly fibronectin interactions. [59] [131]

Other members of the 1-integrin family, including 11, 21, 21, and 61, known to interact with collagen and laminin, are not expressed on normal $CD34^+$ cells. [85] [86] This underlies the observation that normal HPC fail to adhere to collagen or laminin. However, aberrant expression of 1, 2, 3, or 6 on transformed, leukemic $CD34^+$ cells in patients with acute or chronic leukemia may allow leukemic blasts to interact with collagen and/or laminin. This may be implicated in the ability of leukemic blasts to prematurely circulate in the blood.

2-Integrins

$CD34^+$ cells as well as their progeny express 2-integrins, known to interact with endothelial cell surface ligands, such as the intracellular adhesion molecules (ICAMs). [132] [133] As egress from and entry into the BM requires HSC to pass through endothelial barriers, one could hypothesize that 2-integrins play a role in the egress of HSC and HPC from the BM during mobilization or for their return to the BM following transplantation. In vitro, $CD34^+$ cells adhere to tumor necrosis factor- (TNF) activated stroma through the CD11b/CD18ICAM-1 ligand-receptor pair. [133] However, a number of in vivo observations indicate that 2-integrins may not play an important role in the interaction of HSC with the BM microenvironment. Patients with the leukocyte adhesion deficiency syndrome (LAD), [134] [135] in whom 2-integrins are defective, have no obvious defects in hematopoiesis. 2-dependent interactions seem not to be required for steady-state hematopoiesis nor for egress of HSC from the BM in the PB. Likewise, IV administration of anti-CD18 antibodies to baboons does not affect the number of peripheralized HPC although it increases the number of circulating granulocytes. [120] Finally, infusion of anti-CD11a antibodies in vivo in children undergoing mismatched transplantations improves rather than prevents engraftment. [136] Thus, 2-integrins may only be minimally important for the passage of HSC through the capillary sinusoids and for the ordered development of the hematopoietic process.

Other Integrins

Members of the 3- [137] [138] and 7- [139] integrin family can be found on maturing lymphoid and megakaryocytic precursors and blood cells. The av3-integrin on megakaryocytes and platelets allows interaction with fibrinogen, vitronectin, and thrombospondin. [137] [138] There is no evidence that av3 is expressed on $CD34^+$ HPC or HSC. The 47-integrin, known to interact with the addressins MadCAM [140] and VCAM, [141] is found on B-cell and T-cell precursors and mature lymphoid cells and plays an important role in the homing and recirculation of these cells. 47 is also found on mature myeloid cells, including monocytes and eosinophils, and mediates their interaction with the same ligands. [142] There is no evidence that 7-integrins are present on more primitive HPC and HSC.

CD44

Almost all $CD34^+$ cells, precursors, and blood cells express the CD44 receptor. [78] [79] [143] [144] CD44 is an integral membrane protein with an extracellular domain homologous to the cartilage link protein to which sialic acids and glycosaminoglycan side chains are attached. [145] Specificity of interaction depends on the structural heterogeneity of CD44. Ten putative exons can be inserted either alone or in tandem in the extracellular domain. [146] Use of alternative donor or acceptor splice sites within exons may result in an even greater degree of heterogeneity. As for integrins, mere presence of CD44 does not indicate that the receptor has functional significance. Stimulation with cytokines, phorbol esters (PMA) or with specific activating antibodies can modulate the affinity of CD44 for its ligand. [78] [147] [148] [149] The ligands for CD44 include hyaluronic acid, collagen, and fibronectin. CD44 is in part responsible for the adhesion of HPC to the BM microenvironment [77] [150] [151]

Incubation of stromal layers with hyaluronase [151] or BM cells with anti-CD44 antibodies [77] inhibit HPC adhesion to stroma. $CD34^+$ progenitors adhere to immobilized

hyaluronate [78] and fibronectin [150] through CD44. CD44-mediated interactions may also be important to regulate hematopoiesis. Studies in mouse [152] and human [77] [149] [153] have demonstrated that interference with CD44-mediated progenitor-stroma interactions alters hematopoiesis in long-term stroma-dependent marrow cultures. Although some studies have examined the signaling pathways affected by triggering the CD44 receptors, [154] [155] the mechanism(s) through which CD44 regulates hematopoiesis are not as well elucidated.

Selectins

Selectins have a long extracellular domain containing an amino-terminal Ca^{++} binding domain, an epidermal growth factor domain, and a series of consensus repeats like those in complement regulatory molecules. [156] Selectins also have a short membrane spanning domain and a short cytoplasmic tail. As in most adhesion receptors, the selectin cytoplasmic tail is responsible for transferring signals from the extracellular milieu to the cell. [157] The ligands for selectins are sialylated fucosylglucoconjugates present on endothelium, termed adressins. [158] [159] L-selectin is expressed on $CD34^+$ hematopoietic progenitors [160] whereas L-selectin and P-selectin are present on more mature myeloid and lymphoid cells. [158] [159] On neutrophils, selectins serve as adhesion receptors responsible for initial, reversible attachment to the endothelium. Tethering by selectins then allows integrin-mediated firm adhesion to the endothelium. The role of L-selectin present on $CD34^+$ cells is not known. Individuals lacking the fucosyl-transferase gene, responsible for fucosylation of selectin ligands, do not appear to have abnormal hematopoietic function. [161] Thus, if selectins are important for hematopoiesis, their function can be assumed by other adhesion receptors. Expression of L-selectin is upregulated on HPC found in the PB both under steady-state conditions or after mobilization with cytokines. [162] The implications of this for mobilization from and homing to the BM are, however, still unclear.

Sialomucins

As discussed previously, selectins recognize sialylated, fucosylated carbohydrate ligands of the sialyl Lewis X (sLex) type. [163] These carbohydrate ligands are presented by a class of adhesion molecules with mucin-like structure, termed sialomucins. Sialomucins present selectin carbohydrate ligands in a clustered, tissue-specific manner, increasing the avidity of selectin-ligand interactions. A number of sialomucins have been described on HPC; their function in progenitor-microenvironment interactions is not entirely clear.

CD34

CD34 is expressed on HSC and HPC as well as endothelial cells. [164] [165] Its role in hematopoiesis is not clear. Endothelial cell expressed CD34 serves as a ligand for selectins on lymphocytes and neutrophils. [165] [166] [167] CD34 on HPC may play a role in adhesion, although conflicting data is available. [168] [169] Ectopic expression of human CD34 in murine thymic precursors does not affect their growth but significantly increases their ability to adhere to BM stromal feeders. [168] It is unclear if this is CD34-mediated or mediated through another receptor, whose function is activated by the presence of CD34. In addition, several studies have shown that crosslinking of CD34 using antibodies that recognize sialoglycoprotease sensitive epitopes (e.g., QBEND10, ICH3, BI.3C5, MY10) but not other antibodies (9F2, 8G12) induces homotypic aggregation. CD34-mediated homotypic aggregation can be blocked by antibodies against 2-integrins but not by the anti-CD34-antibodies themselves. [170] Thus, some but not all isoforms of CD34 may be involved in HPC adhesion, albeit indirectly through activation of other cell surface adhesion receptors. Most in vitro HPC adhesion studies have used cells selected with anti-CD34 antibodies. No significant differences have been documented between adhesion of CFC and LTC-IC present in unselected mononuclear cells or in $CD34^+$ enriched cell populations [35] [36] [41] [54] [56] [77] [78] [85] [171] [172] This suggests that anti-CD34 antibodies do not usually increase or decrease adhesion of HPC to stromal feeders. Whether CD34-dependent interactions affect HSC or HPC growth is also not known. $CD34^{-/-}$ mice have decreased numbers of progenitors in the marrow. [173] This may indicate that the CD34 antigen is involved in progenitor growth.

CD43

Like CD34, CD43 is a transmembrane sialomucin expressed on the majority of HPC, including myeloid and lymphoid committed and primitive progenitors. [174] [175] Its role in adhesion of HPC to stroma is not well established. However, studies have indicated that it may be important for the regulation of proliferation/survival of committed myeloid progenitors, as crosslinking of CD43 induces apoptosis of CFC [174] [175]

CD164

CD164 has been identified on human $CD34^+$ cells and more mature blood cells. [176] Like the other sialomucins, CD164 is a transmembrane receptor found on HPC and possibly HSC as well as on a number of other cells. [176] [177] Its role in hematopoiesis still needs to be defined, although CD164 may play a role in the adhesion of HPC to stromal feeders and, like CD43, may impart growth inhibitory signals on $CD34^+$ progenitors. [178]

Lectins

Lectin receptors with galactosyl and mannosyl specificity present on HSC are believed to be important for the homing of murine HSC to the BM, but not to the spleen. [179] Addition of synthetic galactosyl and mannosyl probes but not fucosyl probes to murine long-term BM cultures results in disappearance of active hematopoiesis. [180] In contrast to the adhesion receptors described previously, lectin homing receptors may be the only adhesion receptors that are exclusively expressed on HSC. The receptor has not yet been cloned but is thought to consist of a 110 kDa glycoprotein containing approximately 5% N-linked carbohydrate which binds mannosyl and galactosyl residues with low affinity. [181]

The Immunoglobulin Superfamily

Several molecules result in cell-cell interaction and are therefore termed cell-adhesion molecules or CAMs. [182] [183] Almost all of these molecules are members of the immunoglobulin superfamily. This group of molecules receives its name as a result of the presence of large 90100 amino acid repeats which are also seen in immunoglobulins. All receptors within the immunoglobulin superfamily are expressed as plasma membrane spanning molecules, enabling them to affect growth and survival of cells and to affect the activation status of other adhesion receptors.

Vascular Cell Adhesion Molecule (VCAM)

VCAM is expressed on BM endothelial cells, [34] [41] Stro-1 positive BM stromal cells, [36] and dendritic cells. [184] Both myeloid and lymphoid progenitors and more mature cells can interact with VCAM through 41- or 47-integrins. [36] [141] [142] [185] VCAM is a transmembrane receptor. Engagement of VCAM, like engagement of adhesion receptors or cytokine receptors, may signal

stromal cells. [141] Coculture of myeloid and lymphoid progenitors with stromal feeders, to which they adhere chiefly through an 41-VCAM interaction, [126] [129] [130] [131] can induce changes in stromal cytokine production. Although VCAM may be in part responsible for this signaling, other receptors are also involved. [186]

Intercellular Cell Adhesion Molecule (ICAM)

ICAMs are expressed on mesenchymal and hematopoietic cells. [182] [187] [188] Three distinct ICAMs have been cloned. They have a high degree of homology, but their constitutive and induced expression profile differs. ICAM-1 is expressed on BM fibroblasts [189] and can be induced on endothelial cells. [133] It is also present on mature myeloid and lymphoid cells. [190] [191] ICAM-2 is significantly smaller than ICAM-1 and is constitutively expressed by endothelial cells. [188] ICAM-3 is expressed chiefly on cells of hematopoietic origin. [182] Like VCAM, ICAMs are transmembrane receptors capable of activating a number of signal pathways that may affect for instance

cytokine production or the activation state of other cell surface receptors. [149] [191] Although ICAM-1 and ICAM-3 are present on $CD34^+$ cells, their function on these cells is unclear.[192]

Neural Cell Adhesion Molecule (NCAM)

NCAM is present on stromal cells that support hematopoiesis.[193] Its function on stromal cells is unclear. NCAM is also present on mature T lymphocytes and NK cells,[194] but absent from myeloid or lymphoid precursors and progenitors. Abnormal expression of NCAM has been described on myeloid precursors of some patients with chronic myelogenous leukemia[195] and malignant lymphocytes of some patients with lymphoma.[196] Changes in NCAM expression in melanoma and colon carcinoma have been implicated in altered trafficking of the transformed cells. It has not been determined whether the presence of NCAM on HPC in some malignancies correlates with differences in tumor cell phenotype or homing to novel environments.

Platelet Endothelial Cell Adhesion Molecule (PECAM)

PECAM is expressed on endothelial cells, neutrophils, and platelets. [197] [198] Like NCAM, PECAM-mediated adhesion occurs through homotypic interactions. This is responsible for the maintenance of tight contacts between endothelial cells. Its extracellular domain can also bind to glycosaminoglycans, such as heparan sulfate. [199] It is expressed not only on mature hematopoietic cells but also on hematopoietic progenitors. [199] The role of PECAM in HPCstroma interactions is unknown. As has been shown for other receptors such as L-selectin[200] and CD44,[150] there is evidence that adhesive interactions through PECAM may modulate adhesion through 1-integrins.[201] Like L-selectin, PECAM is more highly expressed on $CD34^+$ cells mobilized in the blood by cytokines and chemotherapy. [201] [202] [203] The functional implications of this are unknown.

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EGRESS FROM AND HOMING TO THE BONE MARROW

Egress under Physiological Conditions

Once hematopoietic progenitors have matured, they are released from the BM in the PB. The exact mechanism(s) underlying this process is not well understood (Fig. 13-8). Upon maturation, adhesion receptor expression and/or function changes. For instance, myeloid progenitors express functional 41- and 51-integrins which are thought to retain them in the BM microenvironment attached to VCAM and fibronectin. [36] [54] [85] [130]

Figure 13-8 Egress of mature hemopoietic elements from the BM microenvironment. Little is known concerning the adhesive interactions governing the egress of mature blood cells from the BM microenvironment. Maturing myeloid, [36] [54] [85] [130] [160] erythroid, [126] [127] [128] and B-lymphoid [129] [130] precursors and blood cells express a different array of adhesion receptors than more primitive progenitors. In addition, the functional status of these receptors changes upon maturation. It is unknown whether acquisition of novel, functional adhesion receptors that allow interaction with basement proteins and endothelial cells, such as 11, 21, 31 or 61-integrins, 2 integrins and selectins, or loss of functional receptors such as the 41-integrins, thought to be important for the localization of cells in the BM microenvironment, is responsible for the egress of mature blood elements from the BM. It is also unclear whether similar mechanisms underlie the spontaneous release of the small population of stem and progenitor cells than can be found in the circulation under steady-state conditions.

Even though these progenitors also express members of the 2-integrin family, [85] [86] the role of 2-integrins in attachment of HPC to stromal cells is less clear. [133] [134] [135] [136] Mature neutrophils, in contrast, express functional 2-integrins, which allows them to interact with endothelial expressed ligands, such as members from the ICAM family. [187] [188] In addition, mature neutrophils also express 1-integrins that interact with collagen and laminin, present in basal membranes, [80] [84] and functional L-selectin. [156] [157] [158] [159] Acquisition of functional receptors on more mature progeny that can bind to endothelial barriers may be a prerequisite for egress from the BM microenvironment. Likewise, immature erythroid progenitors express functional 41- and 51-integrins, [126] [127] [128] which allows them to interact with VCAM and fibronectin and may retain them in the BM. These receptors are nonfunctional on more differentiated erythroblasts and normoblasts. Mature red cells no longer express 41- or 51-integrins.

Under steady-state physiological conditions the majority of more primitive HPC and HSC are retained in the BM microenvironment. A small population of $CD34^+$ cells, CFC, LTC-IC and long-term repopulating HSC can, however, be found circulating in the PB, [203] a phenomenon that has been exploited for transplantation. Physiological stressors such as exercise, stress, and infections can increase the number of circulating HSC and HPC. [204] [205] [206] The mechanism underlying the spontaneous mobilization of this HSC/HPC pool is not understood. Some studies

suggest that expression of the 41-integrin on these circulating $CD34^+$ cells may be lower than what is seen on $CD34^+$ cells recovered from the BM. [207] This may contribute to the spontaneous release of these progenitors from the BM. Whether and how these HPC/HSC traffic spontaneously between the PB and BM is not known.

Mobilization of HSC

The observation that HSC and HPC can be mobilized in the blood by treatment with cytokines and/or chemotherapy has led to the now widespread use of HSC from PB rather than BM for transplantation. [201] [202] [208] [209] [210] [211] A number of studies have described differences in adhesion receptor expression and other characteristics between $CD34^+$ cells present in mobilized PB and steady-state BM. For instance, $CD34^+$ present in mobilized PB express less 4 and CD11a/CD18 and more PECAM-1, CD62L, and CD44 than their counterparts from steady-state BM. [162] [202] [203] [212] [213] Furthermore, significantly fewer HPC in mobilized PB are in S-phase than among their counterparts in the BM. [212] [213] Although such differences have been documented, it is still unclear how chemotherapy/cytokines induce these differences and how these differences may be related to or responsible for the mobilization phenomenon (Fig. 13-9). These questions have been addressed in part in studies in mice and baboons in which specific HPC-stromal interactions were targeted by infusion of anti-adhesion receptor antibodies (Fig. 13-7). These studies indicate that alterations in 41-VCAM interactions, [118] [120] [214] but not 41-fibronectin, [215] 51-GRGDSP, [214] or 2-integrin-mediated interactions [120] may play an important role in mobilization of HSC.

A number of questions still remain. For instance, are changes in adhesion receptor expression directly induced by the pharmacological concentrations of cytokines or chemotherapy themselves? If so, do these differences in expression of adhesion receptors induced by the mobilization regimen have functional implications for the subsequent in vivo behavior of HSC? Is mobilization induced by chemotherapy also in part due to alterations in the BMblood barrier as a result of chemotherapy-induced damage to endothelial cells? Are the phenotypic differences observed between steady-state BM $CD34^+$ cells and $CD34^+$ cells present in the PB following mobilization reflective of intrinsic differences in the characteristics of these two cell populations? If so, how does this impact on the short-term and long-term engraftment and differentiation potential of HSC?

Homing and Engraftment

Although stem cell transplantations have successfully been done for more than 30 years, it remains somewhat of a mystery how HSC infused intravenously home to the BM microenvironment (Fig. 13-9). As outlined previously, except perhaps for lectins, [179] [180] [181] no adhesion receptor has yet been identified that is exclusively present on HSC. In addition, no adhesive ligand, except perhaps for hemonectin, [87] [88] [89] has been identified that is exclusively present in the BM microenvironment. So, what underlies the specificity of the HSCBM microenvironment interaction? When infused intravenously, HSC lodge initially in the microvasculature of the lung and liver. [216] [217] [218] [219] From there they colonize the BM. To enter the BM space and provide permanent hematopoiesis, HSC must pass through sinusoids, migrate through the

Figure 13-9 Mechanisms of mobilization and homing. Under steady-state conditions, a small population of progenitor and stem cells can be found circulating in the blood. What the differences in adhesive characteristics are between the progenitors found spontaneously in the blood or present in the BM is unknown. Under conditions of stress, following infection, or following administration of cytokines, alone or in combination with chemotherapy, a significantly larger population of progenitor and stem cells is recovered from the circulation. [201] [202] [203] [204] [205] [206] [207] [208] [209] [210] In vivo animal studies have demonstrated an important role for the 41-integrin in mobilization of murine and baboon stem and progenitor cells in the blood. It is not clear whether decreased function or expression of this integrin is responsible for the mobilization of human hemopoietic progenitor and stem cells in the blood. It is also not clear whether changes in other adhesion receptors such as L-selectin or PECAM-1 contribute to the aberrant premature circulation of progenitors. Finally, it is also not known whether changes in the microenvironment itself caused by exposure to chemotherapy and/or cytokines contribute to the mobilization of cells that under steady-state conditions are found almost exclusively in the BM. Although transplantation of stem cells has been performed for over 30 years, little is known concerning mechanisms underlying their homing to the BM microenvironment. For engraftment to occur, multiple different steps are required: cells have to

diapedese (*transmigration*) through the endothelial lining of BM sinusoids, migrate through the extracellular space of the marrow (*migration*), and adhere to niches and start proliferation (*adhesion*). It is likely that this requires multiple sequential migration and adhesion events involving more than one adhesion receptor. Animal studies have shown an important role of 41-integrins in homing and engraftment.[118] [119] [120] [214] [215] The nature of the specificity that adhesive interactions provide to the homing of hemopoietic stem and progenitor cells to the BM but not to other organs is not known. It is possible that lectins with mannosyl and galactosyl specificity serve to guide progenitors to the bone marrow microenvironment. [87] [88] [89] As for mobilization, the role of L-selectins, PECAM-1 or 2-integrin, which allow interaction with endothelial cell expressed ligands such as ICAM-1, adressins and basement membrane proteins, in the transmigration process through the endothelial barrier is unknown. It is also not known whether 1-integrins or other receptors are required for migration through the BM space. Finally, there is evidence that exposure of the BM microenvironment to chemotherapy or radiation therapy alters the microenvironment. What role this has in homing and engraftment remains to be determined.

BM extracellular space, and attach to the so-called stem cell niches. Passage through endothelial barriers requires that cells attach to the endothelium. For neutrophil diapedesis through endothelium, this requires tethering of the neutrophil through selectins [157] [158] [159] [160] to endothelial expressed addressins, [163] followed by firm integrin-mediated attachment. Subsequently, integrins will allow migration through the endothelium and the BM extracellular space. In contrast to adhesion, cell migration depends on the coordinated establishment of adhesion at the leading edge of a cell and simultaneous release at the trailing edge. [220] The rate of migration depends on dynamic changes in the strength of cellligand interactions. [112] [113] [114] [115] Several factors dictate the strength of cellligand interactions including the number and affinity state[109] [110] [111] [171] [172] of the adhesion receptors and the strength of the adhesion receptorcytoskeletal interactions. This can be influenced by cytokine-mediated signals[109] [114] [171] [172] or signals originating from other adhesion receptors. [110] [150] [200] It therefore

follows that successful engraftment will depend not only on the presence of several different adhesion receptors, but also on the functional state of these receptors to accommodate both adhesive and migratory events. Finally, alterations in the microenvironment itself due to the preparative radiation therapy and/or chemotherapy may affect engraftment.[221] [222] [223]

In vivo studies in animal models have demonstrated that 41-VCAM interactions are important for successful engraftment (Fig. 13-7). Infusion of HSC preincubated with anti-4 integrin antibodies results in increased recovery of HSC from the PB and spleen and decreased recovery of HSC in the marrow during the first 24 days after transplantation.[118] Continued presence of the anti-4 antibodies will disallow engraftment. [55] [118] [120] [214] Further, 1$^{-/-}$ HSC cannot compete successfully with 1-expressing HSC for colonization of hematopoietic organs.[11] Thus, it is believed that 1-integrins play an important role in homing/engraftment as well as in mobilization. Decreased 1-integrin-mediated interactions are thought to be responsible for mobilization [118] [120] [207] and integrin-mediated adhesion and migration required for homing.[55] [118] [119] [120] [214] This leaves us with one question that still needs answers: How then do mobilized progenitors, which by definition adhere less, result in earlier engraftment, which requires adhesion, than steady-state marrow?

Lectins with mannosyl-galactosyl specificity expressed on HSC have also been implicated in homing to the BM. [179] In humans, more rapid engraftment has been reported when CD34^{+} cells expressing high levels of L-selectin are used as grafts. [162] It is not known whether this is due to the high density of L-selectin receptors which should allow better HSCendothelial interactions, or due to associated and also not yet understood differences between L-selectin ^{high} and L-selectin^{low} cells.

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CONCLUSION

In embryonic and fetal life, hematopoiesis takes place mainly outside the BM microenvironment. However, at birth, HSC relocate to the BM which becomes almost the exclusive hematopoietic organ. The reasons underlying the migration from organ to organ are unclear. In adult life, the majority of immature progenitors resides in the BM, although small numbers of HSC and HPC can be found in the PB circulation. Certain external factors or stem cell intrinsic factors can affect the localization of HSC and HPC, resulting in the premature circulation of immature cells in the circulation, also termed mobilization. Although this process is now extensively being exploited in the transplant setting, we are only starting to decipher the reasons underlying this phenomenon.

Steady-state hematopoiesis, however, is concentrated in the BM microenvironment (Fig. 13-10). The interactions occurring in the BM microenvironment necessary for the ordered progression of the hematopoietic process under normal steady-state conditions are complex. It is clear that the majority of cells present in the BM, including fibroblasts, osteoblasts, and endothelial cells, not only serve to contain the HSC and HPC in the BM, but play an active role in the regulation of their growth. Fibroblasts, endothelial cells, and osteoblasts produce a number of cytokines. The local concentrations within the BM are unknown, but likely modest. Low concentrations of multiple cytokines are thought to cooperate in the induction of HSC/HPC proliferation, differentiation, or survival. In addition, several cytokines can be presented as membrane-expressed molecules and therefore be more active. Alternatively, they may be concentrated in and protected by ECM molecules such as GAGs. These GAGs may also activate the function of bound cytokines. In addition to GAGs, the stromal cells produce a number of other ECM components. These ECM components combined with other adhesive ligands that are expressed on the surface of stromal cells serve as anchoring places for HSC and HPC. They are likely

Figure 13-10 Interactions in the BM microenvironment that govern hemopoiesis. Steady-state hemopoiesis takes place in the BM microenvironment. Interactions occurring in the BM microenvironment necessary for the ordered progression of hemopoiesis are complex. Stromal cells (A) produce a number of growth-promoting (B) and growth inhibitory (C) cytokines in low concentrations which cooperate in the induction of progenitor proliferation, differentiation, or survival. The activity of cytokines may be greater when the cytokine is present in a membrane expressed form or because it is concentrated in, protected by, or activated by ECM molecules, such as GAGs (D). Production of these cytokines may be regulated by the presence of hemopoietic cells themselves (1). Stromal cells produce ECM components (D) which combined with other adhesive ligands that are expressed on the surface of stromal cells serve as anchoring places for progenitors (2). Adhesive interactions themselves between progenitors and stromal cell- or ECM-expressed adhesive ligands can profoundly affect the growth and survival of progenitors. Every interaction, whether initiated by cytokines, by engagement of an adhesion receptor, or by interaction between progenitor cells and other hemopoietic cells (3), will affect subsequent interactions and signaling events, further increasing the complexity of the network of signals between stem cells, progenitors, mature blood elements, and stromal cells.

responsible for the as yet unexplained observation that HSC and HPC specifically home to the BM. In addition, engagement of specialized transmembrane adhesion receptors on HSC and HPC by these adhesive ligands may, like cytokine receptor engagement, profoundly affect the fate of HSC and HPC. Adhesion may alter the response of progenitors to cytokines as well as directly stimulate signal pathways that will influence progenitor proliferation, differentiation, and survival. Finally, one needs to keep in mind that every interaction, whether initiated by cytokines or an adhesive event, will affect subsequent interactions and signal events, further complicating the network of signals between HSC, HPC, mature blood elements, and the mesenchymal stromal cells. Although significant progress has been made over the last several decades in our understanding of the players in this complex set of interactions, an equally large number of questions remain to be answered.

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Chapter 14 - Growth Factors, Cytokines, and the Control of Hematopoiesis

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INTRODUCTION

The bone marrow, like all mammalian organ systems, has an exquisite capacity to respond to environmental stimuli in ways that protect the host from the attendant hazards of such stimuli. The responsiveness of each lineage of the hematopoietic system results from coordinated increases in the production and functional activity of appropriate hematopoietic cell types, without expansion of irrelevant ones. A mountaineer at high altitude, for instance, will develop a specific expansion of the erythroid bone marrow and subsequent erythrocytosis, but the bone marrow will not increase production of neutrophils, monocytes, eosinophils, mast cells, T lymphocytes, or B lymphocytes. This response is highly focused on protecting the host from the particular threat: hypoxia. Not surprisingly, the hematopoietic response evolves directly from a hypoxia-induced increase in circulating levels of erythropoietin (EPO), a glycoprotein hormone that specifically stimulates the proliferation and differentiation of cells of the erythroid lineage. [1] [2] EPO is but one of about 30 well-characterized hematopoietic growth factors that regulate the production and activity of blood cells. Additional ones will likely be characterized at an increasing pace. With each new discovery it becomes increasingly clear that all lineage-specific and multilineage responses of hematopoietic cells in the steady state and under conditions of environmental stress depend almost exclusively on the availability of proteins in hematopoietic tissues that stimulate or inhibit the production, differentiation, or traffic of mature blood cells or their progenitors. It is also true that certain redundancies exist. Some of these are sufficient to induce growth and differentiation but not necessary to maintain hematopoiesis in the steady state. Others are necessary, and

their deficiency in an animal results in serious hematopoietic deficiencies of one kind or another. The anemia associated with the erythropoietin deficiency of renal failure, and its responsiveness to treatment with recombinant human EPO, [3] is a clear indication that EPO is necessary for optimal red cell production in mammals.

Fully characterized recombinant hematopoietic growth factors and the genes encoding their receptors have recently become available, enabling hundreds of laboratories to test exactly how a given change in the environment incites expression of the right genes at the right time. In vitro experiments in many laboratories using genomic and cDNA clones, in vitro transcripts, and recombinant proteins have uncovered new levels of complexity and provided new explanations of how biologic organisms work. Studies using mice carrying germline disruptions of such genes have been informative and, in some cases, even surprising. In general, however, results of studies on knockout mice, as well as results of therapeutic trials, confirm the hematopoietic responses predicted by scientists using in vitro techniques. Today, there is an excitingly clear picture of a complex and highly efficient intercellular molecular communication system. That system is the subject of this chapter.

Despite the advances of the past decade, research on hematopoietic growth factors and related cytokines is still in its infancy. Each year additional factors are identified and new activities are found for existing ones. Organizing growth factors and interleukins in chronologic order is not useful because the order of discovery doesnt appoint a factor to a functional category. Accordingly, we will present the hematopoietic growth factors by assigning them to one of three groups: those that function largely in support of a specific lineage, those with an effect on multipotential hematopoietic cells (so-called early-acting factors), and those that regulate hematopoiesis indirectly by inducing the expression of direct-acting growth factor genes in auxiliary cells.

This organizational approach also attempts to illustrate how hematopoietic growth factors actually work. There is some arbitrariness to our choice of big picture assignments. Granulocyte-macrophage colony-stimulating factor (GM-CSF), for example, is assigned to the granulopoietic lineage, not because it has no influence on the behavior of nongranulopoietic cells, but because the biologically dominant effect of the factor, when administered to humans, is on the production and activation state of phagocytic leukocytes. GM-CSF does have effects on progenitors of other lineages. M-CSF is similarly assigned because a major manifestation of M-CSF deficiency is monocytopenia. [4] Certain recurrent themes will become evident that permit a more holistic view of hematopoietic control; these themes are outlined in Table 14-1 . It will become obvious that most of the hematopoietic growth factors do more than one thing and can act on more than one cell type. Consequently, because the lineage assignments we have made are sometimes arbitrary (e.g., interleukin [IL]-5 is in the granulopoiesis section, but has clear effects on lymphoid cells too), we have listed many of the heterogeneous biologic activities of these proteins in Table 14-2 . Some factors influence the production of blood cells directly by binding to receptors on progenitor cells; others influence the process indirectly by binding to receptors on auxiliary cells, which then respond by releasing growth factors; some do both. Some factors can induce cell division; others only permit the survival of progenitors of a given lineage. Some factors, particularly lineage-specific ones, influence the replication or survival of primitive cells and also activate the function of the terminally differentiated cells of that lineage. Some factors act synergistically with other cytokines. Auxiliary cells and progenitor cells can cross-talk and exhibit signal amplification circuits. Finally, the reader will find that hematopoietic growth factors and their receptors also have a number of structural elements in common. Each of these principles will be emphasized later.

TABLE 14-1 -- Repetitive Themes in Hematopoietic Growth Factor Biology

1.Hematopoietic growth and differentiation factors (HGFs), even ones with a high degree of lineage specificity, have multiple biologic activities (reviewed in Table 14-2).
2.HGFs influencing the growth or differentiation of hematopoietic progenitor cells can do so directly or indirectly. For this reason, the role of any given factor in regulating hematopoiesis cannot be fully assessed by simply challenging progenitor cells in vitro or administering the recombinant HGF to an animal. Loss of function analyses (i.e., in HGF-deficient [knockout] mice) are also essential.
3.Cytokines that induce proliferation of hematopoietic precursor cells often have the capacity to enhance the functional activity of the terminally differentiated progeny of these precursor cells.
4.Most lineage-specific factors inhibit programmed cell death of the progenitors of the specific lineage they influence.
5.HGFs commonly act synergistically with other cytokines (examples reviewed in Table 14-3).
6.Hematopoietic regulatory cytokines are organized in a highly complex ordered network.
7.The cytokine network exhibits many signal amplification circuits.

8.HGF receptors share important structural features.

9.Structural abnormalities of HGFs or their receptors may result in clinically significant abnormalities of hematopoiesis.

10.Certain HGFs are of substantial value in clinical practice.

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HEMATOPOIETIC GROWTH FACTOR RECEPTORS

The structures of hematopoietic growth factors and cytokines are quite variable. The receptors for these factors are less so. There are at least six receptor superfamilies involved in controlling the behavior of hematopoietic cells. Most receptors for hematopoietic growth factors are members of the type I cytokine receptor family.

The Hematopoietic Growth Factor Receptor Superfamily (Type I Cytokine Receptors)

The receptors for the majority of hematopoietic growth and differentiation factors are members of this family. The receptors do not possess intrinsic kinase activity but lead to phosphorylation of cellular substrates by serving as docking sites for adaptor molecules that do have kinase activity. The characterization of receptors for many of the growth factors has permitted the identification of a group that includes receptors for leukemia-inhibitory factor (LIF), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-13, IL-18, GM-CSF, G-CSF, EPO, prolactin, growth hormone, ciliary neurotrophic factor, and c-mpl. [5] [6] As shown in Figure 14-1 , there are a number of repetitive structural and functional themes in this unique integral membrane protein superfamily, including:

1. Four cysteine residues in the extracellular domain
2. The sequence WSXWS in the ligand binding extracellular domain that may optimize the tertiary structure of the receptors [7]
3. A capacity for enhanced binding and/or signal transduction when expressed as a heterodimer or homodimer
4. Lack of a known catalytic domain in the cytoplasmic portion of the molecule
5. The presence of fibronectin type III domains [8] in the extracellular regions.

Apart from these shared homologous domains, there is minimal sequence homology among these receptors, and their cytoplasmic domains are immensely variable in size. Nonetheless, these homologies are likely of substantial functional importance and also provide clues to the evolution of this family. For

TABLE 14-2 -- Heterogeneous Biologic Activities of the Hematopoietic Growth Factors and Interleukins

Factor	Bioactivities
Erythropoietic factors	
EPO	Stimulates clonal growth of CFU-E and a subset of BFU-E
	Suppresses apoptosis in erythroid progenitor cells
	Induces release of reticulocytes from marrow
	Induces globin synthesis in erythroid precursor cells
	Stimulates murine megakaryocyte colony growth and terminal maturation in vitro but has no apparent thrombopoietic activity in vivo
	Mitogen for neonatal rat cardiac myocytes
SF	Promotes the proliferation and differentiation of pre-CFCs
	Acts synergistically with IL-3, GM-CSF, and EPO to support clonal growth of CFU-GEMM, BFU-E, and CFU-Mk
	Enhances hematopoietic colony growth in cultures of marrow cells from patients with congenital marrow failure states
	Stimulates the proliferation and differentiation of mast cell precursors
	Chemotactic for mast cells
	Independently stimulates mast cell degranulation and enhances IgE-dependent mediator release from mast cells
	Stimulates expansion of committed progenitor cell compartment in vivo
	Stimulates mast cell hyperplasia in vivo
	Supports melanocyte development and migration
	Supports gametogenesis
IGF-1 (hematopoietic effects of)	Induces erythroid colony formation at high doses in absence of EPO
	Induces DNA synthesis in erythroid progenitor cells
	Anti-apoptotic effects in erythroid progenitor cells and IL-3-dependent cells
Granulopoietic factors	

GM-CSF	Stimulates multilineage hematopoietic progenitor cells
	Stimulates BFU-E growth
	Stimulates granulocyte, macrophage, and eosinophil colony growth
	Stimulates functional activity of eosinophils, neutrophils, monocytes, and macrophages
	Induces IL-1 gene expression in neutrophils and peripheral blood mononuclear leukocytes
	Costimulates T-cell proliferation with IL-2
	Induces or co-induces TNF- gene expression with IFN- in monocytes
	Stimulates proliferation of myeloid leukemic cells
	Stimulates growth of certain nonhematopoietic cancer cells in vitro
	Induces migration and proliferation of vascular endothelial cells in vitro
G-CSF	Stimulates growth of progenitor cells committed to the neutrophil lineage
	Stimulates neutrophil maturation of certain leukemic cells
	Activates phagocytic function of mature neutrophils
	Stimulates quiescent pluripotent hematopoietic progenitor cells to enter G_1-S phase
	Stimulates mobilization of stem cells and progenitors from hematopoietic niches into peripheral blood
	Maintenance of steady-state neutrophil numbers
M-CSF	Induces monocyte/macrophage growth and differentiation
	Activates macrophage phagocytic function
	Activates macrophage secretory function
	Maintenance of steady-state monocyte levels
	Maintenance of steady-state osteoclast numbers
IL-5	Stimulates eosinophil production and activation
	Activates cytotoxic T cells
	Induces or co-induces immunoglobulin secretion
Megakaryocytopoietic factors	
TPO	Stimulates in vitro growth of CFU-Mk, megakaryocytes, and platelets
	Stimulates clonal growth of individual CD34+CD38 cells
	Synergizes with SF, IL-3, and FL
	In single CD34+CD38+ cells, TPO synergized with SF and IL-3 but not Flt3 ligand; no increase in colony growth nor colony size is seen when TPO is added to multicytokine combinations
	Increases megakaryocyte ploidy in vitro and in vivo
	Enhances the proliferation and differentiation of yolk-sac erythroid lineage precursors
	Stimulates production of PDGF, platelet factor 4, and -thromboglobulin from megakaryocytes
	Supports continuous growth of cytokine-dependent human leukemic cell lines
	Stimulates adhesion of hematopoietic progenitor cells to fibronectin by activation of VLA-4 and VLA-5
	No direct effect on platelet aggregation but primes the response to ADP, epinephrine, and thrombin; also increases platelet release of ATP and thromboxane B_2 production and platelet expression of CD62 (P-selectin)
	Stimulates proliferation of c-mpl-positive AML blasts
IL-11	Stimulates proliferation of murine plasmacytoma and hybridoma cell lines
	Stimulates CD4+ T-cell-dependent proliferation of antigen-specific plaque-forming B cells
	Shortens the duration of G_0 of primitive hematopoietic progenitor cells
	Acts synergistically with IL-3 or SF to stimulate the clonal growth of erythroid (BFU-E and CFU-E) and primitive megakaryocytic (BFU-Mk) progenitors
	Increases the ploidy of cultured megakaryocytes
	Increases peripheral platelet and neutrophil counts
	Increases the numbers and cycling activity of committed progenitor cells
	Hastens hematopoietic recovery following cytotoxic chemotherapy, ionizing radiation, and bone marrow transplantation
	Acts as an autocrine growth factor for certain megakaryoblastic cell lines
	Stimulates hepatic acute-phase reactant production
	Suppresses adipogenesis in pre-adipocytes
Lymphopoietic factors	
IL-7	Induces clonal growth of pre-B cells
	Stimulates growth of pre-T cells
	Stimulates growth of CLL, acute leukemia, and Sézary cells
	Enhances IL-3 and GM-CSF production by activated T cells
	Induces expression of IL-6, IL-1, TNF-, and IL-8 in peripheral blood monocytes
IL-2	Induces proliferation and activation of T lymphocytes
	Induces proliferation and activation of B lymphocytes
	Induces proliferation and activation of NK cells
	Induces expression of IL-1 in monocytes and macrophages
	Co-induces (with IL-1) expression of interferon- in T cells

IL-15	Induces proliferation and activation of T cells
	Synergizes with IL-12 to induce proliferation and activation of T cells
	Induces proliferation and activation of B cells
	Induces proliferation and activation of NK cells
	IL-15 (as a single factor) induces differentiation of CD3-CD56+ NK cells from CD34+ HPC
	SF synergizes with IL-15 to increase expansion without altering differentiation state of expanded NK cells
	Induces antitumor responses in animal models in which the tumor is also responsive to IL-2 treatment
	Induces angiogenesis in vivo
	Stimulates accumulation of contractile proteins in muscle fibers of differentiated myocytes
	Stimulates the expansion of PBMC anti-HIV-specific CTL
	Stimulates mast cell proliferation
IL-4	Induces proliferation of activated B cells
	Inhibits IL-2-stimulated proliferation of B cells
	Co-induces immunoglobulin secretion and isotype switching
	Induces proliferation of T cells
	Induces proliferation of fibroblasts
	Co-induces (with PMA) IL-2 receptor expression in T cells
	Inhibits induction and function of LAK cells
	Inhibits IL-1 release
	Induces expression of M-CSF and G-CSF genes in monocytes
IL-10	Inhibits monocyte/macrophage-dependent synthesis of T_H 1-derived cytokines (IL-2, IFN-, lymphotoxin) in humans and mice
	Inhibits monocyte/macrophage-dependent synthesis of T_H 2-type (IL-3, IL-4, IL-5) and NK-derived cytokines (IFN- and TNF-)
	Inhibits monocyte/macrophage-dependent T-cell proliferation
	Inhibits proliferation of and IL-2 production by purified T cells
	Acts as co-stimulator of B-cell proliferation
	Represses constitutive and IFN-induced MHC class II antigen expression on mononuclear phagocytes
	Inhibits the production of IL-1, TNF-, IL-6, IL-8, G-CSF, GM-CSF, and IL-10 by mononuclear phagocytes
	Inhibits the production of reactive oxygen species and NO by mononuclear phagocytes
IL-12	Induces the differentiation of naïve T-helper cells into T_H 1 cells
	Augments functional activity of NK cells
	Acts synergistically with TNF- to stimulate IFN- production by NK cells
	Induces LAK activity in NK cells
IL-13	Shares many biologic activities with IL-4 but is not known to influence T lymphocytes
	Enhances expression of lymphocyte antigens in B cells
	Enhances B-cell proliferation
	Promotes isotype switching to permit IgE expression
	Enhances production of IgG4 and IgM
	Inhibits IFN- production by NK cells exposed to IL-2
	Induces VCAM-1 in endothelial cells but not E-selectin or ICAM-1
IL-14	Stimulates the proliferation of anti-m or *Staphylococcus aureus* Cowan-treated B cells
	Synergizes with IL-2 to stimulate proliferation of anti-m B cells
	Stimulates proliferation of pre-B-cell ALL, hairy cell leukemia cells, prolymphocytic leukemia, and CLL cells
	Inhibits secretion of immunoglobulin by activated B cells
	Autocrine growth factor for some B-cell lymphoma cell lines
IL-16	Chemotactic for CD4+ T cells, monocytes, eosinophils at nanomolar concentrations
	Growth factor for CD4+ T cells
	Induces functional IL-2 receptors on CD4+ T cells
	Inhibits HIV-1 replication in vitro
	Inhibits proliferation in mixed lymphocyte reactions
Multipotential factors (early-acting factors)	
IL-3	Stimulates multilineage colony growth
	Stimulates growth of primitive hematopoietic cell lines with multilineage potential
	Stimulates BFU-E proliferation in vitro
	Stimulates proliferation of murine CFU-S
	Induces B-lymphocyte differentiation
	Co-stimulates T-cell proliferation with IL-2
	Induces macrophages to express M-CSF (this may explain reports that IL-3 induces clonal growth of pulmonary alveolar macrophages)
	Stimulates growth of myeloid leukemic cells in vitro
	Primes hapten-specific contact hypersensitivity responses

FL	Stimulates proliferation of some AML and ALL blasts
	Stimulates proliferation and differentiation of dendritic cells in vitro and in vivo
	Addition of FL to multicytokine combinations augments retroviral transduction of HPC
	Stimulates antitumor responses in murine models of syngeneic cancer
	Mobilizes peripheral blood stem cells weakly as a single agent but markedly synergizes with G-CSF
	Weak colony-stimulating activity as single agent but synergizes with IL-3, GM-CSF, SF, IL-11, IL-6, G-CSF, IL-7, and multicytokine combinations
IL-9	Stimulates clonal growth of BFU-E in combination with EPO
	Stimulates clonal growth of fetal CFU-Mix and CFU-GM
	Augments IL-3-induced growth of murine bone marrow-derived mast cells
	Stimulates proliferation of preactivated PBMC-derived T-cell lines
IL-6	Synergistic with IL-3 in CFU-GEMM colony growth
	Synergistic with M-CSF in macrophage colony growth and with GM-CSF in granulocyte colony growth
	Synergistic with IL-4 in inducing T-cell proliferation, immunoglobulin secretion, and hematopoietic colony formation
	Synergistic with IL-2 and IL-1 in inducing T-cell proliferation
	Co-induces differentiation of B cells
	Induces terminal differentiation of myeloid leukemic cell lines
	Induces neuronal differentiation in certain pheochromocytoma cell lines
	Co-induces cytotoxic T cells in vitro
	Stimulates plasmacytoma growth
	Induces acute-phase responses in vivo
	Induces acute-phase protein synthesis in hepatocytes
	Stimulates megakaryocytopoiesis in vitro and in vivo
Indirect acting factors	
IL-1	Induces expression of GM-CSF, G-CSF, IL-6, and IL-1 in fibroblasts, endothelial cells, keratinocytes, and thymic epithelial cells
	Induces proliferation of preactivated T cells
	Induces acute-phase protein synthesis
	Induces fever and sleep in vivo
	Stimulates release of ACTH
	Promotes transendothelial passage of neutrophils
	Synergizes with IL-3 in stimulating proliferation in primitive hematopoietic progenitor cells in vitro
	Stimulates prostaglandin E production in fibroblasts, monocytes, and neutrophils
TNF-	Modulates EGF receptor expression
	Induces expression of GM-CSF, G-CSF, IL-6, and IL-1 in fibroblasts and endothelial cells
	Enhances mitogen-induced GM-CSF expression in T cells
	Induces release of GM-CSF and M-CSF in vivo
	Inhibits virus replication synergistically with interferons
	Stimulates prostaglandin E production in fibroblasts and neutrophils
	Enhances parasite and tumor cell cytotoxicity of eosinophils and macrophages
	Inhibits proliferation of hematopoietic progenitor cells, lymphocytes, and certain leukemia cell lines in vitro
	Mediates the hemodynamic and toxic effects of endotoxin
	Induces expression of IL-6 in fibroblasts
	Induces expression of adhesion molecules in myeloid cells
	Activates phagocytic function of neutrophils
	Induces expression of IL-8
	Increases production of plasminogen-activator inhibitor in vascular endothelial cells
	Suppresses transcription of the thrombomodulin gene in endothelial cells
	Modulates EGF receptor expression
	Promotes transendothelial passage of neutrophils
	Activates NF B trans-activating protein in lymphoid cells
IL-17	Induces secretion of IL-6, IL-8, PGE_2 , and G-CSF from stromal cells
	Synergizes with TNF- and IFN- to induce stromal cell production of GM-CSF and IL-1
	Up-regulates ICAM-1 expression by fibroblasts
IL-18	Induces IFN- production from T cells, B cells, NK cells, peripheral blood mononuclear cells
	Synergizes with IL-12 to enhance production of IFN- by activated T cells
	Induces GM-CSF production from T cells
	Inhibits production of osteoclast-like multinucleated giant cells in cocultures of osteoblasts and hematopoietic cells
	Augments NK activity in human and mouse PBMC
	Inhibits IL-10 production by T cells
	Enhances *fas*-mediated cytotoxicity of murine T_H 1 but not T_H 0 or T_H 2 cells
The chemokine IL-8	

IL-8	Modulates neutrophil production in the steady state (potential feedback inhibitor of that lineage)
	Stimulates neutrophil chemotaxis, exocytosis, respiratory burst, shape change, adhesion molecule expression, and complement receptor type 1 expression
	Stimulates T-lymphocyte chemotaxis
	Stimulates basophil chemotaxis, histamine release, and leukotriene release
	Stimulates endothelial cell chemotaxis and proliferation
	Stimulates angiogenesis

Figure 14-1 Class I cytokine receptors. This family includes receptors for EPO, TPO, GM-CSF, G-CSF, LIF, prolactin, growth hormone, and interleukins 2, 3, 4, 5, 6, 7, 9, 11, 12, and 15. Shown here are the two components of the high-affinity IL-6 receptor, IL-6R and gp130. Regions of homology are found both in the extracellular and cytoplasmic regions. The conserved extracellular regions of class I receptors contain fibronectin type III regions (rectangles), which contain the ligand binding pockets, four conserved cysteine residues (double lines), and a Trp-Ser-any-Trp-Ser (WSXWS) box (black bar), each of which is essential for receptor function, although in the unique case of the erythropoietin receptor, recent saturation mutagenesis of the WSXWS box revealed no mutation that interdicted signaling. Cytoplasmic domains contain conserved residues as well in regions termed box-1 and box-2 located close to the membrane. Three members of the family, gp130, G-CSFR, and LIFR, also have a third homologous region, box-3. Binding of the ligand causes chain homodimerization or heterodimerization and tyrosine phosphorylation of cellular proteins. Because the members of this family lack their own kinase domains, they transduce signals by associating with cytoplasmic protein tyrosine kinases, often members of the Jak family. Jak molecules phosphorylate sites in the receptor complex that create docking sites for latent cytoplasmic molecules known as signal transducers and activators of transcription (STAT) molecules, which themselves become phosphorylated, dimerize, and move to the nucleus to serve as transcription factors that drive the biologic response. The JAK/STAT pathway is reviewed in Figure 14-5 . Almost all the hematopoietic growth factor receptors are type I cytokine family members. Exceptions include c- *kit* (CD117), the receptor for Steel factor, and M-CSFR, the receptor encoded by c-fms for M-CSF, both of which are receptor tyrosine kinases, and the chemokine IL-8, the receptor for which, located on chromosome 2q35, is a member of the seven transmembrane domain G protein-coupled receptor superfamily.

example, fibronectin type III domains are shared with several cell-adhesion molecules, suggesting that cytokine receptors evolved from ancestors whose products functioned largely to facilitate cellular adhesion to both substrates and other cells.

Another shared feature of many hematopoietic growth factor receptors is their ability to transduce signals that prevent programmed cell death (apoptosis), in some cases without inducing mitogenic signals. This is particularly true of the lineage-specific factors, EPO, M-CSF, IL-5, and IL-7, each of which is reviewed following. [9] [10] [11] [12] [13] Apoptosis is characterized by chromatin condensation at the edge of the nuclear envelope, followed by convolution and fragmentation of the nucleus in terminally differentiated cells or in undifferentiated cells. This process occurs after mild injury, during the aging process of terminally differentiated cells, with factor deprivation, or after exposure to mitotic inhibitors. [14] An emerging view holds that some factors largely transduce anti-apoptotic signals but that additional mitogenic factors are required for a full growth and differentiative response from progenitor cell populations. For example, Krantzs group [15] found that EPO greatly reduced apoptosis in highly purified colony-forming units-erythroid (CFU-E). They also reported that steel factor (SF) and insulin-like growth factor type 1 (IGF-1) served as mitogenic and differentiation-inducing factors, respectively. An alternative view is that the capacity of a given receptor complex to transduce a set of biologic signals depends on discrete functional domains in one or more of the receptor chains. In support of this notion is the observation that discrete sets of signaling pathways can be disrupted by mutagenesis without interdicting all functions of the receptor. [16]

The receptors for many hematopoietic growth and differentiation factors share peptide subunits with other receptors. This kind of swapping is seen in granulopoiesis and lymphopoiesis. For example, IL-5, GM-CSF, and IL-3 have unique low-affinity (-chain) receptors, but they share the same chain, [17] [18] and association of the two chains results in the formation of a specific high-affinity subunit capable of effective signal transduction (Fig. 14-2A). The role of each chain in signal transduction is now being carefully examined. For example, distinct regions within the cytoplasmic domain of the GM-CSFR chain have been identified. [19] A membrane proximal region is necessary for the chain to activate c- *myc* gene expression, while a more distal region, between residues 626 and 763, is required for activation of *ras*, raf-1, and mitogen-activated protein (MAP) kinase and induction of c- *fos* and c-*jun*. [19] Different shared subunits have also been identified for high-affinity complexes that serve as receptors for LIF, IL-6, ciliary neurotrophic factor (CNTF), IL-11, and oncostatin M [20] [21] (Fig. 14-2B). In this case, gp130 functions as a signal transducing component of the IL-6R/gp130, and CNTFR/LIFR/IL-11R:gp130 complexes. Given its capacity to function with so many chains, it isnt surprising that germline disruption of gp130 is incompatible with life. [22] The details of the stimulus-response coupling pathways are not yet known,

Figure 14-2 Receptor chain swapping. Three groups of hematopoietic growth factor receptors share some of their heteromeric receptor chains. **(A)** The heterodimeric IL-3, IL-5, and GM-CSF receptors each have unique chains, and each of them associate with a common beta chain (c) that increases the binding affinity of the complex when compared to the chains alone. **(B)** IL-6 interacts with a complex consisting of an 80 kDa binding subunit (IL-6R) and a second 130 kDa chain (gp130) that is also shared as the second partner with LIFR, OSMR, CNTFR (not shown), and the IL-11R chains. Each of the specific chains serves as a low-affinity binding subunit, the binding affinity of which increases substantially when in a complex with gp130. **(C)** Receptor complexes for many of the factors that influence lymphopoiesis are shared as well; indeed, there are six receptors that are structured this way: IL-2, IL-15, IL-4, IL-7, IL-9, and IL-13. The heterotrimeric IL-2 and IL-15 receptors share two chains.

Figure 14-3 Production of soluble growth factor receptor molecules. Differential splicing of functional (interacting of signaling) domains from high-molecular-weight RNA can result in the production of soluble receptor molecules. This is true for the ligand for c- *kit* (shown here) and the receptors for IL-4, IL-5, IL-7, G-CSF, EPO, and GM-CSF receptors. In some cases the soluble receptors can act as competitive inhibitors for ligand binding. In this figure, the soluble Steel factor molecule in reaction #1 derives from proteolysis of membrane-bound Steel factor at a major cleavage site (MCS). A slightly smaller protein derives from a transcript from which exon 6 has been spliced. This membrane protein has only an inducible cleavage site (ICS) and the released protein is slightly smaller. In the exon 6 + form, the exon6 encoded sequences are represented as a black box within the extracellular domain. The third soluble form is that which is synthesized naturally by mice with the Steel-Dickie mutation. These mutant molecules are not integrated into the membrane, and although the soluble form shows some biologic activity in vitro, these mice are anemic and have bone marrow failure.

but it has been reported that gp130 is phosphorylated on tyrosine residues on stimulation of cells with the ligand. [23] The most highly shared receptor system occurs in lymphopoiesis, in which six different cytokines, IL-2, 4, 7, 9, 13, and 15, share certain subunits and in which IL-2 and IL-15 share two common subunits (Fig. 14-2C). In many instances, soluble forms of the receptor are released, sometimes resulting from translation of differentially spliced mRNA (Fig. 14-3). Soluble forms have been described for IL-4, IL-5, IL-7, G-CSF, c- *kit*, EPO, and GM-CSF receptors.[24] [25] [26] [27] [28] Although the biologic meaning of this phenomenon is not yet fully understood, the soluble forms may act as competitive binding proteins for the ligand and, as is clearly the case for the soluble IL-4 receptor, [28] serve as natural in vivo antagonists for specific cytokines.

Receptors with Cytoplasmic Tyrosine Kinase Domains

Amino acid sequence analysis has permitted the categorization of three subclasses of a tyrosine kinase receptor family. Subclass 1 includes the neu/HER2 proto-oncogene and epidermal growth factor, subclass 2 the insulin and IGF-1 receptors, and type 3 the receptors for M-CSF, SF, Flt3/Flk2 ligand (FL), and platelet-derived growth factor (PDGF). C- *kit*, the flt3 gene, and c-*fms* are genes that encode three related hematopoietic growth factor receptors, the ligands for which are SF, FL, and M-CSF, respectively. Along with PDGF (A and B) receptors, they constitute a subclass of receptor tyrosine kinases with similar topologies. [29] All receptors with tyrosine kinase activity possess large glycosylated extracellular domains, a single transmembrane spanning region, and a cytoplasmic domain that contains one or more tyrosine kinase catalytic sites (Fig. 14-4). These three receptors will be discussed in greater detail following.

Type II Cytokine Receptors

The class II subgroup of cytokine receptors includes the interferon receptors and the receptors for tissue factor and IL-10. Each has an extracellular domain containing a region resembling the fibronectin III domain found in the type I receptors, [30] which may serve as the ligand binding site. While the signaling pathways for IL-10 are not fully elucidated, the interferon signaling pathways have been widely studied and are outlined in Figure 14-5 .

Protein Serine-Threonine Kinase Receptors

The 30 members of the transforming growth factor- (TGF-) superfamily bind to their receptors as homodimers. Although no member of this family of ligands is uniquely hematopoietic, certain of them have profound inhibitory effects on many lineages of cells and function to suppress growth and differentiation signals evolving from auxiliary cells as well. There are three TGF- receptors, a 53 kDa type I receptor (TbRI), a 75 kDa type II receptor (TbRII), and a type III receptor of 200 kDa. Binding of TGF- requires TbRII. Once binding occurs, TbRI associates with the complex and signals are transduced by activation of the serine-threonine kinase cytoplasmic domains of the receptor chains, which, in turn, phosphorylate cytoplasmic signaling molecules known as Smad molecules on serines. [31] For example, after ligation of TGF- receptors, Smad2 and Smad3 are phosphorylated and form heteromeric complexes with Smad4. Phosphorylated Ser465 and Ser467 in Smad2 seems to represent a recognition site for Smad4 binding. [32] As is the case with the signal transducer and activator of transcription (STAT) molecules, phosphorylated Smad complexes translocate to the nucleus, where they act as either inducers or repressors of transcription. The major influence of TGF- on hematopoietic cells is as a mitotic inhibitor. This cytokine governs the activity of cyclinCdk complexes by inhibiting Cdk4; inducing p27/Kip1, an inhibitor of cyclinCdk complexes; inducing p21/Cip1/Waf1, an inhibitor of cyclinECdk2 complexes; inducing p16INK4a and p15INK4b expression, inhibitors of Cdks 4 and 6; and repressing the phosphorylation of the retinoblastoma protein (Fig. 14-6). The importance of the TGF receptor family as braking factors for a variety of cells is clarified by the capacity of inactivated receptors to permit autonomous growth and the frequency

Figure 14-4 The receptor for Steel factor, c-*kit*, is a type III (PDGF-like) cytoplasmic tyrosine kinase receptor. **(A)** The extracellular domain contains no fibronectin-like modules or WSXWS motif, but has five immunoglobulin-like domains (oval-shaped). There are at least six potential sites (indicated with asterisks) for tyrosine phosphorylation in the cytoplasmic region, each of which may serve as a docking site for adaptor proteins containings SH2 domains. **(B)** When growth factors (in this case, Steel factor, SF) bind to this class of receptors, receptor chains dimer-ize. Dimerization induces autotransphosphorylation, which can enhance the catalytic activity of the tyrosine kinase domain. The phosphorylated sites can then serve as docking sites for a variety of adaptor proteins that serve as signal transducers. These adaptor proteins contain SH2 domains that specifically associate with proteins on phosphotyrosines and adjacent carboxyterminal residues. Other adaptor proteins include those with PTB domains (not shown) that differ structurally from SH2 motifs and bind to proteins on phosphotyrosines and adjacent amino terminal residues.

with which these receptors or their downstream targets are inactivated by somatic mutation in a variety of cancers. [33] [34] [35]

Chemokine Receptors

The chemokine receptor family includes receptors for a few factors relevant to blood cell production; IL-8 and mip-1a, for example, both serve, at least in part, as braking factors, slowing progenitor cell proliferation. The chemokine receptors are seven transmembrane-spanning G protein-linked receptors [36] that are divided into three families: or CXC, or CC, and or C.[37] The families are classified based on the variability in cysteine residues. The initial scientific investigation of these molecules focused to a substantial degree on their capacity to influence chemotaxis, but it has become clear that some of these factors (e.g., IL-8 and mip) may serve as feedback inhibitors of hematopoiesis. [38] [39]

Tumor Necrosis Factor Receptor (TNFR) Family

The TNFR family includes TNFR1, TNFR2, *fas*, CD40, nerve growth factor (NGF) receptor, CD27, CD30, and OX40. Each has one or more distinct biologic effects. Both TNF- and *fas* have the capacity to suppress hematopoiesis profoundly by triggering programmed cell death in progenitor cells. [40] [41] [42] [43] *Fas*-mediated apoptosis of stem cells and progenitors may represent a major pathophysiologic mechanism of bone marrow failure in idiopathic aplastic anemia [44] and certain inherited marrow

Figure 14-5 Class II subgroup of cytokine receptors. Shown here is the ligand-binding induced assembly of the IFN- receptor. Each member of this group has an extracellular domain containing a region resembling the fibronectin III domain found in the type I receptors, [30] which may serve as the ligand binding site. **(A)** Binding of the IFN molecule to the IFNR chain, with which jak-1 (Janus kinase 1) is associated, causes the association of with chains of the receptor (the chain is associated with jak-2). **(B)** The Jak-1/Jak-2 complex (dark oval with asterisk) phosphorylates tyrosines on the chain, one of which become a docking site for stat1. When stat1 docks, it too is phosphorylated on tyrosines, dissociates from the chain, and forms a homodimer, which is then transported to the nucleus to bind to activating sequences (GAS elements).

Figure 14-6 The TGF- receptor. There are three TGF- receptors, a 53 kDa type I receptor (TR-I), a 75 kDa type II receptor (TRII), and a type III receptor of 200 kDa (not shown). Binding of TGF- requires TRII. Once binding occurs, TRI associates with the complex and signals are transduced by activation of the serine-threonine kinase cytoplasmic domains (STK) of the receptor chains, which, in turn, phosphorylate cytoplasmic signaling molecules known as Smad molecules on serines. After ligation of TGF- receptors, Smad2 and Smad3 are phosphorylated and form heteromeric complexes with Smad4. Phosphorylated Ser[465] and Ser[467] in Smad2 represent a recognition site for Smad4 binding. Phosphorylated Smad complexes translocate to the nucleus, where they act as either inducers or repressors of transcription. The major influence of TGF- on hematopoietic cells is as a mitotic inhibitor.

Figure 14-7 The TNF-R family. The TNF-R family includes TNFRI, TNFR2, *fas*, CD40, NGF receptor, CD27, CD30, and OX40. Both TNF- and *fas* have the capacity to suppress hematopoiesis profoundly by triggering programmed cell death in progenitor cells. **(A)** *Fas*-mediated apoptosis in lymphoid cells involves the trimeric *fas* complex (containing a cysteine-rich region [CRR] in the extracellular domain and a death domain in the cytoplasmic region) and a set of signaling molecules, many of which are proenzymes. **(B)** After binding of the FasL, the death domains of *fas* and FADD associate, prompting the association of the DED domains of pro-caspase 8 (FLICE) and FADD. Association with FADD induces autocatalytic cleavage of procaspase 8 to produce caspase 8. Caspase 8 cleaves pro-caspase 1 to caspase 1 (IL-1 converting enzyme, ICE) which in turn cleaves pro-caspase 3 to caspase 3 (CPP32). Caspase 3 is responsible for proteolysis of a wide variety of structural cellular proteins, including lamin and poly-ADP ribose polymerase (PARP). **(C)** The TNF receptor can also cause the activation of caspase 8 and apoptosis, but because, in some cells, the death domain (DD) of TNFR1 also associates with TRADD, which activates TRAF2 (which in turn activates NF-B), TNF does not always induce apoptosis on its own (NF-B activation generally protects cells from apoptosis). **(D)** Ligation of more than one TNFR member may influence the apoptosis/survival response substantially. For example, in some cells exposed to TNF, simultaneous ligation of CD30 induces TRAF2 degradation, whic then disconnects the TNF receptor ann NF-B activation. Consequently, when cells are simultaneously stimulated through the TNFRI and CD30 molecules, the cell will undergo apoptosis.

failure states, including Fanconi anemia. [45] Unlike *fas*, TNFRs transduce complex signals via multiple pathways that in some cells induce programmed cell death and in others induce mesenchymal cells to secrete hematopoietic growth factors. The majority of these receptors contain Cys-rich repeats in the extracellular domains and have cytoplasmic portions containing 80 amino acid death domains required for transducing apoptotic signals and for NF B activation. Both TNFR1 and 2 have death domains, as does *fas*. Ligation of more than one TNFR member may influence the type of response substantially. For example, in some cells exposed to TNF, NF B is induced through the activation of TNF receptor associated factor 2 (TRAF2), and the cell survives (NF B protects some cells from TNF-induced programmed cell death[46]). However, ligation of CD30 induces TRAF2 degradation, which then disconnects the TNF receptor and NF B activation. Consequently, when cells are simultaneously stimulated through the TNFR1 and CD30 molecules, the cell will undergo apoptosis. [47] The TNF and *fas* receptors are shown in Figure 14-7 .

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HEMATOPOIETIC CONTROL

Erythropoiesis

Factors involved in effective production of red cells include IL-3, [48] [49] [50] IL-9, [51] [52] IL-11, [53] SF, IGF-1,[54] thrombopoietin (TPO),[55] and GM-CSF,[49] [56] [57] which induce proliferation of primitive erythroid progenitors (Fig. 14-8). Other factors might play a role as well, including angiotensin II. [58] Few of these factors induce erythroid cellular proliferation in the absence of the lineage specific factor EPO, which is the pivotal humoral factor that functions to prevent programmed cell death of the most committed erythroid progenitor cells and their progeny.[15] However, steel factor (SF) and IGF-1 have also emerged as unique regulators of erythropoiesis. [59] These three factors will be reviewed here.

Erythropoietin

Erythropoietin (EPO), an 18 kDa protein [60] (3439 kDa when fully glycosylated [61]) encoded by a gene on the long arm of chromosome

Figure 14-8 Humoral control of erythropoiesis. A number of growth factors control multipotential progenitor cell replication and primitive erythroid progenitor cell growth (BFU-E), but the two most lineage-specific differentiation and survival factors known are erythropoietin and IGF-1. While these two factors are sufficient for erythroid differentiation, the necessary factors seem to be Steel factor and EPO, because deficiencies of either of these factor results in anemia.

7,[62] is expressed largely by cells in the liver in embryonic life, [63] [64] cells of the kidney[65] and, to a lesser extent, liver[63] in adult life, and certain hepatoma cell lines. [66] The production of EPO is induced by hypoxia, a mechanism that may involve the initial activation [66] [67] of heme proteins that stimulate EPO gene expression.[67] EPO stimulates growth, survival (in part by inducing expression of Bcl-x(L) [68]) and differentiation[61] of erythroid progenitor cells and stimulates proliferation and RNA synthesis in more well-differentiated erythroid precursors as well. [69] The EPO receptor (EPO-R) genes, located on murine chromosome 9 and human chromosome 19, encode a classic type I cytokine receptor (Fig. 14-1) that utilizes the Janus activated kinase (JAK)/STAT pathway for signal transduction. [70] Specifically, tyrosine phosphorylation of the EPO-R creates docking sites for SH2 domain(s) in signaling molecules that include STAT2, STAT5, protein tyrosine phosphatases SH-PTP1 and SH-PTP2, and phosphoinositide 3-kinase (PI3 kinase). [71] [72] Subsequent association of EPO-R with SHP-1 terminates signaling.[73] Other signaling pathways, including those involving protein kinase C family members, may also be involved, and the

linkage of these pathways with the JAK/STAT pathway is unclear. [68]

The effects of EPO on progenitor cell proliferation are optimal in the presence of SF, and its effects on terminal erythroid differentiation are optimized by IGF-1. [15] EPO has been reported to stimulate various levels of megakaryocytopoiesis in vitro and platelet production in experimental animals, [74] and high-affinity EPO receptors have been reported in rodent megakaryocytes.[75] However, the physiologic relevance of these observations is unclear because the effect is not seen in serum-free cultures[76] or in cultures of cells enriched for progenitors [77] (and relatively free, therefore, of accessory cells). Moreover, in clinical trials, recombinant human EPO has shown no consistent effects on platelet counts.

EPO was the first hematopoietic growth factor to be identified experimentally, and the use of the recombinant protein has been shown to be effective in the management of anemia associated with renal failure.[3] [78] [79] Other clinical uses of EPO have also emerged. Patients with myelodysplasia, [70] [80] those with progressive anemia in the setting of cancer therapy,[81] and patients who refuse blood transfusions[82] are candidates for erythropoietin therapy, generally given subcutaneously, three times weekly, at 100 units/kg/dose. Patients with acquired immune deficiency syndrome (AIDS) with endogenous erythropoietin levels <500 IU/L need fewer red cell transfusions, have higher hemoglobin levels, and report an improvement in quality of life when treated with EPO. [83]

EPO

Chromosome: 7q21
Gene product: 3439 kDa
Produced by: kidney in adult, liver during development, hepatoma lines
Induced by: hypoxia
Receptor: class I cytokine receptor encoded by a single gene on human chromosome 19p13.3-p13.2 utilizing JAK2 and STAT5 for signaling
Bioactivity: see Table 14-2
Deficiency: anemia in humans; homozygous deletion of EPO or EPO-R in mice is lethal, resulting in embryonic anemia and death

Steel Factor

Steel factor (SF) is a highly glycosylated 2836 kDa protein encoded by a gene on the long arm of human chromosome 12q22. [84] The widely expressed gene gives rise, via alternate splicing, to two mRNA species, one containing exon 6 (exon 6+) and a second in which exon 6 has been spliced out (exon 6). [85] [86] The ratio of exon 6+ to exon 6 transcripts is approximately 3:1.[87] SF expression has been demonstrated in fibroblasts, bone marrow stromal (fibroblast-like) cells, vascular endothelial cells, and Sertoli cells, as well as in various embryonic tissues. [87] [88] [89] No inductive cytokines for SF expression have yet been convincingly documented, [87] although TGF- represses SF expression.[90] SF is elaborated in both membrane-bound and soluble forms, the latter resulting from protease cleavage of the exon 6+ transcript-encoded protein at an exon 6-encoded consensus cleavage site. [86] The membrane-bound and soluble forms of SF display equivalent bioactivity in

clonogenic assays in vitro.[85] However, the particular relevance of the membrane-bound form to normal hematopoiesis, gametogenesis, and pigmentation is clearly illustrated by mice homozygous for the *Sl*d allele, in which a genomic deletion gives rise to a soluble protein lacking the anchoring transmembrane and cytoplasmic domains.[86] [91] Although the soluble protein retains full biologic activity in vitro, these animals are anemic, sterile, and nonpigmented. [86] [91]

Identification of SF and its receptor, the c-*kit* gene product, resulted from years of study of mice carrying mutations in the dominant white spotting (*W*) or Steel (*Sl*) loci.[92] [93] These animals display defects of varying severity in hematopoiesis (macrocytic anemia and reduced numbers of stem cells and tissue mast cells), skin pigmentation, and fertility. Reciprocal marrow transplantation studies with *W, Sl*, and normal mice clearly demonstrated that the hematopoietic defect in *W* mice resides in progenitor cells, while the defect in *Sl* animals was in the hematopoietic microenvironment.[94] [95] Subsequently, *W* mutations were localized to the c-*kit* gene, which encodes a transmembrane protein of the type III tyrosine kinase class of growth factor receptors. [96] [97] In the hematopoietic system, the 145 kDa c-*kit* protein, encoded by a gene on human chromosome 4q11-34[98] [99] and murine 5D-E, is expressed on pre-colony-forming cell (CFC) progenitor cells, [100] CFCs,[101] mast cells and their precursors,[102] interstitial cells of the gastrointestinal tract, [103] and leukemic cell lines. Heterozygous defects in the human c-*kit* gene have been associated with several cases of the piebald trait, a dominantly inherited depigmented state reminiscent of the murine *W* trait,[104] [105] and certain mutations of c-*kit* have been associated with autonomous c-*kit* expression and malignant transformation in mesenchymal tumors of the gut.[103]

SF

Also known as: stem cell factor, kit ligand, mast cell growth factor
Chromosome: 12q22-24
Gene product: approximately 40 kDa
Produced by: fibroblasts, endothelial cells, bone marrow stromal cells, Sertoli cells, hepatocytes, various embryonic tissues
Induced by: expression is constitutive
Receptor: c-kit protein (CD117), a receptor tyrosine kinase encoded by a gene on human chromosome 4q11-q13 (the piebald locus) and murine chromosome 5 (the white spotting locus), certain mutations of which are oncogenic
Bioactivity: see Table 14-2
Deficiency: anemia, mast cell deficiency, reduced stem cell numbers (mice)

SF promotes the proliferation and differentiation of the most primitive hematopoietic progenitor cells (pre-CFCs) into committed progenitor cells (multipotential colony-forming units [CFU-GEMM] erythroid burst-forming units [BFU-E], granulocyte-macrophage colony-forming units [CFU-GM], and megakaryocyte colony-forming units [CFU-Mk].[100] [106] [107] Whether SF promotes self-renewal of pre-CFCs is controversial. While SF has no independent colony-stimulating activity, it acts synergistically with IL-3, GM-CSF, and erythropoietin to promote CFU-GEMM-, BFU-E-, and CFU-Mk-derived colony growth. [108] [109] [110] [111] SF promotes the survival, proliferation, and differentiation of mast cell precursors. [102] [112] In addition, SF is chemotactic for mast cells[113] and enhances the release of mast cell mediators such as histamine.[114] [115] SF administration in vivo induces a marked expansion in the compartment of committed hematopoietic progenitor cells and striking mast cell hyperplasia.[116] [117] Outside the hematopoietic system, appropriate developmentally regulated SF expression is necessary for normal melanocyte development and migration and gametogenesis.[118] Because SF has an influence on primitive hematopoietic cells of other lineages and on multipotential cells, [119] [120] some categorize this factor as an early-acting hematopoietic growth factor. In our

view, however, persuasive evidence that SF plays an essential role in erythropoiesis derives from the anemia that attends SF deficiency in mice, the capacity of SF to enhance DNA synthesis in isolated erythroid progenitor cells, [15] and findings that EPO-R and c-*kit* are co-expressed in erythroid progenitor cells [121] and that these receptors interact in erythroid cells. [122] [123]

Insulin-like Growth Factor

Insulin-like growth factors 1 and 2 [124] (located on chromosomes 12q22-q24.1 and 11p15, respectively [125]) are small peptide (70 and 67 residues, respectively) homologs of proinsulin.[126] Because the role of IGF-2 in hematopoietic cells is unclear, we will focus only on IGF-1. The major site of IGF-1 synthesis is the liver. IGF-1 has multiple effects on a wide variety of cells, and its receptor (IGF-1R) is expressed ubiquitously. The bioactivity of IGF-1 is largely mitogenic and is known to regulate the growth of both normal and malignant cells. [127] [128] IGF-1 knockout mice exhibit extreme embryonic and postnatal growth retardation, neurologic defects, and prenatal mortality.[129] Growth failure and neurologic deficits have also been described in humans with IGF-1 deficiency, [130] but complete IGF-1 deficiency is likely lethal. IGF-1R is a receptor protein kinase encoded by a 100 kb gene on human chromosome 15q26. [131] It is synthesized as a single precursor that dimerizes and is processed into alpha and beta subunits that form a heterotetrameric receptor complex. [132] The activated receptor phosphorylates the Crk adaptor protein, which associates with the guanine releasing proteins Sos and C3G in the *ras* signaling pathway.[133]

First noted by Sawada et al.,[134] the erythropoietic activity of IGF-1 was confirmed by Axelrads group,[135] using serum-free cultures of erythroid progenitor cells from adult human peripheral blood. They discovered that erythroid progenitor cells formed erythroid colonies in the presence of IGF-1, even in the absence of EPO. Moreover, the activities of EPO and IGF-1 overlapped because combinations of these factors resulted in clonal growth that was less than the sum of colony formation with each factor alone.[135] The same group also demonstrated that progenitors from polycythemia vera patients were two orders of magnitude more sensitive to IGF-1 than were normal cells,[136] and that the effect was transduced through the IGF-1R.[137] The effects of IGF-1 are both mitogenic and anti-apoptotic in erythroid progenitor cells,[54] and in IL-3-responsive cell lines as well. [138]

Insulin-like growth factors form complexes with IGF-binding proteins (IGFBP), a group of at least eight important regulatory proteins that bind to IGF-1 and/or IGF-2 differentially and influence their mitogenic activity. [139] Interestingly, certain IGFBP molecules stimulate erythroid colony growth, and plasma IGFBP levels seems to be at least four-fold higher in polycythemia vera patients than in normal volunteers. [140] For these reasons, and notwithstanding the myriad effects of IGF-1 and IGF-2 on many types of cells, we include IGF-1 as an important erythropoietic factor that shares biologic activities with both SF and EPO.

IGF-1

Also known as: somatomedin C
Chromosomal location: human chromosomes 12q22-q24.1
Gene product: 70 amino acids
Produced by: liver
Induced by: growth hormone
Receptor: receptor protein kinase family member, synthesized as single precursor (151 kDa, encoded by a gene on human chromosome 15q25-q26[124]) and processed into (80 kDa) and (70 kDa) subunits that form heterotetramers
Bioactivity: see Table 14-2
Deficiency: embryonic and postnatal growth retardation, neurologic deficits; [129] [130] homozygous inactivating IGF-1 lesions are lethal

Granulopoiesis

Neutrophils

As shown in Figure 14-9 , the production of neutrophilic leukocytes involves a variety of different factors, including GM-CSF, IL-3, M-CSF, and G-CSF. Other factors, including IL-11, SF, and FL, stimulate or enhance neutrophil clonal growth in vitro. Studies on G-CSF knockout mice indicate that the most significant lineage-specific factor for neutrophils is G-CSF. [141]

Granulocyte-Macrophage Colony-Stimulating Factor

GM-CSF is a glycoprotein of 1435 kDa [142] [143] (the molecular weight varies with the degree of glycosylation) encoded

Figure 14-9 Humoral control of granulopoiesis. A number of growth factors control multipotential progenitor cell replication, but production of specific phagocytic lineages depends on specific factors. G-CSF is necessary for neutrophil production. M-CSF is necessary for monocyte production. IL-5 is a strong growth and survival factor for eosinophils. The production of basophils and mast cells is less well understood, but the mast cell deficiency found in Steel factor-deficient mice suggests that Steel factor is a necessary factor for this process, which is also influenced by IL-3 and TGF-.

Figure 14-10 Lineage-specific growth and survival factors influence the functional activity of terminally differentiated cells of the same lineage. GM-CSF activates the functional activity of most phagocytes, including neutrophils. In fact, in GM-CSF-deficient mice, the major morbidity results from the absence of normal phagocytic function rather than a failure to produce phagocytes. M-CSF and IL-5 activate the function of monocytes and macrophages and eosinophils, respectively.

by a gene on the long arm of chromosome 5.[144] GM-CSF stimulates clonal growth of CFU-GEMM, BFU-E, CFU-Mk, CFU-GM, and eosinophil colony-forming cells (CFU-Eo),[145] [146] [147] [148] [149] [150] although some evidence suggests that the effect of GM-CSF on neutrophil colony growth is indirect [151] [152] (e.g., GM-CSF induces auxiliary cells to release neutrophil-specific growth factors [151]). GM-CSF activates the functional activity of most phagocytes, including neutrophils, [153] macrophages,[154] [155] [156] [157] and eosinophils,[158] [159] [160] demonstrating a recurrent theme in hematopoietic control; namely, that lineage-specific growth factors frequently activate the function of the terminally differentiated progeny (Fig. 14-10). In fact, in GM-CSF-deficient mice, the major morbidity results from the absence of normal phagocytic function rather than a failure to produce phagocytes.

The GM-CSF receptor is a type I cytokine receptor with a 44 kDa (CD116) chain and a 96 kDa (CDw131) chain. The receptor is expressed by mononuclear phagocytes, neutrophils, endothelial cells, eosinophils, and fibroblasts. The chain is shared with the chains for IL-3 and IL-5 (Fig. 14-2). The association of the and chains increases the binding affinity of the chain from about 5 nM to about 100 pM. [161]

GM-CSF administered therapeutically enhances the production of neutrophils, monocytes, and eosinophils. [162] In vitro, GM-CSF induces neutrophil, macrophage, and eosinophil colony growth, but there is no strict evidence that GM-CSF alone induces neutrophil differentiation in the absence of G-CSF. [151] In fact, the degree of neutropenia in G-CSF knockout mice is comparable to that of mice nullizygous for both G-CSF and GM-CSF. [163] The observation that white blood cell production in germ-free GM-CSF knockout mice is not perturbed indicates that GM-CSF has no role in steady-state white cell production. [164] Thus, it is likely that neutrophilic leukocytosis in recipients of GM-CSF reflects the capacity of GM-CSF to induce expression of other factors, especially IL-1, [165] [166] which induces expression of G-CSF[167] [168] by a variety of cell types. We have suspected that the putative effects of GM-CSF on eosinophil colony growth might also be indirect through the induction of IL-5 gene expression in auxiliary cells; while this may be the case, it appears as though GM-CSF is capable of activating Jak2 and prolonging the survival of eosinophils.[169] GM-CSF is used in clinical practice, and the indications for its use will be discussed following.

GM-CSF

Chromosome: 5q31.1
Gene product: 1828 kDa
Produced by: mast cells, T lymphocytes, endothelial cells, fibroblasts, and thymic epithelial cells
Induced by: TNF-, IL-1, Lipopolysaccharide (LPS), phorbol esters, calcium ionophore A23187
Receptor: heterodimer composed of a GM-CSF-specific subunit (CD116 on chromosomes Xp22.32, Yp11.3 in the pseudo-autosomal regions) and a subunit (CDw131 on chromosome 22q12.2-13.1) shared with high-affinity IL-3 and IL-5 receptors
Bioactivity: see Table 14-2
Deficiency: susceptibility to infections by obligate intracellular organisms, pulmonary lesions resembling pulmonary alveolar proteinosis

Granulocyte Colony-Stimulating Factor

G-CSF, an 18 kDa protein encoded by a gene on the long arm of chromosome 17,[170] stimulates the proliferation of granulocyte progenitor cells [171] [172] [173] [174] [175] and activates neutrophil function.[176] [177] [178] G-CSF is produced by a wide variety of mesenchymal cells under the influence of inductive factors such as IL-1, [168] endotoxin, [179] and TNF.[180] The 115 kDa G-CSF receptor is a member of the type I cytokine receptor family with one N-terminal immunoglobulin domain, [181] four fibronectin domains in the extracellular region, and a WSXWS motif necessary for signal transduction. [27] [181] [182] There are four forms of the receptor, which result from alternative splicing.[27] Ligand binding results in activation of Jak1, Tyk2, and Jak2 and phosphorylation of stat1, stat3, and stat5. [183] Jak1 is critical for stat activation and Jak2 and Tyk2 are dispensable.[184] Although some have argued that G-CSF has a proliferative influence on pluripotent stem cells, [185] [186] G-CSF-deficient mice show selective chronic neutropenia and have neutrophil counts only 20% those of control animals. [163] G-CSF receptor mutations may cause certain severe congenital neutropenic syndromes,[187] dogs that develop neutralizing antibodies to G-CSF develop neutropenia, [188] and the use of G-CSF in clinical situations has one dominant effect: the induction of neutrophil production and release. Consequently, this gene encodes the clearest neutrophilic granulopoietin yet discovered. Recombinant human G-CSF is widely used in clinical practice, and the indications for its use will be reviewed following.

G-CSF
Chromosome: 17q11.2-q12 *Gene product:* 18 kDa *Produced by:* monocytes, macrophages, endothelial cells, fibroblasts *Induced by:* IL-1, TNF-, endotoxin *Receptor:* G-CSFR (CD114 on chromosome 1p35-34.3), 89.5 kDa polypeptides, four forms each differing only at the C-terminus, probably generated by alternative splicing *Bioactivity:* see Table 14-2 *Deficiency:* neutropenia and failure to develop a neutrophilic leukocytosis response to infections

Granulopoietic Factors in Clinical Practice

G-CSF is one of the most widely utilized hematopoietic growth factors in practice today. G-CSF is approved for mobilizing stem cell traffic in the peripheral blood to support peripheral blood progenitor cell transplantation; the proof of this principle is the successful transduction of pluripotent stem cells obtained using

G-CSF mobilization by retroviral vectors.[189] When peripheral blood is compared with marrow as a source of stem cells for autologous transplantation, it has been shown to result in fewer red cell transfusions, earlier platelet recovery, earlier neutrophil recovery, and earlier discharge from the hospital. [190] G-CSF also reduces the duration of neutropenia after allogeneic and autologous transplantation [191] and is approved for use in patients with severe chronic neutropenia to prevent infectious complications. However, it is most commonly used today to reduce the morbidity of myelosuppressive chemotherapy.

The principal goal of granulopoietin therapies (either G-CSF or GM-CSF) in patients with bone marrow failure is to increase the leukocyte counts to reduce the risk of infection. Most clinical studies using either of these factors after administration of cytoreductive chemotherapy or radiation clearly demonstrate that while the neutrophil counts do decline on time, they recover faster. [192] In addition, neutrophil recovery is also faster (2 vs. 4 days) in patients treated for neutropenic fever with G-CSF.[193] While one would expect, intuitively, that this would reduce the number of patients at risk for infectious complications, studies from the Mayo Clinic, [193] Indiana University,[194] and St. Jude Childrens Research Hospital[195] did not demonstrate this. In other words, such therapy did not reduce the rate of hospitalization for febrile episodes, prolong survival, reduce the number of culture-positive infections, or reduce the costs of supportive care, whether given preemptively [194] [195] or to treat neutropenic fever. The role of either G-CSF or GM-CSF in the management of these common clinical conditions is still uncertain. It is very important to assess the potential for granulopoietin therapies to decrease the risk of infection per se, not to decrease the risk of neutropenic fevers. It is clear that the window of neutropenia is reliably more narrow in the treated population. Thus, it is equally clear, as shown by Crawford et al., [192] that the number of neutropenic fevers will be reduced even if the number of fevers is not. G-CSF is also used in neutropenic patients with AIDS, an issue that also requires further study. [196] [197] [198] G-CSF, when combined with EPO, is effective in the management of selected patients with myelodysplastic syndromes.[199]

GM-CSF has also been used effectively in bone marrow failure states, [162] [200] and to accelerate recovery after marrow transplantation[201] [202] and cytotoxic chemotherapy.[203] It is approved for use in the bone marrow transplant setting and to shorten the duration of drug-induced neutropenia in elderly patients with acute nonlymphocytic leukemia. The use of GM-CSF in the management of patients receiving cytotoxic antineoplastic therapy is associated with the same uncertainties as with G-CSF. The GM-CSF knockout mouse does not have neutropenia, so it is likely that GM-CSF has an indirect effect on the production of neutrophils and eosinophils. It is likely that GM-CSF accomplishes this by inducing IL-1 release, [204] which probably accounts for the incidence of myalgia, flushing, and fever in some patients who receive this agent.

Since most acute myeloid leukemic clones are responsive to GM-CSF, some investigators have hypothesized that the administration of GM-CSF either immediately before or during cytotoxic inductive chemotherapy might put more leukemic cells into cycle and enhance the susceptibility of the leukemic cells to chemotherapeutic agents. Results from a large European trial involving 253 young and middle-aged patients with newly diagnosed acute myeloid leukemia indicated no improvement in outcome.[205] GM-CSF has also been used in patients with AIDS. Although it reduces the incidence and severity of neutropenia in patients treated with interferon and zidovudine,[196] it should be used with antiretroviral therapy [196] because of its potential to enhance the human immunodeficiency virus-1 (HIV-1) load [206] in some patients by inducing proviral gene transcription in latently infected cells.

In summary, in selected cases, G-CSF or GM-CSF treatment is of substantial value and, no doubt, saves lives. However, considering the attendant costs of growth factor therapy, the routine use of G-CSF or GM-CSF to prevent infection in neutropenic cancer patients cannot be encouraged outside the setting of well-designed controlled clinical trials. For nontransplant patients not participating in such studies, it seems most rational to us to utilize these granulopoietic factors in patients undergoing cytotoxic chemotherapy only if the dose-intensity of the chemotherapeutic agents has a demonstrated impact on overall survival (e.g., Hodgkins disease, germ cell neoplasms) and one of the following three criteria apply:

1. The patient has developed, in prior rounds of therapy, potentially life-threatening complications of neutropenia (e.g., documented bacterial infection)
2. The potential for prolonged myelosuppression is high (e.g., HIV-1-seropositive patients [207] [208])
3. The patient has persistent neutropenia between cycles. [209]

Monocytes/Macrophages

Mononuclear phagocytes are phylogenetically the most primitive elements of the blood, being closely related functionally to the phagocytic coloemocytes of invertebrates.[210] [211] In vitro, both GM-CSF and M-CSF regulate survival and function of monocytes, but only M-CSF-deficient mice have monocytopenia and macrophage deficiency.[4] [212] A complete deficiency of mononuclear phagocytes is probably incompatible with life.

Macrophage Colony-Stimulating Factor

M-CSF is encoded by a gene originally assigned to the long arm of chromosome 5, [144] but subsequently reassigned to the short arm of chromosome 1.[213] M-CSF mRNA gives rise to two glycoprotein species (7090 kDa and 4050 kDa) as a result of alternative splicing [144] [214] and stimulates monocyte/macrophage proliferation.[172] M-CSF also activates secretory[215] and phagocytic [216] [217] function. M-CSF also regulates the genesis of osteoclasts and seems to regulate tissue remodeling [218] and placental function.[219] It may also have an auto- or paracrine role in a variety of cancers. [218] M-CSF can be secreted or expressed as an integral membrane glycoprotein on the surface of cells that express the gene. The mononuclear phagocyte is an essential regulatory cell for hematopoietic cells of many lineages. [220] It is, therefore, no surprise that osteopetrotic (op/op) mice with a naturally occurring M-CSF deficiency [4] [212] [221] routinely exhibit bone marrow failure. [212] As is the case with G-CSF for neutrophils and EPO for erythroid cells, M-CSF serves as the major survival factor for mononuclear phagocytes. [222] Specifically, marrow failure and osteopetrosis in M-CSF-deficient mice can be restored by enforcing expression of the anti-apoptotic protein Bcl-2 in mononuclear phagocytes of op/op mice. [222] Consequently, M-CSF augments monocyte survival, permitting them to respond to internal and external cues for their differentiation.

M-CSF
Also known as: CSF-1 *Chromosome:* 1p21-p13 *Gene product:* 4090 kDa *Produced by:* monocytes, macrophages, fibroblasts, epithelial cells, vascular endothelial cells, osteoblasts *Induced by:* IL-3, IL-4, TNF-, endotoxin *Receptor:* a 165 kDa cell-surface receptor tyrosine kinase, encoded by c-fms, a cellular proto-oncogene located on human chromosome 5q33-34, is the cellular homologue of the v-fms oncogene of the McDonough strain of feline sarcoma virus *Bioactivity:* see Table 14-2 *Deficiency:* severe deficiency of macrophages and osteoclasts, hematopoietic failure, osteopetrosis

The M-CSF receptor, the c-fms proto-oncogene product encoded by a gene on human chromosome 5q32-33 and murine chromosome 18, is a type III tyrosine kinase family member. On ligand binding, it forms homodimers that then activate intrinsic tyrosine kinase activity. Activation of the receptor serves to transduce survival, [222] activation, and growth signals to mononuclear phagocytes and their precursors, at least in part by activating c-cbl and protein tyrosine phosphatase-phi. [218]

Eosinophils

Eosinophil production depends on GM-CSF, IL-3, and IL-5, [223] the latter being the more cell-type-specific of the three, at least in granulopoiesis (IL-5 exerts effects in lymphoid cells too). Again, just as G-CSF and EPO are survival factors for their respective lineages, IL-5 prolongs the survival of eosinophils, [12] and incubation of human bone marrow cells in suspension culture with IL-5 induces production of a greater fraction of eosinophils. [224] [225]

Interleukin-5

Interleukin-5, [224] [226] [227] [228] [229] the gene for which is also located on the long arm of chromosome 5, [227] [229] serves as a final example of the notion that growth factors stimulate both proliferation of progenitors and function of the progeny (Fig. 14-10) IL-5 was known as T-cell-replacing factor and eosinophil differentiation factor. [224] [226] [228] IL-5 is produced by T lymphocytes induced by antigen, mitogens, and phorbol esters. [224] [226] [228] [230] [231] The high-affinity IL-5 receptor is a dimer composed of an IL-5-specific ligand-binding chain and a chain also common to the GM-CSF and IL-3 receptors. [17] [18] IL-5 activates cytotoxic T cells [232] and induces immunoglobulin secretion. [233] [234] IL-5 also induces eosinophil production in vitro [226] and activates the functional activity of eosinophils. [235] [236] [237] The role of IL-5 in the production and migration of eosinophils is clearly demonstrated by experiments in which anti-IL-5 antibodies inhibit parasite-induced eosinophilia in mice, [238] and in which mice with IL-5R chain deficiency also failed to mount an eosinophilic response to *N. brasiliensis*. [239]

IL-5
Also known as: T-cell-replacing factor, eosinophil differentiation factor *Chromosome:* 5q31.1 *Gene product:* 5060 kDa *Produced by:* T lymphocytes *Induced by:* antigen, mitogen, phorbol esters *Receptor:* heterodimer composed of IL-5-specific subunit (CDw125, human chromosome 3p26-p24) and subunit (CDw131, human chromosome 22q12.2-q13.1, shared with the high-affinity GM-CSF and IL-3 receptors) *Bioactivity:* see Table 14-2 *Deficiency:* failure to mount an appropriate eosinophilic response

Basophils

The production of basophils and mast cells is induced by IL-3 (reviewed following) and SF (reviewed previously), which seem to be sufficient in vitro to stimulate the production and viability of this cell type, although the relation between these factors and IL-4 (which some investigators find can also induce mast cell production in vitro) has not been clarified. [240] IL-3, capable on its own of inducing both eosinophils and basophils in suspension cultures of bone marrow, when combined with TGF- synergistically induces the specific outgrowth of basophils, at least in part because TGF- inhibits eosinophil production in IL-3-stimulated marrow. [241] The TGF- family is reviewed below under mitotic inhibitory factors.

Megakaryocytopoiesis

IL-3, [242] [243] IL-6, [244] [245] IL-11, [246] [247] [248] LIF, [249] SF, [111] [250] [251] and EPO [74] [252] have been reported to influence the production and/or maturation of megakaryocytes (Fig. 14-11). However, the most profound effects on platelet counts have been seen using TPO and IL-11; while there are likely other stimulators of platelet production, these are the best current candidates for therapeutic agents designed to enhance platelet production. [253] [254] In fact, IL-11 has been recently released for therapeutic use; unlike TPO, levels are consistently elevated in the serum of thrombocytopenic patients. [255]

Thrombopoietin

Thrombopoietin, a 36-kDa protein (6585 kDa when fully glycosylated) [256] encoded by a gene on the long arm of chromosome 3, [256] [257] is constitutively produced by a variety of organs/cell types including hepatocytes, proximal convoluted tubule cells of the kidney, bone marrow stromal cells, muscle, brain, and spleen cells. [258] [259] [260] [261] [262] The TPO receptor is the gene product of c-*mpl*, the human homolog of the murine myeloproliferative leukemia virus, encoded by a gene on human chromosome 1 (1p34) and the D band of murine chromosome 4. [263] The extracellular domain of c-mpl resembles the hematopoietic growth factor superfamily, [264] and the cytoplasmic domain is capable of signal transduction but, like the other members of the family, contains no protein kinase or phosphatase motifs. The TPO receptor can mediate proliferation without activation of the JAK/STAT pathway through the activation of phosphatidylinositol 3 kinase, which pathway is capable of phosphorylating Shc, Vav, and mitogen-activated protein kinase (MAPK) and Raf-1 and induction of c- *fos* and c-*myc* cells expressing the TPO receptor. [16]

Hepatocytes are the primary source of serum TPO in humans. Indeed, serum TPO levels are low to undetectable in patients with cirrhosis and thrombocytopenia and increase within 2 days after orthotopic liver transplantation. [265] [266] Circulating TPO binds via the c-mpl protein to platelets and to a

Figure 14-11 Humoral control of megakaryocyte and platelet production. The two humoral factors that influence this process directly include IL-11 (currently approved for clinical use) and

thrombopoietin (TPO), both of which induce more rapid recovery of platelets after bone marrow injury.

lesser extent megakaryocytes and is internalized and then degraded. [267] [268] [269] TPO levels are inversely related to the platelet count in patients with thrombocytopenia and can be decreased by platelet transfusion. [270] [271] [272] Serum TPO is also inversely related to megakaryocyte mass. [260] [273] The increase in TPO levels seen in thrombocytopenia does not appear to be due to any increase in TPO production but instead results from a decrease in TPO binding to platelets and/or megakaryocytes. [260] [269] [271]

Thrombopoietin stimulates the in vitro growth and differentiation of CFU-Mk, mature megakaryocytes, and even pro-platelets. [274] [275] [276] Megakaryocytes grown in the presence of TPO have increased ploidy. [276] [277] TPO primes mature platelets to respond to aggregation-inducing stimuli such as adenosine diphosphate (ADP), epinephrine, and thrombin and also increases platelet release of adenosine triphosphate (ATP) and thromboxane B_2. [278] [279] [280] In addition to its effects on stimulating in vitro megakaryocytopoiesis, TPO also synergizes with a variety of other hematopoietic growth factors (HGFs), especially SF, IL-3, and FL, to stimulate nonmegakaryocytic progenitor growth. [281] [282] For example, sorted individual CD34+CD38 cells have a limited proliferative response to TPO, but the addition of TPO to SF and FL increases proliferation of BFU-E, CFU-GM, and CFU-MIX by 1040 fold. The addition of IL-3, IL-6, and EPO did not significantly increase colony growth above that seen with the three-factor combination of TPO, SF, and FL. [283] [284] The addition of TPO increased clonal growth in response to SF, FL, IL-3, IL-6, and EPO by as much as 80%;. [283] The primary effect of TPO on these primitive progenitors seems to be in maintaining viability and suppressing apoptosis. [283] [284] [285] [286]

The in vivo role of TPO in regulating hematopoiesis has been demonstrated in mice nullizygous for TPO or its receptor. Such mice have marked thrombocytopenia (platelet counts 510% of normal) but have normal hematocrit levels, white blood cell counts, and peripheral numbers of neutrophils, lymphocytes, monocytes, and eosinophils. [287] [288] [289] The platelets in such knockout mice seem to function normally, demonstrating that TPO/c-*mpl* is not required for the production of normal platelets but that TPO is the primary regulator of platelet mass in vivo. Treatment of TPO ligand- or receptor-deficient mice with IL-6, IL-11, or SF increased platelet counts 0.351.2 fold. [287] TPO- or c-*mpl*-deficient mice had reduced numbers of megakaryocyte, erythroid, granulocyte-macrophage, and multilineage progenitors in bone marrow, peripheral blood, and the spleen. Treatment of TPO knockout mice with recombinant TPO increased the absolute number of bone marrow hematopoietic progenitors. [287] It is not known whether these mice have deficiencies in colony forming units-spleen (CFU-S) or pluripotent stem cells.

Thrombopoietin has been used to date in a limited number of human clinical trials. Treatment with a single dose or with daily doses of TPO results in a dose-dependent increase in platelet count associated with an increase in bone marrow megakaryocytes. [290] [291] [292] No effect of TPO on red cell or white cell counts has been found in these human studies. There are conflicting data on the effect of TPO on bone marrow progenitors, but it is clear that TPO treatment causes mobilization of hematopoietic progenitors. [290] [291] [292]

Preclinical and clinical studies to date have suggested several possible clinical uses for TPO. Although further study is needed, it seems likely that TPO will be useful in the treatment of radiation- or chemotherapy-induced thrombocytopenia, as in the setting of bone marrow transplantation or high-dose chemotherapy. [253] [293] [294] [295] [296] TPO may also be useful in mobilizing peripheral blood progenitors for use in transplantation. [290] [291] [296] Murine models suggest that TPO treatment of donors before stem cell harvest results in more rapid platelet recovery after transplantation. [294] [297] Other potential clinical uses of TPO include pretreatment of platelet apheresis donors to increase the collection yield and treatment of cirrhosis patients with thrombocytopenia due to TPO deficiency. [265] [266] Two human studies have examined the effect of TPO on ameliorating thrombocytopenia after chemotherapy. In a randomized, blinded, placebo-controlled trial, TPO and G-CSF or G-CSF alone was given after treatment with carboplatin and cyclophosphamide. Patients treated with TPO plus G-CSF had faster platelet recovery (day 17 vs. day 22) but no difference in the depth of the platelet nadir compared with patients receiving G-CSF alone. [296] In another randomized, double-blind, placebo-controlled study, TPO or placebo was administered to lung cancer patients being treated with carboplatin and paclitaxel. In the TPO-treated patients, the median platelet nadir was 188,000, versus 111,000 in the placebo-treated patients. The TPO-treated patients also had a faster recovery of platelets to baseline (14 days vs. 21 days) (Fig. 14-12). [253]

One concern in the clinical use of TPO has been the potential for peripheral blood platelet activation and iatrogenic thromboembolic complications. Studies in nonhuman primates and humans have not shown any evidence of platelet activation as assessed by platelet aggregation, ATP release, expression of the platelet activation marker CD62 (P-selectin), or expression of glycoprotein IIb/IIIa. [291] [292] [296] TPO treatment of baboons increased platelet deposition on segments of homologous endarterectomized aorta and vascular grafts in direct proportion to circulating platelet numbers but without significant fibrin accumulation. [296] The reports of a low incidence (<5%) of thrombotic complications in TPO-treated cancer patients suggest a need for further studies to define the risk factors for thromboembolic side effects of therapeutic TPO usage, [253] [296] but the prothrombotic potential of neoplastic cells in cancer patients must also be carefully considered as well.

Another unresolved concern is that TPO may induce myelofibrosis. Myelofibrosis and megakaryocytic hyperplasia are commonly linked, and while short-term TPO therapy does not cause myelofibrosis, [299] lethally radiated mice transplanted with murine marrow transduced with a retroviral vector expressing murine TPO cDNA developed a fatal myeloproliferative disorder that included myelofibrosis. [300] This approach is not a fair test of the long-term consequences of chronic TPO therapy because the levels of TPO were high and uncontrollable and because the model included lethal radiation. Nonetheless, it will be essential for us to rule out myelofibrosis as a complication of long-term therapy in the future.

TPO

Also known as: megakaryocyte growth and development factor (MGDF), *mpl* ligand
Chromosome: human 3q27-28, mouse chromosome 16
Gene product: 6585 kDa
Produced by: wide variety of somatic cells including those of bone marrow stroma, spleen, renal tubule, liver, muscle, brain
Receptor: c-*mpl*, chromosome 1p34
Bioactivity: see Table 14-2
Deficiency: marked thrombocytopenia but no anemia or leukopenia

Interleukin-11

The IL-11 cDNA was isolated from the primate bone marrow stromal cell line PU-34 using an expression cloning strategy based on the capacity of the encoded protein to stimulate the proliferation of a murine plasmacytoma cell line. [247] The 19 kDa protein is encoded by a gene on human chromosome 19. [301] IL-11 is produced by fibroblasts and bone marrow stromal cells, and its production is markedly increased by IL-1. [247] [302] [303] The IL-11 receptor is a type I cytokine receptor heterodimer consisting of IL-11R, structurally related to CD126 (IL-6R) and gp130, [304] [305] the latter being shared with the chains of LIF,

Figure 14-12 In vivo activity of thrombopoietic factors, thrombopoietin, and IL-11. **(A)** Thrombopoietin treatment of baboons. A truncated, pegylated version of human TPO (MGDF) or saline was given by daily subcutaneous injection for 14 days beginning 48 hours after a single dose of 120 mg/kg of 5-FU. [299] Administration of MGDF stimulated more rapid recovery of platelets (closed boxes) than saline alone (closed circles), although the depth of platelet nadir was not affected. The overshoot thrombocytosis seen in the MGDF-treated animals can be abrogated by shorter courses of treatment. Unfortunately, evidence that this particular formulation is immunogenic (neutralizing antibodies have developed in some treated patients) has resulted in a discontinuation of MGDF development. **(B)** IL-11, administrated at two different doses to patients following cancer chemotherapy, reduced the number of patients requiring platelet transfusion. All patients had received platelet transfusions because of severe thrombocytopenia following their previous cycle of chemotherapy. IL-11 (filled bars) or placebo (open bar) was administered subcutaneously beginning one day after

chemotherapy and continued for 1421 days or until the platelet count was 100,000 l.

oncostatin M (OSM), CNTF, and IL-6 (Fig. 14-2). As is the case with the IL-6 receptor, the IL-11R chain binds to IL-11 with low (kDa = 10 nM) affinity and does not activate a signaling pathway. When co-expressed with CD130, the heterodimeric receptor has high-affinity IL-11 binding activity (kDa = 400800 pM), and ligand binding sets off a signaling cascade. [306]

IL-11 is a pleiotropic cytokine with growth stimulatory effects that overlap those of IL-6 on multiple classes of lymphoid and myeloid cells. [303] [307] IL-11 is mitogenic for a number of murine plasmacytoma and hybridoma cell lines, [247] [303] enhances CD4+ T-cell-dependent proliferation of antigen-specific plaque-forming B cells, [247] [308] [309] and shortens the duration of G_0 of primitive hematopoietic progenitor cells, as measured in the blast cell colony assay of Ogawa et al. [310] [311] It acts synergistically with IL-3 or SF to support the clonal growth of erythroid (BFU-E and CFU-E) and primitive megakaryocytic (BFU-Mk) progenitor cells. [53] [105] [246] In combination with IL-3, IL-11 induces an upward shift in the ploidy values of cultured megakaryocytes. [312] In vivo, IL-11 administration stimulates megakaryocytopoiesis, increases peripheral platelet and neutrophil counts, and increases the numbers and cycling activity of all classes of committed hematopoietic progenitor cells. [248] [313] [314] IL-11 hastens hematopoietic recovery after treatment with cytotoxic agents or ionizing radiation and accelerates hematopoietic reconstitution after bone marrow transplantation. [314] [315] In mice, IL-11 stimulates multipotential progenitor cells but not stem cells, [316] but in combination with either SF or FL is capable of inducing in vitro expansion of progenitor cells and can maintain survival of hematopoietic stem cells for up to 21 days in vitro. [317] Taking these important bioactivities into account, it is surprising that IL-11R knockout mice have no hematologic defect, indicating that IL-11 is completely dispensable for hematopoiesis, at least in mice. [318]

Notwithstanding its dispensability, the clear influence of IL-11 on megakaryocytes underscores the potential therapeutic value of this cytokine in the management of thrombocytopenia and chemotherapy- or radiation-induced myelosuppression. Indeed, IL-11 has been approved for the prevention of severe thrombocytopenia and to reduce platelet transfusion requirements for patients receiving cytotoxic chemotherapy for nonmyeloid malignancies (Fig. 14-12B). Studies suggest that IL-11 may act as an autocrine growth factor for certain megakaryoblastic cell lines. [319] Outside the hematopoietic system, IL-11 stimulates hepatic production of acute-phase reactant proteins, [320] suppresses adipogenesis and heparin-releasable lipoprotein lipase activity in murine pre-adipocytes, [302] and, like its homolog IL-6, stimulates neuronal differentiation; however, its most substantial nonhematopoietic function, at least from the standpoint of future therapeutic potential, is its capacity to stimulate recovery of gastrointestinal epithelial cells after cytotoxic therapies. [321]

IL-11

Also known as: adipogenesis inhibitory factor
Chromosome: 19q13.3-13.4
Gene product: 1924 kDa
Produced by: fibroblasts, bone marrow stromal cells
Induced by: IL-1, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187
Receptor: heterodimer consisting of the 43 kDa IL-11R chain encoded by a gene on human chromosome 9p13 and the CD130 (gp130) molecule (Fig. 14-2 **B**) encoded by a gene on chromosome 5q11
Bioactivity: see Table 14-2
Deficiency: no hematologic defect detected [318]

Figure 14-13 Humoral control of B lymphopoiesis. Some of the many factors involved in regulating the growth and differentiation of lymphoid cells are sufficient to induce growth or differentiation or both. However, most of the factors act synergistically; some always need help (IL-6 induces B-cell differentiation only in the presence of IL-2, and IL-10 enhances pro-B-cell differentiation only in the presence of flt3 ligand and IL-7).

Lymphopoiesis

The growth and development of lymphoid cells from the common lymphoid progenitor [322] occurs in multiple anatomic locations, where different factors may influence these processes. Many of the hematopoietic growth factors and interleukins have been shown to play a role in the growth and development of B cells (Fig. 14-13) and T lymphocytes. Natural killer cell control is less well understood, although the role of IL-12 and IL-15 in this process seems clear.

Interleukin-7

Interleukin-7 is a true lymphopoietic factor. A 17 kDa protein encoded by a gene on human chromosome 8q12-13, [323] IL-7 is produced by bone marrow stromal cells [324] and intestinal epithelial cells [325] and binds to a heteromeric receptor that shares a common chain with the receptors for IL-2, 4, 7, 9, and 15 (Fig. 14-2C). The IL-7R chain (CD127) is a type I cytokine receptor expressed on bone marrow lymphoid progenitor cells, thymocytes, mature T cells, and mononuclear phagocytes. The biologic activity of IL-7 is heavily weighted toward the lymphoid lineage, inducing the clonal growth of normal pre-B cells, [324] [326] [327] pre-T cells, [327] and various types of neoplastic lymphoid cells. [328] [329] It enhances differentiation of T-cell-receptor- cells selectively to the CD8+ subset. [330] It acts synergistically with flt3-ligand and IL-10 to augment the growth of primitive B lymphocytes. [331] Like other hematopoietic growth factors, it can act indirectly as well by enhancing the production of other growth factors (IL-3 and GM-CSF production by activated T cells [332] and IL-6, IL-1, TNF-, and IL-8 production by peripheral blood monocytes, [333] [334] for example).

The clear lymphopoietic function of this cytokine is best clarified by findings of lymphopenia and severe combined immunodeficiency in IL-7 [335] or IL-7R [336] knockout mice. Mutant mice have lymphocytopenia and reduced B-cell and T-cell numbers in lymphoid organs, including the spleen and thymus. Marrow B cells could not move from the pro-B stage to the pre-B stage. The lymphoid cells that do exist in these mice respond normally to mitogenic stimuli [335] but have functional defects, including cell-mediated immunity against allogeneic tumor cells. [336] Failure of aging stromal cells to release IL-7 when cocultured with IL-7-dependent murine B cells may serve as a model for B-cell failure in aging. [337] As with most lineage-specific factors, a major biologic effect of IL-7 on lymphoid cells is to prevent programmed cell death. In the case of IL-7, this was confirmed by demonstrating that Bcl-2 can rescue T cells in IL-7R / mice. [338] Finally, IL-7 acts synergistically with earlier-acting factors, in particular FL, which when combined with IL-7 very potently promotes B-cell commitment and differentiation of multipotential murine bone marrow progenitor cells. [339]

IL-7

Also known as: pre-B cell CSF
Chromosome: 8q12-13
Gene product: 17 kDa
Produced by: marrow stromal cells, spleen, and thymus tissue
Receptor: class I cytokine heterodimeric receptor, unique 49 kDa chain (CD127, chromosome 5p13) and a 40 kDa common chain, c (CD132, chromosome Xq13), which the chain shares with those of IL-2, IL-4, IL-9, IL-13, and IL-15
Bioactivity: see Table 14-2
Deficiency: lymphopenia and severe reductions in B-cell and T-cell cellularity of all lymphoid organs including nodes, spleen, marrow, and thymus

Interleukin-2

Interleukin-2 is encoded by a gene on chromosome 4.[340] The 23 kDa gene product is produced by T lymphocytes induced by mitogens, antigens, certain antibodies, phorbol esters, and lectins.[341] [342] [343] The IL-2 receptor (IL-2R), a heterotrimer of 55 ()-, 75 ()-, and 64 ()-kDa subunits, [161] [342] [344] is expressed by T cells,[345] B cells,[346] [347] [348] and natural killer (NK) cells.[349] The biologic activities of IL-2 include induction of growth and activation of T lymphocytes, [341] [342] [345] B lymphocytes,[346] [347] [348] and NK cells[349] both in vitro and in vivo. Intense interest in IL-2 and IL-2-stimulated lymphocytes as effectors of clinical tumor cytotoxicity have provided unambiguous evidence of in vivo immunomodulatory activity of IL-2 in humans.[350] [351] [352] [353] However, it is equally clear that this cytokine is not required for lymphopoiesis per se because IL-2 knockout mice do not suffer lymphopenia or immune deficiency;[354] in fact, the mice develop a syndrome of generalized inflammatory disease that involves multiple organs,[355] [356] often with severe fatal colitis. These surprising results indicate that IL-2 functions most prominently as a modulator or negative regulator of the immune response, and its absence, marked by uncontrolled activation and expansion of CD4+ T cells, [355] disrupts the management of self-tolerance.[355] [356]

IL-2

Also known as: T-cell growth factor
Chromosome: 4q
Gene product: 23 kDa
Produced by: T lymphocytes
Induced by: mitogens, antigens, some antibodies, phorbol esters, lectins, and IL-1
Receptor and subunits: // heterotrimer on T lymphocytes, B lymphocytes, and NK cells. (CD25, chromosome 10p14-p15); (CD122, chromosome 22q11.2-q13); (CD132, chromosome Xq13)
Bioactivity: see Table 14-2
Deficiency: generalized fatal immunoproliferative disorder involving multiple organs; loss of self-tolerance

Responses induced by IL-2 vary among lineages of cells bearing the receptor because permissible gene programs in those cells are different; some of them are also lineage-specific. Each chain of the trimeric IL-2 receptor has been fully characterized. The (p75) chain and the (p64) chain are shared with the receptor for IL-15 (Fig. 14-2C), but the IL-2R chain is unique (as is the IL-15R chain).[357] [358] [359] There is no evidence that differential expression of intermediate- or high-affinity receptors accounts for lineage-specific responses.

Interleukin-15

Interleukin-15 is a 14 kDa polypeptide produced by a variety of cells, including monocytes, macrophages, epithelial cells, muscle cells, fibroblasts, keratinocytes, and hematopoietic stromal cells.[360] [361] The gene for human IL-15 maps to chromosome 4q31, in close proximity to the IL-2 gene (4q26-27).[362] IL-15 and IL-2 exert overlapping effects in lymphopoiesis despite a lack of significant homologies between the IL-2 and IL-15 coding sequences.[361] [362] Instead, the overlapping activities result from receptor homologies and shared domains. The receptors for IL-15 and IL-2 share two subunits (the IL-2R and common chain). Murine and human IL-15-specific subunits have substantial homology (including intron-exon organization) with IL-2R.[359] These two cytokines also swap two receptor subunits. The IL-15 receptor complex is a heterotrimeric complex consisting of an IL-15-specific subunit and the IL-2R and IL-2R subunits.[357] [361] As noted previously, the IL-2R subunit is also used in the receptor complexes for IL-2, IL-4, and IL-7.[363] Not surprisingly, IL-2 and IL-15 induce many of the same biologic responses. IL-15 release is induced by ultraviolet light, Bacillus Calmette-Guerin (BCG), LPS, IL-10, and infection by intracellular organisms.[360] [364] [365] It protects multiple cell types, including T cells and hepatocytes, from apoptosis in vivo[366] resulting from either factor deprivation or fas-dependent programmed cell death. It induces maturation of human natural killer (NK) cells from thymic progenitors.[367]

IL-15, like IL-2, is a potent modulator of NK cell activity. IL-15, used as a single agent, induces differentiation of CD3-CD56+ NK cells from CD34+ hematopoietic progenitor cells. SF synergizes with IL-15 to increase this expansion without altering the differentiation state of expanded NK cells.[368] Indeed, recombinant IL-15 can replace bone marrow stromal cells in the in vitro generation of lytic NK cell activity and is 1050 fold more potent than IL-2.[369] IL-15 induces NK cell activity in peripheral blood mononuclear cell (PBMC) and splenocytes in vitro and in vivo and is chemotactic for NK cells.[361] [370] [371] NK cells are found in IL-2 / mice, whereas IL-2R / mice (lacking the capacity to transduce both an IL-1 and an IL-15 signal) have a marked reduction in circulating NK cells and a total absence of NK lytic activity in vitro.[372] [373] Likewise, in vivo treatment of mice with blocking antibodies to IL-2R results in the disappearance of NK cells. Thus, IL-15 may be required for the generation of functional murine NK cells.[374] Definitive testing of this hypothesis will require the generation of IL-15 / mice or the use of blocking antibodies to the IL-15-specific subunit of the IL-15 receptor complex.

IL-15, like IL-2, is a chemoattractant and growth factor for T cells. IL-15 induces the proliferation of memory (CD45RO+) CD4+ and CD8+ T cells and also of naïve (CD45RO) CD8+ but not naïve CD4+ cells.[375] IL-12 synergizes with IL-15 to increase the proliferation of memory CD4+ T cells.[376] Addition of IL-15 to mixed lymphocyte reactions augments the generation of cytotoxic lymphocytes (CTL) and lymphocyte-activated killer (LAK) cells, and treatment of mice with IL-15 generates CTL and LAK cell activity in vivo. IL-15 also augments antitumor responses in animal models where responses to IL-2 are also seen.[371] [377]

Like IL-2, IL-15 supports the proliferation and maturation of B cells and is costimulatory with anti-m antibody, phorbol ester, or CD40 ligand. B cells treated with IL-15 and CD40 ligand are induced to produce polyclonal IgM, IgG_1, and IgA antibodies, but not IgG_4 or IgE. Indeed, IL-15 can replace IL-2 as a costimulator of B cells and augments the in vitro production of antigen-specific antibodies.[378]

While the bioactivities of IL-2 and IL-15 are similar, there are several important differences between them. First, IL-2 is produced exclusively by activated T cells, whereas IL-15 is secreted by a much broader range of cell types, but not by T cells. The widespread production of constitutive and inducible IL-15 protein may serve to target immune effector cells to sites of peripheral inflammation and infection.[364] [379] Second, only T cells, monocytes, and B cells express mRNA for the IL-2R subunit, whereas expression of mRNA for the IL-15R subunit is fairly ubiquitous. However, it is not clear whether all the cells that express the IL-15R subunit can, under any circumstance, also co-express IL-2R and IL-2R subunits and assemble functional IL-15R complexes,[357] [380] so the biologic meaning of this difference is unclear. The effects of IL-2 are restricted to hematopoietic cells, whereas there is evidence that IL-15 can directly modulate some types of nonhematopoietic cells.[381] For example, IL-15 induces tryosine phosphorylation of endothelial cell proteins in vitro and stimulates angiogenesis in vivo.[382]

IL-15 may be involved in the pathophysiology of certain autoimmune disorders. Elevated levels of IL-15 have been found in the serum and in peripheral blood cells of patients with inflammatory bowel disease and in the synovial fluid of patients with rheumatoid arthritis.[383] [384] TNF-, a central mediator of the inflammation in rheumatoid arthritis, is released by macrophages induced by contact with IL-15-activated T cells. Freshly isolated T cells from rheumatoid synovial fluid also can induce macrophage TNF-, but exogenous IL-15 is required for sustained production of TNF-.[383] Thus, IL-15 may be critical in maintaining synovial inflammation in patients with this disease, and agents designed to modulate IL-15 production may have therapeutic potential for rheumatoid arthritis and other autoimmune disorders.[383]

IL-15

Also known as: IL-T
Gene: human chromosome 4q31 (in close proximity to IL-2 [4q26-27])
Gene product: 14 kDa
Produced by: monocytes, macrophages, epithelial cells, skeletal muscle cells, bone marrow and thymic stromal cells[361]
Induced by: ultraviolet light, BCG, LPS, IL-10
Receptor: heterotrimeric complex consisting of IL-15 specific subunit (chromosome 10p14-p15), IL-2R (CD122, 70 kDa, chromosome 22q11.2-q13), and IL-2R (CD132, 64 kDa, chromosome Xq13) subunits
Bioactivity: see Table 14-2

IL-15 may also function to preserve immune function in HIV-1-seropositive patients. HIV-1 infection results in a decrease in Th1 cells, with an associated loss of IL-2 production. Consequently, peripheral production of IL-15 may be responsible for the maintenance of cell-mediated immunity during the early phases of infection. [379] IL-15 stimulates the NK activity of peripheral blood mononuclear cells in HIV-1-infected patients, enhances mitogen- and antigen-independent lymphocyte proliferation, restores PBMC production of IL-12, augments microbicidal activity of monocytes against *Candida albicans*, [385] and stimulates the expansion of HIV-specific CTL.[386] [387] Is there therapeutic potential for IL-15? IL-15 treatment of mice produces fewer side effects than with IL-2 administration. [371] However,

this finding has not been confirmed in humans. The clinical utility of IL-15 in patients with HIV-1 infections or cancer will depend on an acceptable side effect profile and induction of in vivo responses similar to those produced in vitro.

Interleukin-4

Interleukin-4 was formerly known as B-cell stimulatory factor-1, B-cell differentiation factor, T-cell growth factor-2, and mast cell growth factor-2. The IL-4 gene resides on the long arm of chromosome 5[229] [388] [389] and encodes an 18 kDa protein[390] [391] produced by T lymphocytes induced by phorbol esters, lectins, and certain antigens.[392] There are two receptors for IL-4. The first is a heterodimeric member of the cytokine superfamily consisting of CD124 (87 kDa, chromosome 16p11.2-p12.1) and CD132 (40 kDa, chromosome Xq13). The second consists of CD124 associated with IL-13R (Fig. 142 **C**). [393] IL-4 induces proliferation of activated B cells,[394] T cells,[395] mast cells,[240] and fibroblasts.[396] It induces expression of its own receptor in B cells and T cells, [397] [398] induces isotype switching to IgG and IgE in activated B cells,[234] [399] induces expression of the IL-2 receptor in T cells exposed to phorbol esters, [400] suppresses IL-2-induced B-cell proliferation, [401] [402] and suppresses IL-2-induced IgM secretion.[403] IL-4 can influence nonlymphoid lineages. It induces expression of M-CSF and G-CSF genes in human monocytes[404] and induces expression of TNF- and an inhibitor of hematopoiesis (they may be the same factor) in mixed murine marrow stromal cells. [405] The effects of IL-4 on lymphopoiesis seem most clear when acting in synergy with other factors (Table 14-3).

IL-4 deficient mice are viable and have defective T_H2 cytokine production after nematode infections. IL-4 may be required for induction of optimal gut mucosal antibody responses,[406] although compensatory mechanisms do exist for infections caused by specific challenges, such as *Salmonella* or cholera toxins. [407] In some cases IL-4-deficient mice are more resistant to infectious agents, including schistosomes [408] and immunodeficiency virus. [409] Although IL-4 is capable of inducing IL-9 gene expression, IL-9 secretion in IL-4 knockout mice is only partly suppressed, indicating that IL-9 release is under the control of other factors as well. [410]

IL-4

Also known as: B-cell stimulatory factor-1, B-cell differentiation factor, T-cell growth factor-2, mast cell growth factor-2
Chromosome: 5q23-q21
Gene product: 18 kDa
Produced by: T lymphocytes (both CD4+ and CD8+)
Induced by: phorbol esters, calcium ionophore A23187
Receptor: heterodimeric member of the cytokine receptor superfamily; 87 kDa chain (CD124, chromosome 16p11.2-p12.1) and the 40 kDa c chain (CD132, chromosome Xq13), which is shared with the complex receptors for IL-2, IL-7, IL-9, and IL-15
Bioactivity: see Table 14-2
Deficiency: defective T_H2 cytokine responses, resistance to murine retroviral immunodeficiency syndrome

Interleukin-10

Human IL-10 is an 18 kDa protein[411] that is expressed as a noncovalent homodimer[412] and is encoded by a gene on chromosome 1.[413] The IL-10 receptor is a 90110 kDa class II cytokine receptor family member (Fig. 14-5), consisting of two extracellular fibronectin type III domains but no WSXWS motif and a 318 amino acid cytoplasmic region. IL-10 is truly pleiotropic. It was originally identified as a cytokine elaborated by murine

TABLE 14-3 -- Interleukins, Interferons, and Hematopoietic Growth Factors: Examples of Synergy

1.IL-2, IL-4, and IL-5 synergistically stimulate immunoglobulin isotype switching in B lymphocytes. [346]
2.IL-1 and TNF- stimulate prostaglandin production by fibroblasts. [775]
3.TNF- and IFN- synergistically suppress hematopoiesis in vitro. [645]
4.IL-1 and IL-2 induce interferon production in T lymphocytes. [776]
5.IL-3 and M-CSF stimulate proliferation of murine progenitor cells in clonal assays. [777]
6.IL-1 and TNF- induce differentiation of murine myeloid leukemic cells. [778]
7.IL-4 and IL-2 induce T-lymphocyte proliferation. [779]
8.IL-3 and G-CSF induce proliferation in multipotential progenitor cells. [780]
9.IL-6 and IL-1 activate T lymphocytes. [781]
10.IL-3 and both G-CSF and GM-CSF synergistically stimulate granulocyte and granulocyte-macrophage colony growth. [782]
11.IL-4 and IL-6 induce T-lymphocyte proliferation. [615]
12.IFN- and TNF- induce G-CSF and GM-CSF release from T lymphocytes. [783]

13.IL-3 and IL-2 stimulate T-cell proliferation. [529]
14.GM-CSF and IFN- induce TNF- gene expression by monocytes.[784]
15.IL-3 and EPO stimulate BFU-E growth in vitro.[524]
16.G-CSF and IL-3 synergistically induce megakaryocyte colony growth. [785]
17.IL-1 and TNF- induce IL-1 gene expression in vascular endothelial cells. [786]
18.M-CSF and IL-6 induce macrophage colony growth of human marrow cells.[787]
19.GM-CSF and IL-6 promote granulocyte differentiation. [617]
20.IL-1, IL-3, and M-CSF synergistically support clonal growth of macrophages [788] and primitive myeloid progenitor cells. [789]
21.IL-6, IL-3, EPO, and IL-4 support erythroid and megakaryocytic colony growth. [614]
22.IL-9 augments EPO-induced BFU-E growth,[790] IL-4-induced immunoglobulin production by B lymphocytes, [791] [792] and IL-3-induced growth of murine mast cells. [604]
23.IL-11 acts synergistically with IL-3 and SF to stimulate clonal growth of erythroid (BFU-E and CFU-E) and megakaryocytic (BFU-Mk) progenitor cells. [246] [247]
24.IL-12 synergizes with TNF- to stimulate IFN- production by NK cells. [418]
25.SF acts synergistically with IL-3, GM-CSF, and EPO to support optimal clonal growth of hematopoietic progenitor cells of all lineages [793] [794] [795] and also synergistically functions with IL-6 to augment CFU-S [119] and with IL-9 to stimulate proliferation of the human leukemic cell line MO7E. [796]
26.TPO synergizes with G-CSF in mobilizing hematopoietic progenitor cells in primates. [580]
27.TPO synergizes with SF and IL-3 in vitro to support proliferation of primitive hematopoietic cells. [282]
28.IL-1 and IFN- synergize to induce mip1 gene expression in fibroblasts. [797]
29.Simultaneous administration of FL and G-CSF increases peripheral blood CFU 12- to 20-fold over that seen with either agent alone. [583]
30.TPO and EPO synergistically enhance self-renewal of CFU-E and BFU-E in vitro. [798]
31.FL synergizes with G-CSF to mobilize peripheral blood stem cells. [582]

T_H 2 clones that inhibits the production of cytokines such as interferon-gamma (IFN-) by T_H 1 clones.[414] IL-10 is expressed by all classes of CD4+ T cells, CD8+ T cells, mononuclear phagocytes, and activated B cells. [412]

While IL-10 is in certain circumstances a mitogen, the overall effect of IL-10 on immune function is suppressive. IL-10 knockout mice challenged with *Aspergillus fumigatus* have an exaggerated immune response, including increased release of IL-4, IL-5,

and IFN- and a higher than normal mortality rate. Similar results are found in the same murine model challenged with *Toxoplasma*. [415] Some strains of IL-10-deficient mice also develop chronic enterocolitis, followed by carcinoma of the colon. [416] These findings confirmed earlier studies that first characterized IL-10 as cytokine synthesis inhibitory factor (CSIF) because of its capacity to inhibit the production of cytokines by T lymphocytes. [414] Whereas the murine protein seems to inhibit specifically the production of cytokines (IL-2, IFN-, and lymphotoxin) by T_H 1 cells,[414] [417] human IL-10 inhibits, in addition, the production of T_H 2-type cytokines (IL-3, IL-4, and IL-5)[412] and NK cell-derived cytokines (IFN- and TNF-). [418] [419] [420] This effect is largely indirect, reflecting IL-10-induced inhibition of mononuclear phagocyte antigen-presenting cell function (due, in part, to downregulation of MHC class II antigen expression). [421] [422] In systems in which cells other than mononuclear phagocytes, such as B cells, present antigen, IL-10 is devoid of CSIF activity. IL-10 inhibits mononuclear phagocyte-dependent T-cell proliferation [421] [422] but can also directly inhibit the proliferation of highly purified T cells (due primarily to suppression of IL-2 production). [423] IL-10 is a potent inhibitor of mononuclear phagocyte function, suppressing MHC class II antigen expression, [421] monokine (IL-1, IL-1, TNF-, IL-6, IL-8, G-CSF, GM-CSF, and IL-10 itself) [424] [425] synthesis, and production of reactive oxygen species and NO.[426] [427] As a result of its inhibitory effects on T_H 1-type immune functions, IL-10 appears to play an important role, at least in murine models, in the maintenance and progression of infections by obligate intracellular pathogens that infect macrophages (e.g., *Listeria, Schistosoma mansoni*, and mycobacteria).[428] [429] [430] In such infections, T_H 1-derived cytokines and efficient macrophage function may be crucial in reducing the microbial burden. [431]

Human IL-10 can act as a co-stimulator (e.g., with IL-4) of human B-lymphocyte proliferation. [432] It also plays a major role in regulating B-lymphocyte development in different ways at different differentiation stages. Specifically, IL-10, in concert with FL and IL-7, enhances the generation of CD19 ProB cells from uncommitted bone marrow progenitors but functions to inhibit growth of CDE19+ ProB cells.

IL-10

Also known as: cytokine synthesis inhibitory factor
Chromosome: 1q31-q32
Gene product: 18 kDa
Produced by: T cells, activated B cells and B-cell lymphomas, mononuclear phagocytes, keratinocytes
Induced by: LPS, anti-CD3, PMA
Receptor: class II receptor family, 90110 kDa, human chromosome 11
Bioactivity: see Table 14-2
Deficiency: exaggerated immune responses to obligate intracellular parasites, inflammatory bowel disease

Interleukin-12

Interleukin-12 is a 75 kDa heterodimer composed of disulfide-linked 35 and 40 kDa (p35 and p40, respectively) subunits. [433] [434] [435] Isolation of their respective cDNAs demonstrated that p35 and p40 are encoded by distinct genes and that expression of both is necessary for the production of the biologically active molecule. [436] [437] p35 bears minor sequence homology with IL-6 and G-CSF, while p40 demonstrates homology with the soluble IL-6 receptor, leading to the suggestion that IL-12 represents a cytokinesoluble receptor complex.[438] [439] The two subunits of the IL-12 receptor are IL-12RB2 (human chromosome 1p31.2) and IL-12B1 (human chromosome 19p13.1).[440] The IL-12RB1 chain contains five fibronectin III domains and has a high degree of homology to CD130. [441]

Interleukin-12 expression was originally demonstrated in Epstein-Barr virus (EBV)-transformed B-cell lines. [433] [435] Subsequently, mononuclear phagocytes were shown to be an important source of IL-12. A variety of pathogens or their products (LPS, *Staphylococcus aureus, Mycobacterium tuberculosis, Toxoplasma gondii, Listeria monocytogenes, Leishmania major*) induce IL-12 production by mononuclear phagocytes.[442] [443] [444] As suggested by its original name natural killer cell stimulatory factor, IL-12 has profound effects on NK cells. Exposure to IL-12 augments the functional activity of NK cells and induces LAK activity in NK cells. [433] [445] [446] [447] [448] IL-12 acts also synergistically with TNF- to induce IFN- production by NK cells. [418]

Recent studies have demonstrated the vital role of IL-12 in cell-mediated immunity. IL-12 induces the differentiation of naïve murine CD4+ T-helper cells into T_H 1 cells,[444] a process analogous to IL-4-induced differentiation of naïve T-helper cells into T_H 2 cells.[449] [450] IL-12-mediated generation of antigen-specific

IFN--producing T_H 1 cells is an essential component of the host response to infection by obligate intracellular pathogens, leading to reduction in the microbial burden in several experimental mouse models,[443] [444] [451] [452] and is also involved in the protection against malaria in rhesus monkeys treated, before challenge with sporozoites, with recombinant human IL-12.[453] Accordingly, it is not surprising that IL-12 knockout mice, viable and fertile, are immunologically compromised. Although they display no gross developmental abnormalities, the capacity of these mice to mount a T_H 1 response and to release IFN- in response to endotoxin is impaired, and murine strains ordinarily resistant to *Leishmania major* become sensitive to this organism when deficient in IL-12. [454] [455]

IL-12

Also known as: NK cell stimulatory factor, cytotoxic lymphocyte maturation factor
Chromosome: not reported
Gene product: 75 kDa heterodimer of 35 and 40 kDa subunits
Produced by: mononuclear phagocytes, EBV-transformed B-cell lines
Induced by: LPS, various pathogens
Receptor: single class of high-affinity receptors approximately 110 kDa
Bioactivity: see Table 14-2
Deficiency: unable to mount a T_H 1 response, cannot release IFN- in response to endotoxin, cannot resist leishmanial infection

Interleukin-13

Interleukin-13, a 10 kDa T_H 2 cytokine encoded by a gene on human chromosome 5q31 (in the cluster that also contains IL-3, IL-5, IL-4, and GM-CSF), [456] shares many biologic activities with IL-4. The similarity in biologic activity of IL-4 and IL-13 is probably due to shared signal transduction pathways and receptor structure. The IL-13 receptor complex is a heterodimer consisting of an IL-13-specific subunit, an IL-4-specific subunit, and possibly a third subunit. The c subunit shared by IL-2, -4, -7 , -9, and -15 is not required for IL-13 signal transduction. [457] Consistent with the current model of the IL-13 receptor, a mutant IL-4 protein has been described that competitively antagonizes the activity of both IL-4 and IL-13. [458] Both IL-4 and IL-13 regulate the functional activities of peripheral blood monocytes and bone marrow-derived macrophages. These include alterations in cell surface antigen expression, inhibition of antibody-dependent cytotoxicity, inhibition of nitrous oxide production,

down-regulating production of pro-inflammatory cytokines and chemokines (including IL-1, IL-1, IL-6, IL-8, macrophage inflammatory protein (MIP)-1, TNF-, IL-10, GM-CSF, G-CSF, and IL-12),[459] [460] and increasing production of the IL-1 receptor antagonist. [461] Additionally, IL-13 represses HIV-1 replication in alveolar- and peripheral blood-derived macrophages.[462] [463]

IL-13, like IL-4, increases B-cell expression of various lymphocyte antigens (including CD23, MHC class II and sIgM), enhances B-cell proliferation, promotes isotype switching to allow IgE expression, and enhances production of IgG_4 and IgM. These effects of IL-13 on B cells are seen only in the presence of additional stimuli such as CD4+ T cells and IL-7.[464] IL-13 shares with IL-4 the ability to inhibit IL-2-induced IFN- production by NK cells. Similar to IL-4, IL-13 induces endothelial cell expression of vascular cell adhesion molecule (VCAM)-1 but not that of E-selectin or intercellular adhesion molecule (ICAM)-1. IL-13 may help regulate VCAM-1-mediated accumulation of lymphocytes, basophils, and eosinophils, but not neutrophils at sites of inflammation. [465]

Although the actions of IL-4 and IL-13 are largely redundant, several distinctions can be made. First, IL-13, unlike IL-4, has no significant effect on T cells. [466] Second, the kinetics of IL-13 secretion following T-cell activation differ from those of IL-4. IL-13 production occurs earlier and persists significantly longer (by hours to days) than IL-4 production.[467] Third, IL-13, unlike IL-4, does not regulate pre-B-cell differentiation. This result is consistent with the hypothesis that functional IL-13R are expressed later in B-cell development than functional IL-4R. [468] Currently there is no knockout model of IL-13 deficiency.

IL-13 may have a role in the treatment of macrophage-mediated inflammatory conditions such as septic shock and some autoimmune diseases. In mice, in vivo treatment with IL-13 protects against LPS-induced lethal endotoxemia.[469] In a murine model of experimental autoimmune encephalitis, IL-13 suppressed the development of neurologic disease without altering T- or B-cell immunoreactivity. [470] Another clinical use for IL-13 may be in the treatment of HIV infection, possibly in combination with other immune modulators such as IL-16.[462] [471]

IL-13

Also known as: P600
Chromosome: 5q23-q21 (IL-4 and IL-13 are located within 12 kb in a head-to-tail orientation [472]
Gene product: isoforms of 9 and 17 kDa; 17 kDa probably represents an N-glycosylated isoform[473]
Produced by: T_H 2 T cells, basophils, stromal cells
Induced by: CD28 ligation,[473] Ionomycin, PMA,[474] anti-IgE[475]
Receptor: heterodimer consisting of IL-13-specific subunit, IL-4-specific subunit, and possibly a third subunit; c subunit shared by IL-2, IL-4, IL-7, IL-9, and IL-15 are not required for IL-13 signal transduction
Bioactivity: see Table 14-2

Interleukin-14

Interleukin-14 is a 60 kDa polypeptide produced by normal T cells, T-cell clones, and cell lines generated from patients with T- or B-cell lymphomas. [476] [477] IL-14 secretion is induced by phytohemagglutinin stimulation but not by treatment with LPS, phorbol ester, or concanavalin A. [478] [479] IL-14 binds to a 90 kDa receptor that is expressed only by cells of B-cell lineage. [476] [477] [480] As predicted by the cellular distribution of IL-14R expression, only cells of B-cell lineage respond to IL-14. The IL-14 protein is antigenically related to Bb, the activation fragment of complement factor B, and both are mitogenic for B cells and compete for binding to the IL-14R. However, the biologic responses of B cells to IL-14 and Bb are not identical. This difference may reflect differential activation of IL-14R by the two ligands or the possibility that Bb protein binds to other receptors besides IL-14R. [481] There are no known animal or human models of IL-14 or IL-14R deficiency.

Treatment of activated normal B cells with IL-14 stimulates proliferation but does not increase immunoglobulin secretion. In fact, addition of IL-14 to B cells treated with multicytokine combinations or pokeweed mitogen markedly inhibits immunoglobulin production. In cell fractionation studies, the sIgD-B-cell population is the most responsive to IL-14. Therefore, IL-14 may serve to expand B cells and divert them to a memory cell phenotype. [482] Resting B cells of tonsillar or peripheral blood origin express 50350 surface IL-14R per cell and do not proliferate in response to IL-14. In contrast, B cells activated by *Staphylococcus aureus* or anti-m antibody express 10,00050,000 surface IL-14R per cell and have a marked mitogenic response to IL-14. It is unclear if activation merely increases the IL-14R number to a critical threshold or whether other priming events are required for cellular response to IL-14. [481] [483]

In contrast to the results with normal B cells, neoplastic B cells are often responsive to IL-14 without the need for a comitogenic stimulus. In most cases of pre-B acute lymphocytic leukemia, hairy cell leukemia, prolymphocytic leukemia, or B-cell chronic lymphocytic leukemia, the malignant cells will be stimulated to proliferate in vitro by the addition of IL-14. [476] [480] IL-14 is secreted in some cases of B-cell non-Hodgkins lymphoma. In these cases the malignant cells also expressed functional IL-14R and proliferated in response to IL-14. Decreasing autocrine IL-14 production by neutralizing antibody or antisense IL-14 oligonucleotide inhibited cellular proliferation. Thus, in some patients, autocrine or paracrine production of IL-14 by malignant B cells may play a pathologic role. [484] Therapeutic strategies that block such an autocrine network may be useful in the treatment of certain B-cell neoplasms.

Abnormalities in IL-14R receptor signal transduction may also be involved in certain nonmalignant disease states. B cells isolated from patients with systemic lupus erythematosus are hyperresponsive to IL-14 and, unlike B cells from normal patients, will respond to IL-14 even in the absence of comitogenic stimuli. [485] In contrast, B cells from most patients with common variable immunodeficiency fail to respond to IL-14 even though cell surface expression of IL-14R appears normal. [486]

IL-14

Also known as: high-molecular-weight B-cell growth factor
Chromosome: not assigned
Gene product: 60 kDa
Produced by: T cells, T- and B-lineage lymphoma cells
Induced by: phytohemagglutinin
Receptor: 90 kDa
Bioactivity: see Table 14-2

Interleukin-16

Interleukin-16, formerly known as lymphocyte chemoattractant factor, is unique among the interleukins in that its cognate receptor (CD4) is an immune costimulatory molecule rather than a typical growth factor receptor. [487] IL-16 is a 1618 kDa protein that is formed by caspase 3 proteolytic cleavage of the pro-IL-16 polypeptide. [488] The main cellular source of IL-16 appears to be CD8+ T cells, although eosinophils and mast cells also produce IL-16 protein. [489] [490] [491] IL-16 is a potent chemotactic factor for

Figure 14-14 IL-16 and HIV. The receptor for IL-16 (CD4) is an immune costimulatory molecule, not a typical growth factor receptor. Produced in large part by CD8+ T cells, IL-16 induces cytosolic Ca^{++} and phosphatidylinositol 1,4,5 Tris phosphate (PI 1,4,5, TP) accumulation, activates p56lck, and induces protein kinase C (PKC) translocation to the membrane. IL-16 inhibits HIV-1 promoter activity (the integrated HIV-1 provirus is shown as a white rectangle flanked by black long terminal repeat sequences [LTR] and gray cellular DNA). When expressed in $CD4^{+}$ human cells, IL-16 renders them resistant to HIV-1 infection. IL-16 may have a potential role in treatment of HIV-1 infection.

CD4+ T cells, monocytes, and eosinophils. Additionally, IL-16 can inhibit T-cell activation induced by CD3 ligation or mixed lymphocyte reactions. [487] [492] Currently there are no animal models of IL-16 deficiency. IL-16 receptor-deficient mice (CD4 /) have been described, but the phenotype in these mice is more likely due to a loss of the costimulatory actions of CD4 in T-cell development than from a loss of IL-16 ligand binding activity. [493]

Many investigators have noted that production of CD8+ secreted soluble factor(s) is positively correlated with the clinical status of HIV-1-infected persons and is predictive of resistance to infection by simian immunodeficiency virus (SIV) in primate models. [494] [495] [496] Because IL-16 inhibits HIV-1 replication in vitro, [489] many have speculated that IL-16 is responsible for the bulk of the observed antiviral effect of CD8+ secreted factors. However, this hypothesis has not been rigorously tested.[489] [497] IL-16 does repress HIV-1 promoter activity (quantified using an HIV-1 LTR-reporter sequence), [498] and although IL-16 does not suppress HIV-1 replication in naturally infected peripheral blood mononuclear cells, [499] when expressed in CD4+ human cells, IL-16 renders these cells resistant to HIV-1 infection (Fig. 14-14).[500] The effect of IL-16 on HIV-1 replication does not appear to be related to the modulation of CD4 expression or interference with HIV-1 binding but rather to a direct inhibitory effect of IL-16 on the HIV-1 promoter. [471] [500] Based on studies to date, it appears that IL-16 may have a potential role in the treatment of HIV-1 infection.

IL-16

Also known as: lymphocyte chemoattractant factor[501]
Chromosome: unknown
Gene product: 1618 kDa; production of functional IL-16 requires cleavage of pro-IL-6 polypeptide by caspase 3[488]
Produced by: CD8+ T cells, mast cells
Induced by: serotonin, antigen challenge, C5, PMA, histamine
Receptor: CD4 (55 kDa, human chromosome 12pter-p12)
Bioactivity: see Table 14-2

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FACTORS WITH MULTILINEAGE ACTIVITY (EARLY-ACTING FACTORS)

At least six growth factors, IL-3,[502] [503] GM-CSF,[504] [505] SF,[506] FL,[507] IL-9, IL-6, IL-10,[412] and TPO,[282] [508] have obvious direct effects on multilineage progenitor cells and are thus capable of stimulating hematopoietic precursors before they have become fully committed to one lineage or another. Because of the clear lymphopoietic effects of IL-10, thrombopoietic effects of TPO and IL-11, dominant erythropoietic effects of SF, and granulopoietic effects of GM-CSF, all have been reviewed previously. Only IL-3, FL, IL-9, and IL-6 are reviewed in the category of early-acting factors.

Interleukin-3

Interleukin-3 was one of the earliest recognized multipotential hematopoietic growth factors. [172] [173] [509] The human IL-3 gene (5q31.1, only 9 kb upstream of the gene for GM-CSF) encodes a 1428 kDa protein that influences the growth of multiple lineages. To date, only T lymphocytes (induced by mitogens, phorbol esters, and certain antigens),[510] mast cells, [511] and certain cell types in mouse brain tissue [512] have been found to express the IL-3 gene. Although the GM-CSF and IL-3 genes are separated by only 9 kb of intervening DNA, they are very differently regulated. GM-CSF is produced by stromal cells [168] and IL-3 is not.[149] [513] [514] Although T lymphocytes can be induced to produce both IL-3[510] [515] [516] and GM-CSF,[516] there is clear-cut evidence that each is regulated independently of the other. [517] The high-affinity IL-3 receptor is a heterodimer composed of an IL-3-specific ligand-binding subunit (CDw123, human chromosome Xp22.3, Yp13.3) and a -subunit (CDw131, chromosome 22q12.2-q13.1) shared with the IL-5 and GM-CSF receptors (Fig. 142 **A**). [17] [18] The receptor is expressed on bone marrow progenitor cells, neutrophils, eosinophils, megakaryocytes, BFU-E, and mast cells.[518] Binding of IL-3 to the high-affinity receptor results in rapid phosphorylation of the adaptor molecules CrkL, which associates with Shc, SHP-2, and Cbl.[519] Cbl is phosphorylated, which enhances Cbls association with Grb2, phosphatidylinositol 3-kinase, and fyn.[520] The apoptotic protein BAD is inactivated by phosphorylation in IL-3-stimulated cells. [521] The STAT pathway

may also be activated by binding of IL-3 to its receptor through the association of STAT5 with CDw131. [522]

The biologic activity of IL-3, outlined in Table 14-2 , like many of the hematopoietic growth factors, derives from direct effects on progenitors and from other more indirect effects. The direct growth-stimulatory effects of IL-3 seem to be largely limited to very primitive committed progenitors like BFU-E [147] [523] [524] and hematopoietic progenitor cells with multilineage potential, [147] [525] possibly even CFU-S,[526] but it may be a factor that induces differentiation of multilineage cells because it hastens the development of colony-forming cells from stem cell precursors and is incapable of maintaining stem cell viability in vitro. [527] When expressed by human stromal cells engineered to produce human IL-3, SF, FL, or IL-7, only IL-3-expressing cells were capable of supporting sustained human hematopoiesis in a murine xenograft model.[528]

The fact that IL-3 induces proliferation directly and indirectly by inducing expression of other hematopoietic factors [155] [179] has complicated efforts to clarify the roles of this factor in hematopoiesis. For example, although it is clear that IL-3 induces formation of multilineage colonies in cultured human bone marrow cells, the full biologic potential of the cytokine is apparent only when combined with other factors. Consequently, reports that IL-3 stimulates proliferation or function of certain mature hematopoietic cells[529] [530] [531] [532] may be a result of its ability to induce expression of other factors that actually do the real work. One particularly intriguing issue is the apparent ability of IL-3 to stimulate the full developmental potential of basophils and mast cells in vitro, [532] [533] a phenomenon that seems to be inconsistent in cultures of human marrow cells[534] but invariable in long-term cultures of rodent marrow cells. [535] In fact, administration of IL-3 to nude mice induces marked intestinal and splenic mastocytosis.[536] Nonetheless, the presence of accessory cells (including IL-3-responsive macrophages [155] [179] [537]) in such settings is universal, as is, therefore, the potential for a number of indirect effects. Notwithstanding all its direct and indirect hematopoietic effects, IL-3 is completely dispensible for normal steady-state hematopoiesis because no hematopoietic defects have been found in IL-3 knockout mice. [538]

The place of IL-3 in clinical therapeutics is unknown at this time. Trials of IL-3 therapy for myelodysplasia, acute nonlymphocytic leukemia, Diamond-Blackfan anemia, aplastic anemia, HIV-related cytopenias, and after bone marrow or peripheral stem cell transplantation have been either ineffective or no better than other factors currently approved for clinical use. [539] IL-3 may prove to be useful, however, in combination with other factors. Lemoli et al. [540] reported that the combination of IL-3 and G-CSF was better than G-CSF alone for hastening multilineage recovery, reducing transfusion requirements, shortening hospitalization time, and reducing the number of infections in patients undergoing autologous marrow transplantation for malignant lymphomas.

IL-3

Also known as: multi-CSF, mast cell growth factor-1
Chromosome: 5q31.1
Gene product: 1428 kDa
Produced by: T lymphocytes, mast cells
Induced by: mitogens, phorbol esters, calcium ionophore A23187, IgE receptor activation (mast cells)
Receptor: heterodimer of IL-3-specific subunit (CDw123, chromosome Xp22.3, Yp13.3) and a subunit (CDw131, chromosome 22q12.2-q13.1) shared with IL-5 and GM-CSF receptors
Bioactivity: see Table 14-2
Deficiency: no hematopoietic defect in steady state; deficient delayed-type hypersensitivity [538]

FLT3-Ligand

FLT3-ligand (FL) is the cognate ligand for the flt3/flk2 receptor tyrosine kinase. [541] Flt3/flk2 is a member of the type III receptor tyrosine kinase family that also includes c-*kit* (SF receptor), c-*fms* (M-CSF receptor) and the platelet-derived growth factor receptor.[542] Indeed, the flt3/flk2 gene shares a genomic structural organization with c-*kit* and c-*fms.* [543] Human flt3/flk2 is a 993 amino acid protein with a predicted molecular mass of 110 kDa and contains ten potential sites for

N-linked glycosylation. Cell-associated flt3/flk2 has a molecular mass of 155160 kDa; the difference in apparent versus predicted molecular mass is attributed to glycosylation.[544] [545]

Flt3/flk2 protein is widely expressed in lymphohematopoietic tissues but not in most extrahematopoietic tissues except brain and placenta. [544] [546] [547] Flt3/flk2 protein is not expressed by mature peripheral blood B cells, T cells, monocytes, eosinophils, nucleated red blood cells, or granulocytes, but is expressed by primitive hematopoietic progenitors, including about 20%; of lin-kit+sca-1+ murine bone marrow cells. [548] In humans, 6080% of CD34+ bone marrow cells are flt3/flk2+ and about 90%; of CD34+ umbilical cord blood cells are flt3/flk2+. B-cell precursors, CFU-GM, and HPP-CFC are flt3/flk2+, whereas BFU-E are flt3/flk2. [549] [550]

Flt3/flk2 protein and/or mRNA is expressed by malignant cells in most cases of acute myeloid leukemia (AML) or ALL, [551] [552] [553] but the expression of flt3/flk2 protein or mRNA does not always correlate with a proliferative response to FL. [553] Recently, mutations in the flt3/flk2 gene have been reported in some cases of AML or mylodysplasia (MDS). These mutations involve tandem duplication of sequences in exon 11, which encodes the juxtamembrane domain and the first residues of the N-terminal tyrosine kinase domain. These tandem duplications are translated in frame, always contain Y591 or Y599, and are somatic mutations associated with the leukemic clone. In several series, these mutations were found in up to 20%; of AML cases and 310% of patients with myelodysplasia. [554] [555] [556] The biologic significance of these mutations is unknown but could conceivably involve aberrant receptor activation and/or hypersensitivity to FL.

As might be predicted from the fact that M-CSF, SF, and FL bind to receptors with a common genomic structural organization, these ligands also share a protein structure. Although not homologous at the amino acid level, all are type I transmembrane proteins and have a four helix bundle structure. All three ligands exist as both membrane-bound and soluble isoforms, and some of the soluble isoforms are generated by proteolytic cleavage of membrane-bound precursors. [557] As is the case for M-CSF and SF, both soluble and membrane-bound FL protein isoforms have biologic activity. [558] [559] The relative biologic significance of membrane-bound and soluble isoforms of FL has not been determined.

Variable splicing of the FL gene generates several mRNA species. The most prevalent human mRNA species lacks exon 6 and encodes a transmembrane protein that can be proteolytically cleaved to generate a soluble FL isoform. [541] [559] Another human FL mRNA species contains exon 6 and generates an obligatorily soluble protein because the exon 6 sequence introduces a frame shift and introduction of a stop codon near the end of the extracellular domain. [560] Soluble human FL protein can also be translated from an mRNA in which the transmembrane domain has been spliced out. However, the relative abundance of this FL transcript has not been determined.[559] In mice, the most prevalent mRNA species encodes a membrane-bound protein that is not transmembrane and lacks the extracellular proteolytic cleavage site. To date, this species of FL transcript has not been identified in human cells.

Normal serum levels of FL in humans are <100 pg/ml. Levels of FL are not increased in patients with anemia only, but are

Figure 14-15 Serum FLT3 ligand levels in aplastic anemia (from Lyman, S.D. et al. [799]). Normal (N) serum levels of FL in humans are <100 pg/ml. Serum levels of FL are not increased in patients with anemia only, but increase substantially in patients with bone marrow failure (e.g., aplastic anemia [AA]). AA patients in complete remission (CR) have normal levels, but levels increase again in relapsed (RAA) patients. Little is known about the regulation of FL protein production, and it is not clear why levels increase in patients with pancytopenia, but the reliable increase of serum levels in bone marrow failure states is compatible with the idea that this factor may influence overall bone marrow cellularity in vivo.

increased dramatically in patients with pancytopenia, as seen with aplastic anemia, Fanconi anemia, or chemotherapy- or radiation-induced myelosuppression. [542] [561] For example, serum FL levels averaged 2654 pg/ml in patients with newly diagnosed aplastic anemia and fell to 100200 pg/ml in patients with hematologic remission. In relapsed patients, the FL serum levels increased to 15003000 pg/ml (Fig. 14-15). [561] Little is known about the regulation of FL protein production, and it is not clear why levels increase in patients with pancytopenia. One possibility is a loss of tissue binding sites and a displacement of FL protein into the peripheral circulation. This type of mechanism is believed to be the cause of increased thrombopoietin levels in patients with marrow failure. [269] Alternatively, progenitors or their progeny may produce feedback inhibitors of FL production; such inhibition is lost in marrow failure states, leading to increased FL production by stromal cells or other sources.

The biologic activity of FL is substantial. In general, FL has little in vitro colony-stimulating activity as a single agent but has potent synergism with a variety of other hemopoietins, including SF, [541] [562] IL-3, [541] [562] IL-7, [562] GM-CSF, [563] G-CSF, [563] [564] IL-6, [541] M-CSF, [563] and IL-11, [565] [566] as well as with multicytokine combinations. [567] [568] FL is not a universal hemopoietin: it augments the growth of only certain types of clonogenic colonies. In particular, FL has little or no effect in stimulating BFU-E, CFU-E, BFU-MK, CFU-MK, CFU-mast cell, or CFU-eosinophil. [564] [569] [570] [571] [572] However, FL is a potent stimulator of B lymphopoiesis and granulomonocytic proliferation and differentiation. [339] [551] [566] [573] Additionally, FL has a striking ability to augment dendritic cell proliferation and differentiation in vitro. [574] [575]

FL-responsive hematopoietic cells appear to be more primitive and to have greater lineage restriction than SF-responsive cells. For example, the growth- and differentiation-promoting effects of FL in humans appear to be largely confined to the CD34+CD38 fraction of bone marrow or cord blood. [567] [568] [576] Likewise, the FL-responsive population is contained in linsca-1+ but not linlowsca-1+ or lin-sca-1-purified murine bone marrow cells. [562] [565] Consistent with its action on primitive progenitors, FL supports the in vitro proliferation of long-term culture-initiating cells, CFU-S, and long-term repopulating cells. [317] [572] [576] [577] FL increases recruitment of primitive HPC into the cell cycle and inhibits apoptosis. [577] [578] [579]

The in vivo effects of FL administration have been studied using murine and primate models. FL-treated animals develop dose-related leukocytosis due to increases in lymphocytes, granulocytes, and especially monocytes. Associated with the leukocytosis is an increase in peripheral blood hematopoietic progenitors, especially CFU-GM and CFU-GEMM, with a smaller effect on peripheral blood BFU-E. Minimal decreases in hematocrit and platelet count were seen in mice receiving short courses of FL. Daily treatment with FL also increases peripheral blood primitive hematopoietic progenitors, including CFU-S (200-fold) and lin-kit+sca-1+ (170-fold) and long-term repopulating cells. [580] [581] [582] FL is synergistic with G- or GM-CSF in mobilizing stem cells. For example, simultaneous administration of FL and G-CSF increases peripheral blood CFU 12- to 20-fold over that seen with either agent alone. [583] FL treatment has no significant effect on bone marrow cellularity but does increase immature granulomonocytic cells and immature B cells (B220+sIgM). However, bone marrow immature erythroid cells decrease up to 90% during treatment.

FL has a potent effect on the proliferation and differentiation of dendritic cells in vitro and in vivo. Treatment of mice with FL increases the absolute number of dendritic cells in the spleen, lymph node, and peripheral blood compartments 17-, 4-, and 6-fold, respectively. These FL-stimulated dendritic cells are functionally active, as assessed by their ability to act as antigen presenting cells in vitro and to prime an antigen-specific immune response in vivo. [584] [585] Expansion of dendritic cells by FL administration may allow more effective utilization of dendritic cells in clinical immunotherapy protocols. [584] [586]

Considering the heterogeneous early-acting effects of this cytokine on hematopoiesis, it is surprising, just as it was with IL-11R knockout mice, that no marrow failure state is found in mice with targeted disruptions of the flk2/flt3 gene. [587] These mice, unlike mice nullizygous for SF or c-*kit*, were viable and had no gross abnormalities of bone marrow or spleen cellularity, peripheral blood counts, or morphology. Bone marrow progenitor frequencies for myeloid progenitors (granulocytic, monocytic, erythroid, and mixed colonies) are the same as in heterozygous mice. Disruption of the flt3/flk2 gene does not alter the frequency of day 13 CFU-S or pre-CFU-S. In addition, splenic and thymic cell populations were normal. Interestingly, however, although levels of differentiated bone marrow B cells (B220+CD43-IgM+ and $B220^{bright}$ CD43-IgM+) were normal in nullizygous mice, there was a 50% reduction in pro-B-cell ($B220^{dull}$ CD43+) and a 25% reduction in pre-B-cell (B220+CD43-IgM) populations. In addition, bone marrow from mutant mice generate fewer (by six-fold) B-cell colonies when cultured in the presence of IL-7 alone or IL-7 plus SF. LTCIC for B lymphopoiesis were proportionately reduced in nullizygous animals. [587] Thus, flt3/flk2 is not required for steady-state lymphohematopoiesis, but deficiency does lead to a subclinical defect in B lymphopoiesis.

There is an additional abnormality in these animals that involves hematopoietic stem cells. To assess the effects of flt3/flk2 disruption on stem cells, competitive repopulation studies were performed using homozygous and heterozygous bone marrow. Flt3/flk2 nullizygous marrow was 4.5-fold less efficient in repopulating peripheral blood, 2.5-fold less efficient in repopulating marrow and spleen, and 8-fold less efficient in thymic repopulation. When hematopoietic cell subsets were analyzed, the engraftment potential of mutant mice to generate B cells in marrow (3.6-fold less efficient) or spleen (16.7-fold less efficient) was reduced when compared with bone marrow granulocyte or macrophage repopulation (2.3-fold less efficient). Transplantation experiments utilizing a mutant to heterozygous marrow ratio of 4:1 resulted in an approximately 50% mutant cell engraftment in total bone marrow and peripheral blood cells, but in such mice only 17% of bone marrow B cells, 2% of thymocytes, and 3% of splenic B cells were derived from the

nullizygous donor.[587] Thus, the flt3/flk2 gene appears to be crucial for stem cell transplantation, especially for lymphoid reconstitution.

To assess further the functional role of flt3/flk2 in vivo, the flt3/flk2 nullizygous mice were crossed with W/Wv mice to generate mice nullizygous for flt3/flk2 and with impaired c-kit function. Viability was decreased in the double mutants and there was a 5- to 6-fold reduction in marrow, splenic, and thymic cellularity. The double mutant mice had a 16-fold decrease in bone marrow CFU-GM and a 56-fold decrease in B-cell colony-forming units. After age 3 weeks, the double mutant mice had a marked decrease in viability, with none surviving more than 6 weeks. The reason for the decreased viability is unknown. Combined deficiency of FL and c-*kit* exacerbates the hematopoietic defects seen with single receptor deficiency and is not compatible with long-term survival in mice.[587]

FL will likely prove to be of use in the clinic. In particular, for mobilization of peripheral blood stem cells, the combination of FL plus G- or GM-CSF seems superior to any of the three growth factors used alone.[580] [583] [588] Although preclinical data are lacking, the effects of FL in stimulating the proliferation of primitive progenitors suggest that it may have a role in treating bone marrow failure states such as seen with high-dose chemotherapy, stem cell transplantation, aplastic anemia, or myelodysplasia. FL in vitro synergistically enhances the activity of other growth factors, so optimal clinical use of FL may require tailored use of adjunctive cytokines. FL may also be useful in ex vivo maintenance of primitive progenitors and expansion of CFU for use in transplantation or gene therapy applications.[568] [589] [590] In addition to its use in expanding dendritic cells, FL may be useful clinically as an immune system modulator. In a murine model of syngeneic fibrosarcoma, FL induced complete regressions in more than half the cases and significantly reduced tumor growth in the other mice. Tumor response was dependent on FL dose and was mediated in part by CD8+ T cells.[591] Similar results were reported using a murine breast cancer model. In these studies the effect of FL was transient, as all animals with complete responses developed tumor after a secondary challenge (no FL was given during the rechallenge). FL was even more effective as a tumor vaccine, as it prevented tumors in nearly 90% of animals that were rechallenged with tumor. In 60% of cases, splenocytes harvested from tumor-free mice 4 weeks after the secondary challenge could adoptively transfer immunity to naïve mice. The effectiveness of FL as a tumor vaccine was superior to that of IL-2.[592]

FLT3-Ligand

Also known as: flk-2 (fetal liver kinase 2) ligand
Chromosome: 19q13.3
Gene product: Various isoforms are produced by alternative splicing. Membrane and soluble isoforms exist.
Produced by: mRNA expressed by most tissues examined, including spleen, lung, stromal cells, peripheral blood mononuclear cells, T-cell clones
Induced by: IL-1, pancytopenia
Receptor: Flt3/flk2, a type III receptor tyrosine kinase, human chromosome 13q12-13
Bioactivity: see Table 14-2
Deficiency: 50% reduction in pro-B cells, 25% reduction in pre-B cells, 6-fold reduction in B-cell colony-forming potential, reduced repopulating capacity of stem cells

Interleukin-9

Interleukin-9 [593] [594] was originally identified as a 40 kDa growth factor (P40) for certain murine T-cell clones.[595] [596] The human protein was identified as a factor produced by a human T-cell leukemia virus (HTLV)-1-transformed T-cell line that stimulates the proliferation of MO7E human megakaryoblastic leukemia cells.[597] Sequence comparison of the respective cDNAs[596] [597] demonstrated that P40 was the murine homolog of the human protein, both of which were subsequently designated IL-9. The murine gene is on chromosome 13 and the human IL-9 gene resides on the portion of the long arm of chromosome 5[598] [599] that also carries the genes that encode GM-CSF, IL-3, IL-4, IL-5, and the M-CSF receptor (c-*fms*), and a number of other cytokines and growth factor receptors (Fig. 14-16). IL-9 is produced by activated T lymphocytes, primarily CD4+ lymphocytes.[596] [600] Both IL-4 and IL-10 can induce IL-9 gene expression, but in vivo IL-10 is more important because IL-9 expression is low in IL-10 knockout mice but not low in IL-4 knockout mice.[410] The IL-9 receptor is a member of the hematopoietic growth factor superfamily (Fig. 14-2) and is expressed in membrane-bound and soluble forms.[601] Its unique chain (CD129, human chromosome Xq28, Yq12) consists of a fibronectin type III domain and WSXWS motif[601] and combines with the common chain (CD132, chromosome Xq13). The receptor is expressed on erythroid, myeloid, and lymphoid precursor cells, as well as activated T cells and T-cell lines.

In combination with erythropoietin, IL-9 supports BFU-E-derived colony growth from both unfractionated and highly progenitor-enriched marrow cells.[602] [603] In contrast to its effects on adult progenitors, which appear confined to the erythroid lineage, IL-9 supports the clonal growth of fetal erythroid, multipotential, and granulocyte-macrophage progenitors.[603] Despite its stimulatory effects on MO7E cells, IL-9 lacks megakaryocytopoietic

Figure 14-16 Chromosome 5. A number of hematopoietic growth factors and interleukins are found on the long arm of human chromosome 5. M-CSF, originally assigned to this chromosome, was reassigned to chromosome 1. C-fms is the M-CSF receptor. Other genes clustered in this region include the -2 adrenergic receptor, the monocyte differentiation antigen CD14, interferon response factor-1 (IRF-1), and the platelet-derived growth factor receptor (PDGF-R).

activity. IL-9 acts synergistically with IL-3 in promoting murine mast cell growth.[604] Although originally identified as a murine T-cell growth factor, it has been difficult to demonstrate comparable activity in the human system. Recently, however, IL-9 has been shown to stimulate the proliferation of human peripheral blood-derived T-cell lines and clones that had been preactivated with phytohemagglutinin, IL-2, and irradiated allogeneic feeder cells.[605]

IL-9 is also produced by certain lymphoid cell lines and neoplasms, including HTLV-1- and -2-transformed T lymphocytes,[597] Hodgkins disease cells (particularly by Reed-Sternberg cells),[606] and large cell anaplastic lymphoma cells.[606] It has been suggested that IL-9 may act as an autocrine growth factor in such cases.

IL-9

Also known as: T-cell growth factor P40, mast cell-enhancing activity
Chromosome: 5q31.2-31.3
Gene product: 2030 kDa
Produced by: T lymphocytes
Induced by: phytohemagglutinin, PMA, calcium ionophore A23187, anti-CD3, IL-1, IL-2, HTLV-1 or -2
Receptor: 522 amino acid member of hematopoietic growth factor receptor superfamily
Bioactivity: see Table 14-2

Interleukin-6

The IL-6 gene resides on the short arm of chromosome 7[389] [607] and encodes a 2126 kDa protein. [608] The expression of the gene is seen in heterogeneous cell types, including fibroblasts,[609] endothelial cells,[610] monocyte/macrophages,[609] and T lymphocytes, [611] and is induced by IL-1,[168] TNF-,[612] mitogens,[611] and endotoxin.[613] The IL-6 receptor is a heterodimer consisting of an 80 kDa subunit (CD126, chromosome 1q21) and a 130 kDa signal-transducing subunit (CD130, chromosome 5q11) (Fig. 14-2B). [20] [21] The biologic activity of IL-6 is exceedingly broad (Table 14-2). It functions synergistically with IL-3 in CFU-GEMM and BFU-E growth, [614] with IL-4 and G-CSF in GM colony growth, [614] with IL-4 in inducing T-cell proliferation, [615] and with IL-2 in inducing immunoglobulin secretion, [346] and increases the self-replicative potential of cells in M-CSF- and GM-CSF-stimulated colonies. [168] [614] IL-6 is a potent megakaryocytopoietic factor both in vitro and in vivo. It acts synergistically with IL-3 and GM-CSF in supporting CFU-Mk-derived colony growth, promotes megakaryocytic maturation, and increases peripheral platelet counts when administered to experimental animals.[245] IL-6 may be a humoral mediator of the reactive thrombocytosis that accompanies chronic inflammatory states. IL-6 also acts synergistically with many direct early-acting hematopoietic growth factors, including FL and SF. [616]

Largely a synergistic factor, recombinant human IL-6 has no clearly demonstrable direct effect on the proliferation of any human hematopoietic progenitor cell on its own, although it stimulates murine granulocyte/macrophage colony formation [617] [618] and is unambiguously an autocrine and paracrine growth factor for malignant lymphoid and myeloma cell lines.[619] [620] [621] [622] Thus, in vivo studies in rodents demonstrating that recombinant human IL-6 stimulates replication of CFU-S may reflect the ability of IL-6 to act in concert with other constitutively produced factors. IL-6 also mediates the acute phase response of hepatocytes [623] [624] [625] and influences differentiation of neural cells in vitro, [626] and is a major pyrogen in vivo, an effect that can be blocked by pretreatment with a recombinant soluble IL-6R. [627]

Had it not been for careful studies on IL-6-deficient mice, it would have been difficult to place IL-6 in a category of growth factors because it can act indirectly and can influence multiple hematopoietic lineages and nonhematopoietic cells. Nonetheless, IL-6 deficiency adversely affects survival of hematopoietic stem cells and early progenitor cells of multiple lineages. [628] Therefore, one of its dominant hematopoietic effects is on primitive multilineage cells. A recent study on IL-6-deficient mice suggests strongly that IL-6 also functions as an essential antiinflammatory cytokine that, like IL-10, modulates the intensity of the inflammatory response. [629] Consequently, the placement of this cytokine in one box is admittedly arbitrary.

IL-6

Chromosome: 7p
Gene product: 2126 kDa
Produced by: macrophages, endothelial cells, fibroblasts, T lymphocytes
Induced by: IL-1, mitogens, endotoxin
Receptor: heterodimer consisting of the ligand binding protein IL-6R (CD126), a 450 amino acid low-affinity binding protein encoded by a gene on 1q21, and the protein gp130 (CD130) encoded by a gene located on 5q11, which also serves as a chain for CNTF, IL-11, oncostatin M, and LIF
Bioactivity: see Table 14-2
Deficiency: reduced survival of hematopoietic stem cells and multilineage progenitors, reduced T-cell numbers, reduced proliferation and maturation of erythroid and myeloid cells

FACTORS THAT FUNCTION AS INDUCERS OF GROWTH FACTOR GENE EXPRESSION

Some proteins that regulate hematopoiesis in vivo and in vitro do so indirectly (Table 14-2). In other words, in some cases they incite bystander cells, also known as accessory or auxiliary cells, to release direct-acting factors.

Interleukin-1

Interleukin-1 was formerly known as endogenous pyrogen, lymphocyte-activating factor, and many other names. IL-1 exists in two molecular forms (IL-1 and IL-1), which are encoded by two genes on chromosome 2. Each of these genes encodes 31 kDa precursor molecules that are cleaved by IL-1-converting enzyme [630] to 17 kDa peptides. IL-1, originally thought to be produced only by monocytes and macrophages, is produced by almost all cells. Gene expression is induced by endotoxin, IL-1, GM-CSF, TNF-, and IL-2. There are two high-affinity IL-1 receptors, the 80 kDa type I receptor (CD121a) and the 68 kDa type II receptor (CDw121b), which show differential patterns of tissue expression. CD121a is expressed by T cells, synovial cells, hepatocytes, keratinocytes, and endothelial cells. CDw121b is largely expressed by macrophages, neutrophils, and B lymphocytes.[631] [632] Both are members of the immunoglobulin superfamily and share 28% amino acid homology in their extracellular ligand-binding domains. [631] [633] [634] The bioactivity of IL-1 is tremendously broad, and there is good evidence that it regulates expression of most genes encoding mediators of inflammation.

IL-1 has no colony-stimulating activity itself. However, when administered in vivo, IL-1 universally induces neutrophilic leukocytosis, which results from the induction of G-CSF and GM-CSF expression by other cells, including fibroblasts, [513] [635] endothelial cells,[168] thymic epithelial cells, [636] and T lymphocytes. The broad activity of IL-1 derives, in large part, from its ability to induce the expression of other interleukin and CSF genes, which themselves function as subordinate effector

molecules in the inflammatory process. The temporal pattern of IL-1 gene expression in the inflammatory response and its hierarchic dominance over a variety of other cytokines is shown graphically in Figure 14-18 , in which an activated macrophage produces IL-1 and thereby induces stromal cells (e.g., fibroblasts and endothelial cells) to express IL-1, IL-6, GM-CSF, and G-CSF.

IL-1 knockout mice are viable and do not have marrow failure. They do exhibit hyperresponsive febrile reactions after administration of IL-1 or , and early lethality after infection with influenza virus, but do not have defective immunity against *Listeria* challenges.[637] Because IL-1 is intact in these animals, a better test of the hematopoietic control function would be to knockout both the and forms. In effect, IL-1RI inactivation achieves this. Interestingly, IL-1RI knockout mice are protected against endotoxin-induced lethality [637] [638] but have no other gross defects in immune responses to date except failure to respond to IL-1 in vivo, [638] a finding that indicates the importance of this particular receptor and that other IL-1 receptor forms are insufficient to cover for the loss of IL-1RI.

IL-1 and

Chromosome: 2q
Gene product: 31 kDa precursor, mature 17 kDa cleavage product
Produced by: most cells
Induced by: endotoxin, IL-1, GM-CSF, TNF-, IL-2
Receptors: CDw121b, a 68 kDa protein encoded by a gene on 2q12-q22, and CD121a, an 80 kDa protein encoded by a gene on the same chromosome (2q12). Both are members of the immunoglobulin superfamily
Bioactivity: see Table 14-2
Deficiency: IL-1: minor defects. IL-1RI: no dramatic effects on immune system or hematopoiesis

Tumor Necrosis Factor-Alpha

Tumor necrosis factor-alpha, a 17 kDa protein (a cleavage product of a 29 kDa membrane-associated protein) encoded by a gene stationed on chromosome 6 [639] near the major histocompatibility complex,[640] shares with IL-1 a large number of heterogeneous biologic activities (Table 14-2) and, like IL-1, functions largely to induce the expression of other subordinate genes that, in turn, function as more specific regulators of hematopoietic responses to inflammation. TNF is a member of a family of molecules that influence cell growth and survival, including nerve growth factor (NGF, a 12.5 kDa neurotrophic peptide), CD40L (a 39 kDa integral membrane glycoprotein that forms natural trimeric structures, like TNF), OX40L (a 32 kDa peptide that naturally exists as a trimer), CD27L (CD70, a 50 kDa integral membrane glycoprotein), fas ligand (fasL, a 40 kDa transmembrane glycoprotein that, when bound to its receptor, fas, transduces apoptotic signals to a wide variety of cells), CD30L (a 40 kDa transmembrane glycoprotein that enhances the apoptotic effects of TNF by enhancing degradation of TRAF2 [47]), TNF-related apoptosis-inducing ligand (TRAIL, a 32 kDa molecule that, like fasL, mediates apoptosis), and TNF-like weak inducer of apoptosis (TWEAK). [641] [642] [643] [644]

Receptors for this family are also homologous. They are defined by the consistent presence of cysteine-rich repeats in the extracellular domains and frequently occurring soluble forms generated by proteolytic cleavage. Examples of signaling through this receptor family are shown in Figure 14-7 . Although a good deal of evidence demonstrates that TNF- itself is capable of functioning as a direct inhibitor of progenitor cell growth, [645] [646] [647] some evidence suggests that the ability of TNF to induce expression of other growth factor genes may be of equal importance, at least in the inflammatory response involving lymphopoiesis and granulopoiesis. For example, while TNF- can inhibit CFU-GM-derived colony growth, it also induces the expression of G-CSF and GM-CSF genes in accessory cells, [513] [648] which may function to override the inhibitory function. That EPO gene expression is not induced by TNF- may account for the vulnerability of erythroid cells in anemic patients with chronic inflammatory diseases in which the organism induces TNF- gene expression. [647] [649] Indeed, Roodman[649] has proposed that the anemia of chronic disease results from TNF- production. When factors such as TNF- have confusing double-edged biologic functions, we are obliged to assess the function of the molecule in vivo to assign weight to one or the other in vitro responses. This has been done recently, and it is clear that the stimulatory effect of TNF- dominates in granulopoiesis[650] [651] but inhibitory effects dominate in erythropoiesis. [649] [652] Other examples of the double-edged bioactivity of TNF- exist, some of which have been evaluated by in vivo studies. For example, while the growth of cultured vascular endothelial cells in vitro is inhibited by TNF, TNF administered in vivo is

angiogenic.[653] [654]

The full hematopoietic consequences of TNF-RI and II deficiency have not yet been evaluated, but it is clear that in cells of receptor-deficient mice, TNF- is incapable of inducing IL-6 or GM-CSF, ICAM-1 or VCAM-1, MHC class 1 expression, and CD44.[655] Consequently, it is likely that on-demand hematopoietic responses to inflammatory stimuli will be blunted in vivo as well. Further testing of receptor-deficient mice is clearly warranted, but some preliminary observations support this expectation. Specifically, TNF-RI is necessary for induction by endotoxin of nitric oxide synthase [656] and to prevent lethal complications of bacterial peritonitis. [657]

TNF-

Also known as: cachectin
Chromosome: 6p
Gene product: 17 kDa (cleaved by TNF converting enzyme from a 26 kDa precursor)
Produced by: macrophages, B lymphocytes, NK cells
Induced by: endotoxin, GM-CSF, IL-3, poly(I):poly(C), phorbol esters, calcium ionophore A23187
Receptor: 55 kDa protein homologous to nerve growth factor receptor
Bioactivity: see Table 14-2
Deficiency: TNF-RI-deficient mice: hypersensitive to bacterial infections, failure of endotoxin to induce iNOS, failure of TNF to induce IL-6, GM-CSF, ICAM-1, and VCAM-1

Interleukin-17

Interleukin-17 is a 22 kDa cytokine homologous to the predicted amino acid sequence of open reading frame 13 (ORF13) of Herpesvirus saimiri. This homology is analogous to the presence of an IL-10 homolog in the genome of EBV.[419] The gene encoding the receptor for the human IL-17R has recently been cloned and resides on chromosome 22q11.22-23.[658] The receptor is a transmembrane protein whose extracellular domain does not exhibit homology to other known proteins and whose 521 amino acid cytoplasmic portion lacks recognizable signal transduction domains. mRNA for the IL-17R is ubiquitously expressed, and protein is expressed by all hematopoietic and epithelial cell lines tested. [658] [659]

IL-17 is produced by activated memory T cells and induces stromal cells to secrete inflammatory and hematopoietic cytokines, including IL-6, IL-8, prostaglandin-E_2 (PGE_2), G-CSF and

GM-CSF,[660] and ICAM-1.[659] IL-17 has no direct effect on hematopoietic precursors, but the addition of IL-17 to cocultures of human CD34+ cells and irradiated fibroblasts results in enhanced cellular proliferation and augmented production of mature neutrophils. [660] Some of the indirect effects of T cells on hematopoiesis may be mediated by the capacity of IL-17 to alter stromal cell function.

IL-17

Also known as: CTLA
Chromosome: unknown
Gene product: 15 and 22 kDa isoforms. The 22 kDa protein is produced by glycosylation of the 15 kDa isoform.
Produced by: activated T cells; in mice, IL-17 is produced only by TCR ab+/CD4/CD8 cells.
Induced by: PMA, ionomycin, CD3 ligation, phytohemagglutinin
Receptor: predominant species 128132 kDa, minor species of 105107 kDa (nonglycosylated form); human chromosome 22q11.22-23
Bioactivity: see Table 14-2

Interleukin-18

Interleukin-18, an 18 kDa protein produced by macrophages and keratinocytes, [661] induces IFN- production from lymphoid cells, including unfractionated peripheral blood mononuclear cells as well as isolated populations of T_H1, B cells, and NK cells. IL-18 mRNA is translated to yield an inactive precursor, pro-IL-18, that is cleaved by the IL-1 converting enzyme (ICE, caspase-1) to yield the active protein. [662] [663] The receptor for IL-18 is a member of the IL-1 receptor family, also known as IL-1-receptor related protein (IL-1RRP).[664] IL-18 is one of a number of cytokines induced by endotoxin and may serve to prime the immune system through induction of inflammatory mediators such as TNF-, IFN-, and fas-ligand.[665] Caspase-1 cleavage of pro-IL-18 appears to be the only significant source of active IL-18, as lipopolysaccharide (LPS) treatment of caspase-1 / mice results in negligible induction of IFN-. [662] [663] IL-18 accounts for both TNF and FasL mediated hepatotoxicity in endotoxin-induced liver injury.[666] It activates NFB in murine T-helper cells[667] and induces activation and association of p56-lck and MAPK in murine T_H1 cells.[668] IL-18 induces apoptosis in myelomonocytic KG-1 cells via the fas pathway by inducing expression of p53, IFN-, and fas ligand. [669] No animal models of isolated IL-18 deficiency have been described to date. In vivo administration of IL-18 to animals produces immunologically mediated (primarily NK) antitumor responses. If these studies can be confirmed in humans, IL-18 may be useful in cancer immunotherapy treatment protocols.[670] Pharmacologic inhibition of IL-18 may be useful in treatment of inflammatory and/or autoimmune disorders.

IL-18

Also known as: Interferon--inducing factor, IL-1
Chromosome: unknown
Gene product: 18 kDa
Produced by: keratinocytes, Kupffer cells, activated macrophages, osteoblastic stromal cells
Induced by: LPS
Receptor: 60100 kDa protein (previously known as IL-1R-related protein) [664]
Bioactivity: see Table 14-2

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A HEMATOPOIETIC FACTOR THAT IS DIFFICULT TO CATEGORIZE

Leukemia Inhibitory Factor

The murine cDNA encoding leukemia inhibitory factor[671] (also known as human interleukin for DA cells [HILDA]), a protein first identified by its capacity to induce differentiation and inhibit self-renewal simultaneously in certain murine leukemic cells, [671] [672] [673] was used as a hybridization probe to identify the human homolog [674] located on chromosome 22q12.[675] LIF is expressed by marrow stromal cells,[676] [677] monocytes,[678] and blastocysts,[677] [679] and expression is augmented by IL-1 and TGF. The human gene encodes a glycoprotein with 78% amino acid sequence identity with murine LIF, which, like the murine factor, induces differentiation of murine M1 leukemic cells.[674] Although it has no inhibitory activity on its own against the leukemic cell lines HL60 and U937, LIF, when combined with GM-CSF or G-CSF, reduces the in vitro clonal proliferative capacity of these cell lines [680] and induces differentiation.[681] LIF enhances the efficiency of retroviral mediated gene transfer in hematopoietic cells,[682] expands megakaryocyte mass in mice, [683] and exerts additional in vitro activities. For example, when added to cultures of totipotential embryonic stem cells, LIF suppresses the natural tendency of these cells to commit to differentiation. [684] [685] Whether LIF will function to inhibit commitment in other types of cells remains to be seen. It may prove to play an essential role in regulating the self-replication of not only hematopoietic stem cells, but other progenitors as well. The LIF receptor is a heterodimer consisting of an chain and a second chain, gp130 (CD130) shared with the IL-6R chain. [20] The LIF-R/gp130 heterodimer is also a receptor for oncostatin M (Fig. 14-2B). [20]

Based on the activities described previously, it is difficult to classify this cytokine based on its hematopoietic activity. The difficulty did not improve at all when the LIF knockout mouse was described. These mice have abnormalities of liver function, disrupted placental architecture, decreased bone volume (possibly because of increased osteoclasts), and neural defects, but no hematopoietic defects have been detected to date. [686]

LIF

Also known as: human interleukin for DA cells (HILDA)
Chromosome: 22q
Gene product: 58 kDa
Produced by: peripheral blood mononuclear leukocytes and Krebs II ascites tumor cells
Induced by: IL-1, TGF-
Receptor: a heterodimer consisting of gp130 and LIF-R subunits
Bioactivity: see Table 14-2
Deficiency: no hematopoietic defect

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BRAKING MECHANISMS FOR BLOOD CELL PRODUCTION

Just as mammals have ways of accelerating blood cell production when they need extra cells, there are mechanisms for stepping on the brakes when the need no longer exists. This is accomplished by highly specific internal inactivation steps and two types of environmental cues.

Internal Controls; Signal Modulation Mechanisms

The capacity of growth factor receptors to activate adapter proteins is defined previously. The EPO-R, for example, when bound to EPO, dimerizes, resulting in transphosphorylation of

Figure 14-17 Modulation of growth signal transduction in cells of the erythroid lineage.[72] The erythropoietin receptor (EPO-R) dimerizes on ligand binding, resulting in transphosphorylation of Jak2 molecules. Jak2 activation results in phosphorylation of the EPO-R chains, creating docking sites for STAT molecules (also see Fig. 14-5). Signal termination results, at least in part, from: (a) phosphorylation of the protein tyrosine phosphatase, SHPTP1; (b) binding of SH-PTP1 to a domain on the EPO-R, followed by (c) SH-PTP1-mediated dephosphorylation of the JAK molecules, thus terminating the signal induced by the initial ligand binding event.

Jak molecules, which phosphorylate the EPO-R chains, creating docking sites for STAT molecules. Signal termination results, at least in part, from the subsequent binding of the protein tyrosine phosphatase SH-PTP1 to a domain on the EPO-R, followed by S-PTP1-mediated dephosphorylation of the JAK molecule (Fig. 14-17).[72] STAT molecules themselves are degraded in somewhat similar ways that involve phosphatases[687] and ubiquitin-dependent proteasome-mediated degradation of activated STATs.[688] In addition, a new and very interesting molecular family has been identified that contains STAT-inducible inhibitors of STAT activation. [688] [689] [690] [691] [692] [693] The specific action of these molecules is variable: some clearly function to inhibit Jak kinases, [690] [691] while others bind to STAT molecules themselves (e.g., inhibition of STAT3 activity by PIAS3[693]). Numerous examples of phosphatase-mediated inactivation of activated adaptor molecules can be found in the literature, indicating that the braking capacity of any signaling chain is very broad and set at a high fail-safe level. Phosphatase inhibitors enhance signaling through cytokine receptors.[694] [695] In fact, there are examples in which the failure of regulatory phosphatases results in oncogenic transformation (mitotic signals in cells lacking the capacity for signal termination usually result in tumorigenesis). [696]

Extracellular Cues

The easiest cue to understand is a downturn in growth factor production that deprives the proliferating population of the mitogenic signal. There are, however, occasions when high-level proliferation must be terminated promptly. To effect this, extracellular factors can repress expression of cytokine genes, induce programmed cell death of the proliferating cell population, or inhibit progression through the cell cycle of the proliferating cells. A number of factors can effect one or more of these modulatory responses. Some have already been reviewed. TNF, IL-4, and IL-10, for example, all inhibit cytokine and growth factor release under various circumstances. Three families of factors that modulate hematopoiesis are the interferons (the most clearly suppressive of which are IFN- and -), the TGF- family, and the chemokine family. One example of each family will be presented following.

Interferons

The interferons, a family of related cytokines initially discovered as glycoproteins produced by cells after infection by certain viruses, [697] [698] suppress hematopoietic progenitor cells, largely by inhibiting proliferation or inducing programmed cell death in that population. Since they are capable of inducing expression or release of other biologically active hematopoietic factors, [699] [700] the interferons can accomplish this in two ways. The receptors for each of the three types of interferons (, , and) are members of the cytokine receptor superfamily and after ligand binding cells transduce the IFN signals, at least in part, through the JAK/STAT pathway (Fig. 14-5). Much of the recent work done on the hematopoietic effects of interferons has been done on IFN- and this factor will be discussed in greater detail as a paradigm of the family.

The lymphokine IFN-, a 34 kDa homodimeric protein, directly suppresses the growth of committed progenitor cells and long-term culture-initiating cells (LTCIC) [645] [701] [702] and has even been proposed as a mediator of aplastic anemia.[703] [704] Fairly compelling indirect evidence supports this notion. First, parenteral administration of IFN- suppresses hematopoiesis in vivo.[705] Second, in patients with aplastic anemia, production of IFN- by cultured peripheral blood [706] and bone marrow lymphocytes is increased,[707] [708] [709] and when IFN- gene expression is enforced in bone marrow stromal cells, profound in vitro hematopoietic suppression occurs. [704] Finally, immunosuppressive therapy is often effective in patients with aplastic anemia, [710] [711] and response rates seem to be highest in patients whose bone marrow cells express the IFN- gene.[707]

IFN- induces progenitor cells to undergo programmed cell death, [704] [712] at least in part through its capacity to induce fas expression in these cells. [41] [43] [704] Fas, a 4348 kDa membrane glycoprotein, is a member of the TNF/nerve growth factor receptor superfamily[642] [713] that induces apoptosis when engaged by either its ligand (fas-L, a type II membrane protein homologous to

Figure 14-18 Orchestration of the inflammatory response. **(A)** Establishment of infection. Tissue macrophages are critical for the initiation of the inflammatory response. Macrophages engulf organisms (bacteria in this diagram) and kill them, but while doing so, they release cytokines that begin to induce the early phagocytic phase of the immune response. Release of G-CSF and IL-6 influences phagocyte production and activation in marrow. Release of IL-1 and TNF- also occurs. These indirect-acting cytokines induce expression of additional granulopoietic factors, G-CSF, GM-CSF, IL-6, IL-11, and IL-9, by other mesenchymal cells in the infected tissues and possibly in bone marrow stroma as well. Finally, neutrophil chemotactic factors are released by stromal cells induced by IL-1 and TNF. **(B)** Early phagocytic response. Under the influence of the granulopoietic factors, the chemotactic factor IL-8, and cytokines that induce release of storage-pool granulocytes, neutrophils come onto the scene and more effectively than macrophages begin to kill bacteria and at the same time augment the secretory response by synthesizing more IL-1 in response to GM-CSF. The increase in IL-1 further augments production of granulopoietic and chemotactic factors. G-CSF released from stromal cells and macrophages in the infected tissue activates the functional capacity of neutrophils in situ. **(C)** Multilineage immune response. Immunogenic peptides presented to $CD4^+$ (T_H 1 and T_H 2) and $CD8^+$ T cells result in release of additional granulopoietic

(IL-3, GM-CSF) and macrophage-activating (IFN-, TNF-, GM-CSF) factors, and T $_H$ 2 cells influence the outgrowth of antigen-specific B cells in inflammatory foci and nearby lymph nodes by intercellular communication through CD40/CD40L interactions and by secretion of cytokines (IL-4, -5, -6, -10, and 15) that induce B-cell production and maturation and T-cell expansion. CD8 cells are also influenced by macrophages to release macrophage activating factors and the lymphocyte specific factor, IL-16. **(D)** Late immune response and suppression of phagocyte production. Once the offending bacteria are cleared, production of granulopoietic factors declines but elements of immune stimulation persist through instructed T cells, resulting in suppression of phagocyte production via the inhibitory factors IFN-, TNF-, IL-10, IL-4, and TGF-. B-cell expansion persists for many days beyond the resolution of the infection.

members of the TNF family)[714] [715] or by specific anti-fas antibodies that mimic the effects of fas-L. [716] [717] [718] Fas expression alone is insufficient to account for an apoptotic phenotype. It requires a number of other intact elements, including FADD, [719] [720] a protein that binds to the death domain of fas, and FLICE, [721] an IL-1-converting enzyme (ICE)-like protein that binds to the death domain of FADD and subsequently initiates the caspase [722] cascade that, at least in certain lymphocytes, includes ICE and CPP32.[723] [724] The ordered activation of these death mediators is less well defined in hematopoietic progenitor cells, but the pathway is clearly involved in the high level of programmed cell death found in progenitor cells of children or mice with Fanconi anemia. [45]

Could this family play a modulatory role in the production of cells during infections? We think so, but the issue has not been fully tested yet. In the hypothetical model described in Figure 14-18 , IFN production develops during the recovery phase of infections, at which point the need for highly proliferative progenitor cells has diminished but the need for activated macrophages has not because the organisms are dead but the debris is still present (IFN- is a potent macrophage activator [725] [726]). In this context, IFN molecules may play an important role as a braking factor for neutrophil production. Failure of certain neoplastic hematopoietic clones to respond to braking factors such as IFN might account for the sustained hyperleukocytosis some leukemic patients develop during and after bacterial infections.

Transforming Growth Factor-Beta Family

There are about 30 members of the TGF-1 superfamily, all of which are functionally related. The superfamily has three subfamilies: the TGFs, inhibins and activins, and bone morphogenetic proteins. Most of these molecules act as homodimers joined by disulfide bonds. Although a good number of these molecules may modulate hematopoiesis, the most well studied is TGF-1. This molecule is a disulfide-linked homodimer, each chain of which is processed from a 390 amino acid precursor

to the mature 112 amino acid molecule. The binding molecules and pathways for TGF-1 signaling are reviewed in the previous sections. The effects of TGF on hematopoietic cells are complex. It can inhibit expression of growth factors and their receptors (e.g., c- *kit* and SF[90]) and induce expression of mitotic inhibitory genes. TGF- inhibits mitotic progression in hematopoietic cells and may also induce apoptosis. [727] It governs the activity of cyclin-Cdk complexes by inhibiting Cdk4; inducing p27/Kip1, an inhibitor of cyclinCdk complexes; inducing p21/Cip1/Waf1, an inhibitor of cyclinECdk2 complexes; [728] [729] inducing p16INK4a and p15INK4b expression, [730] inhibitors of Cdks 4 and 6; and by repressing the phosphorylation of the retinoblastoma (Rb) protein (Fig. 14-6). [731] The importance of the TGF receptor family as braking factors for a variety of cells is clarified by the capacity of inactivated receptors to permit autonomous growth and the frequency with which these receptors or their downstream targets are inactivated by somatic mutation in a variety of cancers, [33] [34] [35] and the markedly enhanced inflammatory response and early death seen in mice with targeted disruptions of the TGF-1 gene. [732] [733]

Chemokine Family

Chemokines are a large family of molecules that are distinguished among the cytokines for being the only ones that bind to seven transmembrane-spanning G protein-linked receptors.[36] The chemokines are divided into three families (or CXC, or CC, and or C) [37] on the basis of differences in the content and location of their component cysteine residues. These proteins were originally defined biologically as cytokines that exert effects on chemotaxis. While there are some exceptions, generally the chemotactic activities of the chemokines also sort with the

subtype. In other words, members of the family are chemotactic for granulocytes (neutrophils, eosinophils, and basophils), the family for mononuclear phagocytes and lymphocytes, and the family for lymphoid cells only.[734] Clearly, other activities have been identified for these molecules, including cell adhesion, angiogenesis, HIV-1 suppressive factors, and feedback control of hematopoiesis and the inflammatory response. [36] [734] It is this latter issue that will be reviewed in more detail, using as a paradigm of this family, the chemokine, IL-8.

IL-8 is an 11 kDa chemokine produced by most cells under the influence of inductive cytokines. After its secretion, extracellular N-terminal processing of the mature IL-8 polypeptide yields several active species, ranging from 69 to 79 amino acids in length, with the predominant species consisting of 72 amino acids. [735] [736] IL-8 is produced by mononuclear phagocytes, endothelial cells, fibroblasts, other connective tissue cells, and by neutrophils themselves in response to IL-1, TNF, and a variety of other inflammatory stimuli and proinflammatory cytokines. [737] [738] [739] [740] IL-8 is repressed by cytokines that modulate the inflammatory response (e.g., IL-10 [424]). Neutrophils express two closely related G-protein-coupled IL-8 receptor molecules, which each possess seven membrane-spanning domains and share significant sequence homology with receptors for the neutrophil chemoattractants fMet-Leu-Phe and C5a. [741] [742]

IL-8 is a potent neutrophil agonist, inducing neutrophil chemotaxis, [743] [744] shape change,[745] exocytosis of storage granule proteins,[746] [747] and the respiratory burst.[745] [748] NAP-2, the GRO proteins, and ENA-78 also bind IL-8 receptors and induce similar neutrophil responses. [742] [749] [750] IL-8 is also chemotactic for T lymphocytes, [751] basophils,[752] and endothelial cells[753] and is a potent angiogenic agent. [753] In addition to playing a role in host defense by promoting neutrophil activation and directed migration to sites of infection, IL-8 is also an important mediator of the destructive inflammatory processes that characterize such disease states as rheumatoid arthritis and psoriasis. [754] [755] [756] Observations of extreme neutrophilic leukocytosis in IL-8 receptor knockout mice [39] support the notion that IL-8 is an essential feedback regulator of neutrophil production. [757]

IL-8

Also known as: neutrophil attractant/activation peptide-1
Chromosome: 4q12-21
Gene product: 8 kDa
Produced by: mononuclear phagocytes, fibroblasts, endothelial cells, neutrophils, keratinocytes, synovial cells, chondrocytes
Induced by: IL-1, TNF, LPS, IL-3, GM-CSF, immune complexes, phorbol esters
Receptor: two G protein-coupled receptors (350 and 355 amino acids in length), both on human chromosome 2q35[758]
Bioactivity: see Table 14-2
Deficiency: expansion of lymph nodes, plasmacytosis, and neutrophilia

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ORCHESTRATION OF THE INFLAMMATORY RESPONSE

New students of hematopoiesis are rightly dismayed at the complexity of the growth factor network, with all its synergies and feedback loops. One rule that seems to place the networks into some understandable framework is that most cytokine responses are hierarchic.[166] The central roles of IL-1 and TNF- in hematopoietic regulation, particularly of the inflammatory response, serve as particularly obvious examples of this.[168] Both IL-1 and TNF- genes are expressed early in the inflammatory response (Fig. 14-18). These two gene products induce the expression of a wide variety of subordinate interleukin and growth factor genes that call forth phagocytes by inducing granulopoietic factors, activate the phagocytes through phagocyte activating factors, some of which are the same as the lineage-specific granulopoietic factor that got them circulating in the first place, and then call them to the site of inflammation through the chemotactic factors and adhesion molecules induced by the same orchestrators, IL-1 and TNF. These master switch molecules are also capable of autoamplifying their own signals by enhancing the expression of their own genes[168] and each other (e.g., TNF- induces IL-1 gene expression).[759] More trying complexities exist. IL-1 gene expression is induced by proteins that IL-1 itself induces.[166] Thus, such signal amplification mechanisms can be autocrine, paracrine, or both. Finally, as the need for large numbers of sticky, fast-moving, hungry phagocytes declines during the course of a resolving infection, a set of factors come into play that repress growth factor production and inhibit the response of progenitor cells to such growth factors by causing either apoptosis (e.g., in the case of IFN-) or mitotic inhibition (e.g., TGF-).

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HEMOPOIETINS: AUTOCRINE LOOPS IN NEOPLASTIC CELLS

Certain elements of the hierarchic network of hematopoietic growth factors and interleukins can fail. When cells produce their own growth factor, they are said to exhibit autocrine growth. While there exist some autocrine functions in normal cells, [760] [761] [762] [763] [764] rarely are these functions that directly affect cell growth. For the most part, cells that produce their own growth factors are transformed or neoplastic. An increasing number of neoplastic cells or cell lines, either of the wild type or molecularly engineered, have been found to depend on autocrine growth factors, including IL-1, [765] IL-2,[766] [767] IL-3,[768] [769] IL-4,[395] IL-6,[770] [771] IL-11,[319] IL-14,[484] GM-CSF,[768] [772] M-CSF,[773] [774] and EPO.[2]

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SUMMARY

Our knowledge of hematopoietic control derives, in large part, from five pathways of laboratory and clinical investigation, each of which informs and sometimes surprises us:

1. Cell culture methods that permit studies on hematopoietic regulation in vitro
2. The comprehensive characterization of lineage- and stage-specific cell surface molecules
3. Cloning and characterization of genes encoding proteins with direct and indirect effects on blood cell growth and differentiation
4. The development of mice with targeted disruptions of growth factor and growth factor receptor genes
5. Clinical trials of recombinant hematopoietic growth factors.

As a result of these approaches, we know that cells empowered to produce these soluble regulatory factors do so in response to early inductive stimuli, and these inductive signals are quite predictably responsive to environmental influences. Some are even simple; hypoxia induces EPO gene expression but inflammatory stimuli do not. Most other responses to environmental stressors are more complex, especially those induced by microbial assault. These are met by a multipronged inflammatory response orchestrated by hematopoietic cells under the influence of a complex network of hematopoietic growth factors and interleukins, some of which are expressed early in the response and others of which are expressed late. Despite its complexity, cause-and-effect relationships are clearly testable using a combination of the five approaches. Because many of these

cells and hematopoietic factors have homologs in primitive organisms, and because disruptions of selected components of the control system can result in a wide variety of clinical disorders, from leukemia to aplastic anemia, this extraordinarily efficient molecular network is a paradigm with profound implications for clinicians, developmental biologists, cell biologists, and molecular biologists alike.

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Chapter 15 - Biology of Erythropoiesis, Erythroid Differentiation, and Maturation

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INTRODUCTION

The production of erythroid cells is a dynamic and exquisitely regulated process. The mature red cell is the final phase of a complex but orderly series of genetic events that initiates at the time a multipotent stem cell commits to the erythroid program. Expression of the erythroid program occurs several divisions later in a greatly amplified population of erythroid cells, which have a characteristic morphology, maturation sequence, and function. These maturing cells are termed erythroid precursor cells and reticulocytes. As terminally differentiated cells have a finite life span, they are constantly replenished by influx from earlier compartments of progenitor cells that are irreversibly committed to express the erythroid phenotype. During ontogeny these are successive waves of erythropoiesis in distinct anatomic sites. The erythroid cells developing in these sites have distinguishable phenotypes and intrinsic programs that are dependent on gestational time and their microenvironment. At each site erythroid cells are in intimate contact with other cells (stromal cells and hematopoietic accessory cells) comprising their microenvironment. Within this microenvironment, erythroid development is influenced by cytokines, which are either elaborated by microenvironmental cells or produced elsewhere and then entrapped in the extracellular matrix.

An understanding of the properties of erythroid progenitor and precursor cells and their complex interactions with the microenvironment is essential for understanding the pathophysiology of erythropoiesis. Aberrations either in the generation and/or amplification of fully mature and functional erythroid cells or in the regulatory influences of microenvironmental cells or their cytokines form the basis for various clinical disorders, including aplasias, dysplasias, and neoplasias of the erythroid tissue.

TABLE 15-1 -- Erythroid Progenitors: General Properties

	CFU-GEMM	BFU-E	CFU-E
Self-renewal	++	+	0
Differentiation potential	Multipotent	Committed to erythropoiesis	Committed to erythropoiesis
Cycling status % suicide with ^{3}H Thymidine	1520	3040	6080
Cell density (g/ml)	<1.077	<1.077	<1.077
Incidence/10^{5} cells	25	40120	200600
Circulate in blood	+	+	0
Growth Factor Response			
Erythropoietin	+	+	++
TPO	+	+	+
KL	+	+	+
GM-CSF, IL-3	+	+	+
FL	+	0	0
G-CSF, IL-6, IL-1[a]	+	0	0
Insulin, insulin-like growth factor, activin	0	0	+
TGF-1			++

[a]Most of the cytokines listed exert synergistic effects.

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ERYTHROID PROGENITOR CELL COMPARTMENT

The erythroid progenitor cell compartment, situated functionally between the multipotent stem cell and the morphologically distinguishable erythroid precursor cells, contains a spectrum of cells with a parent-to-progeny relationship, all irreversibly committed to erythroid differentiation. A complete understanding of how erythroid commitment is achieved at the biochemical or molecular level is lacking, although some recent attempts for understanding the molecular basis have been made. [1] [2] [3] Evidence from in vitro cultures of single multipotent progenitor cells allowed to differentiate in competent environments, as well as evidence obtained by studying the phenotype of leukemic cells, suggests that commitment to a specific hematopoietic lineage is accomplished not by acquisition of new genetic information but rather by restriction (probably on a stochastic basis) to specific programs from a wider repertoire available to pluripotent progenitor cells. [4] [5] Recent molecular evidence supports this view. [3] [6] Although the irreversible commitment to express the erythroid phenotype is a feature common to all erythroid progenitor cells, their properties progressively diverge as they become separated by several divisions.

Erythroid progenitor cells are both sparse (Table 15-1) and difficult to isolate in sufficient purity and numbers for study. Their existence and characteristics have been inferred from their ability to generate hemoglobinized progeny in vitro in clonal erythroid cultures (Fig. 15-1). Through this approach two classes of progenitors have been identified. [7] The first, more primitive, class consists of the BFU-E, named for their ability to give rise to multiclustered colonies (erythroid bursts) of hemoglobin-containing cells. BFU-E represent the earliest progenitors committed exclusively to erythroid differentiation and a quiescent reserve, because only 1020% are in cycle at any given time. However, once stimulated to proliferate in the presence of appropriate cytokines, they demonstrate a significant proliferative capacity in vitro, giving rise to colonies of 30,00040,000 cells, which become fully hemoglobinized after 24 weeks, with a peak incidence at 14 to 16 days. They also have a limited self-renewal capacity, because at least a subset of BFU-E is capable of generating secondary bursts, upon replating. In contrast to this class of progenitor cells, a second, more differentiated class of progenitors consists of the CFU-E. Most (6080%) of these progenitors are already in cycle and thus proliferate immediately after initiation of culture to form erythroid colonies within 7 days. Because CFU-E are more differentiated than BFU-E, they require fewer divisions to generate colonies of hemoglobinized cells, and the colonies are small (865 cells).

Although the two classes of committed erythroid progenitors, BFU-E and CFU-E, appear distinct from each other, in reality progenitor cells constitute a continuum, with graded changes in their properties. Only progenitor cells at both ends of the differentiation spectrum have distinct properties. Perhaps

Figure 15-1 Erythroid differentiation. BFU-E and CFU-E occur infrequently in the marrow (approximately 0.3% of mononuclear cells) and cannot be distinguished by morphologic or cytochemical techniques. Their existence is inferred by their ability to differentiate in culture. If marrow is placed in semisolid medium (e.g., methylcellulose) to decrease cell motility, with appropriate nutrients and growth factors (e.g., transferrin, insulin, EPO, and IL-3), CFU-E (after approximately 7 days) differentiate into small clusters of hemoglobinized or red cells termed erythroid colonies. Most BFU-E present in the inoculum differentiate to form multiclustered colonies of hemoglobinized cells, or erythroid bursts, by days 1416. Each erythroid colony or burst derives from one CFU-E or BFU-E, respectively.

TABLE 15-2 -- Erythroid Progenitors: Surface Antigen/Receptors

Receptor/Antigen	CFU-GEMM	BFU-E	CFU-E
CD34	++	++	
CD33	+	+	0
c-kit	++	++	+
HLA-DR (-DP, -DQ)	++	++	+
EPO receptor	+	+	++
Tumor necrosis factor receptor	+	+	++
Ep-1[242]	+	+	++
23.6[243] [a]	0	0	+
CD36	0	±	+
Glycophorin A	0	0	+
ABH, iI[b]	0	+	+
Adhesion Molecules			
VLA-4 (CD49d/CD29)	++	++	++
VLA-5 (CD49e/CD29)	+	+	++
CD41	+	+	
CD11a/CD18	+	+	
CD44	+	+	
HCAM[c]	+	+	

[a] 23.6 (SFL 23.6) is a monoclonal antibody reactive with CFU-E, erythroblasts, and erythrocytes.
[b] ABH and iI are blood group antigens.
[c] Presence of other cytoadhesion molecules, i.e. CD31, L-selectin, P-selectin, E-cadherin, has been described in progenitors (see text). However, the extent of their presence in BFU-E as compared to other cells is not clear.

the earliest cell with the potential to generate hemoglobinized progeny is an oligopotent progenitor, which is capable of giving rise to mature cells of at least one other

lineage (granulocytic, macrophage, or megakaryocytic) in addition to the erythroid. This progenitor, a multilineage CFU called CFU-granulocyte/erythrocyte/macrophage/megakaryocyte (GEMM), and the most primitive BFU-E have physical and functional properties that are shared by both pluripotent stem cells and progenitor cells committed to nonerythroid lineages. These properties include high proliferative potential, low rate of cycling, response to a combination of cytokines, and presence of specific surface antigens or surface receptors (Tables 15-1 and 15-2). In contrast, the latest CFU-E have many similarities to erythroid precursor cells and little in common with primitive BFU-E. Their proliferative potential is limited, they cannot self-renew, they lack the cell surface antigens common to all early progenitors, and they are exquisitely sensitive to erythropoietin (EPO) (Tables 15-1 and 15-2).

Although clonal erythroid cultures are indispensable for the study of erythroid progenitors, they do not faithfully reproduce the in vivo kinetics of red cell differentiation/maturation, and many maturing cells have a megaloblastic appearance and lyse before they reach the end stage of red cell development. In vivo, erythropoiesis probably occurs faster than would be predicted from culture data. For example, studies in dogs with cyclic hematopoiesis, a genetic stem cell defect leading to pulses of hematopoiesis, provide evidence that BFU-E mature to CFU-E over 23 days in vivo (Fig. 15-2), although this process may require 56 days in canine marrow cultures.[8] Erythroid progenitors can be cultured under serum-depleted conditions, [9] as well as in serum-containing media. The effects of recombinant growth factors can be studied in serum-depleted cultures without the complicating influences of multiple or unknown factors present in serum. Conditions that imitate the lower oxygen pressures, found in bone marrow in vivo, favorably influence erythroid development in culture and may be advantageous. [10]

As BFU-E are generated from the multipotent or oligopotent progenitors within the marrow, they are dependent for their survival and proliferation on the presence of cytokines, elaborated by either stromal cells or accessory cells within the microenvironment. A number of cytokines have been shown to influence proliferation and/or survival of early progenitors. Among them kit-ligand, produced by stromal cells and IL-3 produced by a subset of T cells, alone and in synergy, has a profound proliferative effect on BFU-E and its progeny. Other cytokines, such as GM-CSF, IL-11 and thrombopoietin (Tpo) stimulate a subset of BFU-E. [11] [12] [13] Cytokines exert their effects through interaction with specific receptors present on the BFU-E surface. The presence of such receptors has also been documented in the leukemic counterparts of normal BFU-E and in leukemic cell lines. [14] BFU-E cannot survive in the absence of cytokines for more than a few days in culture, and if they are deprived of cytokines for >6 days, >80% of them are lost.[15] In addition to positive regulators (IL-3, GM-CSF, Tpo, KL, or IL-11), substances with negative influences on BFU-E proliferation have been identified. These include tumor necrosis factor- (TNF), transforming growth factor- (TGF-), and interferon- (Inf-). [16] [17]

BFU-E and immediate progeny (but not CFU-E) are motile cells and are found in significant numbers in peripheral blood. As with BFU-E, the ability of stem cells and progenitor cells to circulate is physiologically important for the redistribution of marrow cells when there is local damage to the microenvironment and for the reconstitution of hematopoiesis after transplantation. The spectrum of BFU-E in circulation is probably narrower (consisting mostly of early, quiescent BFU-E) than that of BFU-E in the bone marrow, but otherwise their properties are similar to those of marrow BFU-E. The number of circulating BFU-E (along with other progenitors and stem cells) can be increased to significant levels after cytokine/chemokine treatments and following chemotherapy, and this has been exploited for transplantation purposes.[18]

Surface antigens of BFU-E have been defined through the use of monoclonal antibodies. [19] [20] The antibodies tested thus far include two broad categories: antibodies raised against leukemic cells or cell lines with progenitor cell properties, and antibodies raised against normal, terminally differentiated red cells. Enrichment in BFU-E (or CFU-E) following labeling with these antibodies, or their loss after complement-dependent lysis, have been considered indicative of the presence of test antigens on the BFU-E surface. Reactivities of BFU-E with several antibodies directed against defined surface antigens are listed in Table 15-2 . Like other hematopoietic progenitors, BFU-E display HLA class I (A, B, C) and class II (DP, DQ, DR) antigens on their surface, but class II antigens, especially the products of DR locus, are, in contrast to class I, variably expressed among BFU-E. This may relate to variations in their cycling status, as myeloid progenitors in S phase have been shown to have relatively higher expression of class II antigens. [21] The presence of HLA class II antigens (DR and, to a lesser extent, DP and DQ) most likely allows BFU-E to recognize and interact with the immunoregulatory cells (e.g., T cells, monocytes), which also express class II determinants. [22] In addition to HLA antigens, several other antigenic structures have been found on cells within the BFU-E compartment (Table 15-2). The best representative of these is the CD34 molecule (identified through monoclonal antibodies MY10, 12.3, or HPCA1), which has been successfully exploited for the isolation of BFU-E and other progenitors. CD34 is a highly O-glycosylated cell surface glycoprotein and is expressed in all hematopoietic progenitors and vascular endothelial cells. [23] Its role in hematopoiesis is not clearly defined. A reduction in the number of all hematopoietic progenitors was present in CD34 null murine embryos and adult animals, but no other abnormalities identified.[24] Recently it was shown that expression of CD34 is low or absent in a population of adult long-term repopulating cells in the mouse and man. [25] Furthermore,

Figure 15-2 Studies in a gray collie dog with cyclic hematopoiesis, an inherited disorder of hematopoietic stem cells in which granulocytes, monocytes, platelets, and reticulocyte counts fluctuate (or cycle) at 12-day intervals. It is thought that multipotent stem cells intermittently contribute to hematopoiesis, thus giving rise to pulses of differentiating cells. As shown here, the peak frequency of BFU-E (cycle day 10) occurs 3 days before the peak of CFU-E (cycle day 1), that in turn precedes the peak in the reticulocyte count (cycle day 4). These data confirm that BFU-E are the precursors of CFU-E in vivo and suggest that erythroid differentiation in vivo may occur more quickly than would be predicted from maturation time in culture. *(From Abkowitz et al.,[6] with permission.)*

through the use of antibodies or conjugated ligands it was determined that BFU-E present in enriched progenitor preparations display receptors for KL, EPO, TPO, GM-CSF, IL-3, IL-6, and IL-11 receptors. The great majority of BFU-E, however, in contrast to myeloid progenitors (CFU-GM) do not express the restricted hematopoietic phosphatase CD45RA.[26] [27] Furthermore, BFU-E appear to share with late CFU-MK progenitors the expression of glycoprotein IIb/IIIa (CD41) and of the megakaryocytic receptor c-Mpl.[27] [28]

As BFU-E mature to the CFU-E stage, they begin to express surface proteins characteristic of the morphologically recognizable erythroid cells, the erythroblasts. For example, CFU-E express the erythroid-specific sialoglycoprotein, glycophorin A, and Rh antigens. Blood group antigens of the ABH,iI type are also detectable at least in a subset of CFU-E. In contrast, CD34 molecules and class II antigens and certain growth factor receptors (i.e., IL-3R, c-kit) are greatly diminished or virtually absent at the CFU-E stage (Table 15-2). The most important functional difference between BFU-E and CFU-E is the abundance of EPO receptors on CFU-E and their dependence on EPO for cell survival. CFU-E, in contrast to BFU-E, cannot survive in vitro even for a few hours in the absence of EPO. Although >80% of CFU-E have detectable EPO receptors,[29] only a small proportion of BFU-E have receptors [30] [31] and can terminally differentiate in culture in the presence of EPO alone. [32] Direct binding studies show that the number of EPO receptors peaks at the CFU-E/proerythroblast level and progressively declines when cells mature further [30] (Table 15-2), reflecting the decline in the influence of EPO. In addition to the abundance of EPO receptors, erythroid progenitors are distinguished from other marrow progenitors by the presence of high levels of transferrin receptors. [31] [33] [34] The latter play a unique role in iron transport and in hemoglobin synthesis. Peak levels of transferrin receptors are seen on CFU-E and erythroid precursors, and lower levels are present on reticulocytes. [28] [33] (For a detailed review on iron metabolism and heme synthesis in erythroid cells, see reference [35] .)

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ERYTHROID PRECURSOR CELL COMPARTMENT

The erythroid precursor cell compartment, also termed the erythron, includes cells that, in contrast to the erythroid progenitor cells (BFU-E and CFU-E), are defined by morphologic criteria. The earliest recognizable erythroid cell is the proerythroblast, which after four to five mitotic divisions and serial morphologic changes gives rise to mature erythroid cells. Its progeny include basophilic erythroblasts, which are the earliest daughter cells, followed by polychromatophilic and orthochromatic erythroblasts. Their morphologic characteristics reflect the accumulation of erythroid-specific proteins (i.e., hemoglobin) and the decline in nuclear activity (Fig. 15-3). After the last mitotic division, the inactive, dense nucleus of the orthochromatic erythroblast moves to one side of the cell and is extruded encased by a thin cytoplasmic layer. Expelled nuclei are ingested by marrow macrophages, and the resulting enucleated cell is a reticulocyte. Although all mammals have enucleated cells in their circulation, the evolutionary advantage of enucleation is not readily apparent. It may allow for more red cell deformability when

Figure 15-3 Erythroid maturation sequence. As proliferation parameters (i.e., rates of DNA and RNA synthesis) and cell size decrease, accumulation of erythroid-specific proteins (i.e., heme and globin) increases, and the cells adapt their characteristic morphology. *(Adapted from Granick and Levere, [245] with permission.)*

traveling through the small vasculature or it may minimize the cardiac workload.

It is unlikely that maturation from the proerythroblast to the reticulocyte always adheres to a rigid sequence in which each division is associated with the production of two more differentiated and morphologically distinct daughter cells (i.e., with a basophilic erythroblast giving rise to two polychromatophilic ones). Rather, significant flexibility may be allowed, both in the number and rate of divisions and in the rate of enucleation. Such deviations from the normal, orderly maturation sequence may be dictated by the level of EPO. Thus, when there is an acute demand for red cell production (because of blood loss or hemolysis), the kinetics of formation of new reticulocytes are significantly more rapid. Resulting red cells may be larger (i.e., with increased mean corpuscular volume). This has led to the concept of skipped divisions. [36]

The alterations in cell morphology as erythroid precursor cells mature (Fig. 15-3) are determined by complex biochemical changes, which accommodate the accumulation of erythroid-specific proteins and the progressive decline in proliferation. Compared to erythroid progenitor cells, erythroid precursor cells have been more accessible to study, and considerable information is available about their maturation-related biochemical changes.

The shape and deformability of the red cell is determined by its membrane proteins. Most membrane cytoskeletal proteins (spectrin, glycophorin, band 3, band 4.1, and ankyrin) accumulate after the CFU-E stage (i.e., within the precursor cell compartment). Specifically, expression of membrane glycoproteins such as band 3 and band 4.1 is greatly enhanced at the later stages of erythroid maturation. [37] [38] [39] Likewise, the quantity of polylactosaminoglycan, a specific carbohydrate chain that carries blood group ABH and iI antigenic determinants, is much higher in mature erythrocytes than in erythroblasts. [40] Whereas

a linear, virtually unbranched polylactosamine structure is present in fetal and newborn erythroid cells (reflected by i antigenic reactivity), a branched polylactosaminyl structure is present in adult erythroblasts (reflected by I antigenic reactivity), and branching increases further as maturation progresses. [40] Glycophorins, especially glycophorin A, are expressed fully at the CFU-E or proerythroblast level just before the expression of globin, and there are few changes during maturation. [20] In contrast, the membrane glycoproteins p105 and p95 decline during the later stages of maturation [40] and yet other membrane glycoproteins, such as vimentin (an intermediate filament protein) are totally lost. [37] The loss of vimentin expression at the late erythroblastic stages most likely facilitates enucleation.

In addition to quantitative changes during maturation, there are gradual switches in subunit composition of some cytoskeletal proteins. For example, exclusively erythroid subunits of - and -spectrin are displayed only in end-stage cells. [38] Likewise, multiple transcripts of ankyrin or protein 4.1 have been identified, and the ratios of these transcripts changes during maturation. [41] It is likely that the initial expression of many of these membrane components begins at the progenitor cell level. In these cells, however, final assembly may be discouraged because of the higher turnover of these proteins, which minimizes mutual interactions, or because of asynchrony in their synthesis. Prevention of cytoskeletal assembly at these early stages may secure more membrane fluidity and cell motility needed during this proliferative phase of differentiation. Because molecular probes for many of the red cell cytoskeletal components have been developed, detailed information about the transcription and processing of most of these proteins is beginning to emerge. [39]

Gene activity during erythroid maturation is dominated by the expression of globin. Although globin represents <0.1% of protein at the proerythroblast level, it reaches 95% of all protein at the level of reticulocytes. [42] Its expression has been extensively studied, and its gene regulation is well understood in molecular terms. Major steps in its transcription and processing are now known in considerable detail and are summarized elsewhere in this volume. The globin type synthesized by the adult precursors is hemoglobin (Hb) A($_2$ $_2$). In addition, two other minor globin components, Hb A$_2$ ($_2$ $_2$) and Hb F ($_2$ $_2$) are present. Of significant biologic interest are the low amounts of Hb F that continue to be synthesized throughout life.

This small amount of Hb F, which is present in all normal individuals, has the following characteristics [43] : (1) It is confined to a small fraction of red cells, called F cells, which are detected by sensitive immunofluorescence assays or acid elution techniques and usually constitute 25% of all red cells. Within each F cell, Hb F or -globin constitutes 1425% of total globin. (2) The number of F cells is genetically determined, and gene(s) linked or nonlinked to the -locus are responsible for F-cell formation. (3) F cells do not display other features of fetalness, because their membrane components and enzymes are characteristically adult. (4) Synthesis of Hb F peaks earlier than that of Hb A, so the proportion of fetal hemoglobin is higher in immature cells compared with mature, fully hemoglobinized cells. (5) F cells and cells that contain only Hb A are not derived from distinct stem cell populations but from a common adult stem cell. Whether this cell will form F or non-F (i.e., A) cells is determined at the BFU-E and throughout the CFU-E level. In vitro the great majority of BFU-E have the potential to express Hb F, whereas in vivo only a very small proportion of red cells contain Hb F; this potential appears to be lost during normal cell differentiation and maturation in vivo. This concept links the potential for Hb F expression to the pathway of erythroid differentiation and thus may have implications for interpreting the reactivation of Hb F that occurs in adults under diverse circumstances (e.g., after chemotherapy or with acute bleeding) [43] Many of these circumstances seem to influence Hb F levels by directly or indirectly modifying the kinetics of the normal differentiation/maturation process. [44] [45]

The synthesis of globin appears to be coordinated with the synthesis of heme throughout erythroid maturation, so that functional hemoglobin tetramers are formed

rapidly and spontaneously after the release of newly synthesized globins from polysomes. Information about the accumulation of heme and its synthetic intermediaries has been provided thus far by crude biochemical approaches (Fig. 15-3). However, as the genes for several enzymes in the heme synthetic pathway have now been cloned (i.e., genes for -aminolevulinic acid synthetase, porphobilinogen deaminase, and heme synthetase), information about their regulation is rapidly emerging. [35]

Crucial to the functional response of erythroid precursors is the expression of EPO receptors and transferrin receptors. EPO receptors decrease progressively (from about 1,000 to <300 receptors/per cell) as proerythroblasts mature, and they are undetectable at the reticulocyte level. [29] [46] Through these receptors, EPO exerts its proliferative influence on proerythroblasts and basophilic erythroblasts, but maturation beyond these stages can proceed in the absence of EPO.

Transferrin receptors are found in characteristic abundance in erythroid cells (300800,000/cell). [47] This is a reflection not only of the proliferative needs of erythroid cells but also of their extreme requirements for iron uptake for hemoglobin synthesis. It is for this reason that transferrin receptors persist in maturing, nondividing erythroblasts and in reticulocytes. Transferrin receptors belong to a large group of receptors that internalize their ligand through receptor-mediated endocytosis. This cycle allows for reuse both of the ligand (transferrin) to be resaturated with iron and of the receptor to enter an additional route of endocytosis. [48] Transferrin receptor density decreases with maturation, and after the reticulocyte stage, receptors appear to be shed as small lipid vesicles. [49] There is an inverse relationship between receptor density and the availability of iron. Deprivation of iron results in receptor induction, and excess iron results in receptor suppression. [48] However, the mechanisms that regulate the number of transferrin receptors throughout the maturation of precursors (even within progenitors) are largely unknown. Erythroid precursor cells differ from nonerythroid cells not only by requiring a higher number and higher occupancy of transferrin receptors but by displaying immunologically distinct receptor isoforms. [34] Whether the antigenically distinct form of transferrin receptor in erythroid cells is of physiologic significance to the unique role of erythroid cells in iron metabolism remains to be seen. However, monoclonal antibodies recognizing distinct receptor isoforms are useful in the isolation of erythroid cells from bone marrow. [34] [50]

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ERYTHROPOIETIN AND THE ERYTHROPOIETIN RECEPTOR

EPO, a 35-kd glycoprotein, [51] is the physiologically obligatory growth factor for erythroid development. It is produced in the kidney, by peritubular cells. [52] A heme-containing protein senses oxygen need and then triggers the synthesis of EPO and its release into the bloodstream. [53] Through the interaction of EPO with receptor-bearing cells within the bone marrow, physiologic oxygen demands are translated into increased red cell production. Thus, EPO is a true hormone, manufactured at one anatomic site and transported through the bloodstream to the site of activity.

According to the prevailing model of hematopoiesis, progenitor cells committed to erythroid differentiation (i.e., BFU-E) are generated in a stochastic fashion from pluripotent stem cells.[3] [4] Neither EPO nor other lineage-restricted regulators play any role in determining lineage commitment. According to this model, EPO influences erythroid differentiation by rescuing (from apoptosis) cells that express the EPO receptor and amplifying

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them further. Whether EPO receptors are present on all BFU-E (although only detectable on a subset of BFU-E) is not clear. [30] Thus, it is not known whether the presence of the EPO receptor in BFU-E is synchronous with the initial commitment event or follows it. In addition to the permissive role that the stochastic theory ascribes to EPO, experiments in vivo, in anemic states, or after pharmacologic doses of EPO, suggest that high levels of EPO may hasten the transition from BFU-E to hemoglobin-synthesizing cells either by decreasing the number of divisions required for this transition [36] or by decreasing the resting periods between cell divisions.[54] Autoradiographic studies in purified BFU-E populations indicate that EPO receptors increase as BFU-E mature to CFU-E, with the highest level observed at the CFU-E/proerythroblast boundary.[30] As the transition from BFU-E to CFU-E occurs under the influence of EPO, it may suggest ligand (EPO)-induced receptor up-regulation. Whether the magnitude of such up-regulation is EPO dose-dependent and whether it can modulate the rate of entry of these cells into the maturing compartment is unclear.

BFU-E and CFU-E can be generated in vitro [55] and in vivo,[56] in the absence of EPO or EPO receptor (in EPO or EPO-R null mice), but their survival and terminal maturation is normally dependent on EPO. For CFU-E EPO seems to stimulate all the biochemical processes characterizing erythroid cells (i.e., heme synthesis, globin synthesis, and synthesis of cytoskeletal proteins). However, the precise role of EPO in these processes has not been delineated. Whether EPO only increases the number of cells engaged in these biochemical functions or directly influences the intracellular mechanism of transcription of erythroid-specific proteins is unclear. Indeed, experiments in vitro showing complete maturation of BFU-E in the absence of EPO suggest that other factors or combinations of factors can influence red cell maturation. Activation of gp130 signaling pathway through the use of soluble IL-6 receptor and IL-6 leads to full terminal erythroid maturation (in the presence of SCF and IL-3 but in the absence of EPO), suggesting some form of cross-circuiting in signaling pathways among hematopoietic growth factor receptors. [57] [58] Furthermore, the stimulation by thrombopoietin of erythroid colony formation from yolk sac cells in the absence of EPO-R (in EPO-R / embryos) [59] can be explained by the same reasoning and the fact that there is a very high proportion of bipotent erythroid/megakaryocytic progenitors in yolk sac carrying both EPO and Tpo receptors (c-mpl) compared to adult bone marrow.[60] [61]

Whatever the precise mode of EPO action, it directly affects the number of CFU-E and the maturation of their progeny. This control is achieved by influencing CFU-E survival and not their cycling status.[62] CFU-E are irrevocably lost after one cycle of DNA synthesis if EPO is not present. [63]

With the availability of radiolabeled recombinant EPO and of purified or enriched populations of progenitors and precursors, information about the characteristics of EPO receptors in erythroid cells is continuously emerging. Direct binding studies have shown that as CFU-E and proerythroblasts mature to reticulocytes, there is a progressive decrease in the number of EPO receptors. [30] [31] [46] Pure reticulocyte populations show no detectable binding to EPO. The maturation-associated decline in the number of EPO receptors parallels the declining influence of EPO on erythroid cells during the terminal phase of maturation.

The cloning and expression of the erythropoietin receptor (EPO-R) has allowed a better understanding of the role of EPO in the regulation of erythroid development. The EPO-R polypeptide is a 66 kilodalton membrane protein, which is a member of the cytokine receptor superfamily. [64] [65] Many of the structural features of the cell surface EPO-R have been previously reviewed.[66] Like other members of the cytokine receptor superfamily, which includes the receptors for IL-3, GM-CSF, and IL-5, the EPO-R polypeptide contains four conserved cysteine residues and a WSXWS motif in the extracellular region. Additional extracytoplasmic sequences of the EPO-R determine the specificity for EPO binding. The cytoplasmic region of the EPO-R does not contain a tyrosine kinase catalytic domain, but instead interacts with cytoplasmic tyrosine kinases. Crosslinking of radiolabeled EPO to the cell surface EPO-R results in the formation of at least two major crosslinked protein complexes of 140 kD and 120 kD.[67] The molecular composition of these complexes remains unsolved but suggests that the EPO-R may contain additional subunits or accessory proteins.[68] The extracytoplasmic region of the EPO-R polypeptide contains the EPO binding activity of the receptor. [69] [70] [71] Additional EPO-R subunits may therefore provide other structural and functional elements of the receptor but are not required for high-affinity EPO binding. The extracytoplasmic region of the EPO-R polypeptide has also recently been crystalized. [72] [73] [74] [75] The crystal structure confirms the dimeric structure of the activated receptor. Interestingly, small synthetic peptides are capable of inducing EPO-R dimerization, suggesting a possible avenue of research for EPO-mimetic and EPO antagonist drug design. [75]

The EPO-R mRNA originally isolated from murine erythroblasts cell lines (MEL and HCD57) [76] and from a human erythroid cell line (OCIMI)[77] was found to be present in nonerythroid cells as well. EPO promotes the differentiation of megakaryocytes at physiological concentrations of hormone, suggesting that megakaryocytes have functional cell surface EPO receptors. Rat and mouse placenta also have cell surface EPO-R, detected by radiolabeled EPO crosslinking. EPO promotes a chemotactic effect on endothelial cells,[78] [79] suggesting the presence of a cell surface receptor in these cells as well. Other studies suggest that the EPO-R is expressed in neural cells[80] and smooth muscle cells,[81] though the functional importance of this expression remains unclear. The existence of naturally-occuring splice variants of the EPO-R gene encoding EPO-R polypeptides of variable length and activity [82] [83] [84] [85] has been shown. The soluble secreted form of the EPO receptor[86] binds EPO and thereby competes with the cell surface receptor isoform. The biological function of alternative forms of the cell surface EPO-R, including a truncated form of EPO-R found in early progenitors, [87] remains largely unknown, but may be related to differential EPO signaling and responses (survival, proliferation, differentiation) at different stages in erythroid development.

Signal Transduction by the EPO-R

Considerable progress over the last few years has been made in our understanding of EPO-R-mediated signal transduction. Early studies demonstrated that stimulation of the EPO-R on primary erythroid cells resulted in increased calcium ion flux and increased globin mRNA synthesis. [88] Since the cloning of the EPO-R polypeptide and its stable expression in heterologous cell systems, such as the Ba/F3 cell system, [89] considerable molecular insight has been gained. [90] For instance, it is now clear that EPO induces the homodimerization of the EPO-R polypeptide. [91] [92] Following receptor dimerization at the cell surface, a series of tyrosine phosphorylation events occurs, resulting in a mitogenic signal and a differentiative signal. [93] [94]

Initial studies of the EPO-R signal transduction pathway made use of mutant forms of the EPO-R stably expressed in the indicator cell line, Ba/F3. Ba/F3 cells are a

murine IL-3-dependent pro-B lymphocyte cell line. These cells can be readily transfected with the cDNA for the EPO-R, resulting in stable expression of the receptor on the cell surface. Expression of the full-length, wild-type EPO-R polypeptide in these cells resulted in EPO-dependent growth and partial EPO-induced erythroid differentiation.[93] [94] Expression of truncated forms of the EPO-R polypeptide in these cells resulted in variable growth responses. For instance, truncation of the membrane proximal region of the EPO-R demonstrated a critical positive regulatory domain

of the EPO-R, required for mitogenesis. [89] Furthermore, truncation of the carboxy terminal 40 amino acids of the EPO-R resulted in increased EPO-dependent growth, suggesting that the carboxy terminal region contained a negative regulatory domain normally required for down-modulating EPO-R mitogenic signals. [89]

The biochemical basis for these positive and negative regulatory domains has been elucidated. On the one hand, the membrane proximal positive regulatory region of the EPO-R binds constitutively to the cytoplasmic tyrosine kinase, JAK2. [95] Upon EPO binding to the receptor, the receptor dimerizes, resulting in activation of the prebound JAK2 kinase. The JAK2 kinase next tyrosine phosphorylates multiple signaling proteins in the cell, leading to various mitogenic and differentiative responses. The negative regulatory domain of the EPO-R, on the other hand, is required for recruiting the phosphatase, SHP1, to the EPO receptor. [96] SHP1 binds to an activated tyrosine phosphate on the EPO-R polypeptide and functions to rapidly down-regulate JAK2 kinase activity and to dephosphorylate the EPO-R polypeptide.

Activated JAK2 kinase initiates several events in EPO-R mediated signal transduction. JAK2 initially activates the tyrosine phosphorylation of several tyrosine residues of the cytoplasmic tail of the EPO receptor. These phosphorylated tyrosine residues next serve as docking sites for the binding of other cytoplasmic effector proteins containing SH2 domains, such as the p85 subunit of PI3 kinase, [97] the adaptor protein *Shc*, [98] [99] and the STAT transcription factor, STAT5. [100] [101] Once these proteins have docked on the EPO-R, they become tyrosine phosphorylated and activated for other downstream signaling events. In addition, JAK2 activates the Ras/Raf/MAPKinase pathway, further contributing to the EPO-induced mitogenic signal. [102] [103] The molecular mechanism of Ras activation by JAK2 remains unknown, but may entail direct binding of the proteins and tyrosine phosphorylation. [103] Activation of the JAK2/STAT5 signaling pathway has been studied in considerable detail. Upon EPO-R tyrosine phosphorylation, STAT5 protein binds to a specific phosphorylated tyrosine residue of the EPO-R. [100] [104] Binding is mediated by the SH2 domain of STAT5. Following EPO-R binding, STAT5 itself becomes tyrosine phosphorylated at amino acid Y694. [105] Activated STAT5 then disengages from the EPO-R, undergoes homodimerization, and translocates to the cell nucleus where it activates transcription of EPO-inducible genes. Some EPO-inducible genes, such as *myc* and *fos,* are common to other hematopoietic growth factor signaling pathways. Other EPO-inducible genes are specifically expressed in erythroid cells and are not shared by other growth factor responses. [106]

Other signal transduction pathways downstream from cytokine receptors have also been identified. For instance, EPO and IL-3 activate the tyrosine phosphorylation of the signaling protein, *cbl*, and the subsequent binding and tyrosine phosphorylation of the signal protein, CrkL. [107] The mechanism of activation of this pathway by the EPO-R is not known and the relative role of this pathway in EPO-induced growth and erythroid differentiation remains largely unexplored.

A critical question in the field of EPO-R signal transduction is the mechanism of EPO specificity. Most if not all of the signal transduction pathways activated by the EPO-R (i.e., Ras/Raf/MAPKinase and JAK/STAT pathways) are shared by other hematopoietic cytokine receptors, such as the receptors for IL-3, GM-CSF, and IL-5. How the EPO-R triggers a specific growth factor response resulting in erythroid differentiation remains unclear. Several models are possible. First, the EPO-R may activate a unique but still unknown signaling pathway specific to the EPO-R and distinct from other cytokine receptors. Alternatively, the EPO-R may activate the identical pathways, activated by other cytokine receptors. In this latter model, the specificity of the EPO signal is derived not from the EPO-R itself but instead from interactions with other developmentally programmed events in the erythroid cell, such as the expression of erythroid specific transcription factors.

Activation of the EPO-R in the murine IL-3 dependent cell line, Ba/F3, results in induction of both mitogenesis and -globin accumulation. [93] In contrast, the murine IL-2 dependent cell line, CTLL-2, when engineered to express the heterologous EPO-R, grows in EPO but does not differentiate into globin bearing cells. These data suggest that the expression of the EPO-R is necessary for erythroid differentiation but is not sufficient alone. It is likely that other erythroid-specific markers, such as GATA-1 and NFE-2, or EKLF, are required for cells to differentiate down the erythroid pathway. Other cytokine receptors, such as the IL-3R and the IL-2R, do not drive -globin synthesis in these cell lines. Taken together, these results suggest that the EPO-R generates a differentiation-specific signaling within the context of a proper cellular environment.

Regardless of the mechanism of cytokine specificity, it is clear that each cytokine receptor activates a similar but not identical pattern of signaling events. For instance, the EPO-R shows a preferential activation of the JAK2/STAT5 pathway in cultured erythroid cells in vitro. In contrast, the interleukin-2 receptor shows a preferential activation of the JAK1/JAK3/STAT6 pathway. [105] [108] [109] Interestingly, although EPO activates STAT5a and STAT5b in cultured cells, knockout of the STAT5a or STAT5b gene by homologous recombination results in a normal mouse phenotype with normal erythroid development. [110] This result suggests that, in vivo, other STAT proteins are capable of substituting for STAT5 and functioning downstream of the EPO-R and emphasizes the importance of in vivo studies to confirm the phenotypic relevance of in vitro studies.

Previous studies have also suggested that EPO functions synergistically with other multilineage growth factors, such as KL and IL-3. As discussed in previous sections, EPO and KL function together, resulting in increased erythroid colony cell growth in methylcellulose culture. Recent studies with the EPO-R polypeptide suggest a molecular mechanism for such synergy.[111] Activation of the kit receptor by KL results in a transphosphorylation of the EPO-R at the cell surface. Also, a direct interaction between the EPO-R and the kit receptor has been demonstrated. Taken together, these results suggest that receptor cross-talk at the cell surface may account, at least in part, for the physiologic interaction of some cytokines in controlling hematopoietic cell growth.

The Erythropoietin Receptor in Disorders of Erythropoiesis

As discussed above, the normal role of EPO is to stimulate the cell surface EPO-R in developing erythroid cells. The latter respond to EPO via a proliferative and differentiation response. The EPO-activated signal transduction of the EPO-R is quickly down-regulated in the cell, and continuing presence of EPO is required for optimal differentiation.

In some cells, the EPO-R may become constitutively activated. In these cases, erythroid progenitor cells are placed into a sustained proliferative state. Interestingly, these mechanisms underlie several murine and human examples of erythrocytosis (erythroid overproduction). Multiple mechanisms exist by which the EPO-R may become constitutively activated. First, the Friend Spleen Focus-Forming Virus (SFFV) of the Friend erythroleukemia complex encodes a glycoprotein, F-gp55, which binds and activates the murine EPO-R.[112] [113] [114] F-gp55 appears to bind to the EPO-R via its transmembrane region. The EPO binding site and the F-gp55 binding site are discrete; tertiary complexes of EPO-R, EPO, and F-gp55 have been detected on the surface of Friend Virus-infected cells. [115] Second, the EPO-R can be constitutively activated by a point mutation

(R129C) in the extracytoplasmic region of the polypeptide. [116] This mutation occurs in the dimerization domain of the EPO-R and results in the constitutive homodimerization of the EPO-R polypeptide, presumably through a disulfide bond. This mutation further underscores the importance of receptor dimerization in the initiation of a receptor signaling response. Third, the EPO-R can be constitutively activated in an autocrine manner. Murine erythroleukemia cell lines have been established that coexpress EPO-R and EPO. A fourth mechanism of EPO-R constitutive activation results from EPO-R overexpression. For instance, some murine erythroleukemia cell lines have increased EPO-R mRNA, resulting from SFFV proviral integration within the first intron of the murine EPO-R gene. [117] Overexpression of the normal murine EPO-R polypeptide may thereby contribute to oncogenesis.

Soon after the cloning of the cDNA for the mouse and human EPO-R, the mouse and human genomic structures were identified. The gene for the mouse EPO-R was found to map to mouse chromosome 9, whereas the human gene was found on human chromosome 19p.[118] [119] The mapping of the EPO-R genes led to their implication in various human disease states. For instance, recent studies have demonstrated that a chromosomal breakpoint 3 to the human EPO-R gene results in increased EPO-R expression.[120] The rearranged EPO-R allele appears to encode a mutated EPO-R polypeptide with increased activity, perhaps secondary to loss of the carboxy terminal negative regulatory domain. More recently, the EPO-R has been found to play a role in a rare congenital disease, Familial Erythrocytosis (FE). FE refers to a heterogeneous group of hereditary conditions characterized by an increase in red blood cell mass in the setting of low serum EPO levels. A few families have been identified that demonstrate autosomal dominant inheritance. [121] Interestingly, a linkage has been established between the EPO-R gene and FE. More

recent evidence demonstrates that, in one FE kindred, a mutant EPO-R allele that segregates with the disease contains a nonsense mutation in the coding region of the gene. This mutation results in the synthesis of a truncated EPO-R that lacks the previously described negative regulatory domain of its carboxy terminal region. [122] [123] Additional mutations within the EPO-R gene have also been found, resulting in FE, [124] but have not been identified in acquired disorders of erythropoietin, such as PV (polycythemia vera).

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TRANSCRIPTIONAL CONTROL OF ERYTHROPOIESIS

It is widely believed that lineage-specific transcriptional factors are responsible for regulating the expression of erythroid genes both during ontogeny and the course of erythroid differentiation. Early experiments with somatic cell fusions and with transgenic mice have provided compelling evidence for this belief. [125] [126] [127] More recent studies in mice with targeted gene disruption have provided key insights into the complex molecular pathways that regulate hematopoiesis in general and erythropoiesis in particular.[1] These studies, complemented by in vitro differentiation of mutated ES cells into different lineages, have also provided clear evidence about distinct regulatory requirements of primitive (yolk sac) versus definitive (fetal liver and bone marrow) erythropoiesis, or of early versus late stages of erythroid differentiation.

As erythropoiesis is the first differentiated lineage in embryonic yolk sac hematopoiesis and the predominant lineage in fetal liver hematopoiesis, factors that affect hematopoiesis in general will disturb erythropoiesis during early stages of development and will lead to lethality at different gestational days depending on the defect. Mice lacking the bHLH factor Tal-1/SCL are bloodless and die very early with abrogation of both yolk sac and fetal liver erythropoiesis. [128] Because Tal-1/SCL is preferentially complexed in erythroid cells with the nuclear protein Rbtn2/LMO2, it is not surprising that the targeted disruption of the latter gene also produces a bloodless phenotype,[129] similar to the one seen in Tal-1/SCL knockouts. Furthermore, mice lacking GATA-2 expression , a member of the GATA family of transcriptional factors, also exhibit an early and severe quantitative defect in hematopoiesis that influences all lineages. [130] Other regulatory factors seem to totally spare embryonic (yolk sac) hematopoiesis and have a specific effect only on fetal liver hematopoiesis with death occurring at later days (12.5 days post conception). In this category are the proto oncogene c-myb and the core-binding factors CBF $_2$ /AML-1 and CBF.[131] [132] [133] Embryonic erythropoiesis is spared in mice with targeted ablation of these genes. Both c-myb, the cellular homolog of v-myb proto oncogene and the heterodimeric transcription factor core binding factor (CBF), are abundantly expressed early in normal myelolymphoid cells, decreasing as differentiation proceeds. This expression pattern and their functional influence on growth factor receptor genes (i.e., IL-3, GM-CSF, CSF-1, TCR,) may underlie their importance in the development of all hematopoietic lineages. [1]

Of specific importance for erythropoiesis, however, is the transcription factor, GATA-1, the founder of the GATA family of factors. [1] This protein interacts with the WGATAR DNA motif frequently found in erythroid genes and influences their expression. Both GATA-1 and GATA-2 are expressed early in multipotential progenitors; however, their expression ratios change as the cells differentiate, suggesting that the ratio of these two factors may be important at specific stages of erythroid differentiation. Knockout experiments with both of these genes have borne this out. Thus, in contrast to GATA-2, expressed at high levels in early cells and affecting expansion of all hematopoietic lineages, [130] GATA-1 expression increases as differentiation advances and seems to be the obligatory factor required for survival and terminal differentiation of erythroid cells. In mice with targeted disruption of GATA-1, erythropoiesis proceeds only up to the stage of proerythroblasts, which die early and fail to mature further.[134] [135] Furthermore, transgenic mice with partial loss of function (knockdown alleles) of GATA-1 show that erythroid differentiation is dose-dependent with respect to GATA-1.[136] High levels of GATA-1 may be necessary to form complexes with its co-factor FOG [137] and perhaps with other interacting zinc-finger proteins during terminal erythroid differentiation. Another factor with special importance in the erythroid lineage is the CACCC binding protein, designated erythroid Krüppel-like factor (EKLF), which is expressed at all stages of erythropoiesis, but binds preferentially to CACCC sites in globin promoter. Mice lacking EKLF die from a thalassemic-like defect, because of severe deficiency of globin expression. [138] Other erythroid genes are largely unaffected.

As common transcriptional factors are present in erythroid and megakaryocytic cells, and bipotent erythroid/megakaryocytic progenitors exist both in vitro, in the form of cell lines, and in vivo,[27] recent exciting insights regarding subtleties in the molecular control of these two lineages by the same transcriptional factors have surfaced. By modified gene targeting strategy (knockdown) of GATA-1, a largely unanticipated role for this transcriptional factor in the control of proliferation and maturation of megakaryocytes was uncovered.[139] In addition to GATA-1 other important transcriptional factors essential for terminal megakaryocytic development are NFE2[140] and its partner MatG.[141]

Despite the affiliation of specific transcriptional factors, such as GATA-1, with certain lineages (erythropoiesis, megakaryocytopoiesis), their role in lineage commitment decisions has not been established. Several in vitro experiments would suggest that a dose-related influence of GATA-1 in lineage selection exists. [1] However, these experiments concern only oligopotent or bipotent cell lines with phenotypic plasticity, and the data may

not apply to decisions faced by the hematopoietic stem cells. Nevertheless, the fact that several regulators are necessary for primitive (yolk sac), as opposed to definitive (fetal liver and bone marrow) erythropoiesis, provide evidence that the molecular control between these two hemopoietic sites is indeed different and it may include both ubiquitous and hematopoietic specific factors. In fact, recent evidence suggests that GATA-1 transcription is differentially regulated in yolk sac cells compared to fetal liver erythroid cells, with alternative promoter use and an additional intron element requirement for promoter activation in fetal liver cells. [142]

In addition to transcriptional factors/oncogenes influencing erythropoiesis, targeted ablation and naturally existing mutations of hematopoietic growth factor receptors, especially of the tyrosine kinase family, have disclosed important insights in the control of erythropoiesis. Whereas deletion of the VEGF/flk-1 receptor affects both endothelial and hematopoietic development[143] through its presumed presence in the common endothelial/hematopoietic stem cell, the hemangioblast, mutations affecting the tyrosine kinase kit-receptor (present in hematopoietic cells) or of its ligand (kit ligand, KL, present in stromal cells) seem to predominately affect erythropoiesis in the fetal liver and the adult animal. Mice with kit-mutations (W mutations) leading to absence of or compromised kinase activity, and Steel mice with mutations of KL have disproportionate and severe abrogation of the late erythroid progenitors, CFU-E, and differentiated erythroid precursors resulting in anemia. [144] Recent studies showing cross-phosphorylation of EPO-R following activation of kit/kit ligand signaling may be relevant to the above effect. [111] Mutations or targeted ablations of some downstream signaling substrates for kit or other receptors (i.e., Shp-2 phosphatase or gp 130) seem to produce a hematopoietic picture not unlike the one produced by receptor mutations.[145] [146]

Taken together, these recent studies have significantly expanded our understanding at the molecular level of hematopoietic development in general and of erythropoiesis in particular. The emerging picture is that certain genes, such as SCL, are absolutely required for hematopoietic development, whereas others, such as GATA-2, c-myb, CBF, TEL, and some downstream signal transducing molecules, such as gp130 and Shp-2, are responsible for expansion and maintenance of a normal pool of fetal liver and adult hematopoietic progenitors. The participation of many of these molecules in multicomponent molecular complexes with protein/protein and protein/DNA interactions (i.e., LM02/Lbd1/SCL/E2A/GATA), during the early, proliferative stages of hematopoiesis [147] may underlie their role in the proliferation and maintenance of immature progenitor/precursor pools in erythropoiesis. Other genes such as GATA-1, its partner FOG, and EKLF are necessary to direct high levels of function of erythroid-specific genes in cells already committed to terminal differentiation. Thus, a hierarchical requirement in the expression of specific regulators during early versus late erythroid differentiation or during yolk sac versus fetal liver/adult erythropoiesis is demonstrated. This, however, does not exclude the involvement of some factors (i.e., SCL, TEL) both at early and late stages of erythropoiesis. As information from innovative applications of molecular approaches is becoming available at a fast pace, the list of regulators with a biologic impact on hematopoiesis/erythropoiesis is continuously expanding.

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HEMATOPOIETIC MICROENVIRONMENT

In invertebrates such as worms and sessile marine creatures, erythropoiesis takes place adjacent to peritoneal and endothelial cells. In premammalian species, the spleen is the primary site of erythropoiesis, and with evolutionary advancement, this gradually shifts to the liver and the sinusoidal cavities of bones. [148] These observations suggest that sufficient oxygen, a stagnated flow of blood to avoid the dispersion of factors produced locally, and extensive and redundant surfaces for cell-cell interactions are essential to support red cell production. Similar sites support erythropoiesis during human development, as discussed following. During both phylogeny and ontogeny, the liver and spleen are primarily erythropoietic organs, while granulocytic cells dominate in the bone marrow. [148] Within the bone marrow, hematopoiesis is restricted to the extravascular space, where compact collections of cells are interspersed among venous sinuses. These sinuses originate adjacent to the endosteal bone surface and empty into a central longitudinal vein. Studies in mice demonstrate that BFU-E follow a bimodal distribution with peaks adjacent to the periosteum and midcavity, whereas CFU-E and later erythroid cells have a broad distribution with highest incidence toward the axis of the femur, adjacent to the central vein, [148] [149] and thus suggest that the local anatomy influences the maturation of erythroid cells.

The bone marrow microenvironment consists of three components; stromal cells (e.g., fibroblasts, endothelial cells), accessory cells (monocytes, macrophages, T cells), and extracellular matrix (a protein-carbohydrate scaffold). Accessory cells are progeny of hematopoietic stem cells, and hence after marrow transplantation, these cells are of donor origin, whereas stromal cells are host-derived. [150] [151] Extracellular matrix molecules are synthesized and secreted by microenvironmental cells and include collagens (types I, III, IV, and V), glycoproteins (fibronectin, laminin, thrombospondin, hemonectin, and tenascin), and glycosaminoglycans (hyaluronic acid and chondroitin, dermatan, and heparan sulfate). [152] [153] Besides providing structure to the marrow space, and a surface for cell adhesion, the microenvironment is important for hematopoietic cell homing, engraftment, migration, and the response to physiologic stress and homeostasis.

Although the functional consequences of the microenvironment must ultimately be defined by in vivo studies in mice, dissection of the cellular components of the microenvironment, definition of the cytokines that are produced by individual cells, and the nature of cell-cell interactions have been aided by in vitro models. Long-term bone marrow cultures provide an experimental approach for such studies. [154] [155] In these in vitro conditions, murine hematopoiesis can be maintained for 810 months, and human hematopoiesis for 2 to 3 months.[154] An adherent layer consisting of fibroblasts, adipocytes, and macrophages is a crucial component of the culture system. Progenitor cells adherent to stroma are generally quiescent (dormant) whereas those in the nonadherent cell compartment are in active cell cycle. [155] [156]

With in vitro studies, it has been demonstrated that stromal cells, including endothelial cells and fibroblasts, elaborate cytokines such as GM-CSF, G-CSF, IL-1, IL-3, IL-6, IL-11, KL, flt-3 ligand, activin A, and basic fibroblast growth factor, which influence, alone or in combination, the growth of adjacent marrow progenitors. [154] [155] [156] [157] In addition to positive regulators of replication and differentiation, stromal cells elaborate factors, such as transforming growth factor-beta (TGF), interferon-, and tumor necrosis factor (TNF), which exert a negative influence on proliferation and may help maintain a dormant (noncycling) state. [155] [156] Because some regulators inhibit differentiation along certain lineages, but not others, there is an intriguing possibility that lineage-specific regulation within the microenvironment may be achieved through negative, rather than positive, factors. [158] Several cytokines are expressed in a transmembrane form as well as a soluble (secreted) product. Others bind extracellular matrix, a mechanism that allows for the retention of high concentrations of factor within the microenvironment adjacent to developing progenitors, and that metabolically stabilizes these factors.

Among the factors elaborated by stromal cells, KL has the

most profound effect on erythropoiesis. Mice unable to synthesize KL die in utero because of severe anemia. Steel-Dickie (Sl^{d}) mice that are unable to make the membrane-restricted form of KL are viable, but severely anemic, whereas other lineages in these animals are marginally affected or not affected by this defect. [159] The fact that erythropoiesis is abnormal, despite high levels of circulating Epo and the presence of soluble KL, suggests that normal erythroid differentiation and maturation require both a functional membrane restricted KL/kit and an EPO signaling pathway. Cross-phosphorylation of the EPO receptor by KL may provide a basis for the predominately erythroid effect. [160] Furthermore, recent data suggest that tyrosine cross-phosphorylation of the Epo receptor is sustained longer when cells are cultured on Steel stromal cells engineered to express the membrane restricted form of KL than cells expressing the soluble form. [161]

Besides cell/cytokine (paracrine) interactions, cell/cell adhesion and the adhesion of cells to the extracellular matrix are important functions of the microenvironment. [162] [163] Perhaps most important are the 1 integrins, VLA-4 and VLA-5, which mediate the adherence of hematopoietic cells to stromal cells, to fibronectin, or other components of the extracellular matrix. [163] [164] In mice lacking 1 integrins, hematopoietic stem cells fail to colonize the fetal liver during embryonic development. [165] Antibodies to VLA4 or to the vascular adhesion molecule VCAM-1 (a VLA-4 ligand on endothelial cells) impair homing and lead to the mobilization of progenitor cells and stem cells in adult mice and primates. [166] [167] [168] [169] In in vitro studies, hematopoietic progenitors bind to specific domains of fibronectin in a differentiation-dependent manner (LTCIC and CFU-S12 in mice adhere mainly through the heparin-binding domain and CS-1). BFU-E and other progenitor cells adhere to both the cell-binding (RGDS) and heparin-binding domain, whereas CFU-E preferentially bind the RGDS sequence, and reticulocytes fail to adhere to fibronectin. [153] [170] [171] [172] [173] This differential binding could influence the proliferation and maturation of erythroid progenitor cells, as well as the migration of these cells in and out of the bone marrow cavity. Hematopoietic cytokines present in the microenvironment can also modulate the affinity of 1 integrins for ligand, [174] [175] adding a further complexity to the regulation of erythropoiesis within the marrow microenvironment. In addition, other molecules on hematopoietic cells (e.g., c-kit, [176] CD31, [177] CD44, [178] L-selectin, [179] sialomycins [CD34, [180] CD43 [181]]) may have adhesive functions, but their physiologic roles have not been delineated.

Many observations suggest that hematopoietic progenitor cells at one stage of fetal development may not be supported by a hematopoietic microenvironment of a different ontogenetic stage. For example, cells present in the murine yolk sac are not able to repopulate adult recipients, [182] although they can repopulate newborn recipients with active fetal liver hematopoiesis. [183] Targeted disruption of PBSF/SDF-1, a member of the CXC chemokine family that is constitutively expressed by bone marrow stromal cells, leads to the inhibition of marrow hematopoiesis, although fetal liver hematopoiesis is unaffected, [184] suggesting that this chemokine has a role in the colonization of bone marrow from fetal liver. Recently it was also shown that TEL (translocation-Ets-leukemia) transcriptional factor is critical in the establishment of stable bone marrow hematopoiesis, but was not required for embryonic and fetal liver hematopoiesis. [185]

Accessory cells, such as stromal cells, secrete cytokines and express adhesion molecules, and also may influence marrow hematopoiesis by their nonrandom distribution in the marrow cavity. T cells (along with mast cells) are the only source for IL-3, and through the secretion of TNF- and interferon- may also negatively impact erythropoiesis. In histologic sections of normal marrow, islands of maturing erythroblasts often surround a central macrophage, termed a nurse cell. [186] Adhesion may be mediated through the binding of VLA-4 (on erythroid cells) to VCAM-1 (on central macrophages). [187] Although the specific interactions between macrophages and erythroid cells that promote erythroid differentiation are unknown, tissue macrophages express RNA for erythropoietin, [188] and may influence erythropoiesis through this mechanism.

It is clear that the microenvironment is not only a passive surface for the adherence of progenitor cells, but also exerts a crucial and interactive role in development and maturation. Some interactions are lineage- (red cell-) specific, whereas other interactions affect hematopoiesis more broadly. Stromal and accessory cells secrete cytokines and/or express them in a transmembrane form on their cell surface. Cytokines are retained via binding to components of the extracellular matrix. All components of the microenvironment are involved in adhesive interactions, some of which maintain quiescence, whereas other cell-cell interactions or interactions of cells with matrix components induce proliferation and/or differentiation. It is likely that an individual progenitor cell, in an anatomic niche adjacent to certain stromal cells, accessory cells, and extracellular matrix molecules, responds to the sum of the signals that it uniquely receives. In this way, hematopoiesis, including erythropoiesis, is stochastic, which is in effect a statement of the complexity of the interaction network.

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ONTOGENY OF ERYTHROPOIESIS

During human development distinct anatomic areas for production of erythroid cells are recruited sequentially, in a temporal succession that allows overlap (Fig. 15-4). In addition, there are parallel changes in the morphology and functional properties of the erythroid cells themselves.

During the phase of embryonic erythropoiesis in the blood islands of yolk sac, aggregates of immature erythroid cells undergo maturation synchronously as a single cohort. Before their maturation is completed, they begin to circulate, and by the fifth gestational week they are found in the vascular spaces of the rudimentary liver (Fig. 15-5). At about the same time, foci of immature erythroid cells emerge within the fetal liver as the fetal, or hepatic phase of erythropoiesis commences. [189] From the seventh week onward, the liver is progressively filled with erythroid precursors and becomes the dominant site of erythroid cell production until about the 30th gestational week. Although some red cell production can be found in the thymus, the spleen, or occasionally in the lymph nodes, these other sites are never dominant. From the sixth month onward, the cavities of long bones are invaded by vascular sprouts and become competent to support red cell development. At birth all bone cavities are actively engaged in erythroid production, and the hepatic (fetal) phase of erythropoiesis comes to an end, as the final (adult) phase of erythropoiesis unfolds exclusively within the bone marrow.

In addition to the anatomic shifts in the sites of erythropoiesis, there are associated shifts in the phenotypic characteristics of erythroid cells. Embryonic erythroid cells (derived from the yolk sac) are large (about 200 mm), retain their nuclei at terminal stages of maturation, and have a megaloblastic appearance (Fig. 15-5). Fetal erythroid cells (produced in the fetal liver and later in fetal bone marrow spaces) are smaller than embryonic cells (about 125 m) but have a macrocytic appearance when compared with adult, normocytic red cells (about 80 m). Like adult cells, however, they eject their nuclei during maturation.

Apart from variations in size and morphology, embryonic and fetal erythroid cells differ from each other and from their adult counterparts in several other characteristics, including hormonal or growth factor requirements and proliferative and transplantation potential. For example, whereas fetal erythropoiesis is under the control of EPO,[190] the extent of EPOs influence on embryonic erythropoiesis has been under dispute. Most

Figure 15-4 Sites of hematopoiesis during fetal development and after birth. Only erythroid cells, and possibly lymphocytes, are generated by the yolk sac and early embryo. Significant megakaryopoiesis and granulopoiesis develop at 4 to 5 months. After birth, hematopoiesis occurs in the sinusoidal cavities of the tibias, femurs, and axial skeleton. *(Adapted from Erslev and Gabuzda,[246] with permission.)*

convincing are the results of EPO/EPO-R knockouts[56] that showed only partial effects on embryonic erythropoiesis, in contrast to fetal erythropoiesis. Recent evidence suggests that precursor cells from the extraembryonic mesoderm are dependent on EPO for their proliferation and erythroid differentiation. [191] EPO levels increase between the 9th and the 32nd week of gestation, and fetuses respond to hypoxia or anemia with increased EPO as early as 24 weeks. Fetal erythroid progenitors when studied in vitro appear more sensitive to EPO and kit-ligand than adult progenitors; in contrast, their in vitro response to

Figure 15-5 (A) Section of an 8-mm embryo depicting a portion of hepatic parenchymal cells with embryonic erythroblasts present within primitive sinusoidal cavities. **(B)** At 6 to 8 weeks, discrete aggregates of definitive erythroblasts appear within the liver parenchyma, whereas mature embryonic erythroblasts persist in well-developed sinusoids (PAS stain). **(C)** Later, definitive erythroblasts are spread throughout the liver (100-day fetus) (PAS stain). **(D)**

lymphokines (e.g., IL-3 or G-CSFM) is minimal compared to that of adult erythroid progenitors. [192] [193] It is noteworthy that in the early stages of fetal liver erythropoiesis, mainly erythroid differentiation/maturation is promoted. [194] Although progenitors committed to other lineages are abundant in the fetal liver, very few mature cells (granulocytes, megakaryocytes) from other lineages are seen. In addition to their heightened sensitivity to EPO, fetal erythroid progenitors and precursors are characterized by high proliferative potential and shorter doubling times than adult cells when cultured in vitro. [193] [194] The dependency of stem/progenitor cells on KL also changes during ontogeny. Although the generation of repopulating stem cells (ckit+/Sca-1±/Thy ^{lo} /Lin) and of CFU-S is minimally affected during fetal life in mice that cannot produce KL, adult Steel-Dickie (Sl/Sld) mutant mice (which only produce some soluble KL) display greatly impaired erythropoiesis and hematopoiesis, suggesting that KL/c-kit pathway plays a role in the recruitment and self-renewal behavior of adult stem cells in vivo. [195] [196] Also, the long term transplantation potential is impaired in cells with mutations of c-kit kinase activity. [196] Furthermore, transplantable stem cells from the yolk sac, in contrast to fetal liver cells, cannot engraft adult recipients, either because of altered homing behavior or inability of bone marrow to support their development. [183] Finally, the enzymatic activity of several enzymes in the glycolytic pathway is greater in fetal than in adult red cells. [197] In contrast, carbonic anhydrase levels are very low during intrauterine and early neonatal life. [198] Distinct isozyme patterns for several enzymes (phosphoglycerate kinase, acetylcholinesterase, etc.) also distinguish fetal from adult red cells. [199] [200]

The surface antigenic profiles of erythroid progenitors and precursors are distinct at each ontogenic stage. For example, HLA class I and class II antigens are not detected in embryonic erythroid progenitor cells, but reach adult levels about the ninth week of gestation. [201] In addition, CD34(+) hematopoietic progenitors present in yolk sac express Mac-1, but are negative for the stem cell antigen Sca-1, which is expresed in fetal and adult CD34+ progenitor cells. [202] Adult 34+ progenitors, on the other hand, lack Mac-1 and AA4.1, expressed in fetal CD34+ progenitor cells. Furthermore, fetal BFU-E and CFU-E express similar levels of HLA class II antigens, whereas adult CFU-E are largely devoid of these antigens. [201] [203] Fetal red cells display on their surface a straight, unbranched polylactosaminyl chain (i antigen), whereas this structure, which bears ABH blood group determinants, is highly branched in adult cells (i antigen). [39]

The most widely studied changes during red cell ontogeny are the shifts or switches in globin types. Embryonic erythroblasts are characterized by their avid accumulation of iron, which is stored as ferritin [204] (0.31% of total protein) and by the synthesis of the unique hemoglobins Gower I ($_2$ epsilon$_2$), Gower II ($_2$ epsilon$_2$), and Hb Portland ($_2$ epsilon$_2$). The - and epsilon-globin chains are embryonic -like and -like chains, respectively. [43] These three embryonic types of hemoglobin are most likely synthesized in succession, as the concentration of Gower I is highest in smaller embryos. Thus, a switch from - to - and epsilon- to -globin gene production begins during the embryonic phase of erythropoiesis but is not complete until fetal erythropoiesis is well established. During the transition from yolk sac to fetal liver erythropoiesis (69 weeks), erythroid precursors within the fetal liver co-express embryonic (- or epsilon-) and fetal (- or -) globin both in vivo and in vitro. [205] [206] The

predominant type of hemoglobin synthesized during fetal liver erythropoiesis is Hb F ($_{2}$ $_{2}$), with a high proportion of ^{G} :^{A} = 7:3). Adult hemoglobin A ($_{2}$ $_{2}$), detectable at the earliest stages of fetal liver erythropoiesis, is also synthesized as a minor component throughout this period, but Hb A $_{2}$ ($_{2}$ $_{2}$), a minor hemoglobin in the adult, is undetectable in these early stages. From about the 30th gestational week onward, -globin synthesis steadily increases, so that by term 50%55% of hemoglobin synthesized is Hb A. By 4 to 5 weeks of postnatal age, 75% of the hemoglobin is Hb A, this percentage increasing to 95% by 4 months as the fetal-to-adult hemoglobin switch is completed. Hb F levels in circulating red cells are in a plateau for the first 23 weeks (as a result of the decline in total erythropoiesis that follows birth), but Hb F gradually declines, so that normal levels (<1%) are achieved by 200 days after birth. [207]

Several in vitro and in vivo approaches have been used to study the basis of globin switching through development. Beyond its biologic interest, rigorous research in this area was propelled by the possibility of manipulating globin switching to increase Hb F production in adults and ameliorate the clinical symptoms of disorders of the -globin locus (e.g., sickle cell anemia, thalassemia). Transplantation experiments and ablative endocrine maneuvers in the sheep model have failed to provide convincing support for effects of environmental or humoral factors on the switching process, although some modulation of the rate of switching was seen in these models.[208] [209] The most important determinant of fetal to adult hemoglobin switching seems to be postconceptual age, with the sharpest period for transition between 30 and 52 weeks.[210] The fetal-to-adult switch appears to be unaffected by the time at which birth occurs or by changes in the kinetics of erythropoiesis induced by perinatal hemolysis.[210] A delay in switching is usually observed in cases of general developmental retardation, in patients with certain chromosomal abnormalities (e.g., trisomy 13), and in diabetic infants because of increase in circulating levels of -aminobutyric acid, which directly affects hemoglobin F synthesis. [211] Integration of the data available from in vitro and in vivo approaches indicates that control of globin switching is intrinsic to erythroid cells. Stage-specific transcriptional forces with negative or positive influences (or both) on specific globin genes may provide the molecular basis for differential transcriptional activity during development. This view is favored by experiments in transgenic mice[212] and in heterokaryons (produced by fusion of human with mouse cells),[213] as well as by the isolation of stage-specific transcriptional factors in other erythroid systems (e.g., avian). [214] Furthermore, as -like and -like globin genes are activated sequentially in the order of their location in chromosome 11 or chromosome 16, respectively, it is possible that polarity of the transcriptional activity and globin promoter competition for the locus control region (LCR) and developmental stage-specific transcriptional factors contribute to this regulation. [215]

In summary, throughout human development waves of hematopoiesis are initiated sequentially in newly recruited sites. The first wave of erythropoiesis is seen in yolk sac between days 15 and 18 (7.5 days after conception in mice). In addition to erythroid cells, uncommitted progenitors and progenitors for nonerythroid cells are present in the yolk sac and are thought to be the source of cells colonizing the fetal liver. [216] [217] [218] However, in addition to yolk sac foci of hematopoietic activity have been detected within the embryo around the developing aorta (in Paraortic-Splachnopleura, P-Sp, and the aorta/gonad/mesonephros, AGM, area). [219] [220] [221] In fact, the P-Sp/AGM site in mice was shown to harbor progenitor cells before circulation begins and one day before these cells are found in the yolk sac. Long-term repopulating cells were only detected in the AGM area, leading to speculation that this intraembryonic site is the main or only source of fetal liver colonization, [221] in contrast to earlier experiments implicating the yolk sac for that role. [216] [217] [218] Presence of mesodermally-derived hematopoietic cells in two distinct anatomic sites, one intra-, the other extra-embryonic, has also been seen with explant studies in Xenopus and after analysis of chick-quail chimeras. [222] More recent experiments with human cells have led to similar conclusions. [223] Although the presence of progenitors for definitive hematopoiesis (fetal liver and bone marrow) in two independent sites is indisputable, the extent to which these two sites contribute to fetal liver colonization has been a matter of dispute. The conclusion that only the AGM

area contributes to fetal liver colonization was based on transplantation experiments in adult recipients and has recently been challenged. [183] Transplantation experiments using as recipients newborn mice with active fetal liver hematopoiesis have shown that adult long-term repopulating cells are detectable in the yolk sac at day 9 post-conception and were 37-fold higher than repopulating cells present in the P-Sp/AGM area at the same time. Therefore, failure of yolk sac cells (or AGM cells before day 10 post-conception) to engraft adult recipients may be caused by either compromised homing or impaired survival and proliferation within the adult bone marrow environment (either because of positive regulators or inhibition by negative regulators). In light of this new information, a 30-year-old theory that yolk sac colonizes the fetal liver[216] has been revived. A question that remains unanswered is whether the stem cell activity in 9 days post conception in yolk sac and P-Sp/AGM is generated autonomously and independently or is derived from a common precursor cell with migratory properties. Further murine studies comparing newborn transplant outcomes before the onset of systemic circulation between the yolk sac site and the intra-embryonic AGM site will resolve this issue.

A common precursor cell giving rise to erythroid cells with either yolk sac or fetal liver characteristics has been recently identified during culture of ES in vitro. [224] Also in Zenopus it was recently shown that specification to the primitive or definitive lineages is environmentally regulated. [225] However, since BFU-E present in yolk sac, in fetal liver, and within fetal bone marrow all have a non-yolk sac (non-embryonic) phenotype and these progenitors were not present after ablation of CBF, [226] despite the presence of normal embryonic erythropoiesis, the derivation of embryonic erythroblasts from a distinct progenitor, not present in subsequent life, remains a viable hypothesis.

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CELLULAR DYNAMICS IN ERYTHROPOIESIS

The primary function of the end product of erythropoiesis, the mature red cell, is to transport oxygen efficiently through the circulation to the tissues. To achieve this goal the adult marrow must release approximately 3×10^9 new red cells, or reticulocytes/kg/day.[227] This number of reticulocytes represents 1/100th of the total red cell mass and is derived from an estimated 5×10^9 erythroid precursor/kg. [227] In addition to maintaining homeostasis (i.e., a stable hematocrit), the erythron must be able to respond quickly and appropriately to increased oxygen demands, either acute (e.g., following red cell loss) or chronic (e.g., with hypoxia from pulmonary disease or a right-to-left cardiac shunt). It is now well established that EPO is responsible both for maintaining normal erythropoiesis and for increasing red cell production in response to oxygen needs. The overall marrow response, however, is complex and requires not only the participation of erythroid cells responsive to EPO but also a structurally intact microenvironment and an optimal iron supply within the marrow.

EPO stimulation elicits two types of measurable responses: changes in proliferative activity (including improved survival) and changes in maturation rates. The first detectable response to increased serum EPO is amplification of CFU-E and erythroid precursors, cells that are extremely sensitive to EPO. Because virtually all these cells are already in cycle, increases in their numbers cannot be achieved by increasing their fraction in cycle. Either additional divisions are involved or new cells are recruited to the CFU-E pool (from a pre-CFU-E pool). Additional divisions of CFU-E or precursor cells would increase their transit time within the marrow and potentially delay the delivery of new red cells to the periphery. Because a shortened maturation time has been observed instead and the proliferative potentials of CFU-E and proerythroblasts are finite, high levels of amplification cannot be achieved through this mechanism. Therefore, such needs are met by influx into the CFU-E and precursor pools of newly differentiating cells from earlier progenitor compartments.

Such a surge of newly produced cells has been observed in all previous experiments. [54] [228] [229] A rapid influx of fresh cells was particularly notable in polycythemic mice that were experimentally depleted of CFU-E and erythroid precursors at the time the stimulus was applied. [37] [54] Because of the rapidity of response (e.g., within 24 hours in the polycythemic animals), it appeared that the orderly progression from BFU-E to CFU-E to proerythroblast had been compressed. Such acceleration of differentiation is possible through shortened intermitotic intervals, fewer mitotic divisions, or differentiation without divisions. This short circuiting in differentiation requires high serum levels of EPO and adequate numbers of BFU-E (e.g., these conditions are met in a previously hypertransfused, polycythemic animal stimulated by EPO or in marrow suddenly recovering from acquired pure erythroid aplasia). Once CFU-E and precursors are expanded through this mechanism, most persisting erythropoietic demands can be met through this pool without excess input from pre-CFU-E pools. Thus, acute demands for erythropoiesis are met by influx from pre-CFU-E pools through an accelerated differentiation and maturation sequence. In contrast, chronic demands (i.e., demands in chronic hemolytic anemia) are mainly satisfied through a greatly amplified late erythroid pool and with a minimum distortion in the differentiation sequence. [43] [230] That the kinetics of erythroid differentiation/maturation are different in acute versus chronic marrow regeneration is supported by differing qualitative changes in the newly formed red cells. An increase in i antigen and Hb F expression as well as an increase in cells with higher mean corpuscular volumes is seen with an acute response, whereas these are minimal or less pronounced with chronic responses.[43] [231] When severe anemia persists from birth onwards, erythroid production can increase up to 10-fold above baseline.[230] This is possible not only because of maximally expanded erythropoietic pools but also because the sites of active erythropoiesis may extend to include those that support red cell differentiation during fetal life. Thus, although the marrow space in axial bones (vertebrae, pelvis, ribs, sternum, clavicles) is sufficient for normal erythropoiesis or to respond to moderate anemia, the femur, humerus, spleen and/or liver, and (rarely) thymus may support red cell production in children with congenital hemolytic anemia (e.g., thalassemia major). Expanded erythropoiesis may lead to skeletal deformities, hepatosplenomegaly, or erythropoiesis in the soft tissues adjacent to bone.

Quantitative assessments of changes in erythroid progenitor cell pools in response to EPO stimulation can be made through cultures of bone marrow cells. Despite sampling errors, erythroid cultures can provide rough estimates of relative progenitor abundance within an aspirated marrow specimen and have shown consistent increases in the frequency of CFU-E in proportion to the level of EPO stimulation. [232] [233] Conversely, with increases in the hematocrit or in polycythemic animals, a decrease in CFU-E frequency has been observed.[234] [235] In contrast to CFU-E, the incidence of BFU-E was found to fluctuate less with either acute or chronic expansion of erythropoiesis, probably because a few BFU-E can generate progeny of several thousand cells. Furthermore, BFU-E can increase their fraction in cycle and thus increase the number of differentiated progeny without a significant change in their total numbers. Most BFU-E detectable in marrow or blood erythroid cultures probably represent a reservoir of progenitors not normally participating in day-to-day erythropoiesis. The parameters needed to maintain a healthy or appropriate BFU-E pool in hematopoiesis are not defined. As hematopoietic expansion is curtailed in mice with Steel mutations and anemia develops in mice treated with anti-c-kit Ab antibody,[236] one may suggest that adequate levels of normal KL may be crucial for early erythropoietic expansion. [237]

The rate of red cell production also can be accurately evaluated

through ferrokinetics (i.e., the study of iron incorporation into developing red cells). In addition, a marrow scan, typically with ^{99m}Tc, can document the extent of active erythropoiesis. These approaches, however, are seldom necessary in clinical practice, because estimates of erythropoiesis can be obtained from the reticulocyte index.[230] First, the observed percentage of reticulocytes is normalized for the hematocrit to calculate the total marrow output of reticulocytes. Alternatively, the absolute number of reticulocytes/L can be counted directly, using fluorescent RNA labeling. However, because under conditions of acute need, younger reticulocytes are prematurely released into the circulation, the total number of reticulocytes overestimates the true level of red cell production as measured by iron kinetics. [230] Therefore, a second correction is made to account for the maturation of early circulating reticulocytes, or shift cells (polychromatophilic red cells) when present in the blood smear. The resulting reticulocyte index gives excellent estimates of effective red cell production.

Although the presence or density (or both) of EPO receptors on developing erythroid cells determines the responses to EPO, other properties (e.g., surface antigens on BFU-E versus CFU-E versus end-stage red cells) may provide the basis for selective suppression of CFU-E versus BFU-E or selective immune destruction of red cells versus erythroblasts. For example, suppression of CFU-E or erythroblasts can occur in acquired pure red cell aplasia [238] or B19 parvovirus infection,[239] respectively, whereas BFU-E in both these conditions remain largely unperturbed. Thus, the boundary from BFU-E to CFU-E and erythroblast may be biologically important for the pathophysiology of these disease states. Furthermore, in acquired hemolytic anemia selective destruction at a given stage of maturation (of red cells only or of both erythroblasts and red cells) can be observed depending on the type of antibody produced and the density of its antigen on maturing erythroid cells. Qualitative aberrations in the response of erythroid progenitors to cytokines or EPO may underlie the abnormalities of congenital erythroid aplasia (Diamond-Blackfan syndrome).[240] Analogous qualitative or functional defects can be observed in neoplastic erythropoiesis, as erythroid progenitors from patients with polycythemia vera and other myeloproliferative syndromes have altered sensitivities to EPO. [241]

Therefore, detailed knowledge of the structural and functional properties of erythroid cells throughout their differentiation may provide significant insights into the pathogenesis of hematopoietic disorders affecting the red cell lineage.

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Chapter 16 - Granulopoiesis and Monocytopoiesis

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This chapter describes the process of granulopoiesis (production of neutrophils, eosinophils, and basophils) and monocytopoiesis (production of monocytes and tissue-fixed macrophages). The enormous number of short-lived mature cells that must be produced each day to sustain baseline granulopoiesis, as well as the need for rapid adjustment in rates of production of these cell types in response to acute stress, have necessitated the development of a very intricate cytokine network and a series of progenitor compartments to meet those requirements.[1] [2] The hematopoietic growth factor combinations required for the proliferation, survival, and maturation of these cells are detailed, and the functional similarities in signaling of colony-stimulating factor receptors (CSFR) and interleukin receptors (IL-Rs) are highlighted. Mechanisms of lineage commitment to the granulocyte versus monocyte differentiation pathways are delineated, and the interactions of cytokines with mature cellular elements as well as other accessory cells demonstrated, as are the paracrine and autocrine feedback loops that regulate this complex process. Understanding the properties and regulatory cytokine networks affecting each progenitor compartment within the marrow microenvironment facilitates an understanding of the pathophysiology of disorders of granulopoiesis and monocytopoiesis. These may include aberrant cell proliferation and impaired differentiation in leukemic disorders, excessive precursor production in the setting of inflammation, infectious or allergic diseases, impaired cellular production as in congenital neutropenia, or excessive precursor destruction (or both) due to apoptosis in myelodysplastic diseases.

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STEM CELL AND PROGENITOR HIERARCHY

Growth factors important for myelopoiesis exert their effects at the level of the stem cell or the progenitor cell, or both. Stem cells are defined by their capacity for long-term self-renewal and by their ability to differentiate along multiple lineage pathways. [2] [3] Progenitor cells, on the other hand, retain proliferative potential but are committed to the production of cells from a limited array of lineages. [2] [3] [4] Progenitor cells are often defined as colony-forming cells (CFCs) because of their ability to generate hematopoietic colonies in vitro; such colonies consist of red cells, granulocytes, monocytes/macrophages, eosinophils, basophils, mast cells, megakaryocytes, dendritic cells, natural killer (NK) cells, and T or B lymphocytes. [5] [6] [7] [8] [9] [10] [11] In humans, stem cells are defined by surrogate assays such as the high-proliferative-potential CFC (HPP-CFC), [12] the so-called CFC-blast, [13] the long-term culture-initiating cell assay (LTC-IC), [14] and extended LTCI-C assay. [15] For obvious reasons, an in vivo definition of such a population is not feasible in humans, but studies using transplantation of early human progenitor cells into sublethally irradiated immunodeficient mice can serve as surrogate stem cell assays. [16] [17] [18] Similarly, a human/sheep xenograft model allows the long-term detection of transplanted stem cell progeny, thereby providing the means to study early events in hematopoiesis. [19] [20] [21]

Multipotential cells are largely unresponsive to single growth factors, but they often respond synergistically to combinations of growth factors. [3] For example, the combination of macrophage colony-stimulating factor (M-CSF, CSF-1) and IL-1 stimulates the proliferation of HPP-CFC, [22] [23] whereas the combination of IL-3, IL-6, and granulocyte colony-stimulating factor (G-CSF) is one of many cytokine combinations that promote the proliferation of CFC-blast. [24] [25] Although cytokines influence the growth of these cells, they do not alter the proportion of stem cells that self-renew or differentiate along a given hematopoietic lineage, leading to a stochastic model for hematopoiesis in which a cell randomly becomes committed to differentiate along any given myeloid lineage pathway. [3]

Progenitor Characterization

Early hematopoietic progenitors are CD34+/CD33 and are negative for lineage-specific markers. [26] When CD34+ cells are isolated from bone marrow or blood, most express CD13 and human leukocyte antigen (HLA)-DR surface antigens. Only a minor subset co-express CD33. [27] After 7 days in culture in the presence of GM-CSF and IL-3, CD33 is expressed by 50% of these cells. Concurrently, CD34 expression is lost, and CD13 and HLA-DR expression also diminishes. By 14 days of culture, most cells are CD33+. [28] Patients receiving marrow grafts purged with anti-CD33 have been found to require a significantly longer time to achieve polymorphonuclear neutrophils (PMNs) >500/mm^3 , underscoring the importance of these committed granulocytic progenitors during the early phase of hematopoietic reconstitution after bone marrow transplantation. [29] Marrow grafts depleted of CD33-bearing cells result in durable engraftment after myeloablative treatment, implying that this antigen is absent or expressed in very low amounts on reconstituting stem cells. [29]

The pluripotent stem cell can be found in bone marrow as well as fetal liver and cord and adult peripheral blood, and has been defined by long-term in vivo repopulating assays; it belongs to the CD34+ cell compartment. [30] [31] [32] [33] An earlier stem cell has recently been tentatively described in CD34 cells. [18] [34] Stem cells display the multidrug resistance gene (*mdr-1*) [35] and have a low fluorescent staining using the mitochondrial dye rhodamine 123. [36] [37] They are also characterized by low levels of the Thy-1 antigen (CD90), [38] and display no lineage commitment markers (CD38, CD33, CD20, CD14). [39] [40] [41] [42] These early stem cells display receptors for several hematopoietic growth factors: IL-3R chain (CD123), *c-kit* (CD117) and *FLK-2/FLT3* (CD135), as shown in Figure 16-1 . Surrogate in vitro assays, developed in lieu of reconstitution assays, have also established the presence of a whole continuum of myeloid progenitors of decreasing proliferative potential and multilineage commitment. [2] These include the long-term culture-initiating cells, [15] [43] HPP-CFC-1 and -2, [12] [44] the colony-forming unit (CFU)-blast, [13] [45] and the mixed progenitor assay (CFUgranulocyte/erythroid/ macrophage/megakaryocyte [CFU-GEMM]). [46] Subsets of committed granulocyte and macrophage progenitors can be enumerated in vitro after 714 days in culture, using semisolid

Figure 16-1 Schematic diagram illustrating some of the significant surface antigen features of both progenitor colony-forming cells and of morphologically distinguishable precursors of monocytes and neutrophils. The pluripotent stem cell, which can give rise to both lymphoid and myelocytic progenitors, is CD34 and CD45RO positive but negative for CD45RA. Also shown are natural killer cell precursors and lymphoid and myeloid dendritic precursor differentiation pathways. At the myeloid progenitor level, CD34 persists, but CD33, CD64, and CD13 expression eventually emerges, as do RNA and protein production of myeloperoxidase (MPO). Monocytic precursors develop CD14 expression. The characteristics of the various CD antigens are more extensively defined in the text. CD90, Thy-1; CD123, IL3R; CD117, *c-kit;* CD135, *FLK-2/FLT3;* CD80, B7-1; CD86, B7-2; CD64, FcRI; CD16, FcRIII; CFU, colony-forming unit; GEMM, granulocyte/erythroid/macrophage/megakaryocyte; GM, granulocyte/macrophage; HLA, human leukocyte antigen; LAP, leukocyte alkaline phosphatase.

colony assays and specific histochemical staining techniques, [47] allowing identification of CFUgranulocyte/macrophage (CFU-GM), granulocyte (CFU-G), macrophage (CFU-M), eosinophil (CFU-Eo), and basophil (CFU-Baso). [5] [6] [7] Mast cell and dendritic cell colonies have also been described. Figure 16-2 shows examples of HPP-CFC, CFU-GM, and CFU-M colonies assayed in semisolid media.

The advent of sophisticated flow cytometric cell separation techniques, coupled with improved progenitor and long-term reconstitution assays, has established that the earliest CD34+ stem cell compartment appears not to express CD45RA, as well as antigens associated with lineage commitment (CD33, CD38), but is CD45RO positive. [48] [49] CD34+ cells that express low levels of *kit* protein have been found to be enriched for long-term marrow-engrafting cells. [50] CD34+ progenitors begin to lose myeloid potential and become lymphoid progenitors after acquiring CD10 and CD19 (B-cell lineage) or CD7 and CD45RA+ (T-cell lineage). [51] [52] More definite lineage commitment using a thymic stromal co-culture system has shown that only CD34+ cells expressing the CD20 antigen are destined to become B cells, because they failed to generate myeloid and T-cell progeny in vitro. Similarly, the CD34+, CD13 bright, and the CD34+, CD14+ and CD34+, CD15+ progenitors gave rise to myeloid cells, and were unable to differentiate into the B- or T-lymphoid lineages. [53] The CD34+, CD10, CD19 populations contain mostly LTC-ICs and myeloid progenitors, whereas the CD34+, CD10+, CD19 population gives rise to macrophage colonies and are CD33+. [51] This overlap in B-cell/macrophage lineage development is underscored by the plasticity of hematopoietic cells and is also validated by the identification of bipotential B-cell macrophage progenitors in fetal liver, [54] and by the observed lineage switching between early B cells to macrophages associated with expression of the proto-oncogene *c-fms*, which is the CSF-1 receptor. [55] The earliest bipotential myeloid progenitor, CFU-GM, has been characterized as CD34+, CD33+, CD13+, CD45RA+ and myeloperoxidase positive. [56] Granulomonocytic committed progenitors are CD34 and CD64 (FcRI) positive. [57]

Figure 16-1 recapitulates the salient phenotypic changes that human progenitors undergo as they differentiate into specialized lymphoid and myeloid precursors. A unique subset of marrow progenitor can generate T and B lymphocytes, NK cells, and lymphoid-derived dendritic cells, [58] [59] and a common developmental pathway for thymic NK cells and dendritic cells has been identified. [60] This linkage is further underscored by the dysregulated lymphopoiesis and lymphoid dendritic cell development seen in Ikaros gene knockout mice. [61] Primitive fetal liver progenitors that can give rise to NK progenitors as well as myeloid progenitors are also found in the CD34+, CD38, HLA-DR+ subset. [62] A similar developmental pathway for NK and B lymphocytes applies to embryonic stem cells exposed to vascular endothelial growth factor. [63] More complex differentiation pathways govern dendritic cell ontogeny. As shown in Figure 16-1 , these specialized antigen-presenting cells can arise

from both myeloid (monocyte, granulocyte) and lymphoid precursors. [64] [65] [66]

Figure 16-2 (A) Large, diffuse high-proliferative-potential colony-forming cell (HPP-CFC) at day 25 of culture (original magnification × 8). **(B)** Colony-forming unitgranulocyte/macrophage (CFU-GM) at day 14 of culture (original magnification × 100). **(C)** Smaller CFU-GM at day 14 of culture (original magnification × 160).

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GROWTH FACTOR REGULATION

Synergistic Growth Factors

Hematopoietins exert their effects in concert on primitive stem cells (synergistic early-acting factors) and continue to influence progenitor expansion (intermediate-acting factors); later, one or two factors influence progenitor maturation along specific lineages (late-acting factors). [67] [68] These growth factors were initially defined by colony assays; hence the name colony-stimulating factors. These include G-CSF, GM-CSF, and M-CSF. [5] [69] [70] [71] Other myeloid-active cytokines include the ILs, such as IL-3,[72] IL-4,[73] IL-5,[74] IL-6,[75] IL-7,[76] IL-10,[77] IL-11,[78] IL-12,[79] and IL-13.[80] The *kit*-ligand/stem cell factor (SCF/KL) and the *FLT3* ligand are early-acting growth factors with tyrosine kinase activity and broad synergistic functionality. [81] [82] [83] [84] [85] Also, lineage-specific thrombopoietin (TPO) has shown activity on the early stem cell compartment in vitro [86] [87] and in vivo in TPO receptor-deficient knockout mice. [88]

Although the initial process of stem cell lineage commitment is stochastic (random), stem cells are likely to display multiple cytokine receptors, thereby explaining the synergistic and overlapping or redundant properties of hematopoietic growth factors. [3] [67] These early-acting factors are shown in Figure 16-3 and include G-CSF (produced by stromal cells, fibroblasts, endothelial cells, and monocyte/macrophages), [67] [89] IL-1 (produced by many cell types, including stromal cells, fibroblasts, endothelial cells, keratinocytes, epithelial cells, monocyte/macrophages, and B cells), [2] [90] IL-6 (produced by T and B cells, stromal cells, fibroblasts, endothelial cells, keratinocytes, and mast cells),[91] IL-11 (produced by stromal cells),[92] IL-12 (produced by B cells and macrophages),[93] IL-13 (produced by T cells),[94] and leukemia inhibitory factor (LIF; present in serum and produced by stromal cells, fibroblasts, T cells, astrocytes, and monocytes/macrophages). [95] [96] Other synergistic effects are provided by basic fibroblast growth factor (present in serum, megakaryocytes, platelets, immature granulocytes, and stromal cells, and within an extracellular matrix reservoir).[97] [98] This factor plays an important role in maintaining pluripotential embryonic stem cells together with KL and LIF, [99] and has been reported to act on early cells,[98] but other studies show no effect on CD34+ *c-kit*-positive stem cells,[99] [100] suggesting that its complex effects may depend on indirect stromal accessory cell-derived factors.[101] Last, tyrosine kinase receptor ligands, SCF/KL (produced by stromal cells, and synthesized in a soluble as well as membrane-bound form), [81] [102] and the *FLK-2/FLT3* ligand (stromal cells)[103] [104] are prominent in their ability to stimulate multilineage hematopoiesis [105] [106] [107] [108] as well as enhance granulocyte macrophage progenitor amplification in concert with other cytokines, [108] [109] [110] [111] as shown in Figure 16-3 , whereas hepatocyte growth factor (HGF; made by stromal cells, endothelial cells, fibroblasts, and keratinocytes), which signals through the *c-met* tyrosine kinase receptor, is synergistic in action primarily with IL-11, GM-CSF, G-CSF, and M-CSF against cord blood CD34+ cells. [112] Hepatocyte growth factor, in fact, has been shown to substitute for the defective

Figure 16-3 Various cytokines and chemokines that act at different levels of granulopoiesis and monocytopoiesis. Multiple cyto-kines regulate progenitor cells or mature effector cells at each stage of maturation/differentiation from the primitive pluripotent stem cell to nondividing, terminally differentiated precursors (monocytes, neutrophils, eosinophils, and basophils/mast cells). The cytokines and chemokines also have varying degrees of specificity; some, such as macrophage colony-stimulating factor (M-CSF) and interleukin (IL)-5, act predominantly on the monocytic and eosinophilic pathways, respectively, whereas others, such as granulocyte-macrophage (GM)-CSF, act on multiple granulocytic/monocytic (erythroid not shown) cell types. b-FGF, basic fibroblast growth factor; CFU, colony-forming unit; GEMM, granulocyte/erythroid/macrophage/megakaryocyte; HGF, hepatocyte growth factor; IP-10, interferon--inducible protein; KL, *kit*ligand; LIF, leukemia inhibitory factor; MCP-1, monocyte chemoattractant protein-1; MIP-1, macrophage inflammatory protein-1; NAP-2, neutrophil-activating peptide-2; NGF, nerve growth factor; RANTES, regulated on activation normal T expressed and secreted; SCF, stem cell factor; TPO, lineage-specific thrombopoietin.

SCF/KL signaling in W/W mice. [113] Oncostatin M (made by stroma, fibroblasts), which signals through the gp130 transducer, has been shown to affect early embryonic hematopoiesis.[114] Hematopoietic cytokines participate at various levels in the steady-state regulation of granulopoiesis and monocytopoiesis by promoting the survival of noncycling early progenitors, and by shortening the G0 period of early progenitors and triggering the proliferation and maturation of committed progenitors.[3] [115] These synergistic interactions have been deduced from in vitro early progenitor assays (LTC-IC, HPP-CFC, CFU-blast) as well as intermediate-to-late progenitor assays (CFU-GEMM and CFU-GM). [13] [116] [117] [118] [119] [120] [121] [122] [123] [124] [125] [126] [127] [128] Such interactions occur in vivo when more than one compartment of progenitors is stimulated (see Fig. 16-3).

Lineage-Specific Growth Factors and Chemokines

Cytokine receptor expression becomes more restricted as progenitors differentiate along separate granulocyte or monocyte pathways. Thus, at later stages of lineage commitment they express fewer cytokine receptors, and granulocytic maturation and differentiation is then governed by fewer growth factors, such as GM-CSF and G-CSF for neutrophil production, M-CSF and GM-CSF for monocyte precursor growth, IL-5 for eosinophil growth and maturation, and SCF, nerve growth factor, and IL-3 for basophil and mast cell growth[3] [129] (see Fig. 16-3).

Also as shown in Figure 16-3 , cellular trafficking of early and late leukocytes is regulated by members of a superfamily of chemoattractant proteins termed chemokines.[130] [131] [132] These are small proteins inducible by inflammatory signals and upon cell activation by bacterial, parasitic, or viral antigens. [133] They carry four conserved cysteines (Cys1-Cys3 and Cys2-Cys4) forming two essential disulfide bonds, with distinct structural motifs where the first two cysteine residues are adjacent (C-C branch), separated by an intervening residue (C-X-C branch) [134] or showing only a single cysteine residue, such as lymphotactin (C branch). [135] Yet another novel chemokine with a prominent mucin stalk (fractalkine) displays three amino acid residues between the first two cysteines (CX3C motif). [136] Chemokines direct the migration of mature granulocytic cells (monocyte, neutrophil, eosinophil, and basophil) to sites of inflammation or antigen presentation. [132] [137] Chemokine receptors are seven spanning domain, G-linked structures that mediate calcium flux, actin polymerization, and secondary signaling events. [138] [139] [140] [141] Chemokines have demonstrated activities on dendritic cells, [132] [142] [143] NK cells,[144] T-lymphocyte subsets,[145] [146] and B cells.[147]

Furthermore, chemokine receptors mediate human immunodeficiency virus entry into susceptible cells such as T lymphocytes, monocytes, macrophages, and dendritic and microglial cells. [132] [137] [148] [149] [150] [151] CD34+ hematopoietic stem cells have chemokine receptors such as CCR5 and CXCR4, the receptor for stromal-derived factor-1, which is important in B-cell growth and development and early myelopoiesis. [152] In addition to serving as co-receptor for entry of T-cell tropic human immunodeficiency virus-1, stromal-derived factor-1 is a powerful chemoattractant instrumental in CD34 mobilization and transendothelial migration. [153] [154] [155] [156] Table 16-1 illustrates the complexity of this evolving area, as we have shown positive migration with IL-8, monocyte

TABLE 16-1 -- Chemokine Receptors/Ligands and Cellular Selectivity

Chemokine Receptors	Receptor Cellular Distribution	Chemokine Ligands
CXCR1	Neutrophils	IL-8, GCP-2
CXCR2	Neutrophils, IL-5-primed eosinophils	IL-8, GRO//, NAP-2, ENA78, GCP-2, LIX
CXCR3	Activated T cells, natural killer cells	IP-10, MIG, I-TAC
CXCR4	Neutrophils, monocytes, CD34+ and pre-B-cell precursors, resting and activated T cells, dendritic cells, endothelial cells	SDF-1
CXCR5	B lymphocytes	BCA-1/BLC
CX3CR1	Monocytes, dendritic cells, CD34+ cells, natural killer cells, activated T cells	Fractalkine/neurotactin
CCR1	Monocytes, eosinophils, basophils, CD34+, activated T cells, and dendritic cells	RANTES, MIP-1, MIP-5, MCP-2, -3, -4
CCR2	Monocytes, basophils, activated T cells, dendritic cells, natural killer cells	MCP-1, -2, -3, -4, -5, RANTES, eotaxin-1, -2
CCR3	Eosinophils, basophils, dendritic cells, activated T cells	Eotaxin-1, -2, RANTES, MCP-2, -3, -4, MIP-5
CCR4	Activated T cells, dendritic cells, monocyte-derived dendritic cells, IL-2-activated natural killer cells	TARC MDC
CCR5	Monocytes, CD34+, activated T cells, dendritic cells, natural killer cells	RANTES, MIP-1, MIP-1
CCR6	T cells, dendritic cells	MIP-3/LARC/exodus-1
CCR7	Activated T cells, B cells	SLC/ELC/MIP-3
CCR8	Monocytes	I309, TARC, MIP-1
CCR10	Fetal liver, placenta	MCP-1, -3
Not known	Resting T cells	DC-CK1, PARC
Not known	Resting T cells, natural killer cells	Lymphotactin
CCR1 and CCR3	Broadly active on neutrophils, monocytes, and lymphocytes	Leukotactin-1

IL, interleukin; IP-10, interferon--inducible protein; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; NAP, neutrophil-activating peptide; RANTES, regulated on activation normal T expressed and secreted.

chemoattractant protein (MCP)-1, RANTES (*r*egulated on *a*ctivation *n*ormal *T e*xpressed and *s*ecreted), macrophage inflammatory protein (MIP)1, and fractalkine.[155] [157]

Similarly, chemokines like MIP-1 have been shown to mediate in vitro and in vivo negative regulatory signals on myeloid and erythroid progenitor cells, underscoring a novel mechanism for the alteration in hematopoiesis seen in chronic inflammation and infections. [158] [159] [160] [161] However, their principal mode of action is to control cellular trafficking and tissue transmigration of mature effector cells. [131] Neutrophil migration is mediated by the soluble chemokines like IL-8, neutrophil-activating peptide-2, and Gro- (Gro = growth-related), [131] and membrane-bound neurotactin.[162] Eosinophils and basophils are recruited to sites of allergic stimulation by eotaxin-1 and eotaxin-2.[163] [164] [165] Monocytes are recruited principally by monocyte chemotactic protein(s) elaborated at sites of inflammation by fibroblasts and granulocytes,[166] [167] [168] by an interferon--inducible protein (IP-10), [169] and by T-cell-derived chemokines like RANTES. [170] [171] Table 16-1 illustrates the complexity of this system, and underscores the redundancy of receptors and ligand interactions that regulate the cellular trafficking of granulocytes, monocytes, and immune effector cells such as memory/naive T cells, NK cells, and dendritic cells in normal and pathologic states. [131] [132] [133] [172] [173] [174] [175] [176] [177] [178] [179] [180] [181] [182] [183] [184]

Granulocyte colony-stimulating factor leads to only weak in vitro colony growth by itself, but when it is combined with SCF or GM-CSF in vitro, larger colonies are stimulated.[67] [122] Similarly, G-CSF does not display its maximal range of in vivo activity when administered to W/W mice defective in their *c-kit* signaling, [67] [185] whereas appreciable circulating levels of SCF in normal subjects [186] amplify its effects in vivo, thus explaining its potent activity on neutrophil production and progenitor expansion.[187] This in vivo synergism also occurs on administration of SCF and G-CSF either alone or together, leading to early and committed progenitor expansion and mobilization.[188] [189] [190] Indeed, although commitment decisions of pluripotent cells have been shown to evolve from random asymmetric cell divisions not affected by external growth factors, it is evident that cytokine receptors with overlapping specificities and at times opposing effects govern in large part the lineage-specific maturation of intermediate and late progenitors by promoting the proliferation and survival of precursors expressing the appropriate growth factor receptors.[3] [191] These include the G-CSF receptor (G-CSFR) on neutrophils, the M-CSF receptor (M-CSFR, CSF-1R) on monocytes/macrophages, [192] [193] the IL-5 receptor (IL-5R) on eosinophils and basophils, [74] [194] [195] and the *c-kit* (SCF) receptor (SCF-R) on mast cells [102] (see Fig. 16-3).

Various modes of overexpression or underexpression of growth factors have aided in elucidating their role in lineage commitment. The in vivo lineage specificity of these factors is demonstrated in a canine model of neutropenia. Administration of G-CSF to dogs leads to the generation of neutralizing antibodies to their endogenous G-CSF and, consequently, neutropenia. [196] Transgenic mice expressing IL-5 display hypereosinophilia, [197]

whereas IL-5-blocking antibody administration inhibits helminth-induced eosinophilia in mice. [198] In the op/op mouse osteopetrosis model, the *op* locus encodes the CSF-1 gene on murine chromosome 3. These mice do not produce M-CSF because of a stop codon mutation in the CSF-1 gene; they also have a deficiency in their monocyte/macrophage compartment and suffer from osteopetrosis because of the absence of osteoclasts, which mediate bone resorption. [199] [200] M-CSF is essential for osteoclast proliferation and differentiation, [201] but, in addition, GM-CSF promotes the differentiation of these monocyte-derived precursors into multinucleated osteoclasts.[202] These op/op mice improve over time,[203] suggesting that other factors, such as IL-6, tumor necrosis factor- (TNF-), or GM-CSF, can stimulate macrophage progenitors in lieu of CSF-1. [204] [205] Recently, it has been reported that GM-CSF is not responsible for correction of hematopoietic deficiencies in maturing op/op mice. This was demonstrated in mice lacking both M-CSF and GM-CSF wherein the age-related corrections in hematopoiesis continued to occur. [206] Also, mice lacking the hematopoietic-transcription factor, PU.1, develop osteopetrosis. [207] Finally, animal models such as the W/W have a defective *c-kit* receptor and mast cell deficiency,[208] whereas activating mutations in *c-kit*, the SCFR, have been identified in mast cell leukemia, systemic mastocytosis, and myeloproliferative diseases,[209] [210] [211] underscoring the importance of SCF in terminal mast cell differentiation.

Last, production of knockout mice has served to elucidate the role of GM-CSF and G-CSF as well as that of other cytokines in granulocyte production. For example, mice homozygous for absence of the G-CSF gene[212] are viable, fertile, and superficially healthy but have a chronic neutropenia, CFU-GM deficiency, and impaired neutrophil mobilization. Mature neutrophils were still present in blood and marrow, suggesting that other factors can support neutrophil production in vivo, but that G-CSF may be involved in steady-state and GM-CSF in emergency granulopoiesis. Moreover, during infection, mice lacking G-CSF show impaired monocytopoiesis, underscoring the molecular cross-talk between these receptor/ligand pathways. [213] In contrast, mice lacking GM-CSF have normal hematopoiesis but develop pulmonary alveolar proteinosis. [214] Mice lacking the common chain for the GM-CSF, IL-3, and IL-5 receptors also show a pulmonary alveolar proteinosis [215] and reduced numbers of peripheral eosinophils and lung eosinophils after parasitic challenge. [216] Although they play a major role in expansion of hematopoietic cells in emergency states, the entire function of IL-3/GM-CSF/IL-5 is dispensable for hematopoiesis in both steady states.

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GROWTH FACTOR RECEPTOR STRUCTURE AND FUNCTION

Receptor Structure

The hematopoietic growth factors that regulate granulopoiesis and monocytopoiesis are a diverse group of glycoproteins whose actions are mediated through lineage-restricted specific cytokine receptors. [217] Cytokine receptors can be placed in four subgroups based on their structural characteristics, as shown in Table 16-2 . [218] [219] [220] [221] [222] [223] [224] [225] [226] [227] [228] We restrict ourselves here to the two cytokine families that encompass most of the growth factors. The hematopoietin type I cytokine receptor family includes IL-2R, -3R, -4R, -5R, -6R, -7R, -9R, -11R, -12R , -13R, and -15R, the single-chain erythropoietin receptor (EPOR), G-CSFR, and the TPO receptor, the common signaling subunit KH97 that associates with receptor chains for human GM-CSF, IL-3 and IL-5, the gp130 signaling subunit for IL-6R, the LIF receptor, ciliary neurotrophic factor receptor, and oncostatin M receptor; [218] [219] [220] [221] [222] [223] [224] [225] [226] all of these factors share a structural similarity to the growth hormone/prolactin receptor family. The immunoglobulin superfamily includes the receptors for hematopoietic active growth factors, such as IL-1R, and the tyrosine kinase-containing receptors: CSF-1R, SCF/KLR, FL (*FLT3* ligand), fibroblast growth factor receptor, and platelet-derived growth factor receptor (PDGFR). [218] [226]

High-affinity receptor interactions require homodimeric ligand receptor interactions such as those for G-CSF, [229] M-CSF, [230] [231] SCF/KL,[232] TPO,[233] [234] and EPO,[235] or heterodimeric receptor ligand interactions through multichain receptors like PDGFR [236] as well as receptors with a common signal transducer such as the IL-3/IL-5/GM-CSF common chain,[226] or the gp130 receptor subunit shared by IL-6R, [225] LIF receptor,[225] [237] oncostatin M receptor,[238] ciliary neurotrophic factor receptor,[225] [239] and IL-11R.[240] In addition, shared signaling receptor subunits like the IL-2R subunit common to IL-2, IL-4, [241] IL-7,[242] IL-13,[243] [244] and IL-15[245] may partially account for the functional redundancy of these factors in hematopoiesis and immune regulation. Moreover, in vivo knockout experiments have uncovered a major role for IL-2 in myelopoiesis, highlighting the fundamental importance of T-cell-derived cytokines to normal hematopoietic functions (see Fig. 16-3). [246] The profound neutropenia seen in these IL-2/mice is indeed reminiscent of the leukopenia frequently seen in transplant recipients while on immunosuppressive therapy.

Most cytokines have pleiotropic activities and exhibit multiple functions on multiple cell types, and several CSFs may exert the same function when interacting with a given cell type.[67] [220] Growth factor receptors exist in high- and low-affinity forms on many hematopoietic and nonhematopoietic cells. [218] [223] [247] [248] [249] [250] [251] High-affinity GM-CSFRs are expressed in low numbers on progenitors (50100/cell) and increase as neutrophil and monocyte precursors undergo terminal maturation.[218] [252] All CSF interactions with high-affinity receptors at 37°C are associated with internalization and subsequent degradation of internalized CSF. [230] [248] [253] This can in effect serve as a negative feedback mechanism. [254] [255] Low-affinity receptors have a high rate of ligand dissociation; as such, they may serve as a reserve pool that can be used by new high-affinity receptors as they become available. [256]

Another common growth factor receptor theme is receptor transmodulation.[257] [258] Such modulation of receptor number can occur through a variety of mechanisms.[259] [260] [261] [262] [263] First, several ligands that do not directly interact at the CSF-binding domains of receptors can down-modulate CSFRs. With the M-CSFR, such modulators include lipopolysaccharide, phorbol esters 12-0-tetradecanoyl-phorbol-13-acetate (TPA), and TNF-; for G-CSFR, they include lipopolysaccharide, TPA, and the chemotactic peptide f-met-leu-phe; and for GM-CSFR, they include TPA, f-met-leu-phe, and TNF-. Second, the loss of high-affinity binding sites through internalization is a mode of receptor down-regulation. Third, CSFs can transmodulate other CSFRs. This means that the binding of one CSF to its own receptor at 37°C results in a loss of high-affinity binding sites for another CSF. In the mouse, IL-3R can transmodulate M-CSFR, G-CSFR, and GM-CSFR; GM-CSFRs can modulate M-CSFR and G-CSFR; G-CSFRs can transmodulate M-CSFRs; and M-CSFRs can transmodulate GM-CSFRs. In mice, the multipotential CSFs (IL-3/GM-CSF) seem able to down-modulate lineage-specific CSFRs (G-CSF/GM-CSF), but not vice versa. [218] In humans, the transmodulation between IL-3, GM-CSF, and IL-5 is reciprocal. [256] [260]

Unlike the IL-2R subunit, the GM-CSF/IL-5/IL-3 common subunit does not bind ligand but rather alters the affinity of the ligand for subunits. This finding might help to explain receptor transmodulation: if subunits are limiting, then binding of ligand (IL-3) to its subunit will deplete subunits because the / high-affinity receptor complex will be internalized. [256] The possession of common receptor subunits may help to explain some overlapping activities of these growth factors, but it does not fully explain the unique spectrum of activities of each growth factor or their pleiotropic actions in a given cell

TABLE 16-2 -- Cytokine Receptor Subgroups

Type I Cytokine ReceptorsHematopoietin Receptor Family	Immunoglobulin Superfamily Receptors	Type II Cytokine Receptors	TNF-like Receptors
IL-2R	IL-1RI and II	IFN receptors	TNF receptors
IL-4R	*(Ig repeats)*	*(FBN III domains)*	*(Cys repeats)*
IL-7R			
IL-9R			
IL-15R	Fibroblast growth factor (FGFRs)	IFNR	TNF-R p75
(Shared c chain)			TNF-R p55
		IFNR	TNF-RP
Erythropoietin R	Flt3 (Flk-2)		(LT-R)
Granulocyte CSFR		IFNR	
Thrombopoietin R (Mpl)	*c-kit* (SCF/KLR)		CD40 (gp39)
(Single chain)	CSF-1 receptor (fms, CSFR)		CD27
Granulocyte-macrophage (GM)-CSFR	Platelet-derived growth factor (PDGFRs)		CD30
IL-3R			OX40
AIC2A (IL-3 murine receptor)			4-1BB

AIC2B (murine) or KH97 (human)			Fas
IL-5R			
(Common chain for GM-CSF, IL-3, IL-5)			Low-affinity nerve growth factor receptor
IL-6R			
IL-11R			
Leukemia inhibitory factor (LIFR)			
Oncostatin M (OSMR)			
Ciliary neurotrophic factor (CNTFR)			
Gp130 (common signal transducer)			
IL-12R			
IL-13R			
Growth hormone (GHR)			
Prolactin (PRLR)			

CSF, colony-stimulating factor; FBN, fibronectin; IFN, interferon; IL, interleukin; LT, lymphocyte; SCF/KL, stem cell factor/ *kit* ligand; TNF, tumor necrosis factor.

type. The subunits are responsible for GM-CSF/IL-3/IL-5 signal transduction in responsive cells. [223] [226] [256] It has been reported that mice have an additional subunit of the IL-3 receptor. In mice with a null mutation of the gene encoding the common subunit of GM-CSF, IL-3, and IL-5, high-affinity binding of GM-CSF is abolished whereas that of IL-3 is not.[215] As noted previously, such mice have decreased eosinophils and contract alveolar proteinosis. [216]

Indeed, cross-regulation of cytokine receptors by other factors is a constant theme in hematopoiesis and has been demonstrated with several early- and late-acting cytokines such as SCF, IL-1, IL-6, IL-3, GM-CSF, TNF-, and transforming growth factor- (TGF-). [129] [264] [265] [266] [267] [268] [269] [270] [271] This complex regulation serves as an additional checkpoint to regulate specific precursor survival and maturation after the initial commitment process. [3] [7C] [193] [194] All hematopoietin family receptors have common structural features: two fibronectin type III modules, four conserved cysteine residues in the N-terminal module, and a unique sequence, Trp-Ser-X-Trp-Ser (WSXWS), in the C-terminal module. [219] [226] These structural features ensure appropriate receptor dimerization and ligand-receptor three-dimensional interactions. [272] Last, these receptors have no intrinsic kinase activity of their own, and hence they require activation (phosphorylation of chains) and the additional docking action of intracellular serine threonine/tyrosine kinase(s)/signaling complexes to transduce a mitogenic signal effectively, [226] using a family of signal *t*ransducers and *a*ctivators of *t*ranscription (STATs) proteins that become phosphorylated on tyrosine on activation of the *J*anus *k*inases (JAKs), undergo dimerization by reciprocal *Src* homology domains (SH2), translocate from the cytoplasm to the nucleus, and activate gene transcription via DNA binding domains. [273] [274] [275] As shown in Figure 16-4 , the importance of both and chains to the signaling process has become more apparent as investigators have attempted to map out various regions of the receptor that transduce mitogenic or antiapoptotic signals. [276] [277] [278]

Receptor-Mediated Signaling

In contrast to type I cytokine receptors of the hematopoietin family, tyrosine kinase-containing receptors like M-CSFR (*c-fms*), [279] [280] PDGFR,[281] [282] *FLK-2/FLT3*,[66] [283] and *c-kit*[102] mediate signaling directly after ligand/receptor dimerization and interchain phosphorylation by interacting with multiple signaling molecules with adapter domains for *src*-homology regions (SH2/SH3). [282] M-CSF has also been found to induce STAT1 and STAT3 activation in conjunction with phosphorylation of the nonreceptor tyrosine kinase Tyk2 in macrophages, while leading to Tyk2 and JAK1 phosphorylation in fibroblasts. [284] Table 16-3 illustrates the family of JAK/STAT subtypes that each cytokine receptor activates along with some of the nonreceptor tyrosine kinases that have been associated with these signals. It is also important to note that some of these pathways differ between hematopoietic and nonhematopoietic target cells. [285] [286] [287] [288] [289] [290] [291] [292] [293] [294] [295] [296] [297] [298] [299] [300] [301] [302] [303] [304] [305] [306] [307] [308] [309] [310] [311]

Figure 16-4 Multiple signaling pathways involved in granulopoiesis, including the interleukin (IL)-6R/gp130 receptor and the IL-3R, IL-5R, and GM-CSFR receptors, which possess a common subunit (c). Also shown is the ability of IL-1 to act synergistically with these other growth factors. Complex intracytoplasmic cross-talk between mediators of these pathways occurs, and many aspects remain to be elucidated (denoted by question mark [?]). The chain of the IL-3/GM-CSF/IL-5 receptor family has no intrinsic protein tyrosine kinase (PTK)-activating capacity and can associate with the JAK2 tyrosine kinase. The common chain and gp130 interact with JAK2, and JAK1 and JAK2, respectively, resulting in receptor phosphorylation and activation of STAT5 and STAT3, which act as transcription factors into the nucleus. The chain of these receptors confers specificity for the individual cytokine signals, as shown by receptor reconstitution studies. AA, arachidonic acid; APP, acute-phase protein; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase (also known as MAP, mitogen-activated protein); GM-CSF, granulocyte-macrophage colony-stimulating factor; Grb2-sos, growth factor receptor binding protein and mammalian son of sevenless; IL-1Racp, IL-1 receptor accessory protein; IRAK, IL-1 receptor-associated kinase; JAK, Janus kinase; LT, leukotriene; MEK, MAP kinase/ERK-activating kinase; NF, nuclear factor; PL, phospholipase; SHC, *src* homology domain containing protein adaptor; STAT, signal transducers and activators of transcription.

Cytokine receptor-mediated signal transduction results in cell activation, priming, proliferation, or maturation, depending on the particular target cell being analyzed. [3] [247] [269] [280] A complex cascade of events following GM-CSF/IL-3 high-affinity receptor engagement has been uncovered and includes (1) G-binding protein(s) translocation, [312] [313] [314] [315] [316] [317] (2) phosphatidylinositol pathway activation, [318] [319] [320] (3) phospholipase A_2 and D activation,[321] [322] [323] [324] (4) $p21^{ras}$ activation,[325] [326] [327] (5) *raf* kinase phosphorylation,[328] [329] (6) protein kinase C (PKC) activation, [330] [331] (7) Na^+ /H^+ antiporter activation,[332] [333] and (8) mitogen-associated activated protein kinase (MAPK) phosphorylation.[334] [335] [336] Similar events also occur with M-CSFR-(*fms*)-mediated signaling, leading to Na^+ antiporter activation,[279] [337] *raf* kinase phosphorylation and activation,[279] [338] cell proliferation,[280] and cyclin D1 expression, [339] and activation events such as enhanced phagocytosis, [340] chemotaxis,[341] [342] chemokine gene expression,[343] and integrin expression[344] of *c-fms*-positive target cells. The convergence of these various CSFR-mediated signaling pathways, involving PKC or MAPK (also termed ERK1 and 2, for *e*xtracellular *s*ignal-*r*egulated *k*inase), to the nucleus results in the transcriptional regulation of new genes, such as *EGR-1*, *c-jun*, and *AP1*, in both proliferating and terminally differentiated myeloid cells, [223] [345] [346] [347] [348] [349] with enhanced DNA-binding activity of the transcription factor AP-1, nuclear factor (NF)-AT, and NF-B after induction of MAPK in T cells. [350] The interaction of these signaling events is detailed in Figure 16-4 .

Another important signaling pathway is used by the synergistic factor IL-1. It involves the newly characterized IL-1 receptor accessory protein (IL-1RAcp) and two receptor-associated kinases (IRAK-1 and IRAK-2), which interact with a cytosolic protein, MyD88, and activate the adapter protein, TRAF6, which in turn leads to NF-B activation through the NF-B inducing kinase and two I-B kinases, IKK-1 and IKK-2 [351] [352] [353] [354] [355] (see Fig. 16-4). NF-B is a well characterized transcriptional activator for multiple cytokine and chemokine genes such as IL-6 and IL-8, triggered by proinflammatory cellular activators such as IL-1. [356] [357] [358] [359] [360]

Most hematopoietin receptors, such as G-CSFR and GM-CSFR, are devoid of tyrosine kinase activity and require additional molecules to mediate their mitogenic activity.[219] [223] For example, neither subunit of the GM-CSFR is a tyrosine kinase and neither has domains similar to those found in G-protein-associated receptors. The finding of common substrates phosphorylated on tyrosine residues after activation of several cytokine receptors, as well as the necessity of tyrosine kinase activity to activate $p21^{ras}$, suggested that these receptors were undergoing phosphorylation after ligand interaction by associating with intracytoplasmic signaling complexes with adapter

TABLE 16-3 -- JAKs, STATs, and Nonreceptor PTKs Mediating Signal Transduction

Cytokine	JAK Subtype	STAT Subtype	PTKs
Erythropoietin (EPO)	JAK2	STAT5, STAT1	Fes, Tec
G-CSF	JAK1, JAK2, Tyk2	STAT1, STAT3	Lyn, Syk, c-rel, Tec, PI3K
Thrombopoietin (TPO)	JAK2, Tyk2	STAT1, STAT3, STAT5	Tec
Growth hormone (GH)	JAK2	STAT1, STAT3, STAT5	Tec
Prolactin (PRL)	JAK1, JAK2	STAT1, STAT5	Lyn, Tec, PI3K
Ciliary neurotrophic factor (CNTF)	JAK1, JAK2, Tyk2	STAT1, STAT3, STAT5	
IL-6	JAK1, JAK2, Tyk2	STAT1, STAT3, STAT5	Hck, Btk, Tec, PI3K
IL-11	JAK1, JAK2, Tyk2	STAT1, STAT3	Yes, Src, PI3K
Leukemia inhibitory factor (LIF)	JAK1, JAK2, Tyk2	STAT1, STAT3, STAT5	Hck
Oncostatin M (OSM)	JAK1, JAK2, Tyk2	STAT1, STAT3, STAT5	
GM-CSF	JAK2	STAT5, STAT1, STAT3	Fes, Lyn, Yes, Tec, PI3K
IL-3	JAK2	STAT5, STAT1, STAT3	Lyn, Fyn, Fes, Tec, PI3K
IL-5	JAK2	STAT5, STAT1	Lyn, Syk, PI3K
IL-2	JAK1, JAK3	STAT1, STAT3, STAT5	Lck, Fyn
IL-4	JAK1, JAK3	STAT6	Lyn, Syk, PI3K
IL-7	JAK1, JAK3	STAT1, STAT5	Lyn, Syk
IL-9	JAK1, JAK3	STAT5	Lyn, Syk
IL-15	JAK1, JAK3	STAT5	Lyn, Syk
IL-12	JAK2, Tyk2	STAT3, STAT4	
IL-13	JAK1, JAK2, Tyk2	STAT6	
IFN-/	JAK1, Tyk2	STAT1, STAT2, STAT3	
IFN-	JAK1, JAK2	STAT1	
IL-10	JAK1, Tyk2	STAT1, STAT3, STAT5	
kit ligand (KL)	JAK2	STAT1, STAT2, STAT3	Lyn, PI3K
M-CSF	JAK1, Tyk2	STAT5	Fyn, Src, Yes, PI3K
FLT3 ligand		STAT1, STAT3	Fyn, Src, PI3K
Platelet-derived growth factor (PDGF)	JAK1, JAK2, Tyk2	STAT1, STAT3, STAT5	Fyn, Src, PI3K

CSF, colony-stimulating factor; G, granulocyte; IFN, interferon; IL, interleukin; JAK, Janus kinase; M, macrophage; PTK, protein tyrosine kinase; STAT, signal transducer and activator of transcription.

proteins containing 2 SH2 in a variety of cells. [223] [325] Furthermore, insights into the physiology of proteins with SH2 domains revealed their capacity to act as transducers of several tyrosine kinase signaling pathways. [361] The seminal discovery has been the isolation of an SH2 domain containing transforming protein with a unique sequence (SHC). [362] [363] The SHC cDNA encodes overlapping proteins of 46.8 and 51.7 kd, whereas antibodies recognize three components (46, 52, and 66 kd). SHC proteins are rapidly phosphorylated by *src* kinases and form a complex with the 23-kd polypeptide of the adapter molecule Grb2 with an SH3 domain and its partner binding protein sos (a guanine nucleotide exchange factor). [364] [365] This activated complex mediates ras activation after receptor/ligand interactions, as shown for the epidermal growth factor/erbB-2 receptor [356] [357] [358] [359] [360] [361] [362] [363] [364] [365] [366] [367] [368] [369] and for the insulin/insulin receptor substrate-1. [370] [371] [372] The demonstrated role of the SH2/SH3 domains of Grb2 in coupling epidermal growth factor signaling to the ras activator sos provided the final link for growth factor-mediated p21^{ras} activation. [373] Soon thereafter came the discovery of the JAK tyrosine kinase family and the realization that either JAK1 or JAK2 can serve as receptor-associated tyrosine kinases to transduce multiple growth factor/receptor proliferative signals, [374] as illustrated by the signaling mediated through growth hormone, interferon- (through JAK1), and interferon- (through both JAK1 or JAK2). [375] [376] [377]

This tyrosine kinase pathway linked to receptor subunits and Grb2/sos adapters offered the necessary explanation for the frequently seen p21 ^{ras} activation, which leads to the MAPK extracellular signal-regulated kinase activation [378] seen with GM-CSF, [223] [334] [335] SCF, and IL-3. [223] [325] [326] As shown in Figure 16-4 , hematopoietins such as IL-6, IL-3/GM-CSF/IL-5, and the immunoglobulin-like receptor for IL-1 use distinct but somewhat overlapping intracytoplasmic signaling pathways, eventually leading to new gene (*c-myc*, cyclin D2, *c-jun*, *c-fos*) transcription. [223] Multiple cytokines, with the exception of IL-4, stimulated the phosphorylation of a p97-kd protein (JAK2) and p42 MAPK. [379] [380] [381] [382] GM-CSF and IL-3 phosphorylated the same sets of proteins, including raf kinase and MAPK, in a factor-dependent cell line (MO7), whereas SCF, in addition, phosphorylated phospholipase C on tyrosine, [381] like the kinase-containing receptors such as PDGFR, M-CSFR, and *FLK-2/FLT3* receptor. [280] [281] [282] [283] Distinct cytoplasmic regions of the common subunit mediating the signaling for the IL-3/IL-5/GM-CSF ligands have been mapped by reconstitution experiments using a factor-dependent hematopoietic cell line of 3T3 fibroblasts. [383] [384] [385] [386] [387] [388] [389] [390] The importance of both and chains for GM-CSF, IL-3, and IL-5 signal transduction is illustrated in Figure 16-4 , [277] [292] [386] [388] [391] as is polypeptide signaling to the nucleus via JAK kinase. [392]

Mounting evidence implicating the IL-6 signal transducer gp130 protein in JAK2 kinase activation is now available. [393] In the event that this activation leads to tyrosine phosphorylation of an interferon-stimulated growth factor, this may provide a direct transcriptional signal to the nucleus without activating the *ras-raf*-MEK-MAPK cascade, which is reminiscent of the JAK1/2-mediated tyrosine phosphorylation of several cytoplasmic STAT proteins [393] [394] [395] (see Fig. 16-4). These transduction pathways have also been confirmed by the demonstration of JAK2 kinase association with several type I cytokine receptor/ligand complexes, such as IL-3, [396] [397] EPO, [398] [399] and GM-CSF, [400] [401] which have also been found to be associated with SHC and *sos*, leading to p21^{ras} activation. [384] [385] [386] Similarly, SCF [402] and the p210$^{bcr/ab}$ kinase [403] recapitulate the same events, leading to receptor

subunit phosphorylation and activation of the SHC signaling component of the p21 ^{ras} pathway by tyrosine phosphorylation, underscoring the importance of this cascade and its common utilization by several cytokine-receptor interactions. [362] [403] [404] [405]

Receptor reconstitution experiments showing a divergence in cellular signaling events between hematopoietic cells and nonhematopoietic cells such as 3T3 fibroblasts, as well as subtle differences between SCF, IL-3, and GM-CSF action on the same target cells, require additional explanation. [381] [385] [387] The discovery of the tyrosine phosphorylation of many endogenous cytoplasmic transcription factors with SH2/SH3 domains by JAK1 or JAK2 kinases [406] [407] at last provided a *ras*-independent growth factor signaling pathway. [408] The detailed analysis of signaling complexes has revealed a p80-kd component in cells stimulated by IL-3, GM-CSF, or IL-5, whereas interferon--treated cells activated several transcription factors containing the p91-kd component of epidermal growth factor-3. [407] Thus, the cellular specificity of signaling may be provided by individual receptor subunits (such as chains of CSFR) that activate unique cytoplasmic transcription factors. [406] In addition, the GM-CSFR subunit participates in glucose transport without requiring tyrosine phosphorylation, thus providing a link to the increased survival of precursors induced by GM-CSF. [409]

Another issue in cytokine receptors is the pleiotropic nonreceptor tyrosine kinases (e.g., src kinases) and transforming proto-oncogenes (e.g., *abl*, *myb*, *vav*) that have been identified in hematopoietic cells. [410] [411] Although the exact function of these molecules is far from being totally understood, gene knockout experiments have

started to shed some light on their role in hematopoiesis. [410] [411] For instance, Pim-1 kinase is induced in IL-3/GM-CSF-treated cells, [223] and its expression parallels the growth of cytokine-exposed cells. [412] The tyrosine kinase *c-fps/fes* has been implicated in GM-CSFR and EPOR signaling, [413] [414] [415] the *src* kinase *lyn*, normally associated with the high-affinity Fc receptor I, is regulated by IL-3 in myeloid cells, [416] and the *syk* kinase participates in GM-CSF/IL-5-induced eosinophil differentiation. [307] These potential regulatory kinase complexes are also illustrated in Figure 16-4 , and are highlighted in Table 16-3 .

Granulocyte colony-stimulating factor receptor, which is also devoid of kinase activity, by itself forms a three-component complex with *lyn* and *syk* protein kinases. [417] The study of patients with severe congenital neutropenia offers insights into the role of this receptor, because patients display normal G-CSFR on their neutrophils, [418] but their CFU-GM growth in response to G-CSF alone is poor, whereas a combination of SCF and G-CSF results in normal growth patterns. [419] Indeed, molecular characterization of G-CSFR showed distinct cytoplasmic regions involved in the induction of proliferation or differentiation, [420] and transfection analysis showed cross-phosphorylation of IL-3R by G-CSF stimulation of cells expressing both receptors, indicating potential functional redundancy of cytokine receptor signaling [421] [422] (see Fig. 16-4). Proliferative responses to G-CSF are associated with activation of the p21 ^{ras}/MAPK signaling pathway. [423] The identification of a truncated G-CSF receptor in a case of severe congenital neutropenia [424] offers an example of the important role of receptor-associated signaling complexes leading to p21 ^{ras} and MAPK activation in mediating cell proliferation. [423] [425] The importance of PKC in G-CSF-mediated neutrophil survival underscores the potential for this activation pathway in the cytokine receptor signaling process diagrammed in Figure 16-4 . [426] [427] G-CSF has been found rapidly to activate the STAT3 transcription factor, [428] [429] and distinct regions of the receptor are required for tyrosine phosphorylation of JAK2, STAT3, and p42,p44 ^{MAPK}. [430]

Along with the characterization of protein kinases came the realization that in these dynamic phosphorylation/dephosphorylation processes, distinct protein tyrosine phosphatases were an integral part of turning off or, in some instances, turning on a given signaling cascade. [431] In the case of hematopoiesis, the CD45-associated phosphatase has been shown to modulate IL-3-, GM-CSF-, and SCF-mediated progenitor growth, [432] whereas M-CSF phosphorylates [433] and SCF associates with a tyrosine phosphatase termed hematopoietic cell phosphatase (HCP or SHP-1). [434] This phosphatase associates with the IL-3 receptor chain, mediates the down-regulation of IL-3-induced tyrosine phosphorylation and cell proliferation, and regulates *c-jun* expression in monocytes. [435] [436] HCP is inactive in motheaten mice, [437] which are characterized by hyper-reactivity to multiple cytokines like G-CSF, GM-CSF and IL-3 in vitro, and in vivo suffer from marked hyperproliferation of granulocytes and macrophages. [438] [439] [440] Another negative regulator of cytokine signaling is a 145-kd, SH2-containing inositol-5-phosphatase (SHIP) [441] [442] that is widely expressed in hematopoietic cells and has been shown to down-regulate the antiapoptotic and proliferative signals triggered by several cytokines, including TPO, EPO, IL-3, KL, M-CSF, GM-CSF, and G-CSF. [441] [443] [444] [445] The central role of this phosphatase as a negative regulator of myelopoiesis is underscored by the phenotype of mice with targeted disruption of SHIP, which have increased numbers of GM progenitors in marrow and spleen and die prematurely from extensive lung consolidation by myeloid cells. [446]

Last, the regulation of myeloid proliferation and differentiation may also include small intracellular messenger molecules such as nitric oxide, which has been implicated in the modulation of cell differentiation and *c-myc* and *c-fos* gene expression in the HL60 human myeloid leukemia cell line, adding further complexity to the cytokine transduction pathways regulating myeloid cell proliferation or differentiation, or both. [447]

Lineage Commitment

The mechanisms of lineage commitment toward granulopoiesis and monocytopoiesis and the roles of cytokines, cytokine receptors, and specific myeloid genetic programs are still incompletely understood. [448] [449] In vitro studies of murine embryonic stem cells and embryoid bodies reveal a temporal coordinated expression of growth factor receptor genes during cell maturation. [450] Certain genes are expressed constitutively during development of these cells, such as *c-kit* and the subunits of the growth factor receptor genes. Expression of the common subunit of the IL-3/IL-5/GM-CSF receptors, as well as SCF, are induced early. By contrast, *c-fms*, G-CSFR, and CD34 are induced later, at an intermediate stage of development, reflecting the initiation of myeloid commitment. [450] The extent to which growth factors and cytokine receptor expression determine the differentiation pathway taken by GM progenitors remains largely unknown, as do the genes responsible for cell commitment. There is evidence that multilineage gene expression precedes commitment in the hematopoietic system. [451]

Several in vitro models of differentiation provide clues to the importance of various stimuli to lineage commitment. For instance, IL-3 and GM-CSF act dominantly in a factor concentration-dependent manner to suppress *c-fms* (M-CSFR) expression on their target cell. [452] Overexpression of *c-fms* in an IL-3/GM-CSF factor-dependent murine myeloid leukemia cell line (FDC-P1/MAC) leads to a CSF-1-dependent differentiation pattern. Also, a GM-CSF-activated ribonuclease system down-regulates *c-fms* mRNA in these cells. [453] Furthermore, TGF-1, a known cell growth inhibitor, increases *c-fms* expression in these cells, [454] implying that an important control point in regulating monocyte/macrophage lineage differentiation may be the expression of *c-fms* itself. [455] The HL60 cell line can mature along either granulocytic, monocytic, or eosinophilic pathways, allowing the identification of myeloid-specific genes involved in lineage commitment. [456] [457] HL60 cells exposed to phorbol esters (TPA) or vitamin D_3 are induced along the monocytic lineage, whereas dimethylsulfoxide causes granulocytic maturation, and retinoic

acid induces a biphenotypic maturation of these cells. [458] Such established cell lines are useful models for analyzing the developmental expression of CSFRs, chemokines, and novel genes that regulate or are temporally associated with lineage-specific differentiation. [455] [460] Using these models, cytokine combinations have been shown to inhibit proliferation and promote lineage commitment, [461] whereas sequential exposure to maturation inducers such as vitamin D_3 or retinoic acid and lineage-specific CSFs accelerates the differentiation process. [462] Similar studies have underscored the central role of specific genes in modulating myeloid commitment, such as the *c-myc*, [463] [464] *c-myb*, [465] *SCL*, [466] and *EGR-1* genes. [345] [467] Furthermore, they have demonstrated the importance of *c-myc* expression on cell proliferation [464] and the need to down-regulate its function before the induction of terminal differentiation. [468] [469] PU.1 but not *ets-2* has been found essential for macrophage development from embryonic stem cells, [470] and induction of PU.1 correlates with negative regulation of the *c-myb* promoter, which is essential for continued cell proliferation. [471] Moreover, myeloid development is selectively disrupted in PU.1 null mice. [472] The efficient survival and amplification of macrophage precursors depends on external signals (cytomatrix adhesion and cytokine receptor engagement) as well as the coordinate expression of PU.1 transcription factor, which in turn activates M-CSFR expression. [473]

Granulocyte-macrophage colony-stimulating factor concentration determines myeloid versus monocytic commitment by controlling *c-fms* expression. [204] At low concentrations of GM-CSF, macrophage colony formation is favored because of relatively high expression of *c-fms*, whereas at higher GM-CSF concentrations, mixed colony formation (GM) is preferred because of the lower levels of *c-fms* expression. [204] [452] Similarly, *EGR-1*, a DNA-binding zinc-finger transcription factor, is an early response gene up-regulated by macrophage inducers (TPA and CSFs); [280] [345] it functions by restricting myeloblast differentiation to the macrophage lineage. [467] The essential role of M-CSFR in monocyte/macrophage lineage commitment is also illustrated by a *c-fms*-dependent HL60 cell differentiation model in which monocytic differentiation and retinoblastoma (*Rb*) gene expression depend on the presence of active M-CSFR on the cell surface. [474]

Similar cross-regulation of CSFRs occurs in the 32DC13(G) IL-3-dependent murine cell line, which can be primed by G-CSF to express GM-CSFRs and thereby differentiate into monocytes and granulocytes. [475] Such findings are in keeping with the observed synergistic effects between GM-CSF and G-CSF on highly purified progenitor cells. [476] Moreover, as with *c-fms* and monocytic maturation, expression of G-CSFR promotes granulocytic differentiation in the WEHI-3B D+ cell line, [477] whereas the up-regulation of G-CSFR mRNA appears to be an early commitment event in normal myeloid differentiation that is often dysfunctional in leukemic cells. [478]

A common feature of hematopoietic cells is their capacity to switch lineages. [449] The introduction of *v-fms* into pre-B lymphocytes allows the generation of monocytic leukemic cell lines. [479] The bipotential capability of precursors of B cells and macrophages in murine fetal liver [54] and the expression of *c-fms* by B-transformed B cells such as hairy cell leukemia cells [342] suggest the presence of common transcriptional regulators in each cell type. [480] Hence, it is not surprising that common genes belonging to the *ets* family are active in early macrophage differentiation and B-cell development. These include *ets2* and *PU.1* proto-oncogene(s). [481] [482] Indeed, the *PU.1/Spi-1* proto-oncogene regulates the lymphoid-specific immunoglobulin heavy chain enhancer at the pre-B-cell stage. [483] Other genes such as *fes* and *MZF-1* appear to modulate granulocytic precursor proliferation and maturation. [484] [485]

Other mechanisms for lineage commitment have been identified in the HL60 leukemia model. Retinoic acid-induced cell differentiation can occur through the induction of TGF-1 protein and TGF-1 receptor expression, potentially triggering an autocrine inhibition loop. [486] Another mechanism regulating neutrophilic maturation can be the easing of the dominant negative effect (blocking maturation) exerted by retinoic acid -fusion transcripts in leukemic promyelocytes. [487] Alterations in inositol lipids and phosphates regulate neutrophilic or monocytic lineage maturation of HL60 cells. [488] Finally, whereas PKC activation promotes monocytic maturation of myeloblasts [489] and enhances CFU-GM growth in conjunction with cytokines (IL-6 and G-CSF), [490] inhibitors of myosin light chain kinase have been shown to differentiate the monoblastic leukemia U937 line, an effect antagonized by GM-CSF. [491]

The central role of transcriptional regulators in normal and leukemic cells has been increasingly appreciated as sites of chromosomal translocations have been characterized.[480] For example, embryonic lethality and impairment of hematopoiesis result in mice heterozygous for an *AML-1/ETO* fusion gene. [492] Homeobox genes have also been found to have individual roles in differentiation and proliferation of myeloid progenitors. Undoubtedly, complex regulatory signaling pathways involving receptor and nonreceptor kinases and phosphatases must affect specific genetic programs responsible for the lineage commitment process. [473]

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CELLULAR PRODUCTION

Granulocytes are derived from a common myeloid stem cell and comprise neutrophils, eosinophils, basophil/mast cells, and dendritic cells. Neutrophils (PMNs) and monocytes/macrophages constitute two important elements of the host defense system. Basophils and mast cells contribute to mucosal immunity, and eosinophils, basophils, and mast cells aid in response to allergens and parasitic infections. In this role, they must have the capacity to migrate, phagocytize, and produce inflammation-inducing substances to achieve antimicrobial destruction or contribute to inflammatory reactions. In addition to combating microorganisms, tissue macrophages constitute the resident reticuloendothelial system of many organs such as liver, spleen, and bone marrow. [493] In these sites, they may also serve to eliminate debris and senescent cells, aid in antigen presentation and processing, and, in some cases, effect antitumor cytotoxicity. Because these effector cells are constantly used, the need to maintain appropriate cell numbers in an equilibrium state as well as to have the capacity for increased production in times of stress requires a finely controlled regulation of both neutrophil and monocyte production.

Neutrophil and Monocyte Production

In the adult, the marrow is the source of both neutrophils and monocytes, which circulate until they exit the vascular spaces to the tissues. In the marrow, the CFU-GM is the earliest cell type that can be identified in culture as having commitment to differentiate along the myeloid pathway. This cell type is distinguished by its ability to form hematopoietic colonies when assayed in vitro in semisolid media such as agar or methylcellulose in the presence of CSFs (see Fig. 16-2). CFU-GM, which numbers approximately $50100/10^6$ nucleated cells in a normal human marrow specimen, is a bipotential progenitor and can respond to GM-CSF differentially based on growth factor concentration. As discussed previously, at high concentrations of GM-CSF, the granulocytic pathway is favored, whereas at low concentrations, monocytic/macrophagic differentiation occurs preferentially (see Fig. 16-3). CSF-1 appears to control its own expression on maturing progenitor cells, providing another feedback regulatory pathway to limit unchecked monocyte production, [494] whereas anti-inflammatory agents such as cyclo-sporine and dexamethasone regulate CSF-1 and *c-fms* expression at a post-transcriptional level. [495]

Progenitor cells cannot be recognized morphologically in

marrow, but rather have the appearance of nondescript mononuclear cells (lymphoid appearance). The myeloblast is the first morphologically identifiable precursor. Myeloblasts, promyelocytes, and myelocytes are mitotic, but metamyelocytes, band neutrophils, and mature PMNs constitute what is known as the postmitotic compartment (see Fig. 16-1). Azurophilic granules appear at the promyelocyte stage, but the specific granules containing such substances as lactoferrin and cytochrome b do not appear until the myelocyte stage. The adult human harbors an estimated 7.7×10^9 PMNs/kg. In addition to neutrophils found in the vascular space and tissues, there is a large marginated pool and a large marrow reserve, allowing for rapid recruitment of effector cells in times of need. Neutrophils spend approximately 67 days in the marrow. On egress, they spend only a short time in the circulation (half-life 6.7 hours). The released PMN is differentiated and postmitotic, and neutrophils live in tissues for only a few days.

Monocytic cells, of which the promonocyte is the earliest recognizable stage, spend less time in the bone marrow than do PMNs, so they are released into the circulation with the ability to mature further. No marrow reserve exists. Monocytes spend 13 days in transit through the marrow and 872 hours in the blood. On entry into tissues, further maturation and possible proliferation can occur. Monocytes can survive in tissues for long periods, reportedly >80 days. The tissue-fixed macrophages derived from monocytes include alveolar macrophages, which can proliferate when exposed to IL-3 with M-CSF, [496] the Kupffer cells in the liver, splenic macrophages, [493] and oligodendrocytes/glial cells in the central nervous system, which elaborate cytokines and express GM-CSFRs. [497] [498]

Dendritic cells are specialized cells that appear to be derived from CD34+ progenitors, [499] [500] because colonies with a dendritic appearance appear in the same colonies as macrophages, suggesting a common origin. Dendritic cells are HLA-DR+ and CD4+, but they lack nonspecific esterase and other macrophage surface markers. They are HLA-DQ+ and are able to stimulate allogeneic mixed lymphocyte cultures. In the presence of TNF- and GM-CSF, these cells proliferate and acquire the characteristic morphologic appearance of dendritic cells. [501] Furthermore, dendritic cells in the lungs as well as pleural and peritoneal macrophages are subject to local control by GM-CSF, [502] a process also seen in transgenic mice expressing GM-CSF. [503]

To maintain steady-state hematopoiesis, it is estimated that 8.7×10^8 PMNs/kg and 5.7×10^6 monocytes/kg must be produced every day. The cytokine factors that influence the proliferation and maturation of these cells must thus be finely tuned to be able to accommodate ever-changing and sometimes urgent demands for increased production. CFU-M generally needs two factors, GM-CSF plus M-CSF or IL-6 plus M-CSF, to express full colony-forming potential. [476] GM-CSF synergizes with G-CSF in the formation of granulocytic colonies (CFU-G) with respect to number and size of the colonies. Also, GM-CSF synergizes with M-CSF to increase the number and size of macrophage colonies (CFU-M). [67] [476] A significant proportion of CFU-M growth requires the presence of IL-6, indicating that an intricate regulatory cytokine network probably influences marrow promonocyte production. [504] [505] Additional studies have also identified a monocyte-specific inducer elaborated by activated macrophages undergoing phagocytosis or at sites of inflammation. [506] This cytokine, termed factor-increasing monocytopoiesis, differs from IL-1 and CSF-1 and regulates marrow monocyte production in a specific long-range manner. [507] Its relationship to the newly identified monocyte-active chemokines such as MCP-1, -2, and -3 remains to be determined. [166] The cytopenias frequently associated with infections and other bone marrow disorders reflect a complex regulatory network of soluble factors such as positive regulatory cytokines, inhibitory cytokines (TGF-, TNF-), [271] [507] and chemokines (MIP-1, IP-10, MCP-1, stromal-derived factor and), [130] [158] [159] [508] [509] [510] soluble cytokine receptors, [511] and short-range regulatory signals mediated by leukotrienes, [512] [513] prostaglandins, [514] and cytomatrix-bound factors regulating the interactions between stromal cells and progenitor cells. [515] [516] [517] [518] Furthermore, macrophages developing in the marrow may differ in their function and phenotype. [519] [520] These complex cytokine/cell-cell interactions are illustrated in Figure 16-5 , which recapitulates intramedullary regulatory events occurring during active immune stimulation or chronic infections. [2] [521] Mature mononuclear cells including granulocytes, monocytes, and T lymphocytes release cytokines (Table 16-4) and chemokines (Table 16-5) when exposed to activating agents such as bacterial antigens or inflammatory stimuli, as shown in Figure 16-5 . Tables 16-4 and 16-5 underscore the potential for localized regulation of progenitor output and precursor tissue accumulation by mature cells. [522] [523] [524] [525] [526] [527] [528] [529] [530] [531]

Eosinophil Production

The eosinophil is a type of granulocyte characterized by distinct granules that become apparent at the early myelocyte stage of development. [195] Eosinophils are derived from CD34+ committed progenitors that proliferate and mature in the marrow. Mice deficient in CD34 have reduced eosinophil accumulation after allergen exposure, but otherwise have normal hematopoiesis. [532] As illustrated in Figure 16-3 , eosinophil production requires early-acting factors such as SCF, IL-3, and G-CSF, [533] [534] [535] as well as late-acting lineage-specific factors such as IL-5 [195] [535] and GM-CSF. [536] Eosinophilic colonies in culture have been identified by luxol fast blue or peroxidase staining. [6] [537] Their distinctive morphology and enzymatic granule content account for their staining properties, and are described in greater detail in Chapter 40 . The normal concentration of eosinophils in the blood is $<0.7 \times 10^9$ cells/L. Many diseases are associated with eosinophilia, including parasitic

infestation with tissue-invasive organisms, allergic states (bronchial asthma, allergic rhinitis, pulmonary aspergillosis), vasculitides, various collagen vascular diseases, and paraneoplastic conditions. [538] [539]

The sequential action of eosinophil-active cytokines and their cellular derivation are shown in detail in Figure 16-6 . Fibroblasts and bone marrow vascular endothelial cells release cytokines such as SCF/KL, GM-CSF, G-CSF, and IL-5, [540] all of which affect eosinophilopoiesis. Several growth factors act on early eosinophil progenitors in a synergistic fashion. These include factors such as G-CSF and *kit* ligand (SCF), both of which are not specific for this lineage. [533] [536] IL-3 is capable of inducing colonies from CD34+/CD33 cells, whereas IL-5 by itself does not have this capability. [536] When CD34+/CD33 cells are grown in IL-3 followed by IL-5, CFU-Eo emerge. CD34/CD33+ cells form CFU-Eo if first exposed to G-CSF or to IL-3 and then replated in IL-5. [536] IL-3, GM-CSF, and IL-5 have additional activity at intermediate stages of eosinophilopoiesis, and they are also active on mature eosinophils. [537] [541] It has also been found that hybrid eosinophil/basophil intermediates and immature mononuclear eosinophils exhibit autocrine regulation of viability because of constitutive production of GM-CSF. [542]

In vivo experiments in mice and humans infected with filarial parasites have shown IL-5 to be the principal regulator of eosinophil production. [198] [543] [544] IL-2 administration leads to eosinophilia, which can be blocked by a monoclonal antibody to IL-5. [545] This in vivo IL-2 administration is associated with increased IL-5 RNA expression in the spleens of treated mice. [545] Transgenic mice expressing IL-5 manifest eosinophilia and autoantibody production. [197] Moreover, IL-5 has also been found to be elevated in patients with hypereosinophilia, [546] and has been identified along with GM-CSF and IL-3 in the eosinophilia associated with T-cell lymphoma. [547] Expression of IL-5 in thymocytes/T cells leads to development of massive eosinophilopoiesis in tissues and consequent eosinophilia. [548] In IL-5R-deficient mice, basal levels of eosinophils were

Figure 16-5 Positive and negative regulators of monocytopoiesis and granulopoiesis (neutrophil component). Mediators of both a positive and negative nature are active at all levels of development. Also, certain cytokines at different concentrations and in different isoforms (e.g., transforming growth factor [TGF]-) can act in either inhibitory or stimulatory fashion at various levels of maturation. CFU, colony-forming unit; CSF, colony-stimulating factor; G, granulocyte; GEMM, granulocyte/erythroid/macrophage/megakaryocyte; SGMR, soluable granulocyte-macrophage colony-stimulating factor receptor; HPP-CFC, high-proliferative-potential colony-forming cells; IGF, insulin-like growth factor; IL, interleukin; IP-10, interferon--inducible protein; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; LTC-IC, long-term culture-initiating cell; M, macrophage; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PF4, platelet factor-4; ra, receptor agonist; SCF, stem cell factor; sIL-R, soluble interleukin receptor.

TABLE 16-4 -- Cytokines Released by Activated Mature Precursors

Cytokine	Neutrophil	Monocyte or Macrophage	Eosinophil	Mast Cell or Basophil
IL-1, IL-1	+++	+++	+++	+++
IL-1ra	+++	+++		
IL-3			+++	+++
IL-4		+++		+++
IL-5			+++	+++
IL-6	+++	+++	+++	+++
IL-10		+++		
IL-12		+++		
IL-13		+++		+++
IL-15		+++		
G-CSF		+++		
GM-CSF	+++	+++	+++	+++
M-CSF	+++	+++		
Oncostatin M		+++		
Leukemia inhibitory factor		+++		
Tumor necrosis factor-	+++	+++		+++
Interferon-	+++	+++		
TGF-		+++	+++	
TGF-		+++	+++	
Nerve growth factor		+++		

CSF, colony-stimulating factor; G, granulocyte; IL, interleukin; M, macrophage; ra, receptor agonist; TGF, transforming growth factor.

TABLE 16-5 -- Chemokines Released by Activated Mature Precursors

Chemokine	Neutrophil	Monocyte or Macrophage	Eosinophil	Mast Cell or Basophil
Interleukin-8	+++	+++	+++	+++
Gro-/MGSA	+++	+++		
MCP-1	++	+++	+++	
MCP-2,3		+++		
MCP-5		+++		+++
MIP-1	+++	+++	+++	+++
MIP-1	+++	+++		+++
IP-10	+++	+++		
Lymphotactin				+++
RANTES		+++	+++	
Eotaxin/eotaxin-2		+++	+++	

Gro, growth-related; IP = 10, interferon--inducible protein; MCP, monocyte chemoattractant protein; MGSA, melanoma growth-stimulatory activity; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T expressed and secreted.

produced, but impaired eosinophilopoiesis enhanced the survival of *Angiostrongylus cantonensis*.[549]

T cells and eosinophils are implicated in the pathogenesis of many disease states such as asthma. [537] [538] [539] The role of human types 1 and 2 T-helper-derived cytokines in eosinophilic states is highlighted in Figure 16-6 .[550] [551] The activation of IL-5-producing T cells in atopic states may lead to eosinophilia; additional factors, some of which have not been cloned yet, are also operational in these conditions. [552] Also, in the hypereosinophilic syndrome, there is evidence in some patients for clonal proliferation of type 2 helper T cells. [553] [554] A case has been reported of a patient with hypereosinophilia who had T-cell clones that produced IL-4 and IL-5 and demonstrated clonal rearrangements of the chain of the T-cell receptor. [554] Moreover, the importance of cell-cell intercrine stimulation is illustrated by the finding that GM-CSF-induced LTB4 secretion can lead to increased IL-5 elaboration by T cells, thereby amplifying eosinophilic precursor maturation at the sites of antigenic stimulation.[555] Eosinophils in the circulation and at the sites of allergic inflammation express IL-5, GM-CSF, [556] [557] and eotaxin,[163] and elaborate a potent angiogenic factor, TGF-, as indicated in Tables 16-4 and 16-5 .[558]

Basophil and Mast Cell Production

Both mast cells and basophils develop from the hematopoietic stem cell.[559] Basophils complete their differentiation in the marrow, but precursors of mast cells leave the marrow, invade connective or mucosal tissue, proliferate, and differentiate into mast cells. Mechanisms regulating maturation of these two cell types are also different. The basophil is a granulocyte subtype derived from marrow progenitors that functions in allergic disease. In contrast, tissue mast cells do not circulate in the blood but complete their maturation in tissues, where they provide mucosal immunity as well as serve a source of cytokines and a variety of agents on activation by allergens.[560] Mast cells are derived from CD34+, *c-kit*-positive progenitors, and not from monocytes, as previously thought.[561] The production of basophils as well as tissue-fixed mast cells is regulated by a complex array of cytokines elaborated by T-cell subsets, macrophages, and stromal cells/fibroblasts (see Fig. 16-6). Basophilopoiesis is regulated by overlapping cytokines such as SCF, IL-3, [560] [562] [563] and IL-5,[7] [537] [564] which are active in eosinophilopoiesis, but it also relies on IL-4,[7] [565] IL-9,[566] and IL-10,[567] cytokines with anti-inflammatory roles that do not affect eosinophil production. [7]

Figure 16-6 Regulatory cytokines and accessory cells that modulate eosinophilopoiesis and basophilopoiesis. Factors produced by T-lymphocyte subsets, macrophages, fibroblasts, and bone marrow endothelial cells (BMEC) control the development of these cell types. These cytokines act individually or synergistically within each maturation pathway to promote cell proliferation or inhibit apoptosis. Several cytokines can activate mature precursors and thereby prime them for degranulation, migration, and enhanced effector function. CFU, colony-forming unit; CSF, colony-stimulating factor; IL, interleukin; NGF, nerve growth factor; SCF, stem cell factor; Th, T-helper.

Also, at later stages of development, mast cells are differentially influenced by IL-3 and SCF/KL, whereas basophils are regulated by IL-10, IL-3, and IL-4. [560] Recently, IL-3 has been shown to augment immunity after parasitic challenge.[568] Mouse basophils express little or no *c-kit* receptor on their surface, nor can they survive for long periods in SCF-supplemented cultures. Mast cells are also FcepsilonR+ but develop *c-kit* receptors during their maturation.[569] A rat model with an abnormal *W* locus has been developed that has no mast cells but has normal numbers of basophils, [570] implying that additional regulators such as nerve growth factor and IL-10 can substitute for SCF in basophil development [571] (see Fig. 16-6). SCF/KL also induces mast cell proliferation, maturation, and heparin synthesis. [572] SCF/KL also allows the emergence of mast cells from marrow mononuclear cells in long-term culture.[563] Signaling through the *c-kit* receptor tyrosine kinase is essential for development and survival of mast cells, but not of basophils. [573] SCF influences mast cell development only in the context of other cytokines.[574] Nerve growth factor promotes murine mast cell colony formation[575] and has been found to be synergistic with GM-CSF in normal and leukemic basophilic cell differentiation[564] [576] (see Figs. 16-3 and 16-6).

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CELLULAR DYNAMICS

Cytokine Expression by Mature Cells

Macrophages synthesize and release GM-CSF and G-CSF and display a large spectrum of heterogeneity. [520] [522] Very little CSF is produced by resting macrophages, but stimulation with substances such as endotoxin can increase GM- or G-CSF production, whereas bone marrow-derived macrophages can elaborate SCF in small amounts.[519] Because tissue macrophages are separated from proliferating granulopoietic cells in the marrow, it is uncertain whether CSFs produced by these resident macrophages have any proliferative effects on the marrow progenitor compartment; they may serve only to activate granulocytes/macrophages locally. Monocytes also produce IL-1, IL-6, and TNF-, [577] as well as many chemokines that are instrumental in recruiting effector granulocytes and lymphocytes to areas of inflammation or antigen challenge (see Tables 16-4 and 16-5). Disruption of the microtubule network of human monocytes induces expression of IL-1 but not of IL-6 or TNF-.[578] The IL-1 production is mediated by the stimulation of protein kinase A. [384]

Neutrophils as well as eosinophils express IL-6 mRNA and secrete IL-6 protein. [556] This expression is rapidly down-regulated after cells are removed from the blood. Neutrophils can thus modulate T- and B-lymphocyte functions, granulocyte self-priming, and endothelial interactions through secretion of IL-6. GM-CSF has also been found to induce *c-fos*, *EGR-1*, IL-1, G-CSF, M-CSF, and TNF- genes in neutrophils, again suggesting that neutrophils can play a role in regulating the inflammatory response.[247] [579] [580] [581] [582] One can assume that there are active autocrine loops in both mature neutrophils and monocytes. [583] [584] Mast cells release IL-6 through *c-kit*. Mature eosinophils have been found negatively to regulate eosinophilopoiesis by decreasing IL-5 levels. Basophils produce IL-4 and IL-13 on FcepsilonR1 cross-linking. As with other granulocytic effector cells, eosinophils, basophils, and mast cells also express cytokines and chemokines on activation (see Tables 16-4 and 16-5).

Cytokine Modulation of Mature Effector Cell Function

Growth factors are released at sites of inflammation and can exert both priming and direct effects on mature monocytes and neutrophils. These influences can arm effectors for improved response to other physiologic substances that are also active at sites of inflammation and infection such as chemotactic factors, ILs, and leukotrienes. GM-CSF, as an example, can decrease random migration of macrophages and neutrophils, increase surface expression of Mo1, and increase leukocyte aggregation.[585] [586] These represent only a few of the many effects of GM-CSF on mature neutrophils, which in general include accentuating host defense mechanisms such as adhesion, chemotaxis, phagocytosis, superoxide production, antibody-dependent cell-mediated cytotoxicity, and synthesis and release of inflammatory mediators and cytokines. Many of these effects have clinical significance, such as the profound drop in neutrophil counts after intravenous boluses of GM-CSF and G-CSF, possibly due to sequestration of these activated cells in lungs. [586]

Numerous cytokines also have effects on the mature eosinophil. IL-5 can prevent apoptosis in mature human eosinophils. [566] GM-CSF, IL-5, and IL-3 can activate eosinophils, manifested in many ways. The FcRII is activated in human eosinophils in response to these cytokines, [587] and each one has a priming effect on the eosinophil respiratory burst [588] and on responses to FMLP and to platelet-activating growth factor. [589] Moreover, GM-CSF and IL-3 amplify leukotriene C4 production after Ca^2 + ionophore exposure.[590] Cytokines also modulate cell-surface expression of activation antigens on mature eosinophils. [591] [592] The chemokines RANTES and eotaxin 1 and 2 have been shown to be chemoattractants for eosinophils, [132] [593] a property not shared by MCP-1 MIP-1, or IL-8 [594] (see Fig. 16-3 and Table 16-1). Platelet-activating factor is another potent inflammatory mediator that can activate mature eosinophils and stimulate eosinophil production from precursor cells [595] (see Tables 16-4 and 16-5).

Granulocyte-macrophage colony-stimulating factor, IL-3, and IL-5 have been found to influence basophil functional activity. [7] [560] [596] IL-10 stimulates mast cells and their progenitors,[567] and also regulates cytokine production in mouse marrow-derived mast cells. [597] Finally, the chemokine MIP-1 has also been found to activate basophils and mast cells.[598] In addition to its effects on basophil and mast cell development, SCF/KL potentiates release of mediators from mast cells [599] and activates their adhesion to fibronectin. [600] Both cyclosporine and FK506 have been found to inhibit cytokine production from mast cells [601] [602] and basophils.[603] Tables 16-4 and 16-5 illustrate the panel of cytokines and chemokines released by basophil/mast cells on activation.

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Chapter 17 - Thrombocytopoiesis

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The clinical consequences of alterations in platelet levels are well known, ranging from severe thromboembolic episodes (occurring in some patients with primary thrombocytosis) to hemorrhage resulting from thrombocytopenia. With the exception of instances of increased peripheral destruction (e.g., trauma, infection), most quantitative and qualitative disorders of platelet function result from intrinsic errors occurring within the megakaryocyte. Platelets lack a nucleus and (for the most part) a rough endoplasmic reticulum, and therefore have little ability to alter their biochemical composition or structure. Consequently, an understanding of the biochemical and molecular mechanisms underlying thrombocytopoiesis rests on understanding the development of the platelet within its precursor cell, the megakaryocyte. Until recently, however, megakaryocyte differentiation was difficult to study because the low frequency and fragility of these cells make them difficult to purify in sufficient numbers for biochemical and molecular studies. Recent scientific advances, together with new developments in cytokine therapy and bone marrow transplantation, now provide therapeutic tools with which to alter platelet production.

Megakaryocyte development is a complex process in which a wide variety of regulatory signals work in concert to direct a highly specific response to thrombopoietic demand. The cells of the megakaryocyte lineage are the primitive, actively proliferating progenitor cells as well as the mature postmitotic megakaryocytes undergoing maturational development (Fig. 17-1). The complex nature of this developmental hierarchy is underscored by the wide variety of hematopoietic growth factors that stimulate these cells (i.e., the various colony-stimulating factors [CSFs], interleukins, and thrombopoietin [TPO]). Developing megakaryocytes also interact with surrounding extracellular molecules that further modulate the developmental process. Each of these key elementscells, growth factors, and extracellular moleculesthus defines a highly organized and localized regulatory system known as the megakaryocytic microenvironment. It is this system that coordinately regulates megakaryocyte development and the daily production of approximately 2×10^{11} platelets. [1]

Although the exact makeup of the megakaryocyte microenvironment is still unknown, many of its important elements have been defined. The cellular components are the parenchymal cells (i.e., cells committed to megakaryocyte lineage) and the neighboring stromal cells such as fibroblasts, endothelial cells, and macrophages. These stromal cells produce both membrane-associated and soluble cytokines (growth factors) as well as extracellular molecules important to megakaryocyte function. To date, over 25 hematopoietic growth factors have been identified and molecularly cloned, including interleukin (IL)-1-IL-18, granulocyte (G)-CSF, granulocyte-macrophage (GM)-CSF, macrophage (M)-CSF, leukemia inhibitory factor (LIF), c-kit ligand, erythropoietin, and TPO. A variety of other regulatory factors such as the interferons, tumor necrosis factor, and transforming growth factor- (TGF-) are capable of inhibiting or stimulating (or both) hematopoiesis. Among these 30 or so growth factors, at least four interleukins (IL-3, IL-6, IL-9, and IL-11) as well as c-kit ligand, GM-CSF, and possibly erythropoietin stimulate both in vivo and in vitro megakaryocyte

Figure 17-1 Cellular hierarchy of megakaryocyte development. Megakaryocytes can be conceptually divided into three stages: the proliferating progenitor cells, which have the typical 2C/4C DNA content, the immature megakaryocytes, which have an intermediate DNA content and are transitional between the progenitor cells and the more mature cells, and the mature, postmitotic cells, which have an 8C to 128C DNA content (see text). CFC-Mk-HPP, colony-forming cell-megakaryocyte-high-proliferative potential; BFU-Mk, burst-forming unit-megakaryocyte; CFU-Mk, colony-forming unit-megakaryocyte; PMkB, promegakaryoblast.

development (for a review, see Kaushansky[2]). The final component of the megakaryocytic microenvironment is the extracellular matrix (ECM). Once referred to as basement membrane, ECM is no longer thought to be an inert structural scaffold for various organs. Instead, it is a dynamic, complex cellular substrate whose components stimulate cells to proliferate, differentiate, or migrate (for a review, see Long [3]). Recent studies demonstrate that, like other lineages, megakaryocytes have unique developmental requirements that are modulated by interactions with specific ECM molecules. [4] [5] [6]

Interestingly, megakaryocytopoiesis occurs in a number of locations throughout the body. The primary site of megakaryocyte development (and hence platelet production) is the bone marrow. However, it is known that megakaryocyte precursors and some mature megakaryocytes circulate, [7] [8] which suggests that capillary beds might filter (trap) such cells. If the surrounding microenvironment is appropriate, then megakaryocytes may develop in such extramedullary tissue. This is true for both the spleen and lungs, each of which contains megakaryocytes and produces platelets. [9] [10] However, the contribution of these organs to total thrombocytopoiesis is on the order of only 715%. [1] [10]

An understanding of the disorders of platelet production requires knowledge of the complex regulatory events that occur during normal megakaryocytopoiesis. Moreover, a rational approach to therapeutic interventions that might alter the clinical course of megakaryocyte disorders must take into consideration the nature of the developmental control mechanism(s) being affected. This chapter focuses on current concepts of megakaryocyte/platelet development, examining the various types of megakaryocytic cells, their responses to cytokines and other extracellular influences, and recent observations on the biochemical and molecular control of lineage-specific gene expression.

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BIOLOGY OF MEGAKARYOCYTES

Megakaryocyte Progenitor Cells

The cellular hierarchy of megakaryocytopoiesis is best understood if megakaryocyte development is artificially divided into three stages: progenitor cells, immature megakaryocytes (promegakaryoblasts [PMkB]), and mature megakaryocytes (Fig. 17-1). Megakaryocyte progenitor cells are responsible for the expansion of megakaryocyte numbers and proliferate in response to a number of mitotic growth factors. Promegakaryoblasts are transitional in nature, bridging between the progenitor cells and the more mature postmitotic cells. Mature megakaryocytes no longer proliferate but have the unique ability to continue or increase DNA synthesis, without undergoing mitosis, during maturation (see following). Mature megakaryocytes are markedly larger than other marrow cells and thus have a dramatically increased cell volume. As a result, an individual megakaryocyte produces on the order of 2,0003,000 platelets. [11]

Most of our knowledge concerning hematopoietic progenitor cells comes from in vitro assays of these cells. The most primitive hematopoietic progenitor cells are multi- or pluripotential, giving rise to cells of different lineages. These in turn give rise to committed progenitor cells, which are defined as proliferating cells capable of generating progeny of a given lineage(s). The development of either progenitor type in vitro thus results in a clonal expansion of cells, leading to the formation of a colony in semisolid media. Originally, the entity generating a colony was called a colony-forming unit (CFU) to reflect the concept that it was unclear whether a colony was derived from a single cell; this is now definitively proved.

In vitro studies demonstrate that megakaryocyte progenitor cells, like progenitor cells of other lineages, progressively lose proliferative potential as they develop. The proliferating cells of this lineage are themselves heterogeneous and are of at least three distinct cell populations, each marked by varying degrees of proliferative potential. Reports from animal studies suggest that the earliest detectable cell in this lineage, the megakaryocyte high-proliferative-potential colony-forming cell (Mk-HPP-CFC), is the most primitive progenitor cell of this lineage. Unlike most other progenitor cells, this cell proliferates in vitro to the extent that its colonies are macroscopically visible. The burst-forming unit-megakaryocyte (BFU-Mk) is more mature than the Mk-HPP-CFC but retains a high degree of proliferative potential, developing bursts of individual colony-forming cells (Fig. 17-2). The most mature proliferating cell is the colony-forming cell megakaryocyte (CFU-Mk), which has very limited proliferative potential.

The existence of Mk-HPP-CFC was first established by the groups of Long [12] [13] and Quesenberry. [14] Initially, these cells were observed in murine bone marrow; recently they have been observed in human fetal bone marrow. [15] Nonetheless, the murine data show that these cells are quite primitive and respond to a variety of hematopoietic regulators. Mk-HPP-CFC produce colonies of a few thousand megakaryocytes, demonstrating a proliferative potential of some eight to ten replicative divisions. Mk-HPP-CFC require a minimum of three different mitogenic signals for proliferation. Long and co-workers have demonstrated that these cells have an obligate requirement for IL-3 and simultaneously require co-activation of the protein kinase C- and cyclic adenosine monophosphate (cAMP)-mediated signal transduction pathways. [12] Quesenberrys group confirmed and extended these observations, showing that three to five recombinant hematopoietic growth factors are required to sustain the proliferation of these early megakaryocyte progenitor cells. Interestingly, colonies observed in these latter studies contained cells of multiple hematopoietic lineages, with large numbers of megakaryocytes present.

The BFU-Mk has a high proliferative potential, generating 100500 megakaryocytes per cell, representing a proliferative capacity of some five to seven replicative events. [16] These cells are believed to be the progeny of Mk-HPP-CFC and the immediate ancestors of the colony-forming cell. [13] BFU-Mk colonies morphologically resemble the erythroid burst-forming cell in that the BFU-Mk colonies consist of multiple foci of megakaryocyte development, each of which presumably is due to the presence of a single CFU-Mk (Fig. 17-2). Human BFU-Mk cells have in vitro characteristics similar to those of murine cells and, additionally, are resistant to treatment in vitro with 5-fluorouracil. [17] [18] Proliferation of these cells in chemically defined media (i.e., serum-free cultures) has allowed precise determination of their growth factor responsiveness. BFU-Mk require two categories of growth factors for in vitro development. They have an obligate requirement for mitogenic growth factors such as IL-3 or GM-CSF. Additionally, optimal development of BFU-Mk requires the presence of synergistic co-regulators that augment hematopoietic growth factor-driven proliferation and differentiation (see discussion under Megakaryocyte-Active Cytokines). [16] In humans, these cells respond to IL-3 and GM-CSF as proliferative stimuli. [18] In addition, c-kit ligand (also known as stem cell factor, or mast cell growth factor), IL-11, IL-1, and thrombopoietin synergize with IL-3 to augment BFU-Mk development. [18] [19] [20] A number of other putative megakaryocyte co-regulators (i.e., IL-6, erythropoietin, G-CSF, IL-4) fail to synergize with IL-3, GM-CSF, or Mk-CSF in stimulating BFU-Mk. [18]

The most differentiated of the megakaryocyte progenitor cells is the CFU-Mk cell. This was the first megakaryocyte progenitor cell to be assayed in vitro. This cell and its progenitors have relatively fastidious requirements for in vitro growth. As a result, the first murine CFU-Mk colony was not developed in vitro until 9 years [21] [22] [23] after the first hematopoietic progenitor

Figure 17-2 Megakaryocyte progenitor cells. **(A** & **B)** The cellular hierarchy of the megakaryocyte lineage is believed to begin with a primitive, highly proliferative burst-forming cell, BFU-Mk, which divides, migrates, and differentiates to generate multicentric clusters of megakaryocytes. **(C** & **D)** Each BFU-Mk gives rise to several nonmotile, less proliferative CFU-Mk, each of which generates a small cluster of megakaryocytes in culture. *(Reproduced from Long MW, Gragowski LL, Heffner CH et al.: Phorobol diesters stimulate the development of an early murine progenitor cell: the burst forming unit megakaryocyte. J Clin Invest 67:431, 1985, by copyright permission of the American Society for Clinical Investigation.)*

cell (the granulocyte progenitor cell), [24] and the first human megakaryocyte colony was not reported until 5 years later. [25] [26] The CFU-Mk cell has a restricted proliferative potential, generating only 432 megakaryocytes (i.e., 25 divisions). [27] This progenitor cell responds to a variety of single growth factors (e.g., IL-3, GM-CSF), and also interacts with co-regulators such as c-kit ligand, flt3/flk2-ligans, and thrombopoietin. [19] [28] [29] Interestingly, CFU-Mk cells are not the first cells in the lineage to respond to thrombocytopenia. In fact, increases in CFU-Mk numbers occur as a late response to decreased platelet numbers. [30] [31] This suggests that megakaryocyte progenitor cells somehow sense megakaryocyte (and not platelet) mass, thus increasing in number to supply an increased need for megakaryocytes. This type of regulatory network was first suggested by Ebbe and Phalen, who demonstrated that decreased megakaryocyte numbers resulted in a correcting stimulus that was independent of platelet level. [32]

A number of reports suggest that other types of multi- or pluripotential cells express megakaryocytic potential (e.g., the colony-forming unit-granulocyte/erythrocyte/megakaryo-cyte/macrophage [CFU-GEMM]). Notably, a developmental relationship seems to exist between the erythroid and megakaryocyte lineage. Early work by Nicola and Johnson documented the progressive loss of lineage potential by pluripotential progenitor cells, resulting in a bipotential erythroid/megakaryocytic progenitor cell. [33] This concept was strengthened by observations of Papayannopoulou et al., [34] [35] Long et al., [36] and others [37] [38] showing that cell lines derived from patients with erythroleukemia express some megakaryocyte phenotypic markers or can be induced to undergo megakaryocyte

commitment and differentiation (or both). Other investigators have documented the existence of transplantable megakaryocyte progenitors (Mk-CFC $_s$)[39] and cell size heterogeneity among in vitro colonies (large heterogeneous colonies). [40]

Immature Megakaryocytes (Promegakaryoblasts)

Promegakaryoblasts are transitional cells intermediate between proliferating progenitor cells and postmitotic, mature megakaryocytes. [41] [42] Morphologically, these immature cells are not readily observed in vitro or in bone marrow specimens, but they may be identified by their expression of megakaryocyte/

platelet-specific markers such as platelet peroxidase, platelet glycoprotein IIb/IIIa, von Willebrand factor (vWF), etc. [43] [44] Promegakaryoblasts are restricted in (or lack) proliferative potential. They thus are the developmental stage at which megakaryocytes cease to proliferate but continue to acquire an increased DNA content. As such, they are endomitotic (a mechanism of acquiring polyploid nuclei, see below) and contain an intermediate DNA content. PMkB respond to a variety of hematopoietic growth factors (IL-3, c-kit ligand, IL-6, TPO) in vitro, maturing into single large megakaryocytes. [45] [46] Observations of the early phases of CFU-Mk colony formation demonstrate that progenitor cells pass through a PMkB stage during development, thus confirming the parent-progeny relationship between PMkB and megakaryocytes.[41] Studies in animals document the responsiveness of these cells in vivo. Promegakaryoblasts are highly sensitive to thrombopoietic demand and are the first cells to increase in number after the induction of thrombocytopenia and to decrease under conditions of thrombocytosis. [42] [47] Subsequently, increases and reductions (respectively) are seen in megakaryocyte numbers, again confirming the kinetic and developmental relationship between PMkB and their more differentiated progeny. Promegakaryoblasts also are a heterogeneous group of cells that during development increase in nuclear and cytoplasmic complexity. [41] [46] [48] [49] Three distinct subpopulations of these cells exist, differing in their physicochemical characteristics, morphology, antigen expression, and enzymatic content.[43] [46] [48] [50]

Mature Megakaryocytes

Morphologically recognizable megakaryocytes exist in three maturational stages as defined by their morphology (Fig. 17-3). The megakaryoblast (stage I) is characterized by a high nucleus to cytoplasm ratio and scanty basophilic cytoplasm, reflecting the large amount of protein synthesis occurring in these cells. The promegakaryocyte (stage II) is the cell in which both the cytoplasmic volume and number of platelet-specific granules increase. The granular or platelet-shedding megakaryocyte (stages III and IV) is the most mature of the megakaryocytes and supposedly is the platelet-shedding cell. It should be understood that these morphologic classifications also represent a maturation progression and are themselves heterogeneous with respect to many other developmental characteristics such as antigenic expression, enzymatic content, and DNA content.

Platelets

The final event of megakaryocyte development is the release of platelets into the circulation. Interestingly, platelets were the first element of this lineage to be identified and among the first of the blood cells observed. In the late 1800s both William Oster (in 1874) and Georges Jayem (in 1878) described and illustrated blood platelets.[51] In 1882, Julius Bizzorzero coined the term platelet (*Blutplättchen*) and noted their shape change with activation and their involvement with hemostasis. [51] However, it was not until 1906 that Homer Wright linked platelets with the megakaryocyte, [52] [53] and not until the 1950s that this hypothesis was proved. [11] [54] Insofar as the existence of platelets has been recognized for over a century, it is surprising that the mechanisms by which platelets are produced and released remain poorly understood. During maturation, proliferation and invagination of the megakaryocyte plasma membrane occur, resulting in the development of a tubular network known as the demarcation membrane system (DMS). The DMS is thought to divide the megakaryocyte cytoplasm into platelet fields (Fig. 17-4), although its exact role in the formation of individual platelets remains obscure and controversial. Finally, megakaryocytes seem to extend pseudopods into the sinusoidal lumen, from which platelets are shed into the circulation. [55] Studies using TPO-based in vitro assays of platelet production have allowed the

Figure 17-3 Mature human megakaryocytes. **(A)** Megakaryoblast. **(B)** Promegakaryocyte (stage II). **(C)** Granular megakaryocyte (stage III). **(D)** Granular megakaryocyte (stage IV). *(Courtesy of Dr. Maryann Weller.)*

Figure 17-4 Megakaryocyte demarcation membrane system (DMS). The DMS is thought to evolve from an invagination and proliferation of the plasma membrane, and is involved in platelet formation. **(A** &**B)** Transmission electron micrographs of two separate stage III/IV human megakaryocytes. P, a platelet field within the megakaryocyte cytoplasm (the distance between the vertical bars is approximately 2 m); N, nucleus; n, nucleolus; AG, -granules; arrows, the DMS; arrowheads, opening of the DMS to the extracellular environment. *(Courtesy of Dr. Maryann Weller.)*

beginnings of the biochemical and molecular control of this process to be understood.

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REGULATION OF MEGAKARYOCYTE DEVELOPMENT

There are three areas in which physiologic control over the megakaryocyte lineage is relevant: the expansion of megakaryocyte numbers (proliferation), the regulation of megakaryocyte maturation, and control of platelet shedding. A large number of investigators have detailed the role of cytokines in the first two of these areas. Moreover, increasing evidence suggests the importance of ECM components to megakaryocyte and platelet production. The actual process of both platelet formation and shedding is poorly understood. Given the lack of definitive data, the regulation of platelet production per se will not be discussed. Instead, this section examines megakaryocyte antigenic expression and the regulation of megakaryocytes by cytokines and the ECM.

Markers of Megakaryocyte Development

Megakaryocytes and their precursors express antigenic determinants that are developmentally regulated. The expression of these cell-surface structures allows the isolation and purification of subpopulations of marrow cells that are enriched for the various megakaryocyte progenitor cells. One of the antigens on megakaryocytes and their precursors is CD34, a pan-hematopoietic cell antigen first identified by Civins group (Strauss et al. [56]). This antigen is expressed on all hematopoietic progenitor cells and allows their separation from other bone marrow cells by a variety of immunologic procedures. Subsequent examination of CD34+ subsets for other developmental markers has yielded the beginnings of an immunophenotypic analysis of the megakaryocytic lineage.

As expected, cells of the megakaryocyte lineage from the BFU-Mk to the mature cells express CD34, although its expression is reduced in mature cells. Subsequent characterization of CD34+ cells based on the expression of the human major histocompatibility (MHC) class II complex HLA-DR yields further segregation of progenitor cell phenotypes. In general, expression of the HLA-DR antigen characterizes a more mature subset of hematopoietic progenitor cells (i.e., CD34+ HLA-DR cells are more primitive than CD34+ HLA-DR+ cells). [4] Among megakaryocyte progenitors, this distinction separates BFU-Mk (which are CD34+ DR) from the more mature CFU-Mk (CD34+ HLA-DR+). [57] Importantly, CD34+ HLA-DR cells can sustain long-term megakaryocytopoiesis in growth factor-driven cultures for 1012 weeks, thus demonstrating the marked proliferative potential of these cells. [57] Megakaryocyte CD34+ HLA-DR burst-forming progenitor cells also express the c-kit receptor[58] (a tyrosine-kinase receptor that interacts with the c-kit ligand [KL]). It should be realized, however, that the progression of cells through a differentiation sequence results

in a spectrum of phenotypes (immunologic, morphologic, or otherwise) and that cells do not exist as compartments. Although the majority of cells of a given type may express (or lack) a given antigen, this does not mean that antigenic expression is phenotypically exclusive.

Platelet glycoprotein (GP)IIb/IIIa (CD41) is another important marker of the megakaryocyte lineage. This antigenic structure results from the interaction of two gene products (GPIIb and GPIIIa) on the surface of cells and is expressed on cells of this lineage from the progenitor cells through to the platelets. [43] [59] Co-expression studies demonstrate that CD41+ cells represent approximately 2% of the CD34+ cells subpopulation (i.e., 0.06% of total nucleated bone marrow cells). This number is consistent with the frequency of the assayable megakaryocyte progenitor cells in human bone marrow.

Studies of human PMkB by Vainchenkers group (Debili et al. [50]) show that these cells also express CD34. Three antigenically distinct subpopulations of human PMkB can be identified based on the co-expression of CD34 and platelet GPIIIa (i.e., CD34+ IIIa, CD34+ IIIa+, and CD34 IIIa+). These cells thus are transitional in antigenic expression, being the point at which megakaryocytes alter expression of CD34 and begin to express platelet GPIIIa. Based on proliferative capacity and granular content, CD34 IIIa+ cells are the most mature, having a high granular content and a low proliferative potential. CD34+ IIIa+ cells are intermediate, and the CD34+ IIIa cells are the most primitive, having a higher proliferative capacity and giving rise to more polyploid progeny. Nonetheless, the overall proliferative capacity of PMkB remains low because only 10% of these double-positive cells are capable of forming megakaryocyte colonies. [50] These studies show that CD34 is still expressed on the (polyploid) transitional PMkB, and suggest that CD34 expression occurs in those cells capable of some degree of DNA synthesis (mitosis or endomitosis). Also, platelet GPIIIa is present on both PMkB and those megakaryocyte progenitor cells with a low proliferative capacity.

Immunophenotypic analysis demonstrates that, although mature megakaryocytes express little if any CD34, they do express multiple markers associated with the platelet lineageplatelet factor 4, platelet GPIIb/IIIa, vWF, thrombospondin, and thrombomodulinbut are heterogeneous for the degree of expression of these antigens. The functional significance of such antigenic heterogeneity among the various subpopulations of mature megakaryocytes remains unresolved. Although the exact function of platelet antigen heterogeneity on megakaryocytes is unknown, it may be clinically relevant. For example, Stahl et al. have shown that expression of platelet epitopes on megakaryocytes is important in immune interactions. [60] Thus, not all antibodies to platelet antigens react with megakaryocytes, perhaps owing to lack of exposure of the target antigen. Also, expression of specific (but undefined) antigens occurs in megakaryocyte progenitor cells. [61] Such antigens may be the targets of antibodies that lead to certain autoimmune disorder such as acquired amegakaryocytic thrombocytopenias. [62]

Megakaryocyte-Active Cytokines

The ability to assay megakaryocyte progenitor cells in vitro has gone hand in hand with the ability to identify growth factors important to megakaryocyte development. In 1969, an elegant study by Harker and Finch demonstrated that, in vivo, thrombocytopoiesis was regulated by alterations in both megakaryocyte number and megakaryocyte volume (mass). [1] In the late 1970s and early 1980s, investigators struggling with crude sources of megakaryocyte growth factors (conditioned medium from various tissues or cell lines) discovered the in vitro correlates of the Harker and Finch hypothesis: that distinct factors (bioactivities) seem to regulate the proliferative and maturational events occurring during in vitro megakaryocyte development. [29] [46] [63] [64] The purification of these activities proved difficult, but the rapid identification and cloning of numerous human recombinant hematopoietic growth factors in the mid- to late 1980s and early 1990s markedly improved our understanding of the cytokine control of megakaryocytopoiesis.

A number of cytokines affect megakaryocyte proliferation in vitro, raising the questions of growth factor redundancy and combinatorial control. Given the importance of hematopoiesis it is not surprising that redundant control mechanisms exist for each of the lineages. The concept of combinatorial control is illustrated by studies in which multiple cytokines provide a better (in vitro) stimulus than single growth factors. Studies in which purified progenitor cells are cultured in defined serum-free media demonstrate that as many as 27 recombinant hematopoietic growth factors have additive proliferative effects on megakaryocyte progenitor cell proliferation. [28] [29] [65]

Among the many possible cytokine combinations, the most physiologically relevant class of interactions consists of growth factor combinations that are synergistic (i.e., pharmacologically nonadditive). This type of control is biologically important, as synergistic responses strongly suggest that differing intracellular signal transduction pathways are co-activated, leading to dramatic and rapid increases in proliferation. This has been clearly demonstrated in other developmental systems and for murine as well as human megakaryocytopoiesis. [12] [29] [66] Furthermore, the identification of such synergistic control mechanisms in vitro points to possibly more

effective forms of therapeutic intervention in platelet disorders.

Of the hematopoietic growth factors that affect megakaryocyte proliferation, IL-3 (a 1730-kd glycoprotein) is the most potent. [17] [18] [67] It stimulates each of the three classes of megakaryocyte progenitor cells, and immature cells as well as mature megakaryocytes. However, the physiologic role of IL-3 is not known. Exogenous IL-3 stimulates in vivo expansion of megakaryocyte progenitor cells, but by itself IL-3 has little significant effect on in vivo platelet production. [68] Moreover, IL-3 is produced only by antigen-activated T lymphocytes, which suggests that its role in maintaining basal hematopoiesis or platelet production is minimal. Another pleiotropic cytokine affecting megakaryocyte development is GM-CSF, a glycoprotein of approximately 23 kd. GM-CSF stimulates development of BFU-Mk and CFU-Mk. However, parallel cell culture experiments demonstrate that its megakaryocyte-stimulatory activity is approximately 1/100th that of IL-3. [67] Nonetheless, this protein functions as a megakaryocyte CSF, and its actions are additive to those of IL-3, [28] suggesting a single converging intracellular mitotic signaling pathway.

A number of growth factors have megakaryocyte maturational activities (e.g., TPO IL-6, IL-3, c-kit ligand, IL-1, IL-11, G-CSF, LIF). [18] [19] [67] [68] [69] [70] [71] [72] Although none of these purified or recombinant molecules is megakaryocyte lineage specific, some are synergistic co-regulators. Such auxiliary growth factors were first defined in vitro as cytokines that lack the ability to stimulate megakaryocyte proliferation but do function as co-regulators to augment megakaryocyte size, DNA content, antigen expression, and so forth. [18] [29] [73] Of the hematopoietic growth factors identified, only a few fall into this category: c-kit ligand (stem cell factor), IL-1, IL-6, and IL-11. [18] [20] [71] [74] Each of these growth factors is capable of augmenting the megakaryocytic activity of other growth factors such as IL-3, albeit at differing developmental levels. For example, IL-1, c-kit ligand, and IL-11 each interact with IL-3 at the level of BFU-Mk, [18] whereas IL-6 and c-kit ligand each synergize with IL-3 to modulate colony-forming cell development. [74] IL-11 has multiple effects on in vivo and in vitro megakaryocytopoiesis. [20] It not only affects IL-3-dependent megakaryocyte colony formation, but also has a potent effect

on megakaryocyte maturation. Neben et al. have shown that recombinant human IL-11 when administered in vivo to mice results in increased numbers of megakaryocyte progenitors, increased megakaryocyte DNA content (polyploidy), and increased peripheral platelet counts. [75] Recently, IL-11 was approved for use in humans for the treatment of chemotherapy-induced thrombocytopenia. Another megakaryocyte maturational promoter is IL-6. This cytokine stimulates megakaryocyte maturation and its actions (are partially) additive to those of IL-3. [65] [70] Although in vivo, IL-6 stimulates platelet production (and speeds recovery from thrombocytopenia in animals), its actions may occur via the secondary activation of accessory (stromal) cells. [74] [76]

Finally, a number of cytokines (e.g., TGF-, platelet factor 4, certain interferons) can inhibit megakaryocytopoiesis. Platelets release specific and general inhibitors of megakaryocytopoiesis such as TGF- [77] [78] and platelet factor 4. [79] However, the physiologic role for platelet-derived inhibitors is unclear. Theoretically, it may be hypothesized that increased platelet destruction should stimulate rather than inhibit platelet production, raising the paradoxical situation of increasing inhibitors with increased platelet destruction. Other inhibitors such as the interferons also inhibit megakaryocyte development. [80] [81] Again, the mechanism of action is unknown.

Thrombopoietin

The existence of TPO as a (putative) lineage-specific regulator of platelet production was proposed approximately 36 years ago. [82] [83] It was classically defined as an *activity* in the plasma of thrombocytopenic animals or humans that, when transferred to a secondary recipient, stimulates platelet production (as monitored by radiolabeled amino acid incorporation). [82] [83] [84] For three decades, multiple unsuccessful attempts were made to purify and clone TPO. The search for TPO began in earnest following observations by Wendling and colleagues that a rare and poorly understood member of an orphan-receptor family was involved in megakaryocytic development. Thus, Vigon et al. [85] [86] cloned the human and murine homologues of the v-*mpl* oncogene that is transduced by the *m*yelo*p*roliferative *l*eukemia virus. The c-*mpl* gene encodes a protein with strong homologies with the highly conserved hematopoietin receptor superfamily and is expressed in low levels in cells of hematopoietic origin. [86] As it pertains to thrombocytopoiesis, Methia et al. used reverse transcriptase-based PCR studies to demonstrate c- *mpl* expression in CD34+ cells, megakaryocytes, and platelets. [87] As well, they showed that antisense c-*mpl* oligonucleotides specifically inhibited in vitro megakaryocyte colony formation. These data clearly indicated a role for this receptor in megakaryocytopoiesis and further suggested that the as yet unidentified mpl ligand may be the elusive TPO.

Guided by the observations by Wendlings group, a number of teams finally cloned TPO in 1994. [88] [89] [90] [91] [92] [93] The TPO gene encodes a protein with a predicted molecular weight of 3135 kd that contains two domains, the amino terminal domain having homology with erythropoietin and a carboxyl-terminal domain containing a serine- and proline-rich region. [88] The genomic structure of TPO spans 68 kilobases [94] [95] and consists of approximately 7 exons and 6 introns, with the protein encoded by exons 37. [94] Localization studies have mapped human TPO to chromosome 3, within the region of 3q2628. [94] [96] Analysis of the promoter region of this gene shows binding sites for several important transcriptional activating factors, including GATA-1 (see following) and Ets family members. [97] A splice variant of the TPO gene exists as a result of a 4-amino-acid deletion within the erythropoietin homology domain. [93] [94] [96] These studies also demonstrated a profound effect of thrombopoietin on the megakaryocyte lineage. Administration of TPO results in an increase in the frequency of megakaryocytes in the bone marrow and spleen, an increase in megakaryocyte size and DNA content, an increase in megakaryocyte/platelet-specific antigenic markers, and a 3- to 10-fold increase in circulating platelet concentration.

Numerous studies over the past 30 years have demonstrated an inverse relationship between the levels of circulating TPO (i.e., TPO-like activities) and platelet mass. The arrival of TPO cloning, the availability of recombinant or purified protein, and the availability of gene knockout animals have allowed the dissection of this relationship at the cellular and molecular level. Data from these studies best fit the model in which the predominant feedback mechanism regulating TPO concentration is its binding to platelets or megakaryocytes (or both) (Fig. 17-5). Thus, during periods of normal homeostasis, platelet counts (i.e., mass) remain constant and circulating TPO is at its basal concentration. During thrombocytopenia, platelet mass drops, resulting in a reduction in the binding and degradation of TPO by c-mpl-positive cells and an increased concentration of free TPO. Conversely, during conditions such as rebound thrombocytosis or primary thrombocythemia (other than familial; see following) elevated platelet/megakaryocyte mass serves as a sink, reducing the levels of circulating TPO to achieve homeostasis. Thus, TPO production (in most circumstances) remains constant, and its concentration is regulated by the total mass of platelets/megakaryocytes available to bind and degrade this protein. Gene inactivation studies demonstrate that TPO and its receptor (c- *mpl*) are the primary regulators of megakaryothrombocytopoiesis. [98] [99] [100] [101] [102] Thus, TPO-deficient and c-mpl-deficient mice show an approximately 85% reduction in circulating platelets and markedly reduced bone marrow megakaryocyte numbers. [98] [99] Studies of TPO concentration in c-*mpl*-deficient mice show increased levels, [98] [103] and gene dosage effects in TPO-deficient animals (i.e., TPO+/ vs. TPO/) also demonstrate this relationship. [102] Confirming these in vivo observations, a number of studies demonstrate the interaction of TPO with platelets. Purified native TPO (rabbit) binds to platelets; conversely, its administration during conditions of thrombocytopenia results in a reduction in the elevated levels of TPO. [104] [105] Studies of platelets from c-*mpl*-deficient mice indicate that they fail to bind radiolabeled TPO, whereas normal platelets bind, internalize, and degrade this protein. [103] [104] [106] Likewise, the administration of washed, normal platelets to c- *mpl*-deficient animals causes a transient reduction in their high TPO levels. [103] Consistent with these observations, platelets express high-affinity TPO receptors (i.e., c-mpl) with an affinity of 200560 pM and between 20 and 200 receptors per platelet. [103] [107] Interestingly, TPO levels are low to intermediate in normal individuals as well as in individuals with idiopathic thrombocytopenic purpura at (ITP) and thrombotic thrombocytopenic purpura (TTP). [108] [109] However, they are markedly elevated after chemotherapy and in patients with aplastic anemia or acute lymphocytic anemia. These data, plus data acquired from NF-E2-null mice (see below), demonstrate that megakaryocyte mass also is a major determinant of TPO concentration. Thus, individuals with thrombocytopenia due to peripheral destruction have normal TPO concentrations owing to the presence of increased megakaryocyte numbers, as do NF-E2 mice. [108] [110] TPO is produced by the adult and fetal liver and the adult kidney, [88] [89] and its mRNA levels are not altered in these organs by changes in platelet concentration. [111] [112] [113] However, a secondary or alternative mechanism of regulating TPO concentration seems to exist in the bone marrow and spleen that is activated during conditions of thrombocytopenia. In these organs, TPO is transcriptionally activated during thrombocytopenia, resulting in an increase in TPO mRNA and (presumably) increased protein. [111] [114]

Four organs are reported to produce TPO: the liver, kidney, bone marrow, and spleen. Of these, the data are clearest regarding

Figure 17-5 Regulation of circulating thrombopoietin levels. TPO levels are inversely related to the combined platelet/megakaryocyte mass due to TPOs binding and degradation by these cells (see text).

constitutive production by the liver and kidney, where TPO mRNA is detectable by Northern blot analysis and in situ hybridization. [88] [89] [114] These two organs most

likely account for the greatest part of basal TPO production. In the bone marrow, the expression of TPO mRNA is detectable by semiquantitative RT-PCR [111] and in situ hybridization, [114] with both procedures demonstrating an increase during thrombocytopenia. In the spleen, TPO is undetectable by conventional Northern blot analysis,[88] but RT-PCR and in situ hybridization-based detection demonstrate the presence of TPO mRNA and increased levels during thrombocytopenia. [111] [114] The regulation of TPO concentration, therefore, can be thought of as having both constitutive and inducible components (Fig. 17-6). The liver, kidney, and perhaps the spleen constitutively produce TPO, and its levels are regulated by the total mass of platelets (primarily) and megakaryocytes. However, during times of thrombopoietic stress, increased TPO expression by the spleen and bone marrow presumably contributes to platelet production. The concept of megakaryocyte binding of TPO is important to understanding TPO regulation, as megakaryocyte disorders that affect platelet shedding would be predicted to have normal TPO concentrations in the face of profound thrombocytopenia. Such is the case in animals deficient in the NF-E2 transcription factor (see following).

The cellular targets of TPO are diverse, as c-mpl receptors are expressed on both megakaryocytes and platelets as well as on hematopoietic stem/progenitor cells. In mice, TPO effects on early stem/progenitor cells are shown by its stimulation of the expansion of very immature precursors both in vitro and in vivo. [92] [99] [115] [116] [117] TPO thus stimulates both murine long-term repopulating cells as well as phenotypically defined primitive hematopoietic stem cells. [117] In isolated populations of immunologically defined primitive human hematopoietic cells (i.e., CD34+, Lin, Thy1+ cells), TPO generates CD41+ megakaryocytes (alone or in conjunction with other pluripotent cytokines such as IL-3 or c-kit ligand). [116] This capacity is observed at the single cell level, thus demonstrating the direct effect of TPO on hematopoietic stem/progenitor cells.

Within the megakaryocyte lineage, TPO is required for full maturation, and TPO synergizes with other members of the hematopoietin-receptor superfamily to augment megakaryocyte development. Under serum-free conditions TPO stimulates CD34+ cells (either alone or in combination with other cytokines such as IL-3, erythropoietin, or c-kit ligand) to produce megakaryocytes. [118] [119] Megakaryocyte progenitor cells show variable response to TPO (alone or in combination with other cytokines), suggesting that these cells have a differential sensitivity to this growth factor. [118] [120] More mature progenitor cells (as defined by co-expression of CD34 and CD41) or the translational PMkB (in single cell culture) respond with either maturational development or a limited degree of proliferation. [121] Interestingly, although the resulting single megakaryocytes are mature and of high ploidy, 60% show ultrastructural defects in the formation of the DMS or in -granule synthesis, whereas

Figure 17-6 Regulation of thrombopoietin production. TPO is constitutively produced in the liver, kidney, and perhaps the spleen. During times of increased thrombocytopoietic demand, the bone marrow and spleen seem to increase their expression of TPO, thus contributing to platelet production. TPO in turn is bound by circulating platelets, as well as by megakaryocytes and other c-mpl-positive cells in the bone marrow and other sites of hemato-thrombocytopoiesis (see text).

the remainder are ultrastructurally indistinguishable from normal bone marrow megakaryocytes. It is interesting that, in serum-free culture, TPO fails to support full megakaryocyte polyploidization even when target cells are stimulated with high concentrations of TPO. Thus, a few TPO-stimulated human megakaryocytes reach ploidy levels of 16C or greater.[91] Even the use of more mature CD34+, CD41+ megakaryocytes as a starting population generates CD41+ cells predominantly having a low DNA content.[122] [123] The limited ability of human CD34+ stem/progenitor cells to generate high ploidy megakaryocytes is in sharp contrast to the murine system, where 3555% of megakaryocytes generated ex vivo are high-ploidy cells.[124] [125] [126] Such observations suggest that other growth factors or microenvironmental signals are required for full polyploidization of human megakaryocytes and, hence, for megakaryocyte maturation. The availability of recombinant TPO also allows the generation of intact, functional platelets in vitro, thus allowing studies of this poorly understood aspect of megakaryocyte development. [127] Interestingly, the administration of TPO to platelets in vitro does not stimulate platelet aggregation. [128] Rather, its preadministration (in vivo or in vitro) primes platelet aggregation, increasing platelet responsiveness to various agonists such as ADP, epinephrine, thrombin, and collagen. [129] [130]

Studies of the domain structure of the TPO receptor (i.e., c-mpl) and its ligand binding have determined both the important features of this receptor and the nature of its signal transduction cascade. Intracellular domains of c-mpl proximal to the transmembrane region are required for proliferative response, [131] and these regions also are necessary for activation of the Janus kinases/signal transducers and activators of transcription (JAK/STAT) signal transduction pathway. [131] [132] Conversely, C-terminal regions of the receptor activate both shc-mediated signal transduction and increase c- *fos* mRNA.[131] [133] They are also necessary for proliferative response, whereas a subdomain (amino acids 6983) appears to signal receptor-mediated differentiation. [134] As mentioned, TPO binding to its receptor activates JAK, leading to STAT transcriptional factor phosphorylation and subsequent gene activation. TPO activates JAK2 and STAT 1, 3, and 5, with STAT 5 perhaps being the major component of this signal transduction process,[133] [135] although its activation may be indirect. [134] Additionally, ligand binding of c-mpl results in phosphorylation of the c-mpl receptor itself,[133] as well as activation of phosphotidylinositol-3 kinase and phospholipase C . As mentioned, TPO interacts with a variety of cytokines, yielding additive or, often, synergistic effects on megakaryocyte development, or the proliferation of hematopoietic stem/progenitor cells. Such interacting cytokines often share the gp130 receptor chain, although gp130 is not required for TPO signaling. [135] [136]

A number of animal studies have documented the in vivo effectiveness of TPO administration in models of myelosuppression

or bone marrow/peripheral blood transplantation. The in vivo actions of TPO on megakaryocyte development are seen in the original cloning/function reports and gene inactivation studies (see above). These reports, plus the gene inactivation studies, clearly demonstrate that TPO and c-mpl function as the primary regulators of in vivo megakaryocytopoiesis and document their role in early hematopoiesis. These data predict that TPO administration should have clinical utility. In murine models of chemotherapy- or radiation-induced myelosuppression and in bone marrow transplantation models, TPO accelerates the recovery of hematopoietic progenitor cells, reticulocytes, RBCs, neutrophils, and platelet counts; it often also increases the cell concentration during cytopenic nadirs. [101] [119] [137] [138] [139] [140] Prior administration of TPO to donor or recipient mice increases platelet and erythrocyte recovery in bone marrow transplantation models, suggesting that TPO modulates both the transplanted donor cells as well as the recipients hematopoietic microenvironment. [141] [142] Encouraging results are also seen in TPO administration to nonhuman primates. In general, TPO results in a 3- to 5-fold increase in circulating platelets (in either normal or myelosuppressed animals). This appears to be the result of an increase in both megakaryocyte number and volume (thus yielding a marked increase in megakaryocyte mass). [143] [144] [145] As in mice, TPO decreases the thrombocytopenia and augments neutrophil recovery in nonhuman primates given -irradiation; [146] it has the same effect in other models of myelosuppression.[147] Finally, administration of TPO to human patients with solid tumors results in a 3- to 10-fold increase in circulating platelet concentration (mean elevation, 3-fold). [148] Ex vivo testing of the platelets from these individuals showed no apparent activation or priming of the platelets as a result of TPO administration.

Extracellular Influences and Cell Interactions

The cellular concentration of bone marrow is roughly 10^9 cells/ml. This suggests that blood cells develop within the context of their interactions with neighboring cells and extracellular molecules. In the past decade, a number of investigations have demonstrated that stromal cells and ECM are dynamic and inductive (or permissive) components of all developing cellular systems. With respect to hematopoiesis, numerous studies have shown that hematopoietic progenitor cells interact with growth factors, accessory cells such as T cells, stromal cells, and ECM components (for reviews, see Long [3]). This developmental network is further complicated by observations that stromal cells express membrane-associated growth factors, [149] [150] and that ECM both binds hematopoietic growth factors and presents these cytokines in a biologically functional manner. [151] [152]

Both cell-cell and cell-ECM communications among developing megakaryocytes are poorly understood. Structurally, mature (platelet-shedding) megakaryocytes are located on the abluminal surface of the bone marrow sinusoid. It is postulated that megakaryocytes extend pseudopods through or between sinusoidal endothelial cells, thus allowing shear forces to fragment platelets into the circulation. [55] Both the location and the putative mechanism of platelet shedding imply that megakaryocyte-ECM or megakaryocyte-(endothelial) cell interactions are important to thrombocytopoiesis. Isolated megakaryocytes adhere to bovine corneal endothelial cell-derived ECM and proplatelet-like structures are induced under these conditions. [153] As well, megakaryocytes adhere to collagen and secrete both a collagenase and a gelatinase,[5] [153] suggesting a possible mechanism for pseudopod infiltration of the surrounding ECM.

Studies of megakaryocyte progenitor cells show that cell-ECM relationships are important to megakaryocyte proliferation. Approximately 30% of CFU-Mk cells in bone marrow adhere to the ECM proteins fibronectin or thrombospondin (TSP).[154] Interestingly, 6080% of primitive CD34+, HLA DR BFC-Mk attach to TSP, whereas they fail to bind to fibronectin.[4] Therefore, primitive megakaryocyte progenitor cells show both altered expression of cytoadhesion molecule attachment and altered

responsiveness to complex matrix-cytokine regulatory signals.

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MOLECULAR AND BIOCHEMICAL CONTROL OF MEGAKARYOCYTE COMMITMENT AND DIFFERENTIATION

Transcriptional Control of Megakaryocyte Gene Expression

One of the fundamental goals of molecular hematology is the identification of specific genes, or sets of genes, that direct the activation of lineage-specific differentiation events. A related and equally important aim is to identify the specific nuclear proteins (transcription factors) involved in the (trans-) activation of such master control genes. The activation of lineage-specific gene expression during the commitment of multi- or pluripotent progenitor cells requires a great deal of transcriptional specificity, which is complicated by the observation that DNA-binding transcription factors recognize short (68 bp) DNA sequences. [155] For example, cis-acting DNA regulatory sequences recognized by factors involved in transcription include TATA, which binds transcription factor IID, CCAAT, which is found in many gene promotor regions, and GATA, a regulatory element observed in genes of hematopoietic, endothelial, and neural cells. [155]

To date, the nature of megakaryocyte master control genes remains obscure. Nonetheless, a number of cis-regulatory elements have been identified, and their regulatory motifs are found within the control regions of specific genes. Of these, the GATA motif is an important control element for erythroid and, megakaryocyte development (for a review, see Orkin[155]). The GATA sequence was first observed in the promoter region of chicken globin genes, [156] and later in the enhancer region of the human -globin gene. [157] In fact, all currently identified erythroid genes contain GATA sequences within their control regions. [155] As well, the core regions of the locus control region (LCR) of the human - and -globin gene clusters contain GATA sequences. [155] There exists a family of nuclear DNA-binding proteins that interact with GATA sequences. GATA-1 (also known as NF-E1, GF-1, eryf-1) was the first such protein identified and is an abundant nuclear protein in erythroid cells and megakaryocytes.[158] [159] GATA-1 is a 413-amino acid, 50-kd polypeptide containing two highly conserved zinc finger regions, which are both necessary and sufficient for DNA binding. In hematopoietic tissue, expression of GATA-1 is observed in the erythroid, megakaryocyte, and mast cell lineages. [158] GATA-2 is present in megakaryocytes and mast cells,[155] and GATA-3 expression is restricted to T cells, being found in the enhancer region of the T-cell receptor gene. [160] Interestingly, GATA-1 expression is detectable in CD34+, CD38+ bone marrow cells but not in the more primitive CD34+, CD38 cells, suggesting a role for GATA-1 in the function of more differentiated progenitor cells. [161]

GATA-binding proteins are observed in various tissues or cell lines, but the mere presence of a transcriptional factor does not define its role in lineage-specific gene expression or hematopoietic differentiation. [155] The function of such transcription factors is best examined in animals in which the gene of interest is rendered inoperative. This has been achieved for GATA-1 by Pevny et al., who disrupted the GATA-1 gene in embryonic stem (ES) cells by homologous recombination. [162] The resultant knock-out cells were analyzed both in vitro and in vivo. In vitro differentiation of GATA-1-deficient ES cells failed to generate erythroid cells. Confirming its role in erythropoiesis, the

inoperative gene also failed to contribute to hematopoiesis in null animals. GATA-1-deficient mice either died in utero or had severe anemia. Importantly, these animals sustained white blood cell production and generated clonogenic myeloid progenitor cells, [162] indicating that GATA-1 has a predominant role in erythroid differentiation.

A number of other megakaryocyte/platelet specific genes (thrombomodulin, PF4, platelet GPIIb, platelet GPIX) have GATA sequences in their promoter regions, [163] [164] [165] as do megakaryocytes and megakaryocyte cell lines.[155] A recent study indicated that transfection and 50-fold overexpression of GATA-1 in the murine myeloid cell line 416B restored some of its previously lost megakaryocyte potential. [166] Cells expressing high levels of GATA-1 (re)acquired some, but not all, of the appropriate megakaryocyte characteristics. For example, consistent with the presence of GATA sequences in its promoter, PF4 levels were increased, as was the level of acetylcholinesterase (a marker for megakaryocytes in certain rodents and cats). Likewise, a limited number (2040%) of GATA-transfected cells increased their cell size and volume. In most of these cells the volume increase was modest, but a small proportion (38%) became both large and polyploid. However, the degree of polyploidy appeared to be limited to 8C. [166] These studies suggest that GATA-1 is important to certain aspects of megakaryocyte differentiation, but its solitary overexpression (even in 50-fold excess) is not sufficient to drive full megakaryocyte differentiation. However, the importance of GATA-1 to megakaryocyte development is shown in GATA-1-null mice in which the gene is selectively lost in the megakaryocytic lineage. [167] GATA-1-null mice have marked thrombocytopenia, deregulated megakaryocyte proliferation, and deficient cytoplasmic maturation.

Another transcription factor, NF-E2, is important to megakaryocyte development. NF-E2 is an obligate heterodimer composed of a 45-kd subunit restricted to hematopoietic cells and a widely expressed 18-kd subunit. [110] The p45 subunit is co-expressed with GATA-1 in hematopoietic cells, megakaryocytes, and mast cells. Mice lacking p45 have a high mortality from bleeding secondary to profound thrombocytopenia. [110] Moreover, they show a relative megakaryocytic hyperplasia in which the mature cells undergo a cytoplasmic maturational arrest, lack granules, and show reduced amounts and an abnormal structure of the DMS. Interestingly, the few null animals surviving birth are thrombocytopenic but do not show the expected increase in TPO concentration. Rather, they have normal TPO levels. This apparent paradox was resolved by Shivdasani et al., who used radiolabeled TPO to demonstrate that the megakaryocytes in both the bone marrow and spleen (which are increased in number) as well as large platelet-like particles in the spleen bind TPO and thus regulate TPO concentration. [168] These data also confirm the aforementioned studies demonstrating a role for megakaryocyte mass in regulating TPO. Recently, the role of GATA-1 and NF-E2 in megakaryocyte development has become clearer. GATA-1 specifically interacts with a novel zinc-finger protein known as Friend of GATA (FOG). [169] The high degree of specificity in this interaction suggests that the function of DNA-bound GATA-1 is to recruit FOG (and other factors) for the assembly of cell-specific transcriptional complexes. It is therefore noteworthy that FOG is co-expressed with GATA-1 in megakaryocytic (and erythroid), but not other cells, and that GATA-1 and FOG synergize in activating the p45 NF-E2 gene.[169]

Megakaryocyte Cell Cycle Control and Endomitosis

Unlike other cells, megakaryocytes continue to synthesize DNA during differentiation. During this process megakaryocytes become polyploid, [170] [171] [172] having a DNA content of 8C128C, where 2C is the DNA content of a somatic cell. Megakaryocytes are not multinucleate cells but contain this increased DNA content within a single, albeit highly lobulated, nucleus. [173] [174] Ebbes and Feingendins groups, using tritiated thymidine incorporation, demonstrated that mature (stages II and III) megakaryocytes do not take up this label, thus showing that they are not undergoing DNA synthesis. [175] [176] State I megakaryocytes are the only recognizable cells capable of synthesizing DNA, but only 2040% of these cells do so. On prolonged exposure to tritiated thymidine, 100% of the megakaryocytes become labeled, indicating that the majority of DNA synthesis occurs in the immediate precursor of the megakaryoblast (i.e., PMkB). The cell cycle of megakaryocytes thus is different from that of other cells in that the normal 2C4C2C cell cycle progression is abolished. However, this release from normal cell cycle control does not imply that megakaryocyte DNA synthesis is dysregulated. The acquisition of a polyploid nucleus is tightly or globally controlled, as megakaryocytes show progressive doublings of their DNA content and no intermediate ploidy classes (e.g., 3C, 6C) are seen.

Two observations indicate that formation of a polyploid nucleus requires alterations in the megakaryocyte cell cycle. First, the immediate precursors of the stage I

megakaryocytes (i.e., PMkB) actively synthesize DNA for a prolonged period. [175] [176] Second, morphologic observations of primary cells show that megakaryoblasts do not go through the usual processes of mitosis, as few if any cells reach metaphase and none progress into anaphase or telophase. [173] As important as these historical observations are, they are based either on morphologic evidence or on the analysis of a limited number of megakaryocytes. Indeed, the term that is usually applied to this type of polyploidization, endomitosis, is a morphologic classification. The definition of endomitosis refers to the replication of nuclear elements within an intact nuclear envelope without subsequent chromosomal movement or cytokinesis.[177] This term is best used to describe megakaryocyte polyploidization.[173] Frequently the term endo-reduplication is erroneously applied to megakaryocytes. Endo-reduplication is the mechanism that results in polytenic (diplo- and quatro-chromosome number) cells in insects, and is a chromosome duplication cycle not associated with endomitotic-like changes. [177]

Given that the polyploid nature of megakaryocytes is unique among mammalian bone marrow cells, the question arises as to the biologic significance of this altered DNA content. A related question is whether or not polyploidization occurs as a prerequisite to or as a consequence of the increase in megakaryocyte cell volume occurring within these cells. It is known that megakaryocyte DNA content is related to megakaryocyte cell size and thus to the eventual numbers of platelets produced.[173] [178] A number of studies have documented the effect of thrombocytopenic demand on megakaryocytes. For example, acute thrombocytopenia results in an increased DNA content prior to increased platelet production. [173] Likewise, increases in cytoplasmic volume and cytoplasmic maturation occur predominantly, if not completely, in stages II and III megakaryocytes,[173] [178] which do not appear to synthesize DNA. Therefore, whatever its functional significance, polyploidization precedes the increase in megakaryocyte cell volume. This association of increased DNA content and increased cell volume implies that the large DNA content in megakaryocytes is in some unknown manner relevant to the process of platelet formation. For example, increased DNA content may be associated with increased mRNA expression, which in turn drives the marked degree of biosynthesis required for platelet formation. Although this remains to be proved, it is clear that megakaryocytes synthesize increased amounts of DNA prior to increases in cytoplasmic volume and cytoplasmic maturation.

It is now recognized that two classes or families of proteins control the cell cycle in mammalian cells. These are the cell division kinases (also known as cyclin-dependent kinases, CDK),

and the cyclins, so named for their cyclical synthesis and degradation. Together, these two classes of proteins form a protein-kinase complex in which the catalytic unit is a CDK and the regulatory unit is a cyclin. These proteins are highly conserved, [179] [180] and different CDK complexes regulate cell cycle progression. The role of these kinase complexes in cell cycle control is complex. Currently, seven members of the cyclin gene family are known, [181] [182] as well as at least seven distinct CDK genes. [183] The role of the kinase known as cdc2 and its cognate cyclin (B) is best understood. Together, these proteins form a mitosis-initiating cdc2 kinase complex that is also known as maturation (or mitosis)-promoting factor. The cdc2-cyclin B kinase complex regulates the initiation of mitosis at the G_2/M transition as well as subsequent events such as spindle fiber formation and cytokinesis. [181] Among the CDK proteins, cdk2 plays an important role in regulating both G_1/S transit and S-phase progression. A number of regulatory cyclins are complexed with cdk2: cyclin E plays a role in G_1 progression and in G_1/S transit; cyclin A is essential for both S phase and the initiation of DNA replication. [184] [185] [186] [187] [188] The cdk2-cyclin E complex is assembled in mid-G_1 and its associated kinase activity peaks in late G_1 and early S phase, whereas cdk2-cyclin A activity is maximal in S phase. [184] [189] Another family of cyclins, the G_1 cyclins (D-type cyclins in mammals and the analogous Cln regulators in yeast), together with cdk4 or cdk6, are important in timing G_1 progression, as well as in G_1/S transit.[190] The D-cyclins are partially cell-type specific, and most cells express cyclin D_3 and either D_1 or D_2 .[191] Interestingly, the D-type cyclins also bind with cdk2. [192]

The precise role of CDK and cyclin proteins in megakaryocyte endomitosis is unknown. Nonetheless, a general hypothesis can be put forward concerning the biochemical control of the endomitotic cell cycle. That is that mitosis must be abrogated to retain an increased DNA content within a single nucleus, and that S phase must be modified to prevent (or weaken) its interdependency with the M phase. Not too surprisingly, megakaryocytes show demonstrable alterations at the two control (or restriction) points evident in all cycling cells. They have a prolonged S-phase period and synthesize increased amounts of DNA (i.e., they are altered in G_1 or S phase), and they undergo an abrogation of mitosis (M phase). Interestingly, in normal cells, the cdc2-cyclin B complex kinase activity peaks in early metaphase. [193] This is just the point at which megakaryocytes fail to progress through mitosis. Moreover, recent observations demonstrate that stabilizing mutations of the cyclin B gene (e.g., the loss of its N-terminal domain) results in persistence of this protein, and its sustained presence leads to mitotic arrest. [194] [195]

As mentioned, megakaryocytes are unique in that they leave the diploid (2C) state to differentiate, synthesizing 464 times the normal DNA content within a single nucleus, a process known as endomitosis. Human erythroleukemia (HEL) cells model this process, becoming megakaryocytic during phorbol diester-induced megakaryocyte differentiation.[36] Datta and co-workers have shown that the mitotic arrest occurring in these polyploid cells involves novel biochemical alterations in the cdc2-cyclin B1 complex: a marked reduction in cdc2 protein levels, and an elevated and sustained expression of cyclin B1. [196] As a result, endomitotic cells lack cdc2-cyclin B1-associated H1-histone kinase activity. Constitutive overexpression of cdc2 in endomitotic cells failed to reinitiate normal mitotic events, even though cdc2 was present in 10-fold excess. This was due to an inability of cyclin-B1 to physically associate with cdc2. Thus, during megakaryocytic differentiation of HEL cells, mitosis is abrogated during endomitosis owing to the absence of cdc2 and the failure to form active cdc2-cyclin B kinase complexes, resulting in a disassociation of mitosis from the completion of S phase. Similar results were reported by Zhang and co-workers, who demonstrated a decreased cdc2-cyclin B kinase activity in polyploid megakaryocytes.[197] Consistent with this, Garcia and Cales also demonstrated a lack of cdc2/cyclin B kinase activity in phorbol-stimulated megakaryocytic cell lines (HEL and MEG-01). [198] These data are consistent with studies showing that a loss of the cdc2-cyclin B complex in yeast results in multiple S phases, [199] that cyclin B is absent during multiple rounds of S phase in *Drosophila* embryos,[200] and that endoreduplication in maize endosperm involves both an increase in the amount and activity of S-phase-related protein kinases and active inhibition of cdc2-cyclin B activity. [201]

Polyploid HEL cells also are a useful model for examining endomitotic S-phase control, as their cell cycle machinery must be modulated in order to allow the acquisition of high levels of DNA content (ploidy) within a single nucleus and to allow the re-replication of newly synthesized DNA without an intervening mitosis. In order to evaluate the mechanisms of S-phase control during the process of polyploidization, Datta and Long also investigated the modulations occurring in CDK complexes during the induction of megakaryocyte differentiation in human HEL cells. During polyploidization, megakaryocytic HEL cells undergo a dramatic modulation in the subunit composition of G_1 - and S-phase-associated CDK complexes and an increase in their specific activities. In particular, cyclin D_3 protein levels are increased (owing to both a change in D_3 mRNA levels and a stabilization of its half-life), and there is a marked increase in cdk2-cyclin D_3 kinase activity throughout the period of endomitosis. [202] Moreover, these changes occur within the context of an up-regulated function of cdk2-cyclin E complexes that is associated with both G_1/S-phase transit and S-phase progression. [202] The cyclin D observations are consistent with studies by Wang et al. [203] showing that antisense cyclin D3 oligonucleotides abrogated megakaryocyte development (although ploidy of the megakaryocytes was not examined). Further studies regarding the regulation of these complex CDK interactions will be important to understanding the cell cycle control in such diverse processes as megakaryocyte differentiation or the types of genomic instability occurring in cancer cells. However, it should be noted that data derived from the study of cell lines may not exactly reflect the biochemical changes occurring in primary megakaryocytes. Recent preliminary observations (K. Kaushansky and W. Vainchenker, pers. commun.) indicate that megakaryocyte development (cytokine driven) in vitro differs somewhat from that of megakaryocytic cell lines: the mitotic arrest in cultured megakaryocytes seems to happen nearer to or following the anaphase. A paper by Vainchenker and co-workers demonstrates the similarities and differences between the cell line data and that of megakaryocytes produced ex vivo. Thus, HEL cells and cultured developing megakaryocytes both show an increase in cyclin B1 levels (in megakaryocytes only up to 8C cells, after which levels are sustained), as well as a cyclin B/associated H1 histone kinase activity. [196] [204] In contrast, cultured megakaryocytes do express cdc2, although their physical association with cyclin B or kinase activity was not tested. Nonetheless, this excellent paper clearly demonstrates that endomitosis of human megakaryocytes is due to abortive mitosis and that this is associated with nuclear envelope breakdown and the formation of complex mitotic spindles. It is clear that a better understanding of endomitotic events requires further analysis of the cell line data, data from cultured megakaryocytes, and, eventually, data from purified populations of primary megakaryocytes.

CONCLUSIONS AND PERSPECTIVE

Our increased understanding of the cellular and molecular basis of megakaryocytopoiesis has had an immediate impact on clinical medicine. There are numerous dyscrasias of megakaryocytopoiesis that result in abnormal platelet production, such as amegakaryocytic thrombocytopenia, thrombocytopenia with absent radii, and primary thrombocythemia. Although the molecular and biochemical basis of these disorders often remains

unknown, a number of preliminary studies have documented alterations in the frequency of megakaryocyte progenitor cells in these conditions. [205] [206] [207] [208] As well, amegakaryocytic thrombocytopenia is associated with increased levels of circulating regulatory factors, [62] [64] and familial forms of primary thrombocythemia have been described in which patients have increased megakaryocytes and thrombocytosis attributed to increased TPO production. [210] The marrow fibrosis that characterizes agnogenic myeloid metaplasia (myelofibrosis) is ascribed to mitogenic factors, released from abnormal mega-karyocytes, that stimulate fibroblast proliferation and ECM deposition.[211] Thrombocytopenias are associated with either high or normal TPO levels, depending on the remaining megakaryocyte mass. [108] [110] [168]

In order to effectively control platelet production, both normal and abnormal megakaryocyte development must be examined at the cellular and molecular levels. This requires that the interacting ECM-cytokine complexes that regulate platelet production be defined. Similarly, the megakaryocytic signal transduction pathways activated by these thrombopoietic signals must be understood. Ultimately, understanding megakaryocyte development will require identifying those genes that regulate megakaryocyte commitment and differentiation. This requirement notwithstanding, current clinical trials of IL-11 and of TPO have resulted in the development of effective strategies for the therapeutic management of the thrombocytopopenias.

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Chapter 18 - Inherited Forms of Bone Marrow Failure

Melvin H. Freedman

INTRODUCTION

In the context of this chapter, bone marrow failure is defined as decreased production of one or more of the major hematopoietic lineages on an inherited basis (Table 18-1). The term constitutional is often used interchangeably with inherited and similarly implies that a genetic abnormality causes bone marrow dysfunction, especially when it occurs in two or more members of the same family or is associated with congenital physical abnormalities. The designation congenital has a looser connotation and refers to conditions that manifest early in life, often at birth, but does not connote a particular causation. Congenital marrow failure is not necessarily inherited or constitutional and may be caused by acquired factors such as viruses and environmental toxins.

Because hematopoiesis is an orderly but complex interplay of stem and progenitor cells, marrow stromal elements, and positive and negative cellular and humoral regulators, marrow failure can potentially occur at a number of critical points in the hematopoietic lineage pathways. For the inherited marrow disorders, current theory holds that genetic mutations interfere with hematopoiesis and cause the marrow failure, although the specific molecular basis is not yet known for any of these conditions. Acquired factors may also be operative and may interact with the putative genetic mutations to produce overt disease with varying clinical expression. Hence, the conditions shown in Table 18-1 can be transmitted as a simple Mendelian disorder determined primarily by a single mutant gene with inheritance patterns of autosomal dominant, autosomal recessive, or x-linked types. Alternatively, some or all of these can be multifactorial disorders caused by an interaction of multiple genes and multiple exogenous or environmental determinants.

The incidence of the inherited marrow failure disorders can

TABLE 18-1 -- Inherited Bone Marrow Failure Syndromes

Pancytopenia
Fanconi's anemia
Shwachman-Diamond syndrome
Dyskeratosis congenita
Amegakaryocytic thrombocytopenia
Other genetic syndromes
Down's syndrome
Dubowitz's syndrome
Seckel's syndrome
Reticular dysgenesis
Familial aplastic anemia (non-Fanconi's)
Unilineage Cytopenia
Diamond-Blackfan anemia
Kostmann's syndrome/Congenital neutropenia
Thrombocytopenia with absent radii
Congenital dyserythropoietic anemias
Types I, II, III
Variants

be roughly approximated from careful clinical observations compiled at large centers. For example, data from the Children's Hospital Medical Center, Boston, and the Prince of Wales Hospital, Australia, show that these syndromes comprise 3035% of cases of pediatric marrow failure, respectively, and Fanconi's anemia represents about two-thirds of the total.[1] [2]

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PANCYTOPENIA

Fanconi's (Aplastic) Anemia

Introduction

This classical marrow failure disorder is inherited in an autosomal recessive manner with a heterozygote frequency of about 1 in 200 and is remarkable for its diversity in phenotype. It occurs in all racial and ethnic groups. Although the original report in 1927 by Professor Fanconi described pancytopenia combined with physical anomalies in three brothers, a summary of the large body of published information on approximately 1,000 cases [3] over the ensuing 70 years has underscored the clinical variability of the condition. [4] At presentation patients may have: (a) typical physical anomalies but normal hematology; (b) normal physical features but abnormal hematology; or (c) physical anomalies and abnormal hematology, the so-called classic phenotype that fits the original description. There can also be sibling heterogeneity in presentation with discordance in clinical and hematological findings, even in affected monozygotic twins. All patients show abnormal chromosome fragility, most easily seen in metaphase preparations of peripheral blood lymphocytes cultured with phytohemagglutinin and enhanced by adding clastogenic agents (see Laboratory Evaluation).

About 75% of patients are between 3 and 14 years of age at the time of diagnosis of Fanconi's anemia, with a mean of about 8 years in males and almost 9 years in females.[4] It is noteworthy that 4% are diagnosed in the first year of life and 10% at 16 years of age or older.

Biologic and Molecular Aspects

A breakthrough in the search for Fanconi's anemia genes evolved from the important observation that hybrid cells formed from Fanconi's anemia and normal cells resulted in correction of the abnormal chromosome fragility, a process known as complementation. [5] [6] [7] [8] [9] It was further demonstrated that cell fusion studies from several unrelated Fanconi's patients could also produce the corrective effect on chromosomal fragility by complementation. [7] [8] [9] Currently, eight complementation groups and hence eight separate genes (termed types A, B, C, D, E, F, G, and H) have been distinguished on the basis of these somatic cell hybridization experiments,[6] [10] [11] with Fanconi's anemia type A (FA-A) accounting for over 65% of the cases analyzed. [12] A cDNA for the group C gene was identified in 1992 and localized to chromosome 9q22.3.[13] Using the same successful expression cloning method, a cDNA representing the group A gene on chromosome 16q24.3 has also been reported.[14] Even though the precise functions of the FA-C and FA-A proteins are unclear so far, complementation analysis suggests that they operate in concert with at least three to six additional proteinsthe products from the other Fanconi genes. Although FA-D has not been cloned yet, genetic mapping localizes it to 3p22-26. The FA-A protein localizes primarily to the cell nucleus, whereas the FA-C gene product is located in both the nucleus and cytoplasm. All of the FA proteins may thus control or participate in a conceptually novel biochemical process which must play an important role in the various physiological and cellular processes implicated in the Fanconi phenotype, such as skeletal development, blood cell formation, and maintenance of genomic integrity. Cloning the remaining genes should help researchers to eventually understand this process at the molecular level.

A knockout mouse model of FA-C has provided some insight into the role of that gene. [15] When mice were generated with targeted inactivation of the FA-C gene, the nullizygous genotype did not exhibit developmental abnormalities or gross hematologic defects for up to 9 months. Spleen cells from the knockout mice had increased numbers of chromosomal aberrations in response to two agents that enhance chromosome fragility, mitomycin C and diepoxybutane (DEB). Homozygous male and female mice also had compromised gametogenesis and markedly impaired fertility, mimicking some of the features of the human disease. Although the mice did not exhibit gross hematologic defects, FA-C nullizygous mouse hematopoietic progenitors were markedly sensitive to -interferon, a cytokine implicated in growth arrest and apoptosis. When mitomycin C was administered to homozygous mice they developed either pancytopenia or total bone marrow failure, depending on the dose of the drug.[16]

Further information regarding the importance of FA-C in hematopoiesis was provided by experiments in which normal human lymphocytes and bone marrow cells were exposed to an antisense oligodeoxynucleotide (ODN) complementary to bases 4 to +14 of FA-C mRNA.[17] The mitomycin C assay showed that the antisense ODN repressed FA-C gene expression in lymphocytes. Escalating doses of antisense ODN increasingly inhibited clonal growth of erythroid and granulocyte-macrophage progenitors, thereby demonstrating a seemingly important role for the gene in sustaining normal hematopoiesis.

A recent study [18] examined the phenotypic consequences of mutations of FA-C in patients. Kaplan-Meier analysis showed that IVS4 or exon 14 mutations define poor risk subgroups clinically, as they are associated with earlier onset of hematologic abnormalities and poorer survival compared to patients with the exon 1 mutation and to the non-FA-C population. The molecular biology of Fanconi's anemia and the implications for diagnosis and therapy have recently been reviewed. [19]

Etiology and Pathogenesis

The two theories of pathogenesis of Fanconi's anemia relate to either defective DNA repair or an inability of Fanconi's cells to remove oxygen-free radicals that damage cells. Despite an extensive body of published data, neither theory has prevailed. The strongest evidence supporting an oxygen metabolism deficiency is the G_2 phase cell cycle defect of Fanconi's cells, consisting of both a phase transit delay and/or a complete arrest. [20] This effect is reduced when Fanconi's cells are grown at low

oxygen levels, and a similar defect can be induced in normal cells grown at high oxygen levels. [21] [22] This phenotype cannot be induced by treatment with DNA cross-linking agents, suggesting that it is not related to a DNA repair defect.

The best evidence supporting the theory that the primary defect is in DNA repair comes from experiments in which the frequency of mutations induced by 8-methoxy psoralen (8-MOP) plus near ultraviolet light (320-360 mm) (UVA) at the hypoxanthine phosphoribosyl transferase and $Na^+ + K^+$ -ATPase loci was lower in FA-A and FA-D cells than in controls.[23] [24] These results could indicate that Fanconi's cells cannot repair cross-links through the normal pathway involving either mismatch repair or recombinational repair following bypass of the lesion, or both. Similar studies that assayed the repair of cross-linked herpes simplex virus DNA following transfection found that Fanconi's cells, unlike controls, were able to repair cross-linked DNA only under conditions of multiple infection. [25] Since multiplicity reactivation is dependent on recombinational repair, it can be inferred that Fanconi's cells are defective in an excision repair pathway.

To reconcile the evidence of defective oxygen metabolism with the data demonstrating the involvement of the DNA repair system in the pathology of Fanconi's cells, it may be possible that in Fanconi's anemia a set of proteins, closely involved in DNA repair, is particularly sensitive to oxidative damage. The Fanconi's anemia

mutation could either make the repair pathway sensitive to oxidative damage or cause a transient increase of oxidative damage to which the repair machinery is particularly sensitive.

The bone marrow dysfunction in Fanconi's anemia is evident at the hematopoietic progenitor level in bone marrow and peripheral blood. The frequencies of CFU-E, BFU-E and CFU-GM colony-forming cells are reduced fairly consistently in almost all patients after aplastic anemia ensues, [26] [27] [28] [29] [30] [31] [32] [33] [34] as well as in a few patients before the onset of aplastic anemia.[35] Although marrow cells from Fanconi's patients show normal transcripts for the -and -chains of the GM-CSF/IL-3 receptor and for c-kit protein, there is a deficient growth response in the majority of patients of CFU-GEMM, BFU-E and CFU-GM progenitors to GM-CSF plus stem cell factor (c-kit ligand) or IL-3 plus stem cell factor, a finding not seen in controls. [36] Because all hematopoietic lineages are affected in these studies, the basic defect probably involves the pluripotent stem cell compartment. Confirmatory data for this hypothesis using long-term bone marrow cultures were reported by one group [37] but contradicted by another.[38] Decreased colony numbers in these studies can be interpreted as the result of an absolute decrease in progenitors, or a result of adequate numbers of progenitors that have faulty proliferative properties and cannot form colonies in vitro. Both interpretations may be true.

Cytokine studies in Fanconi's patients have shown varied abnormalities. Although Fanconi's fibroblasts showed no deficiencies in stem cell factor or M-CSF production, variability ranging from diminished production to augmentation of production of IL-6, GM-CSF and G-CSF was seen in different patients. [39] A fairly consistent finding that may relate directly to pathogenesis is diminished IL-6 production in Fanconi's patients and markedly heightened abnormal TNF- generation. [40]

Clinical Manifestations

The presence of characteristic congenital physical anomalies alone or in combination in the setting of bone marrow failure makes a diagnosis of Fanconi's anemia straightforward (Fig. 18-1). It is important to note, however, that Fanconi's anemia patients may lack anomalies. The older historical terms Estren-Dameshek aplastic anemia[41] and constitutional aplastic anemia type II [42] referred to such patients. With the introduction of clastogenic stress-induced chromosomal breakage analysis as a confirmatory test for Fanconi's anemia, data from the International Fanconi's Anemia Registry (IFAR) showed that 39% of 202 patients tested had aplastic anemia and anomalies, but 30% had aplastic anemia only and were without anomalies.[43] Twenty-four percent had anomalies only and 7% had neither. Hence, the Estren-Dameshek cases that lack anomalies should not be considered as a separate entity but as part of the pleiotropic continuum of Fanconi's anemia.

Table 18-2 shows the categories of characteristic physical abnormalities and their approximate frequency. The most common anomaly is skin hyperpigmentation, a generalized brown melanin-like splattering, which is most prominent on the trunk, neck, and intertriginous areas and which becomes more obvious

Figure 18-1 Classical phenotype of Fanconi's anemia. Patient has pigmentary changes around the neck, shoulders and trunk, short stature, absent radii and absent thumbs bilaterally, microcephaly, and low-set ears.

TABLE 18-2 -- Characteristic Physical Anomalies in Fanconi's Anemia

Anomaly	Approximate Frequency (%)
Skin pigment changes	65
Short stature	60
Upper limb abnormalities (thumbs, hands, radii, ulnae)	50
Hypogonadal and genitalia changes (mostly male)	40
Other skeletal findings (head/face, neck, spine)	30
Eyes/lids/epicanthal fold anomalies	25
Renal malformations	25
Ear anomalies (external and internal), deafness	10
Hips, legs, feet, toes abnormalities	10
Gastrointestinal/cardiopulmonary malformations	10

Modified from data compiled by Dr. Blanche Alter, with permission.[4]

with age. Café-au-lait spots are common alone or in combination with the generalized hyperpigmentation, and sometimes with vitiligo or hypopigmentation. The skin pigmentation should not be confused with hemosiderosis-induced bronzing in transfusion-dependent patients who have not been adequately chelated.

The majority of patients are small and have short stature. As summarized by Alter, of 19 published cases who had growth hormone assays performed, 14 showed a deficiency, but only about half of those treated with growth hormone showed a growth response. [4]

Malformations involving the upper limbs are common, especially hypoplastic, supernumerary, bifid, or absent thumbs. Hypoplastic or absent radii are always associated with hypoplastic or absent thumbs in contrast to the thrombocytopenia with absent radii (TAR) syndrome in which thumbs are always present. Less often, anomalies of the feet are seen including toe syndactyly, short toes, a supernumerary toe, clubfoot, and flat feet. Congenital hip dislocation and leg abnormalities are occasionally seen.

Males often have gonadal and genital abnormalities including an underdeveloped penis or micropenis, undescended, atrophic, or absent testes, hypospadias, phimosis, and an abnormal urethra. Female patients occasionally have malformations or atresia of the vagina, uterus, and ovary.

Many patients have a Fanconi facies and unrelated patients can resemble each other almost as closely as siblings. The head and facial changes vary but commonly consist of microcephaly, small eyes, epicanthal folds, and abnormal shape, size or positioning of the ears. About 10% of Fanconi's patients are mentally retarded.

Renal anomalies occur but require imaging for documentation. Ectopic, pelvic, or horseshoe kidneys are detected often, as well as duplicated, hypoplastic, dysplastic, or absent organs. Occasionally, hydronephrosis or hydroureter is present.

Auerbach et al. developed a scoring system[43] for the probability of an accurate diagnosis of Fanconi's anemia using discriminating clinical and laboratory variables in patients enrolled in the IFAR whose diagnosis was confirmed by clastogenic stress-induced chromosomal breakage analysis. The variables used for scoring are shown in Table 18-3A and the scoring system in Table 18-3B . One point is added or subtracted for each variable with a positive or negative coefficient, respectively. A patient with a score of 4+ or greater has a 98% probability of having Fanconi's anemia.

Laboratory Evaluation

A cardinal feature is the gradual onset of bone marrow failure with declining values in one or more hematopoietic lineages. Thrombocytopenia usually develops initially, with subsequent onset of granulocytopenia and then anemia. Severe aplasia eventually develops in most cases but the full expression of pancytopenia is variable and ensues over a period of months to years. The development of aplastic anemia can be accelerated by intercurrent infections or by drugs such as chloramphenicol. Within families, there is a tendency for the hematologic changes to occur at about the same age in affected siblings. [44] Data from 388 patients reported to the IFAR showed that 332 developed hematologic abnormalities at a median of 7 years (range: birth31 years). [45] The actuarial risk of developing hematologic abnormalities rose progressively with age and was 98% by 40 years.

TABLE 18-3A -- Discriminating Clinical and Laboratory Variables in Fanconi's Anemia

Variable	Frequency (%)		Coefficient	Contribution
	FA	Non-FA		
Growth retardation	60.9	24.1	0.70	38.47
Skin pigmentation	58.4	22.2	0.78	27.09
Kidney, urinary	35.1	9.3	0.71	17.56
Thumb, radius	51.0	21.3	0.85	15.24
Microphthalmia	44.1	13.9	0.81	12.80
Learning disability	15.8	18.5	0.66	11.62
Other skeletal	30.2	26.9	0.57	8.47
Thrombocytopenia	80.2	61.1	0.73	7.43

TABLE 18-3B -- Probability of Having Fanconi's Anemia Based on Simplified Score

Simplified Score	FA (N)	Non-FA (N)	Probability
1	0	5	0.00
0	5	20	0.20
1	27	59	0.31
2	53	18	0.75
3	59	5	0.92
4	58	1	0.98
Total	202	108	

Modified from Auerbach et al.,[43] with permission.

The red cells are macrocytic with mean cell volumes often above 100 fl even before the onset of significant anemia. Erythropoiesis is characterized by increased Hb F and increased expression of i antigen, but not necessarily both features in individual cells. The increased Hb F production is not clonal and has a heterogeneous distribution. Ferrokinetic studies indicate that most patients have an element of ineffective erythropoiesis as part of the marrow failure. Red blood cell (RBC) lifespan may be slightly shortened but this is a minor component, if any, of the anemia.

In the early stages of the disease, the bone marrow can show erythroid hyperplasia, sometimes with dyserythropoiesis and even megaloblastic-appearing cells. As the disease progresses, the marrow becomes hypocellular and fatty, sometimes patchily, and shows a relative increase in lymphocytes, plasma cells, reticulum cells, and mast cells. With full-blown marrow failure, the morphology on biopsy is identical to that seen in severe acquired aplastic anemia.

A major finding is abnormal chromosome fragility, seen readily in metaphase preparations of peripheral blood lymphocytes cultured with phytohemagglutinin. The karyotype shows spontaneously occurring chromatid breaks, rearrangements, gaps, endoreduplications and chromatid exchanges in cells from homozygote Fanconi's anemia patients. Cultured skin fibroblasts also show the abnormal karyotype underscoring the constitutional basis for the disorder. The abnormal lymphocyte chromosome pattern, the number of breaks per cell, and the variations in proportion of abnormal cells have no direct correlation with the hematological or clinical course of individual patients.

Spontaneous chromosomal breaks, as described, are occasionally absent in true cases of homozygote Fanconi's anemia. [43] The clastogenic stress-induced chromosomal breakage analysis was introduced, in part, to circumvent this problem and to bring specificity to testing for Fanconi's anemia. Chromosomal breakage is strikingly enhanced compared with controls if clastogenic agents such as DEB are added to the cultures. [46] Indeed, homozygote Fanconi cells are hypersensitive to many oncogenic and mutagenic inducers, such as ionizing radiation, SV40 viral transformation, and alkylating and chemical agents including mitomycin C, cyclophosphamide, nitrogen mustard, and platinum compounds.[4] For definitive diagnostic purposes, the IFAR has defined Fanconi's anemia as increased numbers of chromosome breaks/cell after exposure to DEB[43] [47] with a mean of 8.96 (range: 1.323.9) compared to normal controls of 0.06 (range: 00.36).

The abnormal chromosome picture can be used to make a prenatal diagnosis of Fanconi's anemia. [47] [48] Diagnostic testing can be performed on fetal amniotic fluid cells obtained at 16 weeks' gestation, or earlier on chorionic villus biopsy specimens at 912 weeks. A very high degree of prenatal diagnostic accuracy has been obtained by looking at both spontaneous and DEB-induced breaks. [47] [48]

Predisposition to Malignancy

A major feature of the Fanconi's anemia phenotype is the propensity for cancer. The karyotype data, the defects in DNA repair, and the cellular damage that occur in Fanconi's patients translate into an enormous predisposition for malignancy. More than 80 of 1,000 published patients have been reported with leukemia, about 30 with liver tumors, and 47 with other cancers, giving an overall incidence of malignant transformation of about 20%. [3] [49] Fanconi patients may also develop myelodysplastic syndromes (MDS). These are traditionally defined as clonal refractory cytopenias with characteristic dysplastic changes in marrow cells and a propensity to evolve into AML. Some patients also have clonal cytogenetic findings in marrow cells without overt MDS/AML. The clonal findings of deletions, translocations, and marker chromosomes often involve chromosomes 1 and 7.

To determine the risk of malignant myeloid transformation in Fanconi's anemia patients, Butturini et al. used the observational database of the IFAR in which most patients' diagnoses were confirmed by DEB testing. [45] They defined MDS as 5% to 30% myeloblasts in marrow or 5% to 20% myeloblasts in blood. AML was defined as more than 30% marrow blasts or more than 20% blasts in blood. Marrow dysplastic morphology was not used as a criterion. Of 332 patients in the IFAR who developed varying hematologic abnormalities, 59 subjects (18%) developed MDS or AML with a median interval of observation prior to the transformation of 13 years (1 month to 32 years). Using the authors' strict disease definitions, 34 patients had MDS and 25 had AML. It is noteworthy that 20 of the 59 patients initially presented at diagnosis with established MDS or AML and the diagnosis of Fanconi's anemia was made secondarily. Using the same IFAR data, the actuarial risk of MDS or AML developing over time could be determined. At 5, 10, 20, and 40 years of age, the probability of malignant transformation escalates from less than 5%, to approximately 8%, 25%, and 52%, respectively. The risk of MDS/AML was higher for patients in whom a prior clonal marrow cytogenetic abnormality had been detected. Loss of chromosome 7 (monosomy 7) or rearrangement or partial loss of 7q, rearrangements of 1p36 and 1q2434, and rearrangements of 11q2225 were the most frequently recurring cytogenetic changes.

Alter has compiled published data on 42 patients who developed one or more malignancies other than leukemia or liver tumors. [4] Almost all patients were at least 10 years old when the tumors presented; the average age was 23 years. Most of the tumors were squamous cell carcinomas involving the gastrointestinal tract at any site from the oropharynx to the anorectalcolonic area. Less frequently, gynecologic malignancies were also described, with primaries arising in vulva, cervix, and breast. Unusual combinations were occasionally described, such as Wilm's tumor with medulloblastoma, cancer of the vulva and of the tongue, hepatic carcinoma and cancer of the tongue, and hepatic carcinoma and esophageal carcinoma.

Liver tumors, benign and malignant, as well as peliosis hepatis consisting of blood-filled empty spaces in the liver, occur with increased frequency in Fanconi's anemia.[4] The most common tumors reported were hepatocellular carcinoma followed by hepatomas and adenomas. Because almost all patients reported in the

literature were taking androgen therapy at the time that the liver disease presented, androgens have been implicated in pathogenesis. Indeed, peliosis hepatis is reversible when androgens are stopped, and in three patients with tumors, discontinuation of androgens alone or coupled with bone marrow transplantation effected a regression of the tumors.

Differential Diagnosis

Patients with abnormal hematology and characteristic physical anomalies are not difficult to diagnose, especially if there are previously affected siblings. Distinguishing Fanconi's anemia from acquired aplastic anemia on clinical grounds can be difficult if the Fanconi's patient lacks physical anomalies. In this situation, the clastogenic stress-induced chromosomal breakage analysis using DEB will specifically identify Fanconi's anemia and lead to the correct diagnosis.

Although neutropenia is the consistent feature of Shwachman-Diamond syndrome, anemia and/or thrombocytopenia is seen in more than half the patients and can be confused with Fanconi's anemia. Since growth failure is also a manifestation of Shwachman-Diamond syndrome, differentiating between the two disorders can initially be difficult. The major difference between them is that Shwachman-Diamond syndrome is a disorder of exocrine pancreatic dysfunction which produces gut malabsorption.

This can be confirmed by fecal fat analysis and by pancreatic function studies using intravenous secretin and cholecystokinin, which confirm markedly impaired enzyme secretion. Computed tomography of the pancreas may also demonstrate fatty changes within the body of the pancreas. Other skeletal distinguishing features found in some Shwachman-Diamond patients are short flared ribs, thoracic dystrophy at birth, and metaphyseal dysostosis of the long bones. Chromosomes are normal in Shwachman-Diamond syndrome and no increased breakage is seen after clastogenic stress.

Dyskeratosis congenita shares some features with Fanconi's anemia including development of pancytopenia, a predisposition to cancer, and skin pigmentary changes. However, the pigmentation pattern is somewhat different in dyskeratosis congenita and manifests with a lacy reticulated pattern affecting the face, neck, chest, and arms, often with a telangiectatic component. At some point, usually in the first decade, dyskeratosis congenita patients also develop dystrophic nails of the hands and feet, and, somewhat later, leukoplakia involving the oral mucosa especially the tongue. Other findings, seen only in dyskeratosis congenita and not in Fanconi's anemia are teeth abnormalities with dental decay and early tooth loss, hair loss, and hyperhydrosis of the palms and soles. Although there are contradictory data regarding chromosomal fragility in dyskeratosis congenita, DEB testing has not shown any difference between patients and controls, which contrasts sharply with Fanconi's patients.

Amegakaryocytic thrombocytopenia and TAR syndrome both present in the neonatal period with an isolated decrease of platelets. A neonatal hematologic presentation is atypical for Fanconi's anemia since less than 5% of patients are diagnosed in the first year of life. Neither of the thrombocytopenic syndromes show chromosome fragility, which readily separates them from Fanconi's anemia. In the TAR syndrome, thumbs are always preserved and intact despite the absence of radii, whereas in Fanconi's anemia the thumbs are hypoplastic or absent when the radii are absent.

Therapy and Prognosis

Because of their clinical complexity, Fanconi's anemia patients should be supervised at a tertiary care center using a comprehensive and multidisciplinary approach. On the initial visit, the following should be performed: a careful physical examination with emphasis on physical anomalies; complete blood counts and chemistries, diepoxybutane (and/or mitomycin C) chromosome fragility testing on peripheral blood lymphocytes on patients and siblings; and, HLA tissue typing on patient and family members. Arrangements should then be made for diagnostic studies to search for any internal anomalies that are described herein. When all of the results are catalogued, a follow-up visit with the family is arranged to discuss management options and prognosis.

It is important to emphasize that the prognosis for patients with Fanconi's anemia is improving. Older literature describing an early demise of Fanconi's patients is flawed because it did not take into account the diversity in clinical and hematological phenotype. A diagnosis can now be made before the onset of serious marrow failure or malignant transformation and survival from the time of diagnosis is longer. Also, the newer forms of management, especially marrow transplantation, have dramatically changed prognosis. Data from the IFAR for the 1980s indicate that Fanconi's patients have a median survival of 25 years of age. [50] Indeed, older female Fanconi's patients can be sexually active; at least 17 patients have become pregnant, resulting in 19 births and 18 surviving children. [51]

If the patient is stable and has only minimal to moderate hematological changes and does not have transfusion requirements, a period of observation is indicated. Blood counts should be monitored every 13 months and bone marrow aspirates and biopsies should be performed annually for morphology and cancer cytogenetics to identify the emergence of a malignant clone or overt transformation to MDS/AML. Depending on the types of congenital anomalies, subspecialty consultations, for example with orthopedic surgery, can be arranged during this interval.

Bone Marrow Transplantation (BMT)

Bone marrow transplantation is currently the only curative therapy for the hematological abnormalities of Fanconi's anemia, and the best donor source is an HLA-matched sibling. Initial efforts to transplant Fanconi's patients using standard preparative regimens and graft-versus-host prophylaxis were plagued by two serious and often lethal problems: severe cytotoxicity to chemotherapy, and exaggerated graft-versus-host disease. BMT protocols were subsequently modified for Fanconi's patients and the outcomes improved substantially.

Gluckman et al. analyzed the data of HLA-identical sibling BMT performed on 151 Fanconi's patients from 42 institutions. [52] The two-year survival rate was 66%. Factors associated with a favorable outcome were younger patient age, higher pre-BMT platelet count, use of antithymocyte globulin, and use of low-dose cyclophosphamide (1525 mg/kg) with limited field irradiation for pre-BMT conditioning, and cyclosporine for graft-versus-host disease prophylaxis.

At the Children's Hospital Medical Center in Cincinnati, 30 Fanconi's patients received matched sibling donor BMT, and the survival rate with normal blood counts was about 85%, with a median follow-up of over three years. [53] The successful Cincinnati protocol is comprised of cyclophosphamide 5 mg/kg/day for four days before transplantation (days 5 to 2), followed by thoracoabdominal irradiation 400 cGy (day 1), with ATG (40 mg/kg days 6, 4, 2, and 20 mg/kg days +2, +4, +6, +8, +10, +12), and cyclosporine for six months after transplant for graft-versus-host disease prophylaxis. If a cytogenetic clone or MDS/AML is identified before BMT, the preparative therapy is escalated to cyclophosphamide 10 mg/kg/day for four days followed by total body irradiation 450 cGy. Methylprednisolone is also added post-BMT, 1 mg/kg/day for 33 days.

Excellent results can also be achieved with conditioning regimens that use a reduced dosage of cyclophosphamide but omit radiation and antithymocyte globulin. [54] The Kaplan-Meier survival estimate with this Seattle protocol was 89% with a median follow-up of 285 days.

Despite the clear-cut success in correcting the marrow failure of Fanconi's anemia with BMT, data from a joint Paris and Seattle study of 79 patients indicate that a subset of survivors will develop secondary cancers, particularly of the head and neck. [55] The occurrence of cancer of the tongue in one such patient illustrates the problem.[56] These malignancies reflect the ongoing genetic susceptibility of host non-hematopoietic tissue to cancer despite successful BMT for marrow failure.

For patients who do not have a matched sibling donor for BMT, a search for a matched unrelated donor can be initiated. Because of the heightened graft-versus-host response observed in Fanconi's patients, the survivals and cure rates have not been as good compared to matched sibling donor BMT. Even when matched unrelated marrow is fully compatible with the recipient at the DNA level (molecular match), these transplants are still problematic. In the analysis by Gluckman et al., [52] the two-year probability of survival for 48 Fanconi's patients transplanted with donor marrow comprised of matched unrelated or alternative related sources was only 29%. In another multicenter report of 49 cases of Fanconi's anemia who received unrelated stem cell transplants, survival was only 43% using HLA-matched marrow and 32% in mismatch recipients.[57]

Because of the increased risks associated with alternative donor marrow, it is not recommended that Fanconi's patients undergo

this type of BMT if their clinical and hematological status is stable. Criteria to proceed with an alternative donor BMT include: (a) failure to respond adequately to androgen therapy and/or cytokine treatment, resulting in an impending need for chronic transfusional support; (b) presence of a persistent cytogenetic clone in bone marrow cells, for example, monosomy 7; and (c) overt malignant transformation to MDS/AML.

Cord blood cells, naturally enriched with hematopoietic stem and progenitor cells, are being increasingly used as a donor source. The first cord blood transplantation was performed in 1988 for a Fanconi's anemia patient using a matched sibling donor. [56] Since then, cord blood collections and transplants have increased quickly for a variety of indications [59] [60] [61] [62] [63] including more Fanconi's anemia patients. [63] Using cord cells from related donors, engraftment in Fanconi's patients was about 78% and the incidence of graft-versus-host disease was reported to be lower than in allogeneic BMT. [63] Recently, cord cells from unrelated donors were transplanted into 5 Fanconi's patients. [64] Some of the donor specimens were mismatched at 1 or 2 loci, yet graft failure was only seen in 1 of the 5 cases, there were no instances of lethal graft-versus-host disease, and event-free-survival beyond day 60 after transplantation was 80% (4 of the 5 cases). To date, of 17 Fanconi's anemia patients who have received unrelated cord blood transplants in the United States, 10 are alive. [53]

Hematopoietic Growth Factors

The potential for recombinant growth factor (cytokine) therapy for Fanconi's anemia has not been fully explored but short-term data thus far are encouraging. An important multicenter clinical trial [65] examined the effect of prolonged administration of G-CSF in 12 Fanconi's patients with neutropenia. The patients were treated with varying subcutaneous dosages daily or every other day for 40 weeks. By week 8 of the study, all patients had an increase in absolute neutrophil numbers and 4 had an increase in platelet counts. Additionally, 4 patients who were not being transfused had a significant increment in hemoglobin levels and a fifth patient lost a transfusion requirement. Concurrent with the impressive improvements in hematology, 8 of 10 patients who finished 40 weeks of G-CSF treatment showed increases in the percentage of marrow and peripheral blood CD34+ cells.

Since genomic instability and a marked predisposition to leukemia and cancer are features of Fanconi's anemia, the wisdom of using a growth-promoting cytokine on a long-term basis for this disorder is a central issue. In the G-CSF study, 1 patient had a marrow clonal cytogenetic abnormality (48 XXY, +14) without MDS or AML at week 40 of treatment. Therapy was stopped and within 3 months the +X, +14 clone disappeared but monosomy 7 appeared in 11% of metaphases and increased over the ensuing months, prompting a bone marrow transplantation. Since monosomy 7 manifested and progressed after G-CSF was stopped, it seems unlikely that G-CSF was involved in the transformation event in this case. Also, the appearance of the +X, +14 clone while the patient was receiving G-CSF treatment and its disappearance when cytokine was discontinued must be put into the context of Fanconi anemia, per se, in which clones, seemingly unstable, are known to manifest and disappear spontaneously. [66] Thus, this pilot study showed overall that G-CSF is probably a safe and effective form of treatment for Fanconi anemia. One patient had a low-grade fever that resolved with dosage modification, and no other untoward clinical effects were noted in any patients.

At one institution, patients without a matched sibling donor who did not have a marrow cytogenetic clone were given combination cytokine therapy consisting of G-CSF 5 g/kg with erythropoietin 50 units/kg administered subcutaneously or intravenously three times a week. [53] Androgen therapy was added if the response was inadequate. Of 20 patients treated, all but 1 had improved neutrophil numbers, 20% achieved a sustained rise in platelets, and 33% had an increase in hemoglobin levels. Although more than half of the responders lost the response after one year due to progression of marrow failure, the requirement for androgen therapy could be delayed by about a year.

Androgens

Androgen therapy has been used to treat Fanconi's anemia for almost four decades. The overall response rate in the literature is about 50% [4] heralded by reticulocytosis and a rise in hemoglobin within 12 months. If the other lineages respond, white cells increase next, followed by platelets, but it may take many months to achieve the maximum response. When the response is deemed maximal, the androgens should be slowly tapered but not stopped entirely.

Oxymetholone, an oral 17- alkylated androgen, is used most frequently at 2 to 5 mg/kg/day with the lower dose preferred initially. Corticosteroids are commonly added to counter the androgen-induced growth acceleration and to prevent thrombocytopenic bleeding by promoting vascular stability. For this purpose, 510 mg of prednisone is given orally every second day. If an injectable androgen is preferred to decrease the risk of liver toxicity, nandrolone decanoate, 1 to 2 mg/kg/week is given intramuscularly followed by suitable pressure and ice packs to prevent hematomas.

Almost all patients relapse when androgens are stopped. Those few who successfully discontinue treatment are often in the puberty age range, when temporary spontaneous hematological remissions have been observed to occur. Many patients on long-term androgens eventually become refractory to therapy as marrow failure progresses. Potential side effects include masculinization, especially troublesome in female patients, and elevated hepatic enzymes, cholestasis, peliosis hepatis, and liver tumors. Those receiving androgens should be evaluated serially with liver chemistry profiles and ultrasonography and/or CT scan of liver.

Future Directions

The premise for gene therapy in Fanconi's anemia is based on the assumption that corrected hematopoietic cells have a growth advantage. Strengthening this supposition are recent descriptions of rare Fanconi's anemia patients who show spontaneous disappearance of cells with the Fanconi's phenotype. These so-called mosaic patients appear to have two populations of leukocytes, one sensitive to mitomycin C and the other with normal resistance to mitomycin C. [19] The phenotypically normal cells appear to have undergone an intragenic mitotic recombination generating one allele with both Fanconi's anemia mutations, whereas the other allele has none (equivalent to a heterozygous carrier). These mosaic patients go into hematologic remission suggesting that hematopoiesis was derived from progenitor cells spontaneously revertant to a normal phenotype.

Preclinical studies using retroviral vectors showed that the FA-C gene and the FA-A gene can be successfully integrated into normal cells and Fanconi's anemia cells. [67] [68] [69] This prompted a clinical trial in 3 FA-C patients. [70] The patients underwent peripheral blood stem cell harvesting and CD34+ cell fractionation following G-CSF administration. The CD34+ cells were admixed with a retrovirus containing the corrected copy of the FA-C gene and the cells were reintroduced into each patient intravenously. The stem cell harvesting and virus transduction were repeated every three months for a total of four procedures. Analysis of the peripheral blood and bone marrow cells demonstrated that the FA-C gene was transferred to a small percentage (0.1%) of the total cells but that various lineages expressed the FA-C gene.

Clinically, one patient had improvement in hemoglobin which increased from a pretreatment level of 89 g/dl to 12

A COMPREHENSIVE PLAN OF MANAGEMENT FOR FANCONI'S ANEMIA

Fanconi's anemia patients become complicated clinically, socially, and psychologically and therefore require a comprehensive multidisciplinary management plan. Ideally, the plan should be initiated in a tertiary care center by a consultant and support staff familiar with the disorder and its nuances. At the first visit, in addition to the detailed clinical, psychosocial, and hematological assessment, chromosome fragility testing should be performed on the patient and siblings using PHA-stimulated peripheral blood lymphocytes with and without added diepoxybutane and/or mitomycin C. HLA tissue typing on the patient and all family members should also be arranged to guide future planning. Imaging and other diagnostic investigations can be planned electively to determine the presence of internal anomalies.

If the hematological changes are mild to moderate and transfusional support is not needed, simple observation is appropriate. Blood counts are checked every 13 months, and marrow aspirates and biopsies performed annually to assess cellularity and morphology, and to obtain cytogenetics on hematopoietic cells. The goal of cytogenetic analysis is to identify the appearance of a malignant clone, or overt transformation to MDS/AML, early.

If transfusional support for red cells and/or platelets is imminent or has already started, further intervention is indicated. Bone marrow transplantation (BMT) is currently the only curative treatment for marrow failure. The results are best when the donor source is an HLA-matched sibling. In this setting, plans for BMT should be activated with the patient's and family's permission. There is nothing to gain by waiting if the patient requires transfusions and has a matched sibling donor. Platelet products should be derived from a single donor source, if possible, and show negative testing for cytomegalovirus.

For patients without a matched sibling donor, a search for an alternative donor source should be initiated after serious and detailed discussion with the family. The term alternative currently encompasses sources from matched unrelated donors, family members other than matched siblings, and umbilical cord cells, related and unrelated, matched and mismatched. Since an alternative donor transplantation is more problematic and has inferior survival data compared to a matched sibling BMT, a decision to go ahead has to be determined for each case by weighing risks and benefits. Criteria to proceed include a failure to respond adequately to androgens and/or cytokine therapy necessitating repeated transfusions, the presence of a persistent clonal cytogenetic abnormality in marrow cells such as monosomy 7, and overt transformation to MDS/AML.

If some form of transplantation is not an option, there are two choices for pharmacologic medical management. Androgen therapy is the historical choice but only affects a marrow response in about 50% of patients and is usually restricted to the erythroid lineage. The penalty is masculinization, which can be very troublesome esthetically in female patients. Oxymetholone is the most widely used oral formulation at doses of 25 mg/kg once a day with preference for the lower dose initially. Prednisone, 510 mg orally once every second day is usually added (but without solid evidence of efficacy) to offset the oxymetholoneinduced growth acceleration, and to stabilize vasculature which may prevent thrombocytopenic bleeding. Because oxymetholone is a 17- alkylated androgen and carries a risk of liver toxicity, a safer but much less popular injectable androgen, nandrolone decanoate, can be given intramuscularly once a week at a dose of 12 mg/kg. Suitable pressure and ice packs must follow the injections to prevent hematomas. When a response to androgens occurs, usually heralded by a rise in hemoglobin within 1 to 2 months, white cells and then platelets may increase but it may take several months to get the fullest response. When the effect is thought to be maximal, the androgen should be slowly tapered to the lowest dose that maintains the response. The androgen should not be stopped completely because almost all patients relapse on discontinuing the drug.

Recombinant growth factor therapy is a new option to androgens and has some advantages. G-CSF, 510 g/kg once a day by subcutaneous injection, will induce a neutrophil response in almost all patients within 48 weeks and has potential to effect a trilineage marrow response. Erythropoietin, 50 units/kg subcutaneously three times a week, can be given additionally but the advantage of this over using G-CSF alone has not been proven. When the effect of G-CSF is judged to be maximal, the schedule can be reduced in some patients to an every second day format with sustained results. The main disadvantage of growth factor therapy is the need for repeated injections. Also, G-CSF probably cannot offset progressive marrow failure indefinitely. However, while the cytokine response is operative, the need to commence androgens can be delayed. There have not been any randomized trials to prove that G-CSF plus androgen treatment is superior to G-CSF alone.

g/dl post-treatment. Although that patient received androgens and G-CSF during the initial period of the trial, both these medications were reduced without affecting blood counts. The other two patients demonstrated variable cell marking at lower levels without significant changes in blood counts.

These results, although crude and preliminary, underscore the potential of a futuristic approach to treating Fanconi's anemia.

Shwachman-Diamond Syndrome

Introduction

This is an inherited disorder of exocrine pancreatic dysfunction with additional features of short stature, variable hematological abnormalities and radiological skeletal changes.[71] [72] [73] The gene responsible for this complex, pleiotropic phenotype is not known and no unifying pathogenesis has been confirmed that can account for all of the multisystem features of the Shwachman-Diamond syndrome. Chromosomes are normal and no increased breakage is seen after clastogenic stress. None of these patients have cystic fibrosis, and sweat chloride levels are normal. Many families have been identified with at least two affected children, and published studies of segregation ratios and family pedigrees support an autosomal recessive mode of inheritance.

Etiology and Pathogenesis

Until the molecular genetics are defined for this syndrome, the pathophysiological link between exocrine pancreatic dysfunction, physical anomalies, and partial to complete marrow failure remains speculative. Copper deficiency in utero is one hypothetical unifying etiology for the various phenotypic manifestations of Shwachman syndrome, but has never been confirmed.[74]

Regarding the neutropenia, an early but informative study of marrow function was performed in which granulopoiesis was analyzed in 10 children from the Toronto series.[75] Marrow proliferative activity was normal, as assessed by determination of mitotic indices and tritiated thymidine uptake into granulocytic cells. Assay of bone marrow CFU-GM progenitors demonstrated normal numbers in four patients and reduced numbers in five. Morphologically, the granulocyte colonies were indistinguishable from normal colonies. Production of colony-stimulating activity from patients' peripheral blood leukocytes appeared normal when tested on control marrow. No serum inhibitors against CFU-GM or colony-stimulating activity could be demonstrated using both control and autologous marrow, and co-culture of patients' peripheral blood lymphocytes with control marrow did not inhibit CFU-GM growth. Thus, in ShwachmanDiamond syndrome, committed granulocytic progenitors were proliferative and their frequency in vitro varied widely, as did the clinical neutropenia. The proliferative activity of mitotic granulocytic cells was normal, and neither a deficiency of humoral stimulators nor the presence of serum or cellular inhibitors of granulopoiesis could be demonstrated. Other investigators in subsequent studies[76] [77] [78] [79] showed decreased CFU-GM and CFU-E compatible with a defective stem cell origin for the marrow failure in Shwachman-Diamond syndrome.

Clinical Manifestations

The many manifestations that occur in varying combinations are shown in Table 18-4 . Pancreatic dysfunction of variable severity is a consistent feature of the syndrome. The pancreatic lesion appears to be due to failure of pancreatic acinar development. Pathologic study reveals extensive fatty replacement of pancreatic acinar tissue and normal ductular architecture. Pancreatic function studies using intravenous secretin and cholecystokinin confirm the presence of markedly impaired enzyme secretion, averaging 1014% of normal, but with preserved ductal function. [80] [81]

The vast majority of patients have symptoms of maldigestion, caused by the pancreatic insufficiency, from birth. The absence of steatorrhea, however, does not exclude a diagnosis of Shwachman-Diamond syndrome. If the syndrome is suspected, a quantitative pancreatic function test should be performed. Alternatively, computed tomography of the pancreas may demonstrate

TABLE 18-4 -- Clinical Features of Shwachman-Diamond Syndrome

Pancreatic	Exocrine pancreatic hypoplasia
Hematologic	Neutropenia (persistent or intermittent)
	Red cell hypoplasia
	Thrombocytopenia
	Pancytopenia
	Elevated fetal hemoglobin
	Myelo-lymphoproliferative diseases
Skeletal	Metaphyseal dysplasia
	Long bone tubulation
	Short or flared ribs
	Thoracic dystrophy
	Clinodactyly
Growth	Short stature (normal growth velocity)
Other	Psychomotor delay
	Renal tubular dysfunction
	Diabetes mellitus
	Dental abnormalities
	Ichthyosis
	Hepatomegaly
	Hirschsprung's disease

fatty changes within the body of the pancreas. A low serum immunoreactive trypsinogen concentration is highly suggestive of severe pancreatic exocrine deficiency. [82] Approximately 50% of patients appear to exhibit a modest improvement in enzyme secretion with advancing age. [81] A number of older patients with Shwachman-Diamond syndrome actually develop pancreatic sufficiency with normal fat absorption when assessed by 72-hour fecal fat balance studies.

The most conspicuous physical findings relate to the pancreatic insufficiency and malabsorption, especially short stature, which is another consistent feature of the syndrome. When treated, most patients show a normal growth velocity, yet remain consistently below the third percentile for height and weight. Some patients have evidence of delayed puberty. The occasional adult achieves the 25th percentile for height.

Skeletal abnormalities are quite variable. Some patients present at birth with thoracic dystrophy, while others have short, flared ribs. Metaphyseal dysostosis of the long bones is a common radiological abnormality and is thought to be quite specific, particularly in the femoral head and the proximal tibia. These changes may not be detectable until after 12 months of age. The etiology of the metaphyseal changes is unclear. In most patients these bony lesions fail to produce any symptoms. However, the integrity of the growth plate may be affected, which in turn may result in skeletal growth disturbances and joint deformities, particularly in the knees and hips.

Additional, less frequent, manifestations felt to be associated with Shwachman-Diamond syndrome include psychomotor delay, hypotonia, massive hepatomegaly, elevated transaminase levels in the absence of hepatomegaly, dental abnormalities, endocardial fibrosis, renal tubular acidosis, and diabetes mellitus.

Laboratory Evaluation

Data from two large institutions have recently been published and probably represent the spectrum of hematologic findings fairly accurately. [81] [83] The combined data from 25 patients in the Toronto series and 21 patients in the London series confirmed that neutropenia is present in virtually all patients on at least one occasion and can be chronic, cyclic or intermittent. It has been identified early in some patients in the neonatal period during an episode of sepsis. Anemia, usually normochromic-normocytic, was recorded in up to 66% of patients, [83] and thrombocytopenia in up to 60%. [81] Fetal hemoglobin was elevated in 80% of patients at some stage during the disease course. [83] Whether this reflects stress hematopoiesis and/or ineffective erythropoiesis concomitant with chronic infections, or reflects MDS in transformation has not been clarified. Reticulocyte responses were inappropriately low for the levels of hemoglobin in 75% of patients. [83]

More than one lineage can be affected, and pancytopenia was observed in up to 44% of cases. [81] The pancytopenia can be severe as a result of full-blown aplastic anemia. However, bone marrow biopsies and aspirates vary widely with respect to cellularity; varying degrees of marrow hypoplasia and fat infiltration are the usual findings, but marrows showing normal or even increased cellularity have also been observed. [81] [83] The severity of neutropenia does not always correlate with bone marrow cellularity, nor is the severity of the pancreatic insufficiency concordant with the hematological abnormalities.

Patients with Shwachman-Diamond syndrome are particularly susceptible to severe infections, including otitis media, bronchopneumonia, osteomyelitis, septicemia, and recurrent furuncles. Overwhelming sepsis is a well-recognized fatal complication of this disorder, particularly early in life. Shwachman-Diamond neutrophils may have a defect in mobility, migration and chemotaxis that does not appear to be caused by malnutrition. [84] [85] [86] In the London series, all 13 patients tested showed

defective chemotaxis. [83] Lithium in some manner appears to restore chemotaxis when used in vitro [87] and in vivo. [88] Alterations in neutrophil cytoskeletal/microtubular function may play a prominent role in causing the defective chemotaxis. [86]

Predisposition to Leukemia

Like some other inherited bone marrow failure disorders, Shwachman-Diamond syndrome predisposes patients to MDS and leukemic transformation. [81] [83] [89] [90] [91] [92] [93] In the London series, [83] MDS developed in seven cases (33%). Five of these patients ultimately evolved into AML (M6 in two, M5 in two, and M2 in one) following a period of MDS (RAEBT in two, RA in two, and RAEB in one). During the MDS phase, five cases had clonal marrow cytogenetic abnormalities, mostly structural changes involving chromosome 7. In the Toronto series, [81] 11 of the 25 patients had pancytopenia and three of these developed AML (12%). In the published literature of 165 patients, [49] nine (5%) developed leukemia (three cases of ALL, two of AML [M2], one M4, one M5, one M6, and one JCML). These sporadic reports in the literature are likely a gross underestimate of the true incidence of malignant transformation judging from the London and Toronto data. Clearly, the propensity for leukemic conversion in Shwachman-Diamond syndrome is extremely high compared with the general population but is probably not as high as in Fanconi's anemia.

A new issue is the occurrence of MDS/AML in Shwachman-Diamond patients while receiving G-CSF therapy for severe neutropenia. In the report of 14 patients with congenital disorders of myelopoiesis who developed MDS/AML (n=13) or a clonal cytogenetic abnormality (n=1) while receiving G-CSF, two of the study group had Shwachman-Diamond syndrome. [93] The concern is that G-CSF therapy may have played a role in the malignant transformation. To date, there is no strong evidence to incriminate the cytokine directly in leukemogenesis (see detailed discussion in Malignant Myeloid Transformation in Kostmann's Syndrome/Congenital Neutropenia in this chapter).

Differential Diagnosis

The syndrome of refractory sideroblastic anemia with vacuolization of bone marrow precursors, or Pearson's syndrome, is clinically similar to Shwachman-Diamond syndrome but very different in bone marrow morphology.[94] [95] [96] [97] [98] [99] [100] [101] [102] [103] Severe anemia requiring transfusions rather than neutropenia is often present at birth and by 1 year of age in all cases. In contrast to Shwachman-Diamond syndrome, the major marrow morphologic findings are ringed sideroblasts with decreased erythroblasts, and prominent vacuolation of erythroid and myeloid precursors.

The disorder shares clinical similarities with Shwachman-Diamond syndrome because of exocrine pancreatic dysfunction in both. Malabsorption and resultant severe failure to thrive occurs in about one-half of cases within the first 12 months of life. Qualitative pancreatic function tests show depressed acinar function and reduced fluid and electrolyte secretion. About 50% of reported cases died early in life from sepsis, acidosis and liver failure; the others appeared to improve spontaneously with reduced transfusion requirements. At autopsy, the pancreas shows acinar cell atrophy and fibrosis; fatty infiltration as seen in Shwachman-Diamond syndrome is not a prominent feature. The need for long-term pancreatic enzyme replacement is unclear. These patients have abnormalities of mitochondrial deoxyribonucleic acid (mtDNA).[99] mtDNA encodes enzymes in the mitochondrial respiratory chain that are relevant to oxidative phosphorylation, including the reduced form of nicotinamide-adenine dinucleotide dehydrogenase (NADH), cytochrome oxidase, and adenosine triphosphatase (ATPase), as well as transfer ribonucleic acids (tRNAs) and ribosomal RNAs.

Shwachman-Diamond syndrome shares some manifestations with Fanconi's anemia such as marrow dysfunction and growth failure, but Shwachman-Diamond patients are readily distinguished because of pancreatic insufficiency with a resultant malabsorption syndrome, fatty changes within the pancreatic body that can be visualized by computed tomography, characteristic skeletal abnormalities not seen in Fanconi's patients, and no increase in chromosome breakage after clastogenic stress testing.

Therapy and Prognosis

Patient management is ideally shared by a hematologist and a gastroenterologist. The malabsorption component of Shwachman-Diamond syndrome responds to treatment with oral pancreatic enzyme replacement. When monitored over time, about 50% of patients convert from pancreatic insufficiency to sufficiency due to spontaneous improvement in pancreatic enzyme secretion.[75] This improvement is particularly evident after 4 years of age.

Growth Factors and Other Strategies

G-CSF has been given for profound neutropenia and has been very effective in inducing a clinically beneficial neutrophil response. [93] [104] [105] [106] [107] In one patient, crossover treatment using G-CSF initially, followed later by GM-CSF demonstrated that both cytokines could effect a neutrophil response. [107] Of nine Shwachman-Diamond patients enrolled in the Severe Chronic Neutropenia International Registry, Seattle, three patients have received G-CSF with good responses lasting from 6 months to more than 6 years (David Dale, MD, personal communication).

Patients have also been treated with corticosteroids with hematologic improvement noted in 6 of 12 patients. [4] A smaller number have received androgens plus steroids in the manner of treating Fanconi's anemia and improved marrow function was also noted. One patient improved on cyclosporin therapy. [108]

Bone Marrow Transplantation

At present, the only definitive therapy for severe marrow failure in Shwachman-Diamond syndrome is allogeneic BMT, although the experience has been very limited. As of 1996, only five patients with this disorder who received a marrow allograft had been reported. [109] [110] [111] [112] [113] The small number of transplanted patients is partly explained because the usual clinical presentation is isolated neutropenia, and if severe it can be successfully managed with antibiotics and growth factor therapy like G-CSF. Transfusional management can also be applied effectively on a long-term basis for patients with advanced bi- and trilineage marrow failure.

A note of caution is sounded regarding BMT for Shwachman-Diamond syndrome. Left ventricular fibrosis and necrosis without coronary arterial lesions have been reported in 50% of Shwachman-Diamond patients at autopsy[114] suggesting that there may be an increased risk of cardiotoxicity with the intensive preparatory chemotherapy used in BMT. Indeed, of two patients who underwent BMT, one died as a result of cardiotoxicity ascribed to cyclophosphamide [110] and the other had significant left ventricular dysfunction throughout the clinical course. [111] A third patient[109] and two others who received transplants in London[83] showed no cardiac dysfunction or cardiotoxicity using comparable conditioning protocols. Although these data are limited, it is recommended that cardiac function be carefully assessed before starting chemotherapy for BMT.

Dyskeratosis Congenita

Introduction

This is an inherited disorder of the mucocutaneous and hematopoietic systems. The diagnostic ectodermal component invariably

consists of the triad of reticulate skin pigmentation of the upper body, mucosal leukoplakia, and nail dystrophy. [115] [116] [117] The skin and nail findings usually become apparent in the first 10 years of life, whereas the oral leukoplakia is seen later. These manifestations tend to progress as patients get older.

Dyskeratosis congenita is also an inherited bone marrow failure syndrome in which aplastic anemia occurs in about 50% of cases, usually in the second decade of life. Patients also have a predisposition to cancer. Because of the cluster of abnormalities involving skin and bone marrow and the predilection to cancer, dyskeratosis congenita resembles Fanconi's anemia. However, the genetics and physical abnormalities of patients with both conditions are quite different and the conditions should be considered as totally discrete entities. Two recent reviews highlight the salient features of dyskeratosis congenita. [118] [119]

The inheritance pattern is somewhat complicated. There are about 225 published cases of dyskeratosis congenita [3] and about 85% of the patients are male, compatible with an X-linked recessive trait. Linkage studies in one large family [120] using X-chromosome-specific restriction fragment length polymorphism markers have assigned a gene for the syndrome to Xq28, a finding confirmed in three additional families. [121] In X-linked families it is possible to identify female carriers using informative Xq28-specific polymorphic probes.

Approximately 15% of cases appear to have another mode of inheritance. Sporadic female cases, familial cases with affected male and female siblings in one generation, and cases with known parental consanguinity fit an autosomal recessive inheritance pattern. An autosomal dominant mode best fits other cases in families with affected male and female members in consecutive generations. Clinically, the autosomal dominant group seems to be milder in its manifestations, and the autosomal recessive group appears to have more physical anomalies and a higher incidence of aplastic anemia and cancer. [118] It seems likely that the dyskeratosis congenita phenotype is due to more than one gene.

Etiology and Pathogenesis

Most studies of the pathogenesis of the aplastic anemia in dyskeratosis congenita have been limited to clonogenic assays. A marked reduction or absence of CFU-GEMM, BFU-E, CFU-E and CFU-GM progenitors has been consistently reported. [26] [27] [122] [123] [124] The absence of a serum inhibitor of CFU-GM was documented in one case, and the absence of T-cell mediated hematopoietic suppression in another case. Although Hanada et al. reported one case of T-cell-mediated suppression of CFU-GM, but not of CFU-E, this effect was not seen after splenectomy or recurrence of the pancytopenia. [125]

Marsh et al.[124] used long-term bone marrow cultures to study hematopoiesis in three patients with dyskeratosis congenita. Two had aplastic anemia and the third had normal blood counts and normal marrow cellularity. Hematopoiesis was severely defective in all three patients with a low frequency of colony-forming cells and a low level of hematopoiesis in long-term cultures. The function of marrow stromal cells was normal in their ability to support growth of hematopoietic progenitors from normal marrow seeded onto them in all 3 cases, but generation of progenitors from patient marrow cells inoculated onto normal stroma was reduced, strongly

suggesting that the defect in dyskeratosis congenita is of stem cell origin.

Thus, the marrow failure in this disorder may be due to a progressive attrition and depletion of hematopoietic stem cells which manifests as pancytopenia when patients are in their mid-teens. Alternatively, the marrow dysfunction may not be a simple consequence of a limited stem cell pool but may represent a failure of replication and/or maturation.

Clinical Manifestations

Clinical manifestations in dyskeratosis congenita often appear during childhood. The skin pigmentation and nail changes typically appear first, mucosal leukoplakia and excessive ocular tearing appear later, and by the mid-teens the serious complications of bone marrow failure and malignancy begin to develop. In rare cases the marrow abnormalities may appear before the skin manifestations. The main causes of death relate either to bone marrow failure or to malignancy. The mean age of death is approximately 30 years.

Cutaneous findings are the most consistent feature of the syndrome. Lacy reticulated skin pigmentation affecting the face, neck, chest, and arms is a common finding. The degree of pigmentation increases with age and can involve the entire skin surface. There may also be a telangiectatic erythematous component. Nail dystrophy of the hands and feet is the next most common finding (Fig. 18-2). It usually starts with longitudinal ridging, splitting, or pterygium formation and may progress to complete nail loss. Leukoplakia usually involves the oral mucosa, especially the tongue (Fig. 18-3) but may also be seen in the conjunctiva, anal, urethral, or genital mucosa. Hyperhidrosis of the palms and soles is common, and hair loss is sometimes seen. Eye abnormalities are observed in approximately 50% of cases. [126] Excessive tearing (epiphora) secondary to nasolacrimal duct obstruction is common. Other ophthalmologic manifestations include conjunctivitis, blepharitis, loss of eyelashes, strabismus, cataracts, and optic atrophy. Abnormalities of the teeth, particularly an increased rate of dental decay and early loss of teeth, are common. Skeletal abnormalities such as osteoporosis, avascular necrosis, abnormal bone trabeculation, scoliosis, and mandibular hypoplasia are seen in approximately 20% of cases.[127] [128] Genitourinary abnormalities include hypoplastic testes, hypospadias, phimosis, urethral stenosis, and horseshoe kidney. Gastrointestinal findings, such as esophageal strictures, hepatomegaly or cirrhosis, are seen in 10% of cases. [129]

Several physical findings can be used to distinguish Fanconi's anemia from dyskeratosis congenita clinically. The following abnormalities are seen only in patients with dyskeratosis congenita but not Fanconi's patients [49] : nail dystrophy, leukoplakia, teeth abnormalities, hyperhydrosis of palms and soles, and hair loss.

Laboratory Evaluation

About 50% of the X-linked male dyskeratosis congenita patients and 70% of the autosomal recessive patients develop aplastic anemia, usually in the teenage years. It occurs in the autosomal dominant patients as well, but much less frequently. Most of these patients already have manifestations of dyskeratosis congenita, but some younger patients can develop marrow failure before the clinical onset of the mucocutaneous manifestations. The initial hematological change is usually thrombocytopenia or anemia, or both, followed by full-blown pancytopenia caused by aplastic anemia. The red cells are often macrocytic and the fetal hemoglobin can be elevated. Oddly, early bone marrow aspirations and biopsies may be hypercellular; however, with time the cellular elements decline with a symmetrical decrease in all hematopoietic lineages. Ferrokinetic studies at this point are consistent with aplastic anemia.

As summarized by Dokal,[119] there is a large body of contradictory information regarding chromosomal fragility and instability in dyskeratosis congenita. Spontaneous chromosome breaks in patients' lymphocytes were reported in some studies but were not confirmed in others. Similarly, excessive spontaneous and clastogenic-induced sister chromatid exchange in patients' lymphocytes were observed by some investigators but not by others. However, standard clastogenic stress studies of dyskeratosis congenita cells using the DNA alkylating agents

Figure 18-2 Dystrophic nails in dyskeratosis congenita.

mitomycin C and DEB in several laboratories clearly have not shown any difference in chromosome breakage between patients and controls. [130] [131] [132] [133] [134] [135] This contrasts sharply with Fanconi's anemia cells and distinguishes one disorder from the other.

However, Dokal et al. argue strongly that dyskeratosis congenita is, indeed, a chromosome instability disorder of a somewhat different type than Fanconi's anemia. [136] Dokal et al. found that primary skin fibroblasts in culture were not only abnormal in morphology and doubling rate, but in some patients metaphases in peripheral blood cells, marrow cells, and fibroblasts

Figure 18-3 Leukoplakia of the tongue in dyskeratosis congenita.

in culture showed numerous unbalanced chromosome rearrangements such as dicentrics, tricentrics and translocations in the absence of clastogenic agents. These findings were confirmed by other investigators [119] and provide evidence for a defect that predisposes patient cells to developing chromosomal rearrangements.

Predisposition to Cancer

Cancer develops in about 10% of patients, usually in the third and fourth decades of life. In contrast with Fanconi's anemia in which both malignant tumors and MDS/AML are seen, dyskeratosis congenita patients are not predisposed to leukemia but only to solid tumors. Young and Alter have summarized the literature on cancers in 20 patients, including 15 X-linked males, four autosomal recessive cases, and one autosomal dominant patient. [137] Most of the cancers were squamous cell carcinoma or adenocarcinomas, and the oropharynx and gastrointestinal tract were involved most frequently. One patient had three separate primaries in the tongue, nasopharynx, and rectum, one had esophageal and cheek carcinomas, and one had separate nasal and tongue malignancies. Thus, the sites of most of the cancers involve areas known to be abnormal in dyskeratosis congenita such as mucous membranes and gastrointestinal tract.

Therapy and Prognosis

Management of aplastic anemia is similar to that for Fanconi's anemia. Androgens, usually combined with low-dose prednisone, can be expected to induce improved marrow function in about 50% of patients. If a response is seen and deemed to be maximal, the androgen dose can be slowly tapered but not stopped. As in Fanconi's anemia, patients can become refractory to androgens as the aplastic anemia progresses. There is no published information on the use of immunosuppressive therapy for this disorder.

Other Treatment Options

As recently summarized,[138] 16 patients with dyskeratosis congenita worldwide have been reported who received allogeneic BMT for marrow failure. [138] [139] [140] [141] [142] [143] [144] [145] Eight patients were less than 11 years of age at the time of the procedure; the others were in their twenties and thirties. All received cyclophosphamide-based preparative regimens with or without irradiation. The results were remarkably poor; only 3 of the 16 were alive and appeared cured by BMT, at 8 months, 15 months, and 5 years post-BMT, respectively. The others died of early, transplant-related complications or, notably, of very late complications. Lethal pulmonary fibrosis in four patients and renal failure in two others occurred years after BMT. Gluckman et al. [146] suggested that the epithelial and endothelial cells of these patients might be abnormally sensitive to the preconditioning agents without any relation to chromosomal fragility. This sensitivity may be reflected by raised von Willebrand factor levels in patients' plasma. [119] Gluckman et al. reported two dyskeratosis congenita patients treated with BMT who developed diffuse vasculitis

caused by endothelial activation several years after BMT. [146] Their renal histology at postmortem showed similar lesions characterized by alteration of the capillary walls of glomeruli, mesangiolysis, arteriolonecrosis, and occasional arteriolar luminal fibrosis.

Thus, judging from available information, strong endorsement cannot be made for BMT in dyskeratosis congenita, although there is usually no other recourse for life-threatening aplastic anemia. Perhaps modified conditioning similar to that used for Fanconi's anemia will ensure a safer procedure and a more optimistic outcome.

Three patients were reported who responded to G-CSF therapy with significant increases in absolute neutrophil counts. [147] [148] [149] Similarly, two other patients received GM-CSF therapy, which resulted in improved neutrophil numbers. [150] [151] G-CSF with erythropoietin resulted in a trilineage hematologic response in one patient.[3] Stem cell factor increased the in vitro growth of erythroid progenitors in some patients with dyskeratosis congenita [152] but it has not yet been used clinically. Although the reports are scanty, cytokine therapy appears to offer potential benefit, at least in the short-term, especially for improving granulopoiesis.

In the X-linked form of dyskeratosis congenita, the assignment of a gene to Xq28, and the availability of most of this region as contiguous yeast artificial chromosomes may facilitate positional cloning of the gene. Although this raises hope for gene therapy in the future, the prospects are not imminent.

Amegakaryocytic Thrombocytopenia

Introduction

Amegakaryocytic thrombocytopenia, or congenital amegakaryocytic thrombocytopenia (CAT) is a syndrome that presents in infancy with isolated thrombocytopenia due to reduced or absent marrow megakaryocytes with preservation initially of granulopoietic and erythroid lineages. Aplastic anemia subsequently ensues in about 45% of patients, usually in the first few years of life. The diagnosis depends on the exclusion of all other specific causes for thrombocytopenia in early life. Although most cases are sporadic, familial cases also occur and the syndrome is felt to be an inherited bone marrow failure disorder. Peripheral blood chromosomes do not show increased fragility, which distinguishes CAT from Fanconi's anemia.

Of reported cases, male cases outnumber female, suggesting that some cases may be X-linked and that others may have an autosomal recessive mode of inheritance. Examples of male-only affected sibships as well as mixed male-female affected sibships are reviewed by Alter. [49]

Etiology and Pathogenesis

Serial studies of bone marrow hematopoiesis using clonogenic assays were performed in an infant from Toronto with CAT. [153] Initially, when the only hematological abnormality was isolated thrombocytopenia, the number of clonogenic hematopoietic progenitors was comparable to controls, including the number of megakaryocyte precursors (CFU-Meg). As the disease evolved into aplastic anemia over an 11-month period, the peripheral blood counts declined, and colony numbers from four classes of progenitors also declined in parallel. When added to the marrow cultures, the patient's plasma was not inhibitory to control or to patient's colony growth. Similarly, no cellular inhibition of hematopoiesis was observed when the patient's marrow was cultured after depleting the sample of T lymphocytes or after adding them back. Furthermore, stromal cells established in short-term and long-term cultures of the patient's marrow showed normal proliferative activity and yielded a fertile marrow microenvironment for patient's and control colony growth. The data suggest that the central problem in CAT is an intrinsic hematopoietic stem cell defect rather than an abnormality of the marrow milieu. The findings are consistent with either a progressive, quantitative attrition of progenitors or their inability to proliferate into colonies in vitro and into differentiated, functional cells in vivo.

Guinan et al.[154] produced data that demonstrated assayable numbers of CFU-Meg progenitors in vitro from 5 patients with CAT in response to IL-3, GM-CSF or the combination of both. The presence of megakaryocyte progenitors in these patients fits

with the data from the Toronto patient when studied early in the course of the disease. [153]

Muraoka et al.[155] examined the effect of recombinant human thrombopoietin on CFU-meg colony formation from a patient with CAT and demonstrated a defective response. The patient's serum thrombopoietin level was significantly elevated compared to controls, and the patient's marrow cells did not express mRNA for c-mpl, the thrombopoietin receptor. The pathophysiology of CAT in this case appeared to be directly attributable to these findings.

Clinical Manifestations

Almost all patients present with a petechial rash, bruising, or bleeding in the first year of life. Most cases are obvious at birth or within the first 2 months. Roughly half of the patients have characteristic physical anomalies, although the others have normal physical and imaging features. Some affected sibships manifest both normal and abnormal physical findings in the same family. As in Fanconi's anemia, CAT patients with and without anomalies should be considered as part of the clinical spectrum of one entity.

The commonest manifestations in those with anomalies are neurologic and cardiac. Findings related to cerebellar and cerebral atrophy are a recurrent theme and developmental delay is a prominent feature in this group. Patients may also have microcephaly and an abnormal facies. Congenital heart disease with a variety of malformations can be detected, including atrial septal defects, ventricular septal defects, patent ductus arteriosus, tetralogy of Fallot, and coarctation of the aorta. Some of these can occur in combinations. Other anomalies include abnormal hips or feet, kidney malformations, eye anomalies, and cleft or high-arched palate.

Laboratory Evaluation

Thrombocytopenia is the major laboratory finding with normal hemoglobin levels and white blood cell counts initially. Although there are usually measurable, albeit reduced, numbers of platelets, peripheral blood platelets may be totally absent. Similar to other inherited bone marrow failure syndromes, red cells may be macrocytic. Hb F can be increased and there may be increased expression of i antigen. Bone marrow specimens show normal cellularity with markedly reduced or absent megakaryocytes. In patients who develop aplastic anemia, marrow cellularity is decreased with fatty replacement; the erythroid and granulopoietic lineages are also symmetrically reduced.

Predisposition to Leukemia

Alter described two patients with CAT who developed leukemia. [49] One male with a normal physical appearance had amegakaryocytic thrombocytopenia from day 1 of life, developed aplastic anemia at 5 years of age, responded poorly to androgens and steroids, and then evolved into AMML at age 16 with death at age 17. A female had thrombocytopenia at 2 months of age, pancytopenia at 5 months, and thereafter developed a preleukemic picture with clonal abnormalities involving chromosome 19. The Toronto patient described herein had thrombocytopenia at 6 months of age, developed progressive aplastic anemia over the next 2 years, acquired monosomy 7 in marrow cells at 5 years of age, and then evolved into MDS with an activating ras oncogene mutation in hematopoietic cells. [153] Hence, the current evidence shows that CAT is another inherited marrow failure disorder that is preleukemic. The risk or incidence of malignant conversion is difficult to determine because of the rarity of the disease and paucity of published data dealing with this issue.

Therapy and Prognosis

Historically, treatment has been unsatisfactory and the mortality rate from thrombocytopenic bleeding, complications of aplastic anemia, or from malignant myeloid transformation has been very close to 100%. For that reason, HLA typing of family members should be performed as soon as the diagnosis is confirmed to see if a matched related donor for BMT exists. If not, a search for a matched unrelated donor should ensue as soon as the seriousness of the clinical picture dictates. The need for transfusional support is a cogent indication.

Platelet transfusions should be used discretely. Platelet numbers should not be a sole indication; clinical bleeding is a more appropriate trigger for the use of platelets. Single-donor platelets are preferred to multiple unfiltered random donor platelets in order to minimize sensitization, and if BMT is a realistic possibility all blood

products should be negative in testing for cytomegalovirus.

Corticosteroids have been used for thrombocytopenia with no apparent efficacy. For aplastic anemia, androgens in combination with corticosteroids may induce a temporary partial response but the effect is short-lived and does not prevent mortality.

Based on the in vitro augmentation of megakaryocyte progenitor colony growth in response to IL-3, GM-CSF or both, a phase I/II clinical trial was initiated for five patients with CAT.[154] IL-3 but not GM-CSF resulted in improved platelet counts in two patients, and decreased bleeding and transfusion requirements in the other three. GM-CSF had no observable benefit when given after IL-3 pretreatment. Prolonged IL-3 administration in two additional patients also resulted in platelet increments. This pilot study illustrates that IL-3 may be an important adjunct to the medical management of CAT. Thrombopoietin has not been tried yet for the treatment of CAT.

There is curative potential using BMT. In the original Toronto series of patients with inherited marrow failure studied by clonogenic assays, [26] [27] [156] a case of familial CAT in the aplastic anemia phase received a BMT from his sister in 1974 and is currently alive and cured. More recently, two infants of 22 and 42 months of age, respectively, underwent allogeneic BMT, one of them with an unrelated donor marrow.[157] Both patients were well with good engraftment of donor marrow and normal blood counts at 12 and 31 months after BMT, respectively.

An additional male patient with familial CAT who had a robust response to IL-3 initially and then later to the IL-3:GM-CSF fusion product underwent a matched unrelated BMT when he became refractory to cytokine therapy. He appeared cured 1 year after BMT (Laurence Boxer, MD, personal communication, 1997). The Toronto patient described in the Etiology and Pathogenesis and Predisposition to Leukemia sections received a one antigen mismatched T-cell depleted unrelated donor BMT in desperation because of clinical deterioration. Engraftment ensued readily but the patient died several months after BMT from complications of grade IV graft-versus-host disease (unpublished data). Despite this negative experience, the other successful BMTs described here illustrate the potential for cure by the procedure.

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OTHER GENETIC SYNDROMES

Bone marrow failure can occur in the context of several specific non-hematological syndromes, and also in familial settings that do not exactly correspond with the entities already described.

Down Syndrome

Down syndrome, or trisomy 21, has a unique association with aberrant hematology. Three seemingly related events can occur.

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[158] In the neonatal period, a myeloproliferative blood picture with large numbers of circulating blast cells has been observed in many of these infants. The blasts apparently are clonal but, remarkably, disappear spontaneously over several weeks in most cases. The term transient leukemia is often used to reflect this unusual natural history.

Second, in about 20% of cases, true leukemia recurs and requires oncologic management. Acute lymphoblastic and myeloblastic leukemias are both seen in Down's syndrome, and acute megakaryoblastic leukemia (M7) is the most common form of the myeloblastic leukemias. It has been estimated that the incidence of M7 is 500 times greater in children with Down's syndrome than in normal children. [158] Third, the onset of M7 is frequently preceded by an interval of MDS characterized by thrombocytopenia, abnormal megakaryocytopoiesis, megakaryoblasts in the marrow, and an abnormal karyotype, commonly trisomy 8. [159] In addition to the propensity for leukemia, a few patients have been reported with aplastic anemia. Alter has summarized five of these cases, three of whom died of marrow failure and two who responded to androgen therapy. [49]

Dubowitz's Syndrome

This is an autosomal recessive disorder characterized by a peculiar facies, infantile eczema, small stature, and mild microcephaly. The face is small with a shallow supraorbital ridge, a nasal bridge at the same level as the forehead, short palpebral fissures, variable ptosis, and micrognathia. [160] This is a rare disorder and incidence rates for complications are difficult to establish; however, as reviewed recently, there appears to be a predilection to cancer as well as to bone marrow dysfunction in patients with Dubowitz's syndrome. [161] Patients have developed ALL, AML, neuroblastoma, and lymphoma. [161] [162] [163] About 10% of patients also develop hematopoietic disorders varying from hypoplastic anemia, moderate pancytopenia, and full-blown aplastic anemia. [161] [164]

Seckel's Syndrome

Sometimes called bird-headed dwarfs, patients with this autosomal recessive developmental disorder have marked growth failure and mental deficiency, microcephaly, a hypoplastic face with a prominent nose, and low-set and/or malformed ears. [165] These patients can be distinguished from those with Fanconi's anemia on the basis of a negative DEB clastogenic chromosome stress test. About 10% of cases develop aplastic anemia, usually severe. [166] [167] [168]

Reticular Dysgenesis

This is an immunologic deficiency syndrome coupled with congenital agranulocytosis. [169] The mode of inheritance is probably autosomal recessive but an X-linked mode is also possible in some cases. The disorder is a variant of severe combined immune deficiency (SCID) in which cellular and humoral immunity are absent and patients also have severe lymphopenia and neutropenia. Because of profoundly compromised immunity, the syndrome presents early with severe infection at birth or shortly thereafter. A striking feature is absent lymph nodes and tonsils, and an absent thymic shadow on radiograph. In addition to lymphopenia and neutropenia, anemia and thrombocytopenia may be present. Bone marrow specimens are hypocellular with markedly reduced myeloid and lymphoid elements. Clonogenic assays of hematopoietic progenitors consistently show reduced to absent colony growth, indicating that the disorder has its origins at the pluripotential lympho-hematopoietic stem cell level. [170] [171] [172] [173] The only curative therapy is BMT. [172]

Familial Aplastic Anemia

Bone marrow failure can cluster in families, but many of these cases cannot be readily classified into discrete diagnostic entities such as Fanconi's anemia. The phenotype of these conditions can be complex, with varying combinations of hematologic abnormalities, immunologic deficiency, physical malformations, and development of leukemia. One approach to nosology is to divide the disorders into inheritance patterns, and then subdivide them into those with and without physical anomalies. [4]

Some families show an autosomal dominant mode of inheritance of marrow dysfunction associated with physical anomalies. The *WT syndrome* is characterized by successive generations of affected family members who have radial-ulnar hypoplasia, abnormal thumbs, short fingers, and fifth finger clinodactyly. [174] Pancytopenia or thrombocytopenia, sometimes with leukemia, occurs in some of the affected. The *IVIC syndrome* [175] or *oculo-otoradial syndrome* [176] manifests with radial ray hypoplasia, absent thumbs or hypoplastic radial carpal bones, impaired hearing, strabismus, and sometimes imperforate anus. Mild thrombocytopenia is seen in about 50% of cases. The *ataxia-pancytopenia syndrome* is a combination of cerebellar atrophy and ataxia associated in affected family members with varied manifestations of anemia, aplastic anemia, MDS, AML, monosomy 7 in marrow cells, and immune deficiency. [177] [178] Other autosomal dominant syndromes with anomalies include: a family with marrow failure, ALL, skin pigmentation, warts, immune dysfunction and multiple spontaneous abortions; [179] successive generations of family members with unilineage cytopenia or pancytopenia with vascular occlusions; [180] and, proximal fusion of the radius and ulna, and aplastic anemia or leukemia. [181]

Autosomal recessive inheritance of anomalies and marrow dysfunction also occurs. Consanguinity can result in a syndrome of microcephaly, mental retardation, skin pigmentation, short stature, and pancytopenia, possibly with a clonal cytogenetic marker in bone marrow cells. [182] A second example of this inheritance mode presents with central nervous system anomalies, such as the Dandy-Walker syndrome or ventricular dilatation and asymmetry, and aplastic anemia. [183]

Autosomal dominant inheritance of a wide-ranging pattern of disordered marrow function can be seen without physical anomalies. Successive generations have been described with the following: acquired aplastic anemia in 4 families comprised of 9 patients, with an affected parent, aunt or uncle [184] ; aplastic anemia in a mother and neutropenia and thrombocytopenia in her offspring [185] ; aplastic anemia, AML and monosomy 7 in various family members [186] ; and hypoplastic anemia in a parent and offspring with either myelofibrosis, AML, MDS, or pancytopenia, all associated with the acquired Pelger-Huet anomaly. [187]

Marrow dysfunction can also be inherited without anomalies in an autosomal recessive pattern. One example encompasses pancytopenia, immune deficiency, multiple cutaneous basal cell and squamous cell carcinomas, oral telangiectasias, and neck and chest poikiloderma. [188] Another pattern includes immune deficiency, pure red cell aplasia and/or neutropenia, and unusual crystalloid structures seen by electron microscopy in neutrophils. [189]

An X-linked inheritance is suggested by a syndrome affecting males in successive generations with one or more of pancytopenia, AML, ALL, and light chain disease but without physical anomalies.[190]

Laboratory Evaluation

These genetic disorders are very heterogeneous clinically and hematologically and only generic descriptives can be used in their characterization. These patients have variable manifestations

of cytopenia involving one or more hematopoietic lineages, usually with hypocellular marrows. In the familial syndromes, additional findings such as physical malformations, immunologic deficiency, and marrow cytogenetic markers such as monosomy 7 distinguish them from cases of sporadic acquired bone marrow failure. Patients' marrows generally show reduced numbers of hematopoietic progenitors in clonogenic assays but this is common to almost all marrow failure disorders and does not distinguish genetic and familial cases from acquired aplastic anemia.

Therapy

Because these disorders are rare, broad conclusions about management are difficult to formulate. For full-blown aplastic anemia with a hypocellular, fatty marrow, curative therapy with BMT if a matched donor is identified remains the first choice. In the familial cases, potential marrow donors must be thoroughly assessed clinically, hematologically and by marrow morphology, clonogenic activity, and cytogenetics to ensure that latent or masked marrow dysfunction is not present. If a matched donor is not available, principles of medical management used for Fanconi's anemia and for acquired aplastic anemia should be used.

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UNILINEAGE CYTOPENIAS

Diamond-Blackfan Anemia

Introduction

Diamond-Blackfan anemia (DBA), or congenital hypoplastic anemia, is a constitutional form of pure red cell aplasia. [191] The syndrome is heterogeneous with respect to inheritance patterns, clinical and laboratory findings, in vitro data, and therapeutic outcome. About 80% of cases are sporadic, suggesting new mutations or acquired disease, but there are examples of recessive inheritance (autosomal and possibly X-linked), as well as autosomal dominant patterns. [192] There is suspicion that DBA represents a family of disorders with different etiologies that share the common hematological phenotype of pure red cell aplasia. Based on data from the DBA registries in France[193] and in the United Kingdom,[194] the suspected incidence of the disorder in Europe is 5 to 7 cases per million live births.

The uniform diagnostic criteria for all cases are: (1) normochromic-macrocytic anemia presenting in 90% of cases in the first 12 months of life; (2) profound reticulocytopenia; (3) normocellular marrow with a selective, marked deficiency of red cell precursors; (4) increased serum levels of erythropoietin; (5) normal or slightly decreased white cell counts; and (6) normal or increased platelet counts. Fetal hemoglobin is usually increased with a fetal G/A pattern, is distributed heterogeneously, and is associated with increased expression of red cell i antigen as well as with fetal levels of red cell glycolytic and hexose monophosphate shunt enzyme activities.

Biologic and Molecular Aspects

Most cases of DBA are sporadic. In contrast with Fanconi's anemia, cytogenetic findings are usually normal, although morphologic alterations of chromosomes 1 and 16 have been reported in a few children. [195] [196] A significant proportion of patients, as many as 1020%, have a family history suggestive of a genetic disorder. Out of 21 patients in the Toronto series, 3 presented clear evidence of familial transmission of the disease. [192] Three of four sisters were affected in one family; an affected father and his son were found in another family; and there was an affected mother and her son in the third family. In addition, occasional histories were revealed of anemia in one or both parents of 5 other patients. Although this could not be documented in most cases, bone marrow examination of one mother who was slightly anemic revealed a reduced number of erythroid precursors (7%), which was suggestive of a mild form of DBA. Autosomal recessive transmission is postulated in families in which there is consanguinity, presence of several affected children, or both. Autosomal dominant inheritance is assumed in those kindreds in which cases of DBA have been documented in two or more successive generations.

Sporadic and familial cases do not differ by clinical criteria. Long-term demographic analysis on a large number of families has been hampered by several factors including the rarity of the syndrome, its relatively recent description, its historically fatal outcome, and the limited number of successive generations available for study. Whether the variable pattern of inheritance observed in pedigrees of DBA reflects different genetic determinants of the disease is still uncertain, but there has been progress in the search for the DBA gene.[197] A female patient was identified with a translocation involving 19q. Linkage analysis with chromosome 19 microsatellite markers in DBA families revealed significant linkage to 19q13 for both dominant and recessive modes of DBA inheritance. Within this region, a submicroscopic de novo deletion of 3.3 Mb was identified in a DBA patient. The deletion coincides with the translocation breakpoint and together with key recombinations restricts the putative DBA gene to a 1.8 Mb region. The results suggest that despite its clinical heterogeneity, DBA is genetically homogeneous for a gene in 19q13.

Etiology and Pathogenesis

The cellular basis for most cases of DBA is becoming much clearer due to improved cell fractionation techniques, chemically defined tissue culture medium, recombinant growth factors, and molecular technology. Initial reports [198] [199] [200] [201] [202] [203] of humoral, cellular, or microenvironmental inhibitors of erythropoiesis in DBA could not be confirmed.[192] [204] [205] [206] [207] [208] [209] [210] [211] [212] Claims for immune-mediated erythroid suppression in DBA would need to satisfy these strict criteria: patients must be newly diagnosed, untreated, and not previously transfused; the in vitro testing system must be completely autologous using only patient cells and serum; and any degree of inhibition must be totally selective for the erythroid lineage. Since almost none of the reports met these criteria, the notion of an immune pathogenesis of DBA shifted to alternate possibilities.

A large body of evidence indicates that the erythroid progenitor compartment is intrinsically defective in DBA. [194] [204] [205] [206] [207] [208] [209] [210] [211] [212] Cultures of DBA marrow using standard clonogenic assays for CFU-E and BFU-E progenitors consistently have shown reduced or absent colonies in most DBA patients, and intermediate, normal, or occasionally increased numbers in the rest. The DBA erythroid progenitors are relatively insensitive to EPO in vitro [209] and to burst-promoting activity,[213] but the hyporesponsiveness to EPO can be corrected in some cases by the addition of glucocorticoids in vitro [214] or by clinically administering prednisone.[215]

The data underscore the fact that the intrinsic defect of DBA erythroid progenitors is an inability to respond normally to inducers of erythroid proliferation and/or differentiation. Confirmation of the overall defect was demonstrated by Bagnara et al. who showed that CD34+ DBA progenitors differentiated normally along megakaryocytic and granulocytic pathways but aberrantly along the erythroid lineage. [216] Accelerated programmed cell death (apoptosis) may play a role in this pathogenesis.[217] Based on the various patterns of erythroid colony growth seen with DBA patients, a model [192] for the aberrant erythropoiesis was developed that proposes maturational arrests at varying sites along the differentiation pathway.

Studies indicate that recombinant interleukin-3 (IL-3) [218] and Steel factor (SCF) in combination with EPO[219] [220] [221] may increase

the in vitro clonogenicity of DBA bone marrow progenitors from unfractionated cell preparations. Halperin et al. reported that the size and number of DBA BFU-E colonies were dramatically increased by adding IL-3 to the cultures, [218] and Abkowitz et al.[219] showed a growth response of BFU-E from 4 patients to SCF alone or in combination with IL-3, GM-CSF, or conditioned media. Bagnara et al. [216] described a lack of response of enriched CD34+ progenitors to IL-3, GM-CSF and erythroid potentiating activity (EPA) in 10 DBA patients, but SCF in combination with IL-3 promoted BFU-E colony growth in 3 patients. Olivieri et al. [220] observed heterogeneity in vitro in 10 patients with normal, intermediate or absent responses to SCF. Alter et al. [221] found that 15 of 16 DBA marrow cultures had increased erythropoiesis, often to normal levels, in the presence of SCF compared to cultures lacking SCF. The human ligand for flt-3 apparently has no effect on DBA marrow colony growth. However, addition of IL-9 to SCF, IL-3 and EPO does potentiate DBA BFU-E growth. [222]

Casadevall et al. discovered that there are significant age-related changes in erythroid and granulopoietic progenitors in a large series of DBA patients. [223] Despite profound anemia, 7 of 10 patients studied within 1 year of diagnosis had normal numbers of CFU-E and BFU-E that showed a normal response to cytokines. In contrast, 12 of 14 patients followed for more than 3 years had decreased erythroid progenitors and, in 7 cases, decreased CFU-GM. The data are consistent with the idea that the DBA defect involves a pluripotent progenitor and worsens with time.

Strong support for this conclusion comes from a detailed study that examined the interaction between DBA CD34+ cells and the hematopoietic microenvironment using long-term bone marrow cultures.[224] Stromal adherent layers from DBA patients did not show evidence of any morphological, phenotypic, or functional abnormality and the stroma sustained the proliferation of normal control CD34+ cells. A major finding in this study was an impaired capacity of DBA CD34+ cells in the presence of normal stromal cells to proliferate and differentiate along not only the erythroid pathway but also along the granulocytic-macrophage pathway. These results indicate an intrinsic defect of a hematopoietic progenitor with at least bi-lineage potential that places it earlier than previously suspected and which was only unmasked by testing in long-term cultures. The findings broaden the definition of DBA and can explain some generalized hematological abnormalities and marrow dysfunction in DBA that have puzzled investigators for years.

These in vitro findings raised speculation that DBA is due to one or more receptor-ligand abnormalities involving various growth-promoting cytokines such as EPO, IL-3 and SCF. Thus far, studies have failed to identify any of these putative abnormalities. DBA lymphocytes produce high levels of IL-3, DBA marrow stromal cells express mRNA for SCF, and DBA patients have measurable serum levels of SCF and have SCF receptors on marrow cells.[225] Moreover, molecular studies have not disclosed mutations in genes for c-kit (SCF receptor) or SCF.[226] [227] [228] Thus, a revised hypothesis is that an intracellular defect exists in signal transduction or in a transcription factor acting early in differentiation. Such anomalies could account for the increased tendency of DBA erythroid progenitors to undergo apoptosis upon EPO deprivation in vitro.[217] A role for induction of apoptosis by the fas-fas ligand system in DBA was also recently suggested because of elevated serum soluble fas ligand in patients compared to controls.[229]

Clinical Manifestations

Aside from findings associated with anemia, about one-half of infants at presentation look healthy otherwise and normal physically. Unless the patient develops cardiac failure as a result of anemia, hepatosplenomegaly and edema are absent.

Both sexes are equally affected. Although the majority of reported patients are white, DBA has been recognized in several ethnic groups, including African Blacks, Arabs, East Indians, and Japanese. Pregnancy, birth history, or both are often abnormal. There is a slightly higher incidence of previous stillbirths or miscarriages among patients' mothers than in the general population. Approximately 10% of the mothers present with obstetrical complications, such as preeclampsia, premature separation of the placenta, or breech presentation. Intrauterine growth retardation has been found in more than 10% of reported patients.

Approximately 50% of the patients present with one or more congenital defects.[193] [194] Most of these phenotypic abnormalities belong to the following categories: (a) craniofacial dysmorphism, including hypertelorism, microcephaly, microphthalmos, congenital cataract or glaucoma, strabismus, microretrognathism, and a high-arched palate or cleft palate; (b) prenatal or postnatal growth failure, independent of steroid therapy; (c) neck anomalies; these may consist of a pterygium coli, or the fusion of cervical vertebrae with flaring of the trapezius muscle (Klippel-Feil syndrome) giving a Turner's syndrome appearance; there may also be the Sprengel deformity (congenital elevation of the scapula) as an isolated anomaly, or a combination of the two anomalies; and (d) thumb malformations, such as bifid thumb (Fig. 18-4), duplication, subluxation, hypoplasia, or absence of the thumb. The characteristic association of triphalangeal thumbs with congenital red cell aplasia has been described in a dozen patients (Fig. 18-5). Although often referred to as Aase syndrome or Aase-Smith syndrome, [230] [231] [232] [233] [234] this entity clearly belongs within the clinicohematologic framework of

Figure 18-4 Bifid thumb in Diamond-Blackfan anemia.

Figure 18-5 Radiograph of a triphalangeal thumb in Diamond-Blackfan anemia.

DBA. In addition, some patients have a flat, hypoplastic thenar eminence, weak or absent radial pulses, or both, which probably represent variations of the thumb malformations.

Some patients have a characteristic facial appearance. The facies of DBA is said to consist of tow-colored hair, snub nose, wide set eyes, thick upper lip, and an intelligent expression. Another facies[192] observed in two unrelated girls of markedly different ancestries consists of small heads, almond-shaped eyes with a slight antimongoloid slant, a carp-like smile, and pointed chins. These patients resemble each other more than they resemble their own family members (Figs. 18-6 A and 18-6 B).

Various other anomalies are occasionally reported in association with DBA. There may be urogenital malformations, such as dysplastic or horseshoe kidneys, duplication of ureters, or renal tubular acidosis. There may also be congenital heart disease, mainly ventricular and atrial septal defects, or hypogonadism, ear malformations, mental retardation, congenital hip dislocation, cartilage-hair hypoplasia with T-cell dysfunction, or tracheoesophageal fistula.

Laboratory Evaluation

The main hematological findings in DBA are summarized in Table 18-5 . The anemia is usually profound at the time of diagnosis. Hemoglobin levels average 6.5 g/dl in patients diagnosed in the first 2 months of life (range of 1.79.1 g/dl) and 4.0 g/dl (range of 1.87.4 g/dl) in those diagnosed later. Macrocytosis is seen in the vast majority of patients, the mean corpuscular volume (MCV) being above the expected values for age. The peripheral blood smear may show, in addition to macrocytes, a mild degree of anisocytosis and poikilocytosis, but these findings are not specific. The aregenerative component of the anemia is reflected by the absence of both polychromasia and nucleated red cells on the blood film. Decreased red cell production is confirmed by absence of a reticulocyte response and by characteristic findings on bone marrow examination.

In over 90% of the patients, the bone marrow aspirate is normocellular, but erythroblasts are markedly decreased or absent.

Figure 18-6 Similar Diamond-Blackfan facies in two unrelated girls of different ancestries consisting of a small head, almond-shaped eyes with slight antimongoloid slant, a carp-like smile, and a pointed chin.

TABLE 18-5 -- Hematological Features of Diamond-Blackfan Anemia at Diagnosis

Hematological Parameters	Findings in DBA
Mean (range)	
Hemoglobin value	

Newborns under 2 months of age[a]	6.5 g/dl (range of 1.79.1 g/dl)
Children 2 months of age or older[a]	4.0 g/dl (range of 1.87.4 g/dl)
Mean corpuscular volume (MCV)	Usually increased for age
Reticulocytes	Markedly decreased (<1%)
White cell count	Normal or slightly decreased
Platelet count	Normal or increased
Fetal hemoglobin	Increased (>5% after 6 months of age)
Red cell i antigen	Expression increased beyond first year of life
Red cell enzymes	Fetal pattern
Red cell adenosine deaminase activity	Elevated in 4090% of cases
Bone marrow morphology	
In >90% of cases	Marked reduction or absence of erythroid precursors
In 510% of cases	Slightly reduced or normal number of proerythroblasts with or without maturation arrest
In all patients	Normal cellularity; normal myeloid and megakaryocytic lineages

[a]Toronto series (n = 21).

Proerythroblasts, if present, account for less than 3% of all nucleated elements, with a myeloid to erythroid ratio of >10:1. In 510% of cases, proerythroblasts may be present in normal numbers, with or without a maturation arrest. The other cell lines are normal. White cell counts and platelet counts are usually normal at diagnosis but platelets may be increased and with normal function.[235] Mild to moderate neutropenia, thrombocytopenia, or both may occur later in the course of the disease, particularly in multitransfused patients who have hemosiderosis and secondary hypersplenism. Progression of the single-lineage erythroid deficiency of DBA into pancytopenia and severe aplastic anemia is rare but occurs.[194] [236] In the Toronto series,[192] one patient met all criteria for DBA during the first 2 years of life. This child later developed severe aplastic anemia and succumbed at 3½ years of age after two unsuccessful attempts at bone marrow transplantation.

Erythrocytes in DBA express a number of fetal characteristics.[237] The level of hemoglobin F (Hb F) is increased persistently, even during remission. It remains at a level of 510% after the age of 6 months and has a heterogeneous distribution in red cells. The HbF has a specifically fetal amino-acid profile, with a high glycine to alanine ratio (G to A). Similarly, the i antigen, which normally disappears from the erythrocyte surface by 1 year of age, is expressed at near fetal levels in older patients with DBA. The i antigen, however, can be detected simultaneously at normal adult levels.

The precise cause of this fetal-like erythropoiesis still requires clarification.[238] [239] It is clearly distinct from the fetal erythropoiesis implicated in various types of leukemia, notably in juvenile chronic myelogenous leukemia, in which the fetal red cells presumably arise from the leukemic clone. The situation in DBA may be analogous to that in other forms of bone marrow failure and in the hematological recovery phase following bone marrow transplantation.[238] In all of these conditions, the fetal (or stress) erythropoiesis may represent an accelerated recapitulation of red cell ontogeny in the face of an increased demand for new red cells in peripheral blood.

Red cell enzymes often display an abnormal pattern of activity.[240] Enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase, or glutathione peroxidase have increased activity in patients with DBA compared to those in normal children and adults or in patients with transient erythroblastopenia of childhood. For some enzymes, this increased activity is comparable to that in cord blood red cells. In apparent contradiction, carbonic anhydrase isoenzyme B, which is not normally present in fetal red cells, was detected in hemolysates from three patients with DBA.[237] Also, the red cells of two of the three patients had adult hexokinase isoenzyme distribution by isoelectric focusing.

Abnormalities in purine and pyrimidine metabolism are reflected by increased activity of red cell adenosine deaminase (ADA) in many patients with DBA.[241] [242] [243] [244] Also, increased orotidine decarboxylase (ODC) activity is seen in some patients.[244] ADA activity is raised in DBA erythrocytes, but not in cord blood red cells from normal newborns or from patients with any of several hematologic conditions associated with stress erythropoiesis. Thus, this enzymatic abnormality cannot be due simply to a reversion to fetal erythropoiesis. Red cell ADA activity was initially reported to be elevated in up to 90% of patients with DBA and to be elevated occasionally in some of their nonanemic relatives. However, in subsequent studies, ADA levels were found to be raised in only 40% of patients. Raised ADA activity may also be detected in some hemolytic anemias and acute leukemias, which limits the utility of this assay as a specific diagnostic marker for DBA. However, increased ADA activity does appear to be useful in differentiating DBA from transient erythroblastopenia of childhood on a biochemical basis.[240] [241] [242] [243] [244]

Serum levels of various factors involved in red cell production, such as erythropoietin, iron, vitamin B_{12}, and folate are appropriately elevated in DBA. These findings are compatible with any form of chronic hypoplastic anemia. Riboflavin levels are normal in the serum, but not in the erythrocytes. This observation initially aroused interest since experimental riboflavin deficiency may be corrected by corticosteroids similar to DBA. However, administration of large doses of riboflavin to several DBA patients did not result in a hematopoietic response.

Red cell serology is usually unremarkable at the time of diagnosis, but alloantibodies are frequently detected in chronically transfused patients. Two infants developed hypoplastic anemia, either in conjunction with or following a bout of Rh hemolytic disease.[245] [246] In one of them,[245] the hypoplastic anemia was transient. It was proposed that the Rh antibody had specificity not only for mature red cells but also for reticulocytes and erythroid progenitors, thereby causing temporary erythropoietic suppression of the bone marrow. The second patient[246] presented with a brisk hemolytic anemia that initially masked the presence of DBA. DBA became manifest 5 weeks postnatally when the Rh hemolytic disease had resolved.

Differential Diagnosis

In clinical practice, after excluding a viral etiology, particularly parvovirus B_{19}, transient erythroblastopenia of childhood (TEC) is usually the only diagnosis that is confused with DBA (Table 18-6). Both entities share the same morphologic findings in the bone marrow. However, TEC is a self-limited disorder with an excellent prognosis and needs no specific therapy except for red cell transfusions in the most profoundly anemic patients.[247]

The definition of TEC includes the following features: (1) gradual onset of pallor in previously healthy children 14 years of age (85% of cases), with older and younger exceptions; (2) normochromic-normocytic anemia with varying reticulocytopenia unless recovery has already ensued; (3) marrow erythroid hypoplasia (60% of cases), or aplasia (10% of cases), or a recovery picture (30% of cases); (4) spontaneous recovery usually

TABLE 18-6 -- Distinguishing Features Between Diamond-Blackfan Anemia (DBA) and Transient Erythroblastopenia of Childhood (TEC)

	DBA	TEC
Cause	Congenital/inherited	Acquired
Previous history	None	Viral infection
Anomalies	30%	0%
Neurological findings	None	Occasional

Immune-mediated	Rare	Common
Course	Chronic	Transient
Risk of leukemia	Increased	Not increased
Mean age (range)	6 months (172)	27 months (1192)
Laboratory findings at diagnosis		
RBC size	Macrocytic	Normocytic
Hb F	Increased	Normal[a]
i antigen	Increased	Normal[a]
RBC enzyme activities	Fetal levels	Adult levels[a]
RBC adenosine deaminase	Increased	Normal

[a]During recovery, values may be increased.

within 48 weeks without recurrence, with rare exceptions. [248]

There are some additional important features of TEC. It can occur in siblings simultaneously and in seasonal clusters from June to October and from November to March. Of concern are the transient neurologic changes that can occur with TEC and which appear to be linked to the disorder. Affected children may have one or more of the following: hemiparesis, papilledema, abnormal extraocular movements, seizures, and unsteadiness of gait. The affected patients in the published reports recovered without sequelae, and the precise relationship of these neurologic changes to the pathogenesis of TEC has not been determined.

It was claimed initially that only the erythroid lineage was affected in TEC and all other hematopoietic lineages were normal. Although the mechanism is not clear, significant neutropenia also occurs in many patients with TEC, being associated in some with hypocellular marrows or with a granulopoietic maturational arrest. The neutropenia may be due to a common pathogenetic mechanism that produces anemia. Increased numbers of CD10+ (CALLA) lymphoid cells have been observed in bone marrow of some TEC patients but the interpretation of this finding is not clear. [249] [250] An unusual presentation of TEC as a leukoerythroblastic anemia has been recorded.[251]

There are no data that firmly incriminate parvovirus or other infectious agents in the etiology of TEC, although a history of a preceding viral-like illness can be obtained in more than half of the patients. The most plausible explanation proposed to date is that TEC is due to transient immunosuppression of erythropoiesis, [252] and possibly of granulopoiesis in those with neutropenia. Most supportive evidence for this thesis comes from in vitro studies. Both Koenig et al. [253] and Dessypris et al.[254] demonstrated an inhibitory effect of TEC serum and fractionated IgG on erythroid colony growth that disappeared as TEC improved. Freedman and Saunders also demonstrated an IgG inhibitor of erythropoiesis in one case, but also an IgM inhibitor in a second patient. [252] A summary of other published studies[255] suggests that over 60% of TEC patients have autologous or allogeneic serum inhibitors of erythroid colony formation. Autologous or allogeneic cell-mediated immune suppression of erythropoiesis has also been identified in about 25% of cases. [252] [255] [256] All of the in vitro studies have generated varying patterns of erythroid colony growth in TEC. Colony numbers can be normal, but reduced numbers of BFU-E and CFU-E progenitors have been recorded in 30% and 50% of cases, respectively.

Therefore, TEC is likely to have an immune etiology. The transient nature of TEC is similar to other autoimmune hematological disorders of childhood, such as idiopathic thrombocytopenic purpura and some cases of autoimmune hemolytic anemia. The decreased activities of virtually all red cell enzymes in TEC compared to normal levels probably relate to the aged population of peripheral blood erythrocytes being tested. [247]

Regarding viral causes of red cell aplasia, Epstein-Barr virus, hepatitis virus, human T-cell leukemia virus-1, and human immunodeficiency virus-1 have all been implicated and should be excluded if the etiology of the anemia remains unclear. Parvovirus B_{19} stands out as a major causal agent of red cell aplasia in the context of an underlying chronic hemolytic anemia in infants and children with chronic congenital and acquired forms of immunosuppression. The fetus is uniquely susceptible to parvovirus infection and in utero transmission is a well-documented cause of nonimmune hydrops fetalis. Parvovirus infection should be ruled out in every case of childhood red cell aplasia by serial measurements of serum IgM and IgG, and by bone marrow examination for the characteristic giant pronormoblasts. Parvovirus may also be detected in marrow by gene amplification using the polymerase chain reaction and confirmed by direct in situ hybridization.

Predisposition to Leukemia

Although not generally regarded as a pre-leukemic condition, DBA is also associated with malignant myeloid transformation, like some of the other inherited marrow failure syndromes.[236] As summarized by Lipton and Alter,[257] acute leukemia and/or MDS have been reported in eight patients with DBA. One girl developed ALL at age 13 after a spontaneous remission of DBA at 5 years of age; the leukemia also remitted completely with therapy, and neither disorder was present at age 17. Two patients originally described by Diamond had intermittent remission of DBA but died of AML at ages 31 and 43, respectively. One of them had received thymic and skeletal irradiation during childhood as therapy for DBA. A girl who received cyclophosphamide for treatment of DBA died of acute promyelocytic leukemia at age 13. A boy who developed acute megakaryoblastic leukemia at age 14 months had anemia at 2 months of age; this may have been a long preleukemic phase. Three male steroid-nonresponders developed MDS at ages 13, 21, and 22 years, respectively. One evolved into AMML, one into AML, and the third patient died of complications of MDS. Eight additional patients developed various other cancers. [3]

Thus, of 379 published cases of DBA,[49] the number of cases of myeloid malignant transformation (eight cases, 2% incidence) is inordinately high and may even be higher as new cases[258] are diagnosed and reported. The link between disordered erythropoiesis and myeloid malignant disease is more understandable

now with the advent of new information, described in this chapter, implicating an earlier marrow progenitor than was previously thought in the pathogenesis of DBA.

Therapy and Prognosis

In younger children and infants, it is important to determine whether the red cell aplasia is DBA or TEC (Table 18-6). Until a firm diagnosis is established, initial treatment in children is almost always transfusional. This allows the flexibility to complete the viral work-up and other investigations, and to observe for a spontaneous remission if the anemia is due to TEC or another self-limited condition. The principle to follow if transfusions are used is to aim for a moderate but not full correction of anemia so that erythropoiesis is not suppressed and recovery from TEC not delayed. Most patients with TEC usually recover within a few weeks after receiving only one transfusion. Occasionally, recovery from TEC is slow and starts to mimic DBA in chronicity. If there is confusion about the proper diagnosis, it is appropriate to withhold corticosteroids in favor of a further transfusion in order to allow more observation time.

Transfusions

Before the first transfusion it is recommended that a full red cell phenotype be performed on patients' cells. This information will be valuable for prevention and management of alloantibody formation due to sensitization.

For patients in whom corticosteroids are either ineffective or excessively toxic, a regular program of periodic red cell transfusions is usually required. During the course of this program, a small number of patients may recover sensitivity to corticosteroids, or even proceed to a spontaneous transient or prolonged remission. Packed red cells are given monthly and this treatment is aimed at keeping the hemoglobin concentration at a level compatible with normal activity. Several complications may arise from transfusions, such as cytomegalovirus or other viral infections and sensitization, but the major long-term threat is iron overload which causes delayed puberty, growth retardation, diabetes mellitus, hypoparathyroidism, and eventually liver cirrhosis and cardiac failure. These complications can be

delayed, and possibly prevented, by the early administration of an iron chelator. Deferoxamine, administered as a daily 12-hour subcutaneous infusion, is the main chelator in use today.

There are uncertainties about the optimal age at which to start deferoxamine for patients with transfusion-dependent anemia. There have been reports of abnormal linear growth and metaphyseal dysplasia in thalassemia major patients treated with deferoxamine before the age of 3 years. [259] This adverse event has prompted recommendations for starting therapy later. However, a progressively rising serum ferritin level or, more accurately, excessive hepatic iron concentration obtained by biopsy after 1 year of regular transfusions would be appropriate indications to commence chelation. The daily starting subcutaneous infusion dose of deferoxamine should not exceed 50 mg/kg. Ascorbate supplementation should be considered if there is sustained loss of efficacy of deferoxamine, especially if tissue ascorbate concentrations are reduced. [260]

Corticosteroids

It is estimated that steroid responsiveness is present in 5075% of DBA patients. Upon administration of prednisone at a dose of 2 mg/kg/day in three divided doses, reticulocytosis is usually seen within 14 weeks, and is followed by a rise in hemoglobin concentration. Once the hemoglobin level reaches 9.010.0 g/dl, prednisone can be slowly tapered by reducing the number of daily doses. If a single daily dose of prednisone maintains the desired hemoglobin level, the dose can be doubled and given on alternate days. This schedule presents less risk of steroid-induced growth retardation.

The dose of prednisone can be further reduced by small decrements on a weekly basis, or more slowly, until the minimal effective dose is determined. This dose is extremely variable. A few patients can be maintained on minute, nonpharmacologic doses, whereas other patients need large doses that preclude long-term therapy because of side effects. There is no known predictor of steroid responsiveness, nor any way to anticipate the type of individual responses.

Alter recognized several patterns of response to corticosteroid therapy, some of which may occur at different times in the same patient. [49] Most children who respond to steroids cannot be completely weaned off the medication and become steroid-dependent. A few of these patients, however, enter steroid-free remission after a prolonged period of treatment. Between 15% of cases promptly respond to therapy and enter a durable steroid-independent remission. However, later relapses, sometimes precipitated by an infectious illness or by hormonal changes such as in pregnancy or with the use of birth control pills, are not uncommon. In other cases, a progressive resistance to steroids occurs, requiring escalating doses of prednisone or alternative therapy. Following a relapse, some patients are responsive to steroids again, whereas others are refractory to subsequent trials. Initial insensitivity to steroids is observed in 25% of cases, and in as many as 60% of patients long-term steroid therapy is hampered by the development of resistance or by side effects of the treatment.

High-Dose Methylprednisolone

Long-term experience with megadose steroid therapy for patients refractory to conventional prednisone treatment has been largely confined to a single center, which reported a sustained erythroid response leading to transfusion independence in 8 of 13 DBA patients. [261] Eleven had been treated with 100 mg/kg/day intravenously, and 2 additional patients with 30 mg/kg/day orally. Another study at a second center [262] showed a transient response in 1 of 8 patients after intravenous treatment with 30 mg/kg/day and a sustained response after a higher dosage (100 mg/kg/day) in 3 of 8 patients. In the latter study, side effects were weight gain, oral moniliasis, increase in hepatic transaminases, transient hyperglycemia, and bacteremia related to central venous access. Thus, high-dose steroid therapy may have a therapeutic role but is not without risk.

Cytokine Therapy

Because of the corrective effect on erythropoiesis by IL-3 in vitro cited herein, clinical trials were introduced for steroid-refractory and steroid-dependent DBA patients and for those in whom BMT was considered too risky. [263] [264] [265] [266] [267] The early enthusiasm generated by sustained remissions in some patients [263] has largely been tempered by the realization that IL-3 is likely to be effective in only a very small number of cases of steroid-refractory, transfusion-dependent DBA. [264] [265] [266] [267] To definitively answer questions regarding the therapeutic role of IL-3 for DBA, 49 patients were treated with the cytokine in a European multicenter compassionate-need study. [266] IL-3 was given as a daily subcutaneous injection at a starting dose of 2.5 g/kg, escalating at day 21 to 5 g/kg, and then to 10 g/kg if there was no response, for a total duration of 12 weeks. Three children had a significant response, achieving sustained remissions off therapy. At the time of entry into the study, one was steroid-responsive and transfusion-independent and two were transfusion-dependent. Two adults had a transient reduction in transfusion requirements but could not tolerate the complete course of therapy. Eosinophilia was common; neutrophil and platelet counts were unaffected except in three patients in whom previously-noted mild thrombocytopenia was transiently exacerbated. Clinical response to IL-3 did not correlate with in vitro culture results. A comparison of individual patient

STANDARD TREATMENT OF DIAMOND-BLACKFAN ANEMIA (DBA)

Until the diagnosis of DBA is certain, the initial approach is to transfuse packed red cells for a moderate but not full correction of anemia. If the diagnosis is TEC and not DBA, full correction of anemia may suppress erythropoiesis and delay recovery of TEC. A red cell phenotype should be performed on patients' cells before the first transfusion to guide in the management of potential alloantibody formation in transfusion-dependent patients at a later date.

Oral corticosteroids are the mainstay of therapy for DBA. Initial treatment is usually prednisone, 2 mg/kg/day in three divided doses and should be maintained for at least 4 weeks. A prednisone-equivalent preparation can also be used because no difference in efficacy has been observed with different formulations, but this is a less popular approach. A reticulocytosis is usually seen within 14 weeks, followed by a rise in hemoglobin. The increment in hemoglobin may be partial, whereby transfusion requirements are decreased but not eliminated, or complete. When the hemoglobin value reaches 9.010.0 g/dl, the steroid dose should be gradually reduced to determine the minimum dosage required to sustain the response. If a single daily dose of prednisone is effective, the dose can be doubled and given on alternate days. Prednisone should be stopped in non-and partial-responders as well as full-responders who require a high daily maintenance dose of >0.5 mg/kg/day. Long-term treatment of responders may be precluded by unacceptable side effects including growth retardation, which already can be a concern as part of the clinical phenotype of DBA. Growth curves should be closely monitored and the occurrence of a plateau should prompt substitution of steroid therapy with a transfusion program in order to allow catch-up growth, especially in the first year of life and at puberty.

For DBA patients in whom steroids are too toxic or ineffective, a regular program of periodic red cell transfusions should be introduced. Packed cells, 15 cc/kg, are given monthly, on average, to maintain a hemoglobin level that allows optimal growth and normal activity. Body iron stores must be monitored by serum ferritin levels every 3 months. A progressively rising ferritin level or, more accurately, an excessive liver iron concentration obtained by biopsy after 1 year of regular transfusions, is an appropriate indication to start iron chelation. Deferoxamine, 50 mg/kg, is given daily for this purpose by subcutaneous infusion over 812 hours using a battery-powered infusion pump. Oral vitamin C supplements should be considered (100 mg/day) if there is sustained loss of efficacy of deferoxamine, especially if tissue ascorbate concentrations are reduced.

Except for conventional prednisone therapy or chronic transfusion-chelation management, the other treatments described in the text should still be considered experimental. These include high-dose methylprednisolone intravenously and orally, subcutaneous IL-3 therapy, and BMT. For all of these options, risks must be weighed against benefits on a case-by-case basis.

characteristics with previously reported series confirmed earlier impressions that patients who had never achieved significant in vivo erythropoiesis in response to steroids or during a spontaneous remission were highly unlikely to respond to IL-3. Thus, the overall response rate in all published studies averages 1020%, and there may be a sustained remission off steroids in children who are steroid-dependent and transfusionindependent at the time of IL-3 therapy, suggesting a possible role for a short course of IL-3 earlier in the treatment of children with steroid-responsive DBA.

Trials with recombinant stem cell factor are awaited on the basis of in vitro culture results. However, to extrapolate from experience with IL-3, it is unwise to assume that in vitro results can predict clinical response. It is also likely that the presence of low-level in vivo erythroid activity is a prerequisite for a clinical response to SCF. EPO serum levels are elevated in DBA and attempts at treatment with high-dose EPO have been ineffective.

Bone Marrow Transplantation

BMT is a therapeutic option for DBA but risks must be weighed against benefits on a case-by-case basis. The fundamental issue centers on the defined mortality rate with BMT when used for a non-lethal medical condition, at least one that is non-lethal in the short-term. In steroid-responsive patients on low-dose maintenance, and in properly transfused and adequately chelated patients, quality of life is not threatened by life-threatening complications. Thus, the decision for intervention with BMT in this setting is difficult.

Nevertheless, experience has broadened since the first BMT was introduced for DBA in 1976. [268] Preparative regimens, supportive measures, and graft-versus-host disease management have progressively become more refined, thereby reducing overall risks of the procedure. But there are still major risks. Of 16 reported DBA cases receiving BMT, [268] [269] [270] [271] [272] [273] [274] [275] four patients died. Of these, two had interstitial pneumonia, one had cardiac failure, and one developed lethal complications associated with chronic graft-versus-host disease. Of the survivors, acute and chronic graft-versus-host disease was observed in nine and seven patients, respectively. To circumvent the graft-versus-host disease complications, there have been recent attempts to use cord blood stem cells from HLA-matched siblings as a donor source with apparent success. [276] [277]

Other Therapeutic Options

A number of uncontrolled therapeutic trials have been performed in steroid-refractory patients using various medications and treatments with varying success in a few patients. [278] [279] [280] [281] [282] [283] [284] The medications include: androgens, riboflavin, vitamin B_{12}, folate, iron and other hematinic agents, 6-mercaptopurine, [278] cyclophosphamide with antilymphocyte globulin, [280] antithymocyte globulin alone, [282] and cyclosporin A. [283] [284] Plasmapheresis has also been tried. [281] Splenectomy, employed in the past, shows no effect on erythropoiesis but may be helpful in transfused patients with proven hypersplenism.

Kostmann's Syndrome/Congenital Neutropenia

Introduction

Severe chronic neutropenia (SCN) and recurrent serious infections are features of a heterogeneous group of disorders of myelopoiesis including congenital neutropenia, cyclic neutropenia, and idiopathic neutropenia. Kostmann's syndrome (KS) is a subtype of congenital neutropenia inherited in an autosomal recessive manner with onset in early childhood of profound neutropenia (absolute neutrophil count <200/L), recurrent life-threatening infections, and a maturation arrest of myeloid precursors at the promyelocyte-myelocyte stage of differentiation. Congenital neutropenia and KS have the same hematologic phenotype and clinical presentation. The recessive inheritance of KS is deduced by inference when there is more than one affected child in a family. Congenital neutropenia is the proper designation used for a single sporadic case in a family, and may or may not be inherited in an autosomal recessive manner

like KS. Since the molecular defect is not known for either diagnostic category, the option to lump or split the two disorders remains a subject of argument. In this chapter, the terms KS and congenital neutropenia are used interchangeably.

The Severe Chronic Neutropenia International Registry (SCNIR) was established in 1994 to catalogue the clinical features and to monitor the clinical course, treatment, and disease outcomes in patients with SCN.[285] [286] [287] [288] [289] [290] [291] [292] [293] [294] The Registry is a valuable resource for clinical data because of the large numbers of patients entered into its worldwide database. Patient data are submitted internationally to the coordinating centers at the University of Washington, Seattle, and the Medizinische Hochschule, Hannover. In late 1996, short-term and long-term information dating back to 1987 on a total of 506 patients was available for analysis. Of the total, 249 patients were classified as having congenital neutropenia including KS.

Etiology and Pathogenesis

Initial studies of KS CFU-GM progenitor growth in vitro yielded variable data. [295] [296] [297] [298] [299] [300] [301] [302] [303] [304] [305] [306] [307] [308] [309] [310] [311] [312] [313] [314] [315] [316] [317] [318] [319] CFU-GM numbers can be decreased or increased, but are usually normal, and the colonies are comprised of myeloid elements arrested in differentiation, thereby mimicking the disease in vitro. Some patients, however, yield CFU-GM colonies with mature neutrophils. [301] [304] KS serum is not inhibitory to CFU-GM growth. Long-term cultures from some patients also show decreased myeloid differentiation. [307]

More recent studies of stromal and marrow mononuclear cells from patients with KS suggest that the primary defect is at the level of the myeloid progenitor. In particular, these experiments have shown normal serum G-CSF levels, normal G-CSF production by marrow stromal cells ex vivo, a normal or increased number of G-CSF receptors on hematopoietic cells, and defective myeloid colony growth. [312] [315] [316] [317] The G-CSF receptor contains discrete cytoplasmic domains that transduce proliferative and differentiating signals. [318] [319] [320] Dong et al. found a mutation that removed the carboxy tail of the G-CSF receptor in a congenital neutropenia patient after developing MDS [321] (see section, Predisposition to Leukemia). This truncated receptor retained the ability to stimulate cell growth, but deleted the differentiation domain. The G-CSF receptor mutation was restricted to the myeloid lineage and was not detected in DNA extracted from other tissues. The absence of germ-line mutations of the G-CSF receptor gene is consistent with the concept that the neutropenia of KS is not caused by this mechanism.

Clinical Manifestations

KS, or infantile genetic agranulocytosis as described in 1956 [322] was recognized in an original cohort of 14 cases and in a subsequent 10 additional patients [323] belonging to a large intermarried kinship in northern Sweden. The mode of inheritance in these original patients was clearly autosomal recessive. Subsequently, the disorder has been recognized widely despite its rarity and in various ethnic groups including Asians, American Indians, and Blacks. Consanguinity is not a uniform finding in cases enrolled in the SCNIR.

About half the patients develop clinically impressive infections within the first month of life and almost all others develop them by 6 months. Skin abscesses are common but deep-seated tissue infections and blood-borne septicemia also occur. SCNIR data illustrate examples of every conceivable form of bacterial and sometimes fungal infection in the pre-cytokine era. Especially troublesome in survivors were recurrent episodes of otitis media and pneumonia, advanced gingival-stomatitis, and, in the extreme, gut bacterial flora overgrowth leading to malabsorption requiring total parenteral nutritional therapy. In contrast to some of the other inherited bone marrow failure syndromes, physical malformations are not a feature. Birth weights are generally unremarkable and physical examinations are normal. There are a small number of reports of short stature, microcephaly, mental retardation, and cataracts but the association with KS does not appear to be strong. Data from the SCNIR indicate that some patients with KS develop bone demineralization before and during G-CSF therapy. The underlying pathogenesis is unclear but patients can develop bone pain and unusual fractures.

Laboratory Evaluation

Neutropenia is profound in KS, usually less than 200/L but often absolute. Compensatory monocytosis and sometimes eosinophilia is seen. At diagnosis, platelet numbers and hemoglobin values are normal. In survivors in the pre-cytokine era, anemia of chronic disease associated with recurrent infections and inflammation was common. Aside from neutropenia, humoral and cellular immunology is completely normal.

Bone marrow specimens are usually normocellular. The striking classical finding is a maturation arrest at the promyelocyte or myelocyte stage of granulocytic differentiation. Cellular elements beyond are markedly reduced or totally absent. The other hematopoietic lineages are normal, active, and undisturbed.

Predisposition to Leukemia

There is concern regarding the phenomenon of malignant myeloid transformation in KS patients receiving G-CSF therapy. Of 249 patients in the SCNIR in late 1996 classified as having congenital neutropenia, including KS, 24 developed MDS/AML yielding an overall incidence or crude rate of about 10% with an average follow-up of 5 years. No cases of MDS/AML occurred in the subgroup of congenital neutropenia patients with glycogen storage disease type 1b, nor among those with cyclic or idiopathic neutropenia.

Conversion to MDS/AML in the KS patients was associated with one or more cellular genetic abnormalities which may be useful to identify a subgroup of patients at high risk.[324] Of 24 who transformed in the Registry series, 14 developed partial or complete loss of chromosome 7 (7q- or monosomy 7) in marrow cells; none of the patients who were tested prior to G-CSF therapy had loss of chromosome 7. Activating ras oncogene mutations were discovered in 5 of 10 patients from the series of 24 after the transformation to MDS/AML but not before. Four of these also had monosomy 7. The mutated fragments were cloned and sequenced and showed GGT (glycine) to GAT (aspartic acid) substitutions at codon 12 in all of them (K12ASP in 2 patients and N12ASP in 3 patients). Marrow cells from 5 transformed patients also showed point mutations in the gene for G-CSF receptor resulting in a truncated C-terminal cytoplasmic region of the receptor that is crucial for maturation signalling.[325] [326] Twenty patients without receptor mutations showed no evidence of progression to MDS/AML; however, 4 additional patients have been identified with mutations but without MDS/AML[325] and are currently being closely monitored.

Can G-CSF be implicated in the malignant conversion of congenital neutropenic patients? Development of MDS/AML must be put in the context of the underlying primary problem. Before the availability of G-CSF therapy, it was recognized that leukemic transformation occurs occasionally in patients with KS/congenital neutropenia.[327] [328] [329] [330] However, in the pre-cytokine era, many KS/congenital neutropenia patients died in the first years of life from other causes. Of published cases, 42% of patients died at a mean age of two years secondary to sepsis and pneumonia. [49] Thus, the true risk of congenital neutropenia patients developing MDS/AML was not defined. Currently with G-CSF therapy, most of these patients do not develop life-threatening

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infections and are surviving, but it is not known whether longer survival will allow for the natural expression of leukemogenesis in this population. Moreover, since the long-term effects of G-CSF are barely known beyond 10 years of observation, it is still unclear whether MDS or AML will occur with increased frequency in patients who receive prolonged therapy of G-CSF to correct the neutropenia.

Differential Diagnosis

The commonest cause of isolated neutropenia in very young children is viral-induced marrow suppression. An antecedent history of good health, the occurrence of a viral illness, and the transient nature of the neutropenia distinguishes this disorder from KS/congenital neutropenia. Autoimmune neutropenia of infancy is being recognized more frequently as a fairly specific syndrome of early childhood. Low neutrophil numbers are often discovered during the course of routine investigation for a benign febrile illness. The illness abates but the neutropenia persists, sometimes for months and occasionally longer than a year. A marrow biopsy is normocellular and an aspirate shows active granulopoiesis up to the band stage; neutrophils may be normally represented or reduced. The neutropenia is due to increased peripheral destruction and the diagnosis is confirmed serologically by demonstrating anti-granulocyte antibodies. The prognosis is good, the neutropenia is self-limited albeit protracted, and patients seldom develop serious bacterial infections as a result of it. Other infrequent acquired causes of severe, isolated

neutropenia in this age group include marrow suppression from a drug or toxin, and neutrophil sequestration as part of a hypersplenism syndrome.

Of the inherited forms of neutropenia, Shwachman-Diamond syndrome can also manifest as isolated neutropenia but can be identified because of growth failure, the malabsorption component due to pancreatic insufficiency, fatty changes in the pancreas seen on computed tomography scanning, and characteristic skeletal abnormalities. Neutropenia can also be a prominent part of antibody deficiency syndromes, dysgammaglobulinemia, and agammaglobulinemia; investigation of chronic neutropenia of childhood should include an immunoglobulin electrophoresis. Cyclic neutropenia is distinguished by predictable symptomatology, especially mouth sores every 21 days in classic cases, often associated with chronic gingivitis. A complete blood count three times a week for a month will demonstrate the diagnostic oscillation pattern with the 21 day nadir. Familial cases of neutropenia with an autosomal dominant mode of inheritance are also described; parents of all cases should be screened with a complete blood count.

Therapy and Prognosis

Before the introduction of G-CSF as a specific therapy of KS and other forms of severe chronic neutropenia, there was limited treatment. Antibiotics were the mainstay of management for active infection and for prophylaxis. Attempts to mobilize neutrophils in KS with lithium had limited application. [331] [332]

Cytokine Therapy

G-CSF has supplanted all other forms of management and should be initiated as front-line treatment when the diagnosis is established. [333] GM-CSF in crossover trials with G-CSF for KS is not as effective and does not induce a neutrophil response consistently. The starting dose of G-CSF is 5 g/kg/day subcutaneously and can be escalated until the desired neutrophil number is achieved. Neutrophils higher than 500/L generally afford some protection from infection but counts greater than 1000/L are clearly safer.

Documentation of the efficacy of G-CSF for SCN is described in a definitive phase III randomized controlled trial. [334] The trial enrolled 123 patients (60 congenital, 21 cyclic and 42 idiopathic) of which 120 had evaluable responses. Defining a complete response over the 4 month treatment period and beyond as the maintenance of median neutrophil numbers greater than 1,500/L, 90% of patients showed a complete response to G-CSF therapy, and infection-related events and antibiotic use were significantly decreased. Safety data were analyzed [335] and events related to the treatment were generally mild and consisted of headache, general musculoskeletal pain, transient bone pain, and rash. None of these required the discontinuation of G-CSF. Thus, the vast majority of patients in this phase III trial benefited substantially from G-CSF therapy with minimal adverse or toxic effects and almost all of the originally-treated patients entered a long-term G-CSF maintenance program. The reduction in fevers, infections, and inflammation translated into a well-documented improvement in the quality of life for these patients.

In the SCNIR 1996 annual report (on file at Clinical Safety, AMGEN Boulder Inc., 3200 Walnut Street, Boulder, CO, 80301), the consistent sustained hematological response in patients treated with G-CSF for more than 8 years was confirmed. With therapy, neutrophil counts rose in more than 90% of SCN patients and were maintained at a plateau for protracted periods resulting in vast clinical benefits. In no instance has there been marrow or hematopoietic lineage exhaustion or depletion with G-CSF therapy. The overall safety of long-term administration of G-CSF is reviewed in detail. [333]

Bone Marrow Transplantation

Prior to the use of G-CSF, BMT was tried in a small number of KS patients with mixed success. Data from the International Bone Marrow Transplantation Registry are limited; the small series is heterogeneous with regard to donor source and clinical status of patients at the time of the procedure.

In the SCNIR database, 13 KS patients who developed MDS/AML were transplanted after the transformation. Only three appeared to be cured of MDS/AML and KS. Results may have been better but nine of the procedures were from matched unrelated donors or were performed in desperation using mismatched donors. One of the three who appeared cured by BMT died of liver failure secondary to chronic hepatitis from type C virus infection.

Thrombocytopenia with Absent Radii

Introduction

TAR syndrome was first described in 1929, [336] defined by Hall et al. in 1969, [337] and subsequently reviewed. [338] The two essential features of TAR syndrome are hypomegakaryocytic thrombocytopenia and bilateral radial aplasia. The rest of the phenotype varies widely and can manifest with abnormalities involving skeletal, skin, gastrointestinal, and cardiac systems.

Most of the genetic evidence supports an autosomal recessive mode of inheritance for TAR syndrome because many families have been observed with more than one affected sibling. The possibility of other modes of inheritance for TAR syndrome has been raised. [338] Almost always, parents of TAR patients are phenotypically normal. Females with TAR syndrome can conceive and give birth to hematologically and phenotypically normal offspring.

Aside from the occurrence of one case of acute lymphoid leukemia in a child with TAR syndrome, [339] there is no predisposition to leukemia nor to other bone marrow dysfunction or cancer in this disorder.

Etiology and Pathogenesis

Thrombocytopenia in TAR syndrome is the result of a defect in megakaryocytopoiesis/thrombocytopoiesis. [340] [341] Initial contradictory

data claiming either high levels of megakaryocyte-CSA in sera of TAR patients, [340] [342] or normal levels, [341] have been resolved. Thrombopoietin levels in serum are consistently elevated in TAR syndrome, thereby excluding a cytokine production defect as a cause for thrombocytopenia in this disorder. [343] Also, expression studies of the thrombopoietin receptor, c-mpl, on the surface of platelets from TAR patients is normal and with a similar molecular weight of the receptor compared to controls. [343] Marrow CFU-Meg progenitors are either absent, [340] [342] or are present in normal frequencies but are comprised of colonies in vitro with abnormal morphology. [341] Cells within these colonies are smaller and the number of cells per colony is much higher than in normal CFU-Meg colonies. CFU-GM and BFU-E colony growth is often increased.

Platelet response to adenosine diphosphate or to the thrombin receptor agonist peptide, SFLLRN (TRAP), is normal in TAR patients. [343] However, in contrast to controls there is no in vitro reactivity of platelets from TAR patients to recombinant thrombopoietin as measured by testing thrombopoietin-synergism to ADP and TRAP in platelet activation. [343] Thrombopoietin-induced tyrosine phosphorylation of platelet proteins in this setting is completely absent or markedly decreased. The results indicate that defective megakaryocytopoiesis/thrombocytopoiesis in TAR syndrome is due to a lack of response to thrombopoietin in the signal transduction pathway of c-mpl.

Clinical Manifestations

The diagnosis is made in the newborn period because of the absent radii, and in about half of patients becuase of a petechial rash and overt hemorrhage such as bloody diarrhea. Patients have bilateral radial aplasia (Fig. 18-7) with preservation of thumbs and fingers on both sides. Additional upper extremity deformities include radial clubhands, hypoplastic carpals and phalanages, and hypoplastic ulnae, humeri, and shoulder girdles. Syndactyly and clinodactyly of toes and fingers are also seen. Characteristic findings include a selective hypoplasia of the middle phalanx of the fifth finger and altered palmar contours. Upper extremity involvement ranges from isolated absent radii to true phocomelia, often asymmetric. The lower extremities are involved in about half of cases. Malformations include hip dislocation, coxa valga, femoral torsion, tibial torsion, abnormal tibiofibular joints, small feet, and valgus and varus foot deformities. Abnormal toe placement is commonly seen, especially the fifth toe overlapping the fourth. Like upper limb involvement, lower extremity deformities range from minimal involvement to complete phocomelia. An asymmetric first rib, a cervical rib, cervical spina bifida, and a fused cervical spine can occur, but trunk involvement is usually minimal. Micrognathia

has been associated with the TAR syndrome in up to 65% of cases.

Cardiac abnormalities occur in one-third of the patients. The commonest are atrial septal defect, tetralogy of Fallot, and ventricular septal defect. Facial hemangiomas are common, as well as redundant nuchal folds. Hays et al. [344] described three additional findings in TAR syndrome: dorsal pedal edema, hyperhydrosis, and gastrointestinal disturbances such as diarrhea and feeding intolerance.

There are important clinical differences that distinguish TAR syndrome from Fanconi's anemia. In Fanconi's patients, when radii are absent thumbs are hypoplastic or absent. Fanconi's patients do not have skin hemangiomas like some TAR patients, whereas TAR patients do not show abnormal skin pigmentation like 65% of Fanconi's patients. Confirmation of Fanconi's anemia is made by the clastogenic chromosome stress test showing increased fragility. TAR patients do not have increased chromosomal breakage.

Prenatal diagnosis has been demonstrated readily both by quantitating platelet numbers obtained by fetoscopy or cordocentesis,

Figure 18-7 Radial aplasia with preservation of the thumb in a newborn with TAR syndrome.

and by imaging. In one case after the diagnosis was made, a prenatal in utero platelet transfusion was given to effect a safe delivery. [345]

Laboratory Evaluation

Thrombocytopenia due to bone marrow underproduction is a consistent finding. Marrow specimens show normal to increased cellularity with decreased to absent megakaryocytes. The erythroid and myeloid lineages are normally represented. When a few megakaryocytes can be identified in biopsies they are small, contain few nuclear segments, and show immature nongranular cytoplasm. If platelet counts increase spontaneously in patients after the first year of life, megakaryocytes increase in parallel and appear more mature morphologically. At diagnosis, leukocytosis is seen in the majority of patients and is sometimes extreme to over 100,000/L with a left shift to immature myeloid forms. The cause of this leukemoid reaction is unclear but it is usually transient and subsides spontaneously. If anemia is present, the likeliest etiology is from blood loss due to thrombocytopenia. When platelet numbers are adequate for study, their size is generally normal, with rare exception,[346] and function is unremarkable, [347] [348] [349] although some patients may show abnormal platelet aggregation and storage pool defects. [350] [351] [352] Unlike some of the other inherited marrow failure syndromes, red cell size and fetal hemoglobin levels are normal. Studies of spontaneous and clastogenic-induced chromosome breakage are also normal.

Therapy

The risk of hemorrhage is greatest in the first year of life. Deaths are usually due to intracranial or gatrointestinal bleeding. If patients survive the first year of life, platelet counts spontaneously increase inexplicably to levels that are hemostatically safe and which do not require platelet transfusional support. A minority of patients have sustained, profound thrombocytopenia that does not improve spontaneously.

As in other inherited marrow failure disorders associated with thrombocytopenia, platelet transfusions should be used judiciously. Clinical bleeding or prophylaxis for orthopedic surgical procedures are appropriate indications. Persistent platelet counts below 10,000/L may require preventative platelet transfusions on a regular basis, especially in the first year of life when the expectation is that a spontaneous improvement in platelet number will ensue with time in most infants. Single donor platelets are preferred to multiple unfiltered random donor platelets to minimize the risk of alloimmunization. HLA-partially-matched donors for platelets may be required if patients become refractory to transfusions.

Other Therapies

Because the overall prognosis for survival is good and because patients do not develop aplastic anemia, or leukemia with rare exception, [339] supportive management is the mainstay. In exceptional situations, profound persistent life-threatening thrombocytopenia can be successfully treated by BMT. [353] The role of thrombopoietin in the management of TAR patients is unclear. Elevated serum thrombopoietin levels at baseline [343] may predict for a poor response to cytokine therapy. IL-11, another thrombopoietic cytokine, is yet to be studied in clinical trials; however, endogenous IL-11 serum levels in TAR patients are also elevated. [343] Androgens, corticosteroids, and splenectomy are ineffective therapies for TAR syndrome.

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INHERITED MARROW FAILURE AND MALIGNANT HEMATOPOIETIC TRANSFORMATION

Historically, the inherited marrow failure syndromes were classified as enign hematology, which contrasted sharply with the malignant myeloid disorders. Patients with Kostmann's syndrome/congenital neutropenia, Shwachman-Diamond syndrome, Fanconi's anemia, congenital amegakaryocytic thrombocytopenia, and Diamond-Blackfan anemia often died early in life from complications of their respective disorders. However, in the current era of advanced supportive care and availability of recombinant cytokines and other effective therapeutics, patients with these conditions usually survive the early years of life and beyond. With the extended lifespan of patients, a new natural history for some of these disorders is evident. As recently reviewed, [354] one of the most sobering observations is that most of these benign disorders confer an inordinately high predisposition to MDS/AML. Thus, the distinction between benign and malignant hematology in the context of the inherited marrow failure disorders has become blurred, and a new clinical and hematological continuum is evident.

Carcinogenesis occurs as a sequence of events that is driven by genetic damage and by epigenetic changes. In the traditional view, the initiation of cancer starts in a normal cell through mutations from exposure to carcinogens. In the promotion phase that follows, the genetically altered, initiated cell undergoes selective clonal expansion that enhances the probability of additional genetic damage from endogenous mutations or DNA-damaging agents. Finally, during cancer progression, malignant cells show phenotypic changes, gene amplification, chromosomal alterations, and altered gene expression.

In the inherited marrow failure syndromes described herein, the first hit or cancer-initiating step may be the constitutional genetic abnormality itself that initially manifests as the single-lineage or multiple-lineage myelopathy. The predisposed progenitor, already initiated, could conceptually show decreased responsiveness to the signals that regulate homeostatic growth, terminal cell differentiation, or programmed cell death. The leukemic promotion and progression steps leading to MDS/AML could then ensue readily in the initiated pool of progenitors or stem cells. When the molecular defects that produce the marrow failure syndromes are discovered, the nature of the leukemogenic-initiating events in these conditions should become evident.

Congenital Dyserythropoietic Anemias

Introduction

The designation congenital dyserythropoietic anemia (CDA) refers to a family of inherited refractory anemias characterized by marrow erythroid multinuclearity, ineffective erythropoiesis, and secondary hemosiderosis. [355] [356] The ineffective erythropoiesis is reflected by marrow erythroid hyperplasia, inappropriately low reticulocyte counts for the level of hemoglobin, and intramedullary red cell destruction. Although these disorders are not bone marrow failure syndromes per se, they are genetically transmitted and result in anemia with a blunted erythropoietic response.

Three forms of CDA have been described, as well as a number of variants. An arbitrary classification used in practice is based on the morphologic features and the serologic findings in each case. [357] [358] The distinguishing features of the three main types of CDA are as follows:

Type I: Megaloblastoid changes, macrocytosis, and internuclear bridges.
Type II: Binuclearity, multinuclearity, pluripolar mitoses, karyorrhexis, and positive acidified serum test.
Type III: Multinuclearity with up to 12 nuclei, gigantoblasts, and macrocytosis.

The designation, Type IV, is sometimes used to classify cases of morphologic Type II CDA but with a negative acidified serum test. [359] [360] Reports of some of the variants[361] [362] [363] [364] [365] [366] underscore the complex nature of CDA and the need to reclassify these disorders accurately when the molecular basis for the various types becomes clear. A recent review addresses this issue and other aspects of CDA. [367]

Etiology and Pathogenesis

The defect that accounts for each type of CDA is found in all of the erythroid progenitors rather than in a subpopulation of cells. [368] [369] [370] Some pertinent biochemical abnormalities are related to the etiology of CDA I and CDA III, [371] [372] [373] [374] [375] but little more is known about pathogenesis. A putative gene for CDA III has recently been localized and maps to an 11cM interval within 15q21q25. [376]

In comparison, the pathogenesis of CDA II has been almost completely clarified. At the stem cell level, in vitro culture of CDA II erythroid progenitors produces CFU-E and BFU-E colonies with erythroblast multinuclearity. [368] Initially, studies of peripheral blood CDA II red cells identified a number of chemical abnormalities including unbalanced globin chain synthesis, [373] increased membrane glycolipids, [377] and altered red cell membrane protein patterns demonstrated by two-dimensional electrophoresis. [375] Furthermore, glycoproteins on CDA II red cells were found to have an abnormal carbohydrate structure leading to aberrant reactivity with anti-i sera. [378] Additional data suggested that the IgM antibody responsible for hemolysis in the acidified-serum lysis test recognizes an abnormal glycolipid structure sharing homology with the i and I antigens. [379] Thus, a

variety of data predicted that abnormalities in the glycosylation pathway were involved in the etiology of CDA II.

Fukuda et al. [380] [381] reported two distinct defects in this glycosylation enzymatic pathway that occur in CDA II: alpha-mannosidase II deficiency and N-acetylglucosaminyltransferase II deficiency. As a result of the former abnormality, addition of new sugar moieties to the oligosaccharide core structure is impaired. A defect, probably in the promoter region of the gene encoding for alpha-mannosidase II, has been identified, thus linking a molecular defect with CDA II. Fukuda et al. [380] also claim that multinucleated erythroblasts are formed in vitro when normal bone marrow is cultured with an inhibitor of alpha-mannosidase II. Also, low levels of N-acetylglucosaminyltransferase II, the enzyme responsible for addition of an N-acetylglucosamine residue to one of the arms of the core, results in glycoproteins with truncated oligosaccharides.

A third enzymatic defect has been described in a variant case of CDA II. Low levels of the membrane-bound form of galactosyltransferase, involved at various stages of oligosaccharide synthesis, severely affect processing and lead to the presence of primitive high-mannose core structures on glycoproteins. [382] All three of these enzymatic deficiencies lead to abnormal oligosaccharides on major erythrocyte proteins such as the anion transporter Band 3. [383] [384] In addition to its other functions, this glycoprotein plays a critical role in the organization of the membrane skeleton that determines normal red cell strength, flexibility, and shape. Abnormal glycosylation of Band 3 may cause it to cluster on the cell surface. [385] Such clustering could cause disruption of the structural network of the erythrocyte and its

precursors, thereby leading to their premature demise.

Defective glycosylation on the red blood cell surface may also affect the regulation of complement on the surface of erythrocytes. Enhanced functional activity of the alternative pathway C3 convertase and of the membrane attack complex may result from the improper glycosylation of glycophorin A, which has been proposed to serve as a complement regulatory protein.[386]

Clinical Manifestations

Some features are shared by all forms of CDA and some features are specific for each type (Table 18-7). In general, there is a wide variation in age of onset of clinical problems related to CDA. Most patients are diagnosed in late childhood or adolescence; however, a few CDA cases have now been reported in newborn infants presenting with hydrops fetalis.[387] [388] [389] Clinical manifestations may include intermittent jaundice and dark urine due to increased hemoglobin catabolism, or signs and symptoms of anemia may be present. Rarely, hyperbilirubinemia without anemia may be the initial presentation of CDA patients. The degree of splenomegaly and hepatomegaly is quite variable. Cholelithiasis may be present as a consequence of chronic hyperbilirubinemia. In some older patients, evidence of hemosiderosis (skin hyperpigmentation, diabetes mellitus, hypogonadism, or delay of secondary sexual characteristics) is often present. It is noteworthy that hemosiderosis occurs in both transfused and nontransfused CDA patients. In the latter, iron overload is a direct consequence of ineffective erythropoiesis and increased gastrointestinal absorption of iron.[390] Two recent reports of a large series of patients with CDA I and CDA II, respectively, illustrate the spectrum of clinical manifestations of these disorders.[391] [392]

Laboratory Evaluation

There is a wide range in severity of the anemia in the CDAs. Some patients are completely asymptomatic, whereas others require blood transfusion. Most commonly, however, the anemia is mild to moderate, with hemoglobin concentrations between 8 and 11 g/dl. Normocytic or macrocytic indices are typical (Table 18-7). Abnormalities of mature erythrocytes, including anisocytosis, poikilocytosis, anisochromasia, and punctate basophilic stippling, can be pronounced and may serve as the first clue to an underlying CDA. Reticulocytes are normal to slightly elevated but less than expected for the degree of anemia, reflecting ineffective erythropoiesis. Red cell life span may be normal to moderately shortened. White cells and platelets are quantitatively normal except on rare occasions when cytopenias can be attributed to splenic sequestration. Infrequently, quantitative abnormalities can also be seen, such as abnormal segmentation of neutrophils, cytoplasmic cisternae in both granulocytes and platelets, and budding of nuclei in megakaryocytes.[393] [394] [395]

TABLE 18-7 -- Distinguishing Features of the Congenital Dyserythropoietic Anemias

Feature	CDA Type I	CDA Type II (HEMPAS)	CDA Type III
Inheritance	Autosomal recessive	Autosomal recessive	Autosomal dominant
Organomegaly	Splenomegaly	Splenomegaly, hepatomegaly	Minimal organomegaly
Anemia	Mild-moderate	Mild-severe	Mild
Mean corpuscular volume	Mild-moderate increase	Normal-mild increase	Normal-mild increase
Bone marrow erythroids			
Light microscopy	Megaloblastoid changes; large cells with incompletely divided nuclear segments; double nuclei, internuclear chromatin bridges	Late polychromatophilic and orthochromic erythroblasts often contain two to seven normal-appearing nuclei	Giant erythroblasts, with up to 12 nuclei; prominent basophilic stippling
Electron microscopy	Widened nuclear pores, cytoplasmic invasion of nucleus, spongy appearance of nuclei, disaggregation of ribosomes, and presence of cytoplasmic microtubules	Excess endoplasmic reticulum appearing as a double cell membrane	Clefts and blebs within nuclear region, autolytic areas in cytoplasm, some iron-filled mitochondria, and myelin figures
Acid serum hemolysis	Negative	Positive	Negative
Sugar water test	Negative	Negative	Negative
Red cell agglutination/lysis			
with anti-i serum	±	++++	±
with anti-I serum	+	++++	±

The bone marrow shows marked erythroid hyperplasia, reversal of the M:E ratio, dyserythropoiesis, and a degree of red cell multinuclearity. No specific cytogenetic finding is associated with the CDAs; however, erythroblasts are frequently hyperdiploid reflecting ineffective cell division with a resultant increased complement of chromosomes within the cells.[396] Increased stainable iron is evident in the marrow, but ringed sideroblasts are not a feature. Ferritin concentration is elevated and correlates with iron stores. Ferrokinetic studies similarly reflect the ineffective erythropoiesis with a shortened plasma iron clearance, an increase of plasma iron turnover as much as 10 times normal, and increased retention of marrow iron, but without evidence of impaired iron utilization as occurs in the thalassemia syndromes.[397] Because excess iron is present even in the absence of transfusions or therapeutic supplementation, it is thought to be due to an increased absorption that occurs in the presence of accelerated, but ineffective, erythropoiesis. Reflecting this, elevation of serum lactate dehydrogenase and unconjugated bilirubin along with depression of serum haptoglobin are frequently noted, but most consistently in CDA II.

Differential Diagnosis

Dyserythropoiesis with erythroblast multinuclearity is seen with other hematologic disorders but these can be readily distinguished from CDA. The megaloblastic anemias have a different clinical presentation and are identified in the laboratory by the presence of hypersegmented neutrophils, decreased red cell folate values, or reduced serum levels of vitamin B_{12} . The myelodysplastic syndromes may manifest as an isolated refractory anemia, but they often present with bi- or tri-lineage cytopenias that contrast sharply with the CDAs. The FAB myelodysplastic syndrome subsets may also show marrow granulocytic and megakaryocytic morphologic abnormalities, the presence of myeloblasts, ringed sideroblasts, and clonal cytogenetic changes. Erythroleukemia (AML, M6) is another cause of marked dyserythropoiesis but typically there is pancytopenia, the erythroblasts are avidly positive for the periodic acid-Schiff stain, and the marrow cells may show a clonal cytogenetic marker. The beta-thalassemia syndromes differ from the CDAs by the presence of marked microcytosis with elevated levels of either hemoglobin A_2 or fetal hemoglobin, or both.

Specific Features of Major Types of Congenital Dyserythropoietic Anemia

Type I

CDA I is inherited in an autosomal recessive manner. In several reported families more than one sibling was affected, and the disorder has been seen in fraternal and identical twins. The onset of anemia, jaundice, or other symptoms may be noted at any age. Affected patients often manifest some degree of icterus and splenomegaly. Recently, CDA I has been associated with skeletal malformations of the limbs in four unrelated patients.[398] [399] The abnormalities consist of absence of nails, short or absent phalanges, and polysyndactyly of the fourth metacarpal. One of the patients also had areas of depigmentation. These anomalies are not

common in CDA I patients but do appear to be quite specific for this subtype.

The degree of anemia is usually mild to moderate (hemoglobin in the range of 8 to 12 g/dl), and red cells are macrocytic. Peripheral blood red cell morphology is characterized by anisocytosis and poikilocytosis, and occasionally Cabot rings are seen. Cabot rings appear to be unique to CDA I and are not seen in types II and III. White blood cells and platelets are normal. Examination of the bone marrow reveals erythroid hyperplasia with some megaloblastic erythropoiesis, and a small number of erythroblasts with dyserythropoietic features. The unique morphologic abnormality seen in type I CDA is the presence of interchromatin bridges between nuclei of two separate erythroblasts, a reflection of impaired cellular division (Fig. 18-8). This internuclear bridging of erythroblasts seen with light microscopy is also a common feature in myelodysplastic syndromes. Electron microscopy reveals additional abnormalities that include widening of the nuclear membrane pore space with cytoplasmic invagination into the nucleus, separation of nuclear chromatin, and chromatin condensation, all of which give the general appearance of a spongy nucleus (Fig. 18-9). Dyserythropoiesis seems limited mostly to more mature red cell precursors. In contrast to type II CDA, there are no unique serologic features.

The defect in CDA I is at the stem cell level. The numbers of CFU-E and BFU-E are normal, but the colonies contain a mixture of normal and abnormal cells when examined by electron

Figure 18-8 Bone marrow from patient with type 1 CDA. Erythroblasts are connected by internuclear bridges connecting two cells. *(Provided by Dr. Jean Shafer, Rochester, NY.)*

Figure 18-9 Bone marrow-electron microscopy of CDA type 1. Note the spongy appearance of nucleus resulting from uneven chromatin with cytoplasmic invagination into the nucleus. (*Provided by Dr. Raoul Fresco, Loyola University, Maywood, IL.*)

microscopy. [400] This suggests that the abnormality is expressed variably in the mature progeny of each stem cell.

Type II (HEMPAS)

CDA II is commonly known as HEMPAS, an acronym for *H*ereditary *E*rythroblastic *M*ultinuclearity with a *P*ositive *A*cidified *S*erum test. [401] There is overlap of some clinical and laboratory manifestations between CDA I and CDA II, but there are three major differences. The first difference is that the magnitude of anemia is usually more severe, and patients with CDA II often require red cell transfusions. Peripheral blood red cells are usually normocytic but with anisocytosis and poikilocytosis (Fig. 18-10). The second difference is that the bone marrow in type II CDA reveals more abnormal erythroblasts (30%), with binuclearity, multinuclearity, and abnormal lobulation (Fig. 18-11). These nuclear abnormalities are seen only in the late erythroblasts, not in basophilic erythroblasts. Karyorrhexis is commonly observed, and pseudo-Gaucher cells may be present, representing the ingestion of debris by histiocytic cells from ineffective erythropoiesis. Electron microscopy of late erythroblasts also reveals an excess of endoplasmic reticulum parallel to the cell membrane, giving the appearance of a double cell membrane [402] [403] (Fig. 18-12). A third difference, which is also a pathognomonic finding, is that type II CDA red cells are lysed by acidified (pH 6.8) sera obtained from approximately 30% of normal persons, but there is no lysis when red cells are incubated with the patient's own acidified serum. This lysis is due to a naturally occurring IgM antibody that binds complement, and this antibody can be removed by preincubating normal sera with HEMPAS erythrocytes. However, the specific HEMPAS antigen recognized by this antibody is not known. In contrast to HEMPAS, the erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH) undergo lysis when the acidified serum is from the PNH patient or from normal donors. Another difference is that PNH erythrocytes undergo lysis in isotonic sucrose, whereas HEMPAS red cells do not lyse in sucrose solution.

The erythrocytes from patients with CDA II also exhibit an increased agglutinability and lysis to anti-i and anti-I sera and manifest increased expression of both antigens. These surface antigens are complex carbohydrate structures found predominantly on fetal and adult red cells, respectively. Increased expression of i antigen can be demonstrated on all red cells in CDA II using fluorescent labels. Relatives of patients with CDA II who have normal marrows but increased agglutinability to anti-i appear to be heterozygote carriers of this disorder. [404] HEMPAS erythrocytes bind a normal amount of complement (C1), but more antibody and less C4 than normals.[405] This causes binding of an excess of C3 and hemolysis.

The number of erythroid progenitors is probably normal in marrow and blood. Although one study found only normal

Figure 18-10 Peripheral blood smear from patient with CDA type II. Note the marked variation in size and shape.

Figure 18-11 Bone marrow aspirate from a patient with HEMPAS (type II CDA) showing erythroid hyperplasia and multinucleated erythroblasts. (*Provided by Dr. Jean Shafer, Rochester, NY.*)

morphology of the erythroblasts produced in culture, [406] subsequent studies reported multinuclearity similar to that seen in the bone marrow. [368] [407] As in type I CDA, the defect in type II CDA is in the erythroid stem cell and is expressed variably in more mature erythroblasts.

A number of clinical associations with CDA II have been reported such as mental retardation, Sweet's syndrome, von Willebrand's disease, and Dubin-Johnson syndrome, among others.[408] [409] [410] Rather than true associations, it is likely that the majority represent coincidental occurrence. An adult patient was recently reported with an extramedullary hematopoietic

Figure 18-12 Electron micrograph of bone marrow erythroblast from patients with HEMPAS (type II CDA). Note the appearance of a double cell membrane reflecting an excess of endoplasmic reticulum. (*Provided by Dr. Raoul Fresco, Loyola University, Maywood, IL.*)

mass in the posterior mediastinum that was a result of marrow expansion associated with ineffective erythropoiesis. [411]

Type III

Based on reported CDA cases, type II is the most common, type I is next, and CDA III is the rarest of the three major forms. As of the mid-1990s, only about 30 cases of CDA III have been described,[49] 15 of which come from a single Swedish family.[412] In contrast to the other major forms, CDA III is inherited as an autosomal dominant disorder although sporadic cases have also been described. [413] [414] These latter cases may represent spontaneous dominant mutations.[415] In the Swedish family, an excess number of cases of monoclonal gammopathy and myeloma have occurred.[416] Also, an adult patient with CDA III was recently described with T-cell non-Hodgkin's lymphoma.[417] These cases, plus a case of Hodgkin's disease occurring in an additional patient, may indicate an increased incidence of lymphoproliferative disease in CDA III.

In CDA III, splenomegaly is usually minimal or absent. The anemia is usually mild to moderate, but transfusion-dependent patients have been observed. [418] The circulating red cells can be normal or mildly macrocytic. Bone marrow examination shows erythroid hyperplasia. Giant erythroblasts with up to 12 nuclei are the most distinctive feature of CDA III observed on light microscopic examination of the bone marrow. These may appear similar to some of the large multinucleated cells seen in CDA II (Fig. 18-11). Abnormally large lobulated nuclei and discordance in nuclear maturation are also found. While they are hallmarks of CDA III, these findings

are not pathognomonic and may be seen in erythroleukemia. Electron microscopy demonstrates nuclear clefts and autolytic areas within the cytoplasm.

The acidified-serum lysis test is negative in CDA III. Agglutination and lysis of erythrocytes to anti-i antibody has only been examined in a few cases of CDA III with conflicting findings.[413] [415] Serum thymidine kinase was measured in 20 CDA III patients and 10 healthy siblings.[419] Elevated thymidine kinase was found in all 20 cases but was normal in the siblings. It is suggested that testing thymidine kinase levels can allow clinicians to discriminate between affected individuals and healthy siblings without performing a marrow aspirate.

Precipitation of beta chains has been observed within abnormal CDA III erythroblasts.[420] The defect in the erythroid precursors is intrinsic to the stem cell and can be reproduced in tissue culture in which morphologically normal as well as giant multinuclear erythroblasts are found.[421]

Variants

Cases of CDA have been reported that do not conform with the classification of types I, II, and III,[359] [422] [423] [424] [425] [426] [427] [428] [429] [430] [431] [432] and it has been recommended that one of these variants be termed type IV.[359] [429] The major features of some of the earlier variant cases were reviewed.[426] In two kindreds, CDA was inherited in an autosomal dominant mode. In some cases marrow erythroid multinuclearity resembled that of HEMPAS, but the acidified serum lysis test was negative.[428] [429] Long-lasting erythroblastosis occurring after splenectomy of such patients has been attributed to impairment of the denucleation of erythroblasts.[428] Unbalanced globin-chain synthesis with excess production of -chains was documented in several patients. In one kindred, a disorder with features of both thalassemia and hereditary erythroid multinuclearity was dominantly transmitted.[425] In variant syndromes, there were also differences in the degree of agglutination by anti-i antibodies and in the concentrations of hemoglobins F and A_2. In one case of CDA, the acidified serum lysis test was positive, but erythroid multinuclearity was absent.

A number of other poorly-defined CDA variants probably exist. Cases of lifelong anemia, probably inherited, have been observed.[427] These were characterized by marked aniso- and poikilocytosis and occasional teardrop and fragmented erythrocytes in the blood. Hyperplastic marrows showed megaloblastoid features without multinuclearity or ringed sideroblasts,[427] but a case with prominent ringed sideroblasts was also described.[430] Neutropenia is commonly present and thrombocytopenia has been observed in some patients. Cytogenetic studies of marrow revealed no chromosomal abnormalities. Reticulocyte response to anemia was absent or inappropriately low in all. Most case studies of parents failed to reveal abnormalities, suggesting an autosomal recessive mode of transmission.

Therapy and Prognosis

In general, the CDAs are associated with a favorable long-term prognosis. For example, CDA I was diagnosed for the first time late in life,[433] and followed a relatively benign course in another case over a 30-year follow-up.[434] Anemia is typically mild in most cases of CDA and requires no intervention, but in more severe presentations, especially those requiring transfusional support, splenectomy may be beneficial. Most of the experience showing an improvement in hemoglobin levels after splenectomy has been in CDA II; the benefit of splenectomy in CDA I is less clear.[418] [435] [436] In planning splenectomy, erythrokinetic studies beforehand may be useful to quantitate the magnitude of splenic sequestration and shortened red cell survival.

Treatment of all forms of CDA with androgens, corticosteroids, vitamin E, vitamin B_{12}, folid acid, pyridoxine, or iron is ineffective in ameliorating the anemia. Iron therapy is also contraindicated because of the underlying propensity for hemosiderosis secondary to increased gut iron absorption. Even if regular red cell transfusions are not initiated, patients should be routinely monitored for evidence of iron overload. Iron chelation with daily subcutaneous infusions of deferoxamine has been underused in the CDAs even though deaths have been recorded from hemochromatosis. A solitary case report on the successful use of deferoxamine in CDA I illustrates the potential benefit.[437] Phlebotomy has been carried out to remove iron in CDA patients, but this could result in a worsening of the anemia and theoretically enhance gut iron absorption. An additional concern is that CDA patients seem predisposed to hepatic cirrhosis irrespective of body iron burden.[367] The pathogenesis of this is unclear.

Interferon alpha-2a was recently reported to increase hemoglobin levels to normal in a CDA I patient who required repeated transfusions for moderately severe anemia.[438] Erythrokinetic studies demonstrated a striking reduction of ineffective erythropoiesis while patients were receiving interferon, and electron microscopy showed a reduction in nuclear structure abnormalities.

Asymptomatic extramedullary hematopoiesis may mimic tumors of the mediastinum, abdomen, and vertebral column.[411] [439] [440] Because of the increased red cell production that occurs, the development of sites of extramedullary hematopoiesis may result in an amelioration of the degree of anemia.[441] Technetium-99m sulfur colloid scintigraphy is useful in delineating the extent of these regions.[440]

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Chapter 19 - Aplastic Anemia

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INTRODUCTION

Aplastic anemia (AA), the paradigm of bone marrow failure syndromes, is most simply defined as peripheral blood pancytopenia and a hypocellular bone marrow (Plate 19-1A, B). From epidemiologic and clinical features, pathophysiologic studies, and response to therapy, AA is a distinctive disease. However, the proper diagnosis of AA requires excluding other causes of pancytopenia (Table 19-1). In addition, AA may occur as a primary hematologic disorder, most often idiopathic, or apparently secondary to various proximate causes, including obvious physical and chemical toxins but also medicinal agents and viruses that may act indirectly. Although AA is usually characterized by a severe diminution in bone marrow function that affects all the hematopoietic lineages, granulocyte, platelet, and red blood cell (RBC) levels may not be depressed uniformly, and less severe degrees of marrow hypoplasia and odd combinations of bicytopenias and even monocytopenias may be observed. Aplastic anemia can be especially difficult to distinguish from hypocellular forms of myelodysplasia, a diagnostic confusion that may rest on real biologic similarities. Even typical AA may vary in its clinical presentation and course, from a fulminant illness marked by continuous or recurrent hemorrhage and major infections to an indolent process manageable by transfusions alone.

History

The study of bone marrow failure is traditionally dated to 1888, when Paul Ehrlich described a young woman who died after an explosive short illness marked by severe anemia, bleeding, and high fever.[1] As a pathologist, Erhlich was struck by the absence of nucleated RBCs and the fatty quality of the femoral marrow, opposite findings from the physiologic response to severe anemia, and he inferred from the morphology a mechanism of failed blood cell regeneration. Vaquez and Aubertin in their 1904 case report of pernicious anemia with yellow marrow first named the disease and emphasized a pathophysiology of failed hematopoiesis (anhematopoiesis).[2] Cabot stressed the marrows distinctive pathology and emphasized the need for its examination in diagnosis. [3] Tissue from patients with early cases of AA could only be examined at autopsy, and in practice, as reflected in the medical literature of the early 20th century, pancytopenia was often equated with aplastic anemia. The etymologic root of the term aplastique

TABLE 19-1 -- Differential Diagnosis of Pancytopenia

Pancytopenia with Hypocellular Bone Marrow
Acquired aplastic anemia
Inherited aplastic anemia (Fanconi anemia and others)
Some myelodysplasia syndromes
Rare aleukemic leukemia (acute myelogenous leukemia)
Some acute lymphoblastic leukemias
Some lymphomas of bone marrow
Pancytopenia with Cellular Bone Marrow
Primary bone marrow diseases
Myelodysplasia syndromes
Paroxysmal nocturnal hemoglobinuria
Myelofibrosis
Some aleukemic leukemias
Myelophthisis
Bone marrow lymphoma
Hairy cell leukemia
Secondary to systemic diseases
Systemic lupus erythematosus, Sjögrens syndrome
Hypersplenism
B_{12} , folate deficiency (familial defect)
Overwhelming infection
Alcohol
Brucellosis
Ehrlichiosis
Sarcoidosis

Tuberculosis and atypical mycobacteria
Hypocellular Bone Marrow ± Cytopenia
Q fever
Legionnaires disease
Mycobacteria
Tuberculosis[a]
Anorexia nervosa, starvation
Hypothyroidism

[a]Pancytopenia in tuberculosis only rarely is associated with a hypocellular bone marrow at biopsy or autopsy. Marrow failure in the setting of tuberculosis is almost always fatal; exceptional patients probably had underlying myelodysplasia or acute leukemia.

is the Greek verb ´theta, to create and give shape to (´eta, the adjective, unformed). Of course, inferences about the etiology of the disease were made from the bone marrow appearance. The aplastic variety of pancytopenia was distinguishable from secondary cellular or regenerative pernicious anemias, and AA was recognized as primary and due to failed blood cell production. [2] Pathologically, the watery yellow marrow seen at autopsy uniquely characterized the aplasia, but the limited and rather general clinical signs were less helpful in distinguishing this type of aregenerative marrow failure from other anemias. [4] Inability of the marrow to produce hematopoietic cells is a common feature of many hematologic diseases, many of which are not classified as bone marrow failure states. Conversely, the concept of AA as an intrinsic marrow disorder may be more applicable to inherited marrow failure than to the immunologically mediated form of the disease observed in adults. Some of the early speculation about marrow failure being a consequence of an environmental insult, either infectious or chemical in nature, or occurring indirectly through altered regulation of hematopoiesis may not have been far off the mark.

Classification

AA is a major sequela of radiation and exposure to chemical agents. It has been associated with use of chemicals and drugs, with viral infections, and with other diseases (Table 19-2). Most patients have an idiopathic form of the disease, meaning only that the cause is unknown. Too strict a division of cases into categories can obscure important pathophysiologic relationships. In any event, the relation between clinical associations and causation is neither direct nor reliable. The basis for assigning

TABLE 19-2 -- A Classification of Aplastic Anemia

Acquired Aplastic Anemia
Secondary aplastic anemia
Irradiation
Drugs and chemicals
Regular effects
Cytotoxic agents
Benzene
Idiosyncratic reactions
Chloramphenicol
Nonsteroidal antiinflammatories
Antiepileptics
Gold
Other drugs and chemicals
Viruses
Epstein-Barr virus (infectious mononucleosis)
Hepatitis virus (non-A, non-B, non-C, non-G hepatitis)
Parvovirus (transient aplastic crisis, some pure red cell aplasia)
Human immunodeficiency virus (acquired immunodeficiency syndrome)
Immune diseases
Eosinophilic fasciitis
Hypoimmunoglobulinemia
Thymoma and thymic carcinoma
Graft-versus-host disease in immunodeficiency
Paroxysmal nocturnal hemoglobinuria
Pregnancy
Idiopathic aplastic anemia
Inherited Aplastic Anemia
Fanconi anemia
Dyskeratosis congenita
Shwachman-Diamond syndrome
Reticular dysgenesis
Amegakaryocytic thrombocytopenia
Familial aplastic anemias
Preleukemia (monosomy 7, etc.)
Nonhematologic syndromes (Down, Dubowitz, Seckel)

a particular case to a particular cause depends on the history. How closely is the patient questioned? How biased are the inquiries? How conscientiously is a possibly significant but distant toxic exposure sought? How exaggerated is a brief episode? Ultimately, patients with idiopathic and secondary disease require similar support and definitive therapies; with a few exceptions, there is little basis for major differences in their clinical management.

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EPIDEMIOLOGY

Incidence

The largest and most comprehensive study of the epidemiology of bone marrow failure is the International Aplastic Anemia and Agranulocytosis Study (IAAAS) conducted in Europe and Israel from 1980 to 1984.[5] This study was performed prospectively and applied strict case definition to pathologically confirmed cases. Using stringent criteria, the overall annual incidence of AA was 2 cases/10^6. Other subsequent studies of similar design have confirmed this figure.[6] The incidence rates reported in the IAAAS study are considerably lower (by 3-to 4-fold) than rates reported in many earlier, mainly retrospective and far less well-designed surveys. Although an apparent increase in the incidence of AA during the 1950s and 1960s was reported by some groups, such a trend has not been supported by data collected in the past 20 years, during which the incidence rates have been stable.[5] [7] [8]

Age Distribution

AA has been identified as a disease of the young. Histograms showing the age distribution of patients (Fig. 19-1) demonstrate

Figure 19-1 Distribution of aplastic anemia by age. For the patients at the University of Washington (a major transplantation center), age is at first treatment; for the patients at the National Institutes of Health (where immunosuppressive therapy is offered), age is at the time of diagnostic bone marrow biopsy. Acute disease is defined as <3 months between diagnosis and presentation at NIH, and chronic as >3 months. *(Seattle statistics made available courtesy of Rainer Storb).*

two peaks, with most patients presenting at age 1525 or after age 60, and with a trough in middle life.[9] [10] The peak of occurrence conflicts with mortality data indicating a single major rise in deaths from AA in late middle age;[11] the explanation for this discrepancy is probably misdiagnosis of AA through confusion of AA with myelodysplasia in older people.[12]

Geographic Distribution

The most remarkable feature of the epidemiology of AA is the unexplained geographic variation in its incidence. The long-standing impression of physicians in Asia and their European and American visitors is that AA is far more common in Asia than in the West. The frequency of AA as an admission diagnosis may rival that of acute myeloid leukemia (AML) in these centers;[13] by contrast, in most American hospitals, AML is five to ten times more common as an admitting diagnosis than AA. National surveys in Japan[14] [15] and China[16] [17] published in the 1970s and 1980s suggested very high prevalence and incidence figures for AA. Autopsy series [18] and mortality statistics[19] suggested that the annual death rate from AA might be more than three times higher in the Far East than in the West. More recently, the incidence of AA in Bangkok and two rural regions of Thailand has been accurately determined using the same methods as employed by the IAAAS researchers in Europe and Israel; the annual incidence was 4.0/10^6 in the capital and 5.6/10^6 in the northeastern province of Khonkaen.[20] The incidence in 21 Chinese provinces has been estimated to be 7.4/10^6 annually,[21] and in the Sabah province of Malaysia a retrospective analysis suggested a rate of 5/10^6.[22] From published, hospital-based series, personal communications, and first-hand observations, AA appears most prevalent in specific regions of the world, including Vietnam, Indonesia, Russia, the former Soviet republics, Iran, Iraq, Pakistan, India, Mexico, other regions of Latin America, [23] and Africa, where it is likely underdiagnosed. [24] In these developing countries the rate of AA appears to be two to three times higher than in Europe.

In the United States, there are no major sex or racial differences in the occurrence of AA.[7]

Epidemiologic Clues to Causality

Population-based studies have investigated possible causal associations. Drugs are implicated in only about 25% of cases of AA. [5] The major drug associations in the IAAAS study were with gold salts (relative risk [RR] of 29), antithyroid drugs (RR = 11), and nonsteroidal anti-inflammatory agents (e.g., RR = 8.2 for indomethacin). Benzene, insecticides, and petrochemicals were only modestly related. Other smaller studies have shown associations with chemical exposures, exposures to viruses, hepatitis, and occupation.[25] In Thailand, significant and high RR ratios have been established for a number of independent variables, including low income, [26] rural residence, and prior exposure to hepatitis A virus; perhaps most provocative is a link to rice farming. [27] AA was attributed to drug exposure in only about 15% of cases. Clusters of AA provide further evidence of an environmental etiology. [28] Japanese in Hawaii seem to have a similar incidence of AA as non-Orientals born in North America.[19] Conversely, a high incidence of AA was reported in the Pacific theater during World War II, an observation that was attributed to quinacrine exposure.[29] Thus, the geographic variation in AA is probably due mainly to environmental causes rather than being based on a genetic predisposition.

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GENETIC ASPECTS

More than one case of acquired AA in a single family is a rare occurrence. In children and young adults, acquired AA should be distinguished from the inherited forms of bone marrow failure such as Fanconi anemia. Identification of patients with Fanconi anemia has important therapeutic implications. Patients with Fanconi anemia may lack typical physical anomalies, and the pancytopenia may develop after childhood (see Chap. 18). Occasionally, AA that is not due to Fanconi anemia has been observed in multiple family members.[30]

The host immune response is genetically restricted, as exemplified by familial susceptibility for autoimmune disease based on HLA linkage. A few histocompatibility types have also been associated with AA, most consistently HLA-DR2:[31] in one study of 75 patients, HLA-DR2 was twice as frequent in AA patients as expected in the population.[32] Other associated antigens include A2 and HLA-B14 class I loci and DR4 and Dpw class II antigens. [33] [34] [35] [36] [37] An analysis of 106 AA patients typed for HLA-A and B loci showed an increased frequency of HLA-B7 and HLA-B14 antigens. Typing for HLA-C loci in 96 AA patients revealed an increased representation of HLA-C7 antigens, while in 82 patients in whom class II typing was performed a previously described increased frequency of HLA-DR2 and HLA-DR15 antigens was observed.[38] HLA-DR subtypes have proved useful in predicting response to immunosuppressive therapy in some studies. In Japanese patients, a class II haplotype (DRB*1501) that determines HLA-DR2 presentation has been recorded to be strongly associated with hematologic responses to cyclosporine (CsA),[39] as well as with the probability of relapse[31] and CsA dependence.[40] However, HLA-DR2 has been inconsistently associated with response to immunosuppression in other studies. The haplotypes associated with clozapine-induced agrannlocytosis have been linked to the heat-shock protein variants encoded within the large major histocompatibility gene region.[41] Genetic predisposition may be responsible for some idiosyncratic reactions to drugs and chemicals leading to the development of AA.

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ETIOLOGY AND PATHOGENESIS

Hematopoiesis in Bone Marrow Failure

Stem Cells

A consistent laboratory finding in AA is the very low numbers of assayable blood and bone marrow progenitor cells. [42] [43] [44] Colony formation by bone marrow cells of patients with severe disease remains unresponsive, even to high levels of hematopoietic growth factors. [45] [46] This defect can persist long after successful treatment of the underlying AA. [43] [47] [48] Not only is the total number of progenitors in a marrow sample reduced, but the number of colony progenitor cells assayed from a purified CD34+ population is also low. [43] In humans, a stem cell surrogate assay that assesses cells capable of colony formation after more than a month in long-term bone marrow culture is available; these long-term culture-initiating cells (LTC-IC) share the frequency, phenotype, and kinetic properties of true stem cells. Studies indicate a profound deficiency in LTC-IC numbers in all patients with severe AA. [49] [50] At clinical presentation, the number of LTC-IC is usually <10% of normal; combined with a reduction in total marrow cellularity to 10%, the stem cell number is estimated to be reduced to 1% of normal in AA (Fig. 19-2).

Figure 19-2 CD34+ cells, primary colony-forming cells, and LTC-IC numbers measured in bone marrow of aplastic anemia patients. Each dot represents an individual patients sample studied. Severe AA (sAA) includes patients at presentation, cases refractory to immunosuppressive therapy, and patients who relapsed after a period of recovery. N, controls; mAA, moderate aplastic anemia; rAA, recovered from aplastic anemia; CFC, colony-forming cells. Primary CFCs were measured in short-term methylcellulose cultures. Secondary CFCs after long-term bone marrow culture reflect LTC-IC numbers.

Figure 19-3 Normal stromal cell function in long-term culture of aplastic anemia bone marrow. *(Courtesy of Dr. Judith Marsh).*

The CD34+ cell marrow population contains most of the committed progenitor cells and stem cells. CD34+ cell numbers are also dramatically reduced in most patients with AA. [43] Patients who have recovered from moderate AA may have higher CD34+ counts, but CD34+ cell levels often remain low despite hematologic recovery. [43] [51]

Stroma and Hematopoietic Growth Factors

The survival and proliferation of hematopoietic cells are dependent on stromal cells. Stromal cell function is usually not defective in AA. [42] For example, adherent cells from patients support hematopoiesis by normal CD34+ cells, whereas no hematopoietic colonies develop when patients CD34+ cells are cultured in the presence of normal stroma (Fig. 19-3). [52] [53] Stromal cells cultured from patients bone marrow generally produce normal quantities of hematopoietic growth factors. [54] [55] [56] A few studies have suggested deficiencies in some factors, an observation of uncertain pathophysiologic significance. Stroma isolated from a minority of patients may poorly manufacture granulocyte-macrophage colony-stimulating factor (GM-CSF), [57] interleukin-3 (IL-3), and granulocyte colony-stimulating factor (G-CSF) [58] or interleukin-1. [59] However, serum levels of erythropoietin, [60] thrombopoietin, [61] G-CSF, [62] and GM-CSF are significantly elevated. [63] Cytokines that act at very early stages of hematopoiesis have been studied as possible etiologic factors in the pathobiology of AA. Flt-3 ligand blood levels are highly elevated in AA, [64] while stem cell factor (SCF) levels are modestly decreased, [65] [66] SCF stromal cell production has been reported to be normal. [66] Adequate stroma function is implicit in the success of marrow transplantation in AA because important stromal elements remain of host origin. Treatment with hematopoietic growth factorseven those putatively deficient in patientshas not been very effective in restoring hematopoiesis in severely affected patients. Thus, the combination of clinical and laboratory observations argues against a defect in stroma function as being responsible for AA.

Pathophysiologic Pathways Leading to Aplastic Anemia

Direct Hematopoietic Injury

Although the commonest form of AA is iatrogenic, transient marrow failure often follows treatment with cytotoxic chemotherapeutic drugs or irradiation (Fig. 19-4). Certain chemical or physical agents directly injure both proliferating and quiescent hematopoietic cells. Such agents cause sufficient damage to DNA to result in apoptosis. However, patients with community-acquired AA rarely have a history of exposure to such physicochemical agents. Even benzene, which may act as particularly inefficient cytotoxic chemical, is an infrequent cause of AA in developed countries. [5] As discussed later, medical drugs are frequently associated with acquired AA, and at least in some instances they may directly cause marrow damage. However, in comparison to conventional therapeutic agents, which are delivered in high doses, relatively low total quantities of ingested drug can cause idiosyncratic hematologic reactions.

Although the causes of idiopathic AA remain unclear, viruses have also been hypothesized to be involved in the pathophysiology of AA. In addition to their direct toxic effects, chemicals and viruses may induce complex immune reactions leading to bone marrow failure in AA (Fig. 19-4). Different etiologic factors may serve as inciting events, resulting in a common pathophysiologic pathway leading to marrow aplasia.

Immune-Mediated Marrow Failure

Mathé and colleagues in the 1970s observed unexpected improvement of pancytopenia after failed marrow transplantation. They speculated that the immunosuppressive conditioning regimen, intended to allow engraftment of the donor marrow, might instead have promoted the recovery of host marrow function. [67] Purposeful targeting of the immune system followed these observations. The known mechanisms of action of these medical treatments, the effectiveness of diverse therapies that reduce lymphocyte number or block T-cell function, and the superior results obtained when agents are combined strongly suggest that such therapeutic success is due to the immunosuppressive effects of the drugs employed. In addition, AA shares numerous clinical and pathophysiologic features with other autoimmune disorders, including multiple sclerosis, type I diabetes, autoimmune thyroiditis, uveitis, and idiopathic myocarditis. Each of these disorders is characterized by T-cell-mediated, tissue-specific organ destruction. Like AA, these disorders

Figure 19-4 Possible etiologies of direct and indirect bone marrow failure in aplastic anemia.

tend to occur in younger persons and may occur in greater numbers in specific geographic locales. The late immune events that dominate at the time of clinical presentationcytotoxic lymphocyte activation, cytokine production, and specific target cell eliminationare common to all of these disorders. In AA, chemical and viral antigens are thought to initiate the immune process, whereas in other T-cell-mediated diseases, specific antigens like myelin basic protein in multiple sclerosis or insulin in diabetes have been identified.

Immune system destruction of marrow occurs in animal models of graft-versus-host disease (GvHD) and in humans with transfusion-associated GvHD, in which marrow destruction is the invariable cause of death.[68] Very small numbers of effector cells, which have been conveyed by residual lymphocytes contained within the transfusion product[69] or with solid organ transplants,[70] are sufficient to mediate GvHD under these conditions. Extremely limited antigenic differences in HLA molecules (even single amino acid substitution) are sufficient to induce GvHD. AA is associated with rheumatologic syndromes, such as eosinophilic fasciitis,[71] and with systemic lupus erythematosus, which may appear or worsen with pregnancy.[72] AA occasionally occurs in individuals with hypogammaglobulinemia[73] [74] or congenital immunodeficiency syndrome.[75] This combination of AA and congenital immunodeficiency syndrome is associated with thymoma, thymic hyperplasia,[76] and thymic carcinoma.[77] In one case, suppressor T cells, already implicated in B-cell inhibition, were shown to suppress erythropoietic colony formation.[78] Reduced serum immunoglobulins and thymic dysplasia have also been observed with marrow aplasia in patients with the congenital dyskeratosis congenital syndrome,[79] which may manifest late in life with bone marrow failure.

Laboratory support for the immune hypothesis first came from co-culture experiments in which mononuclear cells from AA patients blood or bone marrow were shown to suppress in vitro hematopoietic colony formation by hematopoietic progenitor cells. Removal of T cells from the patient samples sometimes improved colony formation in vitro.[80] Peripheral blood and bone marrow samples from patients were shown to produce a soluble factor that inhibited hematopoiesis; normal lymphocytes could be stimulated to release a similar activity.[81] [82] [83] This soluble inhibitory activity was ultimately identified as interferon- (IFN-).[84] Patients T cells, in bulk culture[85] [86] or when cloned,[87] overproduced both IFN- and tumor necrosis factor (TNF). These two cytokines inhibit hematopoietic proliferation at all stages of differentiation.[88] Marrow IFN- mRNA was detected in samples from most patients but not in samples from normal control subjects or from patients with other hematologic diseases.[89] [90] Patients blood and marrow also contained elevated numbers of activated cytotoxic lymphocytes.[91] The activity and levels of these cytotoxic cells fell appropriately with antithymocyte globulin (ATG) therapy.[92] [93] It appears that both secretion of cytokines and accumulation of activated cytotoxic lymphocytes are specifically localized to the affected target tissuethe bone marrow.

The lymphokines IFN- and TNF suppress proliferation of early and late hematopoietic progenitor and stem cells.[88] These effects are far more potent when these lymphokines are secreted into the marrow microenvironment than when they are simply added to the cultures.[94] Such findings are consistent with their localization in the marrow of patients. Although IFN- and TNF can suppress hematopoiesis by effects on cell proliferation, an important component of their inhibitory activity is cell death by induction of apoptosis. Both lymphokines also induce expression of the Fas receptor on CD34+ progenitor cells; triggering of the Fas receptor by its ligand initiates a fatal process of apoptosis.[95] [96] Apoptosis of hematopoietic cells in AA is suggested by the findings of high Fas receptor expression[97] and increased numbers of apoptotic cells[98] in patients bone marrow. Apparently, immune-mediated cell cycle blockade and apoptosis can lead to the dramatic elimination of hematopoietic progenitor and stem cells in AA.

The early immune system events that must precede the global destruction of hematopoietic cells are much less clear. Involvement of CD4+ lymphocytes has been suggested. HLA-DR2 is overrepresented among European and American patients with AA,[32] [99] and a more specific class II haplotype has been identified among Japanese patients.[40] Several HLA antigens may be much more common in certain subsets of AA (B8, Cw7, Dr7, DQ2, and Drw53 in marrow failure after hepatitis[38] [100]). The frequency of HLA-DR2 and HLA-DRw51 were both increased in the idiopathic form AA but were decreased in hepatitis-associated AA, whereas the frequencies of HLA-DQ and HLA-DRw53 appeared to be specifically increased.[38] Clones of HLA-DR-restricted T cells derived from a few patients have been shown to proliferate in response to marrow cells.[101] [102] A V21+CD4 T-cell clone has been isolated from an AA patient and shown to lyse DRB1*0405 target cells, including enriched CD34+ cells.[103] Although it has been suggested that an endogenous antigen (heat shock protein 70) could serve as a putative target for these autoreactive clones, the initial antigen recognition event that leads to breached tolerance and the subsequent process of spreading autoimmunity remains undetermined.

Radiation

Marrow aplasia is a major acute toxic effect of radiation:[104] [105] both stem and progenitor cells are damaged (Fig. 19-5). Bone marrow cells are most affected by high-energy -rays, and secondarily by - and -particles. With the exception of lymphocytes, which are directly killed, a round of replication is required for radiation damage to become manifest. Mitotically active hematopoietic tissue is exquisitely sensitive to irradiation, with a rough correlation between mitotic cycling status and radiation sensitivity. The dose-related occurrence of pancytopenia 24 weeks after exposure to radiation is due to injury of actively replicating progenitor cells. Mortality from hematologic toxicity is a function of the marrows ability to tolerate both depletion of hematopoietic cells and damage to the stem cell. The capacity for recovery of hematopoietic function after even massive single irradiation exposures is considerable, reflecting the resistance of the quiescent stem cell to damage and the enormous marrow repopulating potential of even a greatly reduced stem cell pool. At intermediate radiation doses around LD_{50}, where marrow toxicity limits survival, supportive efforts can drastically alter outcome. Autopsies of atomic bomb victims in Japan showed acellular bone marrows in the first weeks of the explosion, but there frequently was regenerating bone marrow in those who survived longer.[106] The histologic picture of radiation-mediated aplasia includes necrosis, nuclear pyknosis and karyorrhexia, nuclear lysis, and ultimately cytolysis; the associated phagocytosis, marked congestion, and hemorrhage are rapidly followed by fatty replacement.[107] Bone marrow hypoplasia occurs with radiation doses above 1.52 Gy to the whole body. Precise LD_{50} figures for humans do not exist, and estimates are based on the limited direct human data and extrapolation from animal experiments. The LD_{50} is highly dependent on the quality of medical care: improved support may double the tolerated radiation dose.[108] From assessment of the outcome of radiation accidents and high-dose therapeutic irradiation, the LD_{50} has been estimated at about 4.5 Gy,[109] [110] an almost mythical figure sometimes termed the Shields Warren number (Fig. 19-5).

The hematologist might be required to care for a patient who has received a dose sufficient to suppress hematopoiesis but not so overwhelming that death from other causes is inevitable. Although the principles of management of pancytopenia after a single large dose of irradiation are similar to those for treating

Figure 19-5 Scale of whole body radiation doses. A Gray (Gy) is a measure of absorbed dose equivalent to 1 J/kg unit mass; 1 Gy = 100 rads. Radiation represents radiant energy. When absorbed by biologic tissue, radiant energy causes release of electrons and molecular ionization, which in turn result in further energy release. Radiant energy can directly break chemical bonds and indirectly damage macromolecules through generation of high-energy free radical forms. The relationship between increased mutation rate and radiation dose is very approximate (hatched bars). Measurement of the phenotype of an autosomal recessive gene like glycophorin would be expected to be a very sensitive indicator. Since malignant transformation is almost certainly a two-step process, increased leukemogenesis is probably an underestimation of the effect of radiation on a single gene. In addition, even the extensive data from the atomic bomb survivors of Hiroshima are subject to statistical error because of the small number of cases; a linear or exponential curve fit gives varying results, and very high doses of irradiation may not be associated with as high a risk of leukemia because of stem cell death. Other data that may bear on mutation frequency lie outside the range shown: in a patient with ankylosing spondylitis who underwent irradiation of the spine, leukemogenesis was observed at relatively low doses (doubling of the leukemia rate can be extrapolated to about 7 mGy), but such individuals may be predisposed to leukemia. On the other hand, an increased risk of thyroid cancer after irradiation of the mediastinum in childhood occurred at about 4 Gy.

AA in general, some unique points should be made concerning immediate evaluation and long-term prognosis. The type and intensity of the source of radiation and the distance and shielding of the subject are the major determinants of radiation injury; owing to the nature of the exposure, these factors are often difficult to assess. Early recognition of the nature of the accident provides the best opportunity for dosimetry by accident reconstruction and may allow employment of blocking, displacement, or chelation agents. Exposure correlates well with the degree of pancytopenia.[111] Because lymphocytes are particularly sensitive to radiation, their rate of decline can be used to estimate the dose of total body exposure to a level of about 3 Gy.[111] At higher doses, the fall in granulocytes and the severity of thrombocytopenia and reticulocytopenia can be used as gauges.[112] After the Chernobyl radiation accident, measurements of dicentric chromosomes were utilized to estimate dose, which correlated better with neutrophil than with lymphocyte kinetics. The cytogenetic alterations in stem cells are dose related, irreversible, and probably cumulative in increasing the probability of leukemic transformation.[113] The survival of some patients who received doses >9 Gy suggests in retrospect that autologous marrow reconstitution may occur in most persons who survive the immediate consequences of radiation exposure.[114]

Pancytopenia may be a late consequence of a single radiation dose, [115] but AA is not well documented as a delayed event following radiation exposure. Out of 156 cases of AA in Japan in the 20 years following the atomic bomb explosions, only 13 individuals had received more than a 1-rad dose, and of the three individuals who had been heavily irradiated, only one had typical AA. [116]

A variety of hematologic abnormalities are associated with chronic low-level radiation exposure, most commonly lymphocytosis, neutropenia, immature or dysmorphic white cells, and giant platelets (Fig. 19-5). Cytogenetic abnormalities accumulate with time following chronic exposure, but they may not be reliably related to dose.[117] Repeated low doses of radiation can damage bone marrow and have been associated with AA under special circumstances, [118] but even under these circumstances only a small proportion of exposed individuals developed hematologic disease. [119] AA does not appear to be especially frequent among nuclear power plant or thorium processing factory workers or residents living in close proximity to the plants. [120] Excessive numbers of deaths from AA were reported after therapeutic irradiation of the spine for ankylosing spondylitis, [121] although later analysis has suggested that the risk may have been overestimated. [122] Despite occasional instances in which marrow failure developed years after radiation therapy

and chemotherapy,[123] AA was not found in unexpected numbers in a large population of cancer patients who had received therapeutic irradiation. [124] Bone marrow failure has not been observed with abnormally high frequency among persons exposed to higher natural background radiation. [125] Excessive deaths from AA did occur among American radiologists who died between 1948 and 1961;[126] AA deaths were estimated to be 43-fold higher in radiologists between 1920 and 1929 but were not elevated among later cohorts. With radiation as with benzene exposure, late marrow failure referred to in the older literature as aplastic might in fact be classified now as myelodysplasia.

Drugs and Chemicals

Medical drug use is frequently associated with AA (Table 19-3). [127] At the end of the 19th century, chemicals were linked to marrow function through observations of benzene effects on workers. Establishment of a relationship between the analgesic amidopyrine and agranulocytosis in the early 20th century, and an apparent epidemic of AA following the introduction of chloramphenicol in the 1960s, also supported this concept. Initially suggested by the accumulation of case reports, drug associations have been established in formal case-control population-based epidemiologic studies. In the IAAAS study, relative risks were established for individual drugs and large classes of pharmaceutical agents, including NSAIDs, drugs affecting thyroid function, certain cardiovascular agents, some psychotropics, and sulfa-based antibiotics (Table 19-4).[5] Approximately 25% of the cases of AA identified in the IAAAS study could be blamed on drug use. Drug use as a risk factor was also assessed by similar methods in Thailand, [128] where the incidence of AA is higher than in the West. [20] Surprisingly, chloramphenicol was not shown to be a risk factor; only sulfonamide exposure reached statistical significance, and the etiologic fraction for drugs in AA was only about 15%. [128]

Associations between drug exposure and AA are conveniently divided into two classes. Drugs used in cancer chemotherapy are selected for their cytotoxicity, and their regular, dose-dependent induction of marrow aplasia is an expected effect. Most AA associated with medical drug use in the community is described as idiosyncratic, meaning that its occurrences are unexpectedly rare. Many of the drugs implicated in AA also appear to cause other, milder forms of marrow suppression like neutropenia. Although difficult to prove, some dose relationship probably does exist even for idiosyncratic reactions. In most case reports, patients received normal or high doses of the agent, usually for a period of weeks to months. As

TABLE 19-3 -- Classification of Drugs and Chemicals Associated with Aplastic Anemia

I.	Agents that regularly produce marrow depression as a major toxic effect when used in commonly employed doses or normal exposures Cytotoxic drugs used in cancer chemotherapy Alkylating agents (busulfan, melphalan, cyclophosphamide) Antimetabolites (antifolic compounds, nucleotide analogs) antimitotics (vincristine, vinblastine, colchicine) Some antibiotics (daunorubicin, adriamycin) Benzene (and less often benzene-containing chemicals: kerosene, carbon tetrachloride, Stoddards solvent, chlorophenols)
II.	Agents probably associated with aplastic anemia but with a relatively low probability relative to their use Chloramphenicol (see text) Insecticides (see text) Antiprotozoals (quinacrine and chloroquine) >Nonsteroidal antiinflammatory drugs (including phenylbutazone, indomethacin, ibuprofen, sulindac, diclofenac, naproxen, piroxicam, fenoprofen, fenbufen, aspirin) Anticonvulsants (hydantoins, carbamazepine, phenacemide, ethosuximide) Gold and arsenic (and other heavy metals, like bismuth, mercury) Sulfonamides as a class Antithyroid medications (methimazole, methylthiouracil, propylthiouracil) Antidiabetes drugs (tolbutamide, carbutamide, chlorpropamide) Carbonic anhydrase inhibitors (acetazolamide and methazolamide, mesalazine) D-Penicillamine 2-Chlorodeoxyadenosine
III.	Agents more rarely associated with aplastic anemia Antibiotics (streptomycin, tetracycline, methicillin, ampicillin, mebendazole and albendazole, sulfonamides, flucytosine, mefloquine, dapsone) Antihistamines (cimetidine, ranitidine, chlorpheniramine) Sedatives and tranquilizers (chlorpromazine, prochlorperazine, piperacetazine, chlordiazepoxide, meprobamate, methyprylon, remoxipride) Anti-arrhythmics (tocainide, amiodarone) >Allopurinol (may potentiate marrow suppression by cytotoxic drugs) Ticlopidine Methyldopa Quinidine Lithium Guanidine Canthaxanin Thiocyanate Carbimazole Cyanamide Deferoxamine Amphetamines

TABLE 19-4 -- Drugs Associated with Aplastic Anemia in the International Aplastic Anemia and Agranulocytosis Study[a]

Drug	Stratified Risk Estimate (95% CI)	Multivariate Relative Risk Estimate (95% CI)
Nonsteroidal analgesics		
Butazones	3.7 (1.97.2)	5.1 (2.112)
Indomethacin	7.1 (3.415)	8.2 (3.320)

Piroxicam	9.8 (3.329)	7.4 (2.126)
Diclofenac	4.6 (2.011)	4.2 (1.611)
Antibiotics		
Sulfonamides[b]	2.8 (1.17.3)	2.2 (0.67.4)
Antithyroid drugs	16 (4.854)	11 (2.056)
Cardiovascular drugs		
Furosemide	3.3 (1.67.0)	3.1 (1.28.0)
Psychotropic drugs		
Phenothiazines	3.0 (1.18.2)	1.6 (0.47.4)
Corticosteroids	5.0 (2.88.9)	3.5 (1.67.7)
Penicillamine		
Allopurinol	7.3 (3.017)	5.9 (1.819)
Gold	29 (9.789)	

From Kaufman DW, Kelly JP, Levy M, Shapiro S: The Drug Etiology of Agranulocytosis and Aplastic Anemia. Oxford University Press, New York, 1991, with permission of Oxford University Press.

[a]Extracted from the IAAAS monograph.[5] The multivariate model included the following factors: age, sex, geographic area, date of interview, reliability of the patient, person interviewed, transfer from another hospital, history of blood disorder or tuberculosis, exposure to benzene and related chemicals, and use of other suspected drugs.
[b]Other than trimethoprim-sulfonamide combination.

for agranulocytosis, most marrow failure complications occurred after a few weeks of initiation and within the first 6 months of treatment.

The mechanisms that lead to the development of AA after drug exposure include direct chemical toxicity and immune-mediated destruction. These pathophysiologic pathways have been best described for agranulocytosis. Unfortunately, drug-induced hematopoietic failure is difficult to study. AA is a rare rather than a common outcome, precluding development of an animal model, and the diversity of implicated drugs and the problem of confidently assigning causation in an individual case make clinical studies impractical. Drug-induced aplasia cannot be distinguished by history from idiopathic forms of the disease; the clinical course, including the favorable response to immunosuppressive therapy, of patients with histories of drug exposure is the same as in idiopathic disease. [129] Serum assays are also unhelpful because antibodies to either drugs or cells have only occasionally been identified in AA. [130]

The low probability of developing AA following a course of drug may be a reflection of the gene frequency for metabolic enzymes (for direct chemical effects) or immune response genes (for immune-mediated marrow failure) in the population. The rarity of idiosyncratic drug reactions would then arise from the infrequent combination of unusual circumstances: exposure, genetic variations in drug metabolism, the physical properties of the agent, enzymatic pathways that chemically alter the drug, and the susceptibility of the host to the action of a toxic compound. Many drugs and chemicals, especially if they have limited water solubility, must be enzymatically degraded before conjugation and excretion. Degradative pathways for xenobiotics are complex, specific, redundant, and interrelated. Intermediate metabolites in complex degradation pathways may be toxic, highly reactive, and responsible for some adverse effects of the primary agents. Examples of detoxifying enzyme systems directly applicable to bone marrow failure and that also demonstrate genetic variability include arylhydrocarbon hydroxylase (benzene toxicity), epoxide hydrolases (phenytoin toxicity), S-methylation (6-mercaptopurine, 6-thioguanine, azathioprine) and N-acetylation (sulfa drugs). The role of genetic background was shown in experiments using cells of a patient with carbamazapine-associated AA: only after generation of reactive metabolites from the incriminated agent by rat microsomes were the patients lymphocytes killed in a dose-dependent, drug-specific pattern. [131] These metabolites were not toxic for normal donors cells and displayed intermediate toxicity toward cells of the patients mother.

An immune basis for agranulocytosis was initially established with the identification of leukoagglutinating antibodies and, quite dramatically, by the rapid reproduction of the syndrome on drug challenge of affected patients or with infusions of plasma from affected patients into normal volunteers. Strong HLA class II linkage of clozapine[41] and thiouracil[132] agranulocytosis in certain ethnic groups suggests early involvement of CD4+T cells in drug-induced marrow failure. The absence of serum antibodies in AA suggests that drugs do not serve as simple haptens in the induction of this type of marrow failure. Possible mechanisms include binding to cellular proteins, leading to the loss of self-tolerance or disturbance of regulatory immune system networks with the same effect. The rarity of idiosyncratic drug reactions could be a function of genetic variation in drug metabolism systems, differences in major histocompatibility antigens and their peptide-binding properties, and the repertoire of potentially self-reactive circulating lymphocytes during the period of drug exposure.

Cytotoxic Agents

AA is predictably produced by the chemotherapeutic agents used in the treatment of cancer. Some agents, like busulfan, can cause profound delayed depression of bone marrow function and reduce marrow regenerative capacity. By contrast, 5-fluorouracil, cyclophosphamide, 6-mercaptopurine, methotrexate, and vinblastine appear to spare the regenerating cell population.

Benzene

Benzene is a ubiquitous chemical most convincingly linked to AA. [133] [134] [135] Benzene myelotoxicity can be placed between the predictable effects of chemotherapeutic agents and idiosyncratic drug reactions. Industrial emissions add greatly to the biologic sources of ambient benzene. Significant benzene exposure can also occur outside of industry. However, the concentrations of benzene to which consumers are exposed are orders of magnitude lower than those observed in industrial workers. The effect of chronic exposure to low doses of benzene is unknown.

Water-soluble products of benzene metabolism such as phenols, hydroquinones, and catechols mediate the toxicity to the marrow. Benzene and its intermediate metabolites covalently and irreversibly bind to bone marrow DNA, inhibit DNA synthesis, and introduce DNA strand breaks. Benzene thus acts both as a mitotic poison and as a mutagen. Acutely, the more mature, actively cycling marrow precursor cells are preferentially damaged over the more primitive progenitors. [136] Intermittent exposure may be more damaging to the stem cell compartment than continuous exposure[136] and suggests stem cell depletion. Marrow stroma can also be damaged by benzene.[137]

The range of hematologic disease attributable to benzene is quite broad, from relatively frequent mild alterations in blood counts to AA or leukemia. Studies of exposed American workers earlier in the 20th century suggested that the risk of AA was 34% in men exposed to concentrations >300 ppm, and that 50% of individuals exposed to 100 ppm showed some blood cell count depression.[138] The prevalence of some form of marrow suppression with heavy exposure can be high: >10% of workers developed leukopenia; with improved hygiene the figure was lowered to 0.5%. [139] Leukopenia, anemia, thrombocytopenia, and lymphocytopenia are common consequences of benzene; other

manifestations include macrocytosis, acquired Pelger-Huet anomaly, eosinophilia, basophilia, and, more unusually, polycythemia, leukocytosis, thrombocytosis, or splenomegaly. The marrow is usually normocellular but may show hypo- or hypercellularity; [138] a hypercellular phase may precede complete aplasia. In addition to hypocellularity, chronically exposed workers may have marrow necrosis, fibrosis, edema, and hemorrhage. [140] Chronic benzene exposure clearly increases the risk of a variety of lymphohematopoietic malignancies. AA and AML have occurred in the same person, [141] and pancytopenia preceded acute leukemia in one-fourth of industrial workers. [142] Both marrow failure and leukemia in benzene workers may manifest decades after exposure, but malignancy may be the more frequent late consequence.

Aromatic Hydrocarbons

The common perception that other molecules resembling benzene or containing a benzene ring may also cause marrow suppression is not well supported. Not all aromatic hydrocarbons share the same biologic activities. Neither the closely related alkylbenzenes nor pure toluene or xylene are established marrow toxins. Often an aromatic hydrocarbon has been implicated by the clinician as a causative agent for AA for lack of another apparent etiology. For some substances, toxicity may be due to the presence of benzene itself, either as a contaminant of the synthesis of the molecule or in the petroleum distillates used to dissolve the compound. Yet the total number of AA cases reported with aromatic hydrocarbon exposures is small when the large populations exposed to this heterogeneous group of chemicals are considered. For example, surveys of AA patients found that only 26% of cases were associated with insecticide exposure. [143] [144] The significance of a handful of case reports associated with insecticide exposure in the context of the vast use of these compounds is questionable. Of course, the very high prevalence of aromatic hydrocarbons in daily life would greatly amplify even a small individual risk. Pesticides and insecticides have been associated with AA for decades, with almost 300 medical case reports appearing in the medical literature. [145] The most frequently cited insecticides are chlordane and lindane or DDT. For the miscellaneous aromatic hydrocarbons, case reports also greatly outnumber series of patients, and systematic epidemiologic surveys have shown mixed results. Significant excesses of cases of AA were found in workers in the printing industry (odds ratio [OR] = 6.2), in lumber and wood products industries (OR = 3.7), in agriculture workers (OR = 2.4), and in construction workers (OR = 2.0).

Chloramphenicol

Structural similarity of chloramphenicol to amidopyrine, a drug known to cause agranulocytosis, led to early prediction of possible hematotoxicity. [146] [147] During the period of its unrestrained use, chloramphenicol was considered the commonest cause of AA in the United States, [148] accounting for 2030% of total cases and 50% of drug-associated cases.[149] [150] [151] Estimates of the risk of AA after a course of chloramphenicol ranged from 1/20,000 [150] to 1/800,000.[152] Based on these figures, a course of chloramphenicol was estimated to increase the risk of AA 13-fold. Although the introduction of chloramphenicol into the American market was perceived as having increased the total number of cases of AA,[148] [153] this assumption was only weakly supported by epidemiologic data, and the mortality from AA remained essentially constant during the period of chloramphenicols introduction and extensive use and following the withdrawal of chloramphenicol from the market. In recent series reported from the United States and Europe, of a total of 394 patients, only one was found to have ingested the drug. [25] [154] Chloramphenicol has not been associated with AA in Thailand, despite its high rate of use there. In Hong Kong, where utilization of chloramphenicol is almost 100 times higher than in the West, drug-associated AA is infrequent.[155] [156] The early epidemiologic surveys stressed excessive dosage, high blood levels, repeated or intermittent courses, young age, and oral route of administration as particular risks for chloramphenicol marrow toxicity. However, in a series of 600 cases, most patients had received a dose of <10 g.[157]

At ordinary doses, a pattern of reversible alterations in erythropoiesis occurs in most patients treated with chloramphenicol. [158] In vitro, chloramphenicol can decrease hematopoietic colony formation[159] or diminish colony size,[160] but usually at doses greater than those achieved in patients. Inhibition of marrow stromal cell proliferation[161] and the production of growth factors[162] have also been reported. There is no consistent evidence of abnormal marrow sensitivity to the drug in affected individuals. Chloramphenicol was also claimed to produce marked chromosomal abnormalities in white blood cells (WBCs). [163] Others have proposed that chloramphenicol toxicity is the result of covalent binding of the drug to reactive oxidative metabolites, such as an oxalic acid derivative produced by P-450 cytochrome-mediated oxidative dehalogenation to produce a chloramphenicol free radical and hydroxylamine intermediate, all capable of acylating proteins.

NSAIDs

In comparison to chloramphenicol, it took far longer to associate phenylbutazone with AA. [164] Mortality estimates have ranged from $1/10^5$ to $1/10^6$ treatment courses.[165] Usage of other NSAIDs is associated with case reports of AA. The large case-control investigation in Europe not only confirmed the risk of AA with phenylbutazone but identified even higher probabilities with other NSAIDs. [166] There was a suggestion of increased risk with drugs taken regularly for a prolonged period at very high doses, and in some cases hematologic reactions were reproduced on re-exposure.

Neuroleptics and Psychotropic Drugs

A variety of drugs used to treat disorders of the central nervous system have been associated with AA: the hydantoins and carbamazapine, anti-depressants, tranquilizers, and most recently Felbamate. The marketing of Felbamate was severely affected by the occurrence of aplasia in more than 30 patients. [167] Monitoring of drug blood levels and peripheral blood counts in patients receiving carbamazepine was recommended despite fewer than two dozen AA cases reported by 1982. [168] Doubt about the validity of many cases reported in the literature, as well as several large series of patients who did not develop hematologic toxicity [169] and an estimated AA case rate of about 1 in 200,000 treated patients,[170] have led many to question the relationship between carbamazepine and AA.

Gold and Other Heavy Metals

Gold salts have an extraordinarily high frequency of fatal adverse reactions, estimated at $1.6/10^4$ prescriptions. Dose-dependent leukopenia is common, but several dozen cases of AA have been reported.[171] In the IAAAS study, exposure to gold salts was the most significant drug association with AA, with an RR of 29 and an excess risk of 23 cases/10^6 users in 1 week.[5] Spontaneous recovery rarely occurs.[172] Other patients have been successfully treated with stem cell transplant or immunosuppressive therapy; chelation therapy has not been generally helpful. [173] High concentrations of gold salts inhibit hematopoietic colony formation in vitro. [174] There is some evidence for a dose relationship in patients, as high doses have been associated with the development of fatal pancytopenia, as opposed to transient neutropenia or thrombocytopenia.[175]

Arsenic poisoning can result in neutropenia, anemia, and thrombocytopenia. [176] Red cell basophilic stippling such as occurs in lead poisoning is a characteristic finding. Organic arsenicals, originally used in the treatment of syphilis (arsphenamine)

and now as antihelminthics (arseneamide), have also been associated with AA. [177] The quality of the older reports of both arsenic and other heavy metal effects do not allow the exclusion of myelodysplasia as a cause of the marrow failure.

Antithyroid Drugs

Antithyroid drugs have been more closely associated with the development of agranulocytosis than with AA. Thiouracil was reported to cause agranulocytosis in about 2% of treated patients;[178] estimates for propylthiouracil and methimazole ranged widely, from 0 to about 10%. [179] [180] In a Japanese series of over 19,000 cases, only 70 patients developed agranulocytosis. [181] Agranulocytosis was also significantly associated with antithyroid drugs in the IAAAS study, where the RR for agranulocytosis with a history of recent use was >100. In comparison, the risk of AA was about 10-fold lower. [182]

Antibiotics

Trimethoprim-sulfamethoxazole (TMX/SM) is associated with a variety of hematologic toxicities. Of cases reported in Sweden over a decade, leukopenia was most common, followed by bicytopenia, pancytopenia, thrombocytopenia, agranulocytosis, and anemia. The overall probability of developing a hematologic disorder with this treatment was estimated to be $5.3/10^6$ defined daily doses.[183] TMX/SM-associated agranulocytosis is the most common reported complication. As a class, antibiotics may be the commonest agents associated with hospital-acquired neutropenia. [184] Virtually every antibiotic has been associated with the development of agranulocytosis. In the IAAAS, exposure to TMX/SM during the 2 weeks prior to onset of agranulocytosis carried an RR of 12 (CI: 3.940). [154] The association of agranulocytosis with the use of sulfonamides, -lactams, tetracyclines, and other antibiotics, however, did not achieve statistical significance, nor was the use of any antibiotic significantly associated with the development of AA. [154]

Viruses

Viral infections are frequently associated with limited marrow suppression, typically neutropenia and less commonly thrombocytopenia. Epidemiologic studies of AA indirectly suggest an infectious agent. In Thailand, poverty [26] and grain farming (with its attendant water and insect exposure) [185] are associated with the development of AA. Viruses can damage bone marrow directly, by infection and cytolysis of hematopoietic cells, or indirectly, through induction of secondary immune pathways, initiation of autoimmune processes leading to depletion of progenitor and stem cells, or destuction of supporting stroma. Under specific circumstances known viruses can also cause bone marrow failure. AA rarely follows an Epstein-Barr virus (EBV) infection. The classic hepatitis/aplasia syndrome appears to be of viral origin but has not been linked to any known hepatitis agent. Other viruses, including a variety of herpesviruses, retroviruses such as human immunodeficiency virus (HIV), and B19 parvovirus, have not been convincingly implicated as general causes of AA. An interesting parallel exists between AA and the hemophagocytic syndrome, in which immune-mediated pancytopenia develops during convalescence from a wide variety of viral infections. [186] The clinical syndromes in which AA is associated with viral infections are discussed later.

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TYPICAL AND ATYPICAL PRESENTATIONS

Classic Aplastic Anemia

History and Physical Examination

Most patients with AA seek medical attention for symptoms that occur as a result of low blood counts (Table 19-5; Plate 19-2AC). All of the blood elements may be depressed, or a

TABLE 19-5 -- Presenting Symptoms of Aplastic Anemia

Symptom(s)	Number of Patients
Bleeding	41
Anemia	27
Bleeding and anemia	14
Bleeding and infection	6
Infection	5
Routine examination	8
Total	101

Adapted from Wiliams DM, Lynch RE, Cartwright GE: Drug induced aplastic anemia. Semin Hematol 10:195, 1973, with permission.

decrease in a single lineage may dominate the clinical picture. It should be remembered that the differential diagnosis of pancytopenia includes a variety of diseases (see Table 19-1). Bleeding is the most alarming manifestation of pancytopenia and most frequently sends the patient to a doctor. Thrombocytopenia is usually not associated with massive bleeding. Instead, the patient reports easy bruisability and the appearance of red spots, especially over dependent surfaces; gum bleeding with tooth brushing and episodic nosebleeds are also common complaints. Heavy menstrual flow or irregular vaginal bleeding can occur in younger women. In classic cases of paroxysmal nocturnal hemoglobinuria there are reports of red or dark urine that is due to free hemoglobin, but visible bleeding from the genitourinary and gastrointestinal tracts is rare on presentation in AA. Massive hemorrhage from any organ may occur but usually late in the course of the disease and almost always associated with infections, drug therapy (corticosteroids), or invasive procedures.

The ability to adapt to a gradual reduction in hemoglobin concentration is remarkable. The patient with insidious onset of anemia may relate fatigue, lassitude, shortness of breath, or ringing in the ears, but some individuals will tolerate astonishingly low hemoglobin levels without complaint. Angina precipitated by anemia may be a presenting symptom in the older patient. Even abrupt cessation of erythropoiesis will lead to only a slow decline in hemoglobin (about 1 g/dl/week). The presence, absence, or severity of anemia is not useful in judging the time of onset of disease as blood counts do not necessarily fall (or rise) concurrently.

Infection is an infrequent presentation of patients with AA. The sore throat of agranulocytosis is not often observed, presumably because other alarming symptoms appear earlier.

Except for complaints referable to the blood counts, most patients do not have systemic symptoms. Weight loss, persistent fever, and loss of appetite point to an alternative diagnosis. Pain is unusual.

Retrospective studies of AA associated with drugs and viruses and the observation of the occasional patient with serially monitored blood counts suggest a latent period of 68 weeks between the inciting event and the onset of pancytopenia. [187] The interval may be more prolonged when pancytopenia is well tolerated or moderate. Performing a careful, persistent, and reiterative history will often reveal exposure to drugs or chemicals or a preceding viral infection. For purposes of management, a history of blood diseases in other family members is important.

The findings on physical examination usually reflect the severity of the pancytopenia (Table 19-6). However, patients with severe disease may look remarkably well. The patient may present with subtle variations from normal or with a dramatic, even toxic appearance. Petechiae are often present over dependent areas, especially the pretibial surface of the lower leg and the dorsal aspects of the forearm and wrist; a few petechiae may be seen in the oropharynx and on the palate. Scattered ecchymoses of various sizes and shades may be seen, typically on areas

TABLE 19-6 -- Aplastic Anemia: Severity Defined by Laboratory Studies

Severe AA
Bone marrow cellularity <30%
Two of three peripheral blood criteria: ANC <500/mm^3
Platelets <20,000/mm^3
Reticulocytes <40,000/mm^3
No other hematologic disease
Moderate AA
Patients with pancytopenia who do not fulfill the criteria of severe disease

exposed to minor trauma. With severe thrombocytopenia, retinal hemorrhages may be present on funduscopic examination, there may be gingival oozing or blood in

the nares, and hemorrhage may be apparent at the uterine cervical os. The stool may contain traces of heme. Pallor is common and is best appreciated on the mucosal membranes and palmar surfaces. The new patient may be febrile, but specific or localizing signs of infection are uncommon on presentation. Cachexia, splenomegaly, and lymphadenopathy are not associated with AA, and these findings should strongly suggest another diagnosis. The examiner should look carefully for café-au-lait spots and other physical anomalies of Fanconi anemia in adults as well as children. Acquired AA is usually a solitary diagnosis, and evidence from the history or physical examination of another major disease suggests secondary pancytopenia; an exception is drug-associated aplasia, as in the patient with chronic rheumatoid arthritis.

Several atypical presentations of the AA should be pointed out. A physician may encounter an elderly patient with pancytopenia in whom subsequent bone marrow examination reveals dysplastic features. This patient may represent a diagnostic challenge to the physician, who must discriminate AA from hypoplastic myelodysplasia (Fig. 19-6 ; Table 19-7 (Table Not Available)). Although the history of the present illness in a newly diagnosed patient will be short, in the range of months, occasional patients recall a long history of bruisability, anemia, and low blood cell counts reported to them by previous physicians during routine examinations.

Figure 19-6 Diagnostic algorithm in aplastic anemia. PB, peripheral blood; BM, bone marrow; AA, aplastic anemia; MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; AMM, agnogenic myeloid metaplasia; DEB, diepoxybutane; MMC, mitomycin C.

TABLE 19-7 -- Bone Marrow Morphologic Findings That Discriminate Myelodysplasia from Aplastic Anemia

(Not Available)

Adapted with permission from Bagby 1990,[279] which provides percentages for myelodysplasia.

These patients may have a moderate disease that is stable for years. Some patients may be diagnosed incidentally and show remarkably few symptoms despite severely depressed blood counts.

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CLINICAL ASSOCIATIONS

A number of clinical syndromes, usually revealed through a careful history and physical examination, are associated with AA (Fig. 19-6 ; Tables 19-2 and 19-3).

Post-transfusion Graft-versus-Host Disease

Almost uniformly fatal AA is a constant feature of transfusion-associated GvHD, a syndrome produced by the transfusion of competent lymphocytes into immune-deficient hosts, including children with congenital syndromes, cancer patients receiving high-dose chemotherapy, and, more recently, with adoptive cellular immunotherapy for leukemia.[68] [188] Rarely, post-transfusion GvHD occurs in an apparently immunocompetent recipient in the special circumstance in which the donor is homozygous for an HLA haplotype also shared by the recipient, as may occur among first-degree family members. Small numbers of lymphocytes are sufficient to produce the syndrome, which is surprisingly resistant to immunosuppressive therapy. Pancytopenia with marrow hypoplasia is an almost constant feature of post-transfusion GvHD. Runt disease in animals is a model of this immune-mediated marrow failure syndrome.

Pregnancy

Pregnancy is common in the age groups most susceptible to marrow failure, and in many cases its association is probably only coincidental. The true frequency of AA in pregnancy is not known, but from the number of cases reported it appears rare, [189] [190] [191] although bone marrow hypoplasia may be relatively common during pregnancy.[192] A causal relationship is suggested by the temporal relationship between the onset of pancytopenia and that of pregnancy, and by resolution following delivery and spontaneous or induced abortion; [193] [194] occasional patients have developed AA that remitted after each delivery. [195] Survival rates for AA in pregnancy have been relatively high for the mother (83%) and baby (75%), with most pregnancies successful (69%). [191] When death occurs during pregnancy or delivery, hemorrhage usually is responsible. The published data

are insufficient to guide the management of the pregnant woman with AA, especially since it is clear that AA in some cases is serendipitously diagnosed and can persist beyond parturition. A woman who desires a child can be maintained with transfusions, with the understanding that any clinical deterioration is a criterion for interruption or termination of the pregnancy. The risk of pregnancy to the woman who has recovered from idiopathic AA is similarly unknown.

Hepatitis/Aplasia Syndrome

The association of AA and acute, apparently viral hepatitis is not rare. Several hundred cases have been reported. [100] [196] [197] A prior history of hepatitis is recognized in 29% of AA patients in Western series [198] [199] and in perhaps a larger proportion of patients in the Far East. [200] [201] Uncomplicated viral hepatitis is frequently associated with mild blood cell count depression, but severe pancytopenia with marrow aplasia is a rare sequela, estimated at <0.07% of total pediatric hepatitis cases[202] and 2% of patients with non-A, non-B hepatitis.[203] [204] However, in patients, usually children, who develop hepatic failure after fulminant seronegative hepatitis, about one-third develop AA at the time of liver transplantation. [205] [206]

Hepatitis-associated AA has several peculiar features. [100] [197] [207] Typically, an uneventful episode of apparent viral hepatitis in a young man is followed in 12 months, during convalescence from the liver inflammation, by severe pancytopenia. Depression of blood cell counts during the course of hepatitis is, of course, common: leukopenia, atypical lymphocytosis, erythroid macrocytosis, and thrombocytopenia mimic in milder forms the hematologic changes of AA. However, posthepatitis AA has a very poor prognosis, with early estimates of mortality at 90% at 1 year, [197] so that a history of hepatitis in AA has been considered an indication for early marrow transplantation.[208] Patients with posthepatitis AA can successfully undergo marrow transplantation without an increased risk of venoocclusive disease. [209] Patients with hepatitis-associated aplasia have markers of immune system activation and also respond well to intensive immunosuppressive therapy. [100] [210]

The putative viral agent in posthepatitis AA is unknown. Almost all well-studied cases have been non-A, non-B, non-C, [211] [212] and most recently non-G.[100] Both hepatitis C and G viruses are common in patients with AA, but usually as a result of their heavy transfusion load. [100] [211] [213] Posthepatitis AA is linked to both fulminant hepatitis of childhood and acute seronegative hepatitis. Acute viral hepatitis that is seronegative differs clinically from hepatitis C disease: parenteral exposure is not a risk factor, liver functions abnormalities are more severe during the acute phase, and late complications are more common. [214]

Postmononucleosis Aplastic Anemia

Acute infection with EBV causes infectious mononucleosis that is commonly associated with neutropenia and other hematologic abnormalities [215] [216] [217] but, like acute hepatitis, is only rarely complicated by AA. [218] [219] [220] However, EBV may be involved in the etiology of AA more frequently than originally appreciated, since a large number of primary EBV infections are unrecognized. [219] [221] [222] Pancytopenia can be first observed during the acute mononucleosis syndrome or shortly after disappearence of symptoms. Some patients have recovered spontaneously; others after therapy with corticosteroids or ATG. EBV can occasionally be demonstrated in the bone marrow cells of patients with apparently idiopathic AA, in association with serologic evidence of a primary or reactivated viral infection. Some patients with EBV-associated AA may respond to antiviral therapy.[219] [223] [224] [225] For those with severe disease, conventional therapeutic interventions should be implemented early.

Hemophagocytic Syndrome/Aplasia Syndrome

The marrow is hypocellular in about a third of cases with hemophagocytic syndrome. In this disorder there may be progression from marrow hypercellularity to aplasia; myelofibrosis is also common. Pancytopenia occurs in 74% of cases. Anemia is a universal finding; thrombocytopenia (91%) and neutropenia (65%) are also frequent.[226] In contrast to typical AA, patients appear systemically ill, with fever and constitutional symptoms, and peripheral blood cell count depression is often associated with abnormalities of other organ systems: hepatosplenomegaly, lymphadenopathy, cutaneous eruptions, and pulmonary infiltrates. [226] [227] [228] Hemophagocytic syndrome is associated with a large variety of diseases, including immunodeficiency, malignancy, and infections. In the infectious category, viral infections are commonest, often with herpesviruses, including especially EBV, and also cytomegalovirus, herpes simplex, or herpes zoster. [226] [229] B19 parvovirus, HIV-1, and bacterial and parasitic infections also have been associated with hemophagocytosis. Hemophagocytosis is often observed on supravital [230] or Wright-Giemsa staining [187] of the marrow of patients with idiopathic AA, and it is also a morphologic feature of graft rejection after marrow transplantation. [231] In virus-associated hemophagocytosis, there is evidence of immune system activation. The sera of patients contain high levels of IFN-, TNF-, and IL-6, soluble CD8 and soluble IL-2 receptor, and T cells overproduce IFN- in vitro. [186] The clinical response to cyclosporin A (CsA) is consistent with a T-cell-mediated pathophysiology of

hematopoietic failure.[232]

Paroxysmal Nocturnal Hemoglobinuria/Aplasia Syndrome

There is a strong association between AA and paroxysmal nocturnal hemoglobinuria (PNH). These diseases frequently are diagnosed concurrently or sequentially in the same individual, and they share similar clinical and pathologic features (pancytopenia and marrow hypocellularity), a striking geographic variation in rate (higher frequency in Asia), and similar responsiveness to immunosuppressive therapy.[233] [234] [235] [236] PNH is characterized by the triad of intravascular hemolysis, venous thrombosis, and marrow failure. More patients, however, appear to die of thrombotic than of bleeding complications.[237] PNH is due to a somatic mutation in the X-chromosome gene termed *PIG-A*; the product of this gene is responsible for an early step in the biosynthesis of the proteoglycan anchor of the class of glycophosphatidylinositol-linked cell-surface membrane proteins.[235] [238] These proteins can now be measured easily and quantitatively using flow cytometric methods, extending and replacing the classic Ham test. The role of CD59 in inactivating complement on the RBC surface adquately explains the intravascular hemolysis of PNH, but the role of the absence of glycophosphoinositol-linked proteins in the pathophysiology of marrow failure is unknown.

Marrow failure may be present at the onset of PNH or may develop after diagnosis. In a retrospective review, marrow failure was especially prevalent among young patients, occurring in 58%.[239] In a cooperative study from France of 220 patients with PNH (defined by a positive Ham test), there was a previous history of AA in 30%, and in the remainder the actuarial risk of developing pancytopenia was estimated at 14% at 4 years.[240] Conversely, AA may evolve into PNH months or years after successful immunosuppressive treatment; many patients will have only a positive Ham test or a population of granulocytes lacking glycophosphoinositol-linked proteins. In one study a presumptive diagnosis of PNH based on flow cytometry was made in a large proportion, nearly 50%, of AA cases at presentation,[241] but this figure is likely an overestimate.[242] [243] On the other hand, flow cytometric analysis of marrow from patients with

AA suggested that PNH clones may be detectable in the marrow even when absent from the circulation.[244] Longitudinal studies of patients with de novo PNH[237] as well as PNH developing from AA[245] indicate a significant probability of spontaneous disappearance of serologic or flow cytometric evidence of disease.

In the setting of marrow failure, PNH could develop as a result of an intrinsic growth or survival advantage for the somatically mutated cell or because of extrinsic selection of cells bearing the *PIG-A* defect, most current evidence favors the latter possibility. Supporting an extrinsic mechanism are the presence of multiple genetically different *PIG-A* mutations in a single patient;[246] apparent in vivo selection for the development of PNH-like lymphocytes in lymphoma patients treated with CAMPATH-1, a monoclonal antibody that incidentally recognizes a glycophosphoinositol-linked protein;[247] lack of a growth advantage for *PIG-A* knockout cells in vitro[248] and in vivo;[249] and similar in vitro growth characteristics of normal and deficient progenitor cells.[242] Evidence for an intrinsic effect comes from experiments demonstrating that PNH cells proliferate better in immunodeficient mice[250] and from the implication of a glycophosphoinositol-linked protein in the interaction between stromal and progenitor cells.[251] A generalized resistance to apoptosis that was initially proposed has not been confirmed.[252] [253] [254] One likely pathophysiology is relative resistance to attack by the immune system of PNH stem cells due to lack of a required recognition protein on the hematopoietic target cell.[255]

Collagen Vascular Diseases

AA is a component of the collagen vascular syndrome called eosinophilic fasciitis.[256] [257] [258] This severe, scleroderma-like disease is characterized by fibrosis of subcutaneous and fascial tissue, localized skin induration, eosinophilia, hypergammaglobulinemia, and an elevated erythrocyte sedimentation rate. The rheumatologic symptoms of fasciitis respond to corticosteroids, but the associated AA has a very poor prognosis. A few patients have survived after bone marrow transplantation or immunosuppressive therapy. More unusually, AA has complicated systemic lupus erythematosus and rheumatoid arthritis, but in many cases the role of concomitant drug therapy is confounding.[259] Also rarely, AA may complicate Sjögrens syndrome,[260] multiple sclerosis,[261] and immune thyroid disease.[261] AA occasionally occurs in individuals with hypogammaglobulinemia[73] or congenital immunodeficiency syndrome,[75] thymoma, or thymic hyperplasia.[76] [77]

LABORATORY EVALUATION

Peripheral Blood

In typical cases of AA, all the blood cell counts are depressed. From the half-life in the circulation of granulocytes, platelets, and erythrocytes, most patients would be predicted to develop neutropenia and thrombocytopenia, but in fact anemia is common and the granulocyte count may fall further during observation. The blood smear usually shows an obvious paucity of platelets and leukocytes but with normal RBC morphology; toxic granulations may be present in neutrophils. Automated cell counting frequently shows erythrocyte macrocytosis and a normal red cell distribution of width indicating little variation in RBC size. Platelet size is normal and not increased as in immune peripheral destruction, but the low number may cause greater heterogeneity of size. Low numbers of reticulocytes may be accurately quantified with automated counting. Inexplicably, a few patients with otherwise typical AA have been reported to have circulating erythroblasts; [262] similar cells have been detected by flow cytometry.[263] Prior transfusions will alter platelet numbers, relative reticulocyte counts, and hemoglobin values. Although relative lymphocytosis is very common, most patients also have decreased absolute numbers of monocytes[264]

DIAGNOSIS OF APLASTIC ANEMIA

While the ultimate diagnosis of aplastic anemia rests on the interpretation of an adequate bone marrow biopsy specimen, important clues to the etiology of pancytopenia can be obtained from the history, physical examination, and laboratory data. Pancytopenia that is not primarily hematologic in origin but secondary to other disease processes is usually an obvious diagnosis. Patients with severe liver disease and splenomegaly, systemic lupus erythematosus, or overwhelming sepsis may all have low blood cell counts, but their clinical presentation is not subtle. Similarly, bone marrow aplasia follows cytotoxic drug therapy for cancers and, in more recent practice, for a variety of nonmalignant diseases, but this is an anticipated and transient toxic effect. In the challenging case, obvious medical causes of pancytopenia usually have already been excluded. Pancytopenia is almost never due to peripheral blood cell destruction alone. In AA, the blood smear will not show reticulocytes, band forms, or the large platelets typical of increased compensatory bone marrow efforts. Acquired AA is a disease of the young, but so is constitutional aplasia. Patients with Fanconi anemia often have physical anomalies or look funny, but the distinction between acquired and constitutional disease depends on the results of a clastogenic-stress culture of peripheral lymphocytes and the appearance of typical chromosomal abnormalities in vitro. Fanconi anemia has been diagnosed in patients in late middle age! However, in most older patients the major differential diagnosis is between aplastic anemia and myelodysplasia. There is a true gray zone between hypocellular myelodysplasia and moderate aplastic anemia in which competent hematologists may not agree on the final diagnosis. Bone marrow cytogenetics should be performed to help in establishing the proper diagnosis. Myelofibrosis can also produce pancytopenia, but the bone marrow is not aspirable, the spleen is often enlarged, and the peripheral blood smear shows characteristic abnormalities. Acute leukemia in both children and the elderly may manifest with bone marrow hypocellularity requiring a careful search for lymphoblasts or myeloblasts, including phenotypic analysis by flow cytometry. Peripheral blood flow cytometry for glycophosphoinositol-anchored proteins should be performed to diagnose PNH. The patients history may have interesting clues: benzene exposure for myelodysplasia and acute leukemia or a suspicious drug history for AA. Discontinuation of exposure to incriminated drugs or chemicals is mandatory, and in some instances patients may then recover. However, given the difficulty of assigning blame with absolute certainty to environmental agents, we treat all patients similarly and do not advocate protracted observation for possible spontaneous recovery. In patients with severe disease (Table 19-6), suitable and early preparation for bone marrow transplant should be undertaken or immunosuppression begun, whereas in those with moderate disease, clinical status should be evaluated and serial blood cell counts are required to assess progression of the disease.

and lymphocytes. The severity of AA can be graded based on the peripheral blood cell counts (Table 19-6).

There is no characteristic pattern of other findings on routine hematologic and clinical chemistry tests. Serum transaminase levels may be mildly elevated in patients with hepatitis-associated AA. The Ham test and the sucrose hemolysis test may be positive. Patients with AA generally have normal immunoglobulin levels and normal antibody titers to common viral antigens. About one-half of patients were reported to be anergic to a common panel of antigens with normal lymphocyte proliferation in vitro.[265]

Bone Marrow

The marrow must be assessed quantitatively and qualitatively, for cellularity and the morphology of residual cells (Plates 19-1A and B , 19-3AF , and 19-4AD). Both marrow aspiration and biopsy should always be performed, with the core specimen at least 1 cm in length. There should be no compromise in obtaining adequate specimens and no hesitation in requesting and performing a second procedure if required.

Bone marrow cellularity is best estimated from the core biopsy. Point counting under microscopic cross hairs in many parts of a histologic section [266] is the most accurate method of determining cellularity, but hematologists commonly rely on visual estimation only; [267] a crude eyeball approximation is almost always adequate in severe aplasia, as the hematopoietic content of the marrow specimen is usually close to zero. Estimates of marrow cellularity based on examination of the aspirate smear and biopsy specimen are correlated, but dilution of the aspirate by sinusoidal blood often occurs, and the aspirate may be hypocellular when the biopsy specimen is hypercellular[268] or may show focal areas of active hematopoiesis.[269] Normal marrow cellularity decreases considerably with age, a variation that is of some importance in assessing the older patient with aplasia or myelodysplasia. In autopsy samples from normal young children, about 80% of the marrow space of the iliac crest is cellular (range, 60100% [270]); marrow from the sternum, vertebrae, and long bones is more usually 100% cellular in infants; [271] for sternal aspirates, cellularity ranges from 35% to 80%.[272] Marrow cellularity gradually falls from age 20 to 70 years and more precipitously in the very elderly, to about 30% in the eighth decade of life.[270] Although for practical purposes, the lower limit of normal marrow cellularity in adults is accepted at about 30%, the differences at the extremes of life should be recalled when evaluating infants and the elderly. In most patients with AA total marrow cellularity is extremely low, but there may be significant residual lymphocytosis. The increase in marrow fat in aplasia is due to an increase in both the size and number of individual fat cells. Hot pockets of hematopoiesis may be present in a minority of patients.[273] The marrow tends to contract centripetally with age, and a similar process can be observed in pathologic states, so the sternal bone marrow may be more cellular than iliac crest samples.

Examination of the bone marrow (Plates 19-1 , 19-3 , and 19-4) is basic for the diagnosis of most primary hematologic causes of pancytopenia. (Table 19-1 ; Fig. 19-6). Information may be gained by observing the marrow aspirate itself. A fatty, even watery specimen can usually be aspirated without difficulty from an aplastic patient, whereas a truly dry tap is more typical of a packed or fibrotic bone marrow. The morphology of individual cells is best seen in the Wright-Giemsa-stained aspirate smear, the architecture of the marrow in the biopsy section. In acellular specimens, the only cells visible are usually lymphocytes, plasma cells, and stromal

elementsfibroblastoid and histiocytic cells. Some degree of dyserythropoiesis is frequent, usually the megaloblastoid features of macrocytosis and some nuclear-cytoplasmic maturation asynchrony, but sometimes more complex degenerative changes in nuclei and cytoplasm can be observed by light [274] and electron [275] microscopy (Plate 19-3). These features are common to AA and myelodysplasia (Table 19-7 (Table Not Available)), which may be very difficult to distinguish prospectively. [276] Hemophagocytosis of RBCs can also be seen in AA. Examination of the cells close to the spicules of a sparse aspirate smear may disclose a distinctive population of leukemic blasts, or the dysmorphic appearance of residual erythroid cells, which will more strongly suggest dysplasia than aplasia. Increased numbers of myeloblasts are not seen in AA and are evidence of aleukemic leukemia or herald the evolution of leukemia from pancytopenia. [277] Lymphoid aggregates, nests of tumor cells, granulomas, and infectious particles may be apparent on examination of the fixed biopsy specimen. Karyotyping of marrow cells is diagnostically important. Unfortunately, the yield of cells from a hypocellular marrow may be inadequate to perform cytogenetic analysis. Chromosome analysis is usually normal in AA [276] but frequently reveals a clonal abnormality in myelodysplasia. [278] [279]

Radiographic Measures of Bone Marrow Function

Ferrokinetic studies of patients with AA are characterized by high plasma iron concentrations, transferrin saturation, and prolonged clearance of radioactively labeled iron. [280] Binding of labeled ^{59}Fe to transferrin prior to injection more specifically distinguishes iron uptake by erythroid cells. [281] ^{111}Indium, which also binds to transferrin, has been employed for bone marrow scintigraphy. Studies of AA patients almost always show markedly decreased ^{111}In uptake; [282] however, the technique has been limited by the lack of availability of the isotope and imperfect correlation with blood cell counts. Myelopoiesis has been mapped in experimental studies using ^{99m}Tc-conjugated monoclonal antibodies to leukocyte antigens (CD67 [283] and a nonspecific cross-reacting antigen [284]), with good correlation with results of ^{59}Fe scanning. Magnetic resonance imaging (MRI) with spin-echo sequences has proved to be most useful in the study of bone marrow disease. On T1-weighted spin-echo images, fatty marrow appears bright and cellular marrow exhibits a lower-density signal (Fig. 19-7). The high fat content of aplastic bone marrow can be readily appreciated on MRI. MR spectroscopy, which detects the type of fat signal, has shown diverse patterns among AA patients. [285] MRI is complementary to tissue sampling, allowing a large area of marrow to be visualized and fat content to be roughly quantified. The technique appears to be worthwhile in diagnosis, because the patterns of fat and cell distribution appear to differ between aplasia and hypocellular myelodysplasia, and in prognosis, to monitor improvements in hematopoiesis after treatment. [286]

DIFFERENTIAL DIAGNOSIS OF PANCYTOPENIA

AA is not the most common cause of pancytopenia (see Table 19-1). In one study, of 100 pancytopenic cases, a diagnosis of AA could be made in only 19, despite prescreening for systemic or malignant diseases. [287] A rational diagnostic algorithm may be very helpful in establishing a correct diagnosis (Fig. 19-6). An uncertain diagnosis jeopardizes the patient, who may be directed to an improper treatment. Pancytopenia can result from peripheral blood processes with relatively normal bone marrow function, but such syndromes usually occur in an obvious context as complications of nonhematologic primary disease. Pancytopenia is unlikely to be the presenting feature of hypersplenism in cirrhosis or of Evan syndrome in systemic lupus erythematosus. Findings on physical examination may point strongly toward another diagnosis. For example, the patient with myelofibrosis usually has splenomegaly, but a large spleen is very unusual in AA. While B_{12} and folate deficiency have been reported to be associated with erythroid hypoplasia, [288] this must be an exceedingly rare event. Well before a bone marrow aspiration

Figure 19-7 Magnetic resonance imaging of bone marrow in **(A)** severe aplastic anemia in a young man, **(B)** severe aplastic anemia in a middle-aged woman, and **(C)** myelodsyplasia in a middle-aged woman.

is performed, the list of possible diagnoses should be shortened by reasoning from the patients history, physical examination findings, and knowledge of the probability of alternative disorders.

For the practicing hematologist, the most important and difficult choice of diagnoses in pancytopenic patients is among primary bone marrow disorders. An empty bone marrow is usually obvious, but several complicating factors should be recognized. Most diagnostic confusion arises from (1) the equivocal character of moderate AA; (2) alterations in marrow appearance in patients with chronic disease; and (3) real overlap between AA, myelodysplasia, and leukemia.

In moderate AA the modest depression of marrow cellularity can muddle the single most reliable diagnostic criterion. Bone marrow cellularity is imprecisely quantitated at best, and further uncertainty is introduced by large sampling errors. Hot spots of hematopoietic activity in an otherwise acellular specimen reflect biologic heterogeneity in the pattern of cell loss. In addition, a syndrome of transient pancytopenia has been recognized in which spontaneous recovery occurs within a few months; although the blood cell counts can be severely depressed, the marrow is much more commonly normal or hypercellular than hypoplastic. [289] In patients with chronic bone marrow failure, serial bone marrow specimens may not be identical, either because of sampling error or because the original disease was misdiagnosed or has changed its character. Occasional patients with AA are not pancytopenic; they do not have uniform depression of red cell, white cell, and platelet production, despite an empty bone marrow, and their clinical course will be dominated by failure in two or a single hematopoietic lineage. Related conditions such as pure red cell aplasia, amegakaryocytic thrombocytopenia, and agranulocytosis, while usually distinctive in their clinical presentation, may evolve into more generalized marrow failure. In the absence of better markers, sometimes the only accurate diagnosis is a description of the clinical features and the specific marrow morphology.

A hypocellular bone marrow often precludes the proper morphologic diagnosis. This problem may be especially evident in the case of a myelodysplastic syndrome with hypoplastic bone marrow (Table 19-7 (Table Not Available)). In addition to the clinical features, more sophisticated laboratory studies may be helpful. Bone marrow cytogenetics, if positive for chromosomal abnormalities, facilitate the diagnosis of leukemia or myelodysplastic syndrome. Conventional bone marrow examination is not helpful in differentiating AA from Fanconi anemia. Fanconi anemia is diagnosed by a positive mitomycin-C resistance test (see Chap. 18).

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TREATMENT

AA should be considered a medical emergency. Lives are lost, mainly because the grave consequences of severe pancytopenia go unrecognized. The ultimate benefits of definitive therapies such as transplantation or immunosuppression will be unrealized if the patient succumbs to early clinical catastrophe. A haphazard transfusion policy increases the risk of graft rejection after a marrow transplant, but too conservative an approach to transfusion can jeopardize the patients life and increase morbidity. Supportive management therefore requires both meticulous attention to the daily problems that occur as a consequence of pancytopenia and appreciation of their impact on the ultimate possibilities for cure or amelioration of AA. AA can be cured by replacement of stem cells, by bone marrow transplantation, and by immunosuppressive therapy. Androgens and hematopoietic growth factors have secondary roles (Fig. 19-8).

Supportive Management

Bleeding

Bleeding was historically a common symptom in AA, and death from hemorrhage was frequent in the premodern era. The effective use of platelet transfusions has substantially improved survival in this disease. Measurable correction of the platelet count by transfusion almost always alleviates the minor mucocutaneous

Figure 19-8 Algorithm-based selection of treatment for patients with aplastic anemia. BMT, bone marrow transplantation; ATG, antithymocyte globulin; CSA, cyclosporine A; IS, immunosuppression.

bleeding common in thrombocytopenic patients. Major bleeding usually is not due to thrombocytopenia alone, and ancillary explanations for massive hemorrhage should be sought. The bleeding time improves after erythrocyte transfusion in patients with anemia, and there is a strong inverse correlation between the hematocrit and bleeding.[290] Therefore, the treatment of serious hemorrhage should include correction of severe anemia and red cell transfusions.

Modern transfusion practice has made platelets readily available and safe to administer. Other than cost and convenience, the major problem related to platelet transfusions is the development of alloimmunization in the recipient. [291] [292] The life span of the transfused platelet in the circulation is dramatically shortened by host antibodies, almost always directed to HLA-A and B antigens. Alloimmunization is suggested by poor recovery of the 1-hour-post-transfusion platelet count and confirmed by finding specific HLA antibodies in serum. [293] [294] Refractoriness can often be overcome by selection of HLA-matched donors, but 639% of perfectly HLA-matched transfusions fail.[294] Alloimmunization can be prevented or delayed by the use of single-donor platelets rather than pooled platelets. [295] Physical leukocyte depletion by filtration or ultraviolet treatment of blood products [296] [297] has been successfully piloted in AA patients. [298] Avoidance of platelet transfusions except when there is active bleeding is another alternative to prevent alloimmunization, but the dose relationship between exposure to different donors platelets and the probability of developing refractoriness is not clearly established, [294] and only after >40 units have been administered does the risk of alloimmunization clearly increase.[299]

Prophylactic transfusion of platelets is controversial. [300] [301] The primary indication for platelet prophylaxis is to prevent intracranial hemorrhage, but the risk of this complication in the chronically thrombocytopenic patient, while real, is low. Prophylactic platelet transfusions have not been shown to alter survival. [300] [301] [302] Nevertheless, the beneficial effects of avoiding bleeding complications and improving the quality of life have been used to justify their routine continuation. [303] Maintenance of platelet counts >20,000/l will decrease bleeding episodes, [304] [305] but the threshold of 20,000/l is based on a very old study showing reduction in days of bleeding and serious hemorrhage among children with acute leukemia. [306] Although the 20,000/l value has long been used to trigger transfusion, several reports have suggested little difference in the risk of bleeding over a wide range of platelet counts between 5,000 and 100,000/l. [307] [308] Until recently, tradition has resisted attempts to lower the transfusion threshold.[309] In one study, a rigorous transfusion policy during induction therapy was proposed: platelets were administered to every patient with a count less than 5,000/l, for patients with 6,00010,000/l platelet count if fresh minor hemorrhages or fever were present, for patients with platelet counts of 11,00020,000/l if a coagulation disorder was present or before a minor procedure, and for patients with 20,000/l platelet counts if major bleeding complications

occurred or surgery was planned. Fatal or severe bleeding episodes were rare and did not occur in patients with platelet counts >10,000/l. The worst bleeding episodes in these cases were associated with refractoriness to platelet transfusions. [310] In a recent randomized trial of patients with AML, the risk of major bleeding was no different when 10,000 or 20,000/l was chosen as the threshold, while the lower value led to a 20% reduction in platelet use. [311] Any prophylaxis program must be modified to address the individual patient, but a goal of maintaining platelet counts >5,000/l is reasonable.

Bone marrow aspirates and biopsy specimens can be obtained without platelet transfusion support. Major surgery can be accomplished in the setting of thrombocytopenia; in one study, blood loss and morbidity were low even at platelet counts <30,000/l. [312]

Anemia

Other than to reduce the risk of graft rejection after allogeneic transplant, there is no reason to allow a patient to suffer the symptoms of anemia. Once equilibrium is achieved, a constant amount of blood will be required to maintain a given hemoglobin concentration. Physically fit individuals are usually not symptomatic at hemoglobin concentrations >7 g/dl; patients with underlying cardiovascular disease should be maintained at a higher level (9 g/dl). Perhaps in contrast to alloimmunization by platelet transfusions, AA patients show a relatively low frequency (about 11%) of alloimmunization due to packed red cell transfusions. [313] In patients with acquired anemia who are likely to remain dependent on erythrocyte transfusions, deferoxamine therapy is effective in chelating iron. Iron chelation should be used in patients with unresponsive chronic anemia who have a reasonable expectation of survival.

Alloimmunization as a result of blood product administration increases the probability of graft rejection and the mortality following marrow transplantation. Blood products from a potential marrow donor such as a sibling or a parent (who will share histocompatability antigens) should be avoided. Small numbers of transfusions do not have a major deleterious effect on survival. The 5% risk of graft rejection following transplantation in entirely untransfused patients was increased to 15% with receipt of 140 units and to >25% only in more heavily transfused patients. [314] An increased rate of graft rejection was observed in patients who had received >10 units of erythrocytes or 40 units of platelets.[315] Speed in arranging tissue typing and transfer to an appropriate center has a greater impact on the survival of the patient

than the judicious transfusion of a few units of RBCs to a severely anemic patient or platelets to a bleeding patient. Transfusions should not be withheld in an older patient in whom immunosuppressive therapy will be first therapy.

Infection

There are very few specific reports of infections and their therapy in patients with AA. [264] [316] The duration of neutropenia is the major difference between the neutropenia of bone marrow failure and that induced by cytotoxic chemotherapy. With longer periods of neutropenia, the probability of serious bacterial or fungal infection increases. A second major difference is that neutropenia is part of a complex of problems associated with malignant disease and its therapy. In AA, the immune system is activated and, with the exception of intravenous catheter placement, the integument is preserved. Studies of cancer patients have usually identified a low-risk category of neutropenia, determined by the relatively brief period of neutropenia; by this criterion, almost all unresponsive patients with AA are high risk.

In classic studies of leukemic children, neutropenia was shown to increase susceptibility to bacterial infections and the number of infectious episodes was quantitatively correlated with the degree and duration of neutropenia: 910% of days at granulocyte levels above 1,500/l was associated with proved infection, but this figure rose to 20% at granulocyte counts of 5001,000/l, to 36% at 100500/l, and to 53% at <100 neutrophils/l. [317] Extreme neutropenia (neutrophils <100/l) was associated with the most serious infections and a very high mortality. [318] Susceptibility to infection is extremely high with an absolute neutrophil count <200/l, and this value has been used to define a category of very severe AA. [319] As severe granulocytopenia becomes prolonged, infection is inevitable.

Similar recommendations for initiation of empiric antibiotic therapy apply to AA patients as to other patients with neutropenia. The cardinal rule is, if the absolute neutrophil count is <500/l and infection is suspected, immediately begin broad-spectrum parenteral antibiotic therapy. Any regimen may need modification based on results of cultures, new symptoms or signs, or a deteriorating clinical course. Bacteremia is present in only 20% of febrile neutropenic episodes, and in only about 40% of those can a microbiologic cause or localizing physical findings be identified. [320] [321] Thus, early discontinuation of antibiotics in instances where cultures are unrevealing in persistently neutropenic patients is dangerous. Sometimes patients remain febrile despite antibiotic therapy; fever may also reappear after a few days or weeks. In the absence of additional microbiologic data or clinical clues from the patients complaints or physical examination, we routinely institute antifungal therapy with amphotericin B in patients who have remained febrile despite adequate antibacterial therapy for more than 3 days. Fungemia during an initial febrile episode is rare, but fungal infection becomes more likely with repeated courses of antibiotics, and ultimately is the major cause of death in AA patients in whom definitive therapy fails. [322] *Candida* and *Asperqillus* species account for almost all fungal disease in AA. [322] Early aggressive treatment of neutropenic patients can reverse fungal disease, as demonstrated in individual cases [323] and by comparison of autopsy series. [324]

Polymorphonuclear cells have a relatively brief life span in the circulation and their major activity is in infected tissue. There are no simple measures of the therapeutic efficacy of WBC transfusions. The absolute neutrophil count is not measurably increased by standard transfusions. Nonetheless, several controlled trials performed in the 1970s reported significantly improved survival in patients who received granulocyte transfusions; the negative studies were criticized because of the relatively low numbers of granulocytes transfused ($<10^{10}$ /m^2 /day). [325] [326] [327] As granulocyte transfusions have fallen out of favor, they have tended to be used solely in desperate clinical circumstances, making them appear even less efficacious. Granulocyte transfusions are expensive and associated with serious toxicity: severe febrile reactions, pulmonary capillary leak syndrome (especially when amphotericin is being concurrently administered), an increased risk of infection, and inevitable alloimmunization. Alloimmunization leads to an inability of infused leukocytes to localize to sites of infection. However, a meta-analysis of published studies suggested that granulocyte transfusions are beneficial in patients with sepsis unresponsive to antibiotics if (1) adequate cell numbers were administered (23×10^{10} /day), (2) donor and recipient compatibility was present, and (3) the patients treated were unlikely to have imminent improvement in marrow function. [328]

Cytokines offer a new strategy for increasing the efficiency of donor harvests. Administration of G-CSF to normal donors greatly increases the yield of leukapheresis without adverse effects on the volunteers. Such large numbers of neutrophils can be obtained as to allow dramatic increases in the absolute granulocyte level in neutropenic recipients. Case reports have suggested that G-CSF-mobilized granulocyte transfusions may be

helpful in the treatment of life-threatening fungal infections in severely neutropenic patients. [329] [330] A randomized trial to test the efficacy of these preparations is needed.

Infections can be prevented. Most important are measures directed toward increasing general hygiene. [331] The physical surroundings of the patient should be well maintained to reduce the risk of nosocomial infection. Dental hygiene is important and sources of infection should be removed under antibiotic and platelet coverage. Nystatin oral rinses prevent thrush. [332] Routine rectal examinations are more likely to be harmful than helpful. Blood should not be taken from fingertips and ear lobes. Early attention to the signs of infection, especially after hospital discharge, can avert many of the complications of initially minor infectious episodes.

Total protective environments, often in combination with nonabsorbable antibiotics or selective gut decontamination to preserve some anaerobic bacteria, [333] have had strong advocates. Laminar flow rooms with gut sterilization are not obviously superior to simpler methods of isolation. [334] Simple reverse isolation accomplishes little more than compulsive hand washing. [335] Sterile diets, avoidance of fresh fruit and vegetables, and no flowers in the room similarly are practices of unproven value.

Definitive Therapy

Bone Marrow Transplantation

Allogeneic bone marrow transplantation from an HLA-matched sibling donor provides curative therapy for AA patients (Figs. 19-9A and B ; Table 19-8). Bone marrow transplantation in AA is the subject of a large number of reports and reviews. [336] [337] [338] [339] [340] [341] Recently, cytokine-primed peripheral blood has been used successfully as a stem cell graft. [342] [343] [344] The convenience of this method likely will lead to its substitution for marrow harvesting as the major method of stem cell collection. [345] Conclusions drawn from marrow transplant studies should be broadly applicable to peripheral blood transplants.

The first studies of allogeneic marrow transplants conclusively demonstrated their value in comparison to conventional supportive therapy. In a controlled trial reported by the International Aplastic Anemia Study Group, patients with severe

THERAPY FOR APLASTIC ANEMIA

After the diagnosis of acquired aplastic anemia has been established, treatment options must be identified, considered carefully, and chosen with alacrity. In patients with moderate disease (Table 19-6) either an expectant approach can be chosen based on stable course and adequate blood counts, or, in patients dependent upon transfusion support, ATG (40 mg/kg/day × 4 days) should be given. Intradermal sensitivity testing with 50 ng/ml ATG solution should be performed and patients showing immediate reaction should be desensitized. After assessment of the response, patients who improve should be monitored for hematologic signs of relapse and non-responders can be offered alternative therapy, such as androgens or CsA (12 mg/kg for adults or 15 mg/kg for children) for 36 months. For severe disease in which the prognosis with blood transfusion and antibiotic support alone is very poor, bone marrow transplantation from a histocompatible sibling or immunosuppression are accepted and effective therapies. Although large retrospective analyses have shown that long-term survival rates from either transplantation or immune therapy are equivalent, each has its own advantages and disadvantages. For children, bone marrow transplantation remains the treatment of choice if an appropriate family donor is available because they have a low rate of GvHD and the marrow disease is cured by stem cell replacement. The risk of later cancers may be increased, especially in children, after marrow transplantation. Adults with aplastic anemia also successfully receive transplants, although both the risk and severity of chronic GvHD and other treatment-related complications increase with age. Bone marrow transplantation has been performed in patients over 50 years of age, and it is certainly a reasonable approach to a younger adult, especially with more severe degrees of neutropenia.

Most patients with aplastic anemia do not have an HLA-matched sibling donor, and immunosuppression is the treatment of choice in these cases. Such patients with severe disease should receive a combination of ATG and CsA. We recommend a regimen consisting of 40 mg/kg/day of ATG at days 14, followed by CsA for 6 months at a dose of 12 mg/kg. Corticosteroids are added in moderate doses (1 mg/kg of prednisone or methylprednisolone) during the first 2 weeks to ameliorate serum sickness. Improvement should be expected within 6 months. This regimen has produced hematologic responses in about 70% of treated patients, who then have an excellent 5-year survival rate.

Although immunosuppressive therapy is relatively nontoxic, patients frequently relapse and require further treatment; however, relapse is not associated with poor prognosis. A more serious problem is the development of late-onset clonal diseases, PNH (which may not be clinically significant), but also MDS, and more rarely AML. Immunosuppression can be considered ameliorative therapy in which bone marrow destruction may continue and abnormal stem cell clones develop. For patients in whom immunosuppressive therapy fails, there are a number of options. For children, alternative donor bone marrow transplantation should be seriously considered; at the best centers, survival rates almost as good as from sibling donors have been achieved. Adults do less well, primarily owing to transplant-related mortality from the intensive conditioning regimen. Some patients will respond to androgen therapy, others to combinations of growth factors. There is no accepted practice for repeated immunosuppression in a patient in whom a first course of ATG and CsA has failed, and perhaps these patients are best served by enrollment in research protocols for refractory disease. High-dose cyclophosphamide may also be effective therapy for both AA at presentation and in patients with disease refractory to more modest immunosuppression in the form of ATG and CsA.

For general management, a few comments are necessary. First, patients with severe AA should not be subjected to useless early trials of corticosteroids or hematopoietic growth factors as primary treatment. Second, for the occasional patient who must decide between bone marrow transplantation and immunosuppression, the advice of experts familiar with this disease and careful counseling of the patient are advisable. Age and severity of neutropenia are decisive factors. A limited number of transfusions may be necessary to optimize the patients condition prior to definitive therapy and are acceptable. Single-donor platelets should be given and may be obtained from HLA-compatible donors. In severely neutropenic patients, G-CSF or GM-CSF therapy may decrease the risk of life-threatening infections. In the absence of overt bleeding, platelet counts as low as 5,000/l appear to be safe.

Figure 19-9 Actuarial survival in aplastic anemia. **(A)** Bone marrow transplantation (data from the University of Washington). **(B)** Bone marrow transplantation versus immunosuppression with ALG. *(Data from the European Group for Bone Marrow Transplantation).* **(C)** Natural history, survival with supportive and other treatments. Two groups are illustrated. Extrapolated survival curves for patients with severe disease are derived from retrospective reviews from the University of Utah of 101 records collected from the late 1940s to early 1970s; patients received blood transfusions and later in this period also platelets. Almost all were treated with corticosteroids and half with androgens as well. Data for patients who did not receive transplants come from a multicenter study of the efficacy of marrow transplantation, performed in the early 1970s; this control group was variably treated with androgens.

disease who received transplants early had an actuarial survival of >60%, compared to about 20% in patients who received androgens and blood transfusions only.[346] Results with marrow transplantation have improved over time, owing to a combination of factors: progressive modification of conditioning regimens and lower procedure-related early mortality, improved transfusion support and antibiotic regimens, and the introduction of CsA as prophylactic therapy for GvHD. Analysis of the International Bone Marrow Transplant Registry data showed that 5-year survival rates climbed from 48% in the years 19761980 to 66% for 19881992. [347] Some hospitals now report very high rates of survival: 89% in Seattle at 8 years, [348] [349] 79% in Baltimore,[350] 89% and 95% at Viennese centers.[351] [352] Registry data support lower survival values as the general experience for the same period, with 64% of patients who received transplants during the period 19851991 being alive at 5 years.[353] For the period 19901994, the European Group for Bone Marrow Transplant (EGBMT) reported 72% survival at 3.5 years. [354] The explanation for these differences is not obvious. Patient selection and reporting bias may be as important as modest differences among conditioning programs or the experience of the transplant team.[340] In the most favorable subgroupuntransfused or minimally transfused, young, uninfected patientsa survival rate of 8090% should be routinely achieved (Fig. 19-9A).[355]

Graft rejection and GvHD are the major complications of allogeneic transplantation in AA. Graft rejection may be a unique problem for transplantation in AA because of the diseases underlying immune pathophysiology as well as the use of more intensive conditioning preparative chemotherapy for malignant diseases like leukemia. Graft rejection is a major predictor of post-transplantation survival. In one study of transplants performed between 1981 and 1986, 29% of deaths were secondary to graft rejection, the single most common cause of mortality.[356] The rate of graft rejection fell with intensification of the immunosuppressive conditioning regimen, from 15% to 4% in Europe[356] and from 35% to 1015% in Seattle,[357] and remained stable in the last decade.[358] Graft rejection might be due to the pathophysiology of AA, a finding supported by the unexpectedly high proportion of failure in unprepared patients receiving syngeneic transplants [359] [360] [361] [362] and even in adequately preconditioned patients receiving syngeneic transplants. [363] [364] In a group of untransfused patients who received allogeneic stem cells, the incidence of graft rejection was 10%, indicating that AA patients may be particularly sensitive to alloimmunization. [365] Nevertheless, the influence of the number of transfusions on graft rejection is relative, and modest numbers of blood donations (<40 units in the International Bone Marrow Transplant Registry experience [314] and <10 units of erythrocytes or 40 units of platelets in Seattle [315]) do not greatly increase the risk of graft rejection.

Intensification of immunosuppressive conditioning regimens with the use of total body [314] [366] [367] or lymphoid irradiation,[368] [369] CsA,[314] [350] or ATG[348] reduces the risk of graft rejection. Such measures, however, have not been shown to influence long-term survival. [314] [370] [371] The effect of the conditioning program on graft rejection is probably through elimination of the recipients lymphocytes and of subsequent mixed hematologic chimerism, which is associated with rejection. [372] [373] More rapid regeneration of bone marrow grafts has been observed when CsA[3] was used,[374] and second transplants have been successful when ATG was added to the conditioning regimen.[375] The effect of ATG or CsA in the conditioning regimen on the rate of graft rejection [348] [376] [377] is currently being tested in randomized studies.

Rates of chronic GvHD vary and are related to patient selection and treatment regimens. Historically, age has been a major risk factor for the development of chronic GvHD. The incidence of chronic GvHD ranges from 19% for patients 010 years old to 46% for those 1130 years old, and to 90% in patients over 31 years old in an older Seattle series.[378] With improved treatment regimens, patients have fared better, but even recent studies

TABLE 19-8 -- Results of Allogeneic Marrow Transplantation for Aplastic Anemia

Study	Years	N	Age, yr (Mean)	Conditioning Regimen	Prophylaxis for GvHD	Graft Rejection or Failure (%)	Acute GvHD (%)	Chronic GvHD (%)	Actuarial Survival (%)	Mean Follow-up (yr)

Hospital of St. Louis	198089	107	546(19)	CY + TAI	MTX, CSA, or MTX + CSA	3	36	35	68 ± 10 (at 5 yr)	3.75
UCLA	198488	290	0.741 (21)	CY + TLI	MTX + CSA	17	21	12	78 ± 15 (at 5 yr)	2.3
Johns Hopkins	198491	24	453 (21)	CY + CSA	CSA	29	5	0	79 ± 8	3
FHCRC	198893	39	252 (24.5)	CY + ATG	MTX + CSA	5	15	34	92 (at 3 yr)	2.5
IBMTR	198087	595	1>40 ()	CY + TLI, TAI, TBI	MTX, CSA, or MTX + CSA	10	40	45	63 (at 5 yr)	
IBMTR	198591	737							64 ± 4 (at 5 yr)	
EGBMT	198089	540							63 (at 5 yr)	
	199094	165							72 (at 3.5 yr)	

Abbreviations: FHCRC, Fred Hutchinson Cancer Research Center; IBMTR, International Bone Marrow Transplant Registry; EGBMT, European Group for Bone Marrow Transplant; TAI, thoracoabdominal irradiation; CY, cyclophosphamide; MTX, methotrexate; CSA, cyclosporine A; TBI, total body irradiation; TLI, total lymphoid irradiation; GvHD, graft-versus-host disease; ATG, antithymocyte globulin.

confirm a higher incidence and more serious consequences of GvHD in older patients. [379] [380] Children have a lower probability of suffering and dying from chronic GvHD.[381] The major difference in survival figures between patients 2030 years old is almost certainly due to the chronic GvHD. In an EGBMT analysis, a significant survival difference was observed between those less than 20 years old (65%) and those more than 20 years old (56%), but there was no survival difference between patients 2130 years old and those 3155 years old. [370] Young adults have fared better in other series, [382] [383] although morbidity from severe GvHD disease was far more prevalent in the young adults than in children (43% vs. 10%). [382] [383]

Very late complications also occur after transplantation. These include effects on growth and development as well as on the function of endocrine, neurologic, and other organ systems.[384] [385] A high rate of secondary malignancies has been recorded after transplantation. In a National Cancer Institute retrospective analysis of almost 20,000 transplants, the risk of late-onset cancer was eightfold higher at 10 years than in the general population and even higher for young patients, in whom the risk of malignancy was increased about 40-fold. [386] The risk was greatest for the development of malignant melanoma, buccal cavity tumors, and cancer of the liver, brain, CNS, thyroid, bone, and connective tissues. Multivariate analysis suggested that high-dose irradiation was a risk factor for the development of malignancies. For AA, among 320 patients who received transplants in Seattle, four developed cancer, leading to a calculated risk seven times higher than in normal controls.[387] In a French survey, four of 147 AA patients developed solid tumors, an 8-year cumulative incidence rate of 22%, equivalent to an RR of 41. [388] Secondary solid tumors developed in the radiation field of five of 147 AA patients whose conditioning regimen included the thorax and abdomen. [389] Immune events such as acute GvHD, treatment with ATG or monoclonal antibodies, and total-body irradiation have been related to the development of secondary malignancies. [387] Patients with these secondary cancers have a poor prognosis. [390]

The risk of cancer after bone marrow transplantation must be evaluated in the context of other therapeutic options, especially immunosuppression, because a significant risk of late malignancy exists in AA patients independent of transplant therapy. The risk of malignancy in the large registry of the EGBMT was equivalent for patients who received immunosuppression and those who underwent transplantation. Compared to the general European population, the RR of malignancy was calculated at 5.15 for AA patients treated with immunosuppression (confidence interval 3.267.94) and at 6.67 (3.0512.65) for patients receiving transplants. [391]

In summary, excellent survival rates and low morbidity in younger patients make allogeneic bone marrow transplantation the treatment of choice for children and adolescents. Patients older than about 40 years have a higher risk of transplant-related morbidity and mortality. Young adults in the intermediate age group have a reasonable opportunity for cure with bone marrow transplantation but also face more complications than children. In addition to age, a prolonged interval between diagnosis and transplantation, multiple transfusions, and serious infections before transplantation are poor risk factors.

Matched Unrelated and Nonhistocompatible Family Donors

Until recently, the lack of an HLA genotypically identical sibling donor precluded marrow transplantation, thus excluding about 70% of patients with AA from access to this therapeutic option. Alternative potential donors include either relatives who are phenotypically matched or partially matched and HLA phenotypically matched but unrelated volunteers. Although phenotypically identical family donors are available in only 12% of cases, mismatched family members and matched but unrelated donors represent a much larger pool.[392]

Haplotype sharing between parents occasionally has allowed identification and successful transplantation between phenotypically matched relatives. [393] [394] Long-term survival after even one-locus-mismatched family donations is inferior to genotypically matched transplants, mainly because of problems with graft rejection and GvHD. In the large European experience, for phenotypically identical family matches the actuarial survival was 45%, for patients with a single-locus mismatch it 25%, and for those with two to three loci mismatched 11%.[395] [396] In a recent report from Seattle, although all patients who received fully HLA-matched transplants survived, those with mismatches at one or more loci had a much poorer outcome, and even with total body irradiation added to the conditioning regimen, survival was only 50%. [394]

Most large studies of unrelated donors have shown inferior long-term survival and higher rates of complications such as graft rejection, [397] GvHD,[398] [399] and delayed immune system reconstitution.[400] Even more than in standard sibling transplants, age is a crucial risk factor in unrelated transplants and probably more important than the level of match, conditioning regimen, or use of T-cell depletion. [392] [396] [401] [402] [403] For patients with AA who received unrelated transplants and who were enrolled in the National Marrow Donor Program, survival at 2 years was 29%. [404] In the EGBMTs 1994 report, survival in 110 recipients of marrow grafts from other than a matched sibling was 34%, about half the rate of standard transplantation. [354] Superior

TABLE 19-9 -- Intensive Immunosuppression in Severe Aplastic Anemia

Study, Year	Regimen	N	Median Age (yr)	Median ANC/ l	Response (%)	Survival (%)	Relapse (%)	Median Follow-up (d)
German multicenter, 1992	ALG + CSA	43	32	0.48	70 (6 mo)	64 (41 mo)	11	516
EGBMT, 1995	ALG + CSA + G-CSF	40	16	0.19	82 (1 yr)	92 (34 mo)	3	428
NIH, 1995	ATG + CSA	51	28	0.34	78 (1 yr)	86 (1 yr)	18 (1 yr)	912
						72 (2 yr)	36 (2 yr)	

Abbreviations: ANC, absolute neutrophil counts; EGBMT, European Group for Bone Marrow Transplant; NIH, National Institutes of Health; ALG, antilymphocyte globulin; antithymocyte globulin; CSA, cyclosporine A.

results were obtained at Childrens Hospital in Milwaukee, where T-cell depletion of the donor graft was combined with a rigorous conditioning program of cytosine arabinoside, cyclophosphamide, and total body irradiation: in 28 transfused and previously treated children with severe AA, a survival rate of 54% was reported at a median follow-up of almost 3 years, with no incidence of chronic GvHD. [405]

In summary, alternative donor transplantation is feasible; the rare phenotypic match from within the family may be equivalent to a sibling donor, but with other family members or unrelated donors there is a high risk of transplant-related mortality. Because unrelated donor transplantation takes months to arrange, it should be considered early. At the best centers, alternative donor transplantation represents an option, especially for the young patient with very severe pancytopenia in whom immunosuppressive therapy has failed.

Immunosuppression

Antithymocyte and Antilymphocyte Globulins

Immunosuppressive therapy is an effective alternative treatment for patients who are not candidates for bone marrow transplantation (Fig. 19-9 , Table 19-9). Immunoglobulin preparations made from the sera of horses (less frequently rabbits) immunized against human lymphocytes are the mainstays of current regimens. In Europe, thoracic duct lymphocytes are the antigens for antilymphocyte globulin (ALG), and in the United States, thymocytes (from children undergoing cardiac surgery) are the immunogen used for production of ATG; ALG dosages are often expressed in lytic units for lymphocytes in vitro and ATG dosages are expressed in milligrams.[340] [406] [407] [408] [409] [410] Only ATG is licensed for use in the United States. Both ATG and ALG are used in clinics elsewhere in the world.

The efficacy of ALG in marrow failure was discovered serendipitously in the late 1960s, when Mathé observed recovery of autologous hematopoietic function in patients who received antilymphocyte serum as conditioning for marrow transplantation. [67] In a collection of European cases from Basel, Paris, and Leiden in which patients were treated with different serum preparations and with a variety of dose regimens, sustained hematologic improvement occurred in 12 (41%) of 29 patients with severe AA, and the 1-year survival of the entire group was 55%. [411] In a multicenter study, Swiss ALG was clearly superior to androgen treatment, with a significantly better response rate (70% vs. 18%) and 1-year survival (76% vs. 22%). [412] Similar results were also obtained in a randomized study of ATG versus supportive care.[413] In a large multicenter American trial, 47% of patients improved. [414] Review of published results from Europe and North America suggests that overall, about half of patients treated with either ATG and ALG will show hematologic improvement, broadly defined as an end to transfusion dependence and an improvement in a neutrophil number to a level protective against infection. [406] Response rates may vary from 20% to 85%.[406] The putative etiology of AA is not a factor that predicts response. Both virus-associated [100] [219] and drug-induced[415] [416] aplasia respond similarly to idiopathic disease. Cytogenetic abnormalities do not preclude a response since both AA with chromosomal abnormalities[417] [418] [419] as well as some cases of frank myleodysplasia[420] may be responsive to immunosuppressive therapy. The response rate to ATG or ALG is not improved by the addition of androgens [421] or very high doses of corticosteroids.[422]

A hematologic response to ATG is usually apparent within several months of therapy; in some cases, all blood counts rise dramatically, in others, increases in platelets or red cells may be delayed (Fig. 19-10). The average time to improvement in

Figure 19-10 Time to response after treatment with ATG. **(A)** Distribution of patients with severe aplastic anemia by time to achieve an increase in the absolute neutrophil count of 1,000/mm ^{3} . **(B)** Distribution of patients with an initial absolute neutrophil count <200/mm ^{3} by time to achieve an absolute neutrophil count of 1,000/mm ^{3} .

neutrophil number is 12 months.[414] Transfusion independence occurs about 23 months after initiatiation of treatment. [423] [424] Continued improvement without further therapy not uncommonly occurs after 3 months; nevertheless, clinical status by 3 months is strongly correlated with long-term survival. [414] [425] Patient selection is important, as the likelihood of response to ALG has been inversely correlated with disease severity, [426] [427] and in particular with a low neutrophil count. [319] [370]

The administration of antilymphocyte sera is associated with three major toxic effects: immediate allergic phenomena, serum sickness, and transient blood cell count depression (Plate 19-5A and B). Fever, rigors, and an urticarial cutaneous eruption are common on the first day or two of ATG or ALG therapy, and these symptoms respond to antihistamines and meperidine therapy. Anaphylaxis is rare, but its occurrence has been fatal. [428] A positive immediate wheal-and-flare reaction to the cutaneous application of a 50 mg/ml stock solution of ATG may be predictive of massive histamine release on systemic infusion, and desensitization with gradually increasing doses of ATG administered intradermally, subcutaneously, and then intravenously has permitted ATG use in allergic individuals. [428] Corticosteroids are usually administered in moderate doses (1 mg/kg of prednisone or methylprednisolone) during the first 2 weeks to ameliorate the symptoms of serum sickness. Doses of ATG and ALG have varied from 5 to 50 mg/kg and the duration of administration has varied from 4 to 28 days. It is more rational to administer equivalent doses of ATG or ALG by the schedule originally employed in Europe, 40 mg/kg/day for 4 days; antiserum will then have reached low levels in the circulation by the time host antibody appears.[429] A short course of therapy is easier to administer, associated with less serum sickness, and equally effective as the same dose given over a more prolonged course.

Antilymphocyte globulins are immunosuppressive. ATG and ALG contain a heterogeneous mix of antibody specificities for lymphocytes, including reactivity to antigens such as CD2, CD3, CD4, CD8, CD25 (the receptor for IL-2), and HLA-DR. [430] [431] [432] [433] Horse sera fix human complement efficiently, and all preparations are T-cell cytotoxic in vitro, with little difference between ATG and ALG or among lots for lymphocyte killing in vitro. [431] In vitro, antilymphocyte globulins efficiently inhibit T-cell proliferation and block IL-2 and IFN- production and IL-2 receptor expression; [434] ATG induces Fas-mediated apoptosis of T cells, especially after activation.[435] In monkeys, ATG and ALG suppress cutaneous allograft rejection.[433] Studies of rabbit ATG in rhesus monkeys undergoing kidney transplantation have indicated that persistence of specific antibodies may be responsible for a chronic anergic state and tolerance induction. [436] In patients, administration of ATG and ALG results in rapid reduction in the number of circulating lymphocytes, usually to <10% of starting values, and lymphocytopenia persists for several days after discontinuing therapy. Although lymphocyte numbers return to pretreatment values by 3 months, reductions in activated lymphocyte numbers in recovered patients persist.[92] [437] [438] [439] It seems likely that these inhibitory effects on T cells are responsible for the efficacy of antilymphocyte globulins in AA. Nevertheless, the ability of ATG and ALG to also stimulate lymphocyte function by acting as a mitogen may also have a role in their therapeutic efficacy. ATG and ALG provoked both IL-2 and hematopoietic growth factor production by peripheral blood mononuclear cells, [440] later identified as GM-CSF[87] [441] [442] and IL-3.[441] T cells cloned after ALG stimulation produced GM-CSF and/or IL-3, and, less frequently, IL-2 and IFN-. [443] Acutely, ALG administration was accompanied by reduced serum GM-CSF levels in one study.[444] In a mouse model, ATG administration was associated with increased circulating levels of colony-stimulating factors. [445] Administration of ATG after human marrow transplantation is associated with increased serum levels of IL-3. [446] ATG also binds to bone marrow precursor[447] and progenitor[448] cells and might modestly directly enhance hematopoiesis, possibly through binding to CD45RO, a molecule capable of promoting tyrosine phosphatase signaling. [449]

Reliable methods to predict which patients will respond to ATG are lacking. In some cases, inhibitory activity of lymphocytes on hematopoiesis is no longer detected after successful ATG therapy.[450] [451] In one recent study, CD8 T cells obtained from patients before treatment inhibited autologous hematopoietic colony formation, whereas CD8 lymphocytes isolated from the same patients after recovery were not active.[452] Good correlations were reported between clinical response to immunosuppression and improvement in hematopoietic colony formation in vitro after T-cell depletion. [82] [450] These promising findings have not been confirmed by others.[230] [425] [456] [457] [458] The lymphocyte stimulatory effects of ATG and ALG have been associated with positive clinical outcomes by some [459] but not by other[441] studies.

Cyclosporin A

Shahidis group[460] and others[461] [462] [463] [464] [465] [466] [467] reported success with cyclosporine therapy combined with androgens in individual patients with AA, in many of whom other therapies had failed. Several studies suggested efficacy of cyclosporine in patients refractory to ALG or ATG alone, with salvage rates of about 50%. [468] [469] [470] [471] [472] [473] The use of CsA as initial therapy was promoted by a large French cooperative randomized study in which low but equivalent response rates were observed in comparison with standard ALG therapy.[474] However, in a recent randomized German trial, cyclosporine was clearly inferior to ALG as measured by response and survival rates.[435]

The optimal regimen has not been determined. In the United States, CsA has usually been employed in high doses, 12 mg/kg/day for adults and 15 mg/kg/day for children, with doses adjusted according to plasma drug concentrations and serum creatinine levels. In Europe, lower doses, 37 mg/kg/day, have been reported to be equally efficacious.[475] [476] Hematologic improvement may occur in a few weeks or months. A 6-month trial is warranted. Remissions, when achieved, usually have been durable, but some patients experience relapse when CsA is discontinued. Most patients who relapse will then respond to the reinstitution of cyclosporine. [475] [476] [477] [478] Some patients require maintenance treatment. [465] [468]

The toxicity associated with cyclosporine therapy is considerable. Hypertension and azotemia are the most common serious side effects; hirsutism and gingival hypertrophy are also frequent complaints. Increasing serum creatinine levels are an indication for dose reduction. Chronic nephropathy characterized by interstitial fibrosis and tubular atrophy due to cyclosporine can be irreversible. The risk of nephropathy is increased by high doses and longer duration of therapy, and occurs more commonly in older than in younger patients. CsA, especially in combination with corticosteroids, converts patients with AA to a temporary immunodeficiency state and puts them at high risk for opportunistic infections. Monthly aerosolized pentamidine prophylaxis will prevent *Pneumocystis* pneumonia in patients receiving

CsA. Convulsions, possibly related to hypomagnesemia, are another serious complication of cyclosporine therapy.

Combined or Intensive Immunosuppressive Therapy

The combination for the treatment of AA of an agent that lyses lymphocytes (ATG or ALG) with a drug that blocks lymphocyte function is rational (Table 19-9). The strategy resulted in a striking increase in the response rate to immunosuppressive therapy observed in a German randomized trial in which patients were treated initially with a combination of ALG and CsA or ALG only. The addition of CsA led to higher hematologic response rates and more complete responses than were observed with

ALG alone: 65% versus 39% and 70% versus 46% at 3 and 6 months, respectively. [479] In an NIH study of combination ATG and CsA therapy[480] and in a multicenter European study of ALG and CsA,[481] the hematologic response rates at 1 year were about 80%. In all the trials, 5-year survival rates for responding patients have been reported to be between 80% and 90%. Intensive immunosuppression has been beneficial to two groups in particular: patients with extreme neutropenia (<200 neutrophils/l) and children. Both of these groups respond poorly to ATG therapy alone but have nearly equivalent response and survival rates with combined treatment.

Immunosuppressive therapy has been intensified in other regimens. In Europe, repeated courses of ALG are commonly given in an effort to induce hematologic improvement.[426] [482] [483] [484] A longer course of ATG (28 days versus 10 days) appeared to produce more complete responses in an American multicenter trial. [414] The addition of high-dose methylprednisolone to ALG has been associated with very high response rates in some trials. [482] [485] [486] Most recently, the experience with 11 patients who received high doses of cyclophosphamide, 45 mg/kg/day for 4 days without stem cell rescue, was reported, with an overall response rate similar to that reported with ATG therapy. Remarkably, following cyclophosphamide therapy, blood cell counts were normal and there was no evidence of relapse or late complications.[487] However, the period of pancytopenia, especially neutropenia, was prolonged after cyclophosphamide therapy. High-dose cyclophosphamide therapy for AA is of great interest and is being tested in several experimental trials.

Corticosteroids

Methylprednisolone in modest doses, usually 1 mg/kg/day, is usually administered during ATG and ALG therapy to ameliorate symptoms due to serum sickness. Low-dose corticosteroids are not effective treatment for AA. Very highindustrial strengthsteroids have been administered as boluses of 6-methylprednisolone given intravenously in dosages of 20 mg/kg/day on days 13, 10 mg/kg/day on days 47, 5 mg/kg/day on days 811, 2 mg/kg/day on days 1220, and 1 mgkg/ day until day 30, followed by maintenance. Such high-dose regimens may be effective, especially in recently diagnosed patients. [230] [484] [488] As reviewed above, high-dose methylprednisolone has also been added to ALG therapy, with inconsistent results. ATG and ALG therapy is associated with better response rates and many fewer associated toxic effects than high-dose steroid therapy and is generally preferable as initial therapy. Modest doses of corticosteroids do not have a role in the treatment of aplastic anemia except in combination with antilymphocyte globulins. Not only is there little evidence of their effectiveness in reversing marrow failure or improving hemostasis, but even limited courses of steroids may contribute to the development of aseptic vascular necrosis, a troubling complication in the pancytopenic patient.[489]

Late Complications of Immunosuppressive Therapy

Relapse following immunosuppressive therapy is common. In the European experience, in 719 patients treated with immunosuppressive therapy, the actuarial rate of relapse among 358 responders was 35% at 14 years; relapse was more common among patients who had shown initial rapid responses.[490] About half the patients who relapsed responded to a second course of immunosuppression, but survival was lower in patients who experienced relapse than in those who did not. In the large NIH cohort of 112 patients, relapse, defined as a need for treatment, was even more common, with a risk estimated at 87% at 7 years; however, survival was unaffected by the occurrence of relapse, and most patients responded to further therapy, usually reinstitution of cyclosporine. [245] These observations are consistent with a view of AA as a chronic immunologic disease that may not be cured by a single course of immunosuppressive therapy.

A much more serious complication is the development of late clonal hematologic disease, often years after apparently successful immunosuppressive therapy. The syndromes include PNH, myelodysplasia, and AML.[491] [492] [493] These events may represent part of the natural history of AA. Before the recent improvements in treatment, leukemia was considered an unusual complication,[494] but late-onset clonal disorders do not appear to simply be the result of the introduction of immunosuppressive therapy. In a series of 156 patients treated with androgens, there was a 10% actuarial probability of developing PNH; in addition, five cases of late-onset myelodysplasia and one of non-Hodgkin lymphoma were observed.[495] For patients in the modern era who undergo immunosuppression, the most accurate figures concerning the risk of developing myelodysplasia or PNH are derived from retrospective analyses performed by the EGBMT. Of 223 long-term survivors after immunosuppression, 19 developed PNH (13% risk at 7 years) and 11 developed myelodysplastic syndrome, which in five later evolved to AML (combined risk 15% at 7 years).[496] In single-center series, estimated rates of late-onset clonal disease have ranged from 9% (at 6 years) [422] to 57% (at 8 years).[497] In our series of NIH patients, the actuarial risk was about 16% at 7 years, PNH almost invariably appeared early, within the first year of diagnosis, whereas myelodysplastic syndrome occurred late and usually in patients in whom treatment had failed and who remained pancytopenic. PNH may be without clinical manifestations and only diagnosed by a positive Ham test or by means of flow cytometric studies. Myelodysplastic syndrome after AA may transform to acute leukemia[491] but may also be surprisingly indolent.[498]

Immunosuppression versus Bone Marrow Transplantation

Both immunosuppression and bone marrow transplantation are effective therapy for AA (Figs. 19-8 and 19-10). [340] Lack of a matched sibling donor, the expense and availability of transplantation, and risk factors such as active infections, advanced age, or a heavy transfusion burden lead most patients to automatically undergo treatment with ALG and CsA.[499] For a few patients with AA, a choice does exist between transplantation and immunosuppressive therapy. Marrow transplantation offers the possibility of permanent cure. Its disadvantages are cost, procedure-related morbidity and mortality, especially GvHD in older patients, and an increased incidence of solid organ malignancies. Immunosuppressive therapy is easier and initially cheaper. However, many patients do not achieve normal blood cell counts and remain at high risk for relapse and the more serious complications of late clonal disease, especially myelodysplasia.

Retrospective analyses of the large number of European patients reported to the EGBMT show consistently improved results with both therapies but have repeatedly failed to demonstrate a survival advantage for transplantation over immunosuppression: [370] [500] [501] [502] the most recent 5-year survival figures were 75% for immunosuppression and 77% for transplantation (A. Bacigalupo, personal communication). Single-center studies are similar. Certain categories of patients, defined by neutrophil number and age, likely benefit from one therapy or the other. In the EGBMT analyses, marrow transplantation yielded superior results in children less than 10 years old and in younger patients with a neutrophil count of <400/l, whereas immunosuppressive therapy was superior for adults 40 years old and older. For patients of intermediate age and a neutrophil count of <300/l, transplantation results in a favorable outcome when compared with immunosuppressive therapy [356] (A. Bacigalupo, personal communication). Superior results with matched sibling transplantation

in children have been reported by others. [319] [503] [504] [505] [506] In contrast, older individuals fare less well than children with transplantation. In a compilation of results of transplantation for AA performed from 1970 to 1989 in Seattle, long-term survival in patients more than 40 years old was only about 10%, and in patients 3039 years old it was 35%.[507] Even its enthusiasts do not recommend transplantation as first-line therapy for patients more than 50 years old; [508] 40 years was adopted as a cutoff for consideration of transplantation in one recent review. [509] In some cases, an unsuccessful trial of immunosuppression may be followed by transplantation of marrow from a sibling donor.[510]

Androgens

Testosterone and synthetic anabolic steroids appeared to be a major advance in the treatment of AA when they were introduced in the 1960s. [511] [512] The high response rates that were reported in some early series may be retrospectively attributed to the inclusion of patients with moderate acquired and constitutional AA. For

severe AA, controlled trials in general have failed to demonstrate efficacy, as measured by survival rates [513] or hematologic improvement.[414] When added to immunosuppressive therapies, androgens failed to result in any increase in response rates. [421] In Europe, a modest survival advantage was observed only in women with severe neutropenia who received combined therapy. [514]

Although marrow transplantation or immunosuppressive therapy is generally preferred, certain androgen regimens have their advocates. These investigators have reported response rates and prolonged survivals [495] similar to those observed with ATG.[515] Androgens continue to be helpful in some patients when used as second-line therapy. Most hematologists have observed patients who appeared to respond or even to develop hormone dependence. [516] [517] Androgen therapy is popular in Asia[518] [519] [520] [521] and Mexico[522] because it is inexpensive and seemingly effective. Various preparations of androgens in different doses have resulted in virtually identical response rates of 3560% after 6 months of therapy. [523]

Popular androgens include nandralone decanoate, oxymetholone, and danazol. The hemoglobin response will frequently be more impressive than improvements in granulocyte or platelet levels. An adequate trial of full doses given for at least 3 months and possibly as long as 6 months is required before interrupting therapy. Despite the multiple toxic effects of androgens, complications are infrequent, although some are serious and may limit effective therapy, especially in the elderly. [524] The associated liver cholestasis is usually reversible. Hepatotoxicity (bile duct proliferation, peliosis, atypical hepatocyte hyperplasia, tumors) can occur but is less frequent with parenteral formulations.[525] Children appear to tolerate high doses of androgens without lasting effects on growth or maturation. [526]

The mechanism of action by which male hormones improve failed hematopoiesis remains unclear.[527] [528] Increased secretion of erythropoietin, the most definitive effect, is probably not a significant factor in mediating hematopoietic recovery in AA. Modest direct effects on progenitor cells [529] [530] [531] are of uncertain clinical significance. In in vitro and in vivo models of autoimmune disease, androgens appear to act as immunomodulators. [532]

Hematopoietic Growth Factors

Although hematopoietic growth factor production is normal or increased in most patients with AA, pharmacologic stimulation with very high doses of cytokines might be effective, either through a direct effect on residual stem cells, promoting marrow recovery, or by increasing progenitor cell activity and allowing patients to survive long enough to respond to other, more definitive therapy. [533] [534]

Neutropenia most often leads to serious and life-threatening infections. Both G-CSF and GM-CSF are capable of increasing neutrophil counts in patients with AA. [535] [536] [537] Controlled trials with these agents have not been performed, so that data demonstrating that growth factor administration either decreases the incidence of serious infections or improves survival are lacking. In general, neutrophil responses to growth factors are transient, dependent on their continuous administration, and usually restricted to patients with quantitatively less severe forms of AA. Nevertheless, occasional bilineage and trilineage responses have been observed. [538] Children may be more sensitive to the effects of prolonged administration of G-CSF. [536] [539]

G-CSF and GM-CSF have some immediate toxic effects, including bone pain and cytokine flu symptoms. GM-CSF may increase eosinophil and monocyte counts, while G-CSF and GM-CSF can cause a concurrent reduction in platelet numbers. Of concern has been the possibility that prolonged administration of G-CSF may increase the probability of late clonal disease, especially monosomy 7. In retrospective analyses of Japanese children [540] [541] and adults[542] with severe AA, this syndrome appeared to occur only among patients who had received growth factor. This experience has not yet been confirmed in Europe or the United States (where long-term use of growth factors may be less frequent). Regardless of toxic or late effects, growth factors, perhaps because of their recent availability, often are inappropriately employed as first-line therapy in AA, where they have not been shown to be useful. Such practices lead to unfortunate delays in the institution of definitive therapy.[340] [543]

IL-1, IL-3, IL-6, and stem cell factor have been tested in small pilot trials, usually in patients with refractory AA. IL-1, the monocyte production of which is deficient in severe AA, is not effective and is highly toxic. [544] [545] IL-3 can increase neutrophil numbers and less frequently platelet levels. IL-3 use is associated with significant side effects.[546] [547] [548] [549] A trial of IL-6 in AA was discontinued prematurely because of an increased incidence of bleeding episodes and worsening of anemia. [550] Anemia[65] [551] and pancytopenia[552] in rare patients have responded to prolonged administration of high doses of erythropoietin.

Clinically meaningful hematologic responses to the administration of single cytokines have been limited in marrow failure syndromes, but combinations of growth factors might be more effective because of physiologic or pharmacologic synergism. Some complete remissions have been reported with GM-CSF and erythropoietin [418] [553] [554] [555] and with IL-3 and G-CSF.[556] In a large randomized protocol, the combination of G-CSF and high doses of erythropoietin improved hemoglobin values, mainly in patients with moderate disease.[557] Although neither was very effective alone, the combination of stem cell factor and G-CSF has led to improvement in blood cell counts, including trilineage recovery, in some patients with refractory AA. [558]

Growth factors have also been combined with definitive medical therapy for the purpose of improving neutrophil counts during the early phase of immunosuppression. Often, therapy with G-CSF or GM-CSF is instituted because the neutropenic patient is febrile and unresponsive to antibiotics; its value under these circumstances is unknown. A brief course of GM-CSF before or concurrent with ALG has not added appreciable benefit in two small trials. [559] [560] A few case reports have suggested that the combination of a growth factor with CsA might rescue patients with refractory disease, [561] [562] [563] but, as noted above, a randomized trial showed G-CSF and cyclosporine to be inferior to ALG and CsA as first-line therapy for AA. [564] Prolonged G-CSF was part of the EGBMTs trial of intensive immunosuppression, [481] but whether the excellent hematologic responses and survival rates were due to the use of growth factor or to the sequential courses of immunosuppression is unclear. A randomized trial to determine the efficacy of G-CSF

therapy has recently been completed and should determine the value of the cytokine in this setting.

In the absence of convincing evidence of benefit, either short or long term, growth factor use in AA will be dictated by the individual physicians judgment. Most severely neutropenic patients who are persistently or seriously infected undergo a therapeutic trial in the hope of achieving clinical benefit. G-CSF may be preferred to GM-CSF because of its more favorable toxicity profile. Some patients with disease refractory to other forms of treatment may also receive prolonged courses of cytokines in the hope of either raising the low neutrophil count or, less likely, of inducing clinical remission or a reduction in transfusion requirements. Preferably, the use of growth factors over long treatment periods, especially in patients who are not severely neutropenic, should be in the context of a formal study. Both physicians and patients should be aware of possibly significant risks associated with such treatment.

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PROGNOSIS

The initial blood cell counts of a patient with AA are the most important indicators of prognosis. The most popular criteria used to define severe disease are the presence of two of the following three: neutrophil count <500/l; platelet count <20,000/l; corrected reticulocytes <1% (<40,000/l). [346] More complicated formulas are less easy to use and often only quantitate the obvious. [565] In a comparative study of prognostic indices, decreasing blood cell counts during the first 3 months of therapy were uniformly associated with death within 5 years, while stable or improved blood cell counts correctly predicted long-term survival in 75% of patients. Of the prognostic indices, the simple criteria noted above were most accurate at 6 months, correctly predicting poor survival in 85% of patients with 100% sensitivity after 6 months.[566] One modification to the standard criteria is useful. Although bleeding was a major cause of death in the past, infection kills the overwhelming proportion of patients today. Accordingly, in the large European cooperative trials, the category of supersevere AA has recently been defined by an extremely low absolute neutrophil count (<200/l).[319]

In contrast to blood cell counts, bone marrow examination is subject to sampling error, and cellularity is not usually quantitated. For these reasons, in pancytopenic states in general, blood cell counts have been more readily correlated with survival than has the gross marrow appearance. [567] A predominance of lymphoid cells in the bone marrow has been associated with a bad prognosis in some studies, [274] the presence of residual hematopoiesis, particularly erythropoiesis, with a good outlook in others.[568] [569] The permanence of marrow depression after an insult may also be predicted by continued diminished marrow cellularity despite blood cell count recovery.[289] Correlations that were initially found between clinical response to immunosuppression and pretreatment improvement in hematopoietic colony formation after in vitro immune cell manipulation have not proved reproducible or practical.

The rate of spontaneous recovery is difficult to estimate, and most current observers believe it to be low. Untreated severe disease is almost invariably fatal. By contrast, moderate AA has a good prognosis, and some patients with minimal blood cell count depression will recover normal blood cell counts with limited or no therapy.[570] Nevertheless, in an older series of pediatric patients treated mainly with transfusions, only 3% of 334 were judged to be cured, [153] and among more recently reported African patients who received transfusions, corticosteroids, and androgens, mortality at 1 year was 56%, and 72% after 18 months. [24] Interpretation of older publications is complicated by uncertainties concerning diagnosis and the inclusion of a large proportion of patients with moderate disease. In a randomized study from the contemporary era, none of 21 patients assigned to supportive care improved during 3 months of observation. [413]

Improved survival after effective treatment has brought with it a series of problems that affect prognosis, especially late-onset clonal hematologic diseases when bone marrow failure is reversed without stem cell replacement and the late development of malignancies after the aggressive conditioning regimens used in bone marrow transplantation.

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Chapter 20 - Paroxysmal Nocturnal Hemoglobinuria

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INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal disorder of the hematopoietic stem cell that results in the production of blood cells with characteristic defects.[1] The first defect detected was the unusual susceptibility of erythrocytes to the hemolytic action of complement; [2] this characteristic was first definitively demonstrated by Ham and his associates in 1938 and has been used as the principle indicator in laboratory diagnosis of this disorder.

The first descriptions of PNH emphasized the hemoglobinuria, which characteristically occurs at night and often in paroxysms, at times initiated by infections. [3] [4] The recognition that hemosiderinuria is perpetual suggested that more hemolysis occurred than was demonstrated by the dark urine. [5] With the advent of diagnostic tests, it became apparent that the disease was much more complex than first realized. In addition to hemolytic anemia, patients often have venous thromboses in unusual places such as the sagittal sinus, the hepatic veins, and the dermal veins. [6] Many patients have granulocytopenia or thrombocytopenia, or both, in addition to the hemolytic anemia.[7] Although most patients have erythroid hyperplasia of the marrow, many have relative or absolute hypoplasia. In addition, a small proportion of patients develop acute myeloid leukemia. [8] [9]

These nonerythrocytic manifestations led to the hypothesis that the other blood elements are also abnormal and showed abnormalities similar to those found in red cells were found in cells of other hematopoietic lineages. [10] These findings suggested that the disorder originated at the level of the hematopoietic stem cell. [11] The finding that most patients have a normal as well as an abnormal population of erythrocytes and that the abnormal cells appeared to be clonal gave rise to the hypothesis that the disorder is the result of a clonal proliferation of an abnormal hematopoietic stem cell. [12]

More recently, the nature of the abnormality has been described. The abnormal cells in PNH lack a number of membrane proteins that have in common fixation to the plasma membrane by a complex glycolipid anchor.[13] In the abnormal cells in PNH, this anchor cannot be synthesized because of a defect early in the biosynthetic pathway,[14] [15] [16] [17] and since the anchor cannot be made, the proteins cannot be affixed to the membrane. Many of the manifestations of PNH can be traced to the lack of these proteins on the abnormal cells.

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PATHOBIOLOGY

In PNH, normal and abnormal erythrocytes, granulocytes, monocytes, and platelets are present in the peripheral blood at the same time, suggesting that the abnormal cells arose as a clone within the marrow. The clonal origin of the abnormal population was supported by the finding in women heterozygous for variants of the enzyme glucose-6-phosphate dehydrogenase that the abnormal PNH erythrocytes were all of one isoenzyme. [18] Because the gene for this enzyme is located on the X chromosome, the assumption is that the abnormal cells are derived from a single precursor that possessed that isoenzyme after meiotic suppression of the other X chromosome.

More recent studies have confirmed this hypothesis. Using probes for detecting polymorphisms in genes located on the X chromosome, a monoclonal pattern was found in the marrow cells in five of five PNH patients, strongly indicating that the abnormal cells had arisen from a single precursor. [19]

Defect in Glycosylphosphatidylinositol Anchor

The nature of the defect in the abnormal cells in PNH was difficult to understand, as evidence of missing membrane proteins accumulated. The observation by Low and colleagues[20] [21] that

Figure 20-1 Structure of the glycosylphosphatidylinositol (GPI) anchor. Phosphatidylinositol (with unusual fatty acids and an extra fatty acid attachment to the inositol) is inserted into the lipid core of the membrane. The glycan core consists of four sugars: one molecule of *N*-glucosamine and three molecules of mannose. Ethanolamine is attached to these sugars; the one on the terminal mannose is attached by an amide bond to the carboxyl terminus of the protein.

acetylcholinesterase (one of the proteins missing on the abnormal cells [22]) could be removed from the membrane by an enzyme, phosphatidylinositol-specific phospholipase C, without injury to its enzymatic activity, suggested that this protein was attached to the membrane in the same way as the variable surface glycoproteins of trypanosomes (i.e., by a glycosylphosphatidylinositol (GPI) anchor rather than by a transmembrane sequence of hydrophobic amino acids). [21] [22] When decay accelerating factor (DAF) was also found to be attached by this anchor, a defect in this anchoring mechanism was proposed as the fundamental defect in PNH.[23]

The structure of the GPI anchor has remained fundamentally unchanged from the simplest life forms (e.g., trypanosomes) to mammals, including humans (Fig. 20-1).[24] [25] It consists of a molecule of phosphatidylinositol to which are attached four sugars (a molecule of *N*-glucosamine and three molecules of mannose); the last mannose is attached to the carboxyl end of the protein through phosphoethanolamine. Phosphoethanolamine molecules may be attached to each of the other two mannose residues but do not attach to the protein. A palmitoyl moiety is added to the inositol but may later be removed. The *N*-glucosamine is derived from *N*-acetylglucosamine that is added and then deacetylated.[26] The source of the mannose residues is dolichyl phosphoryl mannose, which is synthesized from GDP-mannose and dolichol phosphate (a long-chain polymer of isoprene) by a specific enzyme. [27] The source of the phosphoethanolamines is not entirely clear, but they are probably derived from phosphatidylethanolamine.

When the synthesis of the anchor is complete, it is attached to the protein in the cisterna of the endoplasmic reticulum by transamidation of the amino group of the terminal phosphoethanolamine to the carboxyl group in the protein. [28] When the protein is attached to the anchor, it proceeds through the Golgi apparatus and on to the membrane surface.

Evidence for a Biosynthetic Defect

The absence of all GPI-linked proteins on the abnormal cells in PNH results from a defect in the biosynthesis of the GPI anchor at a step prior to the addition of *N*-acetylglucosamine to the phosphatidylinositol moiety. This was shown in two ways: (1) the abnormal cells synthesize little or no biosynthetic intermediates containing *N*-glucosamine or mannose, [14] [16] and (2) when the abnormal cells were fused with mouse cell lines defective in the synthesis of the anchor, those fused with cells of the class A phenotype did not complement (i.e., exhibit GPI-linked proteins

on the surface), whereas those fused with lines of the other phenotypes did complement. [15] [16] [29] This means that the PNH cells were defective at the same step as the class A murine cells. Although the enzyme mediating this step has not been identified, the gene responsible for it has been found and is called the *pig-a*.[30] [31] [32] [33] The defect in this gene has been identified in nearly 100 patients with PNH. [33] In most patients, the defect results in no gene product; this results in PNH III cells. [33] In some patients, the defect results in a missense message and the product is presumably defective in performing the glycosylation necessary for the synthesis of the GPI anchor; this results in cells with small amounts of the anchored proteins on their surface (PNH II cells). Some patients have two or more clones of abnormal cells.[33]

The *pig-a* gene is located on the X chromosome[17] at Xp.22 (at the end of the short arm) near the gene for the glycine receptor; [34] this means that a disabling acquired abnormality cannot be complemented by an allelic chromosome. Thus, it appears likely that the abnormalities in PNH result from a single hit to this gene in a hematopoietic stem cell.

Effects of the Deficiency of the Membrane Proteins

To date, about 15 proteins have been found to be lacking on the abnormal cells of patients with PNH (Table 20-1). [45] Not surprisingly, the deficiency, whether partial or complete, of so many proteins has profound effects on the function of the abnormal cells. These proteins can be divided into several functional groups.

Complement Defense Proteins

The indicator used to define PNH is the unusual sensitivity of the red cells to the hemolytic action of complement. This results from reduced control of the activation of

complement on the cell surface because of the lack of two (perhaps three) membrane proteins.

Complement consists of a group of proteins that circulate in the plasma in an inactive form; these proteins are sequentially activated on cellular sites to mediate some of the cellular destruction of the immune system (Fig. 20-2).[45] [46] [47] Inactivation mechanisms are in place to control the reactions. Several fluid-phase proteins inhibit the enzymatic activity of some of the complexes;[48] other proteins cleave the cell-bound components, thus inactivating them. Membrane proteins on the cell surface

TABLE 20-1 -- Membrane Proteins Missing from the Blood Cells in PNH

Complement defense proteins
Decay accelerating factor (DAF, CD55)[32]
Membrane inhibitor of reactive lysis (MIRL, CD59, protectin) [33]
C8-binding protein[34] (homologous restriction factor)[35]
Immunologic proteins
Fc receptor IIIa[36]
Lymphocyte function-associated antigen-3 (LFA-3, CD58) [37]
Endotoxin-binding protein receptor (CD14) [38A]
CDw52[38B] (Campath-1)
Enzymes
Acetylcholinesterase[22]
Leukocyte alkaline phosphatase[39]
Receptors
Urokinase (plasminogen activator) receptor[40]
Folate receptor
Granulocyte proteins of unknown function
CD14[41] [a]
CD48[42A]
CD66[42B]
Dombrock-Holley/Gregory-bearing protein [42C]

[a]Rothenberg S, personal communication.

Figure 20-2 Activation complexes of complement and their down-regulation by membrane proteins. Activation can take place by three pathways: the classic pathway involving antibody, the alternative pathway involving surface-bound C3b, and the reactive pathway involving the elements of the terminal complex C5b-7, which has been activated elsewhere (not on the target surface). Initiation complexes localize the reaction, amplification complexes amplify the effect by bimolecular enzymes (convertases), and cytotoxicity complexes insert protein into the lipid of the membrane to breach it. Decay accelerating factor inhibits the amplification complexes; membrane inhibitor of reactive lysis inhibits the formation of the membrane attack complex.

are more important for the protection of homologous cells against the attack by activated complement. Such proteins include DAF (CD55), [35] membrane inhibitor of reactive lysis (MIRL, CD59, protectin,[33] and perhaps C8-binding protein [homologous restriction factor]). [37] [38A]

Decay Accelerating Factor

Decay accelerating factor (DAF), a glycoprotein of 68,000 molecular weight, increases the rate at which the convertase complexes (the enzyme complexes responsible for the activation of C3 and C5 C4b2a of the classic pathway and C3bBb of the alternative pathway) are dissociated or decayed. In disrupting the convertase complexes, it reduces the amount of C3 that is cleaved and thus reduces, ultimately, the number of membrane attack complexes that are formed.

The deficiency of CD55 on the abnormal PNH cells results in a greater activity of the convertases; hence, much more C3 is deposited on the membrane when either the classic or alternative pathway is activated.[49] The deficiency of this activity was thought at first to account for the greater sensitivity of PNH red cells to complement, but in fact this deficiency plays a relatively minor role in that phenomenon.

The platelets in PNH also lack DAF but compensate with the release of factor H from internal granules; [50] factor H is a fluid-phase regulatory molecule with the same molecular action as DAF. This release is stimulated by activation of complement on the platelet surface and results in the local down-regulation of the convertase complexes.

Membrane Inhibitor of Reactive Lysis

Membrane inhibitor of reactive lysis (MIRL) (CD59), a glycoprotein of 19,000 molecular weight, is more important than DAF in the cellular regulation of complement action.[51] It prevents the interaction between C8 and C9 in the final steps of complement activation. [52] Lysis of the cell is caused by the insertion of C9 into the membrane after it has been amphipathic by interaction with C8 of the C5b/8 complex that is situated at the membrane surface; CD59 prevents this interaction. [47] The absence of CD59 on PNH red cells is primarily responsible for their increased susceptibility to complement lysis. [53]

Figure 20-3 Effect of polymeric C9 on the platelet membrane. **(A and Inset)** When the membrane attack complex is assembled on the membrane, aggregates of polymeric C9 result. **(B)** These polymeric C9 complexes are gathered into a region of the membrane and are removed by vesiculation. **(C)** The vesicles that result are not able to maintain phosphatidylserine on the internal leaflet of the bilayer. **(D)** The phosphatidylserine molecules become binding sites for the components of the prothrombinase complex, which is able to activate prothrombin.

The absence of CD59 on the abnormal platelets of PNH does not result in their lysis in vivo but may play a role in a serious complication of PNH, venous thrombosis. The response of the normal and PNH platelet to the insertion of the polymeric C9 into the membrane is to gather the offending complexes and remove them by exovesiculation (Fig. 20-3).[54] The vesicles that are thus generated cannot maintain the acidic phospholipids on the internal leaflet and hence externalized phosphotidylserine serves as a site for prothrombinase complexes. These complexes generate thrombin. Because the formation of polymeric C9 complexes is not regulated on the PNH platelet, many more are inserted into the membrane, resulting in many more vesicles, which generate more thrombin.

The abnormal platelets in PNH appear to be specifically sensitive to the aggregating activity of thrombin. Much smaller amounts of thrombin than are needed to aggregate normal platelets cause aggregation of these abnormal platelets. The combination of these two reactionsincreased production of thrombin and increased

sensitivity to thrombins aggregating activity probably accounts in large part for the markedly increased incidence of thrombosis in these patients.

Fc Receptor IIIa (CD16a)

The Fc receptors on phagocytic cells bind to immunoglobulin, which is attached to antigens of the target cell. Several types exist, but only one is linked to the membrane by the GPI anchor Fc receptor IIIa, the primary IgG receptor present on granulocytes. Its absence on PNH granulocytes [39] may contribute to the propensity of these patients to infections, especially blood-borne ones (septicemia, bacterial endocarditis, etc).

Other Proteins Missing from PNH Cells

A number of other proteins are known to be missing from the blood cells in PNH (Table 20-1). Some are enzymes that have no known function in the biology of the cells. Some are related to immunologic function; their absence may play a role in the pathogenesis of the syndrome, but data for this hypothesis are not available. Lack of the folate receptor and urokinase receptor [43] may alter the way in which those molecules are metabolized, but associated pathophysiology has not been identified.

CLINICAL MANIFESTATIONS

Because of the variety of missing proteins on the hematopoietic cells, PNH is a highly protean syndrome with different manifestations in different patients. In addition, because it is clonal and the size of the abnormal clone is different in different patients, the severity of a given manifestation varies greatly from patient to patient. For these reasons, PNH must be considered in a number of hematologic clinical settings.

Hemolytic Anemia

All patients with PNH have some intravascular hemolysis, which can range from barely detectable to massive, requiring repeated transfusions. The hemoglobin concentration of the blood may range from normal to very low levels, and the reticulocyte count is usually elevated but may be lower than expected for the degree of anemia.[7] The red cells are usually quite normal in appearance, although some variation in shape may occur in some patients with more dysplastic hematopoiesis.

The amount of hemolysis depends on a number of factors:

1. The size of the abnormal clone(s): the proportion of complement-sensitive red cells in the circulation can vary from 1 to 90%. Because these are the cells at risk for hemolysis,[55] the amount of hemolysis will be greater according to the number of abnormal cells, all other things being equal.
2. The abnormality of the red cells: the red cells may vary greatly in their abnormality because of differences in the content of the complement defense proteins on the surface. The most abnormal (the so-called PNH III) cells completely lack these proteins and thus are very readily lysed in the circulation by minimal activation of complement. Cells of intermediate sensitivity (PNH II cells) display limited expression of the proteins on the surface and are not as readily lysed by complement activated in vivo.[56]
3. The degree of complement activation: the abnormal cells in PNH can be lysed by complement even when it is activated on other cells or in the plasma. Thus, situations in which complement is activated (infections, transfusion reactions, etc.) result in increased lysis of these red cells. The characteristic nocturnal hemolysis may be due to the activation of complement by the absorption of endotoxin (a potent activator of the alternative pathway) from the gut, resulting in

C5b/7 complexes that initiate attack through the reactive pathway (Fig. 20-2); normal cells are protected against this attack, whereas the defective PNH cells are not. Paroxysms of hemolysis often occur in conjunction with infections that activate complement.

In addition to contributing to the anemia of the patient, the intravascular hemolysis has several other effects, the severity of which is largely related to the degree of hemolysis:

1. Most patients have excessive iron loss both because of the hemoglobinuria as well as the perpetual hemosiderinuria; 20 mg/day of iron (10 times the normal amount) may be lost without evident hemoglobinuria. [57] In the kidney, hemoglobin permeates the glomerulus as a dimer and is actively transported into the cells of the proximal tubule; when the T_{max} is exceeded, hemoglobinuria results. In the proximal tubule, hemoglobin is broken down, but the iron remains and is excreted as hemosiderin.
2. In acute and massive hemolytic episodes, the kidneys may undergo acute tubular necrosis because of the large amount of hemoglobin being passed; this occurs most readily if the patient is also dehydrated, and can be prevented by adequate hydration. [58]
3. In patients with long-lasting hemoglobinuria, defects in kidney function may occur. These may be as mild as renal Fanconi syndrome (excretion of other molecules normally reabsorbed by the proximal tubule). Rarely, mild renal tubular acidosis of the proximal tubular type may be seen. [59] [60] In a few patients, renal failure may intervene after many years of hemoglobinuria.

Hemoglobinemia may also account for two manifestations of PNHesophageal spasm and impotence. Many patients will have achalasia-like esophageal symptoms when they are having hemoglobinuria, and most men are impotent at those times. This may result from the adsorption of nitric oxide (NO) by free hemoglobin; a reduction in NO results in smooth muscle contraction.

Relative and Absolute Bone Marrow Failure

In all patients with PNH, an element of diminished hematopoiesis is present. In its most severe form, it manifests as aplastic anemia with the virtual absence of bone marrow precursors; in these cases, relatively few red cells are being made, so little hemolysis is evident. In many patients, the bone marrow is actively producing cells, particularly erythrocyte precursors, and in fact may be hyperplastic, yet a hematopoietic defect can be demonstrated.

At least two-thirds of all PNH patients have thrombocytopenia or granulocytopenia during the course of the illness. [61] Because the survival of platelets [62] and granulocytes [63] in the circulation in PNH is normal, these diminished numbers are due to deficient or ineffective production of these cells. These cytopenias may be important aspects of the patients clinical syndrome.

The hematopoietic defect can be detected in most if not all patients on the bone marrow culture. Even when the bone marrow is hyperplastic, many fewer hematopoietic colonies result when PNH progenitor cells are assayed in vitro than when normal marrow progenitor cells are assayed. [64] [65] [66] It is this characteristic that places the syndrome in the class of myelodysplastic rather than myeloproliferative syndromes.

The cause of the relative or absolute hematopoietic defect is not clear. It may have to do with missing GPI-anchored proteins on the abnormal stem cells or on the lymphocytes and monocytes that may control hematopoiesis. The presence of the defect is all the more perplexing because the abnormal stem cell must have some proliferative advantage over the normal clones of cells in order to be able to dominate the marrow in most patients.

An alternative theory has been proposed, suggesting that PNH is the result of two processes: the abnormal stem cells are generated from a somatic mutation but are not able to proliferate in a normal marrow environment; clinical PNH arises when the marrow is suppressed by an aplastic process, most likely immunological in nature.[64] The abnormal PNH cells are less suppressed by this process than the normal cells are and hence can come to dominate the marrow. [66B] Although much data are compatible with this theory, more data are needed to confirm it.

The effects of the diminution in hematopoiesis can be dramatic. It magnifies the effects of the hemolysis, since the response to anemia is inadequate. The granulocytopenia and thrombocytopenia may be severe enough to result in infections and bleeding. Bone marrow aplasia may intervene, with fatal results.

Thrombotic Events

Venous thrombosis, one of the most feared complications of PNH, was described in earlier reports and recognized as part of the syndrome by Crosby in 1952. [6] About 20% of patients with PNH from Europe and the United States experience thrombotic episodes; this fraction appears to be much less in Asian populations, which have symptoms related to such events that are difficult to document. These thromboses commonly occur at several specific sites, as described in the following sections.

Hepatic Vein Thrombosis (Budd-Chiari Syndrome)

The hepatic veins are one of the most common sites of venous thrombosis in PNH.[67] Onset may be acute and dramatic, causing severe right upper quadrant pain, jaundice, hepatomegaly, and ascites;[68] this often occurs during a hemolytic crisis, suggesting that the activation of complement is responsible for both. In other instances, the onset is more gradual and insidious. [69] In either case, serum levels of enzymes indicative of hepatic injury (SGPT, AST) are elevated; this elevation must not be confused with the marked elevation of lactate dehydrogenase and significant elevation of SGOT, which are indicative of hemolysis of the red cells. The serum alkaline phosphatase level may also be elevated. The most direct diagnostic test is the demonstration of the clots in the hepatic veins by computed tomography scan, magnetic resonance imaging (MRI), or ultrasound, particularly with Doppler flow measurements. [70] Sometimes the clots may not be demonstrable, but characteristic abnormalities in flow may indicate clots in the smaller vessels. Contrast injection into the hepatic veins may demonstrate the clots but should be done with caution. Imaging of the liver with colloid ^{99m}Tc may show a relative enhancement of the caudal lobe, which is spared the effects of hepatic vein thrombosis because the veins that drain this part of the liver go directly to the inferior vena cava and are not involved. [71] The thrombosis in the hepatic veins may extend to the inferior vena cava in extreme cases.[72]

The process, once initiated, tends to persist, with periodic exacerbations and remissions. Although patients may live for some years with chronic Budd-Chiari syndrome, it is usually ultimately fatal.

Cerebral Vein Thrombosis

The cerebral veins and sinuses are also prone to thrombosis, particularly the sagittal sinus and the veins covering the parietal lobes. [73] [74] [75] This results in severe headache, focal and nonfocal neurologic symptoms (depending on the location of the thrombosis), and, if sufficiently severe, coma and death. The spinal fluid is often xanthochromic or bloody. Careful examination of the flow of the cerebral veins with MRI or Doppler flow ultrasound may be needed to demonstrate the presence of the thrombosed veins. Although less common than hepatic vein

thrombosis, cerebral venous thrombosis also tends to be chronic and does not portend a good prognosis.

Abdominal Vein Thrombosis

The large and small veins of the abdomen may be thrombosed, resulting in a variety of syndromes. The most common is severe abdominal pain, often recurrent, usually lasting 35 days, with or without signs of intestinal obstruction. The symptoms tend to occur in the same part of the abdomen with each attack. The thromboses are difficult to demonstrate radiologically. In some patients, the problem is so severe that intestinal infarction requiring resection occurs. In other patients with recurrent pain, evidence of scarring and thrombosis may be found at operation; removal of the area may relieve the symptoms. [76]

The larger veins of the abdomen may be thrombosed, resulting in symptoms related to their site. Splenic venous thrombosis may result in splenomegaly and even splenic rupture. Portal venous thrombosis may result in ascites, esophageal varices, and other indications of rerouted venous circulation. Thrombosis of veins of the stomach and duodenum may result in lesions that are detectable with gastroduodenalendoscopy.

Dermal Vein Thrombosis

Thrombosis of the veins of the skin may occur in two clinical patterns. [77] [78] Areas of thrombosis may appear as painful, swollen, discolored lesions, usually measuring 510 cm, over various parts of the body. These usually resolve but on occasion may ulcerate. These dermal thromboses are recurrent but do not necessarily occur at the same site. Alternatively, patients may have localized lesions resembling purpura fulminans, with purple discoloration of larger areas of skin, demarcation, and often necrosis. If the lesions are sufficiently extensive, death may result.

Thrombosis at Other Sites

Thromboses may occur at other sites. Thrombosis of the veins of the lower extremity occurs with greater frequency than is common in the general population, but death by pulmonary embolism is rare. Thrombosis of the epididymal veins leads to a syndrome much like acute orchitis. Arterial thrombosis is rare.

Other Symptoms and Complications

Patients with PNH may have other symptoms that are probably related to their disease but for which there is no current explanation.

1. Many patients complain of dysphagia during episodes of intravascular hemolysis; this appears to be due to the generation in the esophagus of very strong peristaltic waves. When the hemolysis is nocturnal, the dysphagia is present in the morning but disappears by noon.
2. Many men with PNH note impotence during the periods of hemoglobinuria. In some cases, this impotence lasts beyond the hemoglobinuric period and becomes permanent. This and the symptoms of esophageal contraction may result from the fact that free hemoglobin binds nitric oxide, causing increased contractility of smooth muscle.
3. Many patients note a feeling of fatigue that may be disabling during periods of hemoglobinuria. This is not related to hemoglobin level, as it disappears when the hemoglobinuria stops.
4. Rarely, patients may have severe low back pain. Blood chemistries done at the time of pain suggest that there may be muscle destruction, and this symptom has been ascribed to small and undetectable thromboses in the muscles.

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DIAGNOSIS

Until recently, the abnormal sensitivity of the red cells to the hemolytic action of complement has been the basis of the diagnostic tests for PNH. In the original Ham test, complement is activated by acidification of the serum to a pH of 6.2; PNH cells will lyse in such serum, whereas normal cells will not. [2] The test is specific (there is no other cause for a positive test other than the very different syndrome hereditary erythroblastic multinuclearity with positive acidified serum test (HEMPAS, congenital dyserythropoietic anemia type II). [79] The sensitivity of this test is dependent on the concentration of Mg^{2+} in serum. The test becomes more sensitive if the Mg^{2+} concentration is raised to 0.005 M; [80] even then, the test may not detect small populations of abnormal cells and cells of intermediate sensitivity. Furthermore, the test seems to be technically difficult, since many false-positive and false-negative reactions occur during routine laboratory practice.

Complement is also activated when the serum is mixed with a medium of low ionic strength; sucrose is the solute usually used to replace the salt solution in maintaining isosmotic conditions, and the test is often called the sugar water test. [81] [82] This test is quite sensitive and usually does not fail to lyse the abnormal PNH cells; however, it is less specific because cells in other conditions (autoimmune hemolytic anemia, leukemia, etc.) may be lysed. [83] Many tests have been proposed in which complement is activated by antibody to red cell antigens. The most quantitative of these is the complement lysis sensitivity test in which limiting amounts of complement are activated by antibody. [12] By careful titration, both the proportion of abnormal cells and their abnormality can be demonstrated.

Tests Based on Detection of GPI-Linked Proteins

Since the finding that the abnormal cells lack the GPI-linked proteins, the demonstration that these proteins are deficient on the blood cells has been used as a diagnostic test for PNH. In general, monoclonal antibodies to CD59 or CD55 (DAF) are used with flow cytometric analysis (Fig. 20-4). [5] [84] [85] These tests have the advantage of being readily performed on granulocytes and platelets; the proportion of abnormal cells in these populations is greater than that of red cells, because the abnormal granulocytes and platelets have a normal survival time in the circulation.

In patients whose cells are partially deficient, some proteins are more deficient than others; in general, CD59 is the most deficient and (on granulocytes) CD16 (the FC receptor IIIa) is the least deficient. [86]

The results of the examination of CD55 and CD59 on lymphocytes obtained from PNH patients are less clear. In some patients, a population of abnormal cells could be detected in all three lymphocyte cell lineages (T cell, B cell, and natural killer cells) [6] [87] [88] [89] (often in surprisingly small numbers), whereas in others, they could not. [85]

At the present time, the demonstration of red cells and/or granulocytes lacking one or more of the GPI-linked proteins by the use of monoclonal antibodies and flow cytometry provides the best means of diagnosing PNH; this method is both sensitive and specific and can readily provide information on the relative proportion of the abnormal populations. [5]

Differential Diagnosis

Hemoglobinuria is a relatively unusual symptom in clinical medicine and is easily confused with two other symptoms, myoglobinuria and hematuria. Myoglobinuria can be identified by examination of the serum (which is without red pigment), differential solubility of the two pigments (hemoglobin is more easily precipitated by ammonium sulfate), or reaction with

Figure 20-4 Demonstration of the abnormal cells of PNH by flow cytometry, using fluorescein-labeled CD59. In each case, the relative fluorescence intensity, a measure of the CD59 content per cell, is described on the abscissa using a logarithmic scale. The relative number of cells with that intensity is shown on the ordinate. The populations of PNH cells (PNH Inormal, PNH IImoderately abnormal, PNH III markedly abnormal) are indicated. Panels A and Berythrocytes; panels CFgranulocytes.

AN APPROACH TO PNH

DIAGNOSIS

The diagnosis of PNH is ultimately made by the laboratory tests demonstrating complement sensitivity of the red cells by the Ham or other test or (preferably) deficiency of the GPI-linked proteins by flow cytometry. Diagnostic tests should be obtained in patients with (1) hemolytic anemia, with or without hemoglobinuria, with normal-appearing red cells, and without other hemolytic diagnosis; (2) signs of marrow dysfunction including aplastic anemia, granulocytopenia, or thrombocytopenia not otherwise explained, or myelodysplastic disease (if the test is initially negative, it should be repeated at intervals as it may become positive); (3) unexplained thromboses, particularly venous thromboses in unusual places; and (4) recurrent bouts of abdominal pain, or neurologic symptoms and headaches.

TREATMENT

The appropriate treatment depends on the symptoms. All patients should receive iron supplements unless they are receiving transfusions.

Anemia

For anemic patients with signs of hemolysis, prednisone should be tried at a dose of 2040 mg every other day; if this is successful, it may be tapered, but not below a dose of about 20 mg every other day. If it is not helpful (the hemoglobin does not rise after 1 month or so), it should be discontinued unless the patient is having thrombotic complications. Some patients respond to androgens; danazol (400 mg/day) is best tolerated. Again, if no response is seen within 2 months, it should be discontinued. Prednisone and danazol may be given simultaneously. Some patients respond to very high doses of recombinant erythropoietin (20,000 U 23 times a week). Transfusions should be given as needed.

Thrombosis

Patients with acute thrombosis should be given thrombolytic agents (streptokinase, urokinase, or tissue plasminogen activator) as rapidly as possible. Full-dose heparin therapy should be instituted for 710 days, followed by appropriate treatment with Coumadin. Patients with documented thrombosis should be maintained on Coumadin for long periods. Antiplatelet agents (aspirin, ibuprofen, etc.) may be useful but their action is unproved.

Deficient Hematopoiesis

If the patient has an identical twin, syngeneic transplantation should be done. If the patient has a histocompatibility antigen-compatible sibling, transplantation should be considered if any complications due to the PNH are present; this is particularly true for children. Transplantation with the marrow of an antigen-compatible unrelated donor should be reserved only for patients with major complications.

Patients with marrow hypofunction (manifested by low reticulocyte count, thrombocytopenia, and/or granulocytopenia) may be candidates for antithymocyte globulin. The usual dose is 15 mg/kg/day for 10 days or 45 mg/kg/day for 4 days; this must be given with high doses of prednisone and usually results in worsening of the platelet count during and shortly after administration. The use of cyclosporine is also being explored. Granulocytic patients may respond to G-CSF (150300 g 12 times per week).

GENERAL COMMENTS

PNH is a chronic disease, and most patients are quickly tired of having it. Careful follow-up and good patient education are important parts of their care. The hope can be held out that patients who do not have serious complications may eventually be rid of their disease.

specific antibodies against myoglobin and hemoglobin. In hematuria, red cells are present in the freshly collected urine and the supernatant does not contain heme-reactive pigments after centrifugation.

Several other hemolytic diseases may cause hemoglobinuria. Severe autoimmune hemolytic anemia, particularly paroxysmal cold hemoglobinuria, may result in enough intravascular lysis to produce hemoglobinuria. Severe traumatic hemolytic anemia from prosthetic cardiac devices or from microangiopathic causes may also lead to hemoglobinuria; schistocytes are always evident on examination of the peripheral blood film. Rarely, toxic hemolytic anemia (such as that occuring during clostridial infection, copper poisoning, oxidant ingestion in a patient deficient in glucose-6-phosphate dehydrogenase, etc.) may result in hemoglobinuria. Other more common and more subtle symptoms of PNH are more readily missed and may only be identified as part of the clinical constellation of PNH by doing the diagnostic tests for PNH. The abdominal pain that patients with PNH experience can be confused with ileitis. Budd-Chiari syndrome can be confused with cirrhosis or other parenchymal liver disease; the elevation of the enzymes (SGOT and lactate dehydrogenase) usually seen in PNH may add to the confusion. The effects of sagittal venous thrombosis (headaches, edema of the optic nerve, etc.) may resemble pseudotumor cerebri or other cerebrovascular events.

Most commonly, PNH is confused with other hematologic syndromes, particularly other stem cell disorders. The relationship to aplastic anemia has been described. In recent surveys,[90] [91] [92] an average of 6.8% (4.120.8%) of untransplanted patients with aplastic anemia evolve to PNH. Most of these patients have clinical symptoms attributable to PNH, but some have only cells with the defects characteristic of PNH. The occurrence of PNH in other stem cell disorders is more rare but too common to be due to chance alone. Erythroleukemia, chronic myeloid leukemia, and myelofibrosis are the most common accompanying diseases, [93] but patients with refractory anemia with excess blasts,[94] myelofibrosis,[93] and polycythemia vera (personal observation) have been associated with cells expressing the PNH defect. Rotoli and Luzzatto[93] have determined that 11 of 113 patients with myelodysplastic syndrome had a positive Ham test, suggesting the presence of the PNH defect in 112% of the cells. In one series, PNH developed before the documentation of an identifiable myelodysplastic syndrome (5 of 47 patients) and after the development of agnogenic myeloid metaplasia (4 patients). [95] The incidence of the defect in this population might be even higher if granulocytes and platelets were examined for the deficiency of GPI-linked cells.

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COURSE AND PROGNOSIS

The clinical outcome in PNH is extremely variable. Estimates of mean life span from the time of diagnosis have varied from

810 years,[96A] to 15 years or more,[96B] [96C] in a Chinese study, the mean life span was 25 years, probably because of the decreased morbidity caused by thrombosis in this population. The most common causes of death are the consequences of thrombosis (particularly of the hepatic and other intraabdominal veins) or of the effects of hypoproliferation of the bone marrow (infections, bleeding).

The population of abnormal cells may decrease with time in patients surviving for prolonged periods (1520 years). [97] The abnormal erythrocytes and granulocytes may disappear completely but a small population of lymphocytes may persist for many years;[1] commonly, a small population of erythrocytes may remain, but the patient is asymptomatic.

Progression to Acute Leukemia

In about 35% of patients with PNH, the illness progresses to acute leukemia. [8] [9] This is usually of myeloid origin but occasionally the leukemic cells are positive for terminal deoxynucleotidyl transferase, suggesting a lymphocytic origin. [98] The onset is typically about 5 years after the onset of symptoms related to PNH. As the leukemic cells appear, the abnormal red cells disappear but will reappear if (in the rare case) the leukemia is successful treated. [99] The leukemic cells have the PNH phenotype of deficient GPI-linked proteins [100] [101] and may show cytogenetic changes suggestive of subclonal evolution. [102]

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THERAPY

Treatment in PNH depends on the symptoms of the patient. In general, several modalities are available:

1. Correction of the anemia by
   a. Interruption of complement activation
   b. Replacement of missing nutrients
   c. Transfusion
2. Treatment and prevention of thrombosis
3. Modification of the bone marrow by transplantation or stimulation

Each of these modalities of therapy may be applied as needed and as circumstances permit.

Correction of the Anemia

Since the hemolytic component of the disorder is ultimately a result of the activation of complement, measures that prevent this event lead to amelioration of the hemolysis. At present, the only agents that do so are glucocorticoids; the dose required is relatively large (0.30.5 mg/kg/day). The action of the steroids is very rapid. Because the dose required is so high, the drug is generally given every other day; on the intervening day, the patient may have hemoglobinuria and its accompanying symptoms. During an acute episode of hemoglobinuria, the dose may be increased to 1 mg/kg/day and be administered every day until the bout is over. Only about 60% of patients with hemolysis respond to this treatment.

Patients with hemolysis in PNH waste large amounts of iron as hemosiderin and hemoglobin in the urine. Unless they are getting iron from other sources (e.g., blood transfusions), most of these patients need to receive iron supplements. Usually, oral iron administration is sufficient, but occasionally the parenteral route is used. When iron is first administered to an anemic and iron-deficient patient, a hemolytic episode may result when the stimulated marrow begins to pour out abnormal cells that are then hemolysed.[103] [104] This can be prevented by the simultaneous administration of prednisone or by suppression of erythropoiesis by transfusion.

Because patients with hemolysis have increased erythropoiesis, they may theoretically become deficient in folic acid. This deficiency seems to be rare; it may be that the intravascular hemolysis releases red cell folate into the serum, where it again becomes available for metabolism.

Some patients require transfusion to maintain an adequate oxygen-carrying capacity, which may result in the lysis of a small number of the patients own cells and resulting hemoglobinuria. Such lysis is probably rarer than commonly thought and may be due to minor sensitization either to red cells or to other blood elements. [104] [105] When it occurs, it is confused with a hemolytic transfusion reaction resulting from incompatibility. The reaction can be prevented by washing the donor cells before transfusion.[106]

Therapy for and Prevention of Thrombosis

When acute thrombosis occurs, it should be treated as an emergency. Thrombolytic agents (streptokinase, urokinase, or tissue plasminogen activator) should be administered at once unless otherwise contraindicated.[3] [107] [108] [108] The results may be dramatic; the enlarged, swollen liver of a patient with acute Budd-Chiari syndrome may reduce in size over a period of a few minutes to a few hours. Although thrombolytic agents are less effective if not used quickly after the clot has formed, they may be of some use even days later and should be tried.

Once it is safe, the patient suffering acute thrombosis should be given heparin in the usual fashion (a bolus of 5,000 U followed by an infusion of 25,000 U/day followed by careful monitoring of appropriate parameters of anticoagulation). Although hemolytic reactions after heparin administration have been reported, [6] [109] they are rare. As the acute episode passes, the patient should be anticoagulated with warfarin derivatives and should be maintained on anticoagulation therapy for 6 months.

These measures for preventing and treating thrombosis are inadequate. Most patients with Budd-Chiari syndrome never entirely eliminate the problem. With the development of better antithrombin agents (e.g., recombinant hirudin) and better ways of controlling the activation of platelets by thrombin, more successful ways of dealing with this complication may be found.

Modification of Hematopoiesis

Until recently, alteration of hematopoietic function was difficult and ineffective. Androgens and modified cogeners may, in some patients, stimulate erythropoiesis. [110] More recently, recombinant cytokines have been used, particularly erythropoietin and colony-stimulating factor-granulocyte (G-CSF) these are usually only moderately effective and are difficult to administer.

Antithymocyte Globulin

Based on the assumption that T lymphocytes modify hematopoiesis and may play a role in its diminution, patients with aplastic anemia were given equine antiserum to human thoracic duct lymphocytes (antithymocyte globulin); remission of the aplastic anemia was seen in about 60% of patients, although the reason for this response is not entirely clear.[111] [112] On the basis of the similarities of PNH and aplastic anemia, antithymocyte globulin was given to patients with evidence of deficient hematopoiesis.[113] About 70% of these patients had correction of their cytopenia; reversal of thrombocytopenia was the most common. In most cases, the increase in the proportion of complement-sensitive cells was not great, and the amount of hemolysis did not usually increase.

Allogeneic Bone Marrow Transplantation

PNH can be cured by replacement of the abnormal cells of the bone marrow with normal bone marrow.[114] [115] In the past, such

replacement has been limited to patients with bone marrow failure who have histocompatible sibling donors, either (optimally) identical twins or HLA-A, -B, and -D

matched siblings. As bone marrow transplantation has become safer, patients with other complications of PNH have undergone bone marrow transplantation, particularly children, in whom transplantation is safer and in whom the prognosis of the disease is known to be relatively poor. [116]

Syngeneic transplantation is easier to perform than allogeneic. In at least one instance, the syngeneic marrow (from an identical twin) was infused without prior conditioning (treatment of the patient with immunosuppressive and marrow-ablative drugs); disappearance of the abnormal PNH cells resulted. [117] In other instances, this has not been the case. In a recent personal case, a patient with severe Budd-Chiari syndrome was successfully given a syngeneic transplant, following which the liver disease improved. [118]

Allogeneic marrow transplantation still carries a considerable risk of complications, including graft-versus-host disease, intercurrent infections, and failure of engraftment. However, when faced with the poor prognosis of the complicated patient, the benefits are worth the risks in many cases. To date, few patients have been given allogeneic transplants from unrelated donors; although some of these transplants are successful, the complications are even greater than those of allogeneic transplantation from a sibling.

Gene Therapy

With the identification of the gene that is abnormal in PNH, treatment by reinsertion of the gene becomes possible. Experiments are currently under way to ascertain the appropriate conditions for this treatment.

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Chapter 21 - Pure Red Cell Aplasia

Emmanuel N. Dessypris

Pure red cell aplasia (PRCA) is a syndrome characterized by normochromic normocytic anemia, reticulocytopenia (reticulocyte count <1%), and an almost complete absence of erythroblasts from the bone marrow (erythroblasts <0.5%). [1] In contrast to aplastic anemia, in which the aplasia involves all three cell lines, in PRCA the aplasia is selective for the erythroid cell line, resulting in severe anemia with normal leucocyte and platelet counts. After its initial description by Kaznelson in 1922, [2] this syndrome has appeared in the literature under different terms, including pure aplastic anemia, erythrophthisis, chronic hypoplastic anemia, aplastic crisis, erythroblastopenia, erythrogenesis imperfecta, Blackfan-Diamond syndrome, pure red cell agenesis, and primary red cell anemia. [1] Today, the term PRCA is used to describe this disorder in adults; congenital hypoplastic anemia (CHA) or Diamond-Blackfan anemia, and transient erythroblastopenia of childhood (TEC) are used for the congenital and acquired forms that occur in infants and children.

Pure red cell aplasia is a relatively uncommon disorder, the exact incidence of which is not known. It may affect any age group and occurs with almost the same frequency in men and women. It has been described in all parts of the world, and there seems to be no ethnic or racial predisposition.

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ETIOLOGY AND PATHOGENESIS

Pure red cell aplasia may present as a congenital disorder early in life or later as an acquired anemia that may be primary or secondary to a variety of neoplastic, autoimmune, or infectious diseases. A classification of PRCA is presented in Table 21-1 . Congenital hypoplastic anemia is a form of erythroid aplasia that manifests itself during the first year of life.[3] It is considered to be an inherited disorder that is frequently associated with a variety of congenital malformations. In approximately 10% of cases, more than one member of the family has been affected, and in a small number of cases, members in successive generations were affected, indicating an inheritance pattern that has been defined in different families as either autosomal dominant or recessive. [1] [3] [4]

Primary acquired PRCA affects people of any age in the absence of any underlying disorder. It may run an acute and usually self-limited course or may persist chronically as a form of refractory anemia. In children, this condition is referred to as TEC, which is usually of limited duration. [101] In contrast, in adults, the acute form of primary PRCA is unusual and the chronic form of this disorder predominates. It is conceivable that many cases of acute PRCA in adults escape diagnosis because acute arrest of erythropoiesis of short duration is not expected to lead to symptoms of anemia because of the long life span of the red cells. Children, however, are quite frequently seen for minor illnesses during which an anemia may be found, the work-up of which leads to the diagnosis of TEC. A large number of cases of both PRCA and TEC have been shown to have an immune pathogenesis and are classified as autoimmune. However, there is a significant number of cases in which no immune pathogenetic mechanism can be established, and they are classified as idiopathic. The failure to demonstrate an immune mechanism, however, does not mean that the case may be of a nonimmune pathogenesis, because the outcome of treatment seems to be the same among autoimmune and idiopathic cases.[1] A small percentage of cases of idiopathic PRCA, usually refractory to treatment, may evolve into acute leukemia, and these cases are classified as preleukemic/myelodysplastic.[102]

Pure red cell aplasia may develop as a hematologic complication in the course of a variety of diseases (Table 211). Among

TABLE 21-1 -- Classification of Pure Red Cell Aplasia

I. Congenital hypoplastic anemia (Diamond-Blackfan syndrome)
II. Acquired PRCA
A. Primary
1. Autoimmune
2. Preleukemic
3. Idiopathic
B. Secondary PRCA, associated with
1. Thymoma[1] [5] [6]
2. Hematologic malignancies
Chronic lymphocytic leukemia
B-cell type [1] [7] [8] [9]
T-cell type [1] [10] [11] [12]
Large granular lymphocyte leukemia [1] [13]
Hodgkins disease [14] [15] [16] [17]
Non-Hodgkins lymphomas[18] [19] [20] [21] [22]
Multiple myeloma[23] [24]
Waldenströms macroglobulinemia [25]
Chronic myelocytic leukemia [26] [27] [28]
Myelofibrosis with myeloid metaplasia [1] [28] [29] [30]
Essential thrombocythemia [31]
Acute lymphoblastic leukemia[32] [33] [34]
3. Solid tumors
Carcinoma of the stomach[35]
Adenocarcinoma of the breast[36] [37]
Adenocarcinoma of the bile duct[38]
Squamous cell carcinoma of the lung[39] [40] [41]
Epidermoid carcinoma of the skin[42]
Carcinoma of the thyroid [43]
Renal cell carcinoma [44]
Carcinoma of unknown primary site[45]
Kaposis sarcoma [6] [46] [47]
4. Infections

	Human B19 parvovirus [48] [49] [50] [51] [52] [53] [54] [55] [56] [57] [58]
	Human immunodeficiency virus[59] [60]
	T-cell leukemia-lymphoma virus[61]
	Infectious mononucleosis [62] [63] [64] [65]
	Viral hepatitis[66] [67] [68]
	Mumps[1] [69] [70]
	Cytomegalovirus[71]
	Atypical pneumonia[1] [69] [70]
	Meningococcemia[1] [69] [70]
	Staphylococcemia[1] [69] [70]
	Leishmaniasis[72]
5.	Chronic hemolytic anemias[1] [48] [49] [50] [51] [52] [53] [54] [55] [56] [57] [58]
6.	Collagen vascular diseases
	Systemic lupus erythematosus [73] [74]
	Rheumatoid arthritis [75] [76]
	Mixed connective tissue disease[77]
	Sjögrens syndrome[78] [79]
7.	Drugs and chemicals (see Table 212)
8.	Pregnancy[80] [81] [82] [83]
9.	Severe renal failure[84]
10.	Severe nutritional deficiencies [85] [86] [87] [88]
11.	Miscellaneous
	PostABO-incompatible bone marrow transplantation[89] [90]
	Angioimmunoblastic lymphadenopathy [91] [92]
	Autoimmune multiple endocrine gland insufficiency [93] [94]
	Autoimmune hypothyroidism [95] [96]
	Autoimmune chronic hepatitis [97] [98]
	Anti-EPO antibodies post-treatment with EPO[99]

EPO, erythropoietin.

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them, thymic neoplasms were the first to be associated with erythroid aplasia.[1] [5] [6] The incidence of PRCA among patients with thymoma was initially estimated to be as high as 15%; however, in more recent series, the incidence was found to be close to 4%.[1] [5] The presence of a thymoma among patients presenting with PRCA was initially reported to be as high as 50%, but in more recent series it was estimated to be close to 9%.[1] [5] [6] [103] PRCA may precede the development of thymoma, coexist with thymoma, or develop even years after the surgical removal of a thymoma.

Various hematologic malignancies have been associated with severe erythroid aplasia [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18] [19] [20] [21] [22] [23] [24] [25] [26] [27] [28] [29] [30] [31] [32] [33] [34] and with chronic lymphocytic leukemia (CLL), with the B-cell, T-cell, or large granular lymphocyte (LGL) types being the most frequently seen.[7] [13] It has been estimated that the incidence of severe erythroid aplasia among patients with CLL may be as high as 6%, with many cases being missed because severe normochromic anemia and reticulocytopenia are frequent manifestations of advanced-stage CLL and are usually attributed to the myelophthisic process in the marrow. The development of erythroid aplasia may depend on the stage of CLL,[104] but this does not affect its prognosis, and in most cases does not seem to be related to previous cytotoxic chemotherapy.[1] [7] PRCA has been also described in association with Hodgkins and non-Hodgkins lymphomas,[18] [19] [20] [21] [22] [23] [24] multiple myeloma and Waldenströms macroglobulinemia,[23] [24] [25] chronic myelocytic leukemia, idiopathic myelofibrosis and essential thrombocythemia,[26] [27] [28] [29] [30] [31] and acute lymphoblastic leukemia.[32] [33] [34]

Pure red cell aplasia has been reported in association with a variety of nonhematopoietic, nonthymic neoplasms.[35] [36] [37] [38] [39] [40] [41] [42] [43] [44] [45] [46] [47] Considering the rarity of reports and the high incidence of malignant neoplasms, it seems obvious that such an association is very rare. On the other hand, it may be that PRCA is underdiagnosed in patients with advanced cancer in whom anemia is such a frequent finding that bone marrow examination is not routinely performed. In both hematopoietic malignancies and solid tumors, PRCA may appear before or after the diagnosis of malignancy and follows a chronic course independent of the evolution of the underlying disease.[1] In those cases in which a real association exists, successful treatment of the underlying malignant disease leads to remission of PRCA.

In a number of infections, acute, self-limited PRCA may develop.[48] [49] [50] [51] [52] [53] [54] [55] [56] [57] [58] [59] [60] [61] [62] [63] [64] [65] [66] [67] [68] [69] [70] [71] [72] Infection with human B19 parvovirus is responsible for the aplastic crisis seen in children and young adults with chronic hemolytic anemia [48] [52] [53] [54] [55] [58] and for chronic PRCA in immunocompromised patients (i.e., acquired immunodeficiency syndrome, post-transplantation, chemotherapy, or immunosuppressive treatment).[56] [57] Viral hepatitis and infectious mononucleosis are two other viral infections that have been reported in frequent association with PRCA.[62] [63] [64] [65] [66] [67] [68] In general, PRCA remits with treatment or resolution of the underlying infection.

Pure red cell aplasia may appear as a hematologic complication in a number of autoimmune diseases, including collagen vascular diseases, such as systemic lupus erythematosus,[73] [74] rheumatoid arthritis,[75] [76] mixed connective tissue disease,[77] and Sjögrens syndrome,[78] [79] in autoimmune hemolytic anemia,[1] [105] [106] [107] multiple endocrine gland insufficiency,[93] [94] autoimmune hypothyroidism,[95] [96] ulcerative colitis,[97] autoimmune hepatitis,[97] [98] angioimmunoblastic lymphadenopathy,[91] [92] postABO-incompatible bone marrow transplantation,[89] [90] and in very rare cases of patients with end-stage renal disease treated with recombinant erythropoietin in whom antibodies to the hormone developed.[99] In many of these conditions, PRCA may appear as the presenting manifestation of the underlying disease.

A continuously increasing number of drugs and chemicals have been reported as causes of PRCA [108] [109] [110] [111] [112] [113] [114] [115] [116] [117] [118] [119] (Table 21-2). Drug-induced PRCA is usually an acute form of erythroblastopenia that remits soon after discontinuation of the drug or cessation of exposure to the chemical. It may appear after the first exposure to the drug or a significant time after its initiation. In most instances, the association of a drug with PRCA is circumstantial and is based on the evidence that PRCA remits after discontinuation of the drug.[1] [109] Diphenylhydantoin, azathioprine, chlorpropamide, and isoniazid have been repeatedly implicated as causes of PRCA, and in certain instances their association with

TABLE 21-2 -- Drugs and Chemicals Associated with the Onset of Pure Red Cell Aplasia

Allopurinol	Halothane
-Methyldopa	Isoniazid
Aminopyrine	Maloprim (dapsone and pyrimethamine)
Anagyrine	Anagyrine
Arsphenamine	Mepacrine
Azathioprine	Methazolamide
Benzene hexachloride	Penicillin
Bromsulphalein	d-Penicillamine
Calomel	Pentachlorophenol
Carbamazepine	Phenobarbital
Cephalothin	Phenylbutazone
Chenopodium	Procainamide
Chloramphenicol	Salicylazosulfapyridine
Chlormadinone	Santonin
Chlorpropamide	Sodium dipropylacetate
Co-trimoxazole	Sodium valproate
Diphenylhydantoin	Sulfasalazine
Estrogens	Sulfathiazol
Fenbufen	Sulindac
Fenoprofen	Thiamphenicol
FK506 (tacrolimus)	Tolbutamide
Fudarabine	Rifampicin
Gold	Zidovudine
Modified from Dessypris,[1] with permission.	

PRCA has been proven by recurrence of anemia on reinstitution of therapy. [1]

Pregnancy also has been associated with PRCA, which usually remits after delivery. [80] [81] [82] [83] Development of PRCA during pregnancy does not necessarily predict recurrence of the disease in subsequent pregnancies. Only in a unique case did PRCA develop in three successive pregnancies in the same person, and two of the fetuses were born with hydrops fetalis and found to have erythroid aplasia on autopsy, whereas the third one, treated with intrauterine red cell transfusions, was born with red cell aplasia that remitted after 3 months. [83]

In rare cases, PRCA has been associated with renal failure, [84] severe malnutrition such as marasmus and kwashiorkor,[85] and riboflavin,[85] [120] vitamin B_{12} ,[86] [87] [88] and folic acid deficiency. [86]

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BIOLOGIC AND MOLECULAR ASPECTS

The erythropoietic failure in red cell aplasia is characterized by absence of mature marrow erythroid precursor cells despite the presence of normal or nearly normal numbers of erythroid progenitor cells. The failure of the erythroid progenitors to mature, differentiate, and give rise to erythroblasts may be due to an intrinsic defect in these cells, to the presence of humoral or cellular inhibitors of erythropoiesis, or to an abnormal bone marrow microenvironment. Different mechanisms seem to operate in each of the various forms of PRCA.

Congenital Hypoplastic Anemia

The pathogenesis of CHA is not yet fully understood. The basic abnormality seems to be an intrinsic defect in the erythroid cells that prevents their development and differentiation into mature erythroblasts. Earlier reports that the arrest of erythropoiesis is due to a microenvironmental defect, [121] to IgG inhibitors,[122] or to cytotoxic lymphocytes/monocytes[123] [124] have not been confirmed in subsequent larger studies. [125] [126] [127] [128] Serum erythropoietin levels are appropriately elevated [129] and the numbers of erythroid progenitors (burst-forming unitserythrocyte [BFU-E] or colony-forming unitserythrocyte [CFU-E]) in the bone marrow of patients with CHA are either normal or decreased. [125] [127] [128] [130] Earlier studies have suggested a block in erythroid differentiation preceding the BFU-E. [128] In more recent studies, however, a normal or near-normal number of BFU-E was reported.[130] In addition, there is evidence for decreased responsiveness of erythroid progenitors to erythropoietin, which is partially correctable by addition of glucocorticosteroids, [131] [132] but this does not seem to be a constant finding among all examined cases. Erythroid progenitors in CHA seem to exhibit an accelerated rate of programmed cell death on withdrawal of erythropoietin compared with normal erythroid cells. [133] In addition, the response of early erythroid progenitors to interleukin-3, granulocytemacrophage colony-stimulating factor, and stem cell factor in vitro may be either normal or diminished.[134] [135] [136] [137] [138] A repetitive theme in all in vitro studies is the fact that both the numbers of marrow erythroid progenitors and their sensitivity to various hematopoietic factors vary from patient to patient, indicating that what is morphologically recognized as CHA may be pathogenetically a number of diverse but clinically indistinguishable disorders.

The observation that decreased erythroid growth in vitro can be restored to normal in a number of cases of CHA by factors affecting early erythroid progenitor cell growth (BFU-E) led to studies for identification of possible defects in the receptors of these factors. In particular, molecular studies on the stem cell factor gene and its receptor failed to show any abnormalities. [139] [140] Thus, in CHA the intrinsic defect detectable in vitro in erythroid cell cultures seems to be variable from case to case. By the use of linkage analysis with chromosome 19 markers, however, a consistent abnormality was detected in 19q13 among patients with CHA of diverse ethnic origin, indicating that the genetic abnormality for this disease may be homogeneous despite its clinical and phenotypic variability. [141]

Primary Acquired PRCA

Primary acquired PRCA and TEC have been studied extensively for the last 30 years, and repeated studies have indicated that the arrest of erythropoiesis is caused by the presence in the patients plasma of an erythropoietic inhibitor. Earlier studies in animals have shown that injection of patients plasma leads to a significant suppression of in vivo erythropoiesis as measured by [59] Fe incorporation into newly formed red cells. [1] [18] [142] [143] [144] Assessment of the response of patients marrow cells to erythropoietin by measuring the amount of heme synthesized in vitro showed that in the presence of normal serum, PRCA marrow responds normally to erythropoietin, but in the presence of the patients autologous plasma, a significant decline in the heme synthesis is observed, suggesting the presence in the patients plasma of an inhibitor acting on erythroid cells. [145] [146] In about 60% of cases, patients marrow cells respond to erythropoietin in a normal way by increasing the rate of the heme synthesis by two- to ninefold, and in approximately 40%, an inhibitor of erythropoiesis can be detected in their plasma. This inhibitor has been localized to the IgG fraction, and it disappears from the plasma after remission of PRCA. [145] [146] [147] [148]

The stage of erythropoiesis at which the arrest occurs can be assessed by assaying PRCA marrow cells in semisolid media for erythroid progenitors. Despite a conspicuous absence of erythroblasts from the PRCA marrow, in at least 60% of patients normal numbers of early and late erythroid progenitors can be detected, indicating that the arrest occurs at any level between CFU-E and basophilic erythroblasts. In the remaining patients, the erythroid cell compartment is affected at a stage earlier than the CFU-E, so that the CFU-E and/or BFU-E marrow pools are significantly reduced. [1] [28] [149] [150] The presence of normal numbers of erythroid progenitors has been associated with a favorable outcome of immunosuppressive therapy. [28] [150] Maturation and differentiation of erythroid progenitors into erythroblasts in vitro is inhibited by the patients serum IgG. The inhibition is dose dependent and is no longer present in the IgG fraction

of the patients plasma collected after remission. The inhibitory effect of the IgG is specific for erythroid cells because no effect on myeloid progenitor cell growth is detected.[149] [151] [152]

The mode of action of the PRCA IgG inhibitor of erythropoiesis has been studied in a number of cases of primary PRCA and TEC. In some cases, the IgG seems to be cytotoxic to CFU-E in the presence of complement; in others, its inhibitory activity is independent of the presence of complement, but its presence is continuously required during the 7-day period of maturation of CFU-E to erythroblasts. [1] [151] [153] In another cohort of patients, the IgG is cytotoxic to mature recognizable erythroblasts,[130] [154] [155] [156] and in rare cases, the inhibitory IgG has the properties of an anti-erythropoietin antibody. [157] [158] [159] In most cases, the target for the IgG antibody is an erythroid cell at a stage of differentiation between BFU-E and mature erythroblast. The nature of the molecule(s) on the erythroid cell membrane with which the PRCA IgG inhibitor interacts has not yet been defined.

Secondary PRCA

Thymoma and PRCA

A number of studies have demonstrated the presence of an inhibitor of erythroid cell development in the plasma of patients with thymoma and PRCA. [1] [15] [18] [46] [141] [142] [143] These studies, however, were performed on either mice in vivo or allogeneic human marrow cells in vitro, and the significance of these findings is unknown because the possibility of detection of heterophile antibodies or antibodies developed as a consequence of alloimmunization secondary to multiple red cell transfusions cannot be excluded.[1] A possible role for T-cell-mediated suppression of erythropoiesis has been suggested by more recent studies. [160] [161] [162] The exact incidence of antibody- or T-cell-induced erythropoietic arrest in PRCA associated with thymoma remains unknown.

Chronic Lymphocytic Leukemia and PRCA

In B-cell CLL, the frequency of PRCA is 6%, and in T-cell as well as in LGL types it may be even higher. [7] [8] [9] [10] [11] [12] [13] All attempts to detect an inhibitor in the

plasma of these patients have provided negative results. [10] [148] [163] Various studies have demonstrated that in T-cell CLL (including the LGL type), the T lymphocytes are responsible for the suppression of erythropoiesis. [10] [11] [12] [61] [164] The suppression is mediated by direct cell-to-cell interaction, mainly between a subset of T cells expressing receptors for the -chain of IgG (T cells) and erythroid progenitors, and it is human leukocyte antigen (HLA)-DR restricted. [12] The suppression is selective for the erythroid cells, and is not detectable after remission of the PRCA. [11] Similar findings have been reported in B-cell CLL, in which there seems to be a progressive increase in the marrow of T cells, which, when they reach a critical concentration, suppress erythropoiesis and cause red cell aplasia. [8] [104] [163]

Viral Infections and PRCA

Human B19 parvovirus, the agent responsible for exanthema infectiosum/fifth disease is the cause of acute PRCA (aplastic crisis) in children and young adults with chronic hemolytic anemias[48] [49] [50] [51] [52] [53] [54] [55] and of chronic PRCA in immunocompromised patients.[56] [57] [165] [166] [167] [168] [169] Human B19 parvovirus infection is not associated with TEC that affects hematologically normal children. [49] [170]

This DNA virus can infect erythroid cells by binding to the P antigen on erythroid cell membrane, [171] is directly cytotoxic to erythroid progenitors and inhibits preferentially normal CFU-E growth and development, but has no significant effects on myeloid cells. [49] [50] The effects of the virus on erythroid cells in vitro can be abolished by the presence of antibodies to the virus. [49]

APPROACH TO THE DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS OF ACQUIRED PRCA

In a patient with moderate to severe normochromic normocytic anemia with a low reticulocyte count, the diagnosis of PRCA is established by examination of the bone marrow. The peripheral blood smear is examined first for presence of red cells with abnormal morphology, monocytosis, leftward shift, bilobed granulocytes, or lymphocytosis with increased large granular lymphocytes. If there is any finding suspect of myelodysplasia, a sample of the bone marrow aspirate is submitted for cytogenetic studies. If there is a suspicion for a B- or T-cell lymphoid malignancy (B, T, or LGL CLL), the blood is submitted for immunophenotyping of lymphocytes by flow cytometry or for T-cell receptor gene rearrangement. The LGL type of CLL may be difficult to diagnose in early phases, when PRCA is the predominant hematologic abnormality. In the bone marrow, the almost complete absence of erythroblasts with normal myeloid and megakaryocytic cells exhibiting normal maturation is sufficient to confirm the diagnosis. The presence of a small number of large, vacuolated proerythroblasts frequently indicates active infection by B19 parvovirus. Special attention is paid during examination of the bone marrow smear to morphologic abnormalities suggesting possible myelodysplasia or an underlying primary bone marrow disorder. Erythroid hypoplasia with megaloblastoid changes in erythroid cells, mononuclear megakaryocytes, shift of the myeloid cell line to the left, hypogranular myelocytes, lymphocytosis, or increased mast cells are not common features of acquired PRCA, and other coexisting marrow disorders should be excluded by appropriate tests. High or low cellularity on bone marrow biopsy, presence of a small number of erythroblastic islands, and focal infiltration by lymphoid or immature cells are considered findings inconsistent with the diagnosis of primary acquired PRCA. Once the diagnosis is established, the clinical history may provide adequate information for the presence of a disease with which PRCA may be associated. If symptoms suggest an autoimmune disorder, appropriate testing is performed to confirm or rule out such a possibility. In all patients diagnosed with primary acquired PRCA, a computed tomography scan of the chest is obtained to rule out the presence of thymoma. In patients considered to be potentially immunocompromised, active B19 parvovirus infection should be excluded by obtaining IgM and IgG antibody titers for B19 parvovirus or using the polymerase chain reaction to detect the virus.

In normal people, infection by B19 parvovirus does not lead to symptomatic anemia because the arrest of erythropoiesis is transient and the survival of red cells normal. However, in the presence of hemolysis (short red cell survival), even transient arrest of erythropoiesis leads to an acute and precipitous decline of the hemoglobin concentration, resulting in clinically symptomatic anemia. Erythropoiesis is usually restored within 515 days with development of immunity to the virus and elimination of the viral infection. In immunocompromised patients, because of their inability to clear the virus, the infection becomes chronic and the arrest of erythropoiesis persists for as long as the infection is active, leading to a chronic form of PRCA. [56] [57] [165] [166] [167] [168] [169] In these patients, administration of high-dose, pooled human IgG provides adequate amounts of specific antibodies to eliminate the chronic infection, thus allowing the restoration

of erythropoiesis. In view of the primary therapeutic role of human IgG in chronic parvovirus infection, all immunocompromised patients with PRCA should be tested for evidence of active parvovirus infection.

Studies on the pathogenesis of PRCA in the course of viral hepatitis, infectious mononucleosis, and human T-lymphotropic virus type 1 infection have suggested that the suppression of erythropoiesis is mediated by cytotoxic T lymphocytes. [61] [64] [67] The pathogenesis of erythroid aplasia in the course of other infections has received limited attention and remains basically unknown.

Autoimmune Hemolytic Anemia and PRCA

It has been suggested that antibodies directed against red cells may in certain cases attack the erythroid precursors and/or progenitors, leading to erythroid aplasia.[105] This hypothesis was confirmed in two cases of autoimmune hemolytic anemia and PRCA in which the IgG antibody eluted from the red cells inhibited in vitro normal and autologous CFU-E growth. [1] [73] In another two cases, two separate autoantibodies, one directed against the erythrocytes and another one inhibiting autologous CFU-E growth in vitro, were detected.[106] [107]

Collagen Vascular Diseases and PRCA

A serum IgG inhibitor of autologous erythroid progenitor cell growth in vitro has been demonstrated in a case of systemic lupus erythematosus and in a case of rheumatoid arthritis.[74] [75]

Drug-Induced PRCA

Limited numbers of studies have addressed the pathogenesis of drug-induced PRCA. A serum IgG inhibitor was detected in a case of diphenylhydantoin-induced PRCA that inhibited erythroid but not myeloid cell growth in vitro in the presence but not in the absence of subtherapeutic concentrations of diphenylhydantoin. Diphenylhydantoin alone in the same concentration had no effect on autologous erythroid progenitor cell growth in vitro. [108] A similar mechanism was shown in a case of rifampicin-induced PRCA.[172] In both cases, the plasma inhibitor disappeared from the patients plasma after remission of PRCA. However, studies performed in cases of isoniazid- and procainamide-induced PRCA failed to demonstrate a similar mechanism. [112] [114] [173] It seems that various drugs can cause erythroid aplasia

through different mechanisms. Studies on direct drug effects on erythroid cell growth in vitro should be interpreted with caution because a large number of drugs may affect hematopoietic colony formation in vitro in a nonspecific way. The pathogenesis of acquired PRCA is summarized in Figure 21-1 . PRCA may result from either injury of erythroid progenitor cells in vivo by IgG antibodies, cytotoxic T lymphocytes, or parvovirus B19, or by an IgG antibody cytotoxic to mature erythroblasts, or an antibody to erythropoietin.

Figure 21-1 Different mechanisms by which erythropoiesis may be inhibited in pure red cell aplasia. *(From Krantz and Dessypris,[70] with permission.)*

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CLINICAL MANIFESTATIONS

Most adult patients present with symptoms of anemia that at the time of diagnosis may be quite severe. With complete arrest of erythropoiesis, the drop in red cell count averages approximately 1% a day, so the development of anemia is slow and progressive, allowing for physiologic compensatory changes. Children with CHA or TEC are most frequently diagnosed during a routine follow-up visit or a visit to the pediatrician for a febrile illness. Physical examination in primary PRCA is usually negative except for pallor and signs of anemia. Hepatosplenomegaly or lymphadenopathy are not findings consistent with primary PRCA. In secondary cases, physical findings consistent with the underlying disease may be present. In children with CHA, physical examination may reveal the presence of one or more congenital abnormalities described in association with this disorder. [174] [175] [176] In patients with long-standing red cell aplasia who have been supported chronically with red cell transfusions, chronic hepatitis or transfusional hemosiderosis may develop with physical findings secondary to iron overload.

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LABORATORY EVALUATION

In acquired PRCA, the erythrocytes are normochromic and normocytic, whereas in CHA they tend to be either normocytic or macrocytic with a mean corpuscular volume of 95 fl or higher.[4] [174] [175] [176] There is a complete absence of polychromatophilic red cells on the smear, and the reticulocyte count is between 0% and 1%. In CHA, the reticulocyte count may be occasionally as high as 5%. [4] [174] [175] [176] In adults, a reticulocyte count greater than 2% should raise serious doubt about the correctness of the diagnosis. The white cell count and the differential count are normal. Occasionally, mild leukopenia, lymphocytosis, or eosinophilia may be present. The platelet count is usually normal. Mild thrombocytopenia of 100,000150,000 platelets per microliter is occasionally seen, and a number of patients may have a thrombocytosis reactive to anemia.

The hallmark of PRCA is the absence of erythroblasts from an otherwise normal marrow. The cellularity of the marrow is normal or slightly increased. Marrow hypercellularity with elimination of fat spaces is not usually seen in PRCA. In classic cases, the erythroblasts are either totally absent, or they constitute <1% on the marrow differential count. In a small number of cases, a few proerythroblasts or basophilic erythroblasts may be seen, not exceeding 5% of the differential count. [101] [105] [146] [176] In TEC, but not in adult PRCA, such a finding may be a sign of early recovery that becomes apparent a few days later by a rise in the reticulocyte count. [174] The presence of a small number of proerythroblasts with vacuolated cytoplasm and pseudopod formation may raise the suspicion of an active B19 parvovirus infection or of severe malnutrition.[50] [85] In CHA presenting during the first few months of life, the erythroblastopenia may not be as severe as later in life, and erythroblasts, mainly proerythroblasts and basophilic erythroblasts, may constitute up to 10% of marrow cells. [176] In 510% of cases of CHA, erythroid hyperplasia with maturation arrest at the proerythroblast stage or megaloblastic changes have been described. [176]

In unusual cases, a period of ineffective erythropoiesis characterized by erythroid hyperplasia with maturation arrest at the stage of proerythroblasts or basophilic erythroblasts in the marrow and reticulocytopenia in the blood may precede the development of PRCA, develop during the course of PRCA, or develop after a partial response to treatment and before the return of erythropoiesis to normal. [177] [178] [179] Although this picture is not diagnostic of PRCA, it is a phase in the natural course of this disease, and in the absence of any dysplastic features in any of the three marrow cell lines or of any bone marrow karyotypic abnormality, it should raise the suspicion of developing PRCA,

and the marrow should be re-evaluated at a later time or the patient managed as a case of PRCA.

The myeloid cells and the megakaryocytes in the marrow are normal and exhibit full maturation. An increased number of lymphocytes on marrow smear, or on marrow biopsy, an increased number of lymphoid aggregates or a mild increase in plasma cells, eosinophils, or mast cells may be seen. [1] Iron stores are increased and normally distributed, but during recovery or the phase of ineffective erythropoiesis an occasional ring sideroblast may be noticed. [1]

Cytogenetic studies on marrow cells in PRCA reveal a normal karyotype with the exception of preleukemic cases, where chromosomal abnormalities may be detected.[13] [102] [150] The presence of an abnormal karyotype in a patient with morphologically classic PRCA carries a poor prognosis regarding response to treatment and may be a harbinger of leukemic transformation.[13] [102] [150] In CHA, chromosomal studies on peripheral blood lymphocytes in the presence of a DNA clastogenic agent (mitomycincardiepoxybutane) are normal and they clearly distinguish this disorder from Fanconis anemia. The demonstration in a patient with CHA of a translocation involving chromosome 19q has led to the identification of the CHA gene. [141]

Vitamin B_{12} , folic acid, ferritin, serum iron, and saturation of transferrin are normal or elevated. Serum erythropoietin levels are increased in proportion to the severity of the anemia.[145] [155] Ferrokinetic studies show a prolonged clearance of[59] Fe, no accumulation of iron in the sacral bone, and almost undetectable iron incorporation into red cells, findings consistent with an almost complete arrest of erythropoiesis. Ferrokinetic parameters return to normal after full hematologic recovery except in adult patients in partial remission, in whom ineffective erythropoiesis may be present. [179] Bone marrow imaging using[59] Fe or[111] In shows little or no uptake, but this returns to normal on remission. In both children and adults with PRCA, [51] Cr red cell survival is mildly shortened to a degree inadequate to explain the severity of the anemia.[175] [179] [180] Patients refractory to treatment who are supported by regular red cell transfusions may acquire a significant hemolytic component after alloimmunization or development of hypersplenism.[1]

In children with CHA, the erythrocytes maintain fetal-like characteristics, such as i antigen, increased fetal hemoglobin, and increased levels of enzymes in red cell glycolysis and the hexose monophosphate shunt pathways.[175] [176] [181] The distribution of fetal hemoglobin among the red cells is uneven and the ratio of glycine to alanine at position 136 of the -chain indicates that the hemoglobin is of fetal and not adult type. [175] The fetal-like erythropoiesis persists during phases of remission. [176] In addition, erythrocytes in CHA have elevated levels of adenosine deaminase (ADA), an enzyme not elevated in cord blood. [182] The increase in red cell ADA is not specific for CHA because it has been noted in cases of acute lymphoblastic leukemia, in some myeloproliferative disorders, in dyskeratosis with pancytopenia, and in megaloblastic anemia.[183] The fetal-like erythropoiesis and the elevated ADA activity are findings helpful in distinguishing CHA from TEC. During recovery from TEC, a transient phase of fetal-like erythropoiesis has been described, but ADA levels remain normal. [184] [185]

A variety of abnormalities of the immune system have been reported in patients with chronic PRCA, [1] including hypogammaglobulinemia, [162] [186] monoclonal gammopathies,[146] pyroproteins,[146] decreased complement,[187] antinuclear antibodies, [188] [189] decreased or increased B cells, and impaired phytohemagglutinin-induced lymphocyte cytotoxicity.[190] More recently, in a number of patients the presence of lymphocytes with the or T-cell receptor gene rearrangement has been described;[161] [191] [192] [193] [194] however, it is not yet clear whether these were cases associated with T-cell CLL or lymphoproliferative syndrome, frequently having very subtle morphologic abnormalities, [13] or cases of primary acquired PRCA. Immunologic abnormalities have also been described in children with CHA, suggesting that in this disorder the defect may not be restricted to the erythroid cells, but may include lymphocytes. [195]

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DIFFERENTIAL DIAGNOSIS

Pure red cell aplasia in adults can be easily diagnosed when isolated anemia with a normal white cell and platelet count is associated with a marrow of normal cellularity in which there is almost a complete absence of erythroblasts but normal myeloid cells and megakaryocytes. It can be easily differentiated from aplastic anemia, where pancytopenia is present and the marrow cellularity is severely decreased, with hypoplasia or aplasia of all three cell lines. The most frequent hematologic disorders from which PRCA has to be differentiated are refractory anemia/myelodysplastic syndromes. In myelodysplastic syndromes presenting with isolated anemia, the reticulocyte count is rarely <1%, the red cells tend to be slightly macrocytic, and there may be some white cell dysplasia. Careful examination of the blood smear may reveal the presence of monocytosis or Pelger-Huët anomaly. In addition, the marrow is hypercellular with dysplastic myelopoiesis, with a shift of the myeloid cells to the left and a possible increase in blasts and the presence of mononuclear megakaryocytes. Erythroblasts may be present in small numbers, but only rarely are they absent or comprise <5% of the marrow cells, and they usually exhibit megaloblastoid features. Cytogenetic abnormalities are frequent and their presence is helpful in differentiating preleukemic/myelodysplastic erythroid hypoplasia from primary autoimmune PRCA. [1] [13] [102] [150]

In children, TEC must be differentiated from CHA/Blackfan-Diamond syndrome. Table 21-3 presents the major clinical and laboratory findings useful in the differential diagnosis of these two types of erythroblastopenia.

TREATMENT

Acquired PRCA

Once the diagnosis of PRCA is confirmed by bone marrow examination, all drugs should be discontinued and any infection present be treated with the appropriate antibiotics. In the presence of laboratory findings indicative of an active or recent B19 parvovirus infection, administration of normal pooled serum

TABLE 21-3 -- Differential Diagnosis of Pure Red Cell Aplasia in Childhood

	Congenital Hypoplastic Anemia	Transient Erythroblastopenia
Pure red cell aplasia	Present	Present
Age	Younger than 1 y	Older than 1 y
Inheritance	Dominant or recessive	Not inherited
Congenital anomalies	Present	Absent
Mean corpuscular volume	Elevated	Normal
Fetal hemoglobin	Elevated	Normal
RBC i antigen	Present	Absent
RBC glycolytic and hexose monophosphate shunt exzymes	Elevated	Normal
RBC adenosine deaminase activity	Elevated	Normal

RBC, red blood cell.

All RBC characteristics except adenosine deaminase activity are helpful only when tested in a reticulocytopenic child. During recovery from transient erythroblastopenia of childhood, a transient wave of fetal-like erythropoiesis may be detected.

IgG provides specific antibodies capable of controlling the viral infection and allowing the recovery of erythropoiesis. [56] [57] [165] [166] [167] [168] [169] If laboratory evidence exists for B_{12} or folate deficiency, these vitamins should be given in adequate doses. In the presence of a malignant tumor, appropriate treatment should be instituted. PRCA associated with drugs or infection usually remits within 13 weeks after elimination of the responsible drug or treatment of the infection. [1]

In the presence of thymoma, thymectomy should be performed before the initiation of any immunosuppressive treatment. In approximately 3040% of patients with thymoma, erythropoiesis returns to normal within 48 weeks after thymectomy.[5] [6] [196] Patients not responding to thymectomy should be treated as having primary acquired PRCA. Removal of the thymoma seems to increase the effectiveness of immunosuppressive therapy.[6] Patients with severe contraindications to surgery or with malignant thymomas may benefit from chemotherapy or radiation.[196] [197] Thymectomy in the absence of thymoma is not recommended.[1]

For primary PRCA as well as secondary PRCA not responding to the treatment of the underlying disease, the therapeutic plan should focus on sequential use of various immunosuppressive therapies until remission is obtained. Corticosteroids are the immunosuppressive drugs of choice and should be tried before any other form of immunosuppressive treatment. Prednisone is administered orally at a dose of 1 mg/kg/day until a remission is induced. In approximately 40% of patients remission usually occurs within 4 weeks, so that continuation of treatment with prednisone longer than 12 weeks is not recommended. [13] [150] [198] The effect of treatment can be assessed by biweekly reticulocyte count and measurement of hemoglobin concentration. A rising reticulocyte count or stabilization of the hematocrit are the first laboratory findings indicating response to treatment. Once the hematocrit reaches 35%, the dose of prednisone can be tapered very slowly and the drug eventually can be discontinued, preferably after 34 months. Rapid tapering of prednisone can result in recurrence of anemia. A number of responders may be prednisone dependent, requiring small doses of the drug for maintaining a normal hematocrit. [199] [200] The dependence of the response on low-dose prednisone can be assessed during the period of slow tapering and the minimum dose required can easily be determined.

In patients not responding to prednisone after a trial of 23 months, the dose should be rapidly decreased to 2030 mg/day because responses seen after this period of treatment are extremely rare.[198] These patients should be considered candidates for an alternate immunosuppressive treatment, including administration of cyclophosphamide or azathioprine, cyclosporine, antithymocyte globulin (ATG), or high-dose -globulin. No data exist favoring one type of treatment over the other. The physician should take into consideration any coexisting systemic disease, the age of the patient, the potential short- and long-term side effects, and the cost of treatment. The advantage of cytotoxic agents is their low cost, but they have long-term potential leukemogenic and carcinogenic effects. [201] [202] [203] [204] Cyclosporine therapy is more expensive and requires monitoring of renal function, but it seems that the average time to induce remission is shorter than with cytotoxic drugs. In most cases, ATG administration requires hospitalization, which increases its cost significantly, but it lacks the leukemogenic potential of cytostatic agents. High-dose intravenous -globulin is the most expensive form of therapy, but lacks any significant side effects.

Cyclophosphamide or azathioprine may be given alone or, preferably, with small doses of prednisone, which seems to increase the effectiveness of treatment. These drugs are given orally at an initial dose of 50 mg/day. If the white blood and platelet counts allow it, the dose is increased by 50 mg weekly or biweekly to a maximum of 150 mg/day until

APPROACH TO THE TREATMENT OF ACQUIRED PRCA

After establishing the diagnosis, all medicines are discontinued and any infection present is treated appropriately. If no return of erythropoiesis occurs within 4 weeks, the patient is considered to have primary acquired PRCA. Patients with thymoma are treated by thymectomy and immunocompromised patients by high-dose intravenous IgG. Patients with PRCA and cytogenetic abnormalities or myelodysplastic features are treated on a trial-and-error basis with a course of corticosteroids. If they do not respond, as is usually the case, no further immunosuppressive therapy is given. Corticosteroids are the first choice of treatment for primary acquired disease. For PRCA associated with an indolent lymphoid malignancy, the combination of corticosteroids and a cytotoxic agent, cyclophosphamide or azathioprine, is preferred. In patients with PRCA not responding to initial therapy, immunosuppression continues by sequential use of low-dose corticosteroids combined with cyclosporine, ATG, high-dose intravenous IgG, cytotoxic agents, plasmapheresis, and, finally, splenectomy, followed, if necessary, by another round of immunosuppressive therapy. Plasmapheresis is used in those patients unresponsive to sequential immunosuppression in whom an IgG inhibitor of autologous erythroid progenitor cell growth in vitro can be demonstrated and in whom no evidence exists for T-cellmediated suppression of erythropoiesis. In primary PRCA, because of their potential leukemogenic and carcinogenic effects, the use of cytotoxic agents is avoided until other means of immunosuppression are found ineffective. Patients are followed biweekly by a reticulocyte count and a complete blood cell count, and are instructed to return to the clinic with any symptom suggestive of an infection. Relapses are treated with the same regimen that induced the first remission. After a second relapse, patients are given maintenance therapy for a period of 2 years. Patients refractory to all forms of treatment receive regular red cell transfusions and iron chelation therapy with deferoxamine, which is usually initiated after the number of transfused red cell units exceeds 50.

remission occurs or bone marrow toxicity develops. The mean time to response is approximately 1112 weeks, with a broad range of 226 weeks and an overall response rate of 4060%.[13] [145] [146] [147] [150] [155] [198] [205] [206] If response occurs, prednisone is tapered and then the dose of cytotoxic agent is progressively decreased and eventually discontinued. If bone marrow toxicity develops, the drug is discontinued and the marrow is allowed to recover. If, after 3 months of treatment, no response and no marrow toxicity is seen, the dose may be increased progressively (by 50 mg biweekly) to a maximum tolerated or to a maximum of 250 mg/day under close monitoring of the white blood cell and platelet count. If reticulocytosis or stabilization of the hematocrit is noticed, the dose is gradually reduced. If the absolute neutrophil count decreases below 1,000/l or the platelet count drops below 100,000/l, the cytotoxic drug is discontinued. In a number of cases, with the return of granulocytes and platelets to normal, a reticulocytosis may be seen followed by a return of erythropoiesis to normal (Fig. 21-2). If no response occurs, another type of immunosuppressive treatment should be initiated.

Cyclosporine is frequently used as a second or third line treatment at a dose of 1012 mg/kg/day in two divided doses,

Figure 21-2 Response of pure red cell aplasia to cyclophosphamide and prednisone simultaneous with the appearance of marrow toxicity. This 38-year-old man did not respond to initial treatment with prednisone. Cyclophosphamide was added and its dose gradually increased until leukopenia developed (white blood cell count = 2,000/l) when reticulocytes first appeared. The dose of cyclophosphamide was reduced. The patient maintained a normal hematocrit despite phlebotomies initiated for reduction of transfusional iron overload. *(From Krantz SB: Implications of studies on pure red cell aplasia for the study of aplastic anemia. In Hibino S [ed]: Aplastic Anemia. University Park Press, Baltimore, 1978, p. 305, with permission.)*

preferably with 2030 mg of prednisone daily, with monitoring of renal function. Remission occurs usually at an average time of 24 weeks. A response to cyclosporine is seen in 6580% of patients receiving this drug as a second- or third-line treatment. [1] [13] [150] [207] [208] [209] [210] [211] [212] [213] Once remission occurs, the dose of prednisone is tapered, followed by tapering of the dose of cyclosporine. If no remission occurs after 34 months of treatment with cyclosporine, the drug should be discontinued and an alternate mode of immunosuppression initiated.

Antithymocyte -globulin is used as another therapeutic modality, usually in patients who have failed other means of immunosuppression. It is given as an intravenous infusion at a dose of 20 mg of horse IgG per kilogram of body weight daily for 7 days, preferably in combination with 2030 mg prednisone orally per day that is tapered within 2 weeks after completion of ATG therapy. The overall response rate to ATG seems to be approximately 50%. [13] [46] [150] [187] [198] [214] [215]

High-dose intravenous -globulin is the treatment of choice for immunosuppressed patients with PRCA in whom the disease may be the result of infection with B19 parvovirus.[56] [57] [165] [166] [167] [168] [169] Responses have been reported, however, in nonimmunosuppressed patients with primary as well as various secondary types of PRCA.[9] [216] [217] [218]

Plasmapheresis is considered in patients having failed all the previously mentioned immunosuppressive therapies. [152] [219] [220] [221] [222] [223] It is performed at least thrice weekly for a minimum of 23 weeks, and occasionally much longer until a response is noted. Considering the fact that IgG is distributed in both intravascular and extravascular space, plasmapheresis should be performed intensively and for a long period of time before any response is seen.

Splenectomy is considered as a final therapeutic maneuver in patients refractory to all other forms of treatment. Responses to splenectomy have been reported in approximately 17% of such cases recalcitrant to treatment within the first 23 postoperative months. [1] [69] [105] [198] [224] [225] Nonresponders to splenectomy should receive another trial of immunosuppression following the same approach used for initial therapy of PRCA. A number of refractory cases have been reported to have responded to immunosuppressive therapy after splenectomy.[69] [198] [226] Sequential, prolonged immunosuppressive therapy should be administered carefully because, although it induces remission in two thirds of patients, it is associated with a high frequency of infection in one third of patients. [198]

Patients unresponsive to all forms of treatment should be maintained on regular red cell transfusions. They usually require approximately 1 unit of packed red cells per week. Because of the frequency of red cell transfusions, after a period of time iron overload, with its detrimental effects on various organs, develops in these patients. Thus, in this patient population institution of iron chelation with deferoxamine should be considered within the first 6 months after the disease is declared refractory to treatment.[1] [227]

Evaluation of the relative effectiveness of each immunosuppressive treatment of PRCA is very difficult because of the infrequency of the disease and its unpredictable clinical course, with spontaneous remissions occurring in approximately 510% of cases of primary PRCA within 4 months to 14 years after the diagnosis. [1] [13] [150] [198] In addition, there is no clinical or laboratory criterion other than observation by which to distinguish between chronic and acute disease. A number of cases treated immediately after diagnosis may represent acute and self-limited PRCA secondary to a viral infection or drugs that the patient did not mention. Comparative studies of different immunosuppressive modalities are not available. The experience with various forms of treatment of 133 patients at three different institutions is presented in Table 21-4 .

Congenital Hypoplastic Anemia

After excluding TEC as the cause of anemia and establishing the diagnosis of CHA, patients should be treated with prednisone

TABLE 21-4 -- Response of Pure Red Cell Aplasia to Various Immunosuppressive Therapies

Study	Dessypris[1]	Lacy et al.[13]	Charles et al. [150]	Total
Number of patients	49	47	37	133
Primary PRCA	32	25	18	75
Secondary PRCA	17	22	19	58
Corticosteroids	18/41[a]	9/29	9/36	36/106 (34%)
Cytotoxic agents[b]	24/54	14/29	8/27	46/110 (42%)
Antithymocyte globulin	2/6	0/1	8/12	10/19 (53%)
Cyclosporine	3/4	4/5	2/3	9/12 (70%)
Intravenous IgG		5/10	2/8	3/10 (30%)
Plasmapheresis			0/2	
Splenectomy	4/23	0/1	0/1	4/25 (16%)
Multiple treatments	35/49	28/47	28/37	91/133 (68%)

Many patients did not respond to treatment or had relapses, so one patient may be included in more than one treatment modality.

[a] Number of responders/number of patients treated.
[b] Includes cyclophosphamide, azathioprine, or methotrexate given alone or in combination with prednisone.

at a dose of 2 mg/kg in three divided doses per day. Treatment continues until the hemoglobin concentration reaches 10 g/dl, at which point the dose is gradually decreased to a single small dose per day, followed by an attempt to switch to an alternate-day schedule. Approximately 70% of patients respond to corticosteroids with reticulocytosis and an increase in the hemoglobin concentration, usually within 46 weeks; however, the pattern of response may vary from patient to patient. [175] [176] A number of patients respond with a rapid rise of the hematocrit and enter a steroid-independent remission, another group shows only intermittent response, others respond but become dependent on low- or high-dose corticosteroids to maintain their remission, and others respond to initial treatment but later acquire a steroid-resistant disease. Approximately 3040% of patients do not respond to steroids. A trial of treatment with high-dose prednisone (46 mg/kg/day) should be given before a patient is declared unresponsive to corticosteroids. Responses seem to be more frequent in patients in whom treatment is initiated a short period after diagnosis who have been transfused to a limited extent. [228] Approximately 25% of patients with corticosteroid-resistant disease may respond to high-dose intravenous methylprednisolone. [229] In contrast to acquired PRCA, CHA is rarely responsive to immunosuppressive therapy. Only isolated cases have been reported in which a response was seen after 6-mercaptopurine, cyclophosphamide, ATG, or plasmapheresis. [129] [230] [231] [232] [233] [234] Treatment with recombinant human erythropoietin has not induced remissions. [235] Treatment with interleukin-3 may induce a response in 25% of patients. [236] Patients with refractory disease may be considered candidates for HLA-matched allogeneic bone marrow transplantation. [237] [238] [239] Patients refractory to all forms of treatment should be maintained on regular red cell transfusions and chronic iron chelation therapy with deferoxamine.

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PROGNOSIS

With sequential immunosuppressive therapy, approximately 68% of patients enter remission. In addition, approximately 510% of patients may remit spontaneously. Relapses are not uncommon, and in one study 80% of patients were found to be at risk for relapse during the 24 months after remission. [198] However, relapses are treated as successfully as the initial disease. [150] [198] A number of patients may require low-dose maintenance immunosuppressive therapy, which, if deemed necessary, should be continued for 12 years. [1] [75] [150] [199] [200] Even after repeated relapses, approximately 60% of patients become transfusion independent for many years. The median survival of patients with primary acquired PRCA has been estimated to be approximately 14 years. [198] Overall, patients with bone marrow cytogenetic abnormalities or low growth of erythroid progenitor cells in vitro have a much worse prognosis because of very low or no response to immunosuppressive agents, and in these patients the persistent use of sequential immunosuppressive therapies is not indicated. [1] [13] [28] [150] [198] Evolution of PRCA into aplastic anemia has been described, but is exceedingly rare. [6] [180] Approximately 35% of patients with refractory disease will develop acute nonlymphocytic leukemia. [1] [151] [198] [240] The prognosis of patients with secondary PRCA depends on the nature of their primary disease.

In CHA, the use of corticosteroids induces a remission in approximately half of the patients , 80% of whom are expected to reach the age of 50 years. Patients unresponsive to corticosteroids and maintained on chronic red cell transfusions and iron chelation therapy may have a shorter long-term survival, primarily related to iron overload over many decades. The overall median survival among 436 patients reported during the period 19361990 was estimated to be 38 years. [176] Acute nonlymphoblastic leukemia and development of myelodysplastic syndrome have been reported in no more than 2% of cases. [176]

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Chapter 22 - Pathobiology of the Human Erythrocyte and Its Hemoglobins

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INTRODUCTION/OVERVIEW

The anemias, polycythemias, and functional derangements of the human erythrocyte together represent a particularly important group of human disorders. The relevance of these conditions to general medicine extends beyond their individual clinical severities or the number of patients affected. A critical added dimension of erythrocyte disorders is the extraordinarily detailed knowledge available about the basic biochemistry, physiology, and molecular biology of the human red cell and its membrane, enzymes, and its major component, hemoglobin. Red cells are especially abundant, relatively simple, and readily accessible for repeated testing in individual patients. These features have facilitated rapid application of the techniques of cellular and molecular biology to studies of the red cell, its component molecules and structures, and syndromes resulting from abnormalities of these entities. Thus, taken as a group, erythrocyte disorders are better understood at the molecular and cellular level than disorders of any other cell or tissue. It is for this reason that these conditions merit particularly thorough and careful scrutiny by students of hematology.

This chapter attempts to introduce concepts about normal red cell homeostasis that form the essential knowledge base for understanding anemias, polycythemias, and functional erythrocyte disorders. The primary focus and object for detailed discussion of this chapter is hemoglobin, the major component, both quantitatively and qualitatively, of the erythrocyte. Hemoglobin molecules dominate the pathophysiology of many red cell disorders and modulate most of the others, in part because of their sheer quantitative predominance in red cell cytoplasm. The other major relevant aspects of human red cellsthe red cell membrane, red cell enzymes used for intermediary metabolism, red cell differentiation and development, and the process of red cell destructionare discussed in detail in the introductory portions of other chapters. This chapter shall survey these areas only briefly. The reader can find detailed descriptions of the red cell membrane in Chapter 4 and Chapter 33 . Red cell enzymes and enzymopathies are described in Chapter 32 ; red cell differentiation and development in Chapter 15 ; regulation of red cell mass by erythropoietin in Chapter 25 ; and the necessary aspects of red cell destruction in Chapters 34 and 35 .

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ESSENTIAL FEATURES OF RED CELL HOMEOSTASIS

As discussed in considerable detail in Chapter 15 , the mature red cell is the product of a complex and orderly set of differentiation and maturation steps beginning with the pluripotent stem cell. By incompletely understood mechanisms involving hierarchic networks of cytokines, a portion of these cells becomes committed to differentiate along the erythroid pathway. Commitment provokes a progressively increasing sensitivity to the stimulatory actions of the hormone erythropoietin as differentiation proceeds, and also involves preprogramming of certain genes whose expression at high levels will be required during the maturation phase of erythropoiesis. Genes coding for molecules defining the red cell phenotype (e.g., globin) are thus poised for activation at later maturation steps.

Intermediate progenitor cells arising during differentiation have been characterized experimentally, including the burst-forming unit erythroid (BFU-E) and the colony-forming unit erythroid (CFU-E) stages. BFU-Es are progenitor cells that produce bursts or clusters of erythroid colonies, are relatively less sensitive to erythropoietin, and are more plastic with respect to important gene expression parameters, such as the synthesis of adult or fetal hemoglobin by their descendants. CFU-Es produce single colonies, exhibit considerably higher sensitivity to erythropoietin, and appear to be more fixed in their potential to express genes, at least among the globin family. As discussed in Chapter 15 , CFU-Es appear to give rise to the first morphologically recognizable erythroid cells, the proerythroblasts. At this primitive morphologic stage, the program of erythroid cell expression has already been essentially predetermined, and the cell is predestined to undergo only a limited additional number of cell divisions, culminating in formation of the enucleate reticulocyte. The terminal maturation stages are morphologically recognizable as erythroblasts exhibiting progressive hemoglobinization of the cytoplasm, condensation and eventual ejection of the nucleus, and remodeling of the plasma membrane. Actual expression of the preprogrammed genes occurs during the 5- to 7-day period of erythroblast maturation.

As discussed in Chapter 15 , both the actual reconfiguration of chromatin for activation of the genes and activation itself appear to require the concerted and complex interaction of a diverse but limited group of transcription factors. These recognize a specific array of promoter and enhancer sequences that are embedded as recurrent motifs in and around the appropriate target genes. Even though an enormous amount of information has been gathered about sequences such as the GATA enhancers and their cognate transcription factors, the precise means by which these sequences and factors cause erythroid differentiation remains largely mysterious. At this time, this information is of limited clinical relevance to anemias or polycythemias; thus, we shall not consider these aspects in additional detail in this chapter. The orderly 14- to 21-day sequence of differentiation and maturation becomes progressively influenced by the levels of erythropoietin available to the progenitor cells, possibly due to increasing density and affinity of erythropoietin receptors on their cell surfaces.

Within 24 hours after enucleation, the reticulocyte is released into the circulation as an immature erythrocyte, the reticulocyte. These cells retain remnants of nucleated precursors in the form of a relatively small number of polyribosomes actively translating messenger RNA (>90% of which is globin messenger RNA), a cell membrane that retains some molecules and structures reminiscent of its earlier stages of differentiation, and the only complement of enzymes, phospholipids, and cytoskeletal proteins that the cell will possess throughout its remaining life span.

During its first 24 hours in the circulation, the reticulocyte spends considerable amounts of time in the spleen, during which its membrane is polished. This is a poorly understood remodeling process by which some proteins, including adhesive molecules such as fibronectin, are removed. The content of polyribosomes and other nucleic acids progressively declines

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so that stainability with methylene blue, until recently the standard method used for clinical enumeration of reticulocytes, is lost by the end of the first day. At this time, the red cell is regarded as a mature erythrocyte, and it circulates largely unchanged for the remainder of its 120-day life span in the circulation.

Perhaps the most remarkable feature of the human red cell is its durability, given that it is an enucleate cell devoid of organelles that appear to be critical for the survival and function of most other cell types. Thus, the red cell has no mitochondria available for efficient oxidative metabolism, no ribosomes for regeneration of lost or damaged proteins, a very limited metabolic repertoire that largely precludes de novo synthesis of lipids, and no nucleus to direct regenerative processes, adaptation to circulatory stresses, or cell division to replenish itself. The 120-day survival of these cells is even more striking when one considers that, given these handicaps, erythrocytes live their life span in an exceedingly hostile environment. Mechanical stresses of the circulation include high hydrostatic pressure and turbulence and the shear stresses inherent in a microcirculation networked with many capillaries having diameters only one-third to one-half that of the normal red cell; biochemical stresses include osmotic and redox fluxes associated with travel through the collecting system of the kidney, the sluggish vascular beds of the spleen, muscle, and bone, and the rapid changes in ambient oxygen pressures occurring in the lungs. All conspire to damage red cells. Their 4-month survival is thus truly remarkable.

The ability of the red cell to persist in the circulation depends on its simple but exquisitely adaptive membrane structures, its pathways of intermediary energy metabolism and redox regulation, and its ability to maintain its largest cytoplasmic component, hemoglobin, in a soluble and nonoxidized state. Indeed, the membrane and enzymes of the red cell appear to be exquisitely crafted to protect the cell from not only the external ravages of the circulation but also the potential internal assaults of the massive amount of iron-rich and potentially oxidizing protein represented by its complement of hemoglobin molecules. For these reasons, a few basic features of these membrane and enzyme systems merit comment before considering the hemoglobin molecule itself.

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MAJOR FEATURES OF THE RED CELL MEMBRANE

Chapter 4 describes the red cell membrane in considerable detail, using it as a model for understanding membrane structure in general. Only a few major aspects of that discussion bear repeating for the purposes of this chapter. See also Figure 4-2 , which shows a schematic diagram of the red cell membrane organization, and will facilitate understanding of the discussion in this section. The red cell membrane and its underlying cytoskeleton have evolved to provide both mechanical strength and the necessary pliability and resilience to withstand the mechanical, osmotic, and chemical stresses of the circulation. Since the lipid bilayer membrane has the physical properties, essentially, of a soap bubble, it would rapidly be emulsified in the circulation. Strength and order are provided to the lipid bilayer by the hexagonal arrays of the highly helical protein spectrin, which forms a latticework underlying the membrane.

As shown in Figure 4-2 , this spectrin meshwork is held together by adaptor molecules, such as protein 4.1, adducin, p55, and ankyrin, arrayed at defined points along the highly coiled, rodlike structure of the spectrin oligomers. These proteinprotein interactions appear to be critical for holding the latticework together in what has been described as the horizontal dimension that permits resistance to shear stress (see Chap. 33). The involvement of intermediate-length actin fibers and the variability of binding affinities by phosphorylation state appear to provide some flexibility and pliability at these points of interaction. Strength in the vertical dimension is provided by additional molecules, or additional binding functions of the same molecule, whereby the latticework is attached to the lipid bilayer. For the most part, the physiologically important attachments appear to be indirect. Linkage is mediated through the interaction of the adaptor proteins, such as ankyrin and protein 4.1, with the cytoplasmic domains of abundant transmembrane proteins. These traverse and are embedded in the lipid bilayer, providing a firm anchor. The two most critical of these molecules appear to be band 3 (the anion transport channel) and a glycophorin, probably glycophorin C/D. The construction of these attachments by multiple hinge or coupling molecules appears to provide for the flexibility and distensibility of the red cell membrane, a property essential to its ability to flow through small capillaries.

As described in Chapters 30 and 33 , the complex structure of the red cell membrane is exquisitely sensitive to perturbations impinging on any of its components. In particular, the membrane cytoskeleton and phospholipid structures are each highly susceptible to oxidation, particularly by partially proteolized molecules of hemoglobin, which denature to form highly toxic compounds called hemipyrroles. This interaction of denatured hemoglobin with the red cell membrane is clinically important, as illustrated by its impact on the pathophysiology of sickle cell anemia (see Chap. 30), or of oxidized and precipitated globin inclusion bodies in thalassemia (see Chap. 29). For present purposes it is sufficient to note that alterations of proteins of the red cell membrane can contribute to shortening of the red cell life span. Damage may result from direct defects in the cytoskeletal proteins themselves or from susceptibility of these proteins to direct oxidation or attack by oxidized or denatured hemoglobin molecules. The reader is referred to the aforementioned chapters for detailed descriptions of the relevant phenomena.

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ENZYMES OF RED CELL INTERMEDIARY METABOLISM

Mammalian erythrocytes possess the highly specialized yet remarkably simplified set of metabolic pathways. As discussed in Chapter 32 and illustrated in Figure 32-1 , there are essentially three relevant sets of pathways. The first two are interconnected by the enzyme glucose-6-phosphate dehydrogenase (G6PD). Glucose entering the red cell is metabolized by an anaerobic pathway, the Embden-Meyerhof pathway, which terminates with the enzyme lactic dehydrogenase, forming lactate. Despite its inefficiency (a net of only 2 adenosine triphosphate [ATP]/glucose molecule), this pathway is the sole source of usable ATP in the cell. Moreover, the pathway generates reduced NADH, a molecule necessary for driving the reduction of methemoglobin to hemoglobin (see Chap. 32). A shunt within this pathway, the Rapopport-Luebreing shunt, generates the compound 2,3-bis(phosphoglyceric acid) (2,3-BPG), an important cofactor that, when bound to hemoglobin, reduces the affinity of hemoglobin for oxygen, as described later. The ATP generated is necessary for kinase reactions controlling phosphorylation of membrane and signaling components, for fueling ion pumps and channels, and for maintaining phospholipid levels.

The anaerobic metabolic pathway generates, as one of its intermediates, glucose-6 phosphate, which is the substrate for G6PD. G6PD, in turn, appears to be the rate-limiting enzyme for a linked pathway called the oxidative hexosemonophosphate shunt. As shown in Figure 32-1 , this pathway involves a cascade of reactions culminating in the reduction of oxidized glutathione to reduced glutathione. Reduced glutathione is utilized to reverse oxidation of critical structures, including hemoglobin, cytoskeletal proteins, and membrane lipids. Anaerobic glycolysis generates NADH for methemoglobin reduction, 2,3-BPG for modulation of hemoglobin oxygen affinity, and ATP for metabolic

energy requirements. Its end product is lactate. The oxidative glycolysis pathway generates NADPH and reduced glutathione for use as the major erythrocyte antioxidant.

The third set of red cell enzymes is involved in interconversions that maintain a balanced nucleotide pool within the erythrocyte. This pool is essential for the synthesis of ATP and other high-energy nucleotides.

During the past decade, most of the enzymes (or at least the erythroid isoforms of these enzymes) involved in red cell intermediary metabolism have been characterized at the molecular level by cloning of their cDNAs and/or genomic loci. Some of the more relevant information arising from this progress is discussed in Chapter 32 . For our purposes, however, a discussion of this information is not appropriate.

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RED CELL SENESCENCE AND DESTRUCTION

As discussed in several of the chapters already mentioned, erythrocytes, despite their impressive adaptations to circulatory stresses, eventually wear out and are destroyed. Red cell survival in humans appears to be remarkably uniform under normal circumstances, spanning 115120 days from release of the reticulocyte into the circulation to sequestration of the senescent red cell in the reticuloendothelial cells of the liver and spleen. The precise signal, or signals, marking red cells for destruction remain unknown, as does the underlying pathophysiology within the red cell or on its surface. However, several interrelated theories have emerged; these will be noted only briefly because they are mentioned in other chapters.

It is well established that red cells accumulate surface blemishes during their lives in the circulation. These appear to result in part from the accumulation of small amounts of oxygen damage to membrane structures. The altered regions are sensed by the reticuloendothelial (RE) cells during passage of the erythrocytes through the liver and spleen. Removal or pitting of these damaged regions from red cell membranes can be documented microscopically; small amounts of normal membrane are also lost during the process.

As noted in other chapters, the biconcave disk shape of the red cell, so important to its distensibility, depends on a high ratio of surface area to surface volume. This requires redundant membrane surface area. Thus, the membrane surface area of the normal biconcave disk is about 140 microns ^{2} ; to enclose a sphere containing a normal red cell volume (about 90 fl), only about 95 microns ^{2} would be needed. Progressive loss of membrane surface via the pitting phenomenon should ultimately cause the aging erythrocyte to assume a more rigid spherical shape that would inevitably be far less distensible and far less capable of passing through small apertures, especially in the sluggish and torturous circulation of the spleen. This geometric mechanism might, then, lead to the eventual destruction of the red cell.

It is also well documented that red cells progressively lose some of the critical enzymes needed for intermediary metabolism and antioxidant capacity. G6PD levels, for example, progressively decline during the circulating life span, as do levels of several other enzymes. Indeed, the decline of certain red cell enzymes can be used as a crude means of estimating the relative age of different red cell populations. The biochemical or oxidative mechanism for red cell destruction postulates that aged red cells are eventually depleted of critical enzymes needed for maintenance of redox status. Oxidation of critical membrane proteins, lipids, and hemoglobin would then ensue, causing distortion and rigidity of the red cell membrane, with accelerated loss as described above. The end product, once again, would be spherocytes incapable of traversing the splenic vascular bed and escaping engulfment by the RE cell.

More recently, it has been proposed that an immune mechanism may contribute to normal and pathologic red cell senescence. This hypothesis is based on the observation that oxygen damage, regardless of cause, promotes a clustering or capping of oligomers of band 3 on the red cell surface. Under normal circumstances, band 3 molecules form monomers, dimers, or tetramers. Higher-order aggregates appear to be recognized by an endogenous iso-antibody possessed by all of us. Any red cell accumulating oxidative damage, from wear and tear in the circulation, from depletion of enzymes, or from internal pathologic processes, such as denaturation of hemoglobin in sickle cell disease or unstable hemoglobin disorders, might accumulate these aggregates, be bound by antibody, and be removed by the RE cells as antigenantibody complexes, employing the same means used by RE cells to recognize any immune complex. This mechanism could also provide for the pitting or polishing of damaged red cell membranes mentioned earlier. Note that all three of the proposed mechanisms are interrelated by their inception with oxidative damage.

Regardless of the mechanism fostering eventual senescence and destruction of red cells, the process itself involves components clinically useful for assessment of anemias associated with accelerated red cell destruction. Chief among these is the generation of indirect or unconjugated bilirubin, the byproduct of heme catabolism occurring within the RE cells. In markedly accelerated states of red cell destruction, hypertrophy of the liver and spleen can also occur, providing a useful physical indicator of hemolytic anemia. Indeed, these indirect clinical features, coupled with the reticulocyte count, remain more useful for detecting clinical hemolysis than complicated studies of red cell kinetics.

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HEMOGLOBIN SYNTHESIS, STRUCTURE, AND FUNCTION

Basic Features

Hemoglobins are the major oxygen-carrying pigments of the body. They are packaged into red cells in quantities sufficient to carry enough oxygen from the lungs to the tissues to meet the needs of those cells for oxidative metabolism. These quantities are enormousnearly two pounds of hemoglobin is present in the body of a reasonably sized man at any given time. Since free hemoglobin in the bloodstream is catabolized and excreted renally in a matter of minutes, packaging in erythrocytes is essential to preserve the newly synthesized molecules for the entire 4-month life span of the red cell. Otherwise, the caloric and biosynthetic resources needed to replace daily losses of hemoglobin would be prohibitive. The red cells major function is to encase hemoglobin and protect it so it can function as an oxygen transporter for a prolonged period.

The cellular content of blood influences its viscosity; in particular, hemodynamics are adversely compromised by the presence of too many circulating erythrocytes, because blood viscosity correlates especially with hematocrit. To provide for adequate oxygen transport (i.e., enough hemoglobin molecules) in a number of red cells compatible with tolerable viscosity, each red cell must enclose a high concentration of hemoglobin (about 3234 gr per 100 ml cytoplasm). This concentration is close to the solubility limit of hemoglobin in physiologic solutions. It follows that even minor perturbations within these molecules (e.g., oxidation) or in the milieu (e.g., changes in pH or ionic strength) can have potentially devastating effects on the solubility of hemoglobin. Since polymerized or precipitated hemoglobins derange intracellular viscosity, trigger proteolytic reactions that lead to oxidative damage of erythrocytes (see Chap. 31), and compromise oxygen transport, it is hardly surprising that the fate of the red cell is inextricably interwoven with the state of its enormous complement of hemoglobin molecules.

Hemoglobin Structure

The hemoglobin tetramer consists of two pairs of unlike globin polypeptide chains, each associated with a heme group. The behavior of hemoglobin is determined by its primary structure, the covalent linking of amino acids to form the polypeptide globin. The higher-order structures of hemoglobin are dependent on the sequence of amino acid residues that make up the globin chain. The -globin chains contain 141 residues, while the -like chains are 146 amino acids long (Fig. 22-1). There is considerable homology among these globins, especially among the non- chains. While the -globin chains result from a very ancient gene duplication, the non--globin chains are the result of more recent gene duplications and thus are more akin to each other than they are to the -like globin genes. Gene conversion events also ensure the similarity of duplicated genes.

Elements of the secondary structure of globin are shown in Figure 22-3 (Figure Not Available) . About 75% of the globin polypeptide chain forms an helix. There are eight helical segments, A through H, separated by short stretches from which the helix is absent. These nonhelical segments permit folding of the polypeptide upon itself and are often dictated by the presence of prolyl residues, which are generally unable to participate in the formation of helices. While the helical segments of the and non- globin chains do not exactly correspond, it is possible to align amino

Figure 22-1 The -globin chain, showing helical and nonhelical segments. The helical segments are labeled AH, while nonhelical segments are designated NA for those residues between the N terminus and the A helix, CD for residues between the C and D helices, and so forth. *(From Huisman THJ, Schroeder WA: New Aspects of the Structure, Function, and Synthesis of Hemoglobin. CRC Press, Boca Raton, FL, 1971, with permission.)*

Figure 22-2 Tertiary structure of a globin chain. Globin folds into a tertiary structure such that polar or charged amino acids are located on the exterior of the molecule and the heme ring resides in a hydrophobic niche between the E and F helices. Linked to the heme are the proximal (F8) histidine and the distal (E7) histidine. *(From Perutz MF: Molecular anatomy, physiology, and pathology of hemoglobin. In Stammatoyannopoulos G, Neinhuis AW, Leder P, Majerus PW (eds): The Molecular Basis of Blood Diseases. WB Saunders, Philadelphia, 1987, p. 127, with permission.)*

acid residues in all globin peptides by their helical and nonhelical residue number, as indicated in Figure 22-3 (Figure Not Available) . This permits greater appreciation of the homology among globins. Some of the amino acids of globin are invariant, or conserved, in the sense that they are preserved during phylogeny. These residues occur at portions of the molecule that are critical for its stability and functionfor example, heme binding residues, hydrophobic amino acids of the interior of the molecule, and certain subunit contacts at the $_{1\,2}$ interface. The introduction of prolyl residues into helical segments by mutation leads to interruption of the helix and instability of the resulting hemoglobin molecule.

The poorly understood laws that govern the folding of proteins are responsible for the tertiary structure of globin, shown in Figure 22-2 . This folding pattern places polar residues exteriorly and provides a hydrophobic niche for the heme ring between the E and F helices. Numerous noncovalent bonds are formed between the heme and surrounding amino acid residues of globin. An iron atom in the center of the porphyrin ring forms an important bond with the F8 or proximal histidine and via the linked oxygen with the E7 or distal histidine residue. Oxygenation and deoxygenation of hemoglobin occur at the heme iron.

Two -globin chains and two non--globin chains fit together specifically to form a hemoglobin tetramer with a molecular weight of about 64,000 and with the quaternary structure shown in Figure 22-3 (Figure Not Available) . The motion of individual globin chains, as well as the movement of globin chains relative to each other during oxygenation and deoxygenation, gives hemoglobin its unique utility as a respiratory protein.

Figure 22-3 (Figure Not Available) Quaternary structure of hemoglobin. The contacts between subunits are shown as circled amino acids. In the front view **(A)** $_{1\,2}$ contacts are shown, while in the side view **(B)** $_{1\,1}$ contacts are depicted. *(From Dickerson RE, Geis I: Hemoglobin: Structure, Function, and Evolution Pathology. Benjamin-Cummings, Menlo Park, CA, 1983, with permission. Copyright Irving Geis.)*

Hemoglobin Function

Evolution has honed the hemoglobin tetramer into a molecule ideally suited for its tasks. Since human hemoglobin must behave differently from that of altitude

dwellers or inhabitants of hypoxic locales, many different variants of the same basic molecular design have evolved. Owing to the exigencies of molecular evolution, we can find in the genome of all animals, including humans, attempts of nature to propagate a variety of different globin genes. The crystallographic studies of Perutz and coworkers have defined the oxygenated and deoxygenated structures of hemoglobin at ängstrom unit resolution and have provided an exquisitely detailed picture of how the globin chains and individual amino acid residues respond to the loading and unloading of oxygen. Thus, we know more about the function of hemoglobin than about virtually any other protein, and our knowledge of this mechanism provides a beautiful and intellectually satisfying culmination to decades of study by many investigators.

The oxygen dissociation curve of hemoglobin, shown in Figure 22-4 (Figure Not Available) , describes the percent saturation of hemoglobin with oxygen at different oxygen tensions. The sigmoidal shape of this curve is a result of interaction among the subunits of hemoglobin. Communication within the tetramer is called hemeheme interaction or cooperativity. This implies that the four heme groups do not undergo simultaneous oxygenation or deoxygenation, but rather that the state of each individual heme unit with regard to the presence or absence of bound oxygen influences the binding of oxygen to other heme groups. Myoglobin, a heme-containing protein with virtually the same tertiary structure as globin, exists in muscle as a monomer. The oxygen equilibrium curve of myoglobin is a rectangular hyperbola; in physiologic terms, it rapidly becomes fully saturated at low oxygen tensions and remains saturated as the oxygen tension plateaus. The difference in the oxygen equilibrium curves of myoglobin and hemoglobin lies in the tetrameric nature of the hemoglobin molecule and the cooperativity permitted by the association of similar but unlike subunits. Myoglobin, as compared with hemoglobin, has a very low P_{50} (the oxygen partial pressure at which the molecule is half saturated). It therefore has an extremely high oxygen affinity and would not be useful for delivering oxygen to tissues. The oxygen in myoglobin is passed on to the mitochondria, where oxidative metabolism occurs. The sigmoidal shape of the oxygen dissociation curve of hemoglobin indicates that the totally deoxygenated hemoglobin tetramer is slow to become oxygenated, but as oxygenation proceeds, the reaction of heme with oxygen accelerates. Perutz has drawn an analogy in which the appetite of heme for oxygen grows with the eating, and conversely, loss of oxygen by heme lowers the oxygen affinity of the remaining heme groups. The Hill coefficient *n*, which can be calculated from plots of oxygen equilibrium curves, is a description of hemeheme interaction or cooperativity that explains in part the oxygen-binding properties of hemoglobin and myoglobin. The Hill coefficient for myoglobin is 1, indicating no cooperativity; *n* is about 3 for the normal hemoglobin molecule.

The oxygen affinity of hemoglobin within the erythrocyte does not depend solely on the intrinsic properties of the tetramer. The position of the hemoglobin oxygen dissociation curve, and therefore the P_{50} , can be influenced by a number of heterotrophic modifiers, including temperature, pH, and small organic phosphate molecules in the cell. The effects of these modifiers on P_{50} are shown in Figure 22-4 (Figure Not Available) .

Hemoglobin is the prototype of an allosteric protein; its structure and function are influenced by other molecules. The major intracellular modulator of hemoglobinoxygen affinity in human erythrocytes is 2,3-BPG, an intermediate product of glycolysis

Figure 22-4 (Figure Not Available) Oxygen dissociation curve of hemoglobin. The percent saturation of hemoglobin with oxygen at different oxygen tensions is depicted by the red sigmoidal curve. The P_{50} (i.e., the oxygen tension at which the hemoglobin molecule is half saturated) is about 27 mmHg in normal erythrocytes (pink dotted lines). Heterotopic modifiers of hemoglobin function can shift the curve leftward by increasing or rightward by decreasing its oxygen affinity. *(From Benz EJ Jr: Synthesis, structure, and function of hemoglobin. In Kelly WN, DeVita VT (eds): Textbook of Internal Medicine. Vol. 1. JB Lippincott, Philadelphia, 1989, p. 236, with permission.)*

that is present within the erythrocyte at concentrations equimolar to hemoglobin. The synthesis of 2,3-BPG is enzymatically regulated, and its levels may change depending on the conditions extant. 2,3-BPG is able to bind stereospecifically within the central cavity of the hemoglobin tetramer. Hemoglobin prepared in the absence of 2,3-BPG has a very high oxygen affinity, but as 2,3-BPG is added to a hemoglobin solution, the oxygen affinity progressively decreases. BPG is a polyanion that binds strongly to the deoxygenated form of hemoglobin but poorly to its oxygenated or other liganded forms. Specific amino acids are involved in the binding of 2,3-BPG; these chain residues include the N-terminal valines, the H21 histidine (position 143), and the EF6 lysine (position 82). In oxyhemoglobin, the H helices of the chains are insufficiently spread to permit firm binding of 2,3-BPG; this, along with other conformational changes, favors the binding of this anion to the deoxygenated rather than the oxygenated form of hemoglobin. The binding of 2,3-BPG stabilizes the T (tense) structure of the deoxygenated form at the expense of the R (relaxed) structure of the oxyhemoglobin tetramer.

Transition from the deoxy (T) to the oxy (R) form of hemoglobin is accompanied by rotation of the dimers along the $_{1\ 2}$ contact region (Fig. 22-5 (Figure Not Available)). The T structure is stabilized by salt bridges, which are broken as the molecule switches into the R structure. Some abnormal hemoglobins with an intrinsically high oxygen affinity, or low P_{50} , occur as a result of an amino acid substitution that leads to loss of bonds that stabilize the tetramer in the T conformation. Hydrogen ions, chloride ions, and carbon dioxide all decrease the affinity of hemoglobin for oxygen by strengthening the salt bridges that lock the molecule into its T conformation. The corollary of the lowering of hemoglobin oxygen affinity by protons is the combination of hemoglobin with protons upon deoxygenation. This is known as the Bohr effect and is responsible for carbon dioxide transport in blood, another critical function of the hemoglobin molecule. Deoxyhemoglobin binds the hydrogen ion liberated by the reaction of carbon dioxide with water, thus increasing the concentration of bicarbonate. Within the lungs, hydrogen ion is lost as hemoglobin binds oxygen; therefore, carbon dioxide leaves solution and is excreted from the body via the lungs. Deoxyhemoglobin can also directly bind carbon dioxide; however, this process involves the minority of carbon dioxide exchanged by the red blood cells.

Recently, it has been found that the hemoglobin molecule is S-nitrosolated on the 93 cysteine residues when oxyhemoglobin forms in the pulmonary circulation. This is in addition to the binding of NO to heme. As deoxyhemoglobin is generated in the tissues and the R state is converted to the T state, this linkage is disrupted and NO is liberated to exert its effects on vascular tone. A new and unique physiologic and homeostatic function for hemoglobin may therefore be to provide NO, a vasodilator molecule, when oxygen consumption increases.

Red cells containing high levels of hemoglobin F have high oxygen affinity because it binds 2,3-BPG poorly. Physiologically this predicts that the hemoglobin of the fetus should be oxygenated at the expense of the maternal hemoglobin A. The high oxygen affinity of hemoglobin F is accounted for by a single change in its primary structure, the presence of a serine residue at helical position H21 in place of the histidine found in the -globin chain. This weakens the binding of 2,3-BPG and leads to stabilization of the molecule in its R state.

In summary, the primary amino acid structure of and non- globin chains dictates the inevitable quaternary structure in which resides the ability of hemoglobin to serve as a respiratory

Figure 22-5 (Figure Not Available) Subunit motion in the hemoglobin tetramer. The relative motion of hemoglobin subunits on oxygenation and deoxygenation is shown. The $_{1\ 1}$ dimer (shown in black) is moving relative to the $_{2\ 2}$ dimer (shaded). The oxyhemoglobin tetramer (R state) is more compact than the deoxyhemoglobin configuration (T state). *(From Dickerson RE, Geis I: Hemoglobin: Structure, Function, and Evolution Pathology. Benjamin-Cummings, Menlo Park, CA, with permission. Copyright Irving Geis.)*

Figure 22-6 Maps of the -like and -like globin gene clusters located on **(A)** chromosome 11 and **(B)** chromosome 16. Within each gene cluster are pseudogenes, which are remnants of previously expressed globin genes that have become inactivated as a result of mutation. Active genes are shown in red boxes filled with clear introns; inactive or pseudogenes genes are shown in solid boxes, and the theta-globin gene is shown as a pink box. While this gene is transcribed, it is not clear whether it is represented in a cellular protein. The distance between the functional - and pseudo--globin gene is variable, owing to the presence of repeated elements. HS, LCR, locus control regions and DNA ase hypersensitive sites; E, enhancer;

, silencer.

protein. Cooperativity ensures rapid binding of oxygen in the lungs and unloading in tissues. Similarly, carbon dioxide is transported from tissues to lungs. The function of hemoglobin may be influenced by mutation as well as by heterotropic effectors such as protons and 2,3-BPG. The molecule itself changes shape as it provides oxygen for metabolism; it is a lung in miniature, breathing as it allows the body to respire.

Globin Gene Clusters

The amounts and types of human hemoglobin produced at any given age are determined primarily by the selective expression of the individual genes encoding each globin chain. The globin genes of humans are located in two clusters (Fig. 22-6): -like genes in about 30 kb of DNA on the short arm of chromosome 16 between band P13.2 and the telomere, and -like genes in about 70 kb of DNA on the terminal portion of the short arm of chromosome 11 (P15) (Fig. 22-6). Each gene shares certain basic organizational features. Each contains three exons separated by two introns. The introns of the -gene are both small (100300 bp); non--genes have one small and one large (1,0001,200 bp) intron. The second exon of each globin gene encodes the major components of the heme-binding pocket, and the third the non-contact points.

Flanking each gene at both the 5 and 3 ends are groups of conserved nucleotides. In conjunction with protein factors, these influence the promotion of gene transcription, ensure the fidelity of the transcript and its translatability, specify sites for the initiation and termination of translation, and improve the stability of the newly synthesized mRNA (Fig. 22-7). Also encoded within the genes are signals that permit the enzymatic machinery within the nucleus to excise precisely the introns from the mRNA precursor and splice together the exons to form a contiguous mature mRNA. The spliced mRNA is transported to the cytoplasm and translated into protein. These conserved signals lie at the junction of the exon and intron as well as within the introns themselves. They are recognized by small nuclear ribonuclear protein particles, which participate in the formation of a spliceosome, or splicing complex. Their preservation is critical for the splicing process to occur. When mutations occur within splice signal sites, globin synthesis is often impaired. The 5 end of the mRNA contains a cap structure, and the 3 end contains a poly(A) tail, as described in Chapter 1 .

Figure 22-7 Pathway of globin biosynthesis. Transcription of the globin gene results in a large pre-mRNA molecule containing intervening sequences. During intranuclear processing of this molecule, the intervening sequences are excised and the coding sequences ligated to form a contiguous stretch of RNA, which codes for the globin protein. The message is further processed by the addition of a CAP and a poly(A) tail. The mature message is transported from the nucleus to cytoplasm, where it is translated on polyribosomes by the addition of activated amino acids to a growing polypeptide chain. Globin acquires heme, /non- dimers are formed, and the hemoglobin tetramer is assembled. *(From Steinberg, MH: Hemoglobinopathies and thalassemias. In Stein JH (ed): Internal Medicine. 4th ed. Mosby-Year Book, St. Louis, MO, 1994, p. 852, with permission.)*

Figure 22-8 Hemoglobin switching during embryonic, fetal, and adult development. The and epsilon genes are transcribed during embryonic development and are soon replaced by the fetal - and adult -globin gene. At birth, fetal hemoglobin forms about 75% and hemoglobin A 25% of the total. Transcription of the gene begins to fall before birth, and by 6 months of age this gene is expressed only at very low levels. Expression of the -globin gene begins near birth. In adults, hemoglobin A makes up about 97%, hemoglobin A_2 about 2.5%, and fetal hemoglobin <1% of the total. *(From Steinberg MH: Hemoglobinopathies and thalassemias. In Stein JH (ed): Internal Medicine. 4th ed. Mosby-Year Book, St. Louis, MO, 1994, p. 852, with permission.)*

Conserved nucleotide clusters 5 to the coding portion of each globin gene are known, in the aggregate, to act as promoters (Fig. 22-7). Globin promoters are modular. Some modules are located relatively close to the initiation site of mRNA translation, and some are more distally placed. Promoters ultimately form the binding sites for the RNA polymerase complexes that catalyze gene transcription. Mutations within the promoter can affect the level of gene transcription and the amount of globin made. Surrounding and within each gene are other sequence elements that play important roles in its transcriptional regulation (Fig. 22-6). These clusters, termed enhancers, and silencers (see Chap. 1) may lie within introns or 5 and 3 to the coding sequences; in some instances, they are quite remote from the gene. The higher-order structure of DNA in chromatin may permit close approximation of these remote enhancers to the gene during transcription. Enhancers play important roles in the tissue-specific regulation of globin gene expression. Representative regulatory sequences near the globin genes are shown in Figure 22-7 (the regions make HS1, . HS5). DNA elements controlling globin genes are described in more detail later.

The -like and -like globin genes are ordered in the 5 to 3 direction in the same sequence expressed during embryonic, fetal, and adult development (Fig. 22-8). The functional significance of this arrangement is unclear. However, recent evidence suggests that the ordering of the epsilon-, -, and -genes could be an important factor influencing the ability of each locus to interact with distant control elements at different developmental stages.

The -like and -like gene clusters are likely the result of an ancient duplication of a primordial globin gene that existed early in the history of vertebrates, approximately 500 million years ago. Each gene cluster probably developed from duplication of ancestral genes and subsequent divergence through eons of evolution. Within the -like gene cluster, the -globin gene is expressed only very early in embryogenesis and participates in the formation of embryonic hemoglobins. The -globin genes are duplicated, a characteristic of most globin genes, and their encoded amino acid sequences are identical; therefore, only a single -globin polypeptide results. Minor differences within the second intervening sequence and the 3 flanking regions of the -globin gene permit identification of transcripts from each gene. The 5, or $_2$ -gene is expressed more efficiently than the 3, or $_1$ -gene, so that abnormalities of this gene are more apt to be clinically apparent. Both clusters contain genes that are actively transcribed, as well as pseudogenes whose defective structures prohibit expression at any time.

The gene 3 to the $_1$ -gene is the theta-gene, a somewhat mysterious element of the -gene cluster. While theta-gene transcripts are found in fetal tissue and adult erythroid marrow, it is unclear whether this genes translation product is able to participate in the formation of a functional tetramer. To date no theta-globin protein has been found, and deletion of the theta-globin gene does not appear to have any implications for the developing fetus.

The -like-globin gene cluster consists of the embryonic epsilon-gene, transcribed only during the first 611 weeks of life; the duplicated -globin genes that code for the dominant non--globin of fetal life; and the - and -globin genes that code for the hemoglobins of the adult. The coding sequences of the two -globin genes are identical, except at codon 136, where the 5 or (G)-gene codes for glutamic acid; the 3 (or A)-gene encodes an alanine residue. These genes are unequally expressed during fetal development. A switch in their relative rates of expression leads to a similar disparity between the amounts of G and A chains in adults. While the G/A switch is interesting from the standpoint of the control of gene expression, it is of little clinical importance. Hemoglobin F in both the fetus and the adult contains a mixture of G and A chains; the functional qualities of these hemoglobins are identical.

The - and -globin genes are probably the result of a duplication event that occurred >40 million years ago. The -globin gene has become the predominant gene, coding for most non- chains of the adult. The -globin gene has undergone mutation in several critical areas, so its expression is greatly curtailed. Its product, minor adult hemoglobin (Hb A_2), has become functionally insignificant by virtue of its very low level in the erythrocyte. It is likely that the -globin gene is a pseudogene in evolution. In time its expression may be totally abolished as it acquires an inactivating mutation. The pseudogenes dispersed

within both globin gene clusters provide interesting glimpses into the evolutionary history of globin genes. Pseudogenes are inactive remnants of previously expressed genes. As a result of relaxed selection, their mutation rate is higher than that of surrounding active genes. Hb A_2 is clinically useful, however, for characterizing hemoglobinopathies such as -thalassemia.

The expression of the human globin genes is highly regulated. Globin is synthesized in only one tissue, erythroid cells, and only during a narrowly defined stage of erythroid progenitor cell differentiationthe 57 days that commence with the proerythroblast stage and end when the enucleate reticulocyte loses the last traces of its RNA. Within the confines of these strict tissue-specific and differentiation stage-specific boundaries, the globin genes are extraordinarily active. By the late normoblast and reticulocyte stages, 9095% of all protein synthesis in these cells is globin synthesis.

Individual globin genes are expressed at different levels in developing erythroblasts of the human embryo, fetus, and adult (i.e., 3738 weeks gestation and beyond). Different subsets of - and non--genes are expressed and silenced at each developmental stage. Moreover, the overall balance of non--globin, -globin, and heme production is maintained throughout each of these complex switching events. The complex mechanisms ensuring the proper tissue-, differentiation stage-, and ontologic stage-specific expression are incompletely defined. Much recent information about relevant DNA control elements and transcription factors is emerging.

These are discussed later. First, however, the ontogeny of hemoglobin should be reviewed as necessary background.

Ontogeny of Hemoglobin

The hemoglobin composition of the erythrocyte varies depending on when in gestation or postnatal development it is measured. This is a result of sequential activation and inactivation (switching) among genes within the and non--globin gene clusters (Fig. 22-8). What controls these switches in globin gene transcription is not currently understood. The two early embryonic hemoglobins consist of and epsilon chains (Hb Gower-1) and and epsilon chains (Hb Gower-2). The -globin gene is akin to the -globin genes but is expressed only during early embryogenesis. The epsilon embryonic globin chain is -like. The combination of - and -globin chains forms hemoglobin Portland. These early hemoglobins are made primarily in yolk sac erythroblasts and are detectable only during the very earliest stages of embryogenesis except in certain pathologic states, in which they may persist until gestation is complete. The major hemoglobin of intrauterine life is fetal hemoglobin, or Hb F, which consists of two - and two -globin chains. -globin gene expression begins early in embryogenesis, peaks during midgestation, and begins a rapid decline just before birth. By 6 months of age in normal infants, only a remnant of prior -globin gene expression remains, and the level of fetal hemoglobin in the blood is <1% of the total. -globin gene expression starts early in the first trimester, peaks quickly, and is sustained for life. -globin gene expression also commences early in gestation and reaches its zenith within a few months after birth. The combination of - with -globin chains forms hemoglobin A, the predominant hemoglobin of postnatal life. Also present in adult cells is hemoglobin A_2 . The -globin gene, which directs synthesis of the non- chain of hemoglobin A_2 is very inefficiently expressed. Thus, only low levels of hemoglobin A_2 are present; defects in the -globin gene are of no clinical consequence. In adult blood, fetal hemoglobin is not evenly distributed among erythrocytes but is present in only a very small number of red cells, termed F cells. Hemoglobin A_2 is present in all red cells, albeit at levels <3.5% of the total hemoglobin in normal adults.

Hemoglobin Biosynthesis and its Regulation

Throughout development, genes coding for - and non--globin and heme exhibit coordinated expression. Nearly equal amounts of each of the moieties that ultimately constitute the hemoglobin tetramer are made. Excess unpaired globin chains and mutant globins are also removed from the cell by ATP-dependent proteases, ensuring a balance between and non- chain accumulation. Balanced chain synthesis and coordination of globin chain production with synthesis of heme are important because hemoglobin tetramers are highly soluble, while the components of hemoglobin (i.e., unpaired chains, protoporphyrin, and iron) are not. Precipitation of any of these is deleterious to cell survival. Erythroblast proteases are not efficient enough to eliminate the substantial excesses of unpaired chains that accumulate when an - or non--gene is selectively impaired by severe thalassemia mutations. The mechanisms regulating heme production, and some of the interactions between heme and globin synthesis, are discussed in Chapters 26 , 27 , and 29 .

The proper production of the individual globin chains within erythroid tissues at the appropriate states of differentiation and development is regulated predominantly at the level of transcription. The onset of phenotypic maturation at the proerythroblast stage is marked by the onset of globin mRNA biosynthesis in dramatically increasing quantities. - and non--globin gene expression began at essentially the same time, although some studies suggest a slightly earlier onset of -globin gene expression. Transcription persists at a high level throughout most of the remainder of erythropoiesis, declines as the nucleus condenses, and is eventually lost in late erythroblasts. Even as the absolute rates of globin gene transcription begin to fall, however, the relative percentage of total transcriptional activity devoted to globin gene expression continues to rise; this reflects the silencing of transcription of nearly every other gene in the erythroblast.

The transcriptional activation of the globin genes is the major event that must be understood to define and manipulate the regulation of hemoglobin biosynthesis and hemoglobin switching. However, post-transcriptional mechanisms contribute to the final distribution of globin and nonglobin mRNAs, and to the balance of - and non--globins within the erythroblasts. When compared with many other mRNAs, such as cytokine mRNAs, globin mRNAs are extraordinarily stable. Their half-lives have been estimated at 3050 hours. Most other mRNAs have turnover rates, or half-lives, measured within the range of a few minutes to 56 hours. Thus, the rise in the percentage of total mRNA that is globin mRNA is greatly accentuated because the newly transcribed globin mRNAs accumulate and remain quite stable in the cell, while nonglobin mRNAs, which are no longer being produced, are also disappearing at a faster rate. Consequently, the mRNA content of the reticulocytes consists of 9095% globin mRNA.

The transcription rates of the - and non--globin genes are not precisely equal. (This phenomenon has been studied in detail only in adult reticulocytes expressing the - and -globin genes.) A slight, but reproducibly detectable, excess of -globin mRNA is present in erythroblasts. However, -globin mRNA is translated somewhat more efficiently than -globin mRNA. These counterbalancing forces result in nearly equal synthesis of - and -globin polypeptide chains. In fact, there is a very slight excess of -globin production, resulting in a small pool of free -chains. Newly synthesized -chains are thus rapidly and completely incorporated into functional hemoglobin tetramers. Hemoglobin tetramers are remarkably stable throughout the life span of the circulating red cell. Only small amounts suffer oxidative or proteolytic damage. Hemoglobin molecules are thus exposed for prolonged periods to chemically active compounds in the milieu of the bloodstream. They often become nonenzymatically modified by such processes as glycosylation,

acetylation, and sulfation. One of these modifications, glycosylation, occurs more extensively during periods of hyperglycemia and leads to elevated levels of the glycosylated form of Hb A, Hb A_{1c} . This phenomenon is the basis of a useful test for control of the blood sugar in diabetics. Other post-translational modifications are of little clinical importance.

Transcriptional Regulation of Globin Gene Expression

The major molecular elements mediating regulation are trans-acting proteinaceous transcription factors and cis-acting short, specialized regions of DNA sequence that are recognized by some of these factors. The interaction causes these DNA elements to act as promoters, enhancers, or silencers of gene activity. Nuclear proteins that interact with the globin gene clusters include DNA-binding proteins and proteins that interact with the DNA-binding proteins by means of specific proteinprotein interactions, such as leucine zipper domains. (See Chap. 6 for background information about the basic features and properties of transcription factors.) Control of the - and non--globin genes is further complicated by the fact that each gene cluster functions as a larger unit, or DNA domain. The open or closed state of the -like globin cluster domain, with respect to access to transcriptional activating machinery, appears to be under the influence of an enhancer sequence, the locus control region (LCR). The -globin gene cluster is influenced by the HS-40 region that, while not a typical enhancer, has some similarities in function. Each of these regions is located several tens of kbs upstream of the epsilon-gene on chromosome 11 and the -gene on chromosome 16.

Locus control regions are modular DNA sequence elements. For example, several DNase 1-hypersensitive sites (see Chap. 1 and Fig. 22-6) can be detected within the - and -globin clusters LCR loci in chromatin from erythroid, but not nonerythroid, cells (Fig. 22-8). Minichromosome constructs, in which globin genes are attached directly to the LCR in the absence of most intervening DNA sequences, are expressed at rates approaching those of endogenous globin genes in cultured erythroid cells and transgenic mice. Some nonglobin genes are also expressed at very high levels in erythroid cells when attached to these LCR sequences. Thus, to a first approximation, the LCR regions can be regarded as master switches that promote access of other regulatory factors to individual globin promoters and enhancers within the clusters. The LCRs contain binding sites for the major erythroid transcription factors, GATA-1 and NFE-2, as well as sites for generic factors found more widely distributed in many cell types.

The LCR is unquestionably essential for the quantitatively high rates of -globin gene expression during erythroid differentiation. The role of the LCR in dictating tissue specificitythat is, shutdown of globin gene expression in other tissuesor the sequential activation of silencing of genes during hemoglobin switching, is less clear. Studies suggest that tissue specificity and qualitatively proper switching of - and -globin gene expression in transgenic mice could be obtained with DNA constructs lacking the LCR. However, these genes were invariably expressed only 0.55% as actively as the endogenous globin genes of the host cell. The degree and fidelity of tissue-specific expression also appeared to be dependent on several factors, such as the site of transgene integration.

The interrelationships among the LCR and individual promoters, enhancers, and silencers during erythroid differentiation and hemoglobin switching remain incompletely understood despite extraordinary experimental scrutiny. Most investigators agree that both tissue and developmental specificity and highly active and efficient expression of individual globin genes require interaction among the LCR, the individual promoter/enhancer elements flanking the globin gene selected for expression, and complexes of interacting transcription factors. A variety of looping and tracking models have been put forward as hypotheses to explain how a distant DNA sequence element and its bound transcription factors can interact with sequences located close to the transcription start site of a globin gene. No model has

explained all the experimental findings or biologic phenomena.

Despite uncertainty as to the precise molecular mechanisms involved, most investigators agree that opening of the globin gene cluster by the LCR is a prerequisite for expression of any globin genes; this change in chromatic configuration is presumably mediated by the interaction of the LCR with specific transcription factors. The essential role of promoters and enhancers located near the globin genes is suggested by the discovery of mutations in these regions that affect hemoglobin switching or that cause thalassemia-like syndromes. Moreover, competition for the stimulating activity of the LCR can be appreciated by manipulation of the number of copies of globin genes attached to the LCR, the order in which they appear, or the orientation in which they are transcribed. These manipulations alter both the relative amounts of globin mRNA produced from each locus and the stages of development at which individual genes are expressed. Evidence for the existence of LCRglobin gene interactions is thus clear, even though the nature of the interaction remains obscure.

The shutdown of the embryonic genes late in the first trimester and, possibly, the decline of -gene expression during the perinatal period may also require the participation of active silencing mechanisms. The predominance of -globin gene expression over -gene expression in adult life is thus not merely the result of preferential stimulation of -gene expression. Active repression of the -genes may also be relevant; these mechanisms, if they exist, may have to be reversed if one hopes to manipulate hemoglobin switching therapeutically.

The nuclei of erythroid cells contain numerous proteins that have been identified as transcription factors. Many of these are found in a wide variety of tissues. At least two factors, GATA-1 and NFE-2, are much more restricted in their range of expression and have been implicated as particularly important for globin gene expression.

GATA-1 is named on the basis of the DNA sequence motif (T/A) GATA (A/G), the GATA motif that it recognizes and binds. It is a zinc finger class DNA-binding protein (see Chap. 6). GATA-1 has been shown to activate promoters containing the cognate DNA sequence motif, even when placed in nonerythroid cells. NFE-2 recognizes the DNA sequence motif (T/C) GCT GA (C/G) TCA (T/C). It is a member of the B-zip class of transcriptional activators. Both GATA-1 and NFE-2 were originally identified and cloned on the basis of their interaction with their cognate sequences in the globin genes. Initially, each was thought to be present only in erythroid cells. However, further work has demonstrated that each protein has a wider range of tissue expression. For example, GATA-1 is also present in other hematopoietic cell lineages, whereas NFE-2 consists of two subunits, one that is widely expressed and one that is expressed in several hematopoietic lineages and the intestine. Erythroid Kruppel-like factor (EKLF) may be the most specific of the erythroid transcription factors yet discovered. EKLF interacts specifically with the -globin gene promoter, and may influence the - switch. Mice homozygous for disruption of the EKLF gene have lethal thalassemia.

Clearly, neither GATA-1 nor NFE-2 alone can be the sole determination of tissue specificity of the globin genes; otherwise globin gene expression should occur at some level in other tissues in which these proteins are present. However, there is no doubt that each of these proteins is indispensable for both globin gene expression and erythropoiesis. Gene knockout studies have shown that absence of either protein results in greatly impaired erythropoiesis.

GATA-1 and NFE-2 probably interact with each other, either

directly or by means of binding to intermediary proteins, to activate the expression of globin genes and other genes necessary for erythroid maturation. Several other proteins have been identified as binding to various control elements in the globin gene cluster. Some of these appear to be stage-specific selector elements that bind predominantly to the - or -globin gene enhancers and promoters. Others are generic transcription factors. Regulation at these sites is probably hierarchic, depending on the appropriate combination of DNA-binding proteins, the types of proteins that interact with them, and the activation state of the bound proteins through such processes as ribylation and phosphorylation. It is also possible that the same sequence element may interact with different combinational sets of proteins, with a different resulting effect on transcriptional activity, depending on the stage of differentiation and/or embryonic/fetal/adult development.

The precise molecular machinery necessary for regulation of transcription of individual globin genes is beginning to be defined. It is important to realize, however, that these molecular mechanics are susceptible to cellular, microenvironmental, and humoral influences affecting the proliferation and differentiation state of primitive erythroid stem cells in yolk sac, fetal liver, or bone marrow. Overwhelming evidence supports the hypothesis that the potential for expression of - and/or -genes is determined in primitive erythroid stem cells (i.e., BFU-E) long before actual expression of the globin genes is initiated at a later stage of differentiation. The relative percentage of maturing erythroblasts that will ultimately express Hb F or Hb A, or both, can be altered by factors such as cytotoxic drugs or bone marrow stress that alter the relative percentages of Hb F-potent or Hb A-potent stem cells undergoing cell cycle events, differentiation, and so forth. Drugs currently in use in an effort to manipulate Hb F switching seem to work primarily through these cellular mechanisms (see Chap. 15).

Post-transcriptional, Translational, and Post-translational Mechanisms

Processed globin mRNA is exported from the nucleus to the cytoplasm by a mechanism that is not yet clearly defined. mRNA translation occurs in the cytoplasm (Fig. 22-7). The triplet codons or mRNA are recognized by the anticodons of specific tRNAs that bring activated amino acid residues to the nascent polypeptide chains. The process of translation, in which an mRNA template directs the synthesis of protein, is typically divided into three phases: initiation, elongation, and termination (see Chaps. 1 and 2). Each phase is regulated by a variety of protein factors.

The globin mRNA molecule becomes associated with four to six ribosomes, forming the polyribosome. At least 11 eukaryotic translation initiation factors interact with the polyribosome. These mediate stabilization of a preinitiation complex, binding of the initiator methionine tRNA to ribosomal subunits, binding of mRNA to the preinitiation complex, stabilization of mRNA binding, recognition of the cap site at the 5 end of mRNA, and release of initiation factors from the preinitiation complex. Several elongation and termination factors have also been defined. Initiation, or an early step in the elongation process, is rate-limiting.

The first post-translational step in tetramer formation is the combination of and non- chains to form dimers, an event that appears to depend on the relative charge of each globin subunit. The dimers then spontaneously form tetrameric hemoglobin. Because of charge differences among non--globin chains, there is a hierarchy or affinity of these chains for chains. The combination of and chains is most favored, followed by a combination of - and - and -globin chains. Certain mutant hemoglobins that have either gained or lost a charge may alter this hierarchic arrangement. This may influence the proportion of variant hemoglobin present, especially when the patient also inherits an -thalassemia syndrome, in which the synthesis of -globin chains is reduced. The supply of available chains is then limited and non- chains compete with one another to form tetramers with the limiting chain pool.

Globin chain biosynthesis and heme synthesis are mutually important. Heme plays a role in the regulation of the initiation complex. A deficiency of heme (e.g., in iron deficiency) is associated with the accumulation of a repressor of translation initiation factors. -mRNA appears to be initiated more efficiently than -globin, conferring on the associated anemia some of the features of mild -thalassemia. This phenomenon occurs because heme deficiency depresses the availability of initiating factors for which the less efficient -mRNA must compete with the more efficient -mRNA.

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HEMOGLOBINOPATHIESNOSOLOGY

Inherited abnormalities of the hemoglobin molecules that cause morbidity are called hemoglobinopathies and thalassemias. Many of these conditions produce disease (e.g., sickle cell anemia, thalassemia, methemoglobinemia) that are especially important to hematologists. A few acquired conditions lead to modification of hemoglobin (e.g., carbon monoxide exposure, producing carboxyhemoglobinemia) that produce clinical abnormalities.

TABLE 22-1 -- Classification of Hemoglobinopathies and Thalassemias

Structural hemoglobinopathiesmutations altering the amino acid sequence of a globin chain and altering physical or chemical properties of the hemoglobin tetramer in such a way that function is deranged.
Abnormal hemoglobin polymerizationsickle hemoglobin (Hb S); hemolysis, vaso-occlusion
Abnormal hemoglobin crystallization (e.g., Hb C)
High oxygen affinitypolycythemia (Hb Zurich)
Low oxygen affinitycyanosis (Hb Kansas)
Hemoglobins that oxidize or precipitate too readilyunstable hemoglobins (Hb Köln)
M hemoglobinsmethemoglobinemia, cyanosis (e.g., Hb Milwaukee)
Thalassemiadefective production of globin chains with hypochromia, anemia, hemolysis, altered erythropoiesis
-Thalassemia
-Thalassemia
-Thalassemias, -thalassemias, -thalassemias
Thalassemic hemoglobinopathiesmutations altering both synthesis and structure or function of the hemoglobin gene products (e.g., Hb E, Hb Terre Haute, Hb Lepore, Hb Constant Spring)
Hereditary persistance of fetal hemoglobinpersistence of high levels of Hb F into adult life
Pancellularhigh HbF levels in all red cells
Nondeletion forms
Deletion forms
Hb Kenya
Heterocellularinherited increases in the percentage of F cells
Acquired hemoglobinopathies
Methemoglobinemia due to toxic exposures
Sulfhemoglobinemia due to toxic exposures
Carboxyhemoglobinemia due to toxic exposures
Hb H in erythroleukemias
Acquired elevations in F cells and Hb F
Erythroid stress [e.g., recovery from bone marrow suppression]
Bone marrow dysplasias
Exposure to agents altering stem cells or gene expression (e.g., hydroxyurea, butyric acid)

These situations are summarized by the term acquired hemoglobinopathies.

Most of the enormous number (nearly 1,000) of mutations of the globin gene that have been described produce no, or only trivial, clinical effects. The remainder can be classified according to the hematologic and clinical phenotypes that cause reduced solubility with hemolytic anemia (unstable hemoglobins and polymerizing hemoglobins, such as sickle hemoglobin), hemoglobins with altered oxygen affinity, methemoglobins, and the thalassemias involving abnormal synthesis of one or more globin chains with anemia, hemolysis, and alterations of erythropoiesis. Some mutations, such as that responsible for Hb E, can alter both the structure and synthesis of the molecule. The classification of hemoglobinopathies and thalassemias is summarized in Table 22-1 . Individual conditions are discussed in the chapters already cross-referenced in earlier sections of this chapter.

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Chapter 23 - Approach to the Adult and Child with Anemia

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ANEMIA IN THE ADULT

Cycle of Erythropoiesis

Anemia represents a reduction in the body's red cell mass. Under normal conditions, this quantity is maintained within prescribed limits through the regulatory feedback stimulus of the humoral factor erythropoietin. [1] [2] A sensing device responsive to tissue oxygen content within the kidney determines the degree of erythropoietin release from renal peritubular cells. [3] [4] Red blood cells (RBCs) circulate in the peripheral blood for approximately 90120 days, necessitating replacement of the approximately 1% of the body's red cells lost each day to senescence. The spleen is the major site for removal of aged RBCs; splenic macrophages may recognize an acquired RBC senescence antigen,

which facilitates trapping of these cells within the microvasculature of the spleen. [5] [6] The erythropoietic feedback loop ensures that the hemoglobin mass for oxygen delivery matches the body's needs and that production equals destruction of red cells under stable conditions.

Mechanisms of Anemia

Anemia, a deficiency in the RBC mass and the hemoglobin content of blood, occurs whenever any malfunction upsets normal coupling of RBC production with destruction. This pathologic condition can have many origins; in some circumstances, especially in patients with complex illnesses, several factors converge to produce anemia (multifactorial anemia). Anemias can be caused by a primary hematologic disorder within the marrow or by an accelerated loss or destruction of RBCs in the periphery. As well, a myriad of systemic derangements may perturb erythropoiesis or RBC survival. The hematopoietic microenvironment is exquisitely sensitive to the macroenvironment of the body. Anemia, like the sedimentation rate, is thus often a sickness index of the body. The diagnostic challenge in the workup of the anemic individual is to recognize it as a sign of an underlying pathology whose recognition requires an approach to the whole patient for delineation of the mechanism and cause(s) of the red cell deficit.

Normal Hematologic Values

Normal hematologic values are established by sampling large populations of men and women (Table 23-1). Adult males tend to have slightly higher hematocrits because androgen enhances both renal erythropoietin secretion and the enlistment of precursor cells into an erythropoietin-responsive state. Geographic differences also exist for these standards because ambient oxygen tensions affect hemoglobin oxygen saturation; the lower oxygen tensions at high altitudes are associated with increased RBC hemoglobin levels.

Three measurements that were previously performed manually, determination of the hemoglobin level (in grams per deciliter), RBC number × 10 [12] L, and hematocrit (in percent), are now determined by electronic counters. Both the RBC number and the mean corpuscular volume (MCV) are directly measured from a gated window pool, and the hemoglobin concentration is determined by chromatographic quantitation of the hemoglobin present in a defined volume of RBCs. These values are used to calculate the hematocrit, the mean corpuscular hemoglobin (MCH), and the mean corpuscular hemoglobin concentration (MCHC). The Coulter counter can also generate a red cell sizing index (the RDW). The RDW is a measure of the heterogeneity of sizes in patients' circulating red cell populations. High values indicate greater variability in size. In addition to this quantitative characterization of RBCs, the Coulter counter measures the white blood cell (WBC) and platelet counts, permitting a rapid and important assessment of the involvement of cell lines other than red cells. When present, multilineage involvement implies that the anemia is part of a stem cell disorder common to all three cell lines or involves exaggerated peripheral destruction of all lineages, as occurs in hypersplenic states.

TABLE 23-1 -- Criteria of Anemia in Adult Men and Women

	Women	Men
RBC × 10^{12} /L	<4.0	<4.5
Hemoglobin (g/dl)	<12	<14
Hematocrit (%)	<37	<40

Hyporegenerative versus Hyperregenerative Anemias

Each day approximately 0.8% of the RBC pool needs to be replaced by young erythrocytes released from the marrow. Reticulocytes are larger than mature RBCs, and because they still contain portions of polyribosomal RNA material, they can be stained selectively by supravital dyes and counted. Their number in the blood reflects the marrow's response to peripheral anemia. When the cause of anemia is blood loss or hemolytic destruction in the periphery, erythropoietin overdrive of the marrow leads to reticulocytosis (>0.52.0%) to compensate for the peripheral RBC deficit, provided that the marrow's capacity to produce more RBCs is intact. An absence of an appropriate reticulocytosis in the setting of anemia indicates that a lesion that interferes with RBC production is responsible. The reticulocyte count provides an easy means of suspecting either the marrow or the periphery as the *primary* source of anemia. This differentiation dictates further investigative workup by narrowing the focus to the marrow in reticulocytopenic states but to peripheral blood loss or hemolytic abnormalities, or both, when reticulocytosis is present.

The reticulocyte count is expressed as a percentage of the total RBCs examined in an individual patient. This number must be corrected for the presence of anemia

because it is spuriously elevated when it is related to the reduced number of RBCs present in an anemic patient (i.e., if RBCs, the denominator, are reduced, the percent reticulocytes will rise even if no additional reticulocytes are being produced). This correction is generated according to the formula in Table 23-2 . An additional correction in this index is necessary because reticulocytes released under intense erythropoietin stimulation remain in the peripheral blood for twice the usual 1-day survival time of nonstress reticulocytes. An alternative to this somewhat cumbersome corrected reticulocyte index is expression of the reticulocyte count in absolute numbers. The original reticulocyte percentage is multiplied by the RBC number to yield an absolute reticulocyte count; any value >100 × 10^{9}/L is considered evidence of an erythroid marrow compartment that is responding appropriately to a hematologic lesion outside the marrow.

Red Blood Cell Size as a Classification of Anemia

The Coulter counter generates a value for the average volume (MCV) of the red cells, which provides an important means of categorizing the types and causes of anemia. The normal MCV is 8095 fl; values above and below this range define macrocytic and microcytic anemias, respectively. The MCV is useful as a first step in approaching an etiologic diagnosis of an anemia because there are relatively few causes of microcytic and macrocytic anemias. The microcytic anemias have their origin in lesions that cause deficient hemoglobin synthesis. These states include iron deficiency anemia and the anemia of chronic disease, respectively the most common anemia overall and the most common anemia encountered in hospitalized patients. (Either of these forms can also be normocytic, however.) Inherited defects in globin synthesis, the thalassemias, as well as abnormalities in heme synthesis, as in the sideroblastic anemias, may also result in a hemoglobin-deficient hypochromic microcytic anemia.

Severely macrocytic anemias (MCV > 115 fl) almost always

TABLE 23-2 -- Reticulocyte Count

Corrected reticulocyte count = % reticulocytes ×
Absolute reticulocyte count = % reticulocytes × RBC count/mm^3
Absolute reticulocyte count > 100,000/mm^3 = hemolytic anemia

have their cause in folate or vitamin B_{12} deficiency, since both vitamins play an essential role in DNA metabolism. Less striking macrocytosis is usually caused by liver disease, some drugs (e.g., antifolates), or alcoholism; hypothyroidism also may be associated with macrocytic indices. Inherited abnormalities in purine/pyrimidine metabolism are rare causes of macrocytic anemias. The development of a refractory macrocytic anemia, especially in the elderly, should raise consideration of a myelodysplastic or leukemic syndrome.

Two causes of pseudomacrocytosis should not be overlooked: (1) since reticulocytes are polychromatophilic macrocytes, macrocytosis increases with increasing reticulocytosis; and (2) agglutination of RBCs in the presence of cold agglutinins or cryoglobulins may lead to false elevations in the MCV because the aggregates may be counted as large RBCs. Reversal of a high MCV by heating the specimen is proof that the latter phenomenon is involved.

The normocytic anemias include: hemolytic anemias of various causes (in the absence of striking reticulocytosis, which can elevate the MCV); aplastic anemias (although some are macrocytic); and the early stages of almost all anemias, including iron deficiency (in these cases the residual normal population of RBCs present when the anemia first develops counterbalances the emerging population of abnormal cells).

It is important to realize that the MCV is an average value representing the arithmetic mean volume of red cells. Combined abnormalities or deficiencies can result in a normal MCV when iron deficiency (decreased MCV) is accompanied by folate deficiency (increased MCV) or pernicious anemia supervenes in thalassemia. Failure to appreciate that the MCV represents an average RBC size creates the potential for overlooking the real cause(s) of the anemia. The RDW now permits recognition of this phenomenon because it demonstrates graphically the heterogeneity of RBC populations.

The MCH and MCHC values do not provide very much additional information for the categorization of anemias. Both values are low in iron-deficient hematopoiesis, but the reduction in MCV is the most commonly used marker of this state and occurs earlier than the reduction in hemoglobin concentration. However, one disease, hereditary spherocytosis, is characterized by an elevation in the MCHC because it is associated with a varying population of dense microspherocytes. The data generated from the Coulter counter can also be used as an internal control on the hemoglobin and hematocrit parameters. The hematocrit is usually about three times the measured hemoglobin value; a departure from this ratio occurs when intravascular hemolysis releases large amounts of hemoglobin beyond that contained in intact RBCs. Clostridial sepsis may advertise itself in this fashion. Agglutination of RBCs due to cold-reactive antibodies may falsely decrease the RBC value without altering the hemoglobin content of the specimen.

Examination of the Peripheral Blood Smear

Examination of the peripheral blood smear is a diagnostic maneuver of overriding importanceit is at least equal in importance and complementary to the machine-generated hematologic data. It confirms the RBC size categorization determined with the Coulter counter and permits recognition of the many variations in RBC size and shape that are frequently signposts of the causes of hemolytic anemias. Since nucleated RBCs are counted as WBCs by the Coulter counter, the smear must be examined to recognize and correct for this contribution to pseudoleukocytosis. Such erythroblastic findings are helpful clues to the diagnosis of severe thalassemia, myelophthisis, or marrow infiltrative disorders.[7] Examination of the WBCs may reveal hypersegmentation of polymorphonuclear cells as the earliest manifestation of a megaloblastic process[8] (Fig. 23-1). This may be the critical marker in pregnancy or in alcoholics, in whom a

Figure 23-1 Hypersegmentation of polymorphonuclear cell and macrocytosis as peripheral blood evidence of an underlying megaloblastic process.

normal MCV may occur because of a mixed iron-folate deficiency. Target cells, Howell-Jolly bodies, and nucleated RBCs may be clues to functional or anatomic asplenia (Fig. 23-2).

The red cell shape is frequently the most helpful aid in the differential diagnosis of hemolytic disorders. Diagnostic findings include RBC fragmentation as a result of traumatic hemolysis on artificial cardiac valves (Fig. 23-3); spherocytes as a marker of hereditary spherocytosis, immune hemolytic anemias, or hypersensitivity (Fig. 23-4); the oak leaf and sickle cells of sickle cell disease (Fig. 23-5); the target cell of thalassemia and hemoglobin C and liver disease (Fig. 23-6); the spur cell of liver failure (Fig. 23-7); the teardrop form of myelophthisic processes (Fig. 23-8); and the blister forms of glucose-6-phosphate dehydrogenase (G6PD) deficiency (Fig. 23-9). In addition, detection of intraerythrocytic parasites may demonstrate that a fever is caused by malaria (Fig. 23-10). The marked elevation in serum proteins in myelomatous states may result in a stacking of RBCs in rouleaux formation (Fig. 23-11A); aggregation of RBCs into crowded masses (as opposed to their stacking into rouleaux) should suggest cold agglutinin disease (Fig. 23-11B).

Figure 23-2 Howell-Jolly bodies in RBCs of asplenic patient.

Figure 23-3 Helmet cells, microspherocytes, and RBC fragments in microangiopathic hemolytic anemia.

Figure 23-4 Spherocytes and polychromatophilia in hereditary spherocytosis.

Figure 23-5 Target, oak leaf, and sickle cells in sickle cell disease.

Figure 23-6 Target cells, acanthocytes, and macrocytes in liver disease.

Figure 23-7 Marked acanthocytosis in advanced liver disease.

Figure 23-8 Teardrop and nucleated RBCs in myelophthisic anemia.

Figure 23-9 Blister RBCs in drug-related hemolytic anemia.

None of the quantitative values generated by the Coutler machine substitute for the plethora of information derived from a careful, informed examination of the peripheral smear. Omission of this simple and inexpensive undertaking deprives the clinician of what is often the critical clue to the cause of the anemia, and even the cause of nonhematologic disease.

Examination of the Bone Marrow

If anemia is caused by a hyporegenerative process, a bone marrow examination permits evaluation of the production fault responsible

Figure 23-10 Intraerythrocytic parasites in malaria.

Figure 23-11 (A) Stacking of RBCs with rouleaux formation in myeloma. **(B)** Aggregation of RBCs in cold agglutinin disease

for reticulocytopenic anemia. A marrow aspiration is usually coupled with a marrow biopsy; the former permits observation of cellular detail, whereas the latter provides a histologic section in which the marrow contents can be viewed in relation to the bony architecture housing the hematopoietic elements.

The marrow is the final arbiter in the diagnosis of iron deficiency anemia; iron-deficient erythropoiesis does not result in anemia until all iron has been mobilized from the marrow so that iron stores are absent in these patients. In microcytic anemias due to all other causes, detectable iron stores are present in the marrow. Marrow examination is essential for the diagnosis of sideroblastic anemias. Ringed sideroblasts can only be recognized on a marrow study. A marrow aspirate will reveal the megaloblastic origin of some macrocytic anemias and concomitantly provide an assessment of iron stores. A biopsy of the marrow permits recognition of general aplasia or hypoplasia, pure RBC aplasia, or hematologic malignancy (leukemia, lymphoma, or myeloma) as a cause of anemia. Infiltrative diseases of the marrow, sometimes heralded by a leukoerythroblastic picture (teardrop RBCs, nucleated RBCs, early WBC precursors, or abnormalities in platelet shape) in the peripheral blood, may be the initial manifestation of malignancy. Small cell carcinoma of the lung, and breast and prostate cancers frequently involve the marrow, creating so-called myelophthisic anemia. Myelofibrosis, a heavy deposition of collagen fibers within the marrow, can be demonstrated with special silver stains of the biopsy specimen; this abnormality is a component of myeloid metaplasia (myelofibrosis and extramedullary hematopoiesis) but may accompany such disparate states as hairy cell leukemia or mastocytosis.

The marrow may contain granulomas in tuberculosis or histoplasmosis: [9] culture of *Mycobacterium avium intracellulare* from the marrow of patients with AIDS is a frequent means of diagnosing this complication. The accumulation of pinkish glassy appearing acid mucopolysaccharide (ceroid) occurs in the marrow of patients with anorexia nervosa who develop anemia and other cytopenias secondary to their caloric deficiency state. [10] The presence of Gaucher cells in the marrow may explain an otherwise perplexing splenomegaly. [11]

The marrow examination is an initial tool in evaluation of the patient with reticulocytopenic anemia. Because it is an uncomfortable and frightening procedure, every effort should be made to minimize the number of times it must be performed in an individual patient. Anticipation of the specific information

desired should dictate handling of the specimen. Culture materials should be on hand if infection such as tuberculosis is a consideration; flow cytometry or chromosomal studies requiring special media should be arranged in advance. If marrow aspiration is not possible, a touch preparation of the biopsy specimen may provide enough cells to allow a diagnosis. Inaspirable marrows are frequent in myelofibrotic states; the very hypercellular marrows of leukemia or megaloblastic anemias may also resist aspiration.

Integration of Laboratory Data with History and Physical Examination Findings

Specific measurements of hematologic parameters are essential for differential diagnosis, but findings from the history and physical examination are equally crucial. The chronology of the process can be a useful clue: a previous record of an abnormal blood examination or a positive family history suggests an inherited, congenital form of anemia. Direct examination of a parent's hematologic values may confirm an autosomal dominant pattern of an inherited blood disorder. A family history of immune disease, including thyroid, adrenal, and skin (vitiligo) disorders, may suggest pernicious anemia because of the congregation of these immune-mediated diseases in family constellations. [12] The patient's occupation (lead and possible toxin exposures), social habits (alcohol and intravenous drug abuse), travel history (malaria), and diet (folate, iron) are all central to discovering the cause(s) of anemia. The presence of nonhematologic disease states such as renal failure, HIV/AIDS, carcinomatosis, autoimmune disease, or hepatic failure must be recognized, because these and other states can cause anemias or alter their behavior. Drug ingestion history is an essential component of any investigation of anemia; some perplexing anemias have been explained only after the contents of the medicine cabinet were individually reviewed.

The rate at which the anemia develops usually determines the symptoms experienced by the patient. Age and cardiovascular condition are also important in this connection. The classic signs of acute blood loss (tachycardia, postural hypotension) may not be present in chronic anemias, even if severe; the total blood volume may actually be increased in the latter situation secondary to an overexpanded plasma volume. Indeed, rapid administration of a blood transfusion to a patient with

severe chronic anemia may precipitate cardiopulmonary decompensation by aggravating an already expanded blood volume. The intersection of the decreased hemoglobin oxygen-carrying capacity with myocardial ischemia can result in elderly anemic patients presenting initially to a cardiologist. Cardiac symptoms may extend from dyspnea and weakness to angina and frank myocardial infarction.

The remainder of the physical examination may suggest or confirm the type and source of anemia. Both folate and vitamin B_{12} deficiency create the same morphologic type of megaloblastic, macrocytic anemias, but vitamin B_{12} deficiency is also responsible for the neurologic lesion of subacute combined degeneration, with loss of vibratory and position sensation in the lower extremities. Telangiectasis on the buccal mucosa may be the external evidence of the internal bleeding telangiectatic lesions in Osler-Weber-Rendu disease; this hereditary hemorrhagic disease usually presents as a chronic iron deficiency anemia. Jaundice may be the clue to hemolysis or liver disease. A caput medusae vascular pattern over the abdominal wall indicates portal hypertension with splenomegaly, resulting in a hypersplenic, pancytopenic state. Leg ulcers may accompany hemolytic anemias, especially sickle cell disease. Thus, the entire body must be examined in an effort to uncover the cause of anemia.

Because the hematologic system acts as a sickness index of the body's condition, hematologic abnormalities are frequently the clue to the presence of systemic disorders. The anemia of chronic disorders is an involvement of erythropoiesis in the host response to a broad range of bodily insults. [13] Infection, inflammation, and malignancy are all accompanied by this form of anemia.[14]

The problem in diagnosing these disorders may be complicated because fever, a common accompaniment of infection, inflammation, or malignancy, may also be caused by the primary hematologic process. Both hemolytic and megaloblastic anemias may have significant fever as a component of their presentation. Additional confusion arises because the classic manifestations of infection, such as Roth spots in endocarditis, may be due to severe anemia without any contribution of endocarditis.

Malignancies are also frequently accompanied by anemia even in the absence of direct marrow involvement by the tumor. Anemia may advertise the silent presence of a hypernephroma or pancreatic neoplasm.[15] Both acute and chronic renal diseases have a direct effect on erythropoiesis, which is not surprising, in light of the kidney's function as the source of erythropoietin. [16] The appearance of anemia in a diabetic individual may be the key indication that renal deterioration secondary to diabetic nephropathy has occurred. Anemia becomes an almost universal cause of morbidity as renal failure advances.

Endocrine disease also involves anemia as a frequent complication because most of the body's hormones have direct effects on erythrocyte production. Hypothyroidism is associated with an anemia that represents a down-tuning of the erythroid mass to parallel the lowered metabolic demands of the body. Parathyroid, adrenal, and pituitary disorders may all contribute to an anemic state. [17] [18]

Mild anemias that accompany primary nonhematologic disorders may be the minor detail that leads to the major diagnosis. Examination of the blood may provide a computer readout of the body, but this information must be integrated with the history and physical examination findings. It is this integration of data within a broad framework of diagnostic possibilities that constitutes the challenge in the approach to the patient with anemia.

Approach to Anemia with Decreased Red Cell Production

Patients with anemia and a low reticulocyte count have inadequate RBC production. Evaluation of the cause of such anemias may be guided by the MCV of the circulating red cells. [19] [20] None of the distinctions made below in characterizing microcytic, normocytic, and macrocytic anemias is absolute; nevertheless, the MCV can provide a useful guide to the analysis and laboratory investigation of the anemic patient.

Evaluation of Microcytic Anemia

Microcytic anemias generally result from inadequate hemoglobin synthesis. The failure of hemoglobin production may be caused by hereditary defects in globin synthesis, as in thalassemia; by defects in heme synthesis, as in sideroblastic anemias; or by inadequate iron incorporation into the heme moiety because of either iron deficiency or failure of iron mobilization, as seen in the anemia of chronic disease.

The laboratory evaluation of microcytic anemia should include determination of iron indices, namely serum iron, iron-binding capacity, and ferritin; examination of the peripheral blood smear for evidence of thalassemia trait or coarse basophilic stippling suggestive of sideroblastic anemia; and hemoglobin electrophoresis to assist in defining a potential thalassemia trait. The definitive diagnosis may require bone marrow aspiration to assess both iron stores and the effectiveness of iron incorporation into developing red cell precursors.

APPROACH TO ANEMIA WITH LOW MCV AND LOW RETICULOCYTES

Differential diagnosis

- Iron deficiency
- Sideroblastic anemia
- Thalassemia trait
- Anemia of chronic disease

Laboratory evaluation

- Iron, iron-binding capacity, and ferritin
- Examination of peripheral blood smear for evidence of target cells, stippling, etc.
- Hemoglobin electrophoresis for evidence of thalassemia
- Bone marrow aspirate for definition of iron stores, presence of ring sideroblasts.

Iron deficiency is by far the most common cause of anemia in general and of microcytic anemia in particular. In iron deficiency, the total serum iron should be low, and saturation of the serum iron-binding capacity should be less than 10%. [21] In patients who are iron deficient but who have recently ingested iron supplements, these measurements are unreliable. By contrast, serum ferritin, which is a reflection of total body iron stores, is essentially always low in iron deficiency regardless of iron therapy.[22] It must also be remembered that early iron deficiency is generally a normocytic anemia. (A useful guide is that the hematocrit usually falls to <30 before the MCV declines to <80.) Moreover, the RDW value is invariably elevated by the time iron deficiency anemia becomes microcytic. Consequently, evaluation of iron indices is indicated in virtually all anemic patients. Because iron deficiency is essentially always a sign of chronic blood loss, confirmation of the diagnosis in adult

men and postmenopausal women commits the physician to a full evaluation of the gastrointestinal tract to rule out a malignant bleeding source.

In sideroblastic anemia and in thalassemia trait, iron levels are usually normal to high, iron-binding capacity is normal, and ferritin levels are normal to high. [23] Although associated with profound microcytosis, thalassemia intermedia and thalassemia major do not present a diagnostic problem in this setting, as they are both associated with a slightly elevated (27%) reticulocyte count and characteristic physical stigmata of massive bone marrow expansion (see Chap. 29). Sideroblastic anemia is confirmed by the presence of ringed sideroblasts in a bone marrow aspirate (see Chap. 27). Confirmation of -thalassemia trait is by hemoglobin electrophoresis. -Thalassemia trait, in which two of the -globin alleles are deleted, causes microcytosis but minimal anemia, if any. If the diagnosis is suspected, it should be evaluated on a peripheral blood smear because the hemoglobin electrophoresis in -thalassemia is normal. Definitive confirmation can only be obtained with DNA analysis of the -globin gene loci. [24]

The most troublesome diagnostic difficulty related to microcytic anemia is in identifying the anemia of chronic disease. Although usually presenting as a normocytic anemia, this disorder must always be considered in the differential diagnosis of microcytic anemia. [25] [26] No definitive test will confirm a diagnosis of anemia of chronic disease; patients with this anemia classically have low serum iron levels, low iron-binding capacity, and elevated serum ferritin levels. In general, the saturation of the iron-binding capacity is >10%. The diagnosis is primarily one of exclusion, and diagnostic studies may involve a bone marrow aspirate confirming adequate iron stores with poor iron incorporation into siderocytes.

Evaluation of Macrocytic Anemia

The macrocytic anemias can be divided into two major groups, the megaloblastic anemias (see Chap. 28) and the nonmegaloblastic macrocytic anemias. The two can be distinguished on examination of the peripheral blood smear. Megaloblastic anemias affect all hematopoietic cell lines, and the peripheral smear shows evidence of hypersegmented neutrophils and large platelets. [27] [28] These cell lines are often depressed in megaloblastic anemia as well, and patients may in fact present with pancytopenia. By contrast, the nonmegaloblastic macrocytic anemias, other than those due to reticulocytosis, usually reflect membrane cholesterol defects related to constitutional abnormalities such as liver disease or hypothyroidism; consequently, the WBCs and platelets should be morphologically normal.

Additional laboratory evaluations include determination of RBC serum folate and serum vitamin B_{12} levels in patients with megaloblastic anemia, and thyroid and liver function tests in patients with isolated macrocytosis.

The main diagnostic challenge in evaluating patients with macrocytic anemia is identifying myelodysplastic syndromes. Although these syndromes are classically associated with megaloblastic changes, evidence of megaloblastic abnormalities in the peripheral blood of patients with myelodysplasia is frequently subtle or absent. [29] [30] In patients in whom megaloblastic anemia occurs despite normal serum folate and vitamin B_{12} levels, bone marrow aspiration and biopsy should be done to determine whether a myelodysplastic syndrome is present. Patients with an unexplained macrocytic anemia should also undergo bone marrow examination. The marrow evaluation should include cytogenetic studies.

In patients with megaloblastic anemia related to vitamin B_{12} deficiency, a Schilling test should be done to evaluate the presence of pernicious anemia. (The measurement of anti-intrinsic factor or antiparietal cell auto-antibodies is supplanting the Schilling test.) If that diagnosis is confirmed, evidence of other endocrine abnormalities, notably thyroid or adrenal insufficiency, or of gastrointestinal disease, should be sought.

Evaluation of Normocytic Anemia

The evaluation of normocytic anemias is complicated by the fact that many of the anemias classically associated with microcytosis or macrocytosis may in fact occur with a normal MCV, especially in the early stages. Iron deficiency, acquired sideroblastic

APPROACH TO ANEMIA WITH ELEVATED MCV AND LOW RETICULOCYTES

- Differential diagnosis
  - Megaloblastic anemia
    - Vitamin B_{12} deficiency
    - Folate deficiency
    - Myelodysplastic syndrome
    - Drug-induced anemia
  - Nonmegaloblastic anemia
    - Liver disease
    - Hypothyroidism
    - Reticulocytosis
  - Laboratory evaluation
    - Serum vitamin B_{12} , RBC folate
    - Examination of peripheral smear for hypersegmented neutrophils, giant platelets
    - Thyroid function tests, liver function tests
    - Bone marrow aspirate for evaluation for myelodysplastic features

APPROACH TO ANEMIA WITH NORMAL MCV AND LOW RETICULOCYTES

Differential diagnosis

- Early or mild iron deficiency
- Primary bone marrow failure
  - Aplastic anemia
  - Constitutional red cell aplasia (Diamond-Blackfan)
  - Acquired red cell aplasia
  - Myelophthisis
- Secondary bone marrow failure
  - Uremia
  - Endocrinology
  - Human immunodeficiency virus infection
  - Anemia of chronic disease

Laboratory evaluation

- Iron, iron-binding capacity, and ferritin
- Examination of peripheral blood smear for evidence of myelophthisis: teardrops, helmet cells, etc.
- Serum creatinine, thyroid function tests, liver function tests, cortisol levels if appropriate
- Erythropoietin level
- Bone marrow aspirate and biopsy to assess iron stores, red cell production, marrow cellularity

anemia, myelodysplasia, and the anemia of chronic disease all frequently manifest as normocytic anemias. In addition, mixed anemias (e.g., anemia in a patient who is both folate and iron deficient) may result in a normal MCV. Examination of the peripheral blood smear in patients with mixed anemias will reveal a mixed population of RBCs, which may help resolve the dilemma. The guide to the differential diagnosis of normocytic anemia, outlined in the box, consequently is not exhaustive. Early stages or mixtures of microcytic and macrocytic anemias must be considered as well.

The initial evaluation of a patient with a normocytic anemia should include iron studies because of the frequency with which iron deficiency presents as a normocytic anemia. Thereafter the evaluation focuses on establishing the presence or absence of a primary bone marrow failure syndrome. The diagnosis of aplastic anemia, red cell aplasia, or myelophthisis ultimately rests on examination of the bone marrow aspirate and biopsy specimen (to assess marrow cellularity, red cell production, and the presence of abnormal cells within the marrow); however, an inappropriately low reticulocyte count is usually a major initial clue. Examination of the peripheral smear may suggest the presence of myelophthisis, which is associated with teardrop cells and other morphologic red cell abnormalities (Fig. 23-8).

Secondary causes of bone marrow failure are sometimes readily recognizable. Most common among these is the anemia associated with uremia. A frequent dilemma in diagnosing uremia as the cause of anemia is the poor correlation between the serum creatinine level and the degree of anemia seen in the patient. This probably reflects the lack of a direct correlation between the loss of erythropoietin production and loss of glomerular function in the failing kidney. [31] [32] With the availability of erythropoietin assays it is now possible to assess the impact of renal dysfunction on RBC production. Individuals with anemia secondary to renal failure have low erythropoietin levels; virtually all other anemic persons have normal or elevated levels.

Many endocrine disorders, including hypothyroidism, hypoadrenalism, and hypopituitarism, are associated with mild anemia. [33] Although hypothyroidism may cause a macrocytic anemia, it is more commonly associated with a normocytic anemia.[34] Hyperthyroidism is also associated with anemia. Serum tests of endocrine function should be performed in keeping with the clinical picture of the patient.

The anemia in patients with human immunodeficiency virus (HIV) infection is multifactorial and is discussed in detail in Chapter 154 . The bone marrow commonly shows features compatible with the anemia of chronic disease. Other findings include plasmacytosis, lymphoid aggregates, and evidence of granulomatous disease in the marrow.[35]

Approach to Anemia with Increased Red Cell Turnover

Patients with anemia and an elevated reticulocyte count usually have adequate marrow function. The red cell turnover rate is increased, owing to acute blood loss, RBC sequestration, or hemolysis. If the reticulocyte response is adequate, bone marrow examination usually is not necessary in these patients because the disease process is extrinsic to the marrow. The initial evaluation should also establish whether there is evidence of acute bleeding or an enlarged spleen. The remainder of the evaluation is directed toward determining the cause of the hemolytic diathesis.

Patients who have spherocytes in the peripheral blood should be studied for the presence of warm antibody-mediated immune hemolysis by a direct and an indirect Coombs test.[36] Patients with a negative Coombs test should be tested for the presence of cold agglutinins and for other, rarer immune hemolytic disorders such as paroxysmal cold hemoglobinuria (see Chap. 34). [37] [38]

The evaluation of nonimmune hemolytic disorders is guided by the peripheral blood smear findings and the clinical history

APPROACH TO ANEMIA WITH ELEVATED RETICULOCYTE COUNT

Differential diagnosis

- Acute blood loss
- Splenic sequestration
- Hemolysis
  - Immune hemolytic anemia
  - Mechanical hemolysis
    - Valve hemolysis
    - Microangiopathic hemolytic anemia (DIC, TTP)
  - Hereditary hemolytic anemia
    - Hemoglobinopathies
    - Enzyme defects: G6PD deficiency, pyruvate kinase deficiency, etc.
    - Membrane defects: spherocytosis
    - Unstable hemoglobins
  - Acquired membrane defects: PNH, spur cell anemia
  - Infection-related hemolysis: *Clostridia* infection, malaria, babesiosis

Laboratory evaluation

- Examination of the peripheral blood smear for evidence of spherocytes, microangiopathic changes, features of hemoglobinopathies, bite cells
- Urinary hemosiderin
- Direct and indirect Coombs test
- Cold agglutinin titer
- Appropriate further tests as indicated by preliminary evaluation: hemoglobin electrophoresis, G6PD screen, Heinz body preparation, isopropanol stability test, P_{50} , sucrose lysis test, bacterial cultures, examination of smear for malaria, babesiosis, bartonellosis

EVALUATION OF HEMOLYTIC ANEMIA

The evaluation of hemolytic anemia can be guided by the findings in the peripheral blood smear. The smear may allow the crucial distinction to be made between immune and nonimmune hemolysis; this determination will then guide further evaluation. Spherocytes and microspherocytes are classic markers of immune hemolytic anemia; fragmented RBCs are markers of intravascular, nonimmune hemolytic anemia. Since extravascular hemolysis within the spleen does not result in hemoglobinemia, hemoglobinuria, or hemosiderinuria, detection of any of these implies an intravascular hemolytic process. Acute severe intravascular hemolysis may cause detectable hemoglobinemia, which can be seen on visual inspection of the patient's plasma. Hemoglobinuria may be detected with recent intravascular hemolysis. Chronic intravascular hemolysis can be detected by assays for urinary hemosiderin.

(see Chap. 35). Microangiopathic changes in the peripheral blood smear should be viewed in the clinical context in which they occur. Low-grade anemia in a patient with a prosthetic heart valve is probably due to a mechanical hemolysis, which may be chronic and insignificant, but an acute increase in hemolysis from a prosthetic valve should lead to evaluation for perivalvular leak or other valvular dysfunction. [39] Other causes of microangiopathic changes, specifically disseminated intravascular coagulation (DIC) and thrombotic thrombocytopenic purpura (TTP), usually present as acute medical emergencies. [40] [41] In both settings the platelet count is usually decreased. The distinction can be made by clinical evaluation for evidence of a basis for DIC and by the presence of other markers of TTP, such as fever, neurologic dysfunction, or renal insufficiency. The diagnosis of DIC can be supported by elevations in the prothrombin time (PT) and partial thromboplastin time (PTT), which are most often normal in TTP. Low-grade chronic DIC, as may occur with malignancy, may require more extensive evaluation of fibrinogen and fibrin degradation products. [42]

The peripheral smear will also provide ample evidence for most hereditary hemolytic anemias. Sickle cell anemia (see Chap. 30) and severe thalassemia are usually readily diagnosed from a peripheral blood smear, and appropriate follow-up with hemoglobin electrophoresis will confirm the diagnosis. Hereditary membrane defects that give rise to spherocytosis or elliptocytosis are also diagnosed by examination of the peripheral smear (see Chap. 33).

Enzyme defects (see Chap. 32) of the RBC, most commonly G6PD deficiency, may manifest quite late in life, usually in the setting of an acute oxidant stress. [43] Evidence of a disorder in the peripheral blood smear is detectable as bite cells, which reflect splenic conditioning of red cells containing precipitated hemoglobin. [44] If this diagnosis is suspected, a Heinz body smear should be done to look for precipitated hemoglobin in circulating red cells, and G6PD levels should be determined. However, in the setting of an acute hemolytic episode, the circulating young red cells will have higher levels of G6PD, and most or all of the older cells with a very low G6PD level will have been cleared. Consequently, both the Heinz body smear and the G6PD assay may be negative. In this case the G6PD level should be

reassayed when the patient has recovered from the acute hemolytic episode.

Heinz body anemia is also seen in patients with unstable hemoglobins (see Chap. 31).[45] [46] The diagnosis can sometimes be made by hemoglobin electrophoresis, although many unstable hemoglobins are electrophoretically normal. Some show decreased stability in isopropyl alcohol, and others show an altered oxygen dissociation curve.

Other rare causes of nonimmune hemolysis include paroxysmal nocturnal hemoglobinuria (PNH) (see Chap. 20), which results in an acquired membrane defect in the red cell, thereby predisposing the cell to complement-mediated lysis. [47] Patients with PNH may be diagnosed by analysis of acid hemolysis, sucrose lysis, or cell surface marker. The leukocyte alkaline phosphatase score in patients with PNH is also low. Another acquired membrane defect that can be readily diagnosed from the peripheral blood smear is spur cell anemia, which is seen in patients with advanced cirrhosis. The smear reveals striking acanthocytosis; hemolysis results from splenic destruction of the abnormal red cells. [48]

Toxic hemolysis can result from parasitic and bacterial infections. Malaria, babesiosis, and bartonellosis cause hemolysis by direct parasitization of RBCs (see Chap. 35).[49] [50] [51] The appropriate diagnosis can be made from smears of the peripheral blood; in malaria and babesiosis, thick smears are frequently necessary to visualize the parasites. Dramatic, overwhelming hemolysis may be seen in patients with clostridial sepsis. The course of these patients is usually one of fulminant hemolytic anemia, shock, and death even before the bacterial diagnosis can be made. [52]

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ANEMIA IN THE CHILD

The classification of anemia (Greek, without blood) can be based on physiologic, morphologic, or etiologic factors. The prospective approach to any individual patient, however, requires integrating the history and physical examination findings with results of laboratory tests (blood count, red cell indices, reticulocyte count, RBC morphology, specific tests [e.g., Coombs], hemoglobin electrophoresis, etc.), all directed toward determining the underlying disease or process. This section discusses only the special aspects of evaluating anemia in children.

The most straightforward approach to the diagnosis of anemia in both children and adults considers several broad categories: (1) decreased production of RBCs or blood loss; (2) size of RBCsnormal (normocytic), too large (macrocytic), or too small (microcytic); (3) knowledge of the relative frequency of the causes of anemia at various ages; and (4) an appreciation that the diagnosis of anemia depends on normal values related to the patient's age, sex (Table 23-3 (Table Not Available)), [53] cardiopulmonary status, and residence. Children from 6 months to 12 years of age appear anemic compared with adults, but children have higher concentration of 2,3-diphosphoglycerate and ATP in their RBCs, with a corresponding shift to the right of the oxygen dissociation curve. Increased tissue oxygenation compensates for the lower hemoglobin concentrations. The apparent anemia is thus a physiologically appropriate adaptation of the red cell mass to the increased efficiency of oxygen delivery.

The approach to a child with anemia differs from that for an adult in several ways, because of factors related to (1) age differences in normal values for hemoglobin/hematocrit (Hb/Hct) (Table 23-3 (Table Not Available)); [53] (2) the much lower incidence of neoplastic disease in childhood as either a direct or an indirect cause of anemia (<7,000 of the 1,000,000* new cases of cancer each year in the United States occur in individuals <15 years of age); (3) iron deficiency, which is almost always secondary to nutritional factors in children and requires less intensive follow-up evaluation; (4) the relative rarity of alcoholism and its related liver disease as an underlying problem in children; (5) anemia associated with thyroid disease, which is much less common in childhood; (6) the rarity of cardiovascular disease other than congenital heart disease, so that valve replacement, malignant

AN APPROACH TO THE DIFFERENTIAL DIAGNOSIS OF ANEMIA IN THE NEWBORN

TABLE 23-3 -- Red Blood Cell Values at Various Ages: Mean and Lower Limit of Normal (2 SD)

(Not Available)

Adapted from Dallman and Siimes,[53] with permission, as appeared in Oski FA: Pallor. In Kaye R, Oski FA, Barness LA (eds): Core Textbook of Pediatrics. 3rd ed. JB Lippincott, Philadelphia, 1989, p. 62, with permission.

hypertension, and the use of certain drugs are usually not factors; and (7) the frequent difficulty of obtaining adequate specimens for evaluation from newborns and neonates, especially for hemolytic processes such as pyknocytosis. Clinically significant anemia in childhood is more often associated with a primary hematologic abnormality (e.g., hypoplastic or hemolytic anemia), whereas in adults, anemia is more often a secondary manifestation of underlying illness. It is often emphasized that anemia is a symptom, not a disease; this is equally true in children, but the primary cause is in the hematopoietic system more frequently in children than in adults.

Anemia of clinical importance is relatively uncommon in children. When it does occur, the patient's age is often a helpful guide in identifying the underlying process. In the newborn, anemia is most often associated with blood loss or hemolysis. During the neonatal period and early infancy, pure red cell aplasia must be considered, and blood loss becomes a less important cause. Physiologic anemia also develops during this time and is accentuated in preterm infants (Fig. 23-12).[54] [55] [56] [57] From 6 months through 2 years, nutritional anemias and those associated with acute inflammation predominate, after which bone marrow infiltration (e.g., acute lymphocytic leukemia) becomes an important factor. Nutritional deficiency, in particular iron deficiency, becomes a major consideration again during adolescence.

Figure 23-12 Hemoglobin concentration and reticulocyte counts in premature and term infants. Mean values (red line) and 95% confidence intervals are indicated for each of three birth weight categories. (Hemoglobin values are from Lundström et al. [55] and Saarinen and Siimes,[56] reticulocyte values are unpublished data on the same infants from whom hemoglobin values were derived.) *(From Dallman,[57] with permission.)*

FEATURES OF THE PERIPHERAL BLOOD SMEAR OF DIAGNOSTIC IMPORTANCE FOR ANEMIA INTERPRETATION

Red Blood Cell Morphology	Interpretation
Normochromic normocytic	Acute inflammation
Hypochromic microcytic	Iron deficiency, thalassemias, lead poisoning, vitamin B_6 deficiency, sideroblastic processes, chronic inflammation
Macrocytic	Normal newborn, shift cells (reticulocytes), folate or vitamin B_{12} deficiency (macro-ovalocytes)
Target cells	Thalassemias, hemoglobins C, E, and S, liver disease, abetalipoproteinemia, postsplenectomy
Basophilic stippling	Hemolytic anemias, iron deficiency, thalassemias, lead poisoning
Heinz bodies	Normal newborn, hexose-monophosphate shunt abnormalities (e.g., G6PD deficiency, unstable hemoglobins, postsplenectomy)
Howell-Jolly bodies	Splenic hypofunction or absence, megaloblastic anemia
Teardrop cells	Bone marrow infiltration (leukoerythroblastosis)
Spherocytes	ABO incompatibility, G6PD deficiency or other hemolytic anemias, hereditary spherocytosis, hypophosphatemia
Schistocytes	Thalassemia, microangiopathic process (DIC)
Nucleated RBCs	Normal for first several days; hemolytic anemia, acute blood loss, or metaplasia
Polychromasia	Shift cells

Children who are anemic infrequently present in extremis; thus, there is ample opportunity to review the history (Table 23-4) and physical examination findings before ordering laboratory tests. In addition, patients who are anemic (in this section, a hematocrit or hemoglobin value 2 SD below normal for age is taken as the definition of anemia) may be functionally normal with respect to oxygen delivery in the microcirculation because of a compensatory shift to the right of the oxygen disassociation curve.

The history can often help focus on a diagnosis, but the physical examination findings are more often nonspecific. In children, signs of anemia include pallor (often misleading), sallow hue, color of the mucous membranes, jaundice, tachycardia, palpitations, wide pulse pressure, fatigue, dyspnea, poor feeding, weight gain, or other evidence of cardiac decompensation or decreased oxygen transport. Bruising, petechial and overt bleeding, splenomegaly, other abdominal organomegaly, and lymphadenopathy may point to an underlying process responsible for the anemia. In children, it is important to be alert to the specific dysmorphic features of Diamond-Blackfan and Fanconi anemias, vascular and skin changes of sickle cell anemia and sickle C disease, and the facies of -thalassemia major, since these congenital defects usually become symptomatic early in life.

The history is particularly important when deciding which patients should undergo further evaluation, especially when deviations of Hb/Hct from the normal range are small. Mild infections are common and recurrent during childhood, especially during the first several years. These mild infections can result in a transient decrease in Hb/Hct of sufficient magnitude

TABLE 23-4 -- Historical Factors That May Be of Importance in the Diagnosis of Anemia in Children

Factors	Newborn1 mo	1 mo1 yr	Older Infants Children, and Adolescents
Prematurity	Blood loss due to procedures, vitamin E deficiency, anemia of prematurity may respond to erythropoietin	Predisposes to iron deficiency	
Maternal			
Drug ingestion			
Dilantin Warfarin	Bleeding due to hypothrombinemia, treated with vitamin K		
Diuretics	Bleeding due to thrombocytopenia		
Sulfa, etc.	Hemolysis due to G6PD deficiency		
Infections			
Toxoplasmosis Syphilis Herpes	Bleeding due to thrombocytopenia or anemia secondary to red cell aplasia, hemolysis, or inflammation		
Rubella Hepatitis Cytomegalovirus Group B streptococcus			
Mechanical			
Placenta previa			
Abruptio placentae	Blood loss	Predisposes to iron deficiency	
Precipitous delivery			
Amniocentesis			
Cesarean section	Fetomaternal hemorrhage		

Hydralazine			Coombs test positive but rarely produces anemia
Socioeconomic		Pica: lead	
Prevalence of intoxication of iron deficiency is inversely related to status			
Systemic illnesses			
Cardiac disease: prosthetic valves or intracardiac patches		Waring blender syndrome and hemolysis	
Endocarditis			Anemia of chronic disease
Gastrointestinal			
Celiac disease, regional enteritis, ileal resection, chronic atrophic gastritis			Iron and vitamin B_{12} deficiency
Ulcerative colitis and Osler-Weber-Rendu disease			Iron deficiency
Cystic fibrosis		Vitamin K and E deficiency and anemia responsive to protein enrichment	
Liver disease			
Active processes		Shortened red blood cell survival and increased Heinz-body formation	
Renal disease			
Uremia		Shortened red blood cell survival Decreased red blood cell production	
Bloody diarrhea		Microangiopathic process (e.g., hemolytic uremic syndrome or thrombotic thrombocytopenic purpura)	
Dialysis			Folate deficiency
Hypothyroidism		Normochromic normocytic anemia with occasional irregularly contracted cells	
Collagen vascular diseases		Anemia of chronic disease with or without iron deficiency	
Infectious diseases			
Mild infections (e.g., otitis media, pharyngitis, gastroenteritis)		Transient mild decrease in hemoglobin/hematocrit (beware of evaluating mild anemias in children with intercurrent infections/inflammation)	
Sepsis (bacterial, viral, *Mycoplasma*)		Hemolytic anemias	

to suggest the presence of iron deficiency anemia, long considered the most likely cause of mild anemia in childhood, [58] [59] [60] even though iron deficiency anemia has recently become much less common in both low-income families and middle-class families. [61] [62] In the latter, a 3% prevalence of iron deficiency anemia was found in healthy children in Minneapolis. [62] The very low prevalence is almost identical to the 2.4% of normal individuals whose Hb/Hct falls to <2 SD in a normal distribution. This makes screening, further evaluation, and subsequent treatment uncertain on the basis of Hb/Hct alone. The decision concerning further evaluation in the setting of mild anemia should include the historical factors listed in Table 23-4 , socioeconomic status, and temporal relationship to mild infections. This will facilitate the differentiation of iron deficiency, thalassemia trait, and acute inflammation, the most common causes of anemia in childhood.

The basic laboratory evaluation for anemia, including accurate interpretation of the blood smear, often confirms the underlying process or suggests what further tests need to be done.

Collection and processing of specimens are considered routine but may introduce confounding variables, especially in very young children and in those who are quite ill. Finger sticks or heel sticks often produce Hb/Hct values greater than values in simultaneously obtained venous samples as a result of stasis of blood in the finger or heel. The extremity must therefore be warmed before the blood sample is drawn. An erroneously low Hb/Hct value can also be obtained if excessive pressure is used or the extremity is milked, thereby diluting the sample. Perfusion problems related to hydration status, cardiac disease, or infection must also be considered.

If the hematocrit is determined after capillary tube centrifugation, plasma trapping may lead to slightly higher values than those obtained by automated electronic methods. This difference can be accentuated by extreme anisocytosis, as in sickle cell disease, severe iron deficiency, and thalassemia.

In practice, the physician usually finds a hemoglobin or hematocrit value that is below the accepted range of normal. If these values are obtained in a laboratory with electronic cell counting, then red cell indices, RBC count, WBC count, and often the platelet count are included. The smear may or may not be noted. The physician must then decide whether to order further investigations (e.g., the free erythrocyte protoporphyrin is a simple, rapid alternative approach to detecting iron deficiency) or, if the history is consistent and there has been no infection within the previous month, undertaking a therapeutic trial of iron (3 mg/kg/day of ferrous sulfate or its equivalent for 1 month) and rechecking the hemoglobin at that time. An increase in hemoglobin of at least 1 g/dl after treatment indicates that iron deficiency was the correct diagnosis. If the patient is unresponsive or the response is incomplete, compliance must be reviewed (color of stools and amount of ferrous sulfate remaining in the bottle), RBC indices obtained, and the smear reviewed. This latter approach is justified because iron deficiency is still a frequent cause of anemia in children.

Microcytosis out of proportion to the reduction in hemoglobin concentration suggests thalassemia trait, which can be confirmed by hemoglobin electrophoresis. The hemoglobin electrophoresis should be deferred until iron stores are deemed adequate, since hemoglobin A_2 production may be affected. In a patient with microcytic red cells, a normal hemoglobin A_2 value, and adequate iron homeostasis, -thalassemia trait is inferred. This inference is strengthened by a brilliant cresyl blue preparation that is positive for Heinz bodies. Depending on location and time of year, lead poisoning is an important consideration, not so much for the correction of anemia but for the prevention of neurologic sequelae. A history of pica and the presence of basophilic stippling and elevated free erythrocyte protoporphyrin levels would suggest lead intoxication.

Careful examination of the peripheral smear often indicates abnormalities that point to a specific diagnosis or underlying

TABLE 23-5 -- Differentiation of Diamond-Blackfan Anemia from Transient Erythroblastopenia of Childhood

Parameter	Diamond-Blackfan	Transient Erythroblastopenia
Frequency	Rare	Common (? increasing)
Age of diagnosis	<1 yr (90% <6 mo)	0.54 yr
Etiology	Probably genetic	Acquired
Antecedent history	None	Viral illness
Physical abnormalities	Present (25%)	Absent
Course	Prolonged transfusion or steroid dependence	Spontaneous recovery in weeks to months
Laboratory		
MCV	Macrocytic	Normocytic
Hemoglobin F	Increased	Normal[a]
i Antigen	Present	Absent[a]
Fetal pattern of RBC glycolytic and HMP shunt enzymes	Present	Absent[a]
Elevated RBC ADA activity	Usually present	Usually absent

Data from Glader[63] and Lanzkowsky.[64]

[a]During recovery phase of transient erythroblastopenia, fetal RBC features may be detected.

abnormalities. Perhaps the most difficult group of patients are those with hemolytic anemias and red cell aplasia. In these patients and in those with hemoglobinopathies, critical information can often be obtained by examining the hemograms and peripheral blood smears from the parents. For these patients, the reticulocyte count, corrected as the reticulocyte index, is an important guide to further evaluation.

The three major causes of pure red cell aplasia in children are Diamond-Blackfan anemia, transient erythroblastopenia of childhood (TEC), and acquired hypoplastic anemia associated with chronic hemolysis.[63] Diamond-Blackfan anemia and transient erythroblastopenia are differentiated by the features listed in Table 23-5 .[63] [64] Aplastic crises associated with chronic hemolytic anemias are characterized by the underlying process, the precipitous decrease in reticulocytes and hemoglobin concentration, and the frequent association of human parvovirus B19 (see Chap. 19).

The hemolytic anemias are a complex group of disorders associated with an elevated reticulocyte index (Table 23-6).[65] The

TABLE 23-6 -- Features of Common Congenital Hemolytic Anemias

Diagnosis	Hemoglobin Electrophoresis Pattern		Pattern of Inheritance	Predominant Ethnic Group	Morphology
	Cord Blood	Adult			
Sickle cell diseases					
Sickle cell anemia	FS	SS	Autosomal recessive	Africans, black Americans, Arabs, East Indians, Hispanics, Mediterraneans	Sickle cells, target cells, elliptocytes, nucleated red blood cells, reticulocytosis
S--thalassemia	FSA	SA (A_2 >3.5%)	Autosomal recessive	Africans, black Americans, Arabs, East Indians, Hispanics, Mediterraneans	Microcytes, occasional nucleated RBCs, reticulocytosis, variable sickle cells
SC	FSC	SC	Autosomal recessive	Africans, black Americans, Arabs, East Indians, Hispanics, Mediterraneans	Target cells, rare sickle cells, mild reticulocytosis
CC disease	FC	CC	Autosomal recessive	West Africans, black Americans	Target cells
Unstable hemoglobin	FA	AA	Autosomal recessive	Varies with type	Occasional schistocytes, spherocytes, basophilic stippling, and polychromatophilia (Heinz bodies after brilliant cresyl blue stain, accentuated post splenectomy)
-Thalassemia major and intermedia	FF	FF (A_2 >3.5%)	Autosomal recessive	Mediterraneans, Chinese, East Asians, East Indians	Hypochromic macrocytes, microcytes, basophilic stippling, target cells, ovalocytes, nucleated RBCs, and marked poikilocytosis, anisocytosis, and reticulocytosis
Hb H disease	FA and Bart's >15%	AH	Autosomal recessive	Asians, Filipinos, Indonesians, occasionally Mediterraneans, rarely Africans	Microcytes, target cells (Heinz bodies after brilliant cresyl blue stain)
Hydrops fetalis	Barts	Nonviable			
Spherocytosis	FA	AA	Autosomal dominant	Northern European whites	Variable number of spherocytes (usually >10%), polychromatophilia
Elliptocytosis	FA	AA	Autosomal dominant	Various	Increased number of elliptocytes (may present in infancy as pyknocytosis)
Pyropoikilocytosis	FA	AA	Autosomal recessive	Black and occasionally white Americans	Bizarre poikilocytes
Stomatocytosis	FA	AA	Autosomal recessive and dominant forms	Unknown	1050% stomatocytes, reticulocytosis

Glucose-6-phosphate	FA	AA	X-linked	Africans, black Americans, Mediterraneans, Asians	Spherocytes, nucleated RBCs, polychromatophilia
Pyruvate kinase deficiency	FA	AA	Autosomal recessive	Northern Europeans	Macrocytosis, polychromatophilia

Adapted from Addiego et al.,[65] with permission.

correct diagnosis in an individual patient may be apparent after the history, physical examination, and routine blood studies. Frequently, however, additional tests, including hemoglobin electrophoresis, haptoglobin determination, red cell osmotic fragility, specific assays of RBC enzymes, acid hemolysis test (Ham) or sugar water test, and tests for unstable hemoglobin, must be performed as outlined in other chapters.

Examination of the bone marrow is important in the diagnosis of relatively few kinds (and causes) of anemia in childhood. These conditions include megaloblastic, sideroblastic, aplastic, and dyserythropoietic anemias; acute leukemia; neuroblastoma; and lipid storage diseases.

Anemia is always an important finding that should prompt the physician to consider what underlying process may be responsible. In children, the most common causes of mild anemia, iron deficiency, acute inflammation, and thalassemia trait can be recognized and appropriately treated after a thorough medical history has been taken and a physical examination and routine laboratory tests have been performed. Acquired acute anemias often produce diagnostic and treatment difficulties that demand the critical application of specialized laboratory tests and clinical expertise for appropriate management.

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Chapter 24 - Anemia of Chronic Diseases

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The long-term realization that diverse systemic illnesses may cause anemias with similar features has led to the concept of an anemia of chronic disease (ACD). This anemia, first described in chronic infections, was originally thought to reflect a block in iron utilization by red cells. [1] It is now appreciated that ACD may be found in a variety of inflammatory conditions,[2] including infection, rheumatologic disorders, and cancer, and that its etiology is multifactorial, involving not only abnormalities in iron utilization but a decreased life span of erythrocytes, a direct inhibition of hematopoiesis, and a relative deficiency of erythropoietin. Since the late 1980s, the biology and the role of cytokines in ACD has been better characterized, and treatment options have been explored. It is thus now realistic to regard ACD as a distinct pathophysiologic entity.

DESCRIPTION AND EPIDEMIOLOGY

Anemia of chronic diseases is an anemia of underproduction that is usually normocytic, normochromic, and relatively mild, with a hematocrit above 30%. [1] However, the anemia can be severe and the mean corpuscular volume (MCV) may be reduced in approximately 30% of patients[3] ; an MCV as low as 61 fl has been described.[4] ACD is one of the most common causes of anemia; over a 2-month period of observation, 52% of hospitalized patients with anemia who were not iron deficient, hemolyzing, or suffering from a hematologic malignancy met laboratory criteria for ACD [5] ; it has been diagnosed in up to 27% of outpatients with rheumatoid arthritis.[6] This anemia was first described in association with infection, [7] and can be induced experimentally by infection or sterile inflammation. [1] [8] Most hospitalized patients with ACD do have active infection, inflammatory conditions, or malignancy. However, others may have alcoholic liver disease, congestive heart failure, thrombosis, chronic pulmonary disease, diabetes, or a variety of medical problems. [5]

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ETIOLOGY AND PATHOGENESIS

Anemia of chronic diseases is marked by low serum iron, but, in contrast to iron deficiency anemia, total iron stores are normal or elevated; these findings appear to parallel disease activity.[9] Early studies emphasized the role of iron in the pathogenesis of ACD.[8] Rats injected intraperitoneally with *Escherichia coli* lipopolysaccharide absorbed significantly less ^{59}Fe in 96 hours compared with control animals.[10] Endotoxin has also been shown to shorten ^{59}Fe serum half-life in rats.[11] Other studies using trace quantities of labeled iron have shown no direct impairment of iron incorporation into red blood cells, suggesting that inflammation might impair the release of iron from storage sites, thus decreasing plasma iron concentrations and also decreasing iron available for hemoglobin synthesis; indeed, sterile inflammation in a dog model impairs only the reutilization of iron from senescent red cells, suggesting that inflammation leads to a defect in the release of iron from tissues to the plasma transferrin pool.[12] Some early studies suggested that increased ineffective erythropoiesis and increased iron turnover occurred in patients with rheumatoid arthritis,[13] but other studies of patients with rheumatoid arthritis and a diagnosis of ACD show that iron turnover is decreased compared with either normal or iron-deficient control subjects.[14] There are several hypotheses of how such a defect in iron turnover could occur in ACD. Decreased transferrin receptors both in the serum[15] and on erythroblasts[16] have been noted in patients with ACD. Others have suggested that increases in the release of lactoferrin from neutrophils[17] or, more convincingly, in the synthesis of apoferritin,[18] lead to a pool of iron trapped in storage form, unavailable for hemoglobin synthesis (Table 24-1).[8]

In theory, any block in iron reutilization would be made more severe with shortened erythrocyte survival. Rats given endotoxin have a reduced erythrocyte half-life,[11] and a mildly shortened erythrocyte half-life has also been noted in patients with rheumatoid arthritis and ACD. However, most studies have found the decrease in survival to be mild. This mild decrease, even in combination with a reduction of hemoglobin precursors, cannot account for the extent of anemia found in most patients. It is probable that other compensatory hematopoietic factors are not functioning in ACD. Indeed, it is now thought that many of the abnormalities in iron are secondary to changes in hematopoiesis.

Two documented changes in hematopoiesis include (1) a direct inhibition of hematopoiesis, and (2) a relative deficiency of erythropoietin. The inhibition of hematopoiesis found in ACD is thought to be due to soluble factors present in the perturbed bone marrow microenvironment, as discussed in greater detail in the section on the biology of the disease. Studies have shown that removal of bone marrow-adherent cells (mostly macrophages and monocytes) from patients with ACD leads to increased erythroid colony formation; this can be reversed by coculture of ACD adherent cells but not by adherent cells from control marrow.[19] In a study of patients with rheumatoid arthritis with ACD, the numbers of erythroid progenitors in control and anemic patients were similar (not accounting for the difference in anemia between the two groups). Serum from patients with rheumatoid arthritis with anemia produced decreased erythroid burst-forming unit (BFU-E) proliferation, whereas serum from non-anemic arthritic patients did not.[20] Peripheral blood mononuclear cells from patients with rheumatoid arthritis also suppress in vitro BFU-E growth.[21]

Most of the supporting data for a direct inhibition in hematopoiesis are derived from in vitro studies. Data from more clinically relevant studies have clearly documented that in many chronic diseases associated with anemia, there is a *relative* deficiency of erythropoietin. Although serum erythropoietin levels may be increased, they are not as elevated as they should be for the degree of anemia present. In a study of 81 patients with solid tumors who exhibited laboratory data compatible with ACD without marrow involvement by tumor, the erythropoietin levels were higher than in control subjects without anemia, but were only half of what are typical of subjects with similar

TABLE 24-1 -- Etiologies of Anemia of Chronic Disease

1.	Block in reutilization of iron by erythrocyte
2.	Shortened erythrocyte survival
3.	Direct inhibition of erythropoiesis
4.	Relative deficiency of erythropoietin

hematocrits due to iron deficiency. In addition, the normal inverse relation between hemoglobin and erythropoietin level was not seen.[22] In 41 anemic patients with rheumatoid arthritis, 14 of whom had plentiful iron on bone marrow aspirate, serum erythropoietin levels for all rheumatoid patients were lower than for those with simple iron-deficiency anemia and comparable hemoglobin levels.[23] Serum erythropoietin has also been shown to be inappropriately low in human immunodeficiency virus (HIV)-positive patients with normochromic normocytic anemia,[24] and in lung transplant recipients.[25]

There are reports of diabetics with anemia, normal renal function, and lower-than-expected erythropoietin levels; this has been hypothesized to be secondary to diabetic neuropathy,[26] although there may also be a direct role of insulin on stem cells.[27] Up to 40% of patients with chronic liver disease are anemic, and although this anemia is clearly multifactorial, some studies have found a blunted erythropoietin response to anemia seen in cirrhosis.[28] However, at least one study of veterans with anemia and alcoholic liver disease found a serum erythropoietin level appropriate for the degree of anemia.[29] As is discussed later, the fact that exogenous erythropoietin can correct ACD supports the role of a relative erythropoietin deficiency as one of its main causes.

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BIOLOGY AND MOLECULAR ASPECTS

An understanding of the role that inflammatory cytokines play in many of the diseases associated with ACD has suggested an underlying mechanism for much of its pathophysiology, including decreased erythrocyte survival, increased apoferritin synthesis, direct inhibition of hematopoietic progenitor growth, and an inadequate erythropoietin response to anemia.

Increased serum levels of cytokines, particularly interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), and interferon- (INF-) and INF-, have been noted in many inflammatory diseases, including acquired immunodeficiency syndrome (AIDS) and other infections, rheumatoid arthritis, and cancer. [30] TNF, as the name implies, has been clearly shown to be elevated in patients with both solid and hematologic malignancies, as have several other cytokines. [31] Peripheral blood mononuclear cells from patients with rheumatoid arthritis secrete TNF-. [21] TNF- levels correlate with disease activity and the degree of anemia in patients with rheumatoid arthritis. [32]

Cytokines can induce many of the intestinal and extraintestinal symptoms of inflammatory bowel disease; the serum and intestinal mucosal concentrations of many cytokines, especially IL-1 and IL-6, have been found to be increased in inflammatory bowel disease, [33] with antigenic levels of IL-6 correlating with disease severity. [34] An increase in INF- mRNA in mucosal T cells has been described in Crohns disease. [35] In addition, patients with inflammatory bowel disease may have defective production of IL-1 receptor antagonists. [36]

In 37 pediatric patients infected with both HIV and *Mycobacterium avium-intracellulare*, TNF was markedly elevated and there was an association between TNF and anemia. [37] INF- has also been noted to be elevated in those with AIDS. [38] Erythrocytes infected with various strains of malaria, as well as peptides of *Plasmodium falciparum*, stimulate macrophages to secrete TNF, and elevated levels of several cytokines have been found in malarial infections. [39]

The fact that cytokines are thought to play such an important role in so many diseases associated with anemia suggests an association between inflammatory cytokines and anemia.

Cytokine-Induced Decreases in Red Cell Survival

Several studies suggest a role for decreased erythrocyte survival in the pathogenesis of the ACD. Early in vivo and in vitro experiments suggested that fever itself within a few days can induce rheologic changes in erythrocytes and lead to increased destruction, with as much as a 15% decline in red cell mass. [40] More recent evidence suggests that cytokines, the cause of fever in many of the diseases associated with ACD, may also alter red cell kinetics. For example, rats receiving chronic intraperitoneal injections of IL-1 and TNF showed a twofold decline in ^{59}Fe radioactivity, suggesting decreased erythrocyte survival; this effect has been postulated to involve cytokine-induced macrophage and reticuloendothelial system activation. [11]

Cytokine-Induced Abnormalities in Iron Metabolism

Many of the cytokines implicated in ACD appear to have in vivo effects on iron metabolism. Nude mice inoculated with Chinese hamster ovary cells that had been engineered to secrete TNF- show an almost 60% decline in serum iron after 3 weeks while maintaining normal bone marrow stores of iron. [41] Rats injected with IL-1 or TNF experienced a 40% drop in serum iron, and TNF also caused a significant decrease in iron incorporation into erythrocytes. [11] These findings are consistent with the observation that iron reutilization is defective in ACD. [12] TNF has also been shown to increase radiolabeled iron uptake by peritoneal macrophages without an increase in iron release, [42] suggesting that macrophage sequestration of iron may be cytokine induced.

Molecular evidence suggests a direct cytokine effect on iron metabolism. Even though IL-1 has no effect on ferritin transcription, it increases the synthesis of ferritin in human hepatoma cells by an increase in mRNA translation. [43] Increased translation depends on a 5 untranslated region, distinct from the well known iron-responsive element, but similar to a 38-nucleotide consensus sequence found in other IL-sensitive acute-phase reactants (see Chap. 26). [44] The increase in ferritin synthesis occurs without increased cellular transferrin receptor transcription or expression, and involves no new iron influx; [43] IL-1 could thus lead directly to the creation of an intracellular iron pool that is not available for hemoglobin synthesis.

Cytokines Leading to Direct Inhibition of Hematopoiesis

Recent data demonstrate that cytokines, including IL-1, INF, and TNF, can have a direct inhibitory effect on hematopoiesis. However, almost all of the evidence supporting such a role for cytokines is derived from in vitro experiments. Clinical relevance is unclear.

The role of IL-6 in the direct inhibition of hematopoiesis is controversial. Exogenous IL-6 given to monkeys causes a dose-dependent, mild, and short-lived anemia within four weeks. [45] Some studies have also shown that IL-6 has direct inhibitory effects on stem cells. [46] Other studies, however, have demonstrated that IL-6, elevated in rheumatoid arthritis, has no direct effect on bone marrow hematopoiesis. [47] Juvenile-onset chronic arthritis is *associated* with increased IL-6 and anemia, but this anemia is one of iron deficiency. [48] IL-6 has also been shown to cause bleeding in the rat intestinal wall. [49]

There is much better evidence for a role for INF in the direct inhibition of erythropoiesis. INF- can inhibit highly purified erythroid colony-forming units (CFU-E) from mice spleens in a dose-dependent manner. [50] [51] [52] Marrow stromal cells, retrovirally engineered to secrete INF-, inhibit hematopoiesis in long-term bone marrow cultures by blocking cell cycle progression and inducing apoptosis in CD34+ cells to a much greater degree than even exogenous INF. [53] INF inhibition can be reversed by exogenous dosing with murine INF- receptors. [51] Inhibition by INF- can be reversed in a dose-dependent manner by erythropoietin, a phenomenon also seen in ACD, [51] [54] and partial reversal can be achieved by stem cell factor. [55] The concentration of

INF- required to suppress BFU-E was less than that needed to suppress CFU-E, suggesting the suppression occurs at the earlier stages of erythroid development. [52]

Like INF-, INF- also appears to act directly on CFU-E: inhibition does not require accessory T cells or adherent bone marrow cells. [30] [56] Inhibition by INF- appears to work indirectly. Inhibition by either INF- or INF- is not reversed by erythropoietin. [56]

Tumor necrosis factor serum levels have been shown to correlate with the degree of anemia in the ACD. Erythroid growth is increased in the bone marrow of control and chronic anemia patients by monoclonal antibodies against TNF. [32] Transplantation of a Chinese hamster ovary cell line transfected with the human TNF gene led to anemia in nude mice within three weeks, with a significant decrease in both BFU-E and CFU-E. [41] Exogenous TNF decreases erythroid colony counts. [32] The

inhibition of BFU-E growth sustained by normal bone marrow cultured in the presence of peripheral blood mononuclear cells of patients with rheumatoid arthritis can be reversed with antibodies to TNF-.[21] However, there is evidence that the effect of TNF on hematopoiesis is an indirect one, mediated by the local release of other cytokines, including INF from accessory cells.[57] The inhibitory effect of TNF on CFU-E was completely abated by neutralizing antibodies against INF-, but not antibodies to INF- or IL-1.[30]

The effects of IL-1 also appear to be indirect. Growth of purified CFU-E is inhibited by recombinant IL-1 only in the presence of adherent T lymphocytes. The inhibition can be reversed by antibodies to INF-, suggesting that IL-1 may lead to lymphocyte secretion of INF.[58]

Cytokines Leading to Decreased Erythropoietin Secretion

Cytokines have also been implicated in the blunted, inappropriately low erythropoietin levels seen in ACD in several in vitro experiments. In an erythropoietin-producing human hepatoma cell line, HepG2, grown in diffusion-limited oxygen conditions, IL-1, IL-1, and TNF- significantly lowered erythropoietin production. IL-1 also inhibited erythropoietin production in perfused rat kidneys. These effects were not accompanied by alterations in -fetoprotein, showing that the effect was specific and not secondary to a generalized inhibitory growth effect.[59] Using another hepatoma cell line, Hep3B, hypoxia-driven erythropoietin production was also inhibited with IL-1, IL-1, and TNF- (with IL-1 being the most potent inhibitor) in a dose-dependent and additive manner. IL-6 stimulated erythropoietin production.[46]

As is discussed later, perhaps one of the strongest arguments for a role of erythropoietin in the etiology of ACD is that exogenous erythropoietin can at least partially reverse ACD (although supraphysiologic erythropoietin overcoming a direct inhibition of erythropoiesis is also possible). It has been shown that inhibition by INF- can be reversed by erythropoietin.[54] Moreover, the capacity of monocytes from patients with inflammatory bowel disease to secrete TNF predicts therapeutic response to exogenous erythropoietin.[60]

DIAGNOSIS

Because ACD is a multifactorial disease and is seen in many clinical settings, unequivocal diagnosis is often difficult. Thus, 2570% of patients with rheumatoid arthritis and iron studies consistent with the ACD are also iron deficient. [61] [62] Up to 70% of the anemia associated with rheumatoid arthritis may be multifactorial. [63] Many chronic illnesses are also associated with other factors leading to anemia, including iron and nutritional deficiency, bleeding, hemolysis, renal failure with absolute erythropoietin deficiency, and marrow fibrosis or infiltration

THE DIAGNOSIS OF ACD

The diagnosis of ACD is one primarily of exclusion, and is often difficult. Various laboratory tests have been suggested as helpful, but few of these are practical or have proven value in the general population. ACD occurs in too large a variety of acute and chronic illnesses. The best way to diagnose ACD, at least provisionally, is to document an anemia of underproduction (low reticulocyte index) with low serum iron and transferrin levels and an elevated serum ferritin in the setting of a systemic, usually inflammatory, illness. A thorough search may be necessary to document the precise underlying illness.

Other causes of anemia, such as hemolysis, nutritional deficiency, or sequestration, should be ruled out (see Chap. 23) and a component of iron deficiency should be strongly considered in a patient with a systemic inflammation and a low or normal serum ferritin. Often these other causes of anemia accompany ACD. A bone marrow examination is usually not helpful, but may occasionally be necessary to rule out malignancy (including a myelodysplastic syndrome), infection, or iron deficiency.

and replacement. The role of frequent phlebotomy in the hospitalized patient should also be fully appreciated. [64] As an example, the anemia of renal failure has traditionally not been thought of as an ACD, but rather as an anemia of absolute erythropoietin deficiency and mechanical marrow replacement by fibrosis; however, more recently it has been appreciated that efficient dialysis may lead to improved marrow responsiveness to erythropoietin. [65] This suggests that other factors, perhaps including cytokines, may play a role.

One of the major problems with prevalence studies is that ACD is primarily a diagnosis of exclusion. This may often necessitate an evaluation of the bone marrow to ensure adequate iron stores, to rule out infiltration by tumor, fibrosis, or infection, and to exclude a myelodysplastic syndrome; however, in a study of mostly older patients with an idiopathic mild anemia (10 ± 0.6 g/dl), a bone marrow aspirate and biopsy was found to add little to an extensive negative serological work-up and physical examination.[66]

The clinical setting in which the anemia is found helps with the diagnosis, yet 30% of the time no chronic illness can be found; acute illnesses can also lead to anemia.[5] Some argue that a normocytic/normochromic anemia may occur in otherwise healthy people; normal elderly people have also been noted to have mild anemia.[67]

Even though its etiology is no longer thought to be primarily due to a decrease in iron utilization, the diagnosis of ACD is usually made on the basis of elevated marrow iron stores (usually assessed by serum ferritin), and a low serum iron, transferrin, and total iron-binding capacity. A low serum ferritin associated with anemia is suggestive of iron deficiency. Some argue that a ferritin >50 ng/ml excludes any component of iron deficiency, even in inflammatory states. [68] But a normal or elevated ferritin level is more difficult to interpret because ferritin is an acute-phase reactant. Nomograms correcting ferritin for the degree of inflammation present have been published.[69] Others have shown that in acute inflammation, serum ferritins as high as 3,765 ng/ml can still exist with absent bone marrow iron stores by aspirate.[70] Most investigators maintain that serum iron studies cannot predictably rule out iron deficiency. [71]

Several algorithms have been generated to diagnose or rule out ACD. In 120 anemic patients with inflammatory, infectious, or malignant diseases with bone marrow aspirates to assess iron

stores, the serum ferritin was significantly lower in those who were iron deficient but still elevated (63.7 vs. 212 ng/ml); the combination of serum ferritin <70 ng/ml and a red cell ferritin 4 ag per erythrocyte had a specificity of 0.97 and a positive predictive value of 0.82, although these values were not validated in a prospective study.[62] A three-step algorithm for patients with rheumatoid arthritis and serologic evidence of ACD has been derived: male patients with hemoglobins <11.0 g/dl, and serum ferritin <40 g/l have iron deficiency, and those with MCV >85 fl, or those with an iron saturation >7%, have ACD. This formula led to a correct diagnosis in 89% of patients, but this finding was not independently validated. [61]

In ACD, zinc is incorporated into protoporphyrin IX instead of iron. Zinc protoporphyrin has been shown to be greatly elevated in ACD and to be corrected with treatment of the underlying inflammatory condition.[72] But zinc protoporphyrin levels fail to differentiate between ACD and iron deficiency. The number and affinity of transferrin receptors on erythroblasts have been found to be lower in rheumatoid arthritis patients with ACD, [16] thus differentiating it from iron-deficient anemia. Combining assessment of serum ferritin and plasma transferrin receptor concentration (although concentrations of the serum transferrin receptor appear to be unchanged[15]) with the erythrocyte sedimentation rate has also been suggested as an algorithm in differentiating iron deficiency and ACD in elderly women. [73]

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TREATMENT

The anemia associated with chronic illness is usually mild. Correction is often not necessary, especially if as many contributing factors as possible are reversed. In fact, there may be a teleologic reason that ACD exists: not only has the fever associated with infections been shown to inhibit bacterial growth, but decreased iron concentrations (as seen in ACD) act synergistically with pyrexia to inhibit bacterial growth. [74] The attempt by hosts to withhold iron from invaders has been termed nutritional immunity, and has been postulated to be an adaptive factor of ACD. [75] However, ACD can be severe, and almost by definition those with ACD have comorbidities; optimizing any reversible process, including hemoglobin and oxygen content and delivery, may sometimes have a dramatic beneficial effect. A large, multicenter trial has shown that in anemic cancer patients not receiving cytotoxic therapy, performance and quality of life improve with increased hemoglobin concentrations. [76]

The optimal treatment of ACD is correction of the underlying disease process, if one can be clearly documented. It has been shown that ACD mirrors laboratory [47] [63] and clinical [9] correlates of disease activity and duration of disease in rheumatoid arthritis. Clearly, definitive treatment of chronic diseases is usually difficult. If the anemia is symptomatic or severe, treatment of the anemia itself may be indicated. Iron chelators such as deferoxamine have been shown in some animal studies to have mild effects on ACD, perhaps by decreasing free radicals and inflammation, but other studies have not duplicated this finding. [77] [78] Transfusion therapy may be the most common form of treatment for symptomatic ACD; unfortunately, red cell transfusions are costly, have the potential detriments of infection, alloimmunization, and graft-versus-host disease, and are a limited resource.

The realization that serum levels of erythropoietin, although often elevated in ACD, are not elevated appropriately for the degree of anemia, as well as the observation that inhibition of in vitro models of hematopoiesis by cytokines can be reversed by erythropoietin, [54] have led to several successful trials in the treatment of ACD with recombinant erythropoietin. This may be more cost effective as well as safer than transfusion. [79] A relatively small, multicenter, placebo-controlled trial monitored the effects of erythropoietin in patients with rheumatoid arthritis and laboratory data consistent with ACD. The results showed

TREATMENT OF ACD

Treating ACD is often not necessary if the patient is asymptomatic. However, the anemia may be severe and quality of life may be greatly improved with treatment, even in those patients who believed they felt well with their anemia.

The first priority should be to correct any reversible contributors to the anemia (e.g., iron deficiency, absolute erythropoietin deficiency). Because the extent of ACD mirrors the underlying diseases activity, all efforts should be made to treat the underlying disease.

Although transfusion is obviously the fastest way to reverse ACD, many studies have shown a 4080% response rate to standard-dose erythropoietin injections. This response may take 48 weeks. A low pretreatment serum erythropoietin level and a serum ferritin that decreases with erythropoietin treatment appear to be good predictors of response. Refinement of the approach to using erythropoietin in these disorders is likely to be attempted during the next several years.

a dose-dependent response to 50150 U/kg of erythropoietin three times a week; 11 of 17 patients had a response rate of almost six hematocrit points. [80] Responses can take up to four weeks. [80] [81]

Anemic patients with AIDS who were treated with recombinant erythropoietin had a significantly decreased transfusion requirement, especially if their endogenous erythropoietin level was <500 IU/l; however, all of these patients were also receiving zidovudine, a potential bone marrow suppressant. [82] The use of recombinant erythropoietin has also been shown to increase hemoglobin in a variety of nonhematologic neoplastic diseases, including squamous cell cancer, [83] breast cancer and colon cancer, [84] [85] and a variety of other malignancies. [76]

Response rates to erythropoietin in patients with cancer appear to range from 40% to 80% and take 48 weeks to occur. A response often significantly affects many aspects of quality of life, including mood, level of activity, pain and nausea, and anxiety. [76] [85] Prognostic indicators to predict response of ACD to erythropoietin include a low pretreatment erythropoietin level. [76] [84] Although baseline duration of malignant disease, previous and current cancer treatment, bone marrow involvement, and type of tumor (hematologic vs. solid tumor) were found to be negative predictors of response to erythropoietin treatment, only the absolute change in hemoglobin value, the absolute hemoglobin value, the serum erythropoietin level, and the ferritin level were found to be independent predictors in a multivariate analysis of 80 patients with chronic anemia of cancer. [86] Median survival in those responding was 12.6 months compared with 4.3 months in nonresponders. These investigators derived an algorithm: if after two weeks the serum erythropoietin level is >100 mU/ml and the hemoglobin concentration has not increased by at least 0.5 g/dl, or if after two weeks of treatment the serum ferritin level is >400 ng/ml, then response is very unlikely.

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SUMMARY AND FUTURE DIRECTIONS

Anemia is common in many chronic inflammatory, infectious, and malignant conditions, and is often multifactorial. The anemia is exacerbated by inflammatory cytokines, which are thought to be one of the more important causative factors in ACD. ACD is difficult to diagnose, but can usually be strongly suspected on the basis of the clinical setting, the elimination of

other causes of anemia, a low serum iron and transferrin, and an elevated ferritin. Cytokines such as INF- and TNF have been shown to have wide-ranging effects, including an increase in ferritin that impairs iron availability for hemoglobin synthesis, and direct and indirect inhibition of hematopoiesis. There is also molecular evidence to suggest that cytokines lead to what may be the most important factor in ACD, a relative deficiency in erythropoietin for the degree of anemia found. ACD can often be reversed with recombinant erythropoietin. Further studies are required to delineate better the molecular and cellular pathways by which cytokines might lead to inhibition of hematopoiesis, and to explore the role of cytokine antagonists in treating ACD [87] as well as its underlying causes.

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Chapter 25 - Erythrocytosis

Jerry L. Spivak

Under normal circumstances, the red cell mass is maintained within narrow limits and is relatively constant in a given person, although it can vary between people of the same age and sex by more than 10%. In the presence of adequate nutrients, the red cell mass is in a dynamic equilibrium in which the red cells lost from the circulation each day through senescence are replaced by newly formed ones. Red cell production is regulated by erythropoietin (EPO), a glycoprotein hormone produced primarily in the kidneys in adults. EPO is a highly conserved protein that bears significant homology (23%) only with the hematopoietic growth factor, thrombopoietin.[1] [2] The EPO gene is located on the long arm of chromosome 7 in humans,[3] and it encodes a protein of 166 amino acids, the terminal amino acid of which, Arg 166, is cleaved during the processing of the mature hormone.[4] In keeping with the constancy of the red cell mass in a given individual, the circulating level of EPO also remains constant unless hypoxia, anemia, or sudden blood loss intervenes. Indeed, EPO appears to be such an important regulatory or trophic factor that it is never absent from the plasma, even in the anephric state or with extreme erythrocytosis. This chapter examines the mechanisms leading to erythrocytosis and the role of EPO in this process.

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PHYSIOLOGY AND PATHOPHYSIOLOGY OF AN ELEVATED RED CELL MASS

Oxygen Transport

The principal function of the blood is to deliver oxygen from the lungs to the tissues. The primacy of oxygen transport in the bodys economy can be easily inferred from the fact that basal metabolic processes consume oxygen at a rate of 4 ml/kg/min, whereas oxygen stores amount to only 20 ml/kg. Overall, oxygen transport is a complex process involving a variety of components, including ventilatory rate and volume, pulmonary diffusing capacity, cardiac output, red cell mass, hemoglobinoxygen affinity, regional blood flow, and tissue capillary density, as well as ambient oxygen tension. The constancy of the red cell mass indicates that acute or transient changes in the tissue oxygen demands or in ambient oxygen tension are met

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primarily by alterations in minute ventilation, cardiac output, distribution of blood flow, and hemoglobinoxygen affinity. [5] [6] [7] A sustained hypoxic stimulus, however, engenders changes in the red cell mass, the plasma volume, and, eventually, in vascularity at the capillary level. [7] [8] Erythrocytosis therefore suggests the presence of either sustained tissue hypoxia, autonomous EPO production, or (in the absence of an appropriate or inappropriate increase in EPO production) autonomous red cell proliferation.

Red Cell Production and Regulation

An understanding of EPO physiology is central to understanding the pathophysiology of erythrocytosis. Under normal circumstances, EPO is produced constitutively by peritubular interstitial fibroblasts in the inner cortex of the kidney, [9] [10] [11] which are the sites not only of EPO production but of the oxygen sensor that stimulates EPO production.[12] The oxygen sensor is a heme protein that up-regulates EPO mRNA transcription through the mediation of a set of nucleoproteins known as hypoxia-inducible factors (HIF) that interact with an enhancer element in the 3 region of the EPO gene. [13] [14] This mechanism is not unique to EPO but is a universal mechanism for oxygen-regulated gene expression.[15]

Erythropoietin production by individual renal peritubular interstitial fibroblasts appears to be an all-or-none phenomenon because anemia or hypoxia recruits additional cells to produce EPO, rather than increasing hormone production in the cells producing it constitutively. [11] As a corollary, the kidney has no preformed stores of EPO, and changes in circulating EPO are a direct consequence of changes in the production of the hormone. [16] With correction of anemia or hypoxia, EPO production ceases except in those cells that were producing it constitutively. Because no evidence has been found to suggest that hypoxia or anemia alters the plasma clearance of EPO, it appears that its concentration in the circulation is regulated at the level of gene expression. Indeed, studies in hypoxic rodents show a close correlation between renal EPO and plasma EPO concentrations, with the latter lagging behind the former. [17]

Erythropoietin is also constitutively produced in the liver in both hepatocytes and interstitial fibroblasts. In contrast to the situation in the kidneys, however, EPO production in hepatocytes is not an all-or-none phenomenon, but can be regulated in each cell. [18] The degree of hypoxia required to up-regulate liver EPO production is greater than that required to induce renal EPO production. Thus, with mild hypoxia, the liver contributes little to the plasma EPO concentration; with more severe hypoxia, the liver becomes a substantial source of EPO because of its bulk. [19]

Small but significant changes in serum immunoreactive EPO can be detected with hypoxia [20] or changes in the hematocrit if serial measurements are obtained [21] ; unequivocal elevations of serum EPO outside the normal range (426 mU/ml) do not occur until the hemoglobin level falls to <10.5 g/dl. [22] Thus, random EPO determinations are not a useful means for establishing the adequacy of EPO production in situations of slight anemia. Whether a similar threshold exists for hypoxia is unclear because no simple means is available for analyzing oxygen delivery at the tissue level. Careful studies of plasma EPO in patients with cyanotic congenital heart disease suggest that such a threshold does not exist. [20]

Changes in the level of circulatory EPO induced by hypoxia are instructive with regard to the bodys mechanisms. Hypobaric hypoxia results in a detectable increase in EPO within a few hours.[23] The elevation is not usually sustained, even though the hypobaric hypoxia persists. [17] [23] [24] [25] [26] The level usually falls back into the normal range unless the hypoxia is extreme.[26] However, because the normal range for plasma EPO is wide (426 mUml), an increase of 1020 mU/ml, although still within the normal range, can constitute a doubling of the plasma EPO concentration and would be expected to elevate basal red cell production.

This unusual behavior can be explained by the intervention of other hemodynamic compensatory responses. Hypoxia generates acute changes in minute ventilation, heart rate, tissue blood flow, and hemoglobinoxygen affinity. Hyperventilation improves alveolar oxygen delivery, increases arterial oxygen tension (PaO_2) and arterial oxygen saturation (SaO_2), and maintains the diffusion gradient between the blood and the tissues. An additional effect of hyperventilation is reduction in carbon dioxide tension, resulting in respiratory alkalosis. The change in arterial pH stimulates the synthesis in erythrocytes of the organic phosphate 2,3-diphosphoglycerate (2,3-DPG). This important compound, which is present in red cells in amounts equimolar with hemoglobin, [27] binds to hemoglobin and reduces its oxygen affinity. Although alkalosis increases hemoglobin oxygen affinitythat is, reduces the P_{50} of the hemoglobin (the partial pressure of oxygen at which hemoglobin is half-saturated with oxygen)the increase in red cell 2,3-DPG not only counteracts the increase in hemoglobinoxygen affinity but actually reduces it (increases P_{50}) and enhances oxygen unloading at the tissue level. The net effect is improved oxygen delivery to tissues.

These adaptive mechanisms can effectively correct hypoxia and improve tissue oxygen delivery, thereby removing the stimulus for EPO production. Plasma EPO then diminishes to the extent that the hypoxia is corrected. If, however, hypoxia is extreme, 2,3-DPG production alone cannot compensate for the respiratory alkalosis; hemoglobinoxygen affinity remains increased and elevated EPO production persists. Hypoxia does not have to be continuous, or even extreme, to stimulate EPO production. Thus, intermittent hypobaric hypoxia can produce erythrocytosis in rodents, [28] whereas supine hypoventilation or sleep apnea has the same effect in humans.[29] [30] [31]

It is worth emphasizing that hypoxia is also associated with a diminution in plasma volume. This abnormality appears to be independent of the cause of hypoxia because it occurs not only at high altitudes [8] but in cyanotic congenital heart disease [32] and in cigarette smokers.[33] Whether the reduction in plasma volume is part of a physiologic adaptation to tissue hypoxia is unclear. When it is extreme (as described later), it can have a deleterious effect.

An interesting counteracting effect of erythrocytosis is suppression of EPO production independent of tissue oxygenation. [34] This is probably a consequence of the hyperviscosity associated with elevation of the red cell mass. The observed reduction in plasma volume associated with hypoxic erythrocytosis probably serves as a means of facilitating this effect, thereby preventing an overexuberant response to hypoxia because elevation of plasma viscosity alone inhibits EPO production. [35] This

explains in part the observation that EPO levels are often within the normal range in patients with secondary erythrocytosis and compensated hypoxia.

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CLINICAL MANIFESTATIONS

The signs and symptoms associated with erythrocytosis are nonspecific. Unless the cause of hypoxia is evident or unless erythrocytosis is extreme, there may be no unequivocal evidence of its presence. Indeed, William Osler, who provided one of the early and important descriptions of polycythemia rubra vera, also cautioned that even anemic patients could appear plethoric (anemia rubra). Thus, a high hematocrit, hemoglobin, or erythrocyte count, rather than a characteristic symptom or physical finding, may be the first clue to an elevation of the red cell mass. Because alterations in the plasma volume can influence these measurements, they cannot be relied on as indicators of the absolute red cell mass. Even when the plasma volume is

CAUSES OF A HIGH HEMATOCRIT

- Relative or spurious erythrocytosis
  - Hemoconcentration secondary to dehydration (diarrhea, diaphoresis, diuretics, deprivation of water, emesis, ethanol), hypertension, preeclampsia, pheochromocytoma, carbon monoxide intoxication
- Absolute erythrocytosis
  - Hypoxia
    - Carbon monoxide intoxication
    - Highoxygen-affinity hemoglobin
    - High altitude
    - Pulmonary disease
    - Supine hypoventilation syndrome
    - Sleep apnea syndrome
    - Right-to-left cardiac shunts
    - Neurologic defects (respiratory center dysfunction)
  - Renal disease
    - Cysts, hydronephrosis
    - Renal artery stenosis
    - Focal glomerulonephritis
    - Renal transplantation
  - Tumors
    - Hypernephroma
    - Hepatoma
    - Cerebellar hemangioblastoma
    - Uterine fibromyoma
    - Adrenal tumors
    - Meningioma
    - Pheochromocytoma
  - Androgen therapy
  - Bartters syndrome
  - Familial erythrocytosis (with normal hemoglobin function)
  - Polycythemia vera

Bartters syndrome
Familial erythrocytosis (with normal hemoglobin function)
Polycythemia vera

not diminished, the hematocrit and the red cell mass are not directly correlated. Therefore, consideration of the causes of a high hematocrit must include a number of possibilities that could artifactually raise the hematocrit, hemoglobin, or erythrocyte values.

When considering these diagnostic possibilities, it is important not to overlook clues provided by the peripheral blood smear and the red cell indices. For example, in true plethora, a properly prepared blood smear obtained from the fingertip shows red cell crowding out of proportion to what is expected from the apparent hematocrit value. Furthermore, an elevated red cell count in association with microcytosis should suggest the presence of autonomous or EPO-driven erythrocytosis because this situation otherwise occurs only with thalassemia minor, a disorder not associated with a high hematocrit or hemoglobin level. [36] The presence of leukocytosis, thrombocytosis, or splenomegaly should also suggest the presence of polycythemia vera, but these abnormalities are not always present early in the course of that illness.

LABORATORY EVALUATION

Red Cell Mass Determination

The first question confronting the physician when encountering a reproducibly high hematocrit or hemoglobin level is to determine whether the red cell mass shows an absolute increase. This can be done accurately only by direct measurement, using the technique of isotope dilution. With this technique, an aliquot of the patients red cells is labeled with a radioactive tracer and reinfused. The red cell mass can then be calculated from the degree of dilution of the isotopically labeled red cells. It is important to remember in this regard that, in erythrocytosis (particularly in patients with splenomegaly), equilibration of the injected labeled red cells with the total blood pool may be delayed relative to the situation when the blood volume is normal. Therefore, serial blood samples should be taken over a period of approximately 90 minutes to ensure that equilibration is complete and that the red cell mass will not be underestimated.

A direct measurement of the red cell mass effectively separates those patients with a reduced plasma volume from those with absolute erythrocytosis (see Chap. 61 for indications for and interpretations of red cell mass assays). Once the presence of erythrocytosis has been established, the cause must be sought. However, depending on the severity of the plethora, it may be necessary to initiate therapeutic phlebotomy even while the diagnostic evaluation is proceeding.

Serum Erythropoietin

Many factors can cause erythrocytosis. Defining the precise cause may not be possible either initially or even after prolonged evaluation; nonetheless, some guidelines can be suggested. Because erythropoiesis is regulated by EPO, a serum EPO level can provide an indication of whether the erythrocytosis is hormonally mediated or autonomous. Although prior assays for EPO in the serum were insensitive and not widely available, the introduction of recombinant EPO has led to development of a specific, sensitive, accurate, reproducible, and commercially available immunoassay. [37]

In patients with erythrocytosis due to uncompensated hypoxia, serum immunoreactive EPO is elevated; in those with compensated hypoxia, the serum immunoreactive EPO level is usually within the range of normal, [20] [38] [39] and in patients with polycythemia vera, serum immunoreactive EPO is usually low but can be normal (see Chap. 61). [22] [40] The number of patients with autonomous EPO production studied with a sensitive and specific EPO assay is too small to permit firm conclusions, but EPO levels in these patients can be either high or normal and may even fluctuate, suggesting that serial measurements may be necessary to detect an abnormality. [40] This would also be important in those patients with the supine hypoventilation syndrome or sleep apnea in whom serum EPO may be only intermittently elevated. [29] Thus, although an elevated serum EPO level suggests that erythrocytosis is a secondary phenomenon and a low EPO level supports the possibility of autonomous erythropoiesis, a normal serum EPO level excludes neither hypoxia nor autonomous EPO production as the cause of erythrocytosis.

Blood Gas Measurements

An elevated EPO level indicates that erythrocytosis is hormonally driven but does not establish the cause. A normal EPO level, however, does not exclude hypoxia as a cause of erythrocytosis. Therefore, a concomitant assessment of PaO_2 and SaO_2 is required in the evaluation of all patients with erythrocytosis because hypoxia is a correctable cause of this disorder. Patients with polycythemia vera invariably have a normal PaO_2 and an SaO_2 of 90% unless there is coexisting pulmonary disease. [41] However, it is important to be aware that the PaO_2 is a relatively insensitive indicator of hypoxia. This is because hemeheme interactions during oxygen binding render hemoglobin fully saturated over a wide PaO_2 range. It is only when the PaO_2 persistently falls to <67 mmHg that an appreciable increase in red cell mass is encountered. [42]

Arterial oxygen saturation is a more sensitive indicator of potential tissue hypoxia than PaO_2 because it is directly related

to red cell mass. [40] In this regard, it is important to remember that in the presence of an elevated red cell mass, the serum EPO level may be normal even if the SaO_2 is low, owing to the compensatory increase in tissue oxygenation due to the erythrocytosis or the development of hyperviscosity associated with erythrocytosis.

There are also two situations causing hypoxic erythrocytosis in which the SaO_2 can be misleading: highoxygen-affinity (low P_{50}) hemoglobins and carbon monoxide intoxication. In the former condition, the SaO_2 is normal, but tissue hypoxia exists because at ambient tissue oxygen tensions, highoxygen-affinity hemoglobins do not release their oxygen. Thus, for detection of a highoxygen-affinity hemoglobin, determination of its oxygen affinity (P_{50}) is mandatory.

The clinician must also be aware of the complexities of interpreting SaO_2 values in carbon monoxide poisoning. If an indirect method for determining SaO_2 is used (e.g., calculating it from the measured PaO_2 and a standard oxygen dissociation curve), a normal value will be obtained when the SaO_2 is in fact low owing to the binding by hemoglobin of carbon monoxide rather than oxygen. [43] Detection of carbon monoxide intoxication requires both a direct determination of SaO_2 by oximetry and quantitation of the percentage of carboxyhemoglobin. Unless carbon monoxide exposure is constant, however, it will be displaced from hemoglobin sufficiently rapidly to yield a normal SaO_2 .

Erythroid Cell Culture

The observation that polycythemia vera erythroid progenitor cells can proliferate in vitro in the absence of EPO led to the application of in vitro erythroid colony-forming assays for the diagnosis of polycythemia vera. Unfortunately, this type of behavior is not specific for polycythemia vera erythroid progenitor cells and cannot be recommended for the routine evaluation of unexplained erythrocytosis. However, studies of progenitor cells may be helpful if performed in laboratories with special expertise in stem cell assays (see Chap. 61 for details).

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ETIOLOGIES

Hypoxic Erythrocytosis

Hypobaric Hypoxia

The prototypical model for hypoxic erythrocytosis is high-altitude hypoxia. Exposure to high altitudes leads acutely to the predictable changes in minute ventilation, heart rate, blood flow, and hemoglobinoxygen affinity described earlier. Serum EPO is elevated initially but eventually falls to within the normal range if the hypoxia is not extreme. This decline, however, does not prevent an increase in red cell mass, which will be sustained. This effect occurs because EPO can potentiate its own effects, because expansion of the erythroid progenitor cell pool is exponential, and because only picomolar quantities of the hormone are required to sustain the red cell mass under normal circumstances. Early erythroid progenitor cells, at least in vitro, require 10 ^{3} times as much EPO as do late erythroid progenitor cells.[44] Thus, the early unsustained increase in serum EPO probably serves to facilitate recruitment of additional erythroid progenitor cells to expand the progenitor cell pool, whereas smaller quantities of the hormone can maintain this pool once it is expanded.

Concomitant with the changes cited previously is a reduction in plasma volume. [8] Although initially this could be the consequence of fluid loss associated with hyperventilation and the low humidity of high altitude, similar changes are also seen at sea level in patients with hypoxic erythrocytosis due to cyanotic congenital heart disease,[32] in those with chronic carbon monoxide intoxication,[33] and in those with end-stage renal disease who are receiving recombinant human EPO. [45] Thus, a decrease in plasma volume appears to be a component of the compensatory EPO-induced erythrocytosis associated with hypoxia. Other noteworthy compensatory changes associated with high-altitude hypoxia are developmental and require sustained altitude exposure; they include an increase in lung volume and capillary density and a blunted ventilatory response to hypoxia. [7] [46] [47] Because the latter also occurs in patients with cyanotic congenital heart disease, [44] it is not peculiar to hypobaric hypoxia.

Chronic mountain sickness is an extreme response to hypobaric hypoxia in which the elevation of the red cell mass fails to compensate adequately for hypoxia. [48] In this situation, the erythrocytosis far exceeds that expected for the degree of hypobaric hypoxia, and there is symptomatic hyperviscosity. Chronic mountain sickness appears to be the consequence of an additional acquired ventilatory defect superimposed on the adaptive blunted ventilatory response that occurs during chronic exposure to high altitude.[47]

Chronic Pulmonary Disease

Hypoxia occurring with chronic lung disease represents a more complicated clinical situation than hypobaric hypoxia because the erythrocytosis expected for the measured degree of hypoxia is not always present. This, however, appears to be more a matter of patient selection than an indication of a new paradigm. [42] [49] For example, in some patients an increase in plasma volume due to fluid retention masks the increase in red cell mass. [50] In other patients, clinical measurements of tissue oxygenation may not accurately reflect the actual level of tissue oxygenation, whereas in still others, inflammation, infection, or renal disease may impair EPO production as well as the marrows responsiveness to EPO. Finally, as observed in certain patients with highoxygen-affinity hemoglobins, there may be tolerance to a particular level of tissue hypoxia.[51] In general, however, patients with impaired pulmonary function demonstrate the expected correlation between SaO_2 and red cell mass and may even have a steeper response than that of healthy people.[42] Their ability to produce EPO is also more vigorous and the threshold for this is reduced.[52]

The sleep apnea syndrome and supine hypoventilation due to premature airway closure are two special situations in which hypoxia can develop without overt evidence of pulmonary disease.[29] [30] [31] In each instance, hypoxia is intermittent. A random blood gas or EPO measurement may thus fail to reveal the underlying cause of the erythrocytosis.

Chronic Carbon Monoxide Intoxication

Chronic carbon monoxide intoxication, usually as the consequence of tobacco use, is probably the most common cause of erythrocytosis encountered clinically. It is responsible for the syndrome commonly referred to as stress erythrocytosis,[53] benign polycythemia, [54] or spurious erythrocytosis.[55] Carbon monoxide is the perfect respiratory poison. Odorless and colorless, carbon monoxide has an affinity for hemoglobin 210 times as great as that of oxygen. This is attributable not to a greater rate of association of carbon monoxide with hemoglobin compared with oxygen but rather to its slower rate of dissociation from hemoglobin. Not only does the binding of carbon monoxide with hemoglobin reduce the quantity of oxygen that can be bound, it also shifts the oxyhemoglobin dissociation curve to the left, thereby reducing the ability of hemoglobin to unload oxygen to the tissues. [56] Furthermore, carboxyhemoglobin has a reduced affinity for 2,3-DPG. At a high carbon monoxide concentration, red cell 2,3-DPG production is also suppressed. [57] Thus, carbon monoxide not only causes tissue hypoxia but subverts the mechanisms designed to compensate for the hypoxia.

An additional, but often unrecognized, effect of carbon monoxide

intoxication is diminution of plasma volume.[33] When combined with an elevation of the red cell mass, this creates a situation in which erythrocytosis occurs with a normal total blood volume, with the attendant problem of hyperviscosity. The variable extent to which elevation of the red cell mass and depression of the plasma volume can occur in patients with carbon monoxide intoxication has led to the description of syndromes of spurious erythrocytosis due to a high normal red cell mass or a low normal plasma volume,[55] but these syndromes probably represent only part of the continuum of carbon monoxide toxicity.

Although the term *benign polycythemia* has been used to describe erythrocytosis-associated carbon monoxide intoxication, the syndrome is anything but benign. Several series have demonstrated a substantial incidence of thromboembolic events, and in one study a mortality rate six times as great as expected for unaffected people of the same age was observed.[58]

Cyanotic Congenital Heart Disease

Congenital heart disease with anatomic right-to-left shunting is a predictable cause of erythrocytosis. [59] Such patients are similar to those with hypobaric hypoxia with respect to having a blunted ventilatory response and a low plasma volume, but they also may have a coagulopathy with a low fibrinogen and a low platelet count. [59] [60] [61] When the erythrocytosis compensates for arterial oxygen desaturation, the serum EPO level will be normal. [20] However, phlebotomy results in a marked increase

in serum EPO owing to the reversal of the compensatory erythrocytosis. In a manner similar to that encountered in hypobaric hypoxia, a syndrome resembling chronic mountain sickness can develop in patients with cyanotic congenital heart disease. This phenomenon results from hypoxia that cannot be compensated for by erythrocytosis.[62]

HighOxygen-Affinity Hemoglobins

Highoxygen-affinity hemoglobins that are unable to unload oxygen to the tissues at an acceptable tissue oxygen tension are an uncommon but interesting cause of hypoxic erythrocytosis. The first high-affinity hemoglobin, hemoglobin Chesapeake, was identified in 1966 in an 81-year-old man who was being evaluated for angina pectoris.[63] To date, over 40 such mutant hemoglobins have been described. These mutants may be stable or unstable; a few are not associated with erythrocytosis either because their increased affinity for oxygen is not marked or because they are too unstable. [64]

Hemoglobin normally exists in an equilibrium between its oxygenated (relaxed or R) state and its deoxygenated (tense or T) state (see Chaps. 22 and 31). The transition between these states involves changes in molecular conformation and intramolecular bonding. In the deoxy or T state, hemoglobin has a high affinity for 2,3-DPG and hydrogen ions, whereas in the oxygenated or R state, these affinities are reduced. In general, highoxygen-affinity hemoglobins arise from amino acid substitutions that prevent the obligatory conformational changes in the molecule during oxygenation and deoxygenation, particularly those that (1) involve the $_{1\,2}$ interface of the globin chains within the hemoglobin tetramer and stabilize it in the oxygenated state; (2) involve the C-terminal end of the -globin chain and prevent the formation of ionic bonds; or (3) impair 2,3-DPG binding. [64]

Clinically, erythrocytosis is the major abnormality associated with a highoxygen-affinity hemoglobin, and its extent is proportional to the P_{50} of the mutant hemoglobin.[65] Interestingly, occasional patients have a low plasma volume. Those affected are heterozygotes, with the mutant hemoglobin accounting for approximately 40% of their total hemoglobin. The inheritance pattern is therefore dominant. A family history can be revealing, if positive. A negative history does not rule out the diagnosis, however, because of the high frequency of spontaneous mutations. Because of the nature or position of the amino acid substitution, highoxygen-affinity hemoglobins cannot always be detected by routine electrophoretic techniques. Moreover, an electrophoretic abnormality alone would not establish that the hemoglobin had a high oxygen affinity. A P_{50} determination is the only reliable means of establishing this. Of course, carboxyhemoglobin or a low red cell 2,3-DPG can cause a low P_{50} . In evaluating the possibility of a highoxygen-affinity hemoglobin, a study of other family members is also important.

Inappropriate Erythropoietin Secretion

Erythrocytosis Due to Renal Disease

Because the kidneys are the major site of EPO production in adults, some forms of renal disease may cause erythrocytosis as well as anemia. Although it has been suggested that a high hematocrit associated with hypertension may be a clue to the presence of renal artery stenosis, [66] it must be remembered that hypertension per se is associated with a reduction in plasma volume.[67] Experimentally, renal artery stenosis does cause an

BEST ESTABLISHED ASSOCIATIONS BETWEEN TUMORS AND INAPPROPRIATE ERYTHROPOIETIN PRODUCTION

Hypernephroma[89] [90] [91] [92] [93]	More common in men. Erythrocyte sedimentation rate is often elevated. Associated with von Hippel-Lindau disease.
Wilms tumor[89]	
Renal adenoma[89]	
Undifferentiated renal carcinoma[89]	
Hepatoma[89] [95] [96]	More common in men. Increase in plasma volume may obscure the elevated red cell mass.
Liver hamartoma[89]	
Cerebellar hemangioblastoma[89] [97] [98]	More common in men. Metastases can be confused with those of a hypernephroma. Associated with von Hippel-Lindau disease.
Uterine fibromyoma[99] [100]	Tumor extracts (only from very large tumors) contain erythropoietic activity.
Pheochromocytoma[89]	Rare. Spurious elevation of the hematocrit can occur owing to decreased plasma volume. Associated with von Hippel-Lindau disease.
Adrenal adenoma or hemangioblastoma[91]	Associated with von Hippel-Lindau disease.
Paraganglioma[101]	
Meningioma[102]	

increase in serum EPO, but this is usually modest and not of the magnitude of the increase associated with anemia. [68] It has been suggested that this effect reflects a decline in renal oxygen consumption as renal blood flow falls. Whatever the mechanism, although examples of renal artery stenosis associated with erythrocytosis have been described, they are not common.[69] [70]

Renal cysts have been implicated as a cause of inappropriate EPO production, [71] but the most striking example of this has been in patients with autosomal dominant polycystic disease and renal failure, in whom anemia is unexpectedly mild while EPO levels are elevated. [72] Acquired cystic disease has also been described in patients undergoing chronic renal dialysis and presenting with a similar clinical picture. [73] More frequently, however, renal cysts develop in these patients without any amelioration of anemia or erythrocytosis.[74] In other patients, the erythrocytosis identified with renal cysts was actually due to polycythemia vera. [75]

Patients with focal glomerulonephritis, with or without the nephrotic syndrome, constitute another group in whom renal disease is associated with erythrocytosis. [76] [77] Indeed, this association has been described with sufficient frequency to suggest that renal function should be examined in patients with unexplained erythrocytosis. In general, the erythrocytosis is a temporary event during the course of the renal disease.

Erythrocytosis can also be a complication of renal transplantation. [78] [79] [80] [81] The mechanism for this is unclear, but it does not appear to involve rejection and has been seen with cadaver kidneys as well as with kidneys from living donors. [82] It has been postulated that the erythrocytosis in this situation does not arise from the transplanted kidney but rather from the patients own kidneys. [83] [84] In some instances, the erythrocytosis may actually be spurious and due to overzealous use of diuretics.[85] Studies suggest that angiotensin-converting enzyme inhibitors are effective in controlling post-transplantation erythrocytosis. [86] However, phlebotomy therapy should not be withheld pending the results of drug therapy in this situation. A rare cause of erythrocytosis associated with renal disease is Bartters

syndrome.[87] [88]

Tumor-Associated Erythrocytosis

Erythrocytosis associated with a tumor is a rare paraneoplastic syndrome that has excited the imagination of physicians far beyond its clinical frequency. [89] Unfortunately, few studies of this phenomenon have used a sensitive and specific assay for EPO. Renal tumors, both benign and malignant, have been associated with erythrocytosis, and the production of EPO mRNA by renal carcinoma cells has been demonstrated. [90] It is of interest that erythrocytosis associated with renal, hepatic, or cerebellar tumors is more common in men.[89] It is also of interest that three of the tumors associated with erythrocytosishypernephroma, cerebellar hemangioma, and pheochromocytomaare part of the constellation of von Hippel-Lindau disease, which suggests a common underlying genetic mechanism. [93] It is also noteworthy that, in patients with hepatomas, an expanded plasma volume can obscure the presence of erythrocytosis. [89] Although one might, on the basis of studies of EPO excretion,[94] expect EPO levels to be inappropriately high in patients with tumor-associated erythrocytosis, this is not usually the case.

Familial Erythrocytosis

Familial erythrocytosis is an uncommon entity for which the identified syndromes and causes are listed in the accompanying box (also see Chaps. 15 and 61 for detailed discussion of EPO and EPO receptor pathophysiology). Depending on the syndrome, inheritance may be dominant or recessive. [103] [104] [105] [106] [107] [108] [109] [110] [111] Few such families have been studied with a sensitive and specific

FAMILIAL ERYTHROCYTOSIS
Highoxygen-affinity hemoglobin
Diphosphoglycerate mutase deficiency
With increased erythropoietin production
With normal erythropoietin production
With a mutated erythropoietin receptor
Polycythemia vera

assay for EPO. When carefully studied, EPO levels were either normal or elevated and not always influenced by phlebotomy. Erythroid progenitor cell sensitivity to EPO was either normal or increased. In several families, abnormalities of EPO receptor gene expression or binding affinity were not observed. [111] In a number of families, erythrocytosis could be associated with mutations of the EPO receptor gene. Plasma EPO levels in this situation were either normal or low. [112]

Interestingly, DNA sequence analysis has revealed that in each case a nonsense or frameshift mutation caused truncation of the receptor because of the generation of stop codons, with loss of the terminal portion of its COOH domain. [113] [114] [115] Experimentally, removal of the terminal portion of the EPO receptor COOH domain increased its sensitivity to EPO,[116] [117] and human EPO receptors truncated in the same fashion as the familial genetic mutants also demonstrated hypersensitivity to EPO in vitro, at least at low concentrations of the hormone. [114] [115] Since hematopoietic cell phosphatase, which down-regulates EPO receptor signal transduction by tyrosine dephosphorylation, binds to its terminal COOH domain, [118] it has generally been held that the hypersensitivity of truncated EPO receptors is due to loss of hematopoietic cell phosphatase binding. However, data have been presented that suggest that the apparent increase in sensitivity to EPO is actually an increase in sensitivity to insulin-like growth factors, which are also present in the plasma and which are known to enhance erythropoiesis. [119] Support for a role of factors other than EPO in this situation is provided by the observation that not every patient with a truncated EPO receptor has erythrocytosis. [114]

The recently described endemic form of congenital polycythemia in the Chuvash population may reflect a defect in oxygen sensor function (see Chap. 61).

In one patient with erythrocytosis and a low erythrocyte 2,3-DPG level, a deficiency of diphosphoglycerate mutase was found. [110] Some patients with familial erythrocytosis, however, had an elevated red cell 2,3-DPG level, suggesting an unrecognized cause for hypoxia, whereas in others, the cause may have been unidentified renal disease or cigarette smoking. Whatever the mechanism, erythrocytosis has been observed to have adverse effects in a number of these patients, and phlebotomy therapy should be used to maintain the red cell mass at a safe level. [103] [104] It should also be remembered that polycythemia vera is occasionally familial.

Polycythemia Vera Rubra

Polycythemia vera is a form of myeloproliferative syndrome in which the granulocyte, monocyte, and platelet counts, as well as the red cell count, are usually elevated in the absence of a definable cause (see Chap. 61). This disorder appears to represent occupancy of the marrow by the progeny of a neoplastic clone of stem cells, which expands with inappropriate exuberance. Because polycythemia vera is discussed in detail in Chapter 61 , it is not considered further here, except to note that elevation of the counts in all three major cell lineages (red blood cells,

white blood cells, and platelets) and the presence of splenomegaly should lead the clinician to suspect this diagnosis strongly as a cause of an unexplained erythrocytosis.

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EVALUATION AND MANAGEMENT

The two goals in dealing with a patient with erythrocytosis are identification of a correctable cause and reduction of the red cell mass. With respect to the medical history, prior blood counts are important in documenting the onset and duration of the erythrocytosis. The blood counts of family members may be useful in establishing an inherited basis for it. In general, with the exception of pruritus, the symptoms associated with polycythemia vera are not different from those associated with secondary erythrocytosis. The occupational, environmental, and social history of the patient may yield important clues, especially with regard to pulmonary disease and carbon monoxide intoxication. The drug history should not be overlooked because androgenic steroids, administered for therapeutic, cosmetic, or competitive purposes or mistakenly to improve libido, can cause erythrocytosis. [120] [121] [122] The physical examination may suggest a cardiac or pulmonary cause and, with respect to the latter, the sleep apnea syndrome must not be ignored. Splenomegaly should suggest the presence of a myeloproliferative disorder, but its absence does not exclude this possibility.

Laboratory evaluation should be dictated by common sense. Leukocytosis and thrombocytosis suggest polycythemia vera, but, once again, their absence does not exclude this disorder. A urinalysis is mandatory, and a serum EPO measurement should always be obtained, but only by an assay using recombinant-derived reagents.[37] If it is negative, a repeat assay may be worthwhile if an EPO-producing lesion is suspected. [40] If supine erythrocytosis is suspected, a blood sample for assay should be obtained under the appropriate circumstances. A direct measurement of SaO_2 should be obtained, as should a carboxyhemoglobin level. Because the half-life of carboxyhemoglobin is approximately 7 hours, the measurement may be normal if exposure has been interrupted before the patient seeks medical attention.

If the evaluation to this point has not been revealing, renal evaluation with respect to kidney size, presence of anatomic abnormalities, and blood flow is indicated. In the absence of neurologic signs or symptoms, an abdominal computed tomography scan should complete the evaluation for paraneoplastic erythrocytosis. Because highoxygen-affinity hemoglobins may not migrate abnormally on electrophoresis, and because even an electrophoretic abnormality does not by itself establish an oxygen-binding abnormality, a P_{50} determination should be obtained if the history is suggestive and no other cause of erythrocytosis is evident.

In the absence of a clinical assay for establishing clonality with respect to erythrocytosis, even after all the previously mentioned tests have been performed, the diagnosis may not be forthcoming. This problem should not influence the management protocol; treatment of unexplained erythrocytosis is no different from that of polycythemia vera. In each instance, the problem is an expanded red cell mass, which in the case of secondary erythrocytosis may be coupled with a reduction in plasma volume. The net result of these abnormalities is an increase in peripheral vascular resistance, a reduction in cardiac output, and a decline in systemic oxygen transport.[123] Cerebral blood flow is invariably reduced with any form of erythrocytosis, [124] [125] [126] and this may result in impaired glucose delivery to the brain. [127] Therefore, there is no such thing as benign erythrocytosis; thromboembolic complications have been recorded with every form of the disorder, and no symptoms may have been exhibited beforehand. It is therefore recommended that all patients with erythrocytosis without a correctable cause undergo a careful phlebotomy regimen.

In patients with pulmonary disease, cyanotic congenital heart disease, or a highoxygen-affinity hemoglobin, the extent of phlebotomy can be dictated by the patients symptomatic response or by the serum EPO level as a measure of tissue hypoxia. In these disorders, even limited phlebotomy has proved symptomatically beneficial.[128] [129] [130] [131] [132] In cyanotic congenital heart disease, proteinuria may be reduced [133] and the coagulopathy improved.[60] In patients with a low plasma volume, phlebotomy is not deleterious and actually stimulates an increase in the plasma volume. If possible, phlebotomy should be continued until the hematocrit is <45%, because anything less vigorous may be associated with a persistent reduction in cerebral blood flow. [125] Contrary to published commentary, it is always possible to control the red cell mass by phlebotomy if venous access is adequate, because sustained phlebotomy induces iron deficiency with certainty. Phlebotomy does not, despite commentary to the contrary, cause hyperviscosity by producing rigid microcytic red cells. In adults, induced iron deficiency can never cause clinically significant hyperviscosity due to the rigidity of iron-poor red cells [134] because with true iron deficiency it is impossible to increase the red cell mass sufficiently to cause hyperviscosity. Furthermore, it has been well demonstrated that chronic iron deficiency in adults in the absence of anemia does not impair functional aerobic performance.[135]

In summary, erythrocytosis presents a diagnostic challenge to the physician, and in some cases no conclusive answer concerning its mechanism is immediately evident. The clinical evaluation should follow a logical progression, and even in the absence of a firm diagnosis, therapeutic phlebotomy can and should be used. Chemotherapeutic agents are not indicated for the sole purpose of reduction of the red cell mass. [136] Because this is true in polycythemia vera as well as in secondary erythrocytosis, lack of a definite diagnosis does not adversely affect prognosis as long as the red cell mass is adequately controlled.

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Chapter 26 - Disorders of Iron Metabolism: Iron Deficiency and Overload

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INTRODUCTION

Iron is an essential nutrient required by every human cell. A transition metal (atomic number 26, atomic weight 55.85), iron can serve as a carrier for oxygen and electrons and as a catalyst for oxygenation, hydroxylation, and other critical metabolic processes, in part because of its ability to reversibly and readily cycle between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states. As a result, iron is quantitatively the most important biocatalytic element in human enzymology, with vital roles in oxidative metabolism, in cellular growth and proliferation, and in oxygen transport and storage.

Iron is transported and stored as a component of a variety of iron compounds, not as a free cation. The very reactivity that is metabolically useful in iron porphyrin complexes and metalloenzymes makes inorganic iron compounds or ionized forms potentially hazardous. Ionic iron can participate in a number of reactions to produce free radical species, which in turn can damage cellular constituents. As a consequence, either a decrease or an increase in body iron may be clinically significant. If too little iron is available (iron deficiency), limitations on the synthesis of physiologically active iron-containing compounds may have deleterious consequences. If too much iron accumulates (iron overload) and exceeds the bodys capacity for safe transport and storage, iron toxicity may produce widespread organ damage and death.

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IRON METABOLISM

Biological and Molecular Aspects

Proteins of Iron Transport, Uptake, Storage, and Regulation

A quartet of proteins provides for the iron requirements of the body. The internal supply and storage of iron is mediated by three principal proteins, transferrin, transferrin receptor, and ferritin, while their supply in turn is regulated by a fourth class of proteins, the iron regulatory proteins (IRPs). *Transferrin*, a transport protein, carries iron in the plasma and extracellular fluid to supply tissue needs. *Transferrin receptor*, a glycoprotein on cell membranes, binds the transferrin-iron complex and is internalized in a vesicle where the iron is released intracellularly; the transferrin-transferrin receptor assemblage then returns to the cell membrane, where apotransferrin is liberated into the plasma. *Ferritin*, an iron storage protein, sequesters iron in a presumably nontoxic form while holding it ready for prompt mobilization in time of need. The *iron regulatory proteins* (IRP-1 and IRP-2; previously known as the iron-responsive element-binding proteins [IRE-BPs], iron regulatory factors [IRFs], ferritin-repressor proteins [FRPs], and p90) are messenger ribonucleic acid (mRNA)-binding proteins that coordinate the intracellular expression of transferrin receptor, ferritin, and other proteins important for iron metabolism. Each of these principal proteins has been isolated, purified, and characterized. The chromosomal locations of the corresponding genes have been identified and their sequences determined. The available information is summarized in Table 26-1 , and the structures of the proteins are shown diagrammatically in Figure 26-1 .

Transferrin

Transferrin (Fig. 26-1A) is the sole physiologic carrier of iron between body tissues. [1] A single gene for apotransferrin has been identified and located at q21-qter on chromosome 3, near the gene for the transferrin receptor. Structural analysis suggests that the transferrin gene originated as the result of an unequal crossover between two primordial transferrin genes. [2] Apotransferrin is a single-chain glycoprotein (M_r 79,570; 6% glucosidic) with 678 amino acid residues and is composed of two homologous N-terminal and C-terminal lobes (Fig. 26-1A). [1] Each lobe can independently bind a single ferric ion, so the molecule can exist as apotransferrin or as monoferric or diferric transferrin. The lobes are in the shape of prolate ellipsoids, and

TABLE 26-1 -- Proteins of Iron Transport, Storage, and Regulation

	Transferrin	Transferrin Receptor	Ferritin	Iron Regulatory Proteins (IRP-1, IRP-2)
M_r	79,570	185,000	440,000	90,000
Chromosomal location of gene(s)	3q21-qter	3q26.2-qter	H subunit: 11 L subunit: 19	IRP-1: 9 IRP-2: 15
Structure	Single-chain glycoprotein with two iron-binding sites	Transmembrane glycoprotein dimer with two transferrin-binding sites	Spherical protein of 24 subunits, binds up to 4,500 iron atoms	Four-domain proteins; IRP-1 contains a [4Fe-4S] cluster
Function	Iron transport in plasma and extracellular fluid	Receptor-mediated endocytosis of ferric transferrin; is recycled	Iron storage	Coordinate translational regulation of critical proteins of iron metabolism

Figure 26-1 Structures of proteins of iron transport, uptake, storage, and regulation. **(A)** Apotransferrin. Schematic representation of the polypeptide chain shows that it is folded into two lobes, each in the shape of a prolate ellipsoid and containing a single iron-binding site, represented by a dot. Each lobe is in turn composed of two dissimilar domains, labeled I and II. The N-terminal and C-terminal lobes are indicated. *(From Bailey et al., [224] with permission.)* **(B)** Transferrin receptor. Schematic representation of the transferrin receptor on the cell surface shows it as a transmembrane glycoprotein dimer composed of two identical subunits linked by a disulfide bond. The transferrin receptor is amphipathic with a small hydrophilic cytoplasmic tail, a large hydrophilic extracellular domain, and a central hydrophobic region anchoring the receptor into the membrane. The receptor can bind two molecules of transferrin. *(From Huebers and Finch, [7] with permission.)* **(C)** Ferritin. Schematic representation of human isoferritins with different proportions of subunits. In the diagram, shaded subunits represent human H chains and plain subunits, L chains. Homopolymers of H chains and L chains are shown at the top and bottom of the figure, respectively, with heteropoly-mers of decreasing H-chain content placed between the homopolymers and the sources of the various ferritins labeled in the column on the right. *(Reprinted from Harrison and Arosio, [12] with permission from Elsevier Science.)* **(D)** Iron regulatory proteins (IRPs). This schematic representation of IRP-1 is based on the polypeptide fold of porcine mitochondrial aconitase provided by Lauble et al. [215] *(with permission from the American Chemical Society; copyright 1992.).* The molecule is folded into four domains; the first three (the lower portion of the molecule in the figure) are closely associated about the Fe-S cluster (shown in red), with the fourth (the upper portion of the molecule in the figure) attached by an extended hinge or linker peptide, creating an intermolecular cleft. The [3Fe-4S] cluster is coordinated by three cysteine residues while in the form of the protein; active as an aconitase, the fourth iron atom is inserted into the corner of the (4Fe-4S) cubane structure.

each is further divided into two dissimilar domains. Each iron-binding site is located within the interdomain cleft, where the iron is bound by two tyrosines, a histidine, and an aspartic acid residue. [3] An anion (bicarbonate or carbonate) is bound with each ferric ion, serving as a bridging ligand between the iron and protein. [3] Transferrin binds ferric iron with high affinity; under physiologic conditions the effective stability constant [1] [3] is about 10^{20} M^{1} . Binding of two atoms of ferric iron by transferrin results in major conformational changes: the interdomain clefts, which are open in apotransferrin, close when iron is bound, [4] making the molecule more compact, more soluble, and more resistant to oxidative or tryptic denaturation. The two iron-binding domains are similar, with more than 40% sequence identity, but differ in their detailed spectroscopic, thermodynamic, kinetic, and chemical properties. [5] The affinity of the C-terminal site for iron is about one order of magnitude greater than that of the N-terminal site, [6] and in vitro, apotransferrin in interaction with bicarbonate first binds iron at the C-terminal site and then undergoes conformational changes that lead to iron uptake at the N-terminal site. [3]

Most apotransferrin is produced by hepatocytes. [1] Although a variety of other sites of synthesis have been identified (including lactating mammary gland, testis, central nervous system [CNS], lymphocytes, and macrophages), none seems to be a quantitatively important source of plasma transferrin in vivo. The total amount of

apotransferrin in humans is about 240 mg/kg, equally divided between the plasma and extravascular fluids. [7] Apotransferrin is a true carrier that is not lost in delivering iron, so its turnover is unrelated to the plasma iron turnover; its half-life is about 8 days. [7] Each transferrin molecule undergoes 100200 cycles of iron binding and release during its lifetime in the circulation.

Transferrin Receptor

The number and stability of transferrin receptors on the cell surface are prime determinants of cellular iron uptake. The transferrin receptor not only provides transferrin-bound iron access into the cell, it also plays a critical role in the release of iron from transferrin within the cell. A single gene for the transferrin receptor has been identified; like the transferrin gene, it is located on the distal portion of the long arm of chromosome 3 in the region q26.2-qter. [8] The transferrin receptor (Fig. 26-1B) is a transmembrane glycoprotein dimer composed of two identical subunits (each with M_r 94,000) linked by a disulfide bond. The transferrin receptor is amphipathic, with a small hydrophilic cytoplasmic tail (62 amino acid units, M_r about 5,000), a large hydrophilic extracellular domain (648 amino acids, M_r about 140,000), and a central hydrophobic region anchoring the receptor to the membrane (28 amino acids). [9] The transferrin receptor can bind two molecules of transferrin; if each transferrin is diferric, the dimeric receptor can carry a total of four atoms of transferrin-bound iron. The affinity of the transferrin receptor for transferrin depends on both the iron content of transferrin and the pH. With amounts of iron-bearing transferrin sufficient to saturate receptors at a physiologic pH of 7.4, the receptor has very little affinity for apotransferrin, an intermediate affinity for monoferric transferrin, and the highest affinity for diferric transferrin, estimated at 2×10^{9} to 7×10^{9} M. Under such physiologic conditions, the affinity of the transferrin receptor for diferric transferrin is about four times that for monoferric transferrin. [7] At a pH of about 5, the affinity of the transferrin receptor for apotransferrin increases to that for diferric transferrin.

Transferrin receptors appear to be expressed on virtually all nucleated cells and are present in large numbers in erythroid precursors, placenta, and liver. The number of transferrin receptors on the cell surface is a prime determinant of cellular iron supply. In nondividing cells the receptor number is constant, but numbers increase markedly in proliferating cells. The number

of receptors seems to reflect cellular iron requirements; in the rat, the estimated number of transferrin receptors was found to be about 300,000 per cell in the early normoblast, about 800,000 per cell in the intermediate normoblast, and about 100,000 per cell in the reticulocyte. [10] The half-time for disappearance of transferrin receptors from the cell has been reported to range from <24 hours to 23 days. [11]

Ferritin

Ferritin, the major iron storage protein, is composed of 24 subunits of at least two types: L (or light: M_r 19,700; more acidic with pI 4.55.0) and H (or heavy: M_r 21,100; more basic with pI 5.05.7). [12] Genes for both the H and L chains belong to multigene families with members on several chromosomes, but most of these are pseudogenes. A functional H gene and a functional L gene have been mapped to chromosomes 11 and 19. [13]

Apoferritin (M_r 440,000) consists of a spherical protein shell about 1 nm thick and 12 nm in diameter that is composed of mixtures of oblong H and L subunits whose proportions depend on the tissue and iron status of the cell (Fig. 26-1C). Both subunits consist of four helix bundles with a fifth short helix at roughly 60 degrees to the bundle axis. Iron storage within ferritin requires oxidation of Fe^{2+}, movement of Fe^{3+} into the interior of the molecule, and the nucleation and growth of the core of ferric hydroxyphosphate. [12] H subunits are involved in Fe^{2+} oxidation, while L subunits participate in core formation. [14] Tissues functioning as major iron storage depots, such as liver and spleen, have isoferritins with a preponderance of the L subunit, while tissues that do not normally act as iron storage sites, such as heart, contain isoferritins with higher proportions of H subunits. Within a specific tissue, greater amounts of storage iron are associated with a greater predominance of L subunits. [15] These patterns suggest that ferritins enriched in L subunits have a long-term iron storage function while ferritins with a predominance of H subunits may be more active in iron metabolism. The ferritin H subunit contains a ferrioxidase site lacking from the L subunit. [16] Recombinant human homopolymers composed solely of H subunits have been found to take up iron at a rate several times that of homopolymers composed only of L subunits. [14] These variations in the proportions of H and L subunits and other factors such as the extent of glycosylation give rise to tissue isoferritins with different isoelectric points.

Ferritin is found in virtually all cells, providing both an accessible reserve of iron for synthesis of functional iron-containing compounds and a means of sequestering iron in a soluble, apparently nontoxic form. It is especially abundant in cells with specialized roles in the synthesis of iron-containing compounds (erythroid precursors) and in iron metabolism and storage (macrophages, hepatocytes). Within the apoferritin shell, the component subunits are arranged so that a total of eight hydrophilic channels are formed near the N-terminal ends of the subunits along the four threefold axes of symmetry, while an additional six hydrophobic channels lie along the three fourfold axes of symmetry, [12] apparently providing routes for movement of iron and small molecules in and out of the interior of the sphere. Each ferritin molecule can reversibly store as many as 4,500 iron atoms within the shell in an inorganic polynuclear core of ferric hydroxyphosphate; typically only about half that amount is present.

The half-life of hepatic ferritin is about 60 hours. Catabolism of cellular ferritin may result in digestion of the protein shell with reutilization of the iron core, or in conversion to hemosiderin, an amorphous water-insoluble storage compound with a higher iron content and slower turnover than ferritin. [12] [17] Ascorbic acid seems to retard ferritin degradation by reducing lysosomal autophagy of the protein. [18] Intracellular ferritin is synthesized by the smooth endoplasmic reticulum in amounts required to replace catabolized ferritin and hemosiderin and to store any additional iron entering the cell. Small amounts of ferritin are also secreted into the plasma. Plasma ferritin is apparently synthesized by the rough endoplasmic reticulum and glycosylated by the Golgi apparatus. Normally, the amount of plasma ferritin synthesized and secreted seems to be proportional to the amount of cellular ferritin produced in the internal iron storage pathway so that the plasma ferritin concentration is related to the magnitude of body iron stores. [12] [19] [20]

Iron Regulatory Proteins (IRP-1 and IRP-2)

The iron regulatory proteins IRP-1 and IRP-2 function as both sensors and controllers of intracellular iron supply. They are trans-acting cytoplasmic RNA-binding proteins (Fig. 26-1D) that modulate the expression of mRNAs containing a cis-acting regulatory structure termed the iron-responsive element (IRE). [21] [22] [23] The IRE is a conserved 28-nucleotide sequence in a hairpin-shaped, stemloop with bulge conformation that serves as the binding site for the iron regulatory proteins (Fig. 26-2B). [23] [24] [25] The two IRPs are structurally and functionally similar but differ in their binding specificities, their pattern of tissue expression, their modes of regulation, and their responses to specific stimuli. These similarities and differences provide redundancy, flexibility, and versatility in meeting the specialized iron requirements of each cell.

IRP-1 has been found to be the cytosolic aconitase (citrate [isocitrate] hydrolyase), an iron-sulfur protein that acts enzymatically within the Krebs cycle in the interconversion of citrate to isocitrate. [26] [27] [28] A cubane [4Fe-4S] cluster forms the catalytically active site of aconitase in which three of the iron atoms are linked to the protein backbone and the fourth is bound to an inorganic sulfur within the cubane cluster. [29] [30] Intracellularly, aconitase exists in both a mitochondrial and a cytoplasmic form. The mitochondrial form of aconitase, encoded by a gene on chromosome 22, is enzymatically functional but lacks IRP activity. The cytoplasmic form, the product of a gene on chromosome 9, can function both enzymatically as an aconitase and as the RNA-binding IRP-1. [29] [31] [32] The 18 active site residues of mitochondrial aconitase and of IRP-1 are identical. [26] IRP-1 can be reversibly converted from an enzymatically active aconitase (with low RNA-binding affinity) to a high-affinity RNA-binding form (with no aconitase activity) by alterations in the Fe-S cluster in response to changes in intracellular iron availability. As shown in Figure 26-2D , physiologic changes in iron availability reciprocally regulate the aconitase activity (increased with iron repletion) and RNA-binding affinity (increased with iron depletion) of the IRP-1, with the Fe-S cluster functioning as a sensor of iron availability. [29]

Human IRP-2 is highly similar to human IRP-1 [22] , but, lacking the amino acids present at the active site of aconitase, it has no enzymatic activity. IRP-2 binds to IREs with an affinity similar to that of IRP-1 and is just as effective as a translational repressor in vitro. Although the two IRPs bind with equally high affinity to IREs, IRP-1 and IRP-2 recognize distinct and different subsets of IREs. [33] The existence of IRP-specific groups of IREs suggests that IRP-1 and IRP-2 may each regulate different assortments of mRNAs. Changes in intracellular iron availability alter the mRNA-binding activity of both IRPs post-translationally but by different means (Fig. 26-2C). [34] The control of IRP-1 involves a switch from the (nonbinding) form with aconitase activity to the IRE-binding form (without aconitase activity), with little change in the amount of IRP-1 protein. By contrast, the binding activity of IRP-2 is regulated by the degradation of the protein in the proteasome when cells are iron replete. [35] With iron deprivation, new IRP-2 is synthesized. The relative proportions of IRP-1 and IRP-2 vary from one cell type to another, but in most cells, the amounts of IRP-1 are greater than those of IRP-2. Most tissues contain mRNA for both, but a mammalian cell line has been identified that fails to express IRP-1 protein or mRNA. [36]

Although all the IRE-binding activity

Figure 26-2 Cellular iron supply and storage. **(A)** Schematic representation of the process of cellular iron uptake and transferrin-receptor recycling. Iron delivery to the cell begins with the binding of up to two molecules of mono- or diferric transferrin to a transferrin receptor in an energy- and temperature-dependent process. Once bound, the iron-bearing transferrin-receptor complex rapidly clusters with other transferrin-receptor complexes in a clathrin-coated pit, which seals over and fuses to form an internal vesicle or endosome. Moving to the interior of the cell, the endosome then fuses with an acidic vesicle whose internal pH is below 5.5. In this acidic environment iron is released from transferrin and is then made available for cellular use or storage. Acidification within the endosome also increases the affinity of the now iron-free apotrans-ferrin for the transferrin receptor, with the result that the apotransferrin-receptor bond remains intact as the complex within the endosome is transported back to the cell surface. On exposure to the neutral pH of plasma, the apotransferrin loses its affinity for the transferrin receptor and is released from the membrane, which makes both the apotransferrin and the receptor available for neutralization. *(Adapted from Irie and Tavassoli,[216] with permission.)* **(B)** Structure of an IRE. The consensus structure for an IRE is shown, as derived from sequences and possible secondary structures of IREs from ferritin and transferrin receptor mRNAs. *(From Klausner et al.,[57] with permission.)* **(C)** Regulation of IRP-1 and IRP-2. The two IRPs are shown schematically as homologous four-domain proteins that bind to IREs. In iron-replete cells, IRP-1 assembles a cubane [4Fe-4S] cluster, becoming enzymatically active as a cytosolic aconitase but losing affinity for IRE binding. With iron deprivation, treatment with the iron chelator deferoxamine (DFO) or with nitric oxide (NO), IRP-1 disassembles the Fe-S cluster, losing aconitase activity, while slowly (over 15 hours) gaining high affinity for IRE binding. Exposure to hydrogen peroxide (H_2O_2) also abolishes aconitase activity with the development of high-affinity IRE binding but more rapidly (in <1 hour). The amounts of IRP-1 are little changed with these treatments. IRP-2 undergoes proteasome-mediated degradation in iron-replete cells, but this effect of iron is opposed by DFO and NO. With iron deprivation, new IRP-2 with high binding affinity for the IRE is synthesized. **(D)** Schematic representation of the coordinate regulation of transferrin receptor, ferritin, and erythroid-specific -aminolevulinic acid synthase (eALAS) synthesis by the iron regulatory proteins IRP-1 and IRP-2. Transferrin receptor synthesis is controlled by adjusting the amounts of cytoplasmic transferrin receptor mRNA. The 3 untranslated region (3 UTR) of transferrin receptor mRNA contains five IREs. Binding of IRPs to the IREs in the 3 UTR retards cytoplasmic degradation, increasing the concentration of cytoplasmic transferrin receptor mRNA and the rate of transferrin receptor synthesis. With an increased number of cellular transferrin receptors, iron uptake is enhanced. By contrast, ferritin and eALAS synthesis are controlled without changes in the amount of ferritin or eALAS mRNA present by repressing translation of ferritin or eALAS mRNA. The 5 untranslated regions (5 UTR) of both ferritin and eALAS mRNA contain a single IRE. Binding of an IRP to the IRE in the 5 UTR (1) arrests translation of ferritin mRNA, so that less ferritin is produced and iron sequestration is diminished and (2) stops translation of eALAS mRNA, decreasing production of eALAS and diminishing utilization of iron in the heme biosynthetic pathway.

in these cells is attributable to IRP-2, changes in ferritin and transferrin receptor synthesis in response to iron exposure or deprivation are appropriate.

IRP-1 and IRP-2 regulate intracellular iron metabolism not only by reacting to changes in iron availability but also by responding to other stimuli in a cell-specific manner. Oxidative stress and nitric oxide, an important mediator of the inflammatory response, influence the IRE-binding affinity of both IRPs but in different fashions for IRP-1 and IRP-2 (Fig. 26-2C).[37] [38] [39] [40] In erythroid cells, erythropoietin enhances the IRE-binding affinity of IRP-1, but this effect is not seen in cells of macrophage origin.[41] IRE-binding affinities of the IRPs are also influenced by the phosphorylation state of the IRPs. [42] In one macrophage cell line, stimulation with interferon (IFN)-/lipopolysaccharide (LPS) resulted in nitric oxide-dependent alterations of both IRPs but in opposite directions. [40] IRP-1 IRE-binding activity slightly increased, while IRP-2 binding activity was greatly decreased in conjunction with increased ferritin synthesis. Overall, these observations indicate that the contributions of both IRPs to overall regulation must be considered in understanding iron-regulated gene expression in individual cells. The existence of two forms of IRP that differ in their binding specificities, in their patterns of tissue and cellular expression, in their modes of regulation, and in their responses to specific signals potentially provides a subtle and adaptable means of meeting the unique iron requirements of each cell.

Cellular Iron Supply and Storage

Molecular Mechanisms of Cellular Iron Uptake

Cellular Iron Uptake via TransferrinTransferrin Receptor-Mediated Endocytosis

The coordinate roles of transferrin, transferrin receptor, and ferritin in cellular iron supply and storage are shown schematically in Figure 26-2A . Iron delivery to the cell begins with the binding of one or two molecules of mono- or diferric transferrin to a transferrin receptor in an energy- and temperature-dependent process [43] [44] that is complete within 23 minutes. At the neutral pH of plasma, the iron-transferrin complex is further stabilized by the binding of transferrin to the transferrin receptor, both for monoferric and for diferric transferrin. [44] [45] The efficiency of iron delivery to the cell depends on the amounts of mono- and diferric plasma transferrin available. With normal erythropoiesis and a normal transferrin saturation of about 33%, the higher affinity of the receptor for diferric transferrin results in most of the iron supply to cells being derived from this form, providing four atoms of iron with each cycle. At a transferrin saturation of about 19%, equal amounts of iron are provided by mono- and diferric transferrin, while at lower saturations, most of the iron is derived from the monoferric form. [7] [46] Whether mono- or diferric, the fate of transferrin bound to the transferrin receptor is the same. The iron-bearing transferrin-receptor complex rapidly clusters with other transferrin-receptor complexes in a clathrin-coated pit; a YXRF (Tyr-X-Arg-Phe) structural motif in the cytoplasmic domain of the transferrin receptor is required for internalization. [47] Phosphorylation of the receptor by protein kinase C enhances the initial rate but not the final extent of internalization. The phosphorylated and nonphosphorylated forms of the receptor seem otherwise to behave identically, [48] and the internalization sequence and phosphorylation site are distinct from the basolateral targeting signal. [49] Once assembled, the clathrin-coated pit is promptly internalized and detaches from the inner membrane. Within the cytoplasm the vesicle is rapidly stripped of clathrin [50] and the uncoated vesicles fuse to become multivesicular endosomes.[51] In the interior of the cell, a proton pump lowers endosome internal pH to about 5.6. [44] [52]

Role of Transferrin Receptor in the Release of Iron from Transferrin Within the Endosome

In the acidic environment of the endosome, the transferrin receptor plays a critical role in iron dissociation from both mono- and diferric forms of transferrin. [44] At the endosomal pH of 5.6, iron release from free transferrin has a half-time of >15 minutes, and release is predominantly from the N-terminal site. [53] For comparison, in endosomes of erythroid cells, iron release from both transferrin sites is virtually complete within 23 minutes. The interaction of transferrin receptor and transferrin in the complex seems to account for this difference in the rapidity and completeness of iron release, perhaps by producing conformational changes in transferrin. [44] [45] [53] At pH 5.6, iron release from unbound monoferric transferrins is three times faster for the N-terminal form (Fe_N Tf) than for the C-terminal species (Fe_C Tf). Binding to the transferrin receptor has little effect on release from the N-terminal monoferric transferrin (Fe_N Tf) but substantially increases release from the C-terminal site (Fe_C Tf). Binding to the transferrin receptor at pH 5.6 alters site-site cooperative interactions between the two sites of doubly occupied transferrin: iron at the N-terminal lobe is stabilized while iron at the C-terminal lobe is labilized. [44] Overall, transferrin receptor binding at endosomal pH seems to substantially enhance both the rate and completeness of iron release from transferrin within the erythroid cell while minimizing differences between the N- and C-terminal sites.

Transport of Iron Across the Endosomal Membrane via Nramp2

After release within the acidified endosome, the iron must be transported across the endosomal membrane. Nramp2 (natural resistanceassociated macrophage protein 2) has recently been identified as the putative transmembrane iron transport protein that provides both for the movement of iron out of the endosome and for normal intestinal iron absorption (Fig. 26-3A, B).[54] [55] [56]

Figure 26-3 Transport of iron across membranes via Nramp2, the putative transmembrane iron transport protein. **(A)** Twelve-transmembrane-domain model of the rat isoform of human Nramp2, DCT1 (divalent-cation transporter). The putative transmembrane domains 1 to 12 are indicated. The consensus transport motif is indicated in the fourth intracellular loop and putative N-linked glycosylation sites are identified in the fourth extracellular loop. *(Reprinted with permission from Gunshin et al.[55] Copyright 1997 Macmillan Magazines Limited.)* **(B)** Model for iron transport pathway in

mammals. In this model, Nramp2 transports iron from the intestinal lumen into enterocytes. The iron then passes by unknown means to the basolateral surface, is loaded onto transferrin, and is transported through the plasma. The iron-loaded transferrin is bound by a transferrin receptor on the surface of an erythroblast, internalized into an endosome, which is then acidified and the iron released. Nramp2 transports iron out of the endosome for cellular utilization. *(Modified from Vulpe and Gitschier,[217] with permission.)*

Nramp is a newly identified family of integral membrane proteins. Nramp1 was initially identified and named for its association with susceptibility to infection by intracellular pathogens and is expressed only in macrophages. The role of Nramp2 in iron transport was first recognized in efforts to identify the causative mutation in mice with microcytic anemia (*mk*), a mutant with severe defects both in iron absorption and in erythroid iron utilization. A missense mutation in Nramp2 was identified.[54] The rat isoform of human Nramp2 has been identified as a divalent-cation transporter (DCT1) with a broad substrate range that includes Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Pb^{2+}.[55] DCT1 mediates active transport that is proton-coupled and depends on the cell membrane potential. DCT1 is ubiquitously expressed in tissues throughout the body, with notably high expression in enterocytes in the upper small intestine. In the 3 untranslated region of DCT1 cDNA, a putative IRE has been identified, suggesting, by analogy to transferrin receptor mRNA, that IRPs may regulate DCT1 mRNA levels by retarding RNA degradation. [55] Subsequent studies in the Belgrade rat, which has an autosomal recessive microcytic anemia with impaired intestinal iron absorption and defective erythroid utilization resembling that in the *mk* mouse, found a similar missense mutation in Nramp2.[56] In aggregate, these results provide strong evidence that Nramp2 is a mammalian iron transporter involved both in the movement of iron out of the endosome and in the absorption of iron from the gastrointestinal tract (Fig. 26-3B). The exact form and fate of the iron derived from the endosome are still unknown, but this newly released iron presumably is available to the mitochondria of the erythroid cell for heme synthesis or to ferritin for storage.

Return of the TransferrinTransferrin Receptor Complex to the Cell Surface

Acidification within the endosome increases the affinity of the now iron-free apotransferrin for the transferrin receptor, with the result that the apotransferrin-receptor bond remains intact as the complex is transported back to the cell surface within the endosome (Fig. 26-2A).[57] Some endosomes are briefly shunted to the Golgi apparatus for resialation and repair before returning to the membrane. [58] On exposure to the neutral pH of the plasma, apotransferrin loses its affinity for the transferrin receptor and is released from the membrane, making both apotransferrin and receptor available for reutilization. [44] [52]

Coordinate Regulation of Cellular Iron Uptake and Storage

Within the cell, IRP-1 and IRP-2 provide the means for the coordinate regulation of the physiologic uptake and storage of iron by translational control of the synthesis of transferrin receptor and ferritin (Fig. 26-2D).[23] [59] [60] [61] Transferrin receptor synthesis is controlled by adjusting the amounts of cytoplasmic transferrin receptor mRNA. The 3 untranslated region (3 UTR) of transferrin receptor mRNA contains five IREs. Binding of IRPs to the IREs in the 3 UTR retards cytoplasmic degradation, increasing the concentration of cytoplasmic transferrin receptor mRNA and the rate of transferrin receptor synthesis. With an increased number of cellular transferrin receptors, iron uptake is enhanced. Ferritin synthesis is controlled without changes in the amount of ferritin mRNA present by repressing translation of ferritin mRNA. The 5 untranslated region (5 UTR) of ferritin mRNA contains a single IRE. Binding of an IRP to the IRE in the 5 UTR arrests translation of the ferritin mRNA, so that less ferritin is produced and iron sequestration is diminished. Altogether, the coordinate regulation of intracellular iron availability by the IRPs has opposite effects on the synthesis of transferrin receptor and ferritin. A *decrease* in intracellular available iron results in an increase in the proportion of high-affinity IRPs. Increased IRP binding to IREs increases transferrin receptor protein production but decreases ferritin protein production. An *increase* in intracellular iron results, for IRP-1, in assembly of [4Fe-4S] clusters with loss of IRP-1 binding activity, and for IRP-2, in specific proteolysis. Fewer IRPs are bound to IREs, decreasing transferrin receptor protein production while increasing ferritin protein production. These balanced and opposing alterations in iron uptake and storage governed by the IRPs serve to maintain consistent intracellular physiologic iron homeostasis and to respond to both oxidative stress and inflammation. IRPs also bind to functional IREs in the 5 UTR of mRNAs of the erythroid-specific -aminolevulinic acid synthase (eALAS) and of mitochondrial aconitase and repress their synthesis under conditions of iron deprivation, linking iron utilization and cellular energy utilization to the regulation of iron homeostasis. [59] [62] [63] [64] [65]

Body Iron Supply and Storage

Body Iron Distribution

The concentration of iron in the human body is normally about 4050 mg Fe/kg body weight; women typically have lower amounts than men (Table 26-2).[66] [67] [68] [69] Most of this iron is contained in essential iron compounds. This functional iron compartment includes about 30 mg Fe/kg as hemoglobin iron contained

TABLE 26-2 -- Distribution of Iron in the Adult

Type of Iron	Concentration (mg/kg)	
	Men	Women
Functional iron		
Hemoglobin	31	28
Myoglobin	5	4
Heme enzymes	1	1
Nonheme enzymes	1	1
Transport iron		
Transferrin	<1 (0.2)	<1 (0.2)
Storage iron		
Ferritin	8	4
Hemosiderin	4	2
Total	50	40

within circulating red cells and an additional 67 mg Fe/kg that is present in tissues throughout the body in myoglobin and in a variety of heme enzymes (cytochromes, catalases, peroxidases) and nonheme enzymes (ribonucleotide reductase, metalloflavoproteins, iron-sulfur proteins). Transport iron consists of the small fraction (<0.5%) of the total body iron that is in transit to supply tissue iron needs. Iron in the transport compartment is bound to transferrin in plasma and extracellular fluid. The remainder of the iron (56 mg Fe/kg in women, 1012 mg Fe/kg in men) is storage iron in the form of ferritin and hemosiderin, principally in hepatocytes and in macrophages in the liver, bone marrow, spleen, and muscle, serving as a reserve in the event of blood loss.

Iron Balance: Iron Absorption and Loss

The major pathways of iron absorption and loss, and of internal iron exchange and storage are shown schematically in Figure 26-4 . *Iron balance* is determined by the difference between the amounts of iron entering and leaving the body. Humans are unique in their lack of any effective means to excrete excess iron. [70] Iron balance is physiologically regulated by controlling iron absorption: iron stores and absorption are reciprocally related, so that as stores decline, absorption increases. [71] Normally, iron exchange with the environment is extremely limited. Less than 0.05% of the total body iron is acquired or lost each day. Humans are unequaled in the effectiveness with which iron is conserved.

Both heme and nonheme iron are absorbed through the brush border of the upper small intestine. [72] [73] [74] The iron content

IRON REQUIREMENTS

Overall, the *iron requirement* for an individual includes not only that iron needed to replenish physiologic losses and to meet the demands of growth and pregnancy but also any additional amounts needed to replace pathologic losses. Physiologic iron losses are generally restricted to the small amounts of iron contained in the urine, bile, and sweat; the shedding of iron-containing cells from the intestine, urinary tract, and skin; occult gastrointestinal blood loss; and, in women, uterine losses during menstruation and pregnancy.[119] In normal men the daily basal iron loss is slightly less than 1.0 mg/day, and in normal menstruating women it is about 1.5 mg/day. The median total iron loss with pregnancy is about 500 mg, or almost 2 mg/day over the 280 days of gestation.[121]

Figure 26-4 Body iron supply and storage. A schematic representation of the routes of iron movement. The major pathway of internal iron exchange is a unidirectional flow from plasma transferrin to the erythron to the macrophage and back to plasma transferrin. Storage iron in the macrophages of the liver, bone marrow, and spleen is derived almost entirely from phagocytosis of senescent erythrocytes or defective developing red cells. The macrophage is virtually unable to take up iron from plasma transferrin, whereas the hepatocyte may either donate iron to or receive iron from plasma transferrin. Normally, the overall magnitude of iron exchange by hepatocytes is only about one-fifth that of macrophages. Other pathways of iron movement involve approximately equal exchanges for iron absorption and losses, for transfer between the plasma and extravascular transferrin compartments, and for movement between extravascular transferrin and parenchymal tissues. In a pregnant woman, iron is taken up via placental transferrin receptors and unidirectionally passed to fetal transferrin for utilization by fetal tissues. *(Data from Finch and Huebers.[68])*

of the diet is a function of caloric intake; typical diets in the United States contain about 7 mg/1,000 kcal. The availability of dietary iron for absorption is determined by the amount and form of the iron, the composition of the diet, and GI factors. Heme iron is usually only a small portion of the dietary iron but is highly available for absorption (2030% absorbable) and little affected by other components in the diet. [74] Most dietary iron (often <90%) is nonheme iron, which enters a common intraluminal pool, whose availability is determined by the balance between inhibitors (phytates, tannates, phosphates, etc.) and enhancers (amino acids, ascorbic acid) of absorption.[72] [73] Frequently, <5% of the nonheme iron is available for absorption. Iron availability is also influenced by GI factors such as gastric secretion, intestinal motility, and the consequences of surgery or bowel disease.

The absorption of available iron is regulated by the mucosal cells of the proximal small intestine. Mucosal regulation of iron absorption might occur by controlling one or more of the following steps: (1) mucosal uptake of iron across the brush border membrane, (2) retention of iron in storage form within the mucosal cell, and (3) transfer of the iron from the mucosal cell to the plasma. The molecular route of iron uptake is now believed to involve Nramp2, [54] [55] [56] but the details of the movement of iron from the gut have not been established with certainty. Evidence has also been developed for a pathway involving mobilferrin and paraferritin, [72] [73] but the relationship between this pathway and Nramp2 is unclear. Physiologically, the major determinants of mucosal iron absorption are the amount of body iron stores and the level of erythropoiesis: absorption increases with diminished storage iron and increased erythropoietic activity. [75] Hypoxia may also increase iron absorption independently of changes in erythroid activity. [76] A maximum of about 3.5 mg Fe/day may be absorbed from the diet, with adequate amounts of bioavailable iron and the enhanced level of absorption found in the iron-deficient state. [71]

Internal Iron Exchange

Once absorbed, iron is bound to plasma transferrin. The daily disposition of this transferrin-bound iron is summarized in Figure 26-4 , which provides an inventory of the distribution of iron in the body and a schematic representation of the patterns of iron utilization. [66] [67] [68] [69] In an iron-replete 70-kg man, the amount of transferrin iron in the plasma at any given time is only about 3 mg, but >30 mg of iron moves through this transport compartment each day. The *erythron* consists of the aggregate of all erythroid elements, including cells at all stages of development, in the marrow, circulation, and extravascular space. Although iron is an essential nutrient required by every human cell, most of the iron in the body is found within the erythron and most of its daily movement cycles through the erythron. Most iron in transit (24 mg Fe/day) is taken up by erythroid precursors in the marrow; the majority of this (17 mg Fe/day) becomes hemoglobin iron in circulating red cells, which are subsequently catabolized by specialized macrophages in the marrow, spleen, and liver. Iron is then released from the hemoglobin and returned to the plasma transferrin. Some of the erythroid marrow iron (7 mg Fe/day) arrives at the macrophage more directly because of phagocytosis of defective erythroid precursors or removal of erythrocyte ferritin, so that the macrophage returns to the plasma transferrin an amount of iron (22 mg Fe/day) almost equivalent to that donated to the erythron. The remainder of daily erythron iron turnover is derived from some of the newly absorbed iron from the GI tract and from the minor fraction (2 mg Fe/day) of hemoglobin iron that is lost into the plasma with enucleation of normoblasts or from intravascular hemolysis. This is bound to haptoglobin or hemopexin and delivered to the hepatocyte for eventual return to plasma transferrin.

Movement of iron to and from the erythron accounts for 80% of the iron flowing through the transferrin compartment each day. The remaining 20% of iron carried by transferrin includes (1) iron exchange with hepatocytes (5 mg Fe/day), (2) movement between the plasma and extravascular transferrin compartments (3 mg Fe/day), (3) exchange between extravascular transferrin and parenchymal tissues (2 mg Fe/day), and (4) limited external exchange of iron through obligatory losses and absorption of iron from the GI tract (1 and 1.5 mg Fe/day for men and women, respectively). The successive movement of iron through the major pathway of internal iron exchangefrom plasma transferrin to the erythron to the macrophage and back to plasma transferrinis summarized below, with particular attention to those factors that regulate the flow of iron through the cycle. [69]

Movement of Iron from Transferrin to the Erythron

The movement of iron from transferrin to the erythron is determined by (1) the aggregate number of transferrin receptors on erythroid precursors in the erythron and on cells in nonerythroid tissues and (2) the amounts of apo-, mono-, and diferric plasma transferrin. The number of transferrin receptors is a prime determinant of cellular iron supply. Because the number of transferrin receptors in nonerythroid tissues is stable under normal circumstances, the rate of erythropoiesis is the major determinant of the number of transferrin receptors in the erythron. As the rate of erythropoiesis increases, greater numbers of erythroid precursors with transferrin receptors are produced, increasing the rate of iron uptake.

Iron Uptake by Erythroid Cells.

The control of the acquisition, utilization, and storage of iron by erythroid cells seems to differ during different phases of erythropoiesis. Although the available evidence is fragmentary and incomplete, studies of human bone marrow and of erythroid cell lines in culture have suggested that erythropoietin acts as an inducer for the transcription of mRNA for the erythroid-specific enzymes -aminolevulinic acid (ALA) synthase and porphobilinogen (PBG) deaminase in the heme biosynthetic pathway and of - and -globin mRNA.[77] Repression of heme oxygenase, the enzyme responsible for heme degradation, has been proposed as an initiating event in the differentiation of erythroid progenitors. [78] In erythroblasts, heme enhances the transcription of globin mRNA and acts to increase the stability of globin mRNA. Other studies, using erythroid cells obtained from chick embryos at early stages of maturation, have found that the transferrin receptor gene is hyperexpressed and regulated at the transcriptional level by mechanisms that are not responsive to the availability of intracellular iron. [79] The rate of iron uptake from transferrin during erythroid cell development correlates closely with the number of transferrin receptors, suggesting that the level of transferrin receptors is the major factor determining the rate of iron uptake during erythroid cell development. The increase in transferrin receptor expression thus follows the increase in the expression of erythropoietin receptors but precedes the onset of heme synthesis. After hemoglobin synthesis is fully established in late erythroblasts and reticulocytes, translational control of the expression of transferrin receptor, ferritin, and heme seems to become the more important regulatory mechanism.

Use of Iron for Heme Synthesis in Erythroid Cells.

Almost all of the iron taken up by the erythron is used for the synthesis of heme, the prosthetic group of hemoglobin, with small amounts reserved for use in iron-containing enzymes in erythroid cells or sequestered within ferritin. In the adult, the erythron produces >85% of the total heme synthesized in the body, with most

of the remainder produced by the liver. Heme (ferrous protoporphyrin IX) is a planar molecule consisting of an atom of ferrous iron in the center of a tetrapyrrole ring. Heme is synthesized in eight biochemical steps, with the first and final three steps catalyzed by mitochondrial enzymes and the four intermediate steps taking place in the cytoplasm. The final step in heme synthesis is the formation of heme from protoporphyrin IX and ferrous iron, catalyzed by the mitochondrial enzyme ferrochelatase. In human erythroid cells, heme synthesis seems to be controlled at the first step in the production of protoporphyrin IX. Feedback inhibition by heme limits one or more steps that lead to the formation of -ALA, the first committed precursor in the porphyrin pathway. [77] In erythroid cells, this first enzymatic reaction in heme biosynthesis is catalyzed by the erythroid-specific form (see below) of 5-aminolevulinate synthase (ALA synthase) and consists of the condensation of glycine and succinyl CoA to form -ALA, a reaction requiring pyridoxal phosphate. Heme does not affect the level of mRNA for erythroid-specific ALA synthase and has no evident direct effect on the enzyme. Instead, heme seems to reduce the activity of the erythroid-specific ALA synthase by an indirect cellular process that has not yet been characterized.

The enzymes of the heme biosynthetic pathway must be expressed during the life of each cell in the body to provide for cytochrome and other vital heme enzymes. In addition to these constitutive or housekeeping enzymes produced during the life of all cells, erythroid-specific isozymes have been identified for ALA synthase, which catalyzes the first step in the heme biosynthetic pathway, and for PBG deaminase, which catalyzes the third. In humans, the two isozymes for ALA synthase are encoded by different genes, with the gene for the constitutive or housekeeping isozyme on chromosome 3p21 and that for the erythroid-specific form on the X chromosome (Xp21-q21).[80] Only a single gene for PBG deaminase is present in the human genome, but this gene has two overlapping transcription units. [77] An upstream promoter is active in all cells, while the downstream promoter is erythroid specific. Alternative splicing produces two mRNAs that encode the PBG deaminase isozymes. Studies of the promoter regions for the erythroid-specific forms of ALA synthase and PBG deaminase have established that both have cis-acting control motifs similar to those in the -globin

gene promoters, including both GATA-1 and NF-E2 binding sites. [77] These observations suggest that globin genes and the erythroid-specific forms of both ALA synthase and PBG deaminase are all subject to developmental control by common trans-acting factors during erythropoiesis. These trans-acting factors may mediate the effects of erythropoietin on the transcription of mRNAs for globin, ALA synthase, and PBG deaminase described above.

Iron-Dependent Translational Regulation of Erythroid ALA Synthase mRNA.

The erythroid ALA synthase gene on the X chromosome has also been found to contain an IRE motif in the 5 UTR that is not present in the housekeeping gene on chromosome 3. The available studies provide strong experimental evidence that the corresponding IRE is functional in vivo and that, if intracellular iron availability is low, binding of an IRP will prevent translation of the erythroid-specific ALA synthase mRNA (Fig. 26-2D). The IRE in the 5 UTR of the erythroid-specific ALA synthase mRNA may help coordinate iron uptake and heme synthesis in erythroid cells. [77] The observation that erythroid ALA synthase activity is decreased in erythroblasts from bone marrow obtained from patients with iron deficiency [81] is consistent with translational regulation of erythroid ALA synthase mRNA by IRPs. Altogether, the available evidence suggests that regulation of the activity of erythroid-specific ALA synthase, and thereby of the rate of heme synthesis in erythroid cells, involves (1) developmental transcriptional control by erythroid-specific trans-acting factors, (2) feedback inhibition by heme through a still undefined mechanism that does not influence the availability of the mRNA for the enzyme, and (3) intracellular iron availability through IRP control of the translation of the erythroid-specific ALA synthase mRNA.

Ferritin in Erythroid Cells.

Iron that is in excess of erythroid requirements for heme synthesis is sequestered within ferritin. The more immature erythroid precursors, the proerythroblasts and basophilic erythroblasts, contain higher concentrations of intracellular ferritin than mature forms and also have greater proportions of the H-type subunit. [12] The major determinants of the ferritin content of the erythroid cell appear to be the plasma transferrin saturation, reflecting the iron supply to the erythroblast, and the rate of hemoglobin synthesis, as an indicator of the erythroid iron requirement. Erythroid cell ferritin is decreased in patients with iron deficiency or with severe inflammatory disorders and increased in patients with other conditions in which hemoglobin synthesis is reduced, such as thalassemic disorders or sideroblastic anemias, or with iron overload. [12]

Other Regulatory Functions of Intracellular Iron Availability.

Iron availability in erythroid cells may not only specifically alter the rates of production of heme, transferrin receptor, and ferritin through the IRPs but may also have more general effects on the metabolism of the developing erythroid cell. The identification of a conserved and functional IRE in the 5 UTR of mammalian mitochondrial aconitase raises the possibility of a further role for iron availability in the regulation of cellular metabolism by coupling iron availability and the activity of the citric acid cycle. [62]

Movement of Iron from the Erythron to the Macrophage

The major pathway of iron movement from the erythron is to a specialized population of macrophages in the bone marrow, liver, and spleen as red cells reach the end of their life span (Fig. 26-4). Whereas the developing erythroid cell is dedicated to the acquisition of iron from transferrin for use in hemoglobin synthesis, these specialized macrophages are devoted to the extraction of iron from hemoglobin for prompt return to transferrin or, if necessary, for storage for future use. Less is known about the molecular mechanisms underlying the disposition of iron by the macrophage than about any other aspect of iron metabolism.

Removal and Catabolism of Senescent or Damaged Erythrocytes.

Senescent or damaged erythrocytes are selectively sequestered by a specialized population of macrophages in the bone marrow, liver, and especially the spleen. Macrophages in the bone marrow also cull defective immature erythroid cells to prevent their release into the circulation and remove some deposits of erythrocyte ferritin from developing red cells. During their time in the bloodstream, red cells undergo oxidant damage, alterations of membrane proteins and lipids, loss of surface sialic acid and electrostatic charge, decreases in ion gradients, and metabolic depletion of glycolytic and other enzymes, including protein kinases. Hemichrome formation with oxidative damage to membrane transport proteins may produce increased membrane permeability, and defective volume regulation and crosslinking by hemoglobin or hemichromes of membrane band-3 molecules and the formation of senescent antigens have been reported. Aged erythrocytes become dehydrated and lose surface area, with a decrease in cell volume and an increase in intracellular hemoglobin concentration, making the cells less deformable. Despite all these known changes in aged red cells, the characteristic or combination of characteristics that is the proximate cause of the removal of senescent erythrocytes from the circulation has yet to be determined.[82] Billions of red cells are normally eliminated from the circulation each day without any evidence of overt erythrophagocytosis, suggesting that some form of erythrocyte fragmentation occurs before ingestion by macrophages. [82]

The metabolism of catabolized hemoglobin in humans has been examined using heat-damaged erythrocytes labeled with radioactive iron. [83] After injection of the labeled red cells, a delay of 40 minutes precedes the appearance of radioiron in the plasma, presumably representing the time needed for phagocytosis of the damaged erythrocytes and catabolism of heme. Radioiron is then released in a biphasic manner. Two-thirds reemerges in an early, rapid phase of release, with a $t_{1/2}$ of slightly more than one-half hour; the remaining third is incorporated into macrophage stores and reappears in a late, slow phase ($t_{1/2}$ 6 days).[83] In iron deficiency, iron release is derived entirely from the recently catabolized erythrocyte. With increasing iron stores, the proportion of the radioiron derived from ingested red cells declines, especially during the early, rapid phase of iron output. The rate of late release is also influenced by the magnitude of iron stores: the greater the amount of storage iron, the longer is the delay in release. Inflammation and ascorbic acid deficiency also increase the proportion of catabolized erythrocyte iron retained within macrophages.[68] [84]

Studies of the catabolism of red cells by macrophages have suggested that each macrophage is able to fully process at least one ingested erythrocyte per hour. [85] Ingested antibody-coated erythrocytes are surrounded by projections of the plasma membrane and moved to the perinuclear area. Macrophage lysosomes fuse with the vacuole containing the phagocytized red cell, forming a network of interconnecting channels that fragment the erythrocyte as its components are digested. The erythrocyte membrane is lysed and the hemoglobin within is oxidatively precipitated. Almost all the hemoglobin is rapidly catabolized, with the globin proteolytically processed to amino acids, releasing heme. The heme is somehow transported to the endoplasmic reticulum of the macrophage to be degraded by heme oxygenase. Erythrophagocytosis induces synthesis of heme oxygenase.[86]

Catabolism of Hemoglobin Iron.

Heme oxygenase (M_r 32,000) is a microsomal enzyme that catalyzes the rate-limiting step in the oxidative catabolism of heme to yield equimolar quantities of biliverdin IX, carbon monoxide, and iron. Three isozymes of heme oxygenase have been identified, designated HO-1, HO-2, and HO-3. Heme oxygenase-1 (HO-1) is the inducible form of the rate-limiting enzyme of heme degradation that regulates the cellular content of heme. [87] HO-2 is the constitutive

form.[88] The function of HO-3 is unknown, but a regulatory role has been postulated. [89] The process of heme degradation by heme oxygenase has been characterized as a series of autocatalytic oxidations with the reaction intermediates as cofactors. [78] A total of three oxygen molecules and six reducing equivalents are required to oxidatively degrade one heme molecule to biliverdin IX, with one iron and one carbon monoxide molecule released. The precise reaction sequence has not been established, but a single heme oxygenase binds a single heme and cleaves the -methane bridge, leaving a biliverdin-iron complex. The -methane carbon is converted to carbon monoxide, the sole physiologic source of carbon monoxide in the body. Iron is then released from the biliverdin-iron complex, generating the linear tetrapyrrole biliverdin IXa, which is then reduced by biliverdin reductase to bilirubin IXa. HO-1 is required for efficient reutilization of iron for erythropoiesis; mice lacking functional HO-1 developed an anemia associated with abnormally low serum iron levels while accumulating hepatic and renal iron that contributed to macromolecular oxidative damage, tissue injury, and chronic inflammation. [90]

The disposition of iron after release from heme by heme oxygenase is incompletely understood. Presumably the iron freed from heme enters an intracellular reservoir where the IRPs monitor iron availability. The expression and regulation of IRP-1, IRP-2, transferrin receptor, and ferritin have been examined in cultures of human peripheral blood monocytes maturing to macrophages. Although peripheral blood monocytes have few transferrin receptors, cultured monocytes and macrophages of a variety of types seem to express surface transferrin receptors and seem to be able to take up iron by this route. By contrast, ferrokinetic investigations in humans have found no evidence for movement of iron from transferrin to the macrophage in vivo, [67] although these studies may be less sensitive than studies of individual cells. Maturation of monocytes to macrophages was associated with marked increases in high-affinity IRP binding and in both mRNA and protein for transferrin receptor and ferritin, probably as a result of increased gene transcription. [9] Most important, exposure of cultured macrophages to iron salts stimulated IRPs activity and seemed to be associated with a translationally mediated increase in both transferrin receptor and ferritin synthesis; treatment with the iron chelator desferrioxamine decreased transferrin receptor synthesis. [9] These results should be contrasted with the findings in erythroid cells, where an increase in iron exposure would diminish IRP activity, with a resultant decrease in transferrin receptor synthesis and an increase in ferritin synthesis; treatment with an iron chelator would result in an increase in transferrin receptor synthesis. Moreover, stimulation of a macrophage cell line with IFN-/LPS resulted in a slight increase in IRP-1 IRE-binding activity but a great decrease in IRP-2 binding activity in association with increased ferritin synthesis. [40] The consequences of these contrasting patterns of response to iron loading in macrophages and erythroid cells would apparently be to maintain a constant and consistent supply of iron for utilization in heme synthesis in the erythroid cell and to increase iron acquisition and storage in the macrophage. Overall, this pattern would serve to move iron to the erythron from the macrophage in times of lack and to store iron within the macrophage system during periods of excess.

Storage of Iron Within the Macrophage.

Under normal circumstances, the macrophages in the liver, spleen, and bone marrow that are dedicated to reprocessing hemoglobin iron from senescent erythrocytes maintain an equilibrium between iron storage and release. Whatever the molecular mechanisms involved, synthesis of ferritin is induced in response to erythrophagocytosis, and, in the absence of iron deficiency, a portion of the iron derived from the ingested erythrocyte is retained within the macrophage as ferritin iron. Based on studies of heat-damaged erythrocytes labeled with radioactive iron, the fraction of radioiron sequestered within the macrophage can vary from virtually none in association with iron deficiency to a maximum of almost 80% in the presence of marrow aplasia and a fully saturated plasma transferrin. [83] No matter what the circumstances, the macrophage does not seem able to retain more than 80% of the iron derived from the catabolized erythrocyte. Within the macrophage, this iron is stored predominantly in ferritin rich in L subunits. [12] Catabolism of cellular ferritin may result from digestion of the protein shell with reutilization of the iron core or conversion to hemosiderin, an amorphous, water-insoluble storage compound with a higher iron content and slower turnover than ferritin that is suitable for long-term storage of iron. [91] The ferritin and hemosiderin iron stored within the macrophage remains available for mobilization in time of need. With increasing amounts of storage iron within the macrophage, the proportion of iron stored within hemosiderin progressively increases. Ascorbic acid seems to retard ferritin degradation by reducing lysosomal autophagy of the protein, thereby increasing the amount of iron stored in cytoplasmic ferritin. [18]

Movement of Iron from the Erythron to the Hepatocyte

The remaining pathway of iron movement from the erythron provides a means for hemoglobin iron that is released into the plasma to be delivered to the hepatocyte for eventual return to the plasma transferrin (Fig. 26-4). With normal erythropoiesis, this portion of the total iron flux is minor, but it can increase substantially in disorders with increased ineffective erythropoiesis or intravascular hemolysis. Delivery of hemoglobin iron from the plasma to hepatocytes depends primarily on two glycoproteins: (1) haptoglobin for the binding and transport of dimers of hemoglobin, and (2) hemopexin for the binding and transport of heme, assisted, if needed, by albumin.

Haptoglobin Binding and Transport of Hemoglobin.

Hemoglobin may escape into the plasma with the enucleation of erythroblasts on their transformation into reticulocytes, with the destruction of defective developing red cells within the marrow or with hemolysis in the circulation. At the low plasma concentration of hemoglobin produced by these processes, most of the hemoglobin A dissociates into dimers. Pairs of dimers are then quickly bound in a symmetric fashion to a single molecule of haptoglobin. The half-life of apohaptoglobin is 5 days, but that of the hemoglobin-haptoglobin complex is only 1030 minutes, as the complexes are cleared by specific hepatocyte receptors. [92] The hemoglobin-haptoglobin complex (M_r 150,000) is too large to be filtered by the kidneys, a feature that helps restrict the renal loss of iron with hemoglobinemia. After binding to the haptoglobin receptor of the hepatocyte, the hemoglobin-haptoglobin receptor complex is then internalized and dissociated symmetrically by limited proteolysis into two subunits of M_r 82,000 having intact hemes. Unlike the transferrin-transferrin receptor complex, the haptoglobin-haptoglobin receptor is degraded and cannot be recycled. The hemes are then released to an unidentified carrier and catabolized by heme oxygenase, releasing iron within the hepatocyte. [92] [93] Consumption of haptoglobin does not induce increased hepatic production, with the result that sustained hemoglobinemia produces hypo- or ahaptoglobinemia. Haptoglobin is a positive acute phase reactant, so that inflammatory or infectious episodes may increase plasma concentrations.

Hemopexin Binding and Transport of Heme.

Hemoglobin leaked into the plasma that is not bound to haptoglobin is soon oxidized to ferrihemoglobin (methemoglobin), which in turn can dissociate into globin and ferriheme (metheme). Ferriheme (but not hemoglobin) can then be bound by hemopexin; a single hemopexin binds a single ferriheme. Hemopexin (M_r 70,000) is a glycoprotein synthesized by hepatocytes. The

half-life of apohemopexin is 7 days but that of the heme-hemopexin complex is 78 hours, as the complexes are cleared by specific hepatocyte receptors. [94] After binding to the hepatocyte hemopexin receptor, the heme-hemopexin-receptor complex is then internalized within clathrin-coated pits. Receptor-mediated endocytosis of the heme-hemopexin results in catabolism of heme but not of hemopexin or the hemopexin receptor, which are recycled to the cell surface for reutilization in a manner analogous to the transferrin-transferrin receptor complex. [92] Delivery of heme to the hepatocyte results in induction of heme oxygenase, which releases the iron from the heme in the cell. Despite recycling, hemopexin may become depleted in patients with hemolysis. Each molecule of human albumin contains two binding sites for ferriheme, but these have a much lower affinity than the binding site of hemopexin. Binding of ferriheme produces methemalbumin, which, after a delay of several days, also delivers the heme to the hepatocyte, where the iron is liberated for reuse.

Movement of Iron from the Macrophage to Transferrin

The final step in the movement of iron through the cycle shown in Figure 26-4 requires the return of iron derived from the catabolism of senescent erythrocytes to plasma transferrin for delivery to the erythroid marrow. The outpouring of iron to plasma apotransferrin from macrophages in the bone marrow, liver, and spleen normally constitutes the largest single flux of iron from cells in the body. Despite the importance and magnitude of this flow of iron, the mechanisms permitting the exit of iron from the macrophage remain an almost complete mystery.[95] Little information is available about (1) the form of iron within the cell before its departure, (2) the manner in which the iron is able to pass through the plasma membrane, (3) the form of the iron on its emergence from the cell, (4) the site and manner of delivery of the iron to plasma apotransferrin, or (5) the physiologic relevance and quantitative importance of iron release from the macrophage that is not mediated by transferrin but involves ferritin or other carriers.

Release of Iron from Macrophages to Plasma.

Variations in iron release from the macrophage have been considered to be responsible for diurnal variations in the plasma iron concentration. The amount of iron released is a function, in part, of the amount delivered to the macrophage. Modest increases in the red cell load to the macrophage without changes in the activity of the erythroid marrow may be matched by similar increases in iron release to the plasma, but with even larger amounts an increased proportion of the iron will be stored within the macrophage.[83]

In some manner, iron release from the macrophage also seems to be influenced by erythroid marrow requirements. With iron deficiency, all the iron derived from hemoglobin catabolism is promptly returned to the plasma and none is diverted to macrophage stores. [83] With the increase in erythropoiesis associated with acute blood loss, macrophage iron stores are mobilized, but the maximal rate of release from this source in the adult is limited to 4060 mg of iron per day. [96] If erythroid marrow activity is increased in association with a sustained hemolytic state in which nonviable cells are returned to the macrophage, as much as 80160 mg of iron per day may be released from the macrophage to transferrin.[96] Decreases in erythropoiesis can be matched by reductions in macrophage iron release to some extent, but a minimum of 20% of the iron load presented to the macrophage must be returned to the plasma daily. [67] [96] Iron release from macrophages may be inadequate to meet erythroid marrow needs in ascorbate deficiency[84] and copper deficiency. In copper deficiency, hypoferremia is corrected by the administration of ceruloplasmin but not by copper itself.[97]

Although the overall patterns of iron release from the macrophage in various circumstances have been described, the underlying mechanisms are almost entirely obscure. The release of iron is temperature dependent, [85] implying that they are energy-dependent processes. After the completion of erythrophagocytosis, iron export is not affected by iodoacetate, chloroquine, colchicine, cytochalasin B, or cyclohexamide, suggesting the lack of any role in the release process for glycolysis, acidification of an intracellular compartment, microtubule function, microfilament function, or protein synthesis. [85] Inflammatory states are associated with delayed processing and release of iron from the macrophage, an effect reproduced when isolated Kupffer cells are incubated with serum from rats with sterile, turpentine-induced abscesses. The exact mediators of this response with the macrophage are uncertain; no influence on the course of iron release from the Kupffer cell was found with interleukin (IL)-1, IFN-, or tumor necrosis factor. [85]

Apotransferrin Is Not Required for the Release of Iron from Macrophages.

Although transferrin acts as the sole physiologic means for the transport of iron to the erythroid marrow, a requirement for the presence of unsaturated transferrin for release of iron from the macrophage has not been established. Apotransferrin does not enter the macrophage and accepts iron only after the release of iron from the cell.[85] Studies in vivo found that release of iron derived from heat-damaged red cells was unaffected by transferrin saturation and that the injection of apotransferrin did not increase iron release. [68] [84] In vitro, the presence of apotransferrin has had little or no effect on the magnitude of iron release in most investigations. [85]

Release of Iron from Macrophages as Ferritin Iron.

Several studies have now found that much of the iron released from macrophages is in the form of ferritin both in experiments in vitro [85] [98] and in vivo, after the administration of heat-damaged red cells to rats, but the physiologic fate of this released ferritin and its iron remains uncertain. [98] Iron-loaded ferritin released by Kupffer cells after erythrophagocytosis in vitro has been shown to be readily taken up by hepatocytes, [85] [99] possibly by specific hepatocyte ferritin receptors. [98] These results have suggested the possibility that ferritin released by Kupffer cells may serve as an intrahepatic carrier of iron to the hepatocyte. Such a mechanism would protect the hepatocyte against iron deficiency but would also create the risk of iron loading with chronic hemolysis.

Other Pathways of Iron Movement.

Storage iron in the macrophages of the liver, bone marrow, and spleen is derived almost entirely from phagocytosis of senescent erythrocytes or defective developing red cells. Ferrokinetic studies have suggested that the macrophage takes up little if any iron from plasma transferrin. By contrast, the hepatocyte may either donate iron to or receive iron from plasma transferrin. At high transferrin saturations, iron moves from the plasma to the liver, while at low saturations, iron is mobilized from hepatocyte stores and supplied to plasma transferrin. Normally, the overall magnitude of iron exchange by hepatocytes is only about one-fifth that by macrophages. Other pathways of iron movement involve approximately equal exchanges: 1 mg Fe/day is absorbed and lost, 3 mg Fe/day is transferred between the plasma and extravascular transferrin compartments, and 2 mg Fe/day moves between extravascular transferrin and parenchymal tissues. In a pregnant woman the placenta is amply provided with transferrin receptors; these can successfully compete with the erythroid marrow for transferrin iron, which is taken up and unidirectionally passed to fetal transferrin for utilization by fetal tissues. Lactoferrin (M_r 76,000) is a glycoprotein that is structurally similar to transferrin, with a single polypeptide chain and two reversible binding sites for iron.[100] Lactoferrin has no established role in iron transport and is found principally in neutrophils and in bodily secretions. Lactoferrin seems to function as an antimicrobial scavenger of iron in acidic conditions, where transferrin binds iron poorly, and may also have

roles as a modulator of myelopoiesis and of mononuclear cell production and function. [101]

Laboratory Evaluation of Iron Status

The continuum of changes in iron stores and distribution in the presence of increased or decreased body iron content is shown in Figure 26-5 , along with characteristic values for clinically available indicators of iron status. The general usefulness of these measures will be considered here and their specific application in the diagnosis of iron deficiency and iron overload described below. Body iron supply and stores may be evaluated by both direct and indirect means, but no single indicator or combination of indicators is ideal for the evaluation of iron status in all clinical circumstances. As body iron content decreases from the iron-replete normal to the amounts found in iron deficiency anemia, or increases to the magnitudes found in iron overload, each available measure reflects in a different manner the continuum of changes shown in Figure 26-5 . In addition, each indicator may be affected by other conditions, such as infection, inflammation, liver disease, malignancy, or malnutrition, and must be interpreted with an awareness of the potential influence of such coexisting disorders.

Direct Measures

The direct measures of body iron status yield quantitative, specific, and sensitive determinations of body or tissue iron stores. Quantitative phlebotomy provides a direct measure of total mobilizable storage iron. [66] [67] [102] Repeated venesection to remove about 500 ml of blood weekly is performed until the hemoglobin concentration falls to <10 g/dl for 2 weeks without further phlebotomy. Mobilizable storage iron may then be calculated as the amount of hemoglobin iron removed, with corrections for the hemoglobin deficit and estimated GI iron absorption during the course of phlebotomy. Quantitative phlebotomy is inapplicable to most anemic disorders but is occasionally useful in the diagnostic evaluation of some forms of iron overload, for example, in patients with hereditary hemochromatosis who do not undergo liver biopsy.

Bone marrow aspiration and biopsy can provide information about (1) macrophage storage iron, by semiquantitative grading of marrow hemosiderin stained with Prussian blue, or if needed, by chemical measurement of nonheme iron; (2) the iron supply to erythroid precursors, by determining the proportion and morphology of marrow sideroblasts (i.e., normoblasts with visible aggregates of iron in the cytoplasm); and (3) the general morphologic features of hematopoiesis. [67] [103] Bone marrow aspiration and biopsy are useful in studies of iron deficiency but of limited applicability in the evaluation of iron overload because no information about the extent of parenchymal iron deposition is provided. In the evaluation of iron overload, liver biopsy is the best direct test for assessing iron deposition, permitting

quantitative measurement of the nonheme iron concentration and histochemical examination of the pattern of iron accumulation in hepatocytes and Kupffer cells. [67] [103] [104] [105] [106]

These direct methods for assessing iron status have the disadvantages of being invasive procedures, with their attendant discomfort, lack of acceptability to patients, and, in the case of liver biopsy, risk. Several noninvasive means of measuring tissue iron stores are under development, including determination of hepatic magnetic susceptibility,[107] [108] [109] computed tomography (CT),[110] and magnetic resonance imaging (MRI),[104] but as yet none is available for clinical use.

Indirect Measures

The indirect measures of body iron status have the advantages of ease and convenience, but all are subject to extraneous influences and lack specificity, sensitivity, or both. The measurement of plasma ferritin provides the most useful indirect estimate of body iron stores. [20] As noted earlier, ferritin is secreted into the plasma in small amounts. Although intracellular ferritin is produced by the smooth endoplasmic reticulum, plasma ferritin is synthesized by the rough endoplasmic reticulum and glycosylated

Figure 26-5 Continuum of changes in iron stores and distribution in the presence of increased or decreased body iron content. Abnormalities indicating the onset of specific stages of negative iron balance are enclosed in boxes. *(Adapted from Herbert,[216] with permission.)*

PLASMA FERRITIN CONCENTRATIONS

Decreased plasma ferritin concentrations are of great value in the detection of iron deficiency. Plasma ferritin concentrations decline with storage iron depletion; a plasma ferritin concentration below 12 g/L is virtually diagnostic of absent iron stores. The only known conditions that may lower the plasma ferritin concentration independently of a decrease in iron stores are hypothyroidism and ascorbate deficiency,[20] but these conditions would only rarely cause problems in clinical interpretation. Increased plasma ferritin concentrations may indicate increased iron stores, but a number of disorders may raise the plasma ferritin level independently of the body iron store. Plasma ferritin is an acute phase reactant, increased ferritin synthesis being a nonspecific response that is part of the general pattern of the systemic effects of inflammation. Thus, fever, acute infections, rheumatoid arthritis, and other chronic inflammatory disorders elevate the plasma ferritin concentration. Both acute and chronic damage to the liver, as well as to other ferritin-rich tissues, may increase plasma ferritin as an inflammatory process or by releasing tissue ferritins from damaged parenchymal cells; these tissue ferritins are not glycosylated.

by the Golgi apparatus. Under normal circumstances, the amount of plasma ferritin synthesized and secreted seems to be proportional to the amount of cellular ferritin produced in the internal iron storage pathway, so that the plasma ferritin concentration is related to the magnitude of body iron stores. [20] The small amounts of ferritin secreted into the circulation can be measured by immunoassay and have a logarithmic relationship to body iron stores in normal individuals. In the absence of complicating factors, plasma ferritin concentrations decrease with depletion of storage iron and increase with storage iron accumulation. A maximum glycosylated plasma ferritin concentration of 4,000 g/L has been postulated, [111] perhaps representing an upper physiologic limit of the rate of synthesis; higher concentrations are believed to be due to the release of intracellular ferritin from damaged cells.

Measurement of the plasma transferrin receptor concentration provides a useful new means of detecting iron deficiency. [112] The soluble transferrin receptor is a truncated form (M_r 85,000) of the tissue transferrin receptor that consists of the N-terminal cytoplasmic domain that has probably been proteolytically released from the cell membrane.[113] Immunoassays that can detect the soluble truncated form of the transferrin receptor in human plasma are becoming clinically available. Although much remains to be learned about the origin and fate of the plasma transferrin receptors, most are derived from the erythroid marrow, and the concentration of circulating soluble transferrin receptor is primarily determined by erythroid marrow activity. [112] Decreased levels of circulating soluble transferrin receptor are found in patients with erythroid hypoplasia (aplastic anemia, chronic renal failure), and increased levels are present in patients with erythroid hyperplasia (thalassemia major, sickle cell anemia, chronic hemolytic anemia). Iron deficiency also increases soluble transferrin receptor concentrations, although it is not clear to what extent this is the result of increased transferrin receptor expression in individual erythroblasts or simply appropriate to the degree of anemia and associated erythropoietin stimulation. Initial clinical experience indicates that the plasma transferrin receptor concentration reflects the total body mass of tissue receptor and that, in the absence of other conditions causing erythroid hyperplasia, an increase in concentration provides a sensitive, quantitative measure of tissue iron deficiency. [112] In particular, measurement of the plasma transferrin receptor concentration may help in differentiating between the anemia of iron deficiency and the anemia associated with chronic inflammatory disorders. Although the plasma ferritin concentration may be disproportionately elevated in relation to iron stores in patients with inflammation or liver disease, the plasma transferrin receptor concentration seems to be unaffected by these disorders and appears to provide a more reliable laboratory indicator of iron deficiency. Measurement of plasma transferrin receptor concentration is of no use in the detection of iron overload.

The measurement of urinary iron excretion with chelating agents, usually either desferrioxamine or diethylenetriamine pentaacetate (DPTA), offers another means of assessing body iron stores.[67] This test is not helpful in the detection of iron deficiency because of the overlap between values in individuals with normal and decreased iron stores; it is applied primarily to the evaluation of iron overload. The usefulness of the measurement of chelated iron in the urine is limited by a lack of correlation between chelatable iron excretion and the results of quantitative phlebotomy with parenchymal iron overload, and by the susceptibility of the test to extraneous influence by infection, inflammation, level and effectiveness of erythropoiesis, extramedullary hematopoiesis, liver disease, and ascorbic acid deficiency.

The erythrocyte zinc protoporphyrin provides an indicator of iron supply to erythroid precursors over a longer term. [114] In heme biosynthesis the final reaction is the chelation of a ferrous ion by protoporphyrin IX. If no iron is available, zinc is chelated instead to form zinc protoporphyrin. Because zinc protoporphyrin formed during development persists throughout the life span of the red cell, the blood concentration changes only as new cells are formed and old cells destroyed, providing a retrospective view of iron supply over the preceding several weeks. An elevated erythrocyte zinc protoporphyrin (sometimes measured as the zinc protoporphyrin/heme ratio) lacks specificity because concentrations increase not only with iron deficiency but also with other conditions that restrict iron availability, such as infection, inflammation, malignancy, and ascorbate deficiency; levels are also increased in many sideroblastic anemias and especially with chronic lead poisoning.[114] The test is useful for detection of lead poisoning but of no value in detecting iron overload.

Examination of the peripheral blood by measurements of hemoglobin concentration, hematocrit, red cell indices, red cell volume distribution, and reticulocyte count and by inspection of erythrocyte morphology reveals abnormalities only after depletion of iron stores restricts the availability of iron for erythropoiesis. The changes are not specific for iron deficiency and may be found in other conditions with defective hemoglobin synthesis, such as thalassemia, infection, inflammation, liver

TRANSFERRIN CONCENTRATION

An indicator of body iron stores that is much less sensitive than plasma ferritin is the transferrin concentration, usually measured in the clinical laboratory as the total iron-binding capacity (TIBC). Transferrin may increase with storage iron depletion and decrease with iron overload but is not a consistently reliable index because of the degree to which levels are altered by other factors. Inflammation, infection, malignancy, liver disease, nephrotic syndrome, and malnutrition all depress transferrin levels, whereas pregnancy and oral contraceptive use produce elevations.

PLASMA IRON AND TRANSFERRIN SATURATION

The plasma 2iron and the transferrin saturation, which equals the ratio of plasma iron to total iron-binding capacity, provide a measure of current iron supply to tissues.[72] After storage iron is depleted, the serum iron falls; a transferrin saturation of <16% is often used as the criterion for iron-deficient erythropoiesis. By contrast, the plasma iron and transferrin saturation are not reliably elevated with increased iron stores within macrophages, as occurs initially with transfusional iron overload, although the transferrin saturation may increase with parenchymal iron loading. Interpretation of the transferrin saturation is complicated by the substantial circadian fluctuations in plasma iron, as well as by day-to-day variations of 30%. Further, the plasma iron is decreased by infection, inflammation, malignancy, and ascorbate deficiency but increased by iron ingestion, aplastic and sideroblastic anemia, ineffective erythropoiesis, and liver disease.

disease, and malignancy. Iron overload does not produce any diagnostic abnormalities in the peripheral blood.

Future Directions

Continued rapid progress in understanding the molecular basis of cellular iron metabolism is anticipated. The identification of Nramp2 as a principal iron transporter both from the GI tract into the enterocyte and intracellularly from the endosome to the cytoplasm constitutes a major advance in our understanding of iron movement that will almost certainly lead to further advances in our knowledge of iron absorption and internal iron exchange. In conjunction with the identification of *HFE* as a candidate gene for hereditary hemochromatosis, we may finally begin to understand the molecular mechanisms involved in the control of iron absorption. Definitive identification of ligands for intracellular low molecular weight iron is still needed, along with an understanding of their interrelationship with the IRPs. Our understanding of the physiologic significance of differences in IRP-1 and IRP-2 in their binding specificities, patterns of tissue and cellular expression, modes of regulation, and responses to specific signals will be refined. The influence of oxidative stress and inflammation on iron homeostasis will be understood more thoroughly. More work is needed to better understand the details and distinctive features of iron metabolism in specialized cells and tissues, e.g., differences in iron metabolism within the hepatocyte and Kupffer cell.

IRON DEFICIENCY

Iron deficiency denotes a deficit in total body iron resulting from iron requirements that exceed iron supply. The continuum of decreased body iron is shown in Figure 26-5 and the changes in body iron movement and storage in Figure 26-6AC (Figure Not Available) . Three successive stages of iron lack may be distinguished. A decrement in storage iron without a decline in the level of functional iron compounds has been termed iron depletion (Fig. 26-6A (Figure Not Available)). After iron stores are exhausted, lack of iron limits the production of hemoglobin and other metabolically active compounds that require iron as a constituent or cofactor, and iron-deficient erythropoiesis (Fig. 26-6B (Figure Not Available)) develops, although the effect on hemoglobin production may be insufficient to be detected by the standards used to differentiate normal from anemic states. Further diminution in the body iron produces frank iron deficiency anemia (Fig. 26-6C (Figure Not Available)).

Epidemiology

Iron deficiency remains the most common cause of anemia, both in the United States[115] and worldwide.[116] In the United States the amounts of bioavailable iron in the diet, together with food fortification and the widespread use of iron supplements, have reduced the overall prevalence and severity of iron deficiency, but iron nutrition remains a problem in some subgroups, namely, toddlers, adolescent girls, and women of childbearing age. [115] Without iron supplementation, most women will become iron deficient during pregnancy.[117] Some minority groups also have an increased risk of iron deficiency. Globally, 30% of the estimated world population of almost 4.5 billion are anemic, and at least half of these, or 500 million people, are believed to have iron deficiency anemia. [116] Populations who have diets low in bioavailable iron, who suffer from chronic GI blood loss due to helminthic infection, or both have the highest prevalences of iron deficiency.

Etiology and Pathogenesis

The foremost task in the evaluation of a patient with iron deficiency is to identify and treat the underlying cause of the imbalance between iron requirements and supply that is responsible for the lack of iron (Table 26-3). The most common cause of increased iron requirements leading to iron deficiency is blood loss; in men and postmenopausal women, iron deficiency almost inevitably signifies GI blood loss. Within the GI tract, any hemorrhagic lesion may be responsible for blood loss, including hiatal hernia, esophageal varices, gastritis, duodenitis, peptic ulcer, cholelithiasis, intrahepatic bleeding, inflammatory bowel disease, diverticulosis, hemorrhoids, and adenomatous polyps. Iron deficiency is often the first sign of an occult GI malignancy. Chronic ingestion of drugs such as alcohol, salicylates, steroids, and nonsteroidal anti-inflammatory agents may cause or contribute to blood loss. Worldwide, the most frequent cause of GI blood loss is hookworm infection; about 1 billion people are believed to be infected. Other helminthic infections, such as *Schistosoma mansoni, Schistosoma japonicum,* and severe *Trichuris trichura* infection, may also cause GI blood loss. *Helicobacter pylori* gastritis has been documented as a cause of iron deficiency in an Alaskan community.[118] Less common causes of GI bleeding must also be considered in individual patients, including vascular purpura with scurvy, aberrant pancreas, Meckels diverticulum, hereditary hemorrhagic telangiectasia, other vascular ectasia of the bowel, and colonic polyposis.

In women of childbearing age, genitourinary blood loss with menses is often responsible for increased iron requirements. [119] Menstrual losses tend to be decreased with the use of oral contraceptives but are increased with the use of intrauterine devices. Other causes of genitourinary bleeding include uterine malignancies or fibroids; stones, infarction, infection with *Schistosoma haematobium* inflammatory disease, or malignancy of the urinary tract; and, rarely, chronic hemoglobinuria or hemosiderinuria resulting from paroxysmal nocturnal hemoglobinuria or chronic intravascular hemolysis.

Uncommonly, respiratory tract blood loss resulting from chronic recurrent hemoptysis of any cause may produce iron deficiency. In two rare conditions, idiopathic pulmonary siderosis and Goodpastures syndrome, hemoptysis and intrapulmonary bleeding may be inapparent but lead to the sequestration of iron in pulmonary macrophages. Although still within the body, this sequestered iron is lost for systemic utilization, and severe iron deficiency anemia may develop. Recurrent blood donation may lead to iron deficiency, particularly in menstruating women. [120]

In infants, children, and adolescents, the need for iron for growth may exceed the supply available from diet and stores. At birth the iron endowment of the infant is primarily determined

Figure 26-6 (Figure Not Available) Changes in body iron supply and storage with iron deficiency and with the anemia of chronic inflammation. **(A)** Iron depletion. Macrophage iron stores in the bone marrow, liver, and spleen are decreased without a decline in the level of functional iron compounds. **(B)** Iron deficient erythropoiesis. Iron stores are exhausted and the lack of iron limits the production of hemoglobin and other metabolically active compounds that require iron. **(C)** Iron deficiency anemia. Macrophage iron stores in the bone marrow, liver, and spleen are absent. **(D)** Anemia of chronic inflammation. Macrophage iron stores in the bone marrow, liver, and spleen are increased. *(Reprinted with permission from Brittenham GM: Red blood cell function and disorders of iron metabolism. Scientific American Medicine, Vol. 2. Dale DC, Federman DD, Eds. Scientific American, Inc., New York, 1997, Sect. 5, Subsect. II, p. I. All rights reserved.)*

by the birth weight and hemoglobin concentration. Mild maternal iron deficiency usually does not restrict iron delivery to the fetus, but moderate to severe iron deficiency anemia in the mother can result in both a decreased hemoglobin concentration and lower birth weight in the neonate. [121] Premature infants,

TABLE 26-3 -- Causes of Iron Deficiency

Increased iron requirements
Blood loss
Gastrointestinal tract
Genitourinary tract
Respiratory tract
Blood donation
Growth
Pregnancy and lactation
Inadequate iron supply
Diets with insufficient amounts of bioavailable iron
Impaired absorption of iron

Intestinal malabsorption
Gastric surgery
Impaired iron transport

with a lower birth weight and a more rapid postnatal rate of growth, have a high risk of iron deficiency unless given iron supplements. With rapid growth during the first year of life, the body weight normally triples, and iron requirements (expressed as micrograms Fe per kilogram body weight) are at a high level. Iron requirements decline as growth slows during the second year of life and into childhood but rise again during the adolescent growth spurt.

Without supplemental iron, pregnancy entails the net loss of the equivalent of 1,2001,500 ml blood. On average, 270 mg of iron is donated to the fetus, an additional 90 mg is contained in the cord and placenta, and 150 mg is present in the lochia and blood lost at delivery, a total of slightly more than 500 mg of iron. [67] During pregnancy the red cell mass increases by more than a third, requiring almost another 500 mg of iron, which is returned to stores after delivery and not included in the net cost of a pregnancy. After delivery, the resumption of menstruation is usually delayed for some months, but if the infant is breast-fed, lactation requires about 0.51.0 mg Fe/day.

In some instances, an insufficient supply of iron may contribute to the development of iron deficiency. In infants [122] or women who have experienced heavy menstrual losses or multiple pregnancies,

the risk of iron deficiency is further increased by the habitual consumption of diets with insufficient amounts of bioavailable iron, such as those with little or no heme iron and with small amounts of enhancers or large amounts of inhibitors of nonheme iron absorption. For older children, men, and postmenopausal women, the restricted availability of dietary iron is almost never the sole explanation for iron deficiency, and other causes, especially blood loss, must be considered.

An impaired absorption of iron infrequently produces iron deficiency. In some patients, intestinal malabsorption of iron may occur as a manifestation of more generalized syndromes such as steatorrhea of various causes, sprue, celiac disease, or diffuse enteritis. Atrophic gastritis and the attendant achlorhydria may impair iron absorption. In persons of all ages and sexes but particularly in pregnant women and children, pica, the compulsive chewing or ingestion of food or nonfood substances, may contribute to iron deficiency if the material consumed inhibits iron absorption. Iron deficiency frequently complicates gastric surgery such as partial or total gastric resection or gastroenterostomy for bypass of the duodenum. Both the loss of gastric acid production and the decreased time available for iron absorption resulting from the increased rate of intestinal transit contribute to poor iron absorption; recurrent bleeding at the surgical site may also produce iron loss. Despite impaired absorption of dietary iron, therapeutic preparations of iron salts are usually well absorbed.

Increased iron requirements and an inadequate supply of iron often work in concert to produce iron deficiency. Infants fed cows milk receive a diet that not only contains small amounts of iron of low bioavailability but that also increases iron losses by causing GI bleeding. Menstruating women, with some of the highest iron requirements, may consume diets that both have a low content of iron and contain inhibitors of iron absorption, such as calcium. Patients with ulcer disease and increased GI blood loss may be treated with antacids, which diminish dietary iron absorption.

Clinical Presentation

Patients with iron deficiency may present (1) with no signs or symptoms, coming to medical attention only because of abnormalities on laboratory tests, (2) with the features of the underlying disorder responsible for the development of iron deficiency, or (3) with the manifestations common to all anemias. Patients may or may not exhibit one or more of the few signs and symptoms considered highly specific for iron deficiency, namely, pagophagia, koilonychia, and blue sclerae. No signs or symptoms have been ascribed to uncomplicated depletion of storage iron, although patients without iron reserves will not respond as rapidly to an increased need for iron resulting from blood loss, growth, or pregnancy.

Greater deficits in body iron, which restrict the synthesis of functional iron compounds and cause anemia, either may be asymptomatic or may produce a variety of clinical manifestations. Iron deficiency anemia produces the signs and symptoms common to all anemias: pallor, palpitations, tinnitus, headache, irritability, weakness, dizziness, easy fatigability, and other vague and nonspecific complaints. The prominence of these signs depends on both the degree and the rate of development of the anemia.[66] Because iron deficiency is often of insidious onset and prolonged duration, adaptive circulatory and respiratory responses may minimize these manifestations, permitting a surprising tolerance of low hemoglobin concentrations. With greater severity, anemia becomes increasingly debilitating as work capacity and tolerance of physical exertion are restricted, and it can eventually produce cardiorespiratory failure and even death.

Iron deficiency may also produce clinical manifestations independent of anemia. These seem to result from the depletion of functional iron compounds in nonerythroid tissues, resulting in impaired proliferation, growth, and function. Epithelial tissues have high iron requirements due to rapid rates of growth and turnover and thus are affected in many patients with chronic iron deficiency. Glossitis, angular stomatitis, postcricoid esophageal web or stricture (which may become malignant), and gastric atrophy may develop. The combination of glossitis, a sore or burning mouth, dysphagia, and iron deficiency is called the Plummer-Vinson or Patterson-Kelly syndrome. The prevalence of these abnormalities seems to vary geographically, which suggests that environmental or genetic factors may also be involved. [67]

Determining whether gastric atrophy is the cause or consequence of iron deficiency may be difficult, particularly in older patients; in some, pernicious anemia and iron deficiency coexist. Changes in the lingual or buccal mucosa have been suggested as factors causing or contributing to the pica that develops in many patients with iron deficiency. Pagophagia, a variant of pica in which ice is the substance obsessively consumed, is a behavioral abnormality that is considered to be a highly specific symptom of iron deficiency, resolving within a few days to 2 weeks after the beginning of iron therapy. [67] Other types of pica, with a variety of nonfoods such as clay, starch, paper, or dirt or with an assortment of foods, may occur in iron deficiency but are not as clearly linked to a lack of iron or as frequently responsive to iron therapy. Koilonychia, fingernails that are thin, friable, and brittle, with the distal half in a concave or spoon shape resulting from impaired nailbed epithelial growth, is considered virtually pathognomonic of iron deficiency but occurs in a small minority of patients. Blue sclerae, or sclerae with a definite or striking bluish hue, were recognized in 1908 by Osler as associated with iron deficiency [123] and have been reported to be both highly specific and sensitive as an indicator of iron deficiency. The bluish tinge is thought to result from thinning of the sclera, which makes the choroid visible; the thin sclera has been postulated to be the result of impairment of collagen synthesis by iron deficiency. Iron deficiency has also been postulated to have other nonhematologic consequences, still under investigation, involving impaired immunity and resistance to infection, diminished exercise tolerance and work performance, and behavioral and neuropsychological abnormalities.

Laboratory Evaluation

A characteristic sequence of changes in the clinically useful indications of iron status occurs as body iron decreases from the iron-replete normal to the levels found in iron deficiency anemia (Fig. 26-5). The patterns shown are those developing in the absence of complicating factors, such as infection, inflammation, liver disease, malignancy, or other disorders. Initially, owing to any of the causes listed in Table 26-3 , iron requirements exceed the available supply of iron. Iron is mobilized from body stores and iron absorption is increased. If the amounts of iron available from body reserves and absorption are inadequate, depletion of storage iron follows. At this stage bone marrow examination shows absent, or nearly absent, hemosiderin iron. The serum ferritin falls, while the total iron-binding capacity rises. Exhaustion of iron reserves then results in an inadequate supply of iron to the developing erythroid cell, and iron-deficient erythropoiesis commences. Plasma transferrin receptor concentrations increase as the total body mass of tissue receptor expands. The plasma ferritin decreases to <12 g/L, reflecting the absence of storage iron, and the total iron-binding capacity continues to rise. The plasma iron declines, and in combination with the increase in total iron-binding capacity, the transferrin saturation falls to <16%. As a result, most iron is derived from mono- rather than diferric transferrin, and most ferric transferrin-transferrin receptor complexes carry only one

or two atoms of iron rather than as many as four with each intracellular delivery cycle. Marrow examination shows, in addition to the absence of hemosiderin iron, a decrease in the proportion of sideroblasts because too little iron is available to support siderotic granule formation. The erythrocyte zinc protoporphyrin progressively increases with reduction of the amount of iron available for heme formation.

As hemoglobin production becomes restricted, frank iron deficiency anemia (Fig. 26-6C (Figure Not Available)) develops. The normocytic, normochromic red cells previously formed under iron-replete circumstances are gradually replaced by a microcytic hypochromic population. Both the time needed to replace the normal population and the extent of the disparity between erythroid needs and available iron supply determine the rate and degree of change in erythrocyte morphology and red cell indices, such as the mean corpuscular volume, mean corpuscular hemoglobin, and measures of the distribution of red cell volumes such as red cell volume distribution width. Chronic, long-standing iron deficiency anemia may produce severe microcytosis and hypochromia, with very pale, distorted red cells and dramatic reductions in the mean corpuscular volume and mean corpuscular hemoglobin. By contrast, some patients with mild iron deficiency anemia may have erythrocyte morphology and indices indistinguishable from those found in normal, iron-replete individuals. Nonetheless, laboratory evaluation of uncomplicated iron deficiency in otherwise healthy individuals is usually not difficult, and the characteristic patterns of indicators of body iron status shown in Figure 26-5 will typically be diagnostic.

Differential Diagnosis

Iron deficiency is the only microcytic hypochromic disorder in which mobilizable iron stores are absent; in all others, storage iron is normal or increased (Table 26-4). Thus the diagnosis of iron deficiency can almost always be verified by direct assessment of body iron stores using bone marrow examination: if no iron stores are present, the diagnosis of iron deficiency is established; if hemosiderin is found, iron deficiency is excluded. In addition, with iron deficiency sideroblasts will be absent or greatly decreased to <10% of normoblasts.[67] Rarely, previous parenteral iron therapy may cause difficulties in interpretation because of the presence of aggregates of iron that is effectively unavailable. Technical errors due to sampling or artifacts can usually be avoided by the examination of biopsy cores and of both stained and unstained aspirates containing ample amounts of stroma.

Difficulties in the evaluation of microcytic hypochromic disorders usually arise when direct assessment of marrow iron is unavailable and the diagnosis depends on indirect indicators of iron status. In otherwise healthy individuals, these indirect measures may be helpful, but in the presence of other disorders, which may include infection, inflammation, malignancy, malnutrition, alcoholism, or liver disease, their interpretation is often

TABLE 26-4 -- Differential Diagnosis of Microcytic Hypochromic Anemia

With decreased body iron stores
Iron deficiency anemia
With normal or increased body iron stores
Impaired iron metabolism
Anemia of chronic disease
Defective absorption, transport, or utilization of iron
Disorders of globin synthesis
Thalassemia
Other microcytic hemoglobinopathies
Disorders of heme synthesis: sideroblastic anemias
Hereditary
Acquired

THERAPEUTIC TRIAL OF IRON

The diagnosis of iron deficiency is often confirmed by the outcome of a therapeutic trial of iron. A specific, orderly response to, and only to, treatment with iron constitutes the final, definitive proof that a lack of iron is the cause of an anemia. The unequivocal diagnostic response consists of (1) a reticulocytosis, which begins about 35 days after adequate iron therapy is instituted, reaching a maximum on the eight to tenth day and then declining; and (2) a significant increase in the hemoglobin concentration, which should begin shortly after the reticulocyte peak, is invariably present by 3 weeks after iron therapy is begun, and persists until the hemoglobin concentration is restored to normal.[66] The result of a therapeutic trial of iron must be evaluated with due regard for possible confounding factors, such as poor compliance with iron therapy, malabsorption of therapeutic iron, continuing blood loss, and the effects of coexisting conditions, especially infectious, inflammatory, or malignant disorders. The therapeutic trial merely aids in the establishment of iron deficiency. The search for underlying causes must persist despite a positive response.

problematic. Of the indirect indicators, the plasma ferritin is most useful if the concentration is <12 g/L, for in the absence of hypothyroidism or ascorbate deficiency, such a low ferritin level is highly specific for iron deficiency. By contrast, a plasma ferritin concentration within the normal range does not necessarily indicate the presence of storage iron, for ferritin concentrations may be increased independently of iron status by infectious, inflammatory, and malignant disorders, liver disease, and other conditions. Measurement of the plasma transferrin receptor concentration may be useful in differentiating the anemia of iron deficiency from the anemia associated with chronic inflammatory disorders. While serum ferritin may be disproportionately elevated in relation to iron stores in patients with inflammation or liver disease, the plasma transferrin receptor concentration seems to be unaffected by these disorders and to provide a more reliable laboratory indicator of iron deficiency anemia. Disorders that restrict the iron supply may further complicate interpretation by lowering the plasma iron and transferrin saturation, increasing the erythrocyte zinc protoporphyrin, and in the peripheral blood by producing reticulocytopenia, microcytosis, hypochromia, and anemia. Because of the lack of sensitivity and specificity of the indirect indicators of iron status, the most reliable means of diagnosing iron deficiency in some patients is a bone marrow examination or a therapeutic trial of iron.

Specific entities to be considered in the differential diagnosis of hypochromic microcytic disorders are listed in Table 26-4; in all of these, body iron stores are normal or increased. In some, the hematologic abnormalities are associated with impaired iron metabolism. The anemia of chronic disease (Fig. 26-6D (Figure Not Available)) is the most common cause of anemia in hospitalized patients, typically developing over several weeks in those with chronic infectious, inflammatory, or malignant disorders.[124] The anemia is usually mild to moderate, the severity being approximately proportional to that of the chronic illness. Erythrocytes are normocytic or slightly microcytic and variably hypochromic. With the onset of the underlying disorder, an impairment of iron release from macrophages results in an accumulation of iron within these cells. The plasma iron level falls, and iron supply to tissue diminishes. Within the bone marrow is found the diagnostic combination of increased iron sequestered in

IRON DEFICIENCY AND COEXISTING DISORDERS

The detection of iron deficiency in the presence of chronic infectious, inflammatory, or malignant disorders is more problematic. Even if iron lack contributes to the anemia of chronic disease, the transferrin concentration will be decreased and the plasma ferritin concentration increased. In this circumstance bone marrow examination is definitive: if iron deficiency is present, iron stores are absent; if the anemia of chronic disease is alone responsible, iron stores are present and typically increased. A therapeutic trial of iron may also be helpful in determining if a lack of iron contributes to the anemia of chronic disease.

macrophage stores with decreased sideroblasts, reflecting the inadequate supply of iron to developing erythroid cells.

Although in uncomplicated iron deficiency the transferrin concentration (total iron-binding capacity) increases, in the anemia of chronic disease the transferrin concentration decreases, a characteristic feature useful in distinguishing between the two causes of anemia. The plasma ferritin may also be diagnostically helpful: concentrations fall to <12 g/L in uncomplicated iron deficiency but rise in the anemia of chronic disease. The plasma transferrin receptor concentration will remain within the normal range with the anemia of chronic disorders but will increase with iron deficiency. The ratio of the serum transferrin receptor concentration to serum ferritin has been advocated as an improved means of recognizing iron deficiency. [125] These indirect indicators of iron status usually allow differentiation of the anemia of chronic disease from uncomplicated iron deficiency.

Microcytosis and hypochromia resulting from defective absorption, transportation, or utilization of iron rarely occur. Congenital atransferrinemia is an autosomal recessive disorder in which plasma transferrin is nearly absent. A severe microcytic hypochromic anemia, present from birth, is refractory to iron therapy but responds to transferrin infusion. Iron absorption is normal or increased, and iron deposits are found in the liver and other parenchymal tissues but are scant or absent in the bone marrow. Patients die without transferrin infusion or blood transfusions and are at risk for the complications of iron overload.

Other rare congenital defects with microcytic hypochromic anemias of unknown cause have also been reported, sometimes in combination with parenchymal iron overload. A single patient was found to have an acquired microcytic hypochromic anemia resulting from an IgM autoantibody to the transferrin receptor, which diminished iron incorporation by erythroid precursors. In this patient serum iron was increased, but marrow iron stores were absent. [126] Defective uptake of iron by erythroid precursors was suggested by the findings in two siblings with a severe hypochromic anemia in conjunction with increased plasma iron, marked hepatic iron deposition, and fibrosis, but no definite cause was established. [127] Three siblings with microcytic hypochromic anemia apparently due to isolated malabsorption and defective utilization of iron have been described, but the exact cause of their disorder was not determined. [128] More recently, two siblings were identified with severe hypoproliferative microcytic anemia and unexplained iron malabsorption. [129] Abnormalities in Nramp2[54] [55] [56] may now be sought as potential explanations for some of the microcytic hypochromic anemias of unknown cause.

Microcytic hypochromic anemias resulting from disorders of globin synthesis (thalassemias, microcytic hemoglobinopathies) and from disorders of heme synthesis (sideroblastic anemias, congenital and acquired) are discussed in Chapters 27 and 29 . In general, the plasma iron, transferrin saturation, plasma ferritin concentration, and bone marrow iron stores are normal or increased in these disorders, and differentiation of such disorders from iron deficiency is not difficult.

Therapy

The goal of therapy for iron deficiency anemia is to supply sufficient iron to repair the hemoglobin deficit and replenish storage iron. Oral iron is the treatment of choice for almost all patients because of its effectiveness, safety, and economy. The risk of local and systemic adverse reactions restricts the use of parenteral iron to those few patients unable to absorb or tolerate adequate amounts of oral iron. Rarely, red cell transfusions are needed to prevent cardiac or cerebral ischemia in severe anemia or to support patients whose chronic rate of iron loss exceeds the rate of replacement possible with parenteral therapy.

Most patients are able to tolerate oral iron therapy without difficulty, but 1020% may have symptoms attributable to iron. [130] The most common side effects are gastrointestinal. The development of either diarrhea or constipation can usually be treated symptomatically because alterations in bowel habits do not appear to be related to the dose of iron and seldom necessitate a change in the oral regimen. Upper GI tract symptoms do seem to be dose related, reflecting the concentration of ferrous iron in the stomach and duodenum. These side effects occur within 1 hour of iron ingestion and may be mild, with nausea and epigastric discomfort, or severe, with abdominal pain and vomiting. Often upper GI side effects can be managed by administering the iron with or immediately after meals. If symptoms persist, reductions in the amount of iron in each dose may be helpful, either by changing to tablets that contain smaller amounts of iron or by using a liquid preparation of ferrous sulfate. Decreasing the amount of iron in each dose is usually effective in controlling side effects, but if symptoms persist, a reduction in frequency to a single dose per day may be helpful. After a time at lower doses, patients may subsequently be able to tolerate more iron. With patience and persistence, an acceptable oral iron regimen can be devised for virtually all patients. Costly iron preparations with other additives, with enteric coating,

ORAL IRON THERAPY

Oral iron therapy should begin with a ferrous iron salt, taken apart from meals in three or four divided doses and supplying a daily total of 150200 mg of elemental iron in adults or 3 mg of iron per kilogram of body weight in children. Simple ferrous preparations are the best absorbed and least expensive; ferrous sulfate is the most widely used, either as tablets containing 6070 mg iron for adults or as a liquid preparation for children. Administration between meals maximizes absorption. In patients with a hemoglobin concentration <10 g/dl, this regimen will initially provide about 4060 mg of iron per day for erythropoiesis, permitting red cell production to increase to two to four times normal and the hemoglobin concentration to rise by about 0.2 g/dl/day. [96] An increase in the hemoglobin concentration of at least 2 g/dl after 3 weeks of therapy is generally used as the criterion for an adequate therapeutic response. [67] For milder anemia, a single daily dose of about 60 mg iron per day may be adequate. After the anemia has been fully corrected, oral iron should be continued to replace storage iron, either empirically for an additional 46 months or until the plasma ferritin concentration exceeds 50 g/L. [130]

PARENTERAL IRON THERAPY

The most widely used parenteral iron preparation is an iron dextran containing about 50 mg of elemental iron per milliliter in a dark brown, colloidal suspension of ferric oxyhydroxide and low molecular weight dextran. The iron dextran may be given either intramuscularly or intravenously in a dose that is calculated from the deficit in body iron, with allowance both for iron to correct the hemoglobin deficit and for iron to rebuild stores.[130] Immediate life-threatening anaphylactic reactions, the most serious risk associated with the use of either intramuscular or intravenous iron dextran, occur in 0.51% of patients[220] and may have a fatal outcome, even with treatment.[221] Delayed but severe serum sickness-like reactions may develop in a substantial proportion of patients, with fever, urticaria, adenopathy, myalgias, and arthralgias.[220] Iron dextran may exacerbate arthritis in patients with ankylosing spondylitis[222] and rheumatoid arthritis.[223] With intramuscular administration, local reactions include skin staining (which may be minimized by using a Z-technique for injection), muscle necrosis, phlebitis, and persistent pain at the injection site. In animals given massive doses of iron dextran, sarcomas have developed at the injection site, but no conclusive evidence of a carcinogenic risk in humans has been presented. Before every intramuscular or intravenous injection of iron dextran, the manufacturer recommends that a 0.5-ml test dose be given at least 1 hour before the therapeutic injection. The value of this precaution is limited because anaphylaxis is not dose dependent and can occur with the test dose, so iron dextran should be administered parenterally only if the facilities and medical expertise for managing anaphylactic reactions are available.[220] In the past, repeated intravenous or intramuscular injections of iron dextran were considered less convenient and more hazardous than administration of the total dose of iron dextran in a single intravenous infusion.[220] At present, however, the manufacturer stipulates that the maximum intravenous or intramuscular daily dose should not exceed 2 ml of undiluted iron dextran, the equivalent of 100 mg of iron, and warns of an increased incidence of adverse effects with larger doses. This 2 ml/day limit may be compared with the total dose of 40 ml of iron dextran, the equivalent of 2,000 mg of iron, suggested by the manufacturer for an iron-deficient 70-kg individual with a hemoglobin level of 7 g/dl. Despite these risks and restrictions, parenteral iron therapy is still preferred for those patients who cannot be managed with oral iron, for the hazards and expense of chronic red cell transfusion are even greater.

or in sustained-release form do not appear to offer any advantages that cannot be achieved by simply reducing the dose of plain ferrous salts. Administering iron with food and decreasing the dose will diminish the amount of iron absorbed daily and thereby prolong the period of treatment, but haste in the correction of iron deficiency is rarely needed.

Parenteral iron therapy, with the risk of adverse reactions, should be reserved for the exceptional patient who (1) remains intolerant of oral iron despite repeated modifications in dosage regimen, (2) has iron needs that cannot be met by oral therapy because of chronic uncontrollable bleeding, or (3) malabsorbs iron.[131] A screening test for iron malabsorption is the administration to the fasting patient of 100 mg elemental iron as ferrous sulfate in a liquid preparation, followed by measurements of the plasma iron 1 and 2 hours later. In an iron-deficient patient with an initial plasma iron of <50 g/dl, an increase of 200300 g Fe/dl is expected; an increase in plasma iron of <100 g/dl suggests malabsorption and is an indication for a small bowel biopsy.[132]

Prognosis

The prognosis for iron deficiency itself is excellent, and the response to either oral or parenteral iron is similar. Frequently, both clinical and subjective impressions of constitutional improvement are present within the first few days of treatment, with an enhanced sense of well-being and increased vigor and appetite. Pica may resolve and soreness and burning of the mouth abate. Hematologically, mild reticulocytosis begins within 35 days, is maximal by days 810, and then declines. The hemoglobin concentration begins to increase after the first week and has usually returned to normal within 6 weeks. Complete recovery from microcytosis may take up to 4 months. With oral iron dosage totaling 200 mg/day or less, the plasma ferritin remains <12 g/dl until the anemia is corrected and then gradually rises as storage iron is replaced over the next several months.[133] Although epithelial abnormalities begin to improve promptly with treatment, resolution of glossitis and koilonychia may take several months. The overall prognosis depends on the underlying disorder responsible for the iron deficiency.

Failure to obtain a complete and characteristic response to iron therapy necessitates a review and reevaluation of the patient. A common problem is an incorrect diagnosis, with the anemia of chronic disease mistaken for the anemia of iron deficiency. Coexisting conditions, such as other nutritional deficiencies, hepatic or renal disease, or infectious, inflammatory, or malignant disorders, may impede recovery. Occult blood loss may be responsible for an incomplete response. With oral iron therapy, the adequacy of the form and dose of iron used should be reconsidered, compliance with the treatment regimen reviewed, and finally, the possibility of malabsorption considered.

Future Directions

The effects of iron deficiency apart from anemia need further investigation, both at the cellular level and more generally in terms of the effects of lack of iron on immunity and resistance to infection, exercise and work performance, and behavior and development, particularly in the infant and child. Important clues to the underlying mechanisms of these effects of iron deficiency apart from anemia have been provided by an improved understanding of the interconnections of iron metabolism with (1) nitric oxide and cytokine-mediated effects of infection and inflammation and (2) cellular energy utilization. In particular, iron deficiency would seem to preferentially affect the activities of IRP-1 and IRP-2 and their targets.[65] Diagnostically, an immunoassay for plasma transferrin receptor should soon be clinically available and should prove useful in distinguishing the anemia of iron deficiency from the anemia associated with chronic inflammation. Therapeutically, carbonyl iron continues to offer an effective means of preventing and treating iron deficiency that avoids the risk of accidental iron poisoning in children. Alternatively, further development of a gastric delivery system (GDS) for iron could potentially provide effective therapy with the virtual elimination of side effects.[134] The iron GDS preparation may be especially useful in patients who have difficulty in tolerating other iron preparations. Although iron nutrition has improved in the United States and other developed countries, iron deficiency remains a widespread problem in the rest of the world, and effective methods of iron fortification still represent an important need.

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IRON OVERLOAD

Iron overload denotes an excess in total body iron resulting from an iron supply that exceeds iron requirements. Because requirements are limited and humans lack a physiologic means of excreting excess iron, any sustained increase in intake may eventually result in accumulation of iron. The continuum of increased body iron is shown in Figure 26-5 . In adults and children, iron overload may be caused by increased absorption of dietary iron, by parenteral administration of iron, or by a combination of the two. In the neonate and infant, iron overload is a rare disorder that apparently results from an abnormality in iron balance between mother and fetus. Focal iron overload may result from sequestration of iron, as in pulmonary hemosiderosis. Whatever the source of excess iron, when the accumulation overwhelms the bodys capacity for safe storage, potentially lethal tissue damage is the result. The toxic manifestations of iron overload depend in part on the amount of excess iron, the rate of iron accumulation, and the partition of the iron burden between relatively benign sites in the macrophage and more hazardous deposits in parenchymal cells. The recent identification of a strong candidate gene for hereditary hemochromatosis, [135] a frequent cause of iron excess, has increased interest in disorders associated with iron overload.

Epidemiology

The most common form of iron overload in the United States is a genetically determined disorder, the homozygous state for hereditary hemochromatosis, occurring in as much as 0.5% of the population or as many as 1 million individuals. [105] [106] [136] [137] Similar frequencies of an iron-loading gene have been found elsewhere. In the United States other forms of iron overload are less frequent but affect several thousand patients with iron-loading or transfusion-dependent anemias such as thalassemia major and acquired refractory anemias. Globally, thalassemia major and other iron-loading anemias are an important public health problem in countries bordering the Mediterranean and in an area extending from Southwest Asia and the Indian subcontinent to Southeast Asia. [138] Dietary iron overload resulting from intake of iron in brewed beverages is a common problem affecting many populations in sub-Saharan Africa and may also have a genetic component. [139] [140] [141] The various forms of neonatal iron overload [142] and the syndromes associated with focal sequestration of iron are all rare disorders.

Genetic Aspects

The forms of iron overload known to be genetically determined include hereditary hemochromatosis and certain of the iron-loading anemias. Hereditary hemochromatosis has an autosomal recessive mode of inheritance, and a strong candidate gene for this disorder has been identified on the short arm of chromosome 6. Only homozygotes are at risk for symptomatic iron overload. The hemochromatosis gene has been found to have a frequency of 57% in various countries, which suggests that 10% or more of their populations are heterozygous and 0.250.5% are homozygous for the gene. [105] [106] [136] [137] The iron-loading anemias with established genetic components include the inherited sideroblastic anemias (Chap. 29) as well as some of the hereditary disorders of globin synthesis (Chap. 27), such as homozygous states for various forms of -thalassemia and compound heterozygous states such as hemoglobin E--thalassemia.

Etiology and Pathogenesis

Iron overload is caused by conditions that alter or bypass the normal control of body iron content by regulation of intestinal iron absorption (Table 26-5). In hereditary (HLA-linked) hemochromatosis, a genetic abnormality results in an inappropriately elevated iron absorption, with a chronic progressive increase in body iron stores. [67] [105] [106] [137] Iron absorption is also increased in the iron-loading anemias, apparently as the result of erythroid hyperplasia with ineffective erythropoiesis. In dietary iron overload of the type found in sub-Saharan Africa, control of iron absorption is overwhelmed by the large amounts of bioavailable iron in the upper small intestine; a genetic component, not linked to the HLA-A locus, may be involved. [139] [140] [141] The iron contained in transfused red cells given to patients with refractory anemias such as thalassemia major circumvents intestinal regulation of body iron content and also produces progressive iron loading.

With each of these causes of systemic iron overload, the timing of the onset of toxic manifestations, the pattern of the organs affected, and the severity of tissue damage are known to be influenced by a variety of factors. These include (1) the magnitude of the body iron burden, (2) the rate of loading, (3) the distribution of the iron load between apparently innocuous storage deposits in tissue macrophages and potentially injurious accumulations in parenchymal cells, (4) ascorbate status, which helps determine the allocation of iron between macrophage and parenchymal cells, and (5) the extent of internal redistribution of iron between macrophage and parenchymal sites. In general, the amount and rate of iron accumulation within parenchymal cells of the liver, heart, pancreas, and other organs seem to be the principal determinants of tissue injury. The exact mechanisms whereby iron produces cellular injury have not been established, but lipid peroxidation of membrane lipids of subcellular organelles, iron-induced lysosomal disruption, or both may be involved. Tissue damage may also be produced by plasma iron not complexed with transferrin (i.e., non-transferrin-bound iron), which appears in the circulation of patients with iron overload. [143] [144] [145]

Iron overload as a result of increased iron absorption may result either from an abnormality in the control of iron absorption with a diet containing normal amounts of bioavailable iron or from consumption of such excessive amounts of bioavailable iron that a normal regulatory mechanism is overridden. In individuals homozygous for hereditary (HLA-linked) hemochromatosis, an inappropriately elevated iron absorption occurs at any level of body iron and produces progressive parenchymal iron accumulation, initially in the liver but later in the pancreas,

TABLE 26-5 -- Causes of Iron Overload

Increased iron absorption
From diets with normal amounts of bioavailable iron
Hereditary (HLA-linked) hemochromatosis
Iron-loading anemias (refractory anemias with hypercellular erythroid marrow)
Chronic liver disease (cirrhosis, portacaval shunt)
Porphyria cutanea tarda
Congenital defects (atransferrinemia and other disorders)
From diets with increased amounts of bioavailable iron
African dietary iron overload (may have genetic component)
Medicinal iron ingestion (?)
Parenteral iron overload
Transfusional iron overload

Inadvertent iron overload from therapeutic injections
Perinatal iron overload
Hereditary tyrosinemia
Cerebrohepatorenal syndrome
Perinatal hemochromatosis
Focal sequestration of iron
Idiopathic pulmonary hemosiderosis
Renal hemosiderosis
Hallervorden-Spatz syndrome

Figure 26-7 (Figure Not Available) Changes in body iron supply and storage with hereditary hemochromatosis and transfusional iron overload. **(A)** Hereditary hemochromatosis. Despite massive iron deposition in hepatocytes, macrophage iron stores in the bone marrow, liver, and spleen may be normal or only modestly increased. **(B)** Transfusional iron overload. Marked macrophage iron deposition is present along with iron overload in hepatocytes. *(Reprinted with permission from Brittenham GM: Red blood cell function and disorders of iron metabolism. Scientific American Medicine, Vol. 2. Dale DC, Federman DD, Eds. Scientific American, Inc., New York, 1997, Sect. 5, Subsect. II, p. I. All rights reserved.)*

heart, and other organs (Figure 26-7A (Figure Not Available)). Despite marked parenchymal iron deposition, macrophage iron in the bone marrow may be scant or even absent.[146] [147] Initially, the excess iron is deposited predominantly within the parenchymal cells of the liver, but subsequently the iron accumulates in the pancreas, heart, and other organs. The classic tetrad of clinical signs in homozygotes for hereditary hemochromatosis who present symptomatically in middle age or later consists of liver disease, diabetes mellitus, skin pigmentation, and gonadal failure. [106] [137] [148] [149] Cardiac failure eventually develops in some 1015% of untreated homozygotes for hemochromatosis. By the time symptoms of parenchymal damage develop, usually in middle or late life, body iron stores have typically increased from the normal range of 1 g or less to 1520 g or more.[148] Further increments in the body iron stores may be fatal, although some patients are able to tolerate a total iron accumulation of as much as 4050 g. Hereditary hemochromatosis is incompletely expressed in heterozygotes, [150] with about one-fourth developing evidence of minor, apparently harmless increases in body iron stores to totals of not more than 45 g.

Identification of the *HFE* Gene

The most important recent advance in understanding the etiology and pathogenesis of hereditary hemochromatosis has been the identification of a strong candidate gene for this disorder.[135] This gene was originally designated as *HLA-H* but, because of prior use of this identifier, is better termed *HFE*. For more than two decades the hemochromatosis locus has been known to be closely linked to the HLA-A locus on the short arm of chromosome 6. Located more than three megabases telomeric to the MHC, *HFE* is a gene with structural similarities to the MHC class 1 family. The HFE protein is a single polypeptide with a short cytoplasmic tail, a membrane-spanning region, and three extracellular domains analogous to the 1, 2, and 3 domains of other MHC class 1 proteins (Fig. 26-8). In the original study, missense mutations were found in *HFE* in 87% of chromosomes from patients homozygous for hereditary hemochromatosis. [135] The most prevalent (homozygous in 83% of patients) was a Cys282Tyr mutation. A second mutation in *HFE*, His63Asp, was enriched in patients who were compound heterozygotes for the Cys282Tyr substitution. Importantly, 13% of the patients examined had neither mutation but were indistinguishable clinically from the others. Further investigations in populations of European descent have found that the proportion of patients with hereditary hemochromatosis who are homozygous for the Cys282Tyr mutation varies from nearly 100% in Scandinavia to 69% in Italy;[151] the mutation seems to be rare in Asians.[152]

Cellular, biochemical, and molecular studies have begun to characterize the HFE protein and to examine the consequences of the Cys282Tyr mutation. Immunohistochemical studies of the pattern of expression in the GI tract of control subjects have found that the HFE protein has a unique perinuclear localization in cells in the deep crypts of the small intestine in proximity to the presumed sites of iron absorption. [153] Wild-type and His63Asp HFE proteins are expressed on the cell surface, while the Cys282Tyr mutant *HFE* is exclusively intracellular. [154] Further studies have found that, as predicted from the structure (Fig. 26-8), wild-type HFE binds $_2$ -microglobulin and that the Cys282Tyr mutation, but not the His63Asp mutation, completely

Figure 26-8 Hypothetical model of the HFE protein based upon its homology with MHC class I molecules. *(Redrawn with permission from Feder et al.[135] Copyright 1996 Macmillan Magazines Limited.)*

abolishes this interaction.[155] In 1994 de Sousa and her colleagues reported that mice deficient in $_2$ -microglobulin developed iron loading, predominantly in hepatocytes,[156] suggesting that a $_2$ -microglobulin gene product is involved in iron homeostasis. Reconstitution of mice deficient in $_2$ -microglobulin with normal hematopoietic cells failed to correct the increased iron absorption from the GI tract, but after transplantation the excess iron accumulated in Kupffer cells rather than in hepatocytes.[157] Targeted disruption of the murine homolog of the *HFE* gene found that the phenotype of *HFE*-deficient mice recapitulates that of human hereditary hemochromatosis, with an elevated transferrin saturation and an increased hepatic iron concentration with iron deposition predominantly in hepatocytes in a periportal distribution.[158] These findings provide strong support for the hypothesis that mutations in the *HFE* gene are responsible for hereditary hemochromatosis.

An important clue to the pathogenesis of hereditary hemochromatosis has now been provided by the findings that wild-type HFE protein forms a stable complex with the transferrin receptor and that wild-type HFE/$_2$ -microglobulin heterodimers decrease the apparent affinity of the transferrin receptor for transferrin. His63Asp HFE protein also formed a stable complex with the transferrin receptor but did not decrease the affinity of the transferrin receptor for transferrin. Moreover, the Cys282Tyr mutation almost completely prevents the association of mutant HFE protein with transferrin receptor. [159] These observations establish a molecular association between HFE and the transferrin receptor and suggest that the pathogenesis of hereditary hemochromatosis may be related to a disturbance of the transferrin-transferrin receptor mechanism, although the exact manner in which this alteration leads to excessive dietary iron absorption remains to be determined.

Specific Conditions

Juvenile hemochromatosis is a rare genetic disorder that causes an iron overload like that in hereditary hemochromatosis but with much earlier onset, often in late adolescence.[160] The clinical course is more severe, and patients often present with cardiac symptoms and endocrine dysfunction. Recent studies of a series of patients with juvenile hemochromatosis found none with the *HFE* Cys282Tyr or His63Asp mutations;[160] segregation analysis of 6p markers closely associated with *HFE* in families with consanguineous parents clearly showed that juvenile hemochromatosis is unlinked to 6p and thus genetically distinct from the *HFE* mutation.

Iron-loading anemias may be associated with excessive absorption of dietary iron that can produce severe iron overload. [67] [75] [149] [161] These refractory disorders, characterized by erythroid hyperplasia with marked ineffective erythropoiesis, include thalassemia major and intermedia, hemoglobin E--thalassemia, congenital dyserythropoietic anemia, pyruvate kinase deficiency, a variety of sideroblastic anemias, and other anemias associated with blocks in the incorporation of iron into hemoglobin. The rate of iron loading is not related to the severity of the anemia, and patients with nearly normal hemoglobin concentrations may develop massive iron overload. Any red cell transfusions will add to the iron burden. The amount and distribution of iron deposits resemble those found in hereditary hemochromatosis, with excess iron located predominantly in parenchymal cells, initially in the hepatocyte but later also in the pancreas, heart, and other organs.

Chronic liver disease with increased absorption of dietary iron may produce mild iron overload in some patients, [162] [163] [164] [165] [166] including those with alcoholic cirrhosis or with portacaval shunts. Patients with alcoholic liver disease are more commonly iron deficient as a result of repeated episodes of GI blood loss, but storage iron is increased in a minority. The gene for hereditary hemochromatosis is not responsible. The cause of the increased absorption is unknown, but alcohol-induced folate and sideroblastic abnormalities with ineffective erythropoiesis and hyperferremia may contribute. Storage iron is usually increased modestly, to

only 24 g. In contrast to the pattern of iron distribution found in hereditary hemochromatosis, iron deposition is predominantly in Kupffer cells rather than in parenchymal cells.

Mild iron overload from increased iron absorption is also found in patients with porphyria cutanea tarda, [149] [167] [168] a hepatic porphyria, described more fully in Chapter 27 , in which the liver produces excessive amounts of photosensitizing porphyrins, which circulate to the skin. In symptomatic patients hepatic iron stores characteristically are increased modestly, to 24 g. Alcoholic cirrhosis coexists in some patients, but more often no cause for the increased iron absorption is found. Clinically, the importance of iron for the clinical manifestations of porphyria cutanea tarda is shown by the dramatic effect of iron removal by phlebotomy, producing clinical and biochemical remission of the disease. Conversely, reaccumulation of iron results in recrudescence of symptoms. A high prevalence of the His63Asp *HFE* mutation has been reported in Italian patients with porphyria cutanea tarda.

Rare congenital defects associated with iron overload have been reported. In atransferrinemia, described earlier as one of the causes of microcytic hypochromic anemia, dietary iron is readily absorbed, but little is used for red cell production because of the lack of a physiologic means of transport to erythroid precursors. Instead, iron is deposited predominantly in the liver, pancreas, heart, thyroid, and kidneys. Little iron is found in the spleen and virtually none in the marrow. Another rare congenital defect, ascribed to defective uptake of iron by red cell precursors, is also associated with marked hepatic iron deposition and fibrosis. [127]

Hereditary aceruloplasminemia is a newly recognized, rare autosomal recessive disorder of iron metabolism resulting from deficiency of ceruloplasmin ferroxidase activity as a consequence of mutations in the ceruloplasmin gene. [169] [170] [171] [172] [173] Ceruloplasmin is an $_2$ -serum glycoprotein and multicopper oxidase that transports 95% of the copper found in the plasma; each molecule can bind up to six atoms of copper. Patients present with progressive neurodegeneration of the retina and basal ganglia and diabetes mellitus in middle age. Marked iron accumulation is evident in the liver, pancreas, and brain with smaller amounts of excess iron found in the spleen, heart, kidney, thyroid, and retina. [171] No abnormal copper deposits are present in the liver or other tissues. The serum copper is low and no detectable ceruloplasmin is present. The serum iron is low, the serum total iron-binding capacity normal, and the serum ferritin moderately elevated. Different mutations have been found in each of the families identified, but all have been in the ceruloplasmin gene on chromosome 3q. [169] [170] [171] [172] [173] For comparison, both Menkes syndrome (copper deficiency) and Wilsons disease (copper overload) are the result of mutations in genes of the cation-transport P-type ATPase family on chromosome 13q.[174] [175] The unique involvement of the CNS distinguishes the pattern of iron overload in aceruloplasminemia from that found in hereditary hemochromatosis, African dietary iron overload, or as a consequence of transfusion or iron-loading anemias. With early diagnosis of hereditary aceruloplasminemia, deferoxamine therapy might be a means of averting neurologic deterioration. [176] Hereditary aceruloplasminemia unequivocally establishes the importance of the copper protein in iron metabolism, but the exact relationships between the two metals are incompletely understood. Interestingly, a homologous copper oxidase has an essential role in iron metabolism in yeast. [177]

African dietary iron overload occurs in sub-Saharan Africa in association with greatly increased dietary iron intake from a fermented maize beverage that is made from locally grown crops and home-brewed in steel drums. Beverage consumption may supply as much as 50100 mg of iron daily, many times

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The role of protracted medicinal iron ingestion as a cause of iron overload remains indeterminate. Equivocal evidence is found in case reports of apparently normal individuals who have taken medicinal iron chronically. The issue is complicated by the possible influence of an unrecognized allele for hemochromatosis in these case reports, although the consequences of increased iron intake in heterozygotes for hereditary hemochromatosis are not known. By contrast, medicinal iron ingestion can undoubtedly be harmful to patients with iron-loading disorders by further increasing their body iron burden.

the 1020 mg of iron in typical American diets. Iron burdens of a magnitude comparable to those found in hereditary hemochromatosis may accumulate, and liver disease (with cirrhosis and hepatoma), pancreatic disease (with diabetes mellitus), endocrine disorders, and cardiac dysfunction may develop. Although increased dietary iron intake was long considered the sole cause of the increased iron absorption in this disorder, pedigree analysis of African families suggested that a non-HLA-linked gene may be involved and might be common in populations of African derivation. [139] Subsequent studies have provided further support for the hypothesis that an iron-loading gene distinct from that for hereditary hemochromatosis may be involved in the pathogenesis of African dietary iron overload. [140] [141] [178] Recently, the presence of severe iron overload has been documented in U.S. patients of African ancestry, [179] [180] but the relationship to the possible genetic abnormality associated with African iron overload is uncertain. Nonetheless, an important clinical consequence of these observations is that individuals of African ancestry should be included in screening programs for iron overload.

Hyperferritinemia with autosomal dominant congenital cataract is a newly recognized disorder of iron metabolism in which affected family members present with early-onset bilateral nuclear cataracts and moderately elevated serum ferritin concentrations (1,0002,500 g/L), [181] [182] with both conditions co-inherited as an autosomal dominant trait. As described earlier, in normal individuals the serum ferritin is composed of a mixture of L and H subunits. By contrast, in patients with hyperferritinemia and autosomal dominant congenital cataracts, the serum ferritin is mostly of the L-type; H-type ferritin concentrations are too low to be detected. [183] Serum iron and transferrin saturation are normal or low, and body iron, as evaluated by phlebotomy, is not increased. On examination, no hematologic or biochemical abnormalities are evident in affected individuals. Molecular studies have identified mutations in the IRE of the L-ferritin mRNA affecting the highly conserved CAGUGU motif that constitutes the IRE loop and mediates the high-affinity interaction with the IRP. [182] [183] [184] In cultured lymphoblastoid cells from affected patients, the mutation was found to abolish the binding of IRP and result in a high constitutive, poorly regulated L-ferritin synthesis. [183] This mutation, the first reported example of a defect in iron-mediated translational regulation in a human disorder, has a surprisingly mild phenotype. The only sign of the mutation seems to be an accumulation of L-type ferritin in the lens, resulting in a cataract, although a direct relationship between the mutation and the lens deposit of ferritin has not been formally demonstrated.

Parenteral iron overload is usually the result of repeated red cell transfusions in patients with chronic anemia, but occasionally it is unintentionally produced by repeated injections of iron dextran or other parenteral iron preparations in patients with anemias unresponsive to iron therapy.

Transfusional iron overload progressively develops in patients with chronic refractory anemia who require red cell support. [109] [185] [186] [187] In patients with severe congenital anemias such as thalassemia major (Cooleys anemia) or the Blackfan-Diamond syndrome, transfusional iron loading begins in infancy. Severe iron loading may also develop in transfusion-dependent anemias that appear later in life, namely, aplastic anemia, pure red cell aplasia, hypoplastic or myelodysplastic disorders, and the anemia of chronic renal failure. If ineffective erythropoiesis and erythroid hyperplasia complicate the underlying anemia, increased absorption may contribute to the iron burden, but an adequate transfusion regimen will help suppress erythropoiesis and may reduce iron absorption to near normal levels. Patients with sickle cell anemia or sickle cell/-thalassemia are also at risk for iron overload if chronically given transfusions for the prevention of recurrent complications such as stroke, severe infections, and incapacitating painful crises, as well as other problems.

Perinatal iron overload develops in some rare metabolic disorders of the newborn, presumably as the result of disturbances in the regulation of fetal or maternal-fetal iron balance. In hereditary tyrosinemia (hypermethioninemia), moderate iron deposition is restricted to the liver, which is typically cirrhotic; renal abnormalities and hyperplasia of the pancreatic islet cells are also present. [188] The cerebrohepatorenal syndrome, or Zellwegers syndrome, is a fatal disorder with an autosomal recessive mode of inheritance, characterized by abnormal facies, hypotonia, and polycystic kidneys. Parenchymal iron deposits are found in the liver, spleen, kidney, and lungs.[189] Perinatal hemochromatosis,[149] [190] [191] [192] also known as neonatal hemochromatosis or neonatal iron storage disease, is a usually fatal disorder of the newborn of unknown cause. It is characterized by hepatic cirrhosis and iron deposition in parenchymal cells of the liver, heart, and endocrine organs but not in bone marrow or spleen.

Focal sequestration of iron[149] in other rare disorders produces various patterns of localized iron deposition. In idiopathic pulmonary hemosiderosis, repeated episodes of alveolar hemorrhage are followed by the uptake and sequestration of iron in pulmonary macrophages. This excess iron is not available for use elsewhere, and iron stores in the liver and bone marrow may be decreased or absent. In conditions with chronic hemoglobinuria, renal hemosiderosis may develop, but the renal iron deposits are harmless and have no apparent effect on kidney structure or function. The Hallervorden-Spatz syndrome [193] is a rare degenerative neurologic

disease with an autosomal recessive mode of inheritance. It is characterized by the juvenile onset of progressive motor abnormalities, dementia, dysarthria, and dysphagia, with optic nerve atrophy. Although the role of iron in the disorder has not been determined, marked iron deposition is found in the globus pallidus and reticular zone of the substantia nigra.

Clinical Presentation

Clinical manifestations of iron toxicity generally develop only in patients with forms of iron overload in which the magnitude of iron accumulation is sufficient to produce tissue damage. At risk are homozygotes for hereditary hemochromatosis and patients with iron-loading anemias, congenital defects in iron metabolism, African dietary iron overload, chronic medicinal iron ingestion, and parenteral iron loading. Heterozygotes for hereditary hemochromatosis and patients with chronic liver disease or porphyria cutanea tarda are usually spared because of the limited extent of the iron excess. Whatever the cause of the systemic iron overload, similar clinical features eventually develop with sufficient iron accumulation. Symptomatic patients may present with any of the characteristic manifestations of iron overload: increased skin pigmentation, hepatic disease, diabetes mellitus, gonadal insufficiency and other endocrine disorders, abdominal pain, cardiac dysfunction, arthropathy, and occasionally neurologic and psychological abnormalities. [106] [137] [148] In

homozygotes for hereditary hemochromatosis first seen in late middle age, the classic clinical tetrad consists of skin pigmentation, liver disease, diabetes mellitus, and gonadal failure, although not all these features occur in each patient. In younger patients with juvenile hemochromatosis, cardiac abnormalities and gonadal failure may be the presenting features.

Adequately transfused patients with thalassemia major or other congenital refractory anemias grow and develop normally during the first decade of life. Thereafter, without treatment for iron excess, growth fails, sexual maturation is delayed or absent, and liver disease, diabetes mellitus, and other endocrine abnormalities develop; patients usually die of heart disease in adolescence. [109] Transfusion-dependent forms of refractory anemia that are acquired later in life, such as aplastic, myelodysplastic, or sideroblastic anemias, ultimately follow a similar course.

Characteristic manifestations of iron toxicity are found in specific organ systems. Increased skin pigmentation, with a bronze hue in some patients and a slate gray coloration in others, often accompanies iron overload, although the change in pigment may be too slight to be readily evident clinically. Increased melanin is responsible for the bronzing, while the slate gray appearance is attributed to iron deposition in sweat glands and in the basal layers of the epidermis. Massive iron overload sometimes produces reverse freckling, with small pigment-free areas scattered over a conspicuous slate gray discoloration of the skin. [194]

Liver disease is the most common complication of systemic iron overload. With hereditary hemochromatosis, the excess iron absorbed from the diet is deposited predominantly within the parenchymal cells of the liver, but subsequently the iron accumulates in the heart, pancreas, and other organs. By the time that symptoms of parenchymal damage develop, usually in middle or late life, body iron stores have typically increased from the normal range of 1 g or less to 1520 g or more. [148] [195] Further increments in the body iron may be fatal, although some patients are able to tolerate a total iron accumulation of as much as 4050 g or more. The iron within transfused red cells progressively accumulates in patients with chronic refractory anemia who are transfusion dependent. Each unit of transfused red cells contains 200250 mg of iron. Most patients with severe refractory anemia require 200300 ml/kg/year of blood, or the equivalent of 610 g of iron per year in a 70-kg adult. [109] Without chelation therapy to remove iron, cardiac iron deposition develops in patients who have received 100 units of blood (20 g of iron) or more [196] and is often accompanied by other evidence of iron-induced damage to the liver, pancreas, and endocrine organs. In all varieties of iron overload, the development and severity of liver damage are closely correlated with the magnitude of hepatic iron deposition. Whether derived from transfused red cells or from increased absorption of dietary iron, progressive iron accumulation eventually produces hepatomegaly, functional abnormalities, fibrosis, and, finally, cirrhosis. Hepatocellular carcinoma seems to be an ultimate complication of cirrhosis in iron overload; the development of cirrhosis increases the risk of hepatoma by more than 200-fold. [148]

Diabetes mellitus is another common complication of all forms of systemic iron overload; [109] [195] the risk may be greater in patients with a family history of diabetes. Virtually all of the secondary manifestations of diabetes may develop, including retinopathy, nephropathy, neuropathy, and vascular disease. Gonadal insufficiency and other endocrine abnormalities also occur. Hypogonadism may result from primary testicular failure or may be hypogonadotrophic in origin. In hereditary hemochromatosis, impotence may be the presenting symptom. Abnormalities in pituitary and end-organ function may develop; hypothyroidism, hypoparathyroidism, and adrenal insufficiency are infrequent complications. During the second decade of life, both growth and sexual maturation are usually retarded in untreated patients with transfusional iron overload. Abdominal pain of unknown origin may be part of the initial complex of symptoms in as many as 25% of patients with hereditary hemochromatosis.[67]

Iron-induced cardiac disease, occurring as a cardiomyopathy with heart failure, as arrhythmias, or as both, may be a fatal complication of all varieties of systemic iron overload. Heart disease is the most frequent cause of death in patients with transfusional iron overload. [197] [198] About 1015% of patients with untreated hereditary hemochromatosis develop congestive heart failure.[67] Particularly severe cardiac disease may be the presenting symptom in young patients with juvenile hemochromatosis.[160]

Chondrocalcinosis and other forms of arthropathy are common complications of hereditary hemochromatosis and may be the presenting feature. [67] [105] [106] [137] The pathogenesis of the joint changes is unknown. In transfusional iron overload, arthropathy occurs but is uncommon. [109] Osteoporosis is found in some patients with hereditary hemochromatosis. Ascorbate acid deficiency occurs in transfusional siderosis and may play a protective role in some patients because a lack of ascorbate prevents the redistribution of iron from macrophage to parenchymal cells. The combination of ascorbic acid deficiency and osteoporosis is particularly common in African dietary iron overload.[67]

The effect of iron excess on the risk of infection remains uncertain; infection is not a characteristic complication of any of the iron overload syndromes. Nonetheless, clinical reports suggest that an increased availability of iron might be pathogenetically related to infections with certain organisms, including *Vibrio vulnificus, Listeria monocytogenes, Yersinia enterocolitica, Escherichia coli*, and *Candida* spp.[67] [106] [199]

Laboratory Evaluation

The characteristic sequence of changes in clinically useful indicators of iron status as body iron increases from the iron-replete normal to the amounts found in iron overload is shown in Figure 26-5 . The most useful of the indirect measures of body iron status are plasma ferritin and plasma iron and iron-binding capacity. In the absence of complicating factors, plasma ferritin rises to a maximum concentration of about 4,000 g/L as the body iron burden increases. In some circumstances the relationship between plasma ferritin and body iron stores is distorted: the plasma ferritin may greatly underestimate the extent of iron accumulation or may even be normal despite a considerable increase in body iron in a small number of patients with hereditary hemochromatosis, but the explanation for this disproportion is unknown.[20] [200] [201] The plasma ferritin may also underestimate the body iron burden in a small number of patients with transfusional iron overload; ascorbate deficiency may be responsible in some of these cases. [202] Conversely, coexisting liver disease, inflammation, infection, or malignancy may increase plasma ferritin concentrations and complicate the assessment of patients with iron-related hepatotoxicity, hepatitis, hepatoma, or other disorders.

The plasma iron, total iron-binding capacity, and transferrin saturation provide indications of the current iron supply to the tissues. An increased plasma iron and transferrin saturation and a decreased total iron-binding capacity suggest parenchymal iron loading but provide no measure of its magnitude. The transferrin saturation may be normal despite increased iron stores within macrophages, as is found in early transfusional iron loading; it is also affected by coexisting disorders such as liver disease, inflammation, or malignancy.

A liver biopsy is the direct and definitive means of determining the extent of parenchymal iron accumulation. Differentiation of the iron overload associated with chronic liver disease, especially of alcoholic origin, from hereditary hemochromatosis may require special study. The presence of liver disease complicates

TESTING FOR IRON OVERLOAD

A direct measure of body iron avoids the uncertainties inherent in the interpretation of indirect indicators of iron status. Liver biopsy is the definitive direct test for assessing iron deposition and tissue damage in iron overload, permitting measurement of the nonheme iron concentration, histochemical determination of the cellular distribution of iron between hepatocytes and Kupffer cells, and pathologic examination of the extent of tissue injury. In patients with hereditary hemochromatosis undergoing therapeutic venesection, quantitative phlebotomy provides an accurate retrospective determination of the amount of storage iron that can be mobilized for hemoglobin formation. When liver biopsy is contraindicated in a patient, quantitative phlebotomy is occasionally useful in establishing the diagnosis of hereditary hemochromatosis. Bone marrow aspiration and biopsy provide no information about the extent of parenchymal iron loading and are of limited value in the evaluation of iron overload. Iron overload produces no specific abnormalities in the peripheral blood.

the interpretation of plasma ferritin and transferrin saturation values. In this circumstance the results of liver biopsy are again definitive. In chronic liver disease iron is deposited principally in Kupffer cells, whereas in hereditary hemochromatosis the iron is found predominantly within hepatocytes. The hepatic iron index (hepatic iron concentration, corrected for the age of the patient) can also be used to distinguish between patients with alcoholic liver disease with iron loading and those with the heterozygous and homozygous forms of hereditary hemochromatosis.[203] [204] [205]

Differential Diagnosis

The detection and diagnosis of iron overload are most problematic in those conditions resulting from an increased absorption of iron (Table 26-5). Hereditary hemochromatosis is potentially detectable either genetically or phenotypically. After the identification of a candidate gene for hereditary hemochromatosis that is found in most (69100%) of patients with hemochromatosis, a diagnostic genotypic test for the *HFE* Cys282Tyr mutation has been developed. At present, the best use of the test is still under evaluation because initial studies have shown that some iron-loaded patients lack the mutation while some patients who are homozygous for the mutation do not have iron overload.[206] Thus, patients who are found to be homozygous for the *HFE* Cys282Tyr mutation are clearly at risk for iron overload and need phenotypic evaluation by liver biopsy or by quantitative phlebotomy to determine the extent of iron loading.

Patients who are not homozygous for the *HFE* Cys282Tyr mutation may still have iron overload and also require phenotypic evaluation. For these patients, the best indirect means of identifying individuals with iron overload is a combination of measurements of the plasma ferritin and of the plasma iron and transferrin saturation. If any of these measurements is abnormal, a definitive diagnosis can be made directly by liver biopsy, with biochemical measurement of the nonheme iron concentration, histochemical evaluation of the pattern of iron deposition, and pathologic determination of the extent of tissue injury. If the diagnosis of hemochromatosis is established, screening of family members at risk for the disease is mandatory, including siblings andbecause of the possibility of homozygous-heterozygous matingsparents and children. HLA typing may be useful in these family studies. Plasma ferritin and transferrin saturations should be determined; in younger individuals, these measurements should be repeated periodically because some affected individuals do not accumulate appreciable iron burdens until well into adult life. Practice guidelines for the detection, diagnosis, and management of hereditary hemochromatosis have recently been published by the College of American Pathologists.[207]

In patients with iron-loading anemias who are not transfusion dependent, the severity of anemia provides no indication of the risk of iron loading from increased dietary iron absorption. Patients with only minor anemia may accumulate major iron loads. In the diagnosis of these patients, examination of the peripheral blood may show changes of the underlying hematologic disorders. Either morphologic or ferrokinetic techniques may be used to estimate the extent of ineffective erythropoiesis and of erythroid marrow hyperplasia and may be useful in assessing the risk of iron loading. Plasma ferritin and transferrin saturations provide the best direct means of screening for iron overload.

The differential diagnosis of the remaining causes of iron overload listed in Table 26-5 pose few problems. Porphyria cutanea tarda is discussed more fully in Chapter 27 and is readily diagnosed by measurement of urinary porphyrins. The source of the iron overload in patients with parenteral iron loading is evident, whether from transfusion or from repeated injections of therapeutic iron. The various causes of perinatal iron overload are clearly distinguished by clinical and pathologic findings. The diagnosis of idiopathic pulmonary hemosiderosis should be considered whenever iron deficiency anemia develops with coexisting pulmonary abnormalities. Previously, demonstration of iron deposits in the basal ganglia of patients with the Hallervorden-Spatz syndrome was possible only at autopsy, but MRI now provides a means of detecting the localized brain iron during life. [193]

Therapy

The goal of therapy for iron overload is the reduction and maintenance of the body iron at normal or near normal amounts. If possible, phlebotomy is the treatment of choice for hereditary hemochromatosis,[106] [195] for iron-loading anemia if the hemoglobin concentration is high enough to permit venesection, [149] for porphyria cutanea tarda,[149] and for African dietary iron overload. [67] Once the diagnosis of iron overload is established, phlebotomy therapy should begin promptly, for any delay extends exposure to potentially toxic iron accumulations. For most patients phlebotomy should remove 500 ml of blood, containing 200250 mg of iron, once weekly, until storage iron is depleted. The regimen should be individualized; for patients with iron-loading anemia, smaller amounts of blood will need to be withdrawn weekly, while for heavily loaded patients with hereditary hemochromatosis, an even more vigorous program of phlebotomy twice weekly has been advocated. [208] The hematocrit or hemoglobin concentration should be measured before each phlebotomy. After an initial fall in the hemoglobin as erythropoiesis accelerates to keep pace with venesection, the hemoglobin will remain at about 90% of its initial value. The progress of iron removal can be followed by periodic measurements of plasma ferritin, iron, and transferrin saturation. The plasma ferritin concentration declines progressively as iron is removed, but the plasma iron and transferrin saturation remain elevated until iron stores near depletion. In a patient with porphyria cutanea tarda, a few weeks of phlebotomy will suffice, while in hereditary hemochromatosis and an initial body iron burden of 25 g, removal of the iron burden may require 2 years or more.

Occasionally, plasma iron falls and hemoglobin regeneration is temporarily delayed despite incomplete removal of excess iron, as indicated by continued elevation of the plasma ferritin

TIMING OF CHELATION THERAPY

In patients who are transfusion dependent from early infancy (i.e., those who have thalassemia major and other congenital refractory anemias), chelation therapy is best started after about 1020 transfusions, usually around 3 years of age.[109] In older patients with acquired refractory anemias who become transfusion dependent, it seems advisable to begin chelation early, after transfusion of 1020 units of blood. In patients with iron-loading anemias and those with sickle cell disease who are chronically transfused for prevention of complications, early therapy also seems prudent, beginning when the hepatic iron concentration or the serum ferritin increases to about two or three times the upper limit of normal. In each of these disorders, delay in beginning chelation therapy only exposes the patient to a greater risk of iron toxicity.

concentration. A brief halt in the phlebotomy regimen, presumably to allow mobilization of the remaining iron, is usually all that is needed before resuming weekly venesection. Eventually, when iron stores are exhausted, the ferritin will decline to <12 g/L, the plasma iron and transferrin saturation will be decreased, and the hemoglobin concentration will fall to <10 g/dl for 2 weeks without further phlebotomy. After complete removal of the iron load, lifelong maintenance therapy is needed, usually requiring phlebotomy of 500 ml every 34 months. Maintenance phlebotomy should preserve a normal transferrin saturation and a plasma ferritin of <50 g/L. Although well-controlled studies are lacking, phlebotomy is not generally recommended for patients with the modest iron overload that sometimes develops with chronic liver disease; removal of the iron load in these cases seems to have no clinical benefit. [209]

For patients with transfusion-dependent refractory anemias, most patients with iron-loading anemias, and the rare patients with hereditary hemochromatosis in whom phlebotomy is impossible, treatment with the iron chelator desferrioxamine is the only means of preventing or removing toxic accumulations of iron. [109] In patients with hereditary hemochromatosis and cardiac failure, a combination of phlebotomy and chelation therapy has been recommended. Unfortunately, desferrioxamine given orally is poorly absorbed; to be effective the drug must be administered by subcutaneous or intravenous infusion with a small portable syringe pump, ideally for 912 hours each day. Compliance with this regimen may be difficult, but a number of studies have now shown that regular chelation therapy with desferrioxamine can remove tissue iron, prevent organ damage, and prolong survival.

In patients with modest iron loads and no evidence of iron toxicity, slow subcutaneous infusion of desferrioxamine for 912 hours daily usually provides adequate therapy. In severely iron-loaded patients and in patients with evidence of iron toxicity, particularly those with cardiac complications, chronic slow intravenous infusions through an indwelling central venous catheter may permit more rapid reduction of the body iron burden. Administration of ascorbic acid can enhance desferrioxamine-induced iron excretion but carries the risk of an internal redistribution of iron from relatively benign storage sites in macrophages to a potentially toxic pool in parenchymal cells. Although the evidence is circumstantial, large doses of ascorbic acid should be regarded as hazardous in patients with iron overload. [210]
Ascorbate supplementation is probably not needed by patients whose diets regularly include ascorbate-rich foods. Desferrioxamine is a generally safe and nontoxic drug in the iron-loaded patient, but systemic complications have been reported, including allergic anaphylactoid reactions, infectious complications, visual abnormalities and auditory dysfunction, and growth retardation. [211] [212] The risk of many of these complications may be minimized by adjusting the desferrioxamine dose to the magnitude of the body iron load. Adequate desferrioxamine therapy should produce a progressive decrease in the body storage iron of almost any patient with iron overload. If no decline is observed, blood and desferrioxamine use, compliance, ascorbate status, and other features of the therapeutic regimen should be thoroughly reassessed.

Prognosis

The prognosis in patients with iron overload is influenced by many factors, including the magnitude, rate, and route of iron loading; the distribution of iron deposition between macrophage and parenchymal sites; the amount and duration of exposure to circulating non-transferrin-bound iron; ascorbate status; and coexisting disorders, especially alcoholism. The magnitude of iron accumulation seems crucial. In hereditary hemochromatosis the minor iron load found in some heterozygotes seems innocuous. [150] [195] Equal numbers of male and female homozygotes are expected, but symptomatic expression is as much as 10 times as common in men, presumably because iron losses during menstruation and pregnancy leave women with lower iron burdens. [148]

The hepatic iron concentration [213] is a major determinant of the risk of cirrhosis of the liver and in turn of hepatocellular carcinoma, now the two major causes of death in hereditary hemochromatosis. The development of cirrhosis increases the risk of hepatocellular carcinoma more than 200-fold. [148] Hepatomas, the ultimate cause of death in 2030% of patients with hemochromatosis, occur almost exclusively in patients with hepatic cirrhosis. Conversely, if the disease is diagnosed before tissue injury has occurred, phlebotomy therapy to remove the excess iron can prevent all the complications of hemochromatosis, including cirrhosis, and return the patients life expectancy to normal. [148]

Even if organ damage is present, further progression is prevented by phlebotomy, [214] and amelioration of some features of the disease is possible. Skin pigmentation diminishes; hepatic function may improve, while fibrosis is arrested or may sometimes regress; and cardiac abnormalities or even failure may resolve. Diabetes and other endocrine abnormalities usually improve only slightly, if at all, although reversal of hypogonadism has occurred. Arthropathy is usually not improved and may even continue to progress despite phlebotomy. In patients with iron overload who cannot be treated by phlebotomy, chelation therapy is effective in reducing the body iron burden and improving the prognosis. Chronic infusion of parenteral desferrioxamine decreases the hepatic iron concentration, improves hepatic function, promotes growth and sexual maturation, and helps protect against cardiac disease and early death. In all forms of iron overload the most effective means of avoiding complications is to prevent iron accumulation, either by early identification and phlebotomy treatment of homozygotes for hereditary hemochromatosis or by early institution of chelation therapy in patients with iron-loading or transfusion-dependent anemias.

Future Directions

Major advances in the understanding and diagnosis of hereditary hemochromatosis can be expected. The identification of *HFE* as a strong candidate for the gene responsible for most hereditary hemochromatosis, together with the identification of Nramp2 as the iron transport protein for GI absorption, has created an opportunity for rapid progress in our knowledge of the molecular basis for iron absorption and its control. The observation

of an association between *HFE* and the transferrin receptor now links the *HFE* Cys282Tyr mutation to the transferrin-transferrin receptor cycle, and the effects of the mutation on the regulation of iron homeostasis may soon be clarified. The availability of a genetic test for most individuals with hereditary hemochromatosis should stimulate the development of population screening programs that will make possible the early detection of affected individuals and the institution of preventive phlebotomy to avoid organ damage. Progress in noninvasive assessment of the body iron burden can also be anticipated. Several methods for the noninvasive diagnosis and measurement of iron overload are under development that would be most useful in the detection and management of patients with iron overload. Therapeutically, efforts to find iron-chelating agents that will avoid the difficulties with compliance associated with prolonged parenteral administration of deferoxamine will continue. The development of a safe, effective, inexpensive, and easily administered iron-chelating agent would be a major advance in the treatment of iron overload that could substantially improve both the quality and length of life of affected patients in the United States and provide important public health benefits worldwide.

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Chapter 27 - Heme Biosynthesis and Its Disorders: Porphyrias and Sideroblastic Anemias

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INTRODUCTION

The porphyrias and the sideroblastic anemias are metabolic disorders that both involve defects in heme biosynthesis. Most forms of porphyria are inherited as Mendelian autosomal dominants, but some types are recessive and others are acquired through exposure to porphyrinogenic drugs and chemicals. A linked group of diseases, the porphyrinurias, are not porphyrias but have in common alterations of heme biosynthesis. Porphyrins are tetrapyrroles, ubiquitous in nature, that exhibit characteristic red fluorescence on exposure to ultraviolet light. The iron-porphyrin complex, heme, is central to all biological oxidation reactions. In plants, the porphyrin molecule is combined with magnesium to form chlorophyll. Porphyrin biosynthesis is thus one of the most essential biochemical processes in the great majority of life-forms. In man, mutations affecting the first enzyme of the heme biosynthetic pathway produce sideroblastic anemia. Inborn errors that occur at subsequent sites in this path way usually result in metabolic disorders known as the porphyrias (Fig. 27-1). Historical analysis of the potential presence of acute porphyria in the British Royal family has been completed, [1] as well as a study of the family of Vincent van Gogh. [2]

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HEME BIOSYNTHESIS

Heme biosynthesis is an essential pathway and occurs in all metabolically active cells that contain mitochondria. It is most active in erythropoietic tissue where it is required for hemoglobin synthesis and in hepatic tissue where the heme forms the basis of various heme-containing enzymes such as the cytochromes P450, catalase, cytochrome oxidase, and tryptophan pyrrolase. The synthetic pathway starts with the condensation of glycine and succinyl CoA to form 5-aminolevulinate (ALA) under the control of the mitochondrial enzyme ALA synthase. This enzyme requires pyridoxal phosphate as a cofactor. A series of enzymes then controls the conversion of ALA first to the monopyrrole porphobilinogen (PBG) and then to the various porphyrins. Finally, iron is inserted into protoporphyrin by the enzyme ferrochelatase to form heme (Fig. 27-1). During the past ten years, cDNA clones have been obtained for all of the enzymes of heme biosynthesis and the structures of the corresponding genes have been determined. These advances are certain to improve both understanding of the pathogenesis of the porphyrias and methods for identification of carriers. The enzymes of the biosynthetic pathway have all been mapped to specific chromosomes (Table 27-1).

Figure 27-1 Pathway of heme biosynthesis in mammalian cells. The first step in the pathway is catalyzed by ALAS and occurs within the mitochondrion using pyridoxal 5-phosphate as a cofactor. ALA then leaves the mitochondrion and is converted by ALA dehydratase to give a monopyrrole, porphobilinogen. Four molecules of this are converted by porphobilinogen deaminase to a linear tetrapyrrole, hydroxymethylbilane. This molecule is then cyclized by uroporphyrinogen III synthase to uroporphyrinogen III, which is then decarboxylated to coproporphyrinogen III. This molecule enters the mitochondrion and is oxidized in succession by coproporphyrinogen III oxidase and protoporphyrinogen III oxidase. The product is protoporphyrin IX, a substrate for ferrochelatase, which catalyzes the insertion of Fe^{2+} to form heme. The defective steps associated with specific porphyrias and X-linked hereditary sideroblastic anemias are shown.

Heme synthesis and its disorders have been the subject of recent reviews. [3] [4] [5] [6] [7]

Control of Heme Biosynthesis

The overproduction of porphyrins and their precursors in the different porphyrias is mainly hepatic or erythropoietic in origin. In the acute porphyrias and in porphyria cutanea tarda, the liver is the main source of overproduction; in congenital porphyria the marrow is the main source; and in erythropoietic protoporphyria porphyrins are over-produced by both the liver and marrow.

Control of hepatic heme biosynthesis is regulated by the rate of the initial enzymatic step, ALA synthase (now designated ALAS1) which is under negative feedback control by heme. This occurs by more than one mechanism. Heme represses transcription of the ALAS-1 gene and also increases the rate of degradation of the mRNA (Fig. 27-2A). In addition, at the post-translational level, heme blocks the translocation of pre-ALAS1 into the mitochondrion. [8] In the mitochondrion the molecular weight of ALA synthase is smaller than that of the cytosolic pre-ALA synthase [9] due to the removal of the mitochondrial targeting sequence.

The erythroid bone marrow is the major heme-forming tissue in the body producing 85% of the daily heme requirement. Heme synthesis in erythroid cells differs from that in hepatocytes; it is linked to tissue differentiation and the half-life of the same end-product of the two is quite different. Heme complexed with globin is preserved in circulating red blood cells for approximately 120 days, whereas heme produced in liver

TABLE 27-1 -- Classification of Porphyrias

Classification	Disease	Biochemistry		Clinical Features	
Acute porphyria	Acute intermittent porphyria	Increased ALA and PBG		Acute attack	
	Variegate porphyria		Increased porphyria		Photo sensitivity
	Hereditary coproporphyria				
Nonacute porphyria	Porphyria cutanea tarda				
	Erythropoietic protoporphyria				
	Congenital porphyria				
Porphyrinurias	Lead Alcohol				
	Iron-deficiency anemia	Varying biochemical and clinical presentation			
	Liver disease				

Figure 27-2 (A) Control of heme synthesis in hepatic and other tissues. The rate of heme synthesis depends on the first and rate-limiting enzymatic step catalyzed by ALAS1. Heme represses transcription of the ALAS1 gene, increases the rate of degradation of its mRNA, and finally blocks the translocation of the ALAS1 isoenzyme into the mitochondrion. **(B)** Control of heme synthesis in erythroblasts. Cytosolic iron enhances the translation of mRNA of the pre-ALAS2 by inhibiting the interaction of a repressor protein with an iron-responsive element in the mRNA. The product of the last step, heme inhibits the uptake of iron from transferrin into the cytosol. Also heme may inhibit translocation of ALAS2 into the mitochondrion. The overall result is that the rate of heme synthesis is tightly linked to the availability of iron for the ferrochelatase reaction.

for cytochromes and enzymes, such as catalase, is subject to much more rapid turnover, measurable in hours. Regulation in the liver is exquisitely sensitive to fluctuations in intracellular heme levels [10] and responds rapidly to the requirements for synthesis as described in Fig. 27-2A . However, heme synthesis in the bone marrow shows a more leisurely response. This fundamental difference is explained by the finding of two different tissue-specific isoenzymes and two different cDNAs for human liver or housekeeping ALA synthase (ALAS1) and an erythroid ALA synthase (eALA.S or ALAS2) expressed exclusively in erythroid cells. [11] [12] [13] The gene for ALAS2 has been mapped to the X-chromosome and that for the hepatic enzyme to chromosome-3.[12] [13] The ALAS2 gene has 11 exons; exons 5 to 11 encode the catalytic domain of the enzyme and include a lysine residue that forms a Schiff base with the pyridoxal phosphate cofactor. Exon 1 contributes to the 5-untranslated region whose structure allows iron to regulate ALAS2 mRNA translation, whereas exons 1 and 2 contribute the sequence that targets the enzyme to the mitochondria

and is cleaved after import.

Enzyme levels of ubiquitous and erythroid isoenzymes of ALAS are controlled by different mechanisms. Ubiquitous ALAS1 levels in liver are regulated by negative feedback by heme that inhibits gene transcription and import of preALAS1 (Fig. 27-2A). In contrast, heme does not affect transcription of the ALAS2 gene, which is under the control of erythroid-specific promoters, e.g., GATA-1. Whether heme inhibits import of pre-ALAS2 into the mitochondrial matrix remains to be unequivocally established. Heme may possibly also prevent the accumulation of intracellular iron by controlling the acquisition of iron from transferrin (Fig. 27-2B).[7] Levels of intracellular iron in turn regulate the translation of ALAS2 mRNA. A specific repressor protein interacts with an iron-responsive element in the 5 untranslated region of the ALAS2 mRNA and prevents translation, and this repression is relieved by high iron levels. [9] Thus, iron uptake by erythroid cells has a positive effect on the enzymatic step catalyzed by ALAS2 (Fig. 27-2B). This effect ensures that protoporphyrin synthesis is coupled to iron availability.

A second rate-limiting step in the overall heme synthetic pathway lies at the level of porphobilinogen deaminase (PBGD), which has a low endogenous activity and is inhibited by proto- and coproporphyrinogen. There are also two forms of PBGD. The PBGD gene encodes two enzymes. This is because the 10kb long gene with 15 exons has two overlapping transcription units. The upstream promoter works in all cell types and produces a protein of size 4244kD whereas the downstream promoter is only active in erythroid cells and by differential splicing produces a smaller protein of size 4042kD. [14]

Erythroid porphobilinogen deaminase (ePBGD) is stimulated by erythropoiesis in vitro and may play a regulatory role in heme biosynthesis during differentiation. Studies of the erythroid promoter for ePBGD have found trans-acting factors that are common to the -globin gene promoter. [15]

The human porphobilinogen deaminase (PBGD) gene has attracted extensive investigation, because of the practical importance of detecting carriers of the gene for acute intermittent porphyria.[16] Studies of the genetic locus of PBG deaminase on chromosome 11 show great molecular heterogeneity[17] [18] [19] as also found in the parents of a homozygous case of AIP.[20] This heterogeneity results in several phenotypic subtypes of the enzyme at the protein level. Most are cross reacting immunological material (CRIM)-negative but some are CRIM-positive.[21] There is a high interspecies conservation of structure of the enzyme. Most human mutations have been described on exons 10 and 12[22] that is consistent with alteration of the binding sites for the dipyrromethane cofactor for the enzyme. [23] The three-domain structure of porphobilinogen deaminase has been defined by X-ray crystallography. The active site is located between domains 1 and 2. The dipyrromethane cofactor linked to cysteine protrudes from domain 3 into the mouth of the cleft. Flexible segments between domains 1 and 2 are thought to have a role in a hinge mechanism, facilitating conformational changes. Biochemical and X-ray crystallographic studies have shown that the enzyme from *E. coli, Euglena gracilis,* rat, and human has 12 conserved arginine residues in the cleft between the three protein domains. This would fit well with the need to bind six PBG molecules each with two carboxyl groupings.

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PORPHYRIAS

Biologic and Molecular Aspects

The porphyrias are classified into acute and nonacute (cutaneous) according to clinical and biochemical features (Table 27-1).

Each of the different types of porphyria is linked to a reduced activity or deficiency of a specific enzyme in the heme biosynthetic pathway (Fig. 27-1). The enzyme deficiency impairs the production of the end product heme and there is overproduction and increased excretion of the heme precursors formed by the steps prior to the enzyme defect. In addition there is a compensatory increase in activity of the initial and rate-controlling enzyme ALA synthase. In the acute porphyrias there is overproduction of all the porphyrins and porphyrin precursors (ALA, PBG) formed proximal to the enzyme defect. The increased excretion of porphyrin precursors in the acute porphyrias is due to the decreased activity of porphobilinogen deaminase (PBGD) in these conditions. The decrease can be caused by genetic mutation of the enzyme (in acute intermittent porphyria) or by inhibition of PBGD by proto- and copro-porphyrinogen in variegate porphyria and hereditary coproporphyria, respectively.[24]

In the nonacute porphyrias there is overproduction of all porphyrins formed prior to the enzyme defect but no overproduction of porphyrin *precursors.* The cause of this lack of overproduction of porphyrin precursors in the nonacute porphyrias is unclear but it may result from a compensatory increase in the activity of the enzyme PBG deaminase in addition to increased activity of ALA synthase and to site-specific heme synthesis. [25] The pattern of overproduction and excretion of porphyrins and porphyrin precursors in the various porphyrias is shown in Table 27-2 . A consequence is that each of the different porphyrias is characterized by a different excretion pattern. Quantitative studies of the different porphyrins and precursors in the urine and feces will usually identify the particular type of porphyria. The porphyrin precursors ALA and PBG and the more water-soluble porphyrins (with multiple carboxyl groups) are excreted mainly in the urine. Other porphyrins are mainly excreted in the feces by way of the bile. Diverse techniques are used to measure porphyrins and precursors such as high pressure liquid chromatography, extraction, and various forms of fluorimetry. A recent addition has been fluorescence emission scanning to differentiate between variegate porphyria, erythropoietic protoporphyria, and other photocutaneous porphyrias. [26] The International Federation for Clinical Chemistry presents diagnostic information on the Internet (http://www.ifcc.org).

The clinical manifestations of an acute attack of porphyria can be explained by dysfunction of the central, peripheral, and autonomic nervous systems. The mechanism by which altered heme synthesis results in dysfunction is unknown. Several explanations for the pathogenesis of the neuropathy have been advanced:

1. Accumulation of porphyrins and their precursorsALA or, less likely, PBG, which are neurotoxic.
2. Deficiency of heme in neural tissues.
3. Depletion of essential substrates or cofactors resulting from the heme biosynthetic pathway defect, e.g., pyridoxal phosphate or zinc or glycine.
4. Abnormal products derived from other pathways, e.g., hemopyrrole, its lactam, peptides and porphobilin.

Perhaps the most likely hypothesis is that the clinical manifestations of acute porphyria arise as a result of heme deficiency within the nerve cells, although this does not exclude the possibility that ALA may also act as a pharmacological agent in these diseases, compounding the effects of heme deficiency. [27] Thus ALA has a pro-oxidant effect on rat brain tissues [28] and generates free radical species during its auto-oxidation. [27] [29] The concept of auto-oxidation or oxidative stress is supported by the hypothesis that manganese excess could contribute to induction of superoxide dismutase [30] and increased indicators of such stress in lead exposure.[31] There is evidence that ALA is incorporated into cells by a pathway common to it and GABA. [32]

Genetic Aspects

The enzymic links and genetic loci in each of the hereditary porphyrias are shown in Table 27-3 . Nearly all are inherited as

TABLE 27-2 -- Changes in Porphyrins, and Their Precursors in the Porphyrias Porphyrinurias and Hereditary Sideroblastic Anemia

	Urine				Feces		Erthrocyte
	ALA	PBG	Uroporphyrin	Coproporphyrin	Coproporphyrin	Protoporphyrin	Protoporphyrin
Acute porphyrias							
1. Acute intermittent porphyria	Raisedvery high in attack	Raisedvery high in attack	Usually raised	Sometimes raised	Sometimes raised	Sometimes raised	Normal
2. Variegate porphyria	Raisedin attack	Raisedin attack	Usually raised in attack	Usually raised in attack	Raised	Raised	Normal
3. Hereditary coproporphyria	Raisedin attack	Raisedin attack	Sometimes raised in attack	Usually raisedalways in attack	Raised	Usually normal	Normal
Nonacute porphyrias							
4. Porphyria cutanea tarda	Normal	Normal	Raisedvery high in attack	Slightly raised	Raised in remission	Raised in remission	Normal
5. Erythropoietic protoporphyria	Normal	Normal	Normal	Normal	Normal	Usually raised	Raisedusually very high
6. Congenital porphyria	Usually normal	Usually normal	Raisedisomer 1	Raisedisomer 1	Normal	Usually raised	Usually raised
Other conditions							
7. Hereditary sideroblastic anemia	Normal	Normal	Normal	Normal	Normal	Normal	Occasionally raised
8. Lead poisoning	Raised	Normal	Normal	Sometimes raised	Normal	Normal	Raised where blood lead >2M

9. Hereditary tyrosinemia	Raised	Normal	Normal	Normal	Normal	Normal	Normal
10. Iron deficiency anemia	Normal	Normal	Normal	Normal	Normal	Normal	Raised

ALA, 5-aminolaevulinic acid; PBG, porphobilinogen.

TABLE 27-3 -- The Porphyrias, Clinical Involvement, Enzymic Etiology, and Chromosomal Location

Porphyria (Synonym)	Acute Attack, Skin Involvement, and Organ	Enzyme of Heme Biosynthesis Affected	Chromosome Location
		Hepatic ALA synthase (ALAS1)	3p21
X-linked sideroblastic anemia	Bone marrow	Erythroid ALA synthase (ALAS2)	Xp11.21
ALA dehydratase deficiency porphyria (plumboporphyria)	Acute Liver	ALA dehydratase (PBG synthase)	9q34
Acute intermittent porphyria (Intermittent acute porphyria)	Acute + Liver	Porphobilinogen deaminase (hydroxymethylbilane synthase)	11q24.1q24.2
Congenital erythropoietic porphyria (Gunthers disease)	Skin Red cells/bone marrow	Uroporphyrinogen III synthase	10q25.2q26.3
Porphyria cutanea tarda (symptomatic porphyria, cutaneous hepatic porphyria)	Skin Liver	Uroporphyrinogen decarboxylase	1p34
Hereditary coproporphyria	Acute + Skin Liver	Coproporphyrinogen oxidase	3q12
Variegate porphyria (porphyria variegata)	Acute + Skin Liver	Protoporphyrinogen oxidase	1q21q23
Erythropoietic protoporphyria (erythrohepatic protoporphyria)	Skin Red cells and liver	Ferrochelatase (heme synthase)	18q21.3

Figure 27-3 The Chester family pedigree. The propositus, Peter Dobson, was a salmon fisherman from a close-knit community living on the bank of the River Dee, which runs through the city of Chester, U.K. Most of the 330 descendents of his marriage in 1888 still live in the city. Many suffered disabling illnesses and psychiatric upsets, which often went unrecognized as porphyria. The family termed their illness Dobsons complaint. *(Courtesy Giles R. Youngs.)*

autosomal dominant traits. Few carriers of the abnormal gene show clinical signs of the disease, but most can be identified by intensive biochemical investigation. The rare congenital porphyria shows autosomal recessive inheritance. The mutations producing each of the acute porphyrias are heterogeneous at the molecular level and include complete or partial gene deletions, alterations of splicing or stability of mRNA, and missense mutations. An exception is variegate porphyria in South Africa in which the founder effect ensures a predominance of the Arg 59 Tryp mutation. [33] [34] Homozygotic or compound heterozygotic inheritance has been found in a number of the porphyrias as has concurrent inheritance of more than one defect. This may present as either two types of porphyria in one family [35] [36] [37] or as two types in one patient. [36] [37] [38] [39] [40] Examples of this concurrent porphyria are the Chester porphyria (acute intermittent porphyria and variegate porphyria) [36] and dual porphyria (variegate porphyria and porphyria cutanea tarda). [41]

The prevalence of the different forms varies widely. For example, in northern Europe and North America, approximately 1/10,000 individuals carries the gene for acute intermittent porphyria. It has been suggested that spontaneous mutation accounts for 3% of acute intermittent porphyria cases. [42] Variegate porphyria occurs in 1/400 white South Africans. There is a reduction in gene frequency in variegate porphyria from generation to generation that suggests that the allele associated with it is selectively deleterious. [43] The same is probably true of the other porphyrias. The pedigree of Chester porphyria is shown in Fig. 27-3 .

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ACUTE INTERMITTENT PORPHYRIA

Clinical and Laboratory Manifestations

Acute intermittent porphyria is the most severe of the acute porphyrias. During an attack, patients display abdominal and neuropsychiatric or neurovisceral disturbances. Onset is in puberty; females exhibit a four-fold greater incidence of attacks. Attacks occur mainly in young adults, and become less frequent after menopause. It is very uncommon to see attacks in children.[44] Crises may vary in duration from several days to months. They are most commonly followed by complete remission, though deaths are still reported, especially with acute intermittent porphyria. [45]

Gastrointestinal symptoms occur in 95% of cases; most patients present with acute colicky central abdominal pain. Examination reveals tenderness, but little rigidity, and patients may also experience limb pain or generalised muscular aches. Severe

Figure 27-4 Bilateral wrist drop caused by peripheral neuropathy in a patient with acute intermittent porphyria.

PRECIPITATING FACTORS IN ACUTE PORPHYRIA: DRUGS

Most patients who have inherited acute porphyria will enjoy normal health and go through life without any knowledge of their disorder or ever experiencing an acute attack. All porphyrics, however, are at risk of developing an attack if exposed to various precipitating factors. Drugs are the most common precipitating agents. Other factors that may trigger attacks include alcohol ingestion, reduced caloric intake (due to fasting or dieting), and infection. Smoking can cause more frequent attacks.

Hormonal status is also important. Attacks are more common in females and rarely, occur before puberty or after menopause. Pregnancy and oral contraceptives may also precipitate attacks. Some women experience regular attacks, commencing in the week prior to the onset of menstruation. These may require Luteinizing Hormone Releasing Hormone (LHRH) antagonists for control.

Drugs	Other Stimuli
Alcohol	Fasting or dieting
Barbiturates	Hormonesstress
ACE inhibitors	Smoking
Anticonvulsants	
Antidepressants	
Calcium channel blockers	
Cephalosporins	
Ergot derivatives	
Erythromycin	
Steroids or anabolic steroids	
Contraceptives, hormone-replacement therapy	
Sulfonamides	
Sulfonylureas	

DRUG LISTS

Before prescribing any medication to a porphyric patient, advice must be sought from an appropriate specialist. Full drug lists are available on the Internet at http:/www.uq.edu.au/porphyria/ It should be borne in mind that such lists are far from encyclopedic, that new drugs are constantly being introduced to the pharmacopoeia, and that any form of combined preparation must be viewed with suspicion, since little is known about metabolic interactions in these diseases.

vomiting may occur, and constipation is usual. Hyponatremia occurs in severe attacks.

Motor neuropathy complicates two-thirds of porphyric attacks and may be the presenting feature. Motor involvement is most common but paresthesias may also occur. Paralysis usually starts peripherally and then spreads proximally; however, in some patients shoulder-girdle involvement may be the first manifestation. The neuropathy may progress rapidly, resulting in respiratory insufficiency. Weakness, usually symmetrical, involves proximal and distal limb muscles more often than

those of the trunk. Upper limbs and proximal muscles are often affected. Involvement of the wrists, ankles and small muscles of the hand may lead to a permanent deformity (Fig. 27-4), and trunk muscle weakness can lead to respiratory embarrassment. Death is usually due to respiratory paralysis. Progressive weakening of the voice may suggest this; treatment requires tracheotomy and intermittent positive pressure respiration. Paresthesias, numbness, and objective evidence of sensory impairment

may occur with loss of pinprick sensation, most marked around the shoulder and hip areas; generalized tonic-clonic seizures occasionally occur.

Severe anxiety, depression, and frank psychosis are the main psychiatric manifestations of porphyric attacks. These psychiatric manifestations may result in a patient being misdiagnosed as suffering from a primary psychiatric disorder. Agitation, mania, depression, hallucinations, and schizophrenic-like behaviour may occur. Psychiatric manifestations may persist between attacks. [46]

The cardiovascular system is involved in approximately 70% of attacks. Sinus tachycardia (up to 160 beats/minute) and hypertension can occur; these usually revert to normal after an attack. There is evidence that hypertension may occasionally be permanent, even in latent cases of acute intermittent porphyria.

Differential Diagnosis

Attacks of acute porphyria must be distinguished from other causes of acute abdominal pain or peripheral neuropathy sometimes associated with psychosis. Thus heavy metal poisoning (lead or arsenic), as well as Guillain Barre syndrome, must be considered as well as paroxysmal nocturnal hemoglobinuria with its characteristic early morning hemoglobinuria and abdominal pain.

During an attack, all patients excrete a massive excess of the porphyrin precursors, 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) in their urine. Urine, when first voided, is clear and darkens on exposure to light as the hexa-hydro porphyrins, the porphyrinogens, are oxidized to porphyrins.

A rapid screening test during an acute attack is to mix equal volumes of urine and Ehrlichs aldehyde reagent and observe for the pink colour of porphobilinogen.

OTHER ACUTE PORPHYRIAS

Hereditary Coproporphyria

Hereditary coproporphyria (HC) combines the clinical features of acute porphyria with photosensitive skin manifestations. Coproporphyrinogen oxidase activity is decreased,[47] leading to overproduction of coproporphyrin.

The porphyrin precursors ALA and PBG and the more water-soluble porphyrins (with multiple carboxyl groups) are excreted mainly in the urine. Other porphyrins are mainly excreted in the feces by way of the bile.

Variegate Porphyria

Variegate porphyria (VP) is similar to HC except that there are more severe skin lesions, sometimes with scarring (Fig. 27-5). Protoporphyrinogen oxidase is the affected enzyme, thus protoporphyrin is the major circulating porphyrin. Conventionally, VP is most readily diagnosed by measurement of fecal porphyrin concentrations. However, it has been reported[48] that biliary porphyrin levels may provide a better discriminator from normal patients in the asymptomatic phase. As in erythropoietic protoporphyria, there is a tendency to cholelithiasis. The mechanism by which gall stones may form is not certain but some studies have suggested that porphyrins are cholestatic.[49] In both HC and VP the pathway intermediates produced in excess, coproporphyrinogen and protoporphyrinogen respectively, are inhibitors of the secondary rate-controlling enzyme PBG deaminase. [24] [50]

Plumboporphyria

In plumboporphyria, the ALA dehydratase activity is depressed, as occurs in lead poisoning. The clinical picture resembles acute intermittent porphyria but very few cases have been

Figure 27-5 Cutaneous lesions and scarring in a patient with variegate porphyria.

MANAGEMENT OF ACUTE PORPHYRIA

TREATMENT OF ACUTE ATTACK

An *oral carbohydrate* intake of 15002000 kcal/24 hours should be maintained throughout the attack to reduce porphyrin synthesis; give this orally or, for more severe attacks, via a fine bore Teflon nasogastric tube. If this cannot be tolerated, intravenous dextrose (e.g., 20% solution, 2 l/day) should be given. If early in the attack, 24 days from onset, give *intravenous hematin*, as heme arginate (NORMOSANG, Leiras), 24 mg/kg over 30 minutes once or twice a day, to further reduce the overproduction of porphyrin and precursors. [55] Hematin (PANHEMIN, Abbott) in similar doses may also be used, although it should be reconstituted in human albumin solution to avoid phlebitis and mild transient prolongation of coagulation times. No renal complications have occurred with the standard recommended dosages, and even patients with renal insufficiency tolerate hematin well, although the dosage should be reduced slightly. The action of heme therapy may be extended by heme oxygenase blockers, such as tin protoporphyrin.[56] [57]

PROPHYLAXIS

Many drugs are contraindicated and the patient must be warned to avoid precipitating factors. Alcohol should be restricted and smoking discouraged. Dieting (<800 kcal/day) must be avoided. Pregnancy should also be avoided if the disease is active. If a patient requires an anesthetic, nitrous oxide, ether and cyclopropane are safe, and suxamethonium appears to be a safe muscle relaxant. The opiates and belladonna derivates can be used for premedication and propofol for maintenance of anesthesia. Infection can precipitate an attack and should, therefore, be sought and treated. Blood relatives of patients should be screened to see if they carry the gene.

LHRH antagonists that suppress ovulation are a valuable form of prophylaxis in menstrually related attacks.[58] Estrogens and progestogens such as those in the contraceptive pill must be avoided in acute porphyria. The same applies to most steroids and receptor antagonists such as mifepristone.[59]

described and the disease only becomes manifest in homozygous cases when there is some precipitating factor. [51] [52]

Concurrent Porphyrias

The concurrent porphyrias are a rare group of conditions in which there is concurrent inheritance of two different defects within the heme biosynthetic pathway. There is good precedent for more than one defect within the pathway. Previous descriptions [35] [36] [37] [38] [39] [40] [41] have shown the presence of concurrent porphyria within a family, and toxicologically there is good evidence that exposure to poisons such as lead will induce multiple changes within the pathway. [53] Chester porphyria, for example, combines the clinical features of acute and cutaneous porphyria. PBG deaminase and protoporphyrinogen oxidase activity are reduced, leading to overproduction of PBG and protoporphyrinogen. This dual enzyme deficiency is linked to a locus on chromosome 11 separate from that for PBG deaminase. [54] However, even when the patients were symptom-free, the excretion pattern in the urine was typical of the pattern in acute porphyria with a rise in urinary porphyrins and precursors.

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NONACUTE OR CUTANEOUS PORPHYRIAS

In all cutaneous porphyrias, porphyrins (which are photosensitizing) are deposited in the upper layers of the skin and these are responsible for the characteristic skin lesions.[60] In the development of these lesions, reactive oxygen species and other radicals are formed and probably induce oxidative membrane damage, in particular to mast cells, which would permit complement activation as one part of the inflammatory reaction. [61]

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PORPHYRIA CUTANEA TARDA OR CUTANEOUS HEPATIC PORPHYRIA

Biologic and Molecular Aspects

Porphyria cutanea tarda (PCT) exists in inherited and acquired forms. An inherited homozygous form of this disease, hepatoerythropoietic porphyria (HEP), has been described.[62] In both inherited and acquired forms there is diminution in the activity of hepatic uroporphyrinogen decarboxylase, which converts uroporphyrinogen to coproporphyrinogen by the stepwise decarboxylation of the acetyl groups to methyl groups. The mode of control of the enzyme has been studied in a number of experimental models. These show that iron alone or chlorinated hydrocarbons can diminish activity of uroporphyrinogen decarboxylase and this effect is greatly potentiated when both are given together.[63] Patients may have clinical and biochemical evidence of liver disease. Hepatic siderosis invariably occurs and iron is one of the causative agents in acquired PCT. An association of PCT with hereditary hemochromatosis has been suggested, and studies have found a 44% incidence of a characteristic mutation (Cys 282 Tyr) found in a MHC class 1-like gene termed HLAH, which is highly associated with hereditary hemochromatosis. [64] [65] These results strongly implicate the HLAH gene as a genetic susceptibility factor in acquired PCT. An apparent association has emerged between HIV infection, hepatitis C infection, and PCT.[66] [67] The etiological role of viral infection in PCT is not yet clear. It is possible that therapy for HIV with zidovudine precipitates the disease but it is more likely that the association is either merely coincidental or that the viral infection unmasks the preexisting uroporphyrinogen decarboxylase defect. [68] [69]

Genetics

As in the other genetic lesions in the porphyrias, there is heterogeneity in the mutations causing PCT and HEP phenotypes. [70] [71] In the inherited form, enzyme activity is reduced in peripheral blood cells; this is not the case in the acquired forms. Little is known about structure-function relationships for the enzyme despite the breadth of other details of its form and function.[72] In different population groups it is difficult to find the relative numbers of acquired and familial disease. In one analysis in Hungary 77.5% of patients were found to suffer from the acquired form, and of the patients with the familial disease, the frequency in females was higher than in males, suggesting that inheritance may predispose patients to estrogen-precipitated disease. [73]

Clinical Features

The most striking clinical feature of both forms of PCT is a bullous dermatosis on light-exposed areas. This starts as erythema and progresses to vesicles that become confluent to form bullae (Fig. 27-6), which may hemorrhage and leave scars; pruritus is often troublesome. Milia are common and may precede or follow vesicle formation. Increased fragility of the skin is important and, in less severe cases, may be the only clinical sign. In severe cases photomutilation can result, usually because of infection of slowly healing lesions.

The thickening and scarring with calcification has been described as pseudoscleroderma. Hyper-pigmentation is common, and women often complain of hirsutism. Neurological change is not observed. Patients may have clinical and biochemical evidence of chronic liver disease, sometimes with cirrhosis. There is an association with hepatocellular carcinoma. Hepatomegaly is particularly common when alcohol intake is excessive.

Precipitating Factors

Alcohol is the most important precipitating agent. In more than 90% of patients, the intake is excessive. However, estrogenic steroids are also sometimes implicated, as are certain halogenated hydrocarbons. PCT may also develop in people treated with hemodialysis for kidney failure. An outbreak of cutaneous hepatic porphyria in southeast Turkey in 1956 was traced to seed wheat dressed with the fungicide hexachlorobenzene. A neoplastic subgroup has been identified secondary to benign or malignant liver tumors.

Differential Diagnosis

Other causes of bullous or vesicular skin lesions should be excluded, such as a drug reaction (see pseudoporphyria) or

Figure 27-6 A bullous skin lesion of porphyria cutanea tarda.

chronic renal failure. The distinction between PCT, VP, and HCP rests on biochemical testing of urine and feces, with the highest levels of urinary uroporphyrin found during attacks of PCT (Table 27-2).

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ERYTHROPOIETIC PROTOPORPHYRIA

Biologic and Molecular Aspects

Although not described until 1961, this form of erythropoietic porphyria, also known as erythrohepatic protoporphyria, is much more common than congenital porphyria. Ferrochelatase activity is reduced in peripheral blood, liver, bone marrow, and skin, and protoporphyrin is synthesized in excess. [74] Thus the erythroid progenitor cells (burst-forming units-erythroid, or BFE) in erythropoietic protoporphyria (EPP) patients show intense fluorescence when viewed under 405 nm light. [75] The gene mutation in EPP shows heterogeneity as in other porphyrias. [76] [77] The last enzyme of the biosynthetic pathway, ferrochelatase, is important since its endogenous activity is relatively low and could act as a control point in the pathway. Ferrochelatase ligates iron in an iron-sulfur cluster bound to three cysteine residues.[78] It is still uncertain whether ferrochelatase exists in a multi-enzyme complex spanning the mitochondrial membrane with coproporphyrinogen oxidase and protoporphyrinogen oxidase.[79] Such a complex may allow for channeling of substrate from the cytoplasm to the mitochondrial matrix and also provide the reducing potential for the formation of intramitochondrial ferrous iron, essential for heme formation, from the extramitochondrial ferric iron. Immunological studies on human protoporphyria show that immunologically reactive ferrochelatase is present but that enzyme activity was only 17% of normal in 3 subjects. [80]

Genetics

EP is inherited as an autosomal dominant and symptoms may occur at any age including infancy and childhood. [81] Rarely, autosomal recessive inheritance has been described.[82] Several models of various complexity have been proposed to account for the expression of erythropoietic protoporphyria (EPP) [83] and a recent family study considered that the EPP phenotype resulted from co-inheritance of a low output but normal ferrochelatase allele and a mutant allele. [84] There is heterogeneity of the molecular defect, as in all other porphyrias. [74] [76] [85]

Clinical Features

The clinical features are mainly cutaneous on exposure to sunlight. They include pruritic urticarial swelling and redness of the skin on exposure to sunlight. The most distressing symptom is an unbearable burning sensation on the affected parts. Remarkably, such features are ameliorated during pregnancy, which has been linked to lowered protoporphyrin levels. [86] Hepatic involvement, which occurs in later life, involves deposition of hepatotoxic protoporphyrin in the liver and thus can lead to fatal liver failure from an active chronic hepatitis with cirrhosis. [87] Such protoporphyrin deposition may also cause cholelithiasis; the gall stones contain high concentrations of protoporphyrin. The liver disease of EPP seems to correlate with erythrocyte protoporphyrin concentrations. [88] Mild microcytic anemia has been reported[89] [90] as well as mitochondrial iron accumulation and ring sideroblasts in about 30% of patients. [91] Paradoxically, the serum iron and ferritin are low or normal.

Differential Diagnosis

Erythropoietic protoporphyria should be distinguished from other causes of a photosensitive skin rash. The distinction can be made by demonstrating fluorescence in a proportion of red cells (fluorocytes) in the peripheral blood and confirmed by measurement of greatly increased erythrocyte and fecal protoporphyrin. Patients with erythropoietic protoporphyria have a relatively high incidence of ring sideroblasts in the marrow. [91] This can lead to diagnostic difficulty since occasional patients with idiopathic sideroblastic anemia have been reported to have increased erythrocyte protoporphyrin. [92] [93] [94] [95] However, erythropoietic protoporphyria can be distinguished by the autosomal dominant inheritance, dermal photosensitivity, normal or low serum iron, and levels of protoporphyrin in red cells and feces.

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CONGENITAL PORPHYRIA

Biologic and Molecular Aspects

Congenital porphyria or Gunthers disease, although extremely rare, was the first porphyria to be described in 1874. [96] Unlike the other porphyrias, it is inherited as a Mendelian autosomal recessive.

MANAGEMENT OF NONACUTE PORPHYRIA

Patients should avoid exposure to sunlight and use sun block and physical barriers such as cotton gloves.

PORPHYRIA CUTANEA TARDA

The clinical features are reversed by removing any precipitating agent such as alcohol, halogenated hydrocarbons, and drugs. The patient should be screened for hepatic neoplasm and hepatitic viral infection. The mainstay of treatment is to remove liver iron by venesection of 500 ml of blood weekly until clinical remission occurs or until the hemoglobin level falls below 12 g/dl. Chloroquine, at low doses of 125 mg twice a week for several months has also been helpful, as it enhances urinary clearance of porphyrin. Where venesection is difficult, desferrioxamine has been used to reduce liver iron stores. It is of value to screen the patient and first-degree relatives for hereditary hemochromatosis.

ERYTHROPOIETIC PROTOPORPHYRIA

Oral beta carotene offers effective protection in EPP against solar sensitivity. It does so by quenching the radical formation that is a feature of the skin damage. Yellowing of the skin (carotenemia) is one side effect. Interruption of the enterohepatic protoporphyrin circulation by bile salt sequestering agents, such as cholestyramine, reduces plasma protoporphyrin levels and may retard the development of the liver disease. Liver transplantation has been reported to be an effective measure in preventing the progression of this disease. [103]

CONGENITAL PORPHYRIA

Erythropoiesis should be reduced by means of erythrocyte hypertransfusion and by hematin or heme arginate infusion. [101] Bone marrow transplantation has given encouraging biochemical and clinical results but insufficient numbers of patients have been treated to be certain of future prospects of this therapy. [104] Splenectomy and chloroquine therapy (125 mg twice weekly) have an ameliorating effect, as does hypertransfusion, but life expectancy is usually severely shortened.

The onset of solar photosensitivity results from gross overproduction of porphyrins, caused in turn by deficiency of uroporphyrinogen III synthase. Like other porphyrias the defective enzyme results from point mutations at multiple sites within the gene. [97] Other enzymes are largely normal although there is an increase in ALA synthase activity. [98] Excess porphyrins, particularly uroporphyrin 1, accumulate in the normoblasts of the bone marrow and are excreted in the urine and feces. They are also deposited in bones and in the teeth, resulting in a pinkbrown discoloration, which fluoresces bright red in light of wavelengths around 400 nm. Dental restoration has been used to correct the esthetic appearance of the teeth. There are frequently profound changes in bone structure in congenital porphyria. This has been linked to vitamin D deficiency because of light avoidance. [99] However bone changes can be seen when vitamin D levels are adequate and one might speculate that the porphyrins deposited in bone are cytotoxic since similar bone changes are features of both homozygous variegate porphyria and hepatoerythropoietic porphyria. [100]

Clinical Features

Typically the onset of the disease is from birth, but occasionally late-onset cases have been reported. [101] The skin reaction is severe and can be devastating and the teeth become brownish-pink due to their high porphyrin content. Severe cutaneous photosensitivity is manifested by blistering of light-exposed areas and fragility of the epidermis. Skin thickening occurs and there is extensive scarring and hypertrichosis. The recurrent damage associated with scarring on the hand may produce a claw-shaped deformity and loss of digits. Dystrophic nails may curl up and drop off. Lenticular scarring may lead to blindness. Hemolytic anemia often occurs and is associated with increased erythrocyte fragility and splenomegaly. Dyserythropoiesis may contribute to the anemia. [102]

Differential Diagnosis

The most characteristic feature of congenital porphyria is the excess production of series 1 porphyrins rather than series 3 isomer produced in the other porphyrias.

Red cells fluoresce in ultraviolet light, as will the brown-stained teeth, because of high porphyrin content.

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PSEUDOPORPHYRIA AND RENAL DIALYSIS

The term *pseudoporphyria* has been used to describe a bullous dermatosis associated with a number of dermatological conditions that bear some resemblance to porphyria.[105] This photosensitivity is often induced by drugs such as the tetracyclines, naproxen, furosemide, oxaprozin, and many others. In these conditions there is no alteration in porphyrin metabolism or excretion. It is, therefore, incorrect to name any of them a porphyria, and the term pseudoporphyria should not be applied to them, but only to conditions in which alterations of porphyrin metabolism can be found such as the bullous dermatosis of hemodialysis. [106] [107] Patients with renal failure can present with many biochemical features of porphyria prior to hemodialysis. These abnormalities normalize after dialysis, especially when electrolyte abnormalities like zinc deficiency are also corrected. [107] In a considerable proportion of patients with chronic renal failure, skin changes resembling porphyria cutanea tarda (PCT) develop some months to years after the onset of maintenance hemodialysis. In a minor proportion, genuine PCT can be diagnosed. [108] In such cases, there are elevated total porphyrin levels in plasma and in urine if the patient is not anuric. These patients present a therapeutic dilemma since they are normally anemic and thus unsuitable for phlebotomy therapy.

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SIDEROBLASTIC ANEMIAS

Sideroblastic anemias are a heterogeneous group of disorders characterized by anemia of varying severity and diagnosed by finding ring sideroblasts in the bone marrow aspirate. The peripheral blood shows hypochromic red cells, which are microcytic in the hereditary forms (Fig. 27-7A), but are often macrocytic in the acquired forms of the disease. The red cell parameters from automated cell counting may show bimodal volume distribution curves or widened range of cell sizes (Fig. 27-7B); however, this dimorphic size distribution is not always present. Tiny inclusions may be visible in the red cells; these can be confirmed as iron-containing Pappenheimer bodies by Prussian blue staining of the blood smear. The diagnostic test is bone marrow examination together with Prussian blue staining of the bone marrow smears. The presence of ring sideroblasts (Fig. 27-7C) is defined as erythroblasts containing iron-positive (siderotic) granules arranged in a perinuclear collar distribution around one-third or more of the nucleus. Electron microscopic examination has shown that these siderotic granules are mitochondria-containing amorphous deposits of ferric phosphate and ferric hydroxide.

Iron overload is a common clinical feature of refractory sideroblastic anemia and, in severe cases, may lead to complications that characterize secondary hemosiderosis (e.g., diabetes, cardiac failure). Marrow examination shows prominent erythroid hyperplasia, which is a sign of the ineffective erythropoiesis and is responsible for increased iron absorption. The sideroblastic anemias have diverse etiologies but have in common an impaired biosynthesis of heme in the erythroid cells of the marrow. Most sideroblastic anemias are acquired as a clonal disorder of erythropoiesis, with varying degrees of myelodysplastic features (Table 27-4). The inherited forms are uncommon and occur predominantly in males with an X-linked pattern of inheritance. A number of drugs have been associated with a reversible sideroblastic anemia, whereas ring sideroblasts may be found in patients with alcohol abuse (Table 27-4). The first descriptions of ring sideroblasts in association with chronic refractory anemias appeared in the late 1950s [109] [110] following an earlier description of familial X-linked hypochromic microcytic anemia. [111]

TABLE 27-4 -- Classification of Sideroblastic Anemias

Hereditary
X-linked[a]
Autosomal dominant or recessive[a]
Acquired
Idiopathic acquired[a] (refractory anemia with ring sideroblasts)
Associated with previous chemotherapy, irradiation, or in transitional myelodysplasia/myeloproliferative diseases
Drugs
Alcohol
Isoniazid
Chloramphenicol
Other drugs
Rare causes
Erythropoietic Protoporphyria
Pearson syndrome
Copper deficiency or zinc overload
Hypothermia

[a]Trial of pyridoxine indicated.

Figure 27-7 (A) Peripheral blood smear in hereditary sideroblastic anemia showing a population of hypochromic and microcytic erythrocytes. **(B)** Erythrocyte volume distribution curve in a patient with hereditary sidero-blastic anemia. A dimorphic size distribution is evident. **(C)** Bone marrow smear stained with Prussian blue, showing ring sideroblasts (arrow).

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HEREDITARY SIDEROBLASTIC ANEMIA

Biologic and Molecular Aspects

Erythroid cells from patients with X-linked forms of hereditary sideroblastic anemia generally exhibit low activity of ALAS. [5] [112] [113] [114] [115] A defect in this enzyme is firmly established in patients whose anemia responds to pyridoxine therapy, since pyridoxal phosphate is an essential cofactor for ALAS. However, even affected females with moderate anemia unresponsive to pyridoxine have been documented to have low levels of ALAS in bone marrow lysates. [113] [115] In some male patients with X-linked pyridoxine-responsive sideroblastic anemia, the low ALAS activity in bone marrow increased to levels above the normal range when the patient took pyridoxine supplements and recovered from the anemia. [112] [113] [116] There are several possible explanations for this enhancement of ALAS activity by dietary pyridoxine supplements. First, pyridoxine (or its phosphate) may stabilize the ALAS during folding of the enzyme following its synthesis. [116] Second, pyridoxine may protect a mutant ALAS from degradation by mitochondrial protease. [112] Finally, the mutant enzyme may have a higher Michaelis constant (K_m) for pyridoxal phosphate so that increased amounts of its cofactor are required for normal activity. [113]

The gene for the ALAS2 isoenzyme has been localized to the X chromosome and this gene is now known to be the site of most but not all mutations giving rise to X-linked pyridoxine-responsive sideroblastic anemia. [12] [117] Over two dozen different mutations have now been identified in individuals or families with hereditary sideroblastic anemia and nearly all have resulted from single base alterations such as a C G substitution that results in an amino acid change from threonine to serine at residue 388 of ALAS2. [116] All known mutations lie between exons 5 and 11 of ALAS2, the region that codes for the catalytic domain, with most lying within exon 9 which contains the lysine at which binding of pyridoxal 5 phosphate occurs. [5] A mutation, Asp 190 Val, has been described in a pyridoxine-refractory patient and appears to affect the proteolytic processing of the ALAS2 during or after import into the mitochondrion. [118] The variety of different mutations in the erythroid ALAS2 gene responsible for X-linked sideroblastic anemia and their pyridoxine responsiveness have been reviewed. [4] [5]

Genetic Aspects

In the great majority of families with hereditary sideroblastic anemia, males are affected with an X-linked pattern of inheritance

Figure 27-8 Pedigree of a family with pyridoxine-responsive sideroblastic anemia showing X-linked recessive inheritance. , affected; carrier;

, unknown status. Diagonal lines indicate deceased members. This pedigree [116] has been abbreviated to show only the affected branches of the family. The arrow indicates the proband.

(Fig. 27-8). Thus, the assignment of the gene for erythroid ALAS2 to the X chromosome [12] [117] as well as the many mutations documented in erythroid ALAS2 provide the genetic basis for this X-linked disease. In several families, co-inheritance of other X-linked traits (e.g., glucose-6-phosphate dehydrogenase [G6PD] deficiency or ataxia with sideroblastic anemia) has been described. [119] [120] [121] There are well-documented families in which the sideroblastic anemia was inherited as an autosomal dominant trait, [122] [123] as well as rare cases that are autosomal recessive. [124] Both sporadic as well as familial cases have been described that affect only females, [125] [126] [127] the latter possibly representing skewed X-chromosome inactivation involving the ALAS2 gene. [128] The absence of affected male members in these pedigrees suggests that the ALAS2 defects identified are lethal in hemizygous males.

Clinical and Laboratory Evaluation

Typically, the anemia presents in infancy or childhood but the milder forms of anemia may not be found until midlife. Even elderly patients have been diagnosed with this anemia. [129] Some cases may only be discovered during family surveys, which should always be undertaken when hereditary sideroblastic anemia is diagnosed. Still other patients may present with features of iron overload, such as diabetes or cardiac failure. Iron overload occurs commonly even with mild anemia and may occasionally be seen with female carriers. Enlargement of the liver and spleen may occur with mild abnormalities of liver function tests.

Anemia is extremely variable, but even when little or no anemia is present, the mean corpuscular volume (MCV) is low and the red cell volume distribution width may be increased. When anemia is severe the MCV may be as low as 50 fl (^{3}). The blood smear shows a population of cells with hypochromic, microcytic morphology (Fig. 27-7), which contrasts with the other normochromic, normocytic cells (dimorphism). Anisocytosis, poikilocytosis, elongated cells, and siderocytes may also be seen. The characteristic erythrocyte dimorphism is most prominent in patients with milder anemia, in female carriers, or in patients in whom pyridoxine has corrected the anemia but not restored the MCV to normal. Leukocyte values are normal, whereas the platelet count is normal or increased.

Serum iron concentration is increased and transferrin shows an increased percentage of saturation with iron. Serum ferritin levels are invariably increased. Ineffective erythropoiesis can be confirmed by ferrokinetic measurements showing that plasma iron clearance is rapid with subnormal retention of the iron

THERAPY FOR HEREDITARY SIDEROBLASTIC ANEMIA

A trial of pyridoxine 100200 mg/day taken orally is indicated for 3 months in all patients with hereditary sideroblastic anemia. Response is variable and ranges from complete correction of hemoglobin to no effect. Even when pyridoxine completely corrects the anemia (Fig. 27-9), the increase in MCV may not reach normal values and a population of hypochromic, microcytic cells remains. About 2550% of patients with hereditary sideroblastic anemia show a full or partial response to pyridoxine and this vitamin should be continued lifelong in the responders. A lower maintenance dose should be determined for each responding patient by progressive dose reduction since long-term therapy with pyridoxine at 100200 mg/day has been associated with peripheral neuropathy. [131] The adult nutritional requirement for pyridoxine is 12 mg/day; some patients have been maintained on as little as 4 mg/day as supplement. [116] Folic acid supplements should also be administered because the erythroid hyperplasia increases demand for this vitamin. There have been two reports of hereditary sideroblastic anemia responding to parenteral pyridoxal-5-phosphate after failing oral pyridoxine. [132] [133]

Transfusions are the mainstay of treatment for severe anemia that is unresponsive to pyridoxine. Regular administration of packed red cells using white cell filters should be given to relieve symptoms and permit normal childhood development. Iron overload and secondary hemosiderosis rapidly progress after transfusions begin; chelation therapy with desferrioxamine should be initiated from the onset.

Iron removal may be of great benefit for patients who have mild or moderate anemia and evidence of iron overload. [127] [130] These patients can often tolerate intermittent phlebotomy, which is preferable to chelation therapy for iron removal and should be continued to reduce ferritin levels to <300 ng/ml. All patients with iron overload should avoid ingestion of ascorbic acid supplements, which not only enhance iron absorption but also increase the tissue toxicity of elemental iron. Alcohol should also be avoided. Splenectomy is contraindicated in this disease.

Figure 27-9 Response of the hemoglobin and MCW to withdrawal and reinstitution of pyridoxine in a responsive hereditary sideroblastic anemia.

isotope in erythrocytes after 1014 days. Some other features of ineffective erythropoiesis may be variably present: mild increase in bilirubin, decrease in haptoglobin, mild increase in lactate dehydrogenase, and normal or slight increase in reticulocyte numbers. The magnitude of iron overload correlates poorly with the degree of anemia in patients who are not transfused. The degree of ineffective erythropoiesis is a better predictor of the degree of iron overload. Where ferrokinetics are unavailable, the extent of erythroid hyperplasia relative to normal acts as a rough measure of the magnitude of ineffective erythropoiesis. Several studies have shown that the relative increase in erythroid activity multiplied by the patients age shows a good correlation with the degree of iron overload, as measured by plasma ferritin. [127] [130]

Differential Diagnosis

Hereditary sideroblastic anemia should be distinguished from idiopathic hemochromatosis, as both have biochemical evidence of iron overload and a similar tissue pattern of iron deposition. Careful hematologic assessment of patient and family members should make the distinction, since hemoglobin and MCV will be normal in idiopathic hemochromatosis.

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ACQUIRED IDIOPATHIC SIDEROBLASTIC ANEMIA

Acquired sideroblastic anemia may either be idiopathic or occur following chemotherapy or irradiation (Table 27-4). The clonal nature of hemopoiesis in this condition was first suggested by Dacie.[110] Nearly all cases show evidence of dyserythropoiesis in the marrow and there may also be dysplastic changes in either the myeloid precursors or megakaryocytes, or both. Acquired idiopathic sideroblastic anemia falls within the diagnostic category of refractory anemia with ring sideroblasts as defined by the French-American-British group.[134] Acquired sideroblastic anemia has also been described as a rare finding in myeloproliferative disorders such as idiopathic myelofibrosis. However, distinguishing between idiopathic myelofibrosis and myelodysplasia is sometimes difficult and some of the reported cases may represent disease that is intermediate or transitional between these two entities. [135] [136] [137]

Biologic and Molecular Aspects

Clonal hematopoiesis has been demonstrated in acquired idiopathic sideroblastic anemia as well as in the related myelodysplastic syndromes. Specific evidence was first provided by finding a single G6PD isoenzyme in erythrocytes, granulocytes, platelets, and B lymphocytes in a woman who was heterozygous for G6PD and thus carried two isoenzymes in her skin and T lymphocytes.[138] [139] This technique is applicable only to the few women who have G6PD heterozygosity, but restriction fragment length polymorphism analysis (see Chap. 1) can now be applied to most women using probes directed at other X-chromosome genes such as phosphoglycerate kinase or to an X-linked variable copy number tandem repeat sequence (see Chap. 1).[140] [141] The results show uniform monoclonality of hematopoiesis in acquired sideroblastic anemia either with or without associated myelodysplastic features. Initial reports of low levels of ALAS in bone marrow of acquired idiopathic sideroblastic anemia have not been confirmed and the cause of the defective heme synthesis in the abnormal clone is unclear. Some indirect evidence exists for a primary mitochondrial lesion, perhaps in the mitochondrial respiratory chain that impairs the reduction of Fe^{3+} since Fe^{2+} is essential for heme synthesis.[142] [143] [144]

Etiology

Clonal chromosomal changes are found in bone marrow cells in approximately 60% of patients with acquired sideroblastic anemia. Characteristic changes are monosomy 7, trisomy 8, deletions involving chromosomes 5, 7, 11, or 20 and a number of balanced translocations.[145] When sideroblastic anemia is acquired secondary to chemotherapy or irradiation, chromosomal changes are nearly always found and tend to be multiple.[145] Among these changes, the loss of an entire chromosome (5 and/or 7), deletion of a long arm [(del)5, (del) 7, or (del) 13] or an unbalanced translocation are typical.[146] [147] Indeed, when karyotype shows loss of material from chromosomes 5 and/or 7, a detailed occupational history may show exposure to potentially mutagenic chemical agents in a proportion of patients.[148] However, the development of visible chromosomal changes is probably a late event in acquired sideroblastic anemia and may be preceded by the expansion of a clone of genetically unstable stem cells.[149] This concept is in accord with the view that multiple genetic events underlie the pathogenesis of other myelodysplastic syndromes as well as acute myeloid leukemia.[139] [149] [150]

Clinical and Laboratory Evaluation

Typically this anemia develops insidiously in a middle-aged or elderly patient with normal or increased MCV and a blood smear showing a population of hypochromic red cells. Hepatosplenomegaly may be present. Leukocyte and platelet counts are usually normal, but some patients have thrombocytosis that occasionally exceeds $1{,}000 \times 10^9$ /l.[151] If leukopenia or thrombocytopenia is present, a careful search should be made for myelodysplastic features, which if present lead to the more descriptive term refractory cytopenia for the condition.[152] [153] An iron stain of the bone marrow aspirate shows ring sideroblasts, which should total more than 15% of all erythroblasts to make the diagnosis of acquired sideroblastic anemia.[134] [153] [154] [155] Iron cannot be assessed in the marrow trephine biopsy core since it may leach out during decalcification. The bone marrow also shows erythroid hyperplasia; although mild dyserythropoiesis (multinuclearity, nuclear budding) and megaloblastoid changes are present, both myelopoiesis and megakaryopoiesis are usually normal. When changes are confined to dyserythropoiesis, the condition has been termed pure sideroblastic anemia.[153] [156] However, dysplasia of myelo- and megakaryopoietic elements may be present (trilineage dysplasia) with the following features: Pelger-Huet-like anomaly, hypersegmentation or hypogranularity of neutrophils, micromegakaryocytes, large mononuclear megakaryocytes, and megakaryocytes with multiple small nuclei.[153] Dysmegakaryopoiesis is more easily detected in trephine biopsies than in marrow smears, while the trephine may also show unsuspected islands of myeloblasts characteristic of myelodysplasia.[157] The overall blast count in marrow smears is, by definition, <5% and peripheral blood monocytes $<1.0 \times 10^9$ l. Cytogenetic analysis of marrow aspirates provides important information, since a normal karyotype predicts long survival in any type of acquired sideroblastic anemia.[158]

Differential Diagnosis

Ring sideroblasts are not limited to acquired sideroblastic anemia; they also occur in other myelodysplastic conditions, such as refractory anemia with excess blasts, in which the blast count is >5%.[159] Careful examination of peripheral blood and bone marrow will distinguish acquired idiopathic sideroblastic anemia from these related myelodysplastic conditions. Family surveys are very useful in distinguishing acquired from hereditary forms of sideroblastic anemia, as the latter may present in late adult life.

Prognosis

Acquired idiopathic sideroblastic anemia and the related entity of refractory anemia have the most favorable outlook among

THERAPY FOR ACQUIRED SIDEROBLASTIC ANEMIA

Transfusions are indicated for relief of symptomatic anemia. A trial of pyridoxine at 100200 mg/day for 3 months is worthwhile in patients who have anemia but who do not display neutropenia or thrombocytopenia. However, few patients with acquired idiopathic sideroblastic anemia respond to this vitamin. If any response is achieved, maintenance therapy with pyridoxine at lower dosage is indicated. Recently, cyclosporin, 56 mg/kg/day has been reported to benefit the anemia of the closely related myelodysplastic condition of refractory anemia. [164]

the myelodysplastic syndromes, with a median survival of 4276 months and 312% incidence of leukemic progression in different series. [145] [160] [161] [162] The prognosis can be correlated with three factors. First is the severity of the anemia, since repeated transfusions markedly increase iron overload and invariably lead to the organ dysfunction characteristic of secondary hemosiderosis (e.g., heart and liver failure, diabetes). The second factor is whether neutropenia or thrombocytopenia, or both, are associated with the anemia. These cytopenias form the basis of a simple prognostic scoring system in which two or more of the following place the patient in a poor prognostic category: (1) hemoglobin <10 g/dl, (2) neutrophils $<2.5 \times 10^{9}$ /l, (3) platelets $<100 \times 10^{9}$ /l, and (4) blasts >5%.[160] [162] Finally, karyotypic analysis of marrow aspirates provides valuable information, since a normal karyotype carries a more favorable prognosis. Conversely, monosomy 7 or a partial loss of the long arm of chromosome 7 as a single defect imparts a high probability of transformation to acute myeloid leukemia. Multiple chromosomal abnormalities or del(20q) are also associated with an increased risk of progression to leukemia; by contrast, trisomy 8 has no adverse prognostic significance. [145] Evolution of acquired idiopathic sideroblastic anemia to other myelodysplastic conditions, such as refractory anemia with excess blasts, has been described. [163]

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SIDEROBLASTIC ANEMIA AND PORPHYRINURIA SECONDARY TO DRUGS

Alcohol

Ring sideroblasts may be found in the bone marrow of malnourished anemic alcoholics, usually in the presence of associated folate deficiency. [165] [166] [167] In contrast, binge drinking or chronic alcohol ingestion in subjects with good nutrition is not associated with sideroblastic abnormality. Sideroblastic change is never the sole cause for the anemia of alcoholism. Alcohol has a direct toxic effect on hematopoiesis. [168] [169] An increased or high normal MCV and vacuolation of red cell precursors is often seen in addition to the ring sideroblast abnormality. Red cells show dimorphic morphology; evidence in the marrow of folate deficiency is present in one-half of the cases.[169] Transferrin saturation and marrow iron stores tend to be increased but may be low if gastrointestinal bleeding is present. The ring sideroblasts gradually disappear over 412 days when alcohol is withdrawn; [167] during this period, there may be a rebound erythroid hyperplasia, reticulocytosis, and thrombocytosis. Folic acid should be given for the associated megaloblastic changes after blood is taken for vitamin B_{12} and folate assays.

Alcohol consumption lowers the plasma concentration of pyridoxal phosphate, a cofactor for ALAS, the first step in heme synthesis. [170] [171] Conversion of ethanol to acetaldehyde is necessary for this effect and it has been shown that acetaldehyde acts by accelerating the degradation of intracellular pyridoxal phosphate in the liver, thus lowering plasma levels of this coenzyme. [172]

Chronic alcoholics have an altered heme metabolism with increased urinary excretion of coproporphyrin, mainly isomer 3 but normal urinary excretion of uroporphyrin, ALA, and porphobilinogen. Acute and chronic ethanol ingestion markedly depresses the activity of ALA dehydratase in peripheral blood. Ethanol administration to normal subjects results in increased activity of leucocyte ALA synthase and erythrocyte porphobilinogen deaminase, the two rate-controlling enzymes of the pathway. The activities of each of the other four enzymes are depressed. Ferrochelatase, the enzyme that inserts iron into protoporphyrin to form heme, shows the most marked depression, and in alcoholism there is prolonged depression of uroporphyrinogen decarboxylase which provides a rationale for the role of ethanol in the etiology of porphyria cutanea tarda. [173] [174] As noted earlier, ethanol is a major precipitating factor in acute porphyria. [175]

Isoniazid

Administration of the antituberculous drug isoniazid has been occasionally associated with development of a sideroblastic anemia after 110 months of therapy. The anemia is both hypochromic and microcytic with a dimorphic blood smear and ring sideroblasts in the marrow. This complication is thought to occur only in slow acetylators of isoniazid, allowing this drug to react nonenzymatically with pyridoxal and to form a hydrazone that is rapidly excreted in the urine. The anemia can be fully reversed by co-administration of pyridoxine, 2550 mg/day with isoniazid or by withdrawing the latter drug. [176] [177]

Chloramphenicol

Chloramphenicol is an antibiotic that produces a reversible suppression of erythropoiesis after several days of therapy (plasma levels 1015 g/ml). This effect is both predictable and separate from the rare idiosyncratic side effect of aplastic anemia in about 1 of 20,000 exposed persons. Nearly all patients given chloramphenicol >2 g/day develop vacuolation of the erythroid precursors, as well as ring sideroblasts. These effects are thought to arise from suppression of mitochondrial respiration. Chloramphenicol inhibits mitochondrial protein synthesis and reduces cytochrome a + a_3 and b levels.[178] [179] Serum iron concentrations are increased and reticulocyte numbers are subnormal, but these changes revert on stopping the antibiotic.

Other Drugs

A reversible acquired sideroblastic anemia has been described with penicillamine therapy and with triethylene tetramine hydrochloride, a copper-chelating agent used in the treatment of Wilsons disease. [180] [181] Acquired sideroblastic anemia has also been precipitated by progesterone given to a patient on two separate occasions 15 years apart, and this anemia promptly reversed on withdrawal of the drug. [182]

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PRESENTATIONS ASSOCIATED WITH SIDEROBLASTIC ANEMIA AND/OR PORPHYRINURIA

Pearsons Syndrome

Pearsons syndrome is a rare entity that presents in early infancy with anemia and exocrine pancreatic dysfunction. The anemia is normocytic or macrocytic, reticulocytes are low, and variable neutropenia and thrombocytopenia is present. The bone marrow shows a striking vacuolation, as well as ring sideroblasts. [183]

Although usually fatal, milder forms of the anemia are consistent with survival into adult life. The syndrome, which is related to the Kearns-Sayre syndrome, is thought to result from deletions, mutations, or duplications of mitochondrial DNA variably affecting multiple tissues of the body. [184] [185] [186]

Copper Deficiency or Zinc Overload

The copper content of a Western diet averages 0.91.6 mg per day which is only a few times greater than the amount needed to maintain homeostasis of this essential element.[187] [188] Copper deficiency has been described only in malnourished premature infants [189] or in patients receiving long-term parenteral hyperalimentation. [190] [191] The syndrome of copper deficiency consists of sideroblastic anemia with hypochromic cells in the blood smear, accompanied by ring sideroblasts and vacuolated erythroid and myeloid precursors in the marrow, and of neutropenia with an absence of late myeloid forms in the marrow. Additional features may also be seen in infants, namely, osteoporosis and long bone changes, depigmentation of skin and hair, and central nervous system abnormalities. Platelet counts remain normal. Serum copper and ceruloplasmin levels are low, whereas serum iron and transferrin saturation are normal. Prompt reversal of the hematologic changes follows therapy with 25 mg/day copper sulfate taken orally or 100500 g/day copper supplement to the intravenous alimentation formula.

Large quantities of ingested zinc interfere with copper absorption and produce the neutropenia and sideroblastic anemia characteristic of copper deficiency. [192] [193] Zinc sulfate is freely available from health food stores, and as little as 450 mg/day for 2 years is sufficient for this effect. Serum zinc levels are high, while serum copper and ceruloplasmin levels are low. Zinc must be discontinued for 912 weeks for full reversal of the anemia and neutropenia.

Iron-Deficiency Anemia

In iron-deficiency anemia there is an accumulation of protoporphyrin in erythrocytes that rarely reaches the level found in erythropoietic protoporphyria. The zinc complex of protoporphyrin is produced, because ferrochelatase utilizes Zn^{2+} during iron-deficient erythropoiesis. [194] Erythrocyte protoporphyrin may be raised before changes appear in peripheral blood and may be helpful in diagnosing iron deficiency when serum iron and ferritin levels are rising as a result of patients having started iron therapy. In iron-deficient erythropoiesis, erythroid ALA synthase activity is reduced. [195]

Lead Poisoning

It has been known for some time that in patients suffering from lead poisoning, there is accumulation of protoporphyrin in erythrocytes and increased urinary excretion of ALA and coproporphyrin.[196] It should be noted that there are sex-related differences in the porphyrin synthetic response to lead with females showing a more profound coproporphyrinuria than men.[197] The elevated protoporphyrin chelated by zinc is retained in the erythrocyte which may explain the absence of photosensitivity. This accumulation of porphyrins and precursors is due to the inhibition by lead of the heme biosynthetic enzymes: ALA dehydratase, coproporphyrinogen oxidase, and ferrochelatase. An increase in the activity of the rate-controlling enzyme ALA synthase results.

Many of the clinical manifestations of lead poisoning may be the result of altered heme biosynthesis. [53] A mild to moderate anemia that can be hypochromic and microcytic occurs in a minority of patients, while basophilic stippling is prominent due to inhibition of pyrimidine 5 nucleotidase in the maturing reticulocyte. Ring sideroblasts have not been reported. The abdominal pain, constipation, and peripheral neuropathy that occur in lead poisoning are also seen in acute attacks of hepatic porphyria. Neuropathy, seen in lead poisoning, may also be the result of disorders of heme biosynthesis, as in the porphyrias. [196] Alterations in porphyrin metabolism have provided a useful means of detecting and assessing the severity of lead exposure and poisoning. The diminution in activity of erythrocyte ALA dehydratase and elevated erythrocyte protoporphyrin levels are the most sensitive measures.

Hypothermia

Thrombocytopenia, erythroid hypoplasia, and ring sideroblasts have been described in patients with hypothermia associated with neurologic disease. [198] These changes reverse slowly as body temperature returns to normal.

Other Conditions

In hereditary tyrosinemia excess urinary ALA is excreted because ALA dehydratase is inhibited by succinyl acetone. Like acute porphyria and lead poisoning, this disease is associated with neurobehavioural disturbance. In liver disease there may be increased urinary excretion of coproporphyrin predominantly the I isomer. [3] In the Dubin-Johnson syndrome the ratio of coproporphyrin isomer I to isomer 3 is markedly increased in the urine (>80%) possibly as a result of deficiency of hepatic uroporphyrinogen III cosynthase and increased activity of PBG deaminase. In Rotor syndrome, total urinary excretion of coproporphyrin is markedly increased and consists predominantly of coproporphyrin isomer I. In the unconjugated hyperbilirubinemia of Gilberts syndrome, depressed activity of protoporphyrinogen oxidase and increased activity of ALA synthase has been noted in peripheral leucocytes. [3] [199]

Chemical Sensitivity

It has been hypothesized that several otherwise unexplained chemical-associated illnesses, such as multiple chemical sensitivity (MCS) syndrome, may represent mild chronic cases of porphyria or other acquired abnormalities in heme synthesis. However, to date evidence for this is absent. [200] [201]

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Chapter 28 - Megaloblastic Anemias

Aok C. Antony

GENERAL CONCEPTS

The term megaloblastic anemia is used to describe a group of disorders characterized by a distinct morphologic pattern in hematopoietic cells. A common biochemical feature is a defect in DNA synthesis, with lesser alterations in RNA and protein synthesis, leading to a state of unbalanced cell growth and impaired cell division. The cell cycle of normal cells involves a coordinated series of events in DNA, RNA, and protein synthesis; a resting phase is followed by rapid doubling of cellular DNA in the S phase followed by mitosis and division into two cells. Therefore, at any given time the majority of cells have DNA values of 2N while a minority have DNA values of 4N (where N is the amount of DNA in the haploid genome). In contrast, the majority of megaloblastic cells are not resting but vainly engaged in attempting to double their DNA, with frequent arrest in the S phase and lesser arrest in other phases of the cell cycle. Thus, an increased percentage of these cells have DNA values between 2N and 4N because of delayed cell division. This increased DNA content in megaloblastic cells is morphologically expressed as larger than normal immature nuclei with finely particulate chromatin, whereas the relatively unimpaired RNA and protein synthesis results in large cells with greater mature cytoplasm and cell volume. The net result of megaloblastosis is a cell whose nuclear maturation is arrested (immature) while its cytoplasmic maturation proceeds normally independently of the nuclear events. The microscopic appearance of this nuclear-cytoplasmic asynchrony (or dissociation) is morphologically described as megaloblastic. Each cell lineage has a limited but unique repertoire of expression of defective DNA synthesis. This is significantly influenced by the normal patterns of maturation of the affected cell line. Additional variables that affect RNA and protein synthesis can lead to the attenuation or modification of megaloblastic expression.

Megaloblastic hematopoiesis commonly presents as anemia, but this feature is only a manifestation of a more global defect in DNA synthesis that affects all proliferating cells. The peripheral blood picture is characteristic and reflective of megaloblastic hematopoiesis within the bone marrow. The diagnosis is therefore usually fairly simple. However, because any condition that specifically perturbs DNA synthesis may lead to megaloblastosis, determination of the precise etiology is necessary before institution of therapy. Inappropriate therapy can lead to disastrous consequences for the patient. Thus, the biochemical basis for megaloblastosis needs to be understood within the context of evaluation of potential and real variables affecting DNA, RNA, and protein synthesis in a given patient. The most common causes of megaloblastosis are true cellular deficiencies of vitamin B_{12} (cobalamin) or folate, vitamins that are essential for DNA synthesis. The pathophysiology of cellular deficiency is most readily discerned by the clinician who approaches megaloblastosis with a clear understanding of the physiology of these vitamins. A detailed discussion of cobalamin (Cbl) and folate therefore follows.

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COBALAMIN (Cbl)

The term *cobalamin* (Cbl) refers to a family of compounds with the structure shown in Figure 28-1 ; Cbl itself lacks a ligand in the cobalt -position. Vitamin B_{12} is called cyano-Cbl because when it was originally isolated from liver, this position was occupied by a cyano group (an artifact generated in vitro). Details of the chemistry, nomenclature, and in vivo substitutions of Cbl are shown in Figures 28-1 , 28-2 , and 28-3 , and excellent reviews are available on the history, chemistry, and biology of Cbl.[1] [2] [3] [4] Cbl is synthesized and utilized by some microorganisms (e.g., bacteria, fungi). [5] Some strains produce Cbl in excess of their requirements, making them excellent commercial sources for Cbl used in therapy (cost, $60/year/patient requiring Cbl replacement in the United States). Cbl analogues with Cbl- or anti-Cbl-like effects (due to alterations in the corrin nucleus or the nucleotide portion) are produced by microbes or pure chemical interactions of Cbl in nature [5] [6] or as byproducts of Cbl metabolism in vivo.[3] [4] [5] [6] [7] [8]

Nutrition

Cbl is only produced in nature by Cbl-producing microorganisms. Humans receive Cbl solely from the diet. Herbivores obtain their dietary quota of Cbl from plants contaminated with Cbl-producing bacteria that grow in roots and nodules of legumes. Exogenous contamination of plants by feces (e.g., manure used in fertilization) may also be a source of Cbl. The ingested Cbl is utilized by animals for Cbl-dependent reactions in muscular and parenchymal tissues, and carnivores obtain their Cbl supply by ingesting these tissues. Although Cbl is produced by bacteria in the large bowel of humans, it is at a site that is too distal for physiologic Cbl absorption.

Although food Cbl is stable to high-temperature cooking processes, it can readily be converted to inactive analogues by ascorbic acid. Animal protein is the major dietary Cbl source.[9] Meats from parenchymal organs are richest in Cbl (>10 g Cbl per 100 g wet weight); fish and animal muscle, milk products, and egg yolk have 110 g per 100 g wet weight. An average Western diet contains 57 g per day of Cbl, which adequately sustains normal Cbl equilibrium. [10]

Cbl is exceptionally well stored in tissues in its coenzyme forms. Of the total body content of 25 mg in adults, 1 mg is in the liver. [11] There is an obligatory loss of 0.1% per day (1.3 g) regardless of total body Cbl content. It takes 34 years to

Figure 28-1 Cobalamin chemistry and nomenclature. Vitamin B_{12} , cyanocobalamin (CN-Cbl), is a complex molecule consisting of two major portions. A planar group consisting of four reduced pyrrole rings (the corrin nucleus) is attached at right angles to an unusual nucleotide. Three of the four reduced pyrrole rings (designated AD) are connected to one another by methylene carbon bridges; the -carbons of rings A and D are, however, directly linked. The pyrrole rings are linked to a central cobalt atom. One coordinate position of cobalt (above the plane of the corrin nucleus) occupied by CN in CN-Cbl can be occupied by various anionic group ligands (-R) in vitro and in vivo. Substitution at this position forms the basis for the specificity of the molecule as a coenzyme in vivo. The second coordination position of the central cobalt atom (below the plane of the corrin nucleus and nearly perpendicular to it) is a linkage with the glyoxalinium nitrogen atom of the imidazole ring of the nucleotide. The nucleotide consists of a base, 5,6-dimethylbenziminazole, attached to ribose phosphate by -glycoside linkages. The ribose is phosphorylated at C-3; the nucleotide is connected to the ring D of the corrin nucleus through an ester linkage. Compounds containing a corrin nucleus are given the generic name *corrinoid.* The addition of cobalt to the corrin nucleus containing standard side chains gives rise to cobyrinic acid. Additional substitutions in terminal carboxyl groups or modified carboxyl groups (designated *ag*) give rise to various compounds such as cobamic acid, cobinic acid, cobinamide, and cobamide. Cobamide is found in vitamin B_{12} , whose systematic name is therefore -(5,6-dimethylbenziminazolyl)cobamide cyanide. *(From Chanarin,[1] with permission.)*

deplete Cbl stores when dietary Cbl is abruptly malabsorbed, but it may take longer to develop nutritional Cbl deficiency, because of an efficient enterohepatic circulation, which accounts for turnover of 510 g/day of Cbl. [10]

Absorption

Cbl in food is usually in coenzyme form [5-deoxyadenosyl Cbl (Ado-Cbl) and methyl-cobalamin (Methyl-Cbl)], nonspecifically bound to proteins (Fig. 28-4). In the stomach, peptic digestion at low pH is a prerequisite for Cbl release from food protein. [10] This is of clinical significance in the 7080-year-old population among whom hypochlorhydria or achlorhydria is frequently present (in 2550%) leading to inadequate release of protein-bound Cbl via proteolysis with pepsin (pepsin requires a low pH for optimum activity). Although these individuals can absorb crystalline CN-[^{57} Co]Cbl, they may be unable to absorb CN-[^{57} Co]Cbl that is incorporated in vitro or in vivo into food protein.[12] [13] This forms the basis for a modified food-Cbl absorption test (to define the mechanism of Cbl malabsorption) in this cohort of individuals who are Cbl-deficient but have normal Stage I Schilling tests [14] (see discussion later in this chapter).

Once released by proteolysis, Cbl preferentially binds a high-affinity Cbl-binding protein called R protein because it migrates more *r*apidly on electrophoresis than the gastric Cbl-binding protein, intrinsic factor (IF). Gastric juice and salivary R proteins (150-kDa) have higher affinity for Cbl than IF at both acidic and neutral pH (50-fold higher at pH 2 and threefold higher at pH 8). Thus, R protein (also called haptocorrin) is the preferred binding protein once Cbl is released from food. [15] Further research using the porcine gastric haptocorrin cDNA should identify the basis for high Cbl-binding properties. [16]

The Cbl-R protein (holo-R protein) complex, along with excess unbound (apo)-R protein and IF pass through into the second part of the duodenum, where pancreatic proteases degrade both holo-R and apo-R proteins but not IF. The degradation of holo-R and apo-R proteins results in a 150-fold decrease in affinity for Cbl, with consequent transfer of Cbl to IF within 10 minutes. Failure to degrade holo-R proteins by pancreatic protease will preclude involvement of IF in Cbl absorption, because ileal IF-Cbl receptors are specific for IF-bound Cbl and not R-bound Cbl. Indeed, 30% of patients with pancreatic insufficiency malabsorb Cbl. [15] [17] Transfer of Cbl from R protein to IF is a physiologic event and once released from R protein, Cbl binds to IF (a 45-kDa glycoprotein) with high affinity ($K_a = 1.5 \times 10^{10} M^{1}$), 1:1 molar stoichiometry, stability, and resistance to proteolysis over a pH range of 3 to 9. [18] [19] Although R proteins bind both Cbl and most Cbl analogues with comparably high affinity, IF only binds Cbl.

IF is produced in the rough endoplasmic reticulum of parietal (oxyntic) cells in the fundus and cardia of the stomach, [18] [19] and release of IF involves membrane-associated vesicular transport as opposed to release from secretory granules. [20] IF has two binding sites: one for Cbl and another for the ileal IF-Cbl receptor. The IF-Cbl receptor binding site is located in the amino-terminal domain of IF [between amino acid 2562 of 399 amino acids], whereas the Cbl binding site is in the carboxyl-terminal end; glycosylation is apparently not necessary for the integrity of either of the two binding sites. [21] [22] IF is produced in far greater excess than is actually required for absorption [10] and the IF in 24 ml of normal gastric juice is all that is necessary to reverse Cbl deficiency in adults who lack IF. In the absence of IF, <2% of ingested Cbl is absorbed, whereas in its presence, 70% is absorbed.

IF is secreted in response to food in the stomach in a manner analogous to secretion of acid (i.e., by vagal and hormonal stimulation). There is, however, an

unexplained, subtle discordance between IF and acid secretion, since IF release is inhibited by long-term intake of H_2 blockers but not by the alternative $H^+ K^+$ -ATPase antagonist omeprazole. [23] [24] However, sustained

Figure 28-2 Cellular uptake and intracellular reactions involving Cbl. A large family of natural and synthetic cobalamins can be generated when the CN moiety (the upper axial ligand in CN-Cbl) is replaced.[402] On exposure to light, CN is gradually lost from CN-Cbl with the production of hydroxocobalamin (hydroxo-Cbl). Another intermediate reaction is the replacement of the hydroxo group by water, resulting in the formation of aquo-Cbl. In vivo substitutions include the replacement of hydroxo-Cbl or CN-Cbl by a 5-deoxyadenosyl group attached by a covalent bond, giving rise to Ado-Cbl. Methyl-Cbl is the main form in plasma. In vivo, 5-methyltetrahydrofolate readily donates its methyl group to Cob(I)alamin in a reaction involving methionine synthase to form Methyl-Cbl. The loci for defects in Cbl mutants *cbl-cblG* are indicated. Studies which indicate that Methyl-Cbl must be converted to Cob(II)alamin before binding and activation of human methionine synthase and other studies with the bacterial enzyme indicate that this diagram is an oversimplification. [55] [56]

achlorhydria from chronic omeprazole administration will reduce absorption of protein-bound Cbl [25] and eventually lead to food-Cbl malabsorption. [26] IF binds both biliary Cbl and newly ingested Cbl upon their transfer from R protein. [27] [28] Biliary Cbl analogues are not transferred from R protein to IF, resulting in an efficient method for fecal excretion of analogues while allowing for reabsorption of biliary Cbl. The stable IF-Cbl complex, in the form of oligomers and dimers, passes through the jejunum to the ileum, where specific membrane-associated IF-Cbl receptors for the IF-Cbl complex are located on microvilli of mucosal cells of the ileum. [18] [19]

The IF-Cbl receptors from several species have been isolated. The canine IF-Cbl receptor, 220-kDa, is composed of two pairs of subunits of 62-kDa and 48-kDa. [29] It requires Ca^{2+} for binding at pH above 5.4, does not bind free IF, Cbl, or R-Cbl, and is therefore highly specific for IF-Cbl (K_a 10^9 M^1). This receptor contains no carbohydrate, is immunologically distinct from IF, and orients itself in artificial bilayers [30] with its ligand binding site (for IF-Cbl) oriented to the luminal surface. The native hydrophobic species can be digested by papain to a hydrophilic form of 180 to 190-kDa, which retains its ligand binding site. The remaining fragment probably represents the membrane anchor of the receptor. This topology differs from that of porcine and human IF-Cbl receptors, which are believed to be composed of several pairs of two types of subunits;[32] a hydrophilic pair () faces the lumen and is enclosed by a pair of membrane-anchored hydrophobic () subunits, which are attached to -subunits by disulfide bonds. The IF-Cbl complex in dimer form apparently binds to the luminal portion of the receptor. The human ileum contains enough IF-Cbl receptors to bind up to 1 g of IF-bound Cbl; this is the rate-limiting factor in Cbl absorption.

The events following binding of IF-Cbl to IF-Cbl receptors on enterocytes have not been fully delineated. Although transport into the cells requires energy, [33] some studies suggest that the IF-Cbl complex is first internalized by endocytosis and then released from Cbl by lysosomal digestion, whereas others suggest that only Cbl, but not IF, is internalized. [34] Human colon adenocarcinoma (Caco-2) cells [35] exhibit many features common to human enterocytes; they express a 280-kDa IF-Cbl receptor on

Figure 28-3 Major pathways involving Cbl-folate interrelationships. Intra-mitochondrial dehydrogenases [403] which preferentially bind H_4 $PteGlu_5$ provide activated formaldehyde (via dimethylglycinesarcosineglycine). The activated formaldehyde nonenzymatically reacts with the enzyme-bound H_4 $PteGlu_5$ to form 5,10-CH_2 -H_4 $PteGlu_n$ for methionine synthase and thymidylate synthase. Another cytosolic dehydrogenase (glycine N-methyltransferase) that synthesizes sarcosine in the presence of S-adenosyl-methionine from glycine (with conversion of S-adenosyl-methionine to S-adenosyl-homocysteine) is activated on phosphorylation but is allosterically inhibited by (trapped and polyglutamated) 5-CH_3 - H_4 $PteGlu_5$ in Cbl deficiency. This effect 5-CH_3 -H_4 $PteGlu_5$ may lead to methionine-sparing, and the methionine could in turn inhibit the formation of more 5-CH_3 -H_4 $PteGlu_5$ from 5,10-CH_2 -H_4 $PteGlu_n$ and channel the latter for DNA synthesis. Alternatively, the phosphorylation of glycine N-methyltransferase itself may provide a mechanism for modulation of enzyme activity. [404] Nevertheless, this is an area where the methylfolate trap and formate starvation hypotheses are conceivably closely interrelated.

the apical brush border as well as transcobalamin II (TC II) intracellularly. Following uptake of IF-Cbl from the apical surface of these cells by IF-Cbl receptor-mediated endocytosis, there is a slow (over 20 hours) intracellular accumulation of Cbl. [36] The internalized IF is degraded with a half-time of 4 hours, and transcytosed free Cbl is detectable in the basolateral media 4 hours after the onset of endocytosis. TC II is secreted unidirectionally across the basolateral surface and Cbl apparently binds TC II within or at the basal surface of the ileal enterocyte. [37] [38] Thus, Cbl bound to IF is transcytosed from apical to basal direction, and during transcytosis, a transfer to TC II is accomplished. [39] After a delay of 3 to 5 hours, Cbl appears in the portal blood largely (over 90%) bound to TC II. Although some Cbl in the enterocyte is converted to Ado-Cbl, the majority is destined for the portal blood and reaches peak levels in 8 hours.

Cbl in large doses can also passively diffuse through buccal, gastric, and jejunal mucosa so that <1% of a large dose of oral Cbl (1 mg) appears in the circulation within minutes; this property is used to advantage in some individuals with Cbl malabsorption in lieu of parenteral replacement [40] (see discussion later in this chapter).

Transport

More than 90% of recently absorbed or injected Cbl is bound to transcobalamin II (TC II), which is a specific transport protein for delivery of Cbl to tissues. TC II, a 38-kDa polypeptide synthesized in many tissues, binds Cbl with a 1:1 molar stoichiometry and high affinity ($K_a = 1 \times 10^{11}$ M^1).[18] [19] [41] [42] [43] Unlike IF, it will also bind a variety of Cbl analogues (as R protein does). It does *not* however belong to the R protein family and is immunologically distinct from two other plasma Cbl-binding R proteins, TC I and TC III. Recent molecular studies suggest that TC II, TC I, and IF genes originated by gene duplications of an ancestral

Figure 28-4 Components and mechanism of cobalamin absorption. See text for details.

gene, but TC II, unlike the other two Cbl-binding proteins, is the product of a housekeeping gene. [44] [45] The TC II-Cbl complex is cleared so rapidly from the circulation (half-life 6 to 9 minutes) [10] that 98% of TC II in plasma is unsaturated. TC II-Cbl is rapidly bound to specific surface receptors for TC II which are present on several cells.[46] High-affinity TC II-Cbl binding to TC II receptors is specific only for holo- and apo-TC II ($K_a = 25 \times 10^{10}$ M^1). (Because some Cbl analogues can bind TC II with high affinity, these also have the same potential for cellular uptake as Cbl [10] .) Once bound to TC II receptors, TC II-Cbl is internalized by receptor-mediated endocytosis.[47] At the low pH extant in lysosomes, TC II dissociates from Cbl; it is then degraded, while the Cbl is reduced and converted to coenzyme forms (Fig. 28-2). The importance of the transport function of TC II is underscored by the fact that TC II deficiency leads to life-threatening cellular Cbl deficiency. [10] [48]

Cbl is not found free in plasma. Binding to TC II accounts for 1030% of the total serum Cbl, and most of the remaining Cbl is bound to the R protein, TC I. TC I has an N-terminal amino acid sequence similar to that of TC III, but differs from TC III by a higher content of sialic acid residues resulting in significant differences in clearance rates.[10] TC I is not a transport protein and Cbl-bound TC I has a slow clearance rate (half life of 912 days). Despite the fact that TC I binds 75% of the circulating Cbl (present predominantly as Methyl-Cbl) in fasting plasma, about half of the total TC I is in apo-form.

Neither the origin nor the physiologic importance of TC I in blood has been defined. Indeed, hereditary deficiency of TC I is apparently of no clinical consequence. [49] TC I may be a plasma storage form of Cbl, since it accounts for >0.5% of total cellular Cbl uptake. TC I is found in secondary granules of mature polymorphonuclear leukocytes with expression restricted to later stages of myeloid development. [50] TC III appears to have a transport function, since it is cleared from plasma within 3 minutes exclusively by hepatic asialoglycoprotein receptors, a mechanism common to a variety of asialoglycoproteins whose terminal -galactosyl end groups are intact. Conversely, desialation of TC I results in hepatic clearance identical to that of TC III. [10] TC III is derived from specific granules of granulocytes and is released during clotting. Because of its rapid clearance, it is predominantly unsaturated. Functionally, TC III binds a wide spectrum of Cbl analogues, which are rapidly cleared by the liver into bile for fecal excretion. [27] [28] In contrast, between 0.5 and 9 g of Cbl taken up by hepatic TC II receptors is secreted into bile, of which 75% is

reabsorbed, analogous to food Cbl.

Cellular Processing

Following TC II receptor-mediated endocytosis (Fig. 28-2) into lysosomes, the release of Cbl by lysosomal degradation of TC II is an obligatory process for further intracellular metabolism.[10] [47] [51] Following specific transport across the lysosome into the cytoplasm via a newly identified specific transport system, [52] >95% of intracellular Cbl is bound to two intracellular enzymes, methylmalonyl-CoA mutase and methionine synthase. [10] Many lines of evidence[53] [54] suggest that Cob(III)alamin, the most oxidized form of Cbl, must be converted to Cob(II)alamin and Cob(I)alamin by two sequential reductase steps (Fig. 28-2).

In mitochondria Cob(I)alamin is subsequently converted to its coenzyme form Ado-Cbl, which acts as a coenzyme in the *intra*molecular exchange of a hydrogen atom attached to one carbon atom with a group attached to an adjacent carbon atom. Methylmalonyl-CoA mutase in the presence of Ado-Cbl thus converts methylmalonyl-CoA to succinyl-CoA, thereby converting the products of propionate metabolism (i.e., methylmalonyl-CoA) into easily metabolized products.

In the cytoplasm Cbl, as Methyl-Cbl, functions as a coenzyme for the reaction involving methionine synthase, which catalyzes the transfer of methyl groups from Methyl-Cbl to homocysteine (Hcys) to form methionine.[55] [56] [57] In this process Methyl-Cbl is converted to Cob(I)alamin. The methyl group of 5-methyltetrahydrofolate (5-methyl-H_4 PteGlu) is donated to Cob(I)alamin, thus regenerating Methyl-Cbl; 5-methyl-H_4 PteGlu is thus converted to tetrahydrofolate (H_4 PteGlu). Folates and Cbl are thus required together for normal one-carbon metabolism. Spontaneous oxidation of Cob(I)alamin to the catalytically inactive Cob(II)alamin form requires reduction back to Cob(I)alamin before it can accept a methyl group since the cobalt atom in Methyl-Cbl has a 1+ valence. Methionine synthase also catalyzes the conversion of S-adenosyl-methionine to S-adenosyl-homocysteine, during which process the methyl group of S-adenosyl-methionine may also be utilized for re-methylation of Cob(I)alamin. Human methionine synthase binds Cob(II)alamin with a 1:1 molar ratio and contains 2 moles of iron per mole of enzyme. Because Cob(II)alamin must somehow be reduced to Cob(I)alamin before a methyl group can be added to form Methyl-Cbl, methionine synthase and S-adenosyl-methionine/ S-adenosyl-homocysteine together with a reducing system, may be involved in this process. [58] The physiologic importance of the key cofactor roles of the two forms of Cbl (Ado-Cbl and Methyl-Cbl) in methylmalonyl CoA mutase and methionine synthase, respectively, is that the products and byproducts of these enzymatic reactions are critical, as discussed later, for DNA, RNA, and protein biosynthesis.

The human methionine synthase gene that encodes for a 140-kDa protein is present on chromosomal location 1q43. [59] [60] Molecular studies have revealed that methionine synthase is a modular protein with four distinct and separate regions for binding Hcys, 5-methyl-tetrahydrofolate (5-methyl-H_4 PteGlu,) the Cbl prosthetic group, and S-adenosyl-methionine. [57] [61] This can explain how this enzyme plays a central role to catalyze the transfer of a methyl group from bound Methyl-Cbl to Hcys, yielding enzyme-bound Cob(I)alamin and methionine following which the free cofactor, Cob(I)alamin, is then re-methylated by [the methyl group from] 5-methyl-H_4 PteGlu, thereby completing the cycle (Fig. 28-2).

Renal conservation of Cbl is poorly understood. A 550-kDa membrane protein called megalin is found in renal proximal epithelial cells, and functions as a multi-ligand receptor for a variety of macromolecules such as plasminogen, protease inhibitor complex, lactoferrin, and polybasic drugs; interestingly, megalin also specifically binds to and mediates endocytosis of transcobalamin-Cbl complexes[62] in microperfused rat kidney proximal tubules. Because it is expressed in placenta and yolk sac and other tissues, its role in Cbl homeostasis warrants further study.

FOLATES

Nutrition

Folates (see Fig. 28-5 for chemistry and nomenclature), [1C] which are widely distributed in nature in reduced and polyglutamated forms, are synthesized by microorganisms and plants. Leafy vegetables (spinach, lettuce, broccoli, beans), fruits (bananas, melons, lemons), yeast, mushrooms, and animal protein (liver, kidney) are rich sources of folate. [1C] Folates are extremely thermolabile; prolonged cooking (for >15 minutes) in large quantities of water in the absence of reducing agents destroys folates. Oxidation of food folate by nitrites reduces its bioavailability. [63] Because some organisms require exogenous folate for growth, bacteriologic methods (using *Lactobacillus casei*) have been a mainstay for measuring the folate content of food and tissues. The minimum daily requirement of folate is 50 g; the recommended daily intake for folate is 3 g/kg/day (adults 100 g, children 50 g, pregnant women 500 g, and lactating women 300 g). A balanced Western diet contains adequate amounts of folate, but the net dietary intake of folate in many developing countries is often insufficient to sustain folate balance. [65]

Absorption

Dietary folates, which are in the form of pteroyl*poly*glutamates ($PteGlu_{(n)}$), are absorbed less efficiently than pteroyl *mono*glutamate (PteGlu). Although folates in some foods (cabbage, lettuce, orange) are not well absorbed, [1C] most other dietary folates are nutritionally available (i.e., bioavailable). Pteroyl-polyglutamates must be hydrolyzed to pteroylmonoglutamate derivatives prior to absorption by pteroylpolyglutamate hydrolase, an enzyme present in the brush border membranes of small intestinal cells. [6C] This enzyme, composed of two polypeptides of 145- and 115-kDa, has maximal exopeptidase activity at pH 5.5 in the presence of zinc, with equal affinity for $PteGlu_n$ of different glutamate chain lengths.

Luminal pteroylglutamate interacts with brush border membrane-associated folate-binding moieties [67] which exhibit rapid equilibrium binding (<5 minutes), pH dependency (pH 5 optimal), high affinity (K_d 0.08 M), and saturability. There is broad specificity for binding pteroyl *tri*glutamate, 5-methyl-H_4 PteGlu, and other reduced folates, with binding enhanced by physiologic (luminal) concentrations of zinc; transport of reduced-folates occurs within seconds in human brush border membrane vesicles. [68] [6C] [70] [71] Jejunal transport sharply increases with decreasing extravesicular pH from 7 to 5 while intravesicular pH is still 7, again supporting its physiologic relevance. The pH effect, which is probably multifactorial, involving the transporting carrier and PteGlu :OH exchange or PteGlu :H^+ transport, is not observed in the ileum. Some of these characteristics are shared by reduced-folate carriers (RFC) (discussed later in this chapter) which probably mediate the majority of intestinal folate absorption. In fact, the functional role of a 2.5-kb RFC cDNA encoding a 58-kDa brush border membrane protein in intestinal cells has been demonstrated, and RFC protein is also found in the brain, heart, liver, and human intestine. [72] Interestingly, RFC-1 gene expression is developmentally regulated, showing higher maximum capacity for 5-methyl-H_4 PteGlu influx in mature (absorptive) rather than proliferative crypt cells. [73]

Considerable gaps still exist in our knowledge of the structure and functional interrelationships of the human intestinal folate transport carrier, the precise mechanism of passage of

Figure 28-5 Folate chemistry and nomenclature. Folic acid (pteroylmonoglutamate, PteGlu) is the commercially available parent compound for over 100 compounds collectively referred to as folates. [1] PteGlu consists of three basic components: (1) a pteridine derivative, (2) a *p*-aminobenzoic residue, and (3) an L-glutamic acid residue. Before PteGlu can play a role as a coenzyme, it must first be reduced at positions 7 and 8 to dihydrofolic acid (H_2 PteGlu) and then to 5,6,7,8-tetrahydrofolic acid (H_4 PteGlu) and one to six additional glutamic acid residues must then be added via -peptide bonds to the L-glutamate moiety (where lower-case n in $PteGlu_n$ denotes polyglutamation). Folate coenzymes either donate or accept 1-carbon units in numerous reactions in amino acid and nucleotide metabolism. The various substitutions in H_4 $PteGlu_n$ occur either at positions 5 or 10 or both; thus position 5 can be substituted by methyl (CH_3), formyl (CHO) or formimino (CHNH), while position 10 can be substituted by formyl or hydroxymethyl (CH_2OH). Positions 5 and 10 can be bridged by methylene ($-CH_2-$) or methenyl (-CH=).

folate within the enterocyte, and interaction with lysosomal pteroyl-polyglutamate hydrolases. This 75-kDa enzyme is maximally active at pH 4.5 and cleaves both terminal and internal -glutamate linkages, in contrast with the brush-border enzyme that catalyzes hydrolysis of only terminal -glutamate linkages. The intracellular enzyme exhibits greatest affinity for $PteGlu_n$ with longer glutamate chains. [74] The basolateral membrane also possesses a carrier-mediated system, which apparently transports folate more efficiently than across brush borders, suggesting adaptation for eventual transport into portal blood. [75]

Ingested human milk, which contains specific folate-binding proteins (FBP) [76] probably regulates the nutritional bioavailability of ingested folate in neonates by binding with and thereby decreasing folate transport in the jejunum. [77] In the ileum, however, there is no alteration in transport between FBP-bound folate relative to free folate. The net result is a more gradual rise in plasma folate level, leading to slower urinary excretion. This is borne out by a clinical study which indicated that breastfed infants had higher tissue folate levels at 6 months than those weaned before 2 months. [78] Another effect of milk FBPs may be to withhold folate uptake by folate-requiring intestinal bacteria. [1C]

Passive diffusion of folate (M_r 441) across the intestinal mucosa is probably the primary mechanism of folate absorption at high pharmacologic concentrations. [79] In this context the small intestine has a large capacity to absorb folate. Peak folate levels in plasma are achieved 12 hours after oral administration. Whereas therapeutically administered PteGlu enters the portal blood unchanged, food $PteGlu_{(n)}$ is hydrolyzed before transport into the enterocyte, where it is completely reduced to H_4 PteGlu and methylated to 5-methyl-H_4 PteGlu before release into plasma.

Plasma Transport and Enterohepatic Circulation

The normal serum folate level is maintained by dietary folate and a substantial enterohepatic circulation that amounts to 90 g per day of folate. [80] Folates are rapidly cleared from plasma (up to 95% in 3 minutes) by tissues, including the liver. Transport of 5-methyl-H_4 PteGlu into isolated hepatocytes and basolateral membrane vesicles is via a concentrative process involving co-transport with H^+ ions and is electroneutral and is inhibited by other natural folates. [81] Biliary drainage results in a dramatic fall in serum folate (to 30% of basal levels in 6 hours) while abrupt interruption of dietary folate leads to a fall in serum folate levels in 3 weeks. In the plasma, one-third of the folate is free while two-thirds is nonspecifically and loosely bound to serum proteins. A small amount of folate is specifically bound to high-affinity, intrinsically soluble hydrophilic 40-kDa folate-binding proteins (FBP); the physiologic significance of this interaction is not clear. [82] [83] [84] In contrast to Cbl uptake, there is no specific *serum* transport protein that enhances cellular folate uptake.

Cellular Folate Uptake

Folate transport involves translocation of the ligand into cells from the extracellular compartment (i.e., cellular uptake mechanisms) and/or across cellular barriers from one compartment to another (i.e., transcellular mechanisms).[83] Two distinct components

are involved in cellular folate transport. The reduced-folate carrier (RFC) is a low-affinity, high capacity system that mediates the uptake of reduced-folates into cancer cells and probably intestinal cells predominantly at pharmacologic (micromolar, M) extracellular folate concentrations. Folate transport into cells is also mediated by 38- to 44-kDa membrane-associated folate-binding proteins (FBP) or folate receptors (FR) (these terms are synonymous) which bind physiologic folates (i.e., serum 5-methyl-H_4 PteGlu) with high affinity in the nanomolar (nM) range.

As highlighted earlier,[83] the pathway(s) for entry of folates and antifolates are likely to be distinct in different cells depending on the relative efficiency of FR- and RFC-mediated mechanisms, as well as the intra- and extracellular concentration of folates and antifolates (Fig. 28-6). There are probably other determinants in some malignant human cells; for example, despite high FR expression, the RFC was the preferential carrier of antifolates in some cells. [85]

Transcellular folate transport systems are operative in the placenta [86] and renal tubular cells.[87] Although choroid plexi contain a significant amount of FR, the role of these proteins in folate transport across the blood brain barrier/blood cerebrospinal fluid barrier is still unclear. [83] [84] A third pathway for cellular folate transport via passive diffusion[83] is also integrally involved (in concert with FR) in transplacental folate transport [86] (see discussion later in this chapter).

Folate Receptors (FR)

The biologic chemistry and function of FR has been comprehensively reviewed. [83] [84] Three human FR cDNA isoforms, FR-,[88] [89] [90] [91] [92] FR-,[93] [94] and FR-,[95] have been cloned. Although the open reading frames and the 3-untranslated regions of the reported FR- cDNA are identical, their 5-untranslated regions are heterogeneous in length and sequence because FR- contains 2 independent, tissue/cell-specific functional promoters (before exon 1 and exon 4). [96] The genomic organization of human FR has been identified in chromosome 11q13.2q13.5, [91] [97] where four FR-related genes were found within a 140 kb region. The FR- and FR- genes were in sequence (<23 kb apart), with two additional FR-related genes or pseudogenes located upstream of the FR- gene. Genomic clones containing FR- [94] [98] and FR- have been isolated.[96] Although FR can be regulated at the transcriptional level in response to low extracellular folate concentrations, the precise role of *cis*- and *trans*-factors in transcriptional control of FR expression has not yet been demonstrated.

Serum 5-methyl-H_4 PteGlu specifically binds with cell surface

Figure 28-6 Pathways for the entry and intracellular disposition of physiologic and pharmacologic folates and antifolates. The participation of each of the components involved in folate uptake are distinct in different cells depending on (1) the relative efficiency of folate receptor (FR)-mediated and reduced-folate carrier (RFC)-mediated mechanisms, (2) the basal intracellular content of FR and folates, as well as (3) the extracellular concentration of folates and anti-folates. *(From Antony,[83] with permission.)*

Figure 28-7 Folate receptor (FR)-coupled folate uptake and intracellular one-carbon metabolism involving folates. See text for details. *(Adapted from Shane and Stokstad,[143] and Rothberg KG, Ying Y, Kolhouse JF et al: The glycophospholipid-linked FR internalizes folate without entering the clathrin-coated pit endocytic pathway. J Cell Biol 110:637, 1990, with permission.)*

FR with 1:1 molar stoichiometry, high affinity (dissociation constants in the nM range), and in the range needed for binding serum folates. [83] [99] [100] [101] FR are glycosyl-phosphatidylinositol (GPI)-anchored. [102] [103] [104] [105] Recent molecular studies on FR- and FR- suggest Ser-234 and Asn-230, respectively, as the preferred sites of GPI-anchor attachment.[106]

FR transfers folate intracellularly by endocytosis via clathrin-coated pits [107] [108] or by caveolae.[109] In the latter model (which is controversial[84] [108] [110]), folate is dissociated from FR at acid pH generated within caveolae by a proton pump, and is transported through a putative folate transporter into the cytoplasm while apo-FR recycle back to the surface to bind more folate (Figs. 28-6 and 28-7).

FR- are predominantly expressed in most normal and malignant epithelial tissues. [111] This is significant since FR- and FR- exhibit differences in relative affinities (compared to folic acid) for the (6*S*) (physiologic) and (6*R*) (nonphysiologic) diastereoisomers of various folates. [112] Thus, the net efficiency of transport of various folates into cells would depend on the relative expression of one FR isoform over the other. Such data may eventually be important in optimizing efficient delivery of antifolates to effect maximal cytotoxicity of target cells while protecting normal cells. In this context, substitution of alanine in position 49 of FR- converted it to FR-. [113]

It is still controversial whether there is anatomic or functional coupling between the FR and RFC. Nevertheless, the RFC- and FR-mediated folate-transport systems do not communicate with one another (i.e., there is no cross-talk), and both RFC and FR are efficient in the transport of methotrexate and function essentially independently of one another.[114] And when enough FR are expressed, they can mediate the uptake of methotrexate and 5-methyl-H_4 PteGlu with comparable rates to cells expressing only the RFC. This has led to the conclusion that FR have both physiologic and pharmacologic importance, thereby directly confirming several earlier studies.[83]

Transfection of FR cDNA into various cells that do not constitutively express FR [115] [116] [117] [118] led to *greater* proliferation and survival when compared to controls cultured in *low* extracellular folate concentrations (i.e., an overall growth-promoting function). However, in cells that constitutively overexpress FR, further overexpression of FR cDNA leads to a reduction in cell proliferation in vitro and in vivo, because of an inverse relationship between cell proliferation and FR. [105] The underlying mechanisms of this relationship are still unclear.

FR are upregulated in response to extra- and intracellular folate concentrations in some cells, [119] [120] through either transcriptional regulation or by post-translational mechanisms (increase in FR mRNA stability),[121] or via a dominant modulation of FR synthesis at the translational level [122] in some malignant cells. Interestingly, translational control involves the interaction of an 18-base *cis*-element in the 5-untranslated region of FR- mRNA with a 46-kDa *trans*-factor that is critical for translation of FR.[123] Conversely, with excess extracellular folate concentrations of folates and antifolates, FR were downregulated with a reduction in FR mRNA [124] or by reduction of the synthesis of FR at the translational level. [122] Recent investigations have determined that perturbation of plasma membrane cholesterol [125] [126] and sphingolipids[127] also modulates the expression of FR through a poorly understood mechanism. In general, the precise mechanism(s) underlying regulation in most tissues are still unclear. The functional role of FR in mediating folate uptake has been extensively shown in vitro, [83] [84] and although they probably play an important role in vivo, this remains to be shown directly.

The functional role of FR, which are expressed in several normal tissues, [84] has been demonstrated in placenta, proximal renal tubular cells, and hematopoietic progenitor cells. And recently, because FR are overexpressed in some malignancies, they have become potential targets for exploitation as Trojan horses for the delivery of folate-tethered liposomes bearing various cargos (chemotherapy, imaging reagents, genes, or other agents). [128] [129]

FR are precursors to the small amounts of soluble (40-kDa) hydrophilic FBP released extracellularly. [76] [103] [130] [131] [132] [133] Conversion of hydrophobic GPI-anchored

FR to (soluble) hydrophilic FR that retain their folate-binding characteristics can be mediated by an FR-directed metalloprotease [133] [134] [135] or by GPI-specific phospholipases C/D. [102] [103] Neither the significance of soluble FR in folate metabolism, nor the influence of these enzymes on modulation of FRs expression in vivo, are known.

Reduced-Folate Carriers (RFC)

A second mechanism for folate uptake, primarily demonstrated in malignant cells, appears to be due to a carrier-mediated, pH- and energy-dependent process, which transports reduced-folates equally efficiently at higher than physiologic concentrations (Fig. 28-6). A RFC cDNA[136] located in chromosome 21q22.2q22.3, [137] which restores sensitivity to a methotrexate transport-resistant cell line functionally deficient in RFC, encodes a 58-kDa polypeptide and resembles the mammalian glucose transporter (GLUT1), which is a member of the 12-transmembrane domain-spanning membrane transporter family. Additional studies have confirmed the functional significance of RFC genes. [137] [138] [139] The acquisition of methotrexate transport-deficiency in some cells results from inactivating mutations of RFC-1 gene alleles. [140] It is of major clinical relevance that acquired transport resistance in relapsed acute lymphocytic leukemia is also associated with RFC expression. [141]

Cellular Retention and Excretion

Polyglutamation of folate is the major factor for intracellular retention, [142] [143] and the interaction of these forms with intracellular membrane-associated FR may also play a role (Fig. 28-6).[83] [130] As a corollary, human malignant cells with reduced folylpolyglutamate synthase are resistant to antifolates, which cannot be (polyglutamated and) retained intracellularly. [144] Recent studies in leukemia cells suggest that it is the intracellular concentration of (anti)-folates achieved that drives polyglutamation irrespective of the route of entry via FR or the RFC. [145] In human erythrocytes (RBC), folate is accumulated at earlier stages within the marrow by FR;[100] [101] upon maturation, >90% of $PteGlu_{(n)}$ molecules interact with hemoglobin, which, due to its high capacity, assists in intracellular folate retention. [146] RBC folate levels (by the original microbiologic assay) correlate with hepatic folate stores. [1] [3] [4] [10] [147] Intracellular FR may influence intracellular folate metabolism by binding 5-methyl-H_4 PteGlu and competing with dihydrofolate reductase for binding polyglutamates (Fig. 28-6).[130] The attenuating effect on methotrexate cytotoxicity in vitro by high pre-existing extracellular folate concentrations [119] appears to be due to the fact that folates can also passively diffuse into cells. [148] This is potentially significant for protocols involving high-dose (suprapharmacologic) antifolate therapy. [149]

Following glomerular filtration, luminal folate binds FR in the brush border membranes of proximal renal tubular cells [150] [151] and is internalized rapidly via FR-mediated endocytosis; in the low pH of endocytotic vesicles, there is dissociation of folates and slow transport across basolateral membranes into the blood, with recycling of apo-FR back to the luminal brush border membrane. [152] [153]

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INTRACELLULAR METABOLISM AND COBALAMIN-FOLATE INTERRELATIONSHIPS

Pteroylpolyglutamates are the natural substrates for the various enzymes involved in one-carbon metabolism (Figs. 28-6 and 28-7). Thus, pteroyl*mono*glutamates must be *poly*glutamated by

folylpolyglutamate synthase before they participate in one-carbon metabolism. [142] This is best appreciated in mutant Chinese hamster ovary cells, which lack folylpolyglutamate synthase. [10] As a consequence of failure in polyglutamation, these cells have <10% of the intracellular folate content of wild-type cells, and an inability to generate reactions of one-carbon metabolism. The mutant cells therefore require that the *products* of one-carbon metabolism (methionine, glycine, purines, and thymidine) be exogenously supplied. In contrast, wild-type cells can grow as long as adequate folate, Cbl, and homocysteine (Hcys) are available (since they can synthesize their own folate polyglutamates, which then participate in one-carbon metabolism and generate these substances intracellularly).

Tetrahydropteroylglutamate (H_4 PteGlu) is the preferred physiologic substrate for folylpolyglutamate synthase and the polyglutamal form (H_4 $PteGlu_n$) plays a central role in one-carbon metabolism. [143] So factors that either limit the supply of H_4 PteGlu or regulate folylpolyglutamate synthase influence polyglutamation and cellular retention of folates. H_4 $PteGlu_n$ can be converted to (1) formate as 10-formyl-H_4 $PteGlu_n$ (used in *de novo* biosynthesis of purines) and (2) formaldehyde as 5,10-methylene-H_4 $PteGlu_n$ (for synthesis of thymidylate). Furthermore, 5,10-methylene-H_4 $PteGlu_n$ and 10-formyl-H_4 $PteGlu_n$ can be interconverted via intermediates (Fig. 28-7).

To understand the functions of folate coenzymes (Figs. 28-3 and 28-7), it is important to recognize at the outset that 5,10-methylene-H_4 $PteGlu_n$ can be utilized in either (1) the *thymidylate cycle* via thymidylate synthase for thymidine and DNA synthesis, or (2) the *methylation cycle* to form 5-methyl-H_4 $PteGlu_n$, which, via methionine synthase, leads to formation of methionine and H_4 $PteGlu_n$. Both methionine and H_4 $PteGlu_n$ are essential for polyglutamate formation (and thus for retention of folates and perpetuation of normal one-carbon metabolism) and for the interconversion to 5,10-methylene-H_4 $PteGlu_n$ (essential for DNA synthesis). Perturbation of either pathway, directly or indirectly, thus affects DNA synthesis. So thymidylate synthase- and methionine synthase-catalyzed reactions, coupled with the factors that control them via product inhibition, are central issues in protection from megaloblastosis. Additional reactions catalyzed by folate coenzymes involve amino acid interconversions and purine synthesis. [143]

Pteroylmonoglutamate (PteGlu, folic acid) is not a biologically active form of folate; when given therapeutically, it requires reduction to H_4 PteGlu by dihydrofolate reductase (20-kDa) in a two-step reaction {pteroylglutamate (PteGlu) *dihydro*pteroylglutamate (H_2 PteGlu)*tetrahydro*pteroylglutamate (H_4 PteGlu)}. The major form of folate transported into the cell by FR is 5-methyl-H_4 PteGlu (Fig. 28-7). The details of intracellular 5-methyl-H_4 $PteGlu_n$ movement in the cytoplasm and the precise order of channeling of folate along reactions by enzymes involved in one-carbon metabolism are not entirely clear. However, evidence suggests that folate-mediated one-carbon metabolism is indeed compartmentalized among intracellular organelles leading to substrate channeling along folate coenzymes. [154] As an example, some of the enzymes distal to H_4 $PteGlu_n$ are multifunctional, allowing sequential channeling of folate coenzyme forms (e.g., H_4 $PteGlu_n$ 5-CHNH-H_4 $PteGlu_n$ 5,10-H_4 $PteGlu_n$). Thymidylate synthase catalyzes the transfer of the formaldehyde from 5,10-methylene-H_4 $PteGlu_n$ to the 5 position of deoxyuridylate; in this process 5,10-methylene-H_4 $PteGlu_n$ is also reduced to dihydrofolate, which inhibits 5,10-methylene-H_4 $PteGlu_n$ reductase. Because methionine, the product of the methionine synthase reaction, also inhibits this enzyme, this illustrates that modulation of 5,10-methylene-H_4 $PteGlu_n$ reductase by levels of dihydrofolate and methionine can determine the degree of channeling of 5,10-methylene-H_4 $PteGlu_n$ into the methylation and thymidylate cycle (Figs. 28-3 and 28-7).

The mechanism whereby Cbl deficiency produces its megaloblastic effects is not precisely known. It is generally agreed that Cbl deficiency causes a functional intracellular deficiency of 5,10-methylene-H_4 $PteGlu_n$ (Figs. 28-3 and 28-7). The methylfolate trap hypothesis centers around the excess buildup (trapping) of 5-methyl-H_4 PteGlu due to inhibition of the Cbl-dependent methionine synthase; 5-methyl-H_4 PteGlu therefore leaks out of the cell, resulting in an intracellular folate deficiency. The formate starvation hypothesis centers around the relatively greater merit of formate (in the form of 10-formyl-H_4 $PteGlu_n$) and the precursor of formate, methionine, which is also decreased when methionine synthase is inhibited by Cbl deficiency. [155] When methionine is in excess intracellularly, its methyl group is oxidized to formate, which can be used to generate 5-formyl-and 10-formyl-H_4 $PteGlu_n$ (reactions 7 and 8, Fig. 28-7). There are articles that support either hypothesis. [156] [157] [158]

CONSEQUENCES OF PERTURBED ONE-CARBON METABOLISM

Thymidylate and DNA Synthesis

In either Cbl or folate deficiency a net decrease in 5,10-methylene-H_4 $PteGlu_n$ interrupts the reaction mediated by thymidylate synthase which converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP).[147] Although there is a salvage pathway via thymidine kinase, which normally accounts for 5 to 10% of thymidine synthesis,[159] this pathway cannot meet the remaining 90% demand for dTMP generation. (Salvage pathways for purine synthesis can be activated to compensate for diminished generation of purines through folate coenzyme-mediated reactions.) The resulting thymidine deficiency causes a marked increase in dUMP/dTMP ratio. Because of the decrease in dTMP [and eventually dTTP], dUMP and thereby deoxyuridine triphosphate (dUTP) increase. Because DNA polymerase cannot distinguish between dUTP and dTTP, this elevated dUTP promotes misincorporation of uridine residues into DNA. [160] [161] An editorial enzyme, DNA uracil glycosylase, recognizes this faulty misincorporation. It excises dUTP, but the inadequate supply of dTTP leads to failure to repair the break in this DNA strand. Repeated DNA strand breaks lead to significant fragmentation of DNA, with consequent leakage of DNA fragments out of the cell. [162] [163] [164]

The foregoing considerations form the basis for the dU suppression test that is occasionally employed clinically. [159] When cells containing normal dUMP/dTMP ratios are incubated with thymidine ^3H and excess unlabeled deoxyuridine (dU), <10% of thymidine ^3H is incorporated into DNA (by the salvage thymidine kinase pathway), since most of the unlabeled dU is utilized by thymidylate synthase to form dTMP. If the dUMP/dTMP ratio is increased (as in Cbl deficiency), unlabeled dU will not be utilized, and >10% of thymidine ^3H is incorporated into DNA. Addition of Cbl (but not 5-methyl-H_4 PteGlu) to another aliquot of these cells will lead to conversion of endogenous (and unlabeled) dUMP to dTMP; the net result is less thymidine ^3H incorporation into DNA via the salvage pathway. Addition of 5-methyl-H_4 PteGlu will facilitate conversion of dUMP to dTMP in folate-deficient cells but will have no effect in Cbl deficiency. Although this method of testing does not simply represent thymidylate synthase-catalyzed pathways but affects additional pathways involving nucleotide metabolism, [165] the reduction of prior increased thymidine ^3H incorporation in Cbl- or folate-deficient cells by the addition of exogenous Cbl or folate, respectively, has been practically useful to differentiate Cbl from folate deficiency; this is the principle of the diagnostic dU suppression test. [159] A modified diagnostic dU suppression test is useful when inborn errors of Cbl/folate metabolism are suspected[166] or when coincident iron deficiency or thalassemia masks the megaloblastic expression; [167] but in the latter instance, measurement of vitamin levels and/or metabolites to determine Cbl and/or folate deficiency is more practical in routine clinical use.

Chromosome and Cell Cycle Defects

Defective DNA synthesis is reflected by numerous chromosomal abnormalities. There is excessive chromosomal elongation with despiralization associated with random breaks and exaggerated centromere constriction, expression of folate-sensitive fragile sites in hematopoietic cells, and reduced biosynthesis, acetylation, and methylation of arginine-rich histone. [10] [147] [168] [169] All this leads to perturbation of the cell cycle with an increased proportion of cells in prophase of the mitotic cycle and G_2 . This arrest in various stages of DNA synthesis causes many cells to have DNA contents between 2N and 4N. [10] In a recent in vitro model of folate-deficient erythroblastosis, apoptosis of erythroblasts in the late stages of differentiation apparently led to decreased erythrocyte production and anemia. [170] In addition, there was persistent accumulation of p53 and p21; because thymidine prevents the apoptosis of folate-deficient erythroblasts in vitro, this implicated decreased thymidylate synthesis as the main cause of apoptosis in the folate-deficient erythroblasts. [160] Parenthetically, in folate-deficient rats, DNA strand breaks and selective hypomethylation of exons within the p53 tumor suppressor gene can somewhat explain how dietary folate deficiency may enhance carcinogenesis. [171]

Morphologic Expression of Megaloblastosis

There is widening disparity in nuclear-cytoplasmic asynchrony as a Cbl- or folate-deficient cell divides, until the more mature generations of daughter cells either die in the marrow or are arrested (as megaloblastic cells) at various stages in the cell cycle. [10] In fact, the plethora of bone marrow morphologic changes may lead the untrained observer to the diagnosis of acute leukemia. [172] All proliferating cells exhibit megaloblastosis, including epithelial cells lining the gastrointestinal tract (buccal mucosa, tongue, small intestine), cervix, vagina, and uterus. [10] However, megaloblastic changes are most striking in the blood and bone marrow. Ineffective hematopoiesis extends into the long bones, and the bone marrow aspirate (which is better than the biopsy

MORPHOLOGY IN MEGALOBLASTOSIS FROM COLBALAMIN AND FOLATE DEFICIENCY IS THE SAME

Peripheral Smear

Increased mean corpuscular volume (MCV) with macroovalocytes (up to 14 m)[a]
Nuclear hypersegmentation of polymorphonuclear neutrophils (PMNs) (1 PMN with 6 lobes or 5% with 5 lobes)
Thrombocytopenia (mild to moderate)
Leukoerythroblastic morphology (from extramedullary hematopoiesis)

Bone Marrow Aspirate

General increase in cellularity of all three major hematopoietic elements
Abnormal erythropoiesisorthochromatic megaloblasts
Abnormal leukopoiesis*giant* metamyelocytes and band forms (pathognomonic), hypersegmented polymorphonuclear neutrophils (PMNs)
Abnormal megakaryocytopoiesispseudohyperdiploidy

[a]Associated with varying anisocytosis and poikilocytosis.

for observing megaloblastosis) exhibits trilineal hypercellularity, especially in the erythroid series. In contrast to what appears as exuberant cell proliferation with numerous mitotic figures, the cells are actually very slowly proliferating. However, megaloblastosis induced in vitro by anti-FR antibodies causes a true increase in cell proliferative capacity initiated at the level of cell surface FR, suggesting a constitutive control of FR in hematopoietic cell proliferation. [83] [84] [101] [105] [173]

Erythroid hyperplasia reduces the myeloid/erythroid ratio from 3:1 to 1:1. Proerythroblasts are not as obviously abnormal as later forms; they may simply be larger (promegaloblasts). Megaloblastic changes are most strikingly displayed in intermediate and orthochromatic stages, which are larger than their normoblastic counterparts. In contrast to the normally dense chromatin of comparable normoblasts, megaloblastic erythroid precursors have an open, finely stippled, reticular, sieve-like pattern. The orthochromatic megaloblast, with its hemoglobinized cytoplasm, continues to retain its large sieve-like immature nucleus, in sharp contrast to the clumped chromatin of orthochromatic normoblasts. The nucleus is often eccentrically placed in these large oval or oblong cells and lobulation or indentation of nuclei with bizarre karyorrhexis is often seen. In cells destined for the circulation as macroovalocytes, the nucleus may occasionally not be completely extruded. Of the potential progeny of proerythroblasts that develop into later megaloblastic forms, 8090% die in the bone marrow. Marrow macrophages effectively scavenge dead/partially disintegrated megaloblasts. This is the basis for ineffective erythropoiesis (intramedullary hemolysis).

Leukopoiesis is also abnormal. There is an absolute increase in these cells, which are large and have similar sieve-like chromatin. Spectacular giant (2030 m) metamyelocytes and band forms which are often seen are pathognomonic for megaloblastosis. There may be bizarre nucleoli with small cytoplasmic vacuoles. It is probable that giant metamyelocytes cannot easily traverse marrow sinuses and their maturation into circulating hypersegmented polymorphonuclear neutrophils (PMNs) is unlikely. Granulation of the cytoplasm remains unaffected.

Megakaryocytes may be normal or increased in numbers and may exhibit additional complexities in megaloblastic expression. The process of complex hypersegmentation (pseudohyperdiploidy) is associated with liberation of fragments of cytoplasm and giant platelets into the circulation. The net output of platelets is invariably decreased in severe megaloblastosis and abnormal but reversible platelet dysfunction has been documented. [174]

In early Cbl or folate deficiency, normoblasts may dominate the marrow with only a few megaloblasts seen. Complete transformation to megaloblastic hematopoiesis is observed in florid cases and is reflected by varying degrees of pancytopenia. *The earliest manifestation of megaloblastosis is an increase in mean corpuscular volume (MCV) with macroovalocytes (up to 14 m).* Because these cells have adequate hemoglobin, the central pallor, which normally occupies about one-third of the cell, is decreased. In severe anemia, poikilocytosis and anisocytosis are evident. Cells containing remnants of DNA (Howell-Jolly bodies), arginine-rich histone, and nonhemoglobin iron (Cabot rings) may be observed. Extramedullary megaloblastic hematopoiesis may also result in a leukoerythroblastic picture.

Ineffective utilization of iron results in an increased percentage of saturation of transferrin and increased iron stores. If there is associated iron deficiency, the MCV may be normal, and only iron therapy will unmask the megaloblastic manifestations in the peripheral blood. In thalassemia, the entire erythrocyte morphology normally expected in megaloblastosis is masked;[167] [175] however, megaloblastic leukopoiesis is still observed. Significant intramedullary hemolysis (ineffective erythropoiesis) involving >90% of megaloblastic precursors is reflected by a lowered

absolute reticulocyte count, increased bilirubin (up to 2 mg/dL), decreased haptoglobin, and increased lactate dehydrogenase (LDH), often above 1,000 units/ml. There is also a modest decrease in circulating RBC life span.

Nuclear hypersegmentation of DNA in PMNs strongly suggests megaloblastosis when associated with macro-ovalocytosis. Normally, <5% of PMNs have >5 lobes and no cells have >6 lobes in the peripheral blood. If megaloblastosis is suspected (>5% PMNs with >5 lobes or a single PMN with >6 lobes), a formal lobe count per PMN (lobe index) above 3.5 may be obtained.

Megaloblastosis in rapidly proliferating cells of the gastrointestinal tract leads to a variable degree of morphologic changes and atrophy of the epithelial cells of the luminal lining. This leads to functional defects, which include a failure in secretion of IF and malabsorption of Cbl and folate in certain subsets of patients. Thus, a vicious cycle whereby megaloblastosis begets more megaloblastosis is established that can only be interrupted by specific therapy with Cbl or folate. This fact must be recognized when interpreting diagnostic tests involving Cbl absorption (discussed later in this chapter). Interestingly, similar hematopoietic changes have recently been induced in a folate-deficient mouse model. [176]

Neurologic Dysfunction with Cobalamin (Cbl) Deficiency

Because megaloblastosis due to either folate or Cbl deficiency leads to a functional folate coenzyme deficiency, the morphologic manifestations of both deficiencies are understandably indistinguishable. However, only Cbl deficiency results in a patchy demyelination process, which is expressed clinically as cerebral abnormalities and subacute combined degeneration of the spinal cord. [177]

The precise role of Cbl in maintaining the integrity of the central nervous system has not been completely defined. Early studies of the Cbl-dependent enzyme, methylmalonyl-CoA mutase, revealed that the inability to adequately metabolize propionate results in accumulation of propionyl CoA, which can compete with acetyl CoA in pathways of fatty acid synthesis. Thus, fatty acids with odd numbers of carbon atoms can be incorporated into lipids; although this has been demonstrated in nerve biopsies from patients with Cbl deficiency, cause and effect have not been proved. Nitrous oxide (N_2O) was later recognized as an inducer of *functional* Cbl coenzyme deficiency in vivo, because individuals exposed to this gas developed both hematologic and neurologic manifestations similar to those with Cbl deficiency. Because methionine (required in numerous methylation reactions, including those in nerve tissue) also ameliorates the neurologic dysfunction in monkeys, this led to a focus on the methionine synthase-catalyzed reaction and on the potential importance of lack of methionine in Cbl deficiency. The potential etiologic role of some natural Cbl analogues[7] [178] [179] which have anti-Cbl effects, and have been shown to accumulate in the brains of N_2O-treated/Cbl-deficient fruit bats, [177] is still under investigation. Because elevated levels of serum methylmalonic acid (MMA) and total homocysteine (tHcys) are both positively correlated with Cbl-dependent neurologic dysfunction in humans, perturbation of the activity of both Cbl-dependent enzymes may be important. [180] [181] [182] [183] [184] [185] Conversely, hereditary defects in methionine synthase-catalyzed reactions are associated with neurologic dysfunction, [186] whereas methylmalonyl-CoA mutase deficiency is not. Moreover N_2O also inhibits methionine synthase much more than methylmalonyl-CoA mutase, thus elevating the importance of methionine synthase in maintaining the integrity of myelin in humans. Alternatively, there may yet be additional undiscovered pathways. Recent reviews have presented balanced views on various arguments. [187] [188]

SOME CLUES TO DISTINGUISHING COBALAMIN AND FOLATE DEFICIENCIES

Although the megaloblastic manifestations of Cbl and folate deficiencies are clinically indistinguishable, certain distinct patterns in mode of presentation provide clues to the type and etiology of deficiency. In general, the cause of folate deficiency will be found in the fairly recent past (within 6 months) primarily from the history and physical examination. By contrast, the cause of Cbl deficiency will be obscure until specific tests to define the etiology (e.g., Schilling test) are carried out. In bygone days, by the time anemia was symptomatic, >80% of patients had neurologic manifestations, and in 50% this led to some incapacity. Perhaps as a result of widespread use of multivitamins containing folic acid among patients (and even in the food given livestock in the West), the hematologic expression of Cbl deficiency is often substantially attenuated, leading to pure neurologic presentations. Studies highlight the apparent inverse correlation between hematologic and neurologic presentations such that in a third of patients with Cbl deficiency, the earliest signs are often purely neurologic, and symptoms related to paresthesias and diminished proprioception cause the patient to see the physician. Based on the multiple potential etiologies (see box, Megaloblastic Anemias), the truism that what the mind does not know, the eyes do not see is a caveat that cannot be taken lightly; failure to recognize Cbl deficiency as the etiology of neurologic disease and treatment of Cbl deficiency with folate or misdiagnosis of megaloblastosis as erythroleukemia represent significant extremes of deviation from the dictum *primum non nocere.* Areas of overlap in symptomatology in Cbl/folate deficiency are related to megaloblastosis (e.g., common cardiopulmonary and some gastrointestinal manifestations). Although pure folate deficiency in the alcoholic with thiamine deficiency (Wernickes encephalopathy) and peripheral neuropathy is almost indistinguishable from and may mimic Cbl deficiency, the remainder of the neurologic manifestations are uniquely due to Cbl deficiency. And as reviewed recently,[225] folate deficiency in adults has not been unequivocally shown to give rise to neurologic findings. Thus, coexistence of folate deficiency with neurologic disease should prompt investigations to rule out Cbl and other nutrient deficiencies arising from dietary insufficiency or malabsorption.

Curiously, in fruit bats and monkeys, the neurologic dysfunction of N_2O-induced Cbl deficiency occurs when hematologic abnormalities are absent.[10] [189] This was verified clinically in humans, where there is an apparent *inverse correlation* between the extent of neurologic defects versus hematologic abnormalities.[190] [191] Recent studies also highlight the greater sensitivity of human glial cells in culture to short-term Cbl deprivation.[192] The demyelinating process involves patchy swelling of the myelin sheath followed by its breakdown (demyelination), leading to axonal degeneration. Microscopic foci coalesce with one another, giving the surface of the spinal cord (on cross-section) a spongy appearance; later there is secondary Wallerian degeneration of long tracts. Patchy demyelination usually begins in the dorsal columns in the thoracic segments of the spinal cord and then spreads *contiguously* to involve corticospinal tracts. These lesions spread throughout the length of the cord and ultimately involve spinothalamic and spinocerebellar tracts. There is also degeneration of the dorsal root ganglia,

celiac ganglia, and Meissners and Auerbachs plexus. Although demyelination may also extend to the white matter of the brain, it is unclear whether the peripheral neuropathy is due to a distinct lesion or is secondary to spinal cord disease; thus, the clinical manifestations may be extremely varied.[177] [193]

Other Effects of Cbl/Folate Deficiency

Cbl deficiency more often than folate deficiency can also result in sterility from the effects on the gonads. An unexplained finding is generalized melanin pigmentation that is reversible by specific nutrient replenishment. The mechanism of defective bactericidal activity and increased susceptibility to *Mycobacterium tuberculosis* in Cbl deficiency[194] remains unexplained. In folate deficiency, other effects include a reduction in lymphocyte subsets,[195] enhanced predisposition to chemical-induced gastrointestinal carcinogenesis[196] [which may have a clinical correlate in patients with ulcerative colitis with low folate status],[197] and delayed tumor growth.[198] Interestingly, severe folate deficiency impairs pancreatic exocrine function in rats;[199] if confirmed in humans, a resulting lack of R protein degradation could lead to Cbl malabsorption and Cbl deficiency (as noted with chronic folate deficiency).

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BIOCHEMICAL INDICATORS OF EVOLVING DEFICIENCY

Early manifestations of negative Cbl balance are an abnormal deoxyuridine suppression test [200] and increased serum methylmalonic acid (MMA) and tHcys levels.[181] [183] [184] [185] This occurs at a time when the total Cbl in serum is still normal. Continued negative Cbl balance leads to an absolute decrease in serum Cbl level. Likewise, metabolic evidence for folate deficiency (i.e., increased tHcys) is often found when serum folates are still in the low-normal range. Thus metabolite tests reflective of decreased intracellular nutrient availability are followed by biochemical evidence of abnormal thymidylate synthesis (impaired conversion of dUMP to dTMP), morphologic expression of perturbed DNA in PMNs (lobe index), macroovalocytosis, and anemia. The biochemical basis for selective nutrient deficiency in various tissues (e.g., cervical [201] or hematopoietic but not gastrointestinal cells [1] [147]) is still unclear.

Biochemical Evaluation of Cbl/Folate Deficiency

Total Serum Homocysteine (tHcys) and Methylmalonic Acid (MMA) Levels

Cellular nutrient *deficiency* of either Cbl or folate is reflected by decreased intracellular concentrations. *Defective utilization* of Cbl/folate due to intracellular Cbl/folate-dependent enzyme deficiency may allow normal intracellular coenzyme levels. Deficient coenzyme and enzyme levels are both reflected by perturbation of the major Cbl/folate-dependent enzyme-catalyzed reactions. Thus, there is reduced activity of methionine synthase, leading to substrate buildup and to elevated serum levels of tHcys, which can be measured by a sensitive assay.[182] In a classic series of studies, 77 of 78 patients with clinically confirmed Cbl deficiency had elevated values of tHcys, correlating with clinical parameters of Cbl deficiency. [183] In this patient group, 74 of the 78 also had increased serum MMA levels, indicating reduced activity of the second Cbl-dependent enzyme, methylmalonyl-CoA mutase, to convert methylmalonyl-CoA to succinyl-CoA. [181] Similarly, 18 of 19 patients with folate deficiency had elevated levels of tHcys due to reduced activity of the methionine synthase-catalyzed reaction. [183]

The combined use of serum tHcys and MMA can distinguish between Cbl and folate deficiency since most patients with folate deficiency have normal MMA levels, and the rest have only mild elevations. [181] [183] [184] [185] These two tests, which are now clinically available, are useful diagnostically and also in following the patients response to replacement since the abnormally high metabolites will only return to normal when replaced with the appropriate (deficient) vitamin. Thus, a positive response to Cbl, documented by falling levels of tHcys and MMA, is evidence of Cbl deficiency. Conversely, therapy with folate results in a decrease in the isolated tHcys level if folate deficiency is present. [181] [183] [184] [185]

Total homocysteine can be measured in either plasma[202] or serum. [203] In general, plasma levels are slightly lower. The normal value for serum tHcys is 5.113.9 M and serum MMA is 73271 nM, and in general, the higher the values, the more severe the clinical abnormalities. [202] However, there is a fairly wide range of normalcy in tHcys values because of age-, creatinine-, gender-, diet-dependent, [202] and race-dependent[204] variables; nevertheless, most investigators believe that the upper limit of normal should not be greater than 14 M. Children usually have very low values (36 M). Basal levels of MMA are usually <500 nM and in renal failure it rarely increases >1,000 nM.[203] [205] [206] (*Note:* if unseparated blood stands at room temperature, tHcys levels will *increase* over 4 to 24 hours. But frozen serum [from measurements of serum folate/Cbl] can be used for serum MMA and tHcys determinations.)

Among 1,160 elderly participants in the original Framingham Heart Study cohort, nearly one-third had high plasma tHcys that inversely correlated most with plasma folate levels, and to a lesser extent with Cbl and vitamin B_6 (pyridoxine; pyridoxal-5-phosphate is the active form). [207] Thus, a substantial majority of the cases of high tHcys in the elderly can be attributed to low vitamin status. The use of methionine-loading to stress the capacity of the trans-sulfuration pathway (whereby homocysteine is irreversibly degraded by a vitamin B_6 -dependent pathway),[208] has further defined the basis for homocysteinemia.

Because of some variables related to age and mild renal dysfunction that can falsely elevate these metabolites, proof of metabolic deficiency requires demonstration of a reduction in metabolite levels with vitamin supplementation. This was demonstrated in another study, where an intramuscular vitamin supplement (containing Cbl, folate, and pyridoxine) reduced initially elevated metabolite concentrations toward normal in a high proportion of elderly subjects. [209] *Thus it appears that metabolic evidence of vitamin deficiency is common in the elderly, even in the presence of normal serum vitamin levels.*

Serum Cobalamin (Cbl) Levels

Serum Cbl levels previously measured by microbiologic assay using the Cbl-dependent organism *Lactobacillus leichmannii* have been replaced by a simpler assay method that relies on the competitive inhibition by serum Cbl of the binding of CN-[^{57}Co]Cbl to IF (rather than R protein, which falsely measured Cbl analogues also). [8] [210] [211] For the most part, serum Cbl is an established biochemical indicator of Cbl deficiency. [212] [213] However, a report of neuropsychiatric disorders attributed to Cbl deficiency in some patients, despite both the absence of anemia and normal or minimally depressed Cbl levels, underscores the caution with which clinicians should proceed with when interpreting this test. [190] [191] [212] [213] [214] [215] A low serum Cbl is not synonymous with Cbl deficiency and several associated diseases and conditions can falsely raise or lower Cbl levels (Table 28-1). Studies have also identified patients with true Cbl deficiency who have Cbl levels in the low normal range (see discussion later in this chapter).

If the serum Cbl test is broadly used as a screening test, by virtue of the way normalcy is defined, 2.5% of nondeficient individuals will have low levels. Because many millions of individuals probably have serum Cbl tests done every year, several thousand will have low Cbl levels, which simply reflects our definition of the lower limit of normal for this test.[193]

TABLE 28-1 -- Serum Cbl Levels[a] False-Positive Tests and False-Negative Tests[b]

Falsely low serum Cbl in the absence of true Cbl deficiency
Folate deficiency (1/3 of patients)
Multiple myeloma
TC I deficiency
Megadose vitamin C therapy
Patients serum contains other radioisotopes (^{99m} Tc or ^{67} Ga and other radiopharmaceuticals used in organ scanning)

Falsely raised Cbl levels in the presence of a true deficiency
Cbl binders (TC I and II) increased (myeloproliferative states, hepatomas, and fibrolamellar hepatic tumors)
TC II-producing macrophages are activated (autoimmune diseases, monoblastic leukemias, and lymphomas)
Release of Cbl from hepatocytes (active liver disease)

[a]Although a low serum Cbl is not synonymous with Cbl deficiency, 5% of patients with true Cbl deficiency will have low normal Cbl levels.
[b] A potentially serious problem since the patients underlying Cbl deficiency will progress if uncorrected.

The serum Cbl is <300 pg/ml in 99% of patients with clinical hematologic or neurologic manifestations of Cbl deficiency. [185] [216] Conversely, a Cbl level of >300 pg/ml predicts for folate deficiency, or another hematologic disease (Table 28-2). However, if the serum folate levels are borderline/normal and the patient has megaloblastic anemia with Cbl levels >300 pg/ml, metabolite tests or a therapeutic trial may be necessary to rule out underlying folate deficiency.

How often do patients with Cbl deficiency have normal Cbl levels? Among 173 unambiguously Cbl-deficient patients, [185] 5% had normal Cbl levels and up to 10% of adults with true Cbl deficiency will have Cbl values in the low normal (200300 pg/ml) range. Although traditionally such patients would have been regarded as not having Cbl deficiency, they should now be tested with the more sensitive metabolite tests, MMA and tHcys, as discussed earlier in this chapter. Recent studies have again highlighted the need to be vigilant for clinical and biochemical evidence of Cbl deficiency in the elderly at normal Cbl concentrations. [217] And a study on 548 surviving members of the original Framingham Study revealed a prevalence of Cbl deficiency of 12%. [218] Nevertheless, 90% of older patients with serum Cbl levels <150 pmol/L show evidence of true tissue Cbl deficiency. [217]

Serum and RBC Folate Levels

Microbiologic assays of folate using *Lactobacillus casei* suffer from the same limitations as assays for Cbl, [10] and these too have been supplanted, primarily for convenience, by various radioisotope dilution assays. However, the clinical validity of these modifications has in many cases not been adequately documented. [10] There is also significant discrepancy in folate levels when a variety of kits are used on the same sample, raising questions as to the reliability of these assays for clinical use, and especially when compared to microbiologic assays. [219] [220] [221]

When combined with a clinical picture of megaloblastic anemia and additional results of Cbl levels, the serum folate is the cheapest and most useful initial biochemical test to diagnose folate deficiency (Table 28-2).[222] Serum folate level is highly sensitive to folate intake and a single hospital meal may normalize it in a patient with true folate deficiency. Rapidly developing nutritional folate deficiency first leads to a decline in the serum folate level below normal (<2 ng/ml) in 3 weeks; thus, it is a sensitive indicator of negative folate balance. [3] [4] However, isolated reduction of serum folate in the absence of megaloblastosis (i.e., a false-positive result) occurs in one-third of hospitalized patients with anorexia, following acute alcohol consumption, normal pregnancy, and those on anticonvulsants; [212] [213] [223] (unfortunately, these are the very groups who are

TABLE 28-2 -- Stepwise Approach to Diagnosis of Cobalamin (Cbl) and Folate Deficiency

Megaloblastic anemia or Neurologic manifestations consistent with Cbl deficiency plus Test results on serum Cbl and serum folate[a]			
Cobalamin (pg/ml)[b]	**Folate (ng/ml)[c]**	**Provisional Diagnosis**	**Proceed with Metabolites?**
>300	>4	Cbl/folate deficiency is unlikely	No
<200	>4	Consistent with Cbl deficiency	No
200300	>4	Rule out Cbl deficiency	Yes
>300	<2	Consistent with folate deficiency	No
<200	<2	Consistent with (1) combined Cbl plus folate deficiency, or (2) isolated folate deficiency	Yes
>300	24	Consistent with (1) folate deficiency or (2) an anemia unrelated to vitamin deficiency	Yes

Test results on metabolites: Serum methylmalonic acid (MMA) and total homocysteine (tHcys)[d]		
MMA (normal = 70270 nM)	**tHcys (normal = 514 M)**	**Diagnosis**
Increased	Increased	Cbl deficiency is confirmed; folate deficiency still possible
Normal	Increased	Folate deficiency is likely; <5% may have Cbl deficiency
Normal	Normal	Cbl deficiency is excluded

[a] Both serum Cbl and folate tests are performed on the same specimen in one tube (cost for each of these tests in Indiana in 1997: $50). RBC folates (which are not recommended; see text) generate an independent charge of $120. If only serum folate levels are reported, call laboratory for the information they have on Cbl levels done at the same time the folate tests were generated.
[b] Serum Cbl levels: abnormally low <200 pg/ml; clinically relevant low-normal range 200300 pg/ml.
[c] Serum folate levels: abnormally low <2 ng/ml; clinically relevant low-normal range 24 ng/ml.
[d]The costs for testing serum MMA is $105 and for testing tHcys is $104. (Although the actual charges for both tests are $125 in one reference laboratory in Denver, the extra cost reflects a local mark-up for shipping and handling.) Any frozen-over sample from serum folate/Cbl determination can be subjected to metabolite tests.

at high risk for folate deficiency and who will also exhibit low serum folate levels when they become folate-deficient). [1] [224] Conversely, in 2550% of cases (predominantly alcoholics) with folate-deficient megaloblastosis, the serum folate levels may be low normal or borderline (i.e., 24 ng/ml by radioisotope dilution assay).[225] [226] [227] Thus, the serum folate level alone should never dictate therapy, and it is important to consider the clinical picture, peripheral smear and bone marrow morphology and to rule out underlying Cbl deficiency.

When negative folate balance is continued, hepatic folate stores are depleted in 4 months. [3] [4] This leads to tissue folate deficiency which was clinically correlated with a decrease in RBC folate (<150 ng/ml) by a microbiologic assay in the 1960s. [10] Thus, a reduction in serum and RBC folate in the setting of megaloblastic anemia was consistent with the diagnosis of folate deficiency. [1] [3] [4] However, RBC folate tests have major limitations in sensitivity and specificity [1] [227] [228] and are notoriously unreliable in alcoholics and in pregnancy; thus, it was normal or border- line (in the low normal range) in 30% of alcoholics [227] and 60% of pregnant patients[10] with clinically significant folate-deficient megaloblastic anemia. Furthermore, a reduction of RBC folate also occurs in 60% of patients with Cbl deficiency, since the trapped folate which cannot participate in the methionine synthase reaction due to Cbl deficiency (see Fig. 28-2) leaks out of the RBC, further limiting its diagnostic utility. [1] Consequently, the serum folate level is falsely low less often (<10%) with Cbl deficiency.

Other compelling evidence argues against the use of RBC folate in the diagnosis of folate deficiency. The original microbiologic assays for RBC folates have been replaced by radioassays currently used for measurement of *serum* folate levels. Apart from a lack of clinical validation of the use of *radio*assays for measuring RBC folates, these radioassays have had a long history of being unreliable (in several geographic areas) with respect to lack of precision and accuracy, generally leading to a lack of confidence in the results. [219] [225] [228] [229] [230] [231] Unlike the serum folate (which is entirely 5-methyl-tetrahydrofolate), RBC folates are a heterogeneous mixture of different coenzyme forms with varying polyglutamate chain lengths which (among other confounding variables) most likely contributes to these problems. In addition, a quality assurance study from Australia involving 134 laboratories identified nine operator controllable factors that could also lead to poor RBC folate results.[220] [221] In addition, a recent analysis of its clinical value led to the conclusion that the RBC folate test added little useful information to that provided by serum folate levels.[232] Together, these clinically relevant issues have considerably dampened enthusiasm for use of RBC folate tests, and have led several experts in the

United States to entirely avoid their use pending a resolution of problems related to reagent batch quality control, quality assurance, and equally important, provision of data on clinical validation by the various manufacturers of these kits. So for the present, based on information from several recent studies, a cost-conscious approach that primarily relies on measurement of the serum Cbl and serum folate levels as initial tests to define Cbl and/or folate deficiency has been developed (discussed later in this chapter; see also Table 28-2). [222]

Summary of the Clinical Usefulness of Tests for Cbl and Folate Deficiency

The sensitivity of the measurement of MMA and tHcys is best illustrated in patients with pernicious anemia who are on replacement therapy with Cbl. When these patients do not receive Cbl for some time, the levels of MMA and tHcys progressively increase much earlier than the drop in Cbl levels. [185]

An important study has conclusively demonstrated that overlooking true Cbl deficiency is highly unlikely when the two metabolites are measured, since one or both metabolites were increased in 99.8% of >400 patients with proven Cbl deficiency. [226] These data collectively indicate that these serum tests (MMA and tHcys) are the now the gold standard for the diagnosis of Cbl deficiency.

Based on the lower costs of serum Cbl and folate compared to serum MMA and tHcys, it is recommended (Table 28-2) to first use the cheaper tests that will assist in diagnosis of the majority of obvious cases of Cbl and folate deficiency. [222] [233] Clinicians should also restrict use of serum MMA and tHcys to patients (1) with borderline Cbl and folate levels; (2) with existing conditions known to perturb folate/Cbl tests leading to difficulties in the interpretation of test results (discussed earlier in this chapter); (3) when both Cbl and folate levels are low, where a high MMA is useful in confirming Cbl deficiency (rather than attributing the condition to folate deficiency alone); and finally, to patients (4) with clearly low serum levels but in whom there is an alternative explanation for the syndrome that led to obtaining a serum Cbl level (e.g., a diabetic or alcoholic with peripheral neuropathy, or an alcoholic with a high MCV and a low serum Cbl without anemia). Here, serum metabolites can assist in the diagnosis of vitamin deficiency.

Other Tests

(1) Alternative tests for Cbl deficiency that measure the percent saturation of TC II have been described. [234] However, there have been strong objections pointing out the imprecision of this test due to flaws in methodology [235] and another study has not been able to clinically validate this test. [236]

(2) Using a more convenient urinary MMA test, screening a total of 809 elderly individuals yielded a prevalence of up to 5.1% with elevated MMA levels (urinary MMA cost = $50). This study also verified earlier results that serum MMA levels are more sensitive than serum Cbl to diagnose Cbl deficiency. [237] However, inadequate clinical data is available (a) as to whether there is a greater sensitivity of measuring urinary MMA (greater convenience and low cost) versus serum MMA in patients with Cbl deficiency with wide ranges of serum MMA; (b) as to what extent renal dysfunction and dehydrationfactors that limit interpretation of serum MMA results [193] influence urinary MMA levels; (c) on the kinetics of reduction of urinary MMA to Cbl replacement (a reduction in serum MMA to Cbl treatment has been demonstrated). So what is urgently needed are prospective, blinded studies on patients with mild to severe Cbl deficiency (with or without folate deficiency) in whom both urine MMA and serum MMA levels are examined to determine the extent of concordance; (if they are the same, the current price and convenience would favor urinary MMA). However, cost-effectiveness studies comparing the relative merits of urine MMA over serum MMA tests must take into account the additional costs for determination of urine creatinine levels (to normalize urine MMA values), and this should be compared to the clinically significant advantages of being able to distinguish folate from Cbl deficiency (or combined folate and Cbl deficiency) using both serum tHcys and serum MMA tests (Table 28-2). Thus, it is somewhat premature at this time to endorse the urinary MMA test over serum MMA (and tHcys) tests.

(3) Although the deoxyuridine suppression test can diagnose folate or Cbl deficiency even earlier than metabolite tests, [200] it is unfortunately not widely available, and information is still insufficient to warrant its routine use in Cbl/folate deficiency.

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PATHOGENESIS OF COBALAMIN (Cbl) DEFICIENCY

Nutritional Cbl Deficiency: Insufficient Cbl Intake

Among 138 vegetarian Indians residing in the United Kingdom (1380 years of age) with megaloblastic hematopoiesis, 95 had

nutritional Cbl deficiency, some with early neuropsychiatric manifestations. [194] The sole Cbl source for these individuals who abstain from consuming meats, eggs, and cheese was boiled milk and yogurt. One-third had evidence of intestinal malabsorption with osteomalacia, associated iron deficiency (attributable to the low bioavailability of iron in these diets) and an inordinately high incidence of tuberculosis. In northwest India, three-fourths of 136 consecutive cases of nutritional megaloblastic anemia[238] had Cbl deficiency with 60% presenting in the second and third decade with pancytopenia, mild hepatomegaly (46%), mild splenomegaly (34%), fever (42%), thrombocytopenic bleeding (80%), and neutropenia (43%). The hemorrhagic diathesis encountered with thrombocytopenia has been reversed within 1224 hours of replacement with Cbl.[174] [238]

Food faddists may also develop nutritional Cbl deficiency [239] when they practice strict vegetarianism for health reasons. However, owing to an efficient enterohepatic circulation, Cbl deficiency normally develops very slowly (over >10 years). Because the staying power of fads is often of shorter duration, this complication is rarely seen. Long-term maintenance therapy requires oral 5 g aqueous hydroxo-Cbl or 50 g Cbl tablets; this approach allows for continued vegetarianism. The breast-fed offspring of strict vegans (or mothers with tropical sprue and subclinical Cbl deficiency) are also recognized as being at high risk for developing nutritional Cbl deficiency.[240]

Intragastric Events Leading to Cbl Malabsorption

Inadequate Dissociation of Cbl from Food Protein

Dietary Cbl is bioavailable only following proteolytic digestion of food by gastric acid and pepsin. Thus, failure to release Cbl from food protein can present as Cbl deficiency even though gastric analysis reveals the presence of IF. [12] [13] [241] Some older patients (>50 years of age) with atrophic gastritis or partial gastrectomy with hypochlorhydria have low serum Cbl levels and normal absorption of crystalline Cbl, but poor absorption of CN- ^{57} CoCbl that is incorporated into food as a food-Cbl absorption test.[14] [241] Some of these patients may progress to overt clinical manifestations of Cbl deficiency. They may also represent a cohort who are in an early stage in the natural history of pernicious anemia. Geriatric patients with low-normal Cbl levels, abnormal food-Cbl absorption tests, and increased metabolite levels consistent with Cbl deficiency have been encountered in 14.5% of patients in some populations. [242]

Congenital Intrinsic Factor Deficiency

Congenital IF deficiency, transmitted as an autosomal recessive trait and expressed in homozygotes by the age of 2 years, is characterized by a pure IF deficiency without other gastric abnormalities. Only 40+ cases have been described; affected children present with irritability, vomiting, diarrhea, and loss of weight with megaloblastic anemia. Except for the absence of IF in gastric juice, gastric histology/function are normal. [243]

Loss or Atrophy of Gastric Oxyntic Mucosa

IF deficiency arising from atrophy of gastric parietal (oxyntic) mucosal cells must be associated with parallel insufficient HCl secretion and can be caused by (1) total or partial gastrectomy; (2) autoimmune destruction as observed in adult Addisonian pernicious anemia (PA) or, rarely, in a similar disease in children (juvenile PA); and (3) destruction of gastric mucosa by caustic (lye) ingestion.

Total gastrectomy that results in removal of IF-producing cells invariably leads to Cbl deficiency in 5 years (range 210 years), often associated with iron deficiency (dimorphic anemia).[10] In fact, one-third of post-gastrectomy individuals have metabolic evidence for Cbl deficiency [244] warranting close follow-up with metabolite measurements and replacement. But another approach is to assume that all total-gastrectomy patients are at risk and therefore begin prophylaxis shortly after surgery.

Cbl deficiency is observed in only 1020% of patients 8 years after partial gastrectomy, although 30% have Cbl malabsorption; [10] up to 6% of these Cbl-deficient patients will develop frank clinical manifestations of Cbl deficiency with megaloblastic anemia. The etiology is multifactorialcontributing factors include decreased IF secretion, hypochlorhydria (from atrophy of residual oxyntic mucosal cells), intestinal bacterial overgrowth of Cbl-consuming organisms, associated nutritional folate deficiency in 20%, and iron deficiency in 50%. The degree of Cbl deficiency depends on the size of the remaining gastric remnant. It is more common in Bilroth II than in Bilroth I surgery, in subtotal than in partial gastrectomy, and in gastric rather than duodenal peptic ulcer disease. [245] Morbidly obese patients treated surgically with gastric bypass also have more food Cbl malabsorption than patients treated with vertical banded gastroplasty. [246]

Absent IF SecretionPernicious Anemia

The most common cause of Cbl malabsorption is pernicious anemia (PA), a disease of unknown origin in which the fundamental defect is atrophy of the gastric (parietal cell) oxyntic mucosa eventually leading to the absence of IF and HCl secretion. Because Cbl is only absorbed by binding to IF and uptake by ileal IF-Cbl receptors, the net consequence is severe Cbl malabsorption leading to Cbl deficiency.

The incidence of PA is 25 new cases/year/100,000 persons older than 40 years. Although the average age of onset is 60 years, [1] it is increasingly encountered in persons 5 to 10 years younger.[247] PA is not a respecter of age, race, or ethnic origin.[248] The predisposition to developing PA may have a genetic basis but neither the mode of inheritance nor the initiating events or primary mechanism is precisely understood. There is a positive family history in 30% of patients, among whom the risk of familial PA is 20 times as high as in the general population; 20% of siblings of patients are projected to develop PA by the age of 90 years. PA developing concordantly in identical twins has been documented. [249]

There is a significant association of PA with other autoimmune diseases [1] [10] (i.e., the polyglandular autoimmune syndrome characterized by multiple endocrine hypofunction with antibodies to endogenous endocrine glands). Thus, PA is associated with Graves disease (30%), Hashimotos thyroiditis (11%), vitiligo (8%), Addisons disease, idiopathic hypoparathyroidism, and adult hypogammaglobulinemia. The histologic appearance of the gastric mucosa (infiltration with plasma cells

and lymphocytes) is also strongly reminiscent of autoimmune-type lesions. There is also a high incidence of anti-parietal cell IgG antibodies in the serum of 90% of patients with PA.[250] These anti-parietal cell IgG antibodies are cytotoxic to canine gastric parietal cells in vitro. [251] When chronically injected into rats, they decrease gastric HCl, IF, and pepsin secretion and lead to gastric atrophy. [10] The major antigen to which antibodies from patients with autoimmune gastritis and PA are directed is the acid-producing enzyme H^+ , K^+ -ATPase (a 92-kDa protein), found on the membrane of parietal cells. [252] The precise interplay of the various mechanisms for anti-parietal cell IgG-mediated dysfunction of parietal cells remains to be shown in vivo. It is not clear whether the target antigen of anti-parietal cell IgG found in up to 8% of the normal 3060-year-old population, 20% of women over 60 years old, and 5060% of patients with simple atrophic gastritis, is also H^+ , K^+ -ATPase, or another antigen. Anti-IF antibodies are found in the serum of 60% of patients with PA and in the gastric juice of 75%; 90% of patients with

PA have anti-IF antibodies in either serum or gastric juice. [10] Similar IF antibodies are quite rare in the general population; anti-IF antibodies are thus highly specific and confirmatory for PA.

There are two types of IF-antibodies: Type I are directed to the Cbl-binding site on IF and are usually of IgG subclass in the serum. In gastric secretions they may be both IgG and secretory IgA subclass. Type II IF antibodies bind to the IF-Cbl complex and prevent binding to ileal IF-Cbl receptors. Type II antibodies, usually not seen in the absence of type I antibodies, are found in 35% of patients with PA, although studies using a technique that detects both antibodies indicates a higher prevalence than previously documented.[253] Because of their functional properties, these intraluminal anti-IF antibodies may hasten the development of PA and interfere with tests for Cbl absorption.[10] Patients with anti-IF antibodies also have a higher T_4/T_8 cell ratio than those without anti-IF antibodies or controls. The significance of this is not clear, nor is the reason why patients with PA have an increased frequency of antibodies in serum directed against antigens from thyroid acinar cells, lymphocytes, renal collecting duct cells, and altered cellular immunity. [10]

The corticosteroid-responsiveness in some patients with PA (i.e., regeneration of atrophic gastric mucosa and increased IF secretion antedating a decrease in anti-IF antibodies) also implicates an autoimmune pathogenesis of PA.[10] The clustering of parietal cell dysfunction among first-degree relatives of patients with PA (in inbred populations), the fact that some individuals (96% of black women with PA) have high titers of blocking anti-IF antibodies but lack anti-parietal cell antibodies, [248] [254] and the fact that neither antibody may be detected in patients with PA and acquired agammaglobulinemia all suggest that the pathogenesis of PA is heterogeneous. Juvenile PA presents in the second decade with severe Cbl deficiency in conjunction with many of the associated endocrinopathies and autoantibodies observed in adults;[10] why these patients present so much earlier with an apparently identical disease is not known. Taken together, these facts suggest that although there is a genetic predisposition to PA, the full expression of the disease, which appears to have an autoimmune basis, may be modified by acquired environmental influences.

A very significant study from southern California determined that undiagnosed PA is common among the free-living elderly (over 60 years of age). [255] Among 729 individuals, 17 subjects were found to have PA, usually with only minimal clinical manifestations of Cbl deficiencyi.e., 1.9% of the survey population had unrecognized and untreated PA. The prevalence was 2.7% in women and 1.4% in men; but 4.3% of the black women and 4.0% of the white women had PA. Thus, undiagnosed PA is common among the elderly, especially among black and white women. It is significant that this study *did not* include elderly patients with Cbl deficiency caused by other disorders, or the still unknown number of younger people with unrecognized PA and other causes of Cbl deficiency. [255] If these findings can be extrapolated, 800,000 elderly people in the United States have undiagnosed and untreated PA! These individuals are at possible risk for masked Cbl deficiency if exposed to foods fortified with excess folate (discussed later in this chapter); this is the basis for calls for substantial caution, and more research, before further increasing the existing levels of folate fortification.

Abnormal IF Molecules

In three siblings, a defective IF molecule was identified by age 2 years that was identical to IF in all but one respectit was markedly susceptible to acid and proteolytic enzyme (pepsin or trypsin) digestion resulting in defective Cbl absorption by ileal IF-Cbl receptors and megaloblastic anemia. [256] In another case, an abnormal IF molecule having a 60-fold lower binding affinity for ileal IF-Cbl receptors resulted in lower (but not absent) Cbl absorption, probably accounting for the delayed clinical presentation with Cbl deficiency at the age of 13 years. [10]

Abnormal Events in the Small Bowel Lumen: Impaired Transfer of Cbl from R Protein to Intrinsic Factor

Insufficient Pancreatic Protease

Because 30% of patients with severe pancreatic insufficiency fail to degrade R proteins, there is no transfer of food Cbl to IF and consequent Cbl malabsorption (R-Cbl does not bind ileal IF-Cbl receptors). R proteins are highly susceptible to proteolysis by even small quantities of pancreatic protease released in response to food. Thus, in partial insufficiency there is abnormal absorption of CN- ^{57} CoCbl on an empty stomach but normal absorption when the CN-^{57} CoCbl is given with food. Administration of pancreatic extract normalizes Cbl malabsorption in those with complete pancreatic insufficiency. [10]

Inactivation of Pancreatic Protease

Pancreatic protease can be inactivated by massive gastric hypersecretion arising from a gastrinoma in Zollinger-Ellison syndrome. [10] The continued low pH of the luminal contents reaching the ileum may also perturb interaction of the IF-Cbl complex with IF-Cbl receptors (which requires a pH above 5.4).

Usurpation of Luminal Cbl

The near sterile condition of the small bowel is maintained by a combination of the mechanical cleansing action of peristalsis and the chemical action of gastric acid. Disorders conducive to relative stasis, impaired motility, and hypogammaglobulinemia are predisposing factors favoring colonization by bacteria. Many of these bacteria take up free Cbl, whereas the uptake of IF-bound Cbl is markedly diminished. However, if colonization extends proximally to the locus at which IF and Cbl interact, significant Cbl may be usurped before it can bind to IF. [10] Possible absorption of inert Cbl analogues produced by these bacteria may allow competition with normal Cbl to produce an additional anti-Cbl effect. [257] This Cbl malabsorption can be corrected to some extent by a 710 day course of antibiotic therapy; definitive surgical correction is indicated if the patient has significant symptoms (weight loss and diarrhea) that are only partially relieved by antibiotics. The malabsorption of food Cbl in patients with atrophic gastritis has also been normalized with antibiotics, thereby incriminating bacterial usurpation of food Cbl at a very proximal level. [258]

Approximately 3% of individuals infested with the fish tapeworm *Diphyllobothrium latum* develop frank Cbl deficiency. In the life cycle of this parasite, tapeworm eggs passed in human feces embryonate in cool freshwater; the ciliated embryos are swallowed by cyclops (the first intermediate host). After burrowing into the body cavity of the cyclops, they mature into the procercoid larval stage. The hapless cyclops (infected with procercoids) are then swallowed by freshwater fish (e.g., pike, perch, trout, salmon found in lakes in Russia, Japan, Switzerland, Germany, and North America) and procercoids then migrate into the fishs flesh (second plerocercoid larval stage). Humans become infected when they eat partially cooked or raw fish or fish roe containing plerocercoids which develop into adult worms in 6 weeks, growing to an amazing length of 10 meters (!) with up to 4,000 proglottids; [10] when these worms lay eggs, the life cycle is repeated. In a given patient the degree of Cbl deficiency is probably related to the number and extent to which worms lodge proximal to ileal IF-Cbl receptors. They are commonly found in the jejunum; poised in this strategic location, they avidly usurp Cbl for growth. [10] In fact, worm extracts

are so rich in Cbl that they have been used successfully to replenish Cbl in Cbl-deficient states. [10] Once ova have been identified in the stools, expulsion of the worms by praziquantel (510 g/kg, single dose, orally) and Cbl replenishment is invariably curative. So the price tag for eating raw fish or fish roe may involve more than the immediate expense for an exotic gastronomic delight.

Disorders of Ileal Intrinsic Factor (IF)-Cbl Receptors or Mucosa

Absence of IF-Cbl Receptors

The distal ileum has the greatest density of IF-Cbl receptors, and removal of only 12 feet of terminal ileum via resection or bypass can reduce ileal IF-Cbl receptor numbers or interaction with IF-Cbl, respectively, to result in Cbl malabsorption. [10] [259] [260] Thus, ileal bypass or diseased ileal architecture may lead to reduction in transenterocytic transport of Cbl (and folate) which is treatable with parenteral Cbl replacement.

Defective IF-Cbl Receptors or Post-IF-Cbl Receptor Defects

A heterogeneous group of congenital disorders in children involving selective Cbl malabsorption leading to megaloblastic anemias associated with mild, persistent, benign, nonspecific proteinuria (in 90% of cases) is collectively known as Imerslund-Gräsbeck syndrome. [10] These patients are heterogeneous with respect to precise biochemical abnormalities involving Cbl malabsorption. In one case an abnormality in ileal IF-Cbl receptors was demonstrated, [10] and recently among four cases from France, reduced IF-Cbl receptor activity was reported. [261] Most other cases appear to involve a post-receptor defect so parenteral therapy with Cbl bypasses the defect and reverses Cbl deficiency. [262]

Drug-Induced Defects

H_2 antagonists, but not omeprazole, inhibit IF secretion. [23] [263] However, the first of potentially several cases of malabsorption of Cbl from long term omeprazole has been reported.[264] Biguanides (i.e., metformin) appear to decrease IF and acid secretion in healthy volunteers; they also probably inhibit transenterocytic transport of Cbl in 7%;[265] given the magnitude of diabetics on metformin, this could lead to a significant problem. Other drugs (e.g., Slow-K, cholestyramine, colchicine, neomycin) probably also impair trans-epithelial transport of Cbl, [18] [19] manifested by a decrease in serum Cbl levels with mild to insignificant Cbl deficiency; these drugs also commonly interfere with the Schilling test.

Disorders of Plasma Cbl Transport

Absence of Transcobalamin I and Transcobalamin III

Congenital R-protein deficiency is not associated with cellular Cbl deficiency. However, because 80% of serum Cbl is bound to TC I, the serum Cbl levels will invariably be low. Two of six patients in one study have developed a biochemically uncharacterized neurologic syndrome, which has led to speculation that TC III deficiency may result in inability to clear Cbl analogues that may have a pathogenic role in the neurologic dysfunction. [10]

Deficiency of Transcobalamin II

Megaloblastic anemia in infancy associated with normal Cbl levels is the characteristic clinical presentation when TC II is absent or markedly deficient. [266] (Cbl levels are normal due to the TC I-bound Cbl, but this Cbl is unavailable for physiologic cellular uptake.) The impairment in intestinal absorption of Cbl in such patients is also due to the absence of TC II, which plays an important role in binding Cbl within the enterocyte prior to its entry into the circulation. In all, 30 patients have been described.[262] [267] They can be successfully treated by exploiting the property of passive intracellular diffusion of Cbl by daily or biweekly injections of 1 mg Cbl, which ensures Cbl delivery into cells. Based on animal studies, circulatory antibodies to TC II-receptor can cause its functional inactivation, suppress Cbl uptake by multiple tissues, and thereby cause severe Cbl deficiency and a failure to thrive. [268]

Defective Transcobalamin II

Defective TC II, also presenting with Cbl deficiency, can be diagnosed when the amount of TC II measured by radioimmunoassay (RIA) is normal but there is a qualitative abnormality based on its inability to bind Cbl. [48] Conversely, in another patient with normal amounts of TC II by RIA, the TC II was functionally active in binding Cbl but unable to facilitate the uptake of Cbl into cells. [269] Now, the molecular basis for these abnormalities can be determined since the human TC II gene has been cloned.[270]

Disorders of Intracellular Cbl Utilization

Congenital Metabolic Defects (Inborn Errors) of Cbl Metabolism, Cbl Mutants AF

The combination of megaloblastic anemia with increased tHcys and/or MMA in serum and urine despite normal Cbl and folate levels should suggest an inborn error of Cbl metabolism.[54] [262] [267] [271] The inherited defects of Cbl utilization (Fig. 28-2) are heterogeneous and are empirically defined as Cbl mutations *A* through *G* (*cblAcblG*) through the use of complementation studies involving in vitro fusion of cultured mutant fibroblasts obtained from skin biopsies. Mutants involving the synthesis of Ado-Cbl within the mitochondria involve defects in the reduction of Cob(II)alamin to Cob(I)alamin (*cblA*) and of Cob(I)alamin to Ado-Cbl by Ado-Cbl transferase (*cblB*). These defects result in an accumulation of excess MMA. The clinical picture in infancy is dominated by acidosis and ketosis associated with lethargy, failure to thrive, vomiting, dehydration, respiratory distress, hepatomegaly, and coma. Biochemical evidence of methylmalonic acidemia, ketonemia, and ketonuria *without* megaloblastosis is found. Because some of these defects are incomplete, treatment with large doses of Cbl may alleviate the defect. [54] [262] [267] [271]

Cloning of methylmalonyl-CoA mutase cDNA from fibroblasts of a patient with *mut*- phenotype where residual enzyme activity is evident in cultured cells exposed to high concentrations of OH-Cbl has identified a single base substitution (Gly ^{717} Val) as the basis for this mutant at the molecular level; moreover, this confirms that Ado-Cbl binds to determinants in the C-terminal portion of the enzyme. [272] Defects involving conversion of Cob(III)alamin to Cob(II)alamin (*cblC, cblD*) present with combined evidence of reduced activity of methylmalonyl-CoA mutase and methionine synthase (i.e., with increased tHcys and MMA in blood and urine). [54] [262] [267] [271] These patients have prominent neuropsychiatric problems with mental retardation, microcephaly, psychosis, delirium, and retinopathy with megaloblastosis. *cblC* mutants have hemolytic uremic syndrome as an integral manifestation of the spectrum of this disorder. [273] Although the disorder usually presents in infancy, some patients manifest problems in later childhood. Hydroxo-Cbl [Cob(III)alamin] appears to be more effective than CN-Cbl in these patients. *cblE* (distinguished by the lack of increase in MMA) is thought to be a defect arising from the inability to maintain Cbl bound to methionine synthase in its reduced state. [274] [275] *cblF* is due to the inability of Cbl to be transported from the lysosome

into the cytoplasm;[276] this has been visualized by quantitative electron microscopic radiography. [277] Recent molecular studies on a *cblG* patient have demonstrated defects in methionine synthase that can result in reduced activity. [59] [278] Among patients with mutant *cblG* defects, one patient presenting at the age of 21 years with a multiple sclerosis-like syndrome was initially undiagnosed simply because the serum Cbl level and studies of Cbl absorption were normal. [186] *This suggests that adults presenting with cerebral, myelopathic, or neuropathic disturbances (occasionally masquerading as multiple sclerosis) should be screened for such defects using the dU suppression test or serum tHcys and MMA levels that may reflect the biochemical disorder.*

Acquired Disorder of Cbl Utilization: Nitrous Oxide (N_2O) Exposure

N_2O inactivates coenzyme forms of Cbl by oxidizing the fully reduced Cob(I)alamin to Cob(III)alamin; this results in a state of *functional* intracellular Cbl deficiency. This syndrome was first identified in patients with tetanus given N_2O for up to 6 days;[279] subsequently, patients exposed to N_2O for open heart surgery, and through chronic (surreptitious, accidental, or occupational) exposure, such individuals have been recognized as being at high risk for developing megaloblastosis and Cbl-deficient neuromyelopathy. [280] [281] [282] Although megaloblastosis develops within 24 hours and lasts <1 week after a single exposure, the neurologic syndrome is usually seen with chronic intermittent exposure. However, severe neurologic deficits have been reported after prolonged intra-operative exposure to N_2O in patients with unsuspected Cbl deficiency.[283] The integrity of the methionine synthase-catalyzed reaction can be tested by measuring serum tHcys even after 75 minutes of exposure during surgery.[284] Reduced enzyme activity explains the metabolic block, which can be bypassed by 5-formyl-H_4 PteGlu (leucovorin).

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CLINICAL EVALUATION OF CbI ABSORPTION

The Schilling Test

Nutritional deficiency and hereditary enzymatic defects of intracellular Cbl utilization account for a minority of cases of Cbl deficiency. Most patients with clinical Cbl deficiency have impaired Cbl absorption. If Cbl absorption is intact, CN- ^{57} CoCbl administered orally will first bind R-protein in the stomach. In the duodenum, the R-protein of the R-CN-^{57} CoCbl complex will be degraded by pancreatic protease resulting in rapid transfer of released CN- ^{57} CoCbl to the patients own IF. The CN-^{57} CoCbl-IF complex will travel down to the ileum where it interacts with specific IF-Cbl receptors. Once taken up by the enterocyte, CN- ^{57} CoCbl will be transported into the portal blood while bound to TC II. If the blood contains an excess of Cbl (from a flushing injection of exogenously administered Cbl), >8% of CN- ^{57} CoCbl will be excreted in the urine within 24 hours (*Stage I test*). If there is a decrease in endogenous IF, as in pernicious anemia (PA), <8% will ultimately be excreted; however, if IF is given exogenously with the CN-^{57} CoCbl, this abnormality will be corrected [1] (*Stage II test*). In situations in which there is usurping of CN- ^{57} CoCbl by bacterial overgrowth, resulting in decreased absorption of CN- ^{57} CoCbl plus IF, prior therapy with antibiotics for 710 days will correct Cbl malabsorption [1] [257] [258] (*Stage III test*); however, antibiotics will not correct Cbl malabsorption caused by fish tapeworm infestation or defects involving net deficiency of IF-Cbl receptors, e.g., ileal resection, fistulas, or diseases of the ileal mucosa (including TC II deficiency). (See Fig. 28-4 and Table 28-3.)

In pancreatic protease deficiency, CN-^{57} CoCbl remains bound to R protein and will not be transferred to IF, resulting in its diminished absorption and overall urinary excretion,[15] [17] an effect that is correctable by simultaneous administration of Pancrease (or Creon), or by carrying out the test while the patients pancreas is optimally stimulated with a meal or secretin. In the rare syndrome in which IF is unusually susceptible to degradation by acid and pepsin, the abnormal Schilling test can be partially corrected by administration of sodium bicarbonate. [256] Patients with hypochlorhydria and inability to proteolytically release food Cbl (with consequent low serum Cbl with or without clinical manifestations of Cbl deficiency) have a normal Stage I Schilling test since they have no problem with absorbing *crystalline* CN-^{57} CoCbl. The appropriate test to order under these circumstances is the food-CN- ^{57} CoCbl absorption test, which determines the patients capacity to release CN- ^{57} CoCbl bound to food (e.g., egg or chicken serum protein) and thereby identify malabsorption of food-bound CN- ^{57} CoCbl.[12] [13] [14] [241]

With rare exceptions, adults with Cbl deficiency have either Cbl malabsorption (from PA, food-Cbl malabsorption, infestation with *D. latum*, bacterial overgrowth, or ileal malabsorption), or in vegetarians, dietary Cbl insufficiency (see box on Etiopathophysiologic Classification of Cbl Deficiency). All of these conditions can be treated similarly with either monthly parenteral Cbl or daily oral Cbl (discussed later in this chapter). Because information on the cause and locus of Cbl deficiency does not appear to alter or direct therapy with Cbl, does the identification of the locus of Cbl malabsorption make any difference to the outcome in adults (i.e., should a Schilling test be done at all)? Two compelling arguments in favor of the Schilling test are: (1) The test results can suggest additional diagnostic tests (intestinal biopsy, examination of stool for malabsorption or

TABLE 28-3 -- Results of Schilling Tests

Condition	Material Administered				
	CN-[^{57} Co]Cbl Plus H_2O	CN-[^{57} Co]Cbl Plus IF	CN-[^{57} Co]Cbl After 710 Days of Antibiotics	CN-[^{57} Co]Cbl Plus Pancreatic Extract	CN-[^{57} Co]Cbl-Food Cbl Absorption Test
	Stage I Test	Stage II Test	Stage III Test		
Normal	N[a]				
Lack of IF	Low[a]	N			
Usurping of Cbl by bacteria	Low	Low	N		
Pancreatic insufficiency	Low	Low	Low	N	
Lack/bypass of ileal IF-Cbl receptors/defective transenterocytic Cbl transport	Low	Low	Low	Low	
Inadequate dissociation of food Cbl	N				Low

[a]N, results indicate normal absorption; low, results indicate less than normal absorption.

ETIOPATHOPHYSIOLOGIC CLASSIFICATION OF COBALAMIN DEFICIENCY

I. Nutritional Cbl Deficiency (insufficient Cbl intake) Vegetarians, vegans, breast-fed infants of mothers with pernicious anemia
II. Abnormal Epigastric Events (inadequate proteolysis of food Cbl)
Atrophic gastritis, partial gastritis with hypochlorhydria, omeprazole, H_2 -blockers
III. Loss/Atrophy of Gastric Oxyntic Mucosa (deficient IF molecules)
Total or partial gastrectomy, pernicious anemia, caustic destruction (lye)
IV. Abnormal Events in Small Bowel Lumen
  A. Inadequate pancreatic protease (R-Cbl not degraded, Cbl not transferred to IF)
    1. Insufficient pancreatic proteasepancreatic insufficiency
    2. Inactivation of pancreatic proteaseZollinger-Ellison syndrome
  B. Usurping of luminal Cbl (inadequate Cbl binding to IF)
    1. By bacteriastasis syndromes (blind loops, pouches of diverticulosis, strictures, fistulas, anastomosis), impaired bowel motility (scleroderma, pseudo-obstruction), hypogammaglobulinemia
    2. By *Diphyllobothrium latum* (fish tapeworm)
V. Disorders of Ileal Mucosa/IF-Cbl Receptors (IF-Cbl not bound to IF-Cbl receptors)
  A. Diminished or absent IF-Cbl receptorsileal bypass/resection/fistula
  B. Abnormal mucosal architecture/functiontropical/nontropical sprue, Crohns disease, TB ileitis, infiltration by lymphomas, amyloidosis
  C. IF-/post IF-Cbl receptor defectsImerslund-Gräsbeck syndrome, TC II deficiency
  D. Drug-induced effects (Slow-K, biguanides [metformin], cholestyramine, colchicine, neomycin)
VI. Disorders of Plasma Cbl Transport (TC II-Cbl not delivered to TC II receptors)
Congenital TC II deficiency, defective binding of TC II-Cbl to TC II receptors (rare)
VII. Metabolic Disorders (Cbl not utilized by cell)
  A. Inborn enzyme errors (rare)
  B. Acquired disorders: (Cbl functionally inactivated by irreversible oxidation)N_2 O inhalation

D. latum infestation) and specific therapy (gluten-free diet, folate, antibiotics, or antihelminthics), and (2) this in turn identifies the potential for reversibility and thereby dictates the duration of Cbl replacement.

The most common cause of an abnormal Schilling test[10] [212] is incomplete collection of urine. Patients with renal impairment will also excrete less than normal amounts of radioactive Cbl. Although this potential pitfall can be avoided by sampling plasma after administration of CN- ^{57} CoCbl, there is significant overlap of results between patients with true PA and normal individuals; and ideally this requires the use of equipment for whole body counting which is not widely available.

The flushing dose of unlabeled Cbl administered during the Schilling test will invalidate further testing of serum Cbl and initiate a hematologic response in Cbl-deficient patients. Technical difficulties (resulting in diagnosis of Cbl malabsorption in the face of normal Cbl status) include (1) renal failure; (2) incomplete urine collection; (3) failure to give either the test or flushing dose; (4) use of decomposed CN- ^{57} CoCbl or inert IF; and (5) simultaneous ingestion of drugs that impair Cbl absorption. False normal values in the presence of true Cbl deficiency can arise from contamination of urine with stool containing unabsorbed CN- ^{57} CoCbl and the presence of another isotope used in diagnostic tests (^{99m} Tc or ^{67} Ga). It is thus crucial to be alert to the timing of the Schilling test relative to other nuclear imaging studies. So with prior administration of ^{99m} technetium-based radiopharmaceuticals and ^{123} NaI wait 1 week; with ^{67} gallium-citrate and various ^{111} indium compounds,^{131} I-MIBG, and ^{201} thallous chloride wait 6 weeks, but with^{131} NaI, depending on residual thyroid uptake, wait 4 months. [285]

Furthermore, it should be re-emphasized that because Cbl/folate deficiency causes megaloblastosis of intestinal cells, the Stage I Schilling test (with CN- ^{57} CoCbl alone) may be abnormal in folate deficiency; or, a patient with IF deficiency (e.g., PA) may be diagnosed as having an abnormal Stage II test from an intestinal cause.[10] This phenomenon occurs often enough (2575% of cases) to warrant a repeat Stage II Schilling test in patients diagnosed as having Cbl malabsorption (from an intestinal cause) after 2 months of Cbl replacement; only patients with PA will correct in the Stage II test (Table 28-3).

Gastric Analysis

A significant titer of IF antibodies of the blocking type in gastric juice will lead to an abnormal Stage II Schilling test. This is overcome by using an augmented Stage II test with a higher dose of IF. Moreover, a clue as to whether these patients have gastric IF antibodies will be found in the serum, which may contain IF antibodies. Gastric analysis for achlorhydria in adults is valuable only in the single situation in which the presence of gastric acidity in response to maximal stimulation (e.g., Histalog) helps exclude the diagnosis of PA. In children, however, gastric analysis for IF and acid can differentiate between congenital IF deficiency (acid present), juvenile PA (acid and IF absent), and Imerslund-Gräsbeck syndrome (acid and IF present).

The assay for anti-IF blocking antibodies depends on the displacement of CN- ^{57} CoCbl binding to IF in vitro. This can be significantly affected by prior injection of Cbl (as given with a Schilling test) or by radioactive isotopes from other diagnostic tests (see discussion earlier in this chapter).

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PATHOGENESIS OF FOLATE DEFICIENCY

Folate deficiency is usually recognized in the course of certain clinical presentations that predispose to negative folate balance and subsequent deficiency. It is instructive, therefore, to conceptualize cellular folate deficiency as arising from etiologic categories of *decreased supply* (reduced intake/absorption/transport/utilization), or *increased requirement* (metabolic consumption/destruction/excretion). However, in the same patient more than one mechanism may result in net folate deficiency. The precise contribution of one mechanism over the other is often not obvious. Specific tests to define each mechanism are not routinely available for clinical use.

Manifestations of folate deficiency may be hematologic (pancytopenia with megaloblastic marrow), cardiopulmonary, gastrointestinal (megaloblastosis with or without malabsorption), dermatologic (skin pigmentation), genital (megaloblastosis of cervical epithelium), infertility (sterility), and psychiatric (Table 28-4). These manifestations are discussed within the context of the history and physical examination. Cases of neuropathy attributed to folate deficiency are rarely encountered; when they are, the possibility of alcoholism with thiamine deficiency must be considered. In any case, every patient with neuropathy, myelopathy, or psychiatric manifestations associated with megaloblastosis

TABLE 28-4 -- Megaloblastic Sequelae of Folate and Cbl Deficiency and Several Clinical Manifestations Are the Same[a] (***However, the neurologic spectrum of dysfunction with Cbl deficiency is distinct***)**

Hematologic	Pancytopenia with megaloblastic marrow
Cardiopulmonary	Congestive heart failure
Gastrointestinal	Beefy-red tongue and added stigmata of broad spectrum malabsorption in folate deficiency[b]
Dermatologic	Melanin pigmentation and premature graying
Genital	Cervical/uterine dysplasia
Reproductive	Infertility/sterility
Psychiatric	Depressed affect/cognitive dysfunction
Neuropsychiatric[c]	Unique to Cbl deficiency with cerebral, myelopathic, or peripheral neuropathic disturbances including optic and autonomic nerve dysfunction

[a]Inadequate hemoglobinization (from inadequate iron stores and/or globin synthesis) will mask the expected erythroid megaloblastic morphology in the bone marrow and peripheral smear, and only specific therapy (e.g., iron) will unmask classic megaloblastic manifestations (*masked megaloblastosis*). Megaloblastic leukopoiesis will be unchanged.
[b]If folate deficiency is uncorrected for >23 years, Cbl deficiency will supervene.
[c]Dorsal tract involvement is earliest manifestation in >70% patients with Cbl deficiency. Neuropsychiatric manifestations are unassociated with megaloblastosis in up to 30% of patients.

must be investigated in detail to rule out Cbl deficiency. It must be remembered that gastrointestinal megaloblastosis begets further folate malabsorption, which propagates a vicious cycle of folate deficiency in the short term and Cbl deficiency in the long term. With the exception of drug-induced defects or inborn errors of folate metabolism that result in decreased utilization of intracellular folates, all etiologies, irrespective of mechanism, result in reduced net delivery of folates to normal proliferating cells. So in pursuing the causes of folate deficiency in a given patient, efforts should be directed toward obtaining positive evidence for all possible conditions predisposing to negative folate balance and deficiency.

Nutritional Causes of Folate Deficiency

Decreased Intake and/or Increased Requirements

The body stores of folate are adequate for only 4 months. [1] [3] [4] [286] The requirement for folate is 100 g/day in adults and 50 g/day in children, which is 5 times the adult requirement on a weight-for-weight basis. Folate stores are probably depleted much earlier in individuals who are chronically in negative folate balance or who have additional conditions that can tip them into true folate deficiency. The incidence of folate deficiency in the setting of general malnutrition in developing countries is very high and is invariably a problem of multiple vitamin deficiencies when associated with protein-calorie malnutrition (38,000 children die *every day* of hunger and starvation-related illness). [287] Decreased availability of folate-rich foods (in winter, after natural disasters, or the wet season in central Africa), poverty, various cultural/ethnic diets (consisting of maize, rice or well-cooked beans and vegetables), and cooking techniques that destroy food folate, coupled with the anorexia that accompanies chronic illnesses, are just a few of the reasons for rapid development of folate deficiency. [1] [3] [4] [65] [288] [289]

The rapidly proliferating tissues in children have an absolute requirement for exogenously supplied folate. Although human or cows milk is barely adequate to maintain folate balance in breast-fed infants, superimposition of associated illnesses that lead to anorexia or folate malabsorption readily shifts them into negative folate balance. Infants fed powdered milk formulas, goats milk (only 6 g/L folate), or milk that has been boiled (over 50% of folate may be destroyed) are at high risk in this regard, as are those on restricted formulas for phenylketonuria and maple syrup urine disease. In Western countries food faddism, alcoholism, and slimming diets usually lead to decreased folate intake in young to middle-aged individuals. [1] [10] Although beer has a higher folate content than other alcoholic beverages, alcoholism may lead to neglect of healthy dietary practices in favor of the high calories and high of alcohol. The edentulous, infirm, or neglected elderly who are too ill to prepare their meals, as well as psychiatric patients, are particularly at risk for nutritional folate deficiency. [147]

Pregnancy, Infancy, and Human Development

Except for malnutrition in children, pregnancy with poor folate intake is the most common cause of megaloblastic anemia in the world. Pregnancy and lactation are associated with significantly higher folate requirements (300400 g/day) for growth of the fetus, placenta, breast, and other maternal tissues; recent studies have identified increased catabolism of folate during pregnancy as yet another cause predisposing to deficiency. [290] This demand for folate must be met by adequate dietary intake. The placenta has a large number of folate receptors (FR), [103] [291] which facilitate binding and transport of folates to the developing fetus (see discussion later in this chapter). In fact, preferential delivery of folate to the fetus can cause or aggravate folate deficiency in the mother. [10] [147] This is observed clinically when a mother with severe folate deficiency gives birth to a baby who has normal folate stores. Although this invariably recalls the metaphor of the fetus as a parasite, [10] it is

also a vivid physiologically relevant experiment of nature that indicates the existence of a clearly defined mechanism to protect the fetus from the consequences of folate deficiency during critical stages in its growth and development. Folate deficiency in the pregnant mother can nevertheless lead to decreased placental weight and premature, low birth weight infants.[10] In this context, it is significant that (folate-responsive)-hyperhomocysteinemia is now recognized as a risk factor for women with unexplained recurrent early pregnancy loss,[292] [293] as well as in placental abruption or infarction;[294] thus the very significant protective role of folates for the fetus.

How do folates traverse the placenta from mother to fetus? Prior studies have determined that although transplacental folate transport involves a system that is concentrative and resistant to decreased maternal folate, it is sensitive to maternal folate loads such that, when presented with higher than normal folate levels, transport to the fetus is enhanced. Recently, an ex vivo human placental cotyledon perfusion model demonstrated that under physiologic conditions, placental FR had a major functional role in transplacental folate transport.[86] These studies (Fig. 28-8) indicate that net maternal-to-fetal folate transfer is a process consisting of two steps: First, is the concentrative component in which circulating 5-methyl-tetrahydrofolate (5-methyl-H_4 PteGlu) is bound to (i.e., captured by) placental FR on the maternally facing chorionic surface. Although kinetics favor binding, a dynamic state exists wherein a gradual release of 5-methyl-H_4 PteGlu from this pool adds to incoming circulating folates to generate an intervillous blood level 3 times that in the maternal blood. In the second step, folates are transferred to the fetal circulation along a downhill concentration gradient. Thus, it turns out that the prodigious, reversible, high-affinity binding of maternal folates by placental FR [103] [291] is *the* key modulator of transplacental folate transport. In fact, once captured, placental FR-bound folates are predestined for transplacental folate transport, since incoming (dietary) folates displace placental FR-bound folates which then passively diffuse down a concentration gradient to the fetus. And so this elegant cycle goes on, ensuring continued unidirectional transplacental folate transport (Fig. 28-8).

The incidence of megaloblastic marrows in the United States, Canada, and the United Kingdom during late pregnancy is

Figure 28-8 Diagrammatic representation of maternal-to-fetal transplacental folate transport involving placental folate receptors (PFR). *(Adapted from Henderson et al.,[86] with permission. Copyright 1990 by Annual Reviews.)*

25% but in southern India it is 55%.[10] Folate deficiency is eight times as high in twin pregnancies. Multiparity (multiple frequent pregnancies with a prolonged state of negative folate balance) and hyperemesis gravidarum commonly lead to folate deficiency. Because the anemia of pregnancy is most frequently caused by iron deficiency, combined iron and folate deficiency (dimorphic anemia) is the more frequent clinical presentation. Increased utilization of folates in newborns leads to a drop in folate levels by 6 weeks. This drop is exaggerated in premature infants, who because of feeding difficulties, infection, or hemolytic disease often develop pure folate deficiency.[1] [65] [147]

Unambiguous evidence now shows that *periconceptional* folate supplementation (800 g/day) for *normal women* reduces the incidence of neural tube defects (spina bifida, meningocoele, anencephaly).[295] The recognition that folate consumption is not optimal in adolescent girls [296] is a matter for concern given the importance of optimizing folate status in women of childbearing age to prevent neural tube defects. The Food and Drug Administration (FDA) has recommended food fortification with folic acid to certain foods to ensure adequate folate intake in order to prevent folate-responsive neural tube defects (discussed later in this chapter). In addition, periconceptional folic acid in higher doses (4 mg per day) also protects 75% of fetuses of women at risk (defined as women who have previously given birth to a child with neural tube defects) who have a 10-fold increased risk for delivering a subsequent child with the same disorder. [297] [298] In addition, unconfirmed data also suggest that folic acid administered periconceptionally in high doses (10 mg/day) may reduce the incidence of cleft lip. [299] [300]

The issue of folate supplementation has been extended to the animal sciences where the dramatic increase in conception rate, live births, and overall reproductive performance achieved[301] [302] verifies many of the clinical observations discussed earlier in this chapter.

From studies on periconceptional folate supplementation, folate is clearly necessary for the developing fetal nervous system. In addition, in newborns with congenital folate malabsorption syndrome (where a concomitant reduction of folate transport into the nervous system is accompanied by mental retardation, cerebral calcifications, seizures, and peripheral neuropathy), several of these complications can be avoided with early recognition and therapy with parenteral folate alone. [10] [225] [303] Interestingly, folate-deficient weanling mice tend to discard and spill more food than controls! [304]

The mechanism whereby periconceptional folate intake reduces both the *occurrence and recurrence* risk of neural tube defects is incompletely understood. A genetic basis for elevation of plasma tHcys levels has been identified in some families with spina bifida offspring involving a common 677CT mutation in the 5,10-methylene-tetrahydrofolate reductase gene, which causes thermolability of the enzyme (thermolabile MTHFR). There was a significantly higher prevalance of a ++ genotype among spina bifida patients and their mothers with the strongest risk if both mother and child were homozygous for the mutation. [304] [306] However, this genetic basis accounts for only 15% of all cases of spina bifida. Of related significance, most subjects with intermediate hyperhomocysteinemia (i.e., >40 M; nearly 3 times more than normal limits) in the general population have the C677T mutation combined with low folate status, and a daily supplement of low-dose folic acid will reduce and often normalize their tHcys level. [307] [308]

Intrinsic Hematologic Disease

Because folate is necessary for hematopoiesis, folate requirements are increased when there is (1) significant compensatory erythropoiesis in response to peripheral red blood cell destruction, (2) abnormal hematopoiesis, or (3) infiltration by abnormal cells in marrow. The recognition that folate deficiency developing in hemolytic disorders can lead to an acute aplastic crisis has led to routine prophylactic administration of folate. An unexpected increase in transfusional requirement or a fall in platelets should also suggest folate deficiency.[1] [65] [147]

Folate Malabsorption with Normal Intestinal Mucosa

Inhibition of brush border pteroylpolyglutamate hydrolase has been suggested but not documented as a mechanism whereby some drugs cause folate deficiency. Further functional studies following purification of the enzyme,[66] which is apparently functionally active,[309] should clarify the issue.

Congenital Folate Malabsorption

Patients with this disorder (usually the progeny of consanguineous marriages) present in the first 3 months with failure to thrive, diarrhea, sore mouth, megaloblastic anemia with low serum-, RBC-, and cerebrospinal fluid folates, normal Cbl levels, progressive mental retardation, seizures, cerebral calcification, athetosis, and ataxia. They do not respond to oral folate or 5-formyl-H_4 PteGlu (5 mg) because of specific intestinal folate malabsorption as well as defective folate transport into the central nervous system.[310] [311] Parenteral therapy in high concentrations

is necessary to ensure passive folate transport. These experiments of nature are the best evidence for (1) a specific, carrier-mediated transport system for folates in the gut and central nervous system, and (2) the occurrence in vivo of passive folate transport at high extracellular folate concentrations. Although the details of the former are incompletely understood, the latter has been experimentally demonstrated in vitro,[148] and in vivo during high-dose antifolate protocols.[149] Furthermore, the mental retardation and peripheral neuropathy in some of these individuals suggest a role of folates in maintenance of normal functioning of the nervous system. To establish the diagnosis, it is necessary to document that the patient has normal gastrointestinal absorption of other nutrients, intact pancreatic function, and normal mucosa on small intestinal biopsy. The differential diagnosis includes congenital IF deficiency, Imerslund-Gräsbeck syndrome, TC II deficiency, and inborn errors of Cbl metabolism.

Folate Malabsorption with Intestinal Mucosal Abnormalities

Tropical Sprue

Residents of, and visitors to, endemic areas in the tropics can acquire a disorder of unknown etiology characterized by small intestinal malabsorption. [10] [147] [312] [313] Generalized, nonspecific small bowel malabsorption leads to a wide spectrum of clinical manifestations arising from defective absorption of fat, carbohydrate, albumin, calcium, folate, and in later stages, Cbl.[10] The endemic nature of this disorder in the tropics, (and in certain households), [10] and the beneficial response to antibiotics [1] all suggest an infectious etiology. However, the dramatic response to folate, which is curative in the first year in 60% of cases (this cure is cited to be almost diagnostic of the disease) has not been explained. [1] [10] [147] It is unlikely that pure folate deficiency is the primary cause, since nutritional folate deficiency does not result in tropical sprue; the clinical response to antibiotics suggests a close interplay between a pathogenic infectious agent, endogenous flora, and the folate status of the enterocyte.

There is a varying degree of villous atrophy and loss of intestinal functional surface. Although less severe than in nontropical sprue, it is more extensive, involving the entire small intestine. There is abrupt onset of explosive, intermittent, or continuous diarrhea, abdominal distension, and pain associated with anorexia, vomiting, and extreme fatigue. Stools are fluid or semisolid and frequently contain mucus and blood. This stage is followed weeks to months later by nutrient deficiency. Later, as steatorrhea continues, megaloblastosis dominates the clinical picture. In the short term malabsorption leads to folate deficiency, but later in the chronic (longer than 3-year) phase of the disease, Cbl malabsorption contributes additional clinical manifestations of Cbl neuropathy. [1] [10] [147]

Coexisting iron deficiency (common in these areas) leads to a dimorphic blood picture; pellagra and beri-beri may also coexist in these patients. Treatment of the chronic phase with folate will cure the hematologic manifestations but exacerbate Cbl-deficient neurologic disease. In Southern India, among 64 patients with megaloblastic anemia from tropical sprue, 21% were due to Cbl deficiency, 33% to folate deficiency alone and 44% to combined Cbl and folate deficiency. [1] [10] [147] After investigations for associated iron, Cbl, and folate deficiency, therapy with folate and a broad-spectrum antibiotic is indicated together with symptomatic treatment of diarrhea, vomiting, fluid, mineral and electrolyte imbalance, and associated additional nutritional deficiencies. [10]

Nontropical Sprue (Celiac Disease, Gluten-Induced Enteropathy)

Nontropical sprue is the most common cause of intestinal malabsorption in temperate zones. It results from a possibly inherited sensitivity to gluten (a glutamine-rich protein found in wheat and other grains) and a related substance, gliadin. [10] The precise mechanism for induction of sensitivity is not known. The intestinal lesion (villous atrophy with hypertrophied crypts and lymphocytic and plasma cell infiltrate of the lamina propria), is more florid than that seen in tropical sprue but occurs to a greater extent in the proximal small intestine with relative ileal sparing; as a result, Cbl malabsorption is less common. The consequences of malabsorption are otherwise the same. Patients present between the ages of 3050 years with intermittent or persistent diarrhea (abrupt in 20%), weight loss, abdominal distension with discomfort, glossitis, and megaloblastic anemia. Diagnosis is established by demonstration of anti-gliadin and anti-endomysial antibodies (*gt;90% specificity), malabsorption, the characteristic radiographic moulage sign, and jejunal biopsy. The symptoms are exacerbated following a challenge with gluten and institution of a gluten-free diet not only is curative but also decreases the risk of subsequent malignancy (small intestinal lymphoma or gastrointestinal carcinoma, especially esophageal). Evidence of iron deficiency may be prominent in children. The megaloblastosis responds well to folate therapy. [1] [10] [147]

Regional Enteritis and Other Small Intestinal Disorders

The distal small intestine is involved in 80% of individuals with Crohns disease, but folate is efficiently absorbed in other more proximal areas. Thus, only with extensive involvement or

ETIOPATHOPHYSIOLOGIC CLASSIFICATION OF FOLATE DEFICIENCY

I. Nutritional Causes
   A. Decreased dietary intake Poverty and famine, institutionalized individuals (psychiatric/nursing homes)/chronic debilitating disease/goats milk (low in folate), cultural/ethnic cooking techniques (food folate destroyed), or special slimming diets or food fads (folate-rich foods not consumed)
   B. Decreased diet and increased requirements
      1. Physiologic: Pregnancy and lactation, prematurity, hyperemesis gravidarum, infancy
      2. Pathologic: *Intrinsic hematologic diseases* involving hemolysis with compensatory erythropoiesis, abnormal hematopoiesis, or bone marrow infiltration with malignant disease *Dermatologic disease*psoriasis
II. Folate Malabsorbtion
   A. With normal intestinal mucosa
      1. Some drugs (controversial)
      2. Congenital folate malabsorption (rare)
   B. With mucosal abnormalities: tropical and nontropical sprue, regional enteritis
III. Defective Cellular Folate Uptake Familial aplastic anemia (rare)
IV. Inadequate Cellular Utilization Folate antagonists (methotrexate) Hereditary enzyme deficiencies involving folate
V. Drugs (multiple effects on folate metabolism) Alcohol, sulfasalazine, triamterine, pyrimethamine, trimethoprim-sulfamethoxazole, diphenylhydantoin, barbiturates
VI. Acute Folate Deficiency (intensive care unit setting, uncertain etiology)

fistulas do these patients develop folate deficiency. Even in this case, the blood picture is more that of an iron deficiency or anemia of chronic disease. Frank, pure megaloblastic anemia occurs rarely enough in this setting to suggest another etiology for folate or Cbl malabsorption. [1] [147] HIV infection results in an enteropathy, in the absence of opportunistic infection, which leads to the malabsorption of folates. [314]

Defective Cellular Folate Uptake (Familial Aplastic Anemia)

A very rare syndrome of familial aplastic anemia thought to result from defective cellular folate uptake is presumed to reflect a defect in the physiologic membrane transporter for folates.[315] Although the precise biochemical defect is uncertain, patients with this syndrome only respond to suprapharmacologic doses of folates (which presumably enter cells by passive diffusion or via the RFC). The disorder presents in young adulthood with pancytopenia, megaloblastosis, and an aplastic (or myelodysplastic) marrow.[316] The diagnosis should be considered when serum Cbl and folate are normal and the red blood cell folate (by microbiologic assay) is low and Cbl metabolic defects have been excluded.

Inborn Errors of Folate Metabolism

Knowledge of the intracellular metabolic pathways involving folates can help predict the net effect of deficiency of a single enzyme (Fig. 28-7). Substrate buildup or product deficiency leads to the clinical manifestations. The precise biochemical defects can be proved by specific enzyme assays of the patients fibroblasts.

Deficiency of 5,10-methylene-tetrahydrofolate reductase (MTHFR) is the most frequent of these rare disorders and presents with excess tHcys in the serum and urine and with hypomethioninemia. The disease is recognized in infancy because of developmental delay/mental retardation, with motor abnormalities and disturbance in gait. It has, however, occasionally remained undiagnosed until the teenage years. Serum folates are normal or low, but Cbl is normal. There is no megaloblastosis, TC II levels are normal, and there is often a poor response to PteGlu. Despite a variety of treatments administered, no consistent pattern of response has emerged (which may reflect significant genetic heterogeneity within this disorder). [329] Excellent reviews of these and other inborn errors of folate metabolism are available. [262] [267]

Acute Folate Deficiency

The cause of acute folate deficiency in some patients in intensive care units is not known. Clinically the presentation is acute megaloblastic arrest of hematopoiesis with thrombocytopenia. These patients are often acutely ill and in subclinical negative folate balance. The combination of additional insults (decreased intake, total parenteral nutrition containing ethanol, dialysis, surgery, sepsis, drugs) appears to provoke frank folate deficiency. The serum folate, remarkably enough, is often normal in the face of megaloblastosis in the bone marrow without obvious peripheral blood abnormalities. The dU suppression test has documented intracellular folate deficiency, but there is no data on MMA and tHcys levels in this condition. Empiric therapy with 5-formyl-H_4 PteGlu (leucovorin) is recommended. Exposure to N_2O [279] should also be considered in the differential diagnosis. [147] [225]

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MEGALOBLASTIC ANEMIA NOT DUE TO FOLATE OR COBALAMIN DEFICIENCY

In Western countries, chemotherapeutic agent-induced megaloblastic anemias may be more common than those caused by folate or Cbl deficiency. Several chemotherapeutic agents (antimetabolites, alkylating agents, etc.) kill malignant cells primarily by interfering with DNA synthesis; megaloblastosis is therefore an expected side effect. Hereditary orotic aciduria usually presents in the first year of life, due to deficiency or absence of enzymes that convert orotic acid to uridine monophosphate via orotidine monophosphate. The net cellular deficiency of uridine monophosphate leads to perturbed synthesis of DNA as well as RNA. [10] Thiamine deficiency in the DIDMOAD syndrome (*d*iabetes *i*nsipidus, *d*iabetes *m*ellitus, *m*egaloblastosis, *o*ptic *a*trophy, and sensorineural *d*eafness) due to an inability to transport physiologic thiamine is another rare cause for megaloblastosis in childhood. [330]

CLINICAL PRESENTATIONS AND EVALUATION FOR FOLATE AND Cbl DEFICIENCY

Clinical presentations and evaluations for folate and Cbl deficiency are shown in Table 28-4 .

Interview

The patients general demeanor and answers to questions may reveal a blunted affect with evidence of depression, irritability, forgetfulness, and sleep deprivation (common in pure folate deficiency). Alternatively, Cbl deficiency may present with paranoid ideation (mimicking paranoid schizophrenia), dementia, cognitive dysfunction, delusions, or lack of energy manifested by slowed responses. Hallucinations or even obtundation may preclude obtaining an adequate history. The family may indicate the progressive evolution of a marked personality change and may be able to help trace the evolution of symptoms and deviations from the time when the patient was last well. Intermittent therapy with multivitamins, liver pills, and/or injections (often given by a well-meaning family member or unregistered practitioner) is a common quick fix remedy in many cultures. The family is a good source for details on the patients dietary habits (food faddism, vegetarianism, alcohol intake), and family history of medical problems (blood diseases, gluten sensitivity, autoimmune diseases).

A past medical history of epilepsy or alcoholism with seizure disorder (anticonvulsant therapy) is important. Rarely, patients with autoimmune hemolytic anemias may be lost to follow-up and return with acute aplastic crises when they run out of folate. A past surgical history of total or partial gastrectomy and/or anastomosis, fistula, or bowel resection will reveal the potential for perturbation of physiologic absorption (loss of IF, bypassing or loss of absorptive surface, blind loop syndromes). Surreptitious

MISCELLANEOUS MEGALOBLASTIC ANEMIAS NOT DUE TO COBALAMIN OR FOLATE DEFICIENCY

I. Congenital disorders of DNA synthesis
   A. Orotic aciduria
   B. Lesch-Nyhan syndrome
   C. Congenital dyserythropoietic anemia
II. Acquired disorders of DNA synthesis
   A. Deficiencythiamine responsive (DIDMOAD)
   B. Malignancyerythroleukemia
      1. Refractory sideroblastic anemias
      2. All antineoplastic drugs that inhibit DNA synthesis (including antinucleosides used against HIV and other viruses)
   C. Toxicalcohol

or accidental inhalation of N_2O in an occupational setting (dental or anesthesiology professionals) and deliberate inhalation of N_2O (cartridges attached to whipped cream dispensers or visits to houses of laughter, where N_2O can be inhaled for a small fee) will be revealed only on direct questioning. Visits to tropical countries and the development of intermittent episodic diarrhea may give a clue to tropical sprue; prolonged (>3 years) chronic gastrointestinal symptoms followed by insidious development of neurologic problems predicts a combined (folate followed by Cbl) deficiency.

Systemic review of symptoms may range from none (incidental increased mean corpuscular volume and/or PMN hypersegmentation) to severe (unstable angina from severe anemia). With slow development of anemia, the patient will often not develop cardiopulmonary symptoms until there is a 50% reduction in hemoglobin concentration, which leads to dyspnea on exertion, palpitation, and generalized fatigue or lethargy. Only when hemoglobin is below 5 g/dL will the patient develop dyspnea at rest and angina on modest exertion or even at rest. Congestive heart failure is heralded by pedal edema, nocturia, orthopnea, and tender hepatomegaly.

Upper gastrointestinal symptoms with anorexia associated with intrinsic gastrointestinal disease or anemia with heart failure must be distinguished from symptoms due to glossitis. The latter may lead to inability to wear dentures, tolerate hot drinks or spicy foods because of burning, and even odynophagia, which may compromise further food intake (seen in both Cbl and folate deficiency). The patient may volunteer that glossitis is relieved by multivitamin ingestion, at which time the interviewer should remember to ask questions related to the subsequent evolution of neurologic symptoms. Weight loss in Cbl deficiency is not as severe as in folate deficiency arising from intrinsic gastrointestinal disease. Episodic or chronic diarrhea with steatorrhea is commonly due to tropical sprue, although it may be brought on by gluten-containing foods. Although these symptoms may be accompanied by abdominal pain, pain in the absence of diarrhea could be due to tabetic crisis (vomiting, abdominal rigidity, absence of leukocytosis, or fever) accompanying spinothalamic involvement in Cbl-deficient myelopathy.

The patient with PA may have two or three semisolid bowel movements per day; although this may be construed as a normal pattern, it may in fact represent a change since the last time the patient was well. Constipation may be related to obstipation arising from involvement of Meissners and Auerbachs plexus within the gastrointestinal tract. Similarly, incipient loss of

DRUGS THAT PERTURB FOLATE METABOLISM

Ethanol in amounts >80 g/day is toxic to hematopoietic precursors and can directly lead to megaloblastosis with abnormal vacuolization of normoblasts as well as to sideroblastic anemias; these toxic changes seen in severe alcoholics are usually associated with significantly higher alcohol consumption and revert to normal with alcohol withdrawal. Patients who have one nutritious meal each day tend to stave off the eventual development of folate deficiency. [317] Alcohol consumption leads to a relatively rapid (24-day) fall in serum folates. This is a result of the combined effects [147] of increased urinary folate excretion (by decreasing the percentage reabsorption and increasing the fractional excretion of 5-methyl-H_4 $PteGlu_n$) leading to net impaired renal conservation; [318] interruption of the enterohepatic circulation (resulting from an effect on hepatic pteroylpolyglutamate synthesis and reduced release of tissue folates into plasma and/or bile); formation of acetaldehyde/H_4 PteGlu adducts (5,10-(CH_3-CH)-H_4 Pte-Glu_n); increased catabolism of 5-methyl-H_4 $PteGlu_n$ folic acid by ethanol acetaldehyde/xanthine oxidase generated superoxide; [319] malabsorption of folate by inhibition of jejunal folate pteroylpolyglutamate hydrolase; [320] perturbation of methionine synthase activity (which results in altered dUTP to dTTP balance, increased apoptosis, and regenerative proliferation of hepatic cells in a model of ethanol-fed micropigs); [321] and increased urinary excretion of formate, which is normally metabolized via folate-requiring enzymes. [10] [312] [322] The relative degree to which each of these mechanisms contributes to folate deficiency remains to be comprehensively determined. Excess alcohol consumption is probably the most common cause of folate deficiency in the United States. [323]

Trimethoprim and *pyrimethamine* bind to bacterial and parasitic dihydrofolate reductase with much greater affinity than to human dihydrofolate reductase but patients with underlying folate deficiency appear to be more susceptible to the drugs. [324] The ensuing megaloblastosis can be reversed by 5-formyl-H_4 PteGlu. *Methotrexate* binds to human dihydrofolate reductase (K_i 7×10^{10} M) and leads to trapping of folate as a metabolically inert form (H_2 PteGlu). This leads to a true depletion of H_4 $PteGlu_n$ within hours and consequently to functional deficiency of 5,10-methylene-H_4 $PteGlu_n$ and reduced thymidylate synthesis. Although megaloblastosis can develop rapidly, the toxic effects of methotrexate can be avoided by administration of 5-formyl-H_4 PteGlu within 12 hours. However, the effects of repletion on tumor 5,10-methylene-H_4 $PteGlu_n$ presents a problem. FR take up methotrexate at lower doses, but RFC-mediated and/or passive diffusion appears to be the main route of cellular uptake at high doses (Fig. 28-6). Once in the cell, methotrexate is polyglutamated and this determines its cytotoxicity. The maintenance of a gradient between the extracellular and intracellular compartment appears to determine polyglutamation and thus efficiency of cytotoxicity. *Sulfasalazine* produces megaloblastosis in up to 67% of patients taking full doses (>2 g/day) by decreasing absorption of folates (decreasing conversion of H_4 $PteGlu_n$ to H_4 PteGlu), and induction of Heinz body hemolytic anemia (increased requirements). It is still not clear whether cases in which megaloblastic anemia develops while patients are receiving *oral contraceptives* represent a cause-and-effect relationship. Oral contraceptives may increase folate catabolism (metabolic consumption) or may weakly interfere with DNA synthesis. It is significant that the megaloblastosis of cervical epithelium often reverses with folate therapy alone. [147] The mechanism for reduction of serum folate by *anticonvulsants* during prolonged therapy is probably due to the combined action on reduced absorption [147] as well as through induction of microsomal liver enzymes. [325] It is unclear how folates provoke a return of seizures in some patients receiving anticonvulsants, but this effect may be mediated by glutamate (or related) receptors. [326] Despite this potential risk, consensus guidelines have stressed the importance of ensuring that pregnant women with epilepsy be prescribed folates together with anticonvulsants. [327] [328] Although *antineoplastics* and *antiretroviral antinucleosides* such as azidothymidine lead to megaloblastosis, the temporal sequence and investigations to rule out Cbl/folate deficiency should easily lead to correct causal assignment.

bladder or bowel control due to Cbl myelopathy may present with urgency or nocturia.

In contrast to musculoskeletal symptoms (arthralgia or frank arthritis) of autoimmune diseases, nocturnal cramps or pain in upper and lower extremities may indicate spinothalamic tract involvement. Hypoparathyroidism or systemic lupus erythematosus, alone or associated with PA, leads to significant overlap of cerebral, musculoskeletal, and neurologic presentations.

Review of skin symptoms may elicit a history of increased diffuse or blotchy generalized brownish skin pigmentation, especially of nail beds and skin creases. This is common in Cbl and folate deficiency; associated vitiligo suggests autoimmune disease.

Although symptoms related to neurologic dysfunction may be volunteered, a complete detailed questionnaire should be formulated during the interview. Questions should be directed to perversions in taste or smell, decreased visual acuity, changes in color vision, and eye pain (neuritis), tinnitus, or headache. Dizziness with orthostatic hypotension and blacking out may be related to severe anemia. Vertigo or difficulty in walking in the dark (loss of proprioception and position sense), difficulty in ambulation (which may feel like walking on cotton wool), stiffness of extremities (corticospinal tracts), or ataxia (spinocerebellar tracts) may be indicative of a serious Cbl myelopathy. Early symptoms are symmetrical tingling (pins and needles), extending from the tips of the toes to a glove and stocking distribution in later stages. Burning feet syndrome, or more commonly, complaints of difficulty in performing simple tasks such as buttoning clothes, may also be a presenting symptom. When loss of bladder and bowel control brings the patient to the physician, advanced neurologic dysfunction is invariably present.

Other genitourinary symptoms such as recurrent cystitis from bladder dysfunction, impotence (Cbl neuropathy) or a recent Pap smear indicative of cervical dysplasia may, rarely, be presenting symptoms. Multiple pregnancies with short intervals between delivery and conception predispose to a high risk for overt folate deficiency. (Cbl deficiency is more often associated with infertility.)

Physical Examination

Physical examination may reveal different features in well nourished patients (Cbl-deficient vegetarians or PA patients) and poorly nourished (folate-deficient) individuals. The latter will show evidence of significant weight loss or other stigmata of multiple deficiency due to broad-spectrum malabsorption. Thus, associated deficiency of vitamins A, D, and K and/or protein-calorie malnutrition may give rise to angular cheilosis, bleeding mucous membranes, dermatitis, osteomalacia, and chronic infections. Varying degrees of pallor with lemon-tint icterus (a combination of pallor and icterus best observed in fair-skinned individuals) are common features of megaloblastosis.

When anemia is severe, the patient may have a low-grade fever. The skin may reveal either a diffuse brownish pigmentation or abnormal blotchy tanning. Special emphasis should be given to pigmentation of skin creases and nail beds. (Mucous membrane pigmentation will not be noted, in contrast to Addisons disease.) Premature graying is observed in both light- and dark-haired individuals.

A blunted mask-like facies is extremely common in folate deficiency. Alternatively, there may be evidence of classic hyper- or hypothyroid facies (associated with PA). Special attention should be given to the eyes and eyebrows for signs of thyroid dysfunction.

Examination of the mouth may reveal glossitis with a smooth (depapillated), beefy red tongue with occasional ulceration of the lateral surface. The neck may reveal thyromegaly (diffuse or with nodules) if there is associated disease. Increased jugular venous distension should alert the examiner to cardiovascular failure, with its attendant gallop, cardiomegaly (with or without pericardial effusions), pulmonary basal crepitations, and pleural effusion, tender hepatomegaly, and pedal edema. Nontender hepatomegaly, but more often mild splenomegaly, may rarely be due to extramedullary hematopoiesis in severe anemia, but a mid-epigastrium mass raises the ominous possibility of gastric carcinoma (three times as likely in PA).

An inverse correlation has been identified between the extent of anemia and neurologic dysfunction. Patients with normal complete blood count values will often have neurologic signs and symptoms. In prolonged Cbl deficiency, neurologic examination will reveal clear-cut evidence of involvement of posterior and pyramidal, spinocerebellar, and spinothalamic tracts. Among the earliest signs of posterior column dysfunction are loss of position sense in the index toes (before great toe involvement), which is elicited by passive movement, and loss of the ability to discern vibration of a high-pitched (256 cycles/sec) tuning fork (a very early elicitable, objective sign) which invariably *precedes* by many months the loss of ability to sense the vibration of a lower-pitched (128 cps) tuning fork. Usually the patient loses vibration sense to 256 cps from toe to hip before loss of 128 cps vibration sense even begins. Because of the slow coalescence of contiguous spinal cord lesions, a constellation of elicitable signs may be obtained. Upper motor neuron disease is indicated by weakness and progressive spasticity with increased muscle tone, exaggerated deep tendon reflexes with clonus, extensor plantar response, and incoordinate or scissor gait, which may progress to spastic paraplegia. The involvement of peripheral nerves may markedly modify these signs to include flaccidity and the absence of deep tendon reflexes. A positive Romberg sign is not uncommon and a positive Lhermitte sign may be elicited. Loss of sphincter and bowel control, altered cranial nerve dysfunction with altered taste, smell, and visual

acuity or color perception, and optic neuritis (unexplained predominance in males) may be other physical signs indicating Cbl deficiency. Inability to carry out serial subtraction of 7 from 100 is a valuable test to document reduced cerebral function (the electroencephalogram often reveals slow wave frequency) in PA.

Recent Insights into the Changing Spectrum of Cbl Deficiency

The biochemical and clinical spectrum of presentations of Cbl deficiency has changed dramatically compared with earlier descriptions. For instance, earlier in developing countries, the majority of cases with nutritional Cbl deficiency presented in the second and third decade with pancytopenia, mild hepatosplenomegaly, fever, and thrombocytopenic bleeding, all of which are in keeping with the concept of ineffective hematopoiesis of megaloblastosis. [238] [231] Implicit in the earlier literature was the dictum that the neurologic and psychiatric syndrome uniquely associated with Cbl deficiency usually developed in 80% by the time anemia was symptomatic, and in 50% of patients, this led to some incapacity. [1] [332] However, from southern Africa among 144 consecutive adults (who were not vegetarian, pregnant, or lactating) with megaloblastic anemia [333] and similar presentations of megaloblastosis as reported from India, there was a high incidence of neurologic disease among patients with mild to moderate anemia due to Cbl deficiency (in 86%), mostly from pernicious anemia (PA). This reverses previous presumptions that the primary cause of megaloblastic anemia in this population in developing countries is invariably folate deficiency.

That such classic hematologic presentations are infrequently observed in the West was initially recognized when anemia and macrocytosis were not invariably associated with Cbl deficiency. [12] [190] [215]

When the clinical spectrum and diagnosis of Cbl deficiency was reevaluated among a cohort of unselected consecutive patients in New York and Colorado who fulfilled criteria for unambiguous Cbl deficiency, normal values in hematocrit, mean cell volume, and LDH were found in over one-third of patients, and 80% had normal WBC and platelets and serum bilirubin levels. [216] Strikingly, 33% of the patients blood smears were not identified as diagnostic when evaluated by laboratory personnel, when compared to 94% when evaluated by the investigators themselves. This fact is important because the majority of physicians in general practice rely heavily on laboratory personnel to flag an abnormal blood smear. Equally striking was the important finding that neuropsychiatric abnormalities were noted in nearly one-third of patients often in the absence of anemia, macrocytosis, or both. [190] These data formed the basis for reevaluating the diagnostic sensitivity and specificity of serum tHcys and MMA in clear-cut Cbl deficiency (406 patients) or folate deficiency (119 patients). [226] In patients with Cbl deficiency, serum MMA levels were elevated in 98.4% and serum tHcys in 95.9%; both metabolites were normal in only one patient (0.2%). For patients with folate deficiency, the serum tHcys was increased in 91% and MMA was elevated in 12.2%, but in all but one, this was attributed to renal insufficiency or dehydration, conditions which are known to falsely raise concentrations of this metabolite. These data allowed for the conclusion that *normal levels of both MMA and tHcys rule out clinically significant Cbl deficiency with virtually 100% certainty.*[226]

The changing pattern of neurologic presentations also deserves special mention. From a classic review of 153 episodes of Cbl deficiency involving the nervous system, the following facts emerged:[191] First and foremost, in over a quarter of these patients, there was no reduction in the hematocrit despite neurologic disease, and only a minority of patients had combined hematologic and neurologic disease. The inclusion of anemic and nonanemic patients who were Cbl-deficient led to the dramatic conclusion that the higher the hematocrit, the more severe is the neurologic disorder! (This has its experimental correlates in monkeys and fruit bats, which have severe neurologic disease in the absence of anemia. [10] [189]) Profoundly anemic patients frequently had no neurologic deficits, and the level of Cbl had no correlation with either the existence or severity of neurologic disease. Although simultaneous consumption of folate may have negated the development of potential hematologic abnormalities in Cbl deficiency, this could not be documented. Among patients studied, 65% had mild, 25% had moderate and 10% had severe neurologic deficits. Paresthesias or ataxia were most commonly the first symptoms and diminished vibratory sensation and proprioception in the lower extremities were the most common objective early signs. Although multiple neurologic syndromes were often seen in the same patient, the spectrum of objective signs could include loss of fine/coarse touch, decreased or increased deep tendon reflexes with spasticity or muscle weakness, urinary or fecal incontinence, orthostatic hypotension, amaurosis, dementia, psychosis, or mood disturbances.[191] Overall, although the neurologic deficits were mild in the majority of cases, the severity was judged related to the duration of symptoms prior to diagnosis; not unexpectedly, those with the shorter duration responded most to appropriate replacement. The demonstration of cognitive improvement in 11 of 18 geriatric subjects with low Cbl and quantitative cognitive dysfunction treated with Cbl, and the observation that there is a limited window of opportunity for effective intervention also highlights the importance of early diagnosis for this population. [334]

Although the basis for these changes in clinical presentation is speculative, it may be due to heightened awareness of Cbl deficiency, better diagnostic tools, or supplementary folates taken by humans and given to livestock (which correct megaloblastosis but aggravate neuropsychiatric disease).

Diagnostic Issues Related to Information from the Peripheral Smear and Bone Marrow

Although not specific for megaloblastic anemia, macroovalocytes are the hallmark of megaloblastosis (Table 28-5). This distinction is important because only 55% of 109 patients with MCV >105 fl had vitamin deficiency[335] (this percentage may be lower or higher depending on the population under study). Thus, in nearly one-half of all cases, macrocytosis per se is not associated with megaloblastosis (see Table 28-5) and additional tests are necessary for complete diagnosis.

The frequency of hypersegmented PMNs (5% with 5 lobes or 1% with 6-lobed PMNs) in patients with megaloblastic hematopoiesis is 98%. [336] The sensitivity decreases to only 78% in alcoholics although the specificity of this finding is 95%. [227] With a combination of hypersegmented PMNs and macroovalocytosis, the specificity is 9698%[225] [227] and the positive predictive value of either folate or Cbl deficiency is 94%. [225] (*Note*: Hypersegmentation of PMNs is insufficiently sensitive, when compared to metabolite levels, to be used as a clinical tool in the diagnosis of *mild* Cbl deficiency.[337])

Is a bone marrow aspirate always necessary to diagnose Cbl/folate-deficient megaloblastosis? With the addition of highly sensitive serum tests for the specific diagnosis of Cbl and folate deficiency, the need for a bone marrow test is often dictated by the urgency to diagnose megaloblastosis (with results available in an hour). For example, in the case of florid hematologic disease with or without neurologic disease suggestive of Cbl or folate deficiency, a bone marrow aspiration carried out as soon as possible is invaluable in assisting in rapid diagnosis of megaloblastosis. However, in the outpatient setting, or when the patient has a characteristic peripheral smear, or with a primary neuropsychiatric presentation, a case can be made to initiate the sequence of diagnostic tests without a bone marrow aspiration by proceeding with measurement of serum levels of vitamins or metabolites (see Table 28-2). In a pregnant patient with pancytopenia with macroovalocytes, hypersegmented PMNs, and reticulocytopenia with a history of noncompliance with prenatal supplements (and no neurologic findings suggestive of Cbl deficiency), a bone marrow aspiration may not be necessary to initiate therapy for a strong presumptive diagnosis of folate deficiency. Of course, if there is no evidence of response within 10 days, a bone marrow aspiration is indicated.

TABLE 28-5 -- Clinical Conditions Not to Be Confused with Megaloblastosis

Macrocytosis[a] without megaloblastosis[b]
Reticulocytosis
Liver disease
Aplastic anemia
Myelodysplastic syndromes (especially 5q)
Multiple myeloma
Hypoxemia
Smokers
Spurious increases in MCV without macroovalocytosis[c]
Cold agglutinin disease
Marked hyperglycemia
Leukocytosis
Older individuals

[a]The central pallor which normally occupies about one-third of the normal red blood cell is *decreased* in macroovalocytes. This contrasts with the finding of *thin macrocytes* where the central pallor is *increased.*
[b]While megaloblastosis implies that a bone marrow test has been performed, with the recent addition of highly sensitive tests for the specific diagnosis of Cbl and folate deficiency, the need for a bone marrow test is often dictated by the urgency to make the diagnosis (see text).
[c]When the Coulter counter readings of a high MCV are not confirmed by looking at the patient or the peripheral smear.

Masked Megaloblastosis

The term masked megaloblastosis is reserved for conditions in which true Cbl/folate deficiency with anemia is not accompanied by classic findings of megaloblastosis in the peripheral blood and bone marrow. This occurs when there is a coexisting condition that neutralizes the tendency to generate megaloblastic cells (usually involving reduction in RBC hemoglobinization, as in iron deficiency or thalassemia). Among the 123 episodes of folate deficiency in 119 patients, the MCV was normal in 25%.[225] [233] A wide RBC distribution width (RDW) on the Coulter counter readout in the presence of a normal mean corpuscular hemoglobin (MCH) or MCV may reflect either megaloblastic anemia[338] or dimorphic (macroovalocytes plus microcytic hypochromic RBC) anemia. Because megaloblastic WBCs and precursors are unaffected by deficient hemoglobinization, these pathognomonic findings (giant myelocytes and metamyelocytes, and hypersegmented PMNs) remain; the latter may persist for up to 2 weeks after replacement with Cbl/folate.[339] Thus, the recognition of masked megaloblastosis should initiate investigations to rule out iron deficiency, anemia of chronic disease, or hemoglobinopathies. Indeed, appropriate replacement with Cbl or folate will elicit a maximal therapeutic benefit only when iron deficiency is corrected. Conversely, if combined iron and Cbl deficiency (total gastrectomy) or iron and folate deficiency (pregnancy) is treated with iron alone, megaloblastosis will be unmasked.

APPROACH TO DIAGNOSIS AND THERAPY OF MEGALOBLASTOSIS

In general, there are three stages in approaching a patient. (1) *recognizing* that megaloblastic anemia is present; (2) *distinguishing* whether folate, Cbl, or combined folate and Cbl deficiencies have led to the anemia; and (3) diagnosing the *underlying disease* and *mechanism* causing the deficiency. Establishing that the patient does have megaloblastosis is, in theory, straightforward. This is easily done by evaluating the complete blood count, mean corpuscular volume, and peripheral smear, followed by a bone marrow aspiration. Clues to whether Cbl or folate deficiency is responsible for megaloblastosis can be obtained by serum Cbl and serum folate levels; additional testing of serum MMA and serum tHcys will define the true nature of the deficiency. [223] However, this ideal and orderly workup is not always feasible in clinical practice, where the patient may (1) present for the first time with or without associated neurologic disease; (2) be referred after a variable workup has already been initiated for possible megaloblastosis; (3) present with symptoms primarily attributed to a disease predisposing to Cbl or folate deficiency, in which case anemia or neurologic dysfunction may only be a minor symptom; (4) present with isolated neurologic disease in the absence of anemia; or (5) be referred after empiric therapy has been given for presumed Cbl and/or folate deficiency. The immediate question therefore pertains to the overall status of the patient.

*If the patient is decompensated or decompensation is imminent***,** obtain serum folate and Cbl levels and a bone marrow aspiration to confirm megaloblastosis and proceed with transfusion of 1 unit of packed RBCs *slowly*, with vigorous diuretic therapy and/or simultaneous phlebotomy (from the other arm) to obviate further congestive heart failure from fluid overload. Both Cbl and folate should be administered simultaneously in full doses. Tests for Cbl absorption can be deferred until the patient is more stable. Transfusion does not apparently alter serum folate/Cbl levels but will alter red cell folate levels. [340]

If the patient is moderately symptomatic (but not in heart failure), the strong likelihood of a dramatic response (in the sense of well-being and relief of sore tongue) within 2 to 3 days before hematologic improvement argues against immediate blood transfusion. [341] Therefore, proceed with appropriate diagnostic workup as for the well compensated patient.

If the patient is well compensated, and in the outpatient setting, the physician has time to develop an orderly sequence of diagnostic tests. First, check the peripheral smear and rule out other macrocytic anemias (thin macrocytes with a normoblastic marrow in contrast to macroovalocytes). Draw blood for Cbl and folate levels (*before* the patients first hospital meal) to sort out whether the problem is due to deficiency of folate and/or Cbl, or some other deficiency (see Table 28-2). Assuming that there is no urgency to make the diagnosis, the physician can elect to wait for the results of these tests before proceeding with the next test in the diagnostic workup. If making the diagnosis is urgent, a cost-effective test is the bone marrow aspirate; results (indicating megaloblastosis or not) will be available within an hour. If a bone marrow aspiration is performed, send samples for special stains (periodic acid-Schiff positivity for erythroleukemia), flow cytometry, and chromosomal analysis (myelodysplastic syndromes can exhibit some megaloblastic changes in the erythroid series but megaloblastic granulopoiesis is not seen). If the marrow is not obviously megaloblastic but the iron stain reveals absent stores, review the morphology again with special emphasis on granulocytic precursors and promegaloblasts, and look for more subtle megaloblastic changes.

If the patient refuses a bone marrow aspirate and serum Cbl and folate levels are equivocal (i.e., in the low normal range), a strong case can be made to test for serum tHcys and MMA. Serum MMA and tHcys are ordered together (the same sample remaining from the serum sent for Cbl and folate may be used if they were frozen away). Integrating the results of serum MMA and tHcys (which will be available after a week or more) with those of the serum Cbl and folate levels will help distinguish Cbl and/or folate deficiency (see Table 28-2). A normal MMA and tHcys level eliminates Cbl deficiency with 100% confidence, and normal tHcys levels suggest that megaloblastic anemia is *not* due to folate deficiency. These tests are particularly useful (1) if the patient has pure neurologic disease (no hematologic manifestations from Cbl deficiency), or (2) if there are associated conditions like iron deficiency or thalassemia that will mask megaloblastosis. Note that administration of folate or Cbl immediately invalidates the diagnostic value of the serum folate and Cbl levels; also, because the elevated serum tHcys and MMA levels begin to drop and normalize only after about 1 week, there is a period of slightly <1 week after administration of folate/Cbl to clinch the diagnosis. [184] In the rare situation when a defect in utilization of Cbl or folate is suspected, consultation with experts who have published in this area is advised.

A reticulocyte count, indicative of a hypoproliferative state, is useful to follow the patients response to appropriate replacement therapy. Additional supporting studies documenting increased serum lactate dehydrogenase, haptoglobin, and bilirubin (evidence for intramedullary hemolysis) may be performed. Later, studies to define the mechanism for Cbl malabsorption with a Schilling test can then be carried out.

When the megaloblastic state is established, try to determine the underlying mechanism of Cbl/folate deficiency. The etiology of folate deficiency is usually sorted out by this time from the history, physical examination, and the clinical setting. If pure folate deficiency has been prolonged, expect associated Cbl deficiency to ensue (special emphasis should be given to identifying subtle manifestations of neurologic disease). If Cbl deficiency is suspected, test for serum anti-IF antibodies (highly specific for PA), and after this, proceed with the Schilling test (Table 28-3) to differentiate PA from other causes of Cbl deficiency. (*Note*: the Schilling test will initiate a reticulocytosis by the second or third day in pure Cbl deficiency and may also give a partial response in pure folate deficiency.) Additional tests for associated autoimmune diseases should be made (if indicated) by this time. If serum IF antibodies are negative, gastric analysis for Histalog-fast

MODIFIED THERAPEUTIC TRIALS

The traditional therapeutic trial using physiologic doses of vitamins (100 g of folate or 1 g of Cbl given daily while monitoring the reticulocyte response) has given way to a modified therapeutic trial: Here, the intention is often not so much to make the diagnosis of a deficiency, but to confirm the clinical suspicion that the patient does not have deficiency.[225] This would be demonstrated by lack of response to full replacement doses of both vitamins (1 mg of folic acid orally for 10 days and 1 mg of Cbl intramuscularly for 10 days). Clinical scenarios in which such trials may be applicable (after drawing blood for serum Cbl and folate) are as follows:

1. When there is a clinical suspicion that the underlying disease is *not* due to a vitamin deficiency, but this is not supported by clinical, morphologic, and biochemical evaluation. Such conditions include anemia with a megaloblastic bone marrow which may be secondary to chemotherapy, myelodysplastic syndromes, or acute myeloid leukemia, when time is of the essence in making the diagnosis, or the levels of Cbl are likely to be falsely abnormal because of these diseases, or there is underlying dehydration or renal dysfunction which predictably gives falsely high levels of metabolites.
2. In other situations (i.e., pregnancy, AIDS, or alcoholism) with a multifactorial basis for anemia, the response (or lack thereof) to full doses can eliminate Cbl/folate deficiency and thereby narrow the (often extensive) differential diagnosis.
3. In instances when severe anemia with megaloblastosis is clinically obvious, but is also so serious that one cannot wait for the results of specific tests for deficiency. Here again, *full doses* of both vitamins are administered, and if there is a response manifested by brisk reticulocytosis by day 57, retrospective assignment of the deficiency is based on the results of blood samples drawn before beginning the trial.

It is important to emphasize that in all therapeutic trials, if there is no evidence of response within 10 days, a bone marrow is indicated to identify another primary hematologic disease.

achlorhydria need not be performed, except in children for whom it is necessary to distinguish congenital IF deficiency from juvenile PA and Imerslund-Gräsbeck syndrome. In adults, a strong presumptive diagnosis of PA can usually be made with the Schilling test (Table 28-3).

Therapy

Routinely, treatment with full doses of parenteral Cbl (1 mg/day) and oral folate (folic acid) (15 mg) before knowledge of the type of vitamin deficiency is established should only be reserved for the severely ill patient. An appropriate regimen for conditions in which Cbl replenishment will correct cellular Cbl deficiency (but will not correct the underlying problem that led to the deficiency [e.g., PA] is 1 mg of intramuscular CN-Cbl per day (week 1), 1 mg twice weekly (week 2), 1 mg/week for 4 weeks, and then 1 mg/month for life (15% or 150 g will be retained 48 hours after each 1 mg Cbl injection).

Because the doses of Cbl are much greater than that required physiologically, any theoretical advantage of OH-Cbl over CN-Cbl (greater binding to Cbl-binding proteins, greater serum levels, and less renal excretion) is of little significance in general clinical practice. For patients who refuse monthly parenteral therapy or prefer daily oral therapy, or in those with disorders of hemostasis, 1 mg/day tablets are recommended for patients with Cbl malabsorption (where Cbl is passively absorbed at high doses).[10] [342] This is applicable for patients with PA and patients with inability to absorb food Cbl. It is important to emphasize that the physician must ensure that (1) the patients depleted Cbl stores are rapidly repleted by parenteral Cbl *before* switching to oral Cbl in the long-term, and (2) the patient is compliant and demonstrates adequate Cbl levels and resolution of hematologic and neurologic abnormalities on follow-up. For nutritional Cbl deficiency (e.g., vegetarians) when the entire circuitry in Cbl absorption is intact, oral tablets or a syrup of Cbl (50 g/day for life) will suffice.

Oral folate (folic acid) at doses of 15 mg per day results in adequate absorption (even where intestinal malabsorption of physiologic food folate is present). Therapy should be continued until complete hematologic recovery is documented. If the underlying cause leading to folate deficiency is not corrected, folate may be continued. Folinic acid (aka 5-formyl-H_4 PteGlu, leucovorin) should be reserved *only* for rescue protocols involving antifolates (methotrexate or trimethoprim-sulfamethoxazole) or for 5-fluorouracil modulation protocols, and in the rare acute folate deficiency syndrome. It is too expensive for conventional repletion in folate-deficient states.

Response to Replenishment

The response of the patient to appropriate replacement is reversion of megaloblastic hematopoiesis to normal hematopoiesis (probably initiated at the stem cell level) within the first 12 hours; by 48 hours normal hematopoiesis is re-established, and the only evidence for a prior megaloblastic state may be the persistence of a few giant metamyelocytes. Because megaloblastosis caused by Cbl/folate deficiency can be reversed in 24 hours by administration of folate (i.e., a nutritious hospital meal), delay of a diagnostic bone marrow aspirate is to be avoided for this reason. Clinically, the first 36 to 48 hours are often highlighted by the awakening of an occasional semi-stuporous individual whose chief complaint is amazement at the remarkably improved sense of well-being experienced, with increased alertness and appetite and reduced soreness of the tongue. The ongoing normoblastic hematopoiesis is evidenced by decreases in plasma iron and potassium (12 mEq/dL drop in 48 hours), MMA, tHcys, and phosphate excretion. The patient must be given supplemental potassium if borderline or low potassium levels are found *before* therapy is initiated to obviate potentially fatal arrhythmias. The elevated serum MMA and tHcys levels will return to normal by the end of the first week.

Accelerated turnover of normal DNA in erythroid precursors is associated with an increase in serum urate, which usually peaks by the fourth day, and with increased cellular phosphate uptake for nucleotide synthesis. This may precipitate an attack of gout if the patient has a gouty predisposition. The reticulocyte count increases by the second to third day and peaks by the fifth to eighth day (the peak reticulocyte count is directly proportional to the degree of pre-existing anemia). This is followed by a rise in RBC count, hemoglobin, and hematocrit by the end of the first week, which eventually normalizes by 2 months regardless of the initial degree of anemia. By the end of the third week, the RBC count should be above 3×10^6 mm^3 ; if it is not, additional causes of underlying iron deficiency, hemoglobinopathy, chronic disease, or hypothyroidism should be considered (Table 28-6).

Hypersegmented PMNs continue to remain in the blood for 1014 days; however, the number of normal PMNs and platelets rises and normalizes within the first week. During this process, there may be a transient left shift to include myeloid

TABLE 28-6 -- Causes for Nonresponsiveness of Megaloblastosis to Therapy with Cbl or Folate

Wrong diagnosis
Combined folate/Cbl deficiency being treated with only one vitamin
Associated iron deficiency

Associated hemoglobinopathy (sickle cell disease/thalassemia)
Associated anemia of chronic disease
Associated hypothyroidism

precursors. The reduced intramedullary hemolysis (as a result of normalized hematopoiesis) leads to a gradual reduction in the serum bilirubin by the end of the first week, and LDH levels will drop concomitantly.

In response to Cbl, progression of neurologic damage and dysfunction is inhibited. In general, the degree of functional recovery (reversal of neurologic damage) is inversely related to the extent of disease and duration of signs and symptoms. As a rough estimate, signs and symptoms that have been present for less than 3 months are usually completely reversible; with longer duration, there is invariable residual neurologic dysfunction. The reversibility of neurologic damage is slow (a maximal response may take 6 months). Substantial increments (in recovery) are unlikely to be gained after the first 12 months of appropriate therapy, which indicates irreversibility at this point. However, the majority of neurologic abnormalities have improved in up to 90% of patients with documented subacute combined degeneration.

Follow-up

Patients with neurologic dysfunction from Cbl deficiency have traditionally been given more frequent doses of Cbl (biweekly rather than monthly therapy for the first 6 months), despite the lack of evidence that this form of therapy is more beneficial. This approach, nevertheless, serves a purpose in that improvement in neurologic status can be carefully documented. Once maximal responses have been established and patients are deemed capable of administering Cbl independently, they can be given vials of Cbl with appropriate instructions for lifelong monthly injections. Follow-up outpatient visits every 6 months should be instituted in order to ensure adequate maintenance of hematopoiesis, as well as early diagnosis of other diseases commonly associated with the Cbl/folate-deficient state. Follow-up of 95 patients with PA indicates that individuals >60 years are prone to developing iron deficiency which arises from poor iron absorption from achlorhydria. [343] Thus, all patients with PA, especially the elderly, should be screened for iron deficiency both at the beginning and during follow-up.

Although patients with PA have a twofold increase in proximal femur and vertebral fractures and a threefold increase in distal forearm fractures, it is unclear whether this is reduced by therapy with Cbl. Because Cbl stimulates proliferation of bone marrow stromal osteoprogenitors and osteoblastic cells, the suppressed activity of osteoblasts may contribute to osteoporosis and fractures in patients with PA. [344]

Studies of a cohort of 5,072 patients with PA registered in Denmark (from 19771989) revealed that in addition to the well-established increased risk for stomach cancer, there was also a twofold increase in the relative risk for cancer of the buccal cavity and pharynx; however, previously reported elevated risks for other digestive tract cancers were not confirmed. Because of the excess risk of gastric cancer and carcinoid tumors in patients with PA, [345] the value of endoscopic surveillance was studied prospectively; in 56 patients, two patients each with early gastric cancer and gastric carcinoids were identified. Gastric carcinoids associated with PA are clinically indolent tumors, particularly when they are smaller than 2 cm. [346] This information can be used to formulate general guidelines for more frequent surveillance of patients with PA who have larger gastric carcinoids.

Routine Supplementation of Cbl and Folate

Routine *periconceptional* supplementation of folate for (1) normal women[295] and (2) in higher doses for women at risk for delivery of babies with neural tube defects[297] provides effective prophylaxis against the development of neural tube defects. Supplementation with folate throughout pregnancy also helps to prevent premature delivery of low birth weight infants,[10] and routine supplementation for premature infants and lactating mothers is also recommended.

In addition to hematologic diseases leading to increased folate requirements (autoimmune hemolytic anemia, -thalassemia, etc.), folic acid supplements appear to reduce the toxicity of methotrexate in rheumatoid arthritis and psoriasis. [347] [348] [349] Table 28-7 summarizes conditions that warrant routine folate or Cbl supplementation. Although a small randomized study demonstrated a beneficial role of Cbl plus pyridoxine and thiamine in somatic and autonomic symptoms of diabetic neuropathy,[350] the independent role of Cbl [351] as well as its role in persistent sleep wake schedule disorders [352] [353] is unclear. Also, the roles of folate/Cbl in reversing vitiligo,[354] aphthous stomatitis,[355] and of high-dose folate for psychiatric disease [356] [357] [358] require further study. Finally, the fact that increased serum tHcys is a risk factor for occlusive arterial disease (see discussion later in this chapter) indicates that future intervention trials related to early reversal of Cbl/folate deficiency-related increase in serum tHcys will be forthcoming.

Because low serum Cbl levels are associated with faster human immunodeficiency virus (HIV-1) disease progression, [359] there have been calls for intervention studies to determine whether correction of low serum Cbl levels in early HIV-1 infection influences the natural history of disease progression.

Public Health Issues in the Debate on Fortification of Foods with Folates

The problem: Despite recommendation for periconceptional supplementation of normal women with folates to prevent neural

TABLE 28-7 -- Indications for Prophylaxis with Cbl/Folate

Prophylaxis with Cbl Infants of mothers with PA[a]
Infants on specialized diets[a]
Vegetarians/vegans[a]
Total gastrectomy[b]
Prophylaxis with folic acid[c]
All women contemplating pregnancy (at least 400 g/day)
Pregnancy and lactation, premature infants
Mothers at risk for delivery of infants with neural tube defects[d] [e]
Hemolytic anemias/hyperproliferative hematologic states
Patients with rheumatoid arthritis or psoriasis on therapy with methotrexate[f]

[a]In food Cbl malabsorption secondary to inability to cleave food Cbl by acid and pepsin, replacement therapy should be 50 g tablets/day orally. In all other conditions involving any abnormality of Cbl absorption, Cbl tablets of 1,000 g/day should be administered orally to ensure that Cbl transport via passive diffusion across the intestine is sufficient to meet daily needs.
[b]Consider late development of Cbl deficiency and iron malabsorption (prophylaxis with oral Cbl and iron).
[c]Ensure that the patient does not have symptoms of Cbl deficiency (most common, paresthesias/numbness) before initiating folate prophylaxis.
[d] Previous delivery of a child with neural tube defects (spina bifida, encephalocele, meningocele) gives a 10-fold greater risk for subsequent delivery of infant with neural tube defects.
[e] Folic acid (4 mg/day) administered *periconceptionally and throughout first trimester*.
[f]To reduce toxicity of the antifolate.

tube defects,[360] this may not have the desired effect for several years until there is widespread education of women and health care providers. In the meantime, because up to 50% of pregnancies are unplanned, such women will continue to be at risk of giving birth to children with neural tube defects. Fortification of foods such as bread/flour and cereals with folates can theoretically overcome this problem, provided there are no untoward effects from such action. However, demonstration of the widespread prevalence of biochemical evidence for Cbl deficiency in the elderly (discussed further later in this chapter), is a matter of significant concern. The fact that these individuals manifest a response to Cbl suggests that they have clinical (albeit subtle) evidence of Cbl deficiency. When such individuals receive additional

folate in their diet, this might quench the expression of Cbl-deficient hematopoiesis while leading to progression of Cbl-deficient neurologic dysfunction. [233] This could go unrecognized unless there were established surveillance programs to identify these cases. Although one solution could be to fortify foods with folate *and* Cbl, this could lead to new problems stemming from alteration of the color of supplemented foods with a potentially unacceptable or unpalatable reddish to pink tinge of Cbl (which is normally a brilliant blood-red color). Finally, there are unresolved questions which relate to the precise dose of folate to be added to foods, the potential for conversion of supplemented folate and Cbl to inactive analogues due to chemical interactions with foods and other nutrients [8] [9] as well as the influence of packing/canning and shelf-life on the bioavailability of supplemented folates.

A progress report: In addition to the continued problem of compliance with consumption of folate-rich foods (which necessitates knowing which foods are rich in folates)[361] and the intrinsically lower bioavailability of food folate, [362] daily supplements of folic acid or fortification of food with folate appeared to be the only reasonable option to ensure adequate folate consumption by women of childbearing age. Accordingly, in March 1996, the Food and Drug Administration issued regulations that folic acid be added to enriched cereal-grain products (flours, corn meals, pasta, breakfast cereals, and rice) by January 1998. This amount of folate fortification (140 g of folate per 100 g) is projected to accomplish some prevention, but it is still a conservative amount and there is a move to consider further increases in the level of fortification (discussed later in this chapter).

There are still several obstacles to nationwide consumption of the recommended 400 g of folate for women of childbearing age. For example, a Gallup Organization-March of Dimes survey in January 1997 [361] determined that 30% of nonpregnant women reported taking daily multivitamin supplements containing folic acid; this number is nevertheless up from the 23% of women who were polled two years before. But of major significance, only 19% of nonpregnant women <25 years (who contribute to 39% of the births in the United States) were compliant with the recommendations, as opposed to 33% of those >25 years. This highlights a pathetically low awareness of folic acid and of the Public Health Service recommendation, and illustrates the need for further aggressive educational strategies directed at women of childbearing age on the beneficial effects of folic acid.

Estimates of potential benefits and risks for the elderly population suggest that, at the current level of folate fortification, the benefits of projected decreases in homocysteine level and heart disease risk (see discussion later in this chapter), greatly outweigh the expected risks. [363] Projections that higher fortification levels would prevent up to 50,000 premature deaths each year from coronary disease need to be tempered by considering whether this could be harmful to other populations. This is not simply a theoretical issue because projections suggest that there may be 800,000 elderly individuals in the United States who have undiagnosed and untreated pernicious anemia (PA).[255] Furthermore, the number of younger individuals (<60 years of age) with PA, as well as undiagnosed individuals with Cbl deficiency who are unable to release dietary Cbl from food, is unclearbut is also expected to be large. This raises the ominous possibility that several of these individuals will develop Cbl-responsive neuropsychiatric and neurologic disease that could go unrecognized if their diets are supplemented with folate through food fortification policies. This issue, and the development of methods to avoid misdiagnosis of Cbl deficiency within the context of intervention studies with higher doses of folate, are under active consideration by the FDA. Nevertheless, once data on the quantification of the actual risks associated with Cbl deficiency are available such analyses could determine whether even higher levels of folic acid fortification would further reduce risks from folate-responsive hyperhomocysteinemia.[363]

Hyperhomocysteinemia

Normally in cells, homocysteine is metabolized by a methylation and a transsulfuration pathway. In the methylation pathway, homocysteine is methylated to methionine in a reaction involving the Cbl-dependent enzyme (methionine synthase) and 5-methyl-tetrahydrofolate (5-methyl-H_4 PteGlu) which donates its methyl group to homocysteine (Figs. 28-2 , 28-3 , and 28-7). Cellular 5-methyl-H_4 PteGlu is provided by the enzyme 5,10-methylene-tetrahydrofolate reductase (MTHFR). The critical nature of the function of this MTHFR (to provide 5-methyl-H_4 PteGlu to drive the methylation cycle) is highlighted by evidence that in some adults with homocysteinemia, a polymorphism within its folate-binding pocket (involving a CT substitution at nucleotide 677 which converts an alanine to valine) results in a dysfunctional thermolabile MTHFR.[364] [365] [366] This leads to a pathologic buildup of homocysteine, which exits from cells into the circulation where it predisposes the patient to premature occlusive vascular disease. In addition, the transsulfuration pathway, which is vitamin B_6-dependent, can also be activated to reduce cellular homocysteine. So in general, the level of plasma tHcys depends on genetically regulated levels of essential enzymes and the intake of folic acid, vitamin B_6 (pyridoxine), and Cbl, as well as other conditions (renal dysfunction, increased age, nitrous oxide, and antifolates).

Chronic hyperhomocysteinemia is now established as a major risk factor in occlusive vascular diseases. [208] [367] [368] [369] These include myocardial infarctions from coronary atherosclerosis,[370] [371] extracranial carotid artery stenosis,[372] and stroke,[373] vascular disease in end-stage renal failure, [374] [375] thromboangitis obliterans, aortic atherosclerosis,[376] venous thromboembolism,[208] [371] [377] and placental abruption or infarction. [294] (*Note*: An increment of 5 mol/L of tHcys elevates coronary artery disease risk by as much as cholesterol increases of 20 mg/dL. [378])

Although there is no question of the adverse role of homocysteinemia in vascular disease, it is still controversial whether the thermolabile MTHFR is, or is not, a risk factor for vascular disease among different populations. For example, there are several reports in favor of the conclusion that there is a correlation of thermolabile MTHFR (i.e., the C677T mutation) with plasma tHcys in patients with premature coronary artery disease. [379] [380] [381] [382] However, other reports indicate that thermolabile MTHFR is not a major risk of premature coronary artery disease or myocardial infarction, or for restenosis after coronary angioplasty, [366] [383] [384] or for typical late-onset vascular disease. [385] [386]

The basis for this controversy is unclear, but it may relate to distinctly different populations (with additional covariables). For example, the extent to which folic acid supplements can lower elevated tHcys is dependent on the initial tHcy and folate, Cbl and vitamin B_6 (pyridoxine) levels, multivitamin use,[387] and whether the individual has the heterozygous or homozygous form of thermolabile MTHFR. In one study, folate was the most important determinant of plasma tHcys, even in subjects with apparently adequate nutritional status of this vitamin. [293]

So how much folate supplementation is necessary to reduce tHcys levels? A trial of daily intake of 1 or 2 mg of folic acid supplements on tHcy levels revealed that both dosages lowered tHcy levels similarly.[388] Interestingly, homozygotes of thermolabile MTHFR had increased susceptibility to high levels of tHcy at marginally low plasma folate levels, and enhanced responses to folic acid. Therefore, one-third of heterogeneity in responsiveness can be attributable to baseline tHcy and folate levels and to multivitamin use. An unknown variable is that there could exist additional polymorphisms (in genes responsible for maintaining homeostasis of homocysteine) that have not been identified but which may account for increased vascular disease. [389]

A notable study among 365 individuals in the NHLBI Family Heart Study[390] raises issues of a gene-environment interaction: Among subjects with lower plasma folate concentrations (<15.4 nmol/L), those with the homozygous thermolabile MTHFR genotype had fasting tHcys levels that were 24% greater than those with the normal genotype. Because a difference between genotypes was not seen among individuals with folate levels >15.4 nmol/L, this suggests that a gene-environment interaction might increase the risk by elevating tHcys, especially when folate intake is low. So homozygotes probably have a higher folate requirement to maintain optimum homocysteine homeostasis, and folate supplementation may be necessary to prevent fasting hyperhomocysteinemia in such persons.

High plasma tHcys levels are a risk factor for deep-vein thrombosis in the general population. [391] Although the available data supports the concept that selected individuals with thromboembolism should have tests for hyperhomocysteinemia, it is doubtful that many will have evidence for thermolabile MTHFR. [392] It is significant that apparently healthy men with coexistent hyperhomocysteinemia and Factor V Leiden mutation are at substantially increased risk of developing future venous thromboembolism.[393] In addition, the risk of venous thromboembolism among doubly affected individuals with Factor V Leiden appears far greater than the sum of the individual risks associated with either abnormality alone. [393]

In a case-control study involving 19 centers in 9 European countries, a total of 750 cases of atherosclerotic vascular disease (cardiac, cerebral, and peripheral) and 800 controls of both sexes younger than 60 years were assessed for fasting plasma tHcys and after a standardized methionine-loading test to stress the transsulfuration pathway (which irreversibly degrades tHcys; this test picks up another 25% individuals at risk). The results of this study led to the conclusion that an increased plasma tHcys level confers an independent risk of vascular disease similar to that of smoking or hyperlipidemia and powerfully increases the risk associated with smoking and hypertension.[208]

To summarize the clinical data, homocysteinemia in adults with occlusive vascular disease can arise from deficiencies in folate, Cbl, and/or vitamin B_6 (pyridoxine), as well as in individuals with thermolabile MTHFR. The majority of subjects with hyperhomocysteinemia >40 mol/L in the general population have thermolabile MTHFR

combined with low folate status. Daily supplement of low-dose folic acid will reduce and often normalize their tHcy levels. [307] This and other studies have uniformly led to a call-to-arms to undertake randomized controlled trials of the effect of vitamins (pharmacologic doses of folic acid, pyridoxine, Cbl, or betaine) that reduce plasma tHcys levels on vascular disease risk.

How does hyperhomocysteinemia contribute to atherogenesis? There are several plausible mechanisms among which platelet activation, hypercoagulability, increased smooth muscle cell proliferation, induction of endothelial cytotoxicity and/or dysfunction, and stimulation of low-density lipoprotein oxidation have been suggested.[369] Total homocysteine can alter the expressivity of multiple genes, including a stress protein and several novel genes that may contribute to atherogenesis.[394] Hcys stimulates vascular smooth muscle cell proliferation, which is a hallmark of arteriosclerosis, by stimulating cyclin A protein levels and cyclin A-associated kinase activity in vitro. [395] Total homocysteine specifically stimulates aortic cyclin-dependent kinase at the transcriptional level, with the possible consequence of proliferation of aortic cells. [396] Furthermore, S-adenosyl-homocysteine appears to be a physiologic modulator of Apo-1-mediated apoptosis APO-1/Fas (CD95) receptor, which mediates apoptosis in various cell types. [397] Whether this pathway is identical to that found in ethanol-fed micropigs which exhibit similar increases in S-adenosyl-homocysteine [321] requires further study.

Neurocristopathies are a group of diseases arising from defects in the proliferation and migration of neural crest cells during critical periods of embryogenesisexperimentally, this can give rise to diverse defects such as cleft lip/palate and defects in heart septation (ventricular septal defects). The biologic basis whereby folate supplementation protects against some neurocristopathies and neural tube defects is still unclear. An avian embryogenesis model has demonstrated that homocysteine causes dysmorphogenesis of the heart and neural tube, but folate supplementation lowered tHcys and prevented the teratogenic effect.[398] Direct rodent embryotoxicity of tHcys is suggested to be through inhibition of transmethylation reactions by increased embryonic S-adenosyl-homocysteine levels.[399]

Renal Failure and Cbl Replenishment

When coupled with a predisposition to chronic disease anemia, the demonstration of a borderline or low Cbl level arising from increased Cbl clearance during high flux hemodialysis is a matter of some concern[400] especially because (1) MMA and tHcys levels are elevated in patients with renal dysfunction, and (2) elevated tHcys represents an independent risk factor for vascular events in patients on peritoneal dialysis and hemodialysis. [374] [375] So should such individuals be treated during hemodialysis with Cbl, pyridoxine, and folate? Although routine low-dose (1 mg folic acid, 10 mg B_6, and 12 g Cbl) supplementation fails to control hyperhomocysteinemia in 75% of dialysis patients, a placebo-controlled eight-week trial [401] showed that adding supraphysiologic dose folic acid (15 mg/day), B_6 (100 mg/day), and Cbl (1 mg/day) led to a significant reduction in homocysteine without toxicity; and one-third had virtually complete correction to within the normative range. Therefore until additional data is forthcoming, it would be prudent to employ this regimen in such patients.

CONCLUSIONS

In this age of spiraling costs for health care delivery, and the ongoing debate on ways to reduce these costs, few instances in internal medicine yield more satisfying dividends than diagnosing and treating Cbl and folate deficiency. These conditions are devastating when undiagnosed, misdiagnosed, or when Cbl deficiency is treated with folate alone. Recognition of changes in the clinical presentation of Cbl deficiency and availability of sensitive and specific tests should reduce uncertainty in diagnosis. The studies on folate supplementation during pregnancy which identified new folate-responsive diseases are a paradigm for identification of hitherto unrecognized roles for other nutrients in human development. Furthermore, the significant impact of supplemental folates in relieving human suffering consonant with reducing costs for intensive- and long-term care of infants with prematurity and/or neural tube defects, are major achievements and outstanding examples of cost-effective preventive medicine. As a result of parallel research in the veterinary sciences, folate supplements have also profited the meat

industry! Finally, decisions on the optimum amount of folates needed for fortification of food to prevent this devastating complication in newborns must also respect the dictum of *primum non nocere* for the millions of elderly with Cbl deficiency who could be adversely affected from folate supplementation alone. But consideration of any adverse effects on these Cbl-deficient individuals also needs to be tempered by the projected beneficial effects in correction of hyperhomocysteinemia and occlusive vascular disease in many more subjects. Resolution of this very complex debate will establish yet another paradigm for fortification of foods with nutrients that can be beneficial for some but harmful for other population groups.

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DEDICATION

This chapter is dedicated to the memory of Professor John Lindenbaum (19331997), a master clinician, scientist, teacherand intellectual giantwhose sustained and focused investigations led to highly significant contributions to our field.

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Chapter 29 - Thalassemia Syndromes

Bernard G. Forget

INTRODUCTION

The thalassemia syndromes are a heterogeneous group of inherited anemias characterized by defects in the synthesis of one or more of the globin chain subunits of the hemoglobin tetramer. The clinical syndromes associated with thalassemia arise from the combined consequences of inadequate hemoglobin accumulation and unbalanced accumulation of globin subunits. The former causes hypochromia and microcytosis; the latter leads to ineffective erythropoiesis and hemolytic anemia. Clinical manifestations are diverse, ranging from asymptomatic hypochromia and microcytosis to profound anemia, which can be fatal in utero or in early childhood if untreated. This heterogeneity arises from the variable severity of the primary biosynthetic defects and co-inherited modulating factors, such as increased synthesis of fetal globin subunits. Palliative treatment of the severe forms by blood transfusion is eventually defeated by the concomitant problems of iron overload, alloimmunization, and blood-borne infections.

As a group, the thalassemias represent the most common single gene disorder known. In many parts of the world, they constitute major public health problems. Laboratory analysis of these disorders has been one of the most productive and enlightening endeavors of biomedical research. Study of the molecular defects underlying the thalassemia syndromes has led to fundamental advances in our understanding of eukaryotic gene structure and function. For each of these reasons, a thorough understanding of thalassemia and its related disorders is essential to the hematologist. This chapter surveys the major features of these syndromes. Readers wishing more detailed information than can be included here are referred to the comprehensive monographs and chapters written by Weatherall and Clegg, [1] Bunn and Forget, [2] Weatherall, [3] Orkin and Nathan, [4] and Forget and Pearson. [5]

The classification, genetic basis, and pathophysiology of the thalassemia syndromes are based on a thorough understanding of the human hemoglobins, their biosynthesis, their encoding

globin gene families, and their function as soluble oxygen-carrying pigments. Therefore, the readers of this chapter should first familiarize themselves with the material presented in Chapters 15 and 22 . The material presented in this chapter is also substantially clarified by prior reading of Chapter 26 , since the principles underlying the pathophysiology of and therapy for thalassemia draw heavily on knowledge of iron metabolism.

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DEFINITIONS AND NOMENCLATURE

The term thalassemia is derived from a Greek term that, roughly, means the sea (Mediterranean) in the blood. [1] It was first applied to the anemias frequently encountered in people of the Italian and Greek coasts and nearby islands. [6] [7] [8] The term is now used to refer to inherited defects in globin chain biosynthesis. Individual syndromes are named according to the globin chain whose synthesis is adversely affected. Thus, -globin synthesis is absent or reduced in -thalassemia, -globin synthesis in -thalassemia, -globin and -globin synthesis in -thalassemia, and so forth. In some contexts it is also useful to subclassify the syndromes according to whether synthesis of the affected globin chain is totally absent (i.e., ^{0} -thalassemia) or only partially reduced (i.e., $^{+}$ -thalassemia).

The most common forms of thalassemia arise from total absence of structurally normal globin chains or a partial reduction in their synthesis. In contrast to the structural hemoglobinopathies (e.g., sickle cell anemia), which are characterized by the production of normal amounts of mutant globin chains having deranged physical or chemical properties, thalassemias are quantitative disorders: the primary lesion lies in the amount of globin produced. However, some rare forms of thalassemia are characterized by the production of structurally abnormal globin chains in reduced amounts. These thalassemic hemoglobinopathies share features of both thalassemia and structural hemoglobinopathies. [9]

Some mutations alter the patterns of fetal to adult hemoglobin switching. These conditions, called hereditary persistence of fetal hemoglobin (HPFH), are not generally associated with clinical symptoms; nonetheless, they merit consideration in this chapter. Their importance lies in their role as modulating factors when co-inherited with other hemoglobinopathies, and in their usefulness both as models for investigating the molecular basis for globin gene regulation during human development and as paradigms for rational therapy for the major -chain hemoglobinopathies, namely, sickle cell anemia and -thalassemia.

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ETIOLOGY, EPIDEMIOLOGY, AND PATHOPHYSIOLOGY

The thalassemias are inherited as pathologic alleles of one or more of the globin genes located on chromosomes 11 and 16 (see Chap. 22). These lesions range from total deletion or rearrangement of the loci to point mutations that impair transcription, processing, or translation of globin mRNA. The precise nature of the defects is summarized in a later section.

Thalassemias have been encountered in virtually every ethnic group and geographic location. They are most common in the Mediterranean basin and equatorial or near-equatorial regions of Asia and Africa. The thalassemia belt extends along the shores of the Mediterranean and throughout the Arabian peninsula, Turkey, Iran, India, and southeastern Asia, especially Thailand, Cambodia, and southern China. [10] [11] [12] [13] The prevalence of thalassemia in these regions is in the range of 2.515%. Like sickle cell anemia, thalassemia is most common in those areas historically afflicted with endemic malaria. Malaria seems to have conferred selective survival advantage to thalassemia heterozygotes, in whom infection with the malaria parasite is thought to result in milder disease and less impact on reproductive fitness.[1] [2] [14] Therefore, the gene frequency for thalassemia has become fixed and high in populations exposed to malaria over many centuries.

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PATHOPHYSIOLOGYGENERAL PRINCIPLES

The pathophysiology and molecular genetics of individual forms of thalassemia are closely intertwined. Therefore, detailed consideration of these syndromes is best deferred to their individual subsections. This section considers only mechanisms common to the pathogenesis of all of these syndromes. [1] [2] [3] [4] [5] [15]

The primary lesion in all forms of thalassemia is reduced or absent production of one or more globin chains. For all practical purposes, the major impact on clinical well-being occurs only when these lesions affect the - or -globin chains necessary for the synthesis of hemoglobin (Hb A; $_{2}$ $_{2}$). (Severe impairment of -, epsilon-, or -globin production is presumably lethal in utero.) One consequence of reduced globin chain production is immediately apparent: reduced production of functioning hemoglobin tetramers. As a result, hypochromia and microcytosis are characteristic of virtually all patients with thalassemia. In the milder forms of the disease, this phenomenon may be barely detectable.

The second consequence of impaired globin biosynthesis is unbalanced synthesis of the individual - and -subunits. Hemoglobin tetramers are highly soluble and have reversible oxygen-carrying properties exquisitely adapted for both oxygen transport and delivery under physiologic conditions. Free or unpaired -, -, and -globin chains are either highly insoluble or form homotetramers (Hb H and Hb Barts) that are incapable of releasing oxygen normally and are relatively unstable and precipitate as the cell ages. For poorly understood reasons, no compensatory regulatory mechanism exists whereby impaired synthesis of one globin subunit leads to a compensatory downward adjustment in the production of the other (partner) globin chain of the hemoglobin tetramer. Thus, useless excess -globin chains continue to accumulate and precipitate in -thalassemia, whereas excess -globin chains form Hb H in -thalassemia. During uterine development, excess -globin chains form Hb Barts in -thalassemic individuals.

The abnormal solubility or oxygen-carrying properties of these chains lead to a variety of physiologic derangements. Indeed, in the severe forms of thalassemia, it is the behavior of the unpaired globin chains accumulating in relative excess that dominates the pathophysiology of the syndrome, rather than the mere underproduction of functioning hemoglobin tetramers. The precise complications of this pathophysiologic phenomenon are diverse and depend on both the amount and the identity of the globin chain accumulating in excess. For the moment, the fundamental principle that must be appreciated is that thalassemias cause symptoms both by underproduction of hemoglobin and by accumulation of unpaired globin subunits. The unpaired subunits are usually the major source of morbidity and mortality.

The predominant circulating hemoglobin at the moment of birth is fetal hemoglobin (Hb F [$_{2}$ $_{2}$]) (see Chap. 22). Although the switch from - to -globin biosynthesis begins before birth, the composition of hemoglobin in the peripheral blood changes much later because of the long life span of normal circulating red cells. Hb F is thus slowly replaced by adult hemoglobin (Hb A), so that infants do not depend heavily on normal amounts and function of Hb A until they are 46 months old. The pathophysiologic consequences of these considerations are that -chain hemoglobinopathies tend to be symptomatic in utero and at birth, whereas individuals with -chain abnormalities are asymptomatic until 46 months of age. These differences in the onset of phenotypic expression arise because -chains are needed to form both Hb F and Hb A, whereas -chains are required only for Hb A.

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-THALASSEMIA SYNDROMES

Nomenclature

Many different mutations cause -thalassemia and its related disorders such as -thalassemia and the silent carrier state. They are inherited in a multitude of genetic combinations responsible for a heterogeneous group of clinical syndromes. -Thalassemia major, also known as Cooleys anemia or homozygous -thalassemia, is a clinically severe disorder that results from the inheritance of two -thalassemia alleles, one on each copy of chromosome 11. As a consequence of diminished Hb A synthesis, the circulating red cells are very hypochromic and abnormally shaped; they contain markedly reduced amounts of hemoglobin. Accumulation of free -globin chains leads to the deposition of precipitated aggregates of these chains to the detriment of the erythrocyte and its precursor cells in the bone marrow. The anemia of thalassemia major is so severe that chronic blood transfusions are usually required.

The term -thalassemia intermedia is applied to a less severe clinical phenotype in which significant anemia occurs but chronic transfusion therapy is not absolutely required. It usually results from the inheritance of two -thalassemia mutations, one mild and one severe; the inheritance of two mild mutations; or occasionally, the inheritance of complex combinations, such as a single -thalassemia defect and an excess of normal -globin genes, or two -thalassemia mutations co-inherited with heterozygous -thalassemia (in this last form, known as -thalassemia, the -thalassemia allele reduces the burden of unpaired -chains). [16] [17] [18] Simple heterozygosity for certain forms of -thalassemic hemoglobinopathies can also be associated with a thalassemia intermedia phenotype, sometimes called dominant -thalassemia. [19] [20]

Thalassemia minor, also known as -thalassemia trait or heterozygous -thalassemia, is due to the presence of a single -thalassemia mutation and a normal -globin gene on the other chromosome. It is characterized by profound microcytosis with hypochromia but mild or minimal anemia.

-Thalassemia is also called by several other names, including Cooleys anemia, Mediterranean anemia, von Jakschs anemia, [1]

TABLE 29-1 -- **Common -Thalassemia Mutations in Different Racial Goups**

Racial Group	Description
Mediterranean	IVS-1, position 110 (G A)
	Codon 39, nonsense (CAG TAG)
	IVS-1, position 1 (G A)
	IVS-2, position 745 (C G)
	IVS-1, position 6 (T C)
	IVS-2, position 1 (G A)
Black	29, (A G)
	88, (C T)
	Poly(A), (AATAAA AACAAA)
Southeast Asian	Codons 41/42, frameshift (CTTT)
	IVS-2, position 654 (C T)
	28, (A G)
Asian Indian	IVS-1, position 5 (G C)
	619-bp deletion
	Codons 8/9, frameshift (+G)
	Codons 41/42, frameshift (CTTT)
	IVS-1, position 1 (G T)

Data from Kazazian and Boehm[21] and personal communication, 1993.

target cell anemia, erythroblastic anemia, and familial macrocytosis.

Molecular Pathology

Forms of -thalassemia arise from mutations that affect every step in the pathway of globin gene expression: transcription, processing of the mRNA precursor, translation of mature mRNA, and post-translational integrity of the -polypeptide chain (Fig. 29-1 and Table 29-1). Large deletions removing two or more non--genes are found in rare cases, as are smaller partial or total deletions of the -gene alone (Fig. 29-1). Most types of -thalassemia are due to point mutations affecting one or a few bases. (The original literature for this section is massive; it

Figure 29-1 Model of human -globin gene showing sites and types of various mutations causing -thalassemia. *(From Kazazian,[23] with permission.)*

is summarized in several reviews.[2] [3] [4] [5] [21] [22] [23] [24] [25] [26]) Of the more than 125 point mutations causing -thalassemia, about 15 account for the vast majority of affected patients, with the remainder responsible for the disorder in only relatively few patients. It has been determined that five or six mutations usually account for more than 90% of the cases of -thalassemia in a given ethnic group or geographic area (Table 29-1).[22] [23]

Transcription

Several mutations alter promoter regions upstream of the -globin mRNA sequence, impairing mRNA synthesis, whereas mutations that derange the sequence used as the signal for addition of the poly-(A) tail (polyadenylation signal; see Chap. 22) have been shown to result in abnormal cleavage and polyadenylation of the nascent mRNA precursor, with resulting reduced accumulation of mature mRNA.[21] [22] [23] [24]

Processing

Many forms of -thalassemia are due to mutations that impair splicing of the mRNA precursor into mature mRNA in the nucleus or that prevent translation of the mRNA in the cytoplasm. The molecular pathology of splicing mutations is complex (Fig. 29-1). Some base substitutions ablate the donor (GT) or acceptor (AG) dinucleotides (see Chap. 22), which are absolutely required at the intron-exon boundaries for normal splicing, and thereby completely block production of mature functional mRNA. Thus, no -globin can be synthesized (^{0} -thalassemia). Other mutations alter the consensus sequences that surround the GT- and AG-invariant dinucleotides and decrease the efficiency of normal splicing signals by 7095%, resulting in $^{+}$ -thalassemia; some consensus mutations even abolish splicing completely, causing ^{0} -thalassemia. A third type of splicing aberration results from mutations that are not in the immediate vicinity of a normal splice site. These alter regions within the gene, called cryptic splice sites, which resemble consensus splicing sites but do not normally sustain splicing (Fig. 29-2). The mutations activate the site by supplying a critical GT or AG nucleotide or by creating a sufficiently strong consensus signal to stimulate splicing at that site 60100% of the time. The activated cryptic sites generate an abnormally spliced, untranslatable mRNA species. Only 1040% of the mRNA precursors are thus spliced at the normal sites, which causes $^{+}$ -thalassemia of varying severity. The mutation responsible for the most common form of -thalassemia among Greeks and Cypriots (Fig. 29-2) activates a cryptic splice site near the 3 end of the first intron (position 110). [27] [28] The determinants that dictate the degree to which each mutation alters splice site utilization remain largely unknown.

Translation

Mutations that abolish translation occur at several locations along the mature mRNA and are very common causes of ^{0} -thalassmia (Fig. 29-1 and Table 29-1). The most common form of ^{0} -thalassemia in Sardinians results from a base substitution in the gene that changes the codon encoding the 39th amino acid of the -globin chain from CAG, which encodes glutamine,

Figure 29-2 $^{+}$ -Thalassemia arising from alternative mRNA splicing due to a mutation activating a cryptic splicing site. **(A)** The g a mutation is shown enclosed in squares, located near the 3 end of intron 1 (IVS-1); it creates a sequence motif closely mimicking a pre-mRNA acceptor splice site. The product of the alternative splicing event is also shown. Note that use of the activated cryptic site generates a mature mRNA that contains an in-frame termination codon and therefore does not encode a functional -globin chain. *(From Benz,[21] with permission).* **(B)** Diagram of the means by which use of the cryptic splice site 90% of the time (the observed value) causes only 10% of the mRNA precursor molecules to be spliced normally into translatable mature mRNA, thus causing $^{+}$ -thalassemia. *(From Bunn and Forget,[2] with permission.)*

Figure 29-3 ^{0} -Thalassemia arising from a mutation changing an amino acid codon to a termination codon (nonsense mutation). *(From Takeshita et al.,[309] with permission.)*

to TAG, whose equivalent (UAG) in mRNA specifies termination of translation (Fig. 29-3).[29] [30] A premature termination codon totally abrogates the ability of the mRNA to be translated into normal -globin. Premature translation termination also results indirectly from frameshift mutations (i.e., small insertions or deletions of a few bases, other than multiples of 3, that alter the phase or frame in which the nucleotide sequence is read during translation). [30] An in-phase premature termination codon is invariably encountered within the next 50 bases downstream from a frameshift.

Other Sites

Rare mutations that affect gene function by intriguing mechanisms have been described. An extremely large deletion of the -globin gene cluster has been described that removes the epsilon-, -, and -genes. [31] The patient has a severe -thalassemia phenotype, but the -globin gene and 500 bases of adjacent 5 and 3 DNA have an entirely normal nucleotide sequence. The -gene functions normally in surrogate cells. The important aspect of this deletion is that it removes the critical locus control region (LCR)[3] [5] [32] (see Chap. 22) located thousands of bases upstream from the beginning of the globin gene cluster at the 5 end of the epsilon-globin gene; loss of this region severely impairs -gene expression. A number of additional deletions involving the LCR and various portions of the -gene cluster, but sparing the -gene itself, have the same phenotype.[3] [4] [5] [32] In other cases of -thalassemia, the -gene and adjacent DNA are structurally normal, and the basis of abnormal gene expression is unknown.[23]

The relationship between an individual mutation and the clinical severity of the -thalassemia phenotype associated with that particular mutation is complex. [22] [23] For example, the A G mutation at position 29 of the -gene promoter commonly encountered in blacks is associated with a different clinical severity than that found in Chinese patients inheriting the same mutation.[33] Clearly, the genetic context of the mutation is different in the two populations. The mutant -globin gene in the two different racial groups probably arose in different chromosomal backgrounds that have different potentials for -gene expression, as discussed in the following paragraph.

Multiple forms or haplotypes of normal non--globin gene clusters exist in various human populations. These are defined by the patterns of restriction fragment length polymorphisms[34] detected when DNA is digested with restriction endonucleases and analyzed by Southern gene blotting for the fragments bearing the non--globin genes. Haplotypes differ according to whether each restriction site is present along the gene cluster. More than 12 haplotypes have been defined by examination of several restriction sites located along the cluster that are present or absent in a polymorphic manner in normal individuals. [34] The clinical variability encountered in two different groups bearing identical primary mutations correlates best with the haplotype or chromosomal background on which the mutation is inherited. The differences in physiologically important functions among haplotypes that modulate severity remain unknown, but a possible explanation lies in the varying ability of the -globin genes on different chromosomes to respond to severe erythroid stress (see Chap. 15) by increased expression during postnatal life. The -globin genes carried on some haplotypes differ in the degree to which they can respond in this manner. [35] Because Hb F synthesis reduces the severity of -chain hemoglobinopathies,[1] the level of -gene expression from a given chromosome can play an important modulating role.

Pathophysiology

The biochemical hallmark of -thalassemia is reduced biosynthesis of the -globin subunit of Hb A ($_{2}$ $_{2}$). In -thalassemia heterozygotes, -globin synthesis is about half-normal (/ synthetic ratio 0.50.7). In homozygotes for ^{0} -thalassemia, who account for about one-third of patients, -globin synthesis is absent. -Globin synthesis is reduced to 530% of normal levels in $^{+}$ -thalassemia homozygotes or $^{+}$ /^{0} -thalassemia compound heterozygotes, who together account for about two-thirds of cases. [1]

Because synthesis of Hb A ($_{2}$ $_{2}$) is markedly reduced or absent, the red cells are hypochromic and microcytic. -Chain synthesis is partially reactivated, so that the hemoglobin of the patient contains a relatively large proportion of Hb F. [1] However, these -chains are quantitatively insufficient to replace -chain production.

In heterozygotes (with -thalassemia trait), relatively little -globin accumulation occurs. Output from the single normal -globin gene supports substantial Hb A formation, thus preventing harmful accumulation of excess -globin chains. Thus, one encounters hypochromia with microcytosis but relatively little evidence of anemia, hemolysis, or ineffective erythropoiesis.

Individuals inheriting two -thalassemic alleles experience a more profound deficit of -chain production. Little or no Hb A is produced; more important, the imbalance of - and -globin production is far more severe (Fig. 29-4). The limited capacity of red cells to proteolyze the excess -globin chains, a capacity that probably exerts a protective effect in heterozygous -thalassemia, is overwhelmed in homozygotes. Free -globin accumulates, and unpaired -chains aggregate and precipitate to form

inclusion bodies, which cause oxidative membrane damage within the red cell, [36] and destruction of immature developing erythroblasts within the bone marrow (ineffective erythropoiesis). [37] Consequently, relatively few of the erythroid precursors undergoing erythroid maturation in the bone marrow survive long enough to be released into the blood as erythrocytes. The occasional erythrocytes that are formed during erythropoiesis bear a burden of inclusion bodies. These abnormal cells are removed

Figure 29-4 Pathophysiology of severe forms of -thalassemia. The diagram outlines the pathogenesis of clinical abnormalities resulting from the primary defect in -globin chain synthesis.

prematurely by the reticuloendothelial cells in the spleen, liver, and bone marrow, producing hemolytic anemia.

Defective -globin synthesis exerts at least three distinct yet interrelated effects on the generation of oxygen-carrying capacity for the peripheral blood (Fig. 29-4): (1) ineffective erythropoiesis, which impairs production of new red cells; (2) hemolytic anemia, which shortens the survival of the few red cells produced; and (3) hypochromia with microcytosis, which reduces the oxygen-carrying capacity of those few red cells that do survive. In the most severe forms of the disorder, these three factors conspire to produce a catastrophic anemia, complicated by the effects of exuberant hemolysis.

The profound deficit in the oxygen-carrying capacity of the blood stimulates production of high levels of erythropoietin in an attempt to promote compensatory erythroid hyperplasia. Unfortunately, the ability of the marrow to respond is markedly impaired by ineffective erythropoiesis. Massive bone marrow expansion does occur, but very few erythrocytes are actually supplied to the circulation. The marrow becomes packed with immature erythroid progenitors, which die from their -globin burden before they reach the reticulocyte stage. Profound anemia persists, driving erythroid hyperplasia to still higher levels. In some cases, erythropoiesis is so exuberant that masses of extramedullary erythropoietic tissue form in the chest, abdomen, or pelvis.

Massive bone marrow expansion exerts numerous adverse effects on the growth, development, and function of critical organ systems. Children with thalassemia develop a characteristic chipmunk facies due to maxillary marrow hyperplasia and frontal bossing (Fig. 29-5). Thinning and pathologic fractures of the long bones and vertebrae arise from cortical invasion by erythroid elements. Profound growth retardation and endocrine malfunction are common.

Hemolytic anemia results in massive splenomegaly and high-output congestive heart failure. Conscription of so many caloric resources for futile sustenance of erythroid hyperplasia leads to inanition, susceptibility to infection, and, in untreated cases, death during the first two decades of life. Treatment with red cell transfusions sufficient to maintain hemoglobin levels at >9.010.0 g/dl improves oxygen delivery, suppresses the excessive ineffective erythropoiesis, and prolongs life. Unfortunately, as discussed in more detail later, complications of chronic transfusion therapy, including iron overload, in the past usually proved to be fatal before age 30. Iron chelation therapy, initiated at an early age, can prolong survival beyond the third decade.

Clinical Manifestations

The advent of modern hypertransfusion therapy has had a major impact on the clinical and laboratory features of thalassemia major. These regimens have ameliorated many of the most striking

Figure 29-5 Thalassemic facies. See text for description. *(From Jurkiewicz et al.,[252] with permission.)*

manifestations of the disease. The disastrous symptom constellation so prevalent in the past is now, at least in North America and most industrialized countries, of largely historical importance. Nonetheless, the clinical manifestations and complications of untreated or undertreated -thalassemia major described in this section best illustrate the principles of the pathophysiology just outlined. These descriptions apply to the

CLINICAL HETEROGENEITY OF THALASSEMIA

The severity of -thalassemia is remarkable for its variability in different patients. Two siblings inheriting identical thalassemia mutations sometimes exhibit markedly different degrees of anemia and erythroid hyperplasia. Many factors contribute to this clinical heterogeneity. Individual alleles vary with respect to severity of the biosynthetic lesion. Other modulating factors ameliorate the burden of unpaired -globin inclusion bodies. Co-inheritance of the -thalassemia trait actually reduces clinical severity because it restricts production of excess -globin. High levels of Hb F expression persist to widely varying degrees in -thalassemia. Since -globin can substitute for -globin, simultaneously generating more functional hemoglobins and reducing the -globin inclusion burden, this is a powerful modulating factor. Patients may also vary in their ability to solubilize unpaired globin chains by proteolysis. Occasional heterozygous patients have had more severe anemia than expected, apparently because of defects in these proteolytic systems; or because of the type of thalassemic mutation. Inheritance of more than the usual complement of -globin genes has also been claimed to increase with severity of -thalassemia because of additional production of unpaired -globin chains. All these factors emphasize the essential role of -globin inclusions in the pathophysiology of -thalassemia.

disease as it was seen before 1965 and is still prevalent in many parts of the world. Current clinical management and associated clinical manifestations and complications have been reviewed in a number of publications. [38] [39] [40] [41] [42] [43]

Protected by prenatal Hb F production, the infant with Cooleys anemia is born free of significant anemia, although deficient -chain synthesis can be demonstrated at birth. Quantitative Hb A determinations of cord blood reveal that thalassemia homozygotes have <2% Hb A; heterozygotes have 6.89.9%, and normals have 20%. [44] Clinical manifestations usually emerge during the second 6 months of life. The diagnosis is almost always evident by 2 years of age. [45] Pallor, irritability, growth retardation, abdominal swelling due to enlargement of the liver and spleen, and jaundice are the usual presenting features. [46] Facial and skeletal changes develop later. Untreated victims die in late infancy or early childhood as a consequence of severe anemia. In a retrospective review from Italy, the average survival of children with untreated thalassemia major was less than 4 years; approximately 80% died in the first 5 years of life. [47]

Clinical and Laboratory Evaluation

Blood

The anemia of thalassemia major is characterized by severe hypochromia and microcytosis. The hemoglobin level decreases progressively during the first months of life. When the child becomes symptomatic, the hemoglobin level may be as low as 34 g/dl. Red cell morphology is strikingly abnormal, with many microcytes, bizarre poikilocytes, teardrop cells, and target cells (Fig. 29-6). A characteristic finding is the presence of extraordinarily hypochromic, often wrinkled and folded cells

(leptocytes) containing irregular inclusion bodies of precipitated -globin chains.

Nucleated red cells are frequently present. The reticulocyte count is 28%, lower than would be expected in view of the extreme erythroid hyperplasia and hemolysis. The low count reflects the severity of intramedullary erythroblast destruction. The white blood cell (WBC) count is elevated. A moderate polymorphonuclear

Figure 29-6 Morphology of the peripheral blood film in severe -thalassemia. Note the many bizarre cells, the hypochromia, nucleated RBCs, target cells, and leptocytes. *(From Pearson and Benz,[308] with permission.)*

leukocytosis and normal platelet count are typical unless hypersplenism has developed. The bone marrow exhibits marked hypercellularity consequent on normoblastic hyperplasia. These red cell precursors also show defective hemoglobinization and reduced amounts of cytoplasm. Prodigious elevations in circulating nucleated RBCs, an increased WBC count, and thrombocytosis all occur after splenectomy.

The osmotic fragility is strikingly abnormal. The red cells are so markedly resistant to hemolysis in hypotonic sodium chloride solution that some are not entirely hemolyzed, even in distilled water. The serum iron is increased, and iron-binding proteins are fully saturated. [48]

The hemoglobin profile reveals predominantly Hb F. In patients with homozygous ^{0} -thalassemia, no Hb A is found. In the newborn with $^{+}$ -thalassemia, about 90% is Hb F; with advancing age Hb F slowly decreases, but it is always considerably higher than normal (by 1090%). Transfusions will confound the estimation of the true Hb F level. The Hb A_2 /Hb A ratio, which in the normal person is about 1:40, is increased to >1:20 in thalassemia trait, but Hb A_2 levels in thalassemia major are variable, probably because of increased numbers of F cells that have a decreased Hb A_2 content.[1] Other biochemical abnormalities of the red cell in thalassemia major include a postnatal persistence of the i antigen and a decrease of red cell carbonic anhydrase; these findings are probably also due to the elevated levels of circulating F cells.

The intraerythrocytic inclusions in the peripheral blood cells, first described by Fessas, [49] are especially prominent after splenectomy. These inclusions, best seen by supravital staining with methyl violet or by phase microscopy, are aggregates of precipitated, denatured -chains. [50] They are also found in large numbers within erythroid precursors in the bone marrow.

The serum is icteric; unconjugated bilirubin levels are in the range of 2.04.0 mg/dl. Hepatitis, biliary obstruction from gallstones, or cholangitis should be considered if the value is higher. Red cell survival in thalassemia major is variable but usually markedly decreased. The ^{51}Cr half-life ranges between 6.5 and 19.5 days, in contrast to the normal half-life of 2535 days.[37] Increased plasma iron turnover and poor utilization of radiolabeled iron indicate ineffective erythropoiesis. [37] Serum SGOT and SGPT are frequently increased, reflecting hepatic damage secondary to hemosiderosis or viral hepatitis. Lactate dehydrogenase levels are markedly elevated as a consequence of ineffective erythropoiesis. Haptoglobin and hemopexin are reduced or absent. [51]

Low levels of serum zinc are present. A relationship between this finding and growth failure has been postulated but not established. [52] [53] Low levels of serum and leukocyte ascorbic acid are common in thalassemic patients because increased metabolism of the vitamin to oxalic acid occurs in the presence of iron overload. [54] Biochemical evidence of folic acid deficiency has been described and is presumably due to excessive consumption secondary to increased requirements. [55] [56] Daily supplementation with 1.0 mg of folic acid is therefore reasonable, especially if the diet is not optimal. The large amounts of iron present in patients with thalassemia major, coupled perhaps with malabsorption secondary to pancreatic and hepatic fibrosis, may lead to decreased levels of vitamin E. Serum levels of -tocopherol are often reduced to less than <0.5 mg/dl, and increased red cell membrane lipid peroxidation has been described. [57] [58] [59] Unfortunately, therapy with large doses of vitamin E neither improves survival of transfused red cells nor decreases transfusion requirements. [60] Nevertheless, supplemental vitamin E may decrease the toxic effects of iron overload on various tissues.

Coagulation abnormalities similar to those found in patients with liver disease of any cause (i.e., lowered levels of factors II, V, VII, IX, X, and XI) occur, particularly in older patients. Only rarely are the abnormalities sufficient to require specific therapy. A general correlation exists between the coagulation status and the other parameters of hepatic function. Both deteriorate in patients with massive iron overload. [61] Six of nine thalassemia patients studied for platelet function had slightly prolonged bleeding times and abnormal platelet aggregation. [62] Although these changes are probably not of great clinical significance, salicylates should be used with caution. On the other hand, thrombocytosis frequently develops following splenectomy and is thought to contribute to pulmonary vascular obstruction, pulmonary hypertension, and hypoxemia.[63] [64] [65] Salicylates or dipyridamole may be beneficial in such a setting. [65]

Skeletal Changes

Skeletal abnormalities (Fig. 29-7) result primarily from hyperplasia and expansion of the erythroid marrow. [66] These cause widening of the marrow space and thinning of the cortex, with consequent osteoporosis.[67] Striking changes in the skull and facial bones include expansion of the frontal bone with prominent frontal bossing. The membranous bones of the skull do not expand adjacent to the sutures, resulting in a hot cross bun configuration of the skull. Radiographs reveal the diploic spaces to be widened. At first the skull has a granular appearance, but later perpendicular bony trabeculae appear, giving the classic hair on end or crew cut appearance (Fig. 29-7).

The maxilla is regularly involved. Pneumatization of the sinusoids is markedly delayed; marked overgrowth of the maxilla results in severe malocclusion, jumbling of the upper incisors, and prominence of the malar eminences.[68] These bone changes produce the classic facies. The earliest skeletal changes are observed in the metacarpals, metatarsals, and phalanges, where expanded medullary cavities produce a rectangular and then a convex shape (Fig. 29-7). Marked osteoporosis and cortical thinning may predispose to pathologic fractures of the extremities; compression fractures of the vertebrae may also occur (Fig. 29-8). Premature fusion of the epiphyses of the long bones is common in patients who are more than 10 years old. Irregular fusion of the epiphyses of the proximal humerus results in characteristic shortening of the upper arms.[69] [70]

Several abnormalities in the ribs may occur, including notching and osteolytic lesions. [66] [71] The ribs become very wide, especially at the points of their attachment to the vertebral column. Marrow masses may extrude from these sites, creating the appearance of paravertebral masses (Fig. 29-9). An unusual complication is expansion of paravertebral hematopoietic tissue into the spinal canal with resultant cord compression. [72] Decompressive laminectomy or radiation therapy may be necessary to prevent permanent paralysis.[73] [74] Although rarely seen today in properly transfused patients with -thalassemia major, it is not infrequently observed in patients with thalassemia intermedia whose marrow is not suppressed by regular transfusions.

The character and degree of the bone lesions change significantly with age. In older children, the bone lesions regress in the more distal portions of the skeleton (hands, arms, and legs), a feature correlating with the normal developmental replacement of red marrow by fatty marrow. The characteristic changes of the hands and other peripheral areas are thus diminished and may disappear in later life. [75] [76] However, in the skull, spine, and pelvis (which are sites of active, persistent erythropoiesis), the radiographic changes become more conspicuous.[75]

Liver and Gallbladder

Hepatomegaly is prominent in severely affected patients. This is a consequence of extramedullary hematopoiesis initially, so that early hepatomegaly can be reduced by hypertransfusion.[77] Later in the course of the disease, hepatomegaly is associated with extensive cirrhosis. Iron deposition, first present in the Kupffer cells, ultimately engorges the parenchymal cells, resulting in an appearance that is indistinguishable from that of idiopathic hemochromatosis. [78] [79] The hepatocellular injury of

Figure 29-7 Bony abnormalities in severe -thalassemia. **(A & B)** Hair-on-end appearance of the skull, especially obvious in the closeup view shown in B. **(C)** Distortion of the maxillary bones, as well as poor development of the sinus cavities due to opaque masses of extramedullary erythropoiesis. **(D)** Squaring and convexity abnormalities of the hands. *(From Pearson and Benz, [308] with permission.)*

Figure 29-8 Compression fracture of L2 vertebra in a patient with severe -thalassemia. *(From Pearson and Benz,[308] with permission.)*

iron overload may be due to the liberation of hydrolases resulting from initiation by the ferrous form of iron of peroxidative damage of lysosomal membrane lipids. [80]

Viral hepatitis may augment liver damage. In Italy, a high incidence of hepatitis B surface markers (HBsAg) and antihepatitis B core antigen (anti-HBc) and anti-HBs antibodies was found in a group of 253 children. [81] In other reports, a high frequency of chronic active hepatitis was observed. Multiply transfused patients also have a high prevalence of seropositivity for anti-hepatitis C antibody, indicating a likely additional factor contributing to chronic liver disease. It has also been postulated that liver iron overload may facilitate persistence of virus-induced progressive liver diseases. [82] The most important abnormalities of liver function include hypergammaglobulinemia, hypoalbuminemia, moderate decreases in the coagulation factors that are synthesized in the liver, and increased levels of transaminases.[61]

Pigmentary gallstones due to high levels of bilirubin production are found in an increasing number of patients more than 4 years old. Two-thirds of patients have multiple calcified bilirubinate calculi after the age of 15. [83] Gallbladder surgery is not

Figure 29-9 Chest radiograph typical of severe -thalassemia. Note widening of the rib ends and cardiac dilation. *(From Pearson and Benz,[308] with permission.) A B*

usually indicated unless biliary colic or obstructive jaundice has occurred.

Heart

Cardiac abnormalities are important causes of morbidity and mortality in patients with thalassemia major. Cardiac dilation secondary to anemia is almost always present in untreated young children (Fig. 29-9). Unless intensive chelation therapy is started in the first decade

of life, myocardial hemosiderosis is inevitable during the second decade, when serious cardiac disorders become frequent. Early electrocardiographic abnormalities include a prolonged PR interval, first-degree heart block, and premature atrial contractions. [84] Later, ST-segment depression and ventricular ectopic beats constitute ominous indicators of myocardial damage. Periodic evaluation of cardiac function is essential. Echocardiographic estimation of left ventricular function may reveal abnormalities before there are any symptoms. The left ventricular end-systolic pressure-dimension relationship (ejection fraction), as determined by echocardiography, provides a noninvasive measure of ventricular contractility. [85] Sterile pericarditis occurs in many patients with massive iron overload. [86] Although pericarditis is most often attributed to hemosiderosis, an association with -hemolytic streptococcal infection has also been suggested. [87] Therapy is symptomatic: bed rest, treatment of infection, management of superimposed congestive heart failure, and salicylates or corticosteroids for pain. Occasionally pericardiectomy may be indicated.

Cardiomegaly and left ventricular dysfunction progress to chronic refractory congestive heart failure. In addition to standard therapy for failure, the hemoglobin level must be maintained at >1012 g/dl and intensive chelation therapy instituted or continued. The iron-overloaded myocardium has little capacity to improve its performance unless excess iron is removed. Arrhythmias may cause sudden death. Supraventricular tachycardia and atrial fibrillation may necessitate the use of antiarrhythmic agents, even though these agents can further depress ventricular function. These complications are the usual cause of death in patients with thalassemia major.

Lungs

Mild abnormalities of pulmonary function are common. Some patients exhibit primarily restrictive defects; [88] [89] others experience mild to moderate small airway obstruction and hyperinflation. [90] [91] Most patients have a decreased maximal oxygen uptake and anaerobic threshold; these do not normalize after transfusion. [92] As previously mentioned, postsplenectomy thrombocytosis may predispose to pulmonary vascular occlusion and pulmonary hypertension. [63] [64] [65] Treatment with high doses of the iron chelator deferoxamine may also be associated with deterioration of pulmonary function. [93]

Kidneys

The kidneys are frequently enlarged, owing in part to extramedullary hematopoiesis and in part to marked dilation of the renal tubules. [94] The urine is often dark brown, reflecting the excretion of products of heme catabolism. [95] The urine also contains large amounts of urates and uric acid.

Growth and Endocrine Status

Growth retardation, including skeletal and dental age, [96] was common even in young children until the use of hypertransfusion regimens restored relatively normal growth during the first decade. The adolescent growth spurt is delayed or absent without intensive chelation therapy; most patients, even those well maintained by transfusion, do not attain normal stature. [45] [97] Menarche is frequently delayed. Breast development may be poor, and many female patients are oligomenorrheic or amenorrheic. Induction of cyclic uterine bleeding with low-dose estrogen and progesterone may be of psychological benefit. Pregnancy may occur even in patients who have been transfusion dependent from infancy, provided that they have been intensively treated with chelation therapy. [98] [99]

Boys are frequently immature, with sparse facial and body hair. Although spermatogenesis may be normal, libido is often decreased. When lack of secondary sexual characteristics is emotionally disturbing, small doses of androgen may be used to produce phallic enlargement, deepening of the voice, and growth of facial and body hair.

A multicenter study of 250 adolescent patients in northern Italy showed that despite hypertransfusion and 710 years of iron chelation therapy, two-thirds of males and one-third of females more than 14 years old were 2 SD below the mean for height. [100] Many adolescents aged 1218 years lacked any secondary sexual changes of puberty. However, the mean serum ferritin level in the entire group was 3,500 ng/ml, indicating persistence of a high level of excess iron burden in most of this group. More intensive chelation therapy started in the first decade of life frequently will allow normal onset of puberty and development of secondary sexual changes.

Growth retardation and hypogonadism are also found in association with zinc deficiency. Because urinary excretion of zinc is increased by hemolysis, a clinical trial of zinc supplementation was conducted in Turkey; [101] it showed enhanced growth velocity in the treated group. Since these patients did not receive adequate transfusion or chelation therapy, the applicability of these findings to comprehensively treated patients is uncertain.

Abnormal carbohydrate metabolism is common in older patients with thalassemia major. Prepubertal children usually have normal insulin-stimulated glucose metabolism, but pubertal patients exhibit impaired responses despite normal steady-state blood glucose levels. Impaired oral glucose tolerance and higher than normal insulin levels in response to hyperglycemia are also encountered. [102] The defect in these patients appears to be related to insulin resistance, with insulin

deficiency developing later in the progression to diabetes. Multiple endocrine abnormalities such as hypoparathyroidism, [103] [104] hypothyroidism,[105] and hypothalamic pituitary insufficiency[106] are usually detectable only by provocative tests.

Spleen and Splenectomy

Massive splenomegaly is unusual in hypertransfused patients. Thrombocytopenia and neutropenia may result from hypersplenism in untreated patients, although infection and bleeding are unusual. The usual indication for splenectomy is a progressive shortening of the survival rate of transfused blood cells, as evidenced by an increased transfusion requirement. The transfusion requirements of splenectomized patients are considerably less than those of patients whose spleens are intact. [107] [108] A transfusion requirement of >180200 ml/kg/year of packed RBCs usually represents excessive red cell breakdown and a need for splenectomy. Occasionally, serologic evidence of isoimmunization may be documented, permitting selection of compatible donor cells that have normal survival. Red cell survival studies and determination of splenic sequestration by the ^{51}Cr method are not usually of value for prediction of response. Splenectomy should be deferred as long as possible, certainly until after 5 or 6 years of age.

After splenectomy, striking thrombocytosis may occur. Increased numbers of nucleated red cells appear in the blood, and the presence of many red cells containing inclusion bodies composed of precipitated -globin chains can be demonstrated. The urine may become considerably darker.

Patients with thalassemia major are at significant risk for the development of overwhelming, often fatal, infection after splenectomy (postsplenectomy syndrome). The problem is most common in young children. *Streptococcus pneumoniae* causes two-thirds of cases; *Hemophilus influenzae* type B and *Neisseria meningitidis* account for most of the remaining infections. Typically, there is a fulminant clinical course, proceeding from mild fever and headache to hyperpyrexia, prostration, shock, and death within 612 hours; in one study, 1030% of splenectomized children with thalassemia major developed this complication, [109] but antibacterial vaccines administered before splenectomy and prophylaxis with antibiotics following splenectomy have dramatically reduced the incidence.

Removal of the spleen impairs immunity in many ways. The monocyte/macrophage tissues of the spleen are uniquely suited for clearance of bacteria from the blood in the absence of specific antibody.[110] The spleen may actively participate in antibody formation, especially in the early hours of an infection, [111] and is probably crucial to the alternate complement pathway needed for opsonization when specific antibody is lacking. Levels of IgM are also low in splenectomized patients. [112] A role for high levels of serum iron and saturation of iron-binding protein, which may predispose to infection, has been suggested but remains controversial. [113] When bacteria gain entrance to the bloodstream of a splenectomized patient, they are not cleared but proliferate rapidly in the circulation. Enormous numbers accumulate in a relatively short time, causing hyperpyrexia, shock, disseminated intravascular coagulation, adrenal hemorrhage, and, potentially, death.

Splenectomy should clearly be deferred as long as possible. Patients are more likely to have developed humoral immunity to a broad range of bacteria if the spleen is present for the first few years of life. Before splenectomy, polyvalent antipneumococcal, antimeningococcal, and anti- *H. influenzae* vaccines should be administered.[114] [115] [116] Oral penicillin therapy, used as prophylaxis against postsplenectomy infection, is now generally given to splenectomized patients with thalassemia. However, pneumococcal sepsis is not totally preventable, because not all pneumococcal strains are represented in the vaccine. In addition, penicillin-resistant strains of *S. pneumoniae* have emerged with a prevalence in the United States of 510%, or even higher in some localities. Following splenectomy, the patients parents should be instructed to seek medical attention immediately if significant fever (>102°F) develops. In addition to postsplenectomy sepsis, *Yersinia enterocolitica* or pseudotuberculosis infections have been noted in patients with thalassemia major receiving the iron chelator deferoxamine. [117] [118] [119] [120]

Transfusion Therapy

Transfusion therapy for thalassemia was once regarded as a palliative measure. [121] If palliation is defined as the prevention of discomfort and the maintenance of as nearly normal a life as possible, the transfusion programs then in general use were unsatisfactory even for those limited purposes. Symptoms of anemia and the cosmetic and other consequences of overgrowth of erythropoietic tissue rendered life unpleasant and uncomfortable. Because many of the problems of children with thalassemia major could be related to anemia, several centers initiated chronic, more aggressive transfusion programs designed to ameliorate these symptoms. The programs were designed to maintain hemoglobin levels at >910 g/dl. These more vigorous regimens have been designated hypertransfusion.

The clinical benefits of hypertransfusion programs were dramatic. The growth of younger children returned to normal percentiles for height and weight. [122] Erythropoiesis was partially but substantially suppressed, as evidenced by decreased numbers of reticulocytes and normoblasts and marked reduction in the level of Hb F. Enlargement of the liver and spleen receded. Abnormal facies and osteoporosis of long bones either did not develop or regressed. Cardiac dilation improved, and normal age-appropriate activities were possible. [76] [123]

Hypertransfusion programs require an increase of 125% in the amount of administered blood. Fears that this would result in accelerated iron overload, more rapid development of complications, and death at an earlier age were not realized. The life expectancy of children maintained by hypertransfusion programs has not been shortened, probably because the amelioration of severe anemia is associated with a significant reduction in gastrointestinal iron absorption; this offsets to some degree the increased transfusional iron load. The clinical superiority of hypertransfusion has led most treatment centers in industrialized countries to adopt it as standard management. Complications of anemia and erythropoietic hypertrophy have thus become uncommon.

More vigorous transfusional programs (supertransfusion) aimed at keeping hemoglobin levels at >12.0 g/dl have been used to suppress erythropoiesis further. After a transient increase in blood consumption during the first several months, [124] the blood requirements are not increased in splenectomized ^{0}-thalassemia patients during these regimens, although they

THERAPY FOR -THALASSEMIA MAJOR

Current therapy for thalassemia major consists of regular red cell transfusions to maintain a baseline hemoglobin level >9 g/dl, coupled with intensive parenteral chelation therapy with deferoxamine. Splenectomy is appropriate when transfusion requirements increase considerably more than expected for growth; these patients should also receive immunization against pneumococcal and *Hemophilus influenzae* B infections, as well as penicillin prophylaxis. Folic acid supplements may be added to meet the needs of increased erythropoiesis. Specific endocrine deficiencies associated with iron overload may require appropriate interventions. Psychosocial problems are common, as is true for many children with chronic disease; early intervention is necessary. Better treatment, screening, and genetic counseling and accurate prenatal diagnosis have shifted the age distribution of patients; a higher proportion of cases are now occurring in adolescents and adults.

commonly do increase in $^{+}$-thalassemia and in unsplenectomized children. A significant reduction in blood volume can be documented. [125] [126] This probably reflects the blood volume needed to support the expanded marrow tissues at lower levels of transfusion. The long-term effect of this more aggressive transfusion strategy on well-being and prognosis has not been determined.

An experimental transfusion program has been designed to reduce transfusion requirements by using a young population of red blood cells (neocytes) prepared by differential centrifugation or cell separators. [126] [127] These neocyte preparations should circulate longer in the recipient because they have a mean cell age of 30 days, compared with the mean cell age of 60 days of unfractionated blood. However, in two prospective clinical trials, blood requirements were reduced only by means of 13% and 16%.[128] [129] Neocyte preparations are two to four times more expensive than ordinary packed or glycerol-frozen red blood cells. Their use also entails

greater risks of isoimmunization and viral infections owing to the increased number of donor units needed. [128] An even more complicated procedure involving the use of neocyte/gerocyte exchange transfusions has been suggested as a method for further reducing iron overload. [126] [130] The method attempts to remove nearly senescent red cells before they are catabolized, thereby adding their iron to the body stores. Unfortunately, these methods are extraordinarily expensive.

Before the first blood transfusion is given to an infant with thalassemia major, a complete genotype of the RBCs should be obtained. This precaution applies to all patients facing long-term transfusion therapy. This information is valuable for identifying minor blood group incompatibility if isoimmunization develops later. In an Italian cooperative study of 1,435 patients, 5.2% had significant red cell alloantibodies (136 antibodies in 74 patients). [131] Group- and type-specific red cells that are compatible as determined by the indirect antiglobulin reaction should be used. Blood should be as fresh as possible, preferably before 2,3-diphosphoglycerate levels decline (45 days). [132]

Febrile reactions are frequent in patients who have had multiple transfusions. Pretransfusion therapy with acetaminophen and diphenhydramine or prior treatment of cells by freezing in glycerol, thawing, and washing may reduce the severity of the reaction. Glycerol-frozen red cell preparations contain very few leukocytes and produce few febrile reactions; [133] filtered blood is equally effective in preventing reactions. [134] Isoimmunization and transfusion reactions may still occur even with scrupulous techniques. [135] A superimposed autoimmune hemolytic process with circulating autoantibodies has been described in thalassemia patients. [136] A syndrome of post-transfusional hypertension, convulsions, and cerebral hemorrhage of uncertain etiology has also been reported. [137]

In practice, it is usually most efficient to give 13 donor units of red cells every 35 weeks, depending on the size of the patient and the destruction rate of transfused cells. Except for very young patients, those with hypersplenism, and those with thalassemia intermedia, a regularly scheduled transfusion every 3 weeks is both possible and convenient without the need of extra visits for measurement of hemoglobin levels. In more severely anemic patients, several initial transfusions may be necessary to increase the hemoglobin level to 1214 g/dl, but no more than 15 ml/kg of packed red cells should be given within a 24-hour period. More detailed guidelines on transfusion therapy for thalassemia major have been published. [38] [138] [139]

Patients receiving chronic transfusion therapy are at particular risk of acquiring viral infections from blood products, including infections with hepatitis B and C viruses, cytomegalovirus, and human immunodeficiency virus (HIV). Immunization against hepatitis B should be given early to thalassemia major patients, particularly in areas where the virus is common. [140] The current use of serologic screening of donor blood for anti-hepatitis C and anti-HIV antibodies greatly reduces the risk of infections. In a 1987 study of patients in Europe who received transfusions for hemoglobinopathy, 1.6% were shown to be HIV positive, including two patients with clinical AIDS. [141] In New York, where there is a higher incidence of AIDS than in Europe, a 1987 study of 70 chronically transfused thalassemia patients indicated that 17.1% were positive for HIV antibody and one had clinical AIDS. [142] In Philadelphia, only two of 60 similar patients were HIV positive; none have developed AIDS. [143]

Hemosiderosis and Chelation Therapy

Iron accumulation in thalassemia major depends directly on both the number of blood transfusions received and the age of the patient. Transfusional hemosiderosis is the major cause of late morbidity and mortality in thalassemia major. Establishment of a more favorable iron balance should lead to improved survival; this belief has motivated detailed study of various approaches for iron chelation or removal.

The calculated daily acquisition of iron in the transfusion-dependent child average 816 mg of iron, given a dose of 250500 ml of RBCs every month. [144] Excessive gastrointestinal absorption of iron adds to this burden, although absorption is reduced when a hemoglobin level of >9 g/dl is maintained. [145] No physiologic way to induce significant excretion has been found. Phlebotomy, the most efficient method of removing iron in other situations, is obviously precluded in this disease, although it may be applicable in thalassemia intermedia. A pharmacologic approach using specific iron-chelating agents has thus been adopted.

Several drugs with chelating properties have been synthesized or recovered from microorganisms. Many lack iron specificity or are inefficient; others cause significant toxicity. Only one has achieved clinical utility. Deferoxamine mesylate (Desferal, DFO) a siderophore isolated from cultures of *Streptomyces pilosus*, was introduced in 1960; it is a nearly specific iron-chelating agent (i.e., no other ions are chelated) with relatively low toxicity. [146]

Increased iron excretion following administration of DFO is proportional to body iron stores. To offset the amount of iron received by way of transfusions and attain negative iron balance, the chelating agent must cause the daily urinary excretion of 115 mg of iron. In the case of DFO, this was initially achieved in a British controlled study by daily intramuscular injections of 0.5 g/day for several years, which led to a reduced

rate of hepatic iron accumulation and hepatic fibrosis in thalassemic patients. [147] An Australian study using an injection of 1.0 g/day showed decreases in cardiac and hepatic size and improved cardiac function in a small group of patients treated for 210 years. [148] These studies were valuable in that they demonstrated potential efficacy, but lifelong daily intramuscular injection is clearly impractical as a method for dispensing the drug. The observation that iron excretion induced by DFO is markedly enhanced by slow intravenous or subcutaneous injection of the drug was an important advance. [149] A prolonged infusion period permits a longer exposure of the drug to a relatively small chelatable iron pool present at any given time in equilibrium with a nearly nonchelatable pool. In iron-overloaded, ascorbic acid-replete patients, urinary iron excretion >300 mg/day can be accomplished with large continuous intravenous doses. [150]

Slow infusion of DFO will achieve negative iron balance in many transfusion-dependent patients over 45 years of age. [38] [151] [152] [153] [154] [155] This involves daily 10- to 12-hour subcutaneous injections of about 2.0 g of DFO using a small battery-driven pump. The pump impels a syringe that infuses an aqueous solution of DFO through a no. 25 butterfly needle placed under the skin of the abdomen or thigh. Most patients use the pump during sleeping hours. In patients who are poorly compliant with subcutaneous therapy or in those with severe iron overload, home administration of DFO in high doses can be accomplished intravenously via a central venous catheter. The injection site is either a totally implantable reservoir or in the external end of the catheter. Intravenous DFO may also be useful for rapidly lowering the total iron burden and even for reversal of short-term cardiac morbidity.

Measurement of serum ferritin levels is a convenient way to assess efficacy. [153] In compliant patients, a clear drop in ferritin should occur after 1 year of treatment, with continued decline to a level of <1,000 ng/ml in 35 years. [151] [153] [156] Although urinary measurements are usually used to evaluate excreted iron, an approximately equal amount of iron is lost in the stool during long-term therapy.

The optimal age for starting this program has not been established. Some reports describe success in children as young as 24 years, [157] but there may be an adverse effect on growth. Many centers wait until the patient is 56 years old, when significant iron excretion can be accomplished and patient cooperation is better. The hard, painful lumps that occur at the injection sites from time to time can usually be avoided by careful placement of the needle for subcutaneous rather than intradermal injection.

Infections, hypersensitivity, and tachyphylaxis are rare. After 10 years of use, results with DFO protocols are very encouraging, especially in younger patients. [38] [151] [152] [153] [158] Children begun on chelation before 810 years of age show significant decreases in serum ferritin levels and may attain normal levels after 35 years of use. With persistence of DFO administration, the onset of cardiac disease may be prevented, particularly in children started on treatment in the first decade of life. [159] [160] [161] [162] [163] Results in older patients have not been as promising. Many patients begun on chelation therapy after 10 years of age have developed evidence of the progressive cardiac dysfunction associated with hemosiderosis. Nonetheless, severe cardiac disease, either congestive heart failure or ventricular arrhythmias, has been reversed in some patients by intensive high-dose (15 mg/kg/hour for 10 hours per day) intravenous DFO treatment. [152] [164] The treatment may be self-administered nightly at home through a central venous catheter.

A relationship between iron overload and ascorbic acid depletion (first suggested by the epidemiology of scurvy among the Bantu) exists in thalassemia major. [165] [166] Enhancement by ascorbic acid treatment of DFO-induced urinary iron excretion in thalassemia major has been well documented. Administration of 100200 mg/kg/day of oral ascorbic acid results in an approximate doubling of DFO-induced urinary iron excretion. However, cardiac toxicity manifested as arrhythmias and decreased ventricular contractility has been attributed to vitamin C therapy. [167] Ascorbic acid should only be used while DFO is being administered and only in patients who are ascorbate depleted. As ferritin levels decrease toward normal, the efficacy of vitamin C for augmenting iron excretion disappears.

In general, DFO is well tolerated. Chronic administration of >100 mg/kg/day of DFO has caused cataracts in dogs, but this effect has not been observed in thalassemic patients. The most serious current concern regarding toxicity is decreasing visual and auditory acuity. Some clinics have found that many patients experience impaired sight and hearing, which is partially or completely reversible on discontinuing DFO; [168] others have encountered a lower incidence. [169] Any

patient receiving DFO treatment long term should undergo periodic tests of vision and hearing. DFO should be discontinued if abnormalities arise, with cautious re-initiation when abnormalities reverse. High doses of intravenous DFO may cause acute pulmonary disease, which is reversible with discontinuation of the drug. [93]

Existing chelation protocols are highly imperfect, expensive, and inconvenient. Chronic infusion requires a great deal of dedication and persistence on the part of the patient and family; noncompliance is frequent. These realities have stimulated a search for oral chelators. 2,3-Dihydrobenzoic acid, another iron-chelating agent, enhances iron excretion when given orally. However, it is not very effective; iron excretion is increased to only about 4.5 mg/day. [170] A more promising source for a long-awaited effective oral iron chelator consists of the 1-alkyl-3-hydroxy-2-methylpyrid-4-ones, which can be easily synthesized from the inexpensive natural plant product, maltol.[171] [172] [173] [174] [175] [176] One compound, 1,2-dimethyl-3-hydroxypyrid-4-one (L1, or deferiprone), has not been toxic in mice or rabbits at doses effective in humans for oral chelation.[173] Initial clinical trials in London in eight iron-loaded patients, four with -thalassemia major (1326 years old) and four with myelodysplasia (4765 years old), showed maximal 24-hour urinary iron excretions of 4699 mg in thalassemia patients with daily L1 doses of 23 g; these results are comparable to those of urinary iron excretion induced by DFO in the same patients. [174] However, L1 does not increase fecal iron excretion, [177] whereas DFO induces substantial fecal iron excretion in addition to the urinary excretion. These findings may limit the efficacy of L1.

The effectiveness of L1 in reducing body iron burden and serum ferritin is still under investigation. One short-term study showed unchanged serum ferritin levels after L1 therapy.[177] However, other studies showed significant decreases in hepatic iron as well as serum ferritin levels. [178] [179] In a series of patients treated with L1 for a mean duration of approximately 3 years, hepatic iron stores either decreased or remained stable below the threshold level associated with increased risk of cardiac disease from iron overload.[180] However, additional follow-up studies are said to indicate that hepatic iron concentrations and fibrosis actually increased in a substantial percentage of patients treated with L1. [163] [181] Another long-term trial indicated no significant overall change in iron stores, with 50% of patients having liver iron content above the level associated with a high risk of cardiac disease. [182] Both mild adverse reactions, including musculoskeletal pain and stiffness, and infrequent but more serious side effects, including agranulocytosis in approximately 2% of patients, have been reported with L1 therapy. [179] [180] [183] [184] [185] Additional clinical trials with L1 will be necessary to determine both efficacy and safety.

A number of other classes of orally effective iron-chelating drugs are being developed and tested. [175] In the United States, however, DFO is the only effective iron-chelating agent approved

by the Food and Drug Administration for use in patients with transfusional hemosiderosis.

Bone Marrow Transplantation

Since 1982, when bone marrow transplantation (BMT) in thalassemia major was first successfully accomplished, [186] much additional experience has been gained, particularly in Italy. [187] [188] [189] [190] [191] [192] [193] [194] [195] [196] [197] [198] [199] [200] Procedure-related deaths and nonengraftment have occurred; the currently reported mortality of 1020% and disease-free survival after 35 years of 7590% depend on age and iron status at the time of the procedure. Impressive recent results of early BMT suggest that it be considered seriously when feasible; however, the persistent 1020% mortality at most centers, coupled with the improving efficacy of conventional treatment, makes decisions regarding bone marrow transplantation very difficult on both clinical and ethical grounds. [201] [202] [203] [204]

The largest experience has been reported by Guido Lucarelli and associates in Pesaro, Italy. [189] [190] [192] [193] [194] [195] [196] [197] [198] [199] [200] This group has performed more than 800 BMTs for thalassemia major. They have reported that when BMT was performed early in life with marrow obtained from an HLA-matched donor relative, there was a 90% probability of cure in the best prognostic category (class 1), i.e., those who lacked hepatomegaly and portal fibrosis and who were receiving adequate iron chelation therapy. In patients with either hepatomegaly or portal fibrosis (class 2), the event-free survival rate was approximately 80%, whereas in more heavily iron overloaded patients with both hepatomegaly and portal fibrosis (class 3), the event-free survival rate was only approximately 50%. [192] [193] [194] A number of smaller series from other medical centers have been reported, with varying success rates. [195] [196] [197]

The remarkable results in class 1 patients reported by Lucarellis group have led some authorities to recommend that BMT be strongly considered in any young patient with thalassemia major who has a compatible donor.[201] [202] [205] This opinion is probably justified on the basis of the high cure rate, great expense of lifelong conventional transfusion-chelation therapy, and the high prevalence of noncompliance with chronic iron chelation therapy with DFO. Nevertheless, there are dissenting views and certain reservations. [201] [202] [203] [204] [205] [206]

At this time, a decision to recommend or not to recommend BMT in a young thalassemia major patient who has a suitable donor is controversial. Families should be apprised of the risks and potential benefits of the procedure and helped to make an informed decision. If a related HLA-compatible donor is not available, BMT is not generally considered an acceptable option. However, with improved results of BMT in other disorders using matched unrelated donors, partially mismatched family donors, or unrelated cord blood donors, such options may be considered for selected patients.

BMT in older children and adults, many of whom have significant hepatic hemosiderosis and fibrosis, has been less extensively attempted and usually has not been very successful. Nevertheless, Lucarelli et al. [198] recently described excellent results of BMT in older patients who were iron overloaded and had significant hepatic abnormalities. Eighty percent of the adult patients had long-term disease-free survival. These results will lead to a re-evaluation of the use of BMT in older class 2 patients with thalassemia major. Results of BMT in class 3 patients are less encouraging. [194]

After successful BMT, some post-thalassemic patients remain severely iron overloaded and therefore need to undergo a phlebotomy program to reduce iron stores.[206] Rare patients develop a state of stable chimerism in which there is a return of recipient thalassemic erythropoiesis along with a stable proportion of normal donor erythropoiesis that can be sufficient to render the patient no longer transfusion-dependent. [207]

Experimental Therapies

Gene Manipulation and Replacement

Much recent effort has focused on stimulation of -globin gene expression or replacement of defective -globin genes. Enhanced -globin gene expression would ameliorate the unbalanced globin chain synthesis. Normal -globin genes, if introduced into stem cells in such a way as to allow them to function normally, would directly correct the -globin chain deficit.

Active -globin genes are hypomethylated in utero but are methylated and inactive after birth. Hypomethylation of the -globin genes can be induced by the drug 5-azacytidine; indeed, short-term administration of this drug produced the predicted effect in vivo. [208] Despite much subsequent experimental work, it remains unclear whether the effect was due to direct stimulation of fetal genes by demethylation or to recruitment and accelerated differentiation of primitive burst-forming unit-erythroid (BFU-E) progenitor cells, which have greater potential to produce Hb F. [209] [210] [211] Hydroxyurea has an effect on BFU-E similar to that of 5-azacytidine and is a safer drug for long-term use. Short-term as well as longer trials with both agents have been reported in a number of patients with thalassemia or sickle cell disease;[209] [210] [211] [212] [213] [214] [215] [216] [217] [218] [219] [220] [221] [222] [223] total hemoglobin and Hb F levels do rise in some patients with thalassemia intermedia, but toxic effects are frequent and long-term efficacy remains unproved. The combination of hydroxyurea with recombinant hematopoietic growth factors such as erythropoietin has also been tested for possible additional potentiating effects on Hb F production. [211] [224] [225] Recombinant erythropoietin alone has also been administered to patients with -thalassemia intermedia and resulted in a sustained increase in total production of RBCs, but without a specific or selective effect on Hb F production. [226]

Butyrate and other short-chain fatty acids have been demonstrated to augment Hb F production in various animal model systems as well as in man. [209] [217] [227] These compounds are thought to act by altering chromatin configuration, perhaps due to increased histone acetylation. Short-term treatment with intravenous infusions of arginine butyrate in a limited number of patients resulted in increased levels of -globin chain production that were quite striking in some cases and resulted in a marked increase in /-globin chain synthetic ratios. [228] One patient who was homozygous for Hb Lepore and could not be transfused because of alloimmunization was treated on a compassionate basis for a longer period of time, with a resulting rise in the total hemoglobin level (virtually all Hb F) from less than 5.0 g/dl to approximately 10.0 g/dl over approximately 60 days and healing of a leg ulcer. [228] [229] In a subsequent longer-term trial, five patients with various other forms of severe -thalassemia did not have a sustained hematologic response. [230] In yet another trial, administration of arginine butyrate intravenously in pulsed fashion to patients with sickle cell anemia and thalassemia resulted in favorable hematologic responses, raising the possibility of hematologic toxicity and suboptimal responses with continuous infusions of the drug. [231] Orally absorbable compounds also have been reported to result in an increase in F cell and -globin chain production, but the

effect was not as sustained or as quantitatively important as that originally obtained with intravenous arginine butyrate. [232] [233] [234] [235] [236] [237] Although no severe toxic side effects were observed in these relatively short-term trials in humans, the infusion of high doses of butyrate into baboons did result in significant neurologic toxic effects.[238] Further studies will be required to assess the long-term safety and efficacy of therapy with butyrate.

Successful gene replacement in thalassemia remains a goal for the future despite intensive investigation in this area. Techniques for isolating specific human genes and inserting them into the DNA of other cells have been developed. [239] However, formidable problems must be solved before it becomes routinely possible to place genes in a human stem cell, ensure their

safe and active expression at effective levels, and preserve normal growth, differentiation, and proliferation of the genetically transformed stem cell. [240] [241] [242]

Thalassemia Intermedia

Approximately 10% of patients with homozygous -thalassemia exhibit a phenotype characterized by intermediate hematologic severity. [1] [2] [3] [4] The / ratios of globin chain synthesis are usually higher than those found in typical thalassemia major, indicating the presence in these patients a less severe defect in -globin chain synthesis. For example, homozygous -thalassemia in blacks, Portuguese, and others may be relatively mild, at least for the first two decades. [243] [244] Homozygotes or mixed heterozygotes for forms of -thalassemia associated with normal Hb A_2 and normal Hb F (silent carrier state) also tend to have mild to moderate disease. [245] [246] Certain patients have a milder clinical phenotype because they have co-inherited a form of -thalassemia, [16] [17] [18] or because they carry one (or two) -thalassemia chromosome(s) with a higher than usual potential for high levels of -globin gene expression.

Individuals with thalassemia intermedia do not need regular blood transfusions and usually experience only moderate splenomegaly. The ability to maintain a hemoglobin level compatible with comfortable survival in the absence of regular transfusion is the generally accepted criterion for the diagnosis of thalassemia intermedia. Some of these patients can survive well into adult life, frequently with normal maturation and sexual development. However, some patients with thalassemia intermedia develop cardiomegaly, osteoporosis, fractures, arthritis, and splenomegaly. Complications of extramedullary hematopoiesis in the thorax, skull, and pelvis may occur, including spinal cord compression and growths resembling tumors or abscesses. [247] Disfiguring facial changes may result in a grotesque appearance that causes considerable emotional and psychological distress. Complications can be prevented by a transfusion program designed to suppress erythropoiesis by maintaining normal hemoglobin levels, in which case the patients must be reclassified as having thalassemia major.

These patients pose a therapeutic dilemma. If transfusions are begun, progressive hemosiderosis will develop. However, failure to institute transfusions may be associated with unacceptable morbidity. Each patients treatment must be individualized with consideration of the patients clinical condition, not just the hemoglobin level. An index of the degree of abnormal bone expansion may be useful, [248] but risks of transfusion and iron overload must also be considered. The further availability of more effective oral chelation therapy may make the therapeutic decision easier.

Whether or not patients with -thalassemia intermedia receive regular transfusions, they ultimately experience progressive iron overload because of increased absorption of dietary iron induced by ineffective erythropoiesis. By the third or fourth decade, the total iron burden may attain the levels seen in transfusion-dependent patients.[249] Regular consumption of tea with meals to reduce iron absorption is advisable (the tannins in tea chelate iron in the gut). [250] Chelation therapy with DFO is indicated when patients become significantly iron loaded. [251] The orally effective iron chelator deferiprone (L1) is also effective in thalassemia intermedia. [178] Reconstruction of the maxilla may be needed to provide cosmetic improvement of facial asymmetry and malocclusion.[252] Gallstones regularly occur by the second decade of life. Leg ulcers often occur in late adolescence or afterward, potentially necessitating transfusions until they heal. Folic acid supplementation is particularly important because of the marked marrow hyperplasia. Aplastic crises associated with parvovirus or other infections may result in life-threatening anemia. Splenectomy usually is required in cases of secondary hypersplenism, a state that can worsen the degree of anemia in the absence of transfusions.

Some patients with heterozygous -thalassemia will have a moderately severe clinical disorder, with anemia, hemolysis, and splenomegaly. Some of these patients carry a greater than normal number of -globin genes, owing to triplication of one of the -globin gene loci: /. [253] [254] However, most heterozygous patients with triplicated -globin gene loci are clinically similar to those with simple -thalassemia trait. Most cases of severe heterozygous -thalassemia have so-called dominant -thalassemia which is due to the inheritance of a gene for a -thalassemia hemoglobinopathy associated with a structurally abnormal, unstable, -globin chain that may form inclusion bodies.[20] [21] For those heterozygotes with disease of unusual clinical severity, splenectomy may be beneficial.

-Thalassemia Minor (Thalassemia Trait)

Inheritance of a single -thalassemia allele results in a mild hypochromic microcytic anemia. The hemoglobin level averages 1 or 2 g/dl lower than that seen in normal persons of the same age and sex. Elevations in Hb A_2 and Hb F occur during the early years of life. [255] [256] [257] Hb F levels decline more slowly than normal; the diagnostic elevated Hb A_2 levels are established by about 6 months of age. Strong intrafamilial correlations of both Hb A_2 and mean corpuscular volume (MCV) are noted. [255] Osmotic fragility is decreased; indeed, a one-tube osmotic fragility test has been used for mass screening. [258] The red cell count is increased or normal. The red cells are characteristically hypochromic (mean corpuscular hemoglobin [MCH] <26 pg) and microcytic (MCV <75 fl). The smear shows varying numbers of target cells, poikilocytes, ovalocytes, and basophilic stippling (Fig. 29-10). The reticulocyte count is normal or slightly elevated. Red cell survival is normal, iron utilization is decreased, and slight ineffective erythropoiesis is present. [259] Most patients are asymptomatic.

During pregnancy the anemia of thalassemia trait often becomes more severe, and transfusions are sometimes necessary. Because iron deficiency may occur during pregnancy, iron supplementation has been advised to avoid compounding the causes of anemia. [260] [261] In many instances, iron deficiency anemia is erroneously diagnosed, and iron therapy is given without significant improvement.

Microcytic anemia refractory to iron therapy should always suggest the possibility of thalassemia trait. Although a variety of indices calculated from blood count parameters have been suggested to differentiate thalassemia from iron deficiency, each has some degree of inaccuracy; most are no better than the MCV alone. [262] Direct tests for iron deficiency are preferable. In general, the MCV is rarely >75 fl or the hematocrit <30 in -thalassemia trait. In iron deficiency, the hematocrit usually falls to <30 before the MCV falls to <80 fl. Free erythrocyte porphyrin levels are normal in thalassemia trait but are elevated in iron deficiency (see Chap. 26).[1]

There may be characteristic racial differences in the hematologic severity of -thalassemia trait. In blacks, the condition is invariably milder, red cell morphologic abnormalities less marked, and / synthetic ratios higher than in whites and Asians with the trait. [263]

The diagnosis of -thalassemia trait is established in most instances by the demonstration of altered proportions of Hb A_2. The level of Hb A_2 in -thalassemia trait averages 5.1% (range, 3.57.0%), approximately twice the normal level (1.53.5%); the Hb A_2 /HbA_1 ratio is 1:20 instead of the normal 1:40. This increase is probably due to a post-translational (assembly) phenomenon with increased opportunity for -globin chains to combine with -globin chains in the face of -globin chain deficiency.[2] If concomitant severe iron deficiency anemia occurs, Hb A_2 levels may fall, sometimes into the normal range.[264]

Figure 29-10 Morphology of the peripheral blood film in **(A)** heterozygous -thalassemia and **(B)** heterozygous -thalassemia. Note the profound hypochromia and microcytosis and the many target cells. *(From Pearson and Benz,[308] with permission.)*

Hb F levels are inconsistently elevated in -thalassemia. In about one-half of cases, Hb F is within the normal range (<2.0%); in the remainder it is moderately elevated

(2.15.0%). However, in almost every instance, a minor population of red cells (F cells) containing substantial amounts of Hb F can be demonstrated by the Kleihauer technique.[1] Rarely, an individual with heterozygous thalassemia, as evidenced by a reduced / synthetic ratio or by virtue of having a child with thalassemia intermedia or major, has normal Hb A_2 , Hb F, and hemoglobin electrophoresis, without (silent carrier) or with (quiet carrier) minor hematologic changes. [246] Rare individuals have been encountered who exhibit characteristically abnormal red cell morphology but normal levels of Hb A_2 and Hb F.[1] [2] [3] [4] Such individuals are probably carriers of -thalassemia.[1] [2] [3] [4] Iron deficiency must be excluded and globin chain synthesis or gene mapping studies done to establish a diagnosis with certainty.

Prenatal Diagnosis

Thalassemia mutations can now be routinely and reliably diagnosed by using fetal DNA obtained between the 8th and 18th weeks of gestation. The most reliable methods are based on identification of the abnormal gene by direct DNA analysis. [22] [23] Both amniocentesis and chorionic villus biopsy have been used with success. In experienced hands, the latter method is preferable because adequate amounts of DNA can be obtained safely at an earlier gestational age. A technique has also been developed for the isolation from maternal peripheral blood of fetal nucleated RBCs as a source of DNA for prenatal diagnosis. [265] The DNA is analyzed by a variety of polymerase chain reaction (PCR)-based or other methods for the presence of the thalassemia mutation. [191] [266]

The heterogeneity of thalassemia mutations complicates the approach to antenatal diagnosis. More than 125 independent mutations can cause -thalassemia. [25] [26] However, three major factors have improved the speed, efficacy, and reliability of DNA diagnosis. First, extensive surveys of most populations in which these alleles are frequent have revealed that about 15 -thalassemia mutations account for more than 90% of individuals afflicted worldwide. Within any given ethnic group, three to six mutations usually account for the vast majority of severe cases (Table 29-1).[22] [23] One can thus customize the search for mutations according to the ethnic origins of the family at risk. Second, PCR techniques and exquisitely precise hybridization assays (allele-specific oligonucleotide hybridization), which detect single base changes with great reliability, can now be combined to permit screening of minute DNA samples for several mutations very rapidly (Fig. 29-11). This procedure can also be done using nonradioactive oligonucleotide probes immobilized to filters, so-called reverse dot blot analysis. [267] [268] Third, amplification and direct sequencing of the -globin genes (in cases where the mutation is unknown) can now be carried out by use of PCR techniques in about the time formerly needed to culture amniocytes and perform Southern blot analysis.

Most cases of severe -thalassemia can be diagnosed using similar approaches. The vast majority of cases of clinically significant -thalassemia syndromes arise from gene deletions that are readily detected. Therefore, genetic counseling and antenatal diagnosis should be offered to all families at risk of either severe - or -thalassemia.

Screening for Thalassemia Trait

Mass screening and genetic counseling programs are active in Italy, Greece, and other areas in which the frequency of thalassemia is very high. Population screening, combined with prenatal diagnosis, has dramatically decreased the incidence of thalassemia

Figure 29-11 Example of the use of allele-specific probes for diagnosis of a common form of -thalassemia. The mutation shown is that discussed in Figure 29-2 and the text. Two oligonucleotide probes are synthesized, differing only at the position of the mutation. When hybridized under sufficiently stringent conditions, each probe will anneal only to the gene that is perfectly complementary by Watson-Crick base-pairing. Thus, a homozygous normal fetal DNA sample (N/N) will anneal only to the normal probe, homozygous -thalassemic DNA (T/T) only to the thalassemic probe, and DNA from a heterozygote (N/T) to both probes, but with a reduced intensity to each. *(From High and Benz,[310] with permission.)*

major births in these countries.[269] [270] These programs require a critical mass of expert professional and laboratory backup. Voluntary informed participation, confidentiality of results, and meaningful counseling must be ensured.

The most definitive methods for diagnosis of thalassemia trait include quantitative determinations of Hb A_2 , Hb F, and globin chain synthetic ratios, as well as DNA studies for specific mutations. These are accurate but too expensive for initial mass screening. Since thalassemia is almost invariably associated with significant hypochromia (MCH <26 pg) and microcytosis (MCV <75 fl), determination of red cell indices has been used as a preliminary indicator of possible thalassemia trait. [271] Microcytosis due to iron deficiency must be excluded.[272] A number of alternatives have been described for follow-up diagnostic tests. Measurement of free erythrocyte porphyrin levels is useful in the evaluation of individuals with microcytosis, as results may be obtained rapidly and inexpensively from a drop of blood. [1] Serum ferritin and iron and iron-binding capacity studies are also important in the diagnosis of iron deficiency.

Most screening programs use a simple but sensitive initial screening test such as red cell MCV or osmotic fragility. These tests exclude the great majority of individuals who do not have thalassemia trait, but they do not differentiate precisely between thalassemia trait and iron deficiency. Hb F determinations and hemoglobin electrophoresis are necessary to diagnose -thalassemia and Hb Lepore traits in microcytic persons. No simple screening procedures will detect the so-called silent carrier.

-Thalassemia, which occurs in the same populations as -thalassemia, makes screening more complicated. This diagnosis is suggested by a mild, familial hypochromic microcytosis, with low or normal levels of Hb A_2 and Hb F and no evidence of iron deficiency. Precise diagnosis of -thalassemia requires demonstration of -globin gene deletions or a high /-globin chain synthetic ratio.

Figure 29-12 Genetic origins of the classic -thalassemia syndromes due to gene deletions in -globin gene cluster. Hb Constant Spring (Hb CS) is an -globin chain variant synthesized in such small amounts (12% of normal) that it has the phenotypic impact of a severe nondeletion -thalassemia allele; however, the - ^{cs} allele is always linked to a functioning -globin gene, so that it has never been associated with hydrops fetalis.

-THALASSEMIA SYNDROMES

The -thalassemias are more difficult to diagnose because characteristic elevations in Hb A_2 or Hb F, seen in -thalassemia, do not occur. However, the gene deletions responsible for the most common varieties are readily detectable by molecular biology methods. [271] [272]

Molecular Pathology and Pathophysiology

The four classic -thalassemia syndromes are -thalassemia-2 trait, in which one of the four -globin gene loci fails to function; -thalassemia-1 trait, with two dysfunctional loci; Hb H disease, with three loci affected; and hydrops fetalis with Hb Barts, in which all four loci are defective. These syndromes are usually due to deletion of one, two, three, or all four of the -globin genes, respectively (Fig. 29-12). Nondeletion forms of -thalassemia, which account for 1520% of patients, arise from mutations similar to those described for -thalassemia. [273] [274] Figure 29-13 (Figure Not Available) illustrates the different -thalassemia mutations and phenotypes. Structurally abnormal hemoglobins have been associated with -thalassemia. The Quong Sze -globin chain (^{125LeuPr}) is an exceedingly labile -globin chain destroyed so rapidly after its synthesis that no hemoglobin tetramers can be formed. [275]

-Thalassemia-2 trait is an asymptomatic silent carrier state

Figure 29-13 (Figure Not Available) Pathophysiology of Hb H disease and hydrops fetalis with Hb Barts. *(From Benz,[21] with permission.)*

that is commonly associated with the deletion of a single -globin gene. Offspring of an individual with -thalassemia-2 whose spouse has -thalassemia-1 trait can inherit a form of -thalassemia more severe than either of these, namely Hb H disease.

-Thalassemia-1 trait results from deletion or nonfunction of two -globin alleles. In Asian and Mediterranean populations, a deletion that removes both loci from the same chromosome (cis deletion) is common;[273] [274] homozygosity for -thalassemia-2 (trans deletion) is also seen. In blacks, both -globin genes are only rarely deleted in cis, whereas homozygosity for -thalassemia-2 (trans deletion) is quite common. [276] Both genotypes produce asymptomatic hypochromia and microcytosis.

Hb H disease usually results from co-inheritance of the cis -thalassemia-1 deletion and -thalassemia-2 trait. -Globin chain production is only 2530% of normal; -globin chains accumulate during gestation and -globin chains during adult life (Fig. 29-13 (Figure Not Available)). The unpaired -globin chains are somewhat more soluble than the -globin chains that accumulate in -thalassemia, forming recognizable -tetramers designated Hb H. Hb H forms relatively few inclusions in erythroblasts; rather, it precipitates slowly within mature circulating red cells. Patients with Hb H disease thus have a moderately severe hemolytic anemia but relatively little ineffective erythropoiesis. Patients usually survive into adult life. These clinical observations illustrate the central role of unpaired globin chains and ineffective erythropoiesis as determinants of clinical severity. Hydrops fetalis with Hb Barts results from the homozygous state for the -thalassemia-1 cis deletion. The -globin genes are totally absent; no -globin is produced, so that no physiologically useful hemoglobin accumulates beyond the embryonic stage. Free -globin accumulates, forming $_4$ tetramers called Hb Barts (Fig. 29-13 (Figure Not Available)). Hb Barts has an extraordinarily high oxygen affinity, comparable to that of myoglobin. It binds oxygen delivered to the placenta but releases almost none of it to fetal tissues. Severe asphyxia occurs at the tissue level, causing profound edema (hydrops), congestive heart failure, and death in utero. These fatal complications do not occur in fetuses with Hb H disease because enough Hb F is made to sustain life.
-Thalassemia-2 trait is very common in blacks, having a gene frequency of 2030% in some populations. However, the cis -thalassemia-1 deletion is rare in blacks. Thus, even though -thalassemia-2 trait and the trans deletion form of -thalassemia-1 are very common, Hb H disease is rarely encountered, and hydrops fetalis has not yet been reported in blacks. [276] [277]

Clinical Manifestations

Silent Carrier (-Thalassemia-2 Trait)

-Thalassemia-2 trait has no consistent hematologic manifestations. The red cells are not microcytic, and Hb A_2 and Hb F are normal. During the newborn period, small amounts (3%) of Hb Barts ($_4$) may be seen by electrophoresis or column chromatography. This condition is most often recognized when an apparently normal individual becomes the parent of a child with Hb H disease after mating with a person with -thalassemia-1 trait. The mild excess of -globin chains is probably removed in erythroblasts by proteolysis. [278] -Thalassemia-2 is particularly common in Melanesia, as well as in Southeast Asia and in American blacks, reaching a prevalence of >80% in north coastal Papua New Guinea. At the molecular level, -thalassemia-2 has been found to be associated with two common gene deletions resulting from different nonhomologous crossing over events between the two linked -globin genes: a 3.7-kb rightward deletion (^{3} .7) resulting in a fused $_{2\ 1}$ -globin gene, and a 4.2-kb leftward deletion (^{4} .2) resulting in loss of the 5 ($_2$) gene.[273] [274] [279] The level of -globin gene expression differs in the two conditions, as discussed in the following section.

-Thalassemia Trait (-Thalassemia-1 Trait)

-Thalassemia-1 trait is characterized by levels of Hb A_2 in the low to low normal range (1.52.5%) and / synthetic ratios averaging 1.4:1. During the perinatal period, elevated amounts of Hb Barts are noted (38%). Microcytosis is present in cord blood erythrocytes.

Studies of newborns from the archipelago of Vanuatu in the southwest Pacific and from Papua New Guinea, indicate that homozygotes for the rightward ^{3} .7III deletion (where only a fused $_{2\ 1}$ -globin gene, mostly of the $_2$ type, remains) have lower Hb Barts levels (3.5 ± 0.8%) than those of infants homozygous for the leftward ^{4} .2 deletion (where only the $_1$ -globin gene remains) (6.0 ± 1.4%). These results suggest that the 5 $_2$ -globin gene has a higher output than the 3 $_1$ -globin gene, a conclusion supported by direct measurement of $_2$ /$_1$ mRNA ratios.[280] [281]

Hb H is not detected in hemolysates of peripheral red cells, probably because of rapid proteolysis of Hb H or free -globin chains. However, about 1% of erythroblasts and marrow reticulocytes have inclusions.[276] When an -thalassemia gene occurs in persons who are also heterozygous for -globin chain variant hemoglobins, such as Hb S, Hb C, or Hb E, the proportion of the abnormal hemoglobin is lower than seen in simple heterozygotes. [282] The lower level of the abnormal hemoglobin is due to post-translational control because of higher affinity of ^{A} chains for a limited pool of -globin chains, [283] coupled with proteolysis of the uncombined ^{varian} chains.

Hb H Disease

Hb H disease is associated with a moderately severe but variable anemia, resembling thalassemia intermedia with osseous changes and splenomegaly; [284] however, the clinical phenotype may be considerably milder. It occurs predominantly in Asians and occasionally in whites (Mediterraneans) but is rare in blacks.

Because Hb H is unstable and precipitates within the circulating red cell, hemolysis occurs. Hb H can be demonstrated by incubation of blood with supravital oxidizing stains such as 1% brilliant cresyl blue. Multiple small inclusions form in the red cells (Fig. 29-13 (Figure Not Available)). Electrophoresis of a freshly prepared hemolysate at alkaline or neutral pH demonstrates a fast-moving component amounting to 330% of the total hemoglobin. Concomitant iron deficiency may reduce the amount of Hb H in the patients red cells. [285] A syndrome of Hb H disease associated with mental retardation, other congenital anomalies, and large deletions on chromosome 16 has been noted in several white families. [272] [274]

Hydrops Fetalis with Hb Barts

Hydrops fetalis with Hb Barts occurs almost exclusively in southeastern Asians, especially Chinese, Cambodians, Thais, and Filipinos. Affected fetuses usually are born prematurely and either are stillborn or die shortly after birth. [1] [2] [3] [4] Marked anascara and enlargement of the liver and spleen are present. Severe anemia usually is present, with hemoglobin levels of 310 g/dl. The red cells are markedly microcytic and hypochromic and include target cells and large numbers of circulating nucleated RBCs. These morphologic abnormalities and a negative Coombs test exclude hemolytic diseases due to blood group incompatibility. Hemoglobin electrophoresis reveals predominantly Hb Barts, with a smaller amount of Hb H. A minor component identified as Hb Portland ($_{2}$ $_{2}$) migrating in the position of Hb A is seen also. Normal Hb A and Hb F are totally absent. [286]

Hydropic infants have massive hepatosplenomegaly. Extreme extramedullary erythropoiesis occurs in response to the

profound hypoxia and hemolytic anemia characteristic of this disease. The universal edema characteristic of the hydrops fetalis syndrome is a reflection of severe congestive heart failure and hypoalbuminemia in utero. This is partly a consequence of anemia, but the strikingly abnormal oxygen affinity of the tetrameric Hb Barts is probably the most important determinant of the severe tissue hypoxia. The oxygen dissociation curve of Hb Barts lacks the normal sigmoid form due to noncooperativity during oxygen loading and unloading and is markedly shifted to the left. The shift is so great that little oxygen is released under conditions of low oxygen concentration in the tissues.

Infants with this syndrome do not die in an earlier trimester of pregnancy because of the presence of Hb Portland ($_{2}$ $_{2}$). This hemoglobin does display cooperativity in a manner similar to that of Hb F and therefore has a much more favorable oxygen dissociation pattern than that of Hb Barts. A high incidence of toxemia of pregnancy has been described in women carrying severely affected infants, providing an increased rationale for prenatal diagnosis of this condition.

Prenatal Diagnosis

Using molecular hybridization technology, Dozy, Kan, and associates detected the complete absence of -globin genes in fetal fibroblasts obtained by amniocentesis in a pregnancy at risk of homozygous -thalassemia-1 and the hydrops fetalis syndrome. [287] The presence of hydrops can also be detected by ultrasonography. DNA studies or globin synthesis evaluation may be used to confirm the diagnosis in utero. PCR-based assays are available for the detection of the common -thalassemia-1 deletions. [288]

Therapy

Fetuses with homozygous -thalassemia-1 usually die in utero because of severe hydrops fetalis and are stillborn. However, some infants have had successful blood exchange immediately after birth and have been maintained by chronic transfusion therapy afterward. [289] [290] [291] [292] It is also possible to salvage affected fetuses by in utero blood transfusions. [293] [294]

Patients with Hb H disease usually require neither red cell transfusions nor splenectomy. Splenectomy can result in a clinically important rise in hemoglobin level and thus should be considered in patients with marked anemia. Oxidant drugs can accelerate precipitation of Hb H and exacerbate hemolysis; they should therefore be avoided.

Infants with heterozygous -thalassemia-1 trait lose their Hb Barts during the first few months of life and are left with the hematologic findings of -thalassemia trait, a mild hypochromic microcytosis that persists throughout life. [1] The degree of morphologic abnormality varies greatly among different individuals. That -thalassemia can be easily diagnosed by hemoglobin electrophoresis at birth gives some impetus to cord blood screening studies.

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THALASSEMIC STRUCTURAL VARIANTS

Certain structural hemoglobin variants are characterized by the presence of a biosynthetic defect as well as abnormal structure. [9] Thalassemic hemoglobinopathies are unusual forms of thalassemia caused by such structural variants.

Hb Lepore

Hb Lepore ($_2$ [$_2$]) is the prototype of a group of hemoglobinopathies characterized by fused globin chains. [1] [2] [3] [4] The chains begin with a normal -chain sequence at their N terminus and end with the normal -chain sequence at their C terminus. These hemoglobinopathies arise by unequal or nonhomologous crossover or recombination events that fuse the proximal end of one gene with the distal end of a closely linked structurally homologous gene (Fig. 29-14 (Figure Not Available)). During meiosis, mispairing and crossover of the highly homologous - and -globin genes can occur, resulting in a Lepore chromosome, which contains (in addition to -globin genes) only the fused gene, and an anti-Lepore chromosome, which contains the reciprocal fusion product (), as well as intact - and -globin genes. [1] [2] [3] [4]

Lepore globin is synthesized in low amounts presumably because it is under the control of the -globin gene promoter, which normally sustains transcription at only 2.5% the level of the -globin gene. [295] Patients with Hb Lepore have the phenotype of -thalassemia, distinguished by the added presence of 515% Hb Lepore. By contrast, the anti-Lepore globin (Miyada) is not associated with a -thalassemia phenotype because of the presence of an intact and functionally normal -globin gene on the same chromosome.

Heterozygotes for Hb Lepore have the clinical phenotype of -thalassemia trait; homozygotes are usually similar to patients with homozygous -thalassemia. Compound heterozygotes for

Figure 29-14 (Figure Not Available) Genetic origins of Hb Lepore, anti-Lepore Hb, and Hb Kenya. *(From Benz,[21] with permission.)*

Hb Lepore and a classic -thalassemia allele usually have severe thalassemia. Hb Lepore thus interacts with thalassemia in the same way as a severe -thalassemia gene does, although occasional cases have a milder phenotype of the thalassemia intermedia variety, perhaps due to an associated higher than usual level of -globin gene expression. The presence of Hb Lepore should be suspected in hypochromic microcytic individuals who also have a small amount of an abnormal hemoglobin migrating in the position of Hb S on routine hemoglobin electrophoresis. Hb Lepore accounts for 510% of the -thalassemias seen in Greek and Italian populations. Several forms of Hb Lepore have been described that differ in the position at which the transition from to DNA and amino acid sequence occurs.

An analogous variant, Hb Kenya ([^{A}]$_2$), arises from nonhomologous crossing over between the ^{A} - and -globin genes[296] (Fig. 29-14 (Figure Not Available)) and is associated with the phenotype of ^{G} HPFH. It is now clear that a DNA sequence about 600 bases downstream from the -globin gene acts as a strong enhancer, promoting the erythroid-specific expression of the -globin genes in adult cells. [3] [4] [24] The fused ^{A} gene as well as the linked upstream ^{G} gene are thought to come under the influence of the enhancer, because of its abnormal proximity, and thus are expressed at high levels in adult life. Hb Kenya is rare.

Hb E

Hb E ($_{2\ 2}$ ^{26GluLys}) is a common variant (1530% of the population) in Cambodia, Thailand, parts of China, and Vietnam. Hb E trait resembles very mild -thalassemia trait. Homozygotes exhibit more microcytosis but are still asymptomatic.[297] Compound heterozygotes for Hb E and a -thalassemia gene (Hb E--thalassemia) resemble patients with -thalassemia intermedia or -thalassemia major. Hb E is very mildly unstable, but this instability does not alter red cell life span significantly. The high frequency of the Hb E gene is due to the thalassemia phenotype associated with its inheritance.

The only nucleotide sequence abnormality found in the ^{E} -gene is a base change in codon 26 that causes the amino acid substitution. This mutation, which occurs in a potential cryptic RNA splice region, alters the consensus sequence surrounding a potential GT donor splice site and thus activates the cryptic site. Alternative splicing at this position occurs approximately 4050% of the time, generating a structurally abnormal globin mRNA, which cannot be translated appropriately. [298] The other mRNA precursors are spliced at the normal site, generating functionally normal mRNA, which is translated into ^{E} -globin because the mature mRNA retains the base change that encodes lysine at codon 26.

Hb E is important because it is so common in Southeast Asian populations. With increased emigration of Southeast Asians to North America, Hb E syndromes are being seen there with increased frequency. Genetic counseling of these individuals should emphasize the potential consequences of the interaction of Hb E with -thalassemia. Hb E is also an instructive example of the pleiotropic effects that point mutations may have on the amounts and types of gene products derived from a single mutant gene.

Hb Constant Spring

Hb Constant Spring (Fig. 29-12) is an elongated -globin variant resulting from a mutation that alters the normal translation termination codon. [299] Polyribosomes read through the usual translation stop site and incorporate an additional 31 amino acids until another in-phase termination codon is reached within the 3 untranslated sequence. The amount of ^{cs} mRNA is markedly reduced, and ^{cs} -globin is synthesized in only minute amounts. [279] [300] Six possible mutations of the normal translation termination codon (UAA) in -globin mRNA could result in the generation of a sense codon. [301] Of these, five variants have been identified, each having a markedly underproduced abnormal variant, indicating that disruption of normal translation termination is in some way associated with abnormal mRNA accumulation, presumably due to instability of the mRNA.[279] The output of -globin from the ^{cs} allele is only about 1% of normal, and the gene is thus rendered -thalassemic. The ^{cs} allele has been identified only on chromosomes containing a cis-linked functionally normal -globin gene. [1] [2] [3] [4] Thus, -thalassemia-2 trait and Hb H disease (/^{cs}) associated with Hb Constant Spring are common, but hydrops fetalis cannot occur in association with this variant. Homozygosity for the variant is associated with a relatively severe form of Hb H disease.[1] [2] [3] [4]

Extraordinarily Unstable Hemoglobins

Rare cases of -thalassemia (e.g., Hb Quong Sze[273]) and -thalassemia (e.g., Hb Indianapolis, recently renamed Hb Terre Haute [302] [303]) arise from mutations that produce extremely labile globin chains. The chains fail to pair with the complementary chain, or they precipitate and are degraded so rapidly that they never form

tetramers. These post-translational lesions have the same pathophysiologic effects on hemoglobin biogenesis as reduction of globin mRNA production or function. Another group of -globin chain variants, usually due to mutations in exon 3 of the -globin gene, are associated with inclusion body formation and a phenotype of dominant -thalassemia intermedia.[19] [20]

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HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN

HPFH consists of a group of rare conditions characterized by continued synthesis of high levels of Hb F in adult life. [1] [2] [3] [4] [304] No deleterious effects on patients are observed, even when 100% of the hemoglobin produced in HPFH homozygotes is Hb F. These patients thus demonstrate convincingly that prevention or reversal of the Hb F to Hb A switch would provide efficacious therapy for sickle cell anemia and -thalassemia.

Two major types of HPFH have been described. Pancellular HPFH is characterized by very high levels of fetal hemoglobin synthesis and uniform distribution of Hb F among all red cells. Heterocellular HPFH results from inherited increases in the number of F cells (see Chaps. 15 and 22).

HPFH shows ethnic differences. In blacks with heterozygous pancellular deletional HPFH, the Hb F is within a range of 1535% and contains ^{G} - and ^{A} -chains in a ratio of 2:3. In Greeks with pancellular nondeletional HPFH, Hb F levels are lower, 1020%, and the Hb F is 90% of the ^{A} type. Hb A_2 levels are lower than normal, but there are no other hematologic abnormalities in these persons, although /-globin chain synthesis ratios may be decreased in some black heterozygotes.

A few black patients have been described with homozygous HPFH. All hemoglobin within the red cells of these patients is Hb F. Mild microcytosis and hypochromia of the red cells are present without anemia. In fact, hemoglobin levels are mildly elevated, presumably due to increased erythropoiesis stimulated by the left-shifted oxygen dissociation curve of red cells rich in Hb F. Globin chain synthesis reveals a / synthetic ratio of approximately 0.5.

Pancellular HPFH can be divided into two classes. The deletional forms arise from large deletions within the -globin gene cluster that remove the - and -globin genes, part of the intergenic DNA, and DNA downstream (to the 3 side) from the -globin genes. [1] [2] [3] [4] [304] The deletions appear to bring enhancer sequences into the proximity of the remaining -globin genes, promoting their high expression. Homozygotes for this condition produce only Hb F. In nondeletional HPFH, the - and -globin genes are present. Single base changes have been shown

to occur in the promoter regions of either the ^{A} - or ^{G} -globin gene, resulting in overexpression of that form of Hb F. [1] [2] [3] [4] [304] In these individuals, Hb F levels rarely account for more than 20% of total hemoglobin.

HPFH/-thalassemia resembles -thalassemia trait except for a higher proportion and regular distribution of Hb F in the red cells. [1] [2] [3] [4]

In both -thalassemia and HPFH, persistence of Hb F after the period of perinatal Hb F to Hb A switching is more marked than in the classic (high Hb A_2) forms of thalassemia. Indeed, -thalassemia and HPFH represent varying degrees of the same genetic phenomenon. Both conditions frequently arise from deletions of DNA that remove or inactivate the -globin gene. [1] [2] [3] [4] [304]

Heterocellular HPFH appears to result in many cases from mutations outside the -globin gene cluster. One controlling locus resides on the X chromosome. [305] [306] Patients with these conditions probably represent the extreme end of a distribution of polymorphic capacities to produce F cells in adult life. Hb F levels are usually much lower than in the pancellular forms. In some situations, elevated levels of Hb F are seen in otherwise normal individuals. In others, the high levels of Hb F become apparent only when other factors producing erythroid stress are present.

ACKNOWLEDGEMENT

Portions of this chapter have been modified and updated from previous chapters we have written. [21] [24] [307] [308]

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Chapter 30 - Sickle Cell Disease

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INTRODUCTION

Sickle cell disease is an inherited disorder that has as its cardinal features chronic hemolytic anemia and recurrent painful episodes. These and all other elements of the disease are the result of mutant sickle cell hemoglobin (Hb S) within the red blood cells. The resultant illness is influenced by the psychosocial adjustment of patients to the multisystem disease. Due to the complexity of the illness, a comprehensive, combined-modality approach offers the best method of management. [1] [2] [3] The further exigency of living with a painful, life-threatening chronic disease in an ethnically diverse society provides additional complexity to the psychosocial aspects of this illness.[4] Traditional concepts of sickle cell pathophysiology ascribe all features of disease to sequential effects of the AG nucleotide substitution in the sixth codon of the -globin gene, substitution of valine for glutamic acid on the outer surface of the Hb S molecule, reduced solubility and polymerization of Hb S when deoxygenated, sickling and poor deformability of polymer-containing erythrocytes, and occlusion by sickle red cells of the microvasculature (Fig. 30-1). [1] [2] [5] [6] [7] [8] [9] [10] While polymerization remains the *sine qua non* of sickle cell disease,[7] [8] a contemporary understanding of pathophysiology requires an integrated perspective derived from physical chemistry, molecular and cellular biology, biochemistry, coagulation, and genetics. [9] [10] [11] [12] [13]

The syndromes (and genotypes) that make up sickle cell disease are mainly sickle cell anemia (Hb SS), sickle cell-° thalassemia, Hb SC disease (Hb SC), and sickle cell-$^{+}$ thalassemia. Sickle cell trait (Hb AS) lacks anemia and recurrent pain, which distinguishes it from the disease. This chapter presents the history, epidemiology, genetics, pathophysiology, clinical manifestations, laboratory diagnosis, and treatment of the sickle cell syndromes. Normal hemoglobin synthesis, structure, and function are described in Chapter 13 ; the thalassemias are considered in Chapter 29 .

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BACKGROUND

The chronicle of sickle cell anemia, sickle cells, and Hb S began in 1910 with a report by Dr. James Herrick of Chicago on the recurrent pain, anemia, and sickled appearance of the red blood

Figure 30-1 A schematic view of the pathophysiology of sickle cell disease. The GAGGTG mutation in the sixth codon of the -globin gene results in the substitution of valine for glutamic acid as the sixth amino acid of -globin. The inclusion of mutant ^{S} chains in the tetramer creates the variant Hb S. The insolubility of deoxygenated Hb S results in its polymerization and the loss of deformability and sickling of deoxygenated sickle erythrocytes. Vessels become occluded as a result of several processes, including the poor deformability of polymer-containing sickle cells.

cells of a dental student from Granada named Noel (Fig. 30-2).[14] [15] Disease manifestations had been recognized long before in Africa, where different tribes had bestowed on the malady various appellations that evoked the basic nature of recurrent pain. [16] Attention first was directed to the importance of deoxygenation of sickle red cells in 1927 when Hahn and Gillespie demonstrated that oxygen deprivation induced a sickle-like deformation, which was reversible on reoxygenation. [17] Later studies showed that the bulk viscosity of sickle cell suspensions rose dramatically and reversibly on deoxygenation. [18] Evidence that the unique pathologic properties of sickle red cells were due to an abnormal hemoglobin was provided in 1940 when a Johns Hopkins medical student named Sherman discovered that sickle but not normal erythrocytes exhibited optical birefringence when deoxygenated. [19] In the same year, Ham and Castle proposed the vicious cycle of erythrostasis hypothesisa synthesis of the combined knowledge regarding the interactions of deoxygenation, acidic pH, and viscosity of sickle cells. [20]

The abnormality of hemoglobin was confirmed in 1949 when Pauling et al. [21] reported that the electrophoretic mobility of Hb

Figure 30-2 Peripheral blood smears showing the peculiar elongated forms of the red corpuscles. *(Originally seen by Herrick,[14] with permission.)*

S differed from that of normal adult hemoglobin (Hb A). In 1950 the importance of Hb S to the pathobiology of sickle red cells was established by Harris description of reversible sol-gel transformation of deoxygenated Hb S solutions [22] and by Perutz and Mitchisons report of the insolubility of deoxygenated Hb S solutions. [23] Unification of the electrophoretic and solubility abnormalities of Hb S was provided by Ingram, who identified the substitution of valine for glutamic acid as the sixth amino acid of the -globin chain. [24] [25] [26] While the polymerization-sickling doctrine had come full circle, the earlier observation by Diggs and Bibb [27] of a population of irreversibly sickled cells (ISCs) that would not revert to discocytes on reoxygenation forewarned that there was more to sickle cell pathophysiology than polymerization alone.

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GENETICS, EVOLUTION, MALARIA, AND G6PD DEFICIENCY

Genetics

The first evidence that sickle cell anemia was inherited was the finding by Huck [28] in 1923 that red blood cells from unaffected parents and relatives could be made to sickle, a phenomenon that led Sydenstricker et al. to suggest active and latent varieties of sickle cell disease. [29] The conclusion that sickle cell anemia results from homozygous inheritance of a genetic determinant from heterozygous parents was reached independently in 1949 by Beet [30] and Neel[31] and was confirmed in the original electrophoretic studies of Pauling and colleagues, which found patients with sickle cell anemia to have virtually all Hb S but their parents to have both Hb S and Hb A.[21] Before these reports, Silvestroni and Bianco[32] had described a variety of sickle cell disease with small sickle cells that was inherited from one parent carrying the sickling disorder and another with constitutional microcytosis. This was the first evidence that the sickle cell and -thalassemia genes were alleles.

Near the -globin gene on chromosome 11[33] are a number of restriction fragment length polymorphisms,[34] [35] distinct constellations of which define -globin haplotypes having specific ethnogeographic origins. [36] The association of the sickle cell gene with different haplotypes constitutes evidence that the sickle cell mutation has arisen at least five times throughout the course of evolution, four times within Africathe Senegal, Benin, Bantu, and Cameroon haplotypesand once withoutthe Arab-Indian haplotype (Fig. 30-3). [37] Despite rigorous scrutiny,[37] [38] no evidence has been obtained to suggest that haplotypes have provided selective evolutionary pressures on the sickle cell gene. [37]

Figure 30-3 Origins of the sickle cell gene mutations. **(A)** Above, a world map showing the incidence of Hb S in the African continent with the approximate locations where the sickle cell mutations occurred independentlyBenin, Senegal, Bantu, and Arab-Indian. Middle, the -globin gene cluster with polymorphic restriction sites identified by arrows. Below, the restriction fragment length polymorphism combinations that dictate the four major haplotypes. *(Adapted from Nagel and Fleming[37] with permission.)* **(B)** Distribution of falciparum malaria in the Old World approximately 150 years ago. *(From Boyd MF (ed): Malariology. Philadelphia, W.B. Saunders, 1949.)*

Malaria Hypothesis

The mystery of a gene with devastating clinical effects having persisted despite an intrinsic negative evolutionary pressure was explained partly by the concept of heterozygous advantagein any population the much greater number of individuals heterozygous for a particular gene compared with those homozygous creates a positive evolutionary pressure, provided the heterozygous condition offers some selective advantage. Allison [39] [40] concluded that the stable frequency of the sickle cell gene existing in areas of hyperendemic falciparum malaria was the result of a balance between gene exclusion due to the premature death of homozygotes and gene selection due to the resistance of heterozygotes against death from malaria, thereby defining the concept of genetic polymorphism. Indeed, children with sickle cell trait have lower rates of parasitemia and cerebral malaria. [41] In this regard, the selection of the sickle cell gene in Africa was related to the introduction 2,000 years ago of slash-and-burn agriculture, which, by destroying the root structure of tropical jungles that had drained standing water and by introducing sunlight into these previously shadowy areas, provided a breeding ground for the mosquito vector *Anopheles gambiae*.[42] [43] As a result of the selective advantage against death from malaria conveyed by the sickle cell gene, its worldwide distribution parallels that of falciparum malaria, with its highest frequencies occurring in the malaria belt (Fig. 30-3).[44]

The mechanism by which the sickle cell gene protects against malaria is not completely understood, [37] [42] but it has been suggested that rigid sickle cell trait red cells repel parasitic invasion.[45] Equal infection rates of sickle cell trait and nonsickle erythrocytes, [46] however, indicate that the inhospitable nature of the host erythrocyte is manifest at some later stage.[47] Consistent with this conclusion is the retarded parasite replication in sickle cell trait red cells at low oxygen tension. [48] Other disruptive influences on the symbiosis of malarial parasite and sickle trait red cell include the more rapid sickling and removal from the circulation of parasitized sickle cell trait erythrocytes related to intracellular oxygen consumption by parasites [49] and to reduced intraerythrocytic pH levels,[50] oxidation of parasite and red cells as a consequence of iron released from denatured sickle cell hemoglobin, [51] cellular potassium loss that inhibits parasite growth, [50] poor nutrition provided parasites by Hb S,[52] and physical disruption of parasite membranes by Hb S polymer.[53] These notions regarding the mechanisms of protection against malaria must be interpreted in the context of evidence that protection from severe malaria is related to the common West African human leukocyte class I antigen HLA-Bw53, [54] which provides the ability to present liver-stage specific malarial antigens to cytotoxic T cells as part of the immune response. [55]

G6PD Deficiency

The influence of G6PD deficiency, another common African polymorphism, on the epidemiology, expression, and frequency of the sickle cell gene has been a matter of debate. G6PD deficiency was reported to have greater frequency among patients with sickle cell disease. [56] [57] More recent reports have emphasized that the detection of G6PD deficiency is easily confounded by the young age of circulating sickle erythrocytes and have not confirmed a higher frequency of the mutant G6PD gene.[58] [59] Neither greater hemolysis nor more frequent pain was found among male subjects having both sickle cell disease and G6PD deficiency. [60]

Prevalence

The distribution and frequency of the sickle cell gene in different areas of the world has been influenced by evolutionary pressures [37] and transmission of the gene via trade routes and slave trade.[61] Among African Americans, the prevalence of sickle cell trait is 810% among newborns, [62] and in this population the frequencies of the sickle cell (0.045), Hb C (0.015), and -thalassemia (0.004) genes [62] indicate that there are 4,000 to 5,000 pregnancies a year at risk for sickle cell disease. [63] The burden of this disease in the United States is dwarfed by that in the rest of the world, as evidenced by a prevalence for the sickle cell gene as high as 2530% in western Africa[64] and an estimated annual birth of 120,000 babies with sickle cell disease in Africa. [64]

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PATHOPHYSIOLOGY

The striking influence of deoxygenation on Hb S polymerization, cellular sickling, and blood viscosity enunciates the pathophysiology of sickle cell diseaseeach of the manifestations of sickle cell disease is attributable to Hb S polymerization. [7] [8] Despite the appeal of this interpretation, a more complete valuation of the several processes involved in sickle cell pathophysiology is needed for a more comprehensive understanding. [1] [2] [5] [6] [9] [10]

Hb S Polymer

Although oxygenated Hb S is as soluble as Hb A, the increased surface hydrophobicity of Hb S tetramers (due to valine rather than glutamic acid as the sixth amino acid) causes decreased solubility of the deoxygenated molecule. [65] Intermolecular bonding of deoxy-Hb S tetramers generates polymer filaments that associate into bundles (Fig. 30-4). [66] The basic polymer structure is a double-stranded filament, seven of which combine into a 21-nm diameter, 14-stranded fiber having one inner and six peripheral double filaments organized so that adjacent double strands have antiparallel orientation. [66] [67] Numerous studies indicate that one of the two ^{6val} forms a lateral contact with the essential [85] phenylalanine and [88] leucine residues within the F helix hydrophobic pocket of an adjacent Hb S tetramer (Fig. 30-5).[67] [68] [69] The second ^{6val} residue does not participate in intermolecular bonding, a fact relevant to considerations of the degree to which $_2{}^S$ mixed tetramers participate in

Figure 30-4 (A and B) Electron micrographs of a centrifuge pellet of deoxyHb S (X 325,000). **(A)** Transfer section showing bundles of Hb S fibers. **(B)** Longitudinal section showing aligned fibers. *(From Finch JT, Perutz MF, Bertles JF, Dobler J: Structure of sickled erythrocytes and of sickle-cell hemoglobin fibers. Proc Natl Acad Sci USA 70:718, 1973, with permission.)* **(C and D)** Electron micrograph of deoxygenated SS erythrocyte. **(C)** Transfer section. **(D)** Longitudinal section. *(Courtesy of Dr. JF Bertles and Dr. J Döbler.)*

Figure 30-5 Close-up view of the deoxyHb S double strand. Lateral contact between strands in Val 6 as donor and the EF pocket of a molecule on the adjoining strand as acceptor. Axial contacts connect molecules vertically along the same strand. *(From Dickerson and Geis,[67] with permission.)*

polymerization when both Hb S and Hb F are present.[70] Many other residues participate in intermolecular bonding to form axial and lateral contacts within the double filament and lateral contacts between double filaments. [67] [69] [71] Critical residues (and the mutant hemoglobins that ablate their bonding) include lateral contacts (6, 73 [Korle Bu], 66 [I-Toulouse], 83 [Pyrgos], 87 [D-Ibadan], axial contacts (121 [O-Arab], 16 [J-Baltimore], 17 [J-Amiens], 19 [D-Ouled Rabah], 22 [G-Coushatta], 16 [Hb I], 116 [O-Indonesia]), and interpair contacts (54 [J-Mexico], 47 [Sealy], 75 [Winnipeg], 78 [Stanleyville II], 6 [Sawara], 11 [Anantharaj], 68 [G-Philadelphia]).

Polymerization

The solubility of Hb S is approximately 17 g/dl, a level far below the intraerythrocytic concentration of hemoglobin of approximately 34 g/dl; deoxygenation results in supersaturation of deoxy-Hb S solutions, aggregation of deoxy-Hb S molecules,

Figure 30-6 Model for the polymerization and alignment of deoxy-hemoglobin S showing homogeneous and heterogeneous nucleation. *(Adapted from Hofrichter et al., [65] with permission.)*

and formation of deoxy-Hb S polymer.[65] Studies of the kinetics of polymerization have determined that the delay time to polymer formation is inversely proportional of the deoxy-Hb S concentration with 15th30th-order kinetics, a finding that demonstrates that the early stages of polymer formation involve aggregation of 15 to 30 tetramers into a single nucleus of polymerizationhomogeneous nucleation. [65] As polymer forms, the fiber provides a surface that provides more nuclei for polymer formationheterogeneous nucleation (Fig. 30-6).[72] Eaton and Hofrichter[7] explained the pathophysiology of sickle cell disease as a function of polymerization, sickling, and perturbations of blood flow that prolong the duration of cellular transit to exceed the delay time to polymerization. A different approach to understanding polymerization derives from measuring the maximal amount of polymer generated under equilibrium conditions, a system driven by thermodynamics rather than kinetics.[8] Both interpretations concur that polymerization is detrimental to sickle cells and strongly influenced by Hb S concentration, but there are important practical differences between the two. The kinetic interpretation is based on oxygenated morphologically normal cells having no persistent polymer providing nucleation sites,[73] and is consistent with vaso-occlusion occurring after a delay time for nucleation in *postcapillary* vessels. The thermodynamic version holds that polymer persists in some cells at arterial oxygen tensions, [74] that deoxygenated cells lose deformability before sickling, [75] and that the site of vaso-occlusion is the *prearteriolar sphincter*. [8] Kinetic considerations pertain to the greater majority of cells with lower mean corpuscular hemoglobin concentration (MCHC), a lesser polymerization tendency, and no persistent polymer when reoxygenated. Thermodynamic interpretations describe the minority of dehydrated sickle cells having very high MCHC, a strong tendency toward polymerization, and persistent polymer at arterial

INHERITANCE PATTERN OF SICKLE CELL DISEASES

The sickle cell diseases are inherited in an autosomal codominant manner. Sickle cell trait results from the simple heterozygous inheritance of a sickle cell gene from one parent and an Hb A gene from the other; the red cells contain Hb A ($_2{}^A{}_2$) and Hb S ($_2{}^S{}_2$) in a 60:40 distribution. Sickle cell anemia results from the homozygous inheritance of a sickle cell gene from each parent; the red cells contain virtually all Hb S. Hb SC disease results from the compound heterozygous inheritance of a sickle cell gene from one parent and an Hb C gene from the other; the red cells contain nearly equal amounts of Hb S and Hb C ($_2{}^C{}_2$). Sickle cell- thalassemia results from the compound inheritance of a sickle cell gene from one parent and a -thalassemic gene from the other; the red cells contain virtually all Hb S in sickle cell-° thalassemia; the red cells contain mostly Hb S with 530% Hb A in sickle cell-$^+$ thalassemia.

Sickle cell-hereditary persistence of fetal hemoglobin (sickle cell-HPFH) results from the compound heterozygous inheritance of a sickle cell gene from one parent and an HPFH gene (i.e., a deletion of the - and -globin genes that allows continued high-level expression of the fetal -globin genes in adult life) from the other. Sickle cell-HPFH is not associated with anemia or vaso-occlusive symptoms because the presence of large amounts (2535%) of Hb F ($_2{}_2$) in all red cells inhibits Hb S polymerization.

Figure 30-7 Hb F decline in children with hemoglobins AA and SS. *(Data from OBrien et al.[686])*

oxygen tensions. Studies of flow ex vivo showed the postcapillary sphincter to be the site of vasoocclusion, [76] as predicted by kinetic models.

Nitric oxide (NO ; NO) is one of the nitrogen monoxides, which are produced in vivo by the actin of NO synthases (NOS) on L-arginine.[77] [78] [79] NOs numerous biologic functions are the result of this free radical forming an ironnirosyl complex in a target protein. [77] Of particular interest to considerations of Hb S polymerization are its unique interaction with the hemoglobin molecule[80] and its complex interactions with heme oxygenase (HO) and carbon monoxide (CO).[81] Oxygen binding to the heme iron of hemoglobin promotes reversible binding of NO to ^{93Cys} to form S-nitrosohemoglobin,[80] which may be the mechanism by which exposure of sickle erythrocytes to NO increases their oxygen affinity.[82] [83] While this effect on oxygen affinity was predicted to *reduce* polymerization, sickling, and vasoconstriction,[82] the effect of NO on sickle cell pathophysiology is influenced by CO liganding to the heme prosthetic groups of NOS, which diminishes the enzymatic activity of NOS, [79] resulting in decreased NO production. Since increased amounts of CO are generated by the action of heme oxygenases [81] during hemolysis[84] [85] [86] [87] , the salubrious influence of NO on Hb S polymerization may be countered. Greater levels of complexity are provided to these interacting second messenger systems by the direct liganding of CO to heme iron, increasing oxygen affinity,[88] and the induction of heme oxygenase activity by hypoxia.[89] The net effects of these compounds on pathophysiology is not clear; however, recognition of their involvement points out the potential incompleteness of hypotheses based solely on the oxygenation/deoxygenation cycle.

Inherited Influences on Polymerization

When the switch from - to -globin synthesis occurs in the fetus, adult hemoglobin (in the case of sickle cell anemia, Hb S) replaces Hb F. [90] [91] Because of the inhibitory effect of Hb F on Hb S polymerization[70] and cellular sickling,[92] the high fraction of Hb F at birth masks the expression of sickle cell disease until Hb S levels increase to 75% at about 6 months of age (Fig. 30-7).[2] [6] Conditions that preserve elevated levels of Hb F into adulthood similarly modulate the course of sickle cell disease.[93] The compound heterozygous conditions sickle-hereditary persistence of fetal hemoglobin (Hb S-HPFH) and sickle cell- thalassemia both have higher Hb F levels and milder clinical courses than are characteristic of sickle cell anemia. [6] [94] Additional mitigating influences in sickle cell- thalassemia are elevated levels of Hb A_2 [7] and, in sickle cell-$^+$ thalassemia, levels of Hb A up to 30%. These affect both the solubility and polymerization of Hb S. Hb F is a more active inhibitor of polymerization than Hb A, as shown by Hb S solutions with 1530% Hb A (resembling sickle cell-$^+$ thalassemia) having delay times 10 to 100 times longer than pure Hb S solutions, and Hb S solutions with 2030% Hb F (resembling Hb S-HPFH) having delay times 1,000 to 1,000,000 times longer. [95] [96] The greater activity of Hb F than Hb A in increasing Hb S solubility is related to an important molecular difference: Hb A is excluded but $_2{}^A$ hybrid tetramers are included in Hb S polymer, while both Hb F and $_2{}^S$ hybrid tetramers are excluded.[70] [97] [98] A further influence mitigating the polymerization, sickling, and clinical aspects of sickle cell- thalassemia is the reduced MCHC, which retards Hb S polymerization. Hematologic values for sickle cell anemia, the sickle cell- thalassemias, and Hb S-HPFH are found in Table 30-1 .

Alpha thalassemia also modulates the clinical expression of sickle cell disease, in part by reducing the MCHC. [99] Prevalences of the silent carrier of thalassemia syndrome (genotype /) and -thalassemia trait (genotype /) among African Americans are approximately 30% and 2%, respectively. [100] The lower intraerythrocytic concentrations of Hb S associated with thalassemia modulate the hematologic, pathophysiologic, and clinical manifestations of disease, [99] [101] [102] [103] [104] [105] [106] particularly the severity of anemia, which is diminished after

TABLE 30-1 -- Hematologic Variables Associated with Sickle Cell Anemia and the Different Sickle Cell- Thalassemia Syndromes

Genotype	Hb [a]	%Hb A [b]	%Hb F [b]	%Hb A2 [a]	MCV [a]	Reticulocytes [a]	No.
Hb SS[c]	7.83	0	4.56	2.87	85.9	10.18	123
Hb S-° thal[c]	8.85	0	5.86	5.02	69.3	7.2	41
Hb S-$^+$ thal, type I[d]	8.37	35	6.8	4.90	63.7	9.7	3
Hb S-$^+$ thal, type II[d]	10.28	814	5.2	4.68	70.0	6.6	14
Hb S-$^+$ thal, type III[e]	11.55	1825	5.1	4.66	73.3	1.27	76
Hb S-HPFH[f]	14.6	0	25.8	1.95	81.7	2.4	4

[a] The mean data for each variable are shown. Units of measure are g/dl for Hb, percentage of total hemoglobin for Hb F and A_2, fl for MCV, and percentage of total red cells for reticulocytes.

[b] Percentage Hb A that defines the Hb S-$^+$ thalassemia type.[659]

[c] Data from Serjeant et al.[662]

[d] Data from Christakis et al.[661]

[e] Data from Serjeant et al.[663]

[f] Data from Friedman et al. [825]

TABLE 30-2 -- Effect of Thalassemia on the Level of Anemia in Sickle Cell Anemia

Reference	/[a]	/	/
Embury et al.[103]	7.8[b] (n = 25)[c]	9.7 (n = 18)	9.2 (n = 4)
Higgs et al.[104]	7.8 (n = 88)	8.1 (n = 44)	8.8 (n = 44)
Steinberg et al.[106]	8.0 (n = 73)	9.0 (n = 39)	9.5 (n = 13)
Felice et al., age 5 years[107]	8.6 (n = 88)	8.4 (n = 52)	8.3 (n = 50)
Felice et al., age 11 years[107]	7.9 (n = 40)	8.5 (n = 34)	9.6 (n = 2)

[a] The different -globin genotypes indicate the presence of four (/), three (/), or two (/) -globin genes.
[b] The mean Hb level (g/dl) for each group is shown.
[c] The number of subjects in each group is denoted by n.

age 7 years with either the / or the / genotype (Table 30-2).[107] [108]

Cellular Sickling

The original assumption that sickled-appearing red cells must be rigid was confirmed by the observation that a dramatic increase in bulk viscosity of sickle cell suspensions occurred when they were deoxygenated.[18] Within 0.5 seconds of deoxygenation, sickle cells accumulate Hb S aggregates and lose deformability despite having normal morphology; by 3 seconds, the biconcave discs contain short linear polymer; by 15 seconds, the discs acquire protuberances and longer linear polymer; and by 30 seconds, the cells gain a holly-leaf shape and considerable amounts of polymer. [75] [109] Reoxygenation results in loss of much polymer within 3 seconds, in very little polymer but persistent cell deformation at 5 seconds, and in no polymer or cell deformation at 10 seconds. During both deoxygenation and reoxygenation, viscosity parallels the presence of polymer. The poor deformability of reversibly sickled cells was confirmed using laser diffraction viscosimetry and filtration methods.[110] [111] [112]

Both the rate of deoxygenation and the presence of shear stress influence the alignment of intracellular polymer domains, which in turn may affect the rheologic properties of sickle cells and blood. The earliest reports of sickle cells described heterogeneous morphologic changesclassic sickle forms, holly-leaf shapes, and granular deformities.[14] Subsequent studies using electron microscopy[113] [114] and linear dichroism[115] found a correlation between sickle cell morphology and the pattern of intraerythrocytic polymer domains: archetypal crescents have a single domain of highly aligned polymer that seems to have arisen by homogeneous nucleation from a single nucleus; holly leaves have a few less well-aligned domains that appear to have arisen by heterogeneous nucleation from a limited number of nuclei; and granular cells have multiple poorly aligned domains that apparently have arisen by heterogeneous nucleation from many nuclei (Fig. 30-8). The rate of deoxygenation influences the formation of polymer domainsslower deoxygenation is associated with homogeneous nucleation, and rapid deoxygenation favors heterogeneous nucleation.[116] [117] Shear stress also influences polymer formation: shear applied during the delay period accelerates the onset of polymerization and increases viscosity, perhaps by creating more nucleation sites; shear applied after polymerization has occurred diminishes viscosity by shattering the solid polymer phase.[118] [119]

The impact of deoxygenation rate and shear remains speculative, because there is little knowledge of the effect of domain patterns on the rheologic properties of sickle cells or blood. One study found that slow deoxygenation of cell suspensions generates crescentic cells containing single, well-aligned polymer domains and a progressive increase in bulk viscosity. In contrast, rapid deoxygenation created granular cells with multiple domains and an abrupt increase in bulk viscosity; continued deoxygenation transformed these cells into elongated cells with one domain of aligned polymer and lowered the bulk viscosity. [120] The influence of polymer domain patterns on individual cell deformability remains to be clarified.

Irreversibly Sickled Cells

The original observation of cells that remain sickled on reoxygenation [27] demonstrated an aspect of pathophysiology that was not immediately oxygenation-dependent. The preponderance of ISCs among the most dense sickle cells [121] [122] suggested that the common pathway of sickle cell destruction is via dehydration. However, Bertles and Milner[123] found among the densest cells a group of younger cells having unexpectedly low hemoglobin F contentcells apparently predestined to early destruction by lacking the protective effect of Hb F against polymerization and sickling. [70] [92] The relative youth of the most dense cell fraction was confirmed using glycosylated hemoglobin as a measure of cell age. [124]

The rheologic disadvantage of ISCs is demonstrated by their poor deformability [125] and short circulatory survival,[126] but the mechanisms responsible for the generation of ISCs are not completely understood. Although shells of membrane skeletal proteins from ISCs have characteristically elongated shapes, [127] their poor cellular deformability is substantially improved by reducing their MCHC with osmotic manipulation, [128] [129] a fact incompatible with rigid membranes causing their rheologic impairment. An alternative understanding of ISCs derives from their elongated (rather than crescentic) shape, which is

Figure 30-8 Schematic showing that in a slowly deoxygenated cell the sickle morphology results from the formation of a single domain of well-aligned fibers. In a more rapidly deoxygenated cell the holly leaf morphology results in the formation of a number of smaller domains of shorter aligned fibers. In the most rapidly deoxygenated cells the granular morphology results from the formation of a large number of very small domains of randomly oriented short fibers. *(Adapted from Eaton and Hofrichter,[822] with permission.)*

probably due to the rapid polymerization of highly concentrated Hb S, [7] [129] generating multiple small polymer domains. This interpretation is consistent with the importance of recurrent sickling and unsickling to ISC formation both in vitro [130] and in vivo.[131] The similarities of ISCs to the calcium-loaded, potassium-depleted, dehydrated nonsickle erythrocytes that have undergone calcium-sensitive potassium loss by the Gardos phenomenon and the ability to generate ISCs by metabolic depletion in vitro suggests a role of ATP depletion. [132] However, metabolic depletion is unnecessary for ISC production, as there is a population of cells that becomes dehydrated by the Gardos phenomenon while energy-replete. [133] [134]

The clinical importance of ISCs relates to their presence as sickled forms on the peripheral blood smear (in contrast with reversibly sickled cells, which by definition become morphologically normal when exposed to ambient oxygen tensions). The ISC number is generally constant in individual patients and correlates mainly with the degree of anemia.[135] The number of ISCs on the peripheral smear does not change with episodic complications of disease and is not a reliable indicator of events such as the acute painful episode.[136] However, painful episodes have been reported following artificial perturbation of their numbers to exceed steady-state levels. [131] The main clinical use of detecting ISCs on the peripheral smear is in diagnosing sickle cell syndromes: ISCs are seen in all sickle cell disease genotypes but not in sickle cell trait.

Cation Homeostasis and Cell Dehydration

During their brief circulatory sojourn, sickle cells become profoundly dehydrated, [123] a process gravely important to ISC generation, the unique pathobiology of Hb SC red cells, and certain disease complications. The extreme dehydration of sickle cells (MCHC ranging up to 50 g/dl) [7] [128] promotes Hb S polymerization by a direct effect of higher MCHC[8] and by an indirect effect related to the peculiar inverse relationship between Hb S concentration and oxygen affinity. [137]

The mechanism by which sickle cells become dehydrated has recently come into clearer focus. Several energy-driven systems, gradient-driven systems, and pathways contribute to the depletion of the major intracellular monovalent cation, potassium, and water from sickle erythrocytes. [138] [139] While certain aspects of this process are the result of deoxygenation, polymerization, and sickling, others are induced by the mere presence of mutant hemoglobin within the cell. Deoxygenation-induced passive cation leak results in balanced potassium efflux and sodium influx that exceed by an order of magnitude that of normal erythrocytes and oxygenated sickle cells; the fixed stoichiometry of three sodium ions pumped out for every two potassium ions pumped into the cell during reparative attempts by

sodiumpotassium ATPase[140] [141] results in a net depletion of cellular monovalent cation and water. [142] [143] These facts notwithstanding, mathematic modeling of sickle cell volume regulation indicates that the major effectors of cell dehydration are calcium-dependent potassium loss (the Gardos pathway) and potassiumchloride cotransport, acting interdependently. [133] Although not consistent with all the data, [139] a reasonable approximation derives from the notion that a population of calcium-sensitive reticulocytes preferentially lose potassium by the Gardos effect, thereby acquiring intracellular acidosis, which potentiates further potassium loss via potassiumchloride cotransport.[134] The calcium content of sickle cells is also elevated, particularly in ISCs. [144] Although most calcium is sequestered in vesicles, [145] it appears that sufficient free calcium is available to participate in the Gardos phenomenon. [134] Factors that contribute to potassium loss include shape transformation [146] and oxidation of membrane transport proteins. [147]

Among the clinical consequences most directly attributable to cell dehydration are the renal complications of sickle cell trait. The loss of urine-concentrating ability and hematuria in these individuals demonstrate that even Hb AS cells can cause vaso-occlusion when severely dehydrated by the hypertonic environment of the renal medulla.[148] [149] [150]

Another demonstration of the clinical impact of cellular dehydration is the clinical morbidity in Hb SC disease. Although sickle cell trait and Hb SC red cells each contain heterozygous amounts of Hb S and another hemoglobin that does not participate in polymerization, [151] individuals with sickle cell trait are asymptomatic carriers, while those with Hb SC disease have anemia and painful vaso-occlusion. [6] This important difference is the result of higher Hb S concentrations within Hb SC cells due to greater fractional content of Hb S (50% vs. 40%) [151] and to the cellular dehydration [151] [152] mediated by Hb C-induced potassiumchloride cotransport activity.[152] [153]

A third clinical correlate of red cell dehydration, the interaction of thalassemia and sickle cell anemia, is perhaps more important for broadening the perspective of sickle cell pathophysiology than for reaffirming the importance of polymerization. Clearly the less severe anemia associated with coexistent thalassemia (Table 30-2)[99] [103] [104] is accompanied by findings that interdependently support the importance of polymerization: the lower MCHC [103] [104] retards polymerization; [7] [8] the fewer dense cells and ISCs[102] [104] [154] extend the known relationship between the severity of anemia and the number of ISCs; [135] and the diminished deoxygenation-induced cation leak[101] is consistent with the better hydration [155] and greater deformability of -thalassemic sickle cells. [102] [156] Despite the internal consistency of this synthesis, [157] exceptions to its strict interpretation demonstrate the need for a pathophysiologic understanding that extends beyond polymerization. The lack of -thalassemia effect on anemia prior to age 7 years (Table 30-2)[107] [108] suggests the existence of an age-dependent, polymerization-independent factor that enables the influence of MCHC on polymerization. In this regard, it has been suggested [99] that persistent splenic conditioning in early childhood may deprive -thalassemic sickle cells of their fundamental advantagethe greater degree of membrane redundancy, [158] which protects them against deformation-induced cation loss[146] during polymerization. The limitations of polymerization-based interpretations are further demonstrated by the detrimental clinical effects associated with thalassemiathere is an increased incidence of osteonecrosis, [99] [159] [160] and the higher hemoglobin levels are associated with a greater frequency of pain, which after age 20 years is associated with a higher mortality rate. [161] Coexistent beneficial and detrimental influences of thalassemia demonstrate the doubtful validity of using polymerization equations to predict disease severity [94] and the need for pathophysiologic interpretations that include the polymerization-independent mechanisms of cell pathobiology[11] and the detrimental rheologic effects of higher hematocrit and viscosity. [125] [156]

Therapeutic strategies aimed at decreasing the MCHC have used methods of improving cellular hydration and of reducing MCH. Induction of hyponatremia in patients was reported to swell red cells osmotically, lower MCHC, and ameliorate painful episodes. [162] Pharmacologic preservation of sickle cell hydration by cetiedil citrate, an agent that inhibits the Gardos phenomenon and induces passive sodium influx, has shown therapeutic potential. [163] [164] [165] Therapeutic options for maintaining cell hydration also are provided by the administration of imidazole inhibitors of the Gardos pathway [166] [167] or of magnesium supplements, which inhibit potassiumchloride cotransport.[168] It also is possible to reduce the MCHC by reducing the MCH with iron deficiency. It has been observed that spontaneous or induced iron deficiency sufficient to reduce the serum ferritin, mean corpuscular volume (MCV), and MCHC resulted in variably improved Hb S polymerization, red cell survival, level of anemia, and clinical status. [169]

Oxidative Damage

In addition to its abnormal electrophoretic mobility and solubility, Hb S is unstable. [170] [171] [172] [173] Consequences of Hb S instability are the increased degeneration of methemoglobin [174] and release of heme,[175] processes that contribute to the increased degeneration of oxidative radicals by sickle red cells. [175] [176] In addition, the normal physiologic processes of hemoglobin oxygenation and deoxygenation generate methemoglobin S and oxidative radicals continuously. [177] These processes perpetuate an oxidative stress within sickle cells that has major pathophysiologic importance to metabolism, membrane lipids, membrane proteins, and the integrity of the Hb S molecule itself.[11] [178]

The effects of oxidative stress on red cell metabolism are particularly important to sickle cell pathophysiology because the resultant impairment of reductive defense mechanisms potentiates other oxidative damage to the cell. The presence of free heme within sickle red cells inhibits the activity of several enzymes necessary for the generation of the NADH and NADPH required for protection against oxidation. [176] Indeed, sickle cells have been found to have decreased NADH redox potential, [179] hexose monophosphate shunt activity, [180] [181] and GSH content.[181] The metabolic disadvantages of sickle red cells provide underappreciated but important contributions to sickle cell pathobiology.

Both the lipids and proteins that make up erythrocyte membranes are targets of oxidative damage. The combination of increased generation of metHb S, [174] impaired metHb S reduction, and absence of metHb S from sickle erythrocytes[182] suggests the rapid disappearance of metHb S, a suggestion consistent with its degradation to hemichrome from which heme is liberated to cause oxidant damage of cytoplasmic and membrane components.[183] [184] Both heme and nonheme iron have been found in increased amounts on sickle cell membranes. [178] The accumulation of hemichrome aggregates on the cytoplasmic portion of band 3 initiates the coclustering of band 3 molecules in the membrane and the assembly of IgG and complement on the extracellular domains of band 3 molecules, [185] [186] which promotes sickle cell recognition by macrophages[187] and adherence to endothelial cells. [188] The uneven distribution of band 3 and of another transmembrane protein, glycophorin A, [189] demonstrates that the membrane skeletons of sickle cells are disrupted. Abnormalities of the proteins that make up this skeleton are demonstrated by the impaired association of the major membrane skeletal protein, spectrin, into sickle cell membranes. [190] There is also direct evidence for oxidative damage to the membrane skeletal proteins spectrin, ankyrin, band 3, and band 4.1. [191]

In addition, there is evidence for oxidative damage to sickle cell membrane lipids. [192] It is probable that similar abnormalities of membrane lipids are related to their reduced lateral mobility in sickle cell membranes[193] and to the loss of phosphatidylinositol-anchored membrane proteins such as DAF and MIRL, as discussed in the Mechanisms of Hemolysis section.[194]

The role of membrane-bound iron in the oxidative damage perpetrated on sickle erythrocytes is supported by the finding that the oral administration of L1, an effective iron-chelating agent, [195] diminishes the heme and nonheme iron bound to erythrocyte membranes, thereby protecting them from lipid peroxidation and hemichrome deposition.[196]

A unifying observation regarding the role of reactive oxygen species in sickle cell pathophysiology is the inverse relationship reported between clinical severity and intraerythrocytic levels of the enzyme that modulates the generation of peroxide and hydroxyl radicals, superoxide dismutase. [197]

Sickle Cell Adherence

The discovery that sickle red cells were more adherent to cultured vascular endothelial cells [189] [198] has contributed greatly to our understanding of sickle cell pathophysiology.[11] [199] [200] The importance of sickle cell adherence was appreciated by noting that the vaso-occlusive severity of individual patients was directly related to the adhesivity of their sickle cells [201] and that the initiation of vaso-occlusion ex vivo is by adherence of sickle cells to vascular endothelium (Fig. 30-9). [76] Subsequent studies have revealed that adherence differs according to subpopulation of sickle cells, source of endothelial cells, cytoadhesive ligands, and cell membrane receptors.[11] [202] In general, the least dense, reticulocyte-enriched fraction of sickle cells is the most adherent. [76] [203]

Adherence of sickle erythrocytes to unstimulated endothelium employs numerous receptors and ligands, the consideration of which reveals the great complexity of the adhesive process. High-molecular-weight von Willebrand factor (vWF) bridges glycoprotein (GP) Ib or integrins of sickle cells to GP Ib of endothelial cells; thrombospondin links CD36 or sulfated glycans of sickle reticulocytes to CD36 or $_{v\,3}$ integrin of endothelial cells; $_{4\,1}$ integrin of sickle reticulocytes ligands to fibronectin of the endothelial glycocalyx; sickle erythrocyte lipids bind to matrix thrombospondin and laminin; exposure of cryptic portions of sickle red cell band 3 provides

adhesivity; and CD44 of sickle cells binds to matrix laminin and fibronectin. [202] [204] [205] [206] [207] [208] [209]

Figure 30-9 Adhesion of sickle erythrocytes in venules. **(A)** Adherent discocytic sickle cells tethered to the endothelial wall of a venule and aligned in the direction of the flow (arrow). **(B)** Increased adherence of sickle cells at venule or bending and at junctions of smaller-diameter postcapillary venules. The postcapillary vessels (small arrows) are totally blocked. Large arrow indicates flow direction. *(From Kaul et al.,[76] with permission.)*

A general scheme of sickle erythrocyte and endothelial receptors and soluble ligands is depicted in Figure 30-10 .

Adherence of sickle erythrocytes to activated or injured endothelial cells [210] [211] involves additional adhesive mechanisms, [199] [212] which, along with the adhesive contributions of platelets [213] [214] neutrophils (PMN), [215] add to the complexity of sickleendothelial cell adherence. Even normal endothelial cells have potential for binding sickle cellslatent adhesiveness, a large array of adhesive membrane glycoproteins, and pronounced adhesogenic responses to stimulation. [199] [212] [216] [217] [218] Endothelial adhesivity may be enhanced by factors that have the capacity to activate or injure them. Elevated levels or activity of many of these in steady-state, and especially during vaso-occlusive crises or infections, underscores their relevance to the pathogenesis of sickle cell disease. Thrombin generation is increased in sickle cell disease, [219] [220] and this enzyme has been observed to enhance endothelial cell adhesivity for sickle erythrocyte by a mechanism involving interendothelial cell gap formation. [221] Interleukin-1 also occurs at high levels in sickle cell disease and promotes expression of receptors for vWF (GP Ib) and vitronectin, secretion of high-molecular-weight vWF, and potentiation of adhesivity for sickle cells. [222] [223] [224] [225] [226] Tumor necrosis factor- levels are increased, and this cytokine induces expression of GP Ib and vascular cell adhesion molecule-1 and enhances sickle cell adherence. [222] [223] [227] Gamma-interferon promotes expression of CD36 and vWF, both of which

Figure 30-10 Receptors and ligands known to mediate sickle erythrocyte adherence to the vascular endothelium. *(Adapted from Bunn,[373] with permission.)*

participate in sickle erythrocyte adherence. [228] [229] Endothelial cells infected with herpesvirus-1 express Fc-like receptors that bind sickle erythrocytes via the abnormal amounts of immunoglobulin on sickle cell membranes. [188] Other viruses and double-stranded RNA also induce vascular cell adhesion molecule-1 expression. [230] The finding of increased adhesivity of viral-infected endothelial cells [188] suggests an explanation for the frequent occurrence of painful episodes during viral illness. [231]

Oxygen deprivation of endothelial cells also has the potential for altering vaso-occlusion via its effects on endothelial cell coagulant properties, synthesis of interleukin-1, and induction of interendothelial cell gap formation, which, by analogy to thrombin, may account for the higher levels of sickle cell adherence reported. [232] [233] [234] [235] Many of the toxic effects of hypoxia on cells and tissues are worsened substantially by reperfusion. [236] Because of its many features resembling ischemia/reperfusion, [237] [238] [239] [240] [241] [242] sickle cell disease appears to be an apt paradigm for this type of potentially adhesogenic injury. It has been proposed that the immune deficiency of sickle cell disease allows the sequence of chronic inflammation, upregulated endothelial cell adhesion molecules, enhanced sickle cell adherence, and cell trapping, all of which potentiate vaso-occlusion. [243]

Therapeutic opportunities derived from these relationships include inhibiting adherence and aggregation with emulsifying agents such as Poloxomer 188 (RheothRx) [244] [245] or with polyethylene glycol (PEG) [246] or PEG-coated red cells [247] and the potential for future interventions using antibodies and oligopeptides that interfere with ligandreceptor interactions.

Role of Neutrophils

The possible role of PMNs in the pathophysiology of sickle cell disease was suggested by a study of the effects of oxygen inhalation in three subjects during steady state. [131] After cessation of oxygen inhalation, the leukocyte counts of all three rose to abnormal levels, and two experienced painful crises at the time of maximal white cell counts. A subsequent example was the association of a severe painful episode with pulmonary infiltrates and hypoxemia with a leukocyte count of 63,000/l following the administration of granulocyte colony-stimulating factor to a patient with sickle cell disease. [248] Additional circumstantial evidence suggesting a role for PMNs in the pathophysiology of sickle cell disease is derived from comparisons of Hb SS and Hb SC disease. In Hb SS, chronic leukocytosis is much more common, [249] pain is twice as frequent, [161] and life expectancy is two decades shorter. [250] Within the Hb SS group, there is a direct relationship of the leukocyte count and mortality rate, [250] hemorrhagic stroke, [251] and acute chest syndrome. [252] The observed correlation of clinical response to hydroxyurea with reduction in PMN count has been interpreted to indicate a pathophysiologic role of PMNs. [253] [254] [255] However, this association may simply reflect better compliance with drug administration rather than a causal effect.

The cellular mechanisms by which PMNs may influence vaso-occlusion include the poor deformability of PMNs compared with normal erythrocytes, [256] the increased adherence to endothelium and the frequent upregulation of CD64 on membranes of PMNs from patients having pain crisis, [257] the activation of PMNs by sickle erythrocytes, [215] and the important role of PMNs in ischemia/reperfusion injury, [236] [258] [259] a process that resembles the intermittent flow of sickle cell disease. [237] Complex blood cell interactions involving PMNs are suggested by observations of plateletsickle erythrocyte aggregates in the blood, [214] plateletleukocyteendothelial cell adherence on the vessel wall, [260] and participation of PMNs in generating the thrombin- or hypoxia-induced interendothelial cell gaps [258] [259] [261] [262] [263] that facilitate sickle cell adherence. [221]

Coagulation and Platelet Perturbations

The state of frequent, perhaps continuous, perturbation and activation of the hemostatic system in sickle cell disease provides evidence that there may be no real steady state. [12] [241] Increased platelet activation in vivo is suggested by several lines of evidenceincreased levels of -thromboglobulin [264] and platelet factor 4, [265] increased activation-associated antigens on platelet membranes, [214] reduced amounts of thrombospondin in circulating platelets, [266] and increased urinary levels of thromboxane. [267] Reduced platelet count and the shortened platelet survival during acute painful episodes suggest that platelet activation occurs during these events and may be responsible for the aforementioned findings. [268] Further support for this conclusion is provided by the finding of decreased platelet thrombospondin levels during pain crises. [269]

Evidence suggests that steady-state levels of procoagulants and of the natural anticoagulants protein C, protein S, and antithrombin III are abnormal in sickle cell disease. [12] There is direct evidence for increased thrombin generation and activity in sickle cell disease, including elevated levels of thrombinantithrombin III complexes, activation fragment 1.2, and fibrinopeptide A. [219] [220] Administration of low-dose acemocoumerol reduced the elevated levels of fragment 1+2, [270] and in a small study administration of minidose heparin reduced hospitalization time by 75%. [271] It is uncertain whether the prefibrinolytic system is abnormal, but increased levels of fibrin degradation product provide evidence for ongoing fibrinolysis. [12] Further evidence for a thrombophilic state is the correlation between hyperhomocysteinemia and the incidence of stroke in sickle cell disease. [272] Despite the evidence for chronic activation of coagulation and fibrinolysis, data have been presented supporting hepatic dysfunction as the cause of the measured abnormalities. [273]

A source of hemostatic activation specific for sickle cell disease is the red cell itself. Both ISCs and deoxygenated reversibly sickled cells express abnormal amounts of phosphatidylserine on the outer membrane leaflet, which results in activation of the coagulation cascade. [274] In addition, cellular sickling results in the exovesiculation of phosphatidylserine-rich membrane vesicles, [275] which have the ability to activate coagulation. [276] Another mechanism by which abnormal sickle cell membrane properties perturb hemostasis pertains to the increased fraction of phospholipids in the hexagonal II phase, which induces antiphospholipid antibodies. [277] Although the high prevalence of these antibodies in sickle cell patients [277] [278] is reported to have no clinical consequences, [278] the occurrence of right atrial thrombosis has been reported with one patient having both sickle cell disease and antiphospholipid antibodies. [279]

Perturbation of vascular endothelial cells is yet another source of hemostatic activation. [280] The increased levels of thrombin found in sickle cell disease [219] [220] [269] stimulate endothelial cells to release vWF, prostacyclin, plasminogen activators, and platelet activating factor, to express tissue factor and cytoadhesive molecules on

the cell membrane, to promote vasoconstriction, and to activate thrombomodulin. Other known endothelial cell agonists active in sickle cell disease may evoke a procoagulant effect of these cells. [280]

The possible occurrence of a microvascular process similar to thrombotic thrombocytopenic purpura is suggested by the case of a patient having life-threatening multiorgan failure resembling thrombotic thrombocytopenic purpura who responded to plasma exchange, [281] and possibly by another patient whose pain crisis responded to fresh-frozen plasma infusion. [282]

Vascular Regulation

Passage of sickle red blood cells and blood through blood vessels is affected by the lumen size of the blood vessels. In addition to established humoral and neurogenic mediators, vascular tone is subject also to regulation from local autocrine and paracrine influences. [283] Certain molecules involved in vascular homeostasis are relevant to the pathophysiology of sickle cell disease. Among the local paracrine processes, endothelial cells produce prostacyclin, which causes vasodilatation and inhibits platelet aggregation and PMN adherence. [284] Prostacyclin production by endothelial cells is increased by the presence of sickle red cells. [267] [285] Endothelin is a potent vasoconstrictive agent whose synthesis by endothelial cells is stimulated by their contact with sickle erythrocytes. [286] Platelet-derived growth factor also is a powerful vasoconstrictor, and its synthesis is increased by hypoxic conditions, which are common in sickle cell disease.

Nitrous oxide, previously referred to as endothelial cell relaxation factor, is produced in endothelial cells by the effect of NOS on arginine. [78] [283] The binding of oxygen to the heme iron of hemoglobin promotes the binding of NO to ^{93}Cys and the formation of S-nitrosohemoglobin. [80] When hemoglobin is deoxygenated in the tissues, the NO is dissociated to cause vasoconstriction [80] by its action on vascular smooth muscle cells. [78] This process allows variations in physiologic oxygen gradients to balance the local blood flow with oxygen requirements. The administration of an NOS inhibitor to decrease NO production in rats caused decreased cerebral flow of infused sickle cell blood and increased adherence of sickle red cells to the cerebrovascular endothelium. [287] Thrombomodulin is a transmembrane protein of endothelial cells that is activated by thrombin to catalyze the effect of protein C on inhibiting clotting. Soluble levels of this molecule have been proposed as indicators of endothelial cell injury or microvascular disease. [288] [289] [290]

Pathophysiology of Vaso-occlusion

Understanding sickle cell vaso-occlusion as the result of multiple interdependent pathophysiologic processes does not refute the fundamental importance of polymerization and sickling to the disorder. [13] A comprehensive concept of pathophysiology evolves from critical reassessment of what is known about polymerization, sickling, and vaso-occlusion. Paramount to this understanding is the realization that sickling is a chronic, ongoing process, rather than a sudden cataclysmic one. Sickle cells are deoxygenated, on average, at least once a minute; there is no reason to believe that patients experience systemic deoxygenation or that hypoxemia accounts for routine pain crises. Moreover, certain polymerization-independent processes associated with vaso-occlusion are chronically activated, which suggests that there may be no real steady-state circulation of sickle cell blood. [241]

While the clinical morbidity associated with high intraerythrocytic concentrations of Hb S in Hb SC disease, [151] the clinical benignity associated with the high amounts and pancellular distribution of Hb F in sickle cell-HPFH, [291] and the clinical complications caused by specific circumstances known to enhance polymerization [292] [293] [294] emphasize the importance of polymerization, there are sufficient exceptions to this association that polymerization and sickling should probably be regarded as necessary but not sufficient for understanding vaso-occlusion. The argument that polymerization tendency is the major determinant of clinical severity [94] is valid only as it pertains to the severity of anemia. This proviso is further exemplified by the interaction of thalassemia with sickle cell anemia, where the lower MCHC and polymerization tendency improves the level of anemia, [104] but the higher blood viscosity [156] is detrimental to vaso-occlusive severity. [161] The major influence of Hb F on polymerization [70] and sickling [92] has only small effects on the frequency of pain. [295] The profound effect of Hb S concentration on polymerization is most important to cells having high MCHC (ISCs), but the frequency of pain fails to correlate with

the fraction of ISCs in the circulation [136] and in fact appears to have an inverse relationship. [296] [297] Ex vivo studies of sickle cell blood flow indicate that vaso-occlusion is initiated by the adherence of younger cells with lower MCHC to the vascular endothelium (Fig. 30-9), and that the role of poorly deformable, polymerization-prone ISCs is to propagate but not initiate the process. [76]

Increasing evidence for the importance of sickle cellendothelial cell adherence, hemostatic activation, vascular reactivity, and leukocyte participation suggests that polymerization considerations provide only a first approximation of the severity of sickle cell disease, [94] moment-to-moment vaso-occlusive changes are better understood by considering a wider variety of processes. [13] It has been suggested that the apparent lack of predictability in the periodic vaso-occlusion of sickle cell disease may not be random but chaotic and, thereby, dictated by specific internal deterministic parameters [298] and that vaso-occlusion may be better understood as a chaotic process than as the result of any one external determinant. [13]

The observation of an inverse relationship between severity of pain and NO levels [299] may reflect the complexity of known actions of NO on sickle cell oxygen affinity, [82] blood flow regulation, [80] vasomotor regulation, [300] [301] inflammation and vascular permeability, [302] [303] and neurotransmission. [77] [304] [305]

Mechanisms of Hemolysis

Premature destruction of sickle erythrocytes occurs both extravascularly and intravascularly. [306] Extravascular hemolysis results from abnormalities of the sickle cell that allow its recognition and phagocytosis by macrophages [307] and from impaired deformability of sickle red cells, which allows their physical entrapment. [308] Changes in sickle cell membranes from oxidative damage by unstable Hb S and from recurrent sickling promote binding to the membrane of increased amounts of IgG, which mediates recognition by macrophages. [307] [309] Oxidatively denatured hemoglobin binds to the cytosolic portion of the transmembrane protein, band 3, which signals adherence of IgG and complement to the band 3 exterior. [185] [186] Impaired complement inactivation on sickle cell membranes [310] allows the presence of complement, which facilitates the recognition and phagocytosis of sickle cells by macrophages. [187] Mechanical trapping of poorly deformable ISCs also contributes to extravascular hemolysis, as evidenced by the poor deformability of ISCs, [125] their brief circulatory survival, [126] and correlation of their numbers with the severity of anemia. [135] The declining fraction of dense cells and slight worsening of anemia during painful episodes, [311] [312] and the selective trapping of dense cells in ex vivo flow systems [308] suggests further that mechanical trapping contributes to extravascular hemolysis.

Elevations of free plasma hemoglobin suggest that one third of the total hemolysis in sickle cell anemia is intravascular. [306] One mechanism of intravascular hemolysis is sickling-associated exovesiculation [275] [313] of vesicles rich in phosphatidylinositol-anchored membrane proteins, [314] depleting the cell of the complement regulatory proteins DAF and MIRL and leaving the cells susceptible to complement-mediated intravascular lysis. [194] Another component of intravascular hemolysis is increased mechanical fragmentation of sickle cells, [315] [316] which accounts for the accelerated hemolysis in sickle cell patients during exercise. [315]

Immune Deficit

The propensity of children with sickle cell disease to contract *Streptococcus pneumoniae* infection is related to impaired splenic function [317] and diminished serum opsonizing activity. [318] Even before the anatomic autoinfarction of the spleen in patients with sickle cell anemia, [319] defective splenic function is demonstrable by Howell-Jolly bodies on the peripheral blood smear, [317] visible pits on the surface of red blood cells, and abnormal results of radionuclide spleen scanning. [320] Splenic function is restorable by transfusion prior to the second decade. [321] Specific syndromes exhibiting greater rates of hemolysis cause loss of splenic function at earlier agessickle cell anemia > Hb SC disease > sickle cell- $^{+}$ thalassemia. [320] Using red blood cell pit counts and radionuclide liverspleen scanning, it has been found that the functional asplenia in Hb SC disease is usually delayed until after 4 years of age, which suggests that the routine administration of prophylactic penicillin to infants and young children with Hb SC disease may not be necessary. [322]

Opsonization defects and abnormalities of the alternate complement pathway coexist in patients with sickle cell disease, [323] but evidence of a causal relationship is lacking. [324] Rather, the opsonic defect is the result of an abnormality in natural antibody response that impairs the effector arm of opsonization assays. [324] The variable opsonic defects for different bacteria may depend on selective antibody requirements for opsonization of the different microbes.

The decreased titer of antibody against *S. pneumoniae* antigens following splenectomy in individuals without sickle cell disease [325] suggests that splenic hypofunction

may mediate the opsonic deficiency of sickle cell disease. This conclusion is consistent with the earlier loss of splenic function in genotypes characterized by more rapid hemolysis,[320] the correlation between free plasma hemoglobin levels and the rate of consumption of alternate complement pathway components, [326] and the suppression of B-lymphocyte activity by phosphatidylserine-rich vesicles, [327] such as those shed by sickle erythrocytes during sickling. [328] It has been suggested that an underlying immunologic defect may lead to chronic inflammation, enhanced endothelial cell adhesivity, and vaso-occlusive complications. [243]

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CLINICAL MANIFESTATIONS

The clinical manifestations of sickle cell disease vary tremendously between and among the major genotypes. Even within the genotype regarded as being most severe, sickle cell anemia, some entirely asymptomatic patients are detected only incidentally, while others are disabled by recurrent pain and chronic complications. Typically, patients are anemic but lead a relatively normal life punctuated by painful episodes. Virtually every organ system in the body is subject to vaso-occlusion, which accounts for the characteristic acute and chronic multisystem failure of this disease. Important clinical features less directly related to vaso-occlusion are growth retardation, psychosocial problems, and susceptibility to infection. This section describes the systemic manifestations of disease and those of specific organ systems. Specific therapeutic recommendations are included. For detailed discussions the reader is referred to the monographs of Serjeant, [2] Mankad and Moore,[329] and Embury et al.[1] Practical monographs recommended include *Management and Therapy of Sickle Cell Disease*[330] and a review of pediatric therapy by Vichinsky and Lubin.[331]

Life Expectancy

Decreased life expectancy is one of the original correlates of sickle cell disease. In 1973 Diggs reported that the mean survival was 14.3 years; [332] in 1994 Platt et al. reported that the life expectancy was 42 years for men and 48 years for women with sickle cell anemia.[250] The remarkably prolonged survival over the past 20 years is more the result of improved general medical care than of successful antisickling therapy. The impact of prophylactic

penicillin therapy on preventing death from pneumococcal sepsis [333] may now be affecting survival.

Chronic Anemia

Chronic hemolytic anemia is one of the hallmarks of sickle cell disease. As discussed above, the major cause of anemia is hemolysis. Sickle erythrocytes are destroyed randomly, with a mean life span of 17 days.[334] The survival of ISCs is much shorter than of other sickle cells, [123] and the overall hemolytic rate reflects the number of ISCs.[135] The degree of anemia is most severe in sickle cell anemia and Hb S-° thalassemia, milder in Hb S- $^{+}$ thalassemia and Hb SC disease,[249] and, among patients with sickle cell anemia, less severe in those who have coexistent -thalassemia (Tables 30-1 and 30-2).[99] There are also inappropriately low erythropoietin levels;[335] this is more severe in adults than in children, [335] suggesting the presence of subclinical renal disease. The lower erythropoietin levels in sickle cell disease may reflect the retardant effect of increased blood viscosity on erythropoietin production. [336]

The level of chronic anemia is of considerable predictive value. Patients with more severe anemia are more likely to develop infarctive and hemorrhagic stroke, [251] to have glomerular dysfunction, [337] [338] and perhaps to give birth to low-birthweight babies. [339] [340] Conversely, they have fewer episodes of acute chest syndrome, [252] less frequent pain crises,[161] and (after age 20 years) a lower mortality rate. [161]

Exacerbations of Anemia

The rather constant level of hemolytic anemia may be exacerbated by any of several events, more commonly aplastic crises and acute splenic sequestration and less commonly sequestration in other organs, chronic renal disease, bone marrow necrosis, deficiency of folic acid or iron, and hyperhemolysis.

Aplastic crises are transient arrests of erythropoiesis characterized by abrupt falls in hemoglobin levels, reticulocyte number, and red cell precursors in the marrow. Although these episodes typically last only a few days, the level of anemia may be severe because the hemolysis continues unabated in the absence of red cell production. While the mechanisms that impair erythropoiesis in inflammation are operative in infections of all types (see Chap. 24),[341] human parvovirus B19 specifically invades proliferating erythroid progenitors, [342] which accounts for its importance in sickle cell disease (see Chaps. 19 and 24).[343] Parvovirus B19 accounts for 68% of aplastic crises in children with sickle cell disease, [344] but the high incidence of protective

SPLENIC SEQUESTRATION CRISIS

Splenic sequestration crisis may cause rapid, severe worsening of anemia accompanied by persistent reticulocytosis and a tender enlarging spleen. There is a risk of hypovolemic shock, particularly in children. Young children with sickle cell anemia (Hb SS) have a 30% incidence of this complication, and patients of all ages with Hb SC disease (Hb SC) and sickle cell- thalassemia (Hb S- thal) are at risk. There is a 15% mortality rate. Sequestration is recurrent in 50% of cases, so splenectomy is recommended after the acute event remits. In young children chronic transfusion can be used to prevent recurrence until splenectomy can be performed safely. These episodes often occur concomitant with a viral illness, so parents are trained to palpate enlarging spleens during viral illnesses to detect this complication in its early stages.

antibodies in adults[345] makes parvovirus a less frequent cause of aplasia in this age group. Other reported causes of transient aplasia are infections by *S. pneumoniae*, [346] [347] salmonella,[348] streptococci,[346] and Epstein-Barr virus.[347] Bone marrow necrosis, characterized by fever, bone pain, reticulocytopenia, and a leukoerythroblastic response, also causes aplastic crisis, [349] [350] which too may be the result of parvovirus infection.[351] [352] Inhaled oxygen therapy also causes transient red cell hypoproduction; supraphysiologic oxygen tensions curtail erythropoietin production promptly and suppress reticulocytosis within 2 days. [131]

The mainstay of treating aplastic crises is red cell transfusion. When transfusion is necessitated by the degree of anemia or cardiorespiratory symptoms, a single transfusion usually will suffice, since reticulocytosis resumes spontaneously within a few days. Transfusion may be avoided by keeping severely anemic patients at bed rest to prevent symptoms and by avoiding supraphysiologic oxygen tensions. [353] A useful guideline for transfusion is the reticulocyte count. A patient having an exacerbation of anemia with an elevated absolute reticulocyte count is less likely to require urgent transfusion than one with a normal or low absolute reticulocyte

count.

Acute splenic sequestration of blood is characterized by acute exacerbation of anemia, persistent reticulocytosis, a tender, enlarging spleen, and sometimes hypovolemia.[354] Patients susceptible to this complication are those whose spleens have not undergone fibrosisyoung patients with sickle cell anemia and adults with Hb SC disease or sickle cell-$^{+}$ thalassemia.[355] Sequestration may occur as early as a few weeks of age[356] [357] and may cause death before sickle cell disease is diagnosed.[358] In one study, 30% of children had splenic sequestration over a 10-year period, and 15% of the attacks were fatal. [354] [359] The basis of therapy is to restore blood volume and red cell mass. Because splenic sequestration recurs in 50% of cases, [354] splenectomy is recommended after the event has abated. Alternatively, chronic transfusion therapy is used in young children to delay splenectomy until it can be tolerated safely. [360] Because recurrence is possible during transfusion therapy,[360] parents should be trained to detect a rapidly enlarging spleen and to seek immediate medical attention in this event. Less common sites of acute sequestration include the liver[361] and possibly the lung.[362]

Hyperhemolytic crisis is the sudden exacerbation of anemia with increased reticulocytosis and bilirubin level. It has been argued that many diagnoses of hyperhemolytic crisis are actually occult splenic sequestration or aplastic crises detected during the resolving reticulocytosis. In one report, seven of the eight children with hyperhemolysis had G6PD deficiency.[363] If hyperhemolysis exists, it is probable that many cases are related to some complicating etiology, such as G6PD deficiency or immune hemolysis.

The onset of chronically more severe anemia may be due to developing renal insufficiency (see Chap. 148) or deficiency of folic acid or iron (see Chaps. 26 and 28). Inadequate erythropoietin production in chronic renal failure [364] results in deficient compensation for sickle cell hemolysis. It often is possible to forestall the inevitable transfusion dependence that results from progressive renal failure by taking measures to diminish the hemolytic rate or to stimulate erythropoiesis. We recommend the initial use of hydroxyurea therapy alone; this can be supplemented as necessary with recombinant human erythropoietin, or erythropoietin can be administered alone.[365] In using recombinant human erythropoietin, caution must be exercised not to elevate the hematocrit to levels that result in hyperviscosity. [365] Chronic hemolysis results in increased utilization of folic acid stores, [366] and megaloblastic crises from folic acid deficiency have been reported. [367] [368] Despite increased intestinal absorption of iron in sickle cell disease, [369] the combination of nutritional deficiency[370] and urinary iron losses[371] results in iron deficiency

in 20% of children with sickle cell disease. [370] The diagnosis of iron deficiency may be obscured by the elevated serum iron levels associated with chronic hemolysis, necessitating the detection of a low serum ferritin or an elevated serum transferrin level for the diagnosis.

An unanticipated worsening of anemia has been reported to occur due to bystander destruction of antigen-negative recipient erythrocytes during a delayed hemolytic transfusion reaction.[372]

Acute Painful Episode

An episode of acute pain was originally called a sickle cell crisis by Diggs, who used the expression crisis to refer to any new rapidly developing syndrome in the life of a patient with sickle cell disease.[231] In modern parlance, the term acute painful episode is favored over crisis, in part to diminish the catastrophic associations with this disease. While most authorities have ascribed the acute painful episodes to vaso-occlusion, [1] [7] [373] [374] one expert has argued that pain is more likely the result of shunting of blood flow.[375] [376]

Acute pain is the first symptom of disease in more than 25% of patients, the most frequent symptom after age 2 years,[377] and the complication for which patients with sickle cell disease most commonly seek medical attention.[378] [379] Although a general correlation of vaso-occlusive severity and genotype has been posited, [94] there is tremendous variability within genotypes and in the same patient over time. In one large study of patients with sickle cell anemia, one third rarely had pain, one third were hospitalized for pain approximately two to six times per year,

TREATMENT OF PAIN

The object of analgesic therapy in patients with acute painful episodes is to relieve pain rapidly and safely. The most successful approach to this end has been achieved by comprehensive centers that provide individualized therapy in a setting apart from the emergency ward. A helpful approach to dealing with the patient in pain is to provide psychosocial support by teams of physicians, social workers, and nurses who are known to the patient. Parents and the extended family of pediatric patients should be included. Some programs using oral medication are successful, but often parenteral narcotics are necessary. Recommended agents, doses, schedules, and drug combinations are shown in Table 30-6 . After initial control of pain using aggressive narcotic regimens, rescue therapy by patient-controlled analgesia is useful for maintenance inpatient management. The use of constant intravenous infusion of narcotics must be monitored closely because of the risk of respiratory depression, which is particularly dangerous for patients with sickle cell disease. The newer agents toradol and Tramadol offer the advantages of less respiratory depression and addictiveness. The switch from parenteral to oral analgesics may be problematic because of inadequate dosing of oral drugs and their variable absorption. Following discharge of children from the clinic, emergency ward, or hospital, it may be helpful to provide school re-entry liaison services. Patients should be discharged from the hospital with a limited supply of analgesics to avoid fostering drug-seeking behavior, which may become detrimental to future pain management. Narcotic addiction is no more frequent in sickle cell patients than in any others requiring analgesia and is more closely related to societal issues than to problems specific to sickle cell disease.

and one third had more than six pain-related hospitalizations per year. [380] The frequency of pain peaks between ages 1939 years; over age 19 years, more frequent pain is associated with a higher mortality rate.[161] Over a 5-year period in the National Cooperative Study of Sickle Cell Disease, 40% of patients had no painful episodes, 5% of patients accounted for one third of the emergency room visits, and pain frequency correlated with high total hemoglobin levels and low Hb F levels.[161] Medical personnel who see patients only in the emergency department gain a biased view of sickle cell disease skewed by a frequently afflicted minority whose severe disease is the result of specific hematologic determinants.[381]

Pain may be precipitated by events such as cold, dehydration, infection, stress, menses, and alcohol consumption, but the majority of painful episodes have no identifiable cause.[239] It can affect any area of the body, most commonly the back, chest, extremities, and abdomen, may vary from trivial to excruciating, and is usually endured at home without a visit to the emergency department. There are often premonitory symptoms.[382] The duration averages a few days.[383] Painful episodes are biopsychosocial events[239] caused by vaso-occlusion in an area of the body having nociceptors and nerves. [384] Pain is an affect and, as such, consists of sensory, perceptual, cognitive, and emotional components.[239] Frequent pain generates feelings of despair, depression, and apathy that interfere with everyday life and promote an existence that revolves around pain. This scenario may lead to a chronic debilitating pain syndrome; fortunately, this is rare. The management of pain is discussed in the Therapy section.

Approximately half the episodes present with objective clinical signs such as fever, swelling, tenderness, tachypnea, hypertension, nausea, and vomiting. [297] Numerous laboratory tests have been found to lack specificity as indicators of acute vaso-occlusionleukocytosis, [385] D-dimer fragments of fibrin,[386] and markers of

platelet activation.[387] The most promising laboratory indicators of acute vaso-occlusion are changes in the density distribution of sickle cell subpopulations and the rheologic properties of the blood. [240] [312] Just before the onset of pain there is an increase in the fraction of dense cells and a decrease in the maximum deformability of sickle cells; during the evolution of pain, the dense cell fraction declines and the overall red cell deformability increases. The evolution of pain is also associated with changes in the levels of acute phase reactants, [241] [388] serum lactic dehydrogenase,[389] interleukin-1, tumor necrosis factor,[233] and serum viscosity.[390] These tests are of clinical utility only if they are readily available and can be compared to baseline data.

Pain is more frequent with the Hb SS genotype, low levels of Hb F, -thalassemia, higher Hb levels, [161] and sleep apnea.[391] The frequency of pain is reduced during chronic transfusion therapy. [392]

Multiorgan Failure

This disastrous acute event involves multiple organ systems, including the lungs, brain, kidneys, liver, hematologic system, and heart, and usually leads to death. [393] It may be precipitated by infection, vaso-occlusion, or fat embolus and consists of a constellation of life-threatening processes including hypoxemia, acidosis, inflammation, vascular permeability, severe anemia, disseminated intravascular coagulation, renal failure, and hepatic failure. [394] [395] [396] In addition to therapy specific for these processes, red cell exchange, plasma infusion or exchange, and corticosteroids should be considered. [281] [282]

Psychosocial Issues

Modern insights into the psychosocial adjustment of patients with sickle cell disease have provided a level of understanding

that allows interventional therapy. [397] [398] [399] While most patients with sickle cell disease are generally well adjusted, [400] there are risks of depression, low self-esteem, social isolation, poor family relationships, and withdrawal from normal daily living. [400] [401] [402] Particular stressors are recurrent pain and the response to it, [403] curtailed activity due to pain,[404] misinterpretation of the meaning of pain,[405] and depression leading to learned helplessness. [406] While some patients with sickle cell disease become addicted to narcotics, this is uncommon[407] and usually is the result of social influences rather than pain therapy. [408] Well-adjusted patients have active coping strategies,[409] family support,[410] and support from the extended family unit common in African-American society.[411] Interventional approaches should emphasize recognizing and reinforcing individual strengths, confronting pathologic behavior, and establishing coping skills through reinterpreting pain, diverting attention from pain, and using support systems. Attention to psychosocial concerns is vital to the psychosocial well-being and integration into society of patients with sickle cell disease.[410] [412]

Growth and Development

By age 2 years, children with sickle cell disease have detectable growth retardation that affects weight more than height and has no clear gender difference. [413] By adulthood normal height is achieved, but weight remains lower than that of controls. More severe growth delay is noted in children with sickle cell anemia and sickle cell-° thalassemia; Hb SC disease is associated with a less severe growth delay. Skeletal maturation is also delayed. [414] Girls with sickle cell disease have retarded sexual maturation that is greater in those with sickle cell anemia and sickle cell-° thalassemia than those with Hb SC disease and sickle cell- $^{+}$ thalassemia; [413] it is associated with elevated gonadotropin levels for the stage of sexual development [415] and delayed menarche.[416] Boys also have delayed sexual maturation, which is more severe in those with sickle cell anemia than those with Hb SC disease. [413] Retarded sexual maturation in males can be due to primary hypogonadism,[417] hypopituitarism,[418] or hypothalamic insufficiency. [419] The association of retarded growth with abnormalities of the growth hormone and insulin-like growth factor-I suggests a therapeutic role for these factors in children with sickle cell disease who have delayed growth. [420] [421]

Delayed growth in sickle cell disease is associated with accelerated erythropoiesis, elevations of whole body protein turnover, and basal metabolic rate. [422] [423] When children have both sickle cell disease and hypersplenism, splenectomy may result in improved protein turnover, metabolic rate, and growth parameters. [422] [424] Similarly, weight gain has been reported with transfusion. [425] It also has been possible to restore normal growth by nutritional supplementation. [426] There have been reports of responses to folic acid[427] and zinc supplementation,[428] but these approaches are not recommended as standard care.

Infections

Infectious complications of sickle cell disease are a major cause of morbidity and mortality. [429] [430] The infections caused by particular organisms are shown in Table 30-3 (Table Not Available) and the specific organisms affecting different target organs are shown in Table 30-4 (Table Not Available) . [431]

Bacteremia in children with sickle cell disease is most commonly caused by *S. pneumoniae*.[430] [432] [433] Before the use of prophylactic penicillin, *S. pneumoniae* bacteremia accounted for 510 episodes per 100 patient-years in infants. [429] This event is accompanied by leukocytosis, a left shift, aplastic crisis, sometimes disseminated intravascular coagulation, and a 2050% mortality rate. [429] The risk of recurrent *S. pneumoniae* sepsis and death in patients who have had previous sepsis is much increased; all patients having a history of pneumococcal sepsis should remain on penicillin prophylaxis indefinitely and are not candidates for outpatient management of febrile episodes (see Therapy section). [434] While concerns about *S. pneumoniae* sepsis are largely for young children, this complication also occurs in adults, often with devastating results. [435] [436] The second most

TABLE 30-3 -- Bacteria and Viruses That Most Frequently Cause Serious Infection in Patients with Sickle Cell Disease

(Not Available)

From Buchanan, [431] with permission.

TABLE 30-4 -- Organ-Related Infection in Sickle Cell Disease

(Not Available)

From Buchanan, [431] with permission.

common organism responsible for bacteremia in these children, *Hemophilus influenzae* type b, has accounted for 1025% of episodes. [437] [438] *H. influenzae* bacteremia affects older children and is less fulminant than *S. pneumoniae* bacteremia, but it may be fatal. [429] [439] Therapeutic advances in childhood bacteremia have resulted from vaccination against *S. pneumoniae* and *H. influenzae* type b, prophylactic penicillin, and the long-acting, broad-spectrum antibiotic ceftriaxone.

While unconjugated pneumococcal vaccination has been generally disappointing, [333] [440] [441] regular booster immunization has been found to improve antibody titers,[442] and new conjugated vaccines with pneumococcal polysaccharides linked to tetanus or diphtheria toxoid afford greater immunogenicity and boosterability. [443] Conjugated *H. influenzae* type b vaccines produce excellent antibody responses in children with sickle cell disease [444] and now are administered in early infancy.

Randomized, double-blind, placebo-controlled studies of prophylactic penicillin beginning in infancy, including the PROPS study, have found that this therapy reduced the incidence of *S. pneumoniae* bacteremia by 84% in children younger than 3 years.[333] [440] Its use is currently recommended (see the Therapy section).[432] Concerns regarding this approach have been the uncertainty regarding the safety of discontinuing prophylaxis as a result of suppression of anti- *S. pneumoniae* antibody production and the emergence of penicillin-resistant organisms. A randomized, double-blind, placebo-controlled study, the PROPS II study, found that it is safe to stop prophylactic penicillin therapy at age 5 years in children who have not had prior severe pneumococcal infection or splenectomy and are receiving regular follow-up care.[445] However, penicillin-resistant strains have emerged in one third of nasal *S. pneumoniae* carriers.[446]

The efficacy of ceftriaxone therapy for *S. pneumoniae* and *H. influenzae* infection[447] [448] has led to new treatment algorithms that recommend outpatient therapy for

most patients, as outlined in the Therapy section. However, in certain geographic areas, resistant *S. pneumoniae* have emerged, necessitating a thorough knowledge of local resistance patterns to guide the choice of alternate antibiotics (in particular, vancomycin, to which resistance has not been observed). [449] Bacteremia in older patients is more likely due to *Escherichia coli* and other gram-negative organisms, as are urinary tract infections. [429] [433] [450]

Meningitis in sickle cell anemia is primarily a problem of infants and young children, is caused most frequently by *S. pneumoniae*, and occurs in the setting of bacteremia.[430] Rapid administration of antibiotics has resulted in a lower incidence of meningitis among bacteremic patients than 20 years ago, when the incidence was 50%.[433] [451] *H. influenzae* type b is a less common cause of meningitis.[430] Antibiotic therapy is discussed in the Therapy section.

Bacterial pneumonia may be the cause of the acute chest syndrome. Patients with any combination of dyspnea, cough, chest pain, fever, tachypnea, or leukocytosis should be evaluated by chest x-ray, arterial blood gases, blood and sputum culture, cold agglutinins, and serologic study for *Mycoplasma pneumoniae, Chlamydia pneumoniae*, and *Legionella. S. pneumoniae* and *H. influenzae* type b are uncommon causes of acute chest syndrome, even in children.[452] *M. pneumoniae* and *C. pneumoniae* account for approximately 20%. [452] [453] Antibiotic therapy for the acute chest syndrome should cover these four agents, as discussed in the Therapy section. Respiratory viruses are common causes of pulmonary infection. General therapy for the acute chest syndrome is discussed in the section, Pulmonary Complications.

Osteomyelitis occurs more commonly in sickle cell disease than in normal individuals, [430] probably as a result of infection of infarcted bone. In this patient population osteomyelitis is commonly caused by *Salmonella* species. [454] *Staphylococcus aureus,* the most common etiology in patients without sickle cell disease, accounts for <25% of sickle cell disease cases.[455] [456] Infection usually affects long bones, often at multiple sites. [457] Diagnosis is confirmed by culture of blood or infected bone, before parenteral antibiotics covering *Salmonella* and *S. aureus* are begun,[454] as discussed in the Therapy section. Articular infection is less common and is often due to *S. pneumoniae*.[454]

The specificity of the parvovirus B19 for erythroid precursor cells, [342] [458] coupled with the accelerated erythropoiesis in hemolytic anemias, leaves sickle cell patients vulnerable to infection by this agent.[459] In sickle cell disease, parvovirus infection is a common cause of aplastic crisis, especially in children. [343] [347] [351] [460] [461] It has been reported to cause bone marrow necrosis,[351] [352] acute chest syndrome, pulmonary fat embolism,[351] [462] [463] [464] hepatic sequestration,[465] and glomerulonephritis.[466]

It has been reported that the rate of bacterial infection is reduced during chronic transfusion therapy. [392]

Neurologic Complications

Neurologic complications occur in 25% of patients with sickle cell disease [467] and include transient ischemic attacks (TIAs), cerebral infarction, cerebral hemorrhage, seizures, unexplained coma, spinal cord infarction or compression, central nervous system infections, vestibular dysfunction, and sensory hearing loss. [468] [469] [470] One study reported that the median age for strokes was age 6 years and that after age 14 years, strokes occurred infrequently. [471] Another described a pattern in which infarctive strokes were common in children and those over age 30 years, while hemorrhagic stroke was most common between ages 20 and 30 years. [251]

The most common signs of cerebrovascular accident (CVA) in patients with sickle cell disease are hemiparesis, seizures, coma, speech defects, and visual impairment. CVA may occur spontaneously or intercurrently with other complications, such as pneumonia, aplastic crises, painful episodes, or dehydration. [472] Risk factors for CVA include more severe anemia,[251] [473] higher reticulocyte counts,[473] lower Hb F levels, [474] higher white cell counts,[251] [471] the Hb SS genotype (rather than Hb SC disease or sickle cell- thalassemia), sleep apnea, [475] migraines,[476] elevated homocysteine levels,[272] and relative systolic hypertension (i.e., those at the high end of the lower-than-normal range characteristic of sickle cell disease). [477] We recommend that those risks that are reversible be treated. Genetic markers of increased risk are the Central African Republic haplotype [478] and the absence of -thalassemia.[478] [479]

Cerebral thrombosis, which accounts for 7080% of all CVAs in sickle cell disease, [467] [480] [481] results from large vessel occlusion (Fig. 30-11)[482] [483] [484] rather than the more typical microvascular occlusion of sickle cell disease. CVAs are heralded by focal seizures in 1033% of cases [467] [468] [473] and by TIAs in 10%.[467] [472] [481] CVAs are fatal in approximately 20% of initial cases, recur within 3 years in nearly 70%, and are the cause of motor and cognitive

ACUTE CNS EVENTS

Both children and adults with sickle cell disease have an increased risk of CVA. Infarctive strokes can be predicted using transcranial Doppler study and prevented using prophylactic chronic transfusion. Patients with hemiparesis, seizures, coma, speech defects, or visual impairment should be evaluated for CVA. Cerebral thrombosis accounts for 7080% of all cases of CVA in sickle cell disease, causes death in approximately 20%, recurs within 3 years in 70%, and causes motor and cognitive impairment in the majority. Thrombosis occurs in all age groups, but hemorrhage is far more common in older patients. Intracranial hemorrhage results in the same signs as thrombosis, but neck stiffness, photophobia, severe headache, vomiting, and coma are more common. The mortality rate may be as high as 50%, but the morbidity of survivors is low.

Patients with symptoms and signs of CVA should be evaluated immediately by CT scanning or MRI to distinguish cerebral thrombosis and hemorrhage. In those with thrombosis, prompt partial exchange transfusion is performed, and chronic transfusion is begun to maintain the Hb S level <30% and to prevent recurrent thrombosis. Those with hemorrhage should have angiography after partial exchange transfusion is performed to avoid complications associated with injected contrast material. Hemorrhages due to ruptured aneurysm are amenable to surgical intervention.

Figure 30-11 Right common carotid arteriogram taken in anteroposterior projection, demonstrating complete occlusion of the origin of the right anterior cerebral artery (arrowhead). *(From Stockman et al.,[484] with permission.)*

impairment in the majority.[467] Thrombosis occurs in all age groups; hemorrhage is confined to older patients. [467]

Intracranial hemorrhage results in the same signs as thrombosis, but neck stiffness, photophobia, severe headache, vomiting, and altered consciousness are more common. Coma suggests hemorrhage rather than thrombosis.[473] A typical presentation is coma and seizures without hemiparesis. Although the mortality rate may be as high as 50%,[485] [486] [487] [488] the morbidity of survivors is low.[489] Hemorrhage may be subarachnoid, intraparenchymal, or intraventricular, which can be differentiated by angiography. [486] [487] The favorable neurosurgical outcome in subarachnoid hemorrhage due to ruptured aneurysm justifies an aggressive approach to diagnosis, transfusion, vasodilatory therapy, and surgery. [486] [487] [490]

Patients with symptoms and signs of CVA should be evaluated immediately using computed tomography (CT) scanning or magnetic resonance imaging (MRI) to distinguish TIA, cerebral thrombosis, and hemorrhage. In those with hemorrhage, angiography is indicated, after partial exchange transfusion is performed to avoid

complications associated with the injected contrast material. In those with thrombosis, prompt partial exchange transfusion is performed, and chronic direct transfusion to maintain the Hb S level below 30% is instituted to prevent recurrent thrombosis [472] [483] and promote resolution of arterial stenoses. [472] [491] Although recurrent CVAs during chronic transfusion have been reported, [492] this therapeutic modality provides the best means of preventing recurrence (Fig. 30-12).[483] [493] This treatment also provides incidental protection against pain crises, bacterial infections, acute chest syndrome, and hospitalization. [392] After 5 years of red cell transfusion, clinical response and blood flow[491] [494] are reassessed to guide the decision regarding discontinuing therapy. Transfusion therapy may be required indefinitely in those with persistent flow abnormalities. For those with recurrences soon after discontinuing therapy, indefinite transfusion is reinstituted. [495]

Subclinical cerebral infarction or ischemia has been detected by MRI, positron emission tomography, transcranial Doppler (TCD) flow studies, and autopsy. [210] [496] [497] [498] [499] In a study of 194 children with sickle cell disease, of whom 4.6% had had a prior

Figure 30-12 Comparison of stroke recurrence over 62 months in a transfused group and in untransfused historical control groups.[467] [823] *(Adapted from Pegelow et al.,[493] with permission.)*

stroke, 17.9% were found to have MRI abnormalities; global and specific intellectual dysfunction and neuropsychiatric impairment correlated strongly with prior stroke but also with silent infarcts detected by MRI. [500] Neurodevelopmental abnormalities resulting in poor school performance may be the outcome of silent cerebral infarcts.[501] Noninvasive imaging is useful also in predicting CVA; 30% of children with evidence of arterial stenosis by TCD developed stroke, while none developed stroke in the group without detectable stenosis.[499] In the Stroke Prevention in Sickle Cell Disease trial, 120 children diagnosed by TCD with clinically silent cerebral artery stenosis on the basis of cerebral flow rates 200 cm/sec were randomized to receive chronic transfusion therapy or not; after 16 months follow-up, 11 of 60 untransfused subjects had developed strokes, but only 1 of 60 transfused subjects had had a minor neurologic event. [502]

In 30% of patients with sickle cell disease, major vessel stenosis results in the formation of friable collateral vessels that appear as puffs of smoke (moyamoya in Japanese) on angiography.[503] [504] Moyamoya disease predisposes to thrombotic and hemorrhagic strokes, seizures, and cognitive disability. [505] Surgical approaches to therapy, such as extracranialintracranial bypass, have been useful in improving the perfusion of affected regions of the brain. [506] [507]

Seizures occur more commonly among patients with sickle cell disease. In one study, 21 of 152 patients in a pediatric clinic had seizures, 4 of which were related to meperidine therapy. Most had nonfocal CT and MRI studies but focal electroencephalographic changes. [470]

Pulmonary Complications

Pulmonary disease is the leading cause of death in sickle cell disease. [250] [508] There are both acute and chronic pulmonary manifestations of sickle cell disease. The acute chest syndrome consists of dyspnea, chest pain, fever, tachypnea, pulmonary infiltrate on radiography, and leukocytosis. It affects approximately 30% of patients with sickle cell disease and may be life-threatening. [452] [509] The usual etiology is vaso-occlusion, infection, or both simultaneously, [510] and antibiotics are often indicated as therapy. Microbial pathogens are more commonly identified in children. [510] Many episodes in which common pathogens are not cultured are due to atypical agents (*Mycoplasma, Legionella*, and *Chlamydia*), [452] [453] suggesting that antibiotic therapy include agents directed at atypical agents.

Pulmonary fat embolus, evidenced by stainable fat in pulmonary macrophages obtained by bronchoalveolar lavage or sputum induction, is found in 4460% of cases of acute chest syndrome.[462] [511] Aspiration pneumonia and cardiopulmonary resuscitation may cause lipid-laden macrophages. [512] Acute chest syndrome due to pulmonary fat embolus is associated with more severe hematologic and clinical abnormalities [513] and elevated plasma levels of free fatty acids and phospholipase A_2 [395] [514]

Acute chest syndrome is often preceded by febrile episodes in children and by vaso-occlusive pain crisis in adults (Fig. 30-13). [513] [515]

ACUTE CHEST SYNDROME

Symptoms and signs of the acute chest syndrome include dyspnea, cough, chest pain, and less commonly abdominal pain, fever, tachypnea, leukocytosis, and a pulmonary infiltrate on the chest radiograph. This event has a mortality rate of approximately 10%. The process is usually due to infection or vaso-occlusion but may also be the result of noncardiogenic pulmonary edema or of pulmonary embolization from a distant thrombus or infarcted bone marrow. Responsible infectious agents include *S. pneumoniae, H. influenzae, Mycoplasma, Chlamydia, Legionella,* and viruses. Infection with pyogenic bacteria is more common in children than in adults, and the urgency to initiate antibiotic therapy is greater in the pediatric population. In adults the decision to initiate antibiotics is a matter of clinical judgment. Antibiotic coverage must include *S. pneumoniae, H. influenzae* type b, *M. pneumoniae*, and *C. pneumoniae*for example, cefuroxime and erythromycin.

Whatever the etiology, the major danger of the acute chest syndrome is hypoxemia and its attendant widespread sickling and vaso-occlusion, which create a risk of multiorgan failure. The tachypneic patient should be evaluated with arterial blood gas measurements to distinguish metabolic acidosis, hypoxemia, and anxiety. Thereafter, hypoxemia can be followed by pulse oximetry. If a p_{AO2} >70 mmHg cannot be maintained by oxygen inhalation, the patient is transferred to intensive care, where an emergency partial exchange transfusion is performed. Again, there is a greater sense of urgency regarding hypoxemia in children than in adults, and many pediatricians elect partial exchange transfusion at the first sign of the acute chest syndrome. After transfusion, there is often rapid improvement in oxygenation despite continued radiographic abnormalities. Severe episodes may not respond to this therapy and require support with artificial ventilation or even extracorporeal membrane oxygenation while awaiting recovery.

RIGHT UPPER QUADRANT SYNDROME

An acute complication manifest by some or all of the following featureshyperbilirubinemia, abdominal pain, fever, right upper quadrant abdominal tenderness, hepatomegaly, abnormal liver function tests, and hepatic failurehas been called the right upper quadrant syndrome. Possible etiologies include cholelithiasis, viral hepatitis, biliary cholestasis, and hepatic ischemia. Hemoglobin levels usually do not change greatly. An asymptomatic syndrome of benign cholestasis associated with severe hyperbilirubinemia that resolves in 710 days has been reported in children. A syndrome more common in adults is associated with fever, leukocytosis, abdominal pain, and deteriorating liver function tests. This hepatic crisis usually progresses to hepatic failure, coagulopathy, encephalopathy, and death. This syndrome appears to be caused by hepatic ischemia, but viral hepatitis may produce the same clinical picture. Autoimmune liver disease may present as hepatitis, fulminant hepatic failure, or cholangitis. Hepatitis C is common. Because of the nearly uniform mortality of this type of hepatic crisis, exchange transfusion and plasmapheresis and liver transplantation have been used as therapy. No controlled data are available to support this approach.

Pulmonary fat embolus is often preceded by an acute painful episode. [462] In adults the mortality rate is four times higher than in children. [513] Noncardiogenic pulmonary edema also has been observed.[516] Some patients have a rapidly progressive course associated with a precipitous decrease in arterial oxygen tension; they may require intensive care treatment.[510] When arterial oxygen tension cannot be maintained above 70 mmHg with inhaled oxygen, partial exchange transfusion is indicated.[408] In extreme circumstances, artificial ventilation and extracorporeal membrane oxygenation have been used. [517] It has been reported that the inhalation of NO was beneficial to two children with severe acute chest syndrome.[518] Acute chest syndrome is less common during chronic transfusion programs.[392]

Figure 30-13 Age-specific associated events within 2 weeks preceding acute chest syndrome. *(Adapted from Vichinsky et al.,[513] with permission.)*

Evaluation of chronic pulmonary status in patients with sickle cell anemia reveals restrictive and obstructive lung disease, hypoxemia, and pulmonary hypertension singly or in combination, not restricted to but more common in those with a past history of acute chest syndrome. [519] [520] [521] High-resolution, thin-section CT scanning of the lungs revealed chronic interstitial fibrosis. [522] Pulmonary hypertension usually occurs in adults and carries a poor prognosis. [523] The condition can be diagnosed by cardiac catheterization or echocardiogramDoppler study. There is no efficacious treatment, and these patients may be considered for hydroxyurea therapy, vasodilators, anticoagulation, or oxygen inhalation.

Airway hyperreactivity occurs in nearly two thirds of children with sickle cell disease not diagnosed as having asthma. [524] Thirty-six percent of 53 children with sickle cell disease were found to have sleep-related upper airway obstruction, 16% had hypoxemia, and all 15 who underwent adenotonsillectomy were symptomatically improved and had improved hypoxemia.[525] Sleep apnea may be associated with surgically reversible exacerbations of painful episodes [391] and strokes.[475] Etiologies unrelated to prior acute episodes may relate to chronic impairment of pulmonary blood flow [241] or to oxidative damage mediated by free plasma Hb S.[526] Blood gas and pulmonary function measurements should be obtained as baseline data for all patients.

Hepatobiliary Complications

The prevalence of pigmented gallstones in sickle cell disease is directly related to the rate of hemolysis. [527] In sickle cell anemia, gallstones occur in children as young as 3 to 4 years of age[528] and are eventually found in approximately 70% of patients. [529] Some have recommended the surgical removal of asymptomatic gallstones to avoid subsequent difficulty in distinguishing gallbladder pain from acute painful episodes. [530] This approach has become more feasible with the availability of laparoscopic cholecystectomy.[531]

Potential causes for the common hepatomegaly and liver dysfunction in sickle cell disease are intrahepatic trapping of sickle cells, transfusion-acquired infection, transfusional hemosiderosis, and autoimmune liver disease. [532] [533] Histologic examination of the liver shows centrilobular parenchymal atrophy, bile pigment, periportal fibrosis, hemosiderosis, and cirrhosis. In addition to infection with hepatitis viruses, there are acute hepatic episodes, some of which are unique to sickle cell disease. In all of these, the combination of hemolysis, hepatic dysfunction, and renal tubular defects results in strikingly high serum bilirubin levels, sometimes >100 mg/dl.[534] One of these syndromes, benign cholestasis of sickle cell disease, results in severe, asymptomatic hyperbilirubinemia without fever, pain, leukocytosis, hepatic failure, or death. [535] A far graver event is the hepatic crisis, [231] in which hepatic ischemia results in fever, pain, leukocytosis, severe hyperbilirubinemia, and abnormal liver function tests. [536] It may progress to liver failure, which has a dismal prognosis. [537] [538] Corticosteroid and azathioprine therapy may induce responses in autoimmune liver diseases, including hepatitis, sclerosing cholangitis, and acute hepatic failure. [533] [539]

Chronic hepatitis C infection in sickle cell disease occurs with a prevalence that is related to the number of transfusions received; it may be a leading cause of cirrhosis.[540] Liver transplantation has been used successfully as therapy for this complication. [541]

Obstetric and Gynecologic Issues

Although gynecologic complications (delayed menarche, dysmenorrhea, ovarian cysts, pelvic infection, and fibrocystic disease of the breast) are more common in women with sickle cell disease,[542] the major reproductive concern in these patients is

pregnancy. The fetal complications of pregnancy, most of which are related to compromised placental blood flow, are the increased incidence of spontaneous abortion, intrauterine growth retardation, pre-eclampsia, low birthweight, and death; maternal complications include increased rates of painful episodes, severe anemia, infections, and death.[543] [544] [545] [546] It is controversial whether the degree of anemia predicts the birth of babies with low birthweight. [339] [340] The course of pregnancy is more benign in Hb SC disease than in sickle cell anemia. [544] [545] Better fetal and maternal outcomes in recent years are largely due to generally improved antenatal and obstetric care.[546] An unresolved specific issue pertains to the role of transfusions in pregnancy. [547] Some experts recommend prophylactic transfusion,[548] but a large controlled study showed no improvement in fetal outcome from this management option.[549] The type of delivery does not appear to represent a problem, both spontaneous delivery and cesarean section being well tolerated.

Some experts advise that hypertonic saline injections are contraindicated for elective termination of pregnancy because of the risk of sickling-induced vaso-occlusion. However, most methods of abortion are well tolerated. Based on personal experience, there is a very high incidence of acute painful episodes after therapeutic abortion; inpatient intravenous hydration before and for the 24 hours after the procedure is recommended.

Oral contraceptives containing low-dose estrogen are a safe and recommended method of birth control. [550] The choice of barrier methods, oral contraception, and injections of medroxyprogesterone every 3 months for birth control must weigh the risk versus benefit of each.

Renal Complications

The kidney is particularly vulnerable to complications in sickle cell disease because of its hypoxic, acidotic, and hypertonic microenvironment. [293] [551] Clinical manifestations result from medullary and distal tubular, proximal tubular, and glomerular abnormalities. Occlusion of the vasa rectae compromise flow to the medulla (Fig. 30-14), resulting in inability to concentrate the urine, papillary infarction with hematuria, incomplete renal tubular acidosis, and abnormal potassium metabolism. Isosthenuria, reversible with red cell transfusion up to age 15 years, accounts for a propensity to dehydration when fluid intake is insufficient. [552] [553] A common complication is hematuria from papillary infarction, which is usually unilateral from the left kidney, may be massive, and appears to be more common in sickle cell trait than disease because of the much greater frequency of the heterozygous condition. [554] Patients with sickle cell disease or trait who have hematuria must be evaluated to exclude life-threatening etiologies. This can be accomplished using ultrasonography, which obviates the risks associated with contrast material in intravenous pyelography. Therapy with hydration, alkalinization of the urine, and diuresis is standard, [555] [556] but epsilon-aminocaproic acid, [557] triglycyl vasopressin, [558] intravenous distilled water, [555] and nephrectomy [559] have been required. Patients with sickle cell disease cannot excrete acid and potassium normally but usually do not develop systemic acidosis or hyperkalemia without an additional acid load such as renal insufficiency. [560] [561] Abnormal proximal tubular function, partly related to chronic use of analgesics, results in increased clearance of uric acid [562] and creatinine and reabsorption of phosphate. [563]

Glomerular abnormalities result from vaso-occlusion, hyperperfusion, immune complex nephropathy, and parvovirus infection. [466] [564] In sickle cell disease, glomerular filtration is not elevated commensurately with renal plasma flow, glomeruli have increased permeability, and progressive loss of filtration rate is associated with reduced glomerular porosity. [337] [565] Chronic renal insufficiency may be predicted by albuminuria, [565] and is usually preceded or accompanied by hypertension, hyperkalemia, or

Figure 30-14 Postmortem microangiographic studies of the vasa recta in a normal individual **(A)** and a patient with sickle cell anemia **(B)**. *(From Bertles and Döbler,[824] with permission.)*

worsening anemia. Risk factors for the development of chronic renal failure include relative hypertension, [477] use of nonsteroidal anti-inflammatory drugs, [566] [567] [568] and genetic predisposition associated with the CAR -globin haplotype; the latter has been suggested as an indication for bone marrow transplantation to prevent this outcome. [569] [570] Glomerular disease may be detected using renal ultrasound to detect a pattern of diffusely increased reflectivity. [571] The nephrotic syndrome occurs in 4% of patients, leads to chronic renal failure in two thirds, and results in death in 2 years in half the patients; chronic azotemia is associated with a much worse prognosis. [572] The average age of onset of chronic renal failure is 23 years in sickle cell anemia and 50 years in Hb SC disease. [569] Therapy with an inhibitor of angiotensin-converting enzyme was used to diminish hyperperfusion and was found to diminish proteinuria but did not improve the glomerular filtration rate. [573] Renal transplantation is recommended for patients with sickle cell and end-stage renal diseases. [570] [574] [575] [576] [577] [578]

Priapism

Priapism has been defined as an unwanted painful erection. It has been reported to affect 6.442% of males with sickle cell disease, [579] has peak frequencies between ages 5 and 13 years and 21 and 29 years, [580] and is most likely to develop in patients with lower Hb F levels and reticulocyte counts, increased platelet counts, and the Hb SS genotype. [581] [582] [583] Its onset can be acute, recurrent, chronic, acute on chronic, or stuttering. [584] The engorgement in priapism affects the corpora cavernosa but spares the glans penis and corpus spongiosum. [585] In a minority of patients, usually postpubertal, the engorgement also affects the corpus spongiosum and glans; [586] tricorporal priapism can be diagnosed using nuclear scanning of the penis. [587] As a result of recurrent priapism, sickle cell patients have abnormal nocturnal penile tumescence and scarred fibrotic corpora, [588] which may eventuate in impotence. [582] Impotence is more frequent in tricorporal priapism. [586]

PRIAPISM

Priapism affects nearly two thirds of males with sickle cell disease. It typically is bicorporal, affecting the corpora cavernosa and sparing the glans penis and corpus spongiosum. Tricorporal priapism is less common, affects also the corpus spongiosum and glans, and more frequently results in impotence. Preventing recurrences may be accomplished by self-administration of the -adrenergic agent etilefrine orally and by intracavernous injection of this agent for episodes lasting >1 hour. Recommended management includes monitoring response to therapy by intercavernous pressure measurements and to transfusion therapy by quantitative hemoglobin electrophoresis. Initial therapy consists of intravenous hydration and analgesia. If priapism persists 12 hours, partial exchange transfusion, intended to reduce the Hb S level to <30%, is performed. If there is no resolution within 12 hours of transfusion, corporal aspiration with saline and -adrenergic agents is recommended. If there is no response within 12 hours of irrigation, surgery is recommended. The Winter procedure of creating a fistula between the glans penis and the corpora cavernosa by inserting a large-bore needle through the glans under general anesthesia is the procedure of choice. If there is no resolution following the Winter procedure, the Grayhack saphenous vein bypass shunt is recommended.

A successful approach to preventing the recurrence of priapism entails self-administration of the -adrenergic agent etilefrine orally and, for episodes lasting over an hour, by intracavernous injection. [589] A more traditional approach [590] involves monitoring response to therapy by intracavernous pressure measurements and transfusion therapy by quantitative hemoglobin electrophoresis. First-line therapy is conservative, consisting of intravenous hydration and analgesia. If priapism persists 12 hours, partial exchange transfusion [591] is performed to reduce the Hb S level to <30%. If no resolution ensues within 12 hours of transfusion, corporal aspiration with saline and -adrenergic agents is recommended. [592] If there is still no response within 12 hours of irrigation, surgery is recommended. [593] The procedure of choice is the Winter procedure, creating a fistula between the glans penis and the corpora cavernosa by inserting a large-bore needle through the glans under general anesthesia. [594] If this is unsuccessful, the creation of a more formal shunt, such as the Grayhack saphenous vein bypass, may be attempted. [595] Whether conservative or aggressive therapy is used, 45% of patients who have priapism develop some degree of impotence. [582] [583] When impotence persists 12 months, a semirigid penile prosthesis may be implanted. [596]

Ocular Complications

Ophthalmologic complications can include anterior chamber ischemia, tortuousity of conjunctival vessels, retinal artery occlusion, angioid streaks, proliferative retinopathy, and retinal detachment and hemorrhage. [597] [598] The retina is particularly vulnerable to vaso-occlusion, and routine retinal examination is part of routine health-care maintenance for patients with sickle cell disease. Superficial retinal hemorrhages have a pink salmon patch appearance that resolves into an iridescent schisis cavity. Deeper retinal hemorrhages have a black sunburst appearance. Postmortem examination using the ADPase flat-embedded retina technique to define the morphometry of retinal vessels found that autoinfarction occurs at the preretinal capillary rather than the feeding arteriole level and that sea fans tend to develop at the site of arteriovenous crossings. [599] Clinically the retinopathy is best seen using fluorescein angiography (Fig. 30-15). The earlier onset and greater frequency of proliferative retinopathy in Hb SC disease and sickle cell- $^{+}$ thalassemia compared to sickle cell anemia and sickle cell-° thalassemia [600] suggest that retinal vessels are more susceptible to occlusion by more viscous blood than by more rigid individual cells. Peripheral sickle retinopathy may require vision-saving therapy with laser

photocoagulation.[601]

Orbital compression syndrome due to vaso-occlusion of the periorbital marrow space and subperiosteal hemorrhage has been observed to result in headache, fever, and palpebral edema.[602] In this situation, culture, CT scan, and MRI should be used to rule out infectious, neoplastic, and other hemorrhagic etiologies. Conservative therapy, including local measures, analgesia, fluids, transfusion, and careful ophthalmalogic surveillance, is recommended, unless compression of the optic nerve ensues, in which case surgical decompression should be considered.

Bone Complications

Chronic tower skull, bossing of the forehead, and fish mouth deformity of vertebrae are the result of extended hematopoietic marrow causing widening of the medullary space, thinning of the trabeculae and cortices, and osteoporosis. [603] Osteonecrosis may cause a steplike depression of vertebrae, selected shortening of the cuboidal bones of the hands and feet, and acute aseptic or avascular necrosis. [604] [605] The excruciating pain of bone infarction in the hand-foot syndrome that occurs around age 2

Figure 30-15 Fluorescein angiography demonstrating a sea fan appearance of sickle proliferative retinopathy. *(Kindly provided by WC Mentzer.)*

years is often the first symptom of sickle cell disease (Fig. 30-16).[606] This dactylitis resolves spontaneously and is treated with hydration and analgesia. Bone infarcts are demonstrable using nuclear medicine scintigraphy or MRI. [607] [608] [609] Serial scans specific for bone osteoclasts, bone marrow macrophages, and inflammatory cells may be useful adjuncts for distinguishing bone marrow infarction from osteomyelitis, [607] but it is essential to obtain cultures directly from the affected tissue before starting antibiotics. Treatment of osteomyelitis is addressed in the Infection and Therapy sections.

Infarction of bone trabeculae and marrow cells causing osteonecrosis occurs in all sickle cell disease genotypes, but

Figure 30-16 Radiogram showing the bone infarctions in the hands of a child with the hand-foot syndrome dactylitis. *(Kindly provided by WC Mentzer.)*

most frequently in sickle cell anemia with coexistent -thalassemia. [159] [610] The process is associated with increased intraosseous pressure, [611] and is most sensitively detected by MRI.[612] Necrosis occurs with equal frequency in the femoral and humeral heads, but the femoral heads more commonly undergo progressive joint destruction as a result of chronic weight bearing [159] [160] [613] [614] Core decompression surgery to relieve increased intraosseous pressure can be used in early-stage osteonecrosis (i.e., no radiographic evidence of bone collapse) to prevent disease progression. [611] In more advanced disease, a choice must be made whether to accept limited joint mobility or to attempt major reconstructive therapy. This decision must take into account the 30% likelihood that a second hip revision will be required within 45 years of prosthetic hip placement in patients with sickle cell disease. [159]

Arthritic pain, swelling, and effusion [615] may be related to periarticular infarction [616] or gouty arthritis. [617] Appropriate therapy is with nonsteroidal anti-inflammatory agents.

Bone marrow infarction causes reticulocytopenia, exacerbation of anemia, a leukoerythroblastic picture, and sometimes pancytopenia. [349] Pulmonary fat embolism is a rare complication of marrow infarction.[618] It is associated with fat globules in the sputum [618] and refractile bodies visible in the optic fundi. [619] It is a life-threatening event that may require prompt exchange transfusion and perhaps the use of heparin and corticosteroids. [618]

Dermatologic Complications

Leg ulcers are major causes of morbidity in sickle cell disease as a result of their frequency, chronicity, and resistance to therapy. Most occur near the medial or lateral malleolus (Fig. 30-17), and they are frequently bilateral. [620] [621] They may begin spontaneously or as a result of trauma and may become infected, most commonly by *S. aureus, Pseudomonas*, streptococci, or *Bacteroides*.[622] Systemic infection, osteomyelitis, and tetanus are rare complications. [620] Ulcers are resistant to healing[623] and tend to be recurrent in well over half the cases.[622] Their incidence has been reported to vary from 0%[624] to 75%.[620] Ulcers rarely occur in patients younger than age 10 years and are most common in sickle cell anemia, less common in sickle cell-° thalassemia, and nonexistent in Hb SC disease and sickle cell- $^{+}$ thalassemia. [621] The incidence in sickle cell anemia patients declines substantially in those who have coexistent thalassemia. [621] Males have a threefold greater risk for developing leg ulcers than females. [620] [621]

Figure 30-17 Chronic leg ulcer near the medial malleolus. *(Kindly provided by WC Mentzer.)*

Treatment of leg ulcers requires persistence and patience; healing usually requires weeks. Therapy begins with gentle debridement to remove nonviable, superficial tissue from more vital areas. Wet-to-dry dressings and Duoderm hydrocolloid dressings facilitate devitalization. Once debridement is complete, zinc oxide-impregnated Unna boots are used to promote healing. Bed rest speeds healing, [625] and topical antibiotics may be required. [626] It may be necessary to use elastic wraps or leg elevation to control edema. Oral zinc, local hyperbaric oxygen, chronic transfusion, recombinant erythropoietin, hydroxyurea, propionyl-L-carnitine, skin grafts, and pentoxifylline are unproven modalities that may be tried.

Myofascial syndromes consist of soft-tissue swelling in subcutaneous edema that may have a peau dorange appearance. These may be large or discrete lesions a few centimeters in diameter.

SKIN ULCERS

Leg ulcers occur in patients with sickle cell disease beginning in the teenage years. They usually occur near the lateral or medial malleolus and may become chronic and debilitating. Their pathogenesis presumably is related to vaso-occlusion in the skin microvasculature leading to tissue necrosis. Ulcers occur most commonly in males, those with more severe anemia, and those with lower Hb F levels. Treatment of leg ulcers begins with cleansing, debridement, and topical antibiotics. Gentle debridement removes nonviable, superficial tissue from more vital areas. Wet-to-dry dressings and Duoderm hydrocolloid dressings facilitate autodebridement of nonviable surrounding tissues. Once debridement is complete, zinc oxide-impregnated Unnas boots are used to promote healing. Leg edema retards healing of ulcers and can be treated using elastic wraps or leg elevation. Trauma to the area is to be minimized, and shoes must be selected accordingly. Transfusions may be required. Skin grafting has been successful in the most recalcitrant cases.

These lesions are probably the result of dermal or subdermal vaso-occlusion. Treatment is symptomatic.

Cardiac Complications

Although there is no convincing evidence for a specific cardiomyopathy in sickle cell disease, there are important cardiac considerations in the management of patients with sickle cell disease. The chronic anemia of sickle cell disease is compensated by high cardiac output, [627] which results in chronic chamber enlargement and cardiomegaly,[628] even in young children.[629] While the exercise capacity of sickle cell patients is diminished, [630] congestive heart failure is uncommon,[631] and restriction of activity is seldom necessary. An age-dependent loss of cardiac reserve [632] creates a greater risk of heart failure in adult patients during fluid overload, transfusion, reduced oxygen-carrying capacity, or hypertension. [628] Cardiac function can be improved by transfusion. [633] The electrocardiogram shows evidence of left ventricular hypertrophy, and less often first-degree block and nonspecific ST-T wave changes. [634] Acute myocardial infarction in the absence of coronary disease has been reported,[635] and in one autopsy series 9.7% of 72 consecutive patients with sickle cell disease had myocardial infarction. [636] It appears that myocardial infarction may occur with normal coronary arteries as a result of increased oxygen demand exceeding limited oxygen-carrying capacity, or as a result of microcirculatory impairment. In the latter regard, second-degree A-V block associated with septal hypoperfusion affecting the A-V node and His bundle has been observed during a pain episode.[637] It has been suggested that the increased rate of sudden death observed in sickle cell disease may be related to cardiac autonomic dysfunction, as detected by abnormal heart rate variability in response to selected postural maneuvers. [638]

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VARIANT SICKLE CELL SYNDROMES

The sickle cell syndromes that result from inheritance of the sickle cell gene in simple heterozygosity or in compound heterozygosity with other mutant -globin genes are sickle cell trait, Hb SC disease, and sickle cell- thalassemia. These and sickle cell anemia with coexistent -thalassemia are reviewed in this section.

Sickle Cell Trait

The prevalence of sickle cell trait is approximately 810% in African-Americans [62] and as high as 2530% in certain areas of western Africa.[64] There are approximately 2.5 million people in the United States and 30 million in the world who are heterozygous for the sickle cell gene. Sickle cell trait is a benign carrier condition [148] with no hematologic manifestationsred cell morphology, red cell indices, and the reticulocyte count are normal, and sickle forms (ISCs) are not seen on the peripheral blood smear. The usual partition of Hb A and Hb S in sickle cell trait is 60:40 due to a greater post-translational affinity of chains for ^{A} than for ^{S} chains. [639] When thalassemia is coinherited with sickle cell trait, the preferential affinity results in a decreased percentage of hemoglobin S relative to the number of -globin genes deleted (i.e., / 40% Hb S; / 35% Hb S; / 29% Hb S; / 21% Hb S).[99]

Few clinical complications of sickle cell trait have been reported. [148] Splenic infarction occurs at high altitude, [640] curiously more often in Caucasians than in those of African ancestry.[641] In evaluating the etiology of hematuria, it must be remembered that sickle cell trait is a common cause among African Americans. [642] Impaired urine-concentrating ability is directly related to the intraerythrocytic concentration of Hb S and, therefore, to the number of -globin genes inherited. [149] [150] The degree of

hyposthenuria in children progresses with age, and up to a certain age is reversed by transfusion. [643] The frequency of urinary tract infection may be increased in sickle cell trait.[644] [645] There is no increased incidence of anesthetic complications associated with sickle cell trait. [646] The 30-fold increased incidence of unexplained sudden death during basic training of military recruits was apparently related to exercise-induced vaso-occlusion and rhabdomyolysis. [647]

Despite the known complications, past experiences with discrimination in the employment market and health insurance industry provide reminders that the rare clinical events in sickle cell trait provide no real justification for regarding it as anything but a benign carrier condition. [648] Newborn screening programs detect a large number of infants with sickle cell trait; for these parents, genetic counseling is essential. Parents should understand that their child has a benign hereditary condition, not a disease, but that there is a risk for a subsequent child to be born with sickle cell disease.

In individuals who appear to have sickle cell trait but are symptomatic, the laboratory diagnosis must be verified. Hemoglobins other than S that polymerize may account for reports of sickle cell trait associated with clinical problems. Examples are heterozygous Hb S ^{Antilles64§} and Hb Quebec-CHORI.[650] In the latter case, the hemoglobin variant was distinguished from Hb A using mass spectroscopy.

Hb SC Disease

The gene for Hb C ($_{2\ 2}$ ^{6GluLys}) is approximately one-fourth as frequent among African Americans as the sickle cell gene, [62] accounting for a corresponding prevalence of Hb SC disease and sickle cell anemia. Although oxygenated Hb C forms crystals, [651] Hb C does not participate in polymerization with deoxy-Hb S. [151] As discussed above in the section Cation Homeostasis and Cell Dehydration, the red cell desiccation that results from potassiumchloride cotransport sustained by the presence of Hb C[152] [153] raises the intraerythrocytic concentration of Hb S [151] [152] to levels that support polymerization, sickling, and clinical symptoms. Compound heterozygosity for Hb S and C results in a disease that is less severe than sickle cell anemia. [652] [653] Splenomegaly may be the only physical finding, and clinical complications may be less frequent than in sickle cell anemia. As a result of a longer circulatory survival of Hb SC red cells compared to Hb SS cells (i.e., 27 vs. 17 days), [334] the degree of anemia and reticulocytosis is frequently mild: 75% of the patients have a milder level of anemia (hematocrit level >28%) than is usually seen in sickle cell anemia.[249] The predominant red cell abnormality on the peripheral smear is an abundance of target cells; folded (pita bread) cells, ISCs, billiard ball cells, and crystal-containing cells also may be seen. [654]

The great clinical heterogeneity within this genotype notwithstanding, the clinical course is generally milder than that of sickle cell anemia. The frequency of acute painful episodes is approximately half that of sickle cell anemia, [161] and the life expectancy is two decades longer. [250] The incidence of fatal bacterial infection is less than in sickle cell anemia, [429] but there is still an increased risk of *S. pneumoniae* and *H. influenzae* infection.[655] Leg ulcers are uncommon.[621] There is a higher incidence of peripheral retinopathy in Hb SC disease than in sickle cell anemia, [600] but the higher incidence of osteonecrosis reported [656] has been disproven.[159]

Sickle Cell- Thalassemia

The gene frequency of thalassemia among African Americans is 0.004, one-tenth that of the sickle cell gene, [62] and hence there is one-tenth the prevalence of compound heterozygous sickle cell- thalassemia is this population. Sickle cell- thalassemia is divided into sickle cell- $^{+}$ thalassemia and sickle cell-° thalassemia, which have, respectively, reduced or no amounts of Hb A present. [657] [658] Most -thalassemia mutations among African Americans result in $^{+}$ thalassemia,[659] [660] and sickle cell-$^{+}$ thalassemia is subclassified according to the percentage of Hb A present: type I has 35%; type II has 814%; and type III has 1825%. [659] [661] Eighty percent of African-American -thalassemia mutations are due to the promoter region mutations [88 (CT) and 29 (AG)] that result in a type III phenotype. [659] [660] Compound heterozygous sickle cell-° thalassemia occurs infrequently.

The hematologic and clinical severity is a function of the amount of Hb A inherited. [661] [662] In sickle cell- thalassemia, the red cells are hypochromic and microcytic, and the ISCs present on the peripheral blood smear are more numerous in sickle cell-° thalassemia than in sickle cell- $^{+}$ thalassemia. The hematologic severity is a function of the amount of Hb A inherited, as demonstrated in Table 30-1 .

The more benign clinical nature of sickle cell- $^{+}$ thalassemia compared to sickle cell-° thalassemia is reflected by its threefold higher incidence of incidental diagnosis (26% vs. 9%), later mean age of presentation (3.2 years vs. 8.2 years), approximately threefold more frequent leg ulcers (23% vs. 8%), approximately twice as frequent acute chest syndrome (24% vs. 14%), priapism in 4 of 28 versus 0 of 27, and aplastic crises in 2 versus 0. [663] Splenomegaly occurred in approximately one third of both groups. There was a higher incidence of proliferative retinopathy in sickle cell- $^{+}$ thalassemia (18% vs. 10%), consistent with the notion that sickle cell syndromes having higher hematocrits are more likely to be associated with ocular complications. Growth and development was more retarded in sickle cell-°

thalassemia than in sickle cell- $^{+}$ thalassemia.[413]

Sickle Cell Anemia with Coexistent Thalassemia

The use of gene mapping to detect the -globin gene deletions responsible for thalassemia [664] [665] demonstrated a gene frequency of 0.16 for the -thalassemia-2 haplotype () among African Americansnearly 1 in 3 are silent carriers of thalassemia (genotype /), and 2% have -thalassemia trait due to homozygous -thalassemia-2 (genotype /).[100] This high frequency, combined with the powerful effect of Hb S concentration on kinetics [7] and extent[8] of Hb S polymerization, suggested that the lower MCHC of thalassemia would influence the pathophysiology in a large number of patients with sickle cell anemia. This prediction was substantiated by the finding of milder anemia in subjects with sickle cell anemia who had the deletion of either one (genotype /) or two (genotype /) -globin genes (Table 30-2).[103] [104] [106] This effect was related to a diminished hemolytic rate[105] and was not demonstrable until approximately 7 years of age (Table 30-2).[107] [108] The mechanisms by which thalassemia benefits the hematologic aspects of sickle cell anemia were discussed in the Cation Homeostasis and Cell Dehydration section.

Besides milder anemia, thalassemia is associated with fewer reticulocytes [103] [104] [106] and ISCs.[102] [104] The peripheral blood smear contains less polychromasia and fewer sickle forms and more hypochromia and microcytosis, commensurate with the numbers of -globin genes deleted. There are increased Hb A $_2$ levels associated with increasing -globin gene deletions; the Hb F levels are not consistently affected. [103] [104] [106] The prediction of increased affinity of for chains, resulting in higher levels of Hb F when chains are limiting, is not consistently realized, apparently as a result of the selective survival of -thalassemic sickle cells precluding the Hb F effect.[666]

The clear-cut clinical effects of this epistatic interaction are of inconsistent advantage. The reported decreased incidence of acute chest syndrome [104] was not substantiated in another study;[106] there is a decreased incidence of leg ulcers [104] [621] but an increased incidence of osteonecrosis, [155] [160] and the original suggestion

of a decreased incidence of peripheral retinopathy [597] was found to correlate with the higher hemoglobin, MCV, and MCHC levels. [667] The frequency of retinal vessel closure is higher, but not the incidence of retinopathy. [668] In a large multicenter study there was a higher incidence of acute painful episodes associated with higher hemoglobin levels, but the influence of thalassemia did not extend beyond its influence on level of anemia. [161] While one group reported longer life expectancy in subjects having sickle cell anemia and thalassemia, [154] [669] a large multi-institutional national study documented an increased mortality rate associated with higher hemoglobin levels after age 20 years.[161] The interaction of thalassemia with sickle cell anemia has shown clearly the imprudence of extrapolating broadly from polymerization formulas to assumptions of disease severity.[94]

Sickle Cell-° Thalassemia

The -thalassemia locus usually is one of several large deletions of the - and -globin genes that fail to retard the switch from fetal to adult hemoglobin production, thereby allowing an attempted switch from the expression of -globin to that of deleted genes (see Chaps. 22 and 29). This is an uncommon compound heterozygous condition associated with Hb S, F, and A $_2$, with the 1525% Hb F distributed in a heterocellular fashion. Anemia and reticulocytosis are mild and clinical complications infrequent.[670]

Sickle Cell-HPFH

Hereditary persistence of fetal hemoglobin results from one of several large deletions of the - and -globin genes that retard the switch from the production of Hb F to adult hemoglobin (see Chaps. 22 and 29). A more recently discovered variety of HPFH is not due to a deletion, but to one of many point mutations that upregulate the expression of the -globin gene. The clinical expression of deletional and nondeletional HPFH differs in that the 1535% Hb F in the former is distributed in a pancellular fashion, the 15% Hb F in the latter is distributed in a heterocellular fashion, and certain mild types of nondeletional HPFH express high Hb F levels not in simple heterozygosity but only in conditions of erythropoietic stress, such as compound heterozygosity with the sickle cell gene. It is likely that many cases of apparent sickle cell anemia with unexplained elevations of Hb F are the result of a nondeletion HPFH mutation.

The gene frequency of the deletional HPFH locus is 0.0005 among African Americans, [671] resulting in a calculated incidence for compound heterozygous sickle cell-deletional HPFH of 1/100 that of sickle cell anemia. Sickle cell-deletional HPFH provided the first evidence that Hb F was a potent inhibitor of Hb S polymerizationindividuals with pancellular distribution of 25% Hb F were generally neither anemic nor afflicted with vaso-occlusive manifestations (Table 30-1). [291] Hemoglobin electrophoresis revealed only Hb S, F, and A $_2$, which resembles sickle cell anemia, sickle cell-° thalassemia, and sickle cell-° thalassemia. Notable differences, however, are the pancellular distribution of 1535% Hb F, Hb A $_2$ levels <2.5%, and the absence of anemia.[672] The generally benign course of sickle cell-deletional HPFH is uncommonly associated with vaso-occlusive complication. [291]

Sickle Cell-Hb Lepore Disease

The Hb Lepore gene is a crossover fusion product of the - and -globin genes, the product of which, in the case of Hb Lepore Boston, has the same alkaline electrophoretic mobility as Hb S. Because of the thalassemic expression of the fusion gene, individuals with simple heterozygosity for Hb Lepore Boston resemble on hemoglobin electrophoresis sickle cell trait, with only 12% Hb S. Compound heterozygous Hb S-Hb Lepore Boston resembles sickle cell anemia or sickle cell-° thalassemia electrophoretically but has less severe anemia, resembling that of sickle cell- $^{+}$ thalassemia. The combination of predominantly Hb S with microcytosis suggests sickle cell- thalassemia, but the diagnosis is suggested by the low to low-normal Hb A $_2$ levels that result from the incapacitation of one -globin gene by the crossover. Hb F levels vary. The peripheral smear shows microcytosis, hypochromia, and ISCs. Vaso-occlusive complications occur, and splenomegaly is common.[673]

Sickle Cell-Hb D Disease

Because Hb D Punjab or Hb D Los Angeles ($_2$ $_2$ ^{121GluGln}) has a similar electrophoretic mobility to Hb S under alkali conditions, Hb SD disease was first reported as an unusual case of sickle cell anemia.[674] Hb D can be distinguished from Hb S by acid electrophoresis or isoelectric focusing. There is moderately severe hemolytic anemia, and the peripheral smear shows marked anisocytosis and poikilocytosis, target cells, and ISCs. The clinical manifestations of this syndrome are similar to those of sickle cell anemia.[675]

Sickle Cell-Hb O Arab Disease

Although Hb O Arab ($_2$ $_2$ ^{121GluLys}) was first described in an Israeli Arab family, [676] its distribution is widespread. Sickle cell-O Arab disease resembles Hb SC disease on alkaline electrophoresis, but Hb O Arab can be distinguished from Hb C by acid electrophoresis or isoelectric focusing. This syndrome is associated with moderately severe hemolytic anemia, and the peripheral smear shows anisocytosis, poikilocytosis, and ISCs. [677]

Sickle Cell-Hb E Disease

Hb E ($_2$ $_2$ $^{26Glu Lys}$) is a -thalassemic hemoglobinopathy found predominantly in southeast Asia (see Chap. 29). The structural mutant has an electrophoretic mobility similar to Hb E and C under alkaline conditions [678] [679] but can be resolved by acid electrophoresis or isoelectric focusing. The GAGAAG mutation in codon 26 activates a cryptic splice site within the first intron of the ^{E} gene, causing alternate splicing and decreased expression of the structural mutant. [680] As a result, Hb E makes up only 30% of the total hemoglobin in compound heterozygosity for the sickle cell and Hb E genes. Hb SE disease is reported to cause mild hemolysis, no vaso-occlusive complications, and no remarkable abnormality of red blood cell morphology. [681] [682] [683] However, one report documented hematuria and a probable splenic infarct during air travel, [684] and another described moderately severe hemolysis, jaundice, bone pain, splenic infarction, recurrent pneumonia, and ISOs on the peripheral blood smear of three siblings having Hb SE. [685] Patients with Hb SE disease having vaso-occlusive complications justify the inclusion of this syndrome among the sickle cell diseases.

DIAGNOSIS

Both the methods of diagnosing sickle cell syndromes and the goals of diagnostic programs vary depending on the developmental stage of the subject. Characterization of adult hemoglobins is challenging in the fetal and newborn periods because of the predominance of Hb F, which confounds reliable detection of Hb S by solubility testing. In infancy, the diagnosis of sickle cell disease is also influenced by the absence of anemia. As Hb S increases and Hb F declines in the first months of life (Fig. 30-7), the clinical manifestations of sickle cell disease emerge. [686] ISCs can be seen in the peripheral blood of children with sickle cell anemia at 3 months of age, and by 4 months of age moderately severe hemolytic anemia is evident. Useful diagnostic

Figure 30-18 Comparative analyses of several mutant hemoglobins using alkaline electrophoresis, acid electrophoresis, and thin-layer isoelectric focusing. On the right are shown the components of the standard (at the top) and the phenotypes of the other six samples. Their analyses are shown by alkaline hemoglobin electrophoresis in the left panel, acid electrophoresis in the center panel, and thin-layer isoelectric focusing in the right panel. Locations of the various hemoglobin bands are shown below the left and center panels. *(Kindly provided by MH Steinberg.)*

SICKLE CELL DISEASE DIAGNOSIS

Diagnosis of sickle cell syndromes (genotypes) involves examination of the peripheral smear, solubility testing, and hemoglobin electrophoresis (or thin-layer isoelectric focusing).

- The peripheral smear is normal in sickle cell trait (Hb AS), but sickle cells are seen in each of the major sickle cell disease syndromessickle cell anemia (Hb SS), Hb SC disease (Hb SC), and sickle cell- thalassemia (Hb S- thal).
- Solubility tests are abnormal in all syndromes having at least one sickle cell genesickle cell trait (Hb AS), sickle cell anemia (Hb SS), Hb SC disease (Hb SC), and sickle cell- thalassemia (Hb S- thal).
- Hemoglobin electrophoresis (or thin-layer isoelectric focusing) provides definitive diagnostic information.
  - Newborns with sickle cell anemia (Hb SS) and sickle cell-° thalassemia (Hb S-° thal) have Hb S and F (SF pattern); older patients have predominantly Hb S (SS pattern).
  - Newborns with Hb SC disease (Hb SC) have Hb S, C, and F (SCF pattern); older patients have Hb S and C (SC pattern).
- Both sickle cell trait (Hb AS) and sickle cell-$^{+}$ thalassemia (Hb S-$^{+}$ thal) have Hb S and A, but in different proportions. In the former Hb A > S; in the latter Hb S > A.
  - Newborns with sickle cell trait (Hb AS) have Hb A, S, and F (ASF pattern); older patients have Hb A and S (AS pattern).
  - Newborns with sickle cell-$^{+}$ thalassemia (Hb S-$^{+}$ thal) have Hb S, A, and F (SFA pattern); older patients have Hb S and A (SA pattern).

methods include those that separate species according to amino acid composition (i.e., hemoglobin electrophoresis or thin-layer isoelectric focusing) (Fig. 30-18), solubility testing, and examination of the peripheral blood smear (Fig. 30-2).[6] [687] [688]

Older Children and Adults

The purpose of correct diagnosis in this age group is to identify those who need therapy for sickle cell disease and counseling for the disease or the trait. Cellulose acetate electrophoresis at pH 8.4 is a standard method of separating Hb S from other variants. However, Hb S, G, and D have the same electrophoretic mobility with this method. Using citrate agar electrophoresis at pH 6.2, Hb S has a different mobility than Hb D and G, which comigrate with Hb A in this system. A solubility test such as the Sickledex also distinguishes Hb D and G from Hb Sonly Hb S precipitates. Thus, the combination of cellulose acetate electrophoresis with either citrate agar electrophoresis or a solubility test allows a definitive diagnosis of a sickle cell syndrome. Alternatively, thin-layer isoelectric focusing will separate Hb S, D, and G and can replace the two electrophoretic methods. Even with thin-layer isoelectric focusing, it is still necessary to use a confirmatory solubility test for Hb S. The sickle cell prep using metabisulfite or dithionite is not in routine clinical use at this time and is of historical interest only.

Results from electrophoresis or thin-layer isoelectric focusing are similar in sickle cell anemia and sickle cell-° thalassemianearly all the hemoglobin consists of Hb S. While differences in the Hb F and Hb A_2 levels may be useful in distinguishing these syndromes, the presence of microcytosis or one parent without sickle cell trait are more useful indicators of sickle cell-° thalassemia. The diagnosis of Hb SC disease is straightforward; nearly equal amounts of Hb S and Hb C are detected. Sickle cell-$^{+}$ thalassemia and sickle cell trait both have substantial amounts of Hb A and Hb S. This superficial electrophoretic similarity does not provide an obstacle to diagnosis: sickle cell trait is associated with neither anemia nor microcytosis and has an Hb A fraction >50%, [639] and sickle cell-$^{+}$ thalassemia is associated with anemia, microcytosis, and an Hb A fraction that ranges from 5% to 30%. [6] Solubility tests are positive

in both sickle cell disease and sickle cell trait, but sickled forms (ISCs) occur on the peripheral smear only in the sickle cell diseases and not in sickle cell trait. In sickle cell anemia, ISCs predominate and target cells may be few; in either variety of sickle cell- thalassemia, ISCs, target cells, and hypochromic microcytic

discocytes are prominent; in Hb SC disease, target cells predominate and ISCs are rare.

Newborn Screening

The use of prophylactic penicillin [333] and the provision of comprehensive medical care during the first 5 years of life have reduced mortality from approximately 25% to <3%, thereby providing impetus for early identification of infants with sickle cell disease. [437] Based on its economy and superiority of detection, universal screening of all newborns is preferred [689] over ethnically targeted approaches. [690] Blood samples for testing are obtained by heel stick and spotted onto filter paper for stable transport and subsequent electrophoresis or thin-layer isoelectric focusing. [691] [692] Results of solubility testing may be invalid due to the large amount of Hb F present.

A requirement for tests used in newborn screening is the capability to distinguish Hb F, S, A, and C. According to convention, the patterns of hemoglobins present are described in descending order according to the quantities detected. Newborns with sickle cell anemia have predominantly Hb F, with a small amount of Hb S and no Hb A (FS pattern). An FS pattern is obtained also in newborns who have sickle cell-° thalassemia, sickle cell-HPFH, and sickle cell-Hb D or sickle cell-Hb G (i.e., Hb D and E have the same electrophoretic mobility as Hb S). Family studies are confirmatory; in the newborn with sickle cell-° thalassemia, one parent has sickle cell trait and the other -thalassemia minor. When family members are not available, the diagnosis is established by DNA-based testing or repeat hemoglobin analysis at age 34 months.

The newborn with sickle cell trait will have Hb F, Hb A, and Hb S (FAS pattern). The quantity of Hb A is greater than that of Hb S. If the quantity of Hb S exceeds that of Hb A, the presumptive diagnosis is sickle cell- $^{+}$ thalassemia (FSA pattern). It may not be possible to distinguish FAS and FSA patterns in newborns, so DNA-based testing or repeat hemoglobin testing at age 36 months is recommended.

In the future, polymerase chain reaction-based diagnosis from blood spotted onto a filter paper may be used to detect sickle cell genes directly. [693]

Prenatal Diagnosis

The limited efficacy of available treatment options for sickle cell disease accounts for the importance of prenatal diagnosis to its overall management. This endeavor is best performed by a team of obstetricians, geneticists, counselors, and support staff that provides a comprehensive approach. [694] One large survey found that parents at risk for having a child with sickle cell disease were interested in prenatal diagnosis and would consider termination of pregnancy for an affected fetus. [695] Community acceptance of reproductive genetic services depends on the effectiveness of education and counseling. [696] Despite the considerable attention being paid to the social, ethical, and legal issues pertaining to prenatal diagnosis, these remain controversial. [697] [698] [699] [700] [701] One major ethical issue pertains to our diagnostic skills having outstripped our ability to predict the severity of diagnosable conditions. The lack of reliable markers for clinical severity in sickle cell disease was confirmed in a search for such markers in children from Jamaica. [702]

The first successful prenatal diagnoses relied on obtaining fetal blood samples for analysis of globin chain synthesis. [703] Discovery of a restriction fragment length polymorphism in linkage disequilibrium with the sickle cell gene established DNA-based

Figure 30-19 PCR-based restriction analysis for the sickle cell gene. The genotypes of the DNA samples tested are shown below. The size in base pairs for the undigested PCR product and the products resultant from Oxa NI are shown at the left in base pairs. The fragments from normal -globin DNA (AA) shows complete Oxa NI cleavage, from sickle cell trait DNA (AS) shows partial cleavage, and from sickle cell anemia (SS) shows no cleavage. *(Adapted from Chehab et al., [710] with permission.)*

testing as a method not influenced by the presence of Hb F [34] and spurred the development of second-trimester amniocentesis for obtaining fetal DNA for testing. [704] Direct detection of the GAGGTG mutation responsible for the sickle cell gene employed allele-specific hybridization with labeled synthetic DNA probes [705] or restriction endonuclease analysis to determine whether the cleavage site of the enzyme Mst II was ablated by the mutation. [706] The development of the polymerase chain reaction (PCR) for amplifying DNA sequences of interest in vitro [707] allowed testing of minute quantities of DNA [708] and motivated the development of several new methods for detecting the sickle cell generestriction analysis (Fig. 30-19), [709] [710] allele-specific hybridization, [711] reverse dot-blotting, [712] and allele-specific fluorescence PCR. [713] PCR-based diagnosis for Hb SC disease is possible using specific molecular methods for detecting the Hb C gene, [714] [715] and the diagnosis of sickle cell- thalassemia can be made using reverse dot-blot methodology to screen the many African-American -thalassemia mutations, [659] [660] [716] [717] as well as the Hb S and Hb C mutations, in a single hybridization reaction. [718] Fetal DNA samples are obtained by chorionic villus sampling at 810 weeks gestation. [704] Preimplantation diagnosis [719] and testing of fetal cells isolated from the maternal circulation [720] are under development.

Other Laboratory Testing

The chronic hemolytic anemia of sickle cell disease is usually associated with mildly to moderately low PCV, hemoglobin and red cell levels, reticulocytosis of approximately 315% (accounting for high or high-normal MCV), unconjugated hyperbilirubinemia, and elevated lactate dehydrogenase and low haptoglobin levels. There may be polychromasia indicative of reticulocytosis and Howell-Jolly bodies demonstrating hyposplenia on the peripheral blood smear. The red cells are normochromic unless there is coexistent thalassemia or iron deficiency. If the age-adjusted MCV is not elevated, the possibility of sickle cell- thalassemia, coincident thalassemia, or iron deficiency must be considered.

The Hb F level is usually slightly to moderately elevated; the degree varies among patients. The amount of Hb F present is a function of the number of reticulocytes that contain Hb F, the

extent of selective survival of Hb F-containing reticulocytes to become mature Hb F-containing erythrocytes (F cells), and the amount of Hb F/F cell. [721] The -globin cluster haplotypes mentioned in the Pathophysiology section [722] appear to be related to factors that regulate production of Hb F. The Arab-Indian and Senegal haplotypes are associated with higher levels of Hb F than the others, [722] probably as a result of linkage with important -globin regulatory sequences in the locus control region. [723]

A comprehensive analysis of the clinical laboratory data collected from 2,600 subjects with sickle cell disease provides a background for interpreting laboratory abnormalities in this patient group. [249] In particular, normal ranges were established according to genotype, age, and gender. White cell counts are higher than normal in sickle cell anemia, particularly in patients under age 10 years. Mean white cell counts are not elevated in Hb SC disease or sickle cell- $^{+}$ thalassemia. Mean platelet counts are elevated in sickle cell anemia, particularly in patients under age 18 years, but are normal in those with Hb SC disease and sickle cell- $^{+}$ thalassemia.

The serum bilirubin level is higher in sickle cell anemia than in Hb SC disease or sickle cell- $^{+}$ thalassemia as a result of a greater hemolytic rate. The level rises after the first decade, possibly as a result of chronic hepatobiliary dysfunction. AST and ALT levels are often elevated, particularly in adult patients with sickle cell anemia, but mean levels are normal. Alkaline phosphatase levels are elevated in all genotypes until puberty, which occurred later in males and in those with sickle cell anemia.

Serum creatinine levels are low in all genotypes until age 18 years, when males experience a rise, apparently related to increasing muscle mass. Creatinine levels increase with age in all genotypes, presumably due to declining renal function.

THERAPY

Treatment recommendations specific for sickle cell disease can be divided into general health-care maintenance and specific indications or modalities for therapy. [408]

Health-Care Maintenance

Patients with sickle cell disease should have routine office appointments to establish baseline physical findings, laboratory values, and relationships with health-care professionals. These steady-state values provide standards for comparison at times of clinical exacerbations. Red cell phenotyping is performed and individualized blood bank files are instituted during chronic-phase visits. Education regarding the nature of the disease, genetic counseling, and psychosocial assessments of patients and their families are best accomplished during these routine visits. Parents of small children are instructed regarding early detection of infection and palpating enlarging spleens. Children are immunized against *S. pneumoniae, H. influenzae*,[440] [441] [444] hepatitis B, and influenza. For children under age 5 years, prophylactic penicillin recommendations are 125 mg penicillin V orally twice daily until age 2 to 3 years, and 250 mg thereafter. [432] It has been recommended that penicillin prophylaxis be discontinued at age 5 years. [445] Folic acid, 1 mg orally per day, is administered.[366] The routine assessment of cerebral blood flow using TCD is a recommendation based on the evidence that CVA in children can be predicted by TCD and prevented by chronic transfusion therapy. [502] Retinal evaluation is begun at school age and continued routinely, more frequently in the event of retinopathy. Sexually active women should have routine pelvic exams and birth control instructions; oral contraception with low-dose estrogen can be administered safely. [550] An obvious conclusion that follows from these considerations is that continuity of care by an expert caregiver is essential for optimizing the clinical state of these patients.

Infections

The most critical aspect of infectious illness in sickle cell disease is the evaluation and treatment of the febrile child. Routine evaluation includes a physical examination, a complete blood count, blood and urine cultures, a lumbar puncture if meningitis is suspected, and a chest x-ray to evaluate for pneumonia. Results of the complete blood count are compared to baseline values. A left shift in the differential count suggests bacterial infection. Due to the high mortality rate of bacteremia, hospitalization, blood and CSF cultures, and parenteral antibiotics has been the standard of care for children with fevers >38.5°C. [724] [725] However, the recent demonstration of the efficacy of ceftriaxone[447] [448] has led to a different set of guidelines. Those with sickle cell anemia or sickle cell-° thalassemia who appear toxic, have temperatures >40°C, or are not receiving prophylactic penicillin are hospitalized for administration of intravenous ceftriaxone (75 mg/kg). For those with sickle cell anemia or sickle cell-° thalassemia who have temperatures <40°C and are compliant with prophylactic penicillin, and those with Hb SC disease or sickle cell-$^{+}$ thalassemia who have temperatures >38.5°C, the recommended approach is to obtain blood cultures, administer intramuscular ceftriaxone (75 mg/kg to a maximum dose of 2 g), observe for several hours in the emergency department before discharge, and follow the patient closely as an outpatient. For those with Hb SC disease or sickle cell-$^{+}$ thalassemia who have temperatures <38.5°C, therapy is as indicated symptomatically. [447] [448] Therapy for documented *S. pneumoniae* bacteremia is with parenteral penicillin or ceftriaxone for several days.

Meningitis therapy should cover *S. pneumoniae* and probably *H. influenzae* type b[451] and should be continued for at least 2 weeks.

THE CHILD WITH A HIGH FEVER

The most important infectious disease consideration of sickle cell disease is the child with a high fever. Evaluation includes a physical examination, complete blood count, blood and urine cultures, lumbar puncture if meningitis is suspected, and a chest x-ray. A left shift in the differential count suggests bacterial infection. In the past the standard of care has been hospitalization, blood and CSF cultures, and administration of parenteral antibiotics for children with fevers >38.5°C. This remains a reasonable approach in areas where antibiotic-resistant strains of *S. pneumoniae* have emerged; the antibiotic recommended in these areas is vancomycin. In areas lacking antibiotic-resistant *S. pneumoniae*, the following alternative approach may be employed. Only those with sickle cell anemia or sickle cell-° thalassemia who appear toxic, have temperatures >40°C, or are not receiving prophylactic penicillin are hospitalized for administration of intravenous ceftriaxone (75 mg/kg). Those with sickle cell anemia or sickle cell-° thalassemia who have temperatures <40°C and are compliant with prophylactic penicillin and those with Hb SC disease or sickle cell-$^{+}$ thalassemia who have temperatures >38.5°C are given intramuscular ceftriaxone (75 mg/kg to a maximum dose of 2 g) after blood cultures are obtained, observed for several hours prior to discharge, and followed closely as outpatients. For those with Hb SC disease or sickle cell-$^{+}$ thalassemia who have temperatures <38.5°C, therapy is as indicated symptomatically.

Therapy for documented *S. pneumoniae* bacteremia is parenteral penicillin or ceftriaxone for several days. Vancomycin is recommended in areas where *S. pneumoniae* may be antibiotic-resistant.

When antibiotics are used to treat the acute chest syndrome, they should cover *S. pneumoniae, H. influenzae* type b, *M. pneumoniae*, and *C. pneumoniae*. The combination of cefuroxime and erythromycin is recommended.

For osteomyelitis, the diagnosis is confirmed by culture of blood or infected bone. Parenteral antibiotics that cover *Salmonella* and *S. aureus* are given, and antibiotic therapy is based on culture results. Parenteral antibiotics are continued for 2 to 6 weeks. [454] Surgical drainage or sequestrectomy may be required. Most patients are

cured by this approach, but there may be recurrences. [454]

Transfusion Therapy

Patients with sickle cell disease have the same indications for transfusion as do those without hemoglobinopathies, oxygen-carrying capacity and blood volume replacement (aplastic crisis, splenic sequestration), as well as indications that are unique to the special needs of their disease, protection from imminent danger (e.g., acute chest syndrome, septicemia, metabolic acidosis) and improved rheologic properties of blood (e.g., prevention of recurrent cerebral thrombosis, priapism, probably preoperatively). [726] [727] [728] Transfusion complications include alloimmunization, iron overload, and transmission of viral illness. The 30% incidence of alloimmunization in transfused sickle cell patients is due in part to minor blood group incompatibilities in racially mismatched blood. [729] [730] Accordingly, antibodies against the Rh (E, C), Kell (K), Duffy (Fya, Fyb), and Kidd (Jk) antigens present the greatest problem in transfusing these patients. [729] [730] Approaches to minimizing this complication include transfusing extended-matched, phenotypically compatible blood, [729] [731] [732] PEG-coated red cells whose antigens are masked from antibodies, [247] or artificial blood substitutes, such as perfluorocarbon emulsions and hemoglobin-based substitutes. [733] In the latter regard, infusion of a polymerized bovine hemoglobin-based oxygen carrier into asymptomatic adult sickle cell disease subjects resulted in no complications and improved exercise performance compared to control subjects. [734]

Despite earlier impressions to the contrary, [735] as sickle cell patients live longer, iron overload has become a problem in those chronically transfused. [736] Chelation with deferoxamine is recommended when the total body iron level is elevated. [737] Chelation guidelines for patients with sickle cell disease are similar to those for other chronically transfused, iron-overloaded patients; serum ferritin levels should not be allowed to exceed 2,000 g/ml. [738] This therapy is inconvenient and expensive, and the development of oral chelating agents [739] [740] will provide a tremendous advantage to these patients.

Transfusion transmission of HIV, hepatitis B and C, and human T-cell leukemia/lymphoma virus-1 has diminished with improved screening of banked units [741] but remains a problem in patients with sickle cell disease. [726] [727] In addition to better screening programs, the use of leukocyte-depleted red cell transfusions may be of value in reducing this hazard. [742]

Simple transfusion is used for single transfusions to restore oxygen-carrying capacity or blood volume, but partial exchange transfusions are recommended for emergencies and chronic transfusion because of the improved viscosity effects and reduced iron burden of this approach. [726] [727] The volumes required for simple and exchange transfusions (Table 30-5 (Table Not Available)) are particularly important for transfusing children. For normal-size adults, the general rule is that each unit of red cells infused increases the hemoglobin level approximately 1 g/dl. Partial exchange transfusion in adults is accomplished by phlebotomizing 500 ml, infusing 300 ml normal saline, phlebotomizing another 500 ml, and infusing 45 units of packed red cells. [735]

We have chosen to include the indications for transfusion in the discussions of specific disease complications, except for the issue of prophylactic preoperative transfusion. The controversy

TRANSFUSION THERAPY

Transfusion of red cells has been used for almost every complication of sickle cell disease, but its value has been demonstrated for few. Indications for transfusion include the need for oxygen-carrying capacity (i.e., aplastic crisis), blood volume (i.e., splenic sequestration), improved blood rheology (i.e., prevent CVA recurrence, leg ulcers, priapism), and protection from imminent danger (i.e., acute chest syndrome with p_{AO2} <70 mmHg, acute CVA, septicemia, metabolic acidosis). Simple transfusion is sufficient to supply oxygen-carrying capacity and blood volume. Partial exchange transfusion is recommended for acute indications and for chronic programs, where avoiding hyperviscosity and iron overload are important. Hemoglobin levels much above 10 g/dl must be avoided during transfusion because they may result in hyperviscosity and vaso-occlusive complications. A formula useful for manual partial exchange transfusion in adults is to phlebotomize 500 ml whole blood, infuse 300 ml normal saline, phlebotomize another 500 ml whole blood, and infuse 45 units packed red blood cells. In typical patients with sickle cell anemia having hemoglobin levels of 8 g/dl, this generally results in hemoglobin levels of 10 g/dl with <50% Hb S.

Preoperative simple transfusion sufficient to raise the Hb level to 10 g/dl is recommended for patients with Hb SS. For those with Hb SC, partial exchange preoperative transfusion is effective.

Each patient should be characterized according to red blood cell antigen phenotype, and this record should be maintained in the blood bank. Alloimmunization is diminished by transfusing extended-matched red cells.

surrounding this practice [743] [744] has largely been resolved by the results of a multicenter cooperative study. [745] The findings of this study were that prophylactic preoperative transfusion resulted in lower rates of surgical mortality and acute chest syndrome than no transfusion, and that direct transfusion to achieve Hb of approximately 10 g/dl and Hb S <60% was as effective and caused less morbidity than aggressive partial exchange transfusion to achieve Hb S <30%. [745] The efficacy of preoperative partial exchange transfusion in patients with Hb SC disease undergoing abdominal surgery suggests that this type of transfusion be performed preoperatively in this group of patients. [746]

TABLE 30-5 -- Transfusion Formulas

(Not Available)

Pain Management

The acute painful episode is the most frequent reason for which patients with sickle cell disease seek medical attention. When a patient presents complaining of pain, the physician is charged with ruling out etiologies other than vaso-occlusion (e.g., an infectious cause); maintaining optimal hydration by oral or intravenous fluid resuscitation (particularly in children); and providing aggressive relief of pain using narcotics, other analgesics, or other modalities. [747] [748] Patients are often undertreated for pain because many physicians and other health-care providers are unfamiliar with the pharmacology of analgesia and overly concerned with the potential for addiction. Consequently, the duration of painful episodes may be prolonged, a drug-seeking(pain-relieving?) behavior pattern is encouraged, and a pain-oriented personality may evolve. Prompt administration of appropriate doses of analgesia will diminish this potential. The existence of individuals with sickle cell Munchausen syndrome [749] must not interfere with this recommended approach. The absence of ISCs on the peripheral smear provides an immediate means of determining whether a patient has sickle cell disease.

Patients in pain are treated optimally in a familiar ambulatory setting that avoids the hectic environment of the emergency ward. [750] Comprehensive management of the biopsychosocial pain experience includes the use of psychosocial support systems, local anesthetics, epidural anesthetics, combinations of nonsteroidal anti-inflammatory agents and narcotics, and antidepressant drugs. [748] [751]

Hospitalization and intravenous administration of fluid and narcotics are often required for the treatment of severe pain. Table 30-6 presents a description of analgesics and recommendations for their use in the treatment of the painful episodes of sickle cell disease. [747] [748] [752] We recommend an aggressive approach to obtaining prompt pain relief such as intravenous morphine, followed by rescue or maintenance analgesia such as patient-controlled analgesia. [753] The use of patient-controlled analgesia precludes the risk of respiratory depression associated with constant-infusion narcotics, which has been reported to result in death due to respiratory depression.[754] In assessing patient responses to conventional doses of analgesia, it must be remembered that those with sickle cell disease metabolize narcotics rapidly.[755] [756]

Newer approaches to the management of acute pain include

TABLE 30-6 -- Recommended Dose and Interval of Analgesics Necessary to Obtain Adequate Pain Control in Sickle Cell Disease

	Dose/Rate	Comments
Severe/Moderate Pain		
1. Morphine	Parenteral: 0.10.15 mg/kg q 34 h. Recommended maximum single dose 10 mg. P.O.: 0.30.6 mg/kg q 4 h	Drug of choice for pain; lower doses in the elderly and infants and in patients with liver failure or impaired ventilation
2. Meperidine	Parenteral: 0.751.5 mg/kg q 24 h. Recommended maximum dose 100 mg. P.O.: 1.5 mg/kg q 4 h	Increased incidence of seizures. Avoid in patients with renal or neurologic disease or those who receive monoamine oxidase inhibitors.
3. Hydromorphone	Parenteral: 0.010.02 mg/kg q 34 h	
4. Oxycodone	P.O.: 0.040.06 mg/kg q 4 h P.O.: 0.15 mg/kg/dose q 4 h	
5. Ketorolac	Intramuscular: Adults: 30 or 60 mg initial dose, followed by 1530 mg Children: 1 mg/kg load, followed by 0.5 mg/kg q 6 h	Equal efficacy to 6 mg MS; helps narcotic-sparing effect; not to exceed 5 days. Maximum 150 mg first day, 120 mg maximum subsequent days. May cause gastric irritation.
6. Butorphanol	Parenteral: Adults: 2 mg q 34 h	Agonist-antagonist. Can precipitate withdrawal if given to patients who are being treated with agonists.
Mild Pain		
1. Codeine	P.O.: 0.51 mg/kg q 4 h. Maximum dose 60 mg.	Mild to moderate pain not relieved by aspirin or acetaminophen; can cause nausea and vomiting
2. Aspirin	P.O.: Adults: 0.36 mg q 46 h. Children: 10 mg/kg q 4 h.	Often given with a narcotic to enhance analgesia. Can cause gastric irritation. Avoid in febrile children.
3. Acetaminophen	P.O.: Adults 0.30.6 g q 4 h. Children: 10 mg/kg.	Often given with a narcotic to enhance analgesia
4. Ibuprofen	P.O.: Adults: 300400 mg q 4 h. Children: 510 mg/kg q 68 h.	Can cause gastric irritation
5. Naproxen	P.O.: Adults: 500 mg/dose initially, then 250 q 812 h. Children: 10 mg/kg/day (5 mg/kg q 12 h).	Long duration of action. Can cause gastric irritation.
6. Indomethacin	P.O.: Adults: 25 mg q 8 h. Children: 13 mg/kg/day given 3 or 4 times.	Contraindicated in psychiatric, neurologic, renal diseases. High incidence of gastric irritation. Useful in gout.

Adapted from Charache et al.,[330] with permission.

the use of potent nonsteroidal anti-inflammatory drugs, opioid receptor-binding agents, surfactants that inhibit cell adherence and aggregation, inhaled NO, anticoagulants, glucocorticoids, and epidural anesthesia. Ketorolac (Toradol) is a potent nonsteroidal anti-inflammatory that can be given by injection or orally. As a single parenteral agent, it provides analgesia superior to parenteral meperidine, causes no respiratory depression, and is especially effective for relieving bone pain.[757] Because of its severe gastrointestinal side effects, its administration should be limited to a 5-day course, it should be administered with H_2 blockers, and it may be used best with other analgesics to provide a narcotic sparing effect. [3] Tramadol is a centrally acting analgesic that is administered orally, binds to the -opioid receptor, inhibits norepinephrine and serotonin reuptake, induces minimal respiratory depression, has a low potential for abuse or addiction, relieves postoperative pain efficaciously, and is useful in the management of chronic painful conditions. [758] [759] Poloxomer 188 is an artificial polyoxyethylene/polyoxypropylene surfactant that reduces cellcell adherence;[244] given as a 48-hour infusion, it reduced the narcotic requirement of patients having acute painful episodes. [245] It may gain a role in the initial management of painful episodes.

It is difficult to define which of the many biologic effects of NO may result in pain relief, but based on its ability to increase the oxygen affinity of sickle erythrocytes in vitro and in vivo, its predicted use for relieving vaso-occlusive pain, [82] and its apparent efficacy in treating severe acute chest syndrome, [518] the utility of inhaled NO in relieving acute sickle cell pain is under study. On the basis of evidence for a hypercoagulable state in sickle cell disease, acemocoumerol was administered in low doses that achieved mean International Normalized Ratios of 1.64 and reduced the elevated levels of prothrombin activation fragment (fragment 1+2) to 50% of pretreatment levels.[270] No attempt was made to assess the efficacy of this treatment on pain. Minidose heparin, 5,0007,500 units every 12 hours, administered to four patients for 26 years, reduced hospitalization and emergency room time by 75%, and pretreatment pain frequency recurred after heparin was discontinued. [271] Methylprednisolone given as an intravenous infusion of 15 mg/kg to a total dose of 1,000 mg on admission and again 1 day later was found to provide pain relief superior to that of placebo, but there was an unacceptable rate of rebound pain after discontinuation of this therapy. [760] Epidural analgesia is a useful adjunct for the treatment of severe painful episodes.[761]

The rare chronic sickle cell pain syndrome may require therapy similar to that used for the management of the pain of terminal cancer, [762] such as long-acting morphine and fentanyl patches.

Hydroxyurea

Hydroxyurea (HU) is an inhibitor of ribonucleotide reductase without known carcinogenicity. [763] It also induces therapeutically significant increases in Hb F synthesis, [425] [764] [765] [766] an effect that is potentiated by conjoint administration of recombinant human erythropoietin. [767]

A double-blind, placebo-controlled, intention-to-treat multicenter study of HU as treatment of pain crisis in sickle cell disease found that HU produced definite hematologic changes. There were significant declines in the mean level of leukocytes, PMN, reticulocytes, and dense sickle cells and significant increases in the levels of hemoglobin, PCV, MCV, Hb F, F cells, and F reticulocytes [255] (Table 30-7 (Table Not Available)). The significant clinical changes were decreased rate of acute painful episodes, longer interval to first and second acute painful episode, fewer episodes of acute chest syndrome, and diminished number of subjects and units transfused[253] (Table 30-8). No significant changes were observed in the incidence of stroke, hepatic sequestration,

TABLE 30-7 -- Hematologic Effects of Hydroxurea Therapy

(Not Available)

Adapted from Charache et al.,[255] with permission.

or death. No short-term toxicity due to HU was observed. One child born to a subject taking HU and two born to partners of subjects taking HU were normal at birth. The possible long-term effects are under investigation.

A persistent concern pertaining to the use of HU in sickle cell disease is its putative leukemogenic effect. This concern derives from reports on HU treatment of myeloproliferative diseases, conditions associated with an inherent propensity for leukemic conversion. While the use of HU combined with ^{32}P or alkylating agents is associated with increased leukemic conversion,[768] reports claiming a leukemogenic effect for HU alone either lacked controls[769] or were not designed to provide useful information on this issue.[770] In the only study in patients lacking the potential for leukemic conversion, children with erythrocytosis secondary to inoperable cyanotic congenital heart disease, no leukemic conversion was observed.[771]

Studies of HU as therapy for children with sickle cell disease have begun. In one such study, the degree of anemia was lessened, the frequency of pain may have decreased, and there were no short-term side effects.[772]

The mechanism of action of HU remains an enigma. The original interest in this agent was based on the premise that it induced Hb F production. However, before the trial began, there was evidence of therapeutic response preceding changes in Hb F levels.[765] Other changes in sickle erythrocytes that may be independent of Hb F include increased water content,[773] improved deformability,[774] and decreased adherence to the vascular endothelium,[775] the last of which may relate to the decreased $_{4\,1}$ and CD36 molecules on sickle reticulocytes induced by HU.[776] Much remains unknown about the mechanisms by which HU influences sickle cells and vaso-occlusion. However, at this time it appears that myelosuppression may be as important to the beneficial influence of HU as any putative switching effect.

TABLE 30-8 -- Clinical Effects of Hydroxyurea Therapy

Variable	Hydroxyurea	Placebo	p Value
Acute pain crisis rate	2.5/year	4.5/year	<0.001
Hospitalization rate for acute pain crisis	1.0/year	2.4/year	<0.001
Interval to 1st pain crisis	3.0 months	1.5 months	<0.001
Interval to 2nd pain crisis	8.8 months	4.6 months	<0.001
Acute chest syndrome	25	51	<0.001
Subjects transfused	48	73	= 0.001
Blood units transfused	336	586	= 0.004

Adapted from data in Charache et al.[253]

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NEW THERAPEUTIC MODALITIES

The cure of sickle cell disease has been an elusive goal. Many agents have been proposed and tested, but, short of bone marrow transplantation, no cure has been found. As new therapies are developed, it is important to offer cautious optimism but not false hope to patients with sickle cell disease. Newer therapeutic compounds, classified according to their mechanisms of action, are described below.

Membrane-Active Agents

As discussed previously, membrane-active agents that improve sickle cell hydration and deformability, such as cetiedil citrate, [163] [164] [165] have shown limited therapeutic success in a controlled clinical trial. [163] Oral administration of agents that inhibit the cellular dehydration caused by the Gardos and potassiumchloride cotransport pathways,[133] [134] such as the imidazole compound clotrimazole, which inhibits the Gardos pathway [166] [167] or magnesium supplements, which inhibit potassiumchloride cotransport,[168] have been shown to improve the pathobiology of sickle erythrocytes. Compounds that improve membrane deformability are not effective.[777]

A nonionic block copolymer (Pluronic F-68, RheothRx, or Poloxamer 188), the emulsifying agent in the perfluorochemical blood substitute Fluosol DA, improves the rheologic properties of sickle red cells and inhibits their adherence to endothelial cells as a result of a lubricating effect on cell surfaces. [244] [778] [779] Results of a pilot study indicated that compared to placebo, a 48-hour infusion diminished the narcotic requirement significantly and the duration of pain and hospitalization less so. [245] A more extensive multicenter study is underway to determine the efficacy of this approach in the initial treatment of acute painful episodes.

Vasoactive Compounds

Vasodilating agents that improve membrane deformability have not been found effective. [780] However, as discussed above, inhalation of NO at 80 ppm for up to an hour was found to increase the oxygen affinity of sickle cells in vivo [82] and to improve the clinical status of two patients having acute chest syndrome. [781] This treatment is undergoing trial as therapy for sickle cell vaso-occlusion.

Antioxidant Agents

A systematic study of antioxidant agents intended to counter the detrimental effects of oxidation on sickle cells has not been accomplished. Such a study would be complicated by the relative redox potentials of participants in the auto-oxidative processHb S, oxidative radicals, free heme, hemichromes, nonheme iron, membrane lipid, membrane protein, and intermediate metabolites. An agent capable of reducing one component may oxidize another, and a clinical study would have to be carefully controlled to dissect the complex interdependence of oxidation-reduction substrates.

Induction of Hb F Synthesis

The experience with HU was discussed above. The basis and active agents of pharmacologic modulation of Hb F production are reviewed in reference 782 . Butyric acid appears to block the switch from fetal to adult hemoglobin, [783] but enthusiasm for its apparent ability to induce increased numbers of Hb F-containing reticulocytes[784] is tempered by concerns regarding uncertain efficacy, [785] [786] neurologic toxicity, [785] and unacceptably severe nausea and vomiting. [787] New formulations of this agent, some of which are absorbable in oral form, and a host of other Hb F-inducing agents are being evaluated. [788] [789]

Bone Marrow Transplantation

The success of allogeneic bone marrow transplantation (BMT) in curing -thalassemia major [790] encouraged this approach in sickle cell disease. The application of BMT to sickle cell disease is challenged by the selection of appropriate recipients, procurement of compatible donor sources, minimization of procedural risks, and consideration of cost-effectiveness.[791] [792] [793] [794] [795] [796] The choice of BMT candidates presents a dilemma. Transplanting only those who have had serious complications may allow serious disability prior to BMT, [791] [797] while transplanting those predicted by genetic polymorphisms to have severe disease may subject those misidentified to unnecessary risks. [478] The extremely poor prognosis of sickle cell disease in Africa supports the widespread use of BMT, [798] [799] but decisions in better-developed countries hinge on less absolute considerations. In the United States, the increasing life expectancy [250] and large fraction of asymptomatic patients[161] [380] suggest that BMT should be reserved for those with severe disease. These considerations influence the relatively better results of European groups who transplant healthier African patients, compared to American groups who transplant only more severely afflicted patients. [800] [801] [802] [803] The use of standard supportive regimens resulted in serious neurologic complications, including stroke and seizures, in 7 of 21 sickle cell patients undergoing allogeneic BMT, [804] presumably as a result of prior cerebral vasculopathy. Adjusting the procedural approach to BMT in these patients, including altering the preparative regimen, maintaining the hemoglobin >11 g/dl and the platelet count >50,000/ml, and aggressively treating elevated blood pressure, has remedied this risk. [803] [804]

The usual expectation of one HLA-compatible acceptable donor per three siblings [805] is reduced further to approximately one HLA-matched, acceptable donor in five for sickle cell disease.[806] After patients are counseled on the 10% risk of mortality and the 25% risk of graft-versus-host disease (GVHD), approximately 10% of patients with sickle cell disease accept the option of BMT. [797] Solutions to this problem include broadening the ethnic composition of the donor registry pool, improving BMT results using partially matched donors, correcting the genetic defect in autologous marrow erythroid precursors by gene therapy (see following section), and using peripheral blood stem cells as a donor source.

An abundant source of peripheral blood stem cells is umbilical cord blood (UCB). [790] [807] Blood from a single cord and placenta contains enough stem cells to reconstitute the marrow of an adult recipient.[808] Transplantation of nonsickle cell recipients with UCB results in excellent rates of engraftment, recovery, GVHD, and survival.[808] [809] Most importantly, the use of UCB allows crossing the HLA barrier using HLA-disparate stem cells without an increased risk of GVHD. [809] [810] This outcome apparently is the result of the less alloreactive lymphocytes contained in UCB compared to marrow. [811] It permits less aggressive prophylaxis for GVHD.[808] UCB is being used to transplant subjects with sickle cell disease. [812]

The issue of the cost effectiveness of BMT gains perspective from the comparative costs of $150,000 to $200,000 for an uncomplicated BMT in the United States versus up to $112,000 annually for conventional medical care of a chronically transfused, iron-overloaded patient. [792]

Gene Therapy

Recent advances in gene therapy technology provide promise for the future of sickle cell disease. [813] [814] The singular importance of a single nucleotide substitution to

a disease so thoroughly understood renders the sickle cell gene an ideal candidate for

this technology. Efficient gene transfer has been accomplished using replicative defective retroviral vectors. [815] High-level expression is attained by including in the vector regulatory sequences from the locus control region [816] and by inserting the normal gene into its native environment by homologous recombination (an approach that offers the additional advantage of knocking out the mutant gene by inserting the normal). [817] Sustained expression of the corrected gene is gained by transfer into self-replicating stem cells. [818] Successful expression of the human sickle gene in transgenic mice demonstrates the potential feasibility of this approach. [819] Its safety remains to be determined. [820] (See Chap. 95.)

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Chapter 31 - Hemoglobin Variants Associated with Hemolytic Anemia, Altered Oxygen Affinity, and Methemoglobinemias

Edward J. Benz Jr.

INTRODUCTION

Hemoglobinopathies are inherited diseases due primarily to mutations affecting the globin genes. Nearly 1,000 mutations that alter the structure, expression, or developmental regulation of individual globin genes, and the hemoglobins they encode, have been described; most do not produce clinical disease. Many are highly instructive for students of gene structure, function, and regulation, but further consideration of most is not warranted in a clinically oriented textbook. Sickle cell anemia and the thalassemia syndromes are by far the most important mutations that cause clinical morbidity, in terms of both the complexity of the clinical syndromes they cause and the number of patients affected. These conditions are considered in detail in other chapters (see Chaps. 29 and 30). This chapter reviews other abnormalities of the hemoglobin molecule that produce clinical syndromes. Each variant is uncommon. In the aggregate, however, hemoglobinopathies represent important problems for hematologists because they must be considered as possible etiologies for conditions about which hematologists are often consulted: hemolytic anemia, cyanosis, polycythemia, jaundice, rubor, splenomegaly, or reticulocytosis.

The major hemoglobinopathies producing clinical symptoms, other than sickle cell anemia and thalassemias, can be classified as those hemoglobins exhibiting altered solubility (unstable hemoglobins), hemoglobins with increased oxygen affinity, hemoglobins with decreased oxygen affinity, and methemoglobins (Table 31-1). A few acquired conditions, in which toxic modifications of the hemoglobin molecule are important (e.g., carbon monoxide poisoning), are also considered briefly.

The sections that follow emphasize hemoglobinopathies that produce the most severe or dramatic alterations in clinical phenotype and those in which a single clinical abnormality (e.g., hemoglobin precipitation) predominates. However, it is important to emphasize at the outset that of the >100 mutations altering globin chain structure that have been shown to affect solubility or affinity, or both, only a few are clinically important.

TABLE 31-1 -- Classification of Hemoglobinopathies

Structural hemoglobinopathieshemoglobins with altered amino acid sequences that result in deranged function or altered physical or chemical properties
Abnormal hemoglobin polymerizationHb S
Altered oxygen affinity
High affinitypolycythemia
Low affinitycyanosis, pseudoanemia
Hemoglobins that oxidize readily
Unstable hemoglobins, hemolytic anemia, jaundice
M hemoglobinsmethemoglobinemia, cyanosis
Thalassemiasdefective production of globin chains
-Thalassemias
-Thalassemias
-, -, -Thalassemias
Structural hemoglobinopathiesstructurally abnormal Hb associated with coinherited thalassemia phenotype
Hb E
Hb Constant Spring
Hb Lepore
Hereditary persistence of fetal hemoglobinpersistence of high levels of Hb F into adult life
Pancellularall red cells contain elevated Hb F levels
Nondeletion forms
Deletion forms
Hb Kenya
Heterocellularonly specific subpopulation of red cells contain elevated levels of Hb F
Acquired (see below)
Acquired hemoglobinopathies
Methemoglobin due to toxic exposures
Sulfhemoglobin due to toxic exposures
Carbonoxyhemoglobin

Hb H in erythroleukemia
Elevated Hb F in states of erythroid stress and bone marrow dysplasia, usually heterocellular

The abnormal functional properties of most mutant hemoglobins can be detected readily in sophisticated research laboratories, but do not produce laboratory or clinical abnormalities relevant to clinical practice. Moreover, many mutations are pleiotropic, affecting several functional properties of the hemoglobin molecule. Thus, a single mutation can increase oxygen affinity and reduce solubility, or produce methemoglobinemia and reduce solubility.

Table 31-2 (Table Not Available) summarizes the major forms of structurally abnormal hemoglobin, with examples. This table serves as a point of reference for the remaining sections of the chapter.

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UNSTABLE HEMOGLOBINS

The term unstable hemoglobins refers to hemoglobins exhibiting reduced solubility or higher susceptibility to oxidation of amino acid residues within the individual globin chains. More than 100 unique unstable hemoglobin mutants have been documented. Most exhibit only mild instability in in vitro laboratory tests and are associated with minimal clinical manifestations. Both - and -globin variants can cause this condition. Approximately 75% of the mutations described, however, are -globin variants. This probably reflects the potential for -globin variants to exert pathologic effects in utero. Clinical symptomatology of unstable hemoglobins also depends in part on the quantitative proportion of the abnormal hemoglobin. Because the -globin genes are duplicated, mutations in an individual locus generally produce only 2535% abnormal globin. By contrast, a simple heterozygote at the single -globin locus produces 50% of the abnormal variant.

The mutations that impair hemoglobin solubility usually disrupt hydrogen bonding or the hydrophobic interactions that retain either the heme moiety within the heme binding pockets or hold the tetramer together (Fig. 31-1 (Figure Not Available)). Some alter the helical segments (Hb Geneva [^{28LeuPrc}]), others weaken contact points between the - and -subunits (Hb Philadelphia [^{35TyrPhe}]), whereas still others derange interactions of the hydrophobic pockets of the globin subunits with heme (e.g., Hb Köln [^{98ValMe}]). The common pathway to reduced solubility ultimately leads to weakening of the binding of heme to globin. Actual loss of heme groups can occur, for example, in Hb Gun Hill, in which five amino acids, including the F8 histidine, are deleted. In other cases, mutations that introduce prolines into helical segments disrupt the helices and interfere with normal folding of the polypeptide around the heme group. Another feature of these mutations is disruption of the integrity of the tetrameric structure of globin chains. Only the intact hemoglobin tetramer can remain dissolved at the high concentrations that must be achieved within the circulating red cell (see Chap. 29).

Pathophysiology of Unstable Hemoglobin Disorders

The mechanisms by which unstable hemoglobin mutations produce hemoglobin precipitation remain incompletely understood. However, the major outlines of the process have been described (Fig. 31-2). The fundamental step in the pathogenesis appears to be derangement of the normal linkages between heme and globin. Loss of appropriate globin chain folding and interaction may ultimately destabilize the heme-globin linkage or lead to partial proteolysis of the chain, thereby releasing heme from that linkage. Once freed from its cleft, heme probably binds nonspecifically to other regions of the globin molecule, forming precipitated hemichromes, which lead to further denaturation and aggregation of the globin subunits to form a precipitate containing - and -globin chains, globin fragments, and heme, called the Heinz body.

Heinz bodies interact with delicate red cell membrane components (see Chap. 33), thereby reducing red cell deformability. These cells tend to be trapped in the splenic microcirculation and pitted, reflecting attempts by the splenic macrophages to remove the Heinz bodies. Red cell damage may be aggravated by the release of free heme into the red cell. Several biochemical perturbations correlate with the presence of free heme, such as generation of reactive oxidants (i.e., hydrogen peroxide, superoxide, and hydroxyl radicals). The end result of this process is premature destruction of the red cell, producing the hemolytic anemia.

Individual unstable hemoglobins vary in their propensity to generate Heinz bodies and hemolysis. For example, Hb Zurich exhibits relatively mild insolubility. Hemolysis is virtually absent in patients with this variant. Hemolysis may become clinically apparent only in the presence of additional oxidant stresses, such as infection, fever, or the ingestion of oxidant agents. Because of the propensity of these molecules to be hypersensitive to oxidation, some patients with unstable hemoglobins can exhibit episodic hemolysis in response to the same oxidative stressors as those exacerbating the clinical phenotype of glucose-6-phosphate dehydrogenase (G6PD)-deficient patients.

Patterns of Inheritance and Clinical Manifestations

Unstable hemoglobins are usually inherited as autosomal dominant disorders. However, the rate of spontaneous mutation appears to be high, so that the absence of affected parents or sibs does not rule out the presence of an unstable hemoglobin in an individual family. Nonetheless, the presence of a positive family history can be a useful adjunct to diagnosis, and should

TABLE 31-2 -- **Mutations Producing Abnormal Hemoglobin Moleculesa**

(Not Available)

Adapted from Dickerson RE, Geis I: Hemoglobin: Structure, Function, Evolution, and Pathology. Benjamin-Cummings, Menlo Park, CA, 1983, with permission. Copyright Irving Geis.

Figure 31-1 (Figure Not Available) Hemoglobin tetramer showing the position of the more common, clinically significant hemoglobin mutants. Most of those that have been described occur on the -chain at invariant residue sites, near critical intermolecular contacts, or in proximity to the prosthetic heme-binding site. *(Modified from Dickerson RE, Geis I: Hemoglobin: Structure, Function, Evolution, and Pathology. Benjamin-Cummings, Menlo Park, CA, 1983, with permission. Copyright Irving Geis.)*

Figure 31-2 Presumed mechanisms by which denaturation of hemoglobin leads to erythrocyte destruction. The rate of travel through the various pathways probably differs for the different hemoglobin variants and for a variety of stresses to which the protein is subjected. *(From Wynngaarden JB, Smith LH Jr, Bennett JC (eds): Cecil Textbook of Medicine. WB Saunders, Philadelphia, 1992, with permission.)*

provoke consideration of an unstable hemoglobin as the cause of the familial hemolytic diathesis.

The clinical syndrome associated with unstable hemoglobin disorders is often called congenital Heinz body hemolytic anemia. This term derives its origins from the fact that only the most severe cases were detected before the widespread availability of sophisticated methods for detecting and characterizing abnormal hemoglobins. Clinical manifestations are highly variable, ranging from a virtually asymptomatic state in the absence of environmental stressors to severe hemolytic

anemia presenting at birth. Patients with chronic hemolysis present with variable degrees of typical symptoms, including anemia, reticulocytosis, hepatosplenomegaly, jaundice, leg ulcers, and propensity to premature biliary tract disease.

For hemoglobin variants with a given degree of reduced solubility, the degree of anemia may vary because some of these variants also exhibit altered oxygen affinity. Thus, Hb Köln has increased oxygen affinity, resulting in relatively higher levels of tissue hypoxia and erythropoietin stimulation at any given level of hematocrit (see next section); therefore, patients with Hb Köln tend to have higher hematocrits than expected on the basis of hemolytic severity because of increased erythropoietin stimulation. By contrast, Hb Hammersmith exhibits decreased oxygen affinity, improving oxygen delivery and allowing patients to function at a lower hematocrit. Hb Zurich possesses, for complex molecular reasons, a higher-than-normal affinity for carbon monoxide. A high hemoglobin carbon monoxide level develops in patients with Hb Zurich who also smoke. Binding of carbon monoxide protects Hb Zurich from denaturation, thus reducing hemolysis, so these people tend to exhibit lesser degrees of hemolytic anemia than do nonsmoking relatives.

Diagnosis

The presence of an unstable hemoglobin should be suspected in patients with one or more stigmata of accelerated red cell destruction: chronic or intermittent hemolytic anemia or jaundice, premature development of bilirubin gallstones or biliary tract disease (due to accelerated red cell turnover), unexplained reticulocytosis, or bouts of intermittent symptoms that can be related to exposure to oxidant drugs or infections. Other suggestive symptoms include dark urine, transient jaundice, or leg ulcers.

Laboratory diagnosis depends on identification of a mutant hemoglobin that precipitates more easily than normal hemoglobin. The peripheral blood smear may or may not show evidence of hemolysis, (i.e., poikilocytosis, polychromasia, or shift cells). The morphologic evidence for precipitated hemoglobin is the Heinz body, the intraerythrocytic inclusion body detected by staining the peripheral blood smear with a supravital dye, such as brilliant cresyl blue or new methylene blue.

The spleen removes Heinz bodies efficiently, especially if hemolysis is not particularly acute or brisk. Thus, Heinz bodies may not be demonstrable at all times. Two provocative laboratory maneuvers are used to aid detection, both of which unmask the tendency of unstable hemoglobins to precipitate: the heat instability test (heating of a hemoglobin solution to 50°C) or the isopropanol instability test (insolubility in 17% isopropanol).

Hemoglobin electrophoresis should be performed but *should not be relied on as the major diagnostic criterion for ruling in or ruling out a hemoglobinopathy.* Many amino acid substitutions that have a profound effect on solubility do not change the overall charge on the hemoglobin molecule. For example, Hb Köln, the most common of the unstable hemoglobin mutations, arises from a mutation changing the valine at position 98 to a methionine. This mutation is electrically neutral; it does not alter electrophoretic mobility. Therefore, these variants do not form an abnormal band on an electrophoresis gel. Demonstration of an abnormal band would clearly add strong evidence in support of the diagnosis. A normal electrophoretogram, however, should never be regarded as strong evidence against the presence of a mutant hemoglobin, especially if the clinical picture or family history otherwise supports the diagnosis.

Sophisticated analyses of hemoglobin can be obtained from reference laboratories if detailed characterization seems warranted. For example, abnormal hemoglobin or globin bands migrating to novel positions on an isoelectric focusing gel can result from hemoglobin or globin moieties lacking heme in groups. When heme is added to the sample and the proteins are reanalyzed, these bands disappear. This behavior is nearly diagnostic of an unstable variant.

Detection of unstable hemoglobins is occasionally compromised by the selective precipitation of the unstable variant into Heinz bodies. Because most patients are heterozygotes, this phenomenon greatly reduces the apparent percentage of the variant in soluble form. Thus, even a variant possessing altered electrophoretic mobility may be very difficult to detect. Indeed, some unstable hemoglobins, such as Hb Geneva or Hb Terre Haute, are so unstable that no mutant gene product can be detected in the steady state. These abnormal hemoglobins actually produce a thalassemic phenotype (see Chap. 29). They are detectable only by isotope labeling studies or direct analysis of the globin genes.

The differential diagnosis of unstable hemoglobin variants is usually straightforward if the general category of hemolytic disorders is suspected. The most common form of G6PD deficiency can also present with bouts of intermittent or chronic

hemolysis exacerbated by oxidant drugs or infection (see Chap. 32). This diagnosis should be considered, as should other causes of chronic or intermittent hemolytic anemia, such as red cell membrane disorders (e.g., hereditary spherocytosis) or immune hemolytic anemias. Spherocytes are relatively rare in unstable hemoglobin disorders; this is sometimes a useful discriminant.

Management

The severity of the clinical complications of unstable hemoglobins varies enormously. Many patients can be managed adequately by observation and education to avoid agents that provoke hemolysis. Some patients may require transfusions during bouts of severe acute hemolytic anemia. Patients who have significant morbidity because of chronic anemia or repeated episodes of severe hemolysis should be considered candidates for splenectomy, especially if hypersplenism has developed. Children with severe hemolysis may require transfusion support until they are old enough (age 3 or 4 years) to undergo splenectomy without unacceptable immunologic compromise. Splenectomy is usually effective for abolition or reduction of symptoms. Infection often exacerbates hemolysis. Fever should therefore prompt close monitoring of patients for evidence of hemolysis or anemia.

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HEMOGLOBINS WITH INCREASED OXYGEN AFFINITY

Oxygen transport by hemoglobin depends on the sigmoid shape of the hemoglobin-oxygen affinity curve. During the transition from the fully deoxygenated to the fully oxygenated state, the initial oxygenation steps occur with difficulty. In fact, the act of binding the first oxygen molecule increases the affinity of the molecule for subsequent oxygen-binding events, thus creating the sigmoid shape of the curve. The necessary intramolecular reorganization occurs only when the proper arrangement of hydrogen bonds, hydrophobic interactions, and salt bridges are broken and formed in the proper sequence.

Mutant hemoglobins exhibiting altered oxygen affinity arise from amino acid substitutions at the interface between - and -chains or in regions affecting the hydrogen bonds, hydrophobic interactions, or salt bridges that influence the interaction of heme with oxygen. A second major class of mutations alters binding to 2,3-diphosphoglycerate (2,3-DPG; see Chap. 22), which in turn alters oxygen affinity when bound to hemoglobin.

Pathogenesis and Pathophysiology

High-affinity hemoglobins exhibit higher avidity for oxygen, causing the oxygen dissociation curve to shift to the left; an example is Hb Kempsey (^{99AspAsn}) (Fig. 31-3). These hemoglobins bind oxygen more readily than normal and retain more oxygen at lower PO_2 levels. They thus deliver less oxygen to tissues at normal capillary oxygen pressures. The PO_2 in the normal lung (PO_2 = 90100 mmHg) is well above that needed to saturate hemoglobin fully with oxygen (60 mmHg). These variant hemoglobins cannot acquire any additional oxygen in the lung despite their higher affinity. At capillary PO_2 (3545 mmHg), however, high-affinity hemoglobins deliver *less* oxygen. At normal hematocrits, a mild tissue hypoxia results; this stimulates erythropoietin release, causing elevated red cell production and polycythemia. In extreme cases, hematocrits of 6065% can be encountered.

Many types of mutations can increase oxygen affinity. Some alter interactions within the heme pocket, others disrupt the Bohr effect or the salt-bond site, and still others impair the binding of Hb A to 2,3-DPG. Loss of 2,3-DPG binding results in increases in oxygen affinity (see Chap. 22). These and numerous other examples that have been analyzed at the molecular level

Figure 31-3 Hemoglobinoxygen dissociation curves are illustrated for normal hemoglobin (Hb A) and for model abnormal hemoglobins with high and low oxygen affinities. On the abscissa, the partial pressure of oxygen is indicated in millimeters of mercury. On the left ordinate, the saturation of hemoglobin with oxygen is indicated as a percentage; on the right ordinate, the oxygen content of the hemoglobin is expressed as volumes percent. The three inverted arrows show the P_{50} for the three hemoglobins (the partial pressure of oxygen at which the hemoglobin is 50% saturated). This value is lowest for the high-affinity hemoglobin. As the partial pressure of oxygen drops from 100 (arterial) to 40 (tissues), hemoglobin desaturates, giving up a portion of its bound oxygen; the numbers on the brackets indicate the amount of oxygen unloaded by the three hemoglobin types expressed as volumes percent. Note that the high-affinity hemoglobin delivers less than one-half the oxygen that Hb A gives to the tissues, resulting in tissue anoxia, increased erythropoietin secretion, and erythrocytosis. Conversely, the low-affinity hemoglobin is even more efficient than Hb A in supplying tissues with oxygen, resulting in diminished erythropoietin production and anemia. *(From Wynngaarden JB, Smith LH Jr, Bennett JC (eds): Cecil Textbook of Medicine. WB Saunders, Philadelphia, 1992, with permission.)*

have greatly aided our understanding of the molecular basis for reversible oxygen binding.

Diagnosis

High-affinity hemoglobins are a cause of familial unexplained erythrocytosis (see Chap. 25). Functional testing of the hemoglobin is the key to diagnosis. Oxygen affinity is usually measured as P_{50} , the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen (Fig. 31-3). The hemoglobin preparation is exposed to increasing oxygen pressures and the relative percentages of oxyhemoglobin and deoxyhemoglobin are determined. The values are plotted on a curve, and the 50% saturation point is determined. A shift to the left means that the hemoglobin reaches 50% saturation at a lower partial pressure of oxygen. High-affinity variants are thus associated with a lower-than-normal P_{50} value. Hemoglobin electrophoresis can, but may not, reveal an abnormal band.

The most common etiology of a low P_{50} value is carbon monoxide. Carbon monoxyhemoglobin has an extremely left-shifted oxygen affinity curve. Carbon monoxide (CO) stabilizes hemoglobin in the R oxy state without the need for oxygen binding. The clinical consequences of mild chronic CO poisoning are the

same as those seen with high-affinity hemoglobin variants. The most common cause of carbon monoxide toxicity is cigarette smoking, although chronic carbon monoxide exposure can elevate the hematocrit in people such as caisson workers or tunnel toll collectors. Severe acute CO poisoning can cause rapid death due to tissue hypoxia.

Management

Most patients with high-affinity hemoglobins have mild erythrocytosis; they do not require intervention. Very rarely, the hematocrit is very high (>5560). The blood viscosity is then sufficiently elevated to require therapeutic phlebotomy.

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HEMOGLOBINS WITH DECREASED OXYGEN AFFINITY

Pathogenesis

Low-affinity hemoglobin variants, such as Hb Kansas (^{102AsnThr}), arise from mutations that impair hemoglobin oxygen binding or reduce cooperativity. In Hb Kansas, the threonine position, ^{102}, cannot form a hydrogen bond with aspartic acid at position ^{94}. Because this aspartate residue stabilizes the R (oxy) state, Hb Kansas binds oxygen less well and exhibits a right-shifted P_{50} value (Fig. 31-3).

Most low-affinity variants possess enough oxygen affinity to become fully saturated in the normal lung. At the low capillary PO_2 in other tissues, these hemoglobins deliver *higher*-than-normal amounts of oxygen. They become more desaturated than normal hemoglobins. Two abnormalities result from this high level of oxygen delivery. First, because tissue oxygen delivery is so efficient, normal oxygen requirements can be met by lower-than-normal hematocrits. This situation produces a state of pseudoanemia, in which the low hematocrit is deceiving because both oxygen deliveryand the patientsare completely normal. Second, the amount of desaturated hemoglobin circulating in capillaries and veins can be >5 g/dl. Cyanosis may thus be associated with these variants. This usually ominous finding is entirely misleading in these individuals, since it reflects no morbidity.

Diagnosis

Patients with unexplained anemia or cyanosis who appear to be entirely well in all other respects should be evaluated, especially if there is a positive family history. Testing for the abnormal variant follows the same reasoning as that just described for high-affinity variants. The O_2 dissociation curve will be shifted to the right, and the numeric value of the P_{50} will be higher than normal.

Management

Patients with low-affinity hemoglobins are usually asymptomatic. No treatment is required. It is important to document that a low-affinity hemoglobin is the cause of an apparent anemia or cyanosis to preempt inappropriate work-ups and provide reassurance to the patient. Cyanosis in some patients can pose a cosmetic problem, but correction with transfusions is rarely justified.

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METHEMOGLOBINEMIAS

Methemoglobin results from oxidation of the iron moieties in hemoglobin from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. Normal oxygenation of hemoglobin causes a partial transfer of an electron from the iron to the bound oxygen. Iron in this state thus resembles ferric iron and the oxygen resembles superoxide (O_2). Deoxygenation returns the electron to the iron, with release of oxygen. Methemoglobin forms if the electron is not returned. Methemoglobin constitutes 3% of the total hemoglobin in normal humans. These levels in humans are in fact maintained at 1% by the methemoglobin reductase enzyme system (nicotinamide adenine dinucleotide [NADH]-dehydratase, ADH-diaphorase, erythrocyte cytochrome b_3).

Pathogenesis and Clinical Manifestations

Methemoglobinemias of clinical interest arise by one of three distinct mechanisms: (1) globin chain mutations that result in increased formation of methemoglobin, (2) deficiencies of methemoglobin reductase, and (3) toxic methemoglobinemia, in which normal red cells are exposed to substances that oxidize hemoglobin iron such that normal reducing mechanisms are subverted or overwhelmed (see Chap. 32 ; Table 31-3).

Abnormal hemoglobins producing methemoglobinemia (M hemoglobins) arise from mutations that stabilize the heme iron in the ferric state. Classically, a histidine in the vicinity of the heme pocket is replaced by a tyrosine; the hydroxyl group of the tyrosine forms a complex that stabilizes the iron in the ferric state (Fig. 31-4 (Figure Not Available)). The oxidized heme iron is relatively resistant to reduction by the methemoglobin reductase system.

Methemoglobin has a brownish to blue color that does not revert to red on exposure to oxygen. These patients thus appear to be cyanotic. In contrast to truly cyanotic people, however, PaO_2 values are usually normal. Patients with these hemoglobins are otherwise asymptomatic because methemoglobin is rarely >3050%, the levels at which symptomatology becomes apparent.

Hereditary methemoglobinemia resulting from methemoglobin reductase deficiency (cytochrome b_5 reductase deficiency) is very rare. Some patients also exhibit neurologic defects. The mutation might thus affect isoforms of the enzyme common to both erythrocytes and other tissues, including brain. Other patients exhibit only the methemoglobin abnormality.

Like patients with M hemoglobins, patients with methemoglobin reductase deficiency exhibit slate-gray pseudocyanosis. Even homozygotes, however, rarely accumulate >25% methemoglobin, a level compatible with absence of symptoms. Heterozygotes can have normal methemoglobin levels but are especially sensitive to agents causing methemoglobinemia.

A third toxic form of methemoglobinemia is caused by exposure to certain chemical agents and drugs that accelerate the oxidation of methemoglobin (Table 31-4). Nitrite compounds are especially notorious and common. Some of these compounds also have a propensity to exacerbate G6PD deficiency and the precipitation of unstable hemoglobins.

Nitrates are a frequent environmental cause of toxic methemoglobinemia. Nitrates do not directly interact with either hemoglobin or the reductase pathway, but are converted to nitrites in the gut. Well water is a frequently encountered source of excessive nitrates. In general, substantial intake of these agents is required before significant amounts of methemoglobin

TABLE 31-3 -- Types of Methemoglobinemia

Congenital
Defective enzymatic reduction of Fe^3 +-hemoglobin to Fe^2 +-hemoglobin
NADH-methemoglobin reductase (cytochrome b_5 reductase) deficiency
Cytochrome b_3 deficiency
Abnormal hemoglobins resistant to enzymatic reduction (M hemoglobins)
Acquired
Excessive (toxic) oxidation of Fe^2 +-hemoglobin
Environmental chemicals
Drugs

Figure 31-4 (Figure Not Available) Modifications of the heme and its environment that account for two common M hemoglobins. **(A)** Hemoglobin A has a His residue at the 58(E7) position. **(B)** In hemoglobin M-Boston, the histidine is replaced by a tyrosine, the phenolic side chain of which is capable of covalently binding to the heme iron, resulting in stabilization in the oxidized form. **(C)** Hb A has a Val residue at position 67(E11). **(D)** Hb M-Milwaukee has a glutamic acid substitution for the 67 valine. The carboxylic side chain of the Glu forms a bond with iron, shifting the equilibrium toward the ferric state. *(Modified from Dickerson RE, Geis I: Hemoglobin: Structure, Function, Evolution, and Pathology. Benjamin-Cummings, Menlo Park, CA, 1983, with permission. Copyright Irving Geis.)*

are generated. Very young infants are more susceptible to these agents than are adults, but all age groups are at risk given sufficient exposure.

Acquired methemoglobinemia is virtually the only situation in which life-threatening amounts of methemoglobin accumulate.

TABLE 31-4 -- Drugs and Chemicals Having Toxic Effects on Hemoglobin Molecule

Agent	Hemoglobin-Derivative Observed	
	Methemoglobin	Sulfhemoglobin
Acetanilid, phenacetin	+	+

Nitrites (farryl, amyl, sodium, potassium, nitroglycerin)	+	+
Trinitrotoluene, nitrobenzene	+	+
Aniline, hydroxylamine dimethylamine	+	+
Sulfanilamide	+	+
p-Aminosalicylic acid	+	
Dapsone	+	
Primaquine, chloroquine	+	
Prilocaine, benzocaine, lidocaine	+	
Menadione, naphthoquinone	+	
Naphthalene	+	
Resorcinol	+	
Phenylhydrazine	+	+

In general, the only symptom produced when methemoglobin comprises <30% of total hemoglobin is the cosmetic effect of cyanosis. As levels of methemoglobin rise to >30%, however, patients begin to exhibit symptoms of oxygen deprivation, such as malaise, giddiness, and other alterations of mental status. The symptoms reflect a true lack of oxygen availability at the tissue level. Methemoglobin is a markedly left-shifted hemoglobin that delivers little oxygen to the tissues. When methemoglobin is >50% of total hemoglobin, loss of consciousness, coma, and death can rapidly ensue. At this level, the blood is chocolate brown.

Diagnosis

Methemoglobinemia should be suspected in patients with unexplained cyanosis. It is obviously a medical emergency when any patient has cyanosis and altered mental status; a normal PaO_2 should trigger a consideration of methemoglobinemia. The ingestion of nitrites as a suicide gesture, especially in people knowledgeable with respect to chemistry, medicine, or pharmacology, should be considered. Methemoglobinemia can be suspected from the brownish color of blood when it is drawn. Laboratory detection is simple; methemoglobin exhibits characteristic peaks of absorption at 630 and 502 nm, rendering it easily distinguishable from normal hemoglobin. The inherited M hemoglobin mutants are frequently detectable by altered electrophoretic mobility, especially if ferricyanide treatment in vitro

is used to convert all the hemoglobin solution to methemoglobin.

In the case of toxic methemoglobinemia, recognition of exposure to an appropriate agent provides the most important historical clue. Acute poisoning can represent a life-threatening emergency; therefore, laboratory evaluation for methemoglobin should be requested for any person displaying atypical cyanosis or cyanosis occurring along with normal blood gas values. Methemoglobin due to deficiencies of the reductase system can be further evaluated in reference laboratories by direct analysis of these enzymes.

Management

Patients with M hemoglobins are usually asymptomatic and require no management. The secondary cyanosis can present a cosmetic problem. The cyanosis is not reversible because ascorbic acid and methylene blue are usually ineffective.

Patients with deficiency of the reductase system usually do not require treatment. Cyanosis in these cases can be improved by treatment with oral methylene blue, 100300 mg/day, or 500 mg/day of oral ascorbic acid. Riboflavin (20 mg/day) has also been reported to be effective and may be the preferred agent, because methylene blue produces discolored (blue) urine, and ascorbic acid can cause sodium oxalate stones.

Emergency treatment of high levels of toxic methemoglobinemia begins with 12 mg/kg of intravenous methylene blue as a 1% solution in saline. It is usually infused rapidly (1015 minutes); the dose may be repeated if necessary. This treatment is usually effective. Methylene blue acts through the NADPH reductase system, which in turn requires G6PD activity. The method is therefore ineffective in patients who also have G6PD deficiency. These patients, or patients who are severely affected, may require exchange transfusion. Oral ascorbic acid is not useful for emergency situations because it acts too slowly. Follow-up maintenance management, however, can be accomplished with either ascorbic acid or oral methylene blue.

Mild cases of methemoglobin intoxication do not require treatment. The patient can be monitored for 13 days, during which time methemoglobin levels gradually return to normal if the offending agent is eliminated. The most important follow-up therapy for patients with toxic methemoglobinemia involves a thorough search for the offending agent and its removal from the environment.

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Chapter 32 - Red Cell Enzymopathies

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Red cell enzymes have received the intense attention of hematologists, biochemists, and physiologists principally because they are easily accessible. Simple laboratory methods for the rapid purification of erythrocytes facilitated studies of congenital and acquired erythrocyte disorders.

Clinically significant abnormalities of red cell enzymes cause diverse phenotypes including hemolytic anemia, polycythemia, and methemoglobinemia. In other instances, although there is no detectable red cell phenotypic abnormality, red cell enzymes can be used to diagnose systemic disorders such as galactosemia, adenosine deaminase deficiency, and some vitamin deficiencies. In addition, the study of red cell enzymes helped uncover important biological principles such as X-chromosome inactivation and provided the groundwork for important principles of population genetics, biochemistry, and molecular biology.

Mature erythrocytes are the product of a highly specialized cellular process involving loss of the nucleus prior to release from the bone marrow and loss of mitochondria and ribosomes after 12 days in circulation. Unable to carry out oxidative phosphorylation and protein synthesis, the erythrocyte still has to sustain an active metabolism to maintain its viability and to preserve hemoglobin in its functional form to ensure adequate oxygen delivery to tissues.

Red cell enzymes support two important metabolic pathways: glycolysis and the pentose shunt (Fig. 32-1). These two pathways generate three important metabolic intermediates: NADH, NADPH, and adenosine triphosphate (ATP). Under physiological conditions, approximately 90% of glucose is consumed in the glycolytic pathway while about 10% is utilized in the pentose shunt. However, in conditions of increased oxidant stress the contribution of the pentose shunt may be significantly increased.

In order to provide proper oxygen delivery, the erythrocyte must (1) maintain the flexibility and integrity of the red cell membrane using ATP from glycolysis, (2) maintain a high ratio of NADH to NAD for continuous reduction of methemoglobin to reduced hemoglobin, (3) maintain a high concentration of 2,3-BPG

Figure 32-1 Glycolysis (Embden-Meyerhof pathway). GSH, reduced glutathione; GSSG, oxidized glutathione; G6PD, glucose- 6-phosphate dehydrogenase; 6PG, 6-phosphogluconate; 2,3-BPG, 2,3-Bisphosphoglycerate; DHAP, dihydroxyacetone-P; GAPD, glyceraldehydephosphate dehydrogenase.

generated in the Rapoport-Luebering shunt of glycolysis to ensure optimal oxygen delivery to the tissue, and (4) maintain a high ratio of NADPH to NADP (generated in the pentose shunt from the glycolytic intermediate glucose-6-phosphate) for reduction of glutathione and protection against oxidant damage.

Other erythrocyte enzymes participate in nucleotide degradation and salvage and are essential for the removal of nucleotide precursors that may be toxic to erythrocytes, e.g., pyrimidine 5 nucleotidase. In addition, erythrocytes contain enzymes, such as glutamine-oxaloacetic transaminase (GOT), with no obvious physiological function; these may be remnants of its nucleated past.

This chapter is divided into four sections. The first three sections are based on the phenotype of the enzymopathy: methemoglobinemia, hemolytic anemia, and polycythemia. The final section of the chapter addresses heterogeneous issues not associated with a specific red cell phenotype, such as acquired enzyme abnormalities associated with nutritional deficiencies, use of red cell enzymes for the diagnosis of systemic diseases, and uses of red cell enzymes for population genetics and other studies.

METHEMOGLOBINEMIA

Methemoglobin

Methemoglobin Formation

Methemoglobin is a derivative of hemoglobin in which the ferrous (Fe^{++}) irons are oxidized to the ferric (Fe^{+++}) state. The ability of hemoglobin to transport oxygen is dependent on the

Figure 32-2 Auto-oxidation of hemoglobin. Iron is in the ferrous state (Fe^{++}) in deoxyhemoglobin. When oxygen is bound, an electron is partially transferred from the iron moiety to the bound oxygen, forming a ferric-superoxide anion complex (Fe^{+++}-O_2). During deoxygenation, some of the oxygen leaves as a superoxide (O_2) radical. The partially transferred electron is not returned to the iron moiety, leaving the iron in the ferric state (Fe^{+++}) and forming methemoglobin (metHb).

oxidation state of its iron moiety. Oxygen binds easily to the ferrous form of iron present in deoxyhemoglobin. In the formation of oxyhemoglobin, one electron is partially transferred from iron to the bound oxygen, forming a ferric-superoxide (Fe^{+++}-O_2) anion complex. [1] During deoxygenation, some of the oxygen leaves as a superoxide (O_2) radical. The partially transferred electron is not returned to the iron moiety, thus leaving the iron in the ferric state and forming methemoglobin (Fig. 32-2). This autooxidation of hemoglobin occurs spontaneously at a slow rate, creating 0.53% methemoglobin per day. [2] [3]

Methemoglobin is also formed from the oxidation of hemoglobin in other reactions with endogenous compounds and free radicals, including hydrogen peroxide (H_2O_2), nitric oxide (NO), O_2, and hydroxyl radical (HO). [4] [5] Exogenous compounds may oxidize hemoglobin to methemoglobin directly, by a metabolic derivative, or by

generating O_2 and H_2O_2 during their metabolism.

The ferric hemes of methemoglobin are unable to bind oxygen. In addition, the oxygen affinity of the accompanying ferrous hemes in the hemoglobin tetramer is increased.[6] As a result, the oxygen dissociation curve is left-shifted and oxygen delivery is impaired.

Methemoglobin Reduction

Although several potential mechanisms exist to reduce methemoglobin back to hemoglobin, only the NADH-dependent reaction catalyzed by cytochrome b_5 reductase (b5R) is physiologically important. Cytochrome b5R, previously known as diaphorase [7] and methemoglobin reductase, [8] contains a noncovalently-bound prosthetic flavine adeninedinucleotide (FAD) group that acts as an electron acceptor. [9] [10] [11] NADH generated from glycolysis reduces FAD to $FADH_2$, which then reduces the heme protein cytochrome b_5. Electrons from the reduced cytochrome b_5 are in turn transferred to methemoglobin, reducing iron back to the ferrous state (Fig. 32-3).

Figure 32-3 NADH-dependent methemoglobin reduction. NADH is generated during glycolysis in the reaction mediated by glucose-3-phosphate dehydrogenase (G3PD). A pair of electrons from NADH is transferred to the FAD prosthetic group of cytochrome b_5 reductase, reducing it to $FADH_2$. Two molecules of ferric (Fe^{+++}) cytochrome b_5 are then sequentially bound and reduced, forming ferrous (Fe^{++}) cytochrome b_5. An ionic complex between ferrous (Fe^{++}) cytochrome b_5 and a ferric (Fe^{+++}) subunit of a hemoglobin (methemoglobin) tetramer is formed and an electron transferred between the two hemes, creating ferrous (Fe^{++}) hemoglobin. G3P, glyceraldehyde-3-phosphate; 1,3-BPG, 1,3-bisphosphonate glyceraldehyde.

Figure 32-4 NADPH-dependent methemoglobin reduction. NADPH methemoglobin reduction can be activated by exogenously administered methylene blue. G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; G6PD, glucose-6-phosphate dehydrogenase.

An alternative pathway mediated by NADPH-flavin reductase uses NADPH generated by glucose-6-phosphate dehydrogenase (G6PD) in the hexose monophosphate shunt as a source of electrons to reduce redox dyes, such as methylene blue, and flavin (Fig. 32-4).[12] [13] [14] Reduced methylene blue, or leukomethylene blue, nonenzymatically reduces methemoglobin. Since these electron acceptors are not physiologic, this electron transfer is not significant under normal conditions and NADPH-flavin reductase deficiency does not cause methemoglobinemia.[15] However, methylene blue administered in the treatment of methemoglobinemia serves as an exogenous electron acceptor, bypassing blocks in the cytochrome b_5 reductase pathway. Since the reduction of methemoglobin by methylene blue is dependent on NADPH generated by G6PD, methylene blue may be ineffective in the treatment of methemoglobinemia in individuals with G6PD deficiency. [16] In addition, the use of methylene blue in these patients is potentially dangerous because it may produce hemolysis. [16]

Methemoglobin can also be reduced directly by ascorbic acid, reduced glutathione, reduced flavin, tetrahydropterin, cysteine, cysteamine, 3-hydroxyanthranilic acid, and 3-hydroxykynurenine;[4] however, these reactions occur slowly and play a minor role in methemoglobin reduction.

Methemoglobinemia

Normally, the formation and reduction of methemoglobin by the mechanisms described above act to maintain a steady-state level of methemoglobin of 1% of total hemoglobin. Methemoglobinemia occurs when an imbalance due to either increased methemoglobin production or decreased methemoglobin reduction is present.

Acquired or Acute Toxic Methemoglobinemia

Most cases of methemoglobinemia are acquired, resulting from increased methemoglobin formation by various exogenous agents. Many drugs and toxins have been implicated (see box, Substances Associated with Methemoglobinemia).[17] [18]

Methemoglobinemia may occur as a result of overdoses or poisoning but may also occur at standard doses, particularly in individuals with partial deficiencies of b5R.[19] [20] Infants are particularly susceptible because their erythrocyte b5R activity is normally 5060% of adult activity. [21] [22] [23] Activity in the premature infant is even lower. Although b5R levels rise to those of an adult within months of birth, young infants are unusually vulnerable to developing toxic methemoglobinemia upon ingestion of nitrates.[24] Nitrates do not oxidize hemoglobin directly but are converted to nitrites by intestinal bacteria. Methemoglobinemia caused by nitrates in well water continues to be a problem in infants in rural areas. [25]

Methemoglobinemia has also been associated with diarrheal illnesses in infants without known toxin exposure. [26] [27] The exact mechanism leading to methemoglobinemia is unknown but may involve increased endogenous nitrite production. [28] An association with weight in the lower percentiles has been reported. [29] [30]

Although individuals with congenital, chronically elevated methemoglobin concentrations are generally asymptomatic, acute acquired methemoglobinemia may be associated with symptoms at similar methemoglobin levels. The cyanosis or slate-blue color of the skin and mucous membranes results from the different absorbance spectrum of methemoglobin compared to oxyhemoglobin. Cyanosis is clinically discernible when the absolute level of methemoglobin exceeds 1.5 gm% (1015% methemoglobin at normal hemoglobin concentrations).[31] Symptoms of methemoglobinemia are related to impaired O_2 delivery to tissues. Early symptoms include headache, fatigue, dyspnea, and lethargy. At higher levels, respiratory depression, altered consciousness, shock, seizures, and death may occur. [18]

Hereditary Methemoglobinemia

Francois[32] probably reported the first case of congenital methemoglobinemia in 1845 when he described one of his patients with chronic congenital cyanosis without obvious cardiac or pulmonary disease. A familial incidence of autotoxic cyanosis and methemoglobinemia was later described by Hitzenberger in 1932. [33]

There are three types of hereditary methemoglobinemia. Most cases are due to a deficiency of the red cell enzyme b5R. This enzyme deficiency is inherited in an autosomal recessive pattern and was the first hereditary condition in which an enzyme deficiency was identified. [8]

Deficiency of cytochrome b_5 is a rare disorder that also causes congenital methemoglobinemia. Only one well-documented case of cytochrome b_5 deficiency has been described [34] compared to over 500 reported cases of b5R deficiency. This patient, a product of an Israeli consanguineous marriage, was also a male pseudohermaphrodite. Further analysis revealed that he was homozygous for a splicing mutation in the cytochrome b_5 gene,

SUBSTANCES ASSOCIATED WITH METHEMOGLOBINEMIA

Acetaminophen (nitrobenzene derivative)

Acetanilide

Aniline dyes

Local anesthetics

- Benzocaine
- Lidocaine
- Prilocaine

Dapsone

Flutamide

Metoclopramide

Nitric oxide

Nitrites

- Amyl nitrite
- Isobutyl nitrite
- Sodium nitrite
- Nitrates (bacterial conversion to nitrites)

Nitrobenzenes/nitrobenzoates

Nitroethane (nail polish remover)

Nitrofurans

Nitroglycerin

Paraquat/monolinuron

Phenacetin

Phenazopyridine (pyridium)

Primaquine

Sulfamethoxazole

resulting in a premature stop codon and truncated protein.[35] Another family with probable cytochrome b_5 deficiency, as other causes of methemoglobinemia were excluded, was described prior to the recognition of this entity.[36] However, the inheritance pattern in this family was autosomal dominant.

The third type of hereditary methemoglobinemia, hemoglobin M (Hb M) disease (see Chap. 31), is due to an abnormal globin and is autosomal dominant. It is associated with an abnormal pattern of spectrophotometric absorbance and sometimes with abnormal migration on hemoglobin electrophoresis.

Cytochrome b5 Reductase

Function of Cytochrome b_5 Reductase

B5R, a housekeeping enzyme and a member of the flavoenzyme family of dehydrogenases-electron transferases, is involved in the transfer of electrons from the NADH generated by glyceraldehyde 3-phosphate in the glycolytic pathway to cytochrome b_5.[37] [38] Cytochrome b_5 serves as an electron donor in a variety of reactions; in erythrocytes, it transfers electrons to methemoglobin to reduce it to hemoglobin. In other cells, a reaction catalyzed by stearyl-CoA desaturase transfers electrons from cytochrome b_5 to stearyl-CoA in the endoplasmic reticulum.[39] This reaction plays an important role in fatty acid desaturation and drug metabolism.

b5R Gene

The b5R gene locus (DIA1) has been mapped to chromosome 22.[40] [41] [42] Only the rat b5R gene has been comprehensively studied;[43] [44] [45] this gene has several potential transcripts generating multiple isoforms (Fig. 32-5). Differences at the 5 ends of rat liver and reticulocyte cDNAs suggest the use of alternative promoters for the production of all forms of b5R.[43]

Two putative promoters with different characteristics have been well-described. A constitutive promoter region has similarities with housekeeping genes as it does not contain a TATA box or CAAT box but instead contains five GC box sequences (GGGCGG and CCGCCC), representing potential binding sites for the transcription factor Sp1.[46] The erythroid-specific promoter region contains several possible regulatory elements found in erythroid promoter regions, including a TATA box, CAAT-like sequences, two binding sites for the erythroid-specific transcription factor GATA-1, and a GT box.[44] Two additional promoter regions have been identified but not yet fully characterized.[45]

Figure 32-5 Cytochrome b5 reductase gene. The transcription and translation of the b5R gene is complex and utilizes multiple promoters and multiple AUG translation initiation sites (arrowheads). In addition to a strong constitutive promoter (C) and a separate erythroid specific promoter (E), weaker promoters (X and Y) are also present. Four transcripts, utilizing alternate first exons and a common downstream exon, have been reported. The strong constitutive promoter initiates transcription at the first exon, which encodes a myristylation consensus sequence and a 14-amino-acid stretch of uncharged amino acids. This transcript generates a ubiquitously expressed isoform with a 300-amino-acid residue product containing a myristylation group (closed rectangle) covalently attached to the N terminus (open diamond). The erythroid-specific promoter initiates transcription downstream at an alternate exon. This transcript contains at least two translation initiation start sites (arrowheads);

the stronger start site generates a soluble isoform consisting of 275 amino acid residues that are identical to those found in the membrane-bound ubiquitously expressed form (open rectangle). The weaker start site generates a unique membrane-bound form with an N-terminus sequence (closed diamond) that differs from that found in the membrane-bound ubiquitously expressed isoform. Two other transcripts have also been detected, albeit in small amounts; these arise from the transcription of alternate exons and are ubiquitously expressed. While the peptide sequences have been inferred from the cDNA sequences, the identity of all of the isoforms has not been unambiguously confirmed.

b5R Isoforms

Multiple isoforms of b5R are believed to be generated from a single gene by a combination of alternative promoters and alternative initiation of translation. [44] [45] One isoform, found in nonerythroid cells and reticulocytes but not in erythrocytes, is a membrane-associated isoform located on the endoplasmic reticulum (ER) membrane and the outer mitochondrial membrane (OMM).[47] [48] The ER-bound isoform participates in cholesterol biosynthesis, [49] desaturation and elongation of fatty acids, [50] [51] and drug metabolism;[52] the function of b5R on the OMM is unknown. This membrane-bound enzyme is an amphophilic protein consisting of a 275 amino acid hydrophilic moiety that contains the active site and a hydrophobic domain at the NH_2 -terminal end that anchors the protein to the membrane.[53] [54] [55] This isoform is myristylated at its amino terminus.[56] The myristylate residue appears to function in targeting this b5R isoform to the OMM; the targeting mechanism for the ER membrane remains to be determined.[57]

Two isoforms are found solely in erythroid cells. The isoform involved in methemoglobin reduction is soluble and consists of the same 275 hydrophilic amino acids found in the ubiquitously expressed membrane-associated isoform.[58] [59] The other erythroid-specific isoform is membrane-associated with an N-terminal hydrophobic sequence that differs from the sequence found in the ubiquitously expressed isoform.[44] This isoform is not myristylated and may play a role in vitamin E recycling.[60] Although this isoform contributes only 2025% of erythocyte b5R activity in adult humans, it represents a greater proportion in infants due to a decreased cytosolic form.[61] A fourth soluble isoform has been detected in rat and rabbit liver and in human placenta.[62] [63] [64]

Cytochrome b5 Reductase Deficiency

Clinical Features

Deficiency of b5R is inherited in an autosomal recessive pattern and a history of consanguinity may be present. There are two clinical types.

Type I b5R Deficiency

The majority of cases of enzymopenic congenital methemoglobinemia are type I, in which the functional deficiency of b5R is limited to erythrocytes. Homozygotes or compound heterozygotes[65] [66] have methemoglobin concentrations of 1035% and appear cyanotic but are usually asymptomatic even with levels up to 40%.[31] Some patients have reported nonspecific symptoms of headache and easy fatigability. Life expectancy is not shortened and pregnancies occur normally. Significant polycythemia (erythrocytosis) is only rarely observed. The cyanosis is of cosmetic significance only, but can be treated with methylene blue or ascorbic acid, both of which facilitate the reduction of methemoglobin through alternate pathways.[67]

In contrast to the asymptomatic, chronically methemoglobinemic homozygotes (or compound heterozygotes), heterozygous individuals are at risk for developing acute, symptomatic methemoglobinemia after exposure to exogenous methemoglobin-inducing agents. The classic description by Cohen and colleagues [19] of acute toxic methemoglobinemia in US military personnel receiving malarial prophylaxis in Vietnam demonstrated for the first time that heterozygotes for an autosomal recessive disease can, under certain conditions, develop a disease state that is more clinically significant than that of their asymptomatic homozygous peers.

Type I b5R deficiency is distributed worldwide but is endemic in the Athabascan Alaskans,[68] [69] Navajo Indians,[70] and Yakutsk natives of Siberia.[71] [72] The Navajo Indians and the Athabascan Indians of Alaska are known to share a common ancestor; the high frequency of b5R deficency in these groups suggests a common origin for all three of these populations. However, it remains to be determined whether the molecular defect resulting in b5R deficiency is identical in these populations. In other ethnic and racial groups the defect occurs sporadically. Although cyanosis is difficult to detect due to skin pigmentation, type I deficiency has been reported in two unrelated African-American families.[73]

Type II b5R Deficiency

In type II b5R deficiency, which represents 1015% of cases of enzymopenic congenital methemoglobinemia, b5R is deficient in all cells. Type II b5R deficiency has a sporadic distribution. In addition to methemoglobinemia and cyanosis, patients exhibit mental retardation and developmental delay with failure to thrive.[74] Other neurological symptoms may be present, including microcephaly, opisthotonus, athetoid movements, strabismus, seizures, and spastic quadriparesis. Life expectancy is significantly shortened. The mechanism resulting in the neurological problems is currently unknown but may involve abnormal lipid elongation and desaturation in the central nervous system.[75]

Methylene blue or ascorbic acid improves the cyanosis, as in type I b5R deficiency; however, this therapy has no effect on the neurologic aberrations. Theoretically, a bone marrow or liver transplant would alleviate the neurological problems of type II individuals if they are caused by a problem with circulating fatty acids; however, these approaches have not yet been tested. Because the enzyme defect is found in fibroblasts, analysis of b5R activity in cultured amniotic cells for prenatal diagnosis is possible.[76] [77] [78]

Assays of Enzyme Activity

Types I and II b5R deficiency are distinguished by their clinical phenotype and by analysis of enzymatic activity in erythroid and nonerythroid cells. Reports of decreased b5R activity are difficult to compare since several different assays of b5R activity, varying in their substrate and in their normal values, have been used.[2] [65] [66] [68] [72] [73] [79] [80] [81] These assays also vary in their technical difficulty. The first widely accepted b5R activity assay used a difficult to produce methemoglobin-ferrocyanide complex and its reduction by an enzyme containing tissue homogenate.[82] The most rigorous b5R enzyme activity is based on partial purification of the enzyme by an ultracentrifugation and uses the physiological enzyme substrate (cytochrome b_5 prepared by a recombinant DNA technology);[54] this assay is not readily available and is too complex for nonspecialized research laboratories. A more recent assay uses readily available ferricyanide [83] and readily differentiates type I and type II b5R deficiency because patients with type I deficiency have normal enzyme activity in platelets, fibroblasts, EBV-transformed lymphocytes, and granulocytes, while in type II deficiency the activity in nonerythroid tissues is markedly to moderately decreased.[73] [83] [84] [85]

Two families with type III deficiency, in which b5R activity was decreased not only in erythrocytes but also in platelets and leukocytes, have been described.[86] [87] However, the existence of this entity is difficult to accept since these individuals did not exhibit the neurological abnormalities characteristic of type II deficiency. Re-evaluation of one of the patients using the complex recombinant cytochrome b_5 assay confirmed b5R activity in the platelets, leukocytes, and fibroblasts consistent with type I deficiency.[88]

b5R Gene Mutations

Four point mutations in patients with type I b5R deficiency have been reported; all are missense mutations, resulting in an amino acid substitution (Table 32-1). Three of these mutations are found in the 5 end of the b5R gene.[89] [90] A fourth mutation, a G

TABLE 32-1 -- Mutations in Type I Cytochrome b5 Reductase Deficiency

Reported By	Nucleotide Change	Mutation	Protein Effect	Exon	Ethnic Origin	Comment

Katsube et al.[90]	T C	Missense	L148P	5	Japanese	Previously classed as type III
Shirabe et al.[89]	G A	Missense	R57Q	3	Japanese	
Shirabe et al.[89]	G A	Missense	V105M	4	Italian	
Jenkins and Prchal[91]	G A	Missense	E212K	8	African American	

to A transition in exon 8 (E212K) in an African-American family, occurs in the 3 end. [85]

An additional mutation, resulting in the replacement of threonine by serine at codon 116, was found in exon 5 (T116S) in an African-American family with type I b5R deficiency.[91] However, this mutation was not responsible for the enzyme deficiency because the amino acid substitution causes no readily appreciable disruption of the b5R secondary structure. This missense mutation has been determined to be a high-frequency polymorphism of b5R specific for African Americans.

Type II mutations have been reported in both the 5 and 3 ends of the b5R gene (Table 32-2).[80] [81] [84] [92] [93] [94] [95] Type II mutations are heterogeneous, including deletions, point mutations, splicing site mutations, and premature stop codons, whereas all the mutations found in the type I individuals have been missense mutations.

B5R enzyme activity is affected only in erythrocytes in type I deficiency. The mutant enzyme is heat-labile and presumably is unstable and easily degraded. [96] Thus, although b5R is abnormal in all cells, only mature red cells, which cannot synthesize proteins and replace the enzyme, are significantly affected in type I b5R deficiency. Since b5R is coded by a single gene, it is hypothesized that type I b5R deficiency results from mutations producing an unstable enzyme while mutations in type II deficiency either affect the catalytic function or cause underproduction of the enzyme, resulting in a generalized decrease in functional b5R activity.

Diagnosis of Methemoglobinemia

Methemoglobinemia may be clinically suspected when cyanosis occurs in the presence of a normal PaO_2, as obtained by arterial blood gases. Pulse oximetry is inaccurate in monitoring oxygen saturation in the presence of methemoglobinemia. The blood in methemoglobinemia is dark red or a characteristic chocolate color and the color does not change with the addition of oxygen. Methemoglobinemia causes clinically discernible cyanosis when the absolute level of methemoglobin exceeds 1.5 gm%; this correlates with approximately 1015% methemoglobin.[31] Sulfhemoglobin in concentrations greater than 0.5 gm% also causes cyanosis with a normal PaO_2 and may be erroneously measured as methemoglobin. The laboratory diagnosis of methemoglobinemia is based on analysis of its absorption spectra. A fresh specimen should always be obtained because methemoglobin levels tend to increase with storage. Methemoglobin has peak absorbance at 631 nm. The standard method of assaying methemoglobin utilizes a microprocessor-controlled, fixed wavelength co-oximeter. This instrument interprets all readings in the 630 nm range as methemoglobin; thus false positives may occur in the presence of other pigments including sulfhemoglobin and methylene blue. [97] [98] Methemoglobin detected by co-oximeter should be confirmed by the specific Evelyn-Malloy method.[99] In this assay, cyanide (CN) is added and binds to the positively charged methemoglobin, eliminating the peak at 630635 nm in direct

TABLE 32-2 -- Mutations in Type II Cytochrome b5 Reductase Deficiency

Reported By	Nucleotide Change	Mutation	Protein Effect	Exon	Ethnic Origin	Comment
Kobayashi et al.[92]	T C	Missense	S127P	5	Japanese	
Shirabe et al.[94]	3 bp deletion (in frame)	Deletion	F298	9	Japanese	
Mota Viera et al.[80]	G C	Exon skipping	Truncation	Intron 5	Algerian	Exon 5 skipping; frameshift
Mota Viera et al.[80]	C T	Nonsense	R218stop	8	Algerian	
Mota Viera et al.[80]	T C	Missense	C203R	7	French/Spanish	Compound heterozygote
Mota Viera et al.[80]	3 bp deletion (out of frame)	Deletion	M272	9	French/Spanish	Compound heterozygote
Shirabe et al.[94]	G T at splice acceptor site	Unknown	Unknown	Intron 8	Italian	No immunologically detectable b5R in blood cells/fibroblasts
Jenkins and Prchal[91]	G C	Missense	R60P	3	Caucasian	
Manabe et al.[93]	C A	Nonsense	Y42stop	2	Caucasian (UK)	Compound heterozygote
Manabe et al.[93]	C A	Missense	P95H	4	Caucasian (UK)	Compound heterozygote
Owen et al.[95]	A C at splice acceptor site	Exon skipping	Deletion 28 amino acids	Intron 5	not reported	Exon 6 skipping

proportion to the methemoglobin concentration. The subsequent addition of ferricyanide converts the entire specimen to cyanomethemoglobin for measurement of the total hemoglobin concentration. Methemoglobin is expressed as a percentage of the total concentration of hemoglobin.

Distinguishing the hereditary forms of congenital methemoglobinemia requires interpretation of family pedigrees as well as biochemical analyses. Cyanosis in successive generations suggests the presence of the autosomal dominant Hb M disease, whereas normal parents but possibly affected siblings imply the very rare autosomal recessive b5R or cytochrome b_5 deficiencies. Incubation of blood with methylene blue distinguishes b5R deficiency from Hb M disease because this treatment results in the rapid reduction of methemoglobin through the NADPH-flavin reductase pathway in b5R deficiency but not in Hb M disease. [100] [101] [102] To distinguish b5R deficiency from cytochrome b_5 deficiency, measurement of the level of b5R activity or cytochrome b_5 is required; however, these assays are not commercially available.

Treatment

Offending agents in acquired methemoglobinemia should be discontinued. No other therapy may be required, but if the patient is symptomatic, which is often the case in deliberate or accidental overdoses or toxin ingestion, specific therapy is indicated. Methylene blue, 12 mg/kg over five minutes, provides an artificial electron acceptor for the reduction of methemoglobin via the NADPH-dependent pathway. Response is usually rapid; the dose may be repeated in one hour, but frequently is unnecessary. Caution should be exercised to avoid overdosage because large (>7 mg/kg) cumulative doses have been reported to cause dyspnea, chest pain, and hemolysis.[103] [104] Since co-oximetry detects methylene blue as methemoglobin, it cannot be used in determining methemoglobin levels. Methylene blue should not be administered to patients with G6PD deficiency; pretreatment screening of populations with a high incidence of G6PD deficiency (e.g., African Americans, Mediterraneans, and Southeast Asians) is reasonable. If methylene blue is contraindicated, ascorbic acid may be given.

Treatment of the cyanosis in individuals with types I and II b5R deficiency is indicated for cosmetic reasons only. Treatment options include methylene blue (100300 mg/day orally) or ascorbic acid (3001,000 mg/day orally in divided doses), although this therapy has been associated with renal calculi formation. The use of riboflavin (2030 mg/day) has also been reported.[105] Unfortunately, there is currently no therapy for the neurological disorder associated with type II deficiency.

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RED CELL ENZYMOPATHIES ASSOCIATED WITH HEMOLYTIC ANEMIA

The red cell enzyme deficiencies causing hemolytic anemia may be divided arbitrarily into those directly or indirectly involved with the maintenance of a high ratio of reduced to oxidized glutathione, those participating the glycolytic (Embden-Meyerhof) pathway, and pyrimidine 5 nucleotidase deficiency (nucleotide degradation and salvage pathway).

Inheritance

G6PD and phosphoglycerate kinase (PGK) are encoded on the X chromosome and are subject to dosage compensation by X chromosome inactivation. The remaining enzymes are present on the autosomal chromosomes; deficiencies of these enzymes may cause a clinical phenotype if the subject is either homozygous or doubly heterozygous for separate defects affecting both alleles.

Clinical and Laboratory Manifestations

The hemolysis associated with a red cell enzyme deficiency may be chronic or acute intermittent. Acute intermittent hemolysis is typically seen in enzyme disorders affecting glutathione metabolism, e.g., G6PD. During the hemolytic episodes, either normal red cell morphology or nonspecific abnormalities (anisocytosis, polychromasia) may be observed. In defects of enzymes affecting glutathione metabolism, the morphological sequelae of the oxidative denaturation of hemoglobin, i.e. Heinz bodies, may be seen either directly on microscopic evaluation of the blood film or after the red cells are preincubated with oxidants such as phenylhydrazine.[106] The only enzyme deficiency associated with hemolysis that causes consistent morphological abnormalities is 5 nucleotidase deficiency wherein basophilic stippling is observed. Although laboratory screening tests such as the autohemolysis test [107] were previously advocated to detect enzyme disorders causing hemolysis, they are not based on sound physiological basis and should play no role in modern hematology practice. [108] [109]

Enzymopathies of Glutathione Metabolism

Glutathione Metabolism

Erythrocytes contain a remarkably high concentration of glutathione (2 mM),[110] more than any other cell in the body. Oxidant damage converts reduced glutathione (GSH) to the oxidized form (GSSG) and also forms mixed disulfides of proteins (such as hemoglobin) containing free SH groups.

A high GSH to GSSG ratio is the major mechanism protecting the red cell proteins from oxidant damage. Enzymes involved in this metabolic pathway (Fig. 32-6) include -glutamyl cysteine synthase and GSH synthase, which synthesize glutathione; glutathione reductase, which reduces GSSG to GSH; and G6PD, which generates the NADPH required for the reduction of GSSG. Although GSH is actively synthesized in the red cells, its half-life is only three to four days due to the efflux of oxidized glutathione by an active transport mechanism. This transport mechanism provides additional protection from severe oxidative stress. [111] [112]

Although G6PD deficiency is very common, deficiencies of

Figure 32-6 Glutathione pathway. GSH, reduced glutathione; GSSG, oxidized glutathione; G6PD, glucose-6-phosphate dehydrogenase; G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate.

other enzymes of the glutathione pathway are rare. Deficiencies of these enzymes are expected to resemble G6PD deficiency with precipitation of hemolysis and formation of Heinz bodies after exposure to certain drugs, infections, and other oxidant stresses, but these enzyme defects are sometimes also associated with additional clinical manifestations.

G6PD Deficiency

History

The discovery of G6PD deficiency represents a legendary example of clinical investigation that led to the delineation of important biochemical and genetic principles. Ernest Beutler, a young physician from the University of Chicago, and his colleagues were contracted by the US Armed Forces to investigate a newly developed promising malaria drug, primaquine.[113] [114] As was standard in those years, the drug was tested on inmate volunteers in an Illinois state prison. [109] [114] [115] While the drug was tolerated by the majority of male volunteers, it was noted that some inmates developed acute hemolytic episodes. These episodes were short lasting and typically did not recur after immediate reintroduction of the drug, but could recur when the drug was reintroduced after a period of several months. These drug-induced reactions were seen in subjects of particular ethnic groups including African Americans and individuals of Mediterranean extraction. Biochemical analysis of erythrocytes during an acute hemolytic episode revealed a decrease in reduced glutathione. [114] Shortly thereafter, Carson[116] found that the cause of these unique hemolytic episodes was a deficiency of the first step of pentose shuntG6PD. Subsequent studies of carrier females by these investigators and others led to the co-discovery of X chromosome inactivation.[117] [118] [119] Further investigations of this phenomenon have played a pivotal role in our understanding of the hierarchy of hematopoiesis,[120] [121] [122] clonality of malignant neoplasms,[123] and the mechanism of X chromosome inactivation.[117] [124]

Biology and Molecular Aspects

G6PD is a housekeeping enzyme that plays an essential role not only in the reduction of NADPH from NADP but also in the generation of five carbon sugars. In the reaction catalyzed by G6PD, electrons generated by the conversion of glucose 6 phosphate to 6-phosphogluconate are transferred to NADP. The G6PD gene has been cloned;[125] [126] [127] it spans approximately 20 kilobases with 13 exons. The first exon is noncoding [127] and the second intron is unusually long, about 10 kilobases.[125] The enzyme locus *Gd* has been assigned to chromosome Xq28 near the telomeric region of its long arm [128] and is in close proximity to another important disease causing gene, factor VIII, which is only about 100 kilobases upstream. [129] G6PD activity decreases significantly as erythrocytes age, with a half-life of about 60 days.[130] Reticulocytes have five times higher enzyme activity than the oldest erythrocyte subpopulation. [131]

G6PD Deficient Variants

More than 300 G6PD variants have been defined; most are sporadic but some occur at a high frequency. G6PD variants can be divided into three categories: (1) those associated with chronic hemolytic anemia, (2) those associated with acute intermittent hemolytic anemia, and (3) those associated with no obvious risk of hemolysis. A detailed list of these variants is continuously up-dated and can be found elsewhere. [132]

In the past, new variants were defined by their enzyme activity, thermal stability, K_M for the normal (G6P, NADP) and artificial (2-deoxy G6P, deamino-NADP) substrates, K_i for NADPH, and electrophoretic mobilities under various conditions. [133] However, when the nucleotide structure of the wild-type G6PD enzyme, designated G6PD B, was established,[127] some of these previously described different variants were found to represent identical mutations [134] although in some instances they arose independently on different haplotypes of the G6PD gene. [135]

The vast majority of the mutations are missense. Mutations associated with chronic hemolysis tend to cluster in the vicinity of the NADP-binding domain of the G6PD gene while those associated with acute intermittent hemolysis or no hemolysis are scattered throughout the gene. Unlike disease-causing mutations of other genes, deletions and insertions causing frame shift and stop codon mutations are not observed; these events would be expected to be fatal since G6PD is a housekeeping gene essential for basic cellular functions. [136] One intronic mutation of the G6PD gene (G6PD Varnsdorf) has been described; [137] this mutation is associated with chronic hemolysis but the molecular consequences of this mutation have not been fully elucidated. [138]

Of the G6PD deficient variants that occur at a high frequency, the best-known are African G6PD A and the Mediterranean variants; several variants are also present in Southeast Asia. All of these variants cause acute intermittent hemolysis. African G6PD A and the Mediterranean variant of G6PD deficiency have been particularly well-studied. G6PD A+, another well-studied isoenzyme variant, is associated with no obvious hematological phenotype and has a gene frequency similar to that of G6PD A among African Americans.[139] The G6PD A mutation (G202A) arose on a G6PD A+ chromosome (A376G).[140]

Epidemiology

G6PD deficiency is one of the most prevalent disease-causing mutations worldwide, affecting hundreds of millions of people. [137] [141] Most of the G6PD isoenzymes with decreased activity are associated with only moderate health risks without a significant effect on longevity. [142] [143]

In the United States, G6PD deficiency is seen frequently among African Americans (G6PD A), affecting about 10% of males. The Mediterranean variant of G6PD deficiency is common in the southern part of Italy, Greece, Spain, and Corsica, and among Arabs and Kurdish Jews. In some populations of Kurdish Jews the gene frequency exceeds 50%. This variant is heterogeneous at the nucleotide level and is composed of several distinct mutations. [144] [145] Several variants are pandemic in Asia. G6PD Mahidol is common in Thailand and India; [146] G6PD Chinese-1, G6PD Chinese-2, G6PD Chinese-3, and G6PD Canton are frequent in China and Southeast Asia.[147] [148] [149] [150] It is assumed that malaria provided a positive selection pressure accounting for the high gene frequency of these G6PD variants (see discussion following), whereas mutations that arose in parts of the world not plagued by malaria are sporadic.

Acute Hemolysis and G6PD Deficiency

Hemoglobin is maintained in solution in a high concentration in the cytoplasm of the erythrocyte. Oxidant damage leads to the oxidation of free -SH groups of hemoglobin, forming disulfide bridges that in turn lead to decreased hemoglobin solubility. The precipitated hemoglobin may be morphologically recognized as Heinz bodies. The high ratio of reduced-to-oxidized glutathione represents the major defense against oxidative damage of hemoglobin. The enzymatic activity of G6PD generates NADPH that is utilized for the glutathione reduction (see Fig. 32-6). Reduced glutathione (GSH) reconstitutes SH groups of hemoglobin, maintaining the solubility of hemoglobin.

In G6PD deficiency, this process is variably impaired depending on the type of the G6PD mutation. In a subject with an acute hemolytic G6PD deficient variant, there is no clinical or laboratory evidence of hemolysis unless the individual is exposed to certain clinical situations. However, in subjects with a chronic hemolytic G6PD variant, the same clinical situations

lead to an acute hemolytic deterioration of a pre-existing chronic hemolytic anemia.

An acute insult, most commonly *drugs, infections,* or *fava bean ingestion,* typically precipitates hemolysis. In spite of continuation of the drug or persistence of infection, hemolysis is short-lasting, presumably due to the elimination of a subpopulation of red cells with very low G6PD activity. The younger red cells and reticulocytes, which have higher G6PD activity, are typically not hemolyzed.

Pharmaceutical agents are the best-defined precipitants of hemolysis. Although many drugs have been alleged to precipitate hemolysis in G6PD-deficient subjects, relatively few have withstood scrutiny.[108] Many of these agents are now obsolete and unlikely to be used. However, some newer chemotherapy agents, such as Adriamycin,[151] can precipitate a hemolytic episode. A selected number of these agents follow.

Substances to Be Avoided in G6PD Deficiency

Acetanilid

Doxorubicin [151]

Isobutyl nitrite[152]

Methylene blue

Naphthalene[153]

Nitrofurantoin (Furadantin)

Phenazopyridine (Pyridium)[154]

Phenylhydrazine

Primaquine

Sulfacetamide

Sulfamethoxazole (Gantanol)

Sulfanilamide

Sulfapyridine

Methylene blue administration to subjects with acute toxic methemoglobinemia is a well-known cause of severe hemolysis [108] that may be life-threatening in a subject with already compromised oxygen delivery due to methemoglobinemia (see section on methemoglobinemia earlier in this chapter).

Infections have been widely recognized as a cause of hemolysis in G6PD-deficient subjects. It is assumed that diffusion of oxidants from neutrophils undergoing oxidative bursts leads to the formation of disulfide bridges of hemoglobin.

Fava beans are a staple food in many parts of the world where G6PD deficiency is found at a high gene frequency. The hemolysis precipitated by fava bean ingestion, favism, has been widely recognized in the Mediterranean region [155] [156] and occurs only in subjects who are also G6PD deficient. However, not all G6PD-deficient variants are prone to favism. Subjects with the G6PD A variant are typically not susceptible (although a rare exception to this rule was reported) [157] and not all subjects with the more severely deficient Mediterranean variant of G6PD deficiency are sensitive. It is assumed that the hemolytic risk of fava beans depends on the source and preparation of the beans [158] as well as individual variation due to an additional genetic component that has not yet been characterized. [159] Two fava bean metabolites, divicine and isouramil, are presumed oxidants and have been reported as possible causes of favism. [158]

Evolutionary Benefit of G6PD Deficiency

Shortly after the discovery of G6PD deficiency it became apparent that the geographic distribution of populations with a high gene frequency of deficient variants overlapped closely with the prevalence of malaria. Based on this association, it was suggested that G6PD deficiency might be protective against malaria. [160] [161] Unexpectedly, no protective effect of the G6PD deficiency was found for hemizygous G6PD-deficient males. [162] However, an apparent protection was found for heterozygous females[162] who, because of X-chromosome inactivation, are mosaics for deficient and nondeficient cells. While the malaria parasite does not initially thrive in a G6PD-deficient environment, [142] it adapts after several days of residence in a G6PD-deficient erythrocyte by increased production of malarial G6PD. [163] [164] This strategy counteracts the evolutionary defense of G6PD deficiency in males; however, when the parasites infect the variably deficient and nondeficient erythrocytes of heterozygous females, malarial G6PD production is not efficient. [164]

Diagnosis

The diagnosis of G6PD deficiency is based on the generation of NADPH from NADP as detected by either quantitative spectrophotometric analysis [110] or, more conveniently, by a rapid fluorescent screening test. [165] However, false negative results are not unusual, especially if enzymatic analysis is performed shortly after resolution of acute hemolytic episodes. [115] After acute hemolysis, reticulocytes and young erythrocytes, which have much higher enzymatic activity, predominate. These false negative tests are more likely to occur when a screening test rather than a quantitative spectrophotometric analysis of the enzyme activity is used. Furthermore, a high proportion of young red cells can be conveniently estimated by concomitant spectrophotometric analysis of hexokinase, another red cell enzyme whose activity is markedly dependent on red cell age.

Because of the natural mosaicism for X-chromosome enzymes present in females (G6PD-deficient erythrocytes of heterozygous females are also prone to hemolysis), females heterozygous for G6PD deficiency are particularly difficult to diagnose. [115] In populations of African ancestry, the faster electrophoretic mobility of G6PD A discriminates it from the wild-type G6PD B isoenzyme, but electrophoresis does not differentiate G6PD A from the other African polymorphic nondeficient isoenzyme G6PD A+.[115] However, now that the nucleotide substitutions of many G6PD-deficient isoenzymes have been established, molecular diagnostic methods can be more reliably used for the accurate diagnosis of females who are heterozygous for G6PD deficiency. [143] [166]

Therapy

Drugs that are known to precipitate hemolysis in G6PD-deficient subjects should be avoided. In subjects with G6PD A-deficiency, hemolysis is typically short-lasting in spite of continuous use of the offending agent. This is not always the case in the more severe Mediterranean variant of G6PD deficiency and the precipitating agent should be always be withdrawn. When anemia is severe and symptomatic, blood transfusion may be necessary.

-Glutamyl Cysteine Synthase Deficiency

-Glutamyl cysteine synthase catalyzes the first metabolic step of glutathione synthesis. Only two families with a defect of this enzyme have been reported. [167] [168] The first family had hemolytic anemia and progressive neurological defects; the other family had hemolytic anemia but was neurologically normal. [168]

GSH Synthase Deficiency

More than a dozen families with GSH synthase deficiencies have been diagnosed. There are at least two phenotypes. In one phenotype, hemolytic anemia is the only clinically relevant defect. [169] However, in the more common phenotype both hemolytic anemia and severe metabolic acidosis [170] due to the accumulation of 5-oxoproline (see Fig. 32-6) are present. Deficiency of this enzyme in neutrophils has also been alleged to

cause intermittent neutropenia in some families. [171] One affected African American was also an albino, [172] suggesting the participation of this enzyme in melanin

metabolism. Although therapy with vitamin E has been reported to be beneficial in one patient with this disorder, [171] it was not confirmed in another study of a different family.[172]

Glutathione Reductase Deficiency

Glutathione reductase requires the riboflavin-derived co-factor FAD for full activity. Its in vitro activity with and without exogenously added FAD can be conveniently used for diagnosing riboflavin deficiency. [110] The only reported case of an inherited deficiency of this enzyme was a Dutch family with two affected siblings who had unstable GSH and developed a hemolytic crisis after eating fava beans. [173] Acquired deficiency of this enzyme secondary to riboflavin deficiency does not appear to have any hematological phenotype.

Glutathione Peroxidase Deficiency

Glutathione peroxidase contains selenium. Selenium deficiency is particularly common in New Zealand where multiple individuals with decreased enzyme activity have been found.[174] These acquired decreased activities were not associated with a recognizable phenotype; no clear-cut congenital deficiency of this enzyme has been reported.

Enzymopathies of the Glycolytic Pathway

The enzyme deficiencies of glycolytic pathway differ from those enzyme abnormalities of glutathione pathway metabolism in several ways: (1) they do not have any characteristic morphological abnormalities such as Heinz bodies, (2) they are not subject to hemolytic crisis after exposure to oxidants, and (3) their mechanism of hemolysis is not clearly understood. Furthermore, the clinical phenotype of each of these disorders differs and is unpredictable; the phenotype appears to be specific for each enzymatic defect. The diagnosis of these disorders depends on the quantitation of enzyme activity in a hemolysate prepared from leukocyte- and platelet-depleted washed red cells.[110]

Pyruvate Kinase Deficiency

Pyruvate kinase deficiency is the most common erythrocyte enzyme defect causing congenital chronic hemolytic anemia. Although this disorder is far less common than G6PD deficiency, the vast majority of patients with G6PD deficiency never suffer an acute hemolytic episode and those G6PD variants associated with chronic hemolytic states are rare.

PK Isoenzymes and Molecular Biology

The pyruvate kinase (PK) isoenzymes, M_1 , M_2 , L, and R, are products of two distinct genes. The PK M gene, which is expressed in muscle, leukocytes, platelets, brain tissue and other cells, is located on chromosome 15q22; [175] M_1 and M_2 isoforms result from differential processing of a single transcript of this gene. [176] PK-M_2 is the dominant fetal form of PK. In adult life it is largely replaced in muscle and brain by PK-M_1 but persists in leukocytes, platelets, and early erythroid progenitors. [177]

A gene on chromosome 1q21 encodes the L and R PK isoenzymes.[178] This gene utilizes two different tissue-specific promoters initiating transcription at different exons.[179] The L isoform is unique to hepatocytes and is 33 amino acids smaller than the R form, which is unique to erythrocytes. The R isoform gradually replaces the M_1 isoform as erythroid cells mature. The complexity of PK isoforms is further accentuated by the fact that the functional enzyme is a tetramer.

An allosteric enzyme with one substrate-binding site, PK converts phosphoenol pyruvate to lactate, generating ATP in the process. PK also interacts with fructose diphosphate (FDP). Similar to the interaction of hemoglobin with 2,3-BPG, this interaction changes the conformational structure of PK and decreases its Michaels enzymatic constant (K_M) for its substrate phosphoenol pyruvate, thus dramatically increasing the activity (V_{Max}) of the enzyme.[180] [181] PK-M_1 is the only isoform that does not allosterically interact with FDP. The PK M variants also have quite different kinetic properties.

Mechanism of Hemolysis and Physiologic Implications

The mechanism of hemolysis is not clear. Although it has been postulated that the defect in ATP generation contributes to the hemolytic process, [182] this explanation is insufficient because ATP deficiency is difficult to demonstrate in many patients, and other disorders with more severe ATP deficiency are not associated with significant hemolysis.[183]

It has been suggested that the anemia of PK deficiency is better tolerated than a comparable level of anemia seen in hexokinase deficiency, since the block in glycolysis occurs after the Rappaport-Leubering shunt (see section in this chapter on 2,3-BPG deficiency). The accumulation of 2,3-BPG in this downstream glycolytic defect leads to higher 2,3-BPG concentrations and thus better oxygen delivery to the tissue and superior tolerance of anemia. [184]

Patients with hemolytic anemia who undergo splenectomy, with a resultant decrease in the hemolytic process and improvement of anemia, have a higher number of reticulocytes than they did before the splenectomy. [185] [186] This perplexing observation indicates that our knowledge of the regulation of erythropoiesis and reticulocyte kinetics remains incomplete. It has been suggested that process of neonacytolysis [187] influences the premature destruction of reticulocytes and young red cells in patients with significant pyruvate kinase deficiency [186] by an as yet unknown mechanism.[187]

Clinical Presentation and Therapy

The severity of hemolysis in PK-deficient patients is highly variable. It ranges from life-threatening transfusion-requiring hemolytic anemia present at birth to a mild fully compensated hemolytic process without anemia. As would be expected with multiple mutations causing the PK deficiency, the degree of severity is similar within a given family. Affected individuals are either homozygous for the same mutation or doubly heterozygous for two different PK defects. PK deficiency is distributed worldwide, but it has been reported that the gene is more common among people of northern European extraction [188] and perhaps Chinese[189] and certain other ethnic and racial groups. In these populations, the frequency of heterozygosity may exceed 1%. [188] [189]

Patients with severe hemolysis may be chronically jaundiced, and the clinical complications of hemolytic states, including gallstones, aplastic anemia (often due to parvovirus infection), folate deficiency, and, infrequently, skin ulcers, have been all observed. As with any autosomal recessive disorder, the defect is more common in a group with a history of inbreeding or consanguinity. A high frequency of this disorder has been well-documented among Pennsylvania Amish. [190]

Splenomegaly is frequently seen but not invariable. The beneficial effect of splenectomy on hemolysis is well-documented; typically, the degree of hemolysis and anemia is ameliorated and in severe cases the transfusion requirement is generally abolished. However, as stated above, reticulocytes invariably rise postsplenectomy. Unless the patient is transfusion dependent, it is advisable to delay splenectomy until after the age of three years when the risk of pneumococcal, *Hemophilus influenzae,* and meningococcal infections declines. Although folic acid

replacement is frequently used, it may not always be necessary; however, it is inexpensive and safe.

Diagnosis

There are no specific clinical findings or morphological abnormalities in PK deficiency. No routinely available laboratory measurements aid in diagnosis, although it was previously proposed that the autohemolysis test might be helpful in the diagnosis of PK deficiency. This test does not have any physiological basis and should not be used in modern hematology practice.[109]

A screening test, utilizing crude hemolysate with one concentration substrate, has been employed for detection of pyruvate deficiency. [110] However, this screening test

misses the PK variants characterized by increased K_M and those with abnormal FDP interaction. Specialized laboratories that can perform quantitative PK enzyme analysis with various concentrations of substrate with and without FDP (the native FDP must first be removed by dialysis) may be required. [110] The mutant enzyme can be further analyzed by comprehensive kinetic and electrophoretic studies of the partially purified enzyme. [191] However, these studies have now been largely replaced by the detection of specific mutations of the enzyme at cDNA or genomic level. [192] Single nucleotide substitutions have been described as well as intronic mutations, deletions, and insertions. [193] The 1529A PK mutation appears to be particularly common even in unrelated individuals. [194]

Glucose Phosphoisomerase (GPI) Deficiency

GPI deficiency is either the second or the third most common red cell enzyme defect causing chronic hemolytic anemia (following pyruvate kinase deficiency and rare sporadic severe variants of G6PD). More than 100 families have been described. In some families, the hemolysis is severe enough to necessitate chronic blood transfusions. [195] Neonatal jaundice is common in this disorder [196] and hydrops fetalis has also been reported. [197] Although no specific therapy is available, it has been suggested that splenectomy may provide a moderate benefit. [198]

Hexokinase Deficiency

Hexokinase catalyzes the initial step of glycolysis and produces glucose 6-phosphate, which is utilized in both glycolysis and the pentose pathway (see Fig. 32-1). Fewer than 50 cases have been reported. Most are of individuals of northern European extraction, [199] although one case was found in a Chinese individual. [200] Since the metabolic defect is proximal to the generation of 2,3-BPG, it has been suggested that the resultant decreased level of 2,3-BPG leads to a left-shifted hemoglobin-oxygen dissociation curve and a more symptomatic anemia than comparable levels of anemia seen in other red cell disorders. [201] Some families have transfusion dependent hemolysis; it has been reported that splenectomy results in partial amelioration of hemolysis. Postsplenectomy, most patients required no transfusions; however, the hemolysis continued in all reported cases.

Phosphofructokinase and Aldolase Deficiencies

Deficiencies of phosphofructokinase and aldolase are rare causes of glycogen storage disease and chronic nonspherocytic hemolytic anemia. As in other rare autosomal recessive disorders, consanguinity is frequent.

Phosphoglycerokinase Deficiency

Deficiency of this X-chromosome encoded enzyme is associated with hemolytic anemia, mental retardation, and myoglobinuria. The cDNA for the phosphoglycerokinase gene has been cloned [202] and several mutants described.

Triosophosphate Isomerase Deficiency

Triosophosphate isomerase deficiency is a rare red cell defect reported to cause congenital hemolytic anemia and other severe organ dysfunctions, including progressive neurological abnormalities and early death. [203] An increased susceptibility to bacterial infections has also been described. [204] No specific therapy for this disorder is available. In a single reported case, splenectomy did not ameliorate hemolysis.

Pyrimidine 5 Nucleotidase Deficiency

Pyrimidine 5 nucleotidase participates in RNA degradation in reticulocytes. The accumulation of pyrimidines in the red cells is presumed to be toxic and a cause of hemolysis. Pyrimidine 5 nucleotidase deficiency is the only congenital hemolytic anemia due to a red cell enzyme deficiency that has a specific, consistent morphological abnormalitybasophilic stippling. [205] Thus, morphologic examination of the peripheral blood smear provides simple and inexpensive screening. Basophilic stippling is also found in hemolytic anemia due to acute lead toxicity. Lead is a powerful inhibitor of pyrimidine 5 nucleotidase [206] and determination of lead levels should be included whenever the constellation of hemolytic anemia, pyrimidine 5 nucleotidase deficiency, and basophilic stippling is found. Lead-induced acquired pyrimidine 5 nucleotidase deficiency is treatable, unlike the congenital deficiency for which no therapy is available.

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POLYCYTHEMIA DUE TO CONGENITAL RED CELL ENZYME DEFICIENCY

The polycythemias (also known as erythrocytosis) comprise a group of etiologically different disorders. [207] Absolute polycythemias may be primarily due to an intrinsic defect of hematopoietic progenitors or, more commonly, secondary due to stimulation of normal hematopoietic progenitors by extrinsic factors such as erythropoietin. While most polycythemias are acquired, some are congenital. [208] One rare cause of congenital secondary polycythemia is familial 2,3-bisphosphoglycerate (2,3-BPG, previously known as 2,3-DPG) deficiency, which results from a deficiency of the red cell enzyme bisphosphoglyceromutase (BPGM). Present in a very high concentration in red blood cells, 2,3-BPG binds hemoglobin, allosterically changing hemoglobins conformation and modulating its ability to bind oxygen. Thus, a decreased 2,3-BPG level shifts the hemoglobin oxygen dissociation curve to the left (increases hemoglobin affinity for oxygen), resulting in decreased delivery of oxygen into the peripheral tissues and compensatory polycythemia.

Biochemistry

The formation of 2,3-BPG constitutes the Rapoport-Luebering shunt, [209] which is often called an energy switch because it bypasses the formation of ATP in the reaction mediated by the glycolytic enzyme phosphoglycerate kinase (Fig. 32-7). A single multifunctional enzyme, bisphosphoglyceromutase (BPGM), with both synthase and phosphatase activity controls this shunt. BPGM is a homodimer of two identical 258-amino-acid subunits. The BPGM gene has been mapped to 7q34-7q22; transcripts of the gene have been detected in higher levels in erythroid cells than in nonerythroid cells, although no erythroid-specific promoter region has been identified. [210]

The conversion of 1,3-bisphosphoglycerate to 2,3-BPG is catalyzed by the synthase activity of BPGM. 2,3-BPG is then metabolized to 3-phosphoglycerate (3-PGA), an intermediate of the glycolytic pathway, by BPGM-phosphatase activity residing in the same protein molecule. [211] [212] [213] [214] [215] The synthase activity of BPGM is activated by alkalosis, while the phophatase activity of BPGM is enhanced by acidosis. [216]

Figure 32-7 Rapoport-Luebering shunt. 2,3-BPG metabolism is regulated by a multifunctional enzyme, bisphosphoglycerate mutase, with both synthase (BPG synthase) and phosphatase (BPG phosphatase) activity. This reaction bypasses the formation of ATP in the glycolytic pathway. G-3-P, Glyceraldehyde-3-phosphate; 1,3-BPG, 1,3-Bisphosphoglycerate; 3-PG, 3-Phosphoglycerate; 2,3-BPG, 2,3-Bisphosphoglycerate; Pi, Inorganic phosphate.

Clinical Manifestations and Inheritance

Deficiency of BPGM leads to a marked decrease in 2,3-BPG levels. The resultant increased hemoglobin oxygen affinity decreases the amount of oxygen released peripherally, leading to a compensatory polycythemia. One family with this deficiency was comprehensively studied; the proband with polycythemia had severely decreased enzyme activity and 2,3-BPG levels while his children had partially reduced enzyme activity and mildly decreased 2,3-BPG levels reflected in a decreased P_5 but were not polycythemic. [217] These findings suggested an autosomal recessive disease. When the family was restudied, the propositus and two of his sisters had complete deficiency of the synthase activity of BPGM and an affected sisters child had partially decreased enzyme activity. [218] One of the sisters and the propositus were more recently found to be compound heterozygotes for two different BPGM mutations. [219] [220] The heterozygous progeny of the enzyme-deficient subjects were subsequently found to have a moderate polycythemia, [218] [220] and similar observations of polycythemia in heterozygous subjects have been reported by others. [79] [221]

Other reports [221] [222] [223] [224] [225] of reduced BPGM activity and decreased 2,3-BPG levels are difficult to interpret or do not pass rigorous biochemical and molecular biologic scrutiny. In some of these reports, the phenotype included hemolysis; however, other causes of hemolysis were not definitively excluded.

Because of the rarity of complete BPGM deficiency, it is not possible to predict the phenotype of heterozygous individuals; however, it is likely that many individuals heterozygous for this enzyme deficiency will have a mild to moderate polycythemia and in these families an apparent autosomal dominant pattern of inheritance may be observed. [79] [218] [220] [221]

Diagnosis and Therapy

The determination of hemoglobin oxygen association kinetics is the best initial-screening laboratory test for congenital secondary polycythemia. If a co-oximeter is not available, the P_{50} can be estimated from a venous blood gas measurement by a simple mathematical formula, [226] providing that both the venous PaO_2 and the hemoglobin oxygen saturation are measured. A decreased P_{50} usually results from a mutant hemoglobin. Once a hemoglobin mutant has been excluded, a biochemical assay of freshly obtained erythrocytes may be used to detect decreased erythrocyte 2,3-BPG. [110] If 2,3-BPG deficiency is found, it should be followed by an assay of BPGM activity and, if desired, analysis for the gene mutation. Because inheritance of this defect may in some families be autosomal recessive, a family history of this rare deficiency may not be present and, as in all rare recessive disorders, consanguinity should be suspected.

Only limited clinical experience with this disorder is available; the polycythemia would be expected to compensate fully for the decreased tissue oxygen delivery and no significant health impairment would be foreseen. However, a superimposed anemia, such as that caused by blood loss or nutritional deficiency, would be expected to be more symptomatic than the same level of anemia seen in subjects with normal enzyme activity and normal levels of 2,3-BPG.

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DIAGNOSTIC AND OTHER USES OF RED CELL ENZYMOPATHIES

Deficiencies of some erythrocyte enzymes, such as LDH or catalase, have no discernible phenotypes. Other red cell enzymopathies do not cause abnormalities of the erythrocyte, but easily obtainable red cells serve as a convenient source of material for diagnosing systemic diseases. The red cell is capable of metabolizing galactose, but abnormalities of galactose metabolism do not have any hematological phenotype. Galactose kinase deficiency is associated with cataracts, and galactose uridinyl-1-transferase is associated with classic galactosemia (i.e., cataracts, congenital deafness, and mental retardation). Adenosine deaminase deficiency is associated with profound immunological disturbance, but no red cell abnormalities. Measurement of adenosine deaminase in erythrocytes is a convenient diagnostic tool. The activity of erythrocyte glutathione reductase, which requires the riboflavin-derived co-enzyme-FAD, can be measured with and without FAD, serving as a convenient indicator of riboflavin deficiency.

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Chapter 33 - Red Cell Membrane Disorders

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INTRODUCTION

Progress in the characterization of the structure and function of red cell membrane proteins and their genes (Table 33-1) has led to considerable advances in the understanding of the molecular pathology of red cell membrane disorders. This, in turn, has led to the definition and characterization of mutations of membrane proteins as a well-defined cause of hereditary hemolytic disease. Likewise, knowledge of the molecular mechanisms underlying changes in red cell deformability, structural integrity, and shape has advanced. Importantly, red cell shape abnormalities often provide a clue to the pathobiology and diagnosis of the underlying disorder. Consequently, this chapter categorizes red cell membrane disorders according to the following morphologic and clinical phenotypes: (1) hereditary spherocytosis (HS); (2) hereditary elliptocytosis (HE), hereditary pyropoikilocytosis (HPP), and related disorders; (3) Southeast Asian ovalocytosis (SAO); (4) hereditary and acquired acanthocytosis; and (5) hereditary and acquired stomatocytosis. Some hypotheses attempting to explain the molecular basis of these shape abnormalities are outlined in the following sections. The structure and function of the normal red cell membrane are described in Chapter 4 ; a review of that chapter will facilitate understanding the material in this chapter.

Vertical and Horizontal Interactions of Membrane Proteins and Disorders of Red Cell Shape

Palek and colleagues[1] [2] first proposed dividing membrane proteinprotein and proteinlipid interactions into two categories, vertical and horizontal interactions. Vertical interactions, which are perpendicular to the plane of the membrane, stabilize the lipid bilayer membrane. These interactions include spectrin-ankyrin-band 3 interactions, the protein 4.1-glycophorin C linkage, and the weak interactions between the skeletal proteins and the negatively charged lipids of the inner half of the membrane lipid bilayer. Horizontal interactions, which are parallel to the plane of the membrane, support the structural integrity of erythrocytes after their exposure to shear stress. Horizontal interactions involve the spectrin heterodimer site, where spectrin heterodimers assemble into tetramers, the principal building blocks of the membrane skeleton, and the contacts of the distal ends of spectrin heterodimers with actin and protein 4.1 within the junctional complex (Fig. 33-1). The interplay between proteins of the erythrocyte membrane is certainly more complex than can be classified by this model of horizontal and vertical interactions. [3] [4] However, the model serves as a useful starting place for understanding erythrocyte membrane protein interactions, particularly in reference to membrane-related disorders.

According to the vertical/horizontal model, HS is considered a disorder of vertical interactions. Although the primary molecular defects in HS are heterogeneous (including deficiencies or dysfunctions of - and -spectrin, ankyrin, band 3, and protein 4.2; see following), one common feature of HS red cells is a weakening of the vertical contacts between the skeleton and the overlying lipid bilayer membrane together with its integral proteins. Consequently, the lipid bilayer membrane is destabilized, leading to release of bilayer lipids from the cells in the form of skeleton-free lipid vesicles. [5] [6] This lipid loss, in turn, results in membrane surface area deficiency and spherocytosis.

In most patients with HE or the closely related disorder, HPP, a severe hemolytic anemia with marked microspherocytosis, poikilocytosis and fragmentation, the principal lesion involves horizontal membrane protein associations, primarily spectrin dimer-dimer interactions. In a smaller subset of HE patients with a deficiency or a dysfunction of protein 4.1 or glycophorin C, the horizontal defect resides in the junctional complex, where the distal ends of spectrin tetramers connect to

TABLE 33-1 -- Major Red Cell Membrane Proteins and Their Involvement in Hereditary Hemolytic Anemias

Band	Protein	MW (gel) (kd)	MW (calc) (kd)	Copies per Cell (10^3)	Total (%)	Gene Symbol	Chromosomal Localization	Amino Acids	Gene Size (kb)	No. of Exons	Involvement in Hemolytic Anemias
1	-Spectrin	240	280	240	16	SPTA1	1q22q23	2,429	80	52	HE, HS
2	-Spectrin	220	246	240	14	SPTB	14q23q24.2	2,137	>100	32	HE, HS
2.1	Ankyrin	210	206	120	405	ANK1	8p11.2	1,881	>100	40	HS
2.9	-Adducin	103	81	30	2	ADDA	4p16.3	737	85	16	N
2.9	-Adducin	97	80	30	2	ADDB	2p132p14	726	100	17	N
3	Anion exchanger 1	90100	102	1,200	27	EPB3	17q21qter	911	17	20	HS, SAO, HAc
4.1	Protein 4.1	80	66	200	5	EL11	1p33p34.2	588	>100	23	HE
4.2	Pallidin	72	77	200	5	EB42	15q15q21	691	20	13	HS
4.9	Dematin	48 + 52	43	140	1		8p21.1	383			N
4.9	p55	55	53	80		MPP1	Xq28	466			N
5	-Actin	43	42	400500	5.5	ACTB	7pterq22	375	4	6	N
5	Tropomodulin	43	41	30		TMOD	9q22	359			N
6	G3PD	35	37	500	3.5	GAPD	12p13.31p13.1	335	5	9	N
7	Stomatin	31	32		2.5	EPB72	9q33q34	288	12	7	HSt
7	Tropomyosin	27 + 29	28	80	1	TPM3	1q31	239			N

PAS-1	Glycophorin A	36		5001,000	85	GYPA	4q28q31	131	40	7	HE
PAS-2	Glycophorin C	32	14	50100	4	GYPC	2q14q21	128	14	4	HE
PAS-3	Glycophorin B	20		100300	10	GYPB	4q28q31	72	>30	5	N
Glycophorin D	23		20	1	GYPC	2q14q21	107	14	4	N	
Glycophorin E					GYPE	4q28q31	59	>30	4	N	

Abbreviations: HE, hereditary elliptocytosis; HS, hereditary spherocytosis; N, no hematologic abnormalities reported; SAO, Southeast Asian ovalocytosis; HAc, hereditary acanthocytosis; HSt, hereditary stomatocytosis; G3PD, glyceraldehyde-3-phosphate dehydrogenase.

Data from Palek and Jarolim.[1]

Figure 33-1 (Top right) Vertical and horizontal interactions of membrane proteins and the pathobiology of the red cell lesion in hereditary spherocytosis and ellipto-poikilocytosis. **(Left)** A defect of vertical interactions as exemplified by the red cell membrane lesion in HS. Partial deficiencies of spectrin, ankyrin, or band 3 protein lead to uncoupling of the membrane lipid bilayer from the underlying skeleton (arrow), followed by a formation of spectrin-free microvesicles of about 0.20.5 mm in diameter (arrowheads). These vesicles can be visualized by transmission electron microscopy, but they are not seen on examination of a peripheral blood film. The subsequent loss of cell surface area and a decrease in the surface/volume ratio lead to spherocytosis. For further details, see Figure 33-3 . **(Bottom right)** Defect of horizontal interactions of skeletal proteins as exemplified by the membrane lesion in hemolytic forms of HS associated with a defect of spectrin heterodimer self-association. The molecular lesion involving a weakened self-association of spectrin heterodimers to tetramers (SpT) represents a horizontal defect of the shear stress-resisting protein interactions. It leads to a disruption of the membrane skeletal lattice and, consequently, to destabilization of the whole cell, followed by cell fragmentation, as seen on peripheral blood films. *(Modified from Palek and Jarolim,[1] with permission.)*

actin, with the aid of the 4.1 protein. In patients with severely dysfunctional spectrin mutations, the weakened spectrin dimer-dimer self-association disrupts the skeletal lattice, leading to a marked skeletal instability and cell fragments. In patients with mildly dysfunctional spectrins, red cell shape is that of biconcave elliptocytes. It is speculated that elliptocytes are permanently deformed cells because the weakened horizontal interactions facilitate a shear stress-induced rearrangement of skeletal proteins, precluding recovery of the normal biconcave shape (for details, see the discussion under Hereditary Elliptocytosis and Related Disorders). This hypothesis is not applicable to all forms of elliptocytosis. For example, in SAO, the elliptocytic/ovalocytic cells containing mutant band 3 protein are rigid and hyperstable rather than unstable. Further details are discussed in the following sections.

Acanthocytosis, Stomatocytosis, and the Bilayer Couple Hypothesis

The mechanism of acanthocytosis and stomatocytosis associated with defects of membrane proteins is much less clear. Most forms of acanthocytosis are associated with either acquired or inherited abnormalities of membrane lipids (e.g., acanthocytosis in end-stage liver disease or abetalipoproteinemia). In rare subjects with acanthocytosis, membrane protein abnormalities have been detected, but the mechanism whereby they lead to acanthocyte formation is unknown. These abnormalities occur in the McLeod phenotype, the chorea-acanthocytosis syndromes, and rare examples of acanthocytosis associated with abnormalities of the band 3 protein. In all forms of acanthocytosis studied to date, the acanthocytic shape is normalized by agents that interact with the lipids of the inner lipid bilayer leaflet. [7] These studies suggest that the shape abnormalities reflect an asymmetry in the distribution of membrane lipids between the two halves of the red cell lipid bilayer as predicted by the bilayer couple hypothesis (Fig. 33-2).[8] [9] [10] [11] This hypothesis is based on the well-established idea that the lipid bilayer is

Figure 33-2 Bilayer couple hypothesis and the discocyte-echinocyte-stomatocyte transformation. Red cell shape is determined by the ratio of the surface areas of the two hemileaflets of the lipid bilayer. Compounds (black triangles) that preferentially accumulate in the outer hemileaflet produce its expansion, followed by red cell crenation (echinocytosis or acanthocytosis). In contrast, expansion of the inner lipid bilayer leaflet leads to a cup shape (stomatocytosis) and surface invaginations. AB

highly asymmetric, with phosphatidylcholine and sphingomyelin the main phospholipids of the outer face of the bilayer leaflet. [12] According to the bilayer hypothesis, red cell shape reflects the ratio of the surface areas of the two hemileaflets of the bilayer. The preferential expansion of the outer leaflet leads to red cell crenation (echinocytosis or acanthocytosis), while expansion of the inner lipid bilayer produces a cup shape (stomatocytosis) and surface invaginations.

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HEREDITARY SPHEROCYTOSIS

Definition, Prevalence, and History

The typical diagnostic features of HS include a dominantly inherited hemolytic anemia of mild to moderate severity, spherocytosis on the peripheral blood film, and a favorable response to splenectomy. However, the clinical spectrum of HS is variable [13] [14] [15] [16] and includes both mild and asymptomatic forms that may first be diagnosed late in life [17] as well as severe forms that are often inherited in a nondominant fashion. [18] [19] [20] [21] [22] The previously reported HS prevalence in Western populations of 1 in 5,000 persons [23] may be an underestimation, because milder forms of HS may be asymptomatic. [24] [25] HS has also been reported in Japanese and African populations, [26] [27] [28] [29] but its prevalence in these ethnic groups is unknown.

HS was first described in 1871 by Vanlair and Masius [30] and rediscovered 20 years later by Wilson [31] and Minkowski. [32] The next major contributions were (1) Chauffards description of increased osmotic fragility, [33] (2) reports of correction of hemolysis by splenectomy, [34] [35] and (3) the studies by Ham and Castle [36] implicating the spleen in the conditioning of hereditary spherocytes. Soon thereafter, HS membranes were found to be leaky to sodium [37] [38] and to exhibit a loss of lipids, leading to surface area deficiency. [39] [40] An abnormality of membrane skeleton was suspected; [41] subsequently several distinct molecular defects of both skeletal and transmembrane proteins have been identified (Table 33-2). [42] [43] [44] [45] [46] [47] [48] [49] [50] [51] [52] [53] [54] [55] [56] [57] [58] [59] [60] [61] [62] [63] [64] [65] [66] [67] [68] [69] [70] [71] [72] [73] [74] [75] [76] [77] [78] [79] [80]

Etiology and Pathobiology

Two major factors are involved in HS pathophysiology: (1) an intracorpuscular red cell defect, and (2) an intact spleen that selectively retains the intrinsically abnormal HS cells. [13] [81] [82] The accelerated red cell destruction in HS is now recognized to be a multistep process resulting from an inherited deficiency or dysfunction of one of the proteins of the erythrocyte membrane. The ensuing destabilization of the lipid bilayer facilitates a release of lipids from the membrane, leading to surface area deficiency and formation of poorly deformable spherocytes that are selectively retained and damaged in the spleen (Fig. 33-3).

Molecular Pathology

The molecular basis of HS is heterogeneous (Table 33-2). Based on a densitometric quantitation of membrane proteins separated by polyacrylamide gel electrophoresis (PAGE), HS can be divided into the following subsets: (1) isolated partial deficiency of spectrin, (2) combined partial deficiency of spectrin and ankyrin, (3) partial deficiency of band 3 protein, (4) deficiency of protein 4.2, and (5) other less common defects. [83]

Isolated Partial Spectrin Deficiency

The reported mutations of isolated spectrin deficiency include defects of both - and -spectrin. Mutations of the -spectrin gene have been identified in a number of patients with dominantly inherited HS associated with spectrin deficiency (Table 33-2). With one exception, -spectrin ^{Houston} , a frameshift mutation identified in several kindreds, [45] these mutations are private,

TABLE 33-2 -- Mutations Associated with Hereditary Spherocytosis

Variant	Mutation	Protein	Gene	Reference
-Spectrin				
Prague	Splicing	1446frameshift	1, AC (ivs 30)	[42]
LEPRA	Splicing	1729frameshift	99, CT (ivs 36)	[42]
-Spectrin				
Promissão	Missense/translation	1 MetVal	ATGGTG	[43]
Guemene-Penfao	Splicing	100frameshift	1, GC (ivs 3)	[44]
Atlanta	Missense	182 TrpGly	TGGGGG	[45]
Ostrava	Deletion	202frameshift	T	[45]
Kissimmee	Missense	202 TrpArg	TGGCGG	[46]
Oakland	Missense	220 IleVal	ATCGTC	[45]
Philadelphia	Insertion	589frameshift	+A	[45]
St. Barbara		638frameshift		[47]
Bergen	Insertion	783frameshift	+A	[45]
Baltimore	Nonsense	845 Glnstop	CAGTAG	[45]
Winston-Salem	Splicing	935frameshift	+1, GA (ivs 17)	[48]
Columbus	Missense	1227 ProSer	CCTTCT	[45]
Durham	Deletion	14921614 123 AA deletion	Exons 22 and 23	[49]
Birmingham	Missense	1684 ArgCys	CGCTGC	[45]
Sao Paulo	Missense	1884 AVal		[47]
Tabor	Nonsense	1946 Glnstop	CAGTAG	[45]

Ankyrin				
Unnamed	Missense	5 untranslated	CG 204	[50]
Unnamed	Missense	5 untranslated	T C 108	[50]
Unnamed	Deletion	5 untranslated	TG 72/ 73	[50]
Bugey	Deletion	146frameshift	C	[51]
Osterholz	Deletion	173frameshift	20 nt deletion	[50]
Stuttgart	Deletion	329frameshift	GC	[50]
Bari	Deletion	426frameshift	G	[52]
Walsrode	Missense	463 ValIle	GTCATC	[50]
Florianopolis	Insertion	506frameshift	+C	[53]
Laguna	Deletion	535frameshift	A	[53]
Napoli	Deletion	573frameshift	T	[54]
Einbeck	Insertion	573frameshift	+C	[50]
Unnamed	Deletion	596frameshift	C	[55]
Unnamed	Nonsense	631 Glustop	GAGTAG	[56]
Unnamed	Nonsense	765 Serstop	TCGTAG	[56]
Marburg	Deletion	797798frameshift	TAGT	[50]
Unnamed	Deletion	907frameshift	G	[56]
Napoli II	Deletion	933frameshift	C	[52]
Anzio	Deletion	983frameshift	CA	[52]
Nara	Missense	1046 LeuPro		[57]
Unnamed	Nonsense	1053 Argstop	CGATGA	[56]
Tubarao	Missense	1075 IleThr	ATCACC	[53]
Porta Westfalica	Deletion	1127frameshift	C	[50]
Bovendem	Nonsense	1436 Argstop	CGATGA	[50]
Unnamed	Nonsense	1488 Argstop	CGATGA	[56]
Prague	Insertion	15121513 67AA insertion	201 nt insertion	
Dusseldorf	Missense	1592 AspAsn	GACAAC	[50]
Saint-Etienne 1	Nonsense	1721 Trpstop	TGGTGA	[58]
Saint-Etienne 2	Nonsense	1833 Argstop	CGATGA	[58]
Bocholt	Substitution	1879 ArgTrp	CGGTGG	[50]
Rakovnik	Nonsense	1669 Glustop	GAATAA	[59]
Duisburg	Splicing	ivs 16	CA	[50]
Unnamed	Missense	ivs 38	CT	[50]
Band 3				
Montefiore	Missense	40 GluLys	GAGAAG	[60]
Foggia	Deletion	5455frameshift	C	[61]
Kagoshima	Deletion	56frameshift	A	[57]
Hodonin	Nonsense	81 TrpStop	TGGTGA	[62]
Napoli I	Insertion	99100frameshift	+T	[61]
Fukayama I	Deletion	112113frameshift	AC	[57]
Nachod	Splicing	117121 GTVLL deleted	3, CA (ivs 5)	[62]
Fukuoka	Missense	130 GlyArg	GGAAGA	[57]
Osnabruck I, Lyon	Nonsense	150 Argstop	CGATGA	[50] [63]
Worcester	Insertion	170172frameshift	+G	[62]
Fukuyama II	Insertion	183frameshift	+A	[57]
Campinas	Splicing	203frameshift	+1, GT (ivs 8)	[64]
Bohain	Deletion	241frameshift	T	[65]
Princeton	Insertion	273275frameshift	+C	[62]
Boston	Missense	285 AlaAsp	GCTGAT	[62]
Tuscaloosa	Missense	327 ProArg	CCCCGC	[66]
Noirterre	Nonsense	330 Glnstop	CAGTAG	[67]
Bruggen	Deletion	419frameshift	C	[50]
Benesov	Missense	455 GlyGlu	GGGGAG	[62]
Bicêtre II	Deletion	456frameshift	G	[65]
Pribram	Splicing	477frameshift	1, GA (ivs 12)	[62]
Coimbra	Missense	488 ValMet	GTGATG	[68]
Bicêtre I	Missense	490 ArgCys	CGCTGC	[65]
Evry	Deletion	492frameshift	T	[65]
Milano	Duplication	498 23AA insertion	69bp duplication	[69]
Dresden	Missense	518 ArgCys	CGCTGC	[50]
Smichov	Deletion	616frameshift	C	[62]

Trutnov	Nonsense	628 Tyrstop	TACTAA	[62]
Hobart	Deletion	646647frameshift	G	[62]
Osnabruck II	Deletion	663664frameshift	ATG	[50]
Most	Missense	707 LeuPro	CTGCCG	[62]
Okinawa	Missense	714 GlyArg	GGGAGG	[57]
Prague II	Missense	760 ArgGln	CGGCAG	[70]
Hradec Kralove	Missense	760 ArgTrp	CGGTGG	[70]
Chur	Missense	771 GlyAsp	GGCGAC	[71]
Napoli II	Missense	783 IleAsn	ATCAAC	[61]
Jablonec	Missense	808 ArgCys	CGCTGC	[70]
Nara	Missense	808 ArgHis	CGCCAC	[57]
Prague I	Insertion	822frameshift	+CACCCAGATC	[72]
Birmingham	Missense	834 HisPro	CACCCC	[62]
Philadelphia	Missense	837 ThrMet	ACGATG	[62]
Prague III	Missense	870 ArgTrp	CGGTGG	[72]
Vesuvio	Deletion	984frameshift	C	[73]
Protein 4.2				
Lisboa	Deletion	89frameshift	C	[74]
Fukuoka	Missense	119 Trpstop	TGGTGA	[75]
Nippon	Missense	142 AlaThr	GCTACT	[76]
Komatsu	Missense	175 AspTyr	GATTAT	[77]
Notame	Splicing	308frameshift	+1, GA (ivs 6)	[78]
Tozeur	Missense	310 ArgGln	CGACAA	[79]
Shiga	Missense	317 ArgCys	CGCTGC	[80]

Figure 33-3 Pathobiology of the lesion in hereditary spherocytosis. The principal abnormality in hereditary spherocytosis (HS) is the deficiency of membrane surface area leading spherocytic erythrocytes. Two distinct pathways lead to reduced membrane surface area. **(A)** Primary defects of spectrin, ankyrin or protein 4.2 lead to reduced density of the membrane skeleton (solid rectangles). This causes destabilization of the lipid bilayer with the resultant loss of band 3-containing microvesicles. **(B)** Primary defects of band 3 (black ovals) result in band 3 deficiency. This leads to the loss of the lipid-stabilizing effect of band 3 with the release of band 3-free microvesicles from the membrane. Both pathways ultimately result in the loss of membrane material with a reduction in membrane surface area. The ensuing decrease in membrane surface area with formation of spherocytes is paralleled by a decrease in erythrocyte deformability that predisposes the cells to splenic entrapment and conditioning. The spleen plays a secondary, but important, role in the pathobiology of HS. Splenic destruction of abnormal erythrocytes with decreased deformability is the primary cause of hemolysis experienced by HS patients.

i.e., they are unique to individual kindreds, and they may be associated with decreased -spectrin mRNA accumulation. -Spectrin ^{Kissimmee}, a point mutation located in the highly conserved region of -spectrin involved in the interaction with protein 4.1, is dysfunctional in its in vitro binding to protein 4.1 and thereby the linkage of spectrin to actin.[46] [84] However, correction of the binding defect by reducing agents suggests that in circulating red cells, which are rich in reduced glutathione, this spectrin/4.1 protein-binding defect is not functionally expressed. [85]

In nondominantly inherited HS associated with isolated spectrin deficiency, the reported defects involve -spectrin. In normal erythroid cells, -spectrin is synthesized in large excess of -spectrin.[86] Thus, subjects with one normal and one defective -spectrin allele are expected to be asymptomatic, because -spectrin production remains in excess of -spectrin synthesis, allowing normal amounts of spectrin heterodimers to be assembled on the membrane. [87] Patients who are homozygotes or compound heterozygotes for -spectrin defects will suffer from severe HS. Wichterle et al. described a patient with severe HS who was a compound heterozygote for two different -spectrin gene defects: in one allele, there was a splicing defect associated with an upstream intronic mutation, ^{LEPRA}; in the other allele there was another mutation, ^{PRAGUE}.[42] The ^{LEPRA} allele produces approximately six times less of the correctly spliced -spectrin transcript than the normal allele. Further studies have shown that in many patients with nondominant, spectrin-deficient HS, including a number of the patients originally described by Agre et al., [19] [20] [22] ^{LEPRA} is in linkage dysequilibrium with $^{Bug\ Hill}$, an amino acid substitution in the II domain.[88] [89] Thus it appears that the combination of the ^{LEPRA} allele with other defects of -spectrin in trans leads to significant spectrin-deficient spherocytic anemia.

In a report of a lethal and near lethal HS associated with dramatic (26% of normal) spectrin deficiency, pulse labeling studies of burst-forming uniterythroid-derived erythroblasts revealed a marked decrease in -spectrin synthesis.[90] Although the underlying molecular basis of this defect is unknown, a family history of a mild dominantly inherited HS in the mother and the finding of slightly increased osmotic fragility in the hematologically normal father suggest that the proband inherited at least two genetic defects that in a simple heterozygote have either minimal or no adverse consequences.

Combined Partial Deficiency of Spectrin and Ankyrin

The biochemical phenotype of combined partial deficiency of spectrin and ankyrin, originally described by Coetzer et al., [21] is the most common abnormality found in the erythrocytes of HS patients.[91] [92] [93] Ankyrin represents the principal binding site for spectrin on the membrane; thus it is not surprising that ankyrin deficiency is accompanied by a proportional decrease in spectrin assembly on the membrane despite normal spectrin synthesis. [92] Typically, like HS associated with -spectrin mutations, most ankyrin defects are private point mutations that are frequently associated with decreased mRNA accumulation (Table 33-2). One frameshift mutation associated with severe HS, ankyrin ^{Florianopolis}, is an exception; it has been identified in HS patients from three different kindreds from different genetic backgrounds. [53] Approximately 1520% of ankyrin gene mutations reported are de novo mutations. Varying clinical severity among affected members of the same HS kindred has been reported, resulting from parental mosaicism for ankyrin mutations in two families. [55]

A number of patients with atypical HS associated with karyotypic abnormalities involving deletions or translocations of the ankyrin gene locus have been described. In one patient, the underlying molecular defect involved an absence of the entire ankyrin gene on chromosome 8 due to a large interstitial deletion. [94] Ankyrin deletions may be part of a contiguous gene syndrome with the clinical manifestations of spherocytosis, mental retardation, typical facies, and hypogonadism.

Partial Deficiency of Band 3 Protein

Partial deficiency of band 3 protein is found in a subset of HS patients who present with a phenotype of a mild to moderate dominantly inherited HS with mushroom-shaped or pincered red cells.[83] [95] Most, if not all, of these patients also have concomitant protein 4.2 deficiency. Nearly 50 different band 3 mutations associated with HS have been reported (Table 33-2). These mutations are spread throughout band 3, occurring in both the cytoplasmic domain as well as in the

membrane spanning domain.[72] Lethal and near lethal HS associated with fetal hydrops fetalis, metabolic acidosis, and severe anemia with complete deficiency of band 3 and protein 4.2 from erythrocyte membranes have been reported in members of a kindred homozygous for a band 3 mutation, band 3 ^{Coimbra} .[96] Alleles have been identified that influence band 3 expression and that, when inherited in trans to a band 3 mutation, aggravate band 3 deficiency and worsen the clinical severity of disease.[63] [65]

In some band 3-deficient HS subjects, the deficiency is considerably greater in the dense reticulocyte-poor cells than in the light reticulocyte-rich cells. This finding, in conjunction with the report of normal band 3 synthesis and normal mRNA levels in one patient, is consistent with the idea that in some patients the band 3 protein is unstable and is susceptible to accelerated release from the cells. The mechanism of such accelerated band 3 loss is unknown, and the hypothetical molecular mechanisms include a weakened binding of band 3 protein to ankyrin as a result of a primary defect of either band 3 protein or ankyrin or as a result of a defective assembly of band 3 on the membrane. In some band 3-deficient HS patients, band 3 gene expression may be reduced or a band 3 mutation may interfere with the proper co-translational insertion of band 3 into membranes of the endoplasmic reticulum or translocation of band 3 to the plasma membrane.

A number of band 3 mutations clustered in the membrane spanning domain that replace highly conserved arginines have been described. [70] These arginines, which are all located at the cytoplasmic end of a predicted transmembrane helix, are thought to aid in maintaining the orientation of transmembrane spanning segments. In these cases, the hypothesis that the mutant band 3 does not fold and insert into the endoplasmic reticulum and ultimately into the erythrocyte membrane after synthesis appears likely.

Finally, three HS kindreds with rapidly migrating band 3 as studied by SDS-PAGE, due to a post-translational defect of band 3 glycosylation, have been described. [97] There was also a more rapidly migrating glycophorin A, which is known to be involved in band 3 glycosylation, in gels of erythrocyte membranes from these patients. The precise molecular defect(s) and its role in the pathogenesis in these patients is unknown.

Deficiency of Protein 4.2

Recessively inherited HS due to mutations in protein 4.2 is relatively common in Japan (Table 33-2).[26] [27] In these cases there is almost total absence of protein 4.2 from the erythrocyte membranes of affected homozygous patients. Protein 4.2-deficient erythrocytes may also be deficient in ankyrin and band 3 protein.

Several reports have identified protein 4.2 deficiency due to reduced membrane binding of protein 4.2 as a result of mutations in the cytoplasmic domain of band 3. [60] [66] Presumably, these mutations involve sites of protein 4.2band 3 interactions. In one report, there was partial band 3 deficiency and total protein 4.2 deficiency in the erythrocyte membranes of an HS patient who was a compound heterozygote for two different band

3 defects. Presumably, one mutant band 3 protein, band 3 ^{Okinawa} , binds all the available protein 4.2 in erythrocyte precursors because the other mutant band 3, band 3 ^{Fukuoka} , contains a mutation in a region of band 3protein 4.2 interaction. Because band 3 ^{Okinawa} cannot be inserted into the membrane, the band 3 ^{Okinawa} protein 4.2 complex is degraded, leading to the observed phenotype.

Molecular Basis of Surface Area Deficiency

Hereditary spherocytes are intrinsically unstable, releasing lipids under a variety of in vitro conditions, including adenosine triphosphate (ATP) depletion or exposure of cells to shear stress. The loss of membrane material occurs through the release of 0.2- to 0.5-m vesicles containing integral proteins devoid of spectrin. [5] [98] [99] During in vitro incubation, the loss of membrane material is of sufficient magnitude to augment the surface area deficiency, as evidenced by increased osmotic fragility of the cells after incubation.[40] [100] [101] It is generally assumed that a similar process takes place in vivo, but its experimental verification has yet to be provided.

Insofar as the molecular basis of HS is heterogeneous, it is likely that surface area deficiency is a consequence of several distinct molecular mechanisms that share a weakening of the vertical connections between the skeleton and the lipid bilayer membrane. Three distinct hypothetical pathways that may lead to surface area deficiency are depicted in Figure 33-3 . In patients with isolated spectrin deficiency or a combined deficiency of spectrin and ankyrin, the surface area deficiency may involve an uncoupling of the lipid bilayer membrane from the underlying skeleton (Fig. 33-3). In normal red cells, the skeleton forms a nearly monomolecular submembrane layer occupying more than one-half the inner surface of the membrane. Consequently, spectrin deficiency leads to a decreased density of this network (Fig. 33-4). As a result, areas of the lipid bilayer membrane that are not directly supported by the skeleton are susceptible to release from the cells in the form of microvesicles.

In HS associated with a deficiency of band 3 protein, two hypothetical pathways may lead to a loss of surface area (Fig. 33-3). One mechanism may involve a loss of band 3 protein from the cells.[95] Since band 3 protein spans the lipid bilayer membrane many times, it is likely that a substantial amount of boundary lipids are released together with the band 3 protein, thus leading to surface area deficiency. Another possible mechanism may involve a formation of band 3-free domains in the membrane, followed by the formation of membrane blebs, which are subsequently released from the cells as microvesicles. Such a hypothesis is based on the observation that aggregation of intramembrane particles (composed principally of the band 3 protein) in ghosts leads to the formation of particle-depleted domains from which membrane lipids bleb off as microvesicles. Additional evidence supporting the latter model comes from the band 3 knockout mouse model. [102] Erythrocytes lacking band 3 spontaneously shed membrane vesicles, leading to severe spherocytosis and hemolysis.

Alterations in Cation Content and Permeability

HS red cells, particularly those collected from the spleen, are somewhat dehydrated [103] [104] and abnormally permeable to monovalent cations,[105] [106] presumably as a consequence of the underlying skeletal defect. The cellular dehydration may be due to activation of pathways causing a selective loss of potassium and water, such as the K^+ , Cl co-transporter, which may be stimulated by the relatively low pH of the spleen or by oxidative damage, [107] [108] possibly in sites at which red cells are in contact with macrophages producing oxygen radicals. Furthermore, the Na^+ /K^+ pump that regulates the intracellular sodium and potassium content is hyperactive in HS cells, compensating for the increased sodium leak into the cells. Increased activity of this pump could lead to red cell dehydration, since for every two atoms of potassium transported inward, three sodium atoms are extruded from the cell. Protein 4.2-deficient HS erythrocytes have increased anion transport, whereas HS erythrocytes deficient in spectrin, ankyrin, or band 3 have normal or decreased anion transport. [106]

Entrapment of Nondeformable Spherocytes in the Spleen

The importance of the spleen in the pathophysiology of hemolysis in HS was appreciated in the original description of the disease

Figure 33-4 Ultrastructure of the membrane skeleton in normal red cells and in red cells from a patient with severe HS associated with a combined deficiency of spectrin and ankyrin. Hemoglobin-free ghosts were sonicated to open a window that allows us to see the intact skeleton at the inner membrane surface. The ghosts were fixed and negatively stained. The skeleton is preferentially stained under these conditions. **(A)** In ghosts from normal red cells, the individual skeletal proteins are in close apposition to each other and form a monomolecular layer. **(B)** In contrast, the HS skeleton is considerably less dense with the individual proteins separated by skeleton-free areas. *(Courtesy of S.C. Liu.)*

and has been substantiated by subsequent studies.[36] [81] Two factors participate in the selective destruction of the HS cell in the spleen: (1) poor deformability of HS red cells[109] [110] [111] and (2) the unique anatomy of the splenic vasculature, which acts as a microcirculation filter. [112] [113]

The poor red cell deformability is principally a consequence of a decreased cell surface/cell volume ratio resulting from the loss of surface material. [109] [114] In contrast to normal discocytes, which have an abundant surface, allowing the cells to deform and pass through narrow microcirculation openings, HS red cells lack this extra surface. Their poor deformability may be further impaired by cellular dehydration. [109] [115] [116] Interestingly, in contrast to the deformability of the intact cell, the

deformability of the HS red cell membrane is normal or even increased.[109] [117]

The principal sites of red cell entrapment in the spleen are fenestrations in the wall of splenic sinuses, where blood from the splenic cords of the red pulp enters the venous circulation.[112] [113] In the rat spleen, the length and width of these fenestrations (23 m and 0.20.5 m, respectively) are about one-half the red cell diameter. Electron micrographs of spleen specimens show that very few HS red cells traverse these slits. [118] [119] [120] Consequently, the nondeformable spherocytes accumulate in the red pulp, which becomes grossly engorged, as seen in anatomic sections of the spleen obtained after splenectomy. [118] [119] [120] [121] [122]

Splenic Conditioning and Destruction

Once trapped in the spleen, HS red cells undergo additional damage marked by further loss of surface area and an increase in cell density, as is evident in cells removed from the spleen at splenectomy.[36] [82] [124] [125] In part, these conditioned red cells re-enter the systemic circulation, as revealed by the tail of the osmotic fragility curve (see later), indicating the presence of a subpopulation of cells with a markedly reduced surface area. [82] [123] [124] [125] After splenectomy, this red cell population disappears.

Earlier studies attempted to simulate splenic conditioning of HS red cells by in vitro incubation. [121] The possible conditioning factors may include a relatively low pH in the spleen[126] [127] as well as in the sequestered red cells that may further compromise the poor HS red cell deformability, [126] [127] and a contact of red cells with macrophages that may inflict additional damage on the red cell membrane.[108] [128] [129] Glucose deprivation and the ensuing intracellular ATP depletion do not appear to be significant factors, because cells collected from the spleen at splenectomy have a normal ATP content.

The conditioning effect of the spleen appears to represent a cumulative injury. The average residence time of HS red cells in the splenic cords is between 10 and 100 minutes, and only 110% of blood entering the spleen is temporarily sequestered in the congested cords, while the remaining 90% of blood flow is rapidly shunted into the venous circulation.

Although most HS red cells are eventually sequestered and destroyed in the spleen, HS cells that have been most severely damaged in the spleen can be destroyed outside this organ as well. The HS red cell surface alterations that trigger red cell phagocytosis by tissue macrophages are unknown. One possible pathway may involve alterations in phospholipid distribution within the two leaflets of the lipid bilayer, leading to the exposure in the external hemileaflet of phosphatidylserine, which promotes red cell attachment to macrophages and phagocytosis in vitro. Although the distribution of phospholipids in the two lipid hemileaflets is normal in most HS patients, altered phospholipid distribution has been demonstrated in red cells from patients with severe forms of HS. It is possible, but not proved, that similar changes in phospholipid asymmetry take place in the end-stage HS cells that are repeatedly conditioned in the spleen. Unlike senescent normal red cells and some abnormal erythrocytes, HS red cells have normal amounts of surface-associated IgG. Thus, it is unlikely that the HS erythrocytes undergo surface alterations leading to an exposure of new antigenic sites on their surface (the senescent red cell antigen).

HS and Nonerythroid Manifestations

In most HS cases, the clinical manifestations are confined to the erythroid lineage, probably because many of the nonerythroid counterparts of the red cell membrane proteins (e.g., spectrin and ankyrin) are encoded by separate genes or because some proteins (e.g., protein 4.1, -spectrin, ankyrin) are subject to tissue-specific alternative splicing. However, there are notable exceptions. Several HS kindreds have been reported with a co-segregating neurologic or muscular abnormality such as a degenerative disorder of the spinal cord, cardiomyopathy, or mental retardation. The observation that both erythrocyte ankyrin and -spectrin are also expressed in muscle, brain, and spinal cord raises the possibility that these HS patients may have a defect in one of these proteins. [130] [131] [132] This hypothesis is further supported by studies of a mouse model of HS, the *nb* mutation. [133] [134] Homozygous *nb*/*nb* mice have severe HS with spectrin and ankyrin deficiencies attributable to a primary molecular defect of ankyrin. They develop a neurologic syndrome whose progression coincides with the degeneration of Purkinje cells in the cerebellum. Purkinje cells normally express erythroid ankyrin, which is absent in *nb*/*nb* mice.[134] [135]

Defects of band 3 have been described in patients with autosomal dominantly inherited distal renal tubular acidosis. [136] [137] Unlike in most patients with heterozygous mutations of band 3, who have normal renal acidification and abnormal erythrocytes, two band 3 mutations, R589H and S613F, have been associated with renal acidification defects and normal erythrocytes. In addition, two kindreds with co-inherited HS and renal acidification defects due to band 3 mRNA processing mutations, band $3^{Pribram}$ and band $3^{Campinas}$, [64] [138] have been described. In all of these cases, the precise pathogenesis of the renal tubular acidosis is unknown.

Inheritance

Because of the heterogeneous molecular basis of HS, the putative HS genes can be assigned to several chromosomes, including chromosome 1 (-spectrin), chromosome 8 (ankyrin), chromosome 14 (-spectrin), chromosome 17 (band 3 protein), and chromosome 15 (protein 4.2) (Table 33-1). In most HS patients (75%), inheritance is autosomal dominant.[13] [14] [15] In the remaining patients, inheritance is nondominant. In these patients, HS is due to a de novo mutation, [15] [51] [52] [54] [139] which tends to occur at CpG dinucleotides and is associated with small deletions or insertions. It may also be inherited in an autosomal recessive fashion. [20] [22]

A number of cases of recessively inherited HS manifesting with severe hemolytic anemia have been reported. The majority of the affected patients were found to be severely deficient in red cell spectrin, and the putative defect has been assigned to -spectrin. [140] [141] [142] The remaining cases characterized by a recessive inheritance pattern are due to a defect in protein 4.2. [74] [76] [80] This deficiency is associated with relatively mild hemolysis, and the red cell morphology reveals the presence of stomatocytes and ovalocytes, which are not seen in other patients with HS.

Only a few cases of homozygous HS have been reported. These patients have a severe hemolytic anemia, while their parents have a very mild form of the disease. [19] [20] In contrast, the homozygous state for the more typical hemolytic form of HS appears to be lethal. [96] In one such kindred, both consanguineous parents and three offspring (presumably HS heterozygotes) had moderately severe HS, one offspring was normal, and there

were two miscarriages, a frequency consistent with the predicted homozygosity for the HS gene.

Although the clinical severity of HS is highly variable among different kindreds, in general, it is relatively uniform within a given family, in which HS is typically inherited as an autosomal dominant disorder.[15] [23] However, HS has been identified in one or more siblings whose parents had no identifiable abnormalities or only slightly abnormal osmotic fragility after incubation. In addition, HS kindreds have been described in which there was great variability in the clinical severity of affected family members.[13] [14] [16] [143] [144] A number of explanations may account for these observations: (1) There may be variable penetrance of the genetic defect. (2) There may be a de novo mutation or possibly a mild form of recessively inherited HS in the kindred. (3) Modifier alleles that influence the expression of a membrane protein leading to the variability in clinical expression may exist in the family. (4) There may be tissue-specific mosaicism of the defect.

Clinical Manifestations

Typical Forms

A typical HS patient is relatively asymptomatic. As noted in the earliest descriptions of HS, mild jaundice may be the only symptom of the disease. Splenomegaly is present in most patients,[13] [16] with the spleen occasionally reaching large dimensions. Anemia, which is usually mild to moderate, may be absent in some cases because of compensatory bone marrow hyperplasia.

Mild Forms and HS Carrier State

In some patients, anemia is absent, the reticulocyte count is normal or only minimally elevated, laboratory evidence of hemolysis is minimal or absent, [13] [14] [15] and the changes in red cell shape may be mild, escaping detection on the peripheral blood film. The presence of HS is detected only by osmotic fragility testing or during evaluation of a relative with a more symptomatic form of the disease. Some patients are first diagnosed during transient infections such as infectious mononucleosis

or parvovirus infection,[145] [146] during pregnancy, or even in the seventh to ninth decades of life as the bone marrows ability to compensate for the hemolysis wanes.

Severe and Atypical Forms

The relatively uncommon patients with spectrin-deficient, nondominant forms of HS may present with a severe life-threatening hemolysis early in life. [18] [19] [20] [21] In one report the parents, who were distant cousins, were asymptomatic HS carriers and their offspring (presumed HS homozygotes) presented in infancy with a life-threatening hemolytic anemia that was only partially improved by splenectomy. Some patients may be transfusion dependent during early infancy and childhood.

Laboratory Evaluation

Most HS patients have either a mild to moderate anemia or no anemia at all, reflecting the facts that the hemolytic rate may be very mild and that the hemolysis is fully compensated for by increased red cell production, as evidenced by reticulocytosis. Some patients, however, particularly those with nondominantly inherited HS, are severely anemic, with a hemoglobin concentration of 46 g/dl.

Despite the increased percentage of reticulocytes, which have a larger volume than mature red cells, the mean corpuscular volume (MCV) of HS red cells is often low normal or even slightly decreased, a finding that, together with a slight increase in mean corpuscular hemoglobin concentration (MCHC), reflects mild cellular dehydration. The finding of an elevated MCHC combined with an elevated erythrocyte distribution width, as determined by automated cell counters, has been found to be an excellent predictor as a screening test for HS. Another screening method utilizes the Technicon H1 counter (Tarrytown, NY) and its successors. These counters, which measure MCV by light scattering, provide a histogram of MCHCs that has been claimed to identify nearly all HS patients. [147] [148] [149] [150]

Evidence of accelerated red cell destruction, as indicated by increased lactate dehydrogenase and unconjugated bilirubin levels and decreased haptoglobin levels, as well as by reticulocytosis, is present in most patients. However, these abnormalities may be absent in individuals with a mild form of the disease.

Blood Film

In typical HS, spherocytes are readily identified by their characteristic shape on the peripheral blood film (Fig. 33-5A). They lack central pallor, their mean cell diameter is decreased, and they appear more intensely hemoglobinized. The hemoglobinized appearance reflects both altered red cell geometry and increased cell density. In a three-dimensional view, some spherocytes have a spherostomatocytic shape that is occasionally appreciated on the peripheral blood film. In mild forms of the disease,

Figure 33-5 Blood films from patients with HS of varying severity. **(A)** Two blood films of typical moderately severe HS with a mild deficiency of red cell spectrin and ankyrin. Although many cells have a spheroidal shape, some retain a central concavity. **(B)** HS with pincered red cells (arrows), as typically seen in HS associated with band 3 deficiency. Occasional spiculated red cells are also present. **(C)** Severe atypical HS due to a severe combined spectrin and ankyrin deficiency. In addition to spherocytes, many cells have irregular contour. **(D)** HS with isolated spectrin deficiency due to a -spectrin mutation.[85] [151] Some of the spherocytes have prominent surface projections resembling spheroacanthocytes. *(Blood film D was provided courtesy of D.L. Wolfe.)*

the peripheral blood smear may appear normal because the loss of surface area may be too small to be appreciated by blood smear evaluation; the cells appear as fat disks rather than as true spherocytes.

Additional morphologic features have been described in some HS patients. A subset of HS patients whose red cells are deficient in band 3 protein have some pincered red cells (Fig. 33-5B) on the peripheral blood film, a finding that is both sensitive and specific for this HS subset. [62] [95] However, pincered cells disappear after splenectomy. Cells with irregular contour were found in two patients with severe combined spectrin and ankyrin deficiency (Fig. 33-5C). Surface spiculations or acanthocytic spherocytes have been described in a number of cases of HS associated with defects in -spectrin (Fig. 33-5D). Another atypical phenotype involves the presence of sphero-ovalocytes and stomatocytes, as reported in Japanese patients with protein 4.2 deficiency.

Osmotic Fragility

The osmotic fragility test (Fig. 33-6) measures the in vitro lysis of red cells suspended in solutions of decreasing osmolarity. The normal red cell membrane is unstretchable and is virtually freely permeable to water. [151] Thus the cell behaves as a nearly perfect osmometer in that it increases its volume in hypotonic solutions progressively until a critical hemolytic volume is reached. At this point the red cell membrane ruptures and hemoglobin escapes into the supernatant solution through a hole. As a result of the loss of membrane and the ensuing surface area deficiency, the critical hemolytic volume of spherocytes is considerably lower than that of normal red cells. Consequently, these cells hemolyze more than normal red cells when suspended in hypotonic NaCl solutions. However, a finding of increased osmotic fragility is not unique to HS and is also present in other conditions associated with spherocytosis on the peripheral blood film.

The slight dehydration of hereditary spherocytes (which in other conditions is associated with decreased osmotic fragility) has no appreciable effect on HS osmotic fragility because of the overriding effect of the markedly diminished cell-surface area. In fact, the densest, most dehydrated cells exhibit the greatest increase in osmotic fragility. [13] [152] The osmotic fragility curve often

Figure 33-6 Characteristic osmotic fragility curves in hereditary spherocytosis. The typical curve of increased osmotic fragility is the most common finding. The tailed curve reveals a second population (a tail) of very fragile erythrocytes conditioned by the spleen. This tail disappears after splenectomy. The diagonal curve is seen in patients with severe HS. *(Modified from Dacie,[16] with permission.)*

reveals uniformly increased osmotic fragility (Fig. 33-6). In addition, in nonsplenectomized HS subjects, a tail of the osmotic fragility curve may be present, indicating a subpopulation of particularly fragile red cells conditioned by splenic stasis. [13] [82] This subpopulation of cells disappears after splenectomy. In subjects with mild HS, osmotic fragility may be normal and abnormalities can be found only after incubation that further augments the loss of surface area; however, the sensitivity of the incubated osmotic fragility test may be outweighed by a loss of its specificity. The relative contributions of cell dehydration and surface area deficiency can be accurately determined by osmotic gradient ectacytometry, available only in specialized laboratories.

Autohemolysis and Other Tests

Red cell autohemolysis, the spontaneous hemolysis of red cells incubated under sterile conditions without glucose, was previously advocated as a sensitive test for the detection of HS. This test is being used less frequently and is probably no more sensitive than the incubated osmotic fragility test. Other tests described in the literature are not widely used (e.g., the glycerol lysis test, [25] [153] [154] [155] the pink test,[156] [157] and the skeleton gelation test [158] [159]). The former two tests, which employ glycerol to retard the osmotic swelling of red cells, are preferred in some laboratories because they are easy to perform and can be adapted to microsamples.

Detection of the Underlying Molecular Defect

Because the most common finding in erythrocytes of patients with HS is a deficiency of one or more of the membrane proteins, initial studies often include polyacrylamide gel electrophoresis of sodium dodecyl sulfate-solubilized red cell membrane proteins (SDS-PAGE) in gel systems of Laemmli and Fairbanks et al., followed by densitometric quantitation. [160] [161] This technique reveals abnormalities in 7080% of patients, defining the distinct biochemical phenotypes discussed previously. Direct quantitation of membrane proteins by classic radioimmunoassay is more sensitive than the densitometric quantitation and permits accurate measurement of the copy number of the individual proteins per red cell. The importance of the latter approach is heightened by the fact that densitometry may underestimate spectrin and ankyrin deficiencies because it involves a normalization to the band 3 protein (i.e., spectrin/band 3 ratio), the loss of which, together with

membrane lipids, may spuriously normalize the spectrin/band 3 ratio. Consequently, the spectrin/band 3 ratio is lower after splenectomy.

Subsequent strategies to search for the underlying defect are complex. Studies of protein function, which remain the reference standard in the detection of spectrin mutations in HE and HPP (see below), have been disappointing in detecting abnormalities in HS, with the exception of HS characterized by a weakened -spectrinprotein 4.1 interaction. Studies of membrane protein synthesis and assembly, using either reticulocytes or erythroblasts derived from peripheral blood burst-forming units in vitro, have been used to detect two defects: (1) a marked decrease in -spectrin synthesis in lethal HS, and (2) a primary defect involving reduced synthesis and expression of ankyrin in HS with a combined spectrin and ankyrin deficiency. [87] [90] However, these tests are likely to be insensitive in detecting mild to moderate abnormalities in membrane protein synthesis and assembly and are technically cumbersome to perform.

Another approach to beginning to identify the precise molecular defect in HS employs genetic linkage analysis. These studies, which are typically PCR-based, employ highly informative markers in or nearby the HS genes either for linkage exclusion or linkage verification. [162] [163] Disadvantages of linkage analysis include the need to study large kindreds to achieve significance and its lack of utility in studying patients with de novo mutations.

The identification of candidate genes, either by biochemical or genetic techniques, is followed by mutation detection. The most widely employed screening technique to rapidly identify candidate mutations is single-strand conformational polymorphism (SSCP) analysis. SSCP analysis identifies candidate regions in HS genes that can then be subjected to direct nucleotide sequence analysis for detection of the precise genetic defect.

Complications

Gallstones

Bilirubin stones are found in 50% of patients with HS, often even in those with a very mild form of the disease. Gallstones have occasionally been detected during infancy, but they are uncommon in very young children. [164] In a study of gallstones in HS that was conducted before the routine availability of ultrasound, approximately half of HS patients developed gallstones between 10 and 30 years of age. [165] Because of their high incidence, HS patients should be periodically examined by ultrasound for the presence of gallstones.

Crises

True hemolytic crises are relatively rare and only occasionally reported in association with infections. [164] [166] Aplastic crises during viral infections are largely attributable to infection by parvovirus B19. [145] [146] [167] [168] [169] [170] [171] This infection (erythema infectiosum, fifth disease [169] [171]) manifests with fever, chills, lethargy, malaise, nausea, vomiting, abdominal pain with occasional diarrhea, respiratory symptoms, muscle and joint pains, and a maculopapular rash on the face (slapped cheek appearance), trunk, and extremities. [167] [168] [169] [170] The virus selectively infects erythroid precursors and inhibits their growth, [165] [165] [170] and the ensuing anemia, often profound, may be the first manifestation of HS. [145] [146] Multiple family members with undiagnosed HS who are infected with parvovirus have developed aplastic crises at the same time. This has led to the description of HS outbreaks or epidemics.

Parvovirus infection has also been associated with neutropenia and thrombocytopenia. Infection with parvovirus is a particular danger to susceptible pregnant women because it can infect the fetus, leading to fetal anemia, hydrops fetalis, and fetal demise.

Rarely, patients may present with megaloblastic crises because of folate deficiency. This typically occurs in patients with increased folate demands, i.e., those recovering from an aplastic crisis, pregnant women, and the elderly. Megaloblastic crisis in pregnancy has been reported as the first manifestation of HS.

Other Complications

In patients with more severe forms of HS, other complications include leg ulcers and/or dermatitis that heal after splenectomy [172] and symptoms of expanded erythroid space, including paravertebral masses of extramedullary hematopoiesis, which may raise the possibility of an underlying neoplasm. [173] [174] Several cases of hemochromatosis in HS patients have been reported. In some, iron overload resulted from repeated transfusions; in others, the patients had two genetic defects, one involving HS and the other involving a hemochromatosis carrier state. [175] [176] [177] [178]

Over a dozen cases of HS and hematologic malignancy, including myeloproliferative disorders and leukemia, have been reported. [179] [180] It is unknown if long-standing hematopoietic stress predisposes to the development of these secondary disorders or if they occurred randomly.

Differential Diagnosis

Because of the relatively asymptomatic presentation of HS, this diagnosis should be considered during an evaluation for unexplained splenomegaly, unconjugated hyperbilirubinemia of unknown cause, gallstones at a young age, severe anemia during pregnancy, or transient anemia during acute infections. The diagnosis of HS can be missed in mild forms of the disease, because spherocytosis may not be apparent on the peripheral blood film. However, an osmotic fragility test of incubated red cells usually provides a diagnostic answer. It should be noted that spherocytosis is transiently improved and both the osmotic fragility and hemolysis are normalized in patients in whom obstructive jaundice develops. This is due to an expansion of red cell surface area that follows an increased uptake of phospholipids and cholesterol from the abnormal plasma lipoproteins. In normal red cells, this leads to target cell formation; in HS, spherocytes are transformed to discocytes. Spherocytosis and the increased osmotic fragility of HS red cells are likewise improved by iron deficiency, but the red cell life span remains shortened. In addition, coexistence of -thalassemia trait and HS partially corrects the HS phenotype.

More typical forms of HS, characterized by relatively uniform spherocytosis with increased MCHC, are usually easily distinguished from other disorders manifesting with spherocytosis, such as immune hemolytic anemias and unstable hemoglobins. In some patients, the spherostomatocytes in the rare Rh deficiency syndrome and the intermediate syndromes of hereditary stomatocytosis may be confused with HS red cells.

Therapy and Prognosis

Splenectomy

Splenectomy is curative in patients with typical forms of HS. Although red cell survival may remain slightly shortened in some patients, hyperbilirubinemia, anemia, and reticulocytosis are no longer present. Spherocytosis and the increase in osmotic fragility persists, but the tail of the osmotic fragility curve, indicating the presence of a subpopulation of cells conditioned by the spleen, disappears. [13] [82] [125] In patients with severe, nondominantly inherited HS, splenectomy produces a dramatic clinical improvement, but hemolysis is only partially corrected. [18] [20] [21]

Indications for Splenectomy

Risks and benefits should be considered carefully in HS patients before splenectomy is performed. A multitude of factors influence the decision for splenectomy in HS patients, including the risk of overwhelming postsplenectomy infection and the emergence of penicillin-resistant pneumococci. Indications for splenectomy include growth retardation, skeletal changes, symptomatic hemolytic disease (e.g., severe constitutional symptoms), anemia-induced compromise of vital organs, the development of leg ulcers, or the appearance of extramedullary hematopoietic tumors. Whether to perform splenectomy in patients with moderate HS without any of the above factors remains controversial. Because of an increased frequency of postsplenectomy infection in young children, splenectomy should not be performed earlier than 35 years of age. Rarely, a child with severe HS and growth retardation and skeletal deformities may need splenectomy earlier. Based on the recent finding that partial splenectomy improves the hemolytic rate while maintaining splenic phagocytic function, this procedure has been proposed for consideration in transfusion-dependent HS infants. In light of the high regenerative potential of splenic tissue, the long-term benefits of this procedure are uncertain.

Several weeks before splenectomy, patients should be immunized with polyvalent vaccine against *Pneumococcus* as well as vaccines against *Hemophilus influenzae*

type b and *Meningococcus*. After splenectomy, prophylactic antibiotics (penicillin V, 125 mg orally twice daily [<7 years of age] or 250 mg orally twice daily [<7 years of age, including adults]) have been recommended for at least 5 years by some, and for life by others. The duration of prophylactic antibiotic therapy after splenectomy

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is unknown. Before splenectomy and, in some cases, after splenectomy, HS patients should take folic acid (1 mg/day orally) to prevent folate deficiency.

Early complications of splenectomy include local infection or bleeding and pancreatitis. When splenectomy is warranted, laparoscopic splenectomy is becoming the procedure of choice in many centers. Laparascopic splenectomy has been associated with less postoperative discomfort, a quicker return to preoperative diet and activities, a shorter hospitalization time, decreased costs, and smaller scars. [181] [182] [183] In general, the morbidity of splenectomy is lower in HS than in other hematologic diseases. However, the benefits of surgery must be weighed against possible complications, such as postsplenectomy infections, including the postsplenectomy sepsis syndrome.[184] [185] [186] [187] Although these complications are rare and their frequency is likely to diminish further with appropriate vaccinations, the indiscriminate performance of splenectomy in all HS patients with splenomegaly is unwarranted. [188] The risk/benefit ratio and the changing trends in indications for splenectomy have recently been reviewed. [189]

Postsplenectomy Failures

Postsplenectomy failures are due either to the presence of an accessory spleen missed during surgery (accessory spleens may be present in 39% of all patients [190] [191]) or to the presence of another superimposed red cell disorder such as pyruvate kinase deficiency. The recurrence of hemolytic anemia several years after splenectomy should raise the suspicion of development of splenunculi, resulting from autotransplantation of splenic tissue during surgery. [18] The presence of an accessory spleen or splenunculus is suggested by the absence of both Howell-Jolly bodies and the pitted cells with crater-like surface indentations readily seen by interference contrast microscopy.[192] [193] A definitive confirmation of splenosis is made by a radiocolloid liver-spleen scan or by a scan using Cr-labeled heated red cells, which are taken up by the ectopic splenic tissue. [194] [195] Spontaneous regression of HS, presumably as a result of development of hyposplenism, has been observed in two family members who had both HS and sickle cell trait.

Other Therapies

HS erythrocytes may lose additional membrane, even under optimal storage conditions, suggesting that preoperative autologous erythrocyte transfusion may be of little benefit to HS patients scheduled to undergo surgery. [196]

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HEREDITARY ELLIPTOCYTOSIS AND RELATED DISORDERS

Definition, Prevalence, and History

Hereditary elliptocytosis designates a group of inherited disorders that have in common the presence of elliptical red cells on peripheral blood films. Elliptocytosis was first described by Dresbach[197] in 1904, and its heritability was firmly established by Hunter. [198] [199] Subsequent reports have revealed a considerable heterogeneity of clinical expression and have defined several distinct syndromes, including hereditary pyropoikilocytosis (HPP) and Southeast Asian ovalocytosis (SAO). [200] [201] [202]

HE is common in people of African and Mediterranean ancestry. In the U.S. population, the prevalence of HE is about 35 per 10,000. [203] [204] [205] The true incidence of HE is unknown because its clinical severity is heterogeneous and many patients are asymptomatic and without anemia. HE is considerably more frequent in areas of endemic malaria. In equatorial Africa, the prevalence of common HE has been estimated at between 0.6% and 1.6%. Worldwide, HE appears to be more common among blacks, particularly those of African origin. In Southeast Asian populations, the prevalence of SAO is as high as 30%. [200] [206] [207] [208]

The molecular basis of HE remained obscure until 15 years ago, when a defect of skeletal proteins was suggested. [209] [210] [211] [212] [213] [214] Subsequently many erythrocyte skeletal protein defects have been described, some characterized at the level of amino acid and DNA sequence.

On the basis of red cell morphology, HE can be divided into three major groups. Common HE, a dominantly inherited condition, is morphologically characterized by biconcave elliptocytes and, in some patients, rod-shaped cells. The clinical severity of common HE is highly variable, ranging from an asymptomatic condition to a severe recessively inherited hemolytic anemia, designated HPP, in which the blood film reveals numerous red cell fragments, microspherocytes, and poikilocytes. Spherocytic HE, also called hemolytic ovalocytosis, is a rare condition in which both round fat ovalocytes and spherocytes are present on the blood film. SAO, a disorder highly prevalent in the malaria-infested belt of Southeast Asia and the Pacific, is characterized by rigid, spoon-shaped cells that have either a longitudinal slit or a transverse ridge (Fig. 33-7).

Figure 33-7 Blood films of subjects with various forms of HE. **(A)** Simple heterozygote with mild common HE Sp I/65. Note the predominant elliptocytosis with some rod-shaped cells (arrow) and virtual absence of poikilocytes. **(B)** Simple heterozygote with severe common hemolytic elliptocytosis (HE Sp I/74). Note the numerous small fragments and poikilocytes. **(C)** Homozygous common HE due to doubly heterozygous state for two mutant -spectrins, Sp I/74 and Sp I/65. Both parents have mild HE. Note the many elliptocytes as well as numerous fragments and poikilocytes. **(D)** HPP, Sp I/74. The patient is a double heterozygote for mutant -spectrin (Sp I/74) and a presumed synthetic defect of this protein. Note the prominent microspherocytosis, micropoikilocytosis, and fragmentation. Only few elliptocytes are present. Some poikilocytes are in the process of budding (arrow). **(E)** Spherocytic HE (hereditary ovalocytosis). Most red cells are oval rather than truly elliptical. Many oval cells are fat, lacking a central concavity, the feature distinguishing spherocytic HE from common HE. **(F)** Southeast Asian ovalocytosis (Melanesian). Most cells are oval, some containing either a longitudinal slit or a transverse ridge (arrow). All blood smears were photographed at the same magnification (× 1,000).

Common Hereditary Elliptocytosis

A possibility that the primary lesion of HE and HPP erythrocytes resides in the proteins of the red cell membrane skeleton was first raised by the findings of thermal instability of HPP spectrin, [209] [210] retention of the elliptical shape in HE membrane skeletons, disintegration of membrane skeletons after exposure to shear stress, defective self-association of spectrin dimers to tetramers, [212] [213] altered susceptibility of spectrin to tryptic digestion, and a deficiency of the skeletal proteins spectrin and protein 4.1. A large number of reports followed that documented either dysfunction or deficiencies of several membrane skeletal proteins. In many cases, the underlying genetic defect has now been defined.

Etiology

Spectrin Mutations

The most common defects in HE, found in about two-thirds of all patients, are mutations of - or -spectrin. [215] [216] Both - and -spectrin are elongated flexible molecules consisting of triple helical repeats connected by nonhelical segments. [217] [218] These polypeptides are associated side to side in an antiparallel position, forming a flexible, rodlike heterodimer (Fig. 33-8) in which the N-terminus of -spectrin and the C-terminus of -spectrin form the head region of the heterodimer. Spectrin heterodimers associate head to head to form spectrin tetramers, the major structural subunits of the membrane skeleton. Spectrin tetramers, in turn, are interconnected into a highly ordered two-dimensional lattice through binding, at their distal ends, to actin oligomers with the aid of protein 4.1. [219] [220]

The contact site between the - and -spectrin chains of the opposed heterodimers is a combined triple helical repetitive segment in which the first two helices are contributed by the C-terminus of -spectrin, while helix 3 is the first helical segment of -spectrin (Fig. 33-9).[221] The spectrin dimer-tetramer interconversion is governed by a simple thermodynamic equilibrium that under physiologic conditions strongly favors spectrin tetramers. Most -spectrin defects described to date reside at or near the N-terminus of -spectrin, which is involved in the heterodimer contact (the so-called I domain defined by limited tryptic peptide mapping; see the discussion under Laboratory Evaluation and Fig. 33-8). Consequently, these mutations impair the self-association of spectrin into tetramers. Most -spectrin mutations reported are point mutations (Fig. 33-9). These mutations create abnormal proteolytic cleavage sites that typically reside in the third helix of a repetitive segment and give rise to abnormal tryptic peptides on two-dimensional tryptic peptide maps of spectrin.

Most of the reported elliptocytogenic -spectrin mutations are C-terminal point mutations or truncations (Fig. 33-9) that disrupt the formation of the combined triple-helical repetitive segment and consequently the self-association of spectrin heterodimers to tetramers. All of these mutations open a proteolytic cleavage site residing in the third helix of the combined repetitive segment, which gives rise to a 74-kd I peptide.

Although most spectrin mutations reside in the vicinity of the -spectrin self-association site, several mutations residing in the II domain have been described. [222] [223] [224] These mutations are asymptomatic in the simple heterozygous state but cause elliptocytic hemolytic anemia, which may be severe, in homozygous patients.

Protein 4.1 Mutations

Another group of elliptocytogenic mutations, although less common than spectrin mutations, are quantitative or qualitative defects of protein 4.1. Protein 4.1 is a

multifunctional protein that contains several important functional sites, including the

Figure 33-8 Schematic representation of the molecular assembly of the membrane skeleton and the molecular defects in HE and HPP. Spectrin is composed of -spectrin and -spectrin heterodimers (SpD) that associate in their head regions into tetramers. At their distal ends, SpD bind to the junctional complexes of oligomeric actin (band 5) and protein 4.1. Additional proteins found in the junctional complex, such as adducin, tropomyosin, and protein 4.9, are shown in the lower enlarged area. The membrane skeleton is attached to transmembrane proteins by interactions of -spectrin with ankyrin (protein 2.1; black arrow designates the ankyrin-binding site in -spectrin), which in turn binds to the cytoplasmic domain of band 3, and by linkage of protein 4.1 to glycophorin C. The known protein dysfunctions in HE and HPP include (1) defects of the SpD head region due to a mutation of either - or -spectrin, causing impaired assembly of SpD into tetramers, and (2) defects of proteins of the junctional complex such as a qualitative or quantitative defect of protein 4.1 or glycophorin C.

spectrin-binding domain, where 4.1 binds to the distal end of the spectrin heterodimer, markedly increasing the binding of spectrin to oligomeric actin, and a basic N-terminal domain, where 4.1 interacts with glycophorin C, phosphatidylinositol, and phosphatidylserine, facilitating the attachment of the distal end of spectrin to the membrane.[225] [226] [227] [228]

Studies of 4.1 mRNA from normal red cells revealed multiple 4.1 isoforms resulting from alternate mRNA splicing. Alternate translation initiation sites are present in the protein 4.1 mRNA. When an upstream AUG is utilized, isoforms greater than 80 kd are synthesized. During erythropoiesis, this upstream AUG is spliced out and a downstream AUG is utilized, leading to the production of the 80-kd mature erythroid protein 4.1 isoform. [229] On SDS-PAGE, protein 4.1 is resolved into two bands of different sizes: 4.1a and 4.1b. The larger band, 4.1a, is typically found in normal red cells, while the shorter one, 4.1b,

Figure 33-9 Mutations of - and -spectrin affecting the association of spectrin dimers into tetramers. Schematic representation of the self-association site between the - and -spectrin chains into heterodimers. Full circles denote point mutations and open circles denote deletions and insertions, all of which affect self-association of spectrin heterodimers into tetramers. The position of open circles corresponds to the last normal amino acid preceding the mutation. The numbering of amino acids and the respective codons of -spectrin is based on the cDNA sequence, including the first six amino acids not detected by protein sequencing.[239] [240] Most mutations either increase tryptic cleavage at normal sites or open new tryptic cleavage sites (short arrows), giving rise to abnormal tryptic peptides. Their sizes are indicated next to the short arrows. The size of these abnormal peptides provides a basis for designation of some of these mutations (e.g., Sp I/46). The tryptic cleavage site creating the normal 80-kd I peptide is denoted by the long arrow. The spectrin repetitive segments are phased according to conformation, rather than sequence homology, e.g., repetitive segment I is formed by helices 1, 2, and 3. Correspondingly, the atypical repeat at the contact site of self-association contains part of an -spectrin repeat (helix 3) and part of a -spectrin repeat (helices 1 and 2). Note that most spectrin mutations are located within helix 3 of each repetitive segment. *(From Palek and Jarolim,[1] with permission.)*

represents the major isoform of reticulocytes. The 4.1b isoform is converted into the 4.1a isoform by deamidation of Asn 502.

A partial deficiency of protein 4.1 is associated with mild dominantly inherited HE, [230] [231] [232] [233] [234] while a complete deficiency (a homozygous state) leads to a severe hemolytic disease.[234] [235] Homozygous 4.1() erythrocytes fragment more rapidly than normal at moderate sheer stresses, an indication of their intrinsic instability.[236] Membrane mechanical stability can be restored by reconstituting the deficient red cells with protein 4.1 or the protein 4.1/spectrin/actin-binding site. [237] Homozygous protein 4.1() erythrocytes also lack p55 and have only 30% of the normal content of glycophorin C. These homozygous 4.1() erythrocytes, as well as glycophorin C() Leach erythrocytes, demonstrate decreased invasion and growth of *Plasmodium falciparum* in vitro.[238]

Most patients with protein 4.1-associated elliptocytosis are of North African descent. [215] [241] Mutations associated with protein 4.1 deficiency have included a deletion that includes the exon encoding the erythroid transcription start size and mutations of the transcription initiation codon. [242] [243] [244] [245] Qualitative defects of protein 4.1 protein include deletions and duplications of the exons encoding the spectrin-binding domain, leading either to truncated or elongated forms of protein 4.1. [233] [246] [247] Electron microscopic studies of homozygous 4.1() erythrocyte membranes revealed a markedly disrupted skeletal network with disruption of the intramembrane particles, suggesting that protein 4.1 plays an important role in maintenance not only of the skeletal network, but also of the integral proteins of the membrane structure.[248]

Glycophorin C Deficiency

Glycophorin C has been found absent due to a variety of molecular defects. In contrast to other forms of HE, which are dominantly inherited, heterozygous carriers are asymptomatic, with normal red blood cell morphology, and homozygous subjects have no anemia and only mild elliptocytosis apparent on the peripheral blood film.

Glycophorin C deficiency with elliptocytosis, the so-called Leach phenotype, [249] [250] caused by reduced expression of glycophorin C, should be distinguished from the immunochemically defined phenotypes Gerbich and Yus, in which abnormal glycoproteins are formed that can functionally substitute for normal glycophorin C and preserve the normal red cell shape.[251] [252] The Leach phenotype is usually due to a large deletion of genomic DNA (7 kb) that removes exons 3 and 4 from the GPC/GPD locus.[253] [254] The genetic basis of the Leach phenotype in one patient has been reported to be due to a nucleotide deletion leading to frame shift with premature chain termination.

Glycophorin C-deficient subjects are also partially deficient in protein 4.1 and lack p55, presumably because these proteins form a complex and recruit or stabilize each other on the membrane.[255] [256] It has been speculated that the protein 4.1 deficiency

in Leach erythrocytes is the cause of the elliptocytic shape. In contrast, subjects deficient in glycophorin A, the major transmembrane glycoprotein, are asymptomatic.

Pathobiology of the Red Cell Lesion

Most of the elliptocytogenic mutations of spectrin reside within, or in the vicinity of, the spectrin heterodimer self-association site, disrupting this region and consequently disrupting the two-dimensional integrity of the membrane skeleton. These horizontal defects are readily detected by ultrastructural examination of membrane skeleton, which reveals disruption of a normally uniform hexagonal lattice (Fig. 33-10).[99] Consequently, membrane skeletons are mechanically unstable, as are whole cell membranes and the cells.[6] [257] In patients with severely dysfunctional spectrin mutations, or in subjects who are homozygous or doubly heterozygous for such mutant proteins, this membrane instability is sufficient to cause red cell fragmentation with hemolytic anemia under conditions of normal circulatory shear stress.

The pathobiology of the elliptocytic shape is less clear. Red cell precursors in common HE are round, and the cells become progressively more elliptical as they age in vivo.[203] [258] [259] Red cells subjected to shear stress in vitro, or red cells flowing through microcirculation in vivo, have an elliptical or parachute-like shape, respectively. [260] It is possible that elliptocytes and poikilocytes are permanently stabilized in their abnormal shape because the weakened spectrin heterodimer contacts facilitate skeletal reorganization, which follows axial deformation of cells resulting from application of a prolonged or excessive shear stress. This reorganization is likely to involve breakage of the unidirectionally stretched protein connections followed by the formation of new protein contacts that preclude the recovery of a normal biconcave shape.[257] This process has recently been shown to account for permanent deformation of irreversibly sickled cells. [261]

In HPP, the recessively inherited form of HE characterized by severe hemolysis, red cells have two abnormalities. They contain a mutant spectrin that characteristically disrupts spectrin heterodimer self-association, and they are also partially deficient in spectrin, as evidenced by a decreased spectrin/band 3 ratio. [216] [262] In some HPP cases, this biochemical phenotype is a consequence of a double heterozygous state for an elliptocytogenic -spectrin mutation and a defect involving reduced -spectrin synthesis. [87] [263] [264] Such synthetic defect of -spectrin is fully asymptomatic in the heterozygous carrier, because under normal conditions, the synthesis of -spectrin is about three to four times greater than that of -spectrin.

Figure 33-10 Electron micrographs of negatively stained, artificially extended membrane skeletons from **(A)** a control subject, **(B)** a patient with moderately severe HS with partial spectrin deficiency, **(C)** a subject with mild heterozygous HE with a mutant spectrin characterized by a defective self-association of spectrin dimers to tetramers, and **(D)** an HPP subject carrying a severely dysfunctional mutant spectrin. The skeletons were uniformly stretched so that their major protein components and their connections are visible. Only one skeletal layer is exposed. Note the uniform, largely hexagonal lattice in the control sample **(A)**. The globular structures represent junctional complexes (JC) composed of oligomeric actin, while the interconnecting fibers represent spectrin tetramers (Sp). **(B)** The skeletons from an HS patient with a mild spectrin deficiency. The skeleton appears morphologically intact, because spectrin is largely in a tetrameric form. **(C)** A carrier of the spectrin mutation having a mild increase in unassembled dimeric spectrin in the crude spectrin extract and a mildly disrupted skeletal lattice. **(D)** The skeleton from the HPP patient is grossly disrupted. This disruption presumably underlies the marked membrane instability of red cells and red cell fragmentation. The junctional complexes are clustered and only few interconnecting spectrin tetramers can be seen. Membranes from this patient have a marked increase in unassembled (dimeric) spectrin and their spectrin content is reduced to about 70% of normal. *(Courtesy of S.C. Liu.)*

When present in conjunction with an elliptocytogenic mutation of -spectrin, such a synthetic defect augments the expression of the mutant spectrin. Because the elliptocytogenic -spectrin mutants are often unstable, [87] [262] the combination of the two defects leads to spectrin deficiency in the cells. Other HPP subjects are homozygous or doubly heterozygous for one or two elliptocytogenic spectrin mutations, respectively. [87] [263] In such cases, the spectrin deficiency may be a consequence of spectrin instability that reduces the amount of spectrin available for membrane assembly. Furthermore, in red cells containing a high fraction of unassembled dimeric spectrin, the spectrin deficiency may in part be related to the stoichiometric ratio of one ankyrin copy per one spectrin tetramer (i.e., two spectrin heterodimers). Consequently, only about one-half of spectrin heterodimers succeed in attaching to the ankyrin-binding sites. The phenotype of HPP, characterized by the presence of fragments and elliptocytes, together with evidence of red cell surface area deficiency (as reflected by the presence of microspherocytes on the peripheral blood film), suggests that the membrane dysfunction involves both vertical interactions (a consequence of spectrin deficiency, see above) and horizontal interactions involving the elliptocytogenic spectrin mutation.

The red cell lesion in protein 4.1 deficiency shows similarities in regard to cell shape and membrane stability to the above elliptocytogenic mutations of spectrin, [257] suggesting that the deficiency principally affects the spectrin-actin contact (i.e., a horizontal interaction) rather than the skeleton attachment to glycophorin C via protein 4.1 (a vertical interaction).

The molecular basis of elliptocytosis and the mechanical instability of glycophorin C-deficient red cells is not fully understood. However, recent studies suggest that the deficiency of glycophorin C is not directly responsible for the altered mechanical properties. Instead, the mechanical instability appears to be related to a concomitant partial deficiency of protein 4.1, as evidenced by a full correction of membrane instability by introduction into the cells of protein 4.1 or its spectrin-binding peptide, which facilitates the contact of -spectrin to actin. The superimposed deficiency of protein 4.1 is likely to be related to the fact that glycophorin C serves as an attachment site for protein 4.1 to the membrane, recruiting protein 4.1 to the red cell membrane. The effects of the above defects on the mechanical stability of glycophorin C-deficient cells appear relatively minor, because glycophorin C-deficient subjects have no detectable hemolytic anemia and the mechanical properties of the red cells are normal when tested by micropipette aspiration.

Previous studies of HE red cells revealed increased utilization of ATP and 2,3-diphosphoglycerate, [265] presumably due to increased transmembrane sodium movements.[266] As a result of the underlying skeletal defect, HE and HPP red cells also exhibit an abnormally increased Na, K, and Ca permeability. [267] [268] The latter defect was proposed to represent the primary molecular lesion in a case of severe microcytic hemolytic anemia associated with thermal instability of red cells, but subsequent studies of this patient identified a spectrin mutation. [267] [268]

Inheritance

In most patients, HE is inherited as an autosomal dominant disorder. The clinical severity is highly variable both among different kindreds (reflecting heterogeneous molecular lesions[219] and, to a lesser extent, within a given kindred, presumably because of other genetic or acquired defects that modify disease expression. [269] [270] [271] Occasionally, HE is inherited as an autosomal recessive condition from an asymptomatic parent who carries the same molecular defect of spectrin as the HE offspring. HE is linked to blood group antigens Rh and Duffy, the genes of which are located on chromosome 1, the Duffy blood group locus being close to the gene for -spectrin,[272] [273] while the Rh gene locus (1p 33) is close to the gene for protein 4.1 (Table 33-1). [205] [274] [275]

The inheritance of HPP, a disorder closely related to HE, is autosomal recessive: one of the parents carries the -spectrin mutation and either is asymptomatic or has mild HE, while the other parent is fully asymptomatic and has no abnormalities detectable by current biochemical approaches. [2] [219] However, several HPP patients have recently been studied who were doubly heterozygous for two -spectrin mutations; in the heterozygous parents, these mutations were either silent or expressed as mild HE.[276]

Clinical Manifestations

In view of the striking molecular heterogeneity of common HE, it is not surprising that the clinical spectrum of this disorder is variable, ranging from an asymptomatic trait without hemolysis to a life-threatening hemolytic anemia.

Mild Hereditary Elliptocytosis and Asymptomatic Carrier State

In most of these subjects, HE is found accidentally during evaluation of the peripheral blood film. Although some HE subjects have a mild compensated hemolytic anemia,[271] [277] [278] others do not have any evidence of hemolysis, their red cell survival is normal, and the peripheral blood film may reveal only modest (15%) elliptocytosis;[2] [279] they even may be blood donors. The molecular basis of mild HE is heterogeneous, and the reported molecular defects include both - and -spectrin mutations, partial deficiency of the 4.1 protein, and the absence of glycophorin C. Some individuals carrying the spectrin mutation are completely asymptomatic, including normal red cell morphology; this is often the case in one of the parents of a patient with HPP. [2] [279] [280] [281]

Hereditary Elliptocytosis with Sporadic Hemolysis

Worsening of hemolysis together with the appearance of poikilocytes on the peripheral blood film has been reported in patients with hypersplenism, infections, or vitamin B_{12} deficiency,[2] as well as in those with microangiopathic hemolysis such as disseminated intravascular coagulation or thrombotic thrombocytopenic purpura.[282] In the latter two conditions, worsening hemolysis may be due to microcirculatory damage superimposed on the underlying mechanical instability of the red cells.

Hereditary Elliptocytosis with Neonatal Poikilocytosis

Neonatal offspring of parents with mild HE present with symptomatic hemolytic anemia and a marked poikilocytosis. During the first year of life, the hemolysis and poikilocytosis abate, and the clinical picture transforms into that of mild HE. [283] [284] [285] Such patients typically carry one mutant -spectrin allele. [2] [279] The severity of the molecular defect, in terms of the percentage of spectrin dimers and the amount of mutant spectrin in the cells, is the same in the neonatal period as it is later in life. The worsening of hemolysis in the neonatal period has been attributed to the presence of fetal hemoglobin, which binds poorly to 2,3-diphosphoglycerate (2,3-DPG).[286] The ensuing elevation of free 2,3-DPG levels has a marked destabilizing effect on the spectrin-protein 4.1-actin interaction, [286] [287] thereby further destabilizing the membrane skeleton.

Hereditary Elliptocytosis with Chronic Hemolysis

Patients with HE with chronic hemolysis present with moderate to severe hemolytic anemia with elliptocytes and poikilocytes on peripheral blood film; some require splenectomy.[269] [270] [271] [288] [289] In some of the kindreds, the hemolytic HE has been transmitted through several generations. [290] [291] In two families, the affected individuals carried a severely dysfunctional spectrin, Sp I/74. In some kindreds, not all of the HE subjects have chronic hemolysis;

some have a mild hemolysis only, presumably because of another genetic factor modifying the disease expression. [269] [271]

Homozygous and Compound Heterozygous Hereditary Elliptocytosis

Several HE individuals have been described who were apparent homozygotes for the HE gene. [2] [279] [292] More recently, these individuals were found to be either homozygotes or compound (double) heterozygotes for one or two - or -spectrin mutations. [263] [293] [294] [295] The clinical severity is variable, depending on the severity of the underlying molecular defect; patients homozygous for the mildly dysfunctional Sp I/65 have a relatively mild hemolytic anemia that does not require splenectomy, while other homozygous or doubly heterozygous patients, such as those with the Sp I/74 mutation, have a severe, life-threatening disease. [263] [293] [296] In addition, peripheral blood films of some homozygous HE subjects (e.g., Sp I/74) contain numerous very small microspherocytes, and the red cells are partially deficient in spectrin. Thus, their clinical presentation is indistinguishable from that of patients with HPP.

Hereditary Pyropoikilocytosis

Under the term HPP, Zarkowsky et al. described an autosomal recessive severe hemolytic anemia with striking micropoikilocytosis and thermal instability of red cells.[297] It is now established that HPP represents a subtype of common HE, as evidenced by the coexistence of both HE and HPP in the same family and by the presence of the same molecular defect of spectrin. Unlike HE subjects carrying the spectrin mutation, the red cells of HPP subjects are also partially deficient in spectrin.[216] [262] Typically, one parent of the HPP offspring carries an -spectrin mutation while the other parent is fully asymptomatic and has no detectable biochemical abnormality. In many such patients, the asymptomatic parent carries a silent thalassemia-like defect of spectrin synthesis, enhancing the relative expression of the spectrin mutant and leading to a superimposed spectrin deficiency in the HPP offspring. [2] [279] Some HPP subjects inherited two -spectrin mutations; either their parents were hematologically normal or one had mild HE.[263] [276] [279] In these HPP subjects, spectrin deficiency may be related to instability of the mutant spectrin. In one such doubly heterozygous proband, the red cell spectrin content (spectrin/band 3 ratio) was normal, the hemolysis was mild, and the peripheral blood smear resembled that of hemolytic HE (poikilocytes and elliptocytes) rather than HPP, further highlighting the overlap between these two disorders. [298] The thermal instability of spectrin originally reported as diagnostic of HPP is not unique for this disorder; it is also found in HE subjects carrying this -spectrin mutation, both in the homozygous and in the heterozygous state.[263] HPP is seen predominantly in black subjects, but it has also been diagnosed in Arabs and whites. [215] [299] [300] [301]

Molecular Determinants of Clinical Severity

The severity of hemolysis in common HE often varies not only among different kindred but within a given family as well. The two principal determinants of severity of hemolysis are the spectrin content of the cells and the percentage of dimeric spectrin in the crude spectrin extract. [5] The fraction of dimeric spectrin in such extracts in turn depends on several factors. The first of them is the degree of dysfunction of the mutant spectrin. Typically, mutations that are either within or near the combined triple helical repetitive segment representing the spectrin heterodimer self-association site produce a more severe clinical phenotype and a more severe defect of spectrin function than are seen with point mutations in the more distant triple helical repeats. [264] Second, the percentage of the dimeric spectrin depends on the fraction of the mutant spectrin in the cells, which in turn is determined by the gene dose (e.g., simple heterozygote versus homozygote or double heterozygote) or the presence of other genetic defects such as the presence, in trans, of a defect leading to a reduced -spectrin synthesis in some subjects with HPP.

The low-expression -spectrin allele ^{LELY} is the best-characterized abnormality affecting spectrin content and clinical severity. Initially, a polymorphism of the V domain, $^{V/41}$, was identified in HE patients who, when they inherited $^{V/41}$ in trans, had more severe HE than expected. Subsequently an amino acid substitution of exon 46, Leu1857Val, and partial skipping of exon 46, linked to the $^{V/41}$ polymorphism, were identified as the characteristics of the ^{LELY} allele. These abnormalities are located within the site at which spectrin monomers assemble into heterodimers (the spectrin heterodimer nucleation site). [264] [302] [303] In vitro studies suggest that the inability of -spectrin chains to assemble into the mature membrane skeleton is due to a combination of decreased dimer-binding affinity and increased proteolytic cleavage of the mutant -spectrin chains.[304] The presence of ^{LELY} in trans diminishes the propensity of the otherwise normal allele to associate with the corresponding -chain, favoring the attachment of the elliptocytogenic -spectrin allele. Conversely, coexistence of the -spectrin mutation in cis and the mutation involving the -spectrin nucleation site diminishes the propensity of the mutant allele to be incorporated into the spectrin heterodimer, thereby ameliorating the clinical severity of this mutation. [264] [303] The ^{LELY} allele is clinically silent by itself, even when inherited in the homozygous state, probably because -spectrin is normally synthesized in three- to fourfold excess.

Certain acquired factors may affect the clinical severity of HE. In neonatal red cells, the weak binding of 2,3-DPG by fetal hemoglobin leads to an increase in free 2,3-DPG, which in turn induces a superimposed destabilization of the spectrin-actin-protein 4.1 interaction. [286] [287] Lastly, hemolytic anemia can be worsened by several acquired conditions, including those that alter the microcirculatory stress to the cells. [282]

Laboratory Evaluation

Blood Film and Laboratory Evidence of Hemolysis

A careful blood smear evaluation is essential both for the diagnosis of HE and for the classification of the disorder into the three major subtypes outlined earlier (Fig. 33-8). In patients in whom elliptocytosis is the only morphologic abnormality, hemolysis is characteristically minimal or absent, with the exception of spherocytic elliptocytosis, in which the presence of round fat ovalocytes is associated with accelerated red cell destruction. In patients with hemolytic forms of common HE, poikilocytosis is characteristically found on the blood film. In severe forms of HE, particularly in homozygous HE, many red cells circulate as cell fragments, producing a marked decrease in MCV. The finding of red cell fragments together with a striking microspherocytosis and often only occasional elliptocytes is characteristic of HPP (Fig. 33-7).

Osmotic, Thermal, and Mechanical Fragility

Osmotic fragility is increased in HPP, in spherocytic elliptocytosis, and in HE subjects with poikilocytosis apparent on the peripheral blood film. [2] [305] In patients with a mild common HE without poikilocytosis on the peripheral blood film, osmotic fragility is normal. [306]

Thermal instability of red cells was originally reported by Zarkowsky et al. as a characteristic feature of HPP. [297] It reflects thermal instability of the mutant spectrin: in normal red cells, spectrin is denatured and red cells fragment at 50°C. HPP red cells fragment and their spectrin denatures at 41°C. However, the diagnostic value of this test is limited, because thermal instability

of red cells is also noted in HE red cells containing mutant spectrin. [263] In contrast, an occasional patient with otherwise typical HPP may have normal thermal stability of both red cells and spectrin. Red cells in common HE have unstable membranes and membrane skeletons when subjected to shear stress. [2] [236]

Electrophoretic Separation of Solubilized Membrane Proteins

In HE and HPP, SDS-PAGE may reveal proteins of abnormal mobility, the origin of which can be subsequently identified by Western blotting (e.g., truncated - or -spectrins in HE and HPP, or elongated or truncated forms of the 4.1 protein, and a partial or, rarely, complete deficiency of the 4.1 protein in HE) (Fig. 33-11A). In HPP, SDS-PAGE reveals a partial deficiency of spectrin, as indicated by a decreased spectrin/band 3 ratio. [216] [262] Spectrin deficiency, in conjunction with an elliptocytogenic spectrin mutation affecting the spectrin heterodimer contact, is invariably found in HPP.

In a rare subject with a mild recessive HE, it is useful to stain the gels with the periodic acid-Schiff reagent. This approach may reveal a deficiency of glycophorin C. Glycophorin C deficiency is also found in patients deficient in the 4.1 protein, possibly because the latter protein plays a role in the recruitment of glycophorin C on the membrane.[242]

Nondenaturing Gel Electrophoresis of Low Ionic Strength Spectrin Extract

Analysis of the ratio of tetrameric and dimeric spectrin in the low ionic strength extracts reveals the most common functional abnormality in HE (i.e., weakened

self-association of spectrin heterodimers into tetramers) (Fig. 33-11B). Because the spectrin dimer-tetramer interconversion has a high activation energy, it is kinetically immobilized at near 0°C. [308] Consequently, the percentage of spectrin dimers and tetramers in the 0°C crude spectrin extract reflects the relative distribution of these species in the red cell membrane in situ. [309] Mutations of - or -spectrin residing within or near the -spectrin heterodimer self-association site invariably lead to an increase in the fraction of dimeric spectrin (normal range, 5 ± 5%) in the crude 0°C spectrin extract. [264] [310]

Tryptic Peptide Mapping of Spectrin and the Detection of the Underlying DNA Defect

As reviewed elsewhere, [264] [310] [311] tryptic digestion of spectrin under controlled conditions followed by electrophoretic separation gives rise to highly reproducible tryptic peptide patterns (Figs. 33-11C and D). Among these peptides, the 80-kd I domain peptide representing the self-association site of the normal

Figure 33-11 Evaluation of defects of the membrane skeleton in HE and HPP. **(A)** Membrane proteins from control red cells (lanes 1 and 3) and red cells of the two patients (lanes 2 and 4) were solubilized in sodium dodecylsulfate and electrophoresed in a Fairbanks nonlinear 3.517% polyacrylamide gradient gel (first two gels) and Laemmli gel (last two gels). Note excellent resolution of -spectrin, -spectrin, and ankyrin bands in the modified Fairbanks system. In the Laemmli system, -spectrin and ankyrin bands overlap. However, the Laemmli system resolves band 4.1 into two bands (4.1a, 4.1b). In lane 2, a truncated -spectrin mutant with abnormal mobility (spectrin Nice) associated with HE was identified in the Fairbanks gel. In lane 4, both protein 4.1 bands are absent from the erythrocyte membranes of a homozygous individual with 4.1() HE as seen in a Laemmli gel. **(B)** Nondenaturing polyacrylamide gels of crude spectrin (Sp) extracts. The extracts in lane 1, from a normal control, are tetrameric (SpT) and oligomeric (SpO). The extracts in lane 2, from one of two parents who both have common HE, exist as dimers (SpD), tetramers, and oligomers. The extracts in lane 3, from a homozygous HE offspring, exist almost exclusively as dimerics. **(C** & **D)** SDS-PAGE of tryptic digests of Sp from members of the same family followed by Coomassie blue staining **(C)** or immunoblotting with an anti-I spectrin antibody **(D)**. Note that the immunoblot from the HE parent (lane 2) contains both normal 80-kd and abnormal 74-kd I domain peptides, implying that the parent is heterozygous for the Sp I/74 mutant. The immunoblot from the homozygous offspring contains only the abnormal 74-kd mutant I domain peptide; no normal 80-kd peptide is present. *(From Palek and Lambert, [307] with permission.)*

-spectrin is the most prominent one. Nearly all - or -spectrin mutations reported are associated with a formation of tryptic peptides of abnormal size and mobility that are generated instead of the normal 80-kd I domain peptide. The cleavage sites of the most common abnormal tryptic peptides were mapped and found to reside in the third helix of a given triple helical repetitive segment (Fig. 33-9). The reported mutations reside in the vicinity of these cleavage sites either in the same helix or, less commonly, in helix 1 or 2 of a given repetitive segment. Consequently, tryptic peptide mapping remains a powerful tool with which to map the site of the underlying spectrin mutation, which can be subsequently defined by polymerase chain reaction amplification and sequencing of the respective region of the genomic DNA or cDNA.

Differential Diagnosis

Various acquired and inherited conditions can be associated with elliptocytosis and poikilocytosis, including iron deficiency, thalassemias, megaloblastic anemias, myelofibrosis, myelophthisic anemias, myelodysplastic syndromes, and pyruvate kinase deficiency. The percentage of elliptocytes in these conditions is seldom >60%.[258] [312] [313] However, this is not diagnostically useful, since some HE subjects may have a relatively low percentage of elliptocytes. [2] [279] In normal subjects, the percentage of elliptocytes is not >5%, [2] although in earlier reports it was listed as high as 15%. [203] [278] Previous diagnostic criteria of HE, based on the percentage of elliptocytes, such as 25%, [205] 33%, [314] or 40%, [203] and their axial ratio, [271] do not appear useful. [307] The most reliable differentiation of HE from the above conditions is based on a positive family history rather than on the percentage of elliptocytes. [307]

Treatment and Prognosis

As in the case of HS, red cells from patients with more severe forms of HE are retained by the spleen, producing a marked engorgement of splenic pulp. [119] [315] Consequently, patients with symptomatic hemolysis benefit from splenectomy. [305] [306] This procedure is virtually never indicated in heterozygotes with autosomal dominant HE because most do not have clinically significant hemolytic anemia. In contrast, splenectomy is required in most patients with homozygous HE and HPP. If hemolysis is still active after splenectomy, folate should be administered daily. Recommendations for antibiotic prophylaxis, immunizations, and monitoring for intercurrent illnesses are similar to those noted earlier for HS patients before and after splenectomy. Serial interval ultrasound investigations to detect gallstones should be performed in patients with significant hemolysis.

Spherocytic Elliptocytosis

Spherocytic elliptocytosis, which shares features of both HS and HE, has been designated spherocytic HE, HE with spherocytosis, or hereditary hemolytic ovalocytosis. [279] [306] [307] [316] The diagnosis is based on the simultaneous presence of elliptical red cells and spherocytes or fat, round sphero-ovalocytes in the peripheral blood film (Fig. 33-7). In contrast to common HE, cells of other shapes, such as rod-shaped cells, poikilocytes, and fragments, are absent. Importantly, hemolysis, despite relatively mild alterations in red cell morphology, and increased osmotic fragility are the main diagnostic features distinguishing this disorder from common HE.

The molecular basis of classic spherocytic HE is unknown. However, patients with mutations, particularly truncations at the C-terminus of -spectrin, have many of the clinical features of spherocytic HE and are probably an example of this disorder. Patients who lack glycophorin C have rounded, smooth elliptocytes and could be classified as having a mild, recessively inherited variant of spherocytic HE. Finally, some patients with recessively inherited defects of protein 4.2 may display some features of spherocytic HE, particularly mild ovalostomatocytosis. However, the erythrocyte morphology and pathophysiology associated with protein 4.2 deficiency more closely resemble HS; thus it is better classified as a variant of HS than HE.

Southeast Asian Ovalocytosis

SAO is characterized by the presence of oval red cells, many containing one or two transverse ridges or a longitudinal slit (Fig. 33-7). The condition is widespread in certain ethnic groups of Malaysia, Papua New Guinea, the Philippines, and Indonesia. [317] [318] [319] Numerous functional abnormalities of ovalocytes have been reported, including increased red cell rigidity, decreased osmotic fragility, increased thermal stability, resistance to shape change by echinocytogenic agents, and a reduced expression of many red cell antigens. [202] [320] [321] [322] [323] [324] [325] A remarkable feature of ovalocytes is their resistance to in vitro invasion by several strains of malaria parasites, including *Plasmodium falciparum* and *Plasmodium knowlesi*. [323] [324] [326] Moreover, in areas of endemic malaria, the ovalocytic subjects have reduced numbers of intracellular parasites in vivo. [206] In these regions, there is a decrease in the prevalence and in the disease severity of malaria in patients with SAO compared to controls. [327] [328]

The finding of a tight linkage of the abnormal proteolytic digest of erythrocyte band 3 protein to the SAO phenotype has led to the detection of the underlying molecular defect. [320] All carriers of the SAO phenotype were found to be heterozygotes, with one band 3 allele normal and the other containing two mutations in cis: the deletion of 9 codons encoding amino acids 400408 from the boundary of the cytoplasmic and membrane domains of band 3, and the 56 Lys to Glu substitution. [329] [330] [331] The 56 Lys to Glu substitution represents an asymptomatic polymorphism known as band 3 Memphis. [332] [333] [334] The SAO phenotype is associated with a tighter binding of band 3 to ankyrin, [320] increased tyrosine phosphorylation of the band 3 protein, [335] [336] [337] inability to transport sulfate anions, [338] [339] and a markedly restricted lateral and rotational mobility of band 3 protein in the membrane. [320] [330]

Laboratory Evaluation

The finding of 30% of oval red cells on the peripheral blood film, some containing a central slit or a transverse ridge, in the context of a notable absence of clinical and laboratory evidence of hemolysis in a subject from the above-noted ethnic groups is highly suggestive of the diagnosis. A useful screening test is the demonstration of the resistance of ovalocytes or their ghosts to changes in shape produced by treatments that produce spiculation in normal cells, such as metabolic depletion or exposure of ghosts to salt solutions. [320] [322] [326] [329] In contrast to normal red cells, which form spicules in response to such stimuli, SAO red cells or ghosts do not change shape after these treatments. The mechanism of this resistance to changes in shape is not clear, and it may reflect the high rigidity of the red cell membrane.

Since the underlying cause of SAO is the deletion of 27 bases from the band 3 gene, isolation of genomic DNA or reticulocyte cDNA with subsequent amplification of the deletion-containing region appears to be the most specific test for establishing the diagnosis of SAO. This amplification produces a single band in control cells and

a doublet with the second band shorter by 27 base pairs in the SAO cells. [329] [330] [340] Interestingly, this mutation appears to be lethal in the homozygous state, as large screens of individuals from indigenous areas have only identified heterozygotes. [328] [341]

Molecular Basis of SAO Membrane Rigidity and Malaria Resistance

SAO red cells are unique among axially deformed cells in that they are rigid and hyperstable rather than unstable. [321] The SAO mutation is the first example of a defect of an integral membrane protein leading to red cell membrane rigidity, an observation previously attributed to properties of the membrane skeleton. [110] Exposure of red cells to various ligands that bind to glycophorin A has been found to decrease membrane deformability. [342] [343] In both conditions, conformational changes of the cytoplasmic domain of the respective proteins may preclude lateral movement (extension) of the skeletal network during deformation. [110] Another putative mechanism of the high SAO membrane rigidity is increased binding of band 3 to ankyrin and thus to the underlying skeleton, or increased propensity of the band 3 protein to aggregate into higher oligomers with increased band 3-ankyrin stoichiometry, which in turn can facilitate band 3 attachment to ankyrin. [320] [344] Yet another proposed mechanism involves a nonspecific adherence of the SAO band 3 to the skeleton, possibly due to denaturation of the membrane spanning domain. [345]

The molecular basis of malaria resistance of SAO red cells is likely related to altered properties of the band 3 protein, which serves as one of the malaria receptors, as evidenced by the inhibition of in vitro invasion by band 3-containing liposomes. [346] In normal red cells, the invasion process is associated with a marked membrane remodeling that involves redistribution of intramembrane particles that contain band 3 protein. [347] Such particles cluster at the site of parasite invasion, forming a ring around the orifice through which the parasite enters the cell. The invaginated red cell membrane, which surrounds the invading parasite, is free of intramembrane particles. The reduced lateral mobility of band 3 protein in SAO red cells [320] [330] may preclude band 3 receptor clustering, thereby preventing the attachment of the parasites to the cells. Decreased exchange of anions across the red cell membrane has also been proposed to contribute to the resistance of ovalocytes to malaria invasion. [338] [339] In addition, SAO red cells also consume ATP at a higher rate than normal cells, and the partial depletion of ATP levels in ovalocytes has been suggested to account, at least in part, for the resistance of these cells to malaria invasion in vitro. [348]

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ACANTHOCYTOSIS AND RELATED DISORDERS

Acanthocytes (from the Greek *acantha*, thorn) or spur cells are red cells with prominent thornlike surface protrusions that vary in width, length, and surface distribution. Spur cells must be distinguished from echinocytes (Greek *echinos*, urchin) or burr cells, characterized by multiple small projections that are uniformly distributed throughout the cell surface (Fig. 33-12 (Figure Not Available)). [349] Acanthocytes should also be distinguished from keratocytes (horn red cells) that have few massive protuberances.

Acanthocytosis was first described in abetalipoproteinemia [350] [351] and subsequently in severe liver disease, [352] [353] the chorea-acanthocytosis syndrome,[354] [355] the McLeod blood group phenotype,[356] and other conditions.[357] [358] [359] [360] [361] The molecular mechanisms leading to acanthocytosis in abetalipoproteinemia and severe liver disease have been extensively studied and have been attributed to changes in composition of membrane lipids and their altered distribution between the two hemileaflets of the lipid bilayer.

Spur Cell Hemolytic Anemia of Severe Liver Disease

Spur cell hemolytic anemia is an uncommon ominous complication of severe liver disease that is manifested by rapidly progressive

Figure 33-12 (Figure Not Available) Morphologic differences between **(A)** acanthocytes and **(B)** echinocytes as demonstrated by scanning electron microscopy. *(Adapted from Bessis,[349] with permission.)*

hemolytic anemia and acanthocytes on the peripheral blood smear. [352] [353] [362] [363]

Pathobiology

The human red cell membrane contains nearly equal amounts of free (unesterified) cholesterol and phospholipids. The free cholesterol in the plasma readily equilibrates with the red cell membrane cholesterol pool. [364] This is in contrast to esterified cholesterol, which cannot be transferred from plasma into the red cell membrane. The plasma of patients with severe liver disease contains abnormal lipoproteins that have a high free cholesterol/phospholipid ratio. [363] The excess free cholesterol readily partitions into the red cell membrane, leading to a marked increase in free cholesterol in the cells. [363] [364] Consequently, normal cells can develop a spur cell shape after their transfusion into a patient with severe liver disease [362] or after incubation with the liver disease patients plasma or cholesterol-enriched liposomes.[365]

Spur cell formation involves two steps (Fig. 33-13). The first step is evident in red cells of splenectomized subjects with spur cell hemolytic anemia: red cells have an expanded surface area with irregular contour and targeting, [98] reflecting accumulation of free cholesterol in the membrane. This extra cholesterol accumulates preferentially in the outer bilayer leaflet, as suggested by findings of increased accessibility of cholesterol to cholesterol oxidase and a selective decrease in lipid fluidity of the outer hemileaflet of the lipid bilayer. [7] [339] [366]

The second step in acanthocyte formation involves red cell remodeling by the spleen. [99] As a result, red cells become spheroidal and the surface projections are considerably longer and more irregular (Fig. 33-13). The end result of these processes is poorly deformable red cells with long bizarre projections that are readily trapped in the spleen,[353] [367] which is often markedly enlarged because of passive congestion due to underlying portal hypertension. Cholesterol also alters membrane permeability and interacts with several membrane skeletal proteins, but the role of these changes in spur cell lesions is unclear. [368] [369]

Clinical Manifestations

Most patients with chronic liver disease have a mild to moderate anemia related to gastrointestinal blood loss, iron and folic acid deficiencies, hemodilution, or as a direct effect of alcohol on red cell precursors. [364] [370] [371] Peripheral blood smears from these patients often reveal target cells that are particularly prominent in obstructive jaundice.

In some patients, particularly those with end-stage liver disease, anemia rapidly worsens and spur cells appear in high percentage in the peripheral blood. [352] [353] [362] [365] This is accompanied by worsening jaundice, rapid deterioration of liver function, hepatic encephalopathy, and hemorrhagic diatheses. A similar clinical syndrome has been described in patients with advanced metastatic liver disease, cardiac cirrhosis, Wilson disease, fulminant hepatitis, and infantile cholestatic liver disease. [372] The development of spur cell hemolytic anemia is an ominous sign in most patients, predicting a survival seldom exceeding weeks to months. [354] In theory, splenectomy could provide a marked improvement, since the spleen is the major sequestration site of nondeformable acanthocytes; in reality, splenectomy is seldom considered because of severity of the underlying liver disease. [98]

Spur cell hemolytic anemia of liver disease must be distinguished from other hemolytic syndromes of liver disease, including (1) transient hemolysis associated with fatty metamorphosis of the liver and hyperglycemia (Zieve syndrome),[364] [373] (2) transient hemolytic anemia with stomatocytosis,[252] [374] (3) hemolytic anemia with rigid spiculated red cells (echinocytes), which can be seen in malnourished patients with severe hypophosphatemia and hypomagnesemia, [375] and (4) a mild hemolysis with some spherocytes, seen in patients with congestive splenomegaly. [364]

Abetalipoproteinemia

Definition and History

Bassen and Kornzweig first described an association of acanthocytosis with atypical retinitis pigmentosa, progressive ataxic neurologic disease, and a celiac disease later attributed to fat malabsorption. [350] [351] Subsequently, several investigators reported a congenital absence of -lipoprotein, accounting for the diverse manifestations of the disorder. [376]

Pathobiology

This autosomal recessive disorder has been detected in people of diverse ethnic backgrounds. [376] The primary molecular defect involves a congenital absence of

-apolipoprotein in plasma.[376] [377] [378] The B apoproteins (B100 and B48) are generated by alternate transcription of a single gene residing on the short

Figure 33-13 Blood film of a patient with liver cirrhosis and spur cell anemia **(A)** before and **(B)** after splenectomy. The latter smear demonstrates the effect of cholesterol acquisition leading to both targeting (indicating increase in surface area) and irregularities in cell contour. The conditioning effect of the spleen (smear A) is demonstrated by the spheroidal shape of the cells and the remodeling of the spicules. *(From Cooper RA, Kimball DB, Durocher JR: The role of the spleen in membrane conditioning and hemolysis of spur cells in liver disease. N Engl J Med 290:1279, 1974, with permission.)*

arm of chromosome 2.[376] Their deficiency is secondary to defective cellular secretion of the apoprotein by liver cells, caused either by aberrant post-translational processing or by defective aposecretion.[376] In some patients, this is due to qualitative or quantitative defects in the microsomal triglyceride transfer protein (MTP), which catalyzes the transport of triglyceride, cholesterol ester, and phospholipid from phospholipid surfaces. [379] [380] [381] [382] MTP is the only tissue-specific component, other than apolipoprotein B, required for secretion of apolipoprotein B-containing lipoproteins. [383] As a result, apoprotein B is absent in plasma, as are the individual lipoprotein fractions that contain this apoprotein. [377] [378] These lipoprotein fractions include chylomicrons and very-low-density lipoproteins that transport triglycerides, as well as the low-density lipoproteins that are products of very-low-density lipoproteins and transport cholesterol. Consequently, preformed triglycerides are not transported from the intestinal mucosa,[377] [384] and they are nearly absent in the plasma. Plasma cholesterol and phospholipids are markedly reduced, with a relative increase in sphingomyelin at the expense of lecithin. [385] [386] [387]

As is the case in acanthocytosis of liver disease, the acanthocytic lesion is acquired from the plasma. Erythrocyte precursors are of normal shape, and the acanthocytic lesion develops as the cells mature and age in the circulation. [377] [388] Normal cells acquire this shape when transfused into the recipient.

The most striking abnormality of red cell membrane lipids involves a net increase in sphingomyelin. Because plasma lipids readily exchange with the lipids of the red cell membrane,[368] it is likely that this change simply mirrors the alterations in plasma lipid composition. In contrast to red cells in spur cell anemia of severe liver disease, the content of membrane cholesterol is normal or only slightly increased. [367] [369] [387] [389]

The role of membrane lipids in the acanthocyte shape transformation was first established by findings of restoration of biconcave shape after extraction of lipids from the cell membrane by detergents.[390] The molecular basis of the acanthocytic shape is unknown, but several indirect observations suggest that it is related to an increase of the surface area of the outer hemileaflet of the lipid bilayer relative to the inner leaflet. In normal red cells, sphingomyelin is present predominantly in the outer bilayer leaflet; thus, it is likely that the extra sphingomyelin is located in the same leaflet. [391] This conclusion is supported by the observation of shape transformation of acanthocytes to discocytes on incubation with chlorpromazine, a compound that preferentially accumulates in the inner half of the lipid bilayer, leading to its expansion, thus normalizing the surface area asymmetry between the two bilayer halves. Furthermore, sphingomyelin is less fluid than the other phospholipids, which presumably accounts for the overall decrease in acanthocyte membrane fluidity. [392]

Several other abnormalities have been noted in abetalipoproteinemia, including a decrease in plasma lecithin cholesterol transferase activity and an increased susceptibility of membrane and plasma lipids to oxidation as a result of malabsorption-induced deficiency of vitamin E. [363] [393] [394] The contributions of these abnormalities to the acanthocyte red cell lesions is unknown. It should be noted that the membrane lesion in abetalipoproteinemia is considerably milder than that in spur cells of severe liver disease, as evidenced by only mild anemia and a moderately shortened red cell half-life of abetalipoproteinemic acanthocytes. [377] [385]

Clinical Manifestations

This autosomal recessive disease may become evident in the first few months of life, manifested by fat malabsorption with normal absorption of other nutrients. [377] [384] Intestinal biopsy is diagnostic, revealing engorgement of mucosal cells with lipid droplets. [352] [377] [384] [395] [396] Other features include retinitis pigmentosa, which may result in blindness, and a progressive ataxia with intention tremors that usually develops at between 5 and 10 years of age, progressing to death in the second or third decade.[396] [397] Occasionally, ocular manifestations are absent. [398] The hematologic manifestations are relatively mild and include mild normocytic anemia with acanthocytosis (5090%) and normal or slightly elevated reticulocyte counts. [350] [351] [376] [377] [388] Occasional patients may have more severe anemia resulting from the nutritional deficiencies (iron and folate) that accompany fat malabsorption. [376] [377] The treatment includes dietary restriction of triglycerides and supplementation with the lipid-soluble vitamins A, K, D, and E. Vitamin E may stabilize or even improve both the retinal and neuromuscular abnormalities. [377]

Autosomal recessive abetalipoproteinemia should be distinguished from the homozygous form of familial hypobetalipoproteinemia. [376] [377] Although the clinical presentation of both disorders is similar, the latter disorder is milder and the parents have occasional acanthocytes on the peripheral blood film and their plasma low-density lipoproteins are decreased. The molecular lesions in familial hypobetalipoproteinemia involve a variety of apoprotein B gene mutations, leading to aberrant apoprotein B gene transcription or translation. [376] [399] [400] [401] [402] [403]

Variable degrees of acanthocytosis without anemia have also been described with isolated deficiency of apoprotein B100. [402] [403] One of two patients reported was ataxic, while the other had some degree of fat malabsorption. Neither had evidence of retinopathy, and plasma triglyceride levels were normal.

Chorea-Acanthocytosis Syndrome

This autosomal recessive syndrome of adult onset is manifest by multiple neurologic abnormalities, including limb chorea, progressive orofacial dyskinesia with tics, tongue-biting neurogenic muscle hypotonia, and atrophy. [354] [355] [404] [405] [406] [407] The hematologic manifestations are minimal and include a variable percentage of acanthocytes on the peripheral blood film without anemia and normal or only slightly decreased red cell survival. [354] [408]

Some patients have developed parkinsonism or pigmentary retinopathy. [409] [410] One patient with acanthocytosis had a stroke-like syndrome due to encephalopathy, chronic lactic acidosis (possibly related to mitochondrial abnormalities of the muscle), and pellagra-like changes on neuropathologic examination.

The mechanism of acanthocytosis in this syndrome is unknown. Studies of plasma and red cell membrane lipids have revealed no abnormalities [354] [404] [408] [411] except for a high content of unsaturated fatty acids, presumably accounting for a reduced red cell membrane fluidity. [411] [412] Additional abnormalities of uncertain significance include an uneven distribution of intramembrane particles, impaired phosphorylation of the erythrocyte actin-bundling protein dematin, and, in one report, altered function and structure of the erythrocyte anion-exchange protein. [413] [414] Recently, a genome screen of 11 families with chorea-acanthocytosis localized a candidate region to a 6-cM region of chromosome 9q21. [415]

McLeod Phenotype

The McLeod syndrome is characterized by a mild compensated hemolytic anemia with a variable percentage of acanthocytes on the peripheral blood film and, in some patients, late-onset myopathy or chorea.[356] [416] [417] The McLeod blood group phenotype is an X-linked anomaly of the Kell blood group system in which red cells, white cells, or both react poorly with Kell antisera. The affected cells lack Kx, the product of the *XK* gene, which appears to be a membrane precursor of the Kell antigens.[418] [419] The *XK* gene encodes a novel 444-amino acid integral membrane transporter. [420] As expected, Kx is defective in McLeod patients.[420] [421] [422] Male hemizygotes who lack Kx have variable acanthocytosis (885%) and mild, compensated hemolysis.

Because of the red cell mosaicism predicted by the Lyon hypothesis of X chromosome inactivation, female heterozygote carriers may have occasional acanthocytes on the peripheral blood film.[417] [423] Lyonized women with more severe symptoms have been described.[421]

The McLeod syndrome has been reported in association with chronic granulomatous disease of childhood, retinitis pigmentosa, and Duchenne muscular dystrophy.[423] [424] [425] This association is due to the close proximity of the genetic loci for the above disorders in the p21 region of the X chromosome (Xp21),

suggesting the occurrence of variable manifestations due to contiguous gene syndromes. [424] [425] [426] [427] [428] This may explain the occasional findings of either echinocytes or stomatocytes in Duchenne dystrophy, or a choreiform disorder in some subjects with McLeod phenotype. [429] [430] [431]

The Kell antigen appears to consist of two protein components: a 37-kd protein that carries the Kx antigen, a precursor molecule necessary for the Kell antigen expression, and a 93-kd protein that carries the Kell blood group antigen. [418] [432] Red cells with the McLeod phenotype have no detectable Kx antigen, and they have a marked deficiency of the 93-kd protein that carries the Kell antigen. McLeod red cells should be distinguished from Kell null (K_0) red cells, which have a normal shape. In K_0 cells, only the Kell antigen carrying 93-kd glycoprotein is absent, while these cells have twice the amount of the Kx antigen. [418] As in the other acanthocytic disorders, the surface projections of acanthocytes may be related to asymmetry of the surface area of the two lipid bilayer hemileaflets, as indicated by correction of the acanthocytosis by agents that expand the inner lipid layer, [433] [434] as well as the finding of an increased rate of exchange of phosphatidylcholine (localized preferentially in the outer lipid hemileaflet) with an exogenous source. [391] [435] Studies of membrane lipid and protein composition, membrane fluidity, and intracellular enzyme and ATP levels were all normal. [430] [436] In contrast, abnormalities were found in the phosphorylation of certain membrane proteins and phospholipids, in the density of intramembrane particles, and in water permeability, along with reduced deformability and mechanical stability of red cells, but the contribution of these abnormalities to the cell lesion is unknown. [437] [438]

Acanthocytosis in Other Conditions

Two of eight subjects carrying the In phenotype, characterized by decreased expression of the Lutheran P1, I, and Aua blood group antigens, were reported to have acanthocytes on their peripheral blood smears. [439] In one report, a dominantly inherited acanthocytosis was found in association with structural alterations of the anion channel protein involving increased molecular size, restricted rotational diffusion, and a decrease in high-affinity binding sites for ankyrin. [440]

Acanthocytes have also been noted in malnourished patients, including those with anorexia nervosa and cystic fibrosis. In these patients, red cell shape normalizes after restoration of the nutritional status. [357] [359] [367] Likewise, a small number of cells with long spicules resembling acanthocytes are found in hypothyroidism, [360] after splenectomy, [361] and in myelodysplasia.

Differentiation of Acanthocytes from Other Spiculated Red Cells

Echinocytes (Burr Cells)

In contrast to acanthocytes, echinocytes, also called burr cells, have rather uniform surface projections. Although early echinocytic forms have a regularly scalloped cell contour, advanced forms of echinocytes have a spheroidal shape and the surface projections appear as short, narrow spikes (Fig. 33-12 (Figure Not Available)). Although the finding of echinocytes on a peripheral blood film is often an artifact related to blood storage, contact with glass, or an elevated pH, [349] [361] several hemolytic anemias have been reported in association with echinocytosis on peripheral blood films. These conditions include mild hemolytic anemia in long-distance runners[441] and in patients with hypomagnesemia and hypophosphatemia (presumably because of decreased intracellular ATP stores), [375] uremia due to an unknown plasma factor,[442] and pyruvate kinase deficiency. [361]

Inspection of wet blood preparations (but not dried blood films) reveals echinocytosis in most patients with liver disease. [443] In contrast to spur cells in patients with severe liver disease, these echinocytes have a normal cholesterol content, and the molecular abnormality may be related to the binding of abnormal echinocytogenic high-density lipoproteins to the red cell surface. [443]

The mechanisms of echinocytosis in these diverse disorders are likely to be heterogeneous, as suggested by findings that many diverse factors, such as exposure of red cells to certain drugs, calcium loading, or ATP depletion, can induce the transformation of discocytes to echinocytes in vitro. [444] [445] [446] However, in vitro studies of the discocyte-echinocyte-stomatocyte equilibrium have suggested a possible common denominator. As discussed in the Introduction, the lipid bilayer of normal red cells is asymmetric in lipid composition: the outer half of the lipid bilayer is relatively enriched in sphingomyelin and phosphatidylcholine, whereas the inner half is preferentially enriched in the negatively charged phosphatidylserine and phosphatidylethanolamine. [391] [435] Agents that preferentially bind to one or another class of these phospholipids dramatically influence red cell shape. Consequently, agents that preferentially accumulate in the outer half of the red cell lipid bilayer, expanding this lipid bilayer, produce an echinocytic shape, presumably by creating an asymmetry between the two surface areas of the two halves of the lipid bilayer (see Fig. 33-2). Conversely, agents that asymmetrically expand the inner half of the lipid bilayer, such as chlorpromazine, lead to stomatocytic shape transformation. [11] In the case of echinocytes produced by ATP depletion or calcium loading, the altered phospholipid distribution between the two bilayer hemileaflets may be a consequence of calcium-induced phospholipid scrambling or a decrease in the activity of aminophospholipid translocase, an ATP-dependent enzyme that actively translocates aminophospholipids from the outer leaflet to the inner hemileaflet. [12]

Keratocytes, Bizarre Poikilocytes, and Schistocytes

Mechanical trauma of circulating red cells has occasionally produced bizarre shapes resembling acanthocytes, such as cells with horny projections (keratocytes). Some acanthocyte-like cells are also seen in splenectomized HE and HS subjects. Similar shape changes are seen in heated red cells, in which spectrin has been damaged by thermal denaturation, suggesting that these cells are bizarre poikilocytes rather than true acanthocytes. [210] Similar shape abnormalities have been described as an asymptomatic trait (Woronets trait). [447]

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RED CELL MEMBRANE DISORDERS MANIFESTED BY TARGET CELL FORMATION

The common feature of target cells is an increase in the ratio of the cell surface area to cell volume. In microcytic red cells of patients with various forms of thalassemia and hemoglobinopathies, the increased surface/volume ratio, and consequently the target cell shape, reflects, at least in part, the relative abundance of cell-surface area. In liver disease and other disorders discussed below, the target cell formation reflects an absolute expansion of the cell-surface area because of a net accumulation of membrane phospholipids and cholesterol.

Liver Disease

The presence of target cells in association with either normal or slightly increased cell volume is characteristically found in patients with obstructive jaundice, including various forms of liver disease associated with intrahepatic cholestasis. [364] [368] These target cells have a normal survival in the peripheral circulation and do not typically account for the anemias often encountered in patients with liver disease.

In these patients, target cell formation is a consequence of a net uptake of both free cholesterol and phospholipids into the red cell membrane from the plasma because of abnormalities in the cholesterol/phospholipid/protein ratios of low-density lipoproteins. [364] [368] Target cells have a decreased osmotic fragility, as the excess of membrane surface area leads to an increase in the critical hemolytic volume.

Lecithin Cholesterol Acyltransferase Deficiency

The lecithin cholesterol acyltransferase (LCAT) enzyme catalyzes the transfer of fatty acids from phosphatidylcholine to cholesterol. [448] It circulates in plasma as a complex with components of high-density lipoproteins. LCAT deficiency is a rare autosomal dominant disorder manifested by hyperlipidemia, premature atherosclerosis, corneal opacities, chronic nephritis, proteinuria, mild anemia, and the presence of target cells on the blood film. The anemia is due to mild hemolysis together with a diminished compensatory erythropoiesis. [448] As in obstructive jaundice, the target cells in LCAT deficiency have a marked increase in both cholesterol and phospholipids. In addition, the membrane phosphatidylcholine is increased at the expense of sphingomyelin and phosphatidylethanolamine. [448] [449] Bone marrow aspiration and biopsy reveals the presence of sea blue histiocytes. Analysis of plasma lipoproteins reveals multiple abnormalities secondary to the underlying enzyme deficiency. Inherited LCAT deficiency should be distinguished from an acquired deficiency of this enzyme, which is found in patients with severe liver disease. [363] [393]

STOMATOCYTOSIS AND RELATED DISORDERS

Stomatocytes were first described in a girl with dominantly inherited hemolytic anemia. [450] On blood films, her red cells contained a wide transverse slit or stoma (Fig. 33-14). In a three-dimensional view, these cells have a shape of a cup or a bowl. [451] The slitlike appearance is an artifact that results from folding of the cells during blood smear preparation.

Stomatocytes are seen in a variety of acquired and inherited disorders. The latter are often associated with abnormalities in red cell cation permeability that lead to changes in red cell volume, which may be either increased (hence the designation hydrocytosis) [452] [453] [454] or decreased (dessicocytosis or xerocytosis), [455] or, in some cases, near normal.

There is no unifying theory to explain this morphologic abnormality. In vitro, stomatocytes can be produced by drugs that preferentially intercalate into the inner half of the asymmetric lipid bilayer, expanding its surface area relative to that of the outer half of the bilayer. [11]

Hereditary StomatocytosisHydrocytosis

Hereditary hydrocytosis designates a seemingly heterogeneous group of hereditary hemolytic anemias that are transmitted in an autosomal dominant mode in most patients. [453] [456] [457] [458] The disorder is characterized by a moderate to severe hemolytic anemia with 1030% stomatocytes, an elevated MCV, and a reduced MCHC. Osmotic fragility of red cells is markedly increased, as some of the swollen red cells approach their critical

Figure 33-14 Peripheral blood smear of a patient with hereditary **(A)** xerocytosis (dessicocytosis) and **(B)** stomatocytosis (hydrocytosis). *(From Lande and Mentzer,[452] with permission.)*

hemolytic volume. For unexplained reasons, red cell membrane lipids and, consequently, membrane surface area are also increased, [454] but this increase in surface area is insufficient to correct the osmotic fragility of the red cells. Red cell deformability is decreased. [454] [459]

The principal cellular lesion involves a marked increase in intracellular sodium and water content with a mild decrease in intracellular potassium as a result of a marked sodium influx into the red cells. [452] [454] [456] [457] [458] [459] Despite a marked compensatory increase in active transport of sodium and potassium by the Na^+/K^+-ATPase (which normally maintains the low sodium and high concentrations in the cells) and an ensuing increase in glycolysis, the pump hyperactivity is unable to compensate for the vastly increased sodium leak. [408] [452] [454] [456] [457] [458] [459] [460] [461] The molecular basis of this permeability defect is unknown. In vitro, the defect is corrected by a bifunctional cross-linking agent, [462] [463] suggesting the involvement of a membrane protein. Analysis of membrane protein was normal in some patients, [452] [454] [456] [457] [458] [459] but in others the presence of a 25-kd protein of an unknown function was reported. [464] However, this protein was also present in subjects with other disorders who had undergone splenectomy and who had elevated reticulocyte counts. [464] Band 7.2b (stomatin), an integral membrane protein, has been reported to be decreased or absent from the erythrocyte membranes of affected patients. [465] [466] [467] [468] This protein has now been cloned and sequenced, but no mutations have been found in two unrelated patients deficient in this protein. [469] [470]

BLOOD FILM EVALUATION: A CLUE TO PATHOPHYSIOLOGY AND DIAGNOSIS OF A RED CELL MEMBRANE DISORDER

Advances in the understanding of the structure and function of red cell membrane have highlighted the importance of blood film evaluation in the diagnosis of a red cell membrane disorder. Red cell shape abnormalities may provide clues both to disease pathobiology and to diagnosis (see table on opposite page).

MICROSPHEROCYTES

The finding of microspherocytes indicates a deficiency of red cell surface area. In autoimmune hemolytic anemia, the IgG-coated red cells attach to macrophages, which remove parts of the membrane material from the cells. In hemolytic anemias associated with the presence of Heinz bodies, membrane material is removed together with the membrane-associated Heinz bodies as a result of the pitting function of the spleen. In the context of a lifelong hemolytic anemia or a positive family history, the finding of spherocytes leads to the diagnosis of hereditary spherocytosis (HS). The surface area deficiency of HS red cells reflects a lipid loss resulting from the underlying deficiency of spectrin or other major membrane proteins.

ELLIPTOCYTES, POIKILOCYTES, AND FRAGMENTS

Elliptocytes and poikilocytes are found both in hereditary elliptocytosis (HE) and in many acquired conditions, including iron deficiency, megaloblastic anemias, myelofibrosis, myelophthisic anemias, and myelodysplastic syndromes. The molecular basis of acquired elliptocytosis is unclear; in our experience, laboratory evaluation of the underlying molecular defect yields negative results. In contrast, many molecular defects have been detected in HE, and the laboratory evaluation is useful both in establishing the diagnosis and in detecting asymptomatic carriers. In more severe forms of the disorder, elliptocytosis is invariably associated with the presence of poikilocytes and red cell fragments. These findings indicate that the membrane is markedly unstable, as in hemolytic forms of HE or hereditary pyropoikilocytosis (HPP), in which a skeletal protein defect alters the two-dimensional integrity of the membrane skeleton. In severe hemolytic forms of HE and in HPP, the mean corpuscular volume is markedly reduced, since many red cells circulate in the form of fragments, which implies a severe defect in the stress-supporting horizontal interactions of the membrane skeleton. The red cell size distribution index is high, indicating a considerable size heterogeneity of the circulating red cell fragments. Such fragments differ from schistocytes produced by a mechanical trauma to the red cells: the former fragments are round, often seen budding from the red cell membrane, whereas schistocytes appear as cuts with sharp edges and often bizarre shapes, while the remaining red cells are relatively intact. Elliptocytes are also seen in the peripheral blood smears of some patients with -thalassemia syndromes.

ECHINOCYTE/DISCOCYTE/STOMATOCYTE EQUILIBRIUM

Echinocytescells with fine, multiple, and uniform spiculesare found in many conditions, including malnutrition associated with mild hemolysis due to hypomagnesemia and hypophosphatemia, uremia, and hemolytic anemia in long-distance runners. They are also a common artifact of elevated pH, blood storage, or contact with glass. Although the mechanism of echinocyte formation in vivo is not clear, studies in vitro suggest that echinocytosis is caused by an expansion of the outer hemileaflet of the lipid bilayer relative to the inner bilayer hemileaflet. By contrast, expansion of the inner lipid bilayer leaflet relative to the outer leaflet leads to cup shape formation (stomatocytosis) and surface invaginations. Stomatocytosis is seen either as an inherited hemolytic anemia associated with cell volume changes or as an acquired condition, particularly in alcohol-induced liver disease.

ACANTHOCYTES

In contrast to echinocytes, acanthocytes contain long, irregular protrusions that suggest both a net accumulation of lipids in the red cell membrane and an asymmetry between the two lipid bilayer hemileaflets with a preferential expansion of the outer hemileaflet. In the spur cell hemolytic anemia associated with severe liver disease, these spurs reflect a net gain of free (unesterified) cholesterol that preferentially accumulates in the outer half of the lipid bilayer. Because of the conditioning effect of the spleen, the cells are also spheroidal, indicating a secondary loss of surface area, yet their surface protuberances are very prominent. In abetalipoproteinemia, acanthocytes contain excess sphingomyelin in the outer lipid bilayer hemileaflet. The mechanisms of acanthocytosis in other conditions (chorea-acanthocytosis syndrome, malnutrition, hypothyroidism, and McLeod phenotype) are unknown.

TARGET CELLS

In contrast to spherocytes, target cells have either a relative or an absolute excess of surface for a given red cell volume. A relative excess of the surface area is typically found in microcytic red cells in most patients with thalassaemia minor and some hemoglobinopathies (hemoglobin C, D, and E). In red cells having normal or even slightly increased volume, targeting indicates an absolute excess of surface area. For example, in a patient with liver disease or obstructive jaundice, red cell surface area is expanded because both phospholipids and cholesterol are transferred from the abnormal plasma lipoproteins into the red cell membrane.

DEHYDRATED RED CELLS

Changes in red cell density, as reflected by increased mean corpuscular hemoglobin concentration, can likewise be appreciated on the blood film. The staining intensity of intracellular hemoglobin of spherocytes, regardless of the mechanism of their formation, is greater than that of normal discocytes, because the hemoglobin layer that is in the optical path between the light source and the observers eye is thicker than in the normal discocyte. However, the staining intensity of intracellular hemoglobin and, hence, the density of hereditary spherocytes are typically greater than that of spherocytes in autoimmune hemolytic anemia, because hereditary spherocytes are also somewhat dehydrated. In some inherited disorders associated with cellular dehydration (xerocytosis), cells may be either stomatocytic or appear as bizarre targets with intracellular hemoglobin puddled into distinct clumps.

Peripheral Blood Film Evaluation in a Patient with a Red Cell Membrane Disorder

Shape	Pathobiology	Diagnosis
Microspherocytes	Loss of membrane lipids leading to a reduction of surface area resulting from deficiencies of spectrin, ankyrin, or band 3 and protein 4.2	HS
	Removal of membrane material from antibody-coated red cells by macrophages	Immunohemolytic anemias
	Removal of membrane-associated Heinz bodies, with the adjacent membrane lipids, by the spleen	Heinz body hemolytic anemias
Elliptocytes	Permanent red cell deformation resulting from a weakening of skeletal protein interactions (such as the spectrin dimer-dimer contact). This facilitates disruption of existing protein contacts during shear stress induced elliptical deformation. Subsequently, new protein contacts are formed that stabilize the elliptical shape	Mild common HE
	Unknown	Iron deficiency, megaloblastic anemias, myelofibrosis, myelophthisic anemias, myelodysplastic syndrome, thalassemias
Poikilocytes/fragments	Weakening of skeletal protein contacts resulting from skeletal protein mutations	Hemolytic HE/HPP
	Unknown	Iron deficiency, megaloblastic anemias, myelofibrosis, myelophthisic anemias, myelodysplastic syndrome, thalassemias
Schistocytes, fragmented red cells	Red cells torn by mechanical trauma (fibrin strands, turbulent flow)	Microangiopathic hemolytic anemia associated with disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, vasculitis, heart valve prostheses

Schistocytes, fragmented red cells	Red cells torn by mechanical trauma (fibrin strands, turbulent flow)	Microangiopathic hemolytic anemia associated with disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, vasculitis, heart valve prostheses
Acanthocytes	Uptake of cholesterol and its preferential accumulation in the outer leaflet of the lipid bilayer	Spur cell hemolytic anemia in severe liver disease
	Selective accumulation of sphingomyelin in the outer lipid leaflet	Abetalipoproteinemia
	Unknown	Chorea-acanthocytosis syndrome, malnutrition, hypothyroidism McLeod phenotype
Echinocytes	Expansion of the surface area of the outer hemileaflet of lipid bilayer relative to the inner hemileaflet	Hemolytic anemia associated with hypomagnesemia and hypophosphatemia in malnourished patients, pyruvate kinase deficiency; in vitro, artifact of low blood storage (ATP depletion), contact with glass or elevated pH
	Unknown	Hemolysis in long-distance runners, renal failure
Stomatocytes	Expansion of the surface area of the inner hemileaflet of the bilayer relative to the outer leaflet	Exposure of red cells to cationic anesthetics in vitro; in vivo, the drug concentrations may not be sufficient to produce similar effect
	Unknown	Alcoholism, inherited disorders of membrane permeability (hereditary stomatocytosis)
Target cells	Absolute excess of membrane lipids (both cholesterol and phospholipids: symmetric lipid gain), followed by an increase in cell surface area	Obstructive jaundice, liver disease with intrahepatic cholestasis
	Relative excess of surface area because of a decrease in cell volume	Thalassemias and some hemoglobinopathies (C,D,E)

Splenectomy may improve, but not fully correct, the hemolysis.[454] [459] [460] In some patients, splenectomy may be deleterious or even contraindicated (see following discussion).[471] [472] [473]

Hereditary Xerocytosis and the Intermediate Syndromes

Hereditary xerocytosis or dessicocytosis describes an autosomal dominant hemolytic anemia characterized by red cell dehydration and decreased osmotic fragility. [455] [474] [475] [476] [477] [478] Affected individuals have characteristically moderate to severe hemolysis with an increased MCHC, reflecting cellular dehydration. Hydrops fetalis with fetal anemia has been reported in a number of xerocytosis kindreds.[476] Frequently, the MCV is mildly increased. In Coulter-type electronic counters, the conversion of pulse height (from the resistance of a cell passing through an electric field) to a cellular volume is dependent on cell shape. Xerocytes do not deform to the same degree as normal cells, which causes the MCV to be about 10% too high. The peripheral blood film does not always reveal stomatocytes (which are more prominent on wet films), but frequently target cells, dessicocytes, and spiculated cells are seen. In some of the cells, hemoglobin is concentrated (puddled) in discrete areas on the cell periphery (Fig. 33-14).

The mechanism of cellular dehydration is unclear and complex, involving a net potassium loss from the cells that is not accompanied by a proportional gain of sodium.[455] [474] [475] [477] Consequently, the net intracellular cation content and cell water are decreased. In some reports, a decrease in red cell 2,3-DPG has also been noted.[478] [479] Analysis of membrane lipids and proteins failed to detect any abnormalities except for an increase in membrane-associated glyceraldehyde-3-phosphate dehydrogenase.[480] This enzyme binds to the band 3 protein.[481]

Some of the reported cases of hereditary stomatocytosis share features of both hereditary stomatocytosis and xerocytosis. Lux and Becker categorized these disorders as intermediate syndromes.[305] These patients characteristically have both stomatocytes and some target cells on the peripheral blood smear. Osmotic fragility is either normal or slightly increased. Sodium and potassium permeability is somewhat increased, but the intracellular cation concentration and the red cell volume are either normal or slightly reduced. These cells were reported to have subnormal glutathione content. [482] [483] In two other reported patients, red cells underwent in vitro hemolysis at 5°C;[483] [484] hence, the disorder was designated cryohydrocytosis. In one patient, hemolysis was worsened by swimming, and the red cells hemolyzed when exposed to shear stress.[485]

Several investigators have also reported a dominantly inherited hemolytic anemia with stomatocytosis, occasional target cells, spherocytes, and a decreased osmotic fragility in which the main red cell membrane abnormality involved a nearly 50% increase in phosphatidylcholine and a corresponding decrease in phosphatidylethanolamine. [452] [486] [487] [488] In wet preparations, 30% of the cells were stomatocytes.[487] The molecular basis of this syndrome is unclear. Because abnormalities in membrane phospholipid composition have not been systematically investigated, it is uncertain whether the disorder represents a distinct disease entity.[489] For additional details on this rare group of disorders, the reader is referred to reviews in the literature. [452] [488]

The results of splenectomy in this group of disorders are variable. In some patients the hemolytic anemia is improved, although often not fully corrected, by splenectomy,[454] [459] [460] [475] [479] while in others the severity of the hemolysis is unchanged. [490] Splenectomy should be carefully considered in patients with hereditary stomatocytosis. Several patients with stomatocytosis (both hydrocytosis and xerocytosis) have developed hypercoagulability after splenectomy, leading to catastrophic thrombotic episodes.[472] [473] In vitro, stomatocytic erythrocytes from an individual with xerocytosis who had undergone splenectomy demonstrated increased endothelial adherence when compared with stomatocytic erythrocytes from unsplenectomized family members without hypercoagulability. [473] In one hypercoagulable stomatocytosis patient, pentoxifylline decreased red cell adherence. [473] Fortunately, the majority of persons with hereditary stomatocytosis are able to maintain an adequate hemoglobin level, so that splenectomy is not required.

Rh Deficiency Syndrome

Rh deficiency syndrome designates rare individuals who have either absent (Rh_{null}) or markedly reduced (Rh_{mod}) Rh antigen expression, mild to moderate hemolytic anemia associated with the presence of stomatocytes, and occasional spherocytes on the peripheral blood film. [430] [491]

The Rh antigens are present in about 20,00030,000 copies per cell and reside on minor transmembrane proteins with an electrophoretic mobility of 2833 kd on SDS-PAGE.[492] Immunoprecipitation of Rh polypeptides by c, D, and E antigen-specific antibodies followed by two-dimensional mapping of the iodine-labeled chymotryptic peptides indicates that these proteins are distinct but closely related, a conclusion further verified by analysis of their cDNA. Furthermore, recent studies of the Rh gene locus are consistent with the existence of two closely linked genes, one encoding the D polypeptide and the other encoding the Cc, Ee proteins, the antigenic expression of which is a consequence of alternate splicing of their pre-mRNA. [493] [494]

Prediction of their structure from hydropathy plots revealed that the Rh proteins span the lipid bilayer multiple times, with the C- and N-termini located at outer and inner surfaces, respectively.[493] The Rh proteins are heavily palmitoylated, forming dimers or oligomers in solutions and, presumably, in the membrane as well. They are linked to the red cell membrane skeleton,[495] [496] but their function is unknown. A previous suggestion that Rh proteins may be involved in the translocation of aminophospholipids across the membrane has been excluded by the finding of normal phosphatidylserine transport in these cells. [497] In Rh_{null} human red cells, two proteins (32 kd and 34 kd) containing extracellular thiol groups are absent.

The genetic basis of the Rh deficiency syndrome is heterogeneous, and at least two groups can be defined. The amorph type is thought to be related to defects

involving the *RH30* locus encoding the RhD and RhE polypeptides, but its precise genetic basis is unknown. [493] [498] The regulatory type of Rh_{null} and Rh_{mod} phenotypes results from suppressor or modifier mutations independent of the *RH30* locus. Abnormalities of *RH50* have recently been described in Rh_{null} individuals. These have included deletion, splicing, and missense mutations of the Rh50 glycoprotein gene. [499] [500] [501]

Red cells of some Rh_{null} patients have increased osmotic fragility, reflecting a marked reduction in membrane surface area. [502] These cells are also dehydrated, as indicated by decreased cell cation and water content and increased cell density. [502] The potassium transport and the Na^+/K^+ pump activity are increased, possibly because of reticulocytosis. [503] [504] Hemolytic anemia is improved by splenectomy. [491] Additional serologic aberrations involve a weakened expression of Ss and U antigens, [505] [506] which, unlike the Rh antigen system, reside on one of the transmembrane glycoproteins, glycophorin B, [507] the level of which is reduced in Rh_{null} cells by 30%, possibly because glycophorin B is, in part, present as a complex with the Rh proteins.

Familial Deficiency of High-Density Lipoproteins

Severe deficiency or absence of high-density lipoproteins leads to accumulation of cholesteryl esters in many tissues, leading to the clinical findings of large orange tonsils and hepatosplenomegaly. [449] Reported hematologic manifestations include a moderately

severe hemolytic anemia with stomatocytosis. [508] Membrane lipid analyses revealed a low free cholesterol content, leading to a decreased cholesterol/phospholipid ratio and a relative increase in phosphatidylcholine at the expense of sphingomyelin. [508]

Acquired Stomatocytosis

Stomatocytes have been noted in diverse acquired conditions, including neoplasms, cardiovascular and hepatobiliary disease, alcoholism, and therapy with drugs, some of which are known to be stomatocytogenic in vitro. [509] [510] In some of these conditions, the percentage of stomatocytes on the peripheral blood smear may approach 100%. However, the clinical significance of the above observation is unclear because stomatocytes are absent in most patients with the conditions listed. Furthermore, some stomatocytes may be found in normal individuals (35%). [511] [512] The most consistent association is that of stomatocytosis and heavy alcohol consumption. [511] [512] Stomatocytosis has also been reported in Mediterranean immigrants in Australia. Its cause is unknown, but it may be related to alcoholism. [513] [514]

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Chapter 34 - Autoimmune Hemolytic Anemias

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INTRODUCTION

The autoimmune hemolytic anemias are a group of disorders in which autoantibodies against antigens on the erythrocyte membrane cause a shortened red blood cell (RBC) life span. The antierythrocyte autoantibodies of these diseases fall into three generic types, each of which has distinctive serologic properties: (1) cold agglutinins, almost always of the IgM isotype, clump RBCs at cold temperatures; (2) the IgG Donath-Landsteiner antibody fixes to RBC membranes in the cold and activates the hemolytic complement cascade when the cells are warmed to 37°C; and (3) IgG warm autoantibodies bind to erythrocytes at 37°C but fail to agglutinate the cells. Each of these three categories of autoantibodies delineates one or more characteristic clinical disorders, known collectively as autoimmune hemolytic anemia. Autoimmune hemolytic anemia may occur as a primary (idiopathic) disorder, may coexist with another disease (secondary autoimmune hemolytic anemia), or may follow administration of certain drugs (Table 34-1). The nomenclature of these conditions is imprecise and sometimes confusing. This chapter refers to the hemolytic diseases associated with warm-reactive IgG autoantibodies as autoimmune hemolytic anemia, to the hemolytic anemias caused by cold-reactive IgM autoantibodies as cold agglutinin disease, and to the syndromes associated with the Donath-Landsteiner antibody as paroxysmal cold hemoglobinuria.

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HISTORICAL BACKGROUND

Any reader interested in the origins of hematology should seek out Dacies superb review of the history of autoimmune hemolytic anemia. [1] Much of this section follows his scholarly account.

TABLE 34-1 -- Classification of Autoimmune Hemolytic Anemia

Warm autoimmune hemolytic anemia
Primary (idiopathic)
Secondary
Lymphoproliferative diseases
Connective tissue diseases
Miscellaneous diseases
Drugs
Hapten type
Immune complex type
Autoantibody type
Cold autoimmune hemolytic anemia
Primary (idiopathic)
Secondary
Lymphoproliferative diseases
Infections
Mycoplasma pneumoniae
Infectious mononucleosis
Other
Miscellaneous diseases
Paroxysmal cold hemoglobinuria
Associated with tertiary syphilis
Postviral infection (self-limited)

An earlier historical analysis by Dameshek and Schwartz is a classic. [2] Rosenfields personal narrative of the history of immunohematology is also strongly recommended.[3]

Paroxysmal cold hemoglobinuria was the first recognized form of hemolytic anemia, probably because its clinical manifestations are so striking; the principal sign of the disease, the passage of black urine after exposure to the cold, can hardly be ignored. Descriptions of patients with attacks of hemoglobinuria after exposure to cold temperatures, including the case of a 10-year-old boy with *chromaturie*,[4] began to appear between 1854 and 1865 in the medical literature. [4] [5] [6] In 1879, Rosenbach reported inducing hemoglobinuria by immersing his patients feet in ice water; [7] 2 years later, Ehrlich reported his observation of hemolysis and erythrophagocytosis in blood obtained from a chilled finger of a patient with the disease. [8]

The association of paroxysmal cold hemoglobinuria with syphilis was first noted in 1884 by Götze, [9] but nothing else was known of the cause of the disease until 1904, when Donath and Landsteiner published their landmark work. [10] They described three cases of paroxysmal cold hemoglobinuria in which (1) an autolysin bound to the patients RBCs in the cold, and (2) a heat-labile serum factor (now known to be hemolytic complement components) lysed the sensitized erythrocytes when the temperature was raised to 37°C. This classic paper presents the first description of an autoimmune disease.

Less obvious forms of hemolytic anemia were not identified until the end of the 19th century, when Le Gendre [11] and Hayem [12] distinguished a form of jaundice with bile in the plasma, but not in the urine (acholuric jaundice). Widal and Abrami were among the first to recognize congenital and acquired forms of hemolytic anemia; in 1907 they reported autoagglutination of erythrocytes in patients with *lictère hémolytique acquis*.[13] The following year, Chauffard and Troisier [14] described cases of severe hemolytic anemia with serum hemolysins, but progress in delineating autoimmune hemolytic anemia ceased until 1938, when Dameshek and Schwartz revived interest in the topic with their report of similar cases. [15] Dameshek and Schwartz also demonstrated that injection of heterologous antierythrocyte antibodies into guinea pigs induced hemolytic anemia, with spherocytosis and increased osmotic fragility of RBCs, thereby establishing the first experimental model of immune hemolytic anemia.[16] Even so, the idea of an autoimmune form of hemolytic anemia was resisted for several reasons, not the least of which was the difficulty in making the diagnosis. Only cold-reactive antibodies, which directly agglutinate erythrocytes, and rare forms of lytic autoantibodies could be recognized in the laboratory. In 1918, the first example of cold agglutinin disease was described, [17] but the validity of such cases was suspect because cold-reactive autoantibodies were also found in normal serum, an observation made 15 years earlier by Landsteiner. [18] Not until 1937 was it recognized that the serum of patients with cold agglutinin disease had markedly elevated titers of cold agglutinins. [19]

The antiglobulin test, designed to detect nonagglutinating antierythrocyte antibodies, was introduced into clinical medicine by Coombs et al. in 1945. [20] The Coombs test revolutionized immunohematology; within 1 year it was applied to the diagnosis

of autoimmune hemolytic anemia. [21] Ironically, essentially the same procedure, developed in 1908 by Moreschi, [22] was ignored for almost 40 years, perhaps because

Moreschis report dealt with animal RBCs.

In 1954, autoimmune hemolytic anemia in dogs was reported,[23] and in 1958, the first easily bred animal model of the disease, the NZB mouse, was described. [24] This discovery was a turning point in the development of a scientific basis for the study of autoimmunization.

The first recorded attempt to treat acquired hemolytic anemia with splenectomy was carried out in 1911, [25] and in 1950 the beneficial effect of corticotropin on autoimmune hemolytic anemia in two patients with lymphoma was reported by Dameshek[26] and in a 5-year-old girl by Gardner. [27]

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IMMUNOLOGIC MECHANISMS OF RED CELL DESTRUCTION

Immunologic Tolerance

Regardless of their underlying cause, autoimmune hemolytic anemias ultimately result from derangement of the mechanism of immunologic tolerance. An understanding of this mechanism is essential. At the end of its life, when it transforms from a biconcave disk to a sphere, the RBC undergoes the initial steps required to trigger an immune response, namely, phagocytosis and proteolysis by a macrophage. There is no reason to doubt that macrophages produce potentially immunogenic peptide fragments from those worn-out spheres and offer them to lymphocytes. This, after all, is a major function of macrophages, and it applies to virtually any particle or molecule they can ingest and digest. However, the lymphocytes of the immune system specifically ignore the numerous self-antigenic determinants on the RBC membrane. Those same antigens would nonetheless engender an immune response if injected into another person with a different constellation of blood groups. The complex mechanisms that prevent autoimmunization, while simultaneously permitting alloimmunization, are known collectively as immunologic tolerance. The rules governing immunologic tolerance are essential aspects of the mechanisms of immune recognition of erythrocytes.

Owens[28] classic study of the RBCs of dizygotic cattle twins, published in 1945, was the first to uncover evidence of this remarkable aspect of the immune system. Owen observed that such twins had in their circulation a mixture of erythrocytes, some with self blood groups and others with the blood groups of the allogeneic twin. These cattle twins were erythrocyte chimeras, a condition caused by the exchange of hematopoietic stem cells through the vascular anastomoses of the common placenta that nourished both fetuses in utero. The immunologic consequences of chimerism did not escape Owen, who emphasized the lack of any alloantibodies in the twins despite the presence of foreign erythrocytes in their blood.

Owens work was a key element in the theory of immunologic tolerance and was advanced 4 years later by Burnet and Fenner. [29] Their sweeping new idea was that contact with any antigen during embryonic life would result in immunologic tolerance (their original term). They attributed self-tolerance to clonal deletion, the lethal consequence of an embryonic lymphocyte that contacts an antigen. One of the main predictions of this theory was substantiated in 1953 by Billingham et al., [30] who showed that fetal mice injected with foreign cells would later tolerate skin grafts from the donors of those cells. The principle established by that historic experiment has been reaffirmed by recent experiments with transgenic mice, which carry a foreign gene implanted in their germline DNA. A foreign protein encoded by the gene, if expressed early in embryonic life, fails to induce any immune response in the transgenic animal. [31]

The thymus is an important seat of this mechanism (see Chap. 11). There is now convincing evidence that the fetal thymus inactivates T cells with the potential to respond to self antigens.[32] The fetal thymus contains macrophages, epithelial cells, and dendritic cells that not only ingest and degrade (process) antigenic material but also bind the resulting peptide fragments to class II MHC molecules. [33] The latter are membrane glycoproteins required for antigen recognition by T cells; the T-cell receptor for antigen recognizes a configuration consisting of an immunogenic peptide and a portion of the class II MHC glycoprotein (Fig. 34-1).[34] Intrathymic antigen-presenting cells can, for example, ingest and digest RBCs within the thymus and bind degraded fragments of self-hemoglobin to their class II MHC molecules.[35]

Each T-cell clone arising within the thymus expresses its own unique receptor, the result of random recombinations of V and V or V and V gene segments. [32] This stochastic mechanism will inevitably yield clones with receptors capable of recognizing a self-peptide/class II MHC complex. Such clones are destined for deletion from the immune repertoire; the fetal thymus eliminates any thymocyte whose receptor binds with high affinity to an antigen-presenting cell. [36]

Tolerance of Blood Group Antigens

The immune systems solution to the problem of distinguishing self from foreign requires an about-face late in fetal development. The dominant function of the immune system during early embryonic life is the acquisition of immunologic tolerance of the antigens bathing its milieu, but as birth approaches, the fetus begins to acquire the capacity to respond to immunogens. This principle is clearly evident in the case of the ABO blood group antigens, oligosaccharides not only expressed on the membranes of erythrocytes and many other kinds of cells, but also circulating in soluble form in the plasma. The immunodominant sugar of the A antigen is *N*-acetylglucosamine; the B determinant, galactose, differs from the A sugar only by a single acetyl group. The core of transfusion therapy is the infallible ability of the immune system to recognize that minute difference. The extreme rarity of autoimmune hemolytic anemia due to anti-A or anti-B antibodies attests to the deletion from the immune repertoire of lymphocytes with the capacity to produce such autoantibodies. Such clones are probably eliminated or inactivated very early in ontogeny, since the embryo begins to synthesize A or B substances within 5 weeks of its implantation in the uterine wall. [37]

Another telling example of the fetal acquisition of immunologic

Figure 34-1 Antigen-specific receptors. Antigen recognition by immunoglobulin variable regions and T-cell receptors. The antibody molecule typically recognizes a three-dimensional configuration on the antigen, whereas the T-cell receptor binds to a compound structure consisting of a peptide fragment of the antigen (produced as the result of antigen processing by macrophages) and a portion of an MHC structure. $CD4^+$ T cells recognize an antigen fragment plus a class II MHC structure; $CD8^+$ T cells bind to processed peptides plus a class I MHC structure.

Figure 34-2 The I/i blood group system in **(A)** the fetus and **(B)** the adult. The fetus lacks the enzymes (branching enzymes) required to produce the branched forms of ceramide and the I antigen. The unbranched (fetal) and branched (adult) variants of the I antigen are immunochemically distinct structures. Shaded structure represents the erythrocyte membrane. The modified N-terminus of band 3 faces the interior of the RBC. *(From Hakomori,[40] with permission.)*

tolerance is the I/i blood group system, originally discovered by Wiener et al. [38] in a case of cold-reactive autoimmune hemolytic anemia. The serum of their patient contained high titers of an autoantibody with a specificity they denoted I. Its counterpart antigen, i, is typical of fetal erythrocytes (Fig. 34-2).[39] The fetus with I $/i^+$ erythrocytes presumably lacks the enzymatic capacity to convert the unbranched ioligosaccharide to the antigenically different branched oligosaccharide structure of I.[40] Thus, the fetus has no opportunity to acquire immunologic tolerance of the branched I ganglioside, and B-cell clones capable of producing anti-I autoantibodies persist in the immune system throughout life. This explains why Landsteiner so easily found anti-I cold-reactive antibody (cold agglutinins) in the samples of normal serum he tested in 1903. By contrast, he never found anti-A or anti-B autoantibodies when he discovered the ABO blood groups. [41] Further evidence of the failure of the immune system to delete anti-I B-cell clones is the occurrence of monoclonal cold-reactive antibodies in 10% of cases of Waldenström macroglobulinemia; [42] such malignant cells presumably arise from their counterparts in the normal B-cell repertoire.

Self-reactive Lymphocytes

It is highly unlikely that the fetal thymus can purge all self-reactive lymphocytes from the immune system. In the first place, B cells do not differentiate in the thymus and their receptors for antigensurface immunoglobulinsdo not depend on recognition of an antigen-MHC complex. Indeed, self-reactive B cells occur in a surprisingly high frequency; about 1 in 10^4 B cells from healthy young mice produce anti-DNA antibodies.[43] B cells from normal humans, when activated by mitogens in vitro, also produce autoantibodies.[44] [45] Furthermore, it is improbable that all the bodys different proteins, estimated at 100,000 in number, [32] find their way into the fetal thymus. Some, like the I antigen, are expressed only after birth; others are sequestered in the brain and other internal organs. The peripheral lymphocyte population must, therefore, contain at least some antiself T cells and B cells. The existence of such cells accounts for the success of the classic experiment of Rose and Witebsky, [46] who were able to induce autoimmune thyroiditis in rabbits by immunization with rabbit thyroglobulin. If all lymphocytes capable of responding to self-thyroglobulin had been deleted, the animals would have been unresponsive to the injected thyroglobulin. With respect to autoimmune hemolytic anemia, the role of helper T cells with anti-RBC autospecificity has been suggested by a recent study demonstrating the existence of T-cell epitopes on Rhesus polypeptides corresponding to antigens commonly involved in autoimmune hemolytic anemia.[47]

Immunoregulation

Growing evidence shows that the antiself immune repertoire is normally constrained by suppressor T cells. In humans, the mature T-cell population consists of $CD4^+$ helper cells, which augment the activity of B cells, and $CD8^+$ cytotoxic and suppressor cells. Two subsets of $CD4^+$ cells have been identified: helper-inducer T cells, identified by the 4B4 membrane glycoprotein, and suppressor-inducer T cells, marked by the 2H4 (or CD45R) glycoprotein. [48] The latter regulate a subset of $CD8^+$ T cells that have been shown to suppress antibody formation in vitro. [49] An imbalance in suppressor (or suppressor-inducer) T cells is a plausible explanation of the origins of some autoimmune diseases.[50] How such an impairment occurs in the first place is enigmatic, but clinical and experimental findings support the concept. Two children with autoimmune hemolytic anemia and a profound deficiency of suppressor cells, probably due to a thymic defect, have been reported. [51] Removal of the thymus from mice within a few days after birth can result in a variety of T-cell-mediated organ-specific autoimmune diseases. The thymectomy apparently prevents entrance of newly formed suppressor cells into the periphery; administration of T cells from intact mice prevents the autoimmune process. [52] Cyclosporine, a potent immunosuppressive drug, can have the paradoxical effect of causing autoimmune diseases,[53] [54] [55] [56] presumably by disrupting immunoregulatory T-cell populations.

Antigen-independent polyclonal activation of self-reactive B cells, another possible mechanism of autoimmunization, could result from an inherent abnormality of B cells, as has been demonstrated in some patients with systemic lupus erythematosus (SLE),[57] or from the excessive production of B-cell-stimulatory lymphokines (e.g., interleukin [IL]-6) by T cells. The case of a patient with an IL-6-secreting atrial myxoma who produced large amounts of immunoglobulins and autoantibodies [58] is instructive, as is the development of thyroiditis and antithyroglobulin autoantibodies in cancer patients treated with large doses of IL-2. [59] [60] The hypergammaglobulinemia and production of autoantibodies in parasitic diseases [61] and in patients with human immunodeficiency virus (HIV) infection[62] [63] [64] [65] probably also originate as a consequence of polyclonal B-cell activation.

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ANIMAL MODELS OF AUTOIMMUNE HEMOLYTIC ANEMIA

Evidence for Genetic Factors

NZB Mice

The inbred NZB mouse is genetically programmed to develop autoimmune hemolytic anemia at around the age of 68 months (the life span of a normal mouse is 2 years). Antierythrocyte

Figure 34-3 Development of autoimmune hemolytic anemia in NZB mice. Splenic enlargement is due to the effects of the hemolytic anemia and also to marked polyclonal proliferation of $CD5^+$ B cells. As the animals age, the polyclonal population of $CD5^+$ B cells converts to a monoclonal population (see text).

autoantibodies begin to appear at around the age of 3 months, and by 9 months the direct antiglobulin test (DAT) is positive in 6080% of the animals. Typical signs of hemolytic anemia develop, with reticulocytosis, spherocytosis, a shortened RBC survival time, and splenomegaly (Fig. 34-3).[66] The autoantibodies are of two types: anti-X, an IgG autoantibody, reacts with the X antigen exposed on the erythrocyte membrane and causes the hemolytic anemia; anti-HB, an IgM autoantibody, reacts with a hidden phosphorylcholine erythrocyte antigen revealed by in vitro enzyme (bromelain) treatment of the RBCs. Both the X and the HB antigens circulate in the plasma.[67] Anti-HB antibodies, found in virtually all normal strains of mice, are not pathogenic. They are produced by $CD5^+$ B cells;[68] their high levels in NZB serum undoubtedly stem from the marked increase in $CD5^+$ B cells in NZB mice.[69] Other antierythrocyte autoantibodies in NZB mice include an IgG antibody with probable specificity for spectrin,[70] and cold-reactive antibodies with a resemblance to anti-I autoantibodies in humans. [71]

The F_1 progeny of crosses between NZB and NZW mice or between NZB and SWR mice do not develop autoimmune hemolytic anemia, and the antiglobulin test is negative. Instead, lupus nephritis and high levels of anti-DNA antibodies dominate the disease in these animals. [72] Mice of the SWR strain are normalthey never develop any evidence of SLE or any other autoimmune disease. Yet in the (NZB × SWR) cross, a substantial fraction of the anti-DNA antibodies arises from genes inherited from the SWR parent.[73] In the (NZB × SWR)F_1 hybrid, therefore, the inherent immunologic abnormalities of the NZB strain [66] can induce pathogenic autoantibodies that originate from the genome of a normal animal. The shift from autoimmune hemolytic anemia in the NZB parent to nephritis in the F_1 hybrid demonstrates that genetic factors influence not only susceptibility to autoimmunization but also its clinical manifestations.

Graft-versus-Host Disease: Graft-versus-Host Model

Conditions are ripe for a graft-versus-host reaction when foreign lymphocytes are injected into hosts that cannot reject them. In the classic graft-versus-host reaction, the grafted T lymphocytes respond to MHC antigens of the host. A chronic graft-versus-host reaction can occur in F_1 hybrid mice following injection of lymphocytes from one of the parental strains: A (A × B)F_1 . The strain A T cells respond to class II MHC antigens inherited by the F_1 animal from its B parent.

Several different autoimmune diseases can develop in mice or rats as a result of a chronic graft-versus-host reaction. The particulars of the outcome depend on the donor host combination.[73] For example, when lymphocytes from BALB/c mice are injected into (BALB/c × A/J)F_1 animals, the recipients develop typical autoimmune hemolytic anemia (Fig. 34-4).[74] If, however, DBA/2 lymphocytes are injected into (DBA/2 × C57B1/6)F_1 mice, the result is severe glomerulonephritis of the type seen in SLE.[75] In each of these examples, both the donors and the F_1 recipients are normal strains of mice.

The principal requirement for triggering the autoimmune disease is a difference in class II MHC antigens between the donor and the recipient. [76] This intense immunogenic stimulus causes a marked activation of the donors T cells, which release large amounts of IL-4 and IL-6, potent polyclonal activators of B cells. [77] The hosts B cells can respond in two ways: by the production of autoantibodies and, in some cases, by a persistent lymphoproliferative reaction that culminates in malignant lymphoma.[78] The graft-versus-host model provides ample evidence of the importance of polyclonal B-cell activation in autoimmunization, but the genetic factors that determine the particulars of disease a given F_1 hybrid develops are unknown.

Several complications of chronic graft-versus-host disease in humans, including immune thrombocytopenia, [79] the sicca syndrome, and scleroderma,[80] may arise on a similar basis, but since bone marrow transplantation is rarely carried out when there is a class II HLA mismatch, other factors are probably involved. The development of autoimmune hemolytic anemia in recipients of renal allografts could be associated with a graft-versus-host reaction caused by donor lymphocytes in the organ graft.[81] However, the role of cyclosporine, which has been associated with the induction of autoimmunization, cannot be discounted in these cases. [82] [83] [84]

Figure 34-4 Autoimmune hemolytic anemia in F_1 hybrid mice, (A × B)F_1 , injected with parental strain (A) T cells. The two lines compare survival of ^{51}Cr-tagged syngenic erythrocytes in control and experimental animals. Red cell life span is markedly reduced, and the antiglobulin tests are positive in animals with the chronic graft-versus-host reaction.

Autoimmune Hemolytic Anemia in Transgenic Mice

Okamoto et al.[85] developed transgenic mice by inserting the V_H or V_L genes for an NZB antierythrocyte autoantibody into the germline of a normal strain of mice. * The NZB V genes were linked to B-cell-specific promoters, restricting their expression to the B cells of the new hosts. Mice harboring either the V_H or the V_L transgenes had no detectable abnormalities. When these two transgenic lines were bred, all their F_1 offspring bore both NZB immunoglobulin V genes, and many had autoimmune hemolytic anemia due to the transgenic NZB antierythrocyte autoantibody. In some doubly transgenic animals, autoimmune hemolytic anemia appeared, only to remit spontaneously, whereas in others the anemia was fatal. It was apparent that not all the antiself B-cell clones in the F_1 animals were deleted, a surprising result in light of other experimental models in which deletion or inactivation of B-cell clones with the potential to secrete pathogenic autoantibodies has been demonstrated.[86] Deletion, anergy, and autoimmunizationall involving the same autoantibodyappeared and disappeared, apparently at random, in individual

transgenic mice. Even more confounding was the finding of severe autoimmune hemolytic anemia in some transgenics.

This interesting model shows some of the features of autoimmune hemolytic anemia in humans; the tendency toward spontaneous remissions and relapses in the F_1 transgenic mice is noteworthy. In subsequent work, Murakami et al.[87] found that the $CD5^+$ B cell is the main source of the hemolytic autoantibody in the F_1 transgenic mice (see Chap. 8 for a discussion of $CD5^+$ B cells). In adult mice, $CD5^+$ B cells reside mainly in the peritoneal cavity. The peritoneal fluid contains many $CD5^+$ B cells but only a few erythrocytes. The transgenic $CD5^+$ B cells in the peritoneum would thus be isolated from self RBCs and would not be susceptible to clonal deletion by autoantigen. However, death of those antiself $CD5^+$ B cells occurs within 12 hours of an intraperitoneal injection of autologous red cells.[87] It is plausible that the erratic course of autoimmune hemolytic anemia in the F_1 transgenics was due to the chance entrance of red cells into the peritoneal cavity, perhaps as the result of casual injury when the animals were handled by the investigators. This model emphasizes the importance of antigen in maintaining self-tolerance: no antigen, no tolerance.

Origins of Antierythrocyte Autoantibodies

The vast improvement in our understanding of what prevents autoimmunization has not yet informed us of the mechanism that causes autoimmunization. Virtually nothing is known of the origins of warm-reactive IgG antierythrocyte autoantibodies, despite the availability of a thoroughly investigated, spontaneous animal model of the disease (the NZB mouse) and stocks of pathogenic autoantibodies, readily obtained from patients with the disease. A major impediment to advances in our perceptions of how autoimmune hemolytic anemia originates is that the autoantigens are for the most part unknown. Even in those cases in which blood group specificity of the autoantibodies has been identified, the relevant structures have not been elucidated. Leddy et al.[88] have succeeded in identifying four proteins on the red cell membrane that bind to antierythrocyte autoantibodies: the band 3 anion transporter, glycophorin A, and two polypeptides, probably related to the Rh family of antigens. Various combinations of those four autoantibody specificities were found in a group of 20 patients with autoimmune hemolytic anemia.

The association of autoimmune hemolytic anemia with SLE and with immune thrombocytopenia (Evans syndrome, see later), the induction of the disease by drugs that seem to perturb immune regulation (see later), and the graft-versus-host model of autoimmune hemolytic anemia all suggest that at least in some cases there is antigen-independent activation of clones of B cells with the capacity to produce IgG anti-RBC autoantibodies. Such polyclonal B-cell activation may account for the production of antierythrocyte autoantibodies in patients with the acquired immunodeficiency syndrome (AIDS).[89] [90] Hypergammaglobulinemia and other signs of nonspecific activation of B cells are prominent in HIV infection.[91]

The immunologic basis of autoimmune hemolytic anemia in patients with chronic lymphocytic leukemia (CLL) or a B-cell lymphoma is equally obscure.[92] In CLL, the autoantibodies are IgG and often polyclonal,[93] whereas the malignant $CD5^+$ B cells of that disease generally produce only IgM antibodies that are monoclonal. It is therefore likely that B cells other than those constituting the leukemia produce the autoantibodies. The large mass of $CD5^+$ B cells in CLL might induce non-neoplastic CD5 B cells to produce IgG autoantibodies, perhaps by a disturbance of immunoregulatory idiotypic networks (Fig. 34-5). The demonstration of the simultaneous presence of autoantibodies and anti-idiotypic antibodies on red cells in autoimmune hemolytic anemia[94] suggests that such networks may indeed have a role in the disease.

By contrast with the autoantigens that bind to warm-reactive autoantibodies, the structures of the autoantigens of cold agglutinin disease, the I/i system, are known (Fig. 34-2);[40] this has clarified our thinking about the immunology of this group of disorders. There is little reason to doubt that the very high levels of monoclonal cold agglutinins found in some patients with B-cell neoplasms are produced by the malignant cells. The demonstration that an idiotypic marker on monoclonal cold agglutinins could be detected not only on the patients neoplastic B cells but also on 310% of normal B cells[95] supports the view that these autoantibodies are part of the normal immune repertoire; malignant transformation of a cold agglutinin-producing B cell results in a lymphoma complicated by chronic cold agglutinin disease.

The basis of the association of paroxysmal cold hemoglobinuria with syphilis may be antigenic mimicry, in which structural similarities between a microbial antigen and a self-antigen trigger an autoantibody response. In the case of paroxysmal cold hemoglobinuria, the infecting organism, *Treponema pallidum*, should possess two antigenic determinants (epitopes): one recognized by T cells (the foreign epitope), the other by self-reactive B cells (the mimicking epitope). Donath-Landsteiner antibodies would be produced only by syphilitic patients whose class II MHC glycoproteins could present the foreign epitope in an immunogenic form to T cells (Fig. 34-6). A similar mechanism could apply to postinfectious acute cold agglutinin disease, in which a cross-reaction involving antigenic determinants of *Mycoplasma pneumoniae* and the I blood group substance has been incriminated.[96]

Structural analyses of monoclonal anti-I and anti-i autoantibodies from patients with B-cell neoplasms are beginning to yield important clues about the origins of chronic cold agglutinin disease. A striking observation is the repetitive use of the same immunoglobulin V_H gene, $V_{H4\text{-}34}$, in monoclonal IgM cold agglutinins, regardless of the anti-I or anti-i specificity of the autoantibody.[97] [98] In each case, the $V_{H4\text{-}34}$ heavy chain gene had a different CDR3 (see Chap. 8 for a discussion of the structure of immunoglobulin-coding regions); the light chains of cold agglutinins with anti-I or anti-i specificity differed as well. The $V_{H4\text{-}34}$ genes of these cold agglutinins contained few or no somatic mutations of the type that would lead to amino acid substitutions (replacement mutations). This, together with the variations in their CDR3s and in the light chains, implies that the

*The DNA constructs for either the heavy or light chain genes were miscroinjected into fertlized eggs of C55B1/6 mice; the eggs were then transferred to pseudopregnant moce. The presence of the transgenes in the newborn animals was verified by Southern blot analysis.

Figure 34-5 Hypothetical link between $CD5^+$ B cells and the immunologic complications of CLL. The idiotype displayed by the IgM surface immunoglobulins of the large mass of monoclonal $CD5^+$ B cells stimulates a population of T cells with a complementary anti-idiotype. In turn, the anti-idiotypic T cells affect normal CD5 B cells, either by inducing suppression (with resulting hypogammaglobulinemia) or polyclonal activation (resulting in autoimmune hemolytic anemia due to IgG autoantibodies).

$V_{H4\text{-}34}$ germline gene segment itself encodes a binding site for the I and i antigens. In contrast to the heavy chain gene, the light chain genes of the cold agglutinins did contain replacement mutations, especially in their hypervariable regions.[98]

It appears from these results that (1) the germline $V_{H4\text{-}34}$ heavy chain encodes the dominant specificity of monoclonal cold agglutinins; (2) the somatic mutations of the light chain genes of the cold agglutinins are the result of an immune response; and (3) the V_H CDR3 and the light chain confer fine specificity (e.g., for I or i) on the cold agglutinin and influence

Figure 34-6 Induction of autoimmunization by antigenic mimicry. **(A)** The usual foreign antigen contains antigenic determinants (epitopes) recognized by both T cells and B cells. The result is a cooperative interaction between helper T cells and B cells. **(B)** A self-antigen may be recognized by B cells, but T/B collaboration does not occur because of the deletion from the immune repertoire of T cells with receptors for the autoantigen. **(C)** A microbe with a self-mimicking epitope could engender T/B collaboration because T cells would recognize the foreign epitope and B cells would bind to the self-mimicking structure.

its affinity. These data make a convincing case that monoclonal cold agglutinins arise as the result of an immune response, perhaps an autoimmune response to an autoantigen on erythrocytes. The results of these molecular studies of cold agglutinins complement other evidence favoring a role for antigen-mediated clonal selection in some types of B-cell neoplasms (see Chap. 66 for a discussion of clonal selection in B-cell neoplasms).

In contrast to monoclonal cold agglutinins associated with chronic cold agglutinin disease, the naturally occurring IgM cold agglutinins that are present in low titers in normal serum are not restricted to the $V_{H4\text{-}34}$ gene segment. They are associated with different genes of the V_{H3} family as well as the $V_{H4\text{-}34}$ gene.[99] It therefore appears that B-cell neoplasia is an important, but not exclusive, element in the association between $V_{H4\text{-}34}$ and cold agglutinins. The correlation with lymphomas has added interest because $V_{H4\text{-}34}$ has been independently linked to B-cell lymphomas that do not secrete cold agglutinins.[100] Both kinds of V_{H434} -related B-cell tumors may originate from an uncontrolled autoimmune response against I or i RBC antigens, one type lacking the capacity for secreting the cold agglutinin, the other able to

secrete it as a monoclonal IgM immunoglobulin.

AUTOIMMUNE HEMOLYTIC ANEMIA

Epidemiology

The incidence of autoimmune hemolytic anemia is estimated to be approximately 10 cases per million population; for comparison, the incidence of acute myeloid leukemia is about 50 cases per million. [101] Autoimmune hemolytic anemia is more common in women than in men. It occurs at all ages, but usually in midlife. About one-half of cases are idiopathic (i.e., they are unassociated with any other disease). Some cases are induced by drugs, and others occur simultaneously with another autoimmune disease, especially SLE. That disorder may begin with Evans syndrome, a rare combination of autoimmune hemolytic anemia and immune thrombocytopenia. [102] A substantial proportion of cases develop in patients with B-cell lymphomas or

TABLE 34-2 -- Diseases Rarely Associated with Autoimmune Hemolytic Anemia

Collagen vascular disease
Rheumatoid arthritis
Scleroderma
Polyarteritis nodosa
Serum sickness
Sjögren syndrome
Lymphoreticular malignancy
Macroglobulinemia
Hodgkin disease
Multiple myeloma
Mycosis fungoides
Other malignancy
Acute leukemia
Thymoma
Carcinoma: colon, kidney, lung, ovary
Miscellaneous diseases
Myelofibrosis with myeloid metaplasia
Ulcerative colitis
Pernicious anemia
Thyroid disease
Ovarian cysts
Mucocutaneous lymph node syndrome (Kawasaki disease)
Evans syndrome (thrombocytopenia and hemolytic anemia)
Congenital immunodeficiency syndromes
Guillain-Barré syndrome
Primary biliary cirrhosis
Multiply transfused patients with hemoglobinopathies

CLL. Treatment of CLL with drugs such as cladribine (2-CdA) and fludarabine can either control or trigger the onset of autoimmune hemolytic anemia. It is theorized that by affecting T cells more than B cells, these types of drugs induce a disturbance in immunoregulatory T cells. [103] [104] A number of other diseases have also been complicated by autoimmune hemolytic anemia, but only as unusual exceptions (Table 34-2). [105]

Clinical Findings

The clinical findings in autoimmune hemolytic anemia are variable. They include jaundice (usually mild) and symptoms and signs of anemia. The spleen may be palpable 28 cm below the costal margin, especially after several months of unremitting hemolytic anemia. The spleen is enlarged in about one-third of patients. The disease can make its appearance acutely, with symptoms due to rapidly developing anemia, or it can develop gradually in a relatively asymptomatic form. Occasionally the blood bank provides the diagnosis through a positive antiglobulin test in a patient referred for transfusion therapy. The physical examination may reveal only pallor or slight jaundice, but signs of congestive heart failure are not unusual in patients with rapidly developing, severe autoimmune hemolytic anemia. Lymphadenopathy, fever, hypertension, renal failure, a rash, petechiae, or ecchymoses should alert the physician to the possibility of an underlying disease.

Laboratory Evaluation

Laboratory findings reflect the intensity of the hemolytic process and the bone marrows response to the anemia. In fulminant cases, with an RBC life span of <5 days, the anemia is severe and erythropoiesis increases 810-fold. As a result, the reticulocyte count rises, sometimes to levels >40%. In less severe cases, the regenerative

capacity of the bone marrow lags only slightly behind the rate of RBC destruction; hence a mild anemia but an elevated reticulocyte count. Between these extremes are many variations. Inspection of the blood smear in a typical case will reveal polychromatophilia, spherocytes, a few fragmented RBCs, nucleated RBCs, and occasionally erythrophagocytosis. Examination of the bone marrow shows erythroid hyperplasia, often with megaloblastoid features.

Patients with severe hemolytic anemia and markedly increased erythropoiesis occasionally develop folate deficiency and frank megaloblastosis. The growth of hematopoietic tissue in the bone marrow also leads to moderate increases in the white blood cell and platelet counts. The absence of reticulocytosis does not exclude the diagnosis of autoimmune hemolytic anemia but portends a serious prognosis. [106] [107] [108] [109] Presumably due to destruction of young erythrocytes by the autoantibody, the reticulocytopenia aggravates the severity of the anemia and increases the need for RBC transfusions; it may last several weeks despite therapy.

Rapid destruction of erythrocytes leads to other laboratory changes, which, like the preceding abnormalities, are not specific for autoimmune hemolytic anemia (see Chap. 23). These changes include increased serum levels of unconjugated bilirubin and lactate dehydrogenase and a reduction in plasma haptoglobin levels. In rare cases with intravascular destruction of erythrocytes, the plasma hemoglobin is elevated and there is hemoglobinuria; hemosiderinuria follows in 7 days, when renal tubular epithelial cells containing absorbed iron defoliate into the urine. Tests of osmotic fragility and autohemolysis are unnecessary for diagnosis and management; measurements of RBC survival are rarely indicated.

The distinction between autoimmune hemolytic anemia and other forms of hemolytic anemia depends on the DAT, often termed the direct Coombs test. This assay detects the hallmark of autoimmune hemolytic anemia, the presence of IgG or complement bound to the RBC membrane (Fig. 34-7). It is performed by washing the patients RBCs free of plasma, adding the antiglobulin reagent, centrifuging, and reading for the presence or absence of agglutination. The DAT thus tests for the presence of immunoglobulin or complement on the patients RBCs. In patients with severe autoimmune hemolytic anemia, the DAT results are usually strongly positive, but the titer of the autoantibody and the strength of the reaction do not always predict the severity of disease.

The IgG autoantibodies in autoimmune hemolytic anemia are predominantly of the IgG1 and IgG3 subclasses, both of which are capable of activating complement. In most cases, either

Figure 34-7 DAT for detection of **(A)** erythrocyte-bound C3d or **(B)** IgG. Hemagglutination occurs when anti-C3d or anti-IgG can create a lattice structure by bridging sensitized RBCs.

IgG or C3d (a proteolytic fragment of C3; see below) is bound to the RBC membrane. Therefore, the screening antiglobulin reagent, made by immunizing rabbits or by blending murine monoclonal antibodies, must contain antibodies against IgG (including IgG1 and IgG3 subclasses) and C3d. [110] [111] [112] [113] [114] [115] [116] [117] Broad-spectrum antiglobulin test reagents may also contain antibodies against other serum proteins that can bind nonspecifically to RBCs. Therefore, when the routine antiglobulin test is positive, it should be repeated using reagents specific for IgG and C3d. IgA and IgM autoantibodies are rare causes of warm autoimmune hemolytic anemia[118] [119] [120] [121] and need not be considered in usual routine tests. [122]

A positive antiglobulin test, confirmed with a specific anti-IgG reagent, is occasionally found in healthy people; approximately 1 in 10,000 blood donors has a positive antiglobulin test without anemia or evidence of increased hemolysis. [123] A positive antiglobulin test with a monospecific anti-C3d reagent is also seen occasionally in healthy subjects,[124] but such a result almost always points to either warm- or cold-reactive autoantibodies. In about 10% of cases of autoimmune hemolytic anemia, erythrocyte-bound C3d occurs in the absence of erythrocyte-bound IgG, whereas in all cases of cold agglutinin disease, erythrocyte-bound C3d is detected. SLE should be suspected in cases of autoimmune hemolytic anemia in which both IgG and C3d are detected. By contrast, -methyldopa-induced autoimmune hemolytic anemia is rarely associated with erythrocyte-bound C3d; only IgG is present.

The unexpected report of a negative antiglobulin test in a patient suspected of having autoimmune hemolytic anemia, although uncommon, may have several explanations. Technical error is infrequent, but it can occur. Other rare causes are IgA autoantibodies or low-affinity IgG autoantibodies. [125] More commonly, the test is not sensitive enough to detect small numbers of erythrocyte-bound IgG molecules; this occurs most often in autoimmune hemolytic anemia associated with a lymphoma or CLL. Manual antiglobulin tests detect 200500 molecules of IgG per cell; amounts below this level, although undetectable, may be clinically important. In such cases, sensitive assays such as the antiglobulin consumption assay [126] or tests with ^{125} I-staphylococcal protein A, which binds avidly to IgG, can reveal the autoantibodies.[127]

The DAT, like any other laboratory test, requires interpretation. As already noted, a positive antiglobulin test can occur in healthy subjects and patients with autoimmune hemolytic anemia can have a negative antiglobulin test. Of equal importance, a positive antiglobulin test does not always signify erythrocyte-bound IgG antibody or complement, nor does a positive result necessarily imply that the individual has hemolytic anemia. The frequency of false-positive tests in hospitalized patients can be as high as 15%.[128] They can stem from tests performed on clotted blood,[129] from the use of silicone gel tubes,[130] from collection of samples from intravenous lines containing low ionic strength solutions, [131] from medications (e.g., cephalin) that cause nonspecific attachment of plasma protein to the RBC surface,[132] [133] and from hypergammaglobulinemia.[134] [135] If the DAT is positive, specific reagents are required to identify the erythrocyte-bound protein.

In 80% of patients with autoimmune hemolytic anemia, the autoantibodies are present in the serum as well as on the red cell membrane. [136] The indirect antiglobulin test (indirect Coombs test) detects the presence of these serum antibodies in the patients serum (Fig. 34-8). The procedure entails incubating the test serum and normal RBCs, usually at 37°C for 1 hour, washing the cells free of serum, adding the antiglobulin reagent, and reading for agglutination. The antibodies detected by the indirect antiglobulin test may be autoantibodies in a patient with autoimmune hemolytic anemia, or they may be alloantibodies induced by blood transfusion or maternal/fetal incompatibility. Alloantibodies, present only in the serum, have specificity for RBC antigens not present on the patients erythrocytes. The DAT is therefore negative in alloimmunization; exceptions may occur if the alloantibodies bind to recently transfused RBCs.

The specificity of the antibodies detected by the indirect antiglobulin test can be assessed by reacting the test serum with a panel of erythrocytes of known antigenic composition. Alloantibodies have definable specificities, whereas the serum autoantibodies of autoimmune hemolytic anemia usually react with all RBCs except those that contain no Rh antigens, the so-called Rh_{null} RBCs.[137] Even so, the identity of the Rh-associated determinant implied by the lack of reactivity of the autoantibodies with Rh_{null} cells is unknown. Occasionally (12% of cases), relative specificity within the Rh system can be demonstrated, and RBCs lacking the corresponding Rh antigen survive better in vivo than those that express the antigen. [138] [139] [140] [141] Specificities of IgG autoantibodies for other blood groups have been described. [142] [143] [144] [145] [146] [147] [148] [149] [150] Specificity for different subunits of the RBC membrane has recently been reported. [151] [152] [153] [154] [155] In Evans syndrome in patients with the lupus anticoagulant (antiphospholipid antibody syndrome), the erythrocyte-bound autoantibody can react with phospholipids. [156] [157] [158] [159] [160] An important practical point is that, with rare exceptions, the autoantigens are present on all normal erythrocytes; in practice, all RBCs are incompatible (see discussion under TherapyTransfusion).

Pathophysiology

Immune Clearance of Erythrocytes

The autoantibodies that cause RBC destruction in autoimmune hemolytic anemia and the antierythrocyte autoantibodies in healthy persons with no discernible signs of abnormal hemolysis may have the same isotype and even identical serologic specificities. Therefore, the pathogenicity of anti-red cell autoantibodies must depend on additional considerations. The amount of the autoantibody, its avidity for the erythrocyte autoantigen, and its ability to fix complement are of particular importance.[161] Each of these factors probably contributes independently and cumulatively to the erythrocyte lesion.

Figure 34-8 Indirect antiglobulin test for detection of antierythrocyte antibodies in serum. The patients serum is mixed with a panel of normal RBCs, some (or all) of which express the antigen(s) recognized by the serum antibodies. After the antibody-coated erythrocytes are washed, an anti-IgG reagent is added. Hemagglutination occurs, as in Figure 34-7 .

Immune hemolysis in vivo begins with opsonization by autoantibodies. The terminal effect can be destruction of the RBC directly within the circulation (intravascular hemolysis) or removal of the cell from the circulation by tissue macrophages (extravascular hemolysis), or both. Opsonized RBCs are recognized and cleared from the circulation by macrophages located primarily in the spleen, and to a lesser extent in the liver. [162] The interaction of macrophages with RBCs coated with IgG or C3b (or both) occurs through receptors specific for the Fc portion of IgG (especially IgG1 and IgG3) and for C3b. [163] [164] Initiation of the events that culminate in the deposition of C3b on the erythrocyte surface requires at least two IgG molecules bound in close proximity on the RBC membrane. Furthermore, the subclass of the IgG autoantibodies is important not only for binding to macrophage Fc receptors (IgG3 > IgG1) but also for complement activation. The potency of subclasses for complement activation is as follows: IgG1 > IgG3 > IgG2 > IgG4. [162] [163] [164] [165] [166] [167]

The presence on the erythrocyte membrane of both IgG and C3b accelerates immune clearance, [168] [169] [170] suggesting that the Fc and C3b macrophage receptors act synergistically. Sequential studies in one subject of the clearance of RBCs coated in vitro with different amounts of the same antibody showed that the amount of erythrocyte-bound IgG is another factor influencing the rate of hemolysis. Little information is available on the minimum number of erythrocyte-bound IgG molecules required for interaction with macrophages; in some cases of autoimmune hemolytic anemia, <200 IgG molecules per erythrocyte (too few for detection by the DAT) is sufficient to cause in vivo hemolysis. [167]

The splenic environment is especially conductive to immune clearance. IgG-coated RBCs are efficiently trapped from the hemoconcentrated circulation within the spleen. The relatively low plasma concentration in splenic sinusoids tends to alleviate competition between plasma IgG and IgG-coated RBCs for Fc receptors, thus providing optimal interactions between opsonized erythrocytes and macrophages. [163] [169] Rarely, RBC autoantibodies cause reticulocytopenia and dyserythropoiesis, thereby contributing to the severity of anemia. [171]

Red Blood Cell Lesion

The macrophage may ingest an opsonized RBC entirely; more likely, proteolytic ectoenzymes on its surface digest away bits of the erythrocyte membrane, producing a spherocyte, an RBC with the lowest possible surface area/volume ratio. [164] Spherocytes are less deformable and more susceptible to osmotic lysis than disk-shaped red cells, so they are especially susceptible to hemolysis during their travels through the sluggish circulation of the splenic sinusoids. This is why the predominant mechanism of destruction of erythrocytes coated with IgG with or without C3b occurs extravascularly in autoimmune hemolytic anemia (Fig. 34-9). By contrast, intravascular hemolysis is rare because regulatory proteins of the complement system (C3b inactivator and $_1$H globulin limit completion of the complement cascade on the surface of the opsonized erythrocyte. [172] The C3b inactivator system contributes to the local arrest of the complement cascade by degrading C3b to C3d; spontaneous elution of the autoantibody (a measure of its avidity for the RBC) leaves an erythrocyte coated only with C3d.

Macrophage-mediated mechanisms predominate in causing the lesion of autoimmune hemolytic anemia, but the participation of cytotoxic lymphocytes (natural killer cells), which cause antibody-dependent cell lysis, has not been excluded. The efficiency of reticuloendothelial function probably also contributes to the degree of immune clearance and may account for exacerbations of autoimmune hemolytic anemia by viral or bacterial infections. In vitro assays of the ability of blood monocytes from patients with viral infections to phagocytose Ig-coated RBCs have shown marked deviations from normal. [173] [174]

Therapy

General Principles

Because the severity of autoimmune hemolytic anemia may range from indolent to life-threatening, the impetus to initiate treatment must begin with a thorough appraisal of symptoms and the extent of the damage. Rapidly developing anemia with

Figure 34-9 Mechanism of extravascular hemolysis in autoimmune hemolytic anemia. **(A)** Macrophage encounters an IgG-coated erythrocyte and binds to it via its Fc receptors. Thus entrapped, the RBC loses bits of its membrane as a result of digestion by the macrophages ectoenzymes. The discoid erythrocyte transforms into a sphere. **(B)** RBC lightly coated with IgG (and therefore incapable of activating the complement cascade) is preferentially removed in the sluggish circulation of the spleen. **(C)** RBC with a heavy coat of IgG; thus, C3b (black circles) can be removed both by the spleen and the liver.

Box 34-1. HAZARDS OF TRANSFUSION THERAPY

The idea that transfusion therapy represents a special hazard in patients with autoimmune hemolytic anemia[183] [184] [185] [186] has been overemphasized; nevertheless, transfusions are not to be undertaken lightly in such patients. The decision to transfuse requires that the clinician and the blood bank work together. The clinician must supervise the transfusions and insist on close observation of the patient. An adverse reaction dictates additional laboratory tests, but even sophisticated serologic techniques do not ensure uneventful transfusions; acute intravascular hemolysis can occur with no evidence of serologic incompatibility. In vivo compatibility testing with ^{51}Cr-tagged RBCs is of no value in the management of most patients.

Transfusion is warranted without delay and, if necessary, before all the serologic tests are completed when cardiac or cerebral function is threatened. Alleviation of signs and symptoms of anemia can usually be accomplished with relatively small quantities of RBCsas little as 0.51 U of RBCs. Overtransfusion in the presence of high-output cardiac failure can easily lead to circulatory overload, another reason to justify careful observation of the patient during the transfusion.

a hematocrit of <20 requires urgent management, but in the less aggressive forms of the disease, especially in the elderly, it may be more prudent to allow mild anemia than to institute risky treatment.

The management of autoimmune hemolytic anemia depends in part on whether the disease is primary or whether it is secondary to such disorders as B-cell malignancies or SLE. [175] [176] This, too, demands a careful assessment before any treatment begins. In some cases of autoimmune hemolytic anemia secondary to lymphoma or CLL, the pathogenic autoantibody (usually monoclonal) is secreted by the neoplastic B cells. Combination chemotherapy or irradiation of the underlying malignancy often brings the hemolytic anemia under control. [177] [178] [179] In other cases, however, the autoantibodies (usually polyclonal) do not originate from the B-cell neoplasm but probably result from abnormal immune regulation instigated by the neoplastic B cells. Treatment of the latter type of secondary autoimmune hemolytic anemia with immunosuppressive agents may improve the anemia, but it may also trigger an exacerbation. [180]

The ultimate goal of therapy is control of the B-cell populations that secrete pathogenic autoantibodies. However, so little is known about such cells [181] [182] that the currently available therapy is, by default, nonspecific. The desired therapeutic effect is eradication of the abnormal hemolytic process, not reversal of the serologic

abnormalities. Indeed, the DAT often remains positive in the face of a hematologic response.

Transfusion

Severe anemia may cause pulmonary edema (in the setting of high-output cardiac failure), somnolence, and even obtundation. These manifestations occur when the hemoglobin falls to <4 g/dl. They are life-threatening and necessitate transfusion with RBCs. [183] [184] [185] [186] The persistence of tachycardia, postural hypotension, dyspnea, and angina also calls for transfusion. Oxygen therapy may be prescribed, but it is no substitute for transfusion.

Alloantibodies, usually with specificity for the Rh or Kell blood group systems, occur in 30% of patients with autoimmune hemolytic anemia who have a history of blood group immunization by maternal/fetal incompatibility or previous transfusions. [187] [188] [189] These alloantibodies can escape notice in a patient with a positive result on the indirect antiglobulin test because of the concomitant presence in the serum of autoantibodies that react with virtually all normal RBCs. Nevertheless, several techniques may help detect suspected alloantibodies. [190] [191] [192] Absorption of the autoantibodies with the patients own RBCs can permit recognition of residual alloantibodies. Before absorption, the autoantibody must be eluted from the patients RBCs; enzyme treatment of the patients cells increases autoantibody absorption. Another method involves absorption of the patients serum with normal RBCs of selected phenotypes and then retesting of the absorbed serum.

The preceding techniques should not be required as pretransfusion tests in patients with autoimmune hemolytic anemia. Transfusions should never be delayed if the tests are not readily available. However, standard antibody detection and identification tests with both the patients serum and an elute prepared from the patients cells should be performed whenever possible. Titration of the eluate and the serum against RBCs of various Rh phenotypes can indicate an auto-antibody specificity (or preference) within the Rh system. Any such specificity should be respected in selecting donors units. [193] [194]

Corticosteroids

Corticosteroids are the mainstay of treatment for patients with symptomatic, unstable autoimmune hemolytic anemia of either

Box 34-2. PREDNISONE THERAPY

Therapy can begin with prednisone (there is no clear advantage to alternative forms of corticosteroids) in a dose of 12 mg/kg/day in divided doses, depending on the severity of the disease. The physician can consider beginning with a lower dose (e.g., 0.6 mg/kg/day) in elderly patients, especially those who are immobilized or who already have osteoporosis, or when faced with infection or other mitigating complications. Whatever the amount selected, it should be continued until a response becomes evident, usually within 3 weeks, by a rise in the hematocrit and a fall in the reticulocyte count.

Autoimmune hemolytic anemia in children is likely to respond to prednisone with a durable remission. By contrast, permanent remissions are infrequent in adults. Therapy for adults therefore requires a plan to manage (or avoid) relapse. Essential elements to consider in formulating the long-term management of a patient with autoimmune hemolytic anemia are the duration of treatment with the initial dose of prednisone and the rate of dosage reduction after a response has been achieved. The tapering schedule depends, in part, on the severity of the initial presentation and on the prominence of side effects of the treatment. In the absence of contraindications, prednisone is continued at the initial dose until the hemoglobin reaches a level of 10 g/dl, by which time transfusions should no longer be necessary. Thereafter, gradual reduction in the dose can begin, usually at a rate of 510 mg/wk. During this second phase of treatment, the divided daily prednisone dose can be consolidated into a single daily dose. If the remission remains stable after a dose of 10 mg/day is reached, further tapering over a 34-month period can proceed cautiously. Some hematologists will continue treatment for many months, at low doses (e.g., 10 mg every other day), but the efficacy of this practice has not been investigated.

the idiopathic or the secondary forms. The clinical response to prednisone results primarily from its ability to disable macrophages from clearing IgG-or C3b-coated erythrocytes. Corticosteroids interfere with both the expression and function of macrophage Fc receptors. This is probably the earliest, and perhaps even the primary, mechanism of the ability of steroids to diminish the immune clearance of blood cells. [195] [196] [197] [198] Prednisone can also reduce autoantibody production, but only after several weeks of therapy.

Splenectomy

Indications for splenectomy in autoimmune hemolytic anemia include failure to respond to prednisone, dependence on prednisone dosages that are >1020 mg/day, or intractable side effects of the corticosteroid. The procedure can be highly effective because along with the removal of the spleen go its phagocytosing macrophages and autoantibody-producing B cells. In most young adults with primary autoimmune hemolytic anemia, the question of splenectomy arises almost inevitably. However, in an elderly patient with a stable but incomplete remission, maintenance therapy with prednisone at a dose of 10 mg/day for an indefinite period may be the better alternative. There is a slight risk that the overwhelming sepsis syndrome may develop immediately following splenectomy, but systemic bacterial infections can occur long after the postoperative period as well. [199] These risks are lessened by immunization with pneumococcal and meningococcal vaccines, always given preoperatively, and by the prompt use of antibiotics for febrile illness.

The response to splenectomy does not correlate with the age of the patient, the presence or absence of an underlying B-cell disorder, the strength of the antiglobulin test, prior response to prednisone, or pattern of sequestration of ^{51}Cr-labeled red cells; these criteria cannot be used to predict the response to splenectomy. [200] About 5060% of patients with classic autoimmune hemolytic anemia will have a good to excellent initial response to splenectomy. They will need <15 mg/day of prednisone to maintain an adequate level of hemoglobin. [201] Information regarding the clinical implications of an accessory spleen in autoimmune hemolytic anemia is meager. Faced with such a rare finding in a patient with relapse, many hematologists would recommend its removal. The role of splenectomy in patients with mixed IgG, IgM, or mixed cold- and warm-reactive IgG antibodies is unclear.

Box 34-3. CYTOTOXIC DRUG THERAPY

The administration of cytotoxic drugs is best reserved for refractory cases: symptomatic patients who have not responded to splenectomy, those in whom splenectomy is an unacceptable medical risk, those who refuse the operation, or patients who have serious side effects from corticosteroids. Cyclophosphamide (2 mg/kg/day) or azathioprine (1.5 mg/kg/day) should be continued for 3 months to ensure maximal inhibition of autoantibody synthesis. Less than one-half of patients treated with these drugs will respond with a rise in the hemoglobin that can be maintained in the face of substantially reduced doses of prednisone. Indeed, this estimate may be overly optimistic, since negative or unfavorable results seldom reach publication. Unfortunately, no controlled clinical trials of cytostatic agents in the treatment of autoimmune hemolytic anemia have been conducted.

Immunosuppressive Therapy

Most experience with immunosuppressive drugs in the treatment of autoimmune hemolytic anemia has been with alkylating agents (cyclophosphamide and chlorambucil) and thiopurines (azathioprine and 6-mercaptopurine). [202] The basis for the clinical use of these drugs is their inhibitory effect on the immune system, possibly affecting both B cells and T cells. [203] [204]

Cyclophosphamide and azathioprine, like prednisone, can induce numerous side effects. Some of these occur concomitantly with their use; others become evident only after sustained administration. The early side effects include bone marrow suppression and impairment of the immune response (particularly T-cell-mediated immunity). In addition, cyclophosphamide damages ovarian function, inhibits spermatogenesis, [205] [206] [207] [208] and causes bladder fibrosis. [209] Acute myeloid leukemia can develop years after sustained use. [203] By contrast, the prolonged use of azathioprine has not been associated with a statistically significant increase in malignant diseases. All these considerations mandate careful monitoring of any patient treated with either cyclophosphamide or azathioprine.

Plasma Exchange

In a normal person, plasma exchange of 11.5 plasma volumes is effective in lowering the serum level of IgG by 50%. However, continuous antibody production and the large extravascular distribution of IgG limit the efficacy of plasma exchange in autoimmune hemolytic anemia. On cessation of therapy, the rate of return of pretreatment levels of autoantibody depends on the rate of autoantibody production. [210] Occasional dramatic responses have been reported in patients being prepared for surgery or when plasma exchange was a temporizing measure following the initiation of immunosuppressive therapy. [211] [212]

Vinca Alkaloids

Responses in a few patients with autoimmune hemolytic anemia have been reported following infusion of vincristine-laden IgG-coated platelets; the platelets serve the drug directly to macrophages, presumably interfering with the ability of the macrophages to bind or ingest IgG-coated RBCs. Reported responses have been of long duration and associated with a delayed fall in titer on the DAT. The therapeutic effects of vinca alkaloids alone in autoimmune hemolytic anemia have not been systematically investigated. [213]

Danazol

Danazol, an attenuated synthetic androgen, is useful in patients with autoimmune thrombocytopenic purpura when given in a dose of 400600 mg/day orally. It has benefited a limited number of patients with refractory autoimmune hemolytic anemia. Remissions of 1 year have been reported. [214] In view of the relatively low risk of long-term side effects (the drug can cause abnormal liver function), danazol may find a secure place in the management of prednisone-dependent patients.

Intravenous -Globulin

Intravenous -globulin has been found effective in managing selected cases of autoimmune thrombocytopenia. The recommended dose is 400 mg/kg/day for 5 days. The soluble IgG in the material may increase the life span of IgG-coated RBCs by saturating Fc receptors on macrophages. In a recent study of patients with autoimmune hemolytic anemia associated with lymphoproliferative disorders, a long-term benefit was observed with a maintenance dose schedule of intravenous IgG every 21 days. A decrease in antiglobulin titer was found in

these patients, suggesting a mechanism other than blockade of Fc receptors by intravenous IgG. [215]

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COLD AGGLUTININ DISEASE

Cold agglutinin disease refers to a group of disorders caused by antierythrocyte autoantibodies (e.g., cold agglutinins) that preferentially bind RBCs at cold temperatures (418°C). Virtually all sera from healthy individuals contain low-titer cold agglutinins, regarded as benign or harmless RBC autoantibodies and considered polyclonal. Similarly, cold agglutinins that arise following certain infections are also polyclonal and usually benign; in rare cases, a transient form of cold agglutinin disease ensues (see following sections). By contrast, monoclonal cold agglutinins are generally pathogenic and are derived from clonal B-cell expansions (as in idiopathic/chronic cold agglutin disease), which may progress to frank lymphoma (Table 34-3).

Chronic Cold Agglutinin Disease

The most common type of cold agglutinin disease, a chronic form characterized principally by a stable anemia of moderate severity and attacks of acrocyanosis precipitated by exposure to cold, constitutes about one-third of all cases of immunohemolytic anemia. Cold agglutinins cause the cardinal abnormalities of the disease. The acrocyanosis stems from intra-arteriolar agglutination of erythrocytes in the relatively cool tips of the fingers, feet, earlobes, and nose. The hemolytic anemia depends on the capacity of the cold agglutinins to initiate activation of the complement cascade on the surface of the RBC (see discussion under Pathophysiology). Most patients with chronic cold agglutinin disease are in the fifth to eighth decades of life, and many of them have a B-cell neoplasmlymphoma, Waldenström macroglobulinemia, or CLL. The cold agglutinin in those latter cases is monoclonal, almost always IgM-, and may show up as a monoclonal band in the region of the serum protein electrophoretic pattern. In the absence of a B-cell neoplasm, the spleen and lymph nodes are rarely enlarged; such findings warrant a search for the neoplasm.

Laboratory Evaluation

The usual laboratory findings of hemolytic anemia (i.e., anemia, reticulocytosis, polychromatophilia, spherocytosis, erythroid hyperplasia in the bone marrow, and elevations in serum bilirubin and lactate dehydrogenase levels) are generally not striking in chronic cold agglutinin disease. Hemagglutination may be visible to the unaided eye in blood drawn from a patient with cold agglutinin disease and can interfere with automated blood counts. The anemia is often mild and stable because the C3b inactivator in serum limits the extent of cold agglutinin-induced complement activation on the erythrocyte membrane. However, exposure to cold may greatly augment the binding of cold agglutinins, exceeding the restraints of the inactivator system. This can result in a sudden drop in hematocrit, with complement-mediated intravascular hemolysis and renal failure. In a

TABLE 34-3 -- Classification of Cold Agglutinin Disease

Monoclonal[a]
Idiopathic/chronic
B-cell lymphoma
Polyclonal
Benign/natural
Postinfectious (e.g., *Mycoplasma pneumoniae*, Epstein-Barr virus, human immunodeficiency virus), collagen vascular disorders

[a]Monoclonal cold agglutinins are derived from a spectrum of clonal B-cell expansions ranging from preneoplastic (e.g., no evidence of malignancy) to frank lymphoma.

distinctive subset of patients with aggressive cold agglutinin disease, the cold agglutinin titer is relatively low, but the autoantibody has a high thermal amplitude. Recognition of patients with this variant of cold agglutinin disease is important because they may respond to prednisone, [216] whereas patients with high-titer cold agglutinin disease usually do not.

In typical cases of chronic cold agglutinin disease, the cold agglutinin titer is very high (>1:10 ^{5} , and occasionally >1:10^{6}). The antibodies are most reactive in the cold, and hemagglutination disappears as the temperature rises toward 37°C. In some cases, however, the antibody is reactive at relatively high temperatures, and occasionally even at 37°C. The reactivity of the cold agglutinin at high temperatures (i.e., its thermal amplitude), not the titer of the antibody, most accurately predicts the severity of the disease. The DAT is positive because of erythrocyte-bound C3d (see discussion under Pathophysiology), but tests with anti-IgG reagents are negative. The indirect antiglobulin test, conducted at 37°C, is negative. In addition to monoclonal IgM cold agglutinins, IgG/IgM mixed cold agglutinins have been reported. [217] [218] [219] Besides the usual high titers of IgM cold agglutinins, some cases of cold agglutinin disease have low titers of IgG and IgA cold agglutinins.

Cold agglutinins are not cryoglobulins. The latter are most often monoclonal IgM immunoglobulins that, in the cold, either self-associate and precipitate from solution (type I cryoglobulinemia) or precipitate as complexes with polyclonal IgG molecules (type II cryoglobulinemia, often due to a monoclonal IgM rheumatoid factor). Type III cryoglobulins consist of a mixture of polyclonal IgM and polyclonal IgG immunoglobulins. The clinical manifestations of the cryoglobulinemic syndromes are highly variable: types I and II cryoglobulinemia occur in B-cell neoplasms (Waldenström macroglobulinemia, multiple myeloma, lymphoma, and CLL); types II and III cryoglobulinemia can produce a picture of immune complex-mediated vasculitis, with vascular purpura, arthritis, and nephritis as the dominant complications. In occasional patients, the cryoglobulin can also be a cold agglutinin. [220] [221] [222] [223]

Transient Cold Agglutinin Disease

A second type of cold agglutinin disease, usually acute and always self-limited, occurs as a rare complication of several infectious diseases, most notably *M. pneumoniae* infection and infectious mononucleosis. Patients with this form of cold agglutinin disease are therefore much younger than those with chronic cold agglutinin disease. The onset is abrupt, appearing as the infection wanes, and the anemia can be severe. Cold agglutinin titers are moderately elevated, and the cold agglutinins are polyclonal. Often these polyclonal cold agglutinins coincide with high-titer warm-reactive IgG RBC autoantibodies.

Targets of Cold Agglutinin Disease

The antigenic specificity of cold agglutinins is usually identified by their degree of reactivity with RBCs from adults (blood group I) and cord blood (blood group i). The cold-reactive autoantibody produced after some cases of *M. pneumoniae* infection has anti-I specificity, [90] whereas the antibody in infectious mononucleosis frequently, but not always, has anti-i specificity. [224] [225] Additional specificities have been identified by tests with rare adult RBCs that lack the I antigen, or with enzyme-treated erythrocytes. Cold agglutinins with these antigenic specificities (I(T), I(F), Pr, Gd, Sa, Lud, Fl) have no distinguishing clinical features. [226] [227] [228] [229]

[230] [231] [232] [233] [234] Rarely cold agglutinins will be specific for the A blood group antigen. [235]

Pathophysiology

The pathogenic IgM autoantibody in cold agglutinin disease is highly efficient in activating the classical complement pathway

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Box 34-4. TRANSFUSIONS AND COLD AGGLUTININ DISEASE

Transfusion in a patient with cold agglutinin disease requires the same prudent safeguards observed with transfusions in autoimmune hemolytic anemia. All compatibility tests must be carried out at 37°C and with IgG-specific antiglobulin reagents to avoid confusion with the serum cold agglutinin and the erythrocyte-bound C3d. The use of inline blood warmers is advisable, and more elaborate measures to perform the entire transfusion process at 37°C are occasionally required.[248] Hypothermia must be avoided during cardiac surgery, and special techniques must be used to avoid lowering the temperature of blood in the coronary arteries.[249]

on the erythrocyte membrane.[236] [237] However, the thermal dependency of the antibody constrains its pathogenic effects. The autoantibody rapidly elutes off red cells at the 37°C temperature of the visceral circulation, but in the cool peripheral circulation of the hands and feet the cold agglutinin remains on the erythrocyte membrane for at least a few seconds. That amount of time is sufficient to activate the complement cascade to the stage of C3b, which adheres to the RBC after it re-enters the central circulation. In the hepatic circulation, $C3b^+$ RBCs encounter macrophages with receptors specific for C3b;[161] [169] [233] however, C3b sensitization is only a weak signal for the activation of phagocytosisthe hepatic clearance of C3b-coated RBCs requires 500800 C3b molecules per RBC. As a result, many $C3b^+$ RBCs escape unharmed into the systemic circulation, where they come under the influence of the regulatory proteins of the complement system. The C3b inactivator system degrades C3b into C3dg or C3d, or both. The result is a cohort of erythrocytes coated with C3d, but not with the IgM autoantibody. [162] Since macrophages bind to C3d with even lower avidity than to C3b, the $C3d^+$ erythrocytes tend to have a near normal survival in vivo despite a heavy coating with that degradation product of C3. [166] [238]

These limits on the pathogenicity of cold agglutinins account for the subdued hematologic picture in most patients with cold agglutinin disease. If, however, the regulatory C3b inactivator proteins are impaired, limiting cleavage of RBC-bound C3b, or if the production of IgM autoantibodies with a high thermal amplitude is impaired, permitting completion of the complement cascade in the visceral circulation, severe extravascular hemolysis can occur. Several patients with high titers of IgA cold agglutinins have been reported. These cases are not associated with cold agglutinin disease, which may relate to the lack of complement activation by IgA antibodies.[239] [240] [241] [242] [243] [244] [245] [246] [247]

Therapy

Chronic Cold Agglutinin Disease

Therapy for the cold agglutinin syndromes depends on the gravity of the symptoms, the serologic characteristics of the autoantibody, and any underlying disease. In the idiopathic, or primary, form of chronic cold agglutinin disease, prolonged survival and spontaneous remissions and exacerbations are not unusual. The anemia is generally mild, and the simple measure of avoiding exposure to cold temperatures can avoid exacerbations, especially when the cold agglutinin has a low thermal amplitude. Prednisone has been beneficial in rare cases showing relatively low titers of cold agglutinins of a high thermal amplitude or in which an IgG cold-reactive antibody is produced. However, prednisone is not useful therapy in most patients with primary IgM-induced cold agglutinin disease, and its administration should not be undertaken lightly, given the chronicity of the disease. [248] [249] Plasma exchange may help as a temporary measure in acute situations.[210] Splenectomy is usually ineffective because the liver is the dominant site of sequestration of red cells heavily sensitized with C3b. However, rare cases with an enlarged spleen have responded to splenectomy; in some of these patients, a localized splenic lymphoma was found, whereas in others only lymphoid hyperplasia was evident.

It is essential to seek evidence of a B-cell neoplasm before initiating therapy for chronic cold agglutinin disease. Oral alkylating agents (chlorambucil or cyclophosphamide) help many patients with the secondary form of cold agglutinin disease because of their effect on the B-cell neoplasm, but only occasionally do they benefit patients with the primary form of the disease. [250] [251] When cold agglutinin disease is part of an established B-cell malignancy, the severity of hemolysis often waxes and wanes in parallel with the activity of the neoplasm.

Transient Cold Agglutinin Disease

Transient cold agglutinin disease is a rare form that is always self-limited. Supportive measures, including transfusions and avoidance of cold, may suffice to tide the patient over the bout of hemolysis. Corticosteroids are usually not helpful, and splenectomy is almost never indicated.

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PAROXYSMAL COLD HEMOGLOBINURIA

Clinical Manifestations

There are two distinct groups of patients with paroxysmal cold hemoglobinuria: persons with tertiary or congenital syphilis, and children or young adults who develop the disease after a viral illness. The syphilitic variant is rarely seen today. Although the Donath-Landsteiner antibody often occurs in tertiary or congenital syphilis, it generally does not cause hemolytic disease. On exposure to cold, an occasional patient experiences paroxysms of hemoglobinuria and constitutional symptoms: fever, back pain, leg pain, abdominal cramps, and rigors, followed by hemoglobinuria. The infrequent postviral form of paroxysmal cold hemoglobinuria [252] [253] [254] is characterized by constitutional symptoms with fulminant intravascular hemolysis and its associated signs of hemoglobinemia, hemoglobinuria, jaundice, severe anemia, and sometimes renal failure. The disease is self-limited, usually lasting 23 weeks.

Laboratory Evaluation

The IgG antibody responsible for paroxysmal cold hemoglobinuria is found in the patients serum by incubation of normal erythrocytes, fresh normal serum as a source of complement, and the patients serum, first at 4°C and then at 37°C, with appropriate controls. The Donath-Landsteiner antibody fixes the first two components of complement in the cold and completes the cascade on warming to 37°C. [255] The DAT is almost always negative, but occasionally weak reactions for erythrocyte-bound complement are manifested. The indirect antiglobulin test is negative. Most Donath-Landsteiner antibodies have specificity for the P blood group system,[256] [257] but other specificities have been described. [258] [259] [260] The diagnosis depends on recognition of the clinical picture, since tests for the Donath-Landsteiner antibody are not routinely performed.

Therapy

No specific treatment for paroxysmal cold hemoglobinuria has been found. Prednisone is not useful. The best approach is supportive care, transfusions to alleviate symptoms, and avoidance of cold temperatures.

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DRUG-INDUCED IMMUNE HEMOLYTIC ANEMIA

Drug-induced immune hemolytic anemia was commonly seen when penicillin was administered in large doses (i.e., >20 million U/day) and when -methyldopa was widely used in the treatment of hypertension. [261] [262] However, the disease is unusual in present-day clinical practice. [263]

Four distinct mechanisms are associated with the disorder. In the first, the patient makes antibodies against a drug (e.g., penicillin) that can bind to the RBC membrane, exposing its haptenic determinant. The antidrug antibodies combine with the erythrocyte-bound drug, opsonizing and preparing the RBC for destruction. In the case of penicillin, [264] hemolytic anemia occurs only when large amounts are administered; in patients treated with lower doses, a positive DAT result without hemolytic anemia is not unusual because the production of low-avidity IgG antipenicillin antibodies is a common event. Discontinuation of the drug brings the hemolytic anemia to a rapid halt. Clues to the diagnosis are the appropriate clinical setting: a positive DAT result, a negative result on the indirect antiglobulin test, and failure of antibodies eluted from the patients RBCs to bind to normal erythrocyte. The diagnosis is established when both the eluate and the patients serum react with penicillin-coated cells.

The second mechanism involves immune complexes. The offending drug, or drug metabolite, binds to a plasma protein, forming an immunogenic conjugate. The resulting antidrug antibody binds to the drug-plasma protein conjugate, forming an immune complex that adheres to RBCs. The antidrug antibody, usually IgM, causes a clinical picture of intravascular hemolysis, hemoglobinemia, hemoglobinuria, and even renal failure by efficiently activating complement on the erythrocyte membrane. This chain of events accounts for most reported examples of drug-induced immune hemolytic anemia. Recent reports concerning the nonsteroidal drug diclofenac have shown that autoimmune hemolytic anemia is induced by sensitization to the glucuronide conjugate of the drug. [265]

Serologic findings with erythrocyte-bound immune complexes are similar to those of the first mechanism, except that

Box 34-5

DRUGS REPORTED TO CAUSE HEMOLYTIC ANEMIA[a]

Mechanism	Hapten	Immune Complex	Autoantibody
Example	Penicillin	Stibophen	Methyldopa
DAT	Positive	Positive	Positive
Anti-IgG	Positive	Rarely positive	Positive
Anti-C3d	Rarely positive	Positive	Negative
Indirect antiglobulin test (drug not present in test system)	Negative	Negative	Negative or positive
Indirect antiglobulin test (drug in test system)	Positive	Positive	No change due to drug
Other drugs	Cephalothin	Qinine	Mefenamic acid
	Cephaloridine	Quinidine	L-dopa
	Ampicillin	Phenacetin	Procainamide
	Methicillin	Hydrochlorothiazide	Ibuprofen
	Carbenicillin	Rifampin	Diclofenac
	Akfluor 25%	*p*-Aminosalicylic acid	Thioridazine
	Cefotaxine	Antihistamines	Interferon-
		Sulfonamides	
		Isoniazid	
		Chlorpromazine	
		Pyramidon	
		Dipyrone	
		Melphalan	
		Insulin	
		Tetracycline	
		Acetaminophen	
		Streptomycin	
		Hydralazine	
		Probenecid	
		Carbimazole	
		Sulfonylurea derivative	
		Chlorinated hydrocarbon insecticides	
		Cianidanol	
		Cephalosporin	
		Nomifensine	

		5-Fluorouracil	
		Tolmetin	
		Fenoprofen	
		Sulindac	
		Cefotaxine	
		Ceftriaxone	
		Radiographic contrast medium	

[a]Mechanism and serologic findings are summarized.

the DAT reveals complement only bound to the RBC; the IgM antibody is presumed to be no longer present after complement activation. The patients serum reacts with RBCs (lacking antidrug antibody) in the presence of the offending drug, and the eluate from the patients RBCs generally does not react with normal erythrocytes.

The third mechanism involves in vivo sensitization to drugs by the formation of immunogenic drug-RBC complexes. In these cases, the specificity of the drug-induced antibodies is contributed not only by the drug (or its metabolites), but also by defined RBC antigens, particularly of the Rhesus and I/i systems. [266]

The fourth mechanism involves the induction of authentic autoantibodies against RBCs by a drug. -Methyldopa is the classic example (Fig. 34-10).[267] In as many as 20% of patients treated with -methyldopa the DAT result turns positive, but few develop hemolytic anemia. The antiglobulin test result may take several months to a year or more after the start of drug therapy to become positive. In patients with hemolytic anemia, discontinuation of the drug results in the gradual cessation of the hemolytic anemia and disappearance of the autoantibody. Curiously, the autoantibody is usually specific for antigens of the Rh system. The serologic findings are indistinguishable from those of primary autoimmune hemolytic anemia; they include a positive DAT result, usually a positive result on the indirect antiglobulin test, and an eluate that reacts with normal erythrocytes. In contrast to the preceding examples, in this case the drug is not required in the test system to demonstrate the presence of the antibodies. Patients taking -methyldopa often have antinuclear antibodies, rheumatoid factor, and antibodies to gastric parietal cells, in addition to the RBC autoantibodies. The mechanism by which -methyldopa induces autoantibodies

Figure 34-10 Drug-induced hemolytic anemia. **(A)** The planted antigen mechanism, of which penicillin-induced hemolytic anemia is the paradigm, entails binding of a drug to the RBC membrane, followed by binding of antidrug antibody to the planted antigen. **(B)** By contrast, in the autoimmune mechanism, the patient produces authentic antierythrocyte autoantibodies.

is unknown but may involve effects on immunoregulatory T cells.

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UNUSUAL FORMS OF IMMUNE HEMOLYTIC ANEMIA

Hemolytic anemia due to heteroantibodies or alloantibodies may be confused with autoimmune hemolytic anemia in certain cases. Some lots of antithymocyte globulin contain heterologous anti-human RBC antibodies, which can precipitate attacks of immune hemolysis. [268] Anti-A alloantibodies in some preparations of intravenous human -globulin have resulted in RBC destruction, [269] [270] and the synthesis of alloantibodies by B cells in transplanted livers and kidneys may cause a hemolytic anemia that masquerades as autoimmune hemolytic anemia.[271]

FUTURE DIRECTIONS

The lifelong symbiotic relationship between the immune system and erythrocytes begins to unfold during the early stages of embryonic life. Lymphocytes and macrophages supply immature erythrocytes with growth-promoting lymphokines and with iron; in turn, mature RBCs furnish the immune system with oxygen. Old erythrocytes, no longer disks but spheres, end their lives within antigen-presenting macrophages. Even so, lymphocytes ignore virtually all the proteins, glycoproteins, and glycolipids on the RBC membrane. Those same components can trigger the production of antibodies with a lethal potential when injected into another person with a different constellation of blood groups, even if the difference is but a single sugar molecule. The molecular biology of this striking aspect of lymphocyte physiologyimmunologic toleranceis not understood, nor are the mechanisms responsible for its induction and maintenance. The developmental immunology of structurally defined human blood groups is an untapped mine of information with considerable relevance to the phenomenon of immunologic tolerance in humans. The advantages of such studies over experiments on specially bred transgeneic mouse for immunologic investigations should be self-evident.

Virtually nothing is known about how the immunologic tolerance of erythrocyte autoantigens breaks downthe cause of autoimmune hemolytic anemia. Research on this question needs to focus on three topics: the structures of the autoantibodies, molecular identification of the autoantigens, and elucidation of the networks of lymphocytes and idiotypes that enforce unresponsiveness to erythrocyte autoantigens. Modern techniques of molecular biology (gene analysis, protein microsequencing, and cell cloning, in particular) are ripe for application to the problem. A particularly fruitful line for future investigations is the mechanism of the production of IgG warm-reactive antierythrocyte autoantibodies in CLL. This is one of the few cases in which a monoclonal population of B cells with obvious relevance to polyclonal autoantibodies can be cloned in vitro and analyzed with the armamentarium of molecular immunology.

The present classification of the autoimmune hemolytic anemias does not accommodate all variants of these disorders, but it is generally useful. Our terminology reflects current practice: most clinicians recognize that the unembellished term *autoimmune hemolytic anemia* refers to hemolytic anemia due to warm-reactive IgG autoantibodies and that cold agglutinin disease means hemolytic anemia associated with cold-reactive IgM autoantibodies. Not all cases fit into these neat categories, however. The thermal behavior of the autoantibody, or its isotype, may not correspond to arbitrary rules, and some patients produce mixtures of IgG, IgM, and even IgA autoantibodies. Furthermore, the serologic specificity of the autoantibody in autoimmune hemolytic anemia is highly variable. Some autoantibodies bind to well-defined blood group antigens, whereas

others show much broader patterns of reactivity. Still others bind to structural membrane proteins or phospholipids with no known relationship to blood group antigens. Additional contributions to the heterogeneity of the autoimmune hemolytic anemias include the complement-fixing ability of the autoantibody and its avidity for the erythrocyte autoantigen. A realistic classification of the autoimmune hemolytic anemias would take this extensive heterogeneity into account, but it might prove too cumbersome for practical application.

The physician encounters additional layers of complexity in deciding about treatment. Is the disorder primary or secondary? Does the patient have SLE or a lymphoma? Could a drug be involved? Are transfusions warranted if a compatible donor cannot be identified? Management of the patient with autoimmune hemolytic anemia in relapse remains a major problem. None of the available tests accurately predicts the efficacy of splenectomy in an individual case. This research problem has a useful potential, not only in the autoimmune hemolytic anemias but in other immune-mediated cytopenias as well. Despite the plausible and detailed description of the in vivo mechanism of antibody-mediated destruction of RBCs, effective therapeutic advances based on this knowledge have made slow progress.

Every patient with an autoimmune hemolytic anemia tests the knowledge, skill, and art of the physician. These qualities are, to be sure, of singular importance, but common sense and good judgment count above all. They make the difference in any rare disease for which the outcome is unpredictable.

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Chapter 35 - Extrinsic Nonimmune Hemolytic Anemias

Stanley L. Schrier

INTRODUCTION

By definition, extrinsic causes of hemolysis are abnormalities in the environment in which the red cells, usually normal themselves, circulate. These abnormalities may occur acutely or may be chronic in nature. These conditions can arise from either congenital or acquired lesions, but mostly the latter. The decision that a patient has an anemia belonging to this category is made by using the approaches described in Chapter 23 and determining that hemolysis with varying levels of compensation is the cause of the anemia. Signs of extrinsic hemolysis with minimal or no anemia can also be valuable clues to diseases of other organ systems. It should be emphasized that among the most important forms of extrinsic hemolytic anemia are those caused by immune mechanisms; these are discussed in Chapter 34 .

Clinical and morphologic findings suggest the many misfortunes that can befall red cells in their travels. They can be trapped in an abnormal marrow stroma network, sheared by jets in an abnormal heart, cut and fragmented by fibrin strands stretched across damaged areas in the microvasculature, or attacked by parasites. They also can undergo stasis and perhaps metabolic depletion in giant hemangiomas or in an enlarged spleen. An abnormally functioning liver or kidney can cause a buildup of substances in plasma that can alter red cell shape and metabolism. Drugs can cause oxidation or other metabolic damage. Oxidant injury provokes degradation of hemoglobin with the formation of hemichromes. Degraded hemoglobin and hemichromes bind avidly to the cytoplasmic tail of the major transmembrane protein, band 3 (see Chaps. 4 and 33) and cause the clustering of band 3 oligomers.[1] Immunoglobulins and complement then bind to the external membrane face over clusters of band 3, promoting immune destruction. Other membrane proteins may be subject to oxidative attack. Toxins, venoms, heat, and mechanical trauma can directly destroy the membrane. These agents may also cause an alteration in the asymmetry of the phospholipid bilayer, causing phosphatidylserine to move from the inner leaflet of the membrane bilayer to the outer leaflet where it can be recognized by macrophages.

In general, only the most devastating damage leads to direct intravascular destruction. Usually, the initial insult leads to an eventual change in the external portion of the red blood cell (RBC) membrane that in turn causes macrophages to retard, hold, remove, or otherwise modify these RBCs. Infection or inflammation can activate these macrophages. Moreover, some RBC changes are accompanied by a decrease in RBC deformability, which retards flow and thereby facilitates the action of macrophages on the affected RBC. All of these changes lead to extravascular hemolysis.

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FRAGMENTATION HEMOLYSISMICROANGIOPATHY

Clinical Manifestations

Patients present with varying degrees of hemolytic anemia and compensation, with evidence of red cell fragmentation on smear (Plate 35-1). Red cell removal is generally extravascular, with minimal or moderately decreased levels of haptoglobin, but if red cell damage is severe, signs of intravascular hemolysis may also be present. Because of the underlying pathology, some of these syndromes may show evidence of platelet removal, leading to thrombocytopenia. Occasionally the underlying cause may also produce procoagulant activation and depletion with consequent activation of the fibrinolytic system, consistent with disseminated intravascular coagulation (DIC) (Table 35-1).

Pathophysiology

Fragmentation hemolysis occurs when mechanical forces disrupt the physical integrity of the red cell membrane. Brain et al. [2] noted a possible relationship between vascular lesions and cases of RBC fragmentation. They postulated that the lesions either produced shearing forces sufficiently strong to fragment the red cells or incited inflammation of small vessel walls, which in turn would generate the fibrin strands that literally cut the passing red cell into irregular pieces. Subsequent studies have documented the shearing forces required to fragment RBCs. In vitro shear stresses in excess of 3,000 dynes/cm ^{2} cause RBC fragmentation. In vivo studies in patients with mitral prosthetic regurgitation and hemolysis show either high peak shear stresses of 4,500 dynes/cm ^{2} or very rapid acceleration or deceleration, or both. [3] A recent report described a baboon model in which injection of C4b-binding proteins and *Escherichia coli* produced a picture of DIC with microangiopathic hemolysis and thromobocytopenia; treatment with the F(ab)$_2$ monoclonal antibody against platelet glycoprotein (GP) IIb/IIIa (7E3) reduced both the microangiopathic hemolysis and renal disease. [4] This result suggests an alternative mechanism for producing

TABLE 35-1 -- Causes of Red Blood Cell Fragmentation Hemolysis

Damaged microvasculature	
	TTP-HUS
	Associated with pregnancy: pre-eclampsia/eclampsia, HELLP
	Associated with malignancy: with or without mitomycin C
	Vasculitis: polyarteritis, Wegeners granulomatosis, acute glomerulonephritis, *Rickettsia*-like infections
	Abnormalities of renal vasculature: malignant hypertension, acute glomerulonephritis, scleroderma, allograft rejection with or without cyclosporine
	Disseminated intravascular coagulation
Atrioventricular malformations	
	Kasabach-Merritt syndrome
	Hemangioendotheliomas
	Atrioventricular shunts, congenital and acquired (stents, coils, TIPS, Levine shunts)
Cardiac abnormalities	
	Replaced valve, prosthesis, grafts, patches
	Aortic stenosis, regurgitant jets (as in ruptured sinus of Valsalva)
	Drugs
	Cyclosporine, mitomycin, ticlopidine, tacrolimus, cocaine

Abbreviations: TTP/HUS, thrombotic thrombocytopenic purpura/hemolytic uremic syndrome; HELLP, hemolysis plus elevated liver enzymes plus low platelet count.

microangiopathic hemolysis that involves platelets and small vessel thrombi in the kidney.

The red cell membrane is viscoelastic and has self-sealing properties (see Chaps. 4 and 33), so that little hemoglobin leaks out as the cell is being cut. However, prolonged distortion of the membrane produces a plastic change; therefore, the smaller red cell fragments usually do not become microspheres or microdisks but continue to display evidence of the shearing event or distortion in the form of the typical irregular shapes. These irregular shapes and the rigidity that they reflect subsequently interfere with the ability of red cells to fold, elongate, and deform sufficiently to pass through 3-m capillaries and even smaller slits in the walls of the sinusoids of the reticuloendothelial system. This sequence leads to their destruction.

DIFFERENTIAL DIAGNOSIS OF EXTRINSIC NONIMMUNE HEMOLYTIC ANEMIAS

There is no simplistic approach to the differential diagnosis of this type of hemolysis; the physician must pay close attention to the clinical setting. Useful clues come from a determination of whether red cell breakdown is predominantly extravascular or intravascular. However, most important in the analysis is the observation of red cell morphology, because an accurate appreciation of morphology focuses and limits the differential diagnosis. Such nonhelpful terms as *aniso* and *poik* should be discarded. The RBCs are spherocytic, stomatocytic, fragmented, echinocytic, acanthocytic, spurred, or bite cells, or mixtures of these. When necessary, the standard Wright-Giemsa-stained peripheral blood smear should be supplemented with a wet preparation in which 1020 l of freshly drawn heparinized whole blood is added to 200 l of 1% glutaraldehyde in phosphate-buffered saline. After several moments to allow for fixation, the RBCs are examined by phase microscopy (phase 2, ×400 or phase 3, ×1,000), and the three-dimensional appearance of the cells can be appreciated. Subtle degrees of stomatocytosis (spherocytes are really stomatocytes), red cell budding, and acanthocytosis become apparent, and artifacts in smear preparation are avoided.

Differential Diagnosis

Generally, the differential diagnosis of fragmentation hemolysis can be deduced from the clinical setting. The presence of a prosthetic heart valve (Waring blender syndrome) can be readily discerned. The clinical picture of thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS) is generally dramatic and acute (see Chap. 126). Atrioventricular malformations may be associated with DIC and platelet removal; the diagnosis requires a high index of suspicion and imaging studies. The presence of pre-eclampsia in a pregnant woman with microangiopathic hemolysis is usually obvious, but the HELLP syndrome (hemolysis, elevated liver enzymes, and a low platelet count) is a serious complication of pregnancy that can occur without other signs of pre-eclampsia or hypertension. [5] [6] This syndrome can produce hepatic rupture, visual failure, DIC, seizures, and congestive heart failure and needs to be treated by prompt delivery of the fetus (see Chap. 149); cancer can be an underlying cause. Vessels supplying malignant tumors are thought to be structurally abnormal. They exhibit the same sort of fibrin stranding that produces fragmentation hemolysis in DIC and TTP/HUS.

The continued utilization of invasive methods for diagnosis and treatment with the insertion of foreign bodies into the circulation has brought with it complications of microangiopathic hemolysis. Transjugular intrahepatic portosystemic shunts (TIPS) can cause the syndrome in 10% of patients. [7] The hemolysis apears to disappear after 1215 weeks. Similarly, the use of coil embolization to seal off a patent ductus arteriosus produced a fall in hemoglobin from 11.6 to 6 g in one patient. [8] Vasculitis has been implicated as a cause.[9]

It now appears that some drugs can produce microangiopathic hemolysis. Cyclosporine [10] and mitomycin C have been implicated as causing an HUS picture. However, for mitomycin C at least, it is frequently difficult to distinguish the action of the drug from that of the cancer being treated. [11] The new antiplatelet agent ticlopidine also seems to be capable of producing a TTP-like syndrome. There are few preliminary reports, and the incidence is probably quite low. [12] The newer immunosuppressive agent tacrolimus (FK506) seems to be as capable as cyclosporine of causing microangiopathic hemolysis. [13] One patient is thought to have had microangiopathic hemolysis following cocaine use. [14] Therapy for cancer may play a role. Of 581 patients treated for breast cancer with cyclophosphamide, cisplatin, and carmustine, 2.6% developed microangiopathic hemolysis.[15]

Therapy

Management is primarily directed toward the underlying disease or event. In addition, compensation of red cell production should be optimized by replacing iron or folic acid if the patient is deficient in these nutrients. Occasionally it is necessary to remove or replace a prosthetic heart valve when the hemolysis produces a disabling transfusion requirement. The HUS of mitomycin C/adenocarcinoma is said to respond reasonably well to use of the staphylococcal protein A immunoperfusion column[16] or to infusion of vincristine, [11] but it may be necessary to proceed to apheresis with fresh frozen plasma replacement.

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OTHER FORMS OF MECHANICAL DAMAGE TO RED CELLS

Heat Denaturation

Normal RBCs will undergo budding and fragmentation when exposed to a temperature of 49°C in vitro. In some of the hereditary hemolytic anemias this occurs at temperatures as low as 46°C (see Chap. 33). Some clinical circumstances have been identified in which temperatures sufficient to cause heat denaturation

Figure 35-1 Change in RBC morphology produced by heating normal RBCs at the indicated temperatures. Note the budding that begins abruptly at 50°C, leading eventually to spherocytosis.

of RBCs have been generated. Occasionally, cell warmers used to bring transfused red cells to body temperature before infusion in treatment of cold agglutinin disease have malfunctioned and cooked the red cells about to be transfused. In one case, a patients mother decided to warm red cells with a hot water bottle, reasoning that such cells would cause less vein irritation to her child. Such transfusion was followed by evidence of both intra- and extravascular hemolysis, and the peripheral smear showed red cell budding and fragmentation (Fig. 35-1). Presumably, similar events can lead to hemolysis in patients who have sustained very extensive burns. In patients suffering from heat stroke, the temperature is usually below 42°C; at this temperature little RBC denaturation occurs.

Mechanical Trauma

The classic example of red cell damage due to mechanical trauma is march hemoglobinuria, which occurs in soldiers after a long march, in joggers after running on a hard road, or in karate or conga drumming enthusiasts following practice. Anemia is rare, and reticulocytosis is uncommon. Evidence of typical intravascular red cell destruction is present and is thought to be caused by direct trauma to the red cells in the vessels of the feet or hands. Switching jogging paths or using better footwear often relieves the problem. In some cases there is evidence of an underlying red cell membrane abnormality. [17] Occasionally, malfunction of the cell savers used during abdominal or thoracic surgery can mechanically injure red cells.

Cardiopulmonary Bypass

The postperfusion syndrome occurs in some patients following cardiopulmonary bypass. The syndrome includes acute intravascular hemolysis and leukopenia as part of a febrile inflammatory clinical picture. Affected patients may go on to develop pulmonary distress and even adult respiratory distress syndrome. Visible hemoglobinemia occurs, with rising plasma hemoglobin levels, associated with an increase in lysed red cell ghosts seen in the whole blood and plasma. These ghosts have been shown to be coated with the complement complex C5bC9 (see Chap. 37). Presumably, the complement pathway is activated as the blood is passed through the oxygenator. Why the complement activation results in lytic attack on red cells (and also granulocytes) is not known. Treatment involves knowledge of the process and requisite support [18] until the situation corrects itself.

Osmotic Attack

Abrupt changes in osmolality can also cause hemolysis. Freshwater drowning may be associated with so much water in the lungs that the red cells swell as they undergo an in vivo osmotic fragility test in the lung vessels. Saltwater drowning, conversely, can cause profound dehydration of red cells, producing a situation analogous to xerocytosis (see Chap. 33). Very rarely, acute hemolysis may occur from mistaken infusion of or exposure to concentrated hypertonic solutions such as those used in hemodialysis. To manage such an event, the physician must recognize its cause, appreciate the shrunken RBCs on peripheral smear, and restore isotonicity as quickly as possible. The use of a hemodialysis device, if available, may be helpful in this regard. [19]

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HYPERSPLENISM

In all organs of the monocyte-macrophage system (reticulo-endothelial system), blood cells leaving the arterial bed are generally unloaded into channels such that the red cell must pass through the wall of the sinus to re-enter the circulation. The sinusoidal wall has slits 23 m in size and is usually endothelialized on one side and has a macrophagic lining on the other. The normal human adult RBC is a discocyte, having a surface area 40% larger than a sphere of that volume (see Chap. 4). It is this excess surface area that allows a red cell of approximately 8 m in diameter to twist, elongate, and deform sufficiently to squeeze through these 2- to 3-m slits. This excess of surface area is critical and is occasionally referred to as the ratio of surface area to volume (SA/V); it is normally about 1.4. Any condition that reduces the SA/V reduces the ability of the red cell to traverse these sinusoidal slits because a plump sphere cannot deform sufficiently.

Factors that interfere with the interaction of the cytosol and the membrane will also impair the red cells ability to deform. Oxidant attack may produce Heinz bodies that come to lie adjacent to the membrane. They interfere with the smooth movement of the membrane over the cytosol, called tank treading. Such cells are selectively blocked from leaving the splenic cords and entering the sinuses. [20] Inflammation or infection may enhance the ability of splenic macrophages to attack and ingest red cells. Although not strictly a mechanism of hypersplenism, Kupffer cell erythrophagocytosis is a prominent finding in patients undergoing the graft-versus-host hemolysis seen after liver transplantation. [21]

The spleen is somewhat more complicated than other RE organs in that the afferent arterioles pass through lymphoid nodules (the white pulp) and then terminate in the cords of Billroth (the red pulp), into which the blood cells are discharged. In the slow flow of the cords of Billroth, the blood cells are selectively attacked by macrophages and are in direct contact with several classes of lymphocytes. The blood cells must then pass through the cordal walls before they can approach the sinus wall, which they must pass through to re-enter the circulation. Thus, the spleen provides a double filter, and the blood cells must be

TABLE 35-2 -- Pathophysiologic Mechanisms of Splenomegaly

Mechanisms	Examples
Neoplasia	Lymphoma, hairy cell leukemia
Infections	Bacterial endocarditis
	Malaria, schistosomiasis
	Tuberculosis
Portal bed obstruction	Alcoholic cirrhosis
	Splenic vein thrombosis
Collagen-vascular disease	Systemic lupus erythematosus, rheumatoid arthritismalignant phase
Chronic inflammatory disease	Rheumatoid arthritis
Chronic hereditary or acquired hemolytic anemias	Severe -thalassemia
	Autoimmune hemolytic anemia
Miscellaneous disorders	
Lipoidosis	Gaucher disease
Amyloidosis	AL and AA types
Tropical splenomegaly syndrome	Hyperreactive malarial splenomegaly syndrome

remarkably deformable to pass through it. Furthermore, this slow passage permits highly selective action by macrophages, which have receptors that can detect several sorts of alterations in these blood cells. These receptors include the Fc receptor for the appropriate portion of the immunoglobulin molecule, receptors for complement components such as C3b, and perhaps receptors that detect alterations in the outer portion of the phospholipid bilayer or in the externally oriented glycopeptides. The macrophage then holds, retards, modifies (pitting function), or removes (culling function) the blood cells so identified. Normally, the pitting function of the spleen allows it to remove Howell-Jolly bodies and normally occurring endocytic vacuoles (called pocks because of their appearance on phase interference or Nomarski microscopy). The normal culling function of the spleen is exemplified by its removal of senescent RBCs. [22]

All the activities of the spleen are presumably markedly accentuated in a large spleen, and if the increased activity is extensive enough, hypersplenism ensues. It is the size of the spleen, not the portal pressure, that is important in determining the degree of red cell sequestration that occurs. [23] Other factors that may play a role are the state of activation of the splenic macrophages and the size of the small slits between the splenic cords and sinuses. Both macrophages and slits seem to be under a degree of control, as evidenced by variations in the splenic removal of red cells in patients with malaria. [24] [25]

The clinical picture of hypersplenic hemolysis is dominated by the specific cause of the splenomegaly. Although causes of splenomegaly are legion, there are several general mechanisms (Table 35-2). Generally, there is a varying degree of anemia, with varying evidence of a compensatory increase in red cell production. Because stasis and trapping in the spleen are associated with macrophagic attack and remodeling of the red cell surface, the reduction in SA/V leads to spherocytosis. If the red cells undergo a prolonged period of distortion in traversing the cordal-sinus barrier, then tailed red cells will be present as the red cell membranes undergo a plastic change (Plate 35-2) (see Chaps. 4 and 33). Presumably, the enlarged spleen can also trap and remove platelets and white blood cells (WBCs), so that variable thrombocytopenia and leukopenia may occur. The bone marrow may show normal to increased cellularity, with erythroid hyperplasia.

Management depends on the causes of the splenic enlargement. The anemia or pancytopenia usually is not very profound, but if the anemia is very severe, splenectomy may be contemplated. In most situations, recognition of the possibility of hypersplenism is most important in guiding the approach to diagnosis of an unexplained anemia. Massive splenomegaly is frequently associated with expansion of the plasma compartment, so that measurement of hemoglobin, hematocrit, or red cell count may give a falsely low value of the actual red cell mass present. In that circumstance the true RBC mass can be determined by a ^{51} Cr assay.

A good example of the previous situation is the tropical splenomegaly syndrome, now renamed hyperreactive malarial splenomegaly syndrome. [26] Diagnostic criteria

include massive splenomegaly, >10 cm below the costal margin, with no other cause identified; immunity to malaria; elevated serum IgM levels; and clinical response to treatment with the antimalarials like chloroquine, proguanil, or pyrimethamine and folic acid. [26] The pathophysiology of the splenomegaly seems to be a poorly controlled B-lymphocytic production of antibodies, and the IgM stimulation may be a response to malarial antigens or an unidentified mitogen. Malarial parasites are almost never found. The apparent anemia is in large part due to plasma volume expansion, although red cell survival is reported to be slightly attenuated. Splenectomy is unnecessary; antimalarial therapy for several months results in a reduction in spleen size.

INFECTION

Pathophysiologically, there are several mechanisms by which infection can cause hemolytic anemia (Table 35-3).

Direct Parasitization

The classic example of direct parasitization is *Plasmodium falciparum, P. vivax,* or *P. malariae* infection. In each of the malarias, sporozoites injected by the mosquito in its saliva make their way to liver cells, where after 12 weeks they become merozoites, which burst out of the liver cells and into the bloodstream. Then, in a remarkable process, the parasite via its apical end and related organelles called rhopteries attaches to a specific receptor on the red cell surface. For *P. vivax,* the Duffy blood group antigen appears to be involved. *P. falciparum* binds to sialic acid residues on the red cell surface that are on glycophorin A. Following specific attachment, a convulsive movement occurs during which the red cell engulfs the parasite by a process resembling receptor-mediated endocytosis. The parasite then immediately co-opts the red cells metabolic machinery, degrades and ingests hemoglobin, and grows, eventually bursting out of the red cell, and the cycle begins again. The red cells are lysed, both intravascularly as a consequence of direct parasitic destruction, and extravascularly, as a consequence of changes in the splenic microvasculature and in the activation state of the monocyte-macrophage system. In addition, acute malarial infection, particularly with P. falciparum, leads to alteration in splenic function and in the structure of nonparasitized red cells that incites their premature destruction. [24] [25] [27] Treatment consists

TABLE 35-3 -- Mechanisms by Which Infection Can Cause Hemolysis

Direct parasitization of red cells
Examples: malaria, babesiosis
Immune mechanisms
Example: cold agglutinin hemolysis following infectious mononucleosis or mycoplasmal pneumonia (see Chap. 34)
Induction of hypersplenism
Examples: malaria, schistosomiasis
Altered red cell surface topology
Example: *Hemophilus influenzae*
Release of toxins and enzymes
Example: clostridial infection causing TTP/HUS; *E. coli* 0197; HIV(?)

of the use of appropriate antimalarials and the support of erythropoiesis.

Other infections that have somewhat similar pathophysiologies include Carrión disease (bartonellosis), in which a bite from the sandfly injects *Bartonella bacilliformis,* which attaches to the red cell surface and somehow causes lysis. It appears that the invasion of red cells is partly dependent on the flagella of bartonellae. Incubation with antiflagellin antiserum reduces invasion of red cells. [28] In babesiosis the parasite is transmitted by ticks and directly invades red cells, producing fever and hemolytic anemia. On the East Coast the disorder occurs in continental Massachusetts and on Nantucket Island. Most cases occur on the West Coast, particularly Washington and California, and in the northern portion of the Midwest. The organisms can be seen invading red cells on smear examination, somewhat like *P. falciparum* malaria, but these organisms produce no pigment. Babesiosis has been described in an immunocompromised patient with AIDS and thus must be considered in the differential diagnosis of such patients in endemic areas who present with persistent fever and evidence of hemolysis. [29] In addition, there are sporadic reports indicating that acquired chronic toxoplasmosis can occasionally be associated with hemolytic anemia. [30]

Alteration of Red Cell Surface by Bacterial Products

Infection can produce hemolysis by altering the red cell surface. An example is the hemolysis caused by *Hemophilus influenzae* type b.[31] Severely affected patients, particularly those with meningitis, have developed hemolytic anemias requiring red cell transfusions. The capsular polysaccharide of the bacterium, composed of polyribosyl ribosyl phosphate (PRP), is released during infection and binds to the red cell surface. Infected patients develop antibodies to PRP. When the balance between PRP-coated red cells and anti-PRP antibodies is correct, an immune sort of hemolysis, requiring complement, occurs. Red cell destruction is thought to be both intra- and extravascular.

Bacterial Products Causing Hemolysis by Direct Damage to Red Cells

The most dramatic example of hemolysis due to bacterial action is clostridial infection, during which the organism releases enzymes that acutely degrade the phospholipids of the membrane bilayer and the structural membrane proteins. In one case the serum phospholipase C level increased 5-fold over 4 hours as the patient died.[32] The setting can be any infection, but my personal experience is limited to acute cholecystitis, surgery of the biliary tree, and infections surrounding an obstetric event, including criminal or self-induced abortion or other infection of the gravid uterus. The signs of infections may be obvious, but fever may be unimpressive.[33] [34] Signs of collapse appear acutely, and the clue is profound intravascular hemolysis, with a spherocytic anemia developing with shocking suddenness. A clue to the severity of the process may be the inability of the laboratory to perform chemical determinations or to type and cross-match the blood because the sample is hemolyzed. With even the slightest suspicion, the physician immediately starts full doses of penicillin, evaluates the patient for DIC (see Chap. 117), and prepares to support the patient for shock, DIC, acute renal failure, and hemolytic anemia. In the case of septic abortion it is not clear that hysterectomy is lifesaving.[34]

Hemolysis Caused by Less Well Understood Infections

HIV infection can cause Coombs-positive autoimmune hemolytic anemia and a TTP-like syndrome and microangiopathic hemolysis. Cytomegalovirus infection has been reported to cause hemolysis associated with thrombocytopenia in an immunocompetent woman by unknown mechanisms.[35] The hemolytic anemia in visceral

leishmaniasis may be in part caused by the generation of oxidative metabolic products. [36]

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HEMOLYSIS ASSOCIATED WITH LIVER DISEASE

Hemolysis in liver disease is usually not of overwhelming clinical importance by itself but might contribute to the severity of anemia when coupled with defects in red cell production and the type of gastrointestinal (GI) blood loss that occurs in several sorts of liver disease. There are several causes of hemolysis in patients with liver disease. The spleen may be enlarged as a consequence of portal hypertension [37] and produce a hypersplenic picture, a phenomenon seen quite commonly in hepatic cirrhosis.

The literature concerning red cell shape change in liver disease is considerable. The target cell in cirrhosis has an increased SA/V that appears to be a consequence of an increase in both the cholesterol and the phospholipid content of the membrane bilayer. The cholesterol increase is usually proportionately greater, resulting in an increase in the cholesterol/phospholipid ratio. It is this increase in lipid that probably accounts for the increase in red cell surface area, which means there is more membrane than usual for the cellular contents. These red cells probably circulate as bell-shaped red cells called codocytes, but on dried blood films they assume the appearance of target cells. Target cells per se do not have a shortened red cell survival. [37] [38] The red cells of patients with liver disease are frequently echinocytes when wet preparations are examined, but these echinocytes are not easily apparent on dried blood smears. [38] The echinocytes seem to be produced by a material in a patients plasma that causes normal red cells to become echinocytic; this material is an abnormal echinocytogenic high-density lipoprotein. Echinocytes per se do not necessarily have a shortened red cell survival. Some forms of echinocytic red cells are normally deformable when studied in the ektacytometer or rheoscope.

A brisk, clinically important hemolysis can occur in some patients with severe liver disease. The peripheral smear in these individuals usually shows acanthocytes (distorted red cells), extreme forms of which are called spur cells. Spur cells are probably acanthocytes additionally remodeled by an enlarged spleen (Plate 35-3) and are considerably enriched in cholesterol. [37] They are rapidly removed in the spleen, which is usually enlarged.

It may be that increased red cell membrane proteolytic activity is a partial explanation of the differences between acanthocytosis and spur cells. [39] Additional pathophysiologic mechanisms may be involved. Although the adult red cell cannot synthesize phospholipids de novo, it can identify and remove peroxidized fatty acid chains that interfere with normal membrane lipid fluidity. [40] When the fatty acid is removed a lyso-derivative remains, which is lytic, and therefore the missing fatty acid chain must be replaced. A store of acyl groups exists in red cell membranes in the form of acylcarnitine. When needed, the fatty acid (acyl group) is transferred to acyl-CoA and the fatty acid is then inserted into the potentially lytic lysophospholipid by the enzyme lysophosphocholine acyl transferase (LAT). LAT is inhibited in spur red cells, and the same inhibition can be produced by heavily loading red cells with cholesterol in vitro. [40]

Thus, in spur cell anemia the red cells have an abnormal membrane SA/V ratio, their membrane fluidity is impaired, and they are unable to remove and repair peroxidatively damaged fatty acids. On occasion, spur cell hemolytic anemia is severe enough to necessitate consideration of splenectomy. The operative morbidity in such cases is considerable, because the underlying alcoholism usually produces problems with thrombocytopenia and leukopenia, while the severity of the liver disease

can cause problems with procoagulants and intolerance to anesthesia.

Acute alcoholism can be associated with hypophosphatemia, defined as levels of <0.2 mg/dl. Such hypophosphatemia presumably interferes with red cell intermediary metabolism (see Chaps. 22 and 32), and red cell ATP levels fall. Very low ATP levels are associated with red cell rigidity. This rigidity leads to fragmentation, loss of surface area, and spheroidicity. These red cells are then further trapped in the spleen. This hypophosphatemia syndrome can also cause neuromuscular disorders, including weakness, paresthesias, tremors, and seizures. It should be treated aggressively with orally and intravenously administered phosphate supplements. Hypophosphatemia also occurs in cirrhotic patients, patients receiving total parenteral nutrition whose phosphate intake is not carefully monitored, and patients taking large amounts of phosphate-binding antacids. [41] [42]

Stomatocytosis can occur in severe liver disease and is also thought to be a sign of acute alcoholic intoxication. This change in red cell shape can also be seen in acute pancreatitis. The stomatocyte is a cell well on its way to becoming a spherocyte. The reduction in SA/V leads to trapping in the microvasculature of the spleen and other organs of the monocyte-macrophage system, producing varying degrees of hemolysis.

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RENAL DISEASE

The anemia in renal disease is multifactorial, with a major component being impaired red cell, production, which is now well-controlled with erythropoietin. The renal disease also impairs platelet function, which may lead to occult blood loss. However, hemolysis can also occur and is also multifactorial. Disease of the small renal arterioles can produce fragmentation hemolysis of the sort seen in TTP/HUS, pre-eclampsia, and malignant hypertension (Table 35-1). In the past, some patients on chronic hemodialysis were exposed to unusual concentrations of chloramine in the tap water and underwent acute oxidative hemolysis. [43] This occurrence has become very rare, if it even still exists. Otherwise it is not clear that uremia per se produces significant shortening of red cell survival. Patients with chronic renal failure on hemodialysis may be particularly susceptible to oxidative damage to their red cells. Red cell GSH is reduced in some patients, and the activity of the enzymes glucose-6-phosphate dehydrogenase (G6PD) and glutathione peroxidase was found to be relatively low. The ability of these red cells to deal with generation of peroxides is probably impaired. [44]

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VENOMS, BITES, STINGS, AND TOXINS

The best-known example of toxin-caused hemolysis was discussed earlier under clostridial sepsis.

Insect, Spider, and Snake Bites

Hemolysis has occurred after bee and wasp stings, snake bites, and spider bites. Isolated cases of acute intravascular hemolysis have been reported after bee and wasp stings. Two kinds of dangerous spiders live in the United States: the southern black widow and the brown recluse spider. Both sexes of the black widow produce the venom, but only the female has fangs capable of penetrating human skin. Black widow spider bites produce generalized muscle pain and muscular rigidity. Hemolysis, if it occurs at all, is not common. Brown recluse spider bites cause a considerable local reaction, called the volcano lesion. Both DIC and hemolysis may occur after a 24- to 48-hour lag period. Corticosteroids may be beneficial. The hemolysis appears to be self-limiting, but in one case red cell transfusion support was needed.[45]

In some parts of the world, cobra bites can cause intravascular hemolysis because the venom contains phospholipases. In the United States the two classes of venomous snakes are pit vipers (rattlesnakes, cottonmouths, moccasins, and copperheads) and coral snakes. Pit viper venom affects hemostasis and may produce DIC with bleeding, but rarely hemolysis. Coral snake venom produces severe neurologic impairment. Therapy consists of support and use of the appropriate antivenin and prophylactic antimicrobials and tetanus injections.

Drugs and Chemicals (Exclusive of Those Producing Oxidative Hemolysis)

Potassium Chlorate

Potassium chlorate ingestion is listed as a cause of hemolysis, but this compound is no longer available in hospital pharmacies and has no currently recognized medical use. Arsine gas (AsH_3) is generated in industrial plants that engage in lead plating, galvanizing, etching, and soldering. Inhalation of a toxic amount produces a severe intravascular hemolysis[46] of unknown pathogenesis.

Copper

The idea that copper can produce human hemolytic disease is best supported by observations of episodes of severe hemolysis in patients with Wilson disease. The patient is usually a child, adolescent, or young adult in whom the diagnosis of Wilson disease has not yet been made. [47] [48] The initial clinical presentation usually is dominated by the hemolytic anemia, accompanied by weakness and dark urine. The red cell morphology has not been well described, but reticulocytosis is present, with an increased serum bilirubin level partly attributable to the underlying liver disease. Because of the hereditary deficiency in the copper-binding protein ceruloplasmin, serum and urine copper levels in patients with hemolysis are very high. Curiously, in one report hemoglobin A_2 levels were also elevated.[47]

Free copper can interfere with glucose metabolism by hexokinase inhibition and alternatively can generate oxidative hemolysis, perhaps by acting as a Fenton reagent. It is important to establish the diagnosis promptly suspecting this possibility, looking for the Kayser-Fleischer rings on physical examination, and measuring serum and urine copper and ceruloplasmin levels. Treatment with penicillamine reduces the serum copper level and stops the hemolysis. Other forms of copper poisoning also may cause hemolysis in patients who do not have underlying Wilson disease. The amount of copper ingested would have to exceed the normal copper-binding capacity of normal ceruloplasmin.

Lead Poisoning

There are at least two general forms of lead intoxication. Occupational exposure is an example of chronic, slow cumulative poisoning (saturnism). The symptoms are predominantly neurologic and nephrologic, with variable degrees of anemia, which may be due to a production defect combined with hemolysis. Relatively acute poisoning occurs when lead inadvertently finds its way into a food source [49] or is consumed as part of an exotic medication. Such subacute lead poisoning leads to central nervous system symptoms, hepatitis, nephrotoxicity, hypertension, and abdominal colic, along with seizures and severe hemolytic anemia. Physical examination may reveal the lead line on the gums. The peripheral smear shows extensive coarse basophilic stippling and reticulocytosis; however, red cell morphology is not otherwise characteristic. Some authors state that intravascular destruction occurs, but no proof has been provided. Bilirubin levels are not significantly elevated.

The diagnosis of lead-related hemolysis can be made from

THERAPY FOR LEAD-RELATED HEMOLYSIS

In an acute situation with multiple organ malfunctions and hemolysis, lead ingestion is immediately stopped and therapy is begun with 0.51.0 g of edetate calcium disodium administered intravenously over 6 to 8 hours every day for a 5-day course, while monitoring urine lead levels. If the symptoms improve and the urine levels fall satisfactorily, the patient can then be started on a regimen of 500 mg/day of oral penicilamine, which is continued for 60 days. If the symptoms do not improve satisfactorily another course of edetate calcium disodium is given.

the history and physical examination finding of the lead line on the gingiva, and from the coarse basophilic stippling on red cells, which reflects the pathologic aggregation of ribosomes. The diagnosis is confirmed by measuring of blood and urine lead levels. The level of acuity determines the therapy.

The cause of the anemia is complex. Lead interferes with several steps in heme synthesis, particularly those involving heme synthetase and -aminolevulinic acid dehydratase (see Chap. 27). The inhibition of heme synthetase probably accounts for the elevation in free erythrocyte protoporphyrin, which provides a useful corroborative diagnostic test for lead toxicity. Inhibition of heme synthesis also probably accounts for the elevated urinary levels of -aminolevulinic acid and coproporphyrin. Lead poisoning mimics the basophilic stippling and accumulation of pyrimidines seen in hereditary deficiency of the enzyme pyrimidine 5-nucleotidase,[50] probably because lead attacks the enzyme (see Chap. 32).

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DRUG-INDUCED OXIDATIVE HEMOLYSIS

General Concepts

The potential for normal red cells to undergo auto-oxidative destruction is great because the cell is loaded with 20 mM hemoglobin, most of which is bonded to oxygen at the iron(II) atom in heme. The bond that allows the reversible association and dissociation of oxygen from the heme moiety of hemoglobin involves partial transfer of an electron from iron(II) to oxygen. That oxygen now has an extra electron, which makes it a superoxide radical. Ordinarily, when oxygen leaves hemoglobin, it returns the electron, but if it does not, a highly reactive superoxide ion is released, leaving behind it an iron(III) moiety otherwise called methemoglobin.

$Hb\ Fe^{2+}\ 0_2\ Hb\ Fe^{3+} + O_2$ [1]

Methemoglobin cannot reversibly bind oxygen. It is not in itself harmful to red cells, but if the oxidative assault persists, methemoglobin is converted to hemichromes, which are variably denatured hemoglobin intermediates in which the distal histidine unit binds to the oxidized heme. This step is associated with conversion of a high to a low spin state, as measured by electron spin resonance. Continued oxidation leads to irreversibility of the hemichrome oxidation, to precipitation, and eventually to the formation of Heinz bodies. Hemichromes and Heinz bodies can destroy membrane function directly or by causing oxidation of membrane proteins and lipids. [51]

It is estimated that each day 3% of hemoglobin is converted to methemoglobin, but since only 1% of hemoglobin normally is in the form of methemoglobin, this indicates that a mechanism that prevents oxidation in red cells is in effect. This mechanism involves the NADH and NADPH-dependent reducing systems, catalase, glutathione, and the glutathione reductase and peroxidase systems. Defects in this defense system against oxidation lead to an enhanced tendency to oxidative hemolysis. An example is the several G6PD deficiency states. Any agent or event that interferes with the smooth offloading of oxygen will enhance the generation of O_2 [1] and methemoglobin (see equation). If the reducing power of the red cell is inadequate, hemichromes and Heinz bodies will subsequently be generated. In fact, many agents appear to cause oxidative hemolysis by interfering with the smooth functioning of the heme cleft.

Pathophysiology

Once the oxidative attack has been initiated, the sequence proceeds along a recognizable track. The oxidative attack is directed at hemoglobin and the red cell membrane. However, these are not clearly separable because the precipitated hemichrome and Heinz bodies come to lie against the cytosolic face of the membrane. Methemoglobin may be detectably elevated, with levels as high as 5060% of total hemoglobin. The hemichromes, by themselves or with their iron portions acting as a Fenton reagent, mediate the generation of hydroxyl free radicals, which add their effect to that of superoxide and hydrogen peroxide. Lipid peroxidation may take place, membrane proteins may be cross-linked, and adducts between spectrin and denatured globin may form. [52] Such red cells are rigid and are susceptible to trapping in sinusoidal structures whether or not they have Heinz bodies lying against the membrane. In addition, in vitro evidence suggests that such oxidized red cells are increasingly susceptible to phagocytosis by macrophages. These features may account for the extravascular destruction. The oxidative lesions can be so severe as to cause intravascular destruction as well, with hemoglobinemia and hemoglobinuria.

The smear may show bite cells (Plate 35-4), which look as if a macrophage had taken a bite, removing a Heinz body- containing segment of membrane. Red cell rigidity may result in irregularly shaped cells, since these undeformable cells are unable to undergo elastic recoil after fighting their way through the sinus wall. Recurrent loss of membrane material may produce spherocytes. Severe hemolysis may produce the kind of circulating ghost or hemighost called a blister cell or bite cell. These red cells have an empty veil of membrane on one side and puddled hemoglobin on the other. [53] [54] A Heinz body preparation may be positive.

The clinical picture is determined by the specific agent used. It may be useful to screen for G6PD deficiency or a related disorder by using an enzyme assay or the ascorbate cyanide test. Although any defect in the anti-oxidant defense mechanisms, such as G6PD deficiency, considerably increases the susceptibility to hemolysis, the agents listed in Table 35-4 can produce oxidant hemolysis even in persons with normal defense mechanisms. Paraquat ingestion has occurred inadvertently and in suicide attempts.[55] Profound cyanosis with methemoglobinemia can occur within hours, with levels of 120%. This can be succeeded

REDUCTION OF DANGEROUS METHEMOGLOBIN LEVELS

Levels of methemoglobin in excess of 2030% can be dangerous in themselves but can be easily treated with methylene blue (12 mg/kg) infused intravenously over 5 minutes as a 1-g/dl solution. In the presence of a functioning intact NADPH-methemoglobin reductase system, methylene blue is reduced to leuko-methylene blue, which in turn reduces methemoglobin to hemoglobin.

TABLE 35-4 -- Agents That Cause Oxidative Hemolysis

Therapeutic agents
Nitrofurantoin (Furadantin)
Sulfasalaine (Azulfidine)
p-Aminosalicylic acid
Phenazopyridine (Pyridium)
Phenacetin
Dapsone and other sulfones
Recreational drugs
Isobutyl nitrate
Amyl nitrite

Miscellaneous
Naphthalene mothballs
Paraquat
Hydrogen peroxide

by hemolysis, with Heinz bodies seen in appropriate preparations of red cells.

Nitrites have been used in suicide attempts,[56] and there have been industrial exposures as well. More recently nitrites have also been inhaled or swallowed as recreational agents.[57] [58] The nitrite may be sold in sex shops under the name locker room, sweat, or rush. Nitrites bind to hemoglobin,[59] producing methemoglobinemia, which may be so profound as to produce coma. If methylene blue infusion does not quickly turn the chocolate color of blood back to normal, one must consider the possibility that the patient is G6PD deficient and therefore unable to generate adequate amounts of NADPH (see previous discussion). In that case, exchange transfusion may be lifesaving.[59] [60] Benzocaine topical anesthesia in the form of a spray or cream can cause methemoglobin levels of 23% with cyanosis and dyspnea requiring methylene blue treatment.[59] [61] [62]

Pyridium (Phenazopyridine) can cause oxidative hemolysis,[63] even in the absence of renal disease.[64] This agent is in common use for the treatment of bladder irritation and the *Physicians Desk Reference* recommends a maximum of 2 days of therapy. However, it is not uncommon to see patients who have been given a prescription for 14 weeks of therapy.[65]

I have recently seen a case of almost fatal oxidative hemolysis, in which a patient with AIDS had injected hydrogen peroxide directly into his Hickman catheter. (Some persons infected with HIV had circulated a pamphlet suggesting that hydrogen peroxide could be used therapeutically to control the HIV infection.)

It has been recognized for more than 130 years that therapy with dapsone causes oxidative hemolysis.[66] In the past, dapsone was used primarily to treat leprosy and dermatitis herpetiformis and thus was not often encountered as a cause of oxidative hemolysis. More recently, dapsone has come into more widespread use in some communities as a very effective prophylactic agent against *Pneumocystis carinii* pneumonia in patients with AIDS. The reduced levels of GSH described in patients with AIDS may enhance dapsone toxicity. In some clinics

We are now seeing AIDS patients with dapsone-induced methemoglobinemia and hemolytic anemia. The methemoglobinemia, if severe, is treated as described previously and in Chapter 31 . One study suggests that the severity of the oxidative attack can be partially ameliorated by administration of 800 U/day of vitamin E, an agent that has antioxidant action.[69]

HEMOLYTIC ANEMIA IN CHRONIC LARGE GRANULAR LYMPHOCYTIC LEUKEMIA

Although large granular lymphocytic leukemia (LGL) usually presents with neutropenia and a rheumatoid-like picture, several patients have been described with a severe Coombs-negative hemolytic anemia in the absence of splenomegaly and, in a case seen by this author, LGL leukemia occurred in a splenectomized subject (see Chap. 66). The mechanism is unknown, but one report identified direct cytotoxicity against red cells by the LGL cell lines.[70] There are no formal studies on therapy. One patient had a partial response to therapy with 60 mg of Prednisone/day and two patients responded well to weekly doses of methotrexate begun at 10 mg/week.[71]

it is the practice to screen potential recipients for the G6PD deficiency (see Chap. 32) and if negative proceed with dapsone therapy. However, dapsone can cause oxidative attack on normal red cells, leading sequentially to methemoglobinemia, then Heinz bodies, and finally hemolysis, all at generally accepted standard doses.[67] Dapsone is metabolized to a hydroxylamine derivative that is directly toxic to red cells.[68]

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MISCELLANEOUS, POORLY CHARACTERIZED CAUSES OF EXTRINSIC HEMOLYTIC ANEMIAS

Interferon- as a Cause of Hemolytic Anemia

There are two case reports of acute and severe hemolytic anemia occurring in patients being treated with interferon- for chronic myeloid leukemia and hepatitis C. The Coombs test was negative in one case that seemed to respond to withdrawal of interferon and treatment with methylprednisolone. The other case underwent severe intravascular hemolysis. No mechanism is described. [72] [73]

Hemolysis with Intravenous Immune Globin G

Strictly speaking, this is a form of immune hemolysis. It is useful to remember, however, that preparations of IgG contain anti-A and anti-B antibodies and rarely cause an alloimmune hemolytic anemia, as described in two young women being treated for idiopathic thrombocytopenic purpua. If this situation occurs and more IV IgG is needed, performing a minor cross-match choosing a preparation of IV IgG that gives no reaction is recommended. [74]

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Chapter 36 - Immunoglobulins: Polyclonal and Monoclonal Antibodies

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INTRODUCTION

In 1845 Dr. Watson, a general practitioner, sent a urine sample obtained from a patient suffering from mollities ossium with the following letter to Professor H. Bence Jones, an English pathologist:

> Saturday, Nov. 1st, 1845
>
> Dear Dr. Jones,The tube contains urine of very high specific gravity. When boiled it becomes slightly opaque. On addition of nitric acid it effervesces, assumes a reddish hue, and becomes quite clear; but as it cools, assumes the consistence and appearance which you see. Heat reliquifies it. What is it?
>
> (Signed) Dr. Watson

In 1847, Jones published the results of his analysis of the specimen. He confirmed Dr. Watsons observation and reported that the sample contained a protein substance (an oxide albumen) that was distinguished from albumin by its solubility in nitric acid and lack of heat coagulability. He demonstrated that a protein in the sample purified by alcohol precipitation retained the properties of solubility in cold water: increased solubility in boiling water, coagulation with continued boiling, and return to solution with further boiling. Acid precipitated the substance, heating solubilized the acid precipitate, but cooling led to reprecipitation. Jones concluded his analysis by noting: Each oz. of this urine contained as much nutritive matter as an oz. of blood. No supply of food could compensate for such a loss. [1]

Over the years the presence of such proteins, subsequently named Bence Jones proteins, became a diagnostic test for multiple myeloma because of the high association between the plasma cell disease and the urinary pattern. One hundred and ten years lapsed, however, before Jones original insight about a possible relationship between the urinary substances and plasma proteins was confirmed by Korngold and Lipari [2] and Deutsch, Kratovil, and Reif[3] who used immunologic techniques to demonstrate the relationship of the urinary microglobulins (Bence Jones proteins) to normal and myeloma immunoglobulins.

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IMMUNOGLOBULINS

Properties and Structure

The mammalian immune system responds to the almost unlimited array of antigens by producing specific antibodies, each of which reacts specifically with the molecule that induced its production. During the immune response, the structure of the inducing antigen is imprinted on the immune system, as subsequent challenges with the same or structurally related molecule(s) will cause a more rapid rise in antibody levels to much greater concentrations than were achieved following the primary antigenic challenge. Thus, the hallmarks of the immune system include induction, specific protein interaction, and memory.

Antibodies belong to the family of proteins called the immunoglobulins. The basic structure of all immunoglobulins consists of a monomer that contains four polypeptide chains: two identical heavy (H) chains and two identical light (L) chains covalently linked by disulfide bonds (Fig. 36-1).[4] A model of the monomeric form of immunoglobulin has been prepared based on x-ray crystallographic data obtained on the IgG myeloma protein Dob (Plate 36-1). [5] The immunoglobulin monomer consists of a Y- or T-like structure. The size of the arms, called Fab (fragment antigen binding) domain of the Y or T, is 80 × 50 × 40 nm and the size of base, called Fc (fragment crystallizable), is 70 × 45 × 40 nm according to models based on x-ray diffraction data. [6] The immunoglobulin molecule exhibits considerable flexibility; the angle between the Fab domains has been observed in electron microscopic, low-angle x-ray scattering, transient electric birefringence, and resonance energy transfer studies to vary from 0° to 180°. All antibodies have two identical combining sites for each antigen located at the ends of the Fab domains.

The Fab and Fc represent functional domains in immunoglobulins.

Figure 36-1 Diagrammatic representation of the structural features of an IgG molecule. NH_2 indicates the NH_2 -terminus and COOH the C-terminus. The V_H , C_{H1} , V_L , and C_L homology domains are shown as boxes. Only the disulfide linkages that join H and L chains are shown. **(Left)** Approximate boundaries of the CDR regions in the V_L and V_H regions. **(Right)** Sequences encoded by V_H , D, J_H , V_L , and J_L segments in the V_H and V_L regions.

These were discovered by performing limited proteolytic digestion of the molecule. Both the H and L chains contribute amino acids that constitute the antigen-binding site in Fab. The Fab will combine with, but will not precipitate, multivalent antigens, in contrast to native IgG. A fragment can be prepared, called Fab2, which is devoid of Fc but still precipitates antigen. This form of immunoglobulin consists of two Fabs disulfide bonded at a part of the molecule called the hinge region. The hinge region is the part of the immunoglobulin molecule that is responsible for the molecular flexibility exhibited by all immunoglobulins. Every immunoglobulin is a glycoprotein, and the carbohydrate is always found attached to the H chain in the Fc domain. The other major function of immunoglobulins, binding to specific receptors on cells and certain effector proteins such as protein A and C1q, is associated with binding site(s) also found in Fc.

The chain structure of immunoglobulins explains neither antibody structural diversity nor antibody binding to antigen. The discovery that there were variable and constant regions of amino acid sequence formed the basis for understanding both phenomena. Thus, in the L chain, the 100 or so amino acids in amino-terminal half of the protein (V_L) vary between antibody molecules, but in the second half (C_L) there is virtual complete correspondence in amino acids, position for position, to the carboxy-terminus. The H chains exhibit the same pattern and can be divided likewise into V_H and C_H . Comparison of the amino acid sequence of many V_L s has revealed that certain parts of the variable region exhibit excess variability, whereas others have lesser variability. The former are called hypervariable (CDR) regions and the latter framework regions. The H chains exhibit the same pattern in the V_H regions. Amino acid sequence analysis of the C_H region, has shown three homologous regions where the amino acid sequences show more similarity than could have occurred by chance. These are called C_H 1, C_H 2, and C_H 3. The Fab region consists of the intact L chain, and the Fd region of the H chain consists of V_H C_H (Fig. 36-1). The combining site for antigen is a trough or a cavity composed of parts of the hypervariable regions of both the H and L chains. It is a small region, representing only 25% of the antibody V region. The region that interacts directly with the epitope on the antigen is even smaller and is formed by the association of the CDR regions, each of which consists of about 20 amino acids. Thus, the variation in a few amino acids accounts for the specificity and diversity of antibodies with respect to antigen binding. [4]

In addition to the amino acid sequence variations at the binding site, immunoglobulins can exhibit additional physical heterogeneity, and this imparts to each immunoglobulin a special effector function that is reflected in unique biologic properties that are additional to the antigen-binding activity. Heterologous and autologous antisera raised against immunoglobulins have been used to classify three types of physical heterogeneity. The first kind is based on the antigenic heterogeneity exhibited by immunoglobulin when it is used as an immunogen in other species. This is called class or isotypic variation. In humans five isotypes can be distinguished based on unique antigenic (isotypic) determinants found on the H chain. These are designated by capital Roman letters: IgG, IgM, IgA, IgD, and IgE. The H chain of each class is designated by the small Greek letter corresponding to the Roman letter of the class. Thus the H chain for IgG is , for IgM is , for IgA is , for IgD is , and for IgE is . Some of the immunoglobulin classes are composed of polymers of the basic monomer. In humans there are two antigenic varieties of the L chain, called kappa, , and lambda, . Each immunoglobulin will have two identical L chains; the and are shared by all classes. The monomeric form of any immunoglobulin is described by its chain structure. The molecular mass of the immunoglobulins can vary from 150 kd to 10^3 kd. This variation is due to polymerization of the basic monomer form. None of the immunoglobulins, however, are polymeric forms of another class. IgG is the most prevalent, constituting 75% of the total immunoglobulin in blood. It is present in normal adults at concentrations of 6001,500 mg/dL. IgG is designated $_{2\,2}$ or $_{2\,2}$. It is the only class of immunoglobulin that will cross the placenta (Fig. 36-2). [7]

The isotype IgM is a pentamer consisting of five monomeric units disulfide linked at the C-terminus of the H chain; each monomer of IgM is 180 kd due to the presence of an additional C_H domain. The complete protein has a sedimentation coefficient of 19 S, which corresponds to a molecular mass of 850 kd. IgM is designated $(_{2\,2})_5$ or $(_{2\,2})_5$. IgM also contains a 15-kd protein called the J chain. In the current structural model of IgM, the J chain forms a disulfide-bonded clasp at the C-terminus of two H chains (Fig. 36-2). [7]

The structure of the other isotypes of immunoglobulins are summarized as follows. The isotype IgA has a variable number of monomeric units and is designated $(_{2\,2})_n$ or $(_{2\,2})_n$ where n is 25. Serum IgA constitutes 20% of the total serum immunoglobulin, and 80% of this is monomeric. The remainder exists as polymers where n is 25. The other form of IgA is found in external secretions such as saliva, tracheobronchial secretions, colostrum, milk, and genitourinary secretions. Secretory IgA consists of four components: a dimer of two monomeric molecules, a 70-kd secretory component that binds noncovalently to the IgA dimer, and the 15-kd J chain that is believed to form a disulfide-bonded clasp at the C-terminus of the H chains (Fig. 36-2). The isotype IgD has a molecular mass of 180 kd. Its serum concentration is very low, approximately 3 mg/dL. IgD apparently functions as a membrane molecule, being associated on mature but unstimulated B cells in association with IgM. IgE

is the homocytotropic or reaginic immunoglobulin and mediates immediate hypersensitivity. It has a molecular mass of 180 kd and, like IgM, has four C domains. The Fc portion of IgE binds strongly to a receptor on mast cells, and this is how this immunoglobulin exerts its particular activity. The overall properties of the immunoglobulins are summarized in Table 36-1 .[7]

In addition, subclasses of the isotypes IgG, IgA, and IgM have been identified. The structural basis for this heterogeneity is antigenic variation (e.g., amino acid sequence differences) in the Fc portion of the H chain of a given class. The subclasses of IgG are the best characterized. These are called IgG1, IgG2, IgG3, and IgG4. Each has a slightly different structure, the most notable difference being the interchain disulfide-bonding pattern (Fig. 36-2 and Table 36-1). IgG1 comprises 70% of the total IgG and IgG2 20%. IgG3 and IgG4 make up 8% and 2%, respectively, of the total IgG. The subclasses of IgG exhibit different catabolic rates, and IgG2 crosses the placenta slightly more slowly than the other three. The other known subclasses of immunoglobulin isotypes are associated with IgM (IgM1 and IgM2) and IgA (IgA1 and IgA2). The properties and function of these subclasses are less well known.

The second type of variation is called allotypic variation. It is due to genetically controlled antigenic determinants found on both the H and L chains. Although each human has all immunoglobulin isotypes, an individual has only one form of each allotype on their immunoglobulin molecules. Allotypes are co-dominantly expressed, but an individual B lymphocyte secretes only one of the parental forms. This phenomenon is called allelic exclusion.

The third type of variation is due to antigenic determinants that are unique to each particular antibody molecule produced by an individual. These markers are called idiotypic determinants and they are associated with a single species of antibody. The anti-idiotypic antibodies that recognize a particular idiotype will not react with any other immunoglobulins in the donor other than the purified antibody that was used to raise the anti-idiotype antibody. In most cases, the immune response to an antigen results in a mixture of several antibodies each of which has identical binding specificity but distinct idiotypic

Figure 36-2 (A) Structure of the four subclasses of human IgG. Constant region domains are indicated by CnN, where n is the subclass and N is the domain. (B) Structure of human IgM. The J chain is shown in this model as being disulfide linked to two -chains. Other models have been proposed. Filled circle () carbohydrate. (C) Structure of human secretory IgA. This model shows the possible arrangement of the two IgA monomers in relationship to the secretory component and J chain. As the IgA molecule passes through the epithelial cells, the secretory components are synthesized and attached covalently to the Fc domain of the -chains that have previously been joined to the J chain with disulfide links. Light chain, pink; heavy chain, red; disulfide bonds, black lines; carbohydrates, black circles. *(From Turner,[43] with permission.)*

determinants. Thus, there can be many idiotypes for a given antigenic specificity, and this has been interpreted as being a reflection of physical heterogeneity in or near the antibody combining site, for example, in the variable region domains. In some species (notably certain strains of mice), the response to antigen results in a predominant idiotype on all antibodies of a given specificity. Because this is an inherited quality, they are called major, cross-reactive, or public idiotypes. Finally, some public idiotypes have been found in certain species (again most notably mice) to be genetically linked to allotypes. Three kinds of

TABLE 36-1 -- Human Immunoglobulins: Properties and Function

	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
H chain	1	2	3	4		1	2		
Molecular weight (kd)	146	146	170	146	970	160	160	194	199
Molecular weight of H chain (kd)	51	51	60	51	65	56	52	70	73
Number of H chain domains	4	4	4	4	5	4	4	6	5
Carbohydrate (%)	23	23	23	23	12	711	711	914	12
Serum concentration (mg/dl)	90	30	10	0.5	15	30	0.5	0.3	0.0005
Classical complement fixation	++	+	+++	+	+++				
Alternative pathway complement activity						+	+		
Placental transfer	+	+	+	+	+				
Binding to mononuclear cells	+		+						
Binding to mast cells and to basophils									+++
Reaction with protein A from *Staphylococcus aureus*	+	+		+					
Half life (days)	21	20	7	21	10	6	6	3	2
Distribution (% intravascular)	45	45	45	45	80	42	42	75	50
Fractional catabolic rate (% intravascular pool catabolized/day)	7	7	17	7	9	25	25	37	71
Synthetic rate (mg/kg/day)	33	33	33	33	33	24	24	0.4	0.002

Data primarily from Golub[7] and Glynn and Steward,[8] with permission.

anti-idiotype antibodies have been described: those that function as an internal image of the original antigen by mimicking the antigen structure, those that recognize antibody combining site-associated idiotypes, and those that are specific for framework-associated determinants. These three types of anti-idiotype antibodies are diagrammatically illustrated in Figure 36-3 . The first type, internal image antibodies, are the ones of clinical interest. The criteria for an internal image anti-idiotype is that the antibody is an immunogen in other species, that the antibody response to the antibody binds as specifically as the mimicked antigen, and that the antibody should be able to act like a natural ligand for a cellular receptor for the antigen.

Genetic Basis for Diversity

The origin of antibody diversity and heterogeneity is found in the genes that code for these molecules. Before the introduction of molecular biologic approaches to the investigation of the immunoglobulins, there was considerable controversy as to how much information for antibody specificity was carried in the germline and how much originated at the level of somatic cells. This question was resolved by the discovery that in antibodies two genes code for a single polypeptide chain. Two kinds of data support this conclusion. The first was the discovery of the V and C regions. The second was the observation that in DNA

Figure 36-3 Types of anti-idiotype antibodies. 1. paratope; 2. idiotope; 3. framework.

isolated from embryos that make no antibody, the coding sequences for the V and C regions for a given antibody were located at separate sites in the DNA but in mature antibody-producing cells for the same antibody, the V and C regions were found in the same part of the DNA.[4] [6] [8] Detailed analysis of the organization of the L and H chain genes has shown that the genes for L chains are located on chromosome 2, for L chains on chromosome 22, and for H chains on chromosome 14.

The organization of the L chain genes is shown in Figure 36-4 . In addition to the V and C genes, there is a set of genes called J segment (for joining) separated from the germline V genes. Each V region is preceded by a leader sequence (L_1 , L_2 . . . L_N). In the antibody molecule the V_L contains three hypervariable regions (CDR_1 ,

CDR_2 , and CDR_3) and three framework regions (FR_1 , FR_2 , and FR_3 ; Fig. 36-1). The CDR_3 region arises from the region where gene segments are joined. [4] The H chain gene likewise has a tandem array of 100200 V_H segments preceded by leader sequences. At an undetermined distance from the V_H and leader sequences, 30 segments of DNA known as diversity (D) regions code for 10 amino acids not found in L chains. Further downstream of the D regions are located six functional joining (J) segments that impart additional variability (Fig. 36-5).

The main function of B cells is to produce antibodies against an almost infinite variety of antigens that might be harmful to the organism. They comprise a clonally diverse population in which each cell has a monospecific antibody receptor bound to its surface. A foreign antigen encounters its cognate antibody receptor bound to a B cell and, with the cooperation of helper T cells, causes the clonal proliferation and subsequent differentiation of this subset of B cells into antibody-secreting plasma cells. A unique feature of B-cell ontogeny is the creation of a functional antibody molecule by somatic recombination of multiple noncontiguous germline gene segments at both the V and C gene loci. In the case of the H chain, two distinct types of somatic DNA rearrangements take place. [9]

V(D)J Recombination

The first wave of somatic DNA rearrangements involves developmentally regulated, site-specific recombination events that fuse V (variable), D (diversity), and J (joining) coding segments

Figure 36-4 Organization of chain gene. The germline DNA is rearranged in a -producing cell so that one V region is brought together with one J region. The mRNA that encodes the protein has the C region adjoined to the V-J region and yields a complete chain. This same motif is used in the organization of the chains. L_1 , L_2 , L_n , leader sequences. CDR, hypervariable regions; FR, framework regions. *(Data from Golub,[7] with permission.)*

to create a functional VDJ gene.[4] Diversity in this case is generated by selection of any one of the 100200 V_H genes (contributing the CDR_1 and CDR_2 regions) as well as the recombinatorial assortment of different D and J regions (resulting in the CDR_3 region). V(D)J recombination is mediated through conserved recombination signal sequences that flank the coding segments and comprise a palindromic heptamer and an A/T rich nonamer separated by a spacer region of either 12 bp or 23 bp. Coding segments can recombine only if they possess recombination signal sequence spacers of opposite type (the 12/23 rule). The mechanism involves a looping out and deletion of the intervening noncoding DNA resulting in the creation of a coding joint between the D and J segments and, subsequent to a second round of recombination, a coding joint between the V and the recombined DJ segments. Prior to ligation of the coding joints, modification of the ends is carried out by an endonuclease activity and the enzyme terminal deoxynucleotidyl transferase, resulting in further diversification.

Substantial progress has been made in establishing the biochemical components of the V(D)J recombination machinery and in understanding the underlying molecular mechanism of recombination. The proteins encoded by the recombination activation genes, RAG1 and RAG2, are essential for V(D)J recombination and are able to impart such activity to nonlymphoid cells. [10] RAG1 and RAG2 are responsible for the initial endonucleolytic cleavage that generates a 3OH at the coding end and a 5P at the signal end, followed by a nucleophilic attack of the 3OH on the phosphodiester backbone of the other strand, resulting in the formation of a hairpin joint at the coding end. This hairpin joint must then be resolved by an endonuclease, the ends processed and ligated. Other gene products have been implicated including components of the DNA-dependent protein kinase (DNA-PK), which is a multimolecular complex comprising a large catalytic domain (PK_{CS}) and two Ku subunits (Ku70 and Ku80). PK_{CS} possesses a serine/threonine protein kinase activity, whereas Ku binds DNA nonspecifically at double-strand

Figure 36-5 Organization and rearrangement of the H chain gene. Developmentally regulated site-specific recombination occurs initially between D and J segments, and is followed by a V to DJ rearrangement. Controlling elements for the VDJ recombination process include the intronic enhancer (E), which is subtended at both its 5 and 3 ends with a matrix attachment region (MAR), and the 3 enhancer complex (3E) located downstream at the C_H genes.

breaks, nicks, and hairpins, possesses helicase activity, and recruits and activates PK_{CS} . SCID mice, which are mutated at the PK_{CS} locus, display defects in coding joints, whereas Ku-deficient mice adversely affect the formation of functional coding and signal joints. The XRCC4 protein involved in double-strand break repair is also implicated in signal and coding joint formation. [11]

Antibody diversity is not completely accounted for by multiple germline V_H genes, V(D)J recombination, imprecise coding joint formation, and the combinatorial assortment of different H and L chains, although calculations indicate that the number of possible different antigen-binding sites is of the order of 10. [11] Additional variation in the V_H and V_L regions of already-expressed antibody molecules is effected by a process known as somatic hypermutation. This refers to a mechanism, the molecular details of which are largely unknown, by which point mutations are introduced into CDR_1 , CDR_2 , and CDR_3 regions of antibody molecules at a very high rate in the germinal centers of secondary lymphoid organs. Thus the ability to mount an antibody challenge to an almost infinite variety of antigens originates from a number of developmentally regulated somatic recombination and mutation events acting on a finite complement of germline gene segments. [12]

The organization of the human C-region genes is now understood. There are four C genes and one C gene and as many C_H genes as there are classes and subclasses. The location of the C-region genes is on the 3 side of the V, D, and J regions. During the proliferation of the lymphocytes in an immune response, there is usually a class switch. This phenomenon refers to the mechanism by which the isotype switches from to another class while retaining the same rearranged V region. The switch occurs in a stretch of DNA called the S region. There are also enhancer elements in the DNA that are tissue specific and function to increase the level of transcription of the DNA to mRNA (Fig. 36-6).

Antibody Class Switching

The second type of somatic rearrangement is the principal mediator of antibody class (or isotype) switching, by which antibodies of a given specificity acquire different effector functions.[13] [14] [15] In contrast to V(D)J recombination none of the

Figure 36-6 The structural dynamics of antibody class switch recombination (using IgG1 as an example). The murine germ line C_H locus comprises eight different isotype sequences (, , 3, 2, 2, epsilon, and) represented as shaded boxes. Upstream of each isotype (except for) is a block of switch sequences (S) that range in length from 1 to 10 kb. The donor S and acceptor S sequences are represented by open and hatched boxes, respectively. The intronic and IgH 3 enhancers are labeled as E and 3E, respectively. The V(D)J recombination process has already occurred in the pictured chromosome as evinced by the juxtaposition of the V, D, and J segments. The upper panel represents the IgC $_H$ germline configuration. On activation (by CD40L:CD40 interaction, for example) and targeting by switch commitment factors (in this case by the cytokine interleukin-4), I_H transcripts are produced at both the C_H and C_H 1 loci, and subsequently processed. The extent to which the 3E is responsible for transcriptional activation of the I_H locus remains to be determined (middle panel). Switch recombination mediated by the S and S2 sequences leads to the reciprocal production of a switch circle and a novel chromosomal configuration (lower panel).

components of the switch recombinase enzymatic machinery have been cloned, and the molecular mechanism of recombination remains to be elucidated. Antibody class switching typically occurs during the secondary immune response that effectively results in the transfer of effector function from IgM to IgG, IgA, or IgE antibodies.[14] The nature of the antigenic challenge defines the particular immunoglobulin isotype or isotypes produced and, as described above, different immunoglobulin classes have different functions. Class switching to non-IgM isotypes is important to good health as evinced by those individuals suffering from

X-linked hyper IgM syndrome. In this condition, which is due to genetic defects in the CD40L:CD40 signaling pathway essential for isotype switching, [15] individuals display an almost complete lack of serum and non-IgM isotypes (except for small amounts of IgG3) and suffer from a variety of opportunistic infections that severely impair their ability to lead normal lives.

The mechanisms for this class switching in the H chain have been most thoroughly studied in mice and are described as follows. The murine CH locus consists of eight functional genes arranged as 5-C-C-C3-C1-C2b-C2a-C-C-3 at the telomeric end of chromosome 12 (Fig. 36-6). Recombinagenic switch (S) sequences are positioned 14 kb upstream of each of these functional C_H gene segments with the exception of C (Fig. 36-6). Switch recombinations within or nearby these S sequences have been documented in a large number of hybridomas, plasmacytomas, and A-MuLV-transformed pre-B lines. S sequences are composed of direct tandem repeats of a basic core structure, which often contains the pentamers GAGCT and GGGGT although other repeats such as TGAGC, GAGCTG (S, S, and S) and GCAGC, ACCAG (S) are also prevalent. S regions vary in length and repeat unit sequence and display different degrees of homology with one another. [16]

Class switch recombination (CSR) occurs when an illegitimate nonhomologous DNA rearrangement event juxtaposes a functional VDJ gene (initially 5 of the C gene in an IgM-bearing B cell) in close proximity to a downstream C_H gene. This occurs (with the exception of IgD switching) by a looping out and deletion mechanism resulting in the loss of all the intervening C_H genes (Fig. 36-6).[13] [15] Although other mechanisms of isotype switching have been proposed, including RNA-mediated events such as processing of long pan-locus transcripts or trans-splicing, these are not believed to represent the physiologically relevant principal mode of isotype switching. An exception to this is IgD, in which the C locus does not possess S sequences, [16] and switching occurs by differential processing of long transcripts or by recombination between specific homologous non-S sequences upstream of C and C. [15]

It is unclear how the tandem repetitive S regions mediate switching and how switching is regulated at the molecular level. It may be that these sequences represent a structural motif that is directly recognized by the DNA-binding domain of a common switch recombinase [14] [17] or there may even be a class of isotype-specific switch recombinases whose substrate activities are under independent control. Alternatively, or in addition, S region binding proteins may act as accessory molecules to recruit the recombination machinery by specific protein-protein interactions. Switching can be regulated in vivo to yield a specific or limited set of isotypes and important regulators of this process include several T-cell-derived cytokines. These include interleukin (IL)-4, IL-5, IL-10, interferon- and tumor growth factor (TGF)-. [18]

Directed class switching may be manifested by the differential transcriptional competence of the C_H genes.[15] Prior to class switch recombination sterile or so-called germline transcripts encompassing a short I_H exon (residing 5 to every S region) spliced to an adjacent C_H region are expressed in a regulated inducible fashion. [17] [19] Initially, transcription initiates within a promoter 5 of the downstream acceptor I_H exon, continues through the S region and terminates at the 3 end of the C_H gene. The S sequences are then spliced out (Fig. 36-6). Similar RNA structures are formed at the donor C locus by the intronic enhancer, E, which acts as a promoter for an initial transcript, which encompasses I, S, and C and subsequently yields an IC moiety after RNA processing (Fig. 36-6).

A number of cytokines are known to modulate germline transcripts (GT).[15] These results are consistent with the notion that transcriptional activity at the donor and acceptor C_H loci are prerequisites for targeting of switch recombination to these loci. This transcribed status is believed to engender a particular C_H gene with the accessibility to undergo switch recombination (hence the so-called accessibility model of CSR [17] [19]) by facilitating the binding of the catalytic or regulatory units of the switch recombination apparatus or activators thereof. Either the change in chromatin configuration induced by transcription or the transcripts themselves may be responsible for the increased accessibility of the S sequences to the recombinase. Switching to a particular isotype is always preceded by the presence of C_H germline transcripts at that C_H locus[13] [15] and there is a direct correlation between cytokine-mediated induction of germline RNAs and enhanced transcriptional rate at a particular locus.[13] Apart from germline transcriptional control by I exon promoters, IgH locus transcriptional control of CSR is also mediated by the E enhancer and a developmentally regulated enhancer complex located 3 of the C gene (3E) and comprising four distinct enhancers (3E; 3E-HS(1,2); 3E-HS3; 3E-HS4) (Fig. 36-6).[20] The E enhancer, on the other hand, is responsible for the generation of the heterogeneously initiating germline I transcripts that are prerequisites for isotype switching from C to downstream C_H loci. [15]

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THERAPEUTIC USE

Immunoglobulin G was one of the first plasma proteins prepared in a purified state as a therapeutic drug for the treatment of clinical disorders. It remains, along with albumin and factor VIII (antihemophilic factor), the most widely therapeutically used plasma derivative. The method for purifying IgG has remained essentially unchanged from the cold ethanol method developed by Dr. Edwin J. Cohn and his colleagues from 1935 through 1945. The Cohn method for purifying IgG yields a 15% protein solution that can only be given intramuscularly. Over the past 15 years, methods have been developed for preparing IgG in a form suitable for intravenous administration. [21] [22]

Intravenous immunoglobulin (IVIG) is prepared from pooled human plasma by the Cohn-Oncley cold ethanol fractionation procedure and retains the antibody repertoire reflecting the combined immunologic experience of the donors. [23] Aggregates and anticomplementary activity must be removed by any of several approaches including limited enzyme digestion or chemical modification. IVIG contains concentrated IgG with normal plasma ratios of IgG1 and 2 but lower percentages of IgG3 and 4, and only trace amounts of IgA and IgM. IVIG products and manufacturers are listed in Table 36-2 .

Intravenous immunoglobulin has proven both safe and effective in restoring protective immunity to patients with agammaglobulinemia for the past two decades. The major risks associated with IVIG administration are the possibility of transmission of disease and the potential risk of anaphylactoid reactions. Standardized testing of donor units and the fractionation procedure have reduced virus load. In addition, many producers of IVIG now add a heat or detergent viral inactivation step to reduce the risk of disease vector transmission to a very small possibility. Aseptic meningitis with severe headache was reported for 11% of recipients of high-dose IVIG and was associated with patient history of migraine headaches. Other complications include pain, chills, fever, headache, nausea, or vomiting. Most adverse events are self-limiting and improve

TABLE 36-2 -- IVIG Products

Manufacturer or US Distributor	Trade Name
Alpha Therapeutics	Venoglobin S
American Red Cross/Baxter	Polygam SD
Bayer	Gammimune N SD
Baxter	Gammaguard SD
Centeon	Gamma P IV
Nabi	WinRho SDF
Novartis Pharmaceutical	Sandoglobulin
Biotest Pharmaceuticals	Intraglobin F
Immuno US/Baxter	Iveegam
Massachusetts Public Health Biologics Laboratories	Cytogam & Respigam

with a slower infusion rate. Selective IgA deficiency or previous anaphylactic response to IVIG are the only contraindications for the use of IVIG. The relative safety of IVIG and the effectiveness of treatment in obvious and not so obvious diseases had led to widespread use of IVIG for a variety of abnormalities, often without sufficient evidence to justify efficacy. Indications for IVIG are listed in Table 36-3 . [24] [25] Controlled studies will be required to define appropriate use of IVIG in areas in which clinical experience has suggested promise. There is a significant drug cost (approximately $10,000 annually for patients with primary immunodeficiency) and because administration is intravenous and requires 23 hours in a clinical setting, added costs are associated with IVIG administration.

The effects of IVIG are explained by mechanisms beyond antibody

TABLE 36-3 -- Clinical Indications for Use of IVIG

Labeled use for all US-licensed IVIG
Primary immunodeficiencies
Labeled uses for selected IVIG products
Kawasaki syndrome
Chronic lymphoid leukemia
Prophylaxis of bacterial infection in children with human immunodeficiency virus infection
Prevention of graft-versus-host and bacterial infection in bone marrow transplantation
Idiopathic thrombocytopenic purpura
Off-label uses for which efficacy as a primary treatment has been demonstrated
Post-transfusion purpura
Guillain-Barré syndrome
Chronic inflammatory demyelinating polyneuropathy
Off-label uses indicated only after standard interventions have not been effective
Autoimmune hemolytic anemia

Dermatomyositis
Epilepsy
Immune-mediated neutropenia
Infectious disease prophylaxis in high-risk neonates
Myasthenia gravis
Polymyositis
Solid organ transplantation to prevent cytomegalovirus infection
Systemic vasculitis
Systemic lupus erythematosus
Thrombocytopenia
Other applications from pilot trials or clinical experience
Multiple sclerosis
Asthma
Juvenile rheumatoid arthritis
Insulin-dependent diabetes
Crohns disease
Recurrent abortions
Acquired von Willebrand disease

recognition of pathogen antigens. IVIG preparations contain up to 30% dimers composed of idiotype-anti-idiotype antibody pairs. These dimers appear to be very effective as a sink for activated complement and can inhibit complement activation. [26] In immune thrombocytopenia purpura the effects of IVIG seem to be mediated by Fc-receptor blockade of reticuloendothelial cells combined with an anti-idiotypic neutralization of antiplatelet antibody. Other indications of antibody neutralization can be seen in IVIG treatment of myasthenia gravis. The dramatic success of IVIG in treating Kawasaki syndrome may be due to several mechanisms, including anti-idiotypic neutralization of antiendothelial antibodies, inhibition of cytokine production and function, and elimination of causative superantigens. [27] [28] IVIG inhibits B-cell activation and autoantibody production by enhancing CD8+ suppressor T-cell function. Cell-mediated immunity is also affected. Hyperimmune IGIV is made from donor plasma selected for high titer for specific antigens. Prophylaxis for cytomegalovirus and respiratory syncytial virus has been successfully demonstrated using hyperimmune IVIG.[28] [29] Future progress with IVIG will include antigen selection and augmentation with monoclonal antibodies. [30]

Possible therapeutic and diagnostic uses for anti-idiotype antibodies are as prophylactic vaccines. Table 36-4 is a list of viruses and bacteria to which immunity has been induced in mice with an anti-idiotype antibody (see review by Walter, Friesen, and Harthus [31]). Recent efforts have been devoted to using this method to make a vaccine against human immunodeficiency virus based on its ability to mimic CD4, the receptor on T cells for the virus. Such antibodies would also prove useful in developing tumor-specific vaccines. Similar efforts are being devoted to developing such antibodies for use in both in vivo and in vitro immunodiagnostic assays where a purified antigen is not readily available.

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POLYCLONAL AND MONOCLONAL ANTIBODIES

A fundamental concept in the immune system is the clonal nature and function of the B lymphocytes, the cell type responsible for synthesis of immunoglobulins (Chap. 8). The constant production by the mammalian immune system of large numbers of B lymphocyte clones that recognize different antigenic structures on different, as well as single, molecules leads to the production of a vast array of antibody proteins. Thus, an antiserum raised by immunization with a single cell type or a homogeneous, purified antigen contains many different antibodies that have in common, specific reactivity with the immunizing cell or antigen, but that are also a mixture of immunoglobulins that are heterogeneous by other criteria such as size, charge, amino acid sequence, and binding affinity. Because such an antiserum represents a mixture of antibodies derived from many clones of B lymphocytes, all of which have produced an antibody reactive with the immunizing antigen, this is called a polyclonal antiserum and purified immunoglobulins with a single antigen specificity derived from such an antiserum are called polyclonal antibodies.

Monoclonal antibody technology as described in 1975 by Kohler and Milstein combines the clonal properties of B lymphocytes with the random segregation of chromosomes in hybrids formed between two cells.[32] [33] This procedure results in a hybrid cell in which there is immortalization of an individual antibody-producing B lymphocyte. Thus, each cell line derived from the hybrid produces a unique or monoclonal, constant antibody molecule. Such cell lines are called hybridomas. The method for obtaining a monoclonal antibody was first developed in a murine system and is summarized as follows.

Following immunization of the mouse, the B lymphocytes are harvested from the spleen or lymph nodes as a single cell suspension, and fused with a mouse myeloma cell (plasmacytoma cellsee Chap. 76). The myeloma cell is immortal and

TABLE 36-4 -- Immunity of Mice Against Viruses and Bacteria Following Immunization with Anti-idiotype Antibodies

Virus/Bacteria	Anti-idiotype Antibodies Produced In	Class	Immunity of Mice Immunized with-Idiotype
Tobacco mosaic	Rabbit	Polyclonal	Neutralizing antibodies
Reo	Mouse	Monoclonal	Cytotoxic T-cell neutralizing antibody
Rabies	Rabbit	Polyclonal	Neutralizing antibody
Hepatitis B	Rabbit	Polyclonal	Neutralizing antibody
Sendai	Mouse	Monoclonal	Cytotoxic T cells; resistance to challenge
Venezuelan equine encephalomyelitis	Rabbit	Polyclonal	Neutralizing antibody
Polio II	Mouse	Monoclonal	Neutralizing antibody
Escherichia coli	Mouse	Monoclonal	Resistance to challenge
Streptococcal pneumonia	Mouse	Monoclonal	Resistance to challenge

From Walter et al.,[31] with permission.

has the cytoplasmic machinery to produce large quantities of immunoglobulin. The tumor cell used for fusion also contains an enzyme deletion that can be used to select for cells that do not have the deletion. In the murine system this is usually hypoxanthine guanine phosphoribosyl transferase (HGPRT), an enzyme required for survival of the cell in the presence of the folic acid antagonist aminopterine. Fusion of the immunized spleen cells with the myeloma cells is triggered by Sendai virus, polyethylene glycol, electric current, or directed fusion. [34] [35] This mixed cell suspension is separated into small aliquots (usually by dilution into a 96-well microtiter plate holding about 200 L/well) and each aliquot is grown in the presence of the selecting agent, hypoxanthine-aminopterine-thymidine (HAT). Only those hybridoma cells that have acquired the HGPRT from the immunized lymphocytes will survive. The hybrids are then cloned by limited dilution, such that there is one cell per well. The growth of the newly fused hybridoma can be initially facilitated by the addition of IL-6, which can be added exogenously or provided by feeder cells. The individual clones, once they have been grown, are characterized for the antibody of desired specificity, titer, and binding affinity before propagation in large-scale tissue culture production or by propagation in the peritoneal cavity of a mouse as an ascites tumor. Once established, the B-cell hybridomas will exhibit autonomous growth and can be adapted to growth in synthetic media containing no serum. Because these cultures produce a single species of immunoglobulin, the antibodies can be purified by standard column chromatographic methods, although frequently immunoaffinity purification methods are used to obtain highly purified antibody devoid of any contaminating proteins. The basic steps in the production of a monoclonal antibody from an immunized mouse are summarized in Figure 36-7 .

The most widely used species for the production of monoclonal antibodies is the mouse, but hybridoma cell lines can also be obtained from rats and humans. Compared with human immunoglobulin purified from pooled plasma, human monoclonal antibodies would be particularly useful as potential therapeutic reagents for the treatment of human disease, because they would have the added advantage of antigen specificity. There are serious ethical considerations about the immunization of humans with potential toxins or tumor cells, and the lack of a suitable, reproducible source of antigen-activated human lymphocytes has been, and still is, the major difficulty in the preparation of human monoclonal antibodies. An early approach to this problem was to use Epstein-Barr virus to transform human B cells obtained from the peripheral circulation, thus allowing for an expansion of the B cells before fusion. This approach depends on first finding an individual who is

Figure 36-7 General scheme for the production of murine monoclonal antibodies.

producing an antibody of the desired specificity and isotype. Another difficulty in producing human monoclonal antibodies is that a human-mouse hybridoma cell (called a heterohybridoma cell) resulting from fusion of mouse myeloma cells with human B cells tends to lose human chromosomes, and this results eventually in the loss of the ability to secrete human immunoglobulin. A mouse cell line has been developed that fuses efficiently with human B cells and has been used to produce stable human monoclonal-secreting heterohybridomas. Simultaneously, several suitable human myeloma fusion partner cell lines have been developed, as well as a human-mouse heterohybridoma cell line.[35]

Production of Human Monoclonal Antibodies by Gene Technologies

Technical achievements in cellular immunology have led to the ability to produce human monoclonal antibodies, but the general approach for production of monoclonal antibodies still does not address the potential problem of using tumor-derived proteins in humans. Attention has turned, therefore, to the use of gene manipulation methods to produce immunoglobulins that potentially overcome the conceptual and practical difficulties of using human tumor cell lines as a source of human therapeutic agents. In this approach, hybridoma technology is combined with molecular biology techniques for the design and development of immunoglobulins that are specifically targeted for use as human therapeutic and diagnostic reagents. The expansion of the repertoire of monoclonal antibody technologies has resulted in a molecular diversification of routine hybridoma technologies.

First, the use of the polymerase chain reaction technique [36] has allowed for the generation of complete V-region gene libraries. This is accomplished by using a 3 primer in the C region and a degenerated 5 primer that corresponds to homologous sequences at the 5 end of most V-region cDNAs. The amplified DNA is then used to construct, in *Escherichia coli*, a V_H library that has been previously screened by antigen binding and found to produce V fragments that exhibited good antigen binding. [37] [38]

Second, the problem of H-L chain pairing has been solved using in vitro recombined lambda phage libraries, where separate V_H and V_L fragments (inserted respectively on the left and right arm of the phage) are randomly combined in vitro using a unique restriction site located near the center of the phage genome. The diversity generated by this system is probably as large as that generated by the immune system (>10^8 antibodies). The resulting antibodies will not be the same as those generated in mammals because phage do not have mechanisms such as the use of alternative V gene fragments (V, D, or J) to form the V regions. Thus, the phage combinatorial library system is most effective when the starting animal has been immunized with the antigen of interest. It is possible, however, to obtain specific antibodies from phage libraries made from nonimmunized animals by enriching for antigen binding by panning the phage-derived immunoglobulin population on immobilized antigen. [38] Thus, the combinatorial phage library methodology represents a significant advance in monoclonal antibody technology.

Third, the problem of modifying an existing antibody to retain desired characteristics, and introduce other characteristics required for the intended use, forms the basis for engineering monoclonal antibody genes. The high somatic mutation rate of V regions in H and L chains during maturation of the immune response is operative in B-cell hybridomas, and this has led to the development of positive screening and selective methods for the detection and enrichment of hybridoma-secreted monoclonal antibodies that have altered characteristics. [39] Another approach to engineering monoclonal antibodies is based on recombinant DNA technology. The H and L chain plasmid cDNAs expressed in hybridoma cells are cloned, sequenced, and appropriately modified by replacing nucleotide sequences. These plasmids are then transfected into nonimmunoglobulin-secreting myeloma cells and the new cell, called a transfectoma, will secrete the engineered antibody. [40] This is the approach used to humanize murine monoclonal antibodies (e.g., to convert a murine immunoglobulin to resemble a human immunoglobulin). [41] Such engineered antibodies are designed to overcome the problem that murine monoclonal antibodies cannot be repeatedly injected into humans without the eventual development of an immune response to the foreign protein. Finally, the combination of the somatic cell method with this type of recombinant DNA method forms the basis of specific gene therapies for the treatment of genetic diseases. [35]

Therapeutic Use of Monoclonal Antibodies

The potential use of monoclonal antibodies as diagnostic reagents and therapeutics is just beginning to be realized, and no attempt will be made here to summarize all of these emerging developments. Two examples, however, of how monoclonal antibodies have proven useful in hematology are the use of monoclonal antibodies to identify cell types that have a common myeloid cell progenitor and their use in the understanding of human myeloid cell differentiation, as revealed by identification of cell surface marker phenotypes.

At one level, monoclonal antibodies have been found that detect antigens expressed uniquely, not only by T and B lymphocytes, but also as more general markers of peripheral blood monocytes or neutrophils. Thus, certain reagents identify cell surface antigens common to (1) monocytes and neutrophils; (2) monocytes, neutrophils, and large granular lymphoid cells; (3) monocytes and platelets; and (4) neutrophils and large granular lymphoid cells (see review by Foon and Todd [42]).

At a second level, monoclonal antibodies have been used to correlate the presence of cell surface antigens with pathways of normal differentiation within the myeloid lineage. The stage in differentiation where a variety of monoclonal antibodies to cell surface markers react is illustrated in Figure 36-8. Detection of the cell surface antigens is based either on complement-dependent monoclonal antibody-mediated cell lysis (negative selection) or on techniques such as fluorescence-activated cell sorting or immune rosetting (positive selection). These methods allow for determination of antigen expression by multipotent stem cells (CFU-GEMM) and stem cells committed to the myeloid (CFU-GM), erythroid (BFU-E, CFU-E), or megakaryocyte/platelet (CFU-Mega) pathways of differentiation. Certain monoclonal antibodies have been used to prepare 50- to 100-fold enriched preparations of myeloid progenitor cells from pooled bone marrow mononuclear cells. Certain determinants have been found to be expressed uniquely by progenitor cells; other cell surface antigens are found on myeloid, erythroid, or platelet precursors that correspond morphologically and histochemically to distinct stages of maturation; some antigens are either lost or acquired as cells progress from myeloblasts to mature neutrophils; and finally, some antigens are maintained on all recognized myeloid cells. [42]

In addition to serving as differentiation markers, many of the cell surface antigens represent functionally significant plasma membrane proteins, glycoproteins, and glycolipids. Cell surface antigens have been given cluster designations (CD or CDw) followed by a number. The complete listing of all cluster designations for cell surface antigens is listed in Chapter 7. This systematic classification of monoclonal antibodies has made these reagents invaluable tools, not only for the understanding of the development of cellular aspects of hematopoiesis, but in the

Figure 36-8 Schematic representation of human myeloid differentiation indicating the phenotype of cell-surface markers as defined by monoclonal antibody reagents.

diagnosis and classification of hematopoietic disorders and have allowed for guidance on therapeutic decisions.

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SUMMARY

The immunoglobulins modulate and mediate many physiologic functions and serve as diagnostic markers of disease and monitors of changing health. The study of these molecules continues to yield new insights about fundamental biologic, biochemical, and genetic mechanisms controlling hematopoiesis and host defense mechanisms. There is currently a rapid increase in new information on the immunoglobulins based on both biologic and physical/biochemical studies as illustrated by the advances in knowledge on immunoglobulin structure and function and monoclonal antibody technology. This may result in further diagnostic and therapeutic uses of immunoglobulins.

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Chapter 37 - Complement Biology

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INTRODUCTION

The complement system includes more than 20 plasma proteins that interact through two enzymatic pathways to generate products with distinct immunoregulatory, opsonic, and inflammatory activity (Table 37-1 and Fig. 37-1 (Figure Not Available)). In addition to these fluid-phase proteins, the complement system also encompasses a variety of cell-surface proteins with receptor function. These membrane complement receptors recognize activated fragments derived from the complement components in plasma, and through this interaction initiate immune adherence and cell activation. Additionally, cellular receptors that bind activated components of complement can function as inhibitors that serve to limit dissemination of the complement reaction cascades and to protect autologous cell membranes from damage by the complement system. In concert, these plasma proteins and cell membrane receptors play a central role in host defense against infection and contribute to virtually every aspect of the inflammatory response. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12]

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OVERVIEW OF PROTEIN STRUCTURE AND FUNCTION

Table 37-1 summarizes the main components of the complement system. Shared structural motifs and related functions among several of these proteins permit a number of distinct protein superfamilies to be identified. [5] [10] [13] Common structural elements include collagen-like domains of the collectin protein family, common to proteins that initiate complement activation, including C1q, C-reactive protein (CRP), and mannose-binding protein (MBP); a serine protease domain (SPD) that is shared among many of the complement enzymes, including C1r, C1s, MASP, C2, factor B, factor D, and factor I; [14] short cysteine-rich consensus repeats (SCRs), representing highly conserved globular domains of approximately 60 amino acids each that confer binding affinity for C3b/C4b (SCRs are found predominantly in those complement proteins and receptors that bind C3b or C4b, but they are also found in several unrelated proteins); [15] [16] and a cytolytic peptide motif (found in C6, C7, C8, and C9) consisting of a putative membrane-spanning domain and a cysteine-rich domain characteristic of the epidermal growth factor receptor precursor. [8] [17] This latter motif is also shared by perforin, a pore-forming cytolytic polypeptide that is contained in the intracellular granules of cytotoxic T cells. [18]

Shared structural and functional homology is also apparent when the reaction mechanisms of the classical and alternative activation pathways are compared (Fig. 37-1 (Figure Not Available)). This homology is most evident for the C3/C5 convertases of both pathways. [3] [5] [13] These enzyme complexes (C4b2a of the classical pathway and C3bBb of the alternative pathway) each represent a Mg^{2+} -stabilized heterodimer formed between a serine protease (C2a or Bb) and a surface-bound cofactor (C4b or C3b, respectively). C2 and factor B are proenzymes containing SPDs and SCRs. When proteolytically activated, they each share specificity for the same substrates (i.e., C3 and C5). C3 and C4 also derive from homologous precursor polypeptides, and each contains an internal thioester bond (between a cysteinyl sulfhydryl and -glutamyl carbonyl) that is destabilized upon peptide cleavage of their -chains. [19] [20] [21] Transacetylation through nucleophilic attack by surface amino or hydroxyl groups provides a mechanism by which the C3b and C4b domains of these components covalently attach to membrane surfaces, thereby localizing the C3/C5 convertase enzyme complexes to the target membrane of complement attack. Membrane-bound C3b also serves an important opsonic function, labeling the target for removal by the reticuloendothelial system.

Common structure and function can also be discerned among the complement regulatory proteins and cellular complement receptors (Tables 37-1 and 37-2). Certain of the cellular C1q receptors (C1qR) are closely related to cellular collagen receptors. [22] [23] The cellular anaphylatoxin receptors for C3a, C4a, and C5a peptides are related to other G-protein-coupled receptors. [24] The cellular C3b/C4b receptors CR1 and CR2 as well as the C3/C4 regulatory proteins factor H, C4b-binding protein

TABLE 37-1 -- Components and Regulatory Proteins of the Complement System

Protein	Serum Concentration (g/ml)	Number of Peptide Chains	Structural Motifs
Components of Activation Pathways			
C1q	100	18 (6 × 3)	Collagen-type helix
C1r	50	1	Serine protease, SCR
C1s	40	1	Serine protease, SCR
C4	640	3	Internal thioester
C2	25	1	Serine protease, SCR
C3	1,200	2	Internal thioester
Factor B	200	1	Serine protease, SCR
Factor D	1	1	Serine protease, SCR
Factor I	35	1	Serine protease, SCR
Components of Membrane Attack Complex			
C5	70	2	Homologous to C3, C4
C6	65	1	Pore-forming protein, SCR
C7	55	1	Pore-forming protein, SCR
C8	55	3	Pore-forming protein
C9	60	1	Pore-forming protein
Plasma Regulatory Proteins			
C1 inhibitor	200	1	Serpin-type inhibitor
Properdin	25	4	
C4b-binding protein	250	8 (7 and 1)	SCR
Factor H	500	1	SCR
S protein (vitronectin)	500	1	Adhesive protein (RGD sequence)
Membrane Regulatory Proteins			
Decay-accelerating factor (DAF, CD55)		1	SCR, GPI anchor
Membrane cofactor protein (MCP, CD46)		1	SCR
20-kd homologous restriction factor (HRF-20, CD59)		1	GPI anchor
Membrane Receptors			
CR1 (CD35)		1	SCR

CR2 (CD21)		1	SCR
CR3 (CD11b/CD18)		2	Leukocyte $_2$ -integrin
CR4 (CD11c/CD18)		2	Leukocyte $_2$ -integrin
C1q receptor		1	
C3a receptor		1	G-protein coupling
C5a receptor		1	G-protein coupling

Abbreviations: SCR, short consensus repeat; GPI, glycosyl phosphatidylinositol; DAF, decay-accelerating factor; MCP, membrane cofactor protein; HRF, homologous restriction factor.

(C4b-bp), membrane cofactor protein (MCP), and decay-accelerating factor (DAF) all share the SCR motif that confers ability to bind C3b and C4b. The genes for these proteins cluster as a linkage group that has been designated regulators of complement activation (RCAs). [15] [16] Two other cellular C3 receptors, CR3 and CR4, belong to the Leu-CAM $_2$ -integrin family of cellular adhesion receptors, which includes LFA-1. [25]

COMPLEMENT ACTIVATION PATHWAYS

Expression of the biologic activity of the complement system requires proteolytic processing of C3 and C5. Accordingly, the reaction mechanisms of this system are focused on the assembly and control of the C3/C5 convertases (Fig. 37-1 (Figure Not Available)). The alternative pathway C3/C5 convertase (C3bBb) is phylogenetically oldest and remains the most important enzyme mechanism for C3/C5 activation. The classical activation pathway utilizing C2 and C4 appears to reflect evolutionary adaptation, initially to utilize the specificity of a lectin as provided through the interaction of MBP with the mannose-rich lipopolysaccharides common to many microbial pathogens, and subsequently for precise immune recognition as provided through C1qs interaction with antigen-bound immunoglobulin. [5] [10] [26]

Classical Pathway

The classical pathway is initiated through the conformational activation of C1q, one of the components of the C1 enzyme complex. [27] [28] C1 is present in plasma largely as a Ca^{2+} -dependent complex between C1q and the proenzymes C1r and C1s with the stoichiometry $C1q,r_2,s_2$. Ultrastructurally, C1q exhibits six globular heads joined by filamentous segments to a helical stem-like core. This helical core is compositionally and structurally similar to collagen and can compete with collagen for binding to plasma membrane collagen receptors. [22] [29] The C1r and C1s enzyme dimers bind to the collagen-like tail of C1q, whereas the

Figure 37-1 (Figure Not Available) Reaction scheme of the complement activation pathways. Components displaying enzymatic activity are denoted by a solid bar above symbol. $C3{*}H_2O$ is generated from C3 by spontaneous hydrolysis of an internal thiol ester bond in the C3 -chain (see Fig. 37-2). Dashed arrow denotes crossover between classical and alternative pathways, through which C3b generated by the classical pathway C3 convertase (C4b2a) serves as the membrane cofactor for assembly of the alternative pathway C3 convertase (C3bBb). Also note potential for self-propagation of the C3bBb enzyme complex through the cycle of the amplification convertase. See text for details. *(From Sims,[244] with permission.)*

TABLE 37-2 -- Specificity and Cellular Distribution of Major Complement Receptors

Receptor	Ligand Specificity	Structural Motifs	Cellular Distribution in Humans
C1qR	C1q (collagen)		Platelets, PMN, B lymphocytes, monocytes, fibroblasts, endothelial cells
C3aR	C3a (C4a)	G-protein coupling	Mast cells, basophils, eosinophils, PMN, monocytes, many tissues
C5aR	C5a, $C5a_{des\ arg}$	G-protein coupling	Mast cells, basophils, eosinophils, PMN, monocytes, many tissues
CR1 (CD35)	C3b (C4b, iC3b, C3c) SCR	SCR	Erythrocytes, B cells, certain T cells, neutrophils, eosinophils, macrophages, follicular dendritic cells
CR2 (CD21)	C3d, C3dg, (iC3b)	SCR	B cells, T cells, follicular dendritic cells
CR3 (CD11b/CD18)	iC3b (C3d)	Leukocyte $_2$ -integrin	Neutrophils, eosinophils, macrophages, K and (CD11b/CD18) NK cells, follicular dendritic cells
CR4 (CD11c/CD18, p150, 95)	iC3b	Leukocyte $_2$ -integrin	Neutrophils, macrophages, monocytes, K and CD11c/CD18, NK cells

C1qR, C1q receptor; C3aR, C3a receptor; C5aR, C5a receptor; CR1CR4, complement receptors 14.

binding of C1 activators generally occurs through the globular heads of C1q. Activation of C1 requires multipoint attachment of at least two globular heads of C1q to the Fc domains of immunoglobulin within immune complexes.[27] [28] This can be achieved by simultaneous attachment to either two closely spaced IgG molecules or to multiple Fc of a single antigen-complexed molecule of IgM. The conformational change induced in C1q upon binding multiple immunoglobulin Fc is transmitted to the $C1r_2\ s_2$ subunits, resulting in proteolytic autoactivation of the C1r dimer, which then proteolytically activates C1s. Activated C1s possess the catalytic site for proteolytic activation of C4 and C2.

In addition to antigen-antibody complexes and IgG aggregates, C1 activation can be initiated by CRP, certain viral and bacterial membranes, endotoxin, DNA, monosodium urate crystals, cytoskeletal proteins, and heart mitochondrial membranes. [28] Whereas immunoglobulins bind to the globular region of C1q, C1 activation by CRP occurs through the binding of this protein to the collagen-like core of C1q. Two domains on the C1q -chain have been identified as the CRP-binding sites. These same sites have been implicated as the binding sites for DNA, which can also initiate the classical pathway by activation of C1. [30] In addition to C1, human plasma contains another enzyme complex that can initiate the reaction cascade of the classical pathway. MBP, a C-type lectin in plasma that binds to glycosidic residues expressed by bacteria and other pathogens, has a structure similar to that of C1q and binds to C1q receptors, enhancing phagocytosis. Upon binding to mannose-rich surfaces, MBP initiates proteolytic activation of C4 and C2 through an associated C1s-like serine protease (see the following sections). [10]

Proteolytic cleavage of the -chain of C4 by C1s generates two fragments, C4a and C4b. C4a is a vasoactive peptide of 77 amino acids, removed from the amino-terminal end of the C4 -chain.[31] [32] In addition to liberating the C4a peptide, C1s cleavage of the -chain of C4 exposes an intrachain thioester bond within the -chain of the C4b domain of the molecule that provides a short-lived and highly reactive carbonyl for covalent attachment of C4b to suitable acceptor residues (primary amines and hydroxyl groups) of the antigen-antibody complex or the target cell surface. [19] [21] [33] Polymorphism of the C4 gene is reflected in two common isotypes, C4A and C4B.[34] [35] These isotypes differ in the reactivity of the internal thioester bond for amines versus hydroxyl groups, which is reflected in functional differences in hemolytic activity and in their interaction with immune complexes. [21] Once covalently bound to the cell surface or immune complex, C4b can bind C2 into a Mg^{2+} -dependent complex. When complexed to C4b, C2 is proteolytically cleaved by C1s to liberate the C2b fragment, the C2a domain of the molecule remaining attached to C4b. C2a complexed to C4b provides the catalytic site for subsequent proteolytic activation of C3 and C5.

Peptide cleavage of the -chain of C3 by the C4b2a enzyme complex liberates the vasoactive peptide C3a and exposes a reactive intrachain thioester bond within the -chain of the C3b portion of the molecule (Fig. 37-2).[19] [21] [31] [32] [33] [36] Multiple C3b molecules are deposited on the cell surface (or within the immune

Figure 37-2 Schematic diagram of the proteolytic processing of C3 through enzyme reactions of the complement system. Native C3 lacks biologic activity. C3*H_2O is a C3b-like molecule that is generated by spontaneous hydrolysis of the internal thiol ester bond of the C3 -chain. C3-to-C3b conversion is mediated by either classical or alternative pathway C3 convertase through proteolytic cleavage between amino acid residues 77 and 78 of the C3 -chain. In addition to generating C3b, this proteolysis liberates the peptide anaphylatoxin C3a. The formation of C3b exposes the carbonyl of the -chain thiol ester, which freely reacts either with H_2O or with residues on the target surface to attach C3b covalently. Inactivation of C3b is initiated by the protease factor I, which cleaves a 3-kd fragment from the C3b -chain to generate iC3b. Proteolysis by factor I requires binding of C3b either to plasma component factor H or to the membrane receptors CR1 or MCP. Further proteolysis by factor I liberates the larger fragment C3c, leaving C3dg (43 kd) covalently bound to the target surface via the carbonyl of the internal thioester. See text for details. R, residue; SS, disulfide bonds.

complex) through reaction of this thioester bond with nearby residues containing primary amines or hydroxyl groups. [21] [33] Those molecules of nascent C3b that fail to react with acceptor residues undergo spontaneous hydrolysis of the internal thioester bond, thereby losing their capacity for covalent attachment to the target surface. The covalently bound C3b functions as a cofactor for subsequent activation of C5, and as cofactor for assembly of the alternative pathway C3/C5 convertase (the C3bBb complex, described following). On permissive surfaces, the recruitment of this amplification convertase of C3bBb provides a positive feedback mechanism for propagating the activation of C3 and C5.

The next step in the classical pathway is the activation of C5, which is also initiated by the C4b2a enzyme complex through proteolytic cleavage of the -chain of C5. C3b that is covalently bound to the C4b subunit of the C4b2a enzyme complex is thought to facilitate presentation of C5 to the active site in C2a, conferring enhanced specificity of the enzyme for C5.[5] Proteolytic cleavage of C5 liberates the chemotactic peptide C5a, leaving the C5b portion of the molecule weakly attached to C3b. Interaction of C5b with C6 initiates assembly of the membrane attack complex, composed of components C5b, C6, C7, C8, and C9. The resulting C5b9 complex has the potential to insert into lipid bilayers and to initiate plasma membrane damage (see discussion under Assembly of the Membrane Attack Complex).

Alternative Pathway

In contrast to the sequentially ordered enzyme cascade of the classical pathway, the alternative pathway entails a positive feedback interaction in which the principal activation product, C3b, serves as the cofactor of the C3-cleaving enzyme complex (C3bBb) that is responsible for its own production (Fig. 37-1 (Figure Not Available)).[1] [5] [37] This system is therefore continuously primed for explosive C3 activation, the rate of C3 activation governed by the stability of the C3bBb enzyme complex (which, analogously to the C4b2a complex, also serves to activate C5). Activators of this pathway, which include immune complexes, bacterial lipopolysaccharides and yeast cell walls, certain viral membranes and virally infected cell lines, and the autoantibody C3 nephritic factor, share the common property of facilitating stability of the assembled C3bBb enzyme, sequestered from the regulatory control of inhibitors found in plasma.

Initiation of the alternative pathway involves the conformational activation of C3, which is thought to be closely linked to rupture of the internal thioester bond within the C3b domain of the protein.[21] [33] [37] One pathway for activation of C3 is by peptide cleavage to C3b (e.g., as initiated by the classical pathway enzyme C4b2a). In this manner, C3b formed through the classical pathway can directly initiate assembly of the alternative pathway enzyme complex (Fig. 37-1 (Figure Not Available)). In the absence of classical pathway activation, C3 conversion to an activated state can also occur by spontaneous hydrolysis of the thioester bond within the intact protein, resulting in a molecule (C3*H_2O) that is conformationally similar to C3b. Slow conversion of C3 to C3*H_2O is believed to proceed continuously under normal plasma conditions. Conversion of C3 to either C3*H_2O or C3b results in expression of a binding site for factor B, the proenzyme of the alternative pathway C3/C5 convertase.

When bound to C3*H_2O or C3b, factor B becomes sensitive to proteolytic cleavage by factor D, a trace plasma enzyme. [5] [38] Factor D is a unique enzyme as it neither requires enzyme cleavage for activation nor is regulated by plasma serine protease inhibitors (serpins). Rather, the activity of factor D is conformationally regulated through binding to its substrate, factor B in complex with C3*H_2O or C3b. [38] Proteolysis of factor B by factor D liberates a 30-kd fragment (Ba), leaving the larger Bb fragment (80 kd) attached to C3*H_2O or C3b. Through a catalytic site in Bb, the complex of C3*H_2O,Bb can proteolytically convert C3 to C3a and C3b. Nascent C3b generated by this mechanism is capable of binding additional factor B (which is then converted by factor D to active C3bBb), thereby amplifying the reaction. Activation of C5 by the alternative pathway convertase occurs by identical peptide cleavage to that initiated by C4b2a, resulting in formation of C5a and C5b. Again, C3b covalently deposited in the vicinity of the C3bBb complex is thought to be required for proper presentation of C5 to the catalytic site of the enzyme.

The Lectin Pathway of Complement Activation in Innate Immunity

An element of humoral immune defense with particular significance in the neonatal period is the capacity to recognize and clear microbial pathogens during the months before development of a full repertoire of immunoglobulin-secreting memory B lymphocytes. It is now recognized that a key element of this innate immunity is a C1-like serum enzyme complex that can activate C4 and C2 and is triggered through interaction with the mannose-rich lipopolysaccharide commonly found on the surfaces of microorganisms.[10] [39] [40] The first component of this complex is designated serum mannose (or mannan)-binding protein (MBP), a Ca^{2+} -dependent lectin that is structurally related to two other serum lectins, conglutinin and CL-43, and to two pulmonary surfactant proteins, SP-A and SP-D. These proteins all exhibit structural similarity to C1q, having globular and collagen-like domains, and collectively make up the collectin protein family. A C-terminal carbohydrate recognition domain with affinity for N-acetylglucosamine confers the capacity for MBP to directly opsonize microorganisms expressing mannose-rich surface coats. In blood, MBP circulates as a stable complex with a C1s-like proenzyme (designated MBP-associated serine protease, or MASP), a 93-kd polypeptide with sequence homology to both C1r and C1s. Binding of the MBP-MASP complex to the appropriate carbohydrate surface results in autoactivation of MASP by internal peptide cleavage, converting MASP to an active serine protease. Like C1s, active MASP exhibits the capacity to proteolytically activate both C4 and C2 to initiate assembly of the C4bC2a enzyme complex. In contrast to C1s, active MASP also exhibits direct C3-cleaving activity, thereby accelerating C3b generation and the recruitment of the alternative complement pathway through assembly of C3bBb. MBP has also been found to associate with the $C1r_2C1s_2$ complex and support C4/C2 activation by this enzyme , although the physiologic significance of the C1 proteases in MBP-initiated activation of complement remains unclear.

MBP-mediated opsonization and complement activation have been demonstrated for a wide range of microorganisms, including both gram-negative and gram-positive bacteria, fungi, mycobacteria, and parasites, as well as certain enveloped viruses, including influenza virus, HIV-1, and HIV-2. The importance of this pathway of complement activation to innate immunity in man is suggested by accumulating evidence that patients with inherited MBP deficiency have increased susceptibility to recurrent infection as a result of a complement-dependent opsonic defect. [10] [39] [40]

Assembly of the Membrane Attack Complex

Membrane damage by the complement system is initiated through the interaction of C5b (generated by either the classical pathway or alternative pathway C3/C5 convertase) with plasma proteins C6, C7, C8, and C9.[8] [41] [42] [43] C5 shares homology with C3 and C4 but does not contain the internal thioester bond that is found in those proteins. C6, C7, C8, and C9 share structural motifs that are also found in the cytolytic polypeptide perforin, a membrane pore-forming protein contained in the intracellular dense granules of cytotoxic lymphocytes. The first step

in assembly of the C5b9 complex is the formation of a complex between C5b and C6, through a short-lived interactional site that is expressed by nascent C5b. Association of C6 with C5b is thought to occur while C5b remains in contact with membrane-bound C3b. Upon reaction of C7 with the C5b6 complex, a membrane interactional site is transiently expressed (primarily by the C7 subunit), enabling the C5b67 complex to deposit directly onto membrane surfaces. Once bound to a membrane surface, the C5b67 complex expresses a stable binding site for a single molecule of C8. Association of C8 with the complex results in insertion of a portion of the molecule into the hydrophobic core of the membrane and expression of a binding site for C9. The interaction of C9 with membrane-bound C5b8 results in its elongation and insertion into the membrane interior, concomitant with a polymerization of multiple C9 into a large torus-ringed tubular structure. This tubule of polymerized C9 is readily detected projecting from the membrane surface in electron micrographs of complement-damaged cells.

Insertion of the C5b9 proteins affects both the structural and the functional properties of the plasma membrane. [41] [42] [43] [44] [45] [46] [47] Intercalation of hydrophobic

domains of C8 and C9 alters the bilayer arrangement of membrane lipid and can thereby increase cell-surface exposure of phosphatidylserine and other acidic phospholipids that are normally sequestered to the inner surface of the plasma membrane. By exposing these phospholipids, the C5b9 complex can potentially provide catalytic membrane surfaces for assembly of those enzymes of the coagulation pathway that are stabilized by binding to acidic phospholipid. In addition to altering membrane structure, the inserted C5b9 proteins increase membrane permeability to electrolytes and other small molecules. In the absence of compensatory mechanisms, insertion of these proteins ultimately leads to a complete collapse of transmembrane electrochemical gradients and lytic rupture of the plasma membrane.

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REGULATORY PROTEINS OF THE COMPLEMENT SYSTEM

The classical and alternative activation pathways are under strict control designed to limit dissemination of the reactions in plasma and to inhibit activation of the membrane attack complexes at the surface of blood cells and on other autologous membranes that are normally exposed to complement. Inhibitors identified to date include specific binding proteins and proteases present in plasma, as well as several important cell-surface proteins. [48] [49] [50]

C1-INH

C1-INH is a serpin that binds irreversibly to activated C1r and C1s, blocking their enzymatic activity and dissociating their attachment to C1q. [51] [52] This inhibitor also plays important enzyme regulatory roles in the kinin-generating, intrinsic coagulation, and fibrinolytic pathways through interaction with plasmin, kallikrein, and factors XIa and XIIa.[5] Inherited deficiency of C1-INH underlies the syndrome of hereditary angioedema. [52] Antibodies to C1-INH have been observed in certain lymphoproliferative disorders and can give rise to an acquired syndrome of angioedema that is analogous to the hereditary angioedema seen in C1-INH-deficient patients.

Carboxypeptidase N

The biologic activity of C3a, C4a, and C5a is regulated by carboxypeptidase N, a plasma enzyme that rapidly removes the carboxyl-terminal arginine residue from each of the anaphylatoxins.[53] In the case of C3a, removal of this amino acid abolishes its biologic activity. By contrast, carboxypeptidase N-treated C5a (designated $C5a_{desarg}$) retains about 510% of its chemotactic and neutrophil-stimulating activity. [54]

C3b/C4b-Binding Proteins

The C3b/C4b-binding proteins serve to bind C3b or C4b and to regulate assembly of the C3/C5 convertases. [15] [16] [48] [55] [56] Additionally, several of these proteins serve as physiologically important plasma membrane receptors (Tables 37-1 and 37-2). The genes for factor H, C4b-bp, DAF, MCP, CR1, and CR2 are all localized to the long arm of chromosome 1 and compose a family of closely related genes designated regulators of complement activation (RCAs). [15] [16] [48] These proteins share a common globular structure consisting of homologous cysteine-rich repeating units (SCRs), each of approximately 60 amino acids. This characteristic structure is also found in enzymes that interact with C3 or C4, including C1s, C2, and factor B, as well as in a number of unrelated proteins. CR3 and CR4 are C3 receptors that belong to the Leu-CAM family of $_2$ -integrin cell adhesion receptors. [25] These receptors do not contain the SCR motif.

Factor H

Factor H is a plasma protein comprised of 20 SCRs that binds C3b and serves to inhibit association of either C3b or C3*H2O with factor B (restricting assembly of the C3bB proenzyme).[48] [57] Additionally, factor H promotes the dissociation of the assembled C3bBb enzyme complexes and serves as a cofactor for proteolytic degradation of C3b by factor I. [58] Limited intrachain proteolysis by factor I initially converts C3b to inactivated C3b (iC3b), which remains covalently bound to its original acceptor surface (Fig. 37-2). Subsequent degradation of the iC3b -chain by factor I releases the large C3c fragment (150 kd), leaving C3dg (41 kd) still attached to the acceptor surface through the C3d domain of the fragment. Bound C3dg is normally the final C3b degradation product remaining on circulating cells in vivo. In contrast to factor H (which promotes dissociation of the C3bBb), factor P (properdin), another regulatory protein found in plasma, acts to stabilize the alternative pathway enzyme complex.[59] [60]

C4b-Binding Protein

C4b-bp is an acute phase reactant protein found in plasma that binds to C4b, serving both to block its association with C2 and to accelerate dissociation of the assembled C4b2a enzyme complex.[61] [62] Additionally, C4b-bp serves as a cofactor to facilitate proteolytic degradation of C4b by the serine protease factor I, which splits the larger C4c fragment from the molecule, leaving C4d bound to the original acceptor surface. [58] [63] Approximately 50% of C4b-bp normally circulates in plasma as a complex with protein S, a vitamin K-dependent protein with regulatory function in the coagulation system. Thus an elevation in circulating C4b-bp, as can occur during the acute phase response to injury, has potential to increase the risk of plasma clotting. [64] C4b-bp is composed of seven -chains, each with eight SCRs that contain the binding site for C4b, and of a single -chain with three SCRs that form the binding site for protein S. [65] [66] [67] [68]

Regulation at Cell Surfaces

Surfaces that promote activation of the alternative pathway are distinguished by their capacity to stabilize the C3bBb enzyme complex, away from regulatory control by factor H in plasma, whereas nonactivating surfaces facilitate factor H binding to C3b. Although the exact chemical and physical properties that distinguish complement-activating from nonactivating surfaces remain to be elucidated, it appears that the total content and chemical modification of cell surface sialic acid residues can be

determining factors.[69] [70] [71] The inhibitory activity of factor H is increased when C3b is bound to a surface containing polyanions such as sialic acid, reflecting an increase in the apparent affinity of factor H for C3b under these conditions. [70] [72] Consistent with these data, a specific polyanion-binding site in factor H has been localized to the 13th SCR domain of the protein. [73] In addition to the nature of the glycosidic residues presented by the cell surface, the propensity for complement activation is also governed by the expression of specific membrane proteins that exhibit avidity for C3b and C4b and that exert complement regulatory function through their interaction with these membrane-bound components of the C3/C5 convertases. [16] [49] [50] [74] [75]

Decay-Accelerating Factor

Decay-accelerating factor (DAF, CD55) is a 70-kd single chain glycoprotein normally expressed on the surface of red cells, platelets, leukocytes, endothelium, and other cells that serves to accelerate the dissociation of the subunits of the membrane-assembled C4b2a and C3bBb enzyme complexes. [49] [50] [74] [75] DAF does not function as a cofactor for the inactivation of C4b or C3b by factor I. [76] The protein is composed of four SCRs, three of which are essential to its function. [77] In addition to containing the SCR motif, DAF belongs to a class of membrane proteins (examples of which include erythrocyte acetylcholinesterase, lymphocyte LFA-1, leukocyte 5-ectonucleotidase, leukocyte Fc III receptors, neutrophil alkaline phosphatase, and complement regulatory protein CD59) that are attached to the cell surface by glycosidic linkage to the membrane lipid phosphatidylinositol. [78] [79] [80] Defective biosynthesis of the glycosyl phosphatidylinositol (GPI) anchor that normally attaches

DAF, CD59, and other GPI-anchored proteins to the cell surface underlies the syndrome of paroxysmal nocturnal hemoglobinuria (PNH; see Chap. 20),[80] [81] [82] whereas polymorphisms in the DAF gene itself account for the Cromer blood group antigens (see Chap. 132).[83]

Membrane Cofactor Protein

Membrane cofactor protein (MCP, CD46) is a 45- to 70-kd glycoprotein distributed on all human leukocytes and platelets but absent on erythrocytes. [16] [48] [84] This protein contains four SCRs that function in binding C3b, C4b, and iC3b. Additionally, MCP exhibits potent cofactor activity for factor I-mediated proteolysis of C3b and C4b. DAF and MCP act in concert on the membrane surface and are complementary in their activity: DAF exhibits only decay-accelerating activity but no cofactor activity for factor I, while MCP serves as cofactor for factor I but does not promote decay of the C3 convertase enzymes.

CR1

CR1 (CD35), the principal cellular receptor for C3b, also serves as an important cofactor for proteolysis of C3b and C4b by factor I. In contrast to MCP, CR1 exhibits DAF-like function and contributes to the decay of the C3 convertases. [48] [49] [85] [86] [87] Recombinant forms of CR1, MCP, and other complement regulatory proteins may have future therapeutic application as inhibitors of complement activation. [48] [50] [88] [89]

Inhibitors of the Membrane Attack Complex

Formation of C5b on human blood cells is regulated through control of the C3/C5 convertases, exerted by DAF and other inhibitors. Furthermore, the C6-binding site that is exposed when C5 is activated to C5b is highly unstable, limiting the rate of formation of C5b6. Once formed, diffusion of the C5b67 complex to innocent bystander cells is limited by the very short lifetime of the membrane-interactional site exposed in this complex. [1] [90] Membrane damage to autologous cells exposed to activated complement is also restricted by inhibitory proteins found in plasma and on cell surfaces.

Vitronectin (S-Protein) and Clusterin

The diffusion of functional C5b9 complexes from the initial target of complement activation to other cells is restricted by several scavenger proteins in plasma that bind newly formed C5b67, thereby preventing its dissemination to other cells. Proteins with this property include vitronectin (also referred to as S-protein), as well as certain serum lipoproteins, including clusterin (SP40,40). [91] [92] [93] [94] [95] Upon binding one molecule each of C8 and C9, the vitronectin-C5b9 complex (SC5b9) circulates inactive in plasma until cleared, providing a serologic marker of intravascular complement activation that has proceeded to assembly of the C5b9 complex. Vitronectin also contains the RGD (Arg-Gly-Asp) peptide sequence found in fibrinogen, fibronectin, and other integrin-binding proteins, which provides a mechanism for interaction of SC5b9 complexes with plasma membrane integrin receptors on a variety of cells. [96] [97]

CD59 (HRF-20) and Other Homologous Complement Restriction Factors

In addition to the protection afforded by vitronectin and other C5b9 scavenger proteins in plasma, human blood cells, vascular endothelial cells, and many other tissues are also protected from lytic damage by human complement through the cell-surface expression of a protein(s) that specifically serves to inhibit intercalation of C9 into the plasma membrane.[49] [98] [99] [100] [101] [102] [103] [104] This cell-surface inhibitor of the membrane attack complex appears to recognize specific domains contained within the human C8 -chain and human C9 that are involved in the transformation of C9 from its globular conformation in plasma to a membrane-embedded homopolymer with cytolytic activity.[102] [105] [106] [107] [108] Proteins with this function have been termed homologous restriction factors, because of their apparent specificity for human C8 and C9, as opposed to these same complement proteins derived from the serum of other species. The best-characterized human complement homologous restriction factor, designated CD59, is an 18- to 22-kd cell-surface glycoprotein that was first isolated from the erythrocyte membrane. [100] [101] [102] [103] [104] [106] In its mature form, this protein is comprised of a 77-residue polypeptide that is folded through five disulfide bonds, the resulting structure showing similarity to that identified in a group of unrelated proteins, designated the Ly6 superfamily. [109] Like DAF, CD59 is tethered at the carboxyl-terminus of the polypeptide to the cell surface by glycosidic linkage to phosphatidylinositol. [110] Deletion of CD59 from the surface of erythrocytes and other blood cells can occur as the consequence of the GPI-anchoring defect that underlies PNH, which accounts for the unusual sensitivity of the defective blood cells that arise in this disorder to lysis by the C5b9 proteins. A putative 64-kd human erythrocyte membrane protein (commonly referred to as HRF, or C8-binding protein) had been reported to exhibit many of the complement-inhibitory properties that are now recognized to reside in CD59. [111] [112] [113] The capacity of nonprimate cells to resist injury by the human C5b9 complex when transfected with cDNA encoding CD59 suggests one potential strategy for genetic engineering of tissues and organs that are inherently protected from the lytic action of complement in human serum.[114] [115] [116]

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CELLULAR RESPONSES MEDIATED BY COMPLEMENT RECEPTORS

Activated components of the complement system contribute to virtually every aspect of inflammatory response, including increased vascular permeability, leukocyte chemotaxis, leukocyte activation, immune adherence and phagocytosis, solubilization

and clearance of immune complexes, and regulation of the lymphocytic immune response. [1] [2] [3] [4] [7] [48] [117] These proteins also play a direct role in mediating cell lysis through both intravascular and extravascular mechanisms. In addition to their role in host defense and inflammation, there is evidence that complement proteins can also interact with components of the coagulation, fibrinolytic, and kinin systems to alter vascular hemostatic mechanisms. [5] [46] [118] [119] In general, the biologic effects of complement are initiated through binding of activated components to specific complement receptors distributed on the surface of various blood and vascular cells. This binding interaction can serve to remove immune complexes and other complement-coated particles from plasma, through direct adherence to circulating blood cells. In many cases, binding of a complement protein to its cell-surface receptor elicits specific cellular responses that are evoked by receptor-coupled signaling across the plasma membrane.

Responses Mediated by C1q Receptors

Two types of widely expressed plasma membrane receptors for the C1q subunit of C1 have been described: a 60-kd calreticulin homolog, designated cC1qR, that binds to the collagen-like stalk of C1q, and gC1qR, a 33-kd glycoprotein with affinity for the globular heads of C1q. [120] [121] [122] [123] [124] [125] [126] Binding of C1q to these receptors prevents the formation of C1 and can be inhibited by either type 1 collagen or the $C1r_2C1s_2$ enzyme complex. In addition to C1q, gC1qR has been shown to bind vitronectin and thus may participate in the clearance of opsonized particles or cooperate with vitronectin in the inhibition of complement-mediated cytolysis. [127] C1q has been shown to bind to blood platelets and to inhibit collagen-induced platelet aggregation by competing with collagen at the platelet collagen receptor. By contrast, multimeric aggregates of C1q can initiate platelet activation and aggregation. Aggregates of C1q have also been shown to elicit oxidative bursts in neutrophils and to potentiate antibody-dependent cellular killing by peripheral blood lymphocytes. These interactions potentially contribute to the inflammatory responses elicited by C1q-containing immune complexes.

Anaphylatoxin Receptors

The amino-terminal peptide fragments that are released from the -chains of C3, C4, and C5 during complement activation, collectively referred to as complement anaphylatoxins, share considerable structural and functional homology and exhibit a spectrum of biologic effects during an inflammatory response. These effects include smooth muscle contraction, histamine release from mast cells, vasodilation, and increased vascular permeability.

C3a/C4a Receptor

C3a, a 77-amino acid peptide, causes increased vascular permeability and stimulates the degranulation of mast cells and basophils, resulting in the release of histamine and other vasoactive compounds. [24] [53] [54] [128] The C3a receptor has recently been cloned. The cDNA encodes a 53-kd G-protein-coupled receptor with 37% nucleotide identity with the C5a receptor. [129] Because many of the biologic effects of C3a are also shared by C4a (77 amino acids), it has been suggested that they bind to the same receptor, although evidence for a separate C4a receptor has also been presented. [130] [131]

C5a Receptor

C5a, a 74-amino acid peptide, interacts with cell-surface receptors that are specific for C5a (and distinct from the C3a/C4a receptors) to stimulate degranulation of mast cells and basophils. [24] [132] C5a also plays a major role in the recruitment and activation of human neutrophils and macrophages at sites of complement activation. C3a and C5a receptors can undergo internalization after stimulation, resulting in a desensitization of the cells to further stimulation. Like the receptor for C3a, the C5a receptor is G protein coupled and is a member of the superfamily of rhodopsin-type receptors that contain seven transmembrane loops. [132] The deduced amino acid sequence of the C5a receptor shows homology with the leukocyte N-formyl peptide receptor, and the gene for the C5a receptor is closely linked to the formyl peptide receptor genes on chromosome 19. [24]

Through activation of its cellular receptors, C5a initiates several important inflammatory responses. First, C5a serves as a potent chemotactic agent for human neutrophil and mononuclear phagocyte migration. Stimulation by C5a has been shown to increase neutrophil adhesiveness and to initiate neutrophil aggregation. C5a also elicits oxidative metabolism, leading to free radical formation and the secretory release of lysosomal enzymes from neutrophils and a variety of phagocytic cells. These pro-inflammatory changes in C5a-stimulated neutrophils are accompanied by increased surface expression and activity of both CR1 (CD35) and CR3 (CD11b/CD18), thereby promoting leukocyte adhesion as well as phagocytosis of opsonized particles. [24] [133] It has been reported that C5a induces rapid expression of endothelial P-selectin, secretion of endothelial von Willebrand factor, and increased adhesiveness of neutrophils to endothelial cells. [134] [135] The putative role of C5a in mediating neutrophil rolling, attachment, and adhesion is of particular interest in light of its implicated role in the pathogenesis of lung disease. Recent evidence also suggests that inflammatory and immunoregulatory responses to C5a may be mediated through induction of interleukin-6 (IL-6) synthesis by human monocytes. [136] The potent chemotactic and leukocyte-activating properties of C5a (and $C5a_{desarg}$) suggest an important role in the vascular response to complement activation in humans. In particular, these peptides have been implicated as mediators of the neutropenia associated with hemodialysis and cardiopulmonary bypass and may give rise to the pulmonary leukostasis associated with adult respiratory distress syndrome and acute transfusion reactions. [137] [138] The role of C5a in the pathogenesis of endotoxic shock syndrome is suggested by studies in primates, in which functionally blocking antibodies against C5a have been shown to attenuate respiratory distress and enhance survival after gram-negative bacteremia. [139]

Expression of receptors for C3a and C5a had generally been thought to be limited primarily to myeloid cells. The recent cloning of both receptors has made it possible to examine cellular expression of mRNA encoding these receptors in greater detail, which has revealed that both receptors are expressed in many tissues distributed throughout the body and are not restricted to blood leukocytes. [24] [129] [140]

C3b Receptors

As noted, cellular receptors that bind C3b and C4b play an important role in the inactivation of these proteins by providing cofactor function for the serum protease, factor I. By contributing to the degradation of C3b deposited onto the plasma membrane, these receptors serve to limit the accumulation of C3b on circulating blood cells and restrict dissemination of the C3/C5 convertase reaction. In addition to this function, it is now recognized that these cellular receptors for C3b (and its

degradation products) play a central role in mediating cellular adherence and ultimate clearance of immune complexes and other C3b-coated particles and cells. [48] [141] [142] [143]

The cellular distribution and ligand specificities of the various C3 receptors are summarized in Table 37-2 . Although it is distributed in the plasma membrane of nearly all blood cells, the preponderance of CR1 found in the blood of humans and other primates is expressed on the surface of erythrocytes.

In humans, the clearance of immune complexes from the circulation is principally mediated by an initial adsorption to red cells that is mediated via interaction of multiple C3b and/or C4b incorporated into the immune complex with erythrocyte CR1 receptors. [48] [144] [145] After adsorption to the erythrocyte surface, proteolytic processing of C3b provides a mechanism for transfer of the bound immune complex to cells of the reticuloendothelial system: Upon conversion of C3b to iC3b and C3dg (by factor I), the reduced affinity of these degraded C3b fragments for CR1 promotes release of the immune complex from the erythrocyte surface (which contains only the CR1 receptor), followed by endocytic uptake of the complexes by phagocytic cells that express CR3 and CR4. Studies with radiolabeled C3b-coated immune complexes suggest that these immune complexes are stripped from the erythrocyte surface during passage through the liver and, to a lesser extent, the spleen. These data implicate hepatic Kupffer cells as the principal scavenger of C3b-containing immune complexes that are initially transported by red cells. [146]

In addition to serving as receptors for cellular adherence to immune complexes (and other C3-containing particles), these complement receptors are also known to provide stimulatory signals for leukocyte activation. [141] [143] [147] [148] Binding of C3b aggregates to leukocyte membrane CR1, either alone or in concert with occupancy of cell surface Fc receptors by IgG, is known to trigger an endocytic response from neutrophils and mononuclear phagocytes, which is accompanied by enzyme secretion and a cell respiratory burst. The capacity of iC3b-coated particles to trigger similar responses from neutrophils and monocytes suggests a similar role for CR3 and CR4. The distribution of CR1 (the C3b receptor) and CR2 (the C3dg receptor) on B lymphocytes suggests possible roles for these receptors in modulating immune responses. For example, interaction of CR1 and CR2 with their respective ligands has been shown to promote B-lymphocyte proliferation and differentiation into memory B cells, although the physiologic significance of these responses remains to be clarified. [7] [149] Aggregated C3-split products have also been reported to augment the response of IL-2-treated helper T lymphocytes, suggesting a co-stimulatory function. [149] [150] Decreased numbers of erythrocyte CR1 receptors have been documented in patients with systemic lupus erythematosus (SLE), AIDS, and lepromatous leprosy, diseases that are also associated with increased levels of circulating immune complexes, reflecting polyclonal B-cell activation. As indicated, CR2 has been identified as the B-lymphocyte receptor for the Epstein-Barr virus, implicating this receptor in the pathogenesis of infectious mononucleosis and Burkitt lymphoma. [7] [151] [152] Finally, CR3 distributed on natural killer lymphocytes has been implicated in promoting adhesion and enhancing the cytotoxic response to target cells bearing surface iC3b. [153] [154]

CR1

CR1 (CD35), also known as the immune adherence receptor, is the principal cellular receptor for C3b. It is a single-chain membrane glycoprotein that is distributed on erythrocytes, neutrophils, eosinophils, monocytes, B lymphocytes, subsets of T lymphocytes, macrophages, follicular dendritic cells, and glomerular podocytes. [48] [142] CR1 is not found on human platelets. Four polymorphic variants (160250 kd) encoded by four codominantly inherited alleles have been identified, and the cellular concentration of this receptor also exhibits genetic polymorphism. [155] [156] [157] [158] [159] The primary role of CR1 is to mediate binding of particles coated (opsonized) with C3b or iC3b to neutrophils and monocytes that express this receptor, for subsequent phagocytosis. In addition to its role in immune adherence and clearance, CR1 also contributes to the regulation of complement by acting as cofactor for the proteolytic degradation of both C3b and C4b by factor I and by accelerating decay of the C3 convertases.[86] [87] The most common allotype of CR1 (220 kd) contains 30 SCRs. The ligand-binding sites have been localized to SCRs 13 for C4b and to SCRs 810 (duplicated in SCRs 1517) for C3b and C4b.[160] More recent studies have further defined amino acids within these regions that are critical for C3b versus C4b binding.[161] [162] [163]

CR2

CR2 (CD21) is a 145-kd glycoprotein found primarily on B lymphocytes, T lymphocytes, and follicular dendritic cells, but not on phagocytic cell types. This protein functions as a receptor for C3d, C3dg, and iC3b, and is the plasma membrane receptor of the Epstein-Barr virus. [7] [142] [143] [164] [165] CR2 contains 15 SCRs. The binding sites for both the Epstein-Barr virus and C3dg have been mapped to a common domain on CR2, which includes the first two SCRs. [151] [152] [166] CR2 on follicular dendritic cells binds C3d- or C3dg-bearing immune complexes. On B lymphocytes, stimulation through CR2 is thought to play a role in proliferation and differentiation. CR2 exerts an important co-stimulatory function within a noncovalent complex with CD19 and CD81, lowering the amount of specific antigen required for optimal activation through the B-cell receptor when the antigen is coated with C3dg. [148] Recent evidence also implicates CR2 in the infection of B lymphocytes by HIV-1 (see discussion under Acquired Immunodeficiency Syndrome).[167] [168]

CR3, CR4

CR3 (CD11b/CD18, Mac-1) and CR4 (CD11c/CD18) belong to the Leu-CAM family of leukocyte adhesion molecules, which also includes LFA-1 (CD11a/CD18). They constitute the $_2$ subfamily of membrane integrin receptors, two-chain glycoproteins consisting of a common -chain (CD18 antigen) and a noncovalently associated -subunit. [25] [169] [170] These receptors do not contain the SCR motif that is common to CR1, CR2, and other C3b-binding proteins. Of note, CR4 originally designated another C3 receptor (the platelet C3dg receptor) that is not related to CD11c/CD18. [171] CR3 and CR4 are cellular receptors for iC3b, and are distributed on neutrophils, monocytes, and other mononuclear phagocytes and natural killer lymphocytes. They are not found on B lymphocytes, glomerular podocytes, or erythrocytes. CR3 participates in immune adherence and phagocytosis by promoting adhesion of granulocytes and monocytes to iC3b-coated cells and particles, and plays a role in lymphocyte-mediated antibody-dependent cellular toxicity. [170] [172] CR4 shares these functions and has been implicated in granulocyte-mediated lysis of erythrocytes.[172] Additionally, CR3 is involved in polymorphonuclear leukocyte homotypic aggregation through an unidentified counter-receptor, in cellular adherence to activated endothelial cells via ICAM-1, and in cellular adhesion to fibrinogen and coagulation factor X. [173] These functions and recognition of iC3b have been attributed to the so-called I-domain of CR3.[174] CR3 also expresses a lectin-like domain that binds certain polysaccharides, including -glucan, providing a recognition site for phagocyte attachment and phagocytosis of unopsonized yeast cells. [175] [176] A lectin-carbohydrate bridge clustering CR3 and certain GPI-anchored receptor glycoproteins (including FcRIIIb and the receptors for urokinase plasminogen activator) has been demonstrated in which CR3 is stimulated when ligand binds to these GPI-anchored receptors.[177] Expression of the receptor function of CR3 and CR4 requires cell activation, which entails both redistribution to the cell surface and increased affinity for their ligand, possibly through phosphorylation of cytoplasmic domains of the CD18 subunits. [131] [178] [179] Congenital deficiency of these receptors gives rise to the disorder leukocyte adherence deficiency, which is associated with defective chemotaxis, reduced adhesion with impaired transmigration across endothelium, and impaired cell-mediated killing of microbial pathogens. [170] [180] [181] Neutrophils from patients with

complete leukocyte adhesion deficiency fail to amplify the phagocytic function in response to stimulation. [182]

Cellular Responses to the C5b9 Complex

The cytolytic activity of the complement system resides in the capacity of the C5b9 proteins to insert into phospholipid bilayers, thereby increasing membrane permeability to aqueous solute. This membrane permeability-inducing function of the C5b9 complex can be observed for a variety of cellular and synthetic lipid membranes, suggesting that it is directly mediated through interaction of these proteins with the lipid bilayer and does not require binding to specific receptors on the surface of the target cell. The affinity of activated C5b9 for phospholipid bilayers confers a capacity to interact with the membrane envelope of virtually every biologic cell, a feature that at least in principle enables the complement system to defend against a limitless variety of potential pathogens.

Although the cytolytic function of these proteins is consistent with their role in immunologic defense and is a prominent feature of their interaction with nonhuman cells, accumulating evidence suggests that some of the biologic manifestations of complement activation relate to the capacity of the assembled C5b9 proteins to evoke specific cellular responses, without lytic consequence for the target cell. [41] [44] [46] [118] [183] [184] [185] It is now recognized that specific inhibitors of this protein complex are expressed on the surface of human blood cells and vascular endothelium. By limiting the extent of activation and membrane insertion of C9, the cytolytic activity of the complement system is markedly attenuated on these cell membranes. In addition to protecting these cells from lysis during intravascular complement activation, the resistance to complement-mediated cytolysis conferred by these plasma membrane inhibitors may have other biologic consequences. For example, it

has been shown that insertion of the C5b9 proteins into the plasma membrane of human platelets, neutrophils, cultured vascular endothelium, and numerous other cells elicits activation-related responses, indicating that these proteins trigger stimulatory intracellular metabolic pathways without lytic breakdown of the plasma membrane. Among the cellular changes that have been reported to result from sublytic plasma membrane damage by C5b9 proteins are initiation of arachidonate metabolism (leading to the biosynthesis of various active prostanoids), activation of intracellular C-kinases, induction of shape change, secretory exocytosis of the contents of intracellular storage granules, an oxidative respiratory burst, induction of mitogenic responses, induction of IL-8 and monocyte chemoattractant protein-1 synthesis, expression of receptor sites for serum lipoproteins, cell binding and activation of plasminogen, vesiculation of cell-surface components from the plasma membrane, and the induction of a transbilayer migration of plasma membrane phospholipids with exposure of membrane-binding sites for coagulation factors and other plasma proteins that exhibit affinity for anionic phospholipid surfaces. [183] [186] [187] [188] [189] [190] [191] [192] [193] [194] The cellular changes initiated by the C5b9 proteins generally show a dependence on the level of extracellular calcium, suggesting that the stimulatory effects observed reflect increased cytoplasmic [Ca^{2+} +], mediated through the influx of this ion across the complement pore formed in the plasma membrane. [195]

COMPLEMENT IN DISEASE

Genetic Polymorphism and Deficiency States

Insight into the functional importance of individual complement components in man has come from the study of complement deficiency states. Additionally, genetic polymorphisms of many of the complement proteins have now been identified. In several cases these polymorphisms have proved to be of serologic and immunohematologic interest, and have also provided clues to specific protein structure-function relationships. cDNA and genomic clones for most of the complement proteins and receptors have been isolated and the chromosomal location of the genes identified (Table 37-3). The discussion in this section is limited to selected examples of complement deficiency states and allelic variants that are of particular hematologic interest. For extensive review of the structure and regulation of the complement genes and of the known complement deficiency states, the reader is referred to the literature. [7] [196] [197] [198] [199] [200] [201]

C1-INH Deficiency

Inherited deficiency of C1-INH underlies the syndrome of hereditary angioedema. The gene for this serpin protease inhibitor is localized to p11.2q.13 of chromosome 11. Deficiency states can arise through subnormal production of functional C1-INH, or by production of dysfunctional protein. [52] [200]

Class III Genes and C4 Polymorphism

The genes for C4, C2, and factor B form a tight cluster (with the gene for enzyme 21-hydroxylase) in the MHC on the short arm of chromosome 6, a region designated MHC class III.[202] Class III lies between the class I and class II genes of the HLA system. C2 deficiency is commonly associated with SLE and is one of the most common complement deficiency states. Deficiency of factor B has not been described, which may reflect a requirement of this primary C3 activator for survival. Whereas genetic polymorphisms of several of the complement proteins have now been described, that of C4 is of greatest immunohematologic interest. Two isotypes, C4A and C4B, with distinct functional and serologic properties have been characterized. [35] The functional distinction between C4A and C4B isotypes relates to differences in the reaction mechanism and the type of bond that is preferentially formed by the internal -chain thioester group. [203] The C4A isotype preferentially transacylates onto amino group nucleophiles, while the C4B isotype reacts with equal efficiency toward amino or hydroxyl groups. The C4B variant shows greater hemolytic activity owing to increased binding to the red cell surface, whereas the C4A variant binds more efficiently to C1-containing immune aggregates, which serves to inhibit immune precipitation of these complexes. [204] The haplotype C4AQO is linked to SLE and other autoimmune diseases, which may reflect this differential processing of immune complexes. An increased incidence of SLE is also associated with other complement gene defects that would potentially affect clearance of immune complexes, including quantitative or functional defects in the classical pathway components C1q, C4, or C2, or in the plasma membrane receptors that function in the clearance of opsonized immune complexes, particularly CR1. [196] [197] [205] [206] [207] At a serologic level, the C4A and C4B isotypes give rise to the Rodgers (Rg) and Chido (Ch) immune serotypes detected in red cell cross-matching. These antisera detect the antigenic differences of the erythrocyte-bound C4d fragment that originate from the C4A and C4B isotypes: most simply, C4A-coated red cells type Rg+, Ch, while C4B-coated cells type Rg, Ch+. For each, numerous allotypic and isotypic variants have been identified, several of which have been characterized at the molecular level, permitting precise epitope mapping. [35] [208]

Deficiencies and Polymorphisms of Plasma Membrane Complement Receptors and Regulatory Proteins

CR1/CR2

The gene for CR1 is located within the RCA group on the long arm of chromosome 1. [205] [209] Several CR1 allotypes (ranging from

TABLE 37-3 -- Complement Components: Gene Location and Deficiency States

Component	Chromosomal Gene Location	Disease Associated with Deficiency
Components of Activation Pathways		
C1q	1	SLE; pyogenic infection; glomerulonephritis
C1r/C1s	12	SLE; infection; nephritis
C4	6 (MHC class III)	SLE; infection; immune complex disease
C2	6 (MHC class III)	SLE; infection; nephritis
C3	19	SLE; infection; nephritis
Factor B	6 (MHC class III)	
Factor D	19	Neisserial infection
Factor I	4	Pyogenic infection
Components of Membrane Attack Complex		
C5	9	Neisserial infection; SLE
C6/C7	5	Neisserial infection; SLE, Sjögrens
C8	and : 1; : 9	Neisserial infection
C9	5	Neisserial infection; also asymptomatic
Plasma Regulatory Proteins		
C1 Inh	11	Hereditary angioedema
Properdin	X	Neisserial infection; other pyogenic infection

C4bp	1 (RCA gene cluster)	
Factor H	1 (RCA gene cluster)	Infection; hemolytic uremic syndrome; glomerulonephritis
Vitronectin (S protein)	17	
Membrane Regulatory Proteins		
DAF (CD55)	1 (RCA cluster)	No symptoms with isolated deficiency Inab phenotype[a]
MCP (CD46)	1 (RCA cluster)	
HRF-20 (CD59)	11	PNH-like symptoms with isolated deficiency[a]
Membrane Receptors		
CR1 (CD35)	1 (RCA cluster)	SLE
CR2 (CD21)	1 (RCA cluster)	
CR3 (CD11b/CD18)	16	LAD
CR4 (CD11c/CD18)	21	LAD

Abbreviations: SLE, systemic lupus erythematosus; RCA, regulators of complement; C1 Inh, C1 inhibitor; C4bp, C4b-binding protein; DAF, decay-accelerating factor; MCP, membrane cofactor protein; HRF, homologous restriction factor; CR, complement receptor; PNH, paroxysmal nocturnal hemoglobinuria; LAD, leukocyte adhesion deficiency.

[a]DAF and CD59 can be deleted due to GPI-anchoring defect, which is associated with PNH.

160 to 250 kd) have been identified, and numerical expression of erythrocyte CR1 is subject to considerable polymorphic variability. [196] [197] Additionally, there is evidence for acquired reduction of CR1 owing to proteolysis of the receptor in vivo. In several conditions associated with immune diseases, a reduction of erythrocyte CR1 is observed, and in certain cases this has been shown to be accompanied by increased plasma levels of the receptor, implying removal from the membrane by surface cleavage.[205] [210] Decreased cell-surface CR1 can alter the capacity of these cells to subsequently bind immune complexes and impair processing of C3b by factor I.[211] Both CR1 and CR2 have been implicated as receptors modulating the immune response of B cells. Although it is unclear how a deficiency of these receptors in man affects the overall immune response, genetically engineered mice lacking CR1 and CR2 showed markedly impaired immune responses, particularly to T-cell-dependent antigens. [212]

CR3/CR4

Genetic deficiency of CR3 (CD11a/CD18), CR4 (CD11b/CD18), and LFA-1 (CD11c/CD18) gives rise to the syndrome of leukocyte adhesion defect (LAD). [181] The genes coding for the -chains of these receptors are each located on chromosome 16, while their common -chain (CD18) is coded by a gene on chromosome 21. LAD is caused by defective -chain synthesis, which affects cell-surface assembly of these three heterodimeric integrin receptors. Many of the clinical manifestations of LAD, including recurrent pyogenic infections and defective wound healing, can be related to dysfunction of the adhesive and opsonic functions of CR3 and CR4.

DAF

The gene for DAF is contained within the RCA group on chromosome 1.[15] Allotypic variants of DAF give rise to the blood group antigens contained in the Cromer blood group.[83] Genetic deficiency of DAF is associated with the Inab erythrocyte phenotype. [213] These cells lack the common antigens of the Cromer blood group (see Chap. 132). This isolated deficiency of DAF is not associated with a clinical disorder, suggesting that other complement regulatory proteins are sufficient to protect these cells.[213] [214]

MCP (CD46)

Four isoforms of MCP have been identified, the result of alternative splicing of a single gene. [215] These isoforms have been shown to differ in their activity in regulating complement activation, which raises the possibility of tissue-specific and individual variability in the activity of this important complement regulatory protein.

CD59

The gene for CD59 has been localized to the short arm of chromosome 11. Isolated genetic deficiency of CD59 has been described in a single individual, and evidence for autosomal recessive inheritance was presented. [216] The individual was reported to experience episodic intravascular hemolysis with a clinical course similar to that observed in PNH. PNH is an acquired stem cell disorder in which somatic mutation in a hematopoietic progenitor cell gives rise to defective biosynthesis of the GPI anchor by which DAF, CD59, and several other cell-surface proteins are normally anchored to the plasma membrane (see Chap. 20). The blood cells produced in this disorder can have several different phenotypes, depending on the extent to which individual GPI-anchored proteins are missing from the cell surface. Often the affected proteins can be detected in plasma and urine. The intravascular hemolysis that is characteristic of this disorder appears to be most directly attributable to those cohorts of erythrocytes that are missing CD59, and therefore cannot regulate the cytolytic activity of the C5b9 complex.

Contribution of Complement to Accelerated Destruction of Blood Cells

The complement system mediates the accelerated destruction of blood cells that can arise in several hematologic disorders (see Chap. 34).

Autoimmune Hemolytic Anemia

Antibody-initiated activation of the complement system can result in increased deposition of C3b, C4b, and potentially C5b9 on the red cell surface. [217] Most commonly, the shortened survival rate of these cells reflects accelerated hepatosplenic clearance as a consequence of endocytic uptake initiated when red cell-associated IgG and C3b (or C4b) interact with Fc and CR1 receptors on phagocytic mononuclear cells of the reticuloendothelial system. The rate and organ site of red cell sequestration and removal appear to depend on whether endocytic recognition is principally mediated through Fc receptor interaction with IgG or through CR1 interaction with C3b and C4b. Sequestration of IgG-coated red cells (generally associated with warm antibody hemolytic anemia) occurs primarily in the spleen rather than liver, except when there is a very high density of cell-surface IgG. The ability of non-complement-fixing IgG antibody (e.g., anti-Rh $_0$ D) to shorten red cell survival suggests that in the case of IgG antibodies against red cell antigens, splenic sequestration and clearance can be directly initiated by Fc receptors on phagocytic cells. Studies in complement-deficient animals and humans nevertheless suggest that complement activation does serve to accelerate the rate of clearance of these IgG-coated cells and to shift the site of clearance to the liver. It has also been suggested that in the case of non-complement-fixing IgG antibodies, the small amounts of C3b (or C3d or C3dg) and C4b (or C4d) that are normally found on the red cell surface may be sufficient to initiate endocytic clearance by splenic macrophages, when presented concurrently with IgG.

In the case of IgM antibodies against erythrocyte antigens (e.g., as found in chronic cold agglutinin disease), activation of the complement system appears to be required for red cell survival to be affected. When complement activation does occur, most of the affected cells (bearing IgM and C3b or iC3b) are initially sequestered in the liver. The sequestration of these cells is presumably mediated by the interaction of CR1 on hepatic Kupffer cells with red cell C3b and C4b (or iC3b). A portion of the sequestered red cells are subsequently returned to the circulation and remain coated with IgM and proteolytically degraded fragments of C3b (mainly C3dg). Sequestered red cells that escape endocytic clearance and return to the circulation survive normally, reflecting the general absence of receptors for IgM on phagocytic cells and the specificity of CR1 receptors for intact C3b (Table 37-2).

In addition to mediating extravascular clearance, complement activation on the red cell surface can lead to intravascular hemolysis. Nevertheless, for both IgG- and IgM-mediated immune hemolytic anemias, clinically significant intravascular hemolysis is rarely observed, even when complement-fixing antibodies are documented. Exceptions include circumstances in which massive complement activation at the erythrocyte membrane occurs because of the nature of the antibody (e.g., alloimmune hemolysis of ABO-mismatched red cells, or for the antibody of paroxysmal cold hemoglobinuria) and in PNH, where a decrease in cell-surface CD59 compromises the normal regulatory control of the terminal complement proteins.

Neutropenia and Thrombocytopenia

The potential role of C5a (and $C5a_{desarg}$) in the neutropenia and vascular leukostasis associated with cardiopulmonary bypass procedures, hemodialysis, blood transfusion, and sepsis has been described. [218] [219] [220] [221] [222] There are also data suggesting that complement activation contributes to shortened platelet survival in cases of immune thrombocytopenia and in circumstances of alloimmune platelet transfusion. In most instances, shortened platelet survival is thought to reflect hepatosplenic sequestration and removal through the reticuloendothelial system, and not intravascular platelet lysis per se. Since deposition of the C5b9 proteins promotes exposure of membrane-binding sites for coagulation factors responsible for thrombin formation, activation of these complement proteins may underlie the increased risk of thrombosis observed in PNH, immune thrombocytopenic purpura, and other immune and inflammatory states associated with intravascular complement activation.

Acquired Immunodeficiency Syndrome

Several reports suggest that interaction of HIV-1 with components of the complement system may contribute to the pathogenesis of AIDS. [223] [224] [225] [226] Activation of the classical pathway of complement by HIV-1 has been shown to occur through both antibody-dependent and antibody-independent mechanisms, including direct binding and activation of C1 by the GP120/GP41 viral membrane glycoprotein complex. The membrane envelope of HIV-1 incorporates the GPI-anchored complement regulatory proteins CD55 (DAF) and CD59 from the host cell plasma membrane, and viral GP120 promotes binding of factor H to the membrane surface. Collectively these various complement regulatory proteins may confer selective resistance of the HIV-1 viral particle to lysis by complement. [225] On the other hand, complement-dependent enhancement of HIV-1 infection of numerous lymphocytic cells has also been demonstrated in vitro, with viral entry facilitated through C3dg interaction with cell-surface CR2 (CD21), either alone or in concert with the attachment of HIV-1 GP120 to cell-surface CD4. [167] [168] [227] The possibility that complement contributes to the depletion of CD4+ lymphocytes expressing HIV-1-derived surface antigens has also been suggested. [228] A decrease in the surface expression of

CD55 and CD59 has also been described for T lymphocytes of HIV-infected individuals, raising the possibility of increased susceptibility of these cells to lysis by complement.[229] Whether complement activation in vivo ultimately ameliorates or exacerbates the cellular and clinical manifestations of this disorder remains to be clarified.

Interactions Between Complement and Coagulation Systems

Interactions among certain components of the complement, kinin, coagulation, and fibrinolytic systems are known to occur, although their physiologic significance is poorly understood.[5] [46] [119] Complement activation has been shown to affect plasma clotting, clot retraction, and clot lysis. Additionally, complement activation can result in platelet activation and increased expression of platelet procoagulant activity. Activated complement also induces the clot-promoting properties of the endothelial surface by mediating release of cell-surface glycosylaminoglycans, [230] by inducing expression of tissue factor,[231] and by causing the cell-surface exposure of phosphatidylserine and other procoagulant phospholipids. [46] Cell-surface exposure of phosphatidylserine and other aminophospholipids by the C5b9 complex may contribute to the pathophysiology associated with lupus anticoagulant antibody and other antiphospholipid syndromes, as these autoantibodies have the potential to activate complement and generally recognize epitopes that are induced when plasma proteins bind to those phospholipids that are known to be exposed when C5b9 deposits on the plasma membrane.[232] [233] In gram-negative bacteremia, episodes of disseminated intravascular coagulation can be temporarily associated with prominent intravascular complement activation, and unregulated complement activation at the platelet surface has been implicated in the recurrent deep venous thrombosis that occurs in patients with PNH.[234] In recent years, there has also been increased awareness of the potential role of the terminal complement proteins in initiating the changes in vascular endothelium that can result in the thrombotic occlusion of blood vessels during episodes of tissue ischemia and reperfusion and hyperacute rejection of transplanted organs. [45] [235] [236]

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FUTURE DIRECTIONS

Research conducted during the past decade has yielded new insights into the structural, functional, and genetic elements that compose the complement system and has enhanced understanding of the molecular and cellular mechanisms by which complement contributes to human health and disease. Despite widespread appreciation of the role of activated complement in a variety of immune and inflammatory disorders, the development of therapeutic agents that can directly intercede to control the complement system remains a challenge for the future. Recent insight into the molecular specificity of the various regulatory elements that normally control production of the biologically active products of the complement reaction cascades, combined with knowledge of the structure and function of the complement receptors that mediate the myriad of cellular responses to activated complement, enables design of inhibitors that are targeted toward individual complement proteins or selected complement receptors. In this regard, recombinant proteins with complement-inhibitory function in vitro have proved effective in ameliorating complement-mediated inflammatory responses in animal models and are now being tested for safety and efficacy in clinical trials. [48] [50] [220] [237] [238] [239] [240] [241] [242] Complement-related disorders for which a single gene defect can be identified (for example, PNH and LAD) may also be future targets for gene therapy. Finally, the production of genetically altered animals that express one or more human complement inhibitors on cells of the vascular tissue may provide a mechanism to suppress hyperacute rejection following xenotransplantation of nonprimate organs into man. [235] [238] [243]

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Chapter 38 - Normal Neutrophil Structure and Function

Robert L. Baehner

The neutrophil is the predominant blood granulocyte participating in phagocytic killing of bacteria and certain fungi as well as in acute inflammatory reactions throughout the body. The cell is referred to as polymorphonuclear or segmented due to its lobulated nucleus. It is energized by anaerobic glycolysis, which provides adenosine triphosphate (ATP) from glycogen stored in the cytosol. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) generated in the pentose shunt provides the substrate for phagocytic oxidase (phox), a unique electron transport chain catalytically reducing oxygen to superoxide anion (O_2), resulting in the production of hydroxyl radical (OH:), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and long-acting chloramines. The fine granularity of the cytosol is neutral, staining with Wright-Giemsa stain, but cine studies spotlight the granules churning within the polymorphonuclear neutrophil (PMN). The granules are membrane-bound organelles made up of discrete subsets of vesicles, each with its own distinct protein profile. The PMN is equipped with >50 distinct granular proteins. These proteins reside either in the granule membrane or in the matrix and can be differentially mobilized when PMNs are stimulated by inflammatory mediators. Depending on the nature of the mediator or secretogogue, one or more of the four granule subsets is translocated to phagolysosomes, or to the cell surface and released from the PMN. PMNs circulate throughout the entire vascular bed without compromising the integrity of the microvasculature; however, at sites of incipient inflammation, the cell must be able to adhere to endothelium, diapedese between endothelial cells, and then migrate from the microvasculature into tissues. Precise signal transduction pathways provide the trigger for PMNs to respond appropriately to a gradient of chemokines generated at the site of infection or inflammation.

PMN are terminally differentiated cells that leave the peripheral blood with a half-life of 6.7 hours, but the process may vary from 4 to 10 hours depending on need. PMN movement slows in response to very high concentrations of chemokines. Finally, PMNs bind the opsonised microbes and ingest and kill them. When microbes are not the stimulation for chemokine generation, PMNs accumulate and remain at the site of tissue injury. Thereafter, PMNs undergo programmed cell death (apoptosis) in tissues over the next 48 hours. Delay of apoptosis may facilitate PMN-mediated organ or tissue damage by extending PMN integrity at sites of inflammation. In contrast, molecules promoting apoptosis may reduce inflammation.

This chapter describes the normal development of the PMN from myeloblast stage, including granulocyte kinetics, morphology, composition, and metabolism. The bimodal functions of PMN as phagocytes and as acute inflammatory cells are considered. Cellular and molecular perturbations occurring during PMN activation, including signal transduction mechanisms required for chemotaxis, ingestion of opsonized microbes, granule mobilization and secretion, the respiratory burst, oxidative and nonoxidative microbicidal killing, and apoptosis of PMN are described in the following sections.

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MORPHOLOGY OF DEVELOPING POLYMORPHONUCLEAR NEUTROPHILS

The morphologic features seen by light microscopy of developing myeloid cells that are used to identify six stages of PMN development are relative size, nuclear volume, chromatin density, prominence of nucleoli, granule number, and cytoplasmic staining. The ultrastructure of granulocytes is of biologic importance but contributes little to identifying the stages of development based on morphologic criteria using the Wright-Giemsa polychrome-stained bone marrow smear. In general, cell maturation is accompanied by reduction in cell size, diminished volume and increased chromatin condensation of the nucleus, loss of nucleoli, appearance of granules, and cytoplasm that is less basophilic and more abundant relative to nuclear size (Fig. 38-1).

Myeloblast Stage

The myeloblast is the youngest myeloid precursor recognizable in the bone marrow. Size is medium (1520 microns in diameter); the nucleus is large and round, with finely granular chromatin and one or two pale-blue nucleoli; granules are absent; and the cytoplasm is scanty. Ultrastructurally, myeloblasts show numerous round or oval mitochondria (<1 micron in diameter), Golgi apparatus with a centriole, vesicles and sacs, many free cytoplasmic ribosomes, and free polyribosomes but no granules.

Promyelocyte Stage

The promyelocyte is larger than the myeloblast and myelocyte (>20 microns). The nucleus and nuclear chromatin and nucleoli

Figure 38-1 A schematic view of PMN development in human bone marrow as seen under high-magnification light microscopy. Myeloblasts are the youngest recognizable myeloid cell in the marrow. They originate from stem cells programmed for neutrophil development called CFU-G. Nuclear chromatin is fine granular (depicted as white) with one or two nucleoli (depicted as black bodies). Cytoplasm is free of granules. Myeloblasts divide into two larger daughter promyelocytes that contain azurophil (primary) granules (depicted as large black granules) identified by myeloperoxidase stain. The nucleus resembles myeloblast nucleus but may be obscured by azurophilic granules as they continue to assemble only at this stage of myeloid development. Promyelocytes give rise to myelocytes, which proliferate several times, during which time specific or secondary granules (depicted as smaller white granules) are produced. Lactoferrin immunohistochemistry is used to identify specific granules. Myelocytes become smaller with each cell division and nucleoli are lost when the cell is no longer able to divide. Metamyelocytes are smaller than myelocytes and are characterized by a bean-shaped nucleus with dark clumped chromatin. Band cells and PMNs represent the final stages of myeloid development. The addition of a third granule subset, identified by gelatinase assay, and the secretory vesicles, identified by alkaline phosphatase stain, complete the granule profile of PMN. See text for more details. (*From Bainton DF et al: The development of neutrophilic PMN leukocytes in human bone marrow. Origin and content of azurophil and specific granules. J Exp Med 134:907, 1971. © The Rockefeller University Press.*)

resemble myeloblast, but the cardinal feature is the presence of many violet granules with either a dense or coarse pattern, often obscuring other cell landmarks. These so-called azurophilic or primary granules, measuring 0.8 microns, are homogeneous, dense, and round to ovoid and are bounded by a unit membrane. Azurophilic granules are made solely by promyelocytes and contain a variety of bactericidal cationic proteins, proteolytic enzymes, and myeloperoxidase. Electron microscopic studies show that the prototypic protein myeloperoxidase is synthesized and packaged into storage granules by the pathway defined for other secretory proteins (i.e., from endoplasmic reticulum to Golgi complex via vesicles to granules).

Myelocyte

The myelocyte is the last myeloid precursor capable of mitosis. Three cell divisions take place in the myelocyte pool, and as expected with each division the cells change in size, shape, and color. Myelocyte progeny are progressively smaller, being reduced in diameter from >20 microns to 14 microns. During this period, specific secondary granules are synthesized, while primary azurophilic granules are shuffled and diluted throughout the myelocyte pool. Secondary granules of neutrophilic origin are barely visible but become more numerous, filling the cytosol with proteins that stain pale pink or lilac. Specific granules are smaller (0.5 microns) and less dense than primary granules, but like primary granules they have a limiting membrane. They are difficult to see on light microscopy. They contain a variety of adhesive proteins, including -2 integrin (CD11/CD18), cytochrome b (a component of NADPH oxidase), a binding protein for cobalamin, lactoferrin, and unique PMN marker antigens. As the myelocyte divides and matures, the nucleus becomes smaller and irregularly round, the nuclear chromatin becomes coarse and clumped, and nucleoli become sparse to absent. A pink puff is evident on the flattened side of the nucleus of smaller, more mature myelocytes. The nuclear to cytoplasmic ratio is reduced.

Metamyelocyte

The metamyelocyte is slightly smaller than the myelocyte. During this stage, the cytoplasm resembles that of the mature PMN and band forms. The background cytoplasm is uniformly pink. Granules are small and fine, blue-black or gray, and are dispersed homogeneously throughout the cytoplasm. The nucleus is indented like a bean, and the nuclear chromatin is coarse, clumped, and condensed peripherally. The metamyelocyte has lost the ability to divide, and further maturation consists predominantly of nuclear elongation and segmentation. The cell lacks nucleoli, polyribosomes, or endoplasmic reticulum necessary for ongoing protein synthesis.

Band Form

This cell is also called the stab or juvenile form because the nucleus is nonsegmented but is elongated into a sausage or horseshoe shape. No nuclear lobulations are evident, although constrictions may be visible. Nuclear chromatin is aggregated into evenly arranged clumps. The cytoplasm is like the mature PMN, with pink staining and fine azure granules. The proportion of azurophilic primary to specific secondary granules based on cytohistochemistry is about one to two. [1] Band forms are considered fully functional phagocytes and make up 35% of the differential count. They should be included when calculating the absolute neutrophil count (ANC).

Polymorphonuclear Neutrophil

The polymorphonuclear neutrophil or segmented neutrophil is the end stage of maturation. The mature neutrophil is of uniform size (13 microns in diameter), with pink cytoplasm and fine azure granules. The predominant specific secondary granules are so finely dispersed that the cytoplasm may stain only faintly pink. Granular detail normally is not seen at the level of definition by light microscopy. The nucleus is segmented into two to five (mean of three) lobes connected by thin chromatin strands. Five to ten percent of PMNs contain nuclear irregularities such as nicks, appendices, fibrillar bodies, and bridges. Three percent of PMNs from normal females display a drumstick oval mass of dense chromatin 1.5 microns in size attached to one of the lobes by a slender filament. [2] The nuclear chromatin is coarse

and clumped and stains deep purple with Wright-Giemsa. Ultrastructurally, PMN granules show considerable heterogeneity in size and density. A submembranous meshwork of microfilaments poses a barrier for them. Peroxidase staining reveals cytoplasm filled with smaller peroxidase-negative granules (secondary specific granules) and larger, dense peroxidase-positive granules (primary granules). The Golgi region is small compared to the promyelocyte and myelocyte, and the rough endoplasmic reticulum and mitochondria are scant.

Figure 38-2 Transmission electron photomicrograph of a peripheral blood PMN. The nucleus is multilobed. The peripheral distribution of heterochromatin is interrupted by channels of euchromatin where it is in continuity with nuclear pores (arrows). Note the heterogeneity of the granules and the fact that the primary granules (more electron dense) represent a minority population compared to the specific and gelatinase granules (less electron dense). There are few mitochondria, ribosomes, and no rough endoplasmic reticulum. The Golgi zone (labeled Go) is very small. The black dots in the cytoplasm represent glycogen particles. The leading edge or pseudopod is always devoid of granules and rich in actin and actin-associated proteins. (× 17,500) (*From Zucker-Franklin D, Greaves MF, Grossi CE, Marmont AM [eds]: Atlas of Blood Cells, Function and Pathology, 2nd ed. Lea and Febiger, New York 1988, with permission.*)

PMNs contain an abundance of glycogen since the cell depends on anaerobic glycolysis for the generation of ATP (Fig. 38-2).

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CONTENT OF POLYMORPHONUCLEAR NEUTROPHIL GRANULES

Polymorphonuclear neutrophils contain four subsets of granules or vesicles identified by marker enzymes or other proteins present in the granule matrix or membranes of granules and secretory vesicles (Table 38-1).

Cytochemical stains of blood and bone marrow smears identify myeloperoxidase-positive azurophil granules in promyelocytes and in all further differentiated stages of PMNs. The matrix of azurophil granules contains microbicidal proteins and acid hydrolases involved in oxidative and nonoxidative killing of bacteria and fungi (see sections on PMN respiratory burst, nitric oxide pathway and nonoxidative microbicidal killing). Azurophil granule membranes contain receptors and proteins for signal transduction (see section on signal transduction). Lactoferrin is localized to specific granules and serves as a convenient marker of that granule subset. [3] Specific granule matrix contains hydrolases distinct from azurophil granules and vitamin B_{12} binding protein, among others. Specific granule membranes contain chemotactic, opsonic, and adhesion protein receptors and the cytochrome b component of the phagocytic NADPH oxidase. Alkaline phosphatase initially was thought to mark secondary specific granules since myelocytes and all further stages of differentiated PMNs stain positive. [4] [5] It is now known that alkaline phosphatase is contained in secretory vesicles scattered throughout the cytoplasm and plasma membrane of myelocytes, metamyelocytes, band forms, and PMNs.[6] Secretory membranes also are a source for cytochrome b and receptors for serum opsonins. Plasminogen activator receptor is also in the vesicular membranes of PMNs. In addition to secretory vesicles, azurophil granules, and specific granules, another subset of granules more recently has been identified by subcellular fractionation techniques combined with double-labeling immunoelectron microscopy. These granules are rich in gelatinase but low in lactoferrin and negative for peroxidase. [7] Gelatinase granules more readily secrete their contents in response to secretogogues compared to specific or azurophil granules. [8] Table 38-1 lists the contents of PMN granules and secretory vesicles based on their function and subcellular localization.

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GRANULOCYTE KINETICS

Polymorphonuclear neutrophils are distributed within the bone marrow, the circulating blood, and in various tissues throughout the body, depending on specific need. The largest compartment of PMNs is within the bone marrow and contains $2.26^{9} \times 10$ cells per kg body weight.[9] The blood compartment is approximately one third the size of the bone marrow compartment (0.7×10^{9} cells/kg), and only half are in the bloodstream, while the other half are tumbling on or loosely adhering to the endothelium of the microvasculature.[10] These two compartments are referred to as the circulating granulocyte pool (CGP) and the marginating granulocyte pool (MGP), respectively, and the two pools are in a constant state of dynamic equilibrium.[11] [12] The size of the extravascular tissue compartment has not been quantified, but it varies depending on the attraction for PMNs at sites of infection or inflammation (Fig. 38-3 (Figure Not Available)).[13]

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BONE MARROW COMPARTMENTS

The bone marrow compartments of developing myeloid precursors are the stem cell compartment; the mitotic compartment, containing replicating myeloblasts, promyelocytes, and myelocytes; and the maturation-storage compartment, containing nonreplicating metamyelocytes, band forms, and PMNs. The size of the stem cell compartment has not been quantified, but it is very small compared to the mitotic compartment (2.11×10^9 cells/kg) and to the almost threefold larger maturation-storage compartment (5.59×10^9 cells/kg).[14] The transit times of developing myeloid cells are derived from ^{3}H-Tdr (tritiated thymidine) studies.[14] [15] These studies provide data on the hours and days that myeloblasts and all subsequent myeloid precursors spend during the process of replication and maturation to PMNs. Under normal conditions, the entire process requires approximately 10 days. The myeloblast stage is 15 hours, the promyelocyte stage 24 hours, and the myelocyte stage 104 hours, during which the myelocyte replicates up to three times. Thus, the replicating phase of myeloid development is about 6 days. Maturation of metamyelocyte to band form and PMN requires an additional 4.5 days. The PMN may spend another 3 or 4 days in the storage compartment before emerging into the circulation.[16] [17] A model of the production and kinetics of PMNs proposed by Athens is provided in Figure 38-3 (Figure Not Available) .

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BLOOD COMPARTMENT

The mechanisms controlling the release of PMNs from the bone marrow are only partially understood. Endotoxin affects the relationship between marrow sinus endothelial cells and the stromal macrophages covering nonluminal sinusoidal surfaces. Endotoxin induces the retraction of macrophages away from the endothelium and facilitates contact between PMNs and endothelial cells. [18] [19] Endotoxin, androgens, glucocorticoids, and a leukocyte mobilizing factor derived from the third component of complement (C3e) induce the release of bone marrow PMNs into the circulation. [20] [21] [22] [23] The cell must be able to deform and migrate to the luminal side of the endothelial sinusoidal surface. In vitro, PMNs and bands readily pass through millipore filters

TABLE 38-1 -- Content of Human PMN Granule Subsets

Granule	Azurophil	Specific	Gelatinase	Secretory Vesicles
Marker enzyme	Myeloperoxidase	Lactoferrin	Gelantinase	Alkaline phosphatase
Membrane	CD63, granulophysin	CD15,CD66,CD67		CD10,CD13,CD45,
	CD68	CD11b/CD18	CD11b/CD18	CD35 (CR1)
				CD11b/CD18
Oxidase receptor		Cytochrome b	Cytochrome b	Cytochrome b
		Rap 1A	Rap 1A	Rap 1A
Other receptors (R)		FMLP R	FMLP R	FMLP R
		C3bi R	C3bi R	CR 1R
		Fibronectin R		C3bi R(CR3R)
		Laminin R		CR4R
		Vitronectin R		C1q R
				FcIII R
				Plasminogen activator R
Signal transduction	Thrombospondin R			
	Gi2 protein subunit			
Others	NB antigen	Diacylglycerol deacylating enzyme	Decay accelerating factor	
	19 kDa and 155 kDa proteins			
Matrix				
Microbicidal	Myeloperoxidase	Lactoferrin		
	Nitric oxide synthase (NOS)	Lysozyme	Lysozyme	
	BPI protein			
	Defensins			
	Serprocidins			
	Elastase			
	Cathepsin G			
	Proteinase 3			
	Azurocidin (CAP 37)			
Hydrolases	Acid glycerophosphatase	Gelatinase	Gelatinase	
		Collagenase	Acetyltransferase	
	-Mannosidase	Histaminase		
	-Glucuronidase			
	-Glycerophosphatase	Heparanase		
	N-acetyl--Glucosaminidase	NGAL		
		Sialidase		
	Sialidase			
Other	Acid mucopolysaccharide	-2 microglobulin	-2 microglobulin	Plasma proteins
	Heparin binding protein	Plasminogen activator		
		Vitamin B_{12} binding protein		

Adapted from Borregaard N, Kledsen L, Lollike K, Sengelov H: Granules and secretory vessels of the human neutrophil. Clin Exp Immunol 101(suppl 1):6, 1995, with permission from Blackwell Science, Ltd.

with pore sizes of 1 micron in response to chemoattractants. Metamyelocytes and late myelocytes are impaired in this response, whereas early myelocytes, promyelocytes, and myeloblasts cannot move or deform.[24] [25] In rabbits, infusion of C5a and tumor necrosis factor- induce leukocytosis, which still occurs when the principal adherence proteins of PMN, -2 integrin, and L-selectin are blocked by specific antibodies. [26]

In most normal persons, the half-life of blood PMNs, as determined by DFP[32] (di-isopropyl fluorophosphate), is about 7 hours. [12] [27] [28] The disappearance of DFP[32] -labeled PMNs from the blood follows a single exponential curve, implying that PMNs are destroyed or leave the blood randomly rather than according to their age (senescence), as is true for erythrocytes. PMNs do not return to the circulation after they leave it. The granulocyte turnover rate is calculated from the total blood pool of PMNs (27128 PMNs × 10^7 /kg) and the half-life of PMNs (410 hours), and is 62400 PMNs × 10^7 /kg/day. [16]

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EXTRAVASCULAR TISSUE COMPARTMENT

Under normal conditions, PMN losses occur on mucosal surfaces throughout the body. For example, isotopically labeled blood PMNs appear in saliva and urine and increase markedly with gingivitis and pyelonephritis, respectively. [29] [30] The size of the extravascular tissue compartment can only be estimated, but current investigations focus on how PMNs die or delay dying on mucosal surfaces or at sites of infection or inflammation. Although PMNs exit the circulation randomly, they age and die at inflammatory sites over the next 72 hours by undergoing selective intracellular programmed cell death or apoptosis.

Figure 38-3 (Figure Not Available) Model of the production and kinetics of human PMNs. The marrow and blood compartments are drawn to show their relative sizes. In the lower third of the figure, the compartment transit times as derived from DFP [32] studies[12] and tritiated thymidine (H^3 -Tdr) studies[14] [15] are compared. (*From Lee GR, Bithell TC, Foester J et al [eds]: Wintrobes Clinical Hematology, 9th ed. Lea and Febiger, New York 1993, with permission.*)

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PMN APOPTOSIS

This process results in characteristic morphologic changes in PMN volume and nuclear chromatin condensation, with the formation of pyknotic nuclei. Endogenous endonuclease activation results in a time-dependent increase in low-molecular-weight DNA cleavage products identified by DNA electrophoresis. The apoptotic PMN remains intact morphologically but loses cytoskeletal functions of attachment, spreading, shape change, and random migration. L-selectin expression decreases and Fc RIIIb is shed, but expression of CD11/CD18 increases.[31] Apoptotic PMNs are no longer capable of phagocytosis, degranulation, or respiratory burst activity. The cell has been effectively isolated from its further role in phagocytic killing of microbes or as a participant in the inflammatory response. The apoptotic PMN likely represents a modulating event in the control of inflammation, marking the PMN for disposal and harnessing its ability to generate and release noxious cytotoxic products that could induce further damage to inflamed tissues.[32] [33] The apoptotic PMN becomes the phagocytic target of tissue macrophages, is recognized via adhesive proteins such as thrombospondin, and is ingested intact by the macrophage.[34] The precise molecular genetic site(s) for initiation of apoptosis in PMNs is not known at present, but sodium butyrate, which directly affects chromatin structure, delays PMN apoptosis.[35] Bcl-2, a proto-oncogene rearranged and deregulated in B-cell follicular lymphoma, resulting in delayed apoptosis, is expressed in early myeloid differentiation but not in PMNs. PMNs from transgenic mice expressing Bcl-2 in PMN in vitro show delayed apoptosis but are still recognized and phagocytized by macrophages.[36] One candidate for a molecular trigger of apoptosis in PMNs is the *Fas* gene. *Fas* protein is expressed on PMN plasma membrane, and *Fas* ligand induces apoptosis in PMNs.[37] *Fas*-mediated PMN apoptosis is suppressed in vitro by biologic molecules and drugs known to delay apoptosis in PMNs. Also, PMNs constitutively release *Fas* ligand, thus providing a paracrine pathway for the PMN to mediate its own programmed cell death.[37] Molecules known to delay the onset of PMN apoptosis include granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, IL-6, and IL-15. Glucocorticoids and G-CSF exert a protective effect on PMN survival in vitro at concentrations that occur at pharmacologic doses of dexamethasone and G-CSF achievable in vivo.[38] [39] Mediators of inflammation such as endotoxic lipopolysaccharide (LPS), human recombinant C5a, TNF- and interferon (IFN)- also inhibit PMN apoptosis in a concentration-dependent fashion in vitro.[40] [41] [42] Lyn kinase, an src family tyrosine kinase, couples to the GM-CSF receptor and is required for the delayed apoptosis induced by GM-CSF. PMNs from respiratory burst-deficient persons and normal PMNs display similar degrees of delayed apoptosis under anaerobic conditions. However, the phagocytic event itself, as well as proteolytic granule enzymes, including elastase, accelerate PMN apoptosis, supporting the notion that PMN activation is a suicidal process.

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CIRCULATING POLYMORPHONUCLEAR NEUTROPHIL INTERACTIONS WITH ENDOTHELIAL CELLS

Polymorphonuclear neutrophils are subjected to strong shear forces as blood flows through the arterial circulatory system at velocities of 3001000 micron/sec. [27] When viewing the microcirculation with the cine microscope, the vast majority of cells, including red cells, appear as a indistinguishable blur. But within this rapid flow, a subset of PMNs is noted to tumble along the periphery of the blood vessel at a velocity about two orders of magnitude slower than the main flow. Loose PMN interaction with endothelium results in tumbling or rolling and reflects early inflammatory responses of PMN and endothelial cells, preceding firm adhesion of PMNs. [43] Adhesion molecules play a major role in mediating the recruitment of circulating PMNs to sites of inflammation. PMN recruitment involves a dynamic series of events between PMNs and endothelial cells orchestrated by several different families of adhesion molecules and their ligands. PMNs and endothelial cells are transformed from a basal state to an activated state by a variety of inflammatory mediators. The process can be divided into four phases:

1. The inactivated or quiescent state of PMNs and endothelium before release of inflammatory mediators

2. The activated state of endothelium, which mediates PMN rolling
3. The activated or priming state, reflected by alterations in the expression of adhesive molecules, rendering PMNs sticky to endothelium
4. The transmigration phase, when PMNs tread between adjacent endothelial cells supported by adhesive molecules expressed on the lateral surfaces of endothelium (Fig. 38-4).

Figure 38-4 Circulating PMN interactions with vascular endothelial cells during recruitment of PMNs to sites of infection or inflammation. At left, PMNs and endothelial cells are represented in their basal states prior to exposure of either cell type to inflammatory mediators. Constitutive ICAM-2 is expressed on endothelial cells and does not affect free-flowing PMNs, which constitutively express L-selectin and its ligand SLeX. The adjacent right section illustrates PMNs rolling onto endothelial cells that express E-selectin after activation by IL-1, TNF-, or endotoxin. E-selectin induces loose attachment of PMNs to endothelial cells mediated by the L-selectin ligand SLeX. Endothelial cells release IL-8, GM-CSF, and PAF, while other chemoattractants may also be sensed by rolling PMNs. One or more of these molecules induce PMNs to express -2 integrin Mac1 (and LFA-1, not shown), which mediates firm adherence of PMNs to ICAM-1 expressed by activated endothelial cells. At right, PMN is illustrated crawling along endothelial cells and squeezing between them, aided by endothelial cell PECAM-1, which binds PMN Mac1. The subendothelial basement membrane is eroded, presumably by secretion of PMN gelatinase B and elastase. See text for details.

Adhesive molecules involved in PMN-endothelial interactions are selectins and their ligands, including sialylated Lewis X blood group glycoprotein, intercellular adhesion molecules (ICAMs), and the CD18 (-2) family of leukocyte integrins.

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SELECTINS AND THEIR LIGANDS

Selectins are found on all leukocytes (L-selectin), on postcapillary venule endothelial surfaces (E-selectin), and in platelet granules and endothelial cell Weibel-Palade bodies (P-selectin). PMNs constitutively express L-selectin, also called Leu-8/TQ-1, DREG, LAM-1, LECAM-1, or gp 90 MEL, since it is expressed also on monocytes, eosinophils, and lymphocytes. E-selectin, also called ELAM-1 or LECAM-2, is not expressed constitutively on endothelial cells, but is transcriptionally induced by inflammatory mediators IL-1, TNF-, and LPS within several hours after exposure. As a result of activated platelet and endothelial release in response to thrombin, substance P or histamine, P-selectin is expressed during acute inflammation or hypercoagulable states resulting in thrombosis. P-selectin, also called LECAM-3, GMP-140, PADGEM, or CD62, mediates PMN, eosinophil, and monocyte adhesion during inflammation associated with allergy or thrombosis. [44] All three members of the selectin family share a structural motif: an N-terminal C-type lectin domain, an epidermal growth factor (EGF)-homologous domain, a variable number of short consensus repeats (SCARS) as found in complement regulatory proteins, and a transmembrane and a C-terminal cytoplasmic domain. They play a major role in PMN adherence to endothelial cells mediated by binding either to ligands on the PMN (E and P) or endothelial cell (L) at their N-terminal site, including lectin and EGF domains. The ligands of selectins share a similar biochemical structure. They contain carbohydrate groups, which are typically found as terminal structures of glycoproteins and glycolipids. One major selectin ligand is a sialylated and fucosylated tetrasaccharide related to the sialylated Lewis X blood group (SleX, CD15). [45] SleX is heavily expressed on inactivated or quiescent PMNs and monocytes. Both the sialic acid and the fucose linkages are critical for binding. [45] [46] Leukosialin (CD43), the predominant sialoprotein expressed by circulating human PMN, has been implicated as an anti-adhesive molecule shed from the plasma membrane after PMN activation.[47]

Intercellular Adhesion Molecules

Three ICAMS share similar structures to immunoglobulin (Ig) supergene family and other Ig-like adhesion molecules such as VCAM-1, and serve as ligands for the -2 integrins. ICAM-1 is a ligand for CD11b/CD18 (Mac1) and LFA-1 present on PMNs and other leukocytes. [48] LFA-1 binds to ICAM-1 domains 1 and 2, while Mac1 binds ICAM-1 domain 3.[49] The distribution and regulation of the three ICAMS are distinct. ICAM-1 is expressed at very low levels on endothelial cells, but expression is markedly increased by inflammatory cytokines such as IL-1, TNF-, LPS, and IFN-. [50] This increased ICAM-1 expression requires 46 hours for mRNA transcription and new protein synthesis. The increased expression of endothelial ICAM-1 lasts several days. [51] ICAM-2 is constitutively expressed on endothelial cells but does not respond to inflammatory cytokines.[52] [53] ICAM-3 is the only ICAM molecule expressed by PMNs[54] and is otherwise restricted to monocytes and lymphocytes. ICAM-1 is chiefly responsible for maintaining firm adhesion between PMN CD11b/CD18 (Mac1) and vascular endothelium. Another member of the endothelial Ig superfamily, PECAM-1 (CD31), serves an important role in mediating transmigration of PMNs from the vascular space into mucosal or other body tissue. CD31 is constitutively expressed and localized to the lateral junctions of endothelial cells, whereas ICAM-1 is expressed over the entire surface area of endothelium. [55] Anti-PECAM-1 monoclonal antibody (Mab) blocks PMN migration across cytokine-activated endothelial monolayers. [55] [56]

Beta-2 Integrins (CD18)

The CD18 -2 integrins were among the first adhesion molecules described on PMNs and other leukocytes. [42] This family of adhesive proteins comprises three membrane glycoproteins with a common subunit, designated -2 (CD18), and three unique subunits, designated CD11a, CD11b, and CD11c. They form three structurally related / heterodimersCD11a/CD18 (LFA-1), CD11b/CD18 (Mac1), and CD11c/CD18. [42] [57] Both the and subunits have a relatively small cytoplasmic domain, which includes regions capable of binding cytoskeletal structures. [58] The extracellular domain is much larger, and the amino terminal portion of the subunit contains the ligand binding region. Both subunits are required for expression of the intact dimer. [59] Mac1 serves as a receptor for the C3bi fragment of complement.[60] [61] The important role played by CD18 integrins for PMN adhesion to vascular endothelium became apparent when the molecular basis of leukocyte adhesion deficiency (LAD) type 1 disease was found to be due to a genetic deficiency in the expression of all three CD18 integrins. [62] [63] LAD type 1 PMNs bind poorly to IL-1-stimulated endothelial cells and cannot

undergo transendothelial migration. [64] [65] Normal PMNs treated with anti-CD18 Mabs show similar effects to LAD type 1 PMNs.[66] PMN -2 integrins are up-regulated by a variety of chemoattractants, including F-Met-Leu-Phe (FMLP), C5a, LTB4, IL-8, and platelet activating factor (PAF). [63] These chemotactic triggers result in a rapid inverse expression of L-selection (decrease) and Mac1 (increase) on PMNs, or a transition from L-selectin-dependent adhesion to Mac1-dependent adhesion. L-selectin is required for PMNs to localize loosely to activated endothelium adjacent to the inflamed tissue, while -2 integrins mediate the movement of PMNs across the vascular endothelium and into tissue. [67] [68] [69] [70] [71]

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MOLECULAR INTERACTIONS BETWEEN ENDOTHELIUM AND POLYMORPHONUCLEAR NEUTROPHILS

Polymorphonuclear neutrophils freely flow over endothelial surfaces when neither cell is activated. Inflammatory cytokines IL-1, TNF-, or IFN- activate endothelial cells to express E-selectin and to release IL-8, GM-CSF, and PAF. [72] PMNs roll onto activated endothelium mediated by loose binding of SLeX and L-selectin on PMN surfaces to E-selectin expressed on the surface of activated endothelium. Endothelial inflammatory cytokines or extravascularly derived chemoattractants at minimal concentrations of 1×10^{9} M bind to selective PMN chemotactic receptors, which in turn induce the expression of CD18 -2 integrins Mac1 and LFA-1 and reduce the expression of L-selectin and SLeX. At this stage PMNs are firmly attached by their -2 integrins to activated endothelial cell ICAM-1 ligand. In addition to becoming more adhesive to endothelium, PMNs exposed to chemotactic factors also adhere to each other. Aggregates of PMN slow blood flow and permit even more PMNs to be recruited. PMN aggregation is a Mac1-dependent event. In contrast to inflammatory cytokines, the attachment of PMN to endothelium stimulated by thrombin involves expression of PAF on the endothelial surface. [74] PMNs have receptors for TNF-, but they are deactivated and down-regulated by PAF, endotoxin, FMLP, LTB4, and C5a and play no direct role in PMN adhesion. [75] Supported by the -2 integrins, Mac1, and LFA-1-ICAM attachment, PMNs move laterally on endothelium and squeeze between endothelial cells by adhering to PECAM-1. PECAM-1 is constitutively concentrated at endothelial cell margins and is expressed on PMNs. PMNs isolated from CD18-deficient patients adhere to IL-1-stimulated endothelium but fail to transmigrate, thus underscoring the essential role of Mac1 and LFA-1 in PMN transendothelial migration. [76] [77] [78] PMN migration across subendothelial basement membranes is thought to be dependent on the degradation of membrane constituents by PMN granule proteinases. [79] Inhibition of gelatinase B, a metalloproteinase capable of degrading type IV collagen, inhibits PMN trans-basement membrane migration without affecting chemotaxis or degranulation. PMN elastase activates a precursor form of gelatinase B released into supernatant fluid by PMNs in vitro, and inhibition of PMN elastase also blocks trans-basement membrane migration, presumably by inhibition of gelatinase B activation. [80] Transepithelial migration of PMNs is a defining characteristic of mucosal surface inflammation and requires the continued expression of CD11b/CD18 as well as the integral membrane glycoprotein CD47 on PMN and epithelial surfaces. This 60 kDa glycoprotein has homology to the immunoglobulin superfamily and is up-regulated on epithelial surfaces by IFN-. [81] Sustained activation of PMN requires continued engagement of chemoreceptors with specific chemoattractants. Also, the type of subendothelial collagen supporting PMN movement affects activation. Type I collagen activates PMN, whereas the -3 region of type IV collagen down-regulates PMN activation. [82] Some of these events are illustrated in Figure 38-4 .

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MOVEMENT OF POLYMORPHONUCLEAR NEUTROPHILS

Polymorphonuclear neutrophils change their shape and crawl in response to chemoattractants by alternating the extrusion and retraction of a broad frontal lamellipodia that determines the direction of locomotion. They move directly up the gradient of chemoattractant by a tractor-like motion as the lamellapodia fills with soluble proteins and then, contracts as the membrane adheres briefly to the substrate, providing the frictional force for movement. The PMN cell body extends and elongates in the axis defined by the lamellar protrusion, creating a tapered cell with a knobby tail. [83] Orientation accuracy is sensitively regulated by specific chemotactic receptor occupancy along the cell membrane. As little as a 2% change in chemoattractant concentration is detected by the PMN. [84] [85] PMNs move at speeds up to 30 micrometers per minute, qualifying them as the fastest cell in the body. [86] Their cell velocity oscillates between 10 and 30 micrometers per minute, with a periodicity of 5055 seconds. [86]

The molecular underpinnings of PMN crawling have been detailed in a model by Stossel. [86] PMN activation induces contraction of the actin filamentous network in the cortical gel, presumably by inducing the phosphorylation of myosin through a transient increase in cytosolic calcium. Mechanical stress studies suggest that the PMN cortex is a weakly cross-linked structure. [87] The hydrostatic force imposed by network contraction forces the membrane bilayer outwards at a region where it becomes detached from the underlying protein network. Simultaneously at the site of membrane expansion, actin filaments assemble from monomeric subunits into relatively short (1 micrometer) linear filaments. These filaments are cross-linked by actin binding protein, fimbrin, villin, fascin, and actinin into a three-dimensional uniform network. Loss of actin binding protein expression results in an immobile cell that responds feebly to chemoattractants by forming temporary blowouts or blebs over the entire cell surface. This dynamic, cohesive network of actin filaments modulates the flow of sol, provides strength to the forming protrusion, and serves as the anchor for adhesion molecules, welding the protrusion to the underlying substrate. In addition, the cross-linking of initially freely diffusing actin filaments into an immobilized state imparts contractility on the solvent included within the gel, and the squeezing action on the solvent can provide additional force to protrude membrane forward at the leading edge, driving the cell forward (Fig. 38-5).

Thereafter, actin filaments must be disassembled rapidly so that the cycle can be repeated. Gelsolin severs actin filaments rapidly and is activated at micromolar calcium concentrations. Gelsolin blocks actin reassembly by capping actin barbed ends. The barbed end of actin filaments serves as the nidus onto which actin subunits are added. Another highly conserved capping protein, CapZ, [88] may also cap barbed ends, thereby preventing actin filament elongation. Another filamentous actin binding protein, leufactin, was found to be overexpressed in the PMNs of a child with PMN actin dysfunction, resulting in immobile cells and serious infections. [89] The next cycle of assembly and disassembly cannot be initiated until these actin filaments are uncapped. Dissociation and uncapping of the gelsolin-actin and profilin-actin complexes is mediated by polyphosphoinositides generated during signal transduction involved in PMN activation. [90] [91] This information provides a unifying picture linking PMN membrane activation to actin assembly and disassembly. [86] During cell stimulation, signal transduction cascades lead to membrane breakdown or synthesis of polyphosphoinositides, the former generating phosphoinositol and release of intracellular calcium and protons promoting actin disassembly, and the latter providing clusters of polyphosphoinositides promoting actin assembly. The actin cytoskeleton and specific actin binding and bundling proteins seem essential

Figure 38-5 Actin-based mechanism for PMN crawling depicted in three phases: vectorial actin assembly in the left panel, leading to a wave of contraction, gelation in the center panel, and early assembly of actin subunits in the right panel. Vectorial direction is indicated by the arrow. See text for details. (*From Stossel TP: The machinery of blood cell movements. Blood 84:367, 1994, with permission.*)

in selective receptor-mediated responses. [92] [93] [94] The myristoylated MARCKS is a major specific substrate of protein kinase C that is phosphorylated during receptor-mediated activation. MARCKS is thought to regulate the cross-bridge between actin and the plasma membrane. [95]

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CHEMOTACTIC RECEPTORS

Polymorphonuclear neutrophils sense chemoattractant signals by either up-regulating specific receptors or using specific receptors constitutively expressed on their cell surface. The first receptors demonstrated on PMNs were the N-formyl-methionyl tripeptide receptors, the most active being f-Met-Leu-Phe. [96] These synthetic peptide products are similar to naturally occurring bacterial-derived factors. [97] Similar for all specific receptors, each demonstrates time-dependent saturable binding kinetics and a high-affinity dissociation constant (Kd) for the specific chemoattractant. [98] Studies using intact human PMNs reveal approximately 50,000 f-Met-Leu-Phe receptors per cell with a Kd of 20 nM.[96] [99] The molecular cloning of the f-Met-Leu-Phe receptor from PMNs occurred in 1980. [100] [101] Thrombospondin is an extracellular matrix protein that has chemotactic activity and binds to PMNs via specific receptors linked to Gi2 proteins, a subpopulation of which is also associated with FMLP receptors.[102] The thrombospondin receptor and the Gi2 protein subunit are stored in azurophil granules, and FMLP receptors are stored in specific granules and vesicles of PMNs.

Polymorphonuclear neutrophils display specific receptors for the chemotactic cleavage product from the fifth component of complement, C5a. [103] Binding of radiolabeled C5a indicates 50,000100,000 receptors per PMN with a Kd of 2 nM. The C5a receptor has been cloned. Oocytes injected with C5a mRNA transcripts [104] [105] obtained from differentiated HL 60 cells result in oocytes with fully responsive transmembrane currents and calcium fluxes inhibitable by pertussis toxin, indicating close coupling to G protein.[106]

Tissue-derived cytokines with potent chemotactic activity for PMNs are called chemokines. Chemokines are small proteins of 7080 amino acids with four conserved cysteines forming two essential disulfide bonds: a short -terminal and a relatively long carboxyl-terminal domain. Chemokines specific for PMNs are distinguished by having their first two cysteines always separated by one amino acid (CXC chemokines). Their genes are clustered on chromosome 4. In contrast, CC chemokines have the first two cysteines adjacent and are inactive on PMNs but stimulate monocytes, basophils, eosinophils, and T lymphocytes. CXC chemokines are produced by many different cell types, including endothelial cells, platelets, PMNs, T lymphocytes, and monocytes. They include (IL-8) (previously known as neutrophil activating peptide 1 [NAP-1]), neutrophil activating peptide 2 (NAP-2), and GRO-. [107] [108] [109] [110] [111] [112]

Polymorphonuclear neutrophils express high numbers of IL-8 receptors of two types, one highly specific for IL-8 (IL-8R1) and the other with high affinity for all CXC chemokines (IL-8R2).[113] [114] There are approximately 20,000 IL-8-specific receptors per PMN, with a Kd of 0.8 nM. [115] GRO-- and NAP-2-specific receptors number 60,00090,000 per PMN, of which 3045% are high affinity, with a mean Kd of 0.3 nM for GRO- and 0.7 nM for NAP-2. The low-affinity receptors of GRO- and NAP-2 display a Kd of 30 nM.[116] cDNA cloning of the two receptors for IL-8 indicates that their transmembrane architecture is similar to all other PMN chemotactic receptors (i.e., seven hydrophobic transmembrane domains).[117] [118] The seven transmembrane domain receptor family includes receptors involved in signal transduction for vision, olfaction, hormone action, cell proliferation, and neurotransmission. In PMNs, the receptors consist of seven hydrophobic transmembrane helices of 2025 amino acids separated by three hydrophilic loops exposed at the cystosolic and the extracellular face of the plasma membrane. A conserved disulfide bridge stabilizes the first and second extracellular loops (Fig. 38-6). The transmembrane domains have several conserved amino acids, which form the agonist-binding pocket. The conserved amino acids in the intracellular domains confer a common conformation to all receptors, ensuring their interaction with Gi2 proteins. The diversity of amino acid structure of the extracellular and transmembrane domains confers ligand specificity. [120] All CXC chemokines require the amino terminal sequence Glu4-Leu5-Arg6 (ELR) for receptor recognition and signaling. [121] A two-site model for receptor activation and for selectivity between CXC and CC chemokines has been proposed, with the initial receptor contact provided by the main body of the chemokine and the activation provided by the amino terminal regions. [122] However, the C5a receptor activation site is located in the C-terminal region, whereas the N-terminal region is involved in receptor binding. [123] [124]

Figure 38-6 Schematic arrangement of the chemotactic seven transmembrane domain receptor employed by all PMN chemotaxins and chemokines. The receptor is stabilized by a conserved disulfide bridge (S-S) linking the first and second extracellular loops. See text for details. (*From Thelen M, Dewald B, Baggiolini M: Neutrophil signal transduction and activation of the respiratory burst. Physiol Rev 73:797, 1993, with permission from the American Physiological Society.*)

TABLE 38-2 -- Properties of PMN Chemotactic Receptors: Seven Transmembrane Domain Family

Specific Receptor	Type	Receptor/PMN	High-affinity kDa (nM)	Low-affinity kDa (nM)
FMLP	Bacterial product	50,000	20	
C5a	Complement fragment	50,000100,000	2	
IL 8R1	CXC chemokine peptide	20,000	0.8	
GRO	CXC chemokine peptide	60,00090,000	0.3 (3045%)	30 (5570%)
NAP-2	CXC chemokine peptide	60,00090,000	0.7 (3045%)	30 (5570%)
PAF	CXC chemokine phospholipid	1,000	0.2	
		270,000		500
LTB4	CXC chemokine phospholipid	4,500	0.4	
		270,000		75

Polymorphonuclear neutrophils display a second group of CXC receptors to secreted products of phospholipid oxidation and oxygenation released from endothelial cells, macrophages, and PMNs, as well as from bacterial toxins and oxidants. PAF is 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine, formed in endothelial cells in response to stimulation of specific receptors for thrombin, bradykinin, histamine, ATP, and angiotensin II. [125] Binding of radiolabeled PAF reveals 1,000 high-affinity and 270,000 low-affinity sites per PMN with Kd of 0.2 nM and 500 nM, respectively. PAF receptor is a CXC receptor with seven membrane spanning domains and belongs to the family of Gi2 protein-coupled receptors. [126] [127] [128] [129] The receptor is activated by short sn-2 residues present in PAF and other diacylphosphatidycholines containing sn-2 polyunsaturated fatty acyl residues susceptible to oxidative fragmentation. [130] Among the chemoattractants, PAF is unique in inducing lipid-rich cytoplasmic inclusions in the PMN, which primes the PMN for enhanced leukotriene B4 (LTB4) release. [131] (See section on PMN priming.)

Leukotriene B4 is formed from arachidonic acid in the lipoxygenase-catalyzed reaction by oxygenation at C-5, followed by further transformation to leukotriene A4.

This unstable epoxide intermediate is converted to LTB4 by hydrolysis. The newly defined eicosatetraenoates (ETEs), 5-oxoETE and 5-oxo-15(oh)-ETE, share structural motifs, synthetic origins, and bioactions with LTB4. [132] [133] Binding of radiolabeled LTB4 reveals 4,500 high-affinity and 270,000 low-affinity sites per PMN with Kd of 0.4 nM and 75 nM, respectively. [134] The LTB4 receptor in PMNs also appears to be a Gi2 protein-coupled receptor with interconvertible high- and low-affinity states modulated by GTP and has a molecular mass of 5361 kDa. [135] [136] [137] Table 38-2 lists the properties of PMN chemotactic receptors.

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SIGNAL TRANSDUCTION

All inflammatory mediators stimulate PMN in a dose-dependent manner by binding to specific receptors of the Gi2 family proteins. These proteins share the ability to bind guanine nucleotides and possess inherent GTPase activity. Gi2 proteins are arranged in a heterotrimeric structure (, , and subunits). The subunit confers specificity for each receptor, binds guanine nucleotide, and serves as the potential substrate for pertussis toxin-catalyzed ADP ribosylation. Each chemoattractant or chemokine binds to its own distinct set of the seven transmembrane domain receptors on the PMN surface. The receptors use this novel pertussis toxin-sensitive Gi2 protein to transduce a cascade of metabolic reactions, which in turn initiate a diverse set of cellular reactions, including PMN shape changes, increased adherence, chemokinesis and chemotaxis, phagocytic ingestion, initiation of the respiratory burst, and granule secretion. Different concentrations of pertussis toxin are required for inhibition of selected metabolic and cellular responses. For example, inhibition of actin polymerization and the rise in intracellular free calcium (Ca2i) requires higher concentrations of pertussis toxin compared with the dose required to inhibit the respiratory burst. [138] Specific binding of any PMN CXC receptor by the appropriate ligand activates a phosphoinositide-specific phospholipase C that generates two second messengers: inositol 1,4,5 triphosphate (IP3) and 1,2 diacylglycerol (DAG) from hydrolysis of phosphoinositide diphosphate (PIP2). [139] [140] [141] IP3 induces the release of calcium from intracellular storage organelles (calciosomes), leading to a transient rise in cytosolic free calcium (Ca2i). [142] Ca2i potentiates PMN degranulation and superoxide anion generation, whereas total sustained intracellular calcium levels are required for receptor expression. [143] Diacylglycerol remains associated with the membrane and participates in the activation of protein kinase C. [144]

Activation of phospholipase D follows activation of phospholipase C. [145] [146] Phospholipase D acts on phosphotidylcholine (PC) to generate phosphatidic acid, which gives rise to diradylglycerides. Diradylglycerol plays an important role in degranulation and activation of the respiratory burst in adherent PMN. PMNs adherent to fibrinogen exhibit a delay in the onset of the respiratory burst that coincides with the later activation of phospholipase D and the generation of high concentrations of diradylglycerol, release of hydrogen peroxide, and secretion of specific granule marker protein lactoferrin from PMNs. [147]

A third phospholipase, phospholipase A2, is activated in PMNs and provides the source for generation of arachidonic acid and lysophosphatidic acid from phosphatidic acid. Arachidonate and lysophosphatidic acid are subsequently lipoxygenated and acylated to LTB4 and PAF, respectively. [148] These reactions are summarized in Figure 38-7 .

Protein kinase C translocates from cytosol to membrane on activation by Ca2i, which leads to phosphorylation of a variety of proteins on their serine and threonine residues. Protein phosphorylation markedly changes the charge and conformation of the proteins, leading to altered enzyme activity, protein binding, and intracellular distribution of proteins and organelles associated with PMN activation. The protein phosphorylation patterns in PMNs stimulated with various chemotactic agonists are similar. [148] [149] [150] Several of the phosphorylated proteins have been identified, including the p47 phox component of the NADPH oxidase and the MARCKS protein involved in PMN motile responses. [151] [152]

Tyrosine phosphorylation of proteins, including tyrosine kinases themselves, occurs in PMN adherent to endothelium or other surfaces and subsequently activated by chemotactic agonists. [153] [154] [155] Thus, the activation of tyrosine kinases involves PMN

Figure 38-7 Phospholipase-mediated signal transduction reactions during activation of PMNs. In PMN suspensions, activation of phospholipase C (PLC) rapidly generates inositol triphosphate (IP-3) and diacylglycerol (DAG) from phosphoinositol 4,5-bisphosphate (PIP_2). Phosphatidic acid (PA) is generated either from DAG kinase or by the slower action of phospholipase D (PLD) from phosphatidylcholine (PC) in adherent PMN. Diradylglycerols form from PA by activation of PA phosphohydrolase. Tyrosine kinases are activated in adherent PMN. Activation of phospholipase A2 (PLA_2) generates lysophosphatidic acid (LYSO-PA) and arachidonic acid (AA) from PA. See text for details.

-2 integrins CD18 for adherence, [156] [157] as well as the chemotactic CXC seven domain transmembrane receptors, or the nonchemotactic inflammatory mediator receptors present on PMNs. [158] [159] [160] [161]

Adenosine and adrenergic agonists attenuate PMN activation by binding to specific adenosine A2 and -adrenergic receptors, which uncouples chemotactic receptors from Gi2 proteins. [162] The signal for this response proceeds via a novel pathway, independent of cAMP but involving a serine/threonine protein phosphatase in the plasma membrane. [163] Adenosine nucleotides, including extracellular ATP, bind to specific binding sites on PMNs and activate signal transduction in PMNs independent of ATP hydrolysis. [164]

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OPSONIC RECEPTOR-MEDIATED PHAGOCYTOSIS

The motile response of PMNs to microbial infection includes emigration out of the vasculature and movement directed toward the source of the inflammatory chemoattractant, culminating in the phagocytic ingestion of opsonized microbes. Engulfment occurs through the advancing broad flat pseudopod as the PMN moves to surround the microbe. The embracing arms of the pseudopod fuse their distal membranes, sealing the microbe into a membrane-lined phagocytic vacuole. The pseudopods are rich in actin, and the dynamic action of the actin machinery is required. Also, signals from the cytoplasm arising from specific ligand receptors seem to direct actin filaments directly beneath the zone of contact of the opsonized microbe. [165] The advancing pseudopods receptors engage a continuous number of specific opsonic ligands coating the microbes, ensuring their solitary confinement in the phagocytic vacuole. [166] The classical opsonins are IgG and C3, corresponding to the heat-stable and heat-labile opsonins. Opsonins bind to bacteria, enhancing their phagocytic uptake by leukocytes. [167] The antigen binding sites (Fab) of IgG bind to the bacteria, exposing the Fc binding site, which in turn is recognized by three classes of receptors, FcRI, FcRII, and FcRIII. FcRIII is constitutively expressed in PMNs with 100,000300,000 copies per PMN and identified by CD16. FcRIII binds IgG subclasses 1 and 3 with intermediate and low affinity, respectively (1,100 nM and 4,700 nM).[168] FcRIIIA and FcRIIIB represent the two genetic isoforms that are structurally similar in their extracellular domains but differ in their transmembrane and cytoplasmic domains. FcRIIIA has the conventional transmembrane and cytoplasmic domain but is not expressed on PMNs. [169] FcIIIB is expressed on PMNs but lacks both transmembrane and cytoplasmic domains and is anchored to the PMN by a glycosyl-phosphatidylinositol (GPI) protein of 5080 kDa. Despite the lack of a cytoplasmic and transmembrane domain, FcRIIIB is capable of triggering a signal transduction cascade leading to granule release but no associated respiratory burst.[170] [171] FcRIIIB and CR3 act synergistically to activate the respiratory burst, but this synergy is dependent on FcRII. Ligand binding of CR3 leads to FcRII association with the actin cytoskeleton on the adherent PMN surface. Coligation of FcRIIIB leads to tyrosine phosphorylation of FcRII, which serves to initiate activation of a signal transduction pathway leading to activation of the respiratory burst. [172] Also, anti-FcRII Fab-coated erythrocytes following cross-linking of FcRIIIB are phagocytized.[173] FcRIIIB has two allotype forms, NA1 and NA2, whereas FcRIIIA is not polymorphic and always types as NA2.[174] A new alloantigen SH has been identified on FcRIII that is independent of NA1 and NA2 and capable of stimulating alloantibodies to it. [175]

FcRII, identified as CD32, is a constitutive low-affinity receptor with a molecular mass of 40 kDa and does not bind monomeric IgG. The expression of IgGRII is low (1,0004,000 copies per cell) and the binding requires dimeric IgG. IgG Ka values are lacking for PMN, but it is considered a low-affinity receptor with the following subclass affinities: IgG1 = IgG3 >> IgG2 = IgG4. [176] FcRII-mediated signaling requires the activation of tyrosine kinases of both Src family kinases and Syk, resulting in tyrosine phosphorylation of Shc, activation of phospholipase C, and an increase in intracellular calcium. [177] Also, FcRII engagement leads to mitogen-activated protein (MAP) kinase phosphorylation and activation, a prerequisite for phagocytosis.

FcRI is not expressed on quiescent or basal state PMNs, but is expressed to a level of 15,00025,000 copies per PMN after exposure to IFN-. Other cytokines, such as G-CSF and GM-CSF, do not induce expression of FcRI in PMNs, although other inflammatory mediators may have the potential. [119] The receptor is a single transmembrane protein receptor identified by CD64 and binds IgG1 and IgG3 with high affinity (50 nM) and promotes phagocytosis of particles or bacteria opsonized with IgG.[119]

Polymeric IgA antibody can function as an opsonin and is recognized by a specific IgA receptor on PMNs (Fc R). [178] Fc R is a 60 kDa protein composed of two Ig-like domains that share homology with FcR. It recognizes monoclonal IgM Ab My43 and is identified as CD89. Fc R mediates signal transduction via G protein-linked phospholipase C activation, leading to phagocytosis and stimulation of the respiratory burst. [179] [180] [181]

Opsonic phagocytosis also involves complement components.

C3 is bound covalently on microbes either by prior-bound IgG or IgM activation of the classical pathway or by antibody-independent activation of the alternative pathway.[182] C3 is proteolytically converted to C3b, which remains covalently bound to the microbe and is recognized by the complement receptor type 1 (CR1) present on PMNs.[183] CR1 binds dimeric C3b with an affinity of 0.5 nM. Although the binding affinity is low, PMNs effectively phagocytize microbes or particles opsonized with C3b because they are coated with such a high density of C3b molecules. The CR1 receptor is a glycosylated protein with a molecular weight of 160250 kDa and can be identified as CD35. CR1 has four allelic forms with molecular weights of 160, 190, 220, and 250 kDa. [184] [185] [186] The alleles are expressed codominantly, accounting for the heterogeneity of molecular weights. To date there is no apparent relationship between the four alleles and functional activity of CR1. The gene for CR1 has been cloned and sequenced.[187] The predicted protein has a single membrane spanning domain and a short cytoplasmic domain, but a very large extracellular domain comprising at least 30 small consensus repeats. [188] CR1 functions as a cofactor in the breakdown of its ligand C3b to inert C3dg, preventing the accumulation of C3bi.[189] [190] When C3b is generated by IgG or IgM, it is labile and is converted to the more stable ligand C3bi, whereas C3b is more stable as a opsonin when its generation occurs via the alternative pathway. [191] In contrast to IgG, the arrangement of C3b and C3bi is clustered on the microbe or particle, coinciding with the observation that the receptor for C3bi (CR3) is also clustered on the surface of PMNs. [192] [193] CR3 recognizes C3bi but not C3b, and the binding requires multimers of C3bi. In contrast to the other opsonic receptors (CR1, FcRI, FcRII, FcRIII), CR3 requires high but physiologic concentrations of divalent cation (about 0.5 mM calcium and magnesium) for effective ligand binding. [194] CR3 is a -2 integrin family member and is designated CD11b/CD18. It is composed of two polypeptide chains, an subunit of 185 kDa and a subunit of 95 kDa. Both chains have been cloned and sequenced. Binding of CR3 to C3bi is enhanced 100-fold by an amphipathic anion lipid called integrin-modulating factor (IMF-1), [73] or by phosphorylation or aggregation of CR3. [188] [193] [195] [196] [197] Although CR3 ligation does not directly trigger the respiratory burst, [198] [199] CR3 plays a critical role in adherence-dependent potentiation of the repiratory burst and secretory responses of PMNs.[200] [201] PMN adhesion-dependent functions are inhibited by neutrophil inhibiting factor (NIF), a glycoprotein produced by the hookworm *Ancylostoma caninum.*[202] C1q, the recognition subunit of the classical complement pathway, interacts with specific PMN surface molecules via C1q collagen-like region (C1q-CLR) to trigger the respiratory burst.[203] Properties of the PMN opsonic receptors are listed in Table 38-3 .

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DEGRANULATION OF POLYMORPHONUCLEAR NEUTROPHILS (SECRETION)

Degranulation of the PMN starts with the onset of phagocytosis. The granules translocate and ultimately fuse their membranes with the newly created phagocytic vacuoles formed from invaginations of the plasma membrane.[204] [205] [206] Specific granules discharge their contents more rapidly than do primary granules, [207] [208] and more of their contents are secreted extracellularly, perhaps through incompletely formed phagosomes. [209] [210] [211] Primary azurophilic granules are most analogous to lysosomes since they contain all the acid hydrolases found in PMNs. [212] Moreover, the acidic environment of the phagolysosome creates a pH optimal for these enzymes, and the acid milieu is essential for further degranulation. [213] [214] Blockade of anion channels impairs ion fluxes of Na^+ and H^+ and prevents degranulation in PMNs, [215] whereas alkalization of granules promotes their secretion. [216] Although microbial killing and digestion takes place within phagolysosomes, [217] [218] PMNs function as a double-edged sword because granule contents secreted extracellularly inflict tissue injury. [219] Degranulation also occurs when PMNs adhere to tissue surfaces and then bind immune complexes, aggregated immunoglobulin, complement components, or certain cytokines. [220] [221] [222] [223] [224] [225] Under these conditions, granule content release occurs by a process of reverse endocytosis [226] or frustrated phagocytosis. [222]

TABLE 38-3 -- Properties of PMN Opsonic Receptors

Specific Receptor	Marker	Type/Mol. Mass	Opsonic Ligand	Binding Affinity (Ka)	Functions
FcRI; expression requires IFN-	CD64	Single transmembrane protein 72 kDa	IgG1	High 50 nM	Activation Phagocytosis
FcRIIa(a,b not expressed); expression is constitutive	CD32	Single transmembrane glycoprotein 40 kDa	IgG1	Low	Activation Phagocytosis
FcRIIIB; Expression is constitutive A isoform not expressed	CD 16	GPI-anchored glycoprotein 5080 kDa	IgG1 complexes	Low 110 nM	Activation
	NA 1 & NA 2 allelle-antigen SH antigen		IgG3 complexes	Low 470 nM	Immune-binding
					Phagocytosis requires CR1 or FcR2
FcR	CD89	Single transmembrane glycoprotein 60 kDa	IgA1, IgA2		Activation
	My43 IgM		Secretory IgA1, IgA2		Phagocytosis
CR1	CD35 4 allelles	Single transmembrane glycoprotein 160, 190, 220, 250 kDa	C3b & C4b dimers	High 0.5 nM	Phagocytosis
CR3	CD11b/	Heterodimeric transmembrane protein	C3bi	High 0.5 nM	Activation
	CD18			Ca+2, Mg+2	Phagocytosis
	Mac1			IMF-1	

Granules fuse with the plasma membrane, discharging their contents directly to the outside of the PMN.

Cytoskeletal proteins are essential for degranulation. These proteins facilitate granule translocation to either the phagolysosome or the plasma membrane. Both microtubules and microfilaments are involved in this process. Thirty seconds after PMNs are activated, their granules bind to polymerized microtubules [227] and inhibition of microtubule polymerization blocks degranulation in PMNs. [215] [228] Actin polymers act like a barrier between the plasma membrane and granules by inhibiting granule secretion. [229] The fungicidal metabolite cytochalasin B greatly facilitates granule secretion by disrupting subplasmalemal cytoplasmic actin microfilaments, enabling fusion between granule membranes and plasma membranes to take place when PMNs encounter either a particulate or soluble stimulus. [230] [231] [232] [233] [234] [235] The precise cause of membrane fusion is unknown but likely involves activation of several calcium-sensitive PMN phospholipases, resulting in altered lipid composition of granule-phagosome or granule-plasma membrane contact points. Phospholipase A_2 promotes fusion of complex liposomes similar in composition to PMN plasma membrane by lowering the calcium requirement to achieve fusion. [236] Phospholipase A_2 is found in granule membranes of quiescent PMNs and a secretory form of PLA2 translocates to phagolysosomes.[237] [236] Specific granules and secretory vesicles fuse more readily at very low calcium concentrations with artificial liposomes containing a phosphatidic acid/phosphatidylethanolamine ratio of 1:3 (500 M) than do primary azurophilic granules. The latter fail to fuse at concentrations of calcium as high as 12 mM. [239] Cholesterol added to either the liposomes or granule preparations enhances fusion. [240] The concentration of free cytosolic calcium(Ca+2i) required for exocytosis of PMN primary, specific, tertiary, and secretory vesicles varies. Secretory vesicles are exceptionally sensitive to very small increments in cytosolic Ca+2i, whereas primary granules require the highest concentration. [241] This hierarchy in mobilization among PMN granules is also observed during exudation of PMNs into skin chambers. Exudates of PMNs demonstrate complete mobilization of secretory vesicles, a 38% release of gelatinase granules, a 22% release of lactoferrin from specific granules, but only a 7% release of myeloperoxidase from primary azurophil granules. [242] In permeabilized PMNs, Ca+2i concentrations of 1 uM (in vivo physiologic range) result in up-regulation of the specific granule marker CD67, whereas for primary azurophilic granules marked by CD63, GTP-S and 1 uM Ca+2i is required. [243] PMN primary granules contain two GTP binding proteins (25 and 26 kDa), whereas specific granules and plasma membranes contain a set of three GTP binding proteins ranging between 20 and 23 kDa. [244] Also, Mg-ATP and other trinucleotides at 0.31.0 mM concentrations stimulate degranulation in permeabilized PMNs, supporting the notion of a shared trinucleotide receptor regulating degranulation in PMNs.[245] [246] Two adenosine receptors are present on PMNs, and adenosine is known to block degranulation as well as the respiratory burst. [247] [248] [249]

Phospholipase D activation in stimulated PMNs converts phosphotidylcholine to phosphatidic acid (Fig. 38-7). This change in membrane composition promotes degranulation by replacing a nonfusogenic phospholipid with a fusogenic one. Phospholipase D lowers the Ca+2i concentration requirement for the fusogenic action of annexin I to promote fusion of PMN granules with artificial liposomes containing phosphatidic acid and phosphoethanolamine. Annexin I and II are two of several proteins that promote fusion between phospholipid vesicles and the cytoplasmic surface of PMN membranes. Intact PMN or PMN membrane preparations treated with trypsin do not bind to liposomes enriched with fusogenic lipids. This indicates a role for cytoplasmic plasma membrane proteins including annexin I in PMN degranulation.[250] [251] Two isoforms of another fusogenic protein, synaptosomal-associated protein of 25 kDa size (SNAP-25), are present in PMNs. SNAP is essential for exocytosis at nerve terminals.[252] Other proteins functioning in the docking and fusion of synaptic vesicles at the plasma membrane and identified in PMN are

secretory vesicle-associated membrane protein-2 (VAMP-2), syntaxin 4, and SCAMP. The latter protein is also found in gelatinase, specific and azurophilic granules, and the secretory vesicles of PMNs.[253]

Secretogogues are soluble molecules that activate PMNs and provoke release of granule contents to the outside of the cell. Complete secretogogues induce the release of both primary and specific granules and elicit a transient rise in Ca+2i and cAMP. Incomplete secretogues cause a rise in Ca+2i and induce release of specific granules without altering cAMP levels.[254] Most chemotactic factors, including C5a, fMLP, PAF, LTB4, and the calcium ionophore A23187, are complete secretogogues, whereas the incomplete secretagogues include phorbol myristate acetate (PMA) and the cell surface reactive lectin concanavalin A (ConA). Several nonchemotactic interleukins, cytokines, and bone marrow growth factors act as either complete or incomplete secretagogues. The inflammatory mediators TNF-, IL-1, and IL6 each induce release of primary and secondary granules in cytochalasin B-treated PMNs,[255] [256] [257] [258] whereas IL4 and chemotactic IL8 effect release of specific granules.[259] [260] [261] GM-CSF causes a release of specific granules from PMN and acts as a complete secretagogue with cytochalasin B-treated PMNs.[262] [263] A single dose of GM-CSF (7.5 g/kg) to human normal volunteers increases expression of the -2 integrin CD11b found in all PMN granule subsets except primary granules. GM-CSF induces the release of chemotactic IL8 but not IL6 or TNF-.[263] A single dose of G-CSF (300 g) also up-regulates CD11b as well as FcRIII, and results in elevation of plasma lactoferrin and elastase. Continued daily administration of G-CSF raises the absolute neutrophil count and markedly elevates TNF- levels without increasing IL8 or GM-CSF plasma levels.

It is possible to deplete PMN totally of gelatinase tertiary granules while leaving intact 50% of lactoferrin-containing specific granules and 90% of myeloperoxidase-containing primary granules within the partially stimulated PMN. This low level of PMN activation in which tertiary granule gelatinase is selectively released is associated with PMN priming.[263] [264]

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PRIMING OF POLYMORPHONUCLEAR NEUTROPHILS

Polymorphonuclear neutrophils exist in one of three states: quiescent, activated, or primed. Primed PMNs are poised to display an exaggerated respiratory burst or secretory response when specific receptors are triggered by their appropriate agonists. Agonists capable of priming PMN are of three main types:

1. The inflammatory mediators that are chemotactic and bind to the seven transmembrane domain family of receptors (Table 38-2)
2. The serum immunoglobulin and complement opsonins, which bind to the single transmembrane receptors that require cross-linking for their full activation (Table 38-3)
3. The inflammatory cytokines and growth factors that bind to their specific single transmembrane receptors (e.g., TNF-, LPS, GM-CSF, G-CSF, substance P, orthovanidate, IL1).

These molecules prime at dose levels that do not elicit a rise of cytosolic free calcium, but do lead to intracellular protein phosphorylation of tyrosine residues in PMNs.[265] [266] Activation of any of the three classes of receptors prime the PMN for the subsequent exaggerated respiratory burst or secretory response. However, only the chemotactic and opsonic phagocytic receptors can trigger the exaggerated response when engaged by the higher level of the appropriate agonist. PMNs can be primed by one agonist and then triggered into the exaggerated response by a second agonist. [267] Priming can occur through either high- or low-affinity receptors of the same receptor class. Depending on the nature of the stimulus, priming may be achieved rapidly

Figure 38-8 Reactions of the respiratory burst pathway. The enzymes responsible for reactions 1 to 9 are as follows: (1) The respiratory burst oxidase (NADPH oxidase); (2) superoxide dismutase or spontaneous; (3) nonenzymatic, Fe^{2} +-catalyzed; (4) myeloperoxidase; (5) spontaneous; (6) glutathione peroxidase; (7) glutathione reductase; (8) glucose-6-phosphate dehydrogenase; (9) glutathione synthetase. (*Adapted from Curnutte JT, Orkin SH, Dinauer MC: Genetic disorders of phagocyte function. In Stamatoyannopoulous G (ed): The Molecular Basis of Blood Diseases, 2nd ed. Saunders, Philadelphia, 1994, p. 505, with permission.*)

over a few minutes, or more slowly over 30 minutes. The primed state may last several hours.[265] [268]

Priming results from the intracellular crosstalk between the calcium signaling system acting via serine/threonine kinases such as a protein kinase C, phospholipase A_2,[269] or calmodulin-dependent kinases, and the calcium-independent tyrosine kinase signaling system acting via tyrosine kinases such as Fgr, Lyn, Fyn, and Syk. [265] The end intracellular result is complete phosphorylation of those proteins involved in achieving an exaggerated respiratory burst and degranulation response of the PMN.

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RESPIRATORY BURST

Activation of PMNs leads within seconds to a dramatic increase in oxygen consumption that is unaffected by mitochondrial inhibitors. [270] This burst of respiration results from activation of a latent enzyme system referred to as NADPH oxidase or the respiratory burst oxidase. [271] [272] [273] The products of the respiratory burst are generated by single electron transfer from NADPH to molecular oxygen (O_2) forming superoxide anion (O2). Superoxide rapidly dismutates either spontaneously or catalyzed by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). H_2O_2 reacts with superoxide anion, forming the highly reactive hydroxyl radical (OH) and with chloride ion (Cl), forming hypochlorous acid (HOCl). Superoxide anion is only weakly bactericidal, but hydroxyl radical kills microbes and injures surrounding tissue. Hypochlorous acid forms longer-lasting chloramines that contribute to oxidative killing of microbes. These oxidative products also inactivate antiproteinases and activate metaloproteinases such as elastase and collagenase during PMN degranulation or secretion. This contributes to surrounding tissue injury. [274] Low concentrations of H_2O_2 react with reduced glutathione (GSH), catalyzed by glutathione peroxidase to form water and oxidized glutathione (GSSG). PMNs are also equipped with other antioxidants, including ascorbic acid, membrane -tocopherol, and soluble catalase. The source of reducing equivalents for the respiratory burst oxidase and the glutathione regeneration is NADPH, which is replenished from $NADP^+$ by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the hexose monophosphate shunt (HMPS) (Fig. 38-8).

The NADPH oxidase is a multicomponent enzyme system composed of at least seven distinct proteins located in various regions of the quiescent PMN. The properties of these proteins are listed in Table 38-4 (Table Not Available) . Some of these proteins translocate to the cortical cytoskeleton and membrane of the phagolysosome or plasma membrane during opsonic phagocytosis or secretagogue activation of the PMN. In the quiescent PMN, p47 phox protein resides in the cytosol along with p67 phox protein, complexed to p40 phox protein. [275] [276] [277] [278] Activation of the NADPH oxidase results from protein kinase C-mediated phosphorylation of p47 phox protein [279] and the subsequent translocation and binding of p47 phox phosphoprotein and the p67 phox-p40 phox complex to the membrane flavocytochrome b558, a heterodimer comprising a 22 kDa -subunit (p22 phox) and a glycosylated 91 kDa -subunit (gp 91 phox). [280] [281] [282] Initiation of phosphorylation of p47 phox is essential for the translocation and assembly of cytoskeletal and membrane-associated NADPH oxidase components from the cytosol. [283] [284] The binding of the three cytosolic components takes place in the cortical cytoskeleton of the PMN, where p47 phox phosphorylation is completed (Fig. 38-9). [285] Eighty percent of the cytochrome b558 is located in the specific granules, gelatinase granules, and secretory vesicles of the PMN and translocates to the cortical cytoskeleton and plasma membrane on PMN activation. [286] [287]

Low-molecular-weight GTP-binding protein Rap1A is tightly associated with the flavocytochrome b558 and may be required for complete function of the NADPH oxidase. [288] [289] Rap1A is found both in the plasma membrane and in the specific granule membrane and is translocated with the cytochrome b558 to the cortical cytoskeleton or to the phagolysosomal membrane during opsonic phagocytosis of microbes. [290] The binding of Rap1A to cytochrome b558 is lost when Rap1A is phosphorylated (Fig. 38-9). [291] Activation of Rap1 occurs independently of the respiratory burst and is normally activated in PMN from patients with chronic granulomatous disease. [292]

A cytoplasmic GTP-binding protein, Rac2, is also transferred

TABLE 38-4 -- Properties of the NADPH Phagocytic Oxidase (PHOX) Components

(Not Available)

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Figure 38-9 Assembly of the seven components of the NADPH phagocytic oxidase during activation of PMNs. Depicted is the docking of the cytoplasmic components, p47 phox phosphorylated and bound to submembrane actin cytoskeleton, p67 phox complexed to p40 phox, and Rac2-GDP after activation and release from Rho-GDI. Rac2 shuttles the cytoplasmic components, which dock with membrane cytochrome b558 dimer composed of gp-91 phox and p22 phox. Rap1A remains bound to cytochrome b558 unless phosphorylated. Also depicted is the transfer of electrons from cytoplasmic NADPH to the flavoprotein to the cytochrome b558. P67 phox facilitates electron flow from NADPH to the flavoprotein and p47 phox is required for electron transfer beyond the flavoprotein to the heme groups of gp-91 phox and p22 phox of cytochrome b558. Electrons flow from cytochrome to oxygen, which reduces oxygen (O_2) to superoxide anion (O_2). The flow of protons is also depicted, indicating the resultant acidification of phagolysosomes or extracellularly to adjacent sites. See text for further details.

with p47 phox and p67 phox-p40 phox complex to the cortical cytoskeleton on PMN activation. Rac1 and Rac2 are normally inactive by being complexed with the GDP dissociation inhibitor Rho-GDI. [293] [294] [295] Both Rap1A and Rac2 appear to regulate oxidase activity by utilizing their preferred substrate GTP, but not GDP. [296] On PMN activation, biologically active lipids such as arachidonic acid, phosphatidic acid, and phosphatidylinositols are generated. These lipids disrupt the binding of Rap1A from cytochrome b558 and Rac2 from Rho-GDI. Rac2 is active in PMNs and is required for translocation of p47 phox and p67 phox-p40 phox complex. It is likely that rac proteins function as shuttle proteins carrying phox proteins to the cytoskeleton in their GTP-bound state and dissociate in their GDP-bound state while returning to the cytosol. [285] During PMN activation, p47 phox becomes heavily phosphorylated on its serine residues. [297] [298] However, only Ser379 at the C-terminus domain of p47 phox is essential for oxidase activity and for p47 phox binding to the cytoskeleton. [299] In a cell-free system, Rac protein is also required for complete activity of p47 phox and p67 phox (Fig. 38-9). [300]

The cytochrome b558 is the terminal component of the microbicidal superoxide generating system. Both - and -subunits contain a heme group and probably share a third heme group. These heme-containing regions of the cytochrome b558 lie within the PMN membrane lipid bilayer. This structure provides an efficient transfer of electrons from cytoplasmic NADPH to the flavoprotein to cytochrome b558 across the plasma membrane to the surface of the phagolysosome or to the extracellular surface, where oxygen is reduced to superoxide anion. [301] The volt potential is from 330 mV for NADPH to 256 mV for flavoprotein to 245 mV for cytochrome b to 160 mV for O_2 to O2 (Fig. 38-9). These midpoint potentials are considered low compared to mitochondrial cellular cytochromes. [272] P67 phox facilitates electron flow from NADPH to the flavoprotein, and p47 phox is required for electron transfer to proceed beyond the flavoprotein to the heme groups in the cytochrome b558 and then on to superoxide and oxygen. [302] [303] Two moles of oxygen are reduced to superoxide anion and one mole of reduced nicotinamide adenine dinucleotide is oxidized: $2O_2$ + NADPH 2O2 + $NADP^+$ + H^+ . The assembly process of the seven components of the phagocytic oxidase and the associated electron shuttle as NADPH is oxidized and oxygen is reduced to superoxide anion during the PMN respiratory burst is illustrated in Figure 38-9 .

Nitric Oxide Pathway

Another microbicidal pathway, independent of PMN phagocytic oxidase, is the nitric oxide synthetase (NOS) generation of reactive nitrogen intermediates. [304] Nitric

oxide (NO) or other labile NO intermediates, derived from L-arginine, are induced in PMNs exposed to mediators of inflammation.[305] [306] Also, superoxide dismutase enhances the inhibition of thrombin-induced platelet aggregation by release of nitrite anions (NO_2) (NO_3) and peroxynitrite from PMNs. Constitutive NOS purified from quiescent human PMNs is a 130 kDa protein whose activity requires L-arginine and NADPH, tetrahydro biopterin, and flavin adenine dinucleotide (FAD) for maximal activity.[307]

Circulating PMNs display very low NOS activity due in part to an endogenous inhibitor to constitutive NOS. [308] Although NOS colocalizes to the primary granule of PMNs, release of nitric oxide intermediates from PMNs is not associated with either degranulation or the respiratory burst. [309] [310] Three isoforms of NOS occur in blood cells: NOS I in PMNs, NOS II in eosinophils, and NOS III in platelets and megakaryocytes. NOS II isoform is inducible in PMNs to almost 50-fold by gram-negative bacterial endotoxins[311] [312] and likely plays the major role in PMN NO-related inflammatory responses.

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NONOXIDATIVE MICROBICIDAL KILLING

Polymorphonuclear neutrophils can kill bacteria using antimicrobial proteins present in primary azurophilic granules and in gelatinase and specific granules (Table 38-1). The most important antimicrobial proteins of PMNs are defensins; serprocidins, including cathepsin G and azurocidin (GAP 37), and bacterial permeability-increasing protein (BPI).[313] Defensins constitute as much as 50% of PMN granule protein content.[314] They are viricidal, fungicidal, and bactericidal to many microbes and are highly cationic. They exert their cidal activity by inserting into hydrophobic channels, forming voltage-dependent ion channels in lipid bilayers.[315] Cathepsin G is a serine protease that kills both gram-positive and gram-negative bacteria. It exerts its bactericidal action by binding to penicillin-binding proteins of bacteria such as *Staphylococcus aureus* and interferes with the synthesis of peptidoglycans.[316] Azurocidin is another serine proteinase that kills *Escherichia coli, Streptococcus faecalis,* and *Candida albicans* at acid pH, achievable in phagolysosomes of PMNs.[317] BPI protein kills gram-negative bacteria by binding to their LPS capsule and altering their bacterial membrane permeability to extracellular solutes. A mutant strain of *Salmonella typhimurium* resistant to BPI protein was also found to be resistant to PMN bactericidal activity under strict anaerobic conditions, pointing to the clinical importance of BPI protein in nonoxidative killing by PMNs.[318] The specific granule marker lactoferrin is bactericidal to some gram-negative bacteria by generating free radicals from iron bound to it. Lysozyme, present in azurophil, specific, and gelatinase granules, likely aids in the digestion of killed bacteria after they have had their capsule altered by both oxidative and nonoxidative mechanisms within phagolysosomes of PMNs.[319]

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Chapter 39 - Monocyte and Macrophage Development and Function

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INTRODUCTION

The functional macrophage was first described approximately 130 years ago, when large cells with unsegmented nuclei were observed in acutely damaged tissues and areas of inflammation. In areas of chronic inflammation, these cells were more frequent and appeared larger, suggesting that the cells were capable of ingesting demolished cells and inflammatory debris. Because these cells appeared to be capable of ingesting large particles, they were termed macrophages. This scavenger function was long thought to be their principal role. It was not until 1882 that Robert Koch noted that these inflammatory phagocytic cells could engulf tubercle bacilli. Metchnikoff concluded that the epithelioid-like cells making up tuberculous granulomas were actually derived from monocytoid cells of the blood. The monocyte was established as a distinct cell type by 1913. Even then, many pathologists were convinced that monocytes and macrophages were cells of different ontogeny. Much later, cells were identified that represented intermediate forms between typical monocytes in the circulating blood and tissue macrophages, and the connection between these two cell types was established.

In the 1960s, the critical role played by monocytes and macrophage in the eradication of certain intracellular microorganisms was discovered. The macrophages in the skin, lung, and gut appeared to provide a first line of defense against microbial infections and parasitic infections. [1] [2] [3] [4] Exposure to environmental pathogens caused macrophages to enlarge, increase their number of lysosomes, and become more phagocytic. Furthermore, macrophages developed strong resistance to these microorganisms with repeated challenge. This altered state was termed activation.

An additional observation was that even after the acute macrophage-mediated inflammation subsided, rechallenge with the same exogenous microorganisms would result in rapid reaccumulation and activation of macrophages. This reactivation appeared to be an antigen-specific event, triggered by memory lymphocytes. These results indicated an important interaction between lymphocytes and macrophages. More recently, the reciprocal interaction, in which macrophages regulate lymphocyte function, has been the focus of intensive study. Current models of the immune system implicate macrophages in all steps of the hosts encounter with microbial pathogens: from the initial processing of exogenous antigens, to their presentation to lymphocytes, to their own subsequent activation by these lymphocytes, and, in turn, their regulation of these lymphocytes. Macrophages thus play an active role in regulating both the afferent and the efferent arms of the immune system. This phylogenetically older, nonspecific defense system has evolved to interact with, and regulate, the newer and more specific cellular and humoral immune defense systems. Even the term resident or sessile macrophage is being challenged because it becomes clear that this cell can circulate, trafficking into and through normal tissues, and into lymphatics as far as the lymph node. [5] Finally, the role of monocytes and macrophage in the host response to tumors, in tissue maintenance, and in disease states such as atherosclerosis are active areas of research that are only now being elucidated.

The activities of monocytes and macrophage can be divided into 3 broad categories (Table 39-1), described in the following sections.

Tissue Maintenance

Tissue maintenance is an activity that comprises the functions of tissue repair and remodeling activities. Examples include the osteoclast remodeling of bone after injury and the osteoclast

TABLE 39-1 -- Spectrum of Macrophage Activity

Tissue maintenance
Regulation of cell proliferation
Fibroblasts
Smooth muscle cells
Endothelium
Lymphoid, myeloid, erythroid cells
Scavenger function
Phagocytosis
Detoxification
Removal of senescent cells
Secretory function
Proteases and antiproteases
Complement components
Arachidonic acid metabolites/prostaglandins
Wound repair
Debridement and phagocytosis
Angiogenesis
Remodeling

Calcium metabolism/bone remodeling
Iron storage
Neuroendocrine regulation
Placental function and implantation
Immune regulation
Antigen processing and presentation
Accessory functions for humoral and cellular immunity
Pathogen control
Antiviral activity
Antimicrobial activity
Antitumor activity

mobilization of bone in calcium homeostasis.[6] [7] [8] [9] Another example is the splenic macrophage, which mediates phagocytosis of senescent red blood cells. [10] Langerhans cells control keratinization of the skin epidermis, [11] and Kupffer cells in the liver manage gut-absorbed endotoxin as well as red blood cell phagocytosis in the neonatal liver,[12] [13] and macrophages in the central nervous system may participate in neuroendocrine control. [14]

Immune Regulation

Monocytes and macrophages play a central role in immune regulation by coordinating the interaction of T and B cells during antigen presentation. [15] [16] [17] The macrophage, especially in its specialized forms, the Langerhans cell and the lymphoid dendritic cell, not only processes antigens, but releases an array of cytokines that modulate lymphocyte function.[18] Immune regulatory function also includes monocyte or macrophage-mediated suppressor activities such as suppression of lymphocyte proliferation[19] [20] and inhibition of lymphokine production. [21]

Pathogen Control

The antimicrobial properties of monocytes and macrophage include antibacterial, [22] [23] antiparasitic,[24] antifungal,[25] and antiviral[26] properties. Monocytes and macrophage may play multiple roles in a single area of inflammation. For example, macrophages may infiltrate an area of damaged tissue, debride the wound, direct fibroblast and connective tissue regrowth, participate in angiogenesis, and destroy invading pathogens. [27] [28] [29] [30] [31] [32] The versatile role that migratory monocytes and tissue macrophages play in areas of inflammation demands that they have the capacity to express a wide range of morphologic, functional, and biochemical phenotypes. Furthermore, the interaction of monocytes and macrophages with immune, hematopoietic, endothelial, or connective tissue cells appears to require cellular communication mediated both by cell-cell contact as well as the elaboration of cytokines, growth factors, and small molecules such as nitric oxide (NO), reactive oxygen intermediates, and prostaglandins (Table 39-2).

ORIGINS OF MONOCYTES AND MACROPHAGES

In fetal life, primitive macrophages develop and differentiate into fetal macrophages before the appearance of fetal monocytes. [33] Monocytes appear in the fetal circulation at approximately the fifth month of gestation and increase in number during the third trimester. [34] The monocyte count reaches a peak at approximately 12 hours of age and remains high during the first 2 weeks of life. Monocytes develop from a pluripotential stem cells in the bone marrow, [35] now designated as colony-forming unitgranulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) and the more committed colony-forming unitgranulocyte, macrophage (CFU-GM; Fig. 39-1). This pluripotent stem cell can commit along both granulocytic and monocytic lineages. Some of the signals necessary for the commitment to the monocytic lineage are known. M-CSF (monocyte colony-stimulating factor, also called colony-stimulating factor-1 [CSF-1]) is the most important of the CSFs in the development of monocytes/macrophages. M-CSF enhances production of the monocyte precursors, stimulates the chemotactic activity or monocytes/macrophages, induces differentiation of monocytes into macrophages, induces proliferation of some monocyte/macrophage subtypes, and is necessary, but not sufficient for activation of monocytes/macrophages.[36] Genetic knockouts or naturally occurring mutants lacking M-CSF in mice result in osteopetrosis (because of impaired osteoclast development),[37] and a severe deficiency but not complete absence of monocytes/macrophages, demonstrating the existence of an M-CSF-independent monocyte/macrophage subpopulation (Kupffer cells,

TABLE 39-2 -- Monocyte/Macrophage Macromolecular Secretory Products

Interleukin-1 (IL-1) family
IL-1
IL-1
IL-1 receptor
IL-1 receptor antagonist
Platelet-derived growth factor (PDGF) family
PDGF-A
PDGF-B/c-*sis*
VEGF (vascular permeability factor)
Transforming growth factor- (TGF-) family
TGF-1
TGF-2
Activin
Immediate-response gene growth factor family
Macrophage inflammatory proteins (MIP)-1, MIP-1, MIP-2
Monocyte chemoattractant protein (MCP)-1, -2, -3
Mig
IL-6
Monocyte colony-stimulating factor (M-CSF)
Melanocyte growth-stimulating activity (MGSA)
Inflammatory protein (IP)-10
IP-8
Proteases
Plasminogen activator
Urokinase
Collagenase
Elastase
Angiotensin convertase
Acid protease
Coagulation factors
Factors V, VII, IX, X, and prothrombin
Prothrombinase
Plasminogen activator
Plasminogen inhibitor
Plasmin inhibitor
Lipoprotein lipase
Adhesion/matrix factors

Thrombospondin
Proteoglycans
Fibronectin
Miscellaneous polypeptide factors
Tumor necrosis factor-
Granulocyte-macrophage colony-stimulating factor
Granulocyte colony-stimulating factor
Leukemia inhibitory factor/Decay inhibiting activity
Erythropoietin
Transforming growth factor-
Basic fibroblast growth factor
Insulin-like growth factor-I
Defensins
Thymosin
Bombesin
Corticotropin
Interferons (and)
Lysozyme
Neutrophil activating factor (IL-8)
Complement factors: C1, C2, C3, and C5
Alternate pathway factors: factors B and D, properidin
Other regulatory products
Prostaglandin E_2
Acidic isoferritin
Nitric oxide
Respiratory burst products
Nitrates

Langerhans cells and microglia). Interleukin-3 (IL-3) and granulocyte-macrophage CSF (GM-CSF) have been shown to mediate differentiation of monocytic cells both in vitro and in vivo.[38] [39] GM-CSF is produced by T cells, endothelial cells, and fibroblasts; M-CSF is produced by monocytes, endothelial cells, and fibroblasts. [40] Bone marrow precursors exposed to GM-CSF produce mixed granulocyte-monocyte/macrophage colonies (CFU-GM); M-CSF produces colonies that are primarily monocytic (CFU-M). Knockout of the GM-CSF gene in mice produces accumulation of surfactant lipids and proteins in the alveolar space, secondary to defective alveolar macrophage differentiation and inadequate macrophage processing of surfactant. [41]

The development of the monocyte lineage demonstrates the close relationship between the origins of monocytes and granulocytes. Once the pluripotential stem cell has committed to the monocyte lineage (CFU-M), the cell develops through morphologically distinct monoblast and promonocyte stages. The mature monocyte, in the G_1 phase of the cell cycle, is released within 24 hours from the bone marrow to the peripheral circulation. Peripheral blood monocytes rapidly partition between the marginating and circulating pools in the peripheral circulation. Although the marginating pool is approximately 3.5 times greater than the circulating pool, [42] the factors regulating monocyte margination are unclear. The monocyte remains in the peripheral circulation for 871 hours (half-life of 17.4 hours) before migrating into peripheral tissues.[42] [43]

Circulating blood monocytes are 1215 m in diameter with a highly convoluted surface and a lobulated (kidney-shaped or folded), foamy nucleus. A Wright-stained smear of peripheral blood monocytes typically demonstrates indistinct nucleoli as well as gray and variably vacuolated cytoplasm. [44] Monocytes contain a single type of granule with staining characteristics suggestive of lysosomes. Monocytes may be further characterized by the presence of a fluoride-sensitive, nonspecific esterase. After the monocyte has migrated into the extravascular tissues, the cell becomes larger and acquires the cytologic appearance of a tissue macrophage. The cell nucleus appears oval and euchromatic with more prominent nucleoli. The cytoplasm stains blue, reflecting high concentrations of RNA. Compared with the circulating cell, in the macrophage there is an appearance of more ribosomes, lysosomes, mitochondria, endoplasmic reticulum, open vesicles, and electron-dense inclusions of varying sizes and shapes. Increased levels of pinocytic as well as phagocytic activity are also a property of some mature macrophages, although the activities and morphologies of tissue macrophages vary widely according to their location and purpose.

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TRANSITION OF MONOCYTES TO MACROPHAGES

The number of monocytes produced, circulating, and extravasating is controlled at a number of levels. Active inflammation can affect the generation and circulating time of peripheral blood monocytes. The production of monocytes from promonocytes can increase up to fourfold within 12 hours of an inflammatory stimulus. [45] In this setting, the average circulation time of monocytes in the peripheral blood may fall to as little as 30 minutes. [42] The young monocyte is capable of producing growth factors, including M-CSF and GM-CSF, which can act in a paracrine or autocrine manner to stimulate monocytopoiesis. [46] [47] [48] Monocytes contain lysosomal hydrolases and the intracellular enzymes elastase and cathepsin, but have little capacity for production of metalloproteases. They express receptors for glycoproteins, apolipoprotein-E, and transferrin. As monocytes differentiate into macrophages, they begin to produce predominantly metalloproteases and metalloprotease inhibitors, lose expression of hydolases and certain cell surface receptors, and express macrophage-specific genes and products such as inducible NO synthase (NOS), interferon (IFN)-, and the complement

Figure 39-1 Hematopoietic cell development. BFU, burst-forming unit; CFU, colony-forming unit; CSF, colony-stimulating factor; E, erythrocyte; EPO, erythropoietin; GEMM, granulocyte-erythrocyte-monocyte-megakaryocyte; GM, granulocyte-macrophage; IL, interleukin; M, monocyte; RBC, red blood cell. *(From Gabrilove JL: Introduction and overview of hematopoietic growth factors. Semin Hematol 26[suppl 2]:1, 1989, as adapted from Griffin JD: Clinical applications of colony stimulating factors. Oncology 2:15, 1988, with permission.)*

component Bf.[49] The movement of the peripheral blood monocyte into the extravascular tissue produces a tissue-specific macrophage. [50] [51] [52] Tissue-resident macrophages are long-lived and (in some cases) slowly self-proliferating [53] and self-sustaining cells.

Tissue macrophages are a heterogeneous population with very different morphologic and functional properties depending on the tissue where they reside. [54] [55] Even within certain tissues, like the spleen, there is distinct, region-specific microheterogeneity of macrophage populations. [36] This tissue heterogeneity is the result of three interacting forces: (1) a lineage- or origin-dependent effect (i.e., whether the macrophage is bone marrow derived or the result of local proliferation); (2) the subpopulation of the marrow-derived macrophages, which is determined by the types of CSFs in the marrow environment and the developmental stage of the monocyte/macrophage; and (3) adaptive influences, in response to the conditions and stimuli in which the macrophage is residing (Fig. 39-2).[56] For example, the presence of M-CSF and expression of c-*fos* can drive monocytes to differentiate into osteoclasts, whereas exposure to GM-CSF and IL-4 facilitates differentiation toward Langerhans and dendritic cells.

Alveolar macrophages, Kupffer cells, osteoclasts, peritoneal macrophages, and synovial type A cells all belong to the resident mononuclear phagocyte system (or reticuloendothelial system). Macrophages of the reticuloendothelial system are found not only at portals of entry, such as pulmonary alveoli and the gastrointestinal tract, but in generally sterile areas of the body, such as the brain, bone, and bone marrow. These mature resident macrophages have a relatively long life, ranging from 14 days to 1 year. Some of these cells perform primarily homeostatic function, whereas others exert survey or acute antipathogen activities. Tissue macrophages retain some propensity for chemotaxis, and have full capacity for phagocytosis and oxygen-dependent microbicidal activity.

In inflammatory infiltrates, mononuclear phagocytes also comprise a heterogeneous group. They consist of macrophages already residing in the tissue before the onset of inflammation (*resident macrophages*), as well as mononuclear phagocytes recruited from the circulation (*exudate macrophages*). The differentiation from circulating monocyte into resident macrophage may include a transitional form called the *exudate-resident macrophage*. Once extravasation has occurred, it is likely that the microenvironment plays a critical role in determining the cells

Figure 39-2 Terminal differentiation pathways of monocytes. GM (M)-CSF, granulocyte-macrophage (monocyte) colony-stimulating factor; IL-4, interleukin-4. *(Adapted with permission from Gordon S, Clarke S, Greaves D et al: Molecular immunobiology of macrophages: recent progress. Curr Opin Immunol 7:24, 1995 [Current Biology, Ltd.].)*

maturation. The tissue-specific levels of lymphokines, monokines, hormones, and toxins can act as microenvironmental mediators of maturation. Macrophages achieve a spectrum of functional phenotypes depending on these microenvironmental signals. IFN--treated macrophages are more bactericidal and tumorcidal, express increased levels of major histocompatibility complex (MHC) class II for antigen presentation to lymphocytes, and are primed for the release of cytokines, such as tumor necrosis factor (TNF)-, in response to bacterial toxins. [57] [58] [59] [60] Alternatively, T-cell-derived cytokines such as IL-4, IL-10, IL-13, and transforming growth factor (TGF)- may inhibit the effects of the activating cytokines, creating a counter-balance to IFN- and GM-CSF. [61] [62] Tissue microenvironments may also regulate reversible changes in macrophage phenotype and function. For example, the heterogeneity in Fc-receptor expression in pulmonary alveolar macrophage can be reversed by in vitro culture.[63] The glycolytic metabolism of the peritoneal macrophage is high, whereas that of the pulmonary macrophage is low. This metabolic state can be reversed when alveolar macrophages are transferred to an oxygen-poor environment. [64] [65] [66] Thus, differentiation in macrophages is not permanent or terminal, but rather exhibits an adaptive or plastic potential. [56]

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MONOCYTE/MACROPHAGE BIOLOGY

Metabolism

Monocytes and macrophages are facultative anaerobes, with the exception of pulmonary macrophages, which appear to be uniquely dependent on aerobic metabolism.[66] [67] Phagocytosis and the phagocytosis-related oxygen burst through NADPH oxidase and the hexose monophosphate shunt are cyanide insensitive, and the energy for these phenomena depends primarily on glycolysis. [68] [69] Only pulmonary macrophages, their energy metabolism more dependent on oxygen and oxidative metabolism, show little postphagocytic respiratory burst beyond their high resting level. [70]

Monocyte/Macrophage Activation

As our appreciation of the wide spectrum of monocyte/macrophage functions grows, it becomes less useful to speak of macrophage activation as the acquisition of a single class of properties. Unlike T cells, macrophage function does not seem to be defined by unique pathways of differentiation, but rather by the balance of cytokines and factors to which the cells are exposed in their environment. [71] It is more accurate to think of macrophage activation in terms of the acquisition of competence to complete specific complex functions,[59] correlating with patterns of specific gene expression, rather than conversion of macrophage to a single activated state. Different macrophage activation states would include the phagocytic/cytocidal state, the immune/antigen-presenting cell (APC) state, and differentiation-specific states, such as that of the activated osteoclast.

Monocytes and macrophages can readily achieve the complex metabolic state that characterizes macrophage-mediated microbicidal and tumorcidal (cytocidal) activities. [59] [72] Agents that can trigger macrophage activation can be divided into two main classes: physiologic factors produced by the host, such as cytokines and metabolites; and environmental factors derived from viruses, bacteria, or chemical synthesis. Although both classes of activators can stimulate the macrophage, combinations of such stimuli are synergistic in activating macrophages, and are required for some effector functions. Furthermore, the activation process in vivo is likely to be a multistep, multipathway process involving a defined sequence of events. A plausible sequence for macrophage activation in situ may include the priming of the macrophage with one agent (e.g., a physiologic macrophage-activating factor), with synergistic cellular activation on exposure to a second factor. [73] [74] This sequence provides an explanation for the combination of physiologic (e.g., IFN-) and environmental (e.g., LPS [lipopolysaccharide] or phorbol ester [e.g., PMA (phorbol myristate acetate)]) signals required to activate macrophage tumorcidal activity. It is likely that different combinations of the stimuli are responsible for the multiple activation phenotypes, and correlated patterns of macrophage-specific gene regulation, that can be observed.

A soluble factor produced by T lymphocytes was the first cytokine identified as a stimulus of the macrophage cytocidal activation process. This activity was termed macrophage-activating factor (MAF)[59] [72] [75] and was later identified as IFN-.[76] [77] [78] Additional, non-IFN forms of MAF activity have been identified, including T-cell products such as IL-2, IL-4, and macrophage migration inhibitory factor, hematopoietic growth factors such as M-CSF and GM-CSF, and a variety of other products. [79] [80] [81] [82] [83] [84] [85] [86] [87] [88] [89] However, many of these factors may work indirectly, through IFN-, because studies of the consequences of deletion IFN- or IFN- receptor genes demonstrate that IFN- is required for many aspects of macrophage function. [90] [91] M-CSF, although not activating in the classic sense, does stimulate monocyte/macrophage proliferation, increases adhesive properties by inducing glycoprotein receptor expression, and facilitates pathogen clearance by stimulating endocytic pathways and the production of proteolytic agents such as urokinase.

The different external stimuli that result in monocyte/macrophage cytocidal-type activation appear to use a variety of signal transduction pathways to produce intracellular superoxide and microbicidal activity. Lectins are thought to trigger production of superoxide through a rise in intracellular calcium, [92] whereas prostaglandin E_2 appears to effect signaling through adenyl cyclase with the generation of cyclic adenosine monophosphate. LPS, binding to the macrophage by the surface receptor CD14,[93] activates an inositol and diacylglycerol messenger system as well as calcium mobilization. Guanine nucleotide binding proteins (G-proteins) may also play a role in some activation pathways.[92] [94]

Oxidative burst stimulation of phagocytes leads to an increase

in oxygen consumption, activation of the hexose monophosphate shunt, and an increase in glucose uptake. This respiratory burst results in the production of toxic oxygen species (reactive oxygen intermediates)[95] [96] that are generated from oxygen through a membrane-bound hemo-flavoprotein, NADPH oxidase. A B-type cytochrome is related to this oxidase, probably as part of a chain of electron carriers. The NADPH oxidase system catalyzes the one-electron reduction of O_2 to superoxide (O_2) through oxidation and reduction steps. O_2 is converted to several other toxic oxygen species such as hydrogen peroxide, hydroxyl radicals, and singlet oxygen. Ferric iron may react with hydrogen peroxide and superoxide to produce hydroxyl radicals with strong oxidant (i.e., microbicidal) properties over relatively short distances, as in a phagosome. In combination with an enzyme from the azurophilic granules (myeloperoxidase, or an equivalent), H_2O_2 plus a halide form a powerful oxidation system resulting in the generation of oxidized halogens, such as hypochlorous acid. A substantial portion reacts with amines, particularly taurine, to yield chloramines. Cytokines such as TNF- and colony-stimulating factors are able to prime monocytes to respond to a second stimulus, with resulting increases in production of oxygen radicals, release of lysosomal enzymes, increases in phagocytic and cytotoxic capacity, and increased adherence at sites of inflammation.[97]

Mononuclear phagocyte cytocidal-type activation results in certain quantitative and qualitative changes in the macromolecular composition of the macrophage cell membrane. These changes include the increased expression of Fc receptors, class II MHC antigens, IL-2 receptors, and membrane-associated TNF. In contrast, macrophage activation results in decreased expression of other receptors such as transferrin, mannose-fucose, and C3bi (CR3).

Whereas the production of IFN- by the T-helper 1 (Th1) lymphocyte population results in a cytocidal state of macrophage activation, the cytokines produced by the Th2 population, specifically IL-4 and IL-13, stimulate the APC type of activation state (sometimes called an alternative activation state). These T-lymphocyte-derived cytokines in turn enhance macrophage stimulation of T cells by inducing class II MHC antigen and B7 co-stimulatory molecule expression, [98] and antigen uptake and expression. Once activated, monocytes/macrophages in turn can produce cytokines that stimulate both types of helper T-cell subsets. IL-12 production in response to IFN-, especially in combination with B7 expression, is a determining factor in the Th1 lymphocyte-mediated immune response, [99] [100] and TNF- also stimulates this helper subset.

There are a number of routes to macrophage deactivation, including the products of lymphocytes and macrophages themselves. The major macrophage inhibitory cytokines include IL-4, IL-10, IL-13, and TGF-, with each one inhibiting some, but not all, macrophage functions, depending on the activation state of the macrophage.[101] [102] All four inhibit NO production by the macrophage. IL-10, in addition, suppresses T-cell responses to monocytes/macrophages by inhibiting the

expression of the B7 co-stimulatory molecule and lymphocyte-activating cytokine production. As noted previously, IL-4 and IL-13 stimulate the APC type of activation state, while simultaneously inhibiting the cytocidal type of activation state. Other monocyte/macrophage inhibitors include prostaglandin E_2.[59] [72] [75] [103] The synthesis and release of prostaglandin E_2 by macrophages is likely to provide a negative feedback loop for the modulation of the immune response. Corticosteroids are also well known anti-inflammatory agents that suppress the induction of activation-associated gene products in macrophages.

Monocyte/Macrophage Surface Molecules

In keeping with their broad range of activities, monocytes/macrophages express a variety of receptors on their surfaces with which they interact with their environment. These receptors can be artificially divided into those that mediate uptake of microorganisms and particles, and those that mediate cell-cell contact, but many of these receptors are in fact able to serve multiple roles.

Receptors Mediating Uptake of Microorganisms and Particles

There are at least five major classes of receptors on the surface of monocytes and macrophages that mediate attachment and entry of microorganisms or other materials into the cell:

1. Macrophage scavenger receptors (MSRs) bind and endocytose low-density lipoproteins (LDL), [104] especially after their oxidation from contact with vascular wall components. MSRs are further subdivided into MSRs-1, -2, and -3, and include MARCO [105] and CD36 (platelet glycoprotein IV), which differ in expression in macrophage subtypes, and some of which can bind extracellular matrix proteins and bacterial wall proteins such as lipoteichoic acid, LPS, and others. [106]
2. The immunoglobulin Fc-receptors (FcR) mediate uptake of immunoglobulin-opsonized particles and immune complexes, [107] [108] and include those with high specificity for monomeric IgG (FcRI, CD64), complexed IgG (FcRII or III, CD32 or CD16), or for IgE (CD23). The FcRI is expressed only on mononuclear phagocytes, in contrast to the other FcR, which are expressed on both neutrophils and mononuclear phagocytes. Ligand binding to the FcR induces the release of numerous products from the macrophage, including TNF and tissue plasminogen activator, and triggers the respiratory burst. [109]
3. Receptors that recognize the third component of complement (CRs) include CR1 (CD35), binding C3b/C4b, and the 2-integrins CR3 (CD11b/CD18) and CR4 (CD11c/CD18), binding iC3b.[110] The CRs are activated or their expression is induced with monocyte/macrophage activation. Complement fragments may not only identify potential pathogens, but may facilitate their phagocytosis. Because the CR endocytotic pathway can bypass the oxidative burst, this path is often used for uptake by intracellular parasites such as *Mycobacteria*, *Legionella*, and *Leishmania* species.
4. Receptors recognizing mannose/fucose-*N*-acetylglucosamine-terminated glycoproteins are termed macrophage mannose receptors (MMRs). The MMR is found on mature, nonactivated macrophages and mediates endocytosis, phagocytosis, and cytotoxicity by reactive oxygen intermediates, during the ingestion of yeast wall particles (such as zymosan) and *Pneumocystis carinii*.[111] [112] [113] These receptors are composed of repeating domains of calcium-dependent or C-type lectin domains. The receptors described so far include receptors composed of eight [114] and ten contiguous (DEC205) [115] C-type lectin domains.
5. CD14 binds LPS and microorganisms bearing polyanionic molecules.

Monocytes and macrophages also express receptors for coagulation factors VII and VIIA, [116] [117] -endorphin, [75] [118] LDL, and very-low-density lipoprotein (VLDL). [119] [120] [121] [122]

Cell-Cell Adhesion Receptors

Macrophages possess abundant cell-cell adhesion receptors and ligands, which can mediate their recruitment into inflamed tissue or their interactions with other immune cells.[123] These can be grouped into three families: (1) integrins, (2) immunoglobulin superfamily, and (3) selectins. The major integrins on monocytes/macrophages are of the 1 and 2 subfamilies, as classified according to their common chain. The 1 family (VLA molecules) mediate binding of monocytes/macrophages

to the extracellular matrix, and include thrombospondin, fibronectin, and laminin. The 2 family consists of the three leukocyte-restricted integrins, LFA-1 (CD11a/CD18), CR-3 (MAC-1, CD11b/CD18), and p150/95 (CD11c/CD18). The immunoglobulin superfamily members include intercellular adhesion molecule (ICAM)-1 (CD54), -2 (CD102), and -3 (CD50), as well as LFA-3 (CD50) and PECAM (CD31). LFA-3 binds to CD2 on lymphocytes. PECAM is required for diapedesis of monocytes.[124] The selectins (lectin-binding molecules) include L-selectin (CD62L), which is the only selectin on macrophages, but also include E-selectin (CD62E) and P-selectin (CD62P), which recognize oligosaccharide ligands on monocytes/macrophages such as CD15 and CD15s. These lectin receptors likely also serve a role in recognition of microorganisms by macrophages. LFA-1 (CD11a/CD18) and one of its many ligands, ICAM-1, [125] are both present on monocytes and mediate homotypic cell interactions[126] [127] as well as the attachment of monocytes to endothelial cells and to lymphocytes bearing the corresponding receptor/ligand, thus facilitating antigen presentation. [125] [126] [127] [128] MAC-1 (CD11b/CD18) appears to have a major role in the attachment and possibly extravasation of monocytes through endothelium that has been activated by stimuli.[129] Inflammatory cytokines and bacterial products can induce and activate pre-existing integrins. Inflammatory cytokines such as TNF and IL-1 induce the expression of ICAM-1, VCAM-1, and ELAM-1 on endothelial cells. [130] VLA-4 and other ligands on monocyte plasma membranes mediate binding to endothelial VCAM-1 and ELAM-1, facilitating monocyte recruitment to inflammatory sites. [131] The integrin adhesion molecules also have a transmembrane signaling function.[132] Cellular activation appears to be triggered by macrophage binding to the extracellular matrix proteins type IV collagen and fibronectin.[133]

Other adhesion molecules include CD44, which binds hylauronin, and CD36, which binds thrombospondin. Sialoadhesin (CD22) is a sialic acid-binding lectin expressed by stromal macrophages that mediates macrophage binding to bone marrow hematopoietic cells, allowing critical cell contact without activation of phagocytosis.[134]

Movement/Extravasation

Under normal conditions, monocytes constitute 16% of nucleated blood cells. Resident tissue macrophages derived from monocytes are recruited in the absence of inflammation at a basal level by unknown mechanisms. Inflammation induces the rapid egress of monocytes from the circulation. Monocyte movement from the blood to the tissue can be divided into somewhat discrete phases mechanistically (i.e., attachment, diapedesis, dissolution of vessel basement membrane, and migration along gradient of inflammatory mediator or chemoattractant), but the stimuli provoking extravasation of monocytes often act on more than one component simultaneously. Chemoattractant factors responsible for inflammatory cell recruitment include *N*-formyl-methionyl-capped bacterial proteins, complement products such as C5a, fibrinopeptides, leukotriene B4, platelet-activating factor (PAF), and a large number of growth factors and cytokines, such as TGF-, platelet-derived growth factor (PDGF), IL-1, TNF, and IL-2.[135] Many of these factors appear to act by stimulation of phospholipase C with subsequent activation of protein kinase C. Proinflammatory factors selectively chemotactic for monocytes or granulocytes, designated chemokines, have recently been characterized. [136] They fall into two families, c-x-c and c-c, based on their structure. The c-x-c family, which is more chemotactically active for granulocytes than monocytes, includes IL-8, neutrophil activating peptide-2, and melanoma growth-stimulating activity (MGSA). The c-c family members, which recruit monocytes more actively than granulocytes, include monocyte chemoattractant proteins (MCP)-1, -2, and -3, macrophage inflammatory proteins (MIP)-1, -1, and -2, and RANTES. [137] [138] [139] [140] The chemokines act through calcium-dependent activation of protein kinase C, but also activate inhibitory cyclic adenosine monophosphate-dependent kinases that may autoregulate their activity. The monocyte specificity of some of these factors may provide an explanation for the macrophage predominance in chronic inflammatory lesions such as tuberculous granulomas. Monocytes/macrophages express two types of chemokine receptors, the MCP-1 receptor and the cc-chemokine receptor-1, the latter of which binds RANTES and MIP-1 and -1.

A number of agents appear to facilitate monocyte extravascular transmigration by inducing endothelial cell adhesivity to circulating monocytes. [141] These factors include IL-1, TNF-, LPS, IFN-, thrombin, and viral infections, including herpes simplex type I and cytomegalovirus. [142] Conversely, stimulation of monocytes with the cytokines IL-3 or GM-CSF, with complement chemotactic factors, with phorbol esters, or with the formylated bacterial products f-met-leu-phe increases adhesion of monocytes to the endothelium through monocyte activation.[128] [129] [142] Monocytes selectively transmigrate through endothelial junctions during inflammatory states. [143] Monocyte migration into the subendothelial space may also occur in response to chemoattractants present in the intima or media of the vessel wall. [144]

Pinocytosis/Phagocytosis

Cells, microorganisms, and proteins can bind to the monocyte or macrophage cell surface without eliciting any active response. Macrophage activation, however,

results in increased membrane adhesiveness and the simultaneous invagination of membrane and extension of pseudopodia that cause the formation of endocytic vacuoles. Small or soluble molecules are taken up by the process known as pinocytosis. Pinocytosis may be bulk, in which the molecules are in solution around the cell and are taken up into pinocytic vesicles, or may be adsorptive, in which the molecules adhere to the monocyte plasmalemma and enter the vesicles attached to it. If the attachment of these molecules is to a specific receptor, the internalization process is termed receptor-mediated endocytosis.

The uptake of larger objects is termed phagocytosis,[145] and is strongly enhanced by activating stimuli, including engagement of macrophage receptors recognizing Arg-Gly-Asp residues. Most pathogens bind by the complement receptors or MMR, or by Fc receptors if antibody-bound. Once a particle is bound to the macrophage surface, the plasma membrane expands along the particle and engulfs it using actin microfilaments lying just under the cell membrane, creating the phagosome. So called zipper phagocytosis, with circumferential, sequential interactions between the ligands on the particle and the receptor on the macrophage, appears to be the most common mechanism of phagocytosis, although coiling phagocytosis and phagocytosis into spacious vacuoles, resembling pinocytosis, have also been described.[146] [147] Cytoplasmic granules and Golgi-derived vesicles fuse with the phagosome. The subsequent phagolysosome (secondary lysosome) plays an important role in the digestion of internalized particles. Macrophage phagocytosis is important to host defenses against many potential pathogens such as *Mycobacteria*, *Leishmania*, and *Salmonella* species. Some of these organisms may survive by preventing phagosome-lysosome fusion and are able to grow within the resident alveolar macrophages without inducing macrophage activation. Intracellular infection with certain *Mycobacteria* or *Leishmania* species actually causes down-regulation of MHC class II antigen and co-stimulator B7 expression, blunting subsequent immune responses and resulting in the energy associated with chronic infections. Cytoplasmic granules and Golgi-derived vesicles fuse with the plasmalemma of the phagosome at the site of microorganism binding and subsequently discharge their granular contents across the membrane barrier, into the extracellular fluids, in a process called degranulation.

Synthesis and Secretion of Cytokines, Growth Factors, and Effector Molecules

Macrophages are capable of secreting over 100 well defined molecules, which fall into a broad number of categories (see Table 39-2).[75] [135] These categories include cytokines (lymphokines, chemokines, and monokines), growth and differentiation factors, vascular permeability factors, eicosanoids, enzymes and enzyme inhibitors, clotting factors and complement components, plasma binding proteins, and low-molecular-weight reactive oxygen and nitrogen products.

Macrophage-derived cytokines (monokines) can be grouped into several classes based on similarities in structure or biologic activities. TNF-, IL-1, and IL-1, although structurally quite distinct, have broadly overlapping activity profiles.[60] [148] [149] Monocytes and macrophages can produce, as well as respond to, a number of the chemokines, including IL-8, MGSA, IP-10, MCP-1, and MIP-1, -2, and -3.[150] [151] [152] [153] [154] [155] Although some of these factors have been reported to exhibit growth or chemoattractant activity, the precise function for each family member is still being defined. One example of the varied activity of these factors is provided by the chemokines MIP-1 and MIP-2. These cytokines are chemotactic for neutrophils in vitro and also elicit a marked inflammatory response when injected in vivo.[156]

Additional cytokines are elaborated during the inflammatory stimulation of mononuclear phagocytes. These include growth factors (PDGF, fibroblast growth factors, TGF, GM-CSF and M-CSF), the IFNs (and), and IL-6.[75] [157] [158] [159] [160] [161] All of these cytokines are produced early after stimulation, are expressed only transiently, and do not appear to depend on the synthesis of any other gene products for their expression. Many of these factors are made in response to the lipid A component of LPS[162] or to cytokines made by other cells.[163] The process is calcium dependent[164] and is generally the result of the synthesis or increased stability of specific mRNA. Of these secreted proteins, only IL-1 is not made with a signal peptide.[165] [166] Some of the TNF produced is attached to the monocyte membrane,[167] but most is secreted into the medium.[168] These factors contribute to the inflammatory and hematopoietic effects of macrophages, but also play a role in effecting and regulating macrophage cytotoxic activity itself.[72] [75] [89] [103] For example, TNF- induces the expression of IL-1 and IL-6 by macrophages.[88] [169] Conversely, IL-1 stimulates the synthesis of TNF-, IL-6, CSF-1, GM-CSF, and IL-1 itself.[88] [170] [171] The IFNs are potent inducers of a number of monokines. IFN- stimulates the release of IL-12, contributing to natural killer cell and T-lymphocyte activation. IL-10, produced by phagocytosing macrophages (and Th2 lymphocytes), feeds back on the macrophage to decrease the production of inflammatory monokines. Combinations of cytokines can thus have additive, synergistic, or antagonistic regulatory effects on monokine production by macrophages.

Monokines may have interrelated functions that affect specific cells involved in inflammation as well as the general processes of host metabolism. For example, monokines can alter endothelial cell function.[172] IL-1 and TNF can cause fever,[165] [173] [174] muscle breakdown,[174] and changes in liver metabolism.[175] IL-1 and TNF both appear to be involved in the cachexia associated with chronic infection and malignancy.[176] TNF may play a role in the anemia of chronic disease.[177] IL-1 indirectly results in the synthesis of acute-phase reactants, through stimulation of IL-6 synthesis and secretion. These acute-phase reactants are secreted into the circulation; they include C-reactive protein, fibrinogen, $_1$ -antitrypsin, haptoglobin, and serum amyloid A. In addition, IL-6 causes a decrease in the synthesis of albumin, fibronectin, and transferrin.[178] The latter effect is responsible for the decrease in total iron-binding capacity associated with inflammation. The CSFs synthesized and secreted by monocytes and macrophages stimulate the appropriate progenitor cells in the bone marrow to make neutrophils and monocytes in response to infection. The interactions of these factors appear to play an important role in the regulation of cell growth, protein synthesis, cell movement, and cell-cell interactions. Activated monocytes/macrophages are potent producers of eicosanoids synthesized from arachidonic acid. The products of the cyclo-oxygenase pathway include thromboxanes and prostaglandins, and the lipoxygenase pathway produces secreted leukotriene B4 (LTB4) and 5-hydroxyeicosatetraenoic acid (5-HETE).

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PATHOGEN CONTROL

Macrophage have been shown to be critical for arresting the proliferation of, or killing, the facultative and obligate intracellular bacteria, protozoa, and fungi. After activation, macrophages synthesize an array of proteins that regulate and catalyze the production of toxic oxygen intermediates. [179] [180] Toxic oxygen intermediates appear in the phagolysosome and kill microorganisms, primarily those that produce pyogenic infections. Intracellular killing in macrophages, as in granulocytes, is mediated by an oxygen-dependent system that uses hydrogen peroxide, a halide such as chloride, iodide, or a bromide, and a myeloperoxidase-like enzyme. [181] Macrophages, in contrast to monocytes, lack the myeloperoxidase enzyme but may have a similar analogous enzyme; alternatively, their cytocidal activity may be related to the direct activity of reactive oxygen species. [182] [183] Highly reactive oxygen radicals, including superoxide, hydrogen peroxide, singlet oxygen, and the hydroxyl radical, are produced by a membrane-associated NADPH oxidase and associated reactions, and these reactive oxygen species are available for the microbicidal process. Ferric ion may react with hydrogen peroxide and superoxide to produce hydroxyl radicals with strong oxidant properties over relatively short distances, as in a phagosome. Superoxide may be released into surrounding tissue or into phagocytic vacuoles. Superoxide leads to alkalinization of the phagolysosome, activating the neutral and cationic proteases to kill ingested organisms. Lysozyme activity has not conclusively been shown directly to kill ingested bacteria, and may be limited to degrading dead organisms.

Oxygen-independent bactericidal mechanisms also exist, as evidenced by bacterial killing under anaerobic conditions or in the absence of an oxidative burst, and antimicrobial activity by phagocytes with impaired oxidated metabolism (e.g., chronic granulomatous disease). [184] Oxygen-independent mechanisms include granule-associated proteins with microbicidal activity (neutral proteases, lysosomal hydrolases, perforin or perforin-like activities), [185] as well as cationic proteins that may kill bacteria and fungi through their action on the microbial plasma membrane. [186] Numerous bactericidal secretory products, such as lysosomal enzymes, lysozyme, and other factors from macrophages, have also been described. [187] Most of the antimicrobial cationic proteins belong to one of two families, the defensins and the serprocidins. [188] [189] The defensins are basic peptides of 2943 amino acids, and are active against a variety of bacteria. The serprocidins include elastase, cathepsin G, proteinase 3, azurocidin, and BPI. The latter two appear specific for gram-negative bacteria by virtue of their interaction with LPS. Oxygen-independent cytocidal mechanisms may be more selective in their expression than oxygen-dependent mechanisms. [181] Although there is in vitro evidence for cytocidal activity against bacteria, fungi, and some viruses, [190] and resistance to defensins has been suggested a mechanism used by organisms like *Salmonella* to survive in phagolysosomes, there are no comprehensive studies of human monocyte and macrophage oxygen-independent cytocidal mechanisms. The biologic importance of oxygen-independent cytocidal mechanisms thus remains unclear.

In addition to microbicidal capacity, macrophages possess microbiostatic activity for a variety of intercellular organisms. [187] [191] [192] [193] [194] The capacity for macrophages to inhibit the growth of intracellular microorganisms depends on previous exposure to the activating agent and, in some cases, to chemical signals originating from the microbe itself. [193] [195] An important contribution to microbiostasis appears to be the synthesis of small, diffusible molecules by the macrophage. The production of NO has recently been recognized as a major mechanism of monocyte and macrophage toxicity toward microorganisms and tumor cells. [196] [197] The inducible, calmodulin-bound isoform of NOS is monocyte/macrophage specific and generates large amounts of NO for long periods of time, in contrast to other isoforms. A cytocidal type of monocyte/macrophage activation program is required for inducible NOS expression, with priming by IFN- followed by a second signal, such as IL-2, TNF-, LPS, or other bacterial polyanions. [198] NO produced by activated macrophages is cytostatic or cytotoxic for a variety of invading microorganisms. The effector functions of NO appear to involve iron-dependent reactions, the inhibition of enzymes, and perhaps formation of perioxinitride. [199] Killing of *Leishmania* amastigotes by IFN--activated macrophages and macrophage fungistasis are both mediated by NO. [200] [201]

The originally identified macrophage-activating factor, IFN-, enhances macrophage killing of intracellular pathogens, including *Candida* species, *Toxoplasma gondii*, and mycobacteria. [202] Therapeutic activation of the circulating blood monocyte has been achieved after intravenous, intradermal, intramuscular, and subcutaneous administration of IFN- in patients with cancer, lepromatous leprosy, acquired immunodeficiency syndrome, and chronic granulomatous disease. Activation of the alveolar macrophage has also been induced in a compartmentalized fashion by aerosolized IFN-. [203]

Monocytes and macrophages express the CD4 membrane molecule and can serve as a reservoir for the human immunodeficiency virus (HIV), and may be responsible, in part, for the persistence of HIV infections. The predominant infected cell found in cerebrospinal fluid from patients with HIV meningoencephalitis is a macrophage. The brain macrophage, the microglia cell, and macrophage-derived multinucleate giant cells have been found to be infected by HIV. [204] [205] [206] [207] HIV can also be found in pulmonary macrophages, epidermal Langerhans cells, and lymph node macrophages. Not all macrophages, however, appear susceptible to HIV infection. Kupffer cells, for example, have not been shown to be infectable by HIV. Cytokines may play a significant role in HIV-macrophage interactions. Whereas proinflammatory cytokines like TNF- can enhance virus production from infected macrophages, the Th1-type cytokines (IFN-) and the Th2-type cytokines (IL-4, IL-10, and IL-13) inhibit HIV in macrophages. [208]

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IMMUNOLOGIC FUNCTIONS

Antigen Presentation

The monocyte lineage gives rise to a variety of cells (APCs) involved in the processing of antigen, which is required for the development of an immune response. Langerhans cells of the skin, interdigitating cells of the thymus, reticular cells of the spleen, and dendritic cells of lymph nodes are all active in the processing of exogenous antigen. The process of presentation of exogenous antigens to T cells requires the antigenic peptides to be recognized in association with MHC class II molecules on the macrophage cell surface. This molecular association occurs within lysosomes, as newly synthesized class II MHC molecules enter the endosomic pathway, are processed, bind noncovalently to the peptide products of degraded antigens previously endocytosed, and are then transported as a complex to the cell surface. Most antigens presented in the context of class I MHC molecules are normal cellular (endogenous) proteins. These are degraded by an adenosine triphosphate-dependent, large proteolytic complex (proteosome). These peptides are then transported to the endoplasmic reticulum, where they bind to class I molecules before movement to the cell surface. T-lymphocyte interaction with antigen presented by mononuclear cells in the context of MHC molecules involves direct cell-cell contact, and this process is further regulated by interaction with co-stimulatory molecules on the macrophage, such as B7, which engages CD28 on the lymphocyte. The activation of the T lymphocyte may be enhanced by the elaboration of monokines such as IL-1, IL-12, and TNF-. Subsequent T- and B-lymphocyte collaboration results in immunoglobulin production. APCs in certain tissues, such as lung, are not phagocytic and may internalize antigen by pinocytosis [209] ; nonetheless, these cells retain Fc and C3b receptors on their surface for interaction with T lymphocytes and antigen. Macrophages can also present processed antigen to B cells to elicit a humoral response. [210] [211]

Dendritic Cells

Steinman and Cohn originally described the dendritic cell in 1973 as a rare cell with a distinctive appearance. [212] The so-called dendritic cell is distinctive because of its long cytoplasmic processes and intracellular endosomes. Dendritic cells are found in virtually all organs except the brain.

The lineage of dendritic cells was originally postulated to be unique from that of macrophages. Dendritic cells express high levels of MHC class II molecules, but also unique membrane molecules such as CD1, CD83, p55, and s100b.[213] Despite their distinctive membrane phenotypes, more recent experimental evidence using cytokines (such as GM-CSF and TNF-) to stimulate the growth of bone marrow progenitors suggests that granulocytes, macrophages, and dendritic cells all share a common lineage.[214] [215] [216]

The in vivo function of dendritic cells is suggested by their ability to stimulate naive T lymphocytes to proliferate in vitro. In a mixed lymphocyte reaction, dendritic cells stimulate a 100-fold stronger proliferative response than unfractionated leukocytes. [217] Dendritic cells appear to be particularly potent at stimulating a primary T-cell immune response. Once T cells are stimulated, they are relatively more responsive to other APCs such as monocytes or B cells. Dendritic cells may be the only cells capable of stimulating a naive T lymphocyte and initiating a primary immune response.

The tissue distribution and antigen-presenting function of dendritic cells have led to the suggestion that dendritic cells initiate the immune response by processing antigen, migrating through tissues, and stimulating naive T lymphocytes. In the periphery of the body, dendritic cells pick up antigen and deliver it to lymphoid organs by selectively migrating through tissues. In the skin, the Langerhans cells appear to provide the dendritic cell function. Langerhans cells are less differentiated than dendritic cells in lymphoid organs. Langerhans cells can, however, mature into dendritic cells with the same immunostimulatory function. Maturation of the Langerhans cell appears to depend on cytokines such as IL-1 and GM-CSF. [218]

Dendritic cells are capable of remarkably efficient processing of extracellular antigen. In most cases, the peptide antigen is taken up and loaded onto MHC class II molecules. In some conditions, dendritic cells appear to be capable of loading peptide antigens onto MHC class I molecules as well. Dendritic cells, particularly immature dendritic cells, are capable of several types of antigen processing. Dendritic cells can pick up soluble antigens by constitutive macropinocytosis. [114] Macropinocytosis is a specialized type of actin-dependent, fluid-phase endocytosis. Using this type of processing, dendritic cells can internalize

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half of their own volume in 1 hour.[114] Dendritic cells are also capable of receptor-mediated endocytosis, which is a more efficient process because it permits the effective uptake of antigen at a 100-fold lower concentration of antigen. An example of receptor-mediated antigen uptake is the interaction of antigen-IgG complexes that are internalized after binding FcRII (CD32). [114] Dendritic cells express FcRI for the internalization of antigen-IgE complexes. [219] Dendritic cells also appear to be capable of a form of receptor-mediated antigen uptake intermediate between nonspecific fluid uptake and antigen-specific immune complex binding. This process is mediated by so-called pattern recognition receptors. The recently described recycling MMRs appear to be responsible for mediating this type of recognition. MMRs allow dendritic cells efficiently to internalize mannosylated proteins. [220] These receptors are functionally useful as pattern recognition receptors because mannosylated proteins are present on the surface of many microbes.

Dendritic cells process the internalized antigen in endosomal/lysomal compartments. The internalized proteins are processed and loaded onto newly synthesized MHC class II molecules.[221] The MHC-peptide complexes are expressed on the cell surface and retained on the membrane for several days. Once on the cell surface, the MHC-peptide complexes are available for ligand binding with antigen-specific T lymphocytes.

When dendritic cells migrate from the periphery into lymphoid organs, they localize in discrete compartments in peripheral lymphoid organs. Dendritic cells appear to home to specific T-cell areas of lymph nodes and the spleen. [213] In these specialized areas, dendritic cells present antigen to T lymphocytes. A second type of dendritic cell appears to be located in B-cell areas of lymphoid organs. These dendritic cells are specialized for the presentation of native antigen as immune complexes to B cells and are found in germinal centers. Because of their unique location and specialized function, these dendritic cells are called follicular dendritic cells.

The clinical implications of a central regulatory role for dendritic cells have centered on the use of dendritic cells as adjuvants to immunotherapy. The ability reliably to isolate and grow dendritic cells ex vivo could provide an opportunity to load dendritic cells with antigen and reinfuse the cells into the body. [222] Such an approach would have implications for the generation of both tumor- and virus-specific immune responses. [217]

Dendritic cells may also play a role in immune deficiency states and provide a potential target for HIV therapy. HIV-1 has recently been shown readily to enter immature dendritic cells. The presence of intracellular HIV-1 becomes important with the formation of dendritic cell-T-cell conjugates. [223] HIV-1 causes dendritic cells

and memory T cells to fuse. The resulting heterokaryons or syncytia are sites of viral replication and cell death. [224]

Cellular Cytotoxicity

Macrophages have been proposed as the first line of defense against neoplastic diseases by virtue of the selective cytotoxicity gained by macrophages against transformed cells, but not normal cells.[225] Toxicity by monocytes and macrophages to tumor cells can be divided into two types.

Antibody-Dependent Cellular Cytotoxicity

Antibody-coated target cells are recognized by the Fc receptor on monocytes. This recognition enables the monocyte to kill selected target cells with efficiencies determined by the immunoglobulin class and the density of immunoglobulin bound to the target cell and by the Fc receptor type involved on the effector cell. This process is termed antibody-dependent cell-mediated cytotoxicity (ADCC), and this tumorcidal activity has been demonstrated on a number of tumor cell lines in culture.[226] [227] [228] Monocyte tumorcidal activity may be induced by a variety of agents, including LPS, IFN-, interleukins, TNF, phorbol esters, [125] and GM-CSF. This antitumor activity appears to be markedly enhanced by M-CSF.[226] [227] [229] In vivo, the importance of tumor-associated macrophages in tumor rejection remains unclear. Tumor-associated macrophages have the potential to inhibit tumor growth and destroy neoplastic cells. [230] [231] [232] Conversely, macrophages can produce growth factors and promote angiogenesis, resulting in possible paracrine stimulation of neoplastic cells. The clinical importance of ADCC is suggested by regression of neuroblastoma and melanoma in culture when murine monoclonal antibodies are added. [233] [234] Studies of human cells engrafted into nude mice also indicate that ADCC may be an important mechanism for tumorcidal activity.[226]

Antibody-Independent Cellular Cytotoxicity

Recognition of specific target cell structures alone by monocytes and macrophages is a possible stimulus for spontaneous cellular cytotoxicity. The ability of monocytes that have not been previously activated to kill tumor target cells remains controversial. [175] Induction of non-receptor-mediated cytotoxicity can be achieved in monocytes using various soluble and particulate mediators. Phorbol esters, zymosan-activated serum, and aggregated IgG [125] [235] are capable of stimulating monocyte-mediated target cell lysis and the oxidative burst. The maturation of monocytes to macrophage in long-term culture enhances tumorcidal activity. [236]

Mechanisms of Cellular Cytotoxicity

The mechanism of tumor cell lysis appears to involve cell contact [237] and the secretion of a variety of cytotoxic mediators. [238] [239] The cytocidal mechanisms for macrophages may differ from those of their monocyte progenitors because myeloperoxidase is absent [240] and the energy needed for killing may depend more heavily on the respiratory chain [241] than on glycolysis. Arginine-derived NO, synthesized by the inducible isoform of NOS, is also a major mediator of tumor cytostasis by activated macrophages.[197] [242] [243] Potential cellular targets of NO in tumor cells include the oxidoreductases in the electron transport chain, the Krebs cycle enzyme aconitase, and ribonucleotide reductase. [242] [243] [244] [245] NO-independent mechanisms that facilitate tumor cell destruction by activated macrophages also exist, and may include TNF and perforin production.[246] The production of TNF by activated monocytes appears to play an important role in some cytotoxicity, [228] [229] although TNF may not be essential in all systems.[226]

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TISSUE MAINTENANCE

Tissue-resident macrophages are capable of playing a wide variety of roles because of their ability to develop specialized functions on extravasation into certain tissues.

Waste Management

Littoral Macrophages

Resident tissue macrophages were formerly referred to as histiocytes. In organs that act as filters for blood and lymph, such as the spleen, liver, lungs, and lymph nodes, the macrophages (littoral macrophages) are situated to facilitate the clearance of pathogenic organisms and senescent cells. This filtering role of the littoral mononuclear phagocyte system led to the older designation of reticuloendothelial system. The recognition of the monocyte/macrophage lineage as well as the phagocytic function of these cells has led to the newer mononuclear phagocyte system nomenclature.

Spleen

Macrophages are found in all parts of the spleen. Microheterogeneous populations of macrophages in the marginal zones serve specific scavenger and pathogen control functions, whereas those in the white pulp, in close association with lymphocytes and germinal centers, regulate B-cell differentiation and antigen presentation.[247] Macrophages in the red pulp and sinuses, where intimate contact occurs between red blood cells and macrophages during the sluggish percolation of blood through this circulation, cull senescent, deformed, and parasitized erythrocytes from the circulation.

Liver

The portal circulation of the liver flows through the spaces of Disse before entering the hepatic venous system. These spaces are lined with liver macrophages, also known as Kupffer cells, allowing intimate contact of the elements of blood with these phagocytic cells. The Kupffer cells may also play a role in clearing ingested and bacterial toxins from the portal circulation.

Lymph Nodes

Macrophages exist in all areas of the lymph nodes and are especially abundant in the medullary zones, adjacent to efferent lymphatic and blood capillaries. Antigen presentation by macrophages to T lymphocytes occurs in these areas.[248]

Bone Marrow

Macrophages are found throughout the bone marrow, in hematopoietic islands and on the walls of the marrow sinuses, where they play a clearance function, especially in states of ineffective hematopoiesis.[249] In lysosomal storage diseases such as Gauchers disease, large inclusions build up within marrow macrophages (as well as hepatic and splenic macrophages) because of the inability of the cells to break down their lysosomal contents.[54]

Lung

In the lung, macrophages reside both in the interstitium of alveolar sacs and freely in the spaces. At least four types of pulmonary macrophages have been identified, including alveolar, interstitial, intravascular, and dendritic or Langerhans cells.[250] The alveolar macrophages lie within the surfactant film, exposed to air, and represent a first line of defense in the lung. These cells have a role in the clearance of inhaled microorganisms and particulate matter,[251] as well as in the removal of endogenous materials like surfactant proteins. Inhaled foreign material such as asbestos or particulates from smoking is found in these alveolar macrophages. Hemosiderin-laden alveolar macrophages may be diagnostic of recurrent pulmonary hemorrhage in idiopathic hemosiderosis or Goodpastures syndrome, in which iron is sequestered in lung tissue.

Other Tissues

Mononuclear phagocytes are also found throughout the alimentary tracts, particularly in the submucosal tissues and villi of the small intestine. They are present in the central nervous system, especially after injury. Mammary gland macrophages appear in the milk during lactation and have been implicated as a potential source of postnatal transmission of the HIV virus.[252]

Lipid Metabolism and Atherosclerosis

Monocytes take up denatured or oxidized LDL and native VLDL by receptor-mediated endocytosis [119] [120] [121] [122] through the MSR. The LDL enters the lysosomal compartments and free cholesterol is liberated and esterified in the cytoplasm. The cholesterol-laden foam cell, which defines the earliest stage in atherosclerosis, is of monocytic origin.[253] [254] Monocytes and macrophages in culture exposed to sufficient quantities of denatured LDL can acquire the appearance of foam cells after engorgement of the lipid.[119] Plasma LDL that has undergone minor chemical oxidative modification can potently stimulate MCP-1 expression in cultured vascular cells, potentially mediating recruitment of circulating monocytes.[255] Lipoproteins in circulating blood may also facilitate adhesion of circulating monocytes to endothelium. The monocyte-derived macrophage plays a significant role in all stages of atherosclerotic plaque formation. From the initial events in atherogenesis (increased vascular permeability and increased monocyte adherence and intimal recruitment) to the advanced disease (monocyte/macrophage-derived foam cells, cell necrosis, and formation of a necrotic plaque), atherosclerotic pathogenesis appears to involve a strong monocyte/macrophage inflammatory component. [256] [257] [258] Proliferation of macrophages in atherosclerotic lesions has been observed.[259] Experimental knockout of the MSR genes have demonstrated the contribution of these receptors to the atherosclerotic process.[256] The primal role of the MSRs was facilitation of the uptake and clearance of microorganisms and senescent cells, but this useful function is suborned in the presence of circulating oxidized lipids to render the monocyte the inciting agent of atherosclerosis.

Clearance of Senescent or Damaged Cells

Senescent or defective red blood cells (such as those formed in hereditary spherocytosis) may be sequestered in the spleen and removed from the circulation by splenic macrophages, with subsequent degradation of cellular material and return of iron to the iron pool. The presence of antibody or complement components on red blood cells enhances macrophage erythrophagocytosis in the spleen and the bone marrow and plays a central role in the pathophysiology of immune-mediated hemolytic anemias.

Macrophages have the ability to recognize circulating proteins or cell-associated proteins that have been modified by nonenzymatic reactions with extracellular glucose over time. These glycated proteins are taken up through a specific cell surface receptor designated the advanced glycation end-product receptor. [260] Binding to this receptor results in activation of the macrophage with release of cytokines. Macrophages are able to recognize certain sugar residues on tumor cells by specific cell surface lectin receptors. An *N*-acetyl galactosamine/galactose-specific receptor on liver macrophages mediates the attachment of desialylated cells to liver macrophages.[261]

Tissue Growth and Repair

Wound Healing and Angiogenesis

Macrophages are required for angiogenesis and wound healing. [31] [262] Monocytes enter a wound within the first or second day (inflammatory phase of wound repair), where they differentiate, and these macrophages become the predominant immune cell in the tissue by days 35 (proliferative phase). [263] The recruitment of the monocytes is determined by a chemoattractant gradient of MIP-1 and -2, MCP-1, and MGSA. Macrophage depletion studies [31] have shown that macrophages are required for clearance of senescent cells and debris in the wound, for deposition of new fibrous tissue (collagen synthesis and fibroblast proliferation, and for stimulation of angiogenesis).[264] Activation of the recruited macrophages can occur by products released from platelets (TGF- and PAF), thus linking the hemostatic and inflammatory phases of wound repair, and also as the result of exposure to, and phagocytosis of, microorganisms in the wound. Macrophages can also be activated by the low PO_2 and high lactate concentrations in inflamed or wounded tissue.

Activated macrophages in wounds secrete large amounts of

fibroblast and epithelial cell growth factors such as TGF-, fibroblast growth factors, epidermal growth factors, PDGFs, and insulin-like growth factors. Many of these growth factors have overlapping angiogenic activities. Wound macrophages also release the inflammatory mediators IL-1, IL-6, and TNF-, which have mixed activities on cell growth and angiogenesis. In addition, macrophages secrete several complement inhibitors such as $_2$ -macroglobulin, $_1$ -protease inhibitor, and C3 inhibitor, and a wide range of proteases as well as neutral or metalloprotease inhibitors that facilitate reduction of inflammation and tissue remodeling, respectively. [135] Macrophages directly and indirectly result in the production of extracellular matrix components necessary for wound repair, including fibronectin, proteoglycans, and thrombospondin-1. Macrophages are able to induce neovascularization, angiogenesis, and endothelial cell mitogenesis. [265]

Hematopoiesis

In the adult bone marrow and fetal liver, resident tissue macrophages express cell surface receptors (sialoadhesin) that bind developing myeloid and erythroid cells without causing phagocytosis.[266] [267] Immature blood cells, especially erythroblasts, are found clustered around a central macrophage that performs a critical but not well characterized nurse function in hematopoiesis. Monocytes and macrophage are capable of secreting a number of hematopoietic growth factors, including GM-CSF, G-CSF, erythropoietin, IL-1, and M-CSF. [135]

Bone and Calcium Metabolism

The multinucleate osteoclast, which is responsible for bone resorption, remodeling, and calcium homeostasis is derived from a monocyte precursor. The earliest studies demonstrating the hematopoietic origin of osteoclasts used a parabiotic union of osteopetrotic mice. [268] Culture of circulating monocytes with bone stroma is sufficient to induce osteoclastic differentiation, and this process is stimulated by parathyroid hormone (PTH) and vitamin D_3, and inhibited by calcitonin. [198] The differentiation process depends on M-CSF and the product of the c-*fos* gene,[269] whereas osteoclast activity depends on the function of the c-*src* gene product, which may mediate PTH signal transduction.[270] Mature osteoclasts express tartrate-resistant acid phosphatase, PTH, and calcitonin receptors. Bone resorption activity by the osteoclast is stimulated by PTH and vitamin D_3, and also by some of the products of monocytes themselves, including IL-1, TNF-, TGF-, M-CSF, and reactive oxygen intermediates. TGF-, estrogens, IL-4, and IFN- inhibit osteoclast activity. Osteoclasts themselves can secrete IL-6, IL-1, and TGF-2.

Nervous System

Monocytes are uniquely privileged to cross the blood-brain barrier, initially in response to programmed neuronal cell death during development, or later through recruitment in inflammatory or degenerative states. Over a period of 46 days, the surrounding neural tissue induces a profound differentiation of these monocytes, resulting in the formation of microglia, with fine-branching processes extending throughout the neural net. [5] In the pituitary, microglia remove fragments from viable neuroendocrine cells, perhaps processing or regulating the release of neuropeptides. [14] Macrophages also appear to play a critical role in nerve regeneration. In addition to the path clearing achieved by removal of axonal and myelin debris, macrophages also appear to have a direct effect on regrowth of neurites and Schwann cells, resulting in the production of nerve growth factor and insulin-like growth factor. [271]

Monocytes and Macrophages as Therapeutic Agents

Because of their unique characteristics, macrophages have long been identified as potential vehicles for the targeting of genes or other therapeutics to the site of disease. Their relatively long life span and their residence in tissue have made them attractive targets for gene therapy in the treatment of storage diseases and enzyme- or hormone-deficiency diseases. Similarly, their ability to travel through the blood to sites of inflammation or malignancy and selectively intercalate into the diseased tissue has suggested a possible role for monocytes and macrophages in the delivery of cytokines, antineoplastic proteins, or toxins specifically to the tumor. The early and critical role of monocytes in atherosclerosis may again facilitate targeting of genes or other products to the nascent atherosclerotic plaque. The technical difficulties, however, of achieving stable, efficient transfection of this very-slow-growing blood cell have hindered the development of such indirect monocyte/macrophage therapies to date.[272]

Monocytes and macrophages may also have potential for manipulation as direct effectors of an anticancer response. The selective cytotoxicity of activated macrophages against transformed cells while sparing normal cells [225] has suggested their use in adoptive transfer immunotherapy protocols. In these instances, monocytes are activated in vivo or in vitro using a combination of cytokines and sometimes exposure to tumor antigens. Such activated monocytes and macrophages have been shown to home preferentially to tumor and to enhance tumor destruction. The use of these unique cells to deliver therapeutics or directly to effect therapy themselves has great potential.[273]

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Chapter 40 - Eosinophilia, Eosinophil-Associated Diseases, and the Hypereosinophilic Syndrome

Steven J. Ackerman
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HISTORICAL BACKGROUND, ETIOLOGY, AND PATHOPHYSIOLOGY OF HYPEREOSINOPHILIA

Following the initial identification of the eosinophilic granulocyte by Paul Ehrlich in 1879, an extensive cataloguing occurred over the next 100 years of diseases and conditions characterized by blood and/or tissue eosinophilia (Table 40-1). However, the specific functional roles of eosinophils in host defense, allergic reactions and other inflammatory responses, tissue injury, and fibrosis have only begun to be fully delineated and understood during the past 25 years. [1] Recent research has now served to characterize the unique cellular characteristics of activated tissue and peripheral blood eosinophils, their preformed granule protein constituents and inducible lipid, oxidative, and cytokine products, focusing on the eosinophils pro-inflammatory and cytotoxic potential in the pathogenesis of allergic, parasitic, neoplastic, and a variety of other idiopathic disease processes. [2] Recognition of the eosinophil as a proinflammatory effector cell in bronchial asthma has in part fueled the surge in interest in this granulocyte in asthma and other respiratory diseases, [3] and the recent epidemic of eosinophil myalgia syndrome related to ingestion of tainted L-tryptophan [4] served to markedly increased public interest and awareness of the eosinophil.

Studies of the biochemistry, biologic activities, and in particular, the localization in tissues of the distinctive enzymatic and non-enzymatic cationic protein constituents of the eosinophils secondary or specific granule provided the first convincing evidence for the role of this granulocyte in the pathogenesis of inflammation and tissue damage in eosinophil-associated diseases. [2] In addition, identification of eosinophil-specific granule constituents in tissues by a variety of sensitive and specific immunochemical methods provided additional evidence for the participation of this leukocyte in diseases not normally associated with tissue or blood eosinophilia, e.g., certain skin diseases. [5] Investigations over the past 1015 years of the biochemistry, functions, and localization in tissues of these unique enzymatic and non-enzymatic cationic proteins have now provided compelling evidence supporting a pathologic effector role for the eosinophil in directly inducing tissue damage. [6] These distinctive cationic granule protein constituents include the major basic protein (MBP), the eosinophil peroxidase (EPO), and the ribonucleases eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). Eosinophils have also been shown to have the capacity to express a variety of toxic oxidative intermediates, [7] eicosanoid and other lipid mediators of inflammation [8] and most recently, inflammatory and hematopoietic cytokines that are integral to the normal and pathologic role of this granulocyte in health and disease. [9] This chapter provides an overview of current understanding of the inflammatory and pathologic activities of this granulocyte in human disease, focusing principally on the consequences of hypereosinophilia in the idiopathic hypereosinophilic syndrome (HES).

Patients presenting with hypereosinophilia provide an often daunting diagnostic challenge to physicians who must navigate multiple medical subspecialties in order to wade through the long and diverse list of potential causes (Table 40-1). This process, an often frustrating experience for both physician and patient, may result in an indeterminate diagnosis of hypereosinophilia of unknown etiology. The hypereosinophilic syndrome (HES) comprises a group of myeloproliferative disorders of unknown etiology that are marked by sustained overproduction of eosinophils and tissue infiltration. In addition to the striking and sometimes profound eosinophilia associated with the syndrome, idiopathic HES is characterized by a predilection for end organ damage most commonly involving the heart, with the development of eosinophilic endomyocardial fibrosis and related cardiac pathologies. However, because heart disease can also develop with eosinophilias of known etiologies, e.g., tropical eosinophilia and certain cancers, [10] [11] the cardiac manifestations of idiopathic HES are not unique to this syndrome. The currently defined criteria for HES as originally presented by Chusid and colleagues, [12] and which serve to distinguish HES patients from those with identifiable (reactive) causes for their eosinophilia include: (1) persistent eosinophilia of >1,500 eosinophils/mm ^{3} for more than 6 months, (2) exclusion of other potential etiologies for the eosinophilia including parasitic, allergic, or other causes, and (3) presumptive signs and symptoms of organ system dysfunction or involvement which appears related to the eosinophilia or is of unknown cause in the clinical presentation. The recent identification of subgroups of patients with profound idiopathic eosinophilia characteristic of HES, but who do not go on to develop end organ damage characteristic of HES (e.g., patients with episodic angioedema with eosinophilia [13] [14]), complicates the diagnosis, treatment, and management of patients with profound eosinophilia of unknown etiology. This chapter reviews the distinguishing as well as more variable hematologic and clinical features of HES that should assist the physician in separating this syndrome from more common (reactive) eosinophilias of known etiology. This will include a discussion of the current understanding of HES etiology and pathogenesis, as well as therapeutic approaches and strategies for managing this complex syndrome.

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EOSINOPHIL MORPHOLOGY

The eosinophil is similar morphologically to a mature or band neutrophil except for a two-lobed nucleus and the presence of numerous bright orange spherical cytoplasmic granules (Plate 40-1). Eosinophils contain three distinct membrane-bound granule populations that are produced during their differentiation from hematopoietic progenitors in the bone marrow (Fig. 40-1). These include round, uniformly electron dense primary granules present mainly at the eosinophilic promyelocyte/myelocyte stages, specific or secondary granules containing an electron dense crystalloid core surrounded by a less dense granular matrix (>95% of granules in mature eosinophils and the

TABLE 40-1 -- Diseases, Syndromes, and Conditions Commonly Associated with Peripheral Blood Eosinophilia and/or Tissue Eosinophils[a]

Infectious Agents	**Reactions to Cytokine Therapies**
Parasitic	IL-2 and IL-2 plus lymphokine activated killer (LAK) cells
Tropical Eosinophilia	GM-CSF for chemotherapy-induced neutropenia
Visceral Larval Migrans (VLM, Toxocariasis)	
Helminth Infections	**Cutaneous Disorders**
Filariasis (Wuchereria bancrofti, Brugia malayi)	Atopic dermatitis
Onchocerciasis	Immunologic skin diseases
Schistosomiasis	Scabies
Fascioliasis	Eosinophilic cellulitis (Wells syndrome)
Paragonimiasis	Episodic angioedema with eosinophilia
Strongyloidiasis	Chronic idiopathic urticaria
Trichinosis	Bullous pemphigoid
Hookworm	Herpes gestationis
Ascariasis	Recurrent granulomatous dermatitis
Echinococcus/hydatid disease	
Nonparasitic	**Immunodeficiency Syndromes**
Coccidiomycosis	Wiskott-Aldrich
Chlamydial pneumonia of infancy	Selective IgA deficiency with atopy
Scarlet fever and pneumococcal pneumonia (convalescent phase)	Hyper-IgE recurrent infection syndrome (Job syndrome)
Cat scratch disease	Swiss-type and sex-linked combined immunodeficiency
Cryptococcus (CSF eosinophilia) HIV	Nezelofs syndrome
	Graft-versus-host disease
Allergic Diseases	**Connective Tissue Diseases**
Asthma (atopic and intrinsic, nasal polyps, aspirin intolerance syndromes)	Vasculitis/collagen vascular disorders
Bronchopulmonary aspergillosis	Hypersensitivity vasculitis
Allergic rhinitis	Allergic granulomatosis with angiitis (Churg-Strauss syndrome)
Urticarias (acute allergic and chronic idiopathic)	Serum sickness
Atopic dermatitis	Eosinophilic fasciitis
Acute drug (hypersensitivity) reactions (interstitial nephritis, cholestatic hepatitis, exfoliative dermatitis)	Sjögren syndrome
	Rheumatoid arthritis (severe)
Respiratory Tract Disorders	**Neoplastic and Myeloproliferative Diseases and Syndromes**
Hypersensitivity pneumonitis	Neoplastic
Allergic bronchopulmonary aspergillosis	Eosinophil leukemia
Eosinophilic pneumonia	Lymphomas (T cell, Hodgkins disease)
Transient pulmonary infiltrates (Löefflers syndrome)	Chronic myelogenous leukemia
Prolonged pulmonary infiltrates with eosinophilia (PIE syndromes)	T-cell lymphocytic leukemia
Tropical pulmonary eosinophilia (TPE)	Myelomonocytic leukemia with bone marrow
Bronchiectasis	Eosinophilia (M4Eo, inversion 16)
Cystic fibrosis	Solid tumors (mucin secreting, epithelial cell origin, ovarian)

Endocrinologic Disorders	Idiopathic hypereosinophilic syndrome (HES)
	Angioimmunoblastic lymphadenopathy
Addisons disease	Angioblastic lymphoid hyperplasia (Kimuras disease)
	Systemic mastocytosis
Gastrointestinal Diseases	
Inflammatory bowel disease (IBD)	**Rare Causes**
Eosinophilic gastroenteritis	Chronic active hepatitis
Allergic gastroenteritis (young children)	Chronic dialysis
Celiac disease (usually tissues)	Acute pancreatitis
	Postirradiation
Toxic Reactions to Injected Agents	Hypopituitarism
Eosinophil myalgia syndrome (L-tryptophan)	
Toxic oil syndrome	

[a] *Modified and updated from Mahanty and Nutman,[84] with permission.*

morphologic trademark of this granulocyte), and less well characterized small granules, which are sites for hydrolytic enzymes such as acid phosphatase and arylsulfatase, may contain catalase, and may be functionally analogous to lysosomes and peroxisomes of other cells. The large specific granule is the major repository for the eosinophils cytotoxic and pro-inflammatory cationic proteins (Fig. 40-1) which also confer the unique and characteristic tinctorial (eosinophilic) properties of this cell as originally identified by Paul Erlich in 1879. Eosinophils also contain lipid bodies, non-membrane-bound and lipid-rich organelles often confused with granules and present in many types of leukocytes and other cells. [8] Normal blood eosinophils contain greater numbers of lipid bodies than neutrophils and the numbers of lipid bodies increase during eosinophil activation in vitro and engagement in inflammatory reactions in vivo. [15] Although the functions of lipid bodies are incompletely understood, they incorporate fatty acids such as arachidonate and likely serve as intracellular depots for its storage and metabolism,

Figure 40-1 Schematic structure of the mature human eosinophil. Primary (1) and secondary (2) granules, small granules (SG) and non-membrane-bound lipid bodies (LB), are shown, as are the major cell surface cytokine, immunoglobulin and complement receptors. The subcellular localizations of the eosinophil granule cationic proteins, MBP, EPO, ECP, and EDN in the secondary granule and the Charcot-Leyden crystal (CLC) protein (galectin-10) in the coreless primary granule, nucleus, and cytosolic space beneath the plasma membrane are also indicated. (*Updated and reproduced from Gleich et al.,[2] with permission from the Annual Review of Medicine, Volume 44, © 1993, by Annual Reviews.*)

as suggested recently by the localization of all the principal eosinophil eicosanoid-forming enzymes including 5-lipoxygenase, leukotriene C4 synthase, and cyclooxygenase in lipid bodies. [16] As reviewed in detail elsewhere, [17] these four organelles, along with vesiculotubular structures and small vesicles involved in transport and secretion in the activated cell, serve as the major subcellular sites for the eosinophils armamentarium of preformed cytotoxic and inflammatory mediators.

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EOSINOPHIL DEVELOPMENT, RECRUITMENT AND ACTIVATION

Eosinophilopoiesis

Eosinophils differentiate in the bone marrow from stem cell-derived, CD34+ multipotential myeloid progenitors in response to a number of T-cell-derived eosinophilopoietic cytokines and growth factors including IL-3, GM-CSF, and IL-5. These cytokines affect the eosinophil lineage at three different levels including (1) commitment, proliferation, and differentiation of hematopoietic progenitors in the bone marrow, (2) priming and activation in the blood and tissues for enhanced functional activities, and (3) recruitment and tissue localization (see discussion later in this chapter). Although activated T cells are likely the primary source for IL-3, IL-5 and GM-CSF pertinent to eosinophil differentiation and the development of reactive eosinophilia in disease, other cell types including mast cells, macrophages, natural killer cells, endothelial cells, and stromal cells such as fibroblasts are also producers of GM-CSF. IL-5, produced primarily by activated Th2 type helper T cells[18] and mast cells,[9] [19] stimulates the proliferation and differentiation of murine activated B cells and regulates the production of eosinophils in vitro and in vivo. [20] [21] [22] Both IL-3 and GM-CSF have activities on other hematopoietic lineages, whereas IL-5 is more eosinophil-specific and plays a crucial role in regulating the terminal differentiation and post-mitotic activation of eosinophils. [23] IL-5 is therefore a late-acting cytokine that demonstrates maximum activity on an eosinophil progenitor pool that is first expanded by the earlier-acting, multipotential cytokines such as IL-3 or GM-CSF. [23] Although IL-3 and GM-CSF participate in the proliferation and commitment of progenitors to the eosinophil lineage, IL-5 is both necessary and sufficient for eosinophil development to proceed in vivo. [23] [24] In humans, IL-5 does not appear to have any effect on B cells or other lymphoid lineages, and its activity, high affinity receptor, and functions are restricted to eosinophils and hematopoietically related basophils. [23] The expression of the high-affinity receptor for IL-5 is an important prerequisite and very early lineage-specific event in the hematopoietic program for these granuloctyes. Overexpression of IL-5 is observed in many eosinophil-associated diseases [25] [26] [27] and IL-5 transgenic mice develop profound eosinophilia[28] [29] indicating that IL-5 plays important roles in promoting the production and function of eosinophils in vivo. These general observations have recently been confirmed and expanded in studies of IL-5 deficient (gene knockout) mice, [30] [31] which produce basal levels of normal eosinophils in the bone marrow, but do not develop blood and tissue eosinophilia, airway hyperreactivity or lung damage in the murine ovalbumin allergic asthma model, [31] nor eosinophilic responses to helminth parasites. [30] For these reasons, IL-5 and its receptor may be excellent targets for therapeutic intervention in eosinophil-associated inflammatory diseases.

Mobilization and Migration of Eosinophils to Sites of Inflammation

In the normal individual, eosinophils produced in the bone marrow reside only briefly in the peripheral circulation in transit to extravascular sites. They tend to localize preferentially in certain tissues and organs exposed to the external environment, principally in the submucous membrane and loose connective tissue of skin, gastrointestinal tract, genital tracts and the lungs. [32] In contrast, the acute and chronic inflammatory recruitment of eosinophils into tissues occurs primarily in response to the early- and late-phase components of immediate hypersensitivity reactions, but also in association with a number of other immunologically mediated reactions, diseases and idiopathic syndromes to be outlined or discussed in detail later in this chapter. In addition, diurnal variations in the eosinophil intravascular compartment are well documented, with minimum

numbers of blood eosinophils early in the morning and highest numbers late at night, mirroring circadian rhythms in circulating adrenal corticosteriods. Likewise, diurnal variations have also been documented to occur in the mobilization, recruitment, and activation state of eosinophils in tissues, e.g., in diseases such as nocturnal asthma.[33] [34]

Although eosinophils may represent only part of an inflammatory infiltrate comprised of neutrophils, monocytes, and/or lymphocytes, the mechanisms by which they are selectively recruited in large numbers in certain reactions are currently under investigation. [35] The mobilization of eosinophils, as for other leukocytes, from the vasculature involves their rolling and adherence to vascular endothelium via L-selectin, followed by interactions with intercellular adhesion molecule-1 (ICAM-1) through a CD18/CD11a,b-dependent mechanisms, and migration in response to specific cytokines or chemoattractants. Adherence via CD18-independent mechanisms involves binding to cytokine-activated endothelial cells utilizing either E-selectin, also known as endothelial leukocyte adhesion molecule-1 (ELAM) or vascular cell adhesion molecule-1 (VCAM). Selective recruitment of eosinophils likely involves adhesion to VCAM via the $_1$ integrin very late activation antigen-4 (VLA-4), which is expressed by eosinophils but not neutrophils. [36] Because no single chemotactic agent identified thus far is uniquely specific for eosinophils, selective eosinophil recruitment likely involves the complex interaction of multiple adhesion pathways and chemotactic gradients. The inflammatory mediators identified to function as potent eosinophil chemoattractants include the complement fragment C5a and platelet activating factor (PAF), along with a number of the eosinophil-active cytokines including IL-3, IL-5, and GM-CSF, which also prime eosinophils for enhanced migratory responses to agents such as PAF, fMLP, LTB $_4$, and IL-8. The most potent eosinophil chemoattractants identified thus far are active in the 10^{-12} to 10^{-11} M range, are 1,000-fold more active than PAF and C5a, and include IL-2, the CD8+ T-cell-derived lymphocyte chemoattractant factor (LCF) that utilizes CD4 expressed on the activated eosinophil as its receptor, and the chemokine eotaxin.[37] The chemokine RANTES, which is chemotactic for certain T-cell subsets and monocytes, but not for neutrophils, is likewise a potent stimulus for eosinophil migration;[38] its production by the CD4+ T-cell component of cutaneous and pulmonary allergen-induced late-phase reactions may contribute to the eosinophil infiltration characteristic of these responses.

Eosinophil-Activation in Disease: Eosinophil Heterogeneity

Eosinophils secrete both their preformed granule constituents and newly synthesized lipid, cytokine, and peptide mediators of inflammation upon stimulation (Fig. 40-2). It has become increasingly clear that both peripheral blood eosinophils from patients with eosinophilia and eosinophils from the inflammatory tissue microenvironment are heterogeneous with regard to their capacity for stimulation and secretion of the previously described mediators. Eosinophils from blood and tissues of patients with eosinophilia differ from their normal counterparts by a variety of morphologic, biochemical, and functional characteristics, [39] some of which appear to be linked to a significant decrease in cell density as measured by separation over density gradients. Thus, a distinction is now made between the populations of normodense (normal density) and hypodense (light density) eosinophil phenotypes isolated from the peripheral blood and tissues. The blood of normal individuals contains <10% of eosinophils with densities less than 1.082 g/ml, whereas patients with eosinophilia can have markedly increased numbers of the hypodense cells.[26] [40] [41] Hypodense eosinophils possess a variety of properties consistent with their being primed and/or activated in vivo, by analogy with classical macrophage activation. Morphologically, activated hypodense eosinophils show increased vacuolization, decreased granule size (and content of granule cationic proteins such as MBP[41]), and increased numbers of cytoplasmic lipid bodies. [16] The numbers of surface receptors for a variety of eosinophil-active agonists including complement cleavage fragments of C3 (CR1 and CR3), immunoglobulins (IgE, IgA), cytokines, and PAF are increased on hypodense eosinophils from eosinophilic patients, as are a variety of membrane surface antigens such as CD4. [37] Functionally, hypodense eosinophils show increased metabolic activity, oxygen consumption, and generation of superoxide anion, as well as an increased capacity for the synthesis and secretion of LTC $_4$ and certain cytokines, enhanced chemotaxis, and augmented cytotoxicity for antibody-coated targets.

Figure 40-2 Eosinophil degranulating stimuli and eosinophil-derived inflammatory mediators. The listed factors have been shown to stimulate the release of inflammatory mediators from the eosinophil. These mediators may be either pre-formed, such as the eosinophil granule-derived cationic proteins, or newly generated mediators, such as oxidative products, lipid mediators (e.g., LTC_4), and inflammatory or hematopoietic cytokines. (*Reproduced with permission from Furuta et al.*[232])

The mechanism by which eosinophils develop the morphologic, biochemical, and functional characteristics of activated cells in vivo has been largely defined through the in vitro analysis of factors capable of prolonging eosinophil survival and inducing the development of the hypodense phenotype. Culture of normal peripheral blood eosinophils with growth factors (GM-CSF), hematopoietic cytokines (IL-3, IL-5), and interferons (IFN and) either alone or in co-culture with endothelial cells or fibroblasts, induces the development of the hypodense activated phenotype characteristic of eosinophils isolated from patients with hypereosinophilic disorders. In addition, the eosinophilopoietic cytokines (IL-3, IL-5, and GM-CSF) also prolong the survival of mature normodense and hypodense eosinophils in vitro (and possibly in vivo in the tissue microenvironment), likely through the prevention of programmed cell death (apoptosis). [42] [43] Of interest, culture of eosinophils with the previously noted factors in vitro does not precisely recapitulate all the functional characteristics of the in vivo activated, tissue-derived cell, suggesting a requirement for additional as yet undefined factors that may include interaction of adhesion receptors with their ligands during eosinophil recruitment and migration into the tissue or stromal cell-derived factors.

The diagnostic and therapeutic significance of eosinophil heterogeneity has not been fully evaluated. Although the percentage of hypodense eosinophils in the blood may correlate with the degree of peripheral blood eosinophilia, [40] precise relationships between the percentage of hypodense eosinophils and the clinical status of an eosinophilic disorder have not been easily defined. Rough correlations have been reported between disease severity and numbers of hypodense eosinophils in circulation for certain hypersensitivity disorders such as allergic asthma and allergic rhinitis. However, there is a significant overlap in the percentage of hypodense peripheral blood eosinophils in hypereosinophilic disorders of greatly varying severity such as bronchial asthma, the hypereosinophilic syndrome, and certain helminth infections. Because eosinophils reside primarily in the tissue microenvironment for the majority of their lifespan, it is not surprising that their heterogeneity in the peripheral circulation is at best a crude reflection of disease severity. In vitro models of eosinophil heterogeneity suggest a number of pathways for the development of eosinophil subpopulations, including acute exposure to mediators generated in immediate-type hypersensitivity reactions (e.g., PAF, eosinophil-active cytokines such as GM-CSF, IL-3, IL-5, IFN, or TNF), or more chronic exposure to eosinophil active cytokines in combination with as yet undefined factors in the tissue microenvironment as discussed previously. Regardless of the specific mechanisms or mediators involved in eosinophil priming and/or activation, the development of eosinophil heterogeneity as a reflection of the functional, pro-inflammatory capacity of the cell in vivo, suggests the possibility that these processes could be manipulated in a therapeutic manner. For example, in disorders such as HES where excessive levels of IL-5 have been occasionally demonstrated in the peripheral circulation (see discussion later in this chapter), [25] therapies aimed at modulating IL-5 levels in order to both normalize eosinophil phenotype and attenuate the eosinophilia may be appropriate. One such example has been the use of cyclosporin A to produce a clinical remission in a patient with steroid-resistant HES. Cyclosporin A has been shown in vitro to inhibit IL-5 production from T-cell clones derived from the patient. [44] In contrast, since eosinophil infiltration of certain types of malignancies including colon cancers or lung adenocarcinomas may be associated with improved prognosis, [45] [46] it may be advantageous to induce the development of tissue eosinophilia and enhanced cytotoxicity as demonstrated in response to local production of IL-4. [47]

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EOSINOPHILIA AND EOSINOPHIL-ASSOCIATED DISEASES, SYNDROMES, AND INFLAMMATORY REACTIONS

Identification and Quantitation

Eosinophils, so named for their unique granular staining with acidic fluorone dyes such as eosin, were first identified by Paul Ehrlich in 1879. Subsequently, histochemical staining with eosin-based stains became the standard for eosinophil identification in both blood and tissues (haematoxylin and eosin), although other more specific histochemical dyes such as chromotrope 2R are more definitive in tissues, and Fast Green or Luxol Fast Blue can be utilized to specifically distinguish the mature eosinophil from other polymorphonuclear leukocytes in blood smears or cytocentrifuge slides, i.e., neutrophils and basophils, for which the granules do not stain. The chemical characteristics of the fluid medium or tissue in which the eosinophil is analyzed, and most importantly the eosinophils degree of maturity, activation, secretion, and/or degranulation can all induce significant variability in histochemical characteristics for the Romanovsky-type metachromatic stains used most widely for detection of eosinophils in blood and bone marrow specimens. For example, eosinophils in urine, as found in eosinophilic cystitis or interstitial nephritis, are best detected by Hansels stain.[48] As a result, it is now understood that the definitive identification of the eosinophils participation in a particular inflammatory response or disease may require immunochemical localization using specific monoclonal or polyclonal antibodies to eosinophil-specific granule proteins such as major basic protein (MBP), eosinophil peroxidase (EPO), or eosinophil cationic protein (ECP), all of which have been used to identify eosinophil infiltration and secretion/degranulation in tissues where they have been missed by more classical histochemical techniques. [5] [49] For peripheral blood, absolute eosinophil counts done manually or utilizing automated counting systems provide a much more reliable estimation of eosinophil numbers than does microscopic differential counting of blood smears combined with total leukocyte counts, especially when there is an absolute leukocytosis or leukopenia for another lymphoid or myeloid lineage. Circulating eosinophils are generally present in low numbers in normal healthy individuals with consensus absolute counts ranging from 35 to 350 eosinophils/mm^3 (mean 125 for adults; 225 for children under 12 years). The broad range of variability in normal blood eosinophil levels reflects the multiple sources of both subtle and more obvious physiologic effects due to diurnal variation in endogenous glucocorticoid levels (see previous discussion), degree of atopy in the general population, and other factors that can include exercise, emotional stress, and hormonal influences of the menstrual cycle.

Clinical Assessment of Eosinophilia: Peripheral Blood versus Tissue Eosinophils

The low numbers of eosinophils in the blood compared to neutrophils and lymphocytes, coupled with their principal localization in tissues, makes accurate quantitation and interpretation of blood eosinophil counts more difficult than that of other cellular elements. As noted earlier in this chapter, 100200 cell differential counts are fraught with the problems inherent in sampling error when eosinophils constitute <35% of total peripheral blood leukocytes. Further, the use of automated cell counters that may not accurately enumerate eosinophils unless they are present in sufficiently high numbers, and that can miss eosinophil peroxidase deficient cells, may nevertheless require a manual differential or absolute count to be performed if modest to moderate eosinophilia is suspected in the clinical evaluation of a patient. The number of circulating eosinophils in the blood reflects a complex interplay of factors including rates of

development and transit from the bone marrow, margination, and subsequent emigration into tissues through postcapillary venules. Because the eosinophil is primarily a tissue-dwelling cell, is present in extravascular sites at levels several hundred-fold more than in peripheral blood in the rat, and blood levels may vary significantly as noted earlier in this chapter, circulating cells reflect only those in traffic between the marrow and their final functional destination. In humans, the marrow to blood ratio for eosinophils is 57:1. Normal adult bone marrow contains 35% eosinophils, with 40% mature cells, and migration of eosinophils from the marrow into the blood takes 3.5 days. The half-life of the eosinophil in the peripheral circulation of normal individuals is 18 hours, with an average blood transit time of 26 hours, similar to that of neutrophils. Daily mean turnover is 0.2×10^9 /kg and the bone marrow has a post-mitotic reserve of 0.1×10^9 eosinophils/kg. Under ideal steady-state conditions, one would anticipate that peripheral blood eosinophil levels are proportional to levels in normal tissue compartments. In contrast, under the dynamic conditions of certain acute inflammatory or immune responses, temporal lags have been observed to occur between infiltration into reactive tissue sites and the induction of eosinophil development and emigration from the marrow, resulting in the development of eosinopenia and/or delayed blood eosinophilia. [50] [51] [52] Moreover, in the pathology of more chronic eosinophil-associated inflammatory conditions, tissue eosinophilia may be prominent in the absence of minimal or any peripheral blood eosinophilia, e.g., in certain skin diseases. [5] Thus, the clinical assessment of peripheral blood eosinophilia, although diagnostically important, must be interpreted with these caveats in mind.

The list of diseases, syndromes, and inflammatory processes associated with peripheral blood and/or tissue eosinophilia is quite extensive (Table 40-1). Of course, the most common associations are infections with parasitic helminths and allergic diseases and their related inflammatory reactions. Effective clinical assessment of patients with eosinophilia requires a careful and detailed history including travel information, where the patient has lived, drug use including intravenous drugs, L-tryptophan and vitamin supplements, and other over-the-counter nonprescription medications, diet, and allergic symptomology. In addition, the systemic nature of many of the nonallergic/noninfectious conditions involving eosinophilia dictates that a careful history of fever, weight loss, myalgias, arthralgias, rashes, lymphadenopathy, etc. should be obtained as well. Symptomatic or asymptomatic eosinophilia in patients taking drugs associated with known eosinophil responses and eosinophil-related drug reactions strongly dictates that their use be discontinued and that follow-up evaluation confirm that the eosinophilia and related symptoms have resolved. Patients with eosinophilia who have resided in or visited areas endemic for helminths or other parasites should be examined for intestinal and bloodborne infections, with additional serologic evaluation as necessary to identify the causative agent. Once the more common and obvious causes of eosinophilia have been excluded, i.e., drug reactions, parasitic infections, and various allergic conditions, the differential diagnosis of eosinophilia becomes more difficult due to the complexity and number of potential organ systems and conditions involved (Table 40-1).

Monitoring of Eosinophil Activity

An understanding of the importance of both numbers of tissue eosinophils and their functional status, as well as differences in functional status between circulating eosinophils and those that have been recruited into and exposed to cytokines and other factors in the tissue microenvironment, has resulted in the development and more routine use of methods to monitor eosinophil activity in situ. Tissue biopsies have become more routinely employed for both the clinical and experimental evaluation of eosinophil function in diseases involving the skin, [5] [50] [53] lung, [34] [54] [55] lymph nodes,[56] heart,[57] and other tissues, greatly contributing to the current understanding of the eosinophils pro-inflammatory role in disease pathogenesis. The difficulties, tediousness, and sampling errors inherent in accurately quantitating the numbers of eosinophils, let alone their functional status in tissue biopsy specimens, even using antibodies that recognize specific cell surface activation markers or secretion products and modern morphometric techniques, makes routine clinical evaluation of tissue eosinophils by these methods somewhat impractical. Alternatives, such as analysis of tissue secretions from affected organs, e.g., bronchoalveolar lavage (BAL) of the lung, have been utilized with great success in the clinical and experimental evaluation of eosinophil function in diseases such as asthma. [33] [58] [59] In addition to the more routine histochemical identification and enumeration of eosinophils, and immunochemical localization of secreted eosinophil granule cationic proteins in tissue biopsies, two additional methods have been extensively used to monitor eosinophil activation, secretion, and involvement in disease pathogenesis. These include the identification of activated eosinophils by staining with a monoclonal anti-ECP antibody EG2 that recognizes a secreted, deglycosylated form of the protein, [60] [61] [62] [63] and measurement of the eosinophil

granule cationic proteins such as MBP, ECP, and eosinophil-derived neurotoxin (EDN) by radioimmunoassay [63] [64] [65] in various body fluids including serum, plasma, urine, sputum, nasal lavage, cerebro-spinal fluid, and BAL fluid. [66] Under controlled sampling conditions, measurements of these eosinophil granule products have served as excellent indicators of eosinophil secretory activity in vivo and eosinophil involvement in a variety of allergic, parasitic, and certain inflammatory and skin diseases not normally associated with blood or tissue eosinophilia. Examples include assays of MBP and ECP in bronchoalveolar lavage [67] and nasal lavage[68] in asthma and allergic rhinitis, [66] in serum and plasma in lymphatic filariasis, [50] [69] and in skin diseases such as chronic idiopathic urticaria. [70] Such measurements likely reflect both in vivo secretion in tissues and/or secretory activity of eosinophils in the fluid sampled [66] and have provided compelling evidence for relationships between eosinophil secretory activity and disease severity. [67] [71] [72]

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IDIOPATHIC HYPEREOSINOPHILIC SYNDROME

The hypereosinophilic syndrome (HES) comprises a group of myeloproliferative disorders of unknown etiology that are marked by a sustained overproduction of eosinophils. In addition to the striking and sometimes profound eosinophilia associated with the syndrome, HES is characterized by a predilection for end organ damage, most commonly involving the heart, with the development of eosinophilic endomyocardial fibrosis and related valvular pathologies. The currently defined criteria for HES as originally presented by Chusid et al. [12] include: (1) persistent eosinophilia of >1,500 eosinophils/mm ^{3} for more than 6 months, (2) exclusion of other potential etiologies for the eosinophilia including parasitic, allergic, or other causes, and (3) signs and symptoms of organ system dysfunction or involvement that appears related to the eosinophilia or is of unknown cause in the clinical presentation. Total leukocyte counts are often less than 25,000/mm ^{3} with 3070% eosinophils. However, extremely high leukocyte counts of (>90,000/mm^3) may be associated with a poor prognosis in some patients. These three primary features of the syndrome, including sustained eosinophilia of unkown etiology or disease association, along with evidence of organ involvement (Table 40-2) comprise the defining characteristics of HES. The clinical features of patients diagnosed with HES as summarized by Spry [73] and most recently reviewed by Weller and Bubley, [74] Brito-Babapulle, [75] and one of

TABLE 40-2 -- Major Organ Involvement and Prominent Clinical Features in Patients with the Hypereosinophilic Syndrome[a]

Primary Organ Involvement[b]	Clinical Manifestations[c]	
Hematologic (100)[d]	Eosinophilic endomyocardial disease	Psychiatric disturbance
Cardiovascular (58)	Skin lesions, e.g., angioedema, urticaria	Myalgia
Cutaneous (56)	Anorexia and weight loss	Arrhythmias
Neurologic (54)	Thromboembolic disease	Renal impairment
Pulmonary (49)	Lymph node and/or spleen enlargement	Splenic infarction
Splenic (43)	Ophthalmologic complications	Diarrhea alone
Hepatic (30)	Fever, excessive sweating	Arthralgia
Ocular (23)	Gastrointestinal involvement, including diarrhea	Pericarditis
Gastrointestinal (23)	Central nervous system disease	
	Pulmonary involvement	

[a] In order of frequency.
[b] *Reproduced from Table 1 of Weller and Bubley,[74] with permission.*
[c] *Reproduced from Spry,[73] with permission from Munksgaard International Publishers, Ltd., Copenhagen, Denmark © 1982.*
[d] Average percentage of 105 patients from American, French, and English studies combined.

us[76] are shown in Table 40-2 in their frequency of presentation. The most commonly encountered feature in 5075% of patients studied in detail to date [77] [78] [79] include the cardiovascular manifestations, especially endomyocardial disease to be discussed later in this chapter, and its associated thromboembolic complications, the major causes of morbidity (and mortality) in the HES.

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DIFFERENTIAL DIAGNOSIS OF HES

As shown in Table 40-1 , a large number of diseases have been identified that are associated with reactive, secondary eosinophilia, and hypereosinophilia. Their clinical presentations vary significantly and must be clearly distinguished from HES. In particular, there are a number of eosinophilic diseases and syndromes of questionable or unknown etiology that must be differentiated from HES based on both clinical and pathological parameters. The pathology of a number of these eosinophilic syndromes is generally restricted to specific organs, e.g., eosinophilic gastroenteritis and eosinophilic pneumonia; these syndromes lack the multiplicity of end organ damage generally seen in HES, and for this reason they can usually be distinguished from HES. For reasons that remain unclear, these syndromes lack the propensity to evolve toward secondary eosinophil-mediated cardiac disease. Because some patients with HES exhibit evidence of vasculitic disease, and the major eosinophil-associated vasculitis is the Churg-Strauss syndrome, this syndrome likewise needs to be ruled out. The Churg-Strauss syndrome is characterized by a history of blood eosinophilia >10%, asthma, pulmonary infiltrates (non-fixed), abnormalities in the paranasal sinuses, mono- or poly-neuropathy, and extravascular eosinophilic infiltrates in biopsied blood vessels. [80] Necrotizing vasculitis of small arteries and veins and extravascular granulomas are characteristic findings in biopsies from most, but not all Churg-Strauss patients, [81] [82] and asthma, peak eosinophil counts of >1,500/mm^3 , and systemic vasculitis involving two or more extrapulmonary organs generally identify these patients. [82] Although neurologic, pulmonary, and possibly paranasal findings may accompany HES, [77] asthma is characteristically absent. [83] Nevertheless, it may be difficult in some patients to make a clear distinction between HES and Churg-Strauss syndrome, especially since responses to high dose corticosteroids would be identical for both at the outset.

Eosinophilic syndromes with cutaneous involvement can generally be distinguished from HES by the histopathology of biopsied skin lesions. These syndromes include Kimuras disease (angiolymphoid hyperplasia with eosinophilia), Wells syndrome (eosinophilic cellulitis), eosinophilic fasciitis, and eosinophilic pustular folliculitis. The more recently described syndrome of episodic angioedema with eosinophilia, characterized by periodic recurring episodes of angioedema, urticaria, fever, and marked blood eosinophilia, is generally not associated with end organ cardiac damage, and has thus been distinguished from HES. [13] [14] Finally, the eosinophil myalgia syndrome, induced by ingestion of tainted L-tryptophan, should also be excluded.

A differential diagnosis of HES requires the exclusion of all identifiable eosinophilias of reactive, secondary etiologies (Table 40-3 and box, Treatment Approach: Diagnosis and Therapy of HES). These especially include eosinophilias due to parasitic infections caused predominantly by helminthic parasites, but also by two enteric protozoans, *Dientamoeba fragilis* and *Isospora belli*. In adults, filarial infections and strongyloidiasis are most likely to elicit pronounced and prolonged eosinophilias, in contrast to *Trichinella spiralis* infections which cause an acute eosinophilia that does not persist without reinfection. [84] Infections with *Strongyloides stercoralis*, which have the capacity to induce marked hypereosinophilia that mimics HES, [77] [85] are particularly important to exclude, especially since HES has been misdiagnosed in patients with unsuspected strongyloidiasis, and treatment of these patients with immunosuppressive glucocorticoids can lead to disseminated, often fatal disease. [86] Serial stool examinations, and in particular serologic enzyme-linked immunosorbent assays (ELISA) for strongyloides infection should be performed. Parasitic helminth infections not amenable to or detectable by routine stool examinations, including tissue- or blood-dwelling helminths causing filariasis, trichinosis or visceral larval migrans [87] (*Toxocara canis* infections in children), should be evaluated by diagnostic examinations of blood, tissue

TABLE 40-3 -- Diagnosis of the Idiopathic Hypereosinophilic Syndromea [a]

Eosinophils >1,500/mm^3 for at least 6 months duration
Reactive causes of eosinophilia excluded
Known eosinophilic disease entities excluded
Evidence of eosinophilic end organ damage
Clonal eosinophilic disorders excluded
Factors favoring a diagnosis of HES
Elevated serum immunoglobulins
Elevated levels of tumor necrosis factor, interleukins (IL-5) and/or IFN-, -, or -
Elevated serum IgE levels
Good therapeutic response to corticosteroids

[a] *Reproduced from Brito-Babapulle,[75] with permission.*

TREATMENT APPROACH: DIAGNOSIS AND THERAPY OF HES[196]

Patients presenting with eosinophilia of unknown etiology should undergo a thorough clinical and laboratory evaluation consisting of the following:

Complete/detailed history and physical examination

Complete blood count with total eosinophil count and review of peripheral blood smear

Hepatic and renal function tests, urine analysis, erythrocyte sedimentation rate, rheumatoid factor, human immunodeficiency virus (HIV) assay and others as indicated

Quantitation of total IgE level

Stool for ova, parasites × 3, duodenal aspirate

Serologic assays for strongyloides, trichinella, toxocara, and others as clinically indicated

Bone marrow aspirate and biopsy (with cytogenetics)

Chest radiograph, computed tomograph scan of chest, abdomen, and pelvis

Electrocardiogram and echocardiogram

Molecular analysis of immunoglobulin and T-cell receptor gene rearrangement status

Patients meeting the diagnostic criteria for HES:

1. persistent eosinophilia of >1,500 eosinophils/mm^3 for more than 6 months
2. exclusion of other potential etiologies for the eosinophilia including parasitic, allergic, or other causes
3. presumptive signs and symptoms of organ system dysfunction or involvement which appears related to the eosinophilia or is of unknown cause in the clinical presentation.

These patients should be managed according to the strategy defined in the following algorithm for HES (
adapted from Schooley et al.,[195] *with permission*). Patients without any overt evidence of organ dysfunction or severe symptoms should be observed periodically without treatment. Periodic reinvestigation of the etiology of the eosinophilia is appropriate and recommended every 3 months to reconfirm the diagnosis of HES.

Treatment of hypereosinophilia secondary to a defined etiology should be directed at the underlying cause. For patients failing to respond to these measures, a trial of corticosteroids is warranted to halt progressive organ dysfunction secondary to the eosinophilia.

Treatment Strategy

Algorithm adapted from Schooley RT, Flaum MA, Gralnick HR, Fauci AS: A clinicopathologic correlation of the idiopathic hypereosinophilic syndrome. II. Clinical manifestations. Blood 58:1021, 1981, with permission.

biopsies, or specific serological tests (ELISAs or reverse transcription polymerase chain reaction [RT-PCR] assays) that are now available.

CLINICAL MANIFESTATIONS OF HES

Hematologic Findings

The definitive hematologic manifestation of HES is sustained eosinophilia of 3070%, with total leukocyte counts ranging from 10,000 to 30,000/mm ^{3} . However, extremely high leukocyte counts of greater than 90,000/mm^3 are not uncommon in some patients with HES, but are often associated with a poorer prognosis. [88] Blood smears generally show more or less normal, mature eosinophil morphology including typical bilobed nuclei and granule-rich cytoplasm. However, eosinophilic myeloid precursors may also be noted, though less commonly, and eosinophils may also exhibit morphologic abnormalities including nuclear hypersegmentation, decreased size and/or numbers of secondary granules and cytoplasmic vacuolization when

TABLE 40-4 -- Clonal Disorders with Peripheral Blood Hypereosinophiliaa[a]

CEL	Chronic eosinophilic leukemia
AEL	Acute eosinophilic leukemia
CGL	Chronic granulocytic leukemia
PRV	Polycythemia rubra vera
ET	Essential thrombocythaemia
AML	Acute myeloid leukemia
MDS	Myelodysplastic syndrome
SM	Systemic mastocytosis
TLL	Acute lymphoblastic leukemia

[a]Reproduced from Brito-Babapulle,[75] with permission.

viewed at the light microscope level.[12] [89] The presence of myeloblasts and/or dysplastic findings in the peripheral blood may suggest an alternative, clonal etiology such as AML or a myelodysplastic syndrome (Table 40-4).[75] Ultrastructurally, HES eosinophils may show a selective loss of secondary granule components (crystalloid MBP-containing core and/or granular matrix), decreased numbers and/or size of granules, and increased numbers of cytoplasmic lipid bodies and tubulovesicular structures of unknown function.[15] [41] [90] [91]

In addition to their hypereosinophilia, patients with HES may also present with an absolute neutrophilia, further contributing to their overall increased leukocyte counts. This may include bands, less mature precursors, as well as alterations in neutrophil nuclear segmentation and cytoplasmic granules. [92] Basophilia, when seen in some HES patients, is usually minimal. Levels of leukocyte alkaline phosphatase may be abnormally elevated or decreased in HES patients, [12] and vitamin B_{12} and B_{12} -binding proteins in serum can be normal or elevated. [93] [94] In the NIH series, platelet counts were found to be decreased or increased in 31% and 16% of patients, respectively.[92] Approximately 50% of HES patients may be anemic, with abnormal and nucleated erythrocytes in circulation. The hypercellular bone marrow in these patients shows significant increases in the percentage of eosinophils (generally from 25 to 75% of marrow elements), with a clear left shift in eosinophil maturation.[92] Myeloblasts are generally normal in number, and myelofibrosis is rare, being observed in only a minority of patients in the NIH series. [92] Splenomegaly has been reported in 43% of HES patients (Table 40-2), and hypersplenism in these individuals may contribute to both thrombocytopenia, anemia, spleen infarction, and splenic pain induced by capsular distention or infarction is a frequent complication of splenic involvement in these individuals. [95] The progressive leukocytosis with hypereosinophilia in these patients, along with the hypercellular bone marrow and lack of increased numbers of myeloblasts, can make it difficult to distinguish HES from other myeloproliferative syndromes.

Cardiovascular Findings

The cardiac manifestations of HES can be frequent and considerable (5060% of patients; Table 40-2). Prior to the advent of early diagnosis, improved management and newer therapies, cardiac disease was the leading cause of both morbidity and mortality in HES patients. The cardiac and thromboembolic manifestations of HES are likely eosinophil-mediated. However, the risks of developing cardiac disease are not necessarily related to the extent or duration of the eosinophilia, [96] [97] since patients that ultimately develop cardiac involvement are more likely to be males with an HLA-Bw44 phenotype, develop splenomegaly and thrombocytopenia, have elevated vitamin B_{12} levels, and have abnormal hypogranular and vacuolated blood eosinophils and circulating early myeloid progenitors. [96] In contrast, HES patients who do not develop heart disease tend to be females with angioedema, hypergammaglobulinemia, and increased serum IgE levels and immune complexes.[96] The cardiac damage seen in HES, progressing from early necrotic changes through thrombosis and fibrosis, is identical to that seen in patients with hypereosinophilias of diverse etiologies. These include tropical eosinophilias due to loiasis or other filarial infections and parasitic infections such as trichinosis and visceral larval migrans,[98] drug reactions or administration of GM-CSF,[99] and eosinophilias of neoplastic origin including eosinophil leukemia, various carcinomas [100] and Hodgkins or non-Hodgkins lymphomas.[101] Because identical forms of cardiac pathology can develop in patients with hypereosinophilias of diverse etiologies, and some patients never go on to develop cardiac involvement, the pathogenesis of eosinophil-associated cardiac disease likely involves both eosinophils and as yet undefined factors required for the recruitment, activation, and secretion of these granulocytes in the heart and associated cardiovascular tissues.

Eosinophilic endomyocardial fibrosis, as first described by Löffler, [102] is pathologically similar to that of tropical endomyocardial fibrosis, [103] [104] save for the frequent absence of eosinophilia in the latter disorder. [102] However, the general absence of hypereosinophilia in patients with tropical endomyocardial fibrosis is thought to be a function of the late stage of helminthic disease in which the heart disease develops. The histopathology of cardiac involvement in HES is well characterized and can evolve through three sequentially defined stages for which eosinophils and secretion of eosinophil-derived mediators may be directly involved in the pathogenesis. These include: (1) an initial acute necrotic stage of short duration (average of 5 weeks) involving active endomyocarditis, (2) a later thrombotic stage (during an average 10 months of eosinophilia) with mural thrombus formation over endocardial lesions, and (3) a late fibrotic stage (after 2 years of illness) with development of endomyocardial fibrosis.[105] The early necrotic stage with damage to endocardium, involves marked eosinophil and lymphocyte infiltration of the myocardium with myocardial necrosis, formation of eosinophilic microabscesses, and eosinophil degranulation. Unfortunately, this early necrotic stage of cardiac disease is usually not

recognized clinically. Echocardiography and angiography may fail to detect abnormalities in this early stage of the disease since ventricular thickening has not yet occurred, and endomyocardial biopsies, generally from the right ventricle, are required to make the diagnosis of cardiac involvement. [78] Treatment of HES patients with corticosteroids during this acute necrotic stage may avert or control the subsequent development of myocardial fibrosis. However, patients often present at the later stages of the cardiac involvement. In the second stage, thrombi form over the damaged endocardium in either of the ventricles or the atrium, generally with sparing of the aortic and pulmonary valves. [106] Although electrocardiographic changes in these patients are common, they are not specific to HES. Progressive scarring at sites of mural thrombus formation ultimately lead to the late fibrotic stage, with endomyocardial fibrosis resulting in a restrictive cardiomyopathy and mitral and/or tricuspid valve regurgitation. The more common clinical manifestations in the later progressive stages of endomyocardial fibrosis include dyspnea, chest pain, signs of left and/or right ventricular congestive heart failure, the murmurs of mitral valve regurgitation, cardiomegaly, and T-wave inversions. [106] Most patients who progress to this stage of HES cardiomyopathy will benefit from standard medical therapies for congestive heart failure, or mitral valve replacement where hemodynamically indicated. Two-dimensional echocardiography is the most sensitive method for detecting cardiac abnormalities in these patients, including visualization of mural thrombi and the various manifestations of fibrosis, including thickening of the mitral valve and its supporting structures. Approximately 80% of HES patients in the NIH series had echocardiographic abnormalities, with thickening of the left ventricular free wall the most common finding (68% of patients). [106] Cardiac catheterization may

also be useful for demonstrating elevated right and left ventricular end diastolic pressures and angiography may be used to visualize valvular incompetence.

Histopathologic evaluation of the heart of HES patients generally shows four dominant features: (1) endocardial fibrosis and thickening, including involvement of mitral valve and supporting structures, (2) mural thrombus and granulation tissue on the endocardium with extensive infiltration by eosinophils, (3) thrombotic and fibrotic involvement of small intramural coronary vessels, including inflammatory cells and eosinophils, and (4) eosinophilic infiltration of the endocardium, and myocardium (in some cases). In a multicenter study of biopsy and postmortem specimens of cardiac tissue from HES patients at various stages of eosinophilic endomyocardial disease, we identified activated eosinophils (identified by staining with the EG2 anti-ECP monoclonal antibody) and marked intracardiac extracellular deposition of eosinophil granule cationic proteins including MBP, ECP, and EDN/eosinophil protein (EP)X in the early necrotic and later thrombotic lesions, mainly in areas of acute tissue damage on and beneath the endocardium, myocardial necrosis, and in the walls of small vessels. [57] The presence of activated eosinophils and toxic granule proteins in the early lesions of this disease suggests an active role for eosinophils and their products in inducing endocardial damage and myofibrillar injury, although the mechanisms involved in eosinophil recruitment into the heart have yet to be identified. In vitro studies have shown that secretion products of activated eosinophils can damage rat heart cell plasma membranes. [107] In addition, EPO in the presence of hydrogen peroxide and bromide or thiocyanate ion can induce damage to the endothelium of the working isolated rat heart [108] and eosinophil granule proteins can impair thrombomodulin activity, [109] findings supporting a role for these eosinophil products in the development of endocardial injury and subsequent thrombotic events in the HES. [110] The in vitro findings that eosinophil secretion products such as EDN can induce fibroblast proliferation, [111] that ECP alters fibroblast proteoglycan synthesis, [112] and that MBP can augment IL-1 or TGF- induced production of inflammatory cytokines (IL-6 and IL-11) by fibroblasts, [113] further implicates the eosinophil in the development of endocardial fibrosis in later stages of the disease.

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OTHER MAJOR CLINICAL MANIFESTATIONS OF HES

Pulmonary Findings

The overall pulmonary involvement reported for HES patients is approximately 50% (Table 40-2), with the most common respiratory problem being a chronic and persistent, usually nonproductive cough. Although the physiologic basis for pulmonary involvement in HES is not known, it may be secondary to aspects of congestive heart failure, or a number of other factors including infiltration and sequestration of eosinophils in lung tissues or pulmonary emboli originating from ventricular thrombi. Although bronchospasm has been noted in some series of patients, [114] asthma is apparently quite rare in HES.[115] Transudative pleural effusions are the commonest abnormality in patients with frank congestive heart failure. [12] In contrast to chronic eosinophilic pneumonia, [116] the pulmonary infiltrates seen in 1428% of HES patients[12] [114] were either diffuse or focal, without any preference for particular regions of the lung. Pulmonary infiltrates in HES may or may not clear with prednisone treatment, and pulmonary fibrosis can develop in patients with endomyocardial fibrosis. [77]

Neurologic Manifestations

As noted in Table 40-2 , neurologic involvement is quite frequent in HES (50% of patients), encompassing three different complications. [117] The first type of neurologic involvement is caused by thromboemboli, which may originate from intracardiac thrombi in the left ventrical. These thromboembolic episodes may occur even before overt cardiac disease is visible by echocardiography. Patients with thrombotic complications may experience embolic strokes or transient ischemic episodes which may be multiple and recurrent, and these episodes may occur even though the patient is adequately anticoagulated with coumadin and antiplatelet agents. [78] [117] The second type of neurologic manifestation involves primary diffuse CNS dysfunction of unkown etiology. HES patients may variably exhibit changes in behavior, confusion, ataxia, and loss of memory.[117] The third neurologic dysfunction noted in HES is the development of peripheral neuropathies, which can occur in 50% of HES patients exhibiting neurologic involvement.[117] These include symmetric or asymmetric sensory polyneuropathies, including sensory deficits, painful paresthesias, or mixed sensory and motor defects.[117] [118] [119] These neuropathies may improve with steroid administration or other treatments, may be stable or continue to progress despite therapy, or may improve or resolve with time. The histopathology of the involved nerves usually shows varying degrees of axonal loss, without evidence of vasculitis or direct or peripheral eosinophil infiltration. [117] [118] [119] The presence in eosinophils of two granule cationic proteins, eosinophil-derived neurotoxin (EDN) [120] and eosinophil cationic protein (ECP), [121] both equally potent in inducing a neurotoxic and paralytic syndrome known as the Gordon Phenomenon [120] [122] when injected experimentally (intrathecally) into rabbits, [123] has led to speculation that these proteins might be responsible for inducing the various neuropathies commonly seen in HES. The histopathology of the cerebellar dysfunction in the brains of experimental rabbits undergoing the Gordon Phenomenon includes a spongiform degeneration of white matter and loss of Purkinje cells,[122] changes not comparable to the peripheral axonal nerve damage seen in HES. Both EDN and ECP, related 16 kD cationic proteins with 70% amino acid sequence homology, [124] [125] are members of the ribonuclease gene family and possess potent ribonuclease activity and cellular toxicities in vitro.[126] However, there is no direct evidence that these ribonucleases and cellular toxins have the capacity to mediate the types of neurologic damage seen in HES, nor have these proteins been visualized by immunochemical means at sites of HES neuropathology. Thus, the pathogenesis of the encephelopathic, CNS, and peripheral manifestations of HES-associated neuropathy remain speculative.

Cutaneous Manifestations

For reasons that remain unclear, the skin is frequently involved in HES pathology, with cutaneous manifestations present in >50% of patients in most series (Table 40-2).[12] [77] [127] The skin lesions associated with HES most commonly fall into three categories, (1) angioedematous and urticarial, (2) erythematous, pruritic papules and nodules and (3) mucosal ulcerations. As noted earlier, patients with angioedema and urticaria are more likely to have a benign disease course that is responsive to corticosteroids, without the development of cardiac or neuropathic complications. [77] [95] A subgroup of HES patients with cyclic angioedema and eosinophilia are now considered to have a syndrome (episodic angioedema with eosinophilia) that is distinct from classical HES. [14] These patients have a disorder with recurrent attacks of angioedema, urticaria, fever, and bodyweight gain that can be quite pronounced. These clinical manifestations are associated with marked leukocytosis and eosinophilia during the episodes. In HES patients that develop papular or nodular lesions, these usually improve concomitant with positive responses to systemic therapy.[127] Dermal biopsies in these patients generally show mixed cellular infiltrates including eosinophils, without signs of vasculitis. [127] Perivascular eosinophilic infiltrates are also found in these lesions. [127] The aquagenic erythematous

pruritic eruptions and indurated papules and nodules in some HES patients have been found to respond to psoralen and ultraviolet light therapy (PUVA), [128] [129] and in some patients, this treatment has interestingly been accompanied by a return of eosinophil counts to normal. [129] PUVA therapy may also be effective for the cutaneous manifestations of HES associated with HIV infection (exfoliative erythroderma and linear flagellate plaques). [130] HES-associated pruritis and nodular lesions have also been controlled with the addition of dapsone (75150 mgday) to prednisone. [131] Oral sodium cromoglycate (cromolyn sodium) given before meals has also been reported to be efficacious,[132] though neither of these agents had any effect on peripheral eosinophilia. Severe and sometimes incapacitating mucocutaneous ulcerations may be a prodrome to HES and indicate a subset of HES patients with a poor prognosis. [133] [134] They can occur at multiple sites including the mouth, nose, pharynx, penis, esophagus, stomach, and anus,[133] [134] and lesions can flare up independently of other clinical manifestations of HES. Biopsies of the ulcerative lesions usually show mixed cellular infiltrates, without a predominance of eosinophils or any evidence of vasculitis or microthrombi. [133] These ulcers have generally been resistant to treatment with topical or systemic corticosteroids, colchicine, and hydroxyurea, but have recently been shown to respond remarkably well to IFN- with complete and durable remissions.[135] [136]

Eosinophils are present in many skin diseases and in certain conditions may constitute part of the diagnostic histology of the lesion. [137] Although eosinophils in general may not appear prominent in many cutaneous diseases, there is now ample evidence to suggest that they may nevertheless participate in the pathogenesis of cutaneous inflammation[5] [138] and contribute to edematous reactions in atopic dermatitis, [53] syndromes associated with acute[139] [140] and chronic urticarias, [70] IgE-mediated late-phase reactions,[141] chronic dermatitis associated with parasitic infections such as onchocerciasis, [50] episodic angioedema with eosinophilia, [14] eosinophilic cellulitis, [142] and skin lesions associated with rIL-2 administration for advanced malignancies. [143] Indirect evidence includes the ability of the eosinophil to elaborate its granule cationic proteins, some of which are capable of inducing release of vasoactive amines from basophils and mast cells and inducing cutaneous wheal-and-flare reactions following direct injection into human skin, [138] and production of lipid mediators such as leukotriene (LT)C_4 and platelet activating factor (PAF), both potent inducers of vasopermeability in vivo. In addition, levels of eosinophil-active IL-5 are elevated in the syndromes of episodic angioedema with eosinophilia, [13] eosinophil-associated toxicity due to IL-2 administration, [143] and in the acute inflammatory response (Mazzotti reaction) in human onchocerciasis patients treated with diethylcarbamazine.[144]

By far, the most direct evidence to date for eosinophil participation in the etiology of the skin diseases listed in the previous paragraph has been the immunochemical detection, generally by immunofluorescent staining of skin biopsies, of prominent extracellular deposits of eosinophil granule cationic proteins such as MBP, ECP, or EDN, but not neutrophil products (neutrophil elastase) in affected but not unaffected skin, and in the absence of prominent numbers of intact eosinophils in lesional

infiltrates. One exception is the IgE-mediated late-phase reaction that follows the wheal-and-flare response characteristic of type I, IgE-mediated hypersensitivity. This classical late-phase reaction characterized clinically by erythema, edema, pruritis and tenderness peaking 612 hours after intradermal antigen or anti-IgE challenge, involves infiltration by mononuclear cells, neutrophils, basophils, and eosinophils presumably in response to mast cell degranulation, along with extensive extracellular deposition of both eosinophil (MBP) and neutrophil (elastase) granule products. [141] The secretion and extracellular deposition of eosinophil-granule constituents shown to possess potent cytotoxic and pro-inflammatory activities in edematous and eczematous lesions clearly suggest that eosinophils may contribute to the pathogenesis of both acute and chronic skin diseases, including the cutaneous lesions associated with HES. [145] However, studies elucidating the mechanisms that regulate eosinophil recruitment, activation, and secretion in the skin are needed to more clearly understand the dynamics of eosinophil participation in these cutaneous disorders and to identify potential therapeutic approaches to selectively blocking their influx and function.

ETIOLOGY OF HES

HES Versus Eosinophil Leukemia

The hypereosinophilic syndrome has historically been confused with eosinophil leukemia, especially since distinguishing between malignant and nonmalignant causes of hypereosinophilia can be quite difficult (Table 40-4, Table 40-5, Table 40-6).[75] Truly malignant acute eosinophil leukemias have a number of distinguishing characteristics such as a marked increase in the numbers of immature blood and/or bone marrow eosinophils, >10% blast forms in the bone marrow and tissue infiltration with immature eosinophilic cells, and a clinical course and findings that tend to resemble other acute leukemias, including anemia, thrombocytopenia, and increased susceptibility to infections (Table 40-5).[49] Because the cardiac and neurologic manifestations of HES can also develop in eosinophil leukemia, they cannot be used as distinguishing clinical features. In contrast, CNS infiltration by eosinophils and the tendency to produce bone myeloblastomas are features more frequently associated with acute eosinophil leukemia.[49] Chromosomal abnormalities may occur with both HES and eosinophil leukemia, but eosinophil leukemia is more frequently associated with chromosomal anomalies characteristic of other acute nonlymphocytic leukemias including 8:21 and 10p+11q-translocations, and trisomy-1. In addition, eosinophil leukemia has been reported as a variant of the M4Eo phenotype of acute myelomonocytic leukemia with eosinophilia, now linked to inversion 16 chromosomal abnormalities.[146] [147] [148] Chronic forms of eosinophilic leukemias have also been described.

TABLE 40-5 -- Major Factors Supporting a Diagnosis of Clonal Disorder Resulting in Hypereosinophiliaa[a]

Major Factors Supporting a Diagnosis of a Clonal Disorder
Cytogenetic abnormalities
FISH in eosinophils
G6PD alloenzymes in heterozygote females
Cultured eosinophil colonies with abnormal cytogenetics
Grade III bone marrow fibrosis
Trilineage blood or bone marrow myelodysplasia
Abnormal localization of immature precursors (ALIP)
Activating mutations in the K ras oncogene
Abnormal nucleotide content of eosinophils
Clonal T-cell proliferation or T-cell receptor gene rearrangement
Minor Factors Supporting a Diagnosis of Clonality
Elevated B_{12} binding
Hepatomegaly/splenomegaly
Normal levels of interleukins and immunoglobulins
Low neutrophil alkaline phosphatase
Markedly abnormal eosinophil morphology
Anemia or thrombocytopenia
Poor therapeutic response to steroid
Factors of Uncertain Significance
Apoptosis on blood film examination
Thrombotic events
Eosinophilic end organ damage

[a] *Reproduced from Brito-Babapulle,[75] with permission.*

TABLE 40-6 -- Diagnosis of Chronic Eosinophilic Leukemiaa[a]

Mature eosinophils in blood
Establish clonality
No dysplastic morphological features
Absence of Ph +/bcr abl +/mbcr rearranged cells
Absence of clonal lymphoproliferation

[a] *Reproduced from Brito-Babapulle.*[75]

Lymphomas and Hypereosinophilia

A number of lymphomas may be associated with the development of eosinophilia including Hodgkins lymphoma,[149] T-cell lymphoblastic lymphoma,[150] and adult T-cell leukemia/lymphoma,[151] but the eosinophilia is generally much more modest than that seen in HES. In contrast, there are a number of reports of patients presenting with classical clinical and hematologic features of HES who have gone on to develop acute lymphoblastic leukemias [152] [153] [154] [155] or T-cell lymphomas.[156] [157] [158] [159] In a number of T-cell lymphoma cases, the accompanying eosinophilias were shown to be associated with the production of eosinophilopoietic cytokines (GM-CSF, IL-3, or IL-5) by the lymphomas themselves.[160] [161]

PATHOGENESIS OF EOSINOPHIL-ASSOCIATED END ORGAN DAMAGE IN HES

Based on current understanding of eosinophil function and hypereosinophilic conditions, sustained eosinophilia due to any cause, regardless of whether it is of reactive, clonal, or idiopathic origin, has the capacity to lead to end organ damage. The multiple manifestations of such eosinophil-associated end organ damage are considerable as presented in Table 40-7 , and their identification and management have been discussed in many reports and reviews. [49] [74] [76] [84] [115] [162] However, not all cases of sustained hypereosinophilia necessarily lead to end organ damage. For example, patients with syndromes such as eosinophilic pneumonia and episodic angioedema with eosinophilia [14] characteristically fail to develop the cardiac damage characteristic of HES patients. Experimentally, IL-5 transgenic mice, which develop extremely high numbers of peripheral blood eosinophils, do not develop significant end organ damage, suggesting that other factors in addition to IL-5 are likely to be necessary for eosinophil recruitment to the tissue, eosinophil activation, and tissue damage. [27] [28] Other factors may be necessary to induce eosinophilic end organ damage, such as secretion of eosinophil-active inflammatory or hematopoietic cytokines (e.g., GM-CSF), genetic predisposition, clonal T-cell dysfunction, development of a Th2 instead of Th1 T-cell profile, or in situ production of cytokines that block eosinophil apoptosis and enhance eosinophil survival and activation. It is not currently known whether true myeloproliferative eosinophilic disorders of clonal origin have the capacity to lead to end organ damage. It is notable that, HES patients in the National Institutes of Health (NIH) series who ultimately developed cardiac disease, exhibited features suggestive of a diagnosis of myeloproliferative disease, including splenomegaly, thrombocytopenia, anemia, elevated B_{12} levels, cytogenetic abnormalities, and circulating myeloid precursors and myeloid dysplasia. [74] [77] As noted earlier, the eosinophil expresses a number of granule cationic protein mediators capable of inducing thrombotic events, [163] endothelial and endocardial damage, [57] and neurotoxicity (the ribonucleases, eosinophil-derived neurotoxin [EDN] and eosinophil cationic protein [ECP]). [124] [125] Eosinophil granule major basic protein (MBP) and eosinophil cationic protein are both potent cellular toxins capable of damaging normal host cells and tissues in a manner reminiscent of end organ damage associated with tissue eosinophilia. [2] [164] In addition, the eosinophil has the capacity to undergo a potent respiratory burst upon activation, generating

TABLE 40-7 -- End Organ Damage Produced by Hypereosinophiliaa[a]

Organ/System	Reference Number
Cardiac	
Constrictive pericarditis	[197]
Endomyocardial fibrosis	[99] [198] [199]
Myocarditis	[200]
Intramural thrombi	[201] [202] [203]
Valve regurgitation	[192]
Cardiomyopathy	[204]
Neurologic	
Thromboembolic	[203]
Peripheral neuropathy	[205]
Central nervous system/dysfunction	[206]
Epilepsy	[207]
Dementia	[207] [208]
Eosinophilic meningitis	[209]
Dermatologic	
Angioedema	[210]
Urticaria	[131]
Papulonodular lesions	[131]
Mucosal ulcers	[133]
Vesico bullous lesions	[211]
Microthrombi	[212]
Pulmonary	
Pulmonary infiltrates	[213]
Fibrosis	[77]
Pleural effusions	[214]
Pulmonary emboli	[215]
Ocular	
Microthrombi	[216]
Vasculitis	[217]
Retinal arteritis	[218] [219]
Joints	
Arthralgia	[220]
Effusions	[221]
Polyarthritis	[222]

Raynauds phenomenon	[223] [224]
Digital necrosis	[225]
Gastrointestinal	
Ascites	[226]
Diarrhea	[78]
Gastritis	[227]
Colitis	[201] [208]
Pancreatitis	[229]
Cholangitis	[229]
Budd-Chiari Syndrome	[230]

[a]*Reproduced from Brito-Babapulle.[75] with permission.*

reactive oxidative species that can directly, or in association with eosinophil peroxidase, induce oxidant-mediated tissue damage. [108] [110] [165] However, the mechanisms by which eosinophils induce thrombosis and the thromboembolic events associated with hypereosinophilic diseases such as idiopathic HES remain unclear, especially since most investigators have failed to identify consistent systemic alterations in coagulation and fibrinolytic pathways in these patients. [77] [78] However, recent studies by Slungaard and colleagues suggest that eosinophil granule cationic proteins have the capacity to alter thrombomodulin activity, suggesting one possible mechanism for thromboembolism in hypereosinophilic heart disease. [109]

THERAPY AND PROGNOSIS FOR HES

The earlier literature on HES is filled with reports of poor to dismal patient prognosis. For example, a 1975 review by Chusid

and colleagues of 57 published cases indicated an average survival time of 9 months, with a 3-year survival of only 12%. [12] The high morbidity and mortality reported in these reviews of prior decades likely reflects the fact that most HES patients tended to present late with more advanced disease, in particular significant cardiovascular problems. Deaths in these patients were generally the result of congestive heart failure and secondary complications of endomyocardial disease including bacterial endocarditis, progressive valvular incompetence, and the thromboembolic sequelae of the syndrome. [49] [115] However, earlier diagnosis of HES, along with improved clinical and echocardiographic methods for monitoring, and use of cardiac medications and cardiothoracic surgical procedures not previously available, has resulted in more successful prevention and management of cardiac disease in these patients, considerably improving clinical outcome and survival. A report in 1989 of 40 HES patients in France reported an 80% survival after 5 years and 42% survival at 10 and 15 years. [114] Thus for many patients, prevention and management of HES end organ damage, in particular the cardiac sequelae, results in prolonged survival over decades. [74] [115] Current, previously unavailable supportive therapies for managing the cardiovascular complications of HES, along with therapies aimed at controlling the eosinophilia to prevent end organ damage in the first place, are the mainstay of HES treatment regimens (Table 40-8). [74] Treatment should focus on controlling end organ damage, not just on suppressing or eradicating the eosinophilia, especially since the severity of cardiovascular complications of HES do not necessarily correlate with the duration or level of eosinophilia. [96] [97] Although more aggressive treatment (e.g., cytotoxic chemotherapy or bone marrow transplant) may be indicated and necessary in selected patients, the current goal is chronic maintenance therapy. In a 1992 review of HES, Spry [115] points out that overaggressive cytotoxic therapy for some HES patients may be more deleterious than the HES itself, with the risk of marrow aplasia. [12] Current therapeutic options for treatment and management of HES are listed in Table 40-8 and described in greater detail later in this chapter. Recent experience with the use of biologic response modifiers, in particular interferon-, hold considerable promise for durable remissions of eosinophilia and clinical signs and symptoms, particularly in patients with resistant disease, but also in HES patients in general as a first-line therapeutic option. [76]

Initial Therapeutic Options

The indication for aggressive initial treatment is evidence of progressive organ involvement and/or symptoms. In asymptomatic eosinophilic patients who lack evidence of end organ involvement, specific therapy need not be administered immediately. However, careful and frequent (36 month interval) clinical and echocardiographic follow-ups should be done to evaluate such patients for the onset of cardiac involvement, which may develop insidiously. It may be valuable in such patients to evaluate the effect of a short course of prednisone (60 mg/kg/day or 1 mg/kg/d) to determine whether the eosinophilia will be responsive to corticosteroid suppression, should organ involvement ultimately develop. Several prognostic

TABLE 40-8 -- Therapies for the Hypereosinophilic Syndrome[a]

Therapy	Indications
None	If no evidence of organ involvement, i.e., those patients with eosinophilia, but without any defining features of HES
Corticosteroids	First-line agent for those with organ involvement; if effective in suppressing peripheral blood eosinophilia, dose may be tapered or changed to every other day; response to corticosteroids associated with a better patient prognosis
Hydroxyurea	Used in those patients with organ involvement and eosinophilia unresponsive to corticosteroids; anemia and/or thrombocytopenia common with chronic therapy
Vincristine	Especially useful for acute reductions when eosinophil counts become excessive (50,00075,000/l); can be administered episodically to control HES, often with amelioration of thrombocytopenia
Alkylating agents	Chlorambucil and others; may be administered in 4-day pulse doses repeated as dictated by the magnitude of blood eosinophilia
Biologic response modifiers and immunosuppressive agents	-Interferon; cyclosporin A. Experience with these agents is somewhat limited, but they have been used with some apparent successes, especially -interferon
Cardiac surgery	Indicated for serious mitral regurgitation with bioprosthetic mitral valve replacement; less commonly, thrombectomy or endomyocardectomy
Other treatments with poorly defined therapeutic or management roles:	
Pheresis	Plasma- and leukapheresis have no defined role in long-term management of HES; use is usually restricted to emergency situations for patients developing profoundly high eosinophil counts; cell counts rebound to prepheresis levels within 1 day
Antiplatelet agents/anticoagulation	Thrombotic/thromboembolic events are a frequent serious HES complication; anticoagulation is administered where thrombo-emboli or intraventricular thrombi have been identified; effectiveness in HES has not been established
Splenectomy	No established role in routine management of HES; may ameliorate platelet sequestration from hypersplenism; relieve pain from splenic distention and infarctions common to those HES patients with splenomegaly
Radiation therapy	Effective for chloromas of eosinophilic leukemias
Bone marrow transplantation	Clinical experience still very limited; risks do not appear to be justified unless patients have an extremely aggressive disease course that is refractory to other preferred modalities listed above

[a] *Modified from Weller and Bubley,[74] with permission.*

features mentioned earlier may predict a good response to corticosteroid therapy including the presence of angioedema and urticaria, elevated serum IgE levels, and a rapid drop in eosinophil counts in response to the initiation of corticosteroids. Initial therapy in patients presenting with evidence of end organ involvement is with prednisone (1 mg/kg/day or 60mg/d in adults). If an adequate suppression of blood eosinophil counts is obtained, then doses may be slowly tapered and ultimately switched to alternate day therapy (1 mg/kg/day). In the NIH series, 38% of patients responded well and 31% partially to prednisone treatment. [92] [95] [106] Patients less likely to respond favorably to prednisone include those with the poor prognostic signs of splenomegaly, cardiac dysfunction, and neurologic symptoms at the time of presentation. The mechanisms of steroid refractory eosinophilia in HES have not been clearly defined; however, Prin and colleagues have reported decreased eosinophil glucocorticoid receptor expression in HES patients and that absence of receptor correlates with steroid resistance. [166]

Chemotherapeutic Agents

In patients with evidence of end organ involvement and steroid-resistant eosinophilia, the decision may be made to use more aggressive cytotoxic chemotherapy. Hydroxyurea (12 grams/day) has demonstrated efficacy in steroid-nonresponsive patients and is given with the goal of reducing total leukocyte counts to a normal range of <10,000/mm^3 . Eosinophil counts begin to diminish after 714 days of treatment, reflecting the kinetics of eosinophil development and turnover in these patients. Hydroxyurea interferes with DNA synthesis, thereby inhibiting the development of all marrow-derived cells, in addition to eosinophils. The development of anemia and thrombocytopenia as a consequence of hydroxyurea may necessitate red cell transfusions and decreased doses or cessation of therapy. Weekly leukocyte counts may be necessary to monitor idiosyncratic fluctuations in red blood cell or platelet counts, and for consequent adjustment of hydroxyurea dosage. [74] Vinca alkaloids are also effective in HES, and vincristine (1.01.5 mg/m^2 at biweekly intervals), which produces a permanent mitotic arrest by disrupting spindle microtubules, has been shown to be beneficial in some patients. [73] [167] [168] [169] However, use of vincristine, which generally spares marrow toxicity for platelets, may become limited by neurologic complications that may be indistinguishable from the peripheral neuropathies associated with HES in some patients. Because vincristine decreases blood eosinophil counts in 13 days, it may be particularly efficacious for acute treatment of patients with marked hypereosinophilia (>100,000 eosinophils/mm^3) (Table 40-8). Other chemotherapeutic agents that have been used with some success in a limited number of patients include etoposide (VP-16) and alkylating agents such as chlorambucil. Etoposide, a podophyllotoxin derivative and topoisomerase II inhibitor that induces DNA damage, has been used successfully in a number of patients to date. Oral followed by parenteral etoposide, administered in one patient after cessation of hydroxyurea treatment, was effective in controlling symptoms and eosinophil counts for a period of 18 months, but was ultimately withdrawn due to marrow suppression. [170] Two additional patients reported by Bourrat et al.[171] responded to etoposide with prolonged and durable remissions, despite discontinuance of the etoposide treatment. In these cases, doses of 300 mg/m^2 were given IV over a 2-day course or 200 mg/m^2 for 7 or 10 days. Use of oral pulses of chlorambucil have been reported by Weller and Bubley [74] at doses ranging from 410mg/m^2 daily for 4 consecutive days approximately every second month for 2 years with some efficacy in patients refractory to steroids and intolerant to hydroxyurea.

Biologic Response Modifiers

A number of biologic response modifiers and immunosuppressive agents have been used more or less successfully in HES patients with a poor response to corticosteroids and hydroxyurea, including interferon- and cyclosporin A. In fact, the most important recent advance in treating resistant HES has been the use of interferon-. [75] Interferon- has been used successfully to treat a number of leukemias including CML, multiple myeloma, and hairy cell leukemia. Experience by one of us with a series of six HES patients treated with interferon- at the Mayo Clinic suggests that interferon may be a valuable agent for HES patients who are unresponsive to or intolerant of conventional corticosteroid or hydroxyurea therapies, and for patients with this syndrome who develop incapacitating mucosal ulcers.[135] [136] In this series, IFN-$_{2b}$ was administered daily in single subcutaneous injections ranging from 1.0 to 6.25 × 10^6 U/day (MU/day) individualized for each patient. Doses from 1.5 MU/day to 8 MU/day were successful in decreasing eosinophil counts to <1,000/mm^3 in five of six patients and all patients were able to taper and discontinue prednisone and hydroxyurea. [135] [136] Two patients in this series with incapacitating mucosal ulcerations had resolution and no recurrence of their lesions. A number of additional reports indicate variable success in suppressing eosinophilia and hepatosplenomegaly, in clinical parameters and echocardiographic improvements in HES patients treated with interferon- as well as sustained disease remissions. [172] [173] [174] [175] [176] [177] Thus, there is a growing body of evidence in the literature that IFN- has a major place in the long-term treatment of HES, particularly in cases that are unresponsive to more conventional therapies, those with intractable mucosal ulcerations, those with cytogenetic abnormalities, and those presenting as myeloproliferative variants of HES. [178] Cyclosporin-A, an immunosuppressant that interferes with multiple aspects of immunocompetent T-lymphocyte function, has been reported to be effective in a series of four HES patients.[44] [179] [180] The rationale for use of cyclosporin-A is the inhibition of production of eosinophilopoietic cytokines (i.e., IL-5) from putative T-cell clones thought to drive the eosinophilia in these patients. [44] [75] [179] Of interest, T-cell clones have been established and characterized from the peripheral blood of a number of patients with HES,[44] providing the rationale for its use. Cyclosporin-A has been used in conjunction with low-dose corticosteroids, although the reported duration of therapy has been limited (10 months) and longer-term efficacy or side effects in HES have not been determined. Indeed, Zabel and Schlaak [180] demonstrated corticosteroid-sparing effects and durable control of eosinophilia using 4 mg/kg cyclosporin-A in a series of three HES patients.

Pheresis

As noted in Table 40-8 , plasma and leukapheresis have no defined role in the long-term management of HES. The use of leukapheresis, or more specifically eosinopheresis, has generally been restricted to emergency situations for patients developing profoundly high eosinophil counts. However, cell counts rebound rapidly to prepheresis levels within 1 day of the procedure. [181] [182] Even multiple repeated sessions of leukapheresis are usually not sufficient to induce more than a transient decrease in blood eosinophil levels. [181] [182] [183] Five repeated plasma- and leukapheresis sessions over a period of two weeks for a single patient was reported to significantly decrease blood eosinophilia, however continued sessions in this patient were ineffective by themselves to lower blood eosinophil counts to acceptable levels.[183] The mechanism by which pheresis transiently decreases eosinophil levels is not known, but has been suggested to involve a temporary removal or decrease in the levels of circulating eosinophilopoietic factors. [181] [183]

Anticoagulation and Antiplatelet Agents

Because thrombotic and thromboembolic events are frequently serious complications of HES, anticoagulants have often been used, especially in those patients with clear evidence of thromboemboli, neurologic symptoms, and cardiac involvement. Commonly used agents include warfarin, antiplatelet agents, or heparin. However, the efficacy of anticoagulation or antiplatelet agents has not been clearly established in HES disease, and a number of patients treated with these agents have continued to have thrombotic events, despite their adequate use. [78] [115]

Splenectomy

As noted earlier in this chapter, splenomegaly has been reported in 43% of HES patients, and hypersplenism in these individuals may contribute to both thrombocytopenia, anemia, and spleen infarction. Splenic pain induced by capsular distention or infarction is a frequent complication of splenic involvement in these individuals.[95] In HES, splenectomy has the capacity to ameliorate platelet sequestration from hypersplenism and to relieve the pain associated with splenic distention and infarctions. However, splenectomy has no established role in the routine management of HES patients.

Bone Marrow Transplantation

There is still very limited experience reported for bone marrow transplantation as a modality for the treatment of HES. [184] [185] [186] [187] [188] However, a number of the recent reports of bone marrow transplantation for refractory HES include two successes with patients apparently in complete remission [186] [188] and a third that was a success except for the development of graft-versus-host disease (GVHD) requiring management with cyclosporin-A. [184] In a fourth report, a relapse occurred 40 months after transplantation with the patient surviving at least through 44 months. [187] In a much earlier case reported in 1988, an HES patient given an allogeneic transplant recovered hematologically, but subsequently died within three months from a diffuse cytomegalovirus (CMV) infection, thus preventing adequate evaluation of the procedure.[185] Only in HES cases with an extremely aggressive course unresponsive to standard therapies, or in young patients with refractory disease and clinical features suggestive of a myeloproliferative disorder (e.g., chromosomal abnormalities), should allogeneic bone marrow transplantation be considered a treatment option at this time. Without greater understanding of the underlying mechanisms responsible for the overproduction of eosinophils and the aggressive development of end organ damage in some of these patients, the risks associated with bone marrow transplantation may outweigh its more routine use in treatment of this syndrome.

Cardiac Surgery

Treatment of the cardiovascular sequelae of HES continues to be one of the major therapeutic challenges of this syndrome. However, the use of cardiac surgery is one of the major advances since the 1970s in treating the varied cardiac complications of HES. [49] Surgical intervention in eosinophilic heart disease does not appear to carry any risk of disease recurrence at the operative sites. For patients who develop significant compromise of valvular function, endomyocardial thrombosis, or fibrosis, cardiac surgery has the capacity to provide substantial clinical and quality of life improvements. [49] Mitral valve[189] or tricuspid valve (or both) repairs or replacements[190] have been reported for >50 eosinophilic patients.[49] Endomyocardectomy and thrombectomy, as performed for advanced stage Löefflers endomyocardial fibrosis, has also been used effectively in some HES patients [78] [191] and ventricular decortication may be performed in combination with valve replacement. For mitral valve replacements, mechanical valves have proven problematic in terms of recurring thrombotic episodes despite adequate anticoagulation,[191] [192] [193] suggesting the deliberate use of porcine bioprostheses whenever possible. [74] [194] HES patients who have received valve replacements have generally experienced long-term improvement in cardiac function and well-being, making it a useful procedure for the long-term care of HES with cardiac involvement.[74]

Ineffective Agents in HES

A number of agents have proven either less useful or totally ineffective for the treatment of HES. These include antihistamines, anabolic steroids, methotrexate and busulfan (alone or combined), azathioprine, and cyclophosphamide.

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SUMMARY AND CONCLUSIONS

The hypereosinophilic syndrome comprises a rather heterogeneous collection of relatively rare disorders distinguished by end organ damage. It shares many clinical features with a variety of eosinophilic syndromes related to vasculitis, myeloproliferative disorders, and certain parasitic infections. The heterogeneity of HES, ranging from patients with clear myelodysplastic features including cytogenetic abnormalities, to patients with more benign clinical courses such as episodic angioedema with eosinophilia, suggest that multiple disease mechanisms are likely involved. Current research aimed at defining the etiology of HES and mechanisms regulating the development of eosinophil-mediated end organ damage in eosinophil-associated diseases in general, should ultimately lead to more selective and improved therapies. The therapeutic targets for these efforts are likely to include IL-5 and its high-affinity receptor, underlying T-cell clones (either immunocompetent or occult T-lymphoid malignancies) that elaborate eosinophilopoietins such as IL-5 or GM-CSF, tissue or organ-specific dysfunctional elaboration of eosinophil-active chemoattractant factors such as eosinophil-selective chemokines (eotaxins) or vascular endothelial adhesion molecules (VCAM/VLA-4), or as yet undefined mechanisms that induce the overproduction of eosinophils and/or serve to recruit and activate eosinophils selectively in certain tissues and organs. The essential absence of end organ damage in some syndromes of hypereosinophilia contrasts starkly with the morbidity (and mortality) associated with the development of endomyocardial fibrosis in HES and certain other eosinophilias. Because HES patients are clearly a heterogenous group, clinical management based on current knowledge must be specifically tailored to the individual, [76] with the overall goal of controlling the eosinophilia, and in particular, the eosinophil-mediated end organ damage. The past 25 years have seen an evolution in clinical and surgical management of patients with hypereosinophilia and HES. Current treatment options are numerous, such that both eosinophilia and end organ damage can be controlled or even eradicated in the majority of HES patients. For patients with resistant forms of HES, IFN- and bone marrow transplantation hold great promise for durable disease remissions in these patients. It is important to note that treatment with IFN- should now be considered as an initial option for the management of HES, either alone or in combination with existing therapies. [76]

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Chapter 41 - Disorders of Phagocyte Function and Number

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Disorders of Phagocyte Function

INTRODUCTION

The defense of the host against pathogenic microbes is the critical responsibility of the immune system. Unfortunately for the host, these microbes are diverse in nature mdash; they may be viruses, bacteria, fungi, or uni- or multicellular parasites and many have evolved an impressive array of mechanisms to penetrate the host, evade immune surveillance, and even neutralize the antimicrobial poisons generated by the host defense systems. In light of this diversity and the evolutionary cat and mouse game played between pathogens and the immune system, it is not surprising that the immune system has evolved into a complex defense network comprising a variety of cell types and humoral mediators. The innate host defense system is responsible for the first line of protection against invading bacteria, fungi, and parasites and is composed of phagocytes (especially neutrophils), natural killer lymphocytes, complement, and other plasma proteins. As reviewed in Chapters 38 and 39 , phagocytes perform their critical functions either as resident cells in a variety of tissues or as circulating marauders in the bloodstream capable of traveling into any infected tissue. Members of the former category include such well-studied cells as the alveolar, hepatic, peritoneal, and splenic macrophages. The circulating phagocytes, on the other hand, are dominated by neutrophils but also include monocytes and eosinophils.

A complex series of cellular regulatory phenomena are involved in the phagocytes role as a primary defender of the host. These are most acute in the case of the circulating neutrophils, which must be able to sense exceedingly weak chemotactic signals from infected tissues, transduce this information rapidly into purposeful movement toward the source of the signals, engulf

the target microbes on arrival at the infected tissue, and then destroy the pathogens with extremely potent and effective agents while sparing surrounding normal tissues. Microbial killing is accomplished by two types of mechanisms: (1) de novo synthesis of highly toxic, and often unstable, derivatives of molecular oxygen by an enzyme known as the respiratory burst oxidase; and (2) delivery into the phagocytic vacuoles containing the ingested microbes of preformed polypeptide antibiotics and proteases stored within several types of lysosomal granules. [1] [2] [3]

Congenital and acquired disorders of each of the steps in phagocyte function have been described. As would be predicted, these disorders are manifested clinically by recurrent bacterial and fungal infections. Interestingly, the converse of this is only rarely observed. Most patients with recurrent infections do not have any identifiable abnormality in their phagocytes. There are at least two explanations for the clinical rarity of phagocyte disorders. First, given their critical role in host defense, nature may be quite intolerant of major abnormalities in phagocytes. Prior to the modern antibiotic era, patients afflicted with severe disorders probably did not survive into their childbearing years. Second, there is a remarkable redundancy in the antimicrobial machinery of the phagocytes that permits one system to compensate for a defect in another. For example, the host does not rely on a single chemotactic signal or neutrophil membrane receptor to ensure that phagocytes accumulate at sites of infection. Instead, multiple chemotactic signals and receptors are used.

This chapter reviews the major functional and quantitative disorders of phagocytes and is divided into two sections. The first focuses on the qualitative abnormalities and is organized according to the cellular functions outlined: disorders of the respiratory burst microbicidal pathway, abnormalities of phagocyte adhesion and chemotaxis, and defects in the structure and function of lysosomal granules. The second section outlines the quantitative abnormalitiesleukocytosis and leukopeniawith a particular emphasis on neutropenia. The chapter is not meant to be an encyclopedic review of the many papers published on phagocyte abnormalities. This chapter focuses on those disorders for which correlations do exist, with particular emphasis on those that are best understood at the molecular level. Many comprehensive reviews are available to the reader who is interested in the less well characterized phagocyte disorders. [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18]

DISORDERS OF THE RESPIRATORY BURST PATHWAY

The unstimulated human neutrophil consumes relatively little oxygen and relies primarily on glycolysis for energy. [19] [20] Within seconds after contact with suitably opsonized microorganisms or a variety of soluble stimuli, the rate of oxygen consumption abruptly increases, usually by a factor of >100, a metabolic event known as the respiratory burst.[21] This oxygen is consumed in a nonmitochondrial reaction in which it is reduced with a gain of one electron to form superoxide (as the O_2 ion) by a plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase referred to as the respiratory burst oxidase (Fig. 41-1 , reaction 1).[22] [23] [24] NADPH is the preferred substrate for this enzyme, and O_2 appears to be the sole metabolite of the oxygen in most instances.[20] [21] [22] NADPH oxidase, along with those enzymes and reactions that are directly involved in the production or metabolism of reactive oxygen species, constitute the respiratory burst pathway [20] as depicted in Figure 41-1 . Five clinically significant defects have been identified in this pathway involving the following enzymes: NADPH oxidase [25] [26] (reaction 1), leukocyte glucose-6-phosphate dehydrogenase (G6PD) [27] (reaction 8), myeloperoxidase [13] [28] (reaction 4), glutathione reductase [7] [29] (reaction 7), and glutathione synthetase

Figure 41-1 Reactions of the respiratory burst pathway. The enzymes responsible for reactions 1 to 9 are as follows: (1) respiratory burst oxidase (NADPH oxidase); (2) superoxide dismutase (SOD) or spontaneous; (3) nonenzymatic; Fe^{2} +-catalyzed; (4) myeloperoxidase; (5) spontaneous; (6) glutathione peroxidase; (7) glutathione reductase; (8) glucose-6-phosphate dehydrogenase; (9) glutathione synthetase.

[7] (reaction 9). These reactions are involved in the production of O_2 (reactions 8 and 1), in the conversion of O_2 and hydrogen peroxide to other toxic derivatives (reaction 4), or in the detoxification of excess hydrogen peroxide needed to protect the phagocyte during the respiratory burst (reactions 6, 7, and 9).

Chronic Granulomatous Disease

Biology

Chronic granulomatous disease (CGD) comprises a heterogeneous group of defects that share in common the failure of neutrophils, monocytes, macrophages, and eosinophils to undergo a respiratory burst and generate O_2.[11] [25] [30] [31] [32] [33] The disorder is relatively rare, with a prevalence of approximately 1/500,000 individuals based on unpublished estimates from large urban populations. Data from the United States CGD Registry encompassing 368 registered patients indicates a rate of 1200,000 live births for the period 19801990. Because of the central role of superoxide and other respiratory burst products in microbial killing, patients with CGD suffer from recurrent severe bacterial and fungal infections. [34] [35] The disease was first described in 1957 in two independent reports, [36] [37] both of which describe severe recurrent infections associated with visceral granulomas containing pigmented histiocytes. The disease was termed fatal granulomatous disease owing to this distinguishing histologic feature and the grim clinical course in most patients. [36] [38] [39] [40] It was not until the late 1960s and early 1970s that the defect in oxygen consumption and O_2 production was identified and a convenient diagnostic assay, the nitroblue tetrazolium (NBT) test, was developed. [41] [42] [43] [44] [45] It was also during that time that it became apparent that CGD is a heterogeneous disorder. Although most patients were male and had inherited the disease in an X-linked manner, [46] a few female patients with CGD were discovered in whom the mode of inheritance appeared to be autosomal recessive. [31] [47] [48] [49] By 1975 it was clear that most, if not all, instances of CGD were caused by defects in the activity (or activation) of NADPH oxidase [50] (reaction 1, Fig. 41-1).

Our current understanding of CGD was reached with the aid of two major findings. The first was that a unique, low-potential (245 mV) cytochrome b was undetectable in nearly all patients with X-linked disease. [51] [52] The second was the discovery of a method by which NADPH oxidase could be activated in a cell-free system in the presence of negatively charged lipids such as arachidonic acid. [53] [54] [55] [56] These studies disclosed that both

TABLE 41-1 -- Cell-Free Activation of NADPH Oxidase in Two Genetic Forms of CGD

Source of Subcellular Fraction		Superoxide Generation[a] [b]	
Cytosol	**Membrane**	**Experiment 1**	**Experiment 2**
Normal	Normal	73.2	58.5
Patient	Patient	0.1	0.3
Normal	Patient	0.1	50.1
Patient	Normal	68.7	40.1
50% patient/50% normal	Normal	65.3	24.4

[a]Numerical data represent nmoles O_2 per minute per 10^7 cell-equivalents of membrane.

[b]The rate of superoxide generation was used to measure the extent of activation of dormant NADPH oxidase in a cell-free activation system using arachidonic acid (82 M) as the stimulus. Experiment 1 was performed with membranes and cytosol from a normal individual and a patient with X-linked cytochrome b-negative CGD, the most common type of CGD. Experiment 2 was performed with a different normal donor and a patient with autosomal recessive, cytochrome b-positive CGD lacking the p47-phox cytosol oxidase component, the second most common type of CGD. Oxidase activation was performed as described in Curnutte et al.[57]

cytosolic and membrane proteins were required for oxidase activation and that all patients had defects involving either the membranes or the cytosol. [53] [57] [58] Table 41-1 demonstrates this point. If cytosol and membranes from a normal individual are combined in the cell-free system and activated with arachidonic acid, large quantities of O_2 are generated. In experiment 1 in the table, cytosol and membranes from a patient with X-linked CGD (who was missing cytochrome b) were combined, and virtually no O_2 was produced following arachidonate stimulation. The cross-mixing experiment indicated that the patients membranes, but not cytosol, were defective. In experiment 2, a female patient with autosomal recessive CGD associated with a normal level of cytochrome b was studied. In contrast to the patient with X-linked CGD, her cytosol was severely defective while her membranes were normal.

It is now appreciated that NADPH oxidase is a complex enzyme consisting of multiple catalytic and regulatory subunits and that the activity of the oxidase is regulated, at least in part, by controlling the assembly of these components. [23] [24] [59] [60] [61] [62] [63] [64] [65] [66] [67] Perhaps as a fail-safe mechanism to prevent inadvertent activation, some of these components are localized in the membrane in the unstimulated neutrophil, whereas others are present in the cytosol (left side of Fig. 41-2). The membrane contains cytochrome b that functions as the catalytic and redox center of the oxidase, and this cytochrome consists of two closely interacting subunits present in a 1:1 stoichiometrygp91-*phox* (glycoprotein 91 kd of the phagocyte oxidase) and p22-*phox*. [68] [69] [70] [71] [72] [73] [74] Each cytochrome b contains one flavin adenine dinucleotide (FAD) group and two hemes. [75] [76] [77] [78] A low-molecular-weight G protein, rap1A, is also closely associated with cytochrome b, but it is not known whether it is a functional part of the oxidase. [79] [80] [81] The four remaining known oxidase components reside in the cytosol in unstimulated neutrophils: p47-*phox*, p67-*phox*, p40-*phox*, and another low-molecular-weight guanosine triphosphate (GTP)-binding protein, rac2 (or rac1 in guinea pig macrophages) (Fig. 41-2).[63] [82] [83] [84] [85] [86] [87] [88] [89] [90] [91] [92] [93] [94] [95] Prior to stimulation of the neutrophil, three of the cytosolic components (p47- *phox*, p67-*phox*, and p40-*phox*) appear to exist in a 260-kd complex that is stabilized by multiple protein-protein interactions between SH3 (src homology region 3) and proline-rich domains within the proteins (Fig. 41-2). [24] [58] [67] [96]

Figure 41-2 Model of NADPH oxidase activation. Current knowledge of the oxidase suggests that in its dormant state (left side of figure) it is composed of both membrane-bound and cytosolic components. The former include gp91-*phox* and p22-*phox,* which together form the cytochrome b heterodimer that also contains the redox centers of the enzyme: FAD and two heme groups (Fe). Rap1A, a low-molecular-weight GTP-binding protein, is also present in the membrane and may functionally associate with the cytochrome. The cytosolic components include p40- *phox,* p47-*phox,* and p67-*phox,* which exist in a complex of 260 kd. A pool of free, monomeric p47- *phox* is also present in the cytosol prepared from resting neutrophils. In its inactive GDP-bound state, the small GTP-binding protein Rac (Rac2 in human neutrophils) is also cytosolic and is bound to RhoGDP-dissociation inhibitor (GDI). On stimulation (right side of figure), p47- *phox,* p67-*phox,* and p40-*phox* become associated with the plasma membrane primarily through interactions between p47-*phox* and the subunits of the cytochrome. This translocation process is accompanied by, and perhaps requires (1) the release of Rac from RhoGDI, its conversion to an active (GTP-bound) state, and its association with the plasma membrane, and (2) the multisite phosphorylation of p47- *phox*. By a mechanism that is not fully understood, binding of the cytosolic components activates the flavocytochrome to catalyze the transfer of electrons from NADPH to oxygen via the FAD and heme redox centers. The compartment labeled inside is the cytoplasmic space; outside refers either to the extracellular or phagosomal space. *(From Heyworth et al.,[24] with permission.)*

TABLE 41-2 -- Classification of CGD

Component Affected	Gene Locus	Inheritance	Subtype Designation[a]	NBT Score (% positive)	O_2 Production (% normal)	Cytochrome b Spectrum (% normal)	Defect in Cell-Free System	Families Evaluated		Frequency (% of cases)
								Scripps[b]	Europe[c]	
gp91-*phox*	Xp21.1	X	$X91^0$	0	0	0	Membrane	147	36	68
			X91	80100 (weak)	330	330	Membrane	11	2	5
			X91	510	510	510	Membrane	2	0	<1
			$X91^+$	0	0	100	Membrane	4	0	1

p22-*phox*	16p24	AR	$A22^0$	0	0	0	Membrane	7	3	4
			$A22^+$	0	0	100	Membrane	1	0	<1
p47-*phox*	7q11.23	AR	$A47^0$	0	01	100	Cytosol	31	13	17
p67-*phox*	1q25	AR	$A67^0$	0	0	100	Cytosol	9	3	5

Abbreviations: X, X-linked inheritance; AR (or A), autosomal recessive inheritance; NBT, nitroblue tetrazolium.

Modified from Curnutte,[104] with permission.

[a]In this nomenclature, the first letter represents the mode of inheritance (X-linked [X] or autosomal recessive [A]), and the number indicates the phox component that is genetically affected. The superscript symbols indicate whether the level of protein of the affected component is undetectable (^{0}), diminished (), or normal (+) as measured by immunoblot analysis.
[b]This group represents 212 kindreds evaluated at the Scripps Research Institute/Stanford University CGD Clinic.
[c]Cooperative study reported in 1992[105] represents 57 kindreds and 63 patients.

On neutrophil activation, p47-*phox* becomes partially phosphorylated and translocates to the membrane along with the other cytosol components in a process that requires GTP, rac2, and docking sites on p22-*phox* and gp91-*phox* [59] [60] [63] [67] [97] [98] [99] [100] [101] [102] (right side of Fig. 41-2). All CGD cases thus far analyzed at the molecular level are caused by mutations involving one of the four components gp91-*phox*, p22-*phox*, p47-*phox*, and p67-*phox*.

The molecular heterogeneity of CGD can be organized into a modern classification scheme based on the oxidase component affected [103] [104] [105] (Table 41-2). Nomenclature has also been adopted for an abbreviated designation for each subtype of CGD, as outlined in the legend for Table 41-2 . Defects in gp91-*phox* are all inherited in an X-linked fashion and account for approximately 74% of all CGD cases. [105] [106] In most X-linked CGD, gp91-*phox* is completely absent, and there is no measurable cytochrome b, NBT reduction, or intact cell superoxide production (the $X91^0$ subtype). In about 10% of X-linked cases, gp91-*phox* can be present at normal levels, but is nonfunctional ($X91^+$), partially deficient (X91), or mutated in such a way that the Michaelis constant (Km) for NADPH is abnormal (X91). [52] [107] [108] [109] [110] [111] [112] [113] [114] Mutations involving p22-*phox* occur in approximately 4% of patients with CGD and usually result in the complete absence of cytochrome b ($A22^0$).[115] [116] [117] [118] [119] This defect is inherited in an autosomal recessive manner. Because the full expression of cytochrome b in the membrane requires the production of both subunits, a primary deficiency of either component leads to a secondary loss of the other. [69] [120] Thus, neither subunit can be detected on immunoblot analysis in either $X91^0$ or $A22^0$ CGD. The second most common form of CGD is caused by a severe deficiency in the cytosol of p47-*phox* ($A47^0$) and is seen in 17% of all patients. [57] [83] [85] [86] [121] (Table 41-2). In the remaining 5% of patients, CGD is caused by the absence of p67-*phox* ($A67^0$).[57] [83] [85] [86] [121] Both cytosol defects are inherited in an autosomal recessive manner and are characterized by a severe deficiency of cytosol activity in the cell-free system. [57] Membrane function is intact in the cell-free system in these two types of CGD, and cytochrome b levels are normal. [57]

Despite the fact that more than 90% of patients with CGD have respiratory burst defects that result in undetectable levels of O_2 production, there is a surprising heterogeneity in the clinical manifestations of the disease. [31] [34] [104] [122] [123] At one end of the spectrum are the patients who suffer from severe bacterial and fungal infections starting in infancy, and who seldom have more than 612 months between serious infections. At the other end of the spectrum are patients who are well for many years and then unexpectedly develop a highly unusual infection such as a staphylococcal hepatic abscess or *Aspergillus* pneumonia. After their first major infection, some of these patients may be relatively healthy again for another 310 years before the next severe infection occurs. Based on experience with over 150 CGD patients, those with $X91^0$ and $A22^0$ CGD tend to have a more severe clinical course, whereas patients with $A47^0$ CGD often enjoy the milder clinical phenotype despite the equal severity of their respiratory burst defect. [123] Individuals with $A67^0$ CGD or partial respiratory burst activity <10% of normal (most X91 patients) tend to have disease of intermediate severity. Because of this heterogeneity, the diagnosis of CGD should be considered, not only in young children with recurrent severe infections, but also in adolescents and young adults who experience exceptionally severe or unusual infections.

Molecular Genetics of CGD

All of the subtypes of X-linked CGD are caused by mutations in the gene encoding the gp91-*phox* subunit of cytochrome b (Table 41-3).[33] [68] [69] [71] [106] [124] [125] [126] [127] [128] This gene, termed CYBB (MIM 306400), contains 13 exons and spans approximately 30 kb in the Xp21.1 region of the X chromosome. [129] [130] [131] [132] [133] [134] Based on the analysis of 261 X-linked CGD kindreds described in a multicenter international database, there is a striking heterogeneity in the mutations seen, with most being family specific.[125] Deletions, missense mutations, and nonsense mutations occur with comparable frequencies, each accounting for approximately 25% of all the mutations (Table 41-3). Splice-site mutations (16%) and insertions (10%) comprise most of the remaining mutations. Two putative regulatory mutations have also been identified to date. [133] [134] Distribution of the mutations within the CYBB gene

TABLE 41-3 -- Summary of Mutations in the CYBB Gene Encoding gp91-*phox* in 261 Kindreds with X-linked CGD

Type of Mutation	Number of Kindreds	Frequency (%)	Phenotype
Deletions	63	24.2	$X91^0$
Insertions	27	10.3	$X91^0$
Splice-site mutations	42	16.1	$X91^0$
Missense mutations	59	22.6	$X91^0$, X91 , $X91^+$
Nonsense mutations	70	26.8	$X91^0$

Data are from a multicenter database.[125]

appears to be more or less random with no apparent mutational hot spots.[106] Based on these large series of X-linked patients, it is clear that most instances of X-linked CGD arise from mutations in the coding and splice regions of CYBB and not from defects in the regulation of the gene. [106] [125] [126] Most of these mutations affect either the stability of the mRNA or the protein (or both), usually to the extent that no measurable cytochrome b is expressed in the phagocytes (Table 41-2).

The diversity of the mutations in gp91-*phox* parallels the clinical heterogeneity seen in X-linked CGD and, in many cases, provides at least a partial explanation for the phenotype observed. For example, large interstitial deletions in the Xp21.1 region can affect not only CYBB, but also a number of flanking genes. Distal to CYBB are the genes that encode a Kell blood group precursor Kx (gene locus XK) and dystrophin (DMD), defects which cause McLeod hemolytic anemia and Duchenne muscular dystrophy; centromeric to CYBB are the genes for ornithine transcarbamylase and retinitis pigmentosa-3. [130] [135] [136] One patient has been reported who suffered from CGD, Duchenne muscular dystrophy, McLeod hemolytic anemia, and retinitis pigmentosa,[130] whereas several others have been identified who have a similar constellation of defects without retinitis pigmentosa. [68] [106] [137] Because the XK locus that is responsible for the McLeod phenotype is closer to CYBB than the DMD locus,[139] it is more common to see McLeod hemolytic anemia in conjunction with CGD.[68] [106] [137] [138] [139] The variant forms of X-linked CGD (X91) in which some residual cytochrome b and respiratory burst activity are retained can also be explained at the molecular genetic level. For example, an in-frame nucleotide triplet deletion leading to the loss of a single amino acid in an extracellular domain of gp91-*phox* (K315) leads to only a 75% deficiency of cytochrome b and O_2 production and an exceedingly mild phenotype.[106] Similarly, two brothers with mild CGD have been identified who have a missense mutation (K161 R) that still allows their neutrophils to express low levels of a functional cytochrome b and generate O_2 at a rate 3% of normal.[106] In other instances, missense mutations predicting less conservative changes result in either absent (G20 R) or nonfunctional (P415 H) cytochrome b and a more severe clinical picture. [106] The severe phenotype is also more frequently seen in those patients with either nonsense or frameshift mutations because these lead to stop codons at either the site of mutation or slightly downstream (in the case of a frameshift). Truncated forms of gp91-*phox* are apparently highly unstable because patients with premature stop codons, even near the end of the gene, all have undetectable levels of cytochrome b and respiratory burst activity. [106] [126]

Mutations in the regulatory regions of the gp91-*phox* gene are uncommon and seen in <1% of X-linked CGD cases. The two that have been described are from unrelated kindreds that involve point mutations 55 and 57 bp upstream (5) of exon 1. [133] [134] Interestingly, the three patients from these two kindreds exhibited the same, highly unusual biochemical and functional phenotype. By both NBT testing and flow cytometric analysis of H_2O_2 production (using the dichlorofluorescin

assay[140]), 5% of each patients neutrophils had full respiratory burst activity while the remaining 95% were devoid of activity. The total amount of cytochrome b was also 5% of normal, presumably due to normal expression levels in the small clone of cells with full respiratory burst activity. It appears that these putative regulatory mutations adversely affect cytochrome b expression in the majority, but not all, of the circulating neutrophils and that this reflects some type of underlying heterogeneity in the way certain subpopulations regulate gp91- *phox* expression.

Mutations in the gene for p22-*phox* subunit of cytochrome b cause one of the three forms of autosomal recessive CGD and account for about 5% of all CGD cases (Table 41-2). The p22-*phox* gene, termed CYBA (MIM 233690), resides at chromosome 16q24 and contains six exons that span 8.5 kb.[116] As with X-linked CGD, the mutations identified in the nine kindreds studied (seven females and four males) are heterogeneous and family specific (Table 41-4).[44] [47] [116] [118] [119] In all but two of the cases listed in Table 41-4 (cases 2 and 9), the patients are homozygous for the mutant allele due to consanguinity of the parents. The mutations in general predict either major defects in p22-*phox* (e.g., case 1) or nonconservative amino acid substitutions. Not surprisingly, these patients fail to express p22- *phox*, are devoid of cytochrome b, cannot generate O_2 , and have clinically more severe disease. The one exception to this is case 6, who has a nonfunctional, yet spectrally normal cytochrome b that is expressed at normal levels. There is a predicted structural defect in the intracytoplasmic C-terminal domain due to the substitution of a Gln for Pro at residue 156.[119] Analogous to the $X91^+$ patients described above, this mutant nonfunctional cytochrome b is unable to support a respiratory burst and results in severe disease.

The gene for p47-*phox*, termed NCF1 (MIM 233700), resides

TABLE 41-4 -- Summary of Mutations in the CYBA Gene Encoding p22-*phox* in Nine Kindreds with Autosomal Recessive CGD

Kindred; Sex	Type of Mutation	Nucleotide Change [a]	Predicted Amino Acid Change	CGD Type[b]	Comments	References
1. F	Deletion > 10 kb deletion	>10 kb deletion	No p22-*phox*	$A22^0$	Homozygous;[c] severe CGD	[44] [116]
2. M	(1) Deletion (2) Missense	(1) Delete C272 (2) G297 2) A	(1) Stop in exon 6 (2) R90 Q	$A22^0$	Compound heterozygote, severe CGD	[47] [116]
3. M/2F	Missense	G297 A	R90 Q	$A22^0$	Homozygous[c]	[118]
4. F	Missense	A309 G	H94 R	$A22^0$	Homozygous[c]	[118]
5. F	Missense	C382 A	S118 R	$A22^0$	Homozygous[c]	[116]
6. F	Missense	C495 A	P156 Q	A22+	Homozygous;[c] nonfunctional cytochrome	[119]
7. M	Splice	gt at intron 4 (5)	Deletion exon 4	$A22^0$	Homozygous[c]	[118]
8. F	Splice	gt ct intron 5 (5)	Deletion exon 5	$A22^0$	Homozygous[c]	[730]
9. M	(1) Missense (2) Insertion	(1) A186 G (2) Insert G between C194 and A200	(1) E53 V (2) Premature stop codon	$A22^0$	Compound heterozygote	[731]

[a] Nucleotide residues are numbered according to the cDNA sequence described in Orkin.[732]
[b] See Table 41-2 for explanation of CGD types.
[c] In these cases, homozygosity of the mutant allele was due to consanguinity in the parents.

on chromosome 7 at q11.23[141] and contains eleven exons spanning 15 kb.[33] Mutations at this locus cause autosomal recessive CGD and account for about 17% of all CGD cases (Table 41-2). In contrast to the diversity of mutations seen in patients with cytochrome b defects, only two different mutations have been reported thus far in the thirty-five unrelated patients studied.[33] [142] [143] In 31 of these 35 cases, the patients are homozygous for a mutant allele with a GT deleted at the beginning of exon 2 that predicts a premature stop codon later in that same exon.[143] The other four patients are apparently compound heterozygotes with one allele containing the GT deletion in exon 2 and the other a distinct second mutation. In one of these four patients, the other mutation has been identified as a deletion of a single nucleotide (G502).[143A] The predominance of the mutant allele containing the GT deletion in the $A47^0$ CGD patient population appears to be caused by the presence of at least one highly homologous NCF1 pseudogene that contains this GT deletion. [143B] Both the NCF1 wild type and pseudogene(s) colocalize to chromosome 7q11.23 and this close association, coupled with the presence of multiple recombination hot spots within each gene, suggest that the high frequency of the GT deletion mutation in $A47^0$ CGD is caused by recombination events between these two genes.[143B]

Mutations in the gene for p67-*phox* account for the remaining 5% of CGD patients (Table 41-2). This gene, referred to as NCF2 (MIM 233710), is located on the long arm of chromosome 1 at position q25[141] and contains 16 exons that span 40 kb.[144] As shown in Table 41-5 , mutations in the p67-*phox* gene have been reported in twelve kindreds with this type of autosomal recessive CGD.[33] [145]

Clinical Manifestations

In approximately two-thirds of patients, the first symptoms of CGD appear during the first year of life with the onset of recurrent, purulent bacterial or fungal infections. Table 41-6 summarizes the types of infections and infecting organisms most frequently encountered in CGD.[34] [146] [147] [148] [149] [150] [151] [152] The most common types of infections involve sites in contact with the outside worldconsistent with the role of neutrophils as the first line of defense against infection. *Staphylococcus aureus*, enteric gram-negative organisms, *Serratia marcescens, Burkholderia cepacia* (formerly known as *Pseudomonas cepacia*), and *Aspergillus* species represent the most frequently encountered pathogens. Most CGD pathogens share the property of being catalase negative and as such, inadvertently lend H_2O_2 secreted from the pathogen to the peroxide-starved CGD phagocyte, which in turn uses it (once converted to HOCl by myeloperoxidase; see Fig. 41-1) to kill the microbe.[153] It also appears that at least some of the CGD pathogens are resistant to the nonoxidative killing mechanisms of the phagocyte, [154] and thus can proliferate relatively unchecked. It is somewhat surprising how often one fails to identify the infecting organism in CGD, perhaps greater than half the time despite aggressive culturing. In this situation, one treats empirically with the antibiotic that should work and if this fails, one then aggressively pursues more invasive diagnostic procedures looking for one (or more) of the less commonly seen microbes such as *Nocardia* species, *Candida*, and a host of other bacteria and fungi (Table 41-6).[152] [155] [156] [157] [158] [159] [160] [161] [162] [163] [164] [165] [166] [167] [168] [169] [170] [171] [172] [173] [174]

Pneumonia is the most common type of infection seen in CGD with *S. aureus, Aspergillus* species, *B. cepacia*, and enteric gram-negative organisms as the major pathogens. It is noteworthy that *B. cepacia* has emerged as one of the most lethal pneumonias in the past 12 years, having been responsible for a number of deaths.[175] [176] [177] [178] [179] It often is not covered with the first line of antibiotics used for *S. aureus* and most gram-negative organisms and can quietly proliferate (with persistent fevers) to the point of quick, explosive collapse due to endotoxic shock. Intravenous trimethoprim-sulfamethoxazole has been most effective in treating patients if given before widespread dissemination of the

TABLE 41-5 -- Summary of p67-*phox* Mutations in 12 Kindreds with CGD

Kindred; Sex	Type of Mutation	Nucleotide Change[a]	Predicted Amino Acid Change	CGD Type[b]	Comments	References
1. F	Nonsense	C304 T	R102 Stop	$A67^0$	Homozygous; no known consanguinity	[733]
2,3. M/2F	Deletion	Delete 11691173	Stop in exon 14	$A67^0$	Homozygous; seen in two unrelated kindreds	[733]

4. F	Splice	gt at intron 4 (5)	Multiple splice defects	A67^0	Homozygous; no known consanguinity; same allele as in kindreds 5 and 6	[733]
5. 2F	(1) Splice (2) Deletion	(1) gt at intron 4 (5) (2) Delete A728	(1) Multiple splice defects (2) Stop in exon 9	A67^0	Compound heterozygote; same allele as in kindreds 4 and 6	[733]
6. F	(1) Splice (2) Deletion (2) Missense	(1) gt at intron 4 (5) (2) Delete 5563 (in frame) (2) C1183 T	(1) Multiple splice defects (2) Delete K19, K20, D21 (2) R395 W	A67^0	Compound heterozygote; same allele as in kindreds 4 and 5 Two mutations in allele 2	[733]
7. F	Missense	G233 A	G78 E	A67^0	Homozygous; no known consanguinity	[734]
8. F	Splice	gt gc intron 3 (5)	Delete exon 3	A67^0	Homozygous[c]	[735]
9. M	Insertion	AG inserted after G399	Stop codon at position 433	A67^0	Homozygous	[736]
10. F	(1) Deletion (2) Deletion	(1) Delete 170172 (in frame) (2) 1113 kb deletion	(1) Delete K58 (2) No p67-phox	A67	Compound heterozygote	[737]
11. M	Splice	gt at intron 9 (5)	Delete exons 8 and 9	A67^0	Homozygous[c]	[738]
12. NR	(1) Missense (2) Missense	(1) A479 T (2) A481 G	(1) D160 V (2) K161 E	A67^0	Compound heterozygote	[739]

NR, not reported.

[a]Nucleotide residues are numbered from the ATG initiation codon with A defined as +1. [88]
[b]See Table 41-2 for explanation of CGD types.
[c]In these cases, homozygosity of the mutant allele was due to consanguinity in the parents.

TABLE 41-6 -- Infections in CGD

Infections	% of Infections	Infecting Organisms
Pneumonia	7080	*Aspergillus, Staphylococcus, B. cepacia, Pseudomonas, Nocardia, Mycobacterium* (including atypical), *Serratia, Candida, Klebsiella, Paecilomyces*
Lymphadenitis	5060	*Staphylococcus, Serratia, Candida, Klebsiella, Nocardia*
Cutaneous infections/impetigo	5060	*Staphylococcus, Serratia, Aspergillus, Klebsiella, Candida*
Hepatic/perihepatic abscesses	2030	*Staphylococcus, Serratia, Streptococcus viridans, Nocardia, Aspergillus*
Osteomyelitis	2030	*Serratia, Aspergillus, Paecilomyces, Staphylococcus, B. cepacia, Pseudomonas, Nocardia*
Perirectal abscesses/fistulae	1530	Enteric gram-negative organisms, *Staphylococcus*
Septicemia	1020	*B. cepacia, Pseudomonas, Salmonella, Staphylococcus, Serratia, Klebsiella*
Urinary tract infections/pyelonephritis	15	Enteric gram-negative organisms
Brain abscesses	<5	*Aspergillus, Staphylococcus*
Meningitis	<5	*Candida lusitaniae, Hemophilus influenzae, B. cepacia*

The relative frequencies of different types of infections in CGD are estimated from data pooled from several large series of patients in the United States, Europe, and Japan.[34] [147] [148] [149] [150] [151] [152] These series encompass approximately 550 patients with CGD after accounting for overlap between reports. Unpublished data from the United States CGD Registry encompassing 368 patients was also used to estimate the relative frequencies of infections and the responsible organisms. The infecting organisms are arranged in approximate order of frequency for each type of infection. Note: *Burkholderia cepacia* (*B. cepacia*) was previously classified as *Pseudomonas cepacia*.

infection. An open lung biopsy is often needed to establish the diagnosis. *Aspergillus* pneumonia is also difficult to treat, but usually responds to 36 months of daily (then thrice weekly) amphotericin B therapy (with interferon-). Surgery has generally not been needed, except for biopsy or resection of large cavitary lesions.

Lymphadenitis is the second most common infection and is usually caused by *S. aureus* or *S. marcescens*. Incision and drainage should not be delayed if the lesion fails to respond to parenteral antibiotics. Cutaneous abscesses should be similarly managed. Recurrent perinatal impetigo is almost a signature infection in CGD and often requires months of therapy (mostly oral antibiotics) to clear. Hepatic (and perihepatic) abscesses are also common in CGD and are usually, but not always, caused by *S. aureus*. [180] [181] Most lesions require drainage (needle or surgical) to permit efficient healing to occur. Bone infections are particularly problematic in CGD and arise from either hematogenous or contiguous spread (as often is the case with *Aspergillus* infections in the lung invading ribs, vertebral bodies, or the diaphragm).[182] [183] [184] [185] Perirectal abscesses are difficult to treat, even with months of therapy, and can lead to fistula formation. [180]

Many of the more problematic complications of CGD result from imperfectly controlled infections in which stalemates develop between the pathogen and the leukocytes. These lesions become granulomas as the host uses lymphocytes and histiocytes to aid the failing neutrophils in containing the pathogens. As a result of this chronic inflammatory stimulation, patients with CGD can suffer from a variety of more chronic complications (Table 41-7). Lymphadenopathy, hepatosplenomegaly, eczematoid dermatitis,[186] and anemia of chronic disease (hemoglobin levels usually 810 g/dl) are common manifestations of this process and are most prominent in the first 510 years of life in CGD. A chronic ileocolitis resembling Crohns disease is seen in 10% of patients and can range from mild diarrhea to a debilitating syndrome of bloody diarrhea and malabsorption that can necessitate a colectomy. [187] [188] [189] [190] Throughout the body, granuloma formation can lead to dysfunction and obstruction in the esophagus, [191] stomach,[180] [192] [193] intestine,[194] and urinary bladder and kidneys. [195] [196] [197] [198] [199] [200] [201] [202] [203] In the stomach, the gastric antral narrowing can be severe enough in infants and children to resemble pyloric stenosis. Granulomatous lesions and inflammatory cell infiltrates in the urinary system can lead to chronic cystitis, dysuria, and hydronephrosis. Other types of chronic inflammation include gingivitis, [204] chorioretinitis, [205] destructive white matter lesions in the brain,[206] [207] and glomerulonephritis[197] [208] In rare circumstances, patients may develop either discoid or systemic lupus erythematosus by a mechanism that is unknown.[209] [210] [211] [212]

Carriers of CGD, whether the X-linked form or any one of the autosomal recessive forms, are usually asymptomatic with two important exceptions. First, X-linked carriers are at risk of developing mild to moderately severe discoid lupus erythematosus characterized by discoid skin lesions and photosensitivity [211] [213] [214] A few will also suffer from arthralgias, polyarthritis, and Raynauds phenomenon. About one-fourth of women are symptomatic, with discoid lupus, with onset usually in the second decade of life. The disease does not progress to systemic lupus nor does one find serologic evidence of even subclinical disease. Severe discoid lupus can be treated with Plaquenil.

TABLE 41-7 -- Chronic Conditions Associated with CGD

Condition	Relative Frequency (%)

Lymphadenopathy	98
Hypergammaglobulinemia	6090
Hepatomegaly	5090
Splenomegaly	6080
Anemia of chronic disease	Common*
Underweight	70
Chronic diarrhea	2060
Short stature	50
Gingivitis	50
Dermatitis	35
Hydronephrosis	1025
Granulomatous ileocolitis	1015
Gastric antral narrowing	1015
Ulcerative stomatitis	515
Granulomatous cystitis	510*
Pulmonary fibrosis	<10*
Esophagitis	<10*
Granulomatous cystitis	<10
Chorioretinitis	<10
Glomerulonephritis	<10
Discoid lupus erythematosus	<10

The relative frequencies of the chronic conditions associated with CGD were estimated from the series of reports listed in Table 41-6 . In some instances (asterisks), the incidence is estimated from the 50 cases of CGD followed at the Scripps Research Institute and Stanford University (unpublished data).

The second important complication of the CGD carrier state is infection in those X-linked carriers who have an unusually high degree of inactivation of the normal X chromosome in their myeloid cells. If the circulating neutrophil population is skewed to the point that <10% of the cells function, then the carrier has an increased risk of infections that fortunately are usually mild. [215] [216] [217] [218]

Diagnosis

The diagnosis of CGD is usually suggested by the unusual clinical histories outlined above or by a family history of CGD. The diagnosis is most easily confirmed using the NBT slide test.[44] A typical result is shown in Figure 41-3 . Figure 41-3A shows the normal positive staining of a group of seven neutrophils and one monocyte. Figure 41-3B shows the complete absence of NBT staining in a patient with $X91^0$ CGD, the classic X-linked form of the disease. Figure 41-3C shows the mixed population of NBT-positive

Figure 41-3 Nitroblue tetrazolium (NBT) slide test. Peripheral blood neutrophils and monocytes from a drop of fresh whole blood were made adherent to glass slides and stimulated with phorbol myristate acetate. **(A)** Normal neutrophils and monocytes, all of which are NBT-positive. **(B)** Neutrophils and monocytes from an X-linked CGD patient, which are all NBT-negative. **(C)** A mixture of NBT-positive and NBT-negative neutrophils from the X-linked carrier mother of the patient in panel B.

and NBT-negative cells observed in that patients mother. Because of random X-chromosome inactivation, some of the female carriers cells are NBT positive and others negative.[216] [217] [219] Because in this test nearly 100% of the normal cells are positive, we are able to reliably detect the carrier state in X-linked CGD, when as few as 5% of the cells are NBT negative. This test also permits detection of diffuse populations of weakly positive cells such as those seen in X91 CGD, which are characterized by a partial deficiency of cytochrome b. Because X-linked CGD can arise by new mutations in the germ lines of the parents, one does not always see NBT-negative cells in the mother. [2C] [106] [22C]

The diagnosis of CGD can also be established by directly measuring respiratory burst activity as oxygen consumption, O_2 production, or H_2O_2 production. The subtyping of CGD also requires measurement of the cytochrome b content in the patients (and parents) neutrophils, usually by a spectrophotometric assay.

Also required in many instances is the measurement of the activity of the patients neutrophil membranes and cytosol in the cell-free oxidase activation system, as depicted in Table 41-1 . Once a cytosol defect is suspected, the determination of which component is defective can be made by either immunoblot analysis [121] or complementation studies in the cell-free system using known cytosols deficient in either p47- *phox* or p67-*phox*. These latter tests are best performed by laboratories specializing in neutrophil biochemistry.

Molecular genetic analysis of either myeloid cell cDNA [112] or genomic DNA [106] [220] [221] can be used to confirm the genetic subtype and define further the molecular properties of the mutant oxidase. Moreover, the molecular genetic data can be used for genetic counseling in identifying carriers and (if genomic sequencing can be used) for prenatal diagnosis using fetal DNA from the chorionic villus or amniocytes. [221] [222] Alternatively, potentially informative polymorphisms exist in the genes for gp91-*phox* and p67-*phox* that can be used for fetal DNA analysis.[33] [144] [221] [223] [224] [225] In the absence of a molecular genetic approach, prenatal diagnosis can be performed using the NBT test and a small sample of fetal blood obtained by percutaneous umbilical sampling. [226] [227] [228] [229] [230] [231]

Therapy

The four cornerstones of therapy in CGD are (1) prevention and early treatment of infections; (2) aggressive use of parenteral antibiotics for most infections; (3) use of prophylactic trimethoprim-sulfamethoxazole (5 mg/kg/day of trimethoprim) or dicloxacillin (2550 mg/kg/day) for patients allergic to sulfa; and (4) use of prophylactic recombinant human interferon- (rIFN-). Several approaches can be used to prevent infections. Patients with CGD should receive all their routine immunizations on schedule, with influenza vaccine administered each year as well. Cuts and skin abrasions should be cleansed promptly with soap and water and a topical antiseptic applied (2% hydrogen peroxide or Betadine ointment or both). Frequent brushing, flossing, and professional cleaning of teeth can help prevent gingivitis. Constipation should be avoided because it can lead to rectal/anal fissures and abscesses. Early anal infections can be treated with soaking in soapy water (with or without Betadine). The frequency of pulmonary infections can be reduced by not using commercially available bedside humidifiers, by avoiding smoking (cigarettes and marijuana), and refraining from handling decaying plant materials, which often contain numerous *Aspergillus* spores (e.g., hay, mulch, rotting sawdust).[232] Use of corticosteroids should generally be avoided, including extensive topical use, except in cases of severe asthma, esophageal strictures, gastric antral narrowing, granulomatous cystitis, or inflammatory bowel disease. Evidence indicates that corticosteroids are beneficial in these clinical

settings because the steroids induce rapid regression of obstructive symptoms at relatively low oral doses (e.g., 1 mg/kg/day of prednisone). [233] [234] [235] In these situations, the physician and patient should be aware of the risks of the additional immunosuppression caused by the corticosteroids. Finally, rare patients with X91 ^{0} CGD have genomic deletions that span the gp91-*phox* gene and the Xk gene, which encodes a 37-kd red blood cell membrane protein necessary for expression of the Kell genes. [236] [237] Absence of the Xk gene product results in the McLeod syndrome, in which red blood cells have acanthocytosis and weak Kell antigens. [236] Treatment of patients with McLeod syndrome by transfusion poses a serious problem because they can develop alloantibodies of wide specificity that can preclude any further transfusions except with McLeod blood products.

Good evidence now indicates that chronic prophylactic trimethoprim-sulfamethoxazole can decrease the number of bacterial infections in CGD patients by more than half, without a concomitant increased risk of fungal infection. [149] [238] Antibiotic prophylaxis of fungal infections has been less successful because ketoconazole has not been found to provide any protection against *Aspergillus* infections. [149] Itraconazole may, however, be effective in this context and a study is currently underway to test this possibility.

One of the most frequent errors in the management of CGD patients is the failure to treat potentially serious infections promptly and aggressively with appropriate parenteral antibiotics. Even the best antibiotics can be rendered ineffective if given too late in the course of an infection in CGD. Therefore, early intervention is advisable. Although many of the minor infections and low-grade fevers in CGD patients can be managed on an outpatient basis, episodes of consistently high fever over a 24-hour period or clearly established infections (such as pneumonia or lymphadenitis) should be treated with parenteral antibiotics that cover, at least initially, *S. aureus* and enteric gram-negative organisms. Reasonable attempts to define the source of the infection and the responsible microbe should also begin promptly. If the infection fails to respond, then more aggressive diagnostic procedures should be instituted (computed tomography, bone and gallium scans; open biopsies if indicated) and empiric changes in the antibiotics used to broaden coverage to *B. cepacia*. If fungus is identified or strongly suspected, amphotericin B is the drug of choice. Even when appropriate antibiotics are being used, certain types of infections respond slowly and may require months of therapy, particularly *Aspergillus* infections. In this instance, 46 months of amphotericin B (given first daily for 23 months and then thrice weekly) followed by a 13-year course of daily oral itraconazole has been effective in clearing most infections and preventing their recurrence. It is important to note that surgical drainage or resection can sometimes play a key role in accelerating healing of certain types of infection such as lymphadenitis, osteomyelitis, and abscesses of visceral organs such as the liver. Finally, granulocyte transfusions may be of benefit in the treatment of stubborn or extremely serious infections. [151] [239] [240] [241] [242]

In a multicenter trial, patients were randomized in a double-blind fashion to receive either placebo or rIFN- (0.05 mg/m ^{2} three times per week). [243] The results from this study, summarized in Table 41-8 , show a substantial decrease in the number of serious infections in the interferon arma 70% reduction in risk when compared with the placebo. Side effects were observed in some of the patients, but these were minimal (mild headaches and low-grade fevers). The highly significant clinical improvements were not accompanied by improvements in phagocyte function, as measured by O_2 production or in vitro *S. aureus* killing. A few patients with rare variant forms of X91 CGD have shown modest to dramatic increases in O_2 production. [244] [245] [246] It now appears that interferon- augments host defense in the vast majority of patients by means other than reversing the respiratory burst defect. [247] [248] This interpretation is consistent with the observation that rIFN- was comparably effective in both X-linked and autosomal recessive types of CGD across a broad range of mutations that would preclude the synthesis of a functional oxidase (see previous discussion). Over 30 CGD patients have been treated with rIFN- for 810 years without interruption of their thrice weekly regimen. No additional adverse reactions have been noted, and the patients continue to have a substantial benefit with approximately five-fold fewer serious infections compared to the placebo group in the phase III multicenter study (Table 41-8). On average, this group of patients is averaging one serious infection per patient every 45 years.

Neutrophil G6PD Deficiency

The primary substrate for the respiratory burst oxidase, NADPH, is generated by the first two reactions of the hexose monophosphate shunt pathway, for which the responsible enzymes are G6PD (Fig. 41-1 , reaction 8) and 6-phosphogluconate dehydrogenase. [20] [27] [249] As would be expected, a severe deficiency of G6PD in neutrophils results in a greatly attenuated respiratory burst and a clinical picture that can resemble CGD. [20] [250] [251] [252] [253] [254] [255] The key features of this extremely rare X-linked disorder are summarized in Table 41-9 . In light of the relatively high frequency of G6PD mutations in the American black and Mediterranean populations, [27] [249] as well as the fact that leukocyte and erythrocyte G6PD are encoded by the same gene, [249] it might be expected that clinically significant neutrophil G6PD deficiency would occur more often than it does. One of the reasons it does not is the short life span of the neutrophil. Because most G6PD mutations cause the enzyme to decay over a period of days and weeks, levels in the short-lived neutrophil usually do not become critically low, even in some of the most unstable G6PD variants. It appears that only those rare and poorly understood mutations that cause congenital nonspherocytic hemolytic anemia (CNSHA) are associated with extremely low (<5% of normal)

TABLE 41-8 -- Efficacy of IFN-in Preventing Serious Infections in CGD

Variable	Clinical Study			
	Phase III Placebo[a]	Phase III IFN-[a]	Phase IV (U.S.) IFN-[b]	Phase IV (Europe) IFN-[c]
No. of patients	65	63	30	28
Average duration of therapy on study (years)	0	0.83	1.03	2.4
Patient-years on study	50.9	52.1	31.0	67.2
Serious infections per patient-year	1.10	0.38	0.13	0.25
Number of hospital days per patient-year	28.2	8.6	2.2	15.0

[a]Results from The International Chronic Granulomatous Disease Cooperative Study Group. [243]
[b]Results from the Scripps Research Institute CGD Clinic. [740]
[c]Results from Weening et al. [741]

TABLE 41-9 -- Summary of Neutrophil G6PD Deficiency

Incidence	Extremely rare
Inheritance	X-linked
Molecular defect	Poorly characterized family of mutations that cause congenital nonspherocytic hemolytic anemia (CNSHA) in erythrocytes and functional failure of G6PD in neutrophils (possibly kinetic mutants); other rare mutants may also be responsible
Pathogenesis	Severe functional failure of neutrophil G6PD (<5% of normal), leading to an extremely low steady-state concentration of NADPH, which serves as the substrate for NADPH oxidase
Clinical manifestations	CNSHA (hemolytic anemia that occurs even in the absence of redox stress) CGD-like syndrome with recurrent bacterial infections
Laboratory evaluation	Neutrophil G6PD activity <5% of normal Severely diminished respiratory burst and abnormal NBT test Associated CNSHA and diminished erythrocyte G6PD activity
Differential diagnosis	CGD Glutathione reductase or synthetase deficiency
Therapy	Prophylactic trimethoprim-sulfamethoxazole Aggressive use of parenteral antibiotics Transfusion support for severe anemia

Prognosis	Not clear because data on too few patients have been reported May be as severe as CGD if neutrophil G6PD levels are extremely low

levels of G6PD in the neutrophil. [250] [251] [252] [253] [254] [255] Thus, a CGD-like syndrome due to neutrophil G6PD deficiency has only been observed in patients who also have CNHSA (hemolysis that occurs in the absence of redox stress). This unique clinical association, coupled with the laboratory demonstration of extremely low G6PD levels in neutrophils and erythrocytes, distinguishes this disease from CGD. [250] The treatment for neutrophil G6PD deficiency is the same as for CGD except that the efficacy of rIFN- has not been demonstrated in the former. The chronic hemolytic anemia is treated by supportive means, including transfusions. [249]

Disorders of Glutathione Metabolism

As depicted in Figure 41-1 (reaction 6), glutathione peroxidase serves to protect the neutrophil from the deleterious effects of hydrogen peroxide on NADPH oxidase and other neutrophil proteins such as those found in microtubules. [256] [257] The effectiveness of glutathione peroxidase depends on having adequate amounts of reduced glutathione (GSH). Adequate intracellular levels of GSH are maintained by recycling oxidized glutathione (GSSG) to GSH by glutathione reductase (Fig. 41-1 , reaction 7) as well as by de novo synthesis of glutathione by glutathione synthetase (Fig. 41-1 , reaction 9). Severe deficiencies in either of these enzymes have been observed to cause mild phagocytic defects.[256] [257] [258] The key features of these disorders are outlined in Table 41-10 . Both are extremely rare and appear to be inherited in an autosomal recessive manner. The precise mutations for the individual disorders have not been established. In glutathione reductase deficiency, the respiratory burst terminates prematurely, presumably owing to the toxic effects of accumulating hydrogen peroxide on NADPH oxidase. [29] This brief burst of O_2 , however, appears to be sufficient for adequate microbial killing, because the few patients reported have not had problems with recurrent infections. [258] They do have a congenital hemolytic anemia due to diminished levels of glutathione reductase in erythrocytes, which becomes clinically evident during periods of oxidant stress. [256]

In glutathione synthetase deficiency the respiratory burst proceeds normally. [257] The patients have some problems with recurrent infections and have a severe metabolic acidosis due to elevated levels of 5-oxoproline. This metabolite is the product of the first step in glutathione synthesis and is present in increased levels because of a lack of feedback of GSH on the synthetic pathway. Patients with glutathione synthetase deficiency also have intermittent neutropenia [259] (perhaps caused by the acidosis) as well as oxidant-induced hemolysis. [257] Therapy with vitamin E (400 IU/day) has been beneficial in patients with severe glutathione synthetase deficiency suffering from hemolysis and infections. [259] Patients with less severe deficiencies usually do not require therapy.

Myeloperoxidase Deficiency

Myeloperoxidase (MPO) deficiency is the most common inherited disorder of phagocytes. [260] Complete deficiency is seen in 1/4,000 individuals, and partial deficiency is even more common (1/2,000 persons).[28] [261] The key features of MPO deficiency

TABLE 41-10 -- Disorders of Glutathione Metabolism

Disease Aspect	Glutathione Reductase Deficiency	Glutathione Synthetase Deficiency
Incidence	One family: three siblings	Several reported cases
Inheritance	Autosomal recessive	Autosomal recessive
Molecular defect	Diminished glutathione reductase levels in neutrophils (1015% of normal) and erythrocytes; mutation not known	Severe deficiency of glutathione synthetase activity (510% normal); mutation(s) not known
Pathogenesis	Brief respiratory burst truncated by toxic accumulation of $H_2 O_2$ in neutrophil caused by diminished catabolism of $H_2 O_2$ by glutathione	Same as with glutathione reductase deficiency; elevated 5-oxoproline due to lack of feedback inhibition by glutathione
Clinical manifestations	No history of repeated infection	Metabolic acidosis due to elevated 5-oxoproline
	Hemolysis with oxidant stress	Otitis media
		Intermittent neutropenia
		Hemolysis with oxidant stress
Laboratory evaluation	Glutathione reductase level diminished	Severe decrease in glutathione synthetase level
	Premature cessation of O_2 production by neutrophils	Normal respiratory burst
Differential diagnosis	CGD	Glutathione reductase deficiency
	G6PD deficiency	
Therapy	None required	Vitamin E for hemolysis and infections
		Treatment of metabolic acidosis
Prognosis	Benign disorder	Relatively benign disorder

are summarized in Table 41-11 . The disorder is inherited in an autosomal recessive [262] [263] [264] [265] [266] manner, although variable expression of the defect has been observed.[267] Acquired forms of MPO deficiency are also seen. The gene that encodes for MPO is located on chromosome 17 at q22q23 near the breakpoint for the 1517 translocation of promyelocytic leukemia. [268] [269] [270] [271] Subpopulations of MPO-deficient cells can be seen not only in the M3 (promyelocytic) form of acute myeloid leukemia but also in the M2 and M4 forms. [28] MPO-deficient cells are also seen in 25% of patients with chronic myeloid leukemia and myelodysplastic syndromes.[272] [273] [274]

A growing understanding of the molecular basis of congenital MPO deficiency has been made possible by the isolation of both cDNA and genomic clones for MPO [13] [28] [275] [276] [277] [278] [279] [280] and gives insights on how MPO gene expression is regulated. [281] [282] In the few patients thus far studied, several different types of genetic lesions have been observed. [283] [284] These generally appear to affect the post-translational processing of a precursor polypeptide for MPO (apoproMPO). [28] [260] [285] [286] One particularly common missense mutation has been identified in MPO deficient patients (Arg 569 Trp) that interferes with the post-translational incorporation of heme into the apoproMPO and results in an enzymatically inactive MPO precursor protein. [13] Other MPO mutations have not yet been reported. Although the levels of MPO are severely deficient in neutrophils and monocytes as a result of these mutations, the level of eosinophil peroxidase is normal in MPO-deficient patients because it is encoded for by a separate gene. [287] (A series of 21 individuals with eosinophil peroxidase deficiency has been reported. [288] As with MPO deficiency, these individuals were asymptomatic.)

One of the most curious features of MPO deficiency is the remarkable lack of clinical symptoms in affected persons. As

TABLE 41-11 -- Summary of MPO Deficiency

Incidence	1/2,000 (partial deficiency) 1/4,000 (total deficiency)
Inheritance	Autosomal recessive with variable expression; MPO gene on chromosome 17 at q22-q23
Molecular defect	Defective post-translational processing of an abnormal MPO precursor polypeptide; eosinophil peroxidase encoded by different gene and levels normal

Pathogenesis	Partial or complete MPO deficiency leads to diminished production of HOCl and HOCl-derived chloramines; MPO products are necessary for rapid killing of microbes (especially *Candida*) but not absolutely required
Clinical manifes-tations	Usually clinically silent Disseminated candidiasis/fungal disease (rare; usually in conjunction with diabetes mellitus) Acquired deficiency in M2, M3, and M4 acute myeloid leukemias (AML) and myelodysplasia
Laboratory evaluation	Deficiency of neutrophil/monocyte peroxidase by histochemical analysis (eosinophil peroxidase normal) Delayed, but eventually normal, killing of bacteria in vitro Failure to kill *Candida albicans* and hyphal forms of *Aspergillus fumigatus* in vitro
Differential diagnosis	Acquired partial MPO deficiency seen in M2, M3, and M4 AML, myelodysplastic syndromes, and Batten disease
Therapy	None in asymptomatic patients Aggressive treatment of fungal infections when they occur Control of blood glucose levels in diabetics
Prognosis	Usually excellent

shown in Figure 41-1 (reaction 4), MPO catalyzes a key reactionthe production of a potent antimicrobial agent, hypochlorous acid. [20] [289] HOCl in turn reacts with a variety of primary and secondary amines to form chloramines, some of which can be toxic. Moreover, HOCl is capable of activating latent metalloproteinases (e.g., collagenase) and inactivating antiproteinases. [20] [28] [260] Thus, one might predict that severe MPO deficiency would cripple important antimicrobial reactions catalyzed by HOCl. In vitro, an impressive defect in killing *C. albicans* and hyphal forms of *A. fumigatus* is observed.[260] [261] [265] Bacterial killing in vitro is also abnormal in being somewhat slower than normal, but eventually it is complete. [261] [262] [263] [264] [265] [267] [291] These in vitro abnormalities, however, are rarely manifested in patients, except for rare individuals who also suffer from diabetes mellitus. [261] [262] [265] In these individuals disseminated fungal infections (usually candidiasis) are seen.

The discrepancy between the in vitro and in vivo manifestations of MPO deficiency in most patients can be explained in several ways. First, the respiratory burst in MPO-deficient neutrophils is substantially augmented in terms of velocity and duration, presumably owing to the absence of the toxic effects of HOCl on NADPH oxidase.[260] Second, other products of the respiratory burst besides HOCl, together with the oxygen-independent antibacterial proteins, appear to have sufficient potency to compensate for the loss of MPO-dependent reactions. [28] Finally, residual amounts of MPO coupled with the normal levels of eosinophil peroxidase may provide at least some degree of peroxidative activity at the sites of infection.

Treatment is usually not required for MPO deficiency except in those individuals suffering from fungal infections. In these patients aggressive use of antifungal antibiotics is indicated. The prognosis is excellent in the majority of patients with MPO deficiency.

DISORDERS OF PHAGOCYTE ADHESION AND CHEMOTAXIS

Since 1970, numerous investigators have found in vitro chemotactic abnormalities in neutrophils from patients suffering from a wide variety of clinical disorders associated with increased susceptibility to bacterial and fungal infections. [6] [8] [292] In most circumstances the chemotactic abnormality identified was only marginal and not always clearly related to the clinical status of the patient. In other instances, however, clear and major defects were identified in vitro that correlated with the in vivo propensity for infection. Extensive classification systems have been devised to categorize the numerous acquired defects in chemotaxis, [6] and several reviews are available on this subject. [6] [292] [293] [294] The problem in many of these reports is that it is unclear whether the infections were caused by the in vitro chemotactic abnormality or by the multiple medical complications of the underlying disorder (e.g., acidosis, malnutrition, or exposure to nosocomial infections). A further complicating factor is that the in vitro chemotaxis assays have inherent limitations. Abnormal migration of purified neutrophils through filter disks in response to in vitro-generated chemotactic gradients is difficult to quantify and is subject to laboratory artifacts. Furthermore, the extent to which these in vitro chemotactic assay systems faithfully reflect prevailing in vivo conditions is not known. Understanding of chemotactic disorders has been hampered by the limitations of these assays, just as the elucidation of respiratory burst defects was obscured when the major available assay was in vitro bacterial killing. In this section the most important and best characterized of the chemotactic disorders, leukocyte adhesion deficiency, is discussed in detail. A brief discussion of several other clinically significant chemotactic disorders is also provided.

Leukocyte Adhesion Deficiency

Biology

Leukocyte adhesion deficiency (LAD) is a relatively rare disorder of leukocyte adhesion and chemotaxis, which results in severe and sometimes fatal bacterial infections. [292] [295] [296] [297] Approximately 20 patients were described in the literature in the 1970s who, in retrospect, probably had LAD and shared the clinical phenotype of recurrent infections, decreased neutrophil motility, and abnormal particle-stimulated respiratory burst activity. [295] Many also had persistent leukocytosis and delayed umbilical cord separation.

The molecular basis for LAD was first suggested by the finding that neutrophils from a patient with this clinical syndrome lacked a high-molecular-weight membrane glycoprotein. [298] The molecular weight was originally thought to be 110,000 but was subsequently found to be 180,000. The variability was likely due to the highly variable electrophoretic mobility of glycoproteins. The patients neutrophils could not be made to adhere to plastic surfaces or to respond to serum-opsonized particles in terms of ingestion and respiratory burst activity. [298] It was hypothesized that the missing glycoprotein was responsible for cell-surface adhesion and for cell-particle interactions. Several other reports followed that described other patients in whom a similar glycoprotein was missing. [299] [300] It was later found that the missing glycoprotein was actually a group of closely related glycoproteins with molecular weights ranging from 95 kd (the $_2$ subunit) to 150180 kd (three distinct subunits) that form three types of - heterodimers. The dominant heterodimer in phagocytes was designated Mo1, [301] [302] which functions as the C3bi receptor (CR3) of human neutrophils and monocytes. [302] [303] [304] [305] [306]

The molecular basis of LAD has now been proven to involve this group of three leukocyte glycoproteins, which are part of the integrin superfamily of adhesion molecules. [302] [305] [306] [307] [308] [309] [310] [311] [312] [313] Integrins are noncovalently linked heterodimeric glycoproteins consisting of an and a subunit. [296] Within each of the eight known integrin subfamilies the subunit is identical (and defines the subfamily), whereas the subunit varies and confers the functional specificity on the integrin. [296] [308] [309] A total of 22 distinct integrin subunits have been defined (14 and 8 chains) that give rise to 20 different integrins that function as mediators of adhesion (cell-cell and cell-extracellular matrix) and receptors for C3bi and a group of coagulation factors (factor X, fibrinogen, and von Willebrand factor). The molecular defect in LAD involves all three members of the $_2$ integrin subfamily: $_{L\,2}$ (CD11a/CD18), $_{m\,2}$ (CD11b/CD18), and $_{x\,2}$ (CD11c/CD18). [296] [302] [305] [306] The molecular weights of these subunits are $_L$ 180,000; $_m$ 170,000; $_x$ 150,000; and $_2$, 95,000. CD11a/CD18 is often referred to as LFA-1 while CD11b/CD18 is called Mo1 or Mac-1. In the dozens of patients with LAD who have been studied thus far at the molecular level, an absent, diminished, or structurally abnormal $_2$ subunit (CD18) has been identified. In the absence of a normal subunit, the three types of chains in the $_2$ integrin subfamily cannot assemble into normal - heterodimers. Thus, all three $_2$ integrins are moderately to severely deficient on all leukocytes in LAD. The $_2$ integrins serve as receptors for the opsonic complement fragment C3bi, the intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2), and fibrinogen. [296]

In all of the patients with LAD reported to date who have been analyzed at the molecular level, mutations have been identified in the gene encoding the $_2$ (CD18) subunit. Mutations in the subunits have not been found thus far in patients with LAD. The gene encoding CD18 is located on the long arm of chromosome 21 at position q22.3 (the genes for $_2$ integrin subunits are clustered on chromosome 16p11.1p13). [314] [315] [316] [317] [318] [319] [320] [321] CD18 is synthesized as a 747 amino acid polypeptide with a 22 amino acid signal sequence that is subsequently cleaved. The glycoprotein has a single transmembrane domain with a 46 residue cytoplasmic tail. Near the N-terminus of the large extracellular domain is a stretch of 250 conserved amino acids, and it is in this region that the majority of LAD mutations are found. [296] [314] [322] [323] [324] [325] [326] [327] [328] [329] Table 41-12 summarizes the CD18 mutations found in LAD. As with X-linked CGD, LAD mutations are heterogeneous in nature, family specific, and can lead to either undetectable or low (920% of normal) levels of - dimer expression that correlates with the clinical severity of the disease. Among the 12 different mutant alleles identified, there are 8 missense mutations, 2 splice defects, 1 interstitial deletion of 230 bp (patient 19), and 1 frameshift due to

a single bp deletion (patient 18) (Table 41-12).

An example of a mutation leading to low levels of CD18 expression and a moderate clinical phenotype is patient 14. Two different missense mutations are present, one in the conserved region of the domain (Lys 196Thr) and the other in an extracellular cysteine-rich area (Arg 593Cys). [327] It is not clear which allele permits the expression of 1020% normal levels of the $_2$ integrins (or whether both do). A similar moderate phenotype is seen in the three related patients 811 in Table 41-12 . These patients have a third-position mutation in a 5 splice site (gtgagtca) that results in aberrant splicing in 97% of the mRNA with the in-frame loss of 90 nucleotides. [323] [324] [325] The $_2$ subunit precursor translated from this defective mRNA was abnormally small and apparently unable to associate normally with the subunit precursors. Roughly 3% of the CD18 mRNA, however, was found to be normally spliced and was probably responsible for the low level of - dimer expression in the patients leukocytes and moderate phenotype. Severe LAD, with undetectable $_2$ integrins, is seen in patient 18. The one mutant allele identified has a frameshift due to a single nucleotide deletion that predicts a premature stop codon and the loss of the last 56 amino acids that encompass the entire cytoplasmic domain and part of the transmembrane region. [328] This mutation fails to permit the synthesis of - dimers. The other mutant allele must also have a severe mutation.

The diminished or absent expression of all three $_2$ integrins in LAD leukocytes results in the failure of phagocytes to emigrate from the bloodstream to sites of infection. [311] The early stages of phagocyte-endothelial cell interactions, termed rolling, is mediated by selectins [330] and occurs normally in LAD. [311] It is the tight adherence of neutrophils and monocytes to cytokine-activated endothelium that is severely defective in LAD because these interactions are mediated by the $_2$ integrins. Transendothelial migration is also impaired. The other major functional defect in LAD is the failure of phagocytes to bind C3bi opsonized microbes. Because CD11b/CD18 is the predominant C3bi receptor for the neutrophil, those phagocytic functions dependent on this opsonin receptor (i.e., C3bi-mediated ingestion, degranulation, and respiratory burst activity) are severely affected in LAD. [295] [296] [297] Thus, LAD neutrophils are not able to efficiently ingest and kill microbes opsonized with C3bi, a defect that contributes to the propensity of these patients to become infected. Despite the absence of CD11a/CD18, patients with LAD rarely have clinical manifestations of impaired lymphocyte function. [292] [296] It is now possible that the role CD11a/CD18 plays in lymphoid cell function can be compensated, at least in part, by other adhesion proteins (CD2, CD4, CD8, etc.) and costimulatory molecules. [296]

Clinical Features

The key features of LAD are summarized in Table 41-13 . Approximately 60 patients have been described in the literature to date. [296] The mode of inheritance is autosomal recessive, consistent with the location of the CD18 gene on chromosome 21. [296] [297] The clinical presentation of LAD is heterogeneous and is related to the severity of the deficiency of the $_2$ integrins. The severe

TABLE 41-12 -- $_2$ Subunit (CD18) Mutations in LAD

Patient	Mutation Type[a]	mRNA Levels	Protein Expression		Clinical Severity	Mutations Identified[a]		References
			Precursor	- Dimer		Nucleotide	Amino Acid Change[b]	
13	NR	0	0	0	Severe	NR	NR	[323] [324]
47	NR	Low	0Trace	0Low	Moderate	NR	NR	[323] [324]
811	Splice/deletion (homozygous)	Normal	Abnormally small	Low	Moderate	g c at position 3 of 5 splice site of intron following exon containing nt 10661155 causing 90 nt deletion in 97% of mRNA	30 AA deletion (residues 332361)	[323] [324] [325]
12	NR	Normal	Abnormally large	0	Severe	Not identified; appears to cause an extra N-glycosylation site		[323] [324]
13	(1) Missense	Normal (2) NR	Normal	0	Severe	(1) G577 A (2) Not identified	(1) Gly 169 Arg (2) Not expressed	[326]
14	(1) Missense (2) Missense	Normal	Normal	1020%	Moderate	(1) A659 C (2) C1849 T	(1) Lys 196 Thr (2) Arg 593 Cys	[327]
15	(1) Missense (2) NR	Normal	Normal	Low	Moderate	(1) T517 C (2) Not identified (probably after nt 965)	(1) Leu 149 Pro (2) Not identified	[326]
16	(1a) Splice/ insertion (1b) Missense (2) Missense	Normal	Normal	1020%	Moderate	(1a) c a in intron 6 forming aberrant splice site and insertion of 12 nt after nt 813 (1b) C 1828 T (? polymorphism) (2) A 1124 G	(1a) In-frame insertion of PSSQ after Pro 247 (1b) Arg 586 Trp (2) Asn 351 Ser	[322]
17	(1) Missense (2) Deletion	Low	Low	9%	Moderate	(1) T74 A (initiation codon) (2) Deletion T 2142	(1) Delete Met 1; low level initiation at codon 2 (Leu) (2) Frameshift with premature stop codon predicting loss of last 56 AA	[328] [328]
18	(1) Deletion (2) NR	NR	NR	0	Severe	(1) Deletion T 2142 (2) Not identified	(1) Frameshift with premature stop codon predicting loss of last 56 AA (2) Not identified	
19	(1) Missense (2) Deletion	Normal	NR	NR	NR	(1) C 605 T (2) Deletion nt 1729 1959	(1) Pro 170 Leu (2) Loss of 78 AA (residues 553630) and then frameshift	[329]

Nucleotides are numbered according to Kishimoto et al. beginning with the 5 end of the $_2$ subunit cDNA. [314]

Abbreviations: 0, undetectable level; AA, amino acid; NR, not reported.

From Curnutte et al., [742] with permission.

[a] The two alleles are indicated by numbers 1 and 2.
[b] Predicated from nucleotide change.

clinical phenotype is associated with <0.3% of the normal amount of these glycoproteins on the leukocyte surface, while the moderate phenotype has 2.56% of normal levels. [292] [297] In both the severe and moderate forms of the disease, persistent granulocytosis (neutrophil count of 12,000100,000/mm ^{3}) is a constant finding, as are recurrent cutaneous abscesses and periodontal infections or gingivitis. [292] [295] [296] [297] Additional clinical features seen more often in the severe clinical phenotype include delayed umbilical cord separation, perirectal cellulitis, severe ulcerative stomatitis, and bacterial sepsis. Because neutrophils are unable to emigrate to tissues, abscesses and other sites of infections are devoid of pus despite the marked neutrophilia.

Diagnosis

The diagnosis of LAD is made by flow cytometric measurement of surface CD11b (or CD18) in unstimulated and stimulated neutrophils using monoclonal antibodies

directed against CD11b.[292] [295] Neutrophils contain an intracellular pool of CD11b/ CD18 in their secondary (specific) and tertiary granules that can be mobilized to the cell surface during stimulation.[296] [331] [332] Therefore, the deficiency of CD11b can be more dramatically demonstrated by using stimulated neutrophils. Carriers of LAD can be identified by this method because they have been found to express 50% of normal levels of CD11b on the surface of their stimulated neutrophils. [297] [331]

Therapy

Treatment for LAD depends on the clinical severity of the disorder. In patients with the moderate clinical phenotype, cutaneous and oral infections can be managed as they occur. The use of prophylactic antibiotics such as trimethoprim-sulfamethoxazole appears to be beneficial, as does aggressive prophylactic

TABLE 41-13 -- Summary of LAD

Incidence	60 patients described in literature
Inheritance	Autosomal recessive
Molecular defect	An absent, diminished, or structurally abnormal subunit (CD18) caused by one of several types of mutations in the gene; in the absence of a normal subunit, the three types of chains in the $_2$ integrin subfamily (CD11a, b, c) cannot assemble into normal - heterodimers
Pathogenesis	All three $_2$ integrins (CD11a/CD18, CD11b/CD18, and CD11c/CD18) are deficient on all leukocytes causing multiple abnormalities in cell function: adherence, chemotaxis, and C3bi-mediated ingestion/degranulation/ respiratory burst
Clinical manifestation	Persistent granulocytosis (neutrophil count of 12,000100,000/mm^3) Severe or moderate phenotypes depending on severity of deficiency Recurrent pyogenic infections with absent neutrophil infiltration Delayed umbilical cord separation Severe gingivitis/periodontitis
Laboratory evaluation	Flow cytometric measurement of surface CD11b in stimulated neutrophils with monoclonal anti-CD11b
Differential diagnosis	CGD May be associated with severe neutrophil actin dysfunction
Therapy	Bone marrow transplant in clinically severe patients (CD11b < 0.3% of normal) Aggressive use of parenteral antibiotics Possible benefit of prophylactic trimethoprim/sulfamethoxazole
Prognosis	Severe: high incidence of death before 2 years Moderate: can survive into twenties and thirties but with recurrent infections

treatment of periodontal disease. It is important to note that even patients with the moderate phenotype can die of overwhelming infection, as evidenced in several recent reviews, and by the absence of any known patients over the age of 40.[292] [295] [296] [297] In patients with severe LAD, aggressive management is indicated because of the high incidence of death before the age of 2 years.[292] [295] [296] At present, bone marrow transplantation is recommended for these patients and has been reported to be successful in several cases.[295] [323] [333] [334] In theory, LAD should be amenable to gene replacement therapy. The technical feasibility of this approach has now been demonstrated by several groups using Epstein-Barr virus (EBV)-transformed lymphocyte lines from LAD patients.[335] [336]

Actin Polymerization Defect Associated with LAD

A subset of patients with severe LAD may suffer from an additional biochemical abnormality characterized by defective neutrophil actin polymerization. [337] An infant boy who suffered from extremely severe infections associated with profound defects in neutrophil chemotaxis/ingestion was described in 1974. [338] The underlying defect appeared to reside in the neutrophil actin because it failed to polymerize normally in vitro in response to 0.6 M KCl. This index patient was subsequently diagnosed as having LAD on the basis of intermediate levels of CD11b found in neutrophils from surviving family members. LAD is not generally associated with defective actin filament assembly.[337] In rare patients with LAD, however, actin polymerization is abnormal and presumably reflects a functional link between the cell surface integrins and the cytoskeleton. Evidence indicates that the cytoplasmic domains of the integrin and chains associate with cytoskeletal proteins such as talin, vinculin, and -actinin.[309] [339] [340] Given the heterogeneity of the mutations in LAD (Table 41-12), it is possible that only certain types of mutations lead to an associated defect in actin function.

LAD Caused by a Deficiency in Sialyl-Lewis X (LADII)

A clinical syndrome closely related to LAD, but caused by a defect in selectin-mediated adhesion events, has been reported in two unrelated boys of Moslem Arab origin and has been termed LADII.[341] The disease appears to be inherited in an autosomal recessive manner because both patients were products of consanguinous matings. The patients had marked neutrophilia, recurrent bacterial infections, and periodontitis like LAD patients, yet their neutrophils expressed normal levels of CD18. A clue as to the molecular cause of LADII came from the observation that the patients had the rare Bombay (hh) erythrocyte phenotype and were negative for the Lewis antigen as well. These antigenic defects share in common the failure to form certain fucose carbohydrate linkages, raising the possibility that the patients had a generalized defect in fucose metabolism leading to not only the erythrocyte defects, but also a failure to synthesize critical Sialyl-Lewis X moieties on E-selectin and P-selectin counterreceptors on neutrophils. As predicted, neutrophils from both boys were devoid of immunoreactive Sialyl-Lewis X structures and were unable to adhere to human umbilical cord endothelial cells activated with interleukin (IL)-1 to induce E-selectin expression. The precise defect in fucose metabolism is still under investigation.

Hyperimmunoglobulin E Syndrome

Biology

The hyperimmunoglobulin E (hyper-IgE) syndrome is a complex disorder characterized by markedly elevated serum IgE levels, serious recurrent infections, and chronic dermatitis.[342] [343] [344] [345] [346] [347] Although technically not a phagocyte defect, neutrophils from patients with this syndrome exhibit a variable and at times profound chemotactic defect.[342] [343] [344] [348] [349] [350] The hyper-IgE syndrome was first described in 1966 and was called Jobs syndrome, in reference to the biblical description of Job as being afflicted with sore boils from the soles of his feet unto his crown. [343] The skin abscesses in patients with hyper-IgE syndrome lack the erythema that is typical of such lesions and are referred to as cold abscesses. The key features of the hyper-IgE syndrome are described in Table 41-14 .

The disease is extremely rare and its mode of inheritance has not been firmly established, although familial occurrence has been noted. [342] [344] [346] Both males and females have been affected, which suggests autosomal inheritance. The molecular basis for the syndrome is not known. The extremely high serum IgE level is believed to reflect a T-lymphocyte imbalance, leading to abnormal regulation of IgE production, as well as decreased production of IFN- and tumor necrosis factor. [342] [347] [351] [352] [353] Further evidence for a more broad-based immune disorder is the finding that patients with hyper-IgE mount abnormal antibody responses to vaccines.[354] The recurrent bacterial infections are thought to arise by two mechanisms: (1) excessive production of IgE directed against *S. aureus* that occurs at the expense of protective antistaphylococcal IgG,[355] and (2) a variable neutrophil chemotactic defect that occurs independently of fluctuations in the serum IgE level. [342] [356] [357] The underlying T-cell defect may be responsible for this chemotactic defect because it may

TABLE 41-14 -- Summary of Hyper-IgE Syndrome

Incidence	Approximately 50 cases have been reviewed in the literature; single institution series of 6, 13, and 23 cases have been described
Inheritance	Autosomal (? dominant) with incomplete penetrance
Molecular defect	Unknown; putative T-lymphocyte defect, in part manifested by diminished production of interferon-, which affects regulation of IgE production as well as other immune functions
Pathogenesis	The following may contribute to the increased risk of infection: high levels of antistaphylococcal IgE and low levels of antistaphylococcal IgG; fluctuating neutrophil chemotactic defect possibly due to an inhibitor from mononuclear cells; poor antibody response in some patients
Clinical manifestations	Staphylococcal pneumonia Pneumatoceles Fungal superinfection of lung cysts Cold cutaneous skin abscesses and furuncles Chronic eczematoid dermatitis Mucocutaneous candidiasis Coarse facies, growth retardation, osteopenia Sinusitis, keratoconjunctivitis
Laboratory evaluation	Serum IgE >2,500 IU/ml Peripheral blood eosinophilia
Differential diagnosis	Atopic dermatitis Wiskott-Aldrich syndrome, DiGeorge syndrome Hypergammaglobulinemia Chronic granulomatous disease
Therapy	Prophylactic anti-*S. aureus* antibiotics Aggressive treatment of acute infections with parenteral antibiotics Surgical drainage of deep infections and resection of lung cysts Plasmapheresis in severe cases (experimental) IFN- (experimental)
Prognosis	Generally good if managed aggressively Some patients develop lymphoid malignancies

cause the release of chemotactic inhibitors from mononuclear cells. [342]

Clinical Manifestations

The onset of clinical manifestations in patients with the hyper-IgE syndrome is generally in the first 2 months of life and is manifested by chronic dermatitis. [342] By 5 years of age, patients have a history of recurrent skin abscesses, pneumonias, chronic otitis media, and sinusitis. As patients grow older, recurrent staphylococcal pneumonia is a common problem and can be complicated by the formation of pneumatoceles. [358] [359] Septic arthritis, cellulitis, and osteomyelitis are also observed, with the offending microbe usually being *S. aureus*, although other bacterial pathogens have also been found. Patients can have chronic mucocutaneous candidiasis and occasionally exhibit keratoconjunctivitis, sometimes complicated by corneal scarring. Osteopenia of unknown etiology is observed in most patients and results in increased risk of fractures to the long bones and vertebral bodies. [346] One feature noted in the majority of patients is the presence of coarse facial features (broad nasal bridge, prominent nose).

Diagnosis

The diagnosis of hyper-IgE syndrome should be entertained in any child or young adult who has the above-described clinical picture or simply a history of recurrent infections. The hallmark laboratory finding is a marked elevation of serum IgE, almost always >2,500 IU/ml. [342] Levels can be as high as 150,000 IU/ml. [342] Despite the impressive elevations in serum IgE seen in the syndrome, this laboratory finding alone is not diagnostic because comparably high serum levels of IgE can be seen in patients with atopic dermatitis. [342] Because many patients with this latter disorder are also afflicted with eczema and superficial skin infections, atopic dermatitis must be considered in the differential diagnosis of hyper-IgE syndrome. The two can be distinguished because of the severe and recurrent nature of the staphylococcal furuncles and pneumonias seen in hyperIgE syndrome. Patients with other primary immunodeficiency syndromes may also manifest elevated IgE levels [342] (Table 41-14).

Therapy

The therapy for hyper-IgE syndrome is largely supportive because there is no known curative treatment. Prophylactic antibiotics (e.g., dicloxacillin or trimethoprim-sulfamethoxazole) can be effective in preventing *S. aureus* infections. [342] Dermatitis can be treated with topical steroids. Intravenous antibiotics are used for deep-seated infections or for resistant cutaneous infections. Surgical resection of persistent pneumatoceles is sometimes indicated to prevent superinfection by fungal and gram-negative organisms. Plasmapheresis has been reported to be effective in treating patients who have not responded to the above-mentioned therapies. [342]

Treatment of hyper-IgE syndrome with recombinant human IFN- has been investigated based on the observations that this cytokine can suppress the synthesis of IgE [360] [361] and that IFN- production in this disease is diminished. [351] [352] In uncontrolled studies, patients treated with rIFN- (50 g/m^2 subcutaneously thrice weekly) report that they feel better; moreover, their neutrophils exhibit improved in vitro chemotaxis [362] [363] and occasionally their serum IgE levels decrease. [364] In light of the results observed with rIFN- in CGD discussed above, there may be a role for IFN- in preventing infections in hyper-IgE syndrome.

Miscellaneous Chemotactic Disorders

One of the most consistently observed chemotactic abnormalities is seen in neonatal neutrophils. [292] [293] [363] [365] [366] [367] [368] [369] These cells exhibit impaired chemotaxis in vitro in response to a wide variety of chemotactic factors. [292] It appears as though this abnormality is due, at least in part, to defects in cellular adhesion as a result of diminished mobilization of intracellular adhesion-promoting molecules to the cell surface. [6] [365] [370] Defective neutrophil chemotaxis can be seen in normal neonates between birth and 5 days of age. [292] In severely ill infants, the defect may persist for a longer time. As with hyper-IgE syndrome, rIFN- may be helpful because this cytokine has been found to increase the chemotactic activity of neonate neutrophils in vitro. [363] [367]

Localized juvenile periodontitis (LJP) is a heterogeneous disorder of unknown etiology characterized by chronic and recurrent periodontal infections and severe alveolar bone loss with onset at the time of puberty. Nearly 75% of patients with LJP have been reported to have defective neutrophil chemotaxis in vitro. [292] [371] [372] [373] [374] [375] [376] [377] [378] [379] The molecular basis for the chemotactic defect is poorly understood. A number of reports suggest that cellular products derived from certain periodontal bacteria can alter leukocyte function and may be responsible for the diminished chemotactic activity. [380] [381] These inhibitors cannot entirely explain the disorder, however, because neutrophils from patients with LJP exhibit abnormal chemotaxis in vitro even in the presence

of normal serum. [292] [372] [373] [375] [376] At present it appears that LJP is an acquired disorder in some patients and a genetic disorder in others. It may also be a combination of both in certain patients because they may inherit an unusual sensitivity to the chemotactic inhibitors released by certain periodontal microorganisms. The diagnosis of the disorder is made on the basis of severe periodontal disease and destructive alveolar bone loss involving the first molars and incisors developing during adolescence. It is important to note that many qualitative and quantitative neutrophil disorders are also associated with severe periodontal disease. [292] [382] Therefore, the differential diagnosis should include neutropenia (both chronic and cyclic), LAD, CGD, and Chediak-Higashi syndrome (CHS).

DEFECTS IN THE STRUCTURE AND FUNCTION OF LYSOSOMAL GRANULES

Two major disorders of neutrophil granules have been described: Chediak-Higashi syndrome and specific granule deficiency (SGD). A great deal has been learned about the structural and functional abnormalities of neutrophils from patients with these conditions. Both disorders are exceedingly rare but are obligatory components in the differential diagnosis for any patient with recurrent bacterial or fungal infections.

CHS

Biology

Chediak-Higashi syndrome is a rare autosomal recessive, multisystem disease characterized by partial oculocutaneous albinism, frequent (and sometimes fatal) bacterial infections, giant lysosomes in leukocytes, a mild bleeding diathesis, progressive peripheral neuropathies, and cranial nerve abnormalities associated with defects at the optic chiasm.[6] [383] [384] [385] [386] [387] [388] [389] [390] Over 85% of those who survive the recurrent infections develop an accelerated phase of the diseasea progressive lymphoproliferative syndrome that usually occurs during the first two decades of life and is eventually fatal due to a profound pancytopenia that develops.[6] [385] Those patients who survive into their teenage years also suffer from the worsening of their peripheral neuropathies. The abnormal granules in neutrophils, monocytes, and macrophages compromise the function of these leukocytes, thereby leading to the consideration of CHS as a phagocyte disorder.

The diffuse symptoms of CHS are due in large part to the widespread presence of abnormal granules in numerous cell types in the CHS patient. In addition to the lysosomal defects seen in the phagocytes, giant granules are seen in melanocytes, Schwann cells, platelets, and certain cells in the liver, kidney, spleen, pancreas, and adrenal gland.[387] [388] The oculocutaneous albinism with light sensitivity characteristic of CHS is caused by grossly abnormal pigment distribution in melanocytes due to the giant melanosomes.[385] The melanocyte defect is also reponsible for the grayish silver sheen and lighter than expected color in the hair of CHS patients as the abnormal melanosomes poorly transfer their pigment to hair follicle keratinocytes. [388] Platelets in this disorder have a storage pool deficiency of adenine diphosphate and serotonin, presumably due to the abnormal granule morphogenesis in the megakaryocytes, leading to a defect in platelet aggregation that manifests itself as easy bruising and epistaxis. [391] [392] [393] [394]

Some of the most dramatic structural and functional lysosomal defects are seen in the phagocytes and contribute substantially to the increased susceptibility of patients with CHS to bacterial infections. Neutrophils contain a highly inhomogeneous population of giant granules that appear to be derived from the coalescence of both azurophilic and specific granules. [395] [396] [397] [398] [399] The giant granules are often more prominent in the bone marrow than in the peripheral blood because many of the abnormal myeloid precursors are apparently destroyed before they leave the marrow, resulting in moderate neutropenia with absolute neutrophil counts ranging between 500 and 2,000 cell/mm^3 . [385] [400] This neutropenia contributes to the propensity of patients with CHS to develop infections, particularly during the accelerated phase. Making matters worse, the neutrophil granules are markedly deficient in the antimicrobial protein, cathepsin G, as well as elastase. [146] Inhibitors of cathepsin G and elastase are present in mature CHS neutrophils (but not normal neutrophils) and may play a role in this deficiency. [401] Phagocyte function is also impaired. Not surprisingly, degranulation is delayed and incomplete in CHS neutrophils, resulting in diminished rates of bacterial killing. [402] [403] [404] [405] Chemotaxis is also defective, perhaps owing to the presence of the giant granules that interfere with the ability of the phagocyte to travel through narrow passages (e.g., between endothelial cells).[384] [406] Monocytes and macrophages exhibit similar giant cytoplasmic granules, [396] with resultant abnormalities in their phagocytic functions. [383]

Other key cells in the immune system are also affected in CHS. Giant granules are seen in lymphocytes and are associated with diminished antibody-dependent cell-mediated cytolysis of tumor cells.[407] [408] [409] More recent studies reveal a marked defect in the ability of CHS CD8+ cytotoxic T lymphocytes to destroy target cells recognized via the T-cell receptor. [410] The CHS cells contain normal levels of the lytic proteins granzyme A, granzyme B, and perforin properly compartmentalized within the appropriate, though abnormally large, lytic granules. The killing defect is due to the failure of the T cells to secrete these cytotoxic proteins onto the target cells.[410] Natural killer cell function is also profoundly abnormal in CHS and may contribute to the development of the accelerated phase of the disease. [407] [411] [412] [413] Epidermal Langerhans cells in a CHS patient have also been reported to have giant cytoplasmic granules; it is not known whether antigen presentation is adversely affected.[414] Eosinophils contain enlarged crystalloid granules, although the functional significance of this defect is not known. [415]

Molecular Genetics

The presence of dysmorphic granules in multiple tissues suggests an underlying global defect in granule morphogenesis. [292] [385] [386] [387] [398] [399] [416] [417] [418] [419] [420] The search for the molecular defect in CHS has been confounded by the rarity of the syndrome in humans (only several hundred cases identified) but has been greatly aided by the presence of a homologous disorder in a broad range of animals (Aleutian mink, blue foxes, killer whales, cats, Hereford cattle, beige rats, and beige mice).[386] [390] [419] [421] It appears that the same gene product is defective in at least humans, beige mice, and mink because fibroblast complementation studies have revealed that normal fibroblasts from any of these three species can restore normal lysosome size and distribution when fused with any of the three types of Chediak fibroblasts; complementation was not observed in any of the cross-species Chediak-Chediak fusion pairs. [422] The gene locus for the granule defect in beige mice has been mapped to the proximal end of mouse chromosome 13.[423] Through the use of yeast artificial chromosome complementation techniques, the gene responsible for the murine beige phenotype, termed Lyst, has been cloned and has been found to encode a lysomal trafficking regulator protein. [424] [425] The distal region of mouse chromosome 13 containing Lyst is highly homologous to the human chromosome 1q42q44. [426] [427] With the aid of this information, the gene responsible for human CHS (termed CHS1) was cloned and mapped to this region, consistent with the observed autosomal recessive inheritance of this disorder. [425] [428] [429] The human CHS protein is highly homologous to the mouse lysosomal trafficking regulation protein: it is a 3801 amino acid polypeptide encoded by a 13.5 kb mRNA. [430] [431] An alternatively spliced mRNA

TABLE 41-15 -- Summary of CHS1 Mutations in Eight Kindreds with CHS

Kindred; Sex	Type of Mutation	Nucleotide Change[a]	Predicted Amino Acid Change	Comments	References
1. M	Deletion	Delete G1467	Premature stop at codon 566	Homozygous	[428]
2. M	Nonsense	C3310 T	R1104 Stop	Homozygous; survived into adulthood	[428]
3. F	Insertion	G inserted after G117	Premature stop at codon 63	Compound heterozygote; mutation on second allele not known	[425] [428]
4. M	Insertion	A inserted in cluster of six adenines (18971902)	Premature stop at codon 638	Homozygous; parents consanguineous and of Kuwaiti Bedouin origin	[431]
5. M	Deletion	A9590 deleted	Premature stop at codon 3258	Homozygous; parents consanguineous and of Turkish origin	[431]
6.	Nonsense	C148 T	R50 Stop	Compound heterozygote; mutation on second allele not known	[432]
7.	Nonsense	C3085 T	Q1029 Stop	Compound heterozygote; mutation on second allele not known	[432]
8.	Deletion	A3073 and A3074 deleted	Premature stop codon	Compound heterozygote; mutation on second allele not known	[432]

[a]Nucleotide residues are numbered from the ATG initiation codon with A defined as +1.

isoform of 5.8 kb has also been identified, the biologic significance of which is still under investigation. [432]

The molecular genetic defects in several patients with CHS have been identified (Table 41-15). One patient was found to be homozygous for a frameshift mutation caused by the deletion of A9590.[431] Similarly, another was found to have a single-base insertional mutation at the other end of the gene leading to a frameshift and premature stop codon after amino acid 62.[425] A third patient in another report had a dinucleotide deletion (nucleotides 3073 and 3074) leading to a frameshift defect.[432] In the same report, two patients with nonsense mutations were also characterized: each had a C T transition leading to R50 stop in one patient and Q1029 stop in the other.

Clinical Manifestations

The key features of CHS are summarized in Table 41-16 . The disease is usually manifested in infancy or early childhood, with infections involving the lungs, skin, and mucous membranes being most commonly encountered.[385] The most frequent offending organism is *S. aureus*.[383] Gram-negative bacteria, *Aspergillus* species, and *Candida* species also are responsible for many

CLINICAL APPROACH TO PATIENTS WITH DISORDERS OF PHAGOCYTE FUNCTION

All the disorders of granulocyte function discussed in this chapter present with recurrent bacterial and fungal infections. The major diagnostic problem faced by the clinician is that patients with recurrent bacterial and fungal infections seldom have an identifiable granulocyte or monocyte defect. Given this low yield and the relative unavailability of laboratories capable of evaluating phagocyte defects, the physician is faced with the difficult question of deciding which patients with recurrent infections merit a complete evaluation. An excellent discussion of this problem has been published by Johnston [9] and by Holland and Gallin.[16] Guidelines are not well defined, and the following comments should be viewed as suggestions.

Our approach is to consider four aspects of each patients infection history: frequency, severity, location, and responsible pathogen. When considering frequency, the patients age and associated medical conditions must be taken into account. For example, recurrent otitis media in a 2-year-old is far less worrisome than a similar history in a 40-year-old. Another recommendation is that the more unusual the infection, the less frequently it has to occur before a phagocyte evaluation is indicated. Similarly, as few as two or three infections of unusual severity might warrant an evaluation. Infections in unexpected anatomic locations should also alert the clinician to a possible underlying immune disorder. Hepatic, pulmonary, and rectal abscesses, as well as disseminated candidal infections, may be indicative of an underlying phagocyte defect. Finally, the identification of certain pathogens (e.g., *S. marcescens, Aspergillus* species, *Nocardia* species, and *P. cepacia*) in children and young adults can provide the strongest indications for pursuing further studies. Certain unusual clinical findings can also be helpful in determining which patients warrant further testing. For example, an infant with a history of delayed separation of the umbilical cord who has also had several bouts of pneumonia should be evaluated for leukocyte adhesion deficiency (LAD), and a child with nystagmus, fair skin, and recurrent staphylococcal infections should be evaluated for CHS.

Once the physician has decided that a phagocyte evaluation is warranted, what is the appropriate sequence of tests to perform? The following algorithm is useful for approaching patients with recurrent infections regardless of the immune defect responsible. The algorithm should be used in conjunction with the published literature relevant to these disorders, and each laboratory finding must always be interpreted in the clinical context of the patient under examination.*

TABLE 41-16 -- Summary of CHS

Incidence	200 cases described
Inheritance	Autosomal recessive
Molecular defect	A defect in granule morphogenesis in multiple tissues resulting from mutations in the CHS1 gene encoding a lysomal trafficking regulator protein
Pathogenesis	Giant coalesced azurophil/specific granules in neutrophils resulting in ineffective granulopoiesis and neutropenia, delayed and incomplete degranulation, and defective chemotaxis
Clinical manifestations	Partial oculocutaneous albinism Recurrent severe bacterial infections (usually *S. aureus*) Cranial and peripheral neuropathies (muscle weakness, ataxia, sensory loss) Hepatosplenomegaly and complications of pancytopenia in the accelerated phase
Laboratory evaluation	Giant granules in peripheral blood granulocytes and in bone marrow myeloid progenitor cells Widespread lymphohistiocytic infiltrates in accelerated phase
Differential diagnosis	Other genetic forms of partial albinism Giant granules can be seen in acute and chronic myelogenous leukemias
Therapy	Prophylactic trimethoprim/sulfamethoxazole Parenteral antibiotics for acute infections Ascorbic acid (200 mg/day for infants; 6 g/day for adults) Bone marrow transplant at beginning of accelerated phase
Prognosis	Most patients die from infection or complications of the accelerated phase during the first or second decade of life. A few patients have survived into their thirties.

infections. Patients who survive into their teenage years and beyond must contend with progressive peripheral neuropathies that can lead to a bed-ridden state. Approximately 85% of CHS patients will develop an accelerated phase of the disease in which lymphohistiocytic proliferation occurs in the liver, spleen, lymph nodes, and bone marrow.[416] This phase of the disease is often seen in the second decade of life but can be seen much earlier. Occasionally children with CHS present in the accelerated phase. The cellular infiltration is not neoplastic by histopathologic criteria, although the prognosis is dismal all patients dying unless curative therapy is administered as discussed below. The accelerated phase resembles the virus-associated hemophagocytic syndrome because the invading histiocytes often exhibit hemophagocytosis.[416] The bone marrow infiltration and progressive hepatosplenomegaly eventually lead to pancytopenia and death due to infection and bleeding. The accelerated phase may be precipitated by EBV infection. [433] [434]

Diagnosis

The diagnosis of CHS is made on the basis of the giant peroxidase-positive lysosomal granules in the peripheral blood granulocytes or in bone marrow myeloid cells. This morphologic approach can also be used to diagnose CHS prenatally. [435] [436] The accelerated phase of the disease is characterized by diffuse infiltrates of lymphohistiocytic cells seen on biopsy and by pancytopenia. [416] Occasionally, giant granules that resemble those of CHS can be seen in both acute and chronic

myelogenous leukemias.[437] [438] [439]

Therapy

The treatment for the stable phase of CHS is similar to that for other neutrophil disorders. Prophylactic antibiotics such as trimethoprim-sulfamethoxazole appear to be beneficial. Parenteral antibiotics are indicated for acute infections. Treatment with high-dose ascorbic acid (200 mg/day for infants, 6 g/day for adults) has improved the clinical status of some patients in the stable phase.[440] [441] Although there is some controversy regarding the efficacy of ascorbic acid,[442] given the safety of this medication it seems prudent to administer it to all patients.

The treatment of the accelerated phase is extremely difficult. The lymphohistiocytic infiltrates respond poorly, if at all, to vincristine and corticosteroids.[385] [408] [409] [410] [411] [412] [413] [414] [415] [416] The only curative therapy appears to be bone marrow transplantation,[383] [443] [444] [445] [446] [447] which is ideally performed before or at the beginning of the accelerated phase. A total of six patients with CHS have been reported to have received bone marrow transplants from human leukocyte antigen (HLA)-compatible donors, of whom five were still alive at the time of the reports.[383] There is a possibility that the accelerated phase could be prevented or delayed by vaccines against EBV.[383] The onset of the accelerated phase may be related to the inability of patients with CHS to contain and control this virus.[416] [433] Intravenous -globulin has been used in an attempt to delay the development of the accelerated phase, presumably by protecting the patient from infection with EBV.

SGD

Neutrophil SGD is a rare congenital disorder characterized by recurrent bacterial and fungal infections, primarily involving the skin and lungs.[292] [383] [448] The key features of this disorder are summarized in Table 41-17 . SGD is quite rare, but the six cases that have been reported[448] [449] [450] [451] suggest that it is inherited in an autosomal recessive manner. The precise molecular defect responsible

TABLE 41-17 -- Summary of Neutrophil SGD

Incidence	Six cases reported
Inheritance	Autosomal recessive
Molecular defect	Deficiencies in lysosomal proteins in both azurophil granules (defensins) and specific granules (lactoferrin, vitamin B_{12} -binding protein) suggest a common defect in the regulation of the production of these proteins. The precise defect is unknown.
Pathogenesis	Recurrent infections result from the combined effect of deficiencies in microbicidal granule proteins, such as defensins and lactoferrin, and abnormal chemotaxis, perhaps due to a failure to up-regulate surface $_2$ integrins from specific granule stores
Laboratory evaluation	Absent or empty specific granules in neutrophils by light or electron microscopy Bilobed nuclei in neutrophils frequently seen Severe deficiency of neutrophil lactoferrin, vitamin B_{12} -binding protein, and defensins
Differential diagnosis	Acquired specific granule deficiency (e.g., thermal burns or myeloproliferative syndromes)
Therapy	Prophylactic antibiotics Granulocyte transfusions Parenteral antibiotics for acute infections Surgical drainage of refractory infections
Prognosis	With appropriate medical management, patients can survive into their adult years.

for the disorder has not been identified, but growing evidence suggests that there is an abnormality in the transcriptional regulation of a closely related group of myeloid lysosomal proteins.[383] [452] [453] Neutrophils from patients with this disorder have multiple deficiencies in lysosomal proteins present in both azurophilic granules (defensins)[146] [451] and specific granules (e.g., lactoferrin, vitamin B_{12} -binding protein, neutrophil procollagenase).[451] [452] Not only are the protein levels of these granule constituents deficient, but so are the levels of the corresponding mRNAs, supporting the hypothesis of a transcriptional regulatory defect in the neutrophils.[452] Further supporting this explanation is the observation that lactoferrin secretion was normal in the glandular epithelia of patients with SGD despite its severe deficiency in the neutrophils.[454] Because there is only a single lactoferrin gene that encodes for protein in both myeloid and secretory cells, this finding suggests that there is a myeloid-specific regulatory defect. Interestingly, granule formation also appears to be abnormal in eosinophils from patients with SGD as these cells are deficient in three eosinophil-specific proteinsmajor basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin.[453] Unlike the neutrophil and its precursors, however, the eosinophil contains mRNA transcripts for these granule proteins.

The recurrent skin and pulmonary infections characteristic of SGD appear to be caused by two fundamental defects in the neutrophils. One is the marked deficiency of at least two important microbicidal granule proteins, lactoferrin[450] and defensins.[146] [451] The other defect is a relatively severe chemotactic abnormality presumably caused by the absence of the intracellular pool of leukocyte adhesion molecules that normally reside in the specific granules.[449] [455] [456] [457] As discussed above, these $_2$ integrins play a key role in phagocyte chemotaxis.

The diagnosis of SGD can be readily made by microscopic examination. Wright-stained neutrophils are devoid of specific granules but contain normal numbers of azurophilic granules.[383] Electron microscopy reveals small peroxidase-negative vesicles, which presumably represent empty specific granules.[383] [458] Thus, the ultrastructural findings are consistent with the putative defect in regulation of protein synthesis discussed above. The diagnosis of SGD can also be established by directly demonstrating a severe deficiency in either lactoferrin or vitamin B_{12} -binding protein. An acquired form of SGD can be seen in burn patients[383] or in individuals with various myeloproliferative disorders.[459] The treatment for SGD is similar to that for other neutrophil disorders. If medical management is aggressive, the prognosis appears quite good, with patients surviving into their adult years.

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Leukocytosis and Leukopenia

INTRODUCTION

Changes in the number of circulating leukocytes can represent a primary disorder of leukocyte production or a secondary response to some disease process or toxin. Leukocytosis or leukopenia should be defined based on age- and population-based means of neutrophils, lymphocytes, monocytes, or eosinophils. The distribution of leukocytes, or differential, undergoes specific developmental changes and genetic differences as well as changes that reflect disease states. In the neonate, there is a predominance of neutrophils, which persists until the second or third week of life when lymphocytes begin to predominate. At about 5 years of age the neutrophil again becomes the predominant leukocyte. This section focuses on abnormal elevation or depression of mature neutrophils. Abnormalities in lymphocytes and eosinophils are considered elsewhere in this text.

Definitions of Leukocytosis and Neutropenia

Leukocytosis refers to white blood cell (WBC) count elevation >2 standard deviations above the mean of circulating WBCs. Neutrophilia refers specifically to elevation of the absolute neutrophil count (ANC). The latter is calculated by multiplying the percentage of neutrophilic granulocytes by the total WBC count (see equation). The usual explanation for leukocytosis is neutrophilia.

Neutrophil counts show considerable variability during the neonatal period with a mean of 11,000/mm ^{3} and a range of 6,00026,000/mm^3 . Total WBC counts may be as high as 38,000/mm^3 at 12 hours of age.[460] The WBC count drops after 12 hours of age, reaching a mean neutrophil count of 5,500/mm ^{3} (1,50010,000) at 1 week. The differential in the first week of life resembles that of the adult with approximately 60% neutrophils. From 1 week until 56 years, however, there is a predominance of lymphocytes. Thereafter neutrophils increase to about 60%. The mean adult total leukocyte count is 7,500/mm ^{3} (4,50011,000) and the mean neutrophil count is 4,400/mm^3 (1,8007,700).[460] Total leukocyte counts of premature infants are about 30% lower than those of term infants. The sequential changes noted after birth also occur in premature infants but with wider variability. [461]

Neutropenia is defined as a neutrophilic granulocyte count of <1,500/mm ^{3} . This definition can be used for all ages, although separate standards should be used for certain groups. Certain populations of blacks and Yemenite Jews normally have lower granulocyte counts. [462] [463]

The ANC is calculated by multiplying the total WBC count by the percentage of bands and mature neutrophils:

Absolute neutrophil count =WBC × (% bands + % mature neutrophils) × 0.01.

Regulation of Granulocyte Counts

The peripheral neutrophil count reflects the equilibrium of several compartments. In the bone marrow, there is a mitotic pool, a maturation pool, and a storage pool. Outside the marrow, there is a circulating pool, a marginated pool of neutrophils adherent to vascular endothelium, and a tissue pool. The clinical assay of the number of neutrophils, the WBC count and differential, only monitors neutrophils in the circulating pool during a brief 36-hour period of transit from marrow to tissue. There is a complex interplay of factors that regulates the production of granulocytes and their movement from one pool to another, but the movement is always from marrow to blood to tissue.[464]

Granulocytes are derived from a common progenitor that also gives rise to erythrocytes, megakaryocytes, eosinophils, basophils, and monocytes. The biology of hematopoeisis is complex and is regulated by a number of cytokines with overlapping activities. The hematopoietic stem cells normally reside in the bone marrow in a state of quiescence/dormancy. Cytokines that lead to proliferation (or exit from dormancy) of the early progenitor/stem cells are stem cell factor, thrombopoietin, FLT3 ligand, IL-11, granulocyte colony-stimulating factor (G-CSF), and IL-6. IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) act on the later progenitor cells, while cytokines that support differentiation along specific lineages are G-CSF, monocyte colony stimulating factor (M-CSF), and erythropoietin, leading to granulocytic, monocytic, and erythroid differentiation, respectively. [465] [466] [467] [468] [469] [470] Some of these cytokines along with components of complement[471] [472] [473] [474] release granulocytes from the marrow storage pool. This can result in

a two- to threefold increase in the granulocyte count within 45 hours. [471] [475] More than half of the granulocytes in the peripheral circulation at a given time are attached to the vascular endothelium.[476] These marginated neutrophils can be released almost immediately at times of stress. Epinephrine in part mediates this effect.[477] There is now clear evidence that margination is mediated by specific receptors. L-selectin is constitutively expressed on the neutrophil surface and tethers the cell to the vessel probably by binding to sulfated glycoconjugates on the endothelium. E-selectin and P-selectin may also be involved in this process. [464] Some experimental evidence suggests a feedback inhibition loop in the regulation of neutrophil production. Lactoferrin, which is contained in neutrophil-specific granules, suppresses production of colony-stimulating factors by monocytes, thus shutting off neutrophil production when granulocyte counts rise. Acidic isoferritins may also participate in this regulation. [478] [479]

Of the 1.2×10^9 granulocytes per kilogram, 20% are in the myeloid precursor pool, 75% in the marrow storage pool, 3% in the marginated pool, and 2% in the circulating blood.[476] [480] Normally, 1.5×10^9 granulocytes/kg body weight are produced per day. Production can be increased in the presence of an inflammatory stimulus. Granulocytes spend about 9 days in marrow, 36 hours in blood, and 14 days in the tissues. [476] Thus, the total granulocyte count as measured from peripheral blood represents a sample of a population that comprises only 5% of the total pool sampled during a fleeting 2% of the total transit time.

One must consider movement of neutrophils from one compartment to another as well as changes in production in assessment of neutrophilia and neutropenia. Thus, neutrophilia can be due to increased production, as in infection or myeloproliferative disorders, or inhibition of egress from the blood, as in certain chemotactic disorders or steroid therapy. [475] Neutropenia may be due to decreased production, increased margination and egress (as in systemic complement activation or burns),[481] [482] or destruction (as in immune neutropenia).

Unfortunately, evaluation of these processes is limited to examination of the peripheral blood and the bone marrow. Although sophisticated radiotracer techniques exist, they are not of much clinical use. Similarly, clinical tests such as hydrocortisone challenge or epinephrine stimulation, which reflect a releasable marrow storage

pool or marginated pool, have limited differentiating power.

CAUSES OF NEUTROPHILIA

Neutrophilia is classified as primary or secondary, as listed in Table 41-18 .

Secondary Causes of Neutrophilia

Leukemoid Reaction

Leukocytosis >50,000/mm^3 is referred to as leukemoid reaction [483] and is characterized by a significant increase in early neutrophil precursors in the peripheral blood. The differential count has a marked left shift evidenced by the presence of myelocyte, metamyelocyte, and band forms. Promyelocytes and myeloblasts may be observed in severe reactions. In contrast to acute leukemia, proliferation and orderly maturation of all normal myeloid elements is observed in the bone marrow. The morphology of the myeloid elements is normal. Chronic myeloid leukemia may be distinguished from a leukemoid reaction by the finding of a low leukocyte alkaline phosphatase score and the presence of the Philadelphia chromosome on karyotypic analysis of the bone marrow. Leukemoid reactions to infections may be accompanied by toxic granulation, Doehle bodies, and cytoplasmic vacuoles in the neutrophils. [483]

TABLE 41-18 -- Classification of Neutrophilia

Primary (no other evident associated disease)
Hereditary neutrophilia
Chronic idiopathic neutrophilia
Chronic myelogenous leukemia (CML) and other myeloproliferative diseases
Familial myeloproliferative disease
Congenital anomalies and leukemoid reaction
Leukocyte adhesion deficiency (LAD)
Familial cold urticaria and leukocytosis
Secondary
Infection
Stress neutrophilia
Chronic inflammation
Drug induced
Nonhematologic malignancy
Generalized marrow stimulation as in hemolysis
Asplenia and hyposplenism

Newborns have unique responses to stress and infection. The relative number of band forms and less mature granulocyte precursors in the differential of newborn infants has been correlated with sepsis and with depletion of the marrow neutrophil storage pool. [484] [485] In the normal infant, the band/neutrophil ratio is <0.14:0.11 in the first 48 hours irrespective of birth weight or gestational age. [486] An immature/total neutrophil ratio >0.8 in the peripheral blood indicates that marrow reserves are depleted and the risk for death in septic infants is significantly increased. [484] Some infants have extreme responses to stress. Extreme leukocytosis >200,000/mm^3 has been seen in a 26-week infant whose mother received steroids. [487] Transient leukemoid reactions have also been seen in phenotypically normal infants who had trisomy 21 limited to their hematopoietic cells. [488] [489]

When the marrow is directly invaded by tumor, or in cases of marrow fibrosis, or granuloma reactions, neutrophilia associated with immature granulocytes, nucleated red blood cells and teardrop-shaped erythrocytes may be seen accompanied by thrombocytosis. This is called a leukoerythroblastic response. A bone marrow aspirate and biopsy should be performed in these cases to look for granuloma, tumor, or marrow fibrosis. Cultures of the marrow for fungus and tuberculosis [490] should be obtained. Leukoerythroblastic reaction may also be seen with recovery from marrow suppression after chemotherapy. Leukoerythroblastosis can also be seen during recovery from severe myelosuppression. Interestingly, transient erythroblastopenia of childhood, which was thought to be related to parvovirus infection, has been reported to present with leukoerythroblastosis. [491]

Pelger-Huët Anomaly

Although technically not leukocytosis, the Pelger-Huët anomaly can raise the question of serious infection because of an apparent high band count. The Pelger-Huët anomaly is a benign dominantly inherited defect of terminal neutrophil differentiation found with a frequency of 1:6,000 live births. Most of these neutrophils have bilobate nuclei and excessively coarse clumping of nuclear chromatin. The two lobes are joined by a thin, hair-like bridge that is much thinner than that seen in a normal band. This gives the characteristic pince nez or spectacle appearance to the nucleus. The function of these cells is normal. [492] Interestingly, Pelger-Huët cells can develop multiple lobes during states of vitamin B_{12} or folate deficiency but return to their bilobate state when the vitamin deficiency is corrected. [493] Colchicine, sulfonamides, ibuprofen, taxoids, and valproate reversibly induce the anomaly. [494] [495] [496] [497] [498] This so-called pseudo Pelger cell has also been reported transiently during certain acute infections, in acute myelogenous leukemia, and in myelofibrosis. In

particular, the defect has been associated with cytogenetic abnormalities of chromosome 17 in leukemias. [499] The homozygous state results in neutrophils that contain a single round eccentric nucleus with clumped chromatin. Eosinophils and basophils may be involved as well. An unusual case of Pelger-Huët anomaly has been reported in four generations of one family with late-onset progressive proximal muscular dystrophy. [500]

Neutrophilia and a left shift can be indicative of disease or may be totally benign. An outline of the major primary and acquired causes of neutrophilia is presented in Table 41-18 . The causes of neutrophilia are divided into two groups, those that seem to be due to intrinsic problems with the neutrophil or regulation of neutrophil production, and those that are secondary to some other disease process.

Chronic Myelogenous Leukemia

The major differential diagnosis of leukemoid reaction is chronic myelocytic leukemia (CML). This topic is considered in detail in Chapter 62 . Leukemoid reactions and CML can be difficult to distinguish. The classical differentiating features are the presence of a low leukocyte alkaline phosphatase (LAP) score and presence of the Philadelphia chromosome in CML. The LAP score is also low in familial myeloproliferative disease [501] and paroxysmal nocturnal hemoglobinuria. [502] Juvenile CML can be distinguished from the usual adult form by the ability of circulating stem cells to form monocyte colonies in vitro [503] and the absence of the Philadelphia chromosome. Leukocyte counts in CML can be extremely high, occasionally >500,000/mm^3 . Patients with granulocyte counts >200,000/ mm^3 require emergent intervention to prevent the vaso-occlusive complications of hyperviscosity related to the markedly elevated WBC counts.

Acute Infection

Modest leukocytosis with a left shift is commonly seen in association with many acute bacterial infections. Certain bacterial agents such as pneumococcus or staphylococcus may cause particularly high leukocyte counts. Leukocytosis is seen in association with acute otitis media with 9% of patients having >20,000 WBC/mm^3 , whereas 27% of culture-proven positive acute otitis media cases have WBC counts below the mean for age. [504] The predictive value of leukocytosis and increased band forms (WBC >15 × 10^9 /L immature/total >0 2) in detecting bacterial disease was increased from 32% to 71% when associated with depressed fibronectin levels (1 SD < mean for age). This is of particular interest in view of the role of fibronectin in promoting phagocytosis by polymorphonuclear cells and monocytes.[505] The risk of positive blood cultures in children between 3 and 36 months of age with fever >39.5°C is directly related to WBC count. If the temperature is >39.5°C and the WBC is >15,000/mm^3 , 16% have a positive culture, whereas 43% of cultures from similar children with a WBC >30,000/mm^3 are positive. In contrast, only 3.7% of children with counts <15,000/mm^3 , and none with counts <10,000/mm^3 had positive blood cultures.[506] Neutrophilic leukocytosis can also be associated with severe viral disease. Patients with Hantavirus infection, for example, have WBC counts as high as 65,000/mm^3 (median 26,000/mm^3) with 67% bands (median 27%).[507]

Chronic Inflammation

Acute changes in the neutrophil count are due to release from the marrow storage pool and the marginated pool. In cases of overwhelming infection, marrow depletion can occur resulting in neutropenia rather than neutrophilia. Chronic inflammatory processes result in stimulation of granulocyte production and are usually more modest in degree and may be associated with increase in monocytes.

Stress Neutrophilia

Modest elevation in the neutrophil count has been associated with many types of stress. Neutrophilia can occur within minutes of exercise [508] or stress[509] or epinephrine injection[477] and is presumed to be related to movement of neutrophils from the marginated pool into the circulating pool. The neutrophilia secondary to catecholamine injection has been related to reduced neutrophil adherence. The neutrophilia as well as the adherence inhibiting ability of plasma can be blocked by pretreatment of the subject with propranolol or in vitro treatment of the adherence assay system with antibody to cyclic adenosine monophosphate. [477]

Studies of exercise-induced neutrophilia failed to show blockade by propranolol, despite measurable increases in plasma epinephrine. [510] The neutrophilia was directly related to the work load and cardiac output, suggesting a larger role for mechanical and flow-related effects on dislodgment of the leukocytes sequestered in the lung, than possible direct effects of catecholamines on the neutrophils. Other studies show no effect of exercise on circulating colony-forming unit granulocyte/macrophage (CFU-GM) or colony-forming unit granulocyte, erythrocyte, monocyte, macrophage (CFU-GEMM) [508] although it has been suggested that the delayed leukocytosis (up to 235% increase at 5 hours after exercise) may be related to marrow release of leukocytes. [511]

Mild neutrophilia and lymphopenia have been associated with unipolar depression but may be related to the use of antidepressant medications. [512]

Neutrophilia is also seen in the postoperative period. There is a doubling of the leukocyte count 3 hours after surgery. This does not seem to be related to the type of anesthesia.[513] [514]

Leukocytosis in the 12,00020,000/mm^3 range has been reported in the postictal state. This can be associated with fever or pulmonary edema and resolves in a few hours to days.[515]

There is an interesting association between leukocyte count and myocardial infarction. Subjects with a leukocyte count >9,000 had a 4.5-fold higher incidence of myocardial infarction than those with a leukocyte count <6,000. [516] It is also not clear whether the WBC count is a risk factor or merely an indicator of stress. [517] However, the WBC count has been shown to be an independent indicator of atherosclerotic cardiovascular disease. [518]

Drug-Induced Neutrophilia

Leukocytosis has been seen in association with a number of drugs and drug reactions. Steroids are known to induce release of neutrophils from the bone marrow [475] and result in a chronic low-grade neutrophilia. Leukocytosis as high as 200,000/mm^3 in neonates has been associated with maternal steroid administration as well. [487] [519] -Agonists produce an acute neutrophilia by releasing neutrophils from the marginated pool. [520] Lithium is known to produce leukocytosis by increasing production of CSF and has been used with varying success to treat several neutropenic states. [521] Tetracycline has also been associated with leukemoid reaction with WBC counts >80,000/mm^3 .[522] G-CSF is now in common clinical use and can cause significant neutrophilia if use is not closely monitored.

Nonhematologic Malignancy

Leukocytosis is frequently seen in large-cell lung cancer. [523] Usually the leukocytosis seen with solid tumors is modest in the 12,00030,000/mm^3 range[523] [524] [525] [526] [527] although it can be as high as 106,000/mm^3 even in the absence of marrow metastasis.[528] Leukemoid

reactions have been seen in patients with marrow involvement with tumor from lung, stomach, and breast, or with neuroblastoma in children. [528] In some instances, solid tumors of lung, tongue, and kidney have been shown to secrete colony-stimulating activity. [529] [530] [531]

Heat Stroke

Leukocyte counts up to 30,000/mm^3 have been seen in heat stroke. Up to 50% of the neutrophils may have botryoid nuclei with nuclear segments smaller than usual, resembling a clustering of grapes around a central stem.[532] [533]

Marrow Stimulation

Significant leukocytosis can be seen in states of chronic stimulation of the bone marrow such as hemolytic anemia or immune thrombocytopenia. [534] Patients with sickle cell anemia commonly have leukocyte counts in the 12,00015,000/mm^3 range and have an exaggerated elevation in WBC counts with infection. This response may be further augmented by functional asplenia. Likewise, significant rebound leukocytosis can occur in the recovery phase of marrow suppression. [535] [536] These reactions can last several weeks. In one instance there were 90% myeloblasts in the marrow and as many as 20% myeloblasts in the peripheral blood. [537]

Asplenia and Hyposplenism

Moderate neutrophilia can be associated with congenital disease-related asplenia (such as in sickle cell anemia) or surgical asplenia. Usually the neutrophilia is mild.[538] [539]

Primary Causes of Neutrophilia

Hereditary Neutrophilia

A family of four was described in 1974[540] with leukocyte counts that were chronically in the 20,00070,000/mm^3 range, splenomegaly, and widened diploe of the skull. There was no apparent propensity to bacterial infection. The defect seemed to be dominantly inherited. The LAP scores were high in the affected subjects. To date, none of the family members have had any serious medical problems other than a bleeding diathesis related to platelet dysfunction. [540] We have studied one of the

members of this family and find normal neutrophil function and normal expression of surface CD18/CD11b so this is not a variant of leukocyte adhesion deficiency (W. Herring, T.D. Coates, unpublished data). We have seen a second family with this syndrome (D. Powars, personal communication). In this case, the father had marked leukocytosis and spenomegaly and eventually had his spleen removed. His son had similar problems with neutrophil counts at times in 100,000/mm ^{3} range and massive splenomegaly. The neutrophil function and CD18/CD11b expression in the affected father and son are also normal (D. Powars and T.D. Coates, unpublished data).

Chronic Idiopathic Neutrophilia

Chronic leukocytosis can occur in patients who are otherwise well. A series of 34 patients with leukocyte counts between 11,000 and 40,000/mm ^{3} were followed for up to 20 years without associated clinical problems. Bone marrow aspirations were generally normal and LAP scores were normal. The remainder of the blood counts were normal, except for occasional thrombocytosis.[541] These data point out that certain normal subjects fall outside of the 95th percentile with respect to WBC counts and should be considered normal, thus sparing them frequent and extensive evaluations.

Familial Myeloproliferative Disease

A syndrome of growth retardation, hepatosplenomegaly, anemia, and leukocytosis has been described. [50C] Some of these children died in early life, whereas others remained stable or improved. All of the affected subjects had low LAP scores. Furthermore, several other members in four generations of the family had low LAP scores but no other findings. Chromosomal analysis showed no significant consistent abnormalities and no subject had a Philadelphia chromosome. Other families have been reported with familial occurrence of polycythemia vera, CML, and myelofibrosis. [542] [543] Furthermore, there is evidence that myeloproliferative syndromes associated with monosomy 7 may have familial features.[544] [545]

Congenital Anomalies and Leukemoid Reaction

Leukemoid reactions have been associated with amegakaryocytic thrombocytopenia and congenital abnormalities such as tetralogy of Fallot, dextrocardia and absent radii, and rudimentary little toes. [546] [547]

Down Syndrome

Infants with Down syndrome may have a transient leukemoid reaction that resembles congenital leukemia. [548] These children can also have an exaggerated leukemoid response to stress.[549] A transient leukemoid reaction has been seen in a phenotypically normal child who expressed trisomy 21 mosaicism in myeloid cells but not in skin fibroblasts. The chromosomal abnormality disappeared after the leukemoid reaction resolved. [489] [550]

LAD

Since the 1970s, several patients have been described with persistent leukocytosis, delayed separation of the umbilical cord, recurrent infections, and a stimulus-dependent activation defect of the neutrophil. The laboratory hallmark of these disorders is leukocytosis. WBC counts may be only mildly elevated but often are in the 18,00030,000/mm^3 range and may be as high as 150,000/mm^3 during infection.[551] [552] There are no specific morphologic abnormalities seen in the granulocytes. The diagnosis is established by demonstration of absence or marked reduction (<10% of control) in the CR3 receptor on granulocytes. These patients have severe functional defects as well and are described in detail earlier in this chapter.

Familial Cold Urticaria and Leukocytosis

An interesting syndrome of leukocytosis, fever, urticaria, rash, and muscle and skin tenderness on exposure to cold has been reported. This syndrome appears to be dominantly inherited. The onset of symptoms is in infancy and symptoms have been provoked by exposure to cold in the delivery room. Urticaria, followed by fever, starts 7 hours after cold exposure. Leukocytosis, sometimes up to 34,000/mm^3 , starts 10 hours after cold exposure and begins to subside 1214 hours later. [553] [554] In contrast to other causes of urticaria, the skin rash in these patients is characterized histologically by a marked infiltration by neutrophils. The leukocytosis and urticaria were blocked when the patients were infused with endotoxin before cold exposure. This has been related to cold activation of clotting system as it is associated with a transient decrease in levels of C1 esterase inhibitor. [555] [556]

APPROACH TO THE EVALUATION OF NEUTROPHILIA

Laboratory error must always be included in the differential diagnosis of unexplained isolated neutrophilia or neutropenia.

With current technology, much of the human error has been removed [557] although some clinicians continue to believe that manual counts are more accurate. Blood counts that do not make sense in the context of the clinical findings should be repeated before extensive evaluation is undertaken. [558]

Factitious leukocytosis can be seen due to blood sampling problems or certain primary disease states. Inadequate anticoagulation of the specimen can result in platelet clumps being counted as leukocytes by automated cell counters. The WBC count is rarely increased >10% and is usually associated with spurious thrombocytopenia.[559] [560] In cryoglobulinemia, a temperature-dependent increase in leukocyte and platelet counts occurs at about 30°C and can result in WBC counts as high as 50,000/mm^3 and a doubling of the platelet count, presumably due to various sizes of precipitated cryoglobulin particles. [561] This effect is increased if the sample is allowed to cool to lower temperatures.

The general approach to the evaluation of the patient with neutrophilia will depend on the degree of neutrophilia and the signs and symptoms of the associated diseases. Neutrophilia is most commonly seen secondarily to acute or chronic inflammatory processes, and the diagnosis and treatment are dictated by the nature of the primary illness. In the absence of clear history or physical examination findings, the sedimentation rate may be very helpful. An elevated sedimention rate suggests a more extensive evaluation for collagen vascular or other occult inflammatory disease is warranted, whereas a normal value suggests watchful waiting may be the best course. Examination of the bone marrow is of little help in mild leukocytosis. In leukemoid reactions or leukoerythroblastosis, direct invasion of the marrow and myelodysplasia must be excluded. Bone marrow biopsy, marrow cytogenetics, and fungal and tuberculosis evaluation should be included. The biopsy may reveal granulomata, which may intensify the search for fungus, or may reveal metastatic tumor that is missed in marrow aspirate. Marrow cytogenetics may reveal mosaic trisomy 21,[488] [489] monosomy 7,[544] Philadelphia chromosome, or other signs of dysplastic states. The LAP score, which is high in infection and low in CML, is also of help. It must be kept in mind, however, that the LAP score can be normal in CML, particularly juvenile CML, making this diagnosis difficult at times.

Many factors contribute to the wide variability in granulocyte counts in normal populations. Particularly in the asymptomatic patient with persistent mild neutrophilia, one must remember that, by definition, the WBC count in 2.5% of the normal population must be >2 standard deviations above the mean. Because regulation of granulocyte production is genetically controlled, [462] [463] [541] [562] examination of the parents or siblings blood counts may be of help in these few cases.

LEUKOPENIA

There are fewer causes of neutropenia than of neutrophilia. Because most clinicians are looking for increased WBC counts, the diagnosis of neutropenia is often overlooked.

Relation of ANC to Propensity to Infection

Propensity to infection in patients whose neutropenia is due to decreased production is related to the ANC (Table 41-19). In contrast, patients with peripheral destruction or margination of neutrophils, such as seen in immune neutropenias, demonstrate no direct correlation between degree of neutropenia and propensity to

infection.

Patients with neutropenia secondary to chemotherapy, marrow failure, or marrow exhaustion are at great risk for overwhelming bacterial infection. [484] [485] [563] In contrast, children with chronic benign neutropenia of infancy and childhood may have neutrophil counts <200/mm ^{3} for months or years and have no

TABLE 41-19 -- Clinically Significant Neutrophil Counts

ANC	Clinical Significance
>1,500/mm^3	Normal
1,000>1,500	No significant propensity to infection. Fevers can be managed on an outpatient basis.
5001,000	Some propensity to infection. Occasionally fever can be managed on an outpatient basis.
<500	Significant propensity to infection. Always should be managed as inpatient with parenteral antibiotics. Few clinical signs of infection.

These rules apply strictly for neutropenia with hypoplastic marrow, early myeloid arrest, and decreased granulocyte reserve pools. There is more latitude for clinical judgment in neutropenias with normocellular marrow. The only regular exception to these rules is documented chronic benign neutropenia of childhood.

serious infectious problems.[563] Similarly, some adults with immune neutropenia may have severe depression of their neutrophil counts and suffer no serious episodes of infection.[564] Both of these entities are characterized by neutropenia and a cellular marrow. The marrow differential usually shows normal early granulocyte precursors but may have a late myeloid arrest.

Many patients with chronic neutropenia have normal or increased monocytes. These cells are functioning phagocytes and may account for the lack of correlation of neutrophil count with propensity to infection in certain patients. [565] [566] Tissue delivery of neutrophils in chronic immune-mediated neutropenia has been shown to be greater than that in chemotherapy-induced neutropenia of equal degree suggesting that the peripheral blood count may not accurately reflect neutrophil availability.[567] It seems that adequacy of the marrow reserve pool of granulocytes may be the most important determinant of propensity to infection in neutropenic patients.

The types of infections encountered in neutropenic patients depend on the degree and chronicity of the neutropenia as well as the nature of the associated diseases. Patients in good clinical condition with severe neutropenia secondary to decreased production can go many weeks without serious infection although infection is inevitable. If the patient is debilitated or receiving suppressive chemotherapy, the frequency of infection is greater. *S. aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Klebsiella* species are common causes of infection in these patients.[565] [566] [568] Patients with less severe chronic neutropenias (ANC >300) may have recurrent sinusitis, stomatitis, perirectal infection, and gingivitis, [565] [566] [569] but they usually do not become septic.

CLASSIFICATION OF NEUTROPENIA

Neutropenia can be due to decreased production of granulocytes, shift of granulocytes from the circulating pool to marginated or tissue pools, peripheral destruction, or a combination of these causes.[566] [570] The mechanisms have been determined using leukokinetic measurements and bone marrow cultures. These assays are not routinely available and do not significantly alter clinical management. Thus, from a practical standpoint, the simple classification in Table 41-20 is preferred. This classification also generally corresponds to marrow cellularity and propensity to infection.

The classification of some of the neutropenias depends on the clinical course. Because the laboratory characteristics of many of the neutropenias overlap, the differential diagnosis can be difficult. If there is no family history of neutropenia and the bone marrow is normocellular or hypercellular, there is no certain way to differentiate the various neutropenias other than to follow the patient clinically. In fact, the syndromes called chronic benign neutropenia, chronic idiopathic neutropenia, chronic benign neutropenia of infancy, and

TABLE 41-20 -- Classification of Neutropenia

Acquired neutropenia
Postinfectious
Drug induced
Benign familial neutropenia
Chronic benign neutropenia of childhood
Chronic idiopathic neutropenia
Autoimmune neutropenia
Isoimmune neutropenia
Neutropenia associated with immunologic abnormalities
Neutropenia associated with metabolic diseases
Neutropenia due to increased margination
Nutritional deficiency
Intrinsic defects
Kostmann syndrome (severe infantile agranulocytosis)
Myelokathexis/neutropenia with tetraploid nuclei
Cyclic neutropenia
Shwachman-Diamond-Oski syndrome
Chediak-Higashi syndrome
Reticular dysgenesis
Dyskeratosis congenita

autoimmune neutropenia are all very similar, differing only in age of onset or association with recognizable immune disease. Differentiating these syndromes is probably not important. It may be better to lump them into a category of neutropenia with adequate marrow neutrophil reserve because this better predicts the clinical behavior.

Acquired Neutropenia

Many acquired neutropenias have decreased survival of neutrophils in the peripheral circulation. Bone marrow examination reveals normal or increased cellularity with a late maturation arrest, usually at the metamyelocyte or band stage. Elevation of serum lysozyme or lactoferrin reflects destruction of neutrophils. These measurements help demonstrate destruction of neutrophils but do not help with clinical management. [481] [571]

Postinfectious Neutropenia

Neutropenia can be commonly seen after viral infections, particularly in children. [572] The neutropenia can start within a few days of the onset of infection and last several weeks. Granulocyte counts usually return to normal as the viremia resolves. Viral diseases that have been implicated include varicella, measles, rubella, hepatitis A and B, infectious mononucleosis, influenza, cytomegalovirus, [573] [574] [575] and Kawasaki disease. The mechanism of the neutropenia can involve decreased production, redistribution, and destruction of neutrophils. [570] [576] [577] [578] In addition, virus-induced antibody can result in protracted immune neutropenia. Parvovirus B19 is commonly associated with transient neutropenia and can cause protracted leukopenia in immunosuppressed patients. [579] Leukopenia is seen in >70% of patients with acquired immunodeficiency syndrome (AIDS) and can be associated with hypersplenism and with the presence of antineutrophil antibodies. [580] [581] [582] [583]

Infection with *S. aureus*, brucellosis, tularemia, rickettsia, and *Mycobacterium tuberculosis*[570] can cause moderate neutropenia. Marked neutropenia can occur in any patient with overwhelming sepsis. This is more likely in debilitated adults or in newborns. The mechanism is probably due to exhaustion of the marrow reserve pool and is particularly well documented in sepsis in newborns. [484] [485] [584] Furthermore, systemic activation of complement during sepsis with production of C5a, a neutrophil activator, can result in increased adherence of neutrophils to the vascular endothelium and neutropenia due to margination of activated cells. [585]

Drug-Induced Neutropenia

Many therapeutic agents cause neutropenia. [586] The mechanism can involve direct bone marrow suppression as seen with many antineoplastic agents, antibody and complement-mediated damage to precursor cells,[587] [588] or peripheral destruction and clearance of neutrophils. [508] [564] [589] [590] The most common presenting symptoms are fever, sore throat, pharyngitis, sepsis, stomatitis, and pneumonia. [591] Mortality rates as high as 1225% have been reported, although full recovery can be expected in >80% of patients.[591] An overall annual risk of agranulocytosis is 3.4 per million population in an ambulatory population from Israel, Europe, and northeast United States. Approximately 72% of all cases of agranulocytosis in the United States are attributed to medications with procainamide, antithyroid drugs, and sulphasalazine being the most commonly implicated.[592] Most drug-related neutropenias are due to dose-dependent marrow suppression. Phenothiazines, semisynthetic penicillins, nonsteroidal anti-inflammatory agents, aminopyrine derivatives, benzodiazepines, barbiturates, gold compounds, sulfonamides, and antithyroid medications are the most common causes.[592] [593] The cardiac drugs propranolol (relative risk 2.5), dipyridamole (3.8), digoxin (2.5), and acetyldigoxin (9.9) are significantly associated with agranulocytosis. The excess risk from these drugs ranges from 1 to 3 cases/10 million persons exposed for up to 1 week. [594] A more extensive list can be seen in Table 4121 . [591] Usually the neutropenia becomes evident within 12 weeks of exposure to drug. [593] Recovery usually starts within a few days of stopping the drug and is preceded by the appearance of monocytes and immature neutrophils in the peripheral blood. [589] Early recovery occurs more frequently in patients with normal or increased marrow cellularity. In patients with pancytopenia, the median leukocyte recovery time was 14 days for patients with marrow hypoplasia and 10 days for those without marrow hypoplasia.[596] Rebound leukocytosis with marrow and peripheral blasts have been reported, simulating a leukemic state. [546] [547]

Although it is always best to stop the drug if neutropenia occurs, often clinical circumstances make it difficult to do this. With certain medications, such as sulfamethoxizole, the neutropenia depends on the dose and duration of therapy. It is often possible to continue the medication with careful observation. Neutropenia secondary to anticonvulsants often places the clinician in the difficult situation of having to balance the risk of neutropenia with stopping the medication and losing control of the seizures. As long as the ANC remains >500700 cells/mm^3 and there are no infections, it may be safe to continue the medication. A bone marrow aspiration can be helpful under these circumstances. A cellular, late-arrested marrow indicates the neutropenia is immune mediated. In this instance, the neutropenia is less likely to be clinically significant.

Chronic Benign Neutropenia

Chronic benign neutropenia is a syndrome occurring in older children and adults. Onset can occur from childhood to late adulthood. The clinical findings and presentation are quite variable. [565] [566] Neutrophil counts are commonly between 200 and 500/mm^3 , and bone marrow examination usually reveals normal to increased numbers of myeloid precursors with an arrest at a late stage of maturation. Often peripheral monocytosis is present. Some of these patients may have moderately hypocellular marrows.[566] Hepatosplenomegaly is not seen, and there is no other infectious, inflammatory, or malignant disease to which the neutropenia can be attributed. Frequently these patients have a benign course despite the degree of neutropenia. This may be due to the fact that they have some marrow reserve, as demonstrated by the response of their neutrophil count to a hydrocortisone stimulation test. [566] They are also able to mobilize more neutrophils to the tissue than patients with acute

TABLE 41-21 -- Causes of Agranulocytosis/Neutropenia

Drug	Agranulocytosis/ Neutropenia Probable[a]		Agranulocytosis/ Neutropenia Possible[b]		Total	Total Agranulocytosis
	A	B	A	B		
Dipyrone	7	5	4	5	21	17
Mianserin	8	2	2	3	15	13
Salazosulfapyridine (sulfasalazine)	6	1	4	2	13	13
Co-trimoxazole	5	1	1	4	11	10
Antiarrhythmic agents[c]	4	1	4	1	10	10
Penicillins[d]	4	1	3	1	9	8
Thiouracil derivatives[e]	4		3	1	8	8
Phenylbutazone	2	2	2	2	8	8
Cimetidine	1	3	4		8	7
Penicillamine	1	2	1	4	8	7
Diclofenac		3	3	1	7	5
Carbamazepine	2	1	4		7	5
ACE-inhibitors[f]	2	1	3		6	6
Hydrochlorothiazide with potassium sparing diuretic		3		3	6	6
Indomethacin	1	1		4	6	3
Cephalosporins[g]	1	1	1	2	5	5
Oxyphenbutazone	1		3	1	5	5
Nitrofurantoin	2	1	1	1	5	4
Salicylic acid derivatives		1		4	5	4
Clozapine	2	1	2		5	4
Carbimazole	1		3	1	5	2
Sulphonylurea derivatives[h]		2		2	4	4

Methyldopa	1	1		2	4	4
Thiamazole	2		2		4	4
Nucleosides				4	4	4
Aminoglutethimide	2	1		1	4	4
Ibuprofen		2	1	1	4	4
Pentazocine		1		3	4	3
Levamisole	2		2		4	3
Promethazine	2	2			4	3
Choramphenicol		2		1	3	3
Paracetamol and combination preparations		3			3	3
Perazine		1	1	1	3	3
Mebhydrolin	2	1			3	3
Ranitidine	1			2	3	2
Imipramine	1		2		3	2
Other drugs (all mentioned twice or less)[i]	14	13	10	12	49	42
Total	81	60	66	69	276	241

206 patients (more than one cause per patient possible). [591]

Agranulocytosis: nadir of neutrophil count 0.5 × 10^9 /L. Neutropenia: nadir of neutrophil count >0.5 × 10^9 /L but 1.5 × 10^9 /L.

A, causal relation certain or probable: drug taken within 10 days of onset of neutropenia, recovery when drug stopped, no other likely cause of the agranulocytosis or neutropenia; B, causal relationship possible: same as A, but more than one possible cause of neutropenia present.

[a]Isolated agranulocytosis or neutropenia: marrow results and complete blood count information available.
[b]Agranulocytosis or neutropenia present but results of hemoglobin, platelet count, or bone marrow aspiration not available.
[c]Procainamide (2), ajmaline (1), tocainide (1), aprindine (5), and amiodarone (1).
[d]Amoxycillin (1), azlocillin (1), benzylpenicillin (3), phenethicillin (1), cloxacillin and penicillin (2).
[e]Methylthiouracil (1), and propylthiouracil (7).
[f]Captopril (5) and enalapril (1).
[g]Cephalexin (1), cephazolin (1), cefuroxime (1), cefitaxime (1), and cephradine (1).
[h]Glibenclamide (1) and tolbutamide (3).
[i]Phenytoin (2), chlorthalidone (2), sulphamethizole (2), norfloxacin (2), naproxen (2), clomipramine (2), trazodone (2), omeprazole (2), alimemazine (2), pirenzepine, ticlopidine, ibopamine, hydralazine, nifedipine, spironocatone, nalidixic acid, doxycycline, clindamycin, gentamicin, fusidic acid, dapsone, azapropazone, combination preparations with aminophenazone, respectively, propyphenazone, sulindac, piroxicam, pirprofen, niflumic acid, allopurinol, glafenine, valproate, levodopa with carbidopa, chlorpromazine, haloperidol, zuclopenthixol, zopiclone, cinnarizine, metronidazole, combination preparations with pyrimethamine, and theophylline.

drug-induced suppression of equal degree. [567] Antineutrophil antibodies, as well as other immunologic abnormalities, have been seen in some patients though these studies are usually normal. [566] [596] [597] [598] The lack of strong evidence for antineutrophil antibodies in these patients does not preclude the possibility that antibodies against myeloid precursors are present. Antibodies against the promyelocytic leukemic line, HL-60, were detected in sera from three patients with idiopathic neutropenia with no detectable antibody to mature neutrophils. [598] Bone marrow cytogenetic studies are normal as is G-CSF concentration. [566] [599]

Corticosteroids, splenectomy, and cytotoxic agents have been successful in increasing neutrophil counts. [566] Recently, human G-CSF has been used successfully to treat a patient with idiopathic neutropenia. [600] This patients course was benign for several years until his marrow became hypocellular. G-CSF has been shown to decrease the rate of infection in patients with chronic idiopathic neutropenia. [601] Because the clinical course of

this disease may be benign, treatment intended to increase the neutrophil count should be reserved for patients with significant recurrent infectious complications.

Chronic Benign Neutropenia of Infancy and Childhood

This interesting childhood syndrome highlights the relationship between marrow reserve pool and propensity to infection. It probably should be considered as a subset of chronic benign neutropenia. Even though these patients have severe neutropenia, they have no significant propensity to infection. Chronic benign neutropenia of infancy and childhood [602] [603] is a chronic state of mature neutrophil depletion with a compensatory increase in immature granulocytes in the bone marrow. [603] The median age of detection is 8 months. Although it can present any time in the first 3 years of life, 90% of patients are detected before 14 months of age There is a slight female predominance (3:2) and no correlation with birth order. Neutrophil counts are usually very low at presentation, although these counts are normal at birth. There is no family history of neutropenia. [563] [602] [603] [604] Antineutrophil antibodies have been detected in 98% of patients when both immunofluorescent and agglutination assays are used. [563] [605] Although the exact mechanism of the neutropenia is unclear, the facts that antineutrophil antibodies are frequently no longer detectable late in the course of the disease [563] and that antiimmune therapy is effective [563] [606] [607] suggest an immune mechanism. [563]

Many children with benign neutropenia have purulent otitis despite a neutrophil count <200/mm^3, emphasizing the fact that neutrophils can go to sites of infection even when they are not seen in the peripheral blood. It is common for these patients to go several months with neutrophil counts <200/mm^3 and have no febrile episodes. Some of these patients have measurable defects in neutrophil movement and have been described as having the so-called lazy leukocyte syndrome. [608]

Autoimmune Neutropenia

Autoimmune neutropenia has been seen as an isolated phenomenon, [564] [609] secondary to other known autoimmune diseases, [564] [609] [610] related to infections, [576] and related to administration of drugs. [564] Furthermore, with increasing sophistication of immunologic evaluations, immunologic mechanisms are being seen in instances of neutropenia that have classified as idiopathic. [563] [564] [598] [601] [610] Immune neutropenia can be seen in association with idiopathic thrombocytopenic purpura and immune hemolytic anemia as well. [564] In the neutropenia associated with Rh hemolytic disease, however, there is evidence of down-regulation of neutrophil production associated with an increase in erythropoiesis. [611]

Patients with autoimmune neutropenia have moderate to severe neutropenia, usually accompanied by monocytosis. Marrow cellularity is increased with a late maturation arrest. The propensity to infection is related to the degree of neutropenia although the correlation is not good. [564] [610] [612] Hepatosplenomegaly has been seen in about half the patients. The age of presentation is wide, ranging from early childhood to old age.

Neutrophil-specific antibodies to the neutrophil antigens NA1, NA2, ND1, ND2, NB1, as well as to antigens shared by erythrocytes and to HLA antigens have been detected. [605] They are detected by a variety of assays including leukoagglutination, [605] [613] opsonization, [520] [610] immunochemical assays, [564] direct antibody binding, [609] complement activation, [564] [614] and various modifications of these techniques. [615] The antibodies are usually of IgG or IgM type. Due to a lack of a good, readily available panel of known neutrophil antigens, most assays detect only the presence of neutrophil-associated antibody. Antibodies to CD13 have been detected in some neutropenic patients with cytomegalovirus. [616] Detection of antineutrophil antibodies is helpful in establishing the diagnosis of immune neutropenia, although a negative assay does not exclude the diagnosis. [564] [605] This may be due to antibody specificity for neutrophil progenitors rather than mature neutrophils. [587] [588] [598] The degree of neutropenia is related to the specificity of the antibody as well as the titer. This probably accounts for the variability of success in correlating the amount of antibody with severity of disease. [564] [605] [610] In addition to neutrophil-associated antibodies, circulating immune complexes have been detected in about one-third of

patients with immune neutropenia[610] and in patients with chronic idiopathic neutropenia. [617] Although immune complexes can cause a positive indirect immunofluorescent test, patients with immune complexes and negative immunofluorescence antineutrophil antibody tests have been reported. [610]

Antineutrophil antibodies of the IgG type have been reported in systemic lupus erythematosus. These antibodies have been seen both before and after correction of neutropenia by therapy. [564] [618] Approximately 50% of patients with lupus are neutropenic, although few have severe enough neutropenia to result in increased in susceptibility to infection. [617] Neutropenia in lupus may also be related to decreased myelopoiesis. [618] Immune neutropenia can also occur in conjunction with autoimmune hemolytic anemia and thrombocytopenia.[601]

The neutropenia of Felty syndrome (rheumatoid arthritis, splenomegaly, and neutropenia) has also been related to the presence of antineutrophil antibodies. In this complex autoimmune disorder, decreased granulocyte survival as well as decreased production have been reported. [619] [620] [621] Infection is the major cause of death in Felty syndrome. Although neutropenia contributes to the predisposition to infection, neutrophil functional defects have also been found. [622] Immune complexes have also been associated with this disorder. [620] [621] More recently, suppressor T cells have been implicated in mediation of the neutropenia. [601] [623] [624] Improvement in neutropenia and decrease in antineutrophil antibody levels with methotrexate treatment provides further evidence that an immune mechanism may play a role in the etiology of this neutropenia. [625] Splenectomy produces resolution of the neutropenia and improvement in symptoms in 80% of cases. Treatment with G-CSF (510 g/kg/day) has been reported to be successful in management of complications of neutropenia due to Felty syndrome. [626] Despite the possible serious nature of this syndrome, about half the patients do not experience any infections. [626]

Treatment of autoimmune neutropenia depends on the severity of the neutropenia-related symptoms and the nature of the underlying disease. Because many of these patients have a benign course, therapy solely to increase the neutrophil count is not indicated. If the patient has significant neutropenia (ANC <500/mm ^{3}) and recurrent or severe infections, high-dose intravenous IgG [606] [607] [627] or steroids may be used. Splenectomy provides only transient correction of the neutropenia and results in a subsequent propensity to infection. Other cytotoxic therapy may be considered, particularly in lupus or rheumatoid arthritis. [618] [625]

Use of G-CSF has been successful in patients with immune neutropenia and should be considered if the patient is having problems with infection. [601] A low-dose regimen has been used in such cases at reduced cost. [628]

Isoimmune Neonatal Neutropenia

Moderate to severe neutropenia can occur in newborn infants secondary to IgG antibodies transferred from mother to infant. The pathogenesis of this disorder is identical to that of Rh hemolytic disease, with prenatal sensitization to neutrophil antigens resulting in production of antibodies that cross the placenta. [590] [605] [629] The incidence has been estimated at 2/1,000 live births. [629] If the infant is septic, or if subsequent episodes of bacterial infection occur, then marrow aspiration should be

performed. If there is early arrest or storage pool depletion, treatment with granulocyte transfusions or G-CSF should be considered.

Neutropenia in Infants of Hypertensive Mothers

Granulocyte counts as low as 500/mm^3 have been reported in infants of hypertensive mothers. [630] Forty-nine percent of infants of hypertensive mothers had neutropenia that lasted from 1 hour to 30 days. [631] Neutropenia was more common in infants who had growth retardation or whose mothers had severe hypertension. Thrombocytopenia to 90,000/mm^3 was also seen. [631] The neutropenia seems to be due to marrow suppression and may be associated with a slightly increased incidence of infection. [630] [631] [632]

Pure White Cell Aplasia

Pure white cell aplasia (PWCA) is a rare syndrome characterized by severe pyogenic infections and neutropenia. Many (70%) of these patients have an associated thymoma. In some patients, PWCA occurred years after thymoma removal. Bone marrow examination shows almost complete absence of myeloid precursors, with normal erythroid precursors and megakaryocytes. This is in contrast to T- neutropenia or Kostmann syndrome where only early myeloid precursors are seen. Marrow inhibitory activity is seen in both IgG and IgM fractions of patients serum and disappears as the marrow recovers. [587] [588] [633] [634] [635] [636] In some instances, the inhibitory activity is in lymphocyte fractions and not in the plasma. PWCA has been seen with ibuprofen therapy. In vitro serum inhibitory activity required the presence of the drug and of complement. The clinical syndrome resolved when ibuprofen was discontinued. [588] It has also been associated with chlorpropamide [637] and certain natural remedies. [638] In PWCA associated with thymomas, removal of the mass is indicated but may not be sufficient. Cytoxan, steroids, as well as cyclosporin A have been effective in treatment of PWCA, as has intravenous IgG. [639]

Neutropenia Associated with Immunologic Abnormalities

Neutropenia has been found in association with a number of immunologic abnormalities. The patients usually present in childhood with frequent bacterial infections, hepatosplenomegaly, and failure to thrive. Some of these children die in the first few years of life. Hypergammaglobulinemia or hypogammaglobulinemia, [640] [641] [642] [643] T-cell defects, [644] [645] natural killer cell abnormalities, [596] and autoimmune phenomena [646] [647] have been reported. Many of the patients had a positive family history of neutropenia. [640] [641] [642] Neutropenia is found in 26% of patients with X-linked agammaglobulinemia and 4050% of patients with X-linked hyper-IgM syndrome and has been associated with decreased marrow production of neutrophils. [643] [647] Chronic diarrhea, skin rashes, and recurrent viral infections may also be seen in these children. The treatment of these disorders depends on the constellation of immunologic abnormalities present. Some of these patients have been treated with bone marrow transplantation. [644]

It is important to distinguish these patients from the much more common syndrome of idiopathic benign neutropenia. In contrast to benign neutropenia, children with global immune defects associated with neutropenia have manifestations of recurrent or unusual infections. If the child has failure to thrive, frequent hospitalizations for pneumonia or sepsis, a full immunologic evaluation is indicated. Furthermore, live-virus vaccines must be withheld, and any blood products irradiated, until a T-cell defect has been carefully ruled out.

The majority of patients with AIDS have neutropenia as well as anemia. About 30% have thrombocytopenia. [648] Neutropenia has been seen in up to 8% of asymptomatic carriers of human immunodeficiency virus (HIV). The bone marrow is usually hypercellular and has lymphoid aggregates and plasmacytosis. There is commonly dysplastic change in the granulocytes. The neutropenia is felt to be due to ineffective hematopoiesis. The immunoglobulin fraction of HIV-positive serum inhibits CFU-GM and burst-forming unit erythroid in vitro and thus may contain antibodies to progenitors.

T- Lymphocytosis and Neutropenia

Approximately 80% of patients with T- lymphocytosis present with neutropenia during the course of evaluation for recurrent infections. [649] The median age of onset is 5565 years, although it has been seen in children. There may also be a history of rheumatoid arthritis. Splenomegaly occurs in many of the patients, although enlargement of liver or lymphandenopathy is not common. The peripheral blood shows lymphocytosis, although rarely >20,000. Most of the lymphocytes are large granular lymphocytes. The bone marrow in these disorders is normocellular, with an arrest at the myelocyte stage and an increase in lymphocytes. The lymphocytes may contain CD2, CD3, Fc, HNK-1 but lack CD5. [650] They may also show the myeloid marker M1. Natural killer cells are a subpopulation of these cells, making this syndrome of particular interest. Most patients follow a benign course for many years. The longest survival is 20 years. Patients die of progressive lymphoproliferation or of sepsis related to neutropenia. [645] [646] [650] Although the course may be relatively protracted and benign, the lymphocytosis does represent a clonal proliferation and is felt by some to be malignant. [646] [651] [652] A decrease in suppressor cell and natural killer cell activity concomitant with improvement in neutropenia after treatment with intravenous IgG [607] [627] provides support to laboratory data [479] [624] suggesting that these cells play a role in the regulation of myelopoiesis and the pathophysiology of neutropenia. Cyclosporin A has also been effective in some patients. [653]

Neutropenia Associated with Metabolic Diseases

Neutropenia can be associated with ketoacidosis in patients with hyperglycemia, hyperglycinuria, orotic aciduria, and methylmalonic aciduria. [654] [655] [656] [657]

Neutropenia is also seen commonly in association with glycogen storage disease type Ib but not with glycogen storage disease type Ia. Recurrent infections are a major source of morbidity in these patients. The degree of neutropenia is variable but commonly counts may be <500 /mm ^{3} . The bone marrow is normocellular or hypercellular. Variable functional defects have been seen in the neutrophils from these patients. [658] [659] [660] [661] Patients with glycogen storage disease Ib have been treated with G-CSF and GM-CSF.[662] Two patients with severe Crohns disease-like symptoms were greatly helped by treatment with GM-CSF.[662] [663] Interestingly, both of these patients had hypercellular marrows with no evidence of arrest. GM-CSF was required twice daily to maintain granulocyte counts, suggesting its role in promoting release of granulocytes from the marrow storage pool.

Neutropenia Due to Increased Margination

Both acute and chronic neutropenia can occur because of complement activation.[571] [572] [585] [664] [665] [666] The mechanism was first recognized in patients undergoing hemodialysis and is associated with pulmonary dysfunction.[665] [666] Generation of C5a activates neutrophils, resulting in increased adherence and aggregation, and subsequent entrapment in the pulmonary vasculature.[572] [585] Similar activation of complement and neutropenia has been found with the use of membrane oxygenators.[667] Complement-mediated neutropenia due to shifts in the neutrophil pools has also been seen with burns [481] and transfusion reactions.[571] Because

lung dysfunction and pulmonary infiltrates have been seen in some of these processes, the neutrophil has been implicated in the pathophysiology of adult respiratory distress syndrome.[481] [571] [572] However, the occurrence of this syndrome and severe neutropenia, with no neutrophilic infiltration of the lungs, suggests that mechanisms other than direct damage by neutrophils are possible.[668] Neutropenia may be related to complement-mediated destruction of neutrophils as seen in paroxysmal nocturnal hemoglobinuria[669] [670] or due to destruction of granulocyte precursors. [587] [588] [614]

Neutropenia Associated with Hypersplenism

Neutropenia can be seen in association with hypersplenism. Usually the neutropenia is not severe enough to cause symptoms, unless there is antibody present. Anemia and thrombocytopenia are usually also present. Improvement in the hypersplenism induced leukopenia in a patient with AIDS was obtained by splenic embolization.[671]

Neutropenia Associated with Nutritional Deficiency

Neutropenia has been seen in association with anemia in nutritional deficiency of vitamin B_{12} , folate, and copper.[672] [673] [674] All of these neutropenias are characterized by ineffective myelopoiesis and megaloblastic changes in the bone marrow. Similar findings have been seen in the inherited deficiency of transcobalamin II.[675] Neutropenia and megaloblastosis has also been seen in association with sideroblastic anemia in the DIDMOAD syndrome (diabetes insipidus, diabetes mellitus, optic atrophy, deafness). The hematologic abnormalities were responsive to thiamine. [675]

Primary Causes of Neutropenia

Ethnic or Benign Familial Neutropenia

Neutropenia has been seen in several ethnic groups and has been referred to as ethnic, pseudo, or benign familial neutropenia. The level of neutropenia is usually mild and is not associated with any increased propensity to serious infection. Benign familial leukopenia is characterized by neutrophil counts in the 2,1002,600/mm ^{3} range and no propensity to infection. The disorder has dominant inheritance and is not associated with any propensity to infection. The bone marrow is normocellular.[676] [677] Ethnic neutropenia has been seen in American blacks,[462] South African blacks,[462] [678] Yemenite Jews,[463] [678] black Bedouin, and Falasah Jews.[678] While this entity is generally benign, [679] [680] there have been reports of periodontal problems in some of these patients. [681] [682] [683]

Kostmann Syndrome (Infantile Agranulocytosis)

Severe infantile agranulocytosis was described by Kostmann in 1956. [684] This is one of several similar disorders that present in early infancy with severe, recurrent infections and neutropenia. This entity has been reported as demonstrating autosomal dominant or autosomal recessive [622] [668] types of inheritance. Bone marrow examination usually reveals myeloid hypoplasia with an arrest at the promyelocyte stage. Ultrastructural examination shows abnormalities in granule production in some patients.[668] Bone marrow culture studies generally show colony growth[599] [685] that is dependent on exogenous colony-stimulating factors. More than 95% of patients with this previously fatal disorder respond well to G-CSF. [15] [686] [687] Patients may need doses of G-CSF of 1020 g/kg/day with some requiring up to 100 g/kg/day.[15] Bone marrow transplantation should be considered in these patients, particularly if response to cytokine therapy is poor.

Myelokathexis/Neutropenia with Tetraploid Nuclei

Several patients have been described with severe recurrent infection, neutropenia, and dysmyelopoieic features. Many of these patients display neutrophil mobility abnormalities as well. [668] [688] [689] [690] Although they have been reported as separate diseases, there is considerable overlap in many of the infantile neutropenia syndromes.[668] [684] [688] [689] Although these syndromes usually present in childhood, a patient with myelokathexis presented at 34 years of age. [691]

One patient had neutrophil counts in the 100450/mm ^{3} range with biploid- and tetraploid-nucleated neutrophils, bands, and metamyelocytes in the bone marrow. All mature neutrophils, 64% of the bands, and 42% of the metamyelocytes were binucleate. In contrast, early precursors were normal, and only rare abnormal neutrophils were found in the peripheral blood. The cellularity and structure of the marrow was normal, except for the binucleate cells. This child had a history of severe recurrent infections. Chemotaxis of neutrophils obtained from the marrow was markedly decreased compared to adult control. [689]

Similar abnormalities have been found in patients with myelokathexis. One of the first reported patients had abnormal chemotaxis as assessed by the Rebuck skin window.[688] Some of these patients have nuclear abnormalities in peripheral blood neutrophils as well. Abnormalities in phagocytosis, chemotaxis, and the respiratory burst were found in another patient with multiple congenital anomalies, neutropenia, and abnormally hypersegmented neutrophil nuclei. [690] These patients have been helped by treatment with G-CSF.[15]

Cyclic Neutropenia

Cyclic neutropenia is a rare dominantly inherited disorder with variable expression characterized by neutropenia that recurs every 1535 days. The patients have associated recurrent fever, pharyngitis, stomatitis, and other bacterial infections. The severity of the infections parallels the severity of the neutropenia and can be variable from patient to patient. Although the disease tends to be benign, several patients have died of infection. [692] Most of the patients present in childhood, but there is an adult-onset form. The disorder can persist for many years but tends to decrease in severity with time. The bone marrow during times of neutropenia is usually hypoplastic with a myelocyte arrest. [692] [693] [694]

Cyclic neutropenia is felt to represent a stem cell regulatory defect. It can be cured by bone marrow transplantation in an animal model [692] and has been transferred from affected human donor to recipient during bone marrow transplantation. [695]

The disorder must be differentiated from cyclic fevers without neutropenia [696] and from other causes of neutropenia. The diagnosis is established by documentation of periodic neutropenia. This requires monitoring of the neutrophil count at least twice and preferably three times a week for 8 weeks. The nadir of the neutropenia usually lasts 35 days and can be missed if the patient is monitored less frequently than two to three times per week. To establish the diagnosis, there must be regular cyclic fluctuations in neutrophil counts with a nadir of <200/mm ^{3} with a periodicity of 1521 days. Interestingly, patients may report an increased sense of well-being as their counts recover to >500/mm^3 . [593] It is not uncommon to see patients whose neutrophil counts cycle with nadirs in the 5001,000/mm ^{3} range. These patients

usually do not have symptoms nor do they have the signs of chronic gingival infection characteristic of patients with chronic severe neutropenia. It is best to reserve the diagnosis of cyclic neutropenia for those patients with granulocyte counts that periodically fall below 200 to 300/mm ^{3} .

Patients with cyclic neutropenia as defined above almost always have, or will soon develop, severe dental, gingival, and

mucosal infection and therefore should be treated with G-CSF. Treatment should include regular and aggressive dental care. The antibacterial mouthwash, Peridex, is useful in decreasing gingivitis. [15] [593] [697] Shwachman-Diamond-Oski syndrome and T- lymphocytosis can also present with cyclic neutropenia.

Shwachman-Diamond-Oski Syndrome

The combination of neutropenia, metaphyseal dysplasia, and pancreatic insufficiency is known as Shwachman-Diamond-Oski syndrome. [698] [699] These patients present in the first 10 years of life with steatorrhea and infections. Severe or fatal infections occur in over half the patients, although many have relatively few problems with infection despite the neutropenia. Although pancreatic insufficiency is detectable by fecal fat determination or trypsin or lipase measurement, some patients have minimal problems with steatorrhea. Half the patients show moderate improvement in fat malabsorption by 58 years of age. [699] Thus, absence of a clear history of bowel problems does not rule out this diagnosis. Physical examination reveals short stature and a variety of other physical anomalies including strabismus, cleft palate, syndactyly, and microcephaly. Twenty-five percent of patients have metaphyseal dysplasia. Magnetic resonance imaging of the pancreas shows it to be normal or enlarged and has signal characteristics of fat. [700]

The neutrophil counts are usually <500/mm ^{3} . Thrombocytopenia may be present in 70% of the patients, and a mildly macrocytic, prednisone-responsive anemia is seen in 10% of patients. [698] In addition to neutropenia, a neutrophil capping and chemotactic defect is present. A partial chemotactic defect may be seen in the parents, [479] consistent with the presumed recessive inheritance. The reported functional defects have been corrected by injection with thiamine in some patients. [701] [702] The mechanism for this response is unknown. The bone marrow is usually hypoplastic, although it can be normal. Sweat chloride determination is normal.

The steatorrhea responds to pancreatic enzyme replacement and resolves by 5 or 10 years of age, although the pancreatic insufficiency remains. Treatment consists of management of infections and bleeding episodes and supplementation with pancreatic enzymes. The anemia may respond to treatment with prednisone. The neutropenia has responded to G-CSF. Because all patients do not have problems with infection, it is not necessary to treat all patients with G-CSF. However, if the patient develops any serious infection related to the neutropenia, G-CSF should be started. The overall expected survival is 50%. As with other constitutional marrow failure syndromes, there is an increased incidence of malignancy. Both lymphocytic and nonlymphocytic leukemias have been reported, [703] [704] accounting for some of the mortality seen with this syndrome.

CHS

As discussed previously, CHS is a rare inherited disorder characterized by oculocutaneous albinism, progressive neurologic impairment, and giant granules in many cells including neutrophils. The patients also develop severe neutropenia, felt to be due to ineffective granulopoiesis. [705]

Reticular Dysgenesis

A syndrome of agranulocytosis, lymphoid hypoplasia, and thymic dysplasia with normal megakaryocyte and erythroid precursors has been called reticular dysgenesis. [706] [707] These patients have low IgM and IgA, and abnormal T cells. [707] The marrow is hypoplastic with very few neutrophilic or lymphocytic precursors. The few early myeloid cells present have decreased granule formation. [707] These patients die in infancy. Early bone marrow transplantation should be considered.

Dyskeratosis Congenita

Dyskeratosis congenita is an X-linked disorder involving abnormalities of integument and usually mild neutropenia, [708] although some patients have pancytopenia. [709] [710] The marrow can be hypocellular. Some studies suggest an immunologic mechanism for the neutropenia. [709]

Other Causes of Neutropenia

Isolated neutropenia can be part of the presentation of other marrow failure syndromes such as myelodysplasia, preleukemia, Fanconi anemia, and aplastic anemia. Usually there are other characteristic features that are associated with the neutropenia.

DIAGNOSTIC APPROACH TO NEUTROPENIA

The approach to the diagnostic evaluation of a patient with neutropenia can be largely guided by the clinical history and physical examination and does not always require an extensive laboratory evaluation from the onset. If the patient is asymptomatic and has no significant history or physical findings to warrant immediate further evaluation, clinical observation is the best approach. The presence of recurrent oropharangeal infection or significant periodontal infection in the face of an ANC <500/mm^3 suggests that the neutropenia is more significant and that marrow examination should be performed. To the contrary, a patient with normal gingiva and no history of recurrent mucosal infection is less likely to have serious long-standing chronic neutropenia, regardless of the ANC. Isolated neutropenia is an unusual presentation of malignancy, so there is usually time to follow the course of the neutropenia before proceeding with an extensive evaluation. This is particularly true in young children where the most common neutropenias are benign. If the ANC is >7501,000/mm ^{3} , it is unlikely that anything will be gained by bone marrow aspiration for isolated neutropenia. If anemia, particularly normocytic or macrocytic anemia, or thrombocytopenia is present, bone marrow aspiration should be performed. If there is a recent history of a viral infection, or if the patient is on a medication known to be associated with neutropenia, several weeks of observation and discontinuation of the medication may reveal resolution of the neutropenia and avoid a bone marrow aspiration. If the neutropenia is persistent, bone marrow aspiration with biopsy, immunologic evaluation, marrow chromosomes, collagen vascular work-up and other tests may be useful in determining the cause of the neutropenia. Bone marrow culture, epinephrine stimulation test, and hydrocortisone stimulation tests are often discussed on rounds, but are of little discriminatory value, and do not alter the approach to treatment.

Based on the history plus marrow cellularity and morphology, the patient can usually be classified as having a secondary neutropenia or a primary marrow defect, and management can be determined.

In summary, asymptomatic patients with isolated neutropenia can be observed clinically for several weeks. Patients with infections attributable to their neutropenia or other clinical manifestations of serious diseases associated with neutropenia should be evaluated completely.

MANAGEMENT OF NEUTROPENIA

The clinical management of neutropenic states depends on the cause and degree of the neutropenia and on associated disease states. The major problem is management of infectious complications.

Patients with severe neutropenia and little marrow reserve tend to have acute severe infection, whereas patients with hypercellular marrows tend to have chronic infection or no infection problems at all.

Table 41-19 gives the relationship of the ANC to propensity to infection. These guidelines should be used in patients with chemotherapy-induced neutropenia and in neutropenia associated with granulocyte hypoplasia such as aplastic anemia, Kostmann syndrome, or familial severe neutropenia. These patients have no marrow

reserve and tend to have decreased marrow cellularity with an early myeloid arrest. Because these patients cannot respond to infection, many of the inflammatory signs of infection may be absent. For example, radiographs may not reveal pneumonias or there may be no abdominal tenderness with an early ruptured viscus. The organisms that cause infection come from the gastrointestinal tract or skin and can result in overwhelming sepsis very quickly. Thus, febrile patients with neutropenia related to marrow suppression should be treated immediately with broad-spectrum parenteral antibiotics. Antibiotics should be continued for several days past the end of fever. If fever and neutropenia persist more than a week, consideration should be given to the empiric use of amphotericin B. Granulocyte transfusions are effective in certain instances of culture-proven gram-negative infections. They should be used in patients with severe neutropenia with culture-proven gram-negative sepsis who have not shown a clinical response to antibiotics within 2448 hours. Enthusiasm for the use of granulocyte transfusion has waned in recent years, in part due to difficulties in procurement and in part due to better antibiotics, although there is some recent increase in interest with the availability to increase yields with G-CSF. [711] [712] Routine reverse isolation procedures are of no benefit, [713] [714] and serve to decrease contact with medical personnel. Although empiric antibiotics are clearly indicated in patients with fever, prophylactic antibiotics should not be routinely used.

Patients with late marrow arrest and normocellular marrow may be able to handle infections reasonably well. Children in whom the diagnosis of chronic benign neutropenia of infancy has been confirmed can be treated like normal children. In older patients with evidence of marrow reserve and a several month history of severe neutropenia without serious infections, less aggressive therapy may be reasonable as well. When these patients are first encountered, however, they should be treated like other patients with more severe forms of neutropenia.

All patients with chronic neutropenia should receive regular dental care. Chronic gingivitis and recurrent stomatitis can be major sources of morbidity. Antibiotic mouthwash, such as Peridex, can be very helpful in preventing gingivitis.

Until recently, therapy to increase neutrophil counts has had only variable success. Corticosteroids have been effective in some instances of immune-mediated neutropenia[566] [571] [590] as has intravenous IgG. Recombinant G-CSF has been effective in correcting neutropenia in cyclic neutropenia and severe infantile agranulocytosis and should be considered in individuals with symptoms referable to their neutropenia. [15] [601] [686] [687] [697] Bone marrow transplantation has been used successfully in certain patients with severe neutropenia and should be considered if an appropriate donor is available.

MONOCYTOPENIA AND MONOCYTOSIS

Alterations in the number of circulating monocytes are seen in a number of clinical situations (Table 41-22), although generally the disorders are less well defined than those of granulocytes. Production of monocytes is regulated by IL-3 and GM-CSF produced by T lymphocytes and M-CSF produced by endothelial cells and by monocytes themselves. IL-3 and GM-CSF selectively induce M-CSF production by monocytes, suggesting one

TABLE 41-22 -- Disorders Associated with Monocytosis

Inflammatory diseases
Infectious diseases
Tuberculosis
Syphilis
Subacute bacterial endocarditis
Fever of unknown origin
Autoimmune/granulomatous
Systemic lupus erythematosus
Rheumatoid arthritis
Temporal arteritis
Myositis
Polyarteritis
Ulcerative colitis
Regional enteritis
Sarcoidosis
Malignant disorders
Preleukemia
Nonlymphocytic leukemia
Histiocytoses
Hodgkin disease
Non-Hodgkin lymphoma
Carcinomas
Miscellaneous
Chronic neutropenia
Postsplenectomy

mechanism for production of the monocytosis in patients with certain chronic infections associated with enhanced T-lymphocyte activity. [715] Monocytes comprise 19% of peripheral leukocytes though the total monocyte count is higher during the first two weeks of life. [716] [717]

The blood monocyte and tissue monocyte-macrophages undoubtedly play an important role in defense against bacteria and fungal invasion; however, there is no clear association between circulating numbers of monocytes and propensity to infection as there is with granulocytes. Decreases in the number of monocytes has been seen with endotoxemia and with glucocorticoid administration. [718] Noncirculating monocytes seem to be relatively resistant to radiation and cytotoxic chemotherapy.[719]

Monocytosis is generally seen in association with chronic inflammatory processes, whether infectious or immune in nature. Monocytosis has been seen with tuberculosis,[720] subacute bacterial endocarditis, syphilis, and fever of unknown origin. [720] [721] Collagen vascular diseases such as rheumatoid arthritis, systemic lupus erythematosus, myositis, periarteritis, and temporal arteritis have been associated with monocytosis, as well as granulomatous diseases such as sarcoid, regional enteritis, and ulcerative colitis. [720] [722]

Monocytosis has been seen with several primary neutropenic syndromes including cyclic neutropenia, chronic idiopathic neutropenia, and Kostmann syndrome. [603] [676] [684] [692] An increase in monocytes usually heralds recovery from agranulocytosis.

Monocytosis is commonly seen in a number of primary hematologic malignancies. Increased promonocytes and monocytes are seen in patients with chronic and acute nonlymphocytic leukemias as well as in preleukemic states. [723] [724] [725] In many of these patients, the monocytes may be malignant but are indistinguishable from normal by light microscopy.[725] In addition to leukemia, monocytosis has been seen in both Hodgkin and non-Hodgkin lymphomas, [9] histiocytoses, as well as nonhematologic malignancies.[726] [727] [728] Monocytic leukemoid reactions have been seen in association with corticosteroids in patients with myelodysplastic syndrome.[729]

ACKNOWLEDGMENT

JTC was supported in part by U.S. Public Health Service grants AI 24838 and RR 00833. TC was supported in part by U.S Public Health Service grants AI 23547 and HL 96002B. TC thanks Dr. Punam Malik and Mrs. Parvin Izadi for their helpful suggestions.

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Chapter 42 - Disorders of Lymphocyte Function

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INTRODUCTION

Disorders of lymphocyte function may be categorized as either primary defects or secondary to, or associated with, some underlying disorder. This chapter emphasizes primary lymphocyte defects[1] [2] [3] [4] [5] (Table 42-1) and only briefly summarizes secondary disorders,[6] many of which are detailed in other chapters of this text. The secondary immunodeficiency diseases, which include malnutrition and acquired immune deficiency syndrome (AIDS) (Chaps. 75 and 154), are far more numerous both in the United States and worldwide. Immunodeficiency secondary to Epstein-Barr virus (e.g., X-linked lymphoproliferative syndrome [XLP]) is discussed in Chapter 45 , malignancy in Part

TABLE 42-1 -- Primary Immunodeficiencies

Disorder	Inheritance	Locus	Gene Defect/Pathogenesis	Associated Features
Predominantly antibody deficiencies				
X-linked agammaglobulinemia	XL	Xq21.3-22	Defective pre-B-cell maturation 2° to abnormality of *Btk,* a B-cell-lineage cytoplasm tyrosine kinase	
Non-X-linked hyper-lgM syndrome	AR,?		Defective B-cell activation 2° to abnormal CD40 expression, function, or signaling	Autoimmune hematologic diseases (neutropenia, thrombocytopenia, hemolytic anemia)
Common variable immunodeficiency (predominant antibody deficiency, predominant cell-mediated immunity defect)	AR,AD,?	?6p21.3	Defect of B-cell maturation/differentiation with intrinsic B-cell defect, decreased B-cell numbers, defective T-helper cell function, antibodies to B cells, or augmented suppressor function	Autoimmunity, malignancy
Ig heavy chain deletion	AR	14q32.3	Chromosomal deletion at Ig heavy chain gene locus	
heavy chain deficiency (autosomal recessive agammaglobulinemia)	AR	14q32.3	Defective pre-B-cell maturation 2° to failure of heavy chain expression	
chain deficiency	AR	2p11	Point mutation at light chain gene locus	
Selective deficiency of IgG subclasses with or without IgA deficiency	?	?	Defects of isotype differentiation	
IgA deficiency	XL,AR,?	?6p21.3	Failure of deletional isotype switching to and terminal differentiaion of IgA-secreting B cells	Autoimmunity, allergy
Antibody deficiency with normal Ig	?	?	?	
Combined immunodeficiencies				
Severe combined immunodeficiency (SCID)				
T B + SCID				
X-linked ($_c$ deficiency)	XL	Xq13.1-13.3	Defect of T lymphocyte and NK cell development 2° to mutation of common chain ($_c$) of IL-2,4,7,9, and 15 receptors.	
JAK3 deficiency	AR	19p13.1	Mutations in Janus associated kinase 3 (JAK3), signaling molecule for $_c$, phenotype identical to X-SCID	
T B SCID				
RAG1/RAG2 deficiency	AR	11p12-13	Maturation defect of both T- and B-cell development 2° to absence of antigen receptor gene recombination from mutations of RAG1 and/or RAG2 recombinase proteins	
Adenosine deaminase (ADA) deficiency	AR	20q13-ter	Defect of T- and B-cell development 2° to toxic metabolites due to ADA deficiency	Cartilage abnormalities in some
Autosomal-SCID variants	AR	?	Maturation defect of both T- and B-cell development, etiology unknown	
Reticular dysgenesis	AR	?	Stem cell defect affecting maturation of lymphoid and myeloid cells	Pancytopenia
MHC class II deficiency	AR	16p13.1-13.2, 1q21,13q,?	Mutation of transcription factors [CIITA (group A), RFX5 (group C), RFXAP (group D), RFX? (group B)] for MHC class II molecules	Diarrhea, severe viral infections
Purine nucleoside phosphorylase (PNP) deficiency	AR	14q13.1	Defect of T-cell development and function 2° to toxic metabolites due to deficiency of PNP	Anemia, neurologic symptoms

X-linked hyper-IgM syndrome	XL	Xq26-27	Defective B-cell activation and T-cell function due to abnormal CD40 ligand expression on activated T cells	Autoimmune hematologic diseases (neutropenia, thrombocytopenia, hemolytic anemia)
T-cell activation defects				
CD3 or CD3epsilon deficiency	AR		Defective CD3 or CD3epsilon transcription	
Defective response to cytokines or expression of cytokine receptors	?		Defect of IL-2, IL-2 receptor, IL-2 signaling	
ZAP-70 deficiency (CD8 T-cell deficiency)	AR	2q12	Mutations in ZAP-70 kinase gene required for TCR signaling	
TAP2 deficiency	AR	6p21.3	Mutation in TAP2 translocator gene affects MHC class I expression	
Other immunodeficiencies				
Wiskott-Aldrich syndrome	XL	Xp11.22-11.3	Defect of cytoskeletal expressed WASP gene	Thrombocytopenia, small platelets, eczema, malignancies, autoimmunity
Ataxia-telangiectasia	AR	11q23.1	Disorder of cell cycle checkpoint pathway and cellular responses to DNA damage 2° to mutation of *ATM* gene	Ataxia-telangiectasia, malignancies, radiation sensitivity
3rd and 4th pharyngeal pouch/arch syndrome (DiGeorge anomaly)	AR,?	22q11.21-11.23	Embryopathy with thymic hypoplasia or aplasia 2° to deletion or unbalanced translocation of chromosome 22	Hypoparathyroidism, abnormalities of cardiac outflow tract, and abnormal facies (CATCH22)

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Figure 42-1 Immunodeficiency diseases associated with maturation of T and B lymphocytes.

IV, Hematologic Malignancies, bone marrow transplantation in Part VII, and the autoimmune lymphoproliferative syndrome secondary to defects in Fas-mediated apoptosis in lymphocytes in Chapter 66 .

The primary defects arise from abnormalities in development, maturation, or function of cells of the immune system, and can be divided into those giving rise to immunodeficiency diseases primarily involving antibody production and those that have combined defects in both antibody production and T-lymphocyte function (Table 42-1). Excluding immunoglobulin (Ig) A deficiency, primary immunodeficiency disorders have a prevalence of approximately 1/10,000 and most commonly present in the first 6 years of life. The immunodeficiency diseases are characterized by an increased frequency or severity of infections or infections with unusual, relatively avirulent opportunistic organisms. Antibody deficiency diseases, which are more common than T-cell disorders, are most frequently characterized by recurrent pyogenic infections. T-cell deficiency is associated with difficulty in handling opportunistic infections and intracellular pathogensviral, fungal, or parasitic infectionson the basis of abnormal T-cell function, as well as pyogenic bacteria, because of impaired production of antibody by lymphocytes due to the T-cell deficiency. Diarrhea and malabsorption, autoimmune disorders, and malignancies frequently occur in primary immunodeficiency diseases. The majority of malignancies are observed in patients with ataxia-telangiectasia, Wiskott-Aldrich syndrome, common variable immunodeficiency, and the hyper-IgM syndrome. [7] [8] [9]

The immunodeficiency diseases reflect defects of lymphocyte development along a maturational pathway, detailed in Chapters 8 and 9 and shown in Figure 42-1 , or in the function and cooperative interaction of mature lymphoid cells, as described in Chapter 10 and shown in Figure 42-2 . Primary defects of lymphocyte function are usually inherited, and the molecular basis for many of these disorders has been described.

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ANTIBODY DEFICIENCIES

X-Linked Agammaglobulinemia

Etiology and Pathophysiology

The defect in X-linked agammaglobulinemia (XLA) is characterized by the failure of mature B cells to develop from precursor B cells or so-called pre-B cells. Mature B cells are absent or markedly decreased in the peripheral blood, plasma cells are absent in lymphoid tissues, and functional antibody is not produced. [10] [11] [12] Pre-B cells, CD34-CD19+ cells that express intracytoplasmic IgM heavy chain (C) but not light chain or surface

Figure 42-2 Receptorligand interactions of T and B lymphocytes.

immunoglobulin (sIg), are in low-normal numbers or absent in the bone marrow.[13] When pre-B cells are present in XLA, a smaller proportion are proliferating than from healthy subjects. In contrast, pro-B cells (C , TdT+, CD19+, CD10+) are present in normal numbers, suggesting a block at the C+ pre-B-cell stage of B-cell development (Fig. 42-2). Rare B cells can be detected in the circulation of patients and, when present, have an immature surface phenotype with a high ratio of surface IgM to IgD and low levels of human leukocyte antigen (HLA) class II gene products. [14] Occasionally, circulating mature B cells and serum IgG levels higher than observed in typical XLA are detected in patients, but deficiency of antibody production usually persists. [15]

The gene defect in XLA is intrinsic to the B cell, maps to Xq21.3-q22, and affects gene expression of the B-cell specific, 77 kDa, cytoplasmic protein Bruton tyrosine kinase (*Btk*), named after the discoverer of human agammaglobulinemia.[10] [11] [12] [16] [17] This Tec-family tyrosine kinase is involved in signal transduction from sIg and other B-cell receptors (e.g., interleukin [IL]-5, IL-10). At least 175 unique genetic events involving all five domains of *Btk* have been shown to give rise to XLA.[18] The most common mutation is a missense mutation at CpG dinucleotides, and the most common domain targeted for mutation is the kinase domain of *Btk.* A direct correlation between clinical phenotype and specific *Btk* mutations does not exist, and phenotypic differences can be observed even among males in a single kindred. The role of *Btk* in signal transduction from a pre-B-cell receptor (IgM heavy chain with surrogate light chain) or other receptors, the substrates phosphorylated by *Btk,* and the mechanism(s) of inhibition of pre-B-cell maturation with *Btk* gene defects are being defined. In female carriers of XLA, only the normal X chromosome and not the XLA-affected X chromosome encoding the defective *Btk* is used as the active X chromosome in B cells. This is believed to be secondary to decreased proliferation or survival of cells expressing the XLA gene. [15] [19]

Non-X-Linked Agammaglobulinemia

Rare disorders of absent B cells and panhypogammaglobulinemia that resemble XLA but are autosomal recessively inherited have been described. [20] Patients with one form express a structural defect in the m heavy chain gene (C deficiency) that inhibits production and expression of membrane m heavy chain and leads to failure of B-cell development[20] (Fig. 42-2). Another gene defect that gives rise to a similar clinical picture is deficiency of human 5/14.1, [21] a component of the pre-B-cell receptor complex that consists of a rearranged m heavy chain, a surrogate light chain composed of 5/14.1 and V pre-B, and the Ig association signal transducing chains Ig and Ig. These patients illustrate the essential requirement for expression of B-cell surface Ig or a pre-B-cell receptor for early B-cell-differentiation beyond the pre-B-cell stage to the stage of mature B cells.

Another non-X-linked or autosomal recessive form of agammaglobulinemia of unknown etiology is characterized by an arrest of B-cell development at the CD34+CD10+ pre-B-cell compartment, with complete absence of pre-B cells in the bone marrow and mature B cells in the periphery. [22]

Clinical Manifestations

Affected boys with XLA have the onset of recurrent pyogenic infections during the latter half of the first year of life as maternal antibody titers wane. The clinical presentation includes chronic or recurrent otitis media, sinusitis, conjunctivitis, pneumonia, or pyoderma. [23] Infections occur at more than one anatomic site, and are usually caused by pyogenic bacteria such as *Streptococcus pneumoniae, Streptococcus pyogenes,* and *Hemophilus influenzae.* On physical examination, patients with XLA are found to have a paucity of tonsils, adenoids, and peripheral lymph nodes. Mono- or oligoarticular arthritis of large joints with sterile effusions caused by infections with an enterovirus or a nonpathogenic commensal *Mycoplasma* strain such as *Urea-plasma urealyticum* may be the presenting problem.[24] Diarrhea from infection with such organisms as *Giardia lamblia* or rotavirus commonly occurs. Vaccine-associated poliomyelitis is a potential complication caused by the persistence and mutation of the attenuated live poliovirus vaccine strain into a virulent neurotropic form. [25] Before institution of therapy with Ig replacement therapy, neutropenia often occurs during infection. Leukemia and lymphoma occur with increased frequency. There may be a family history of death from infection at an early age in maternal male relatives.

Infectious diseases can cause significant morbidity. Chronic infections of the upper and lower respiratory tract with otitis media, pneumonitis, and sinusitis may occur in spite of Ig replacement therapy. In the past, chronic pulmonary disease has been described in almost half of XLA patients and hearing loss, as a result of chronic otitis media or meningoencephalitis, in about one third of patients. [23] Chronic, disseminated enteroviral infection, usually from echovirus, is a potentially fatal complication and may be associated with meningoencephalitis, hepatitis, pneumonitis, or vasculitis, as well as with a dermatomyositis-like syndrome with rash, brawny edema of the subcutaneous tissues, and muscle weakness.[26] [27] These complications, as well as other serious infections, have accounted for a mortality rate of about 15% by the age of 20 years in the past.[23] In recent times, however, earlier diagnosis and more effective Ig replacement therapy, by the intravenous rather than the intramuscular route, has decreased the morbidity and mortality rate.

Laboratory Evaluation

Absent antibody responses after immunization or natural antigenic exposure, low levels of serum Ig, and absence or very low levels of peripheral blood B lymphocytes are characteristic of the disease. The IgG level is usually <100 mg/dl, but exceptions occur. The other Ig isotypes are usually low or absent. Rarely, some patients have a milder form of disease with some antibody production and a low number of B cells. T-lymphocyte

numbers and function are intact. Neutropenia, secondary to infection and release of endotoxin, accompanied by a maturation arrest in the bone marrow, frequently occurs as a presenting feature.[28] Lymph nodes show an absence of plasma cells, lymphoid follicles, and germinal centers. Definitive diagnosis, prenatal diagnosis, and diagnosis of the carrier state can be performed by genetic analysis.

Therapy

Immunoglobulin replacement therapy is the mainstay of treatment and should be started once the diagnosis is made, preferably

THE EVALUATION OF SUSPECTED IMMUNODEFICIENCY

Laboratory studies should be used to confirm a diagnosis of an immunodeficiency disease after performing a thorough history and physical examination to exclude a localized anatomic or physiologic defect that may be predisposing to recurrent infections. An immunodeficiency should be suspected with the occurrence of infections of unusual frequency, type, or severity. Recurrent infections may occur at multiple sites, and infection may be caused by unusual or opportunistic organisms such as *Pneumocystis carinii, Candida, Mycobacterium avium, Pseudomonas, Serratia,* and cytomegalovirus. There is often a lack of history of exposure, excessive severity or complications of infections, and poor response to treatment. With immunodeficiency diseases, organisms may disseminate more widely, multiply to greater numbers, or remain alive in phagocytes. Patients with immunodeficiency diseases often fail to recover to normal health between individual infections, and may have weight loss or poor weight gain. Autoimmune diseases may accompany antibody deficiency states.

Recurrent pyogenic infections such as otitis media, sinusitis, pneumonia, meningitis, arthritis, osteomyelitis from encapsulated bacteria (e.g., *Streptococcus pneumoniae, Hemophilus influenzae* b, *Neisseria meningitidis, Staphylococcus aureus*) suggest a deficiency of antibody or, less commonly, an abnormality of white blood cells or serum complement. Low numbers or poor function of polymorphonuclear white blood cells is also commonly associated with abscesses and infections with nonencapsulated, nonpathogenic bacteria that reside at the skin or mucosal surfaces. Recurrent infections from viruses, fungi, or intracellular bacteria suggest a deficiency of T-cell function. Infection with *P. carinii* suggests T-cell deficiency. Combined deficiency of T- and B-cell function is characterized by infections with bacteria or viruses. Recurrent systemic *Neisseria* infections suggest deficiency of a terminal complement component. Antibody deficiency in the young child does not predispose to infections until the latter half of the first year of life due to the passive protection provided by maternal antibody. T-cell immunity, however, is not present at birth.

Laboratory evaluation should initially be directed to the type of deficiency suspected on the basis of the history and physical examination. Studies should begin with less expensive and more readily available tests. Results must be compared to age-related normal values. Examination of the complete blood count and peripheral blood smear may suggest the diagnosis. Live viral immunization should be withheld, and blood products should be irradiated before administration, to prevent graft-versus-host disease in patients with suspected T-cell deficiency.

before repetitive infections have caused irreversible pulmonary damage. [29] [30] [31] Intravenous Ig should be employed to provide high doses of Ig replacement. The higher levels of IgG provided by intravenous Ig can prevent chronic pulmonary disease and chronic enteroviral meningoencephalitis, but the latter has been described even with intravenous therapy. Pyogenic infections should be treated vigorously with antibiotics to prevent organ damage. Chronic or recurrent infection of the upper and lower respiratory tracts may require continuous prophylactic antibiotic therapy.

Patients with persistent enteroviral infections have been treated successfully with Ig preparations containing high titers of antibody specific for the infecting enterovirus.[26] [32] For the treatment of enteroviral meningoencephalitis, high titer antibodies specific for the infecting virus may need to be administered directly into the ventricles of the brain to obtain sufficiently high levels at that site, [33] but even with this therapeutic approach, infections have not always been cleared. [27] Novel antiviral therapies for enteroviral infections are in development and should be considered for this complication.

Common Variable Immunodeficiency

Etiology and Pathophysiology

Common variable immunodeficiency (CVI), the most common symptomatic primary antibody deficiency syndrome, includes a group of heterogeneous disorders associated with recurrent infections, poor antibody responses, and hypogammaglobulinemia. [34] [35] [36] [37] CVI includes both congenital and acquired defects with variable ages of onset, clinical symptoms, immunologic defects, and underlying basic defects. Both sexes are affected, with the usual age of presentation in either the first 5 years or the second or third decade of life.

Occasionally, the disorder is familial, with or without a clear-cut pattern of inheritance. Offspring and first-degree relatives may have selective IgA deficiency or autoimmune diseases such as systemic lupus erythematosus (SLE), hemolytic anemia, or idiopathic thrombocytopenic purpura (ITP). [38] [39] In some families, CVI is observed in the parent and IgA deficiency in the children with suggestion of dominant transmission without linkage to the major histocompatibility complex (MHC). [40] A CVI susceptibility gene has been proposed to lie within, or near, the MHC class III region on chromosome 6. This is based on common expression of particular extended haplotypes with deletions in the complement C_4 locus in patients with either CVI or IgA deficiency, suggesting a common genetic predisposition underlying both disorders.[41] [42] [43] Viral infections, including congenital rubella and EBV, have been incriminated in the etiology of some instances of CVI. In contrast, human immunodeficiency virus (HIV) and hepatitis virus infections have been reported to restore IgG and antibody production in some patients with CVI. [44] [45] [46]

Although the exact nature of the defect(s) that give rise to CVI are unknown, a number of immunologic abnormalities have been described. [37] [47] [48] [49] Most patients with CVI have normal numbers of mature B cells that fail to differentiate into antibody-secreting cells. Failure of in vivo B-cell maturation and differentiation is usually thought to be primarily due to defective interactions between T and B cells. Both intrinsic B-cell defects and T-cell abnormalities have been described, but the current thinking is that the abnormal interaction between T and B cells is usually caused by a T-cell defect.

The B cells in CVI may express an immature surface phenotype, with surface IgG-bearing cells in CVI more commonly coexpressing surface IgM than B cells from normal controls.[50] [51] Intrinsic B-cell defects include failure to proliferate or secrete one or more immunoglobulin isotypes, most often IgG or IgA, with appropriate

stimuli. CVI has been classified into subgroups

based on defects of CVI B cells in secreting IgM, IgG, or IgA on stimulation with various polyclonal activators (anti-IgM with IL-2, Epstein-Barr virus [EBV], anti-CD40 with IL-4 or IL-10).[47] [52] [53] [54] [55] [56] In part, these in vitro defects reflect defective in vivo deletional isotype switching to the respective Ig heavy chains.

Defects of CD4 T lymphocytes have been incriminated as the primary defect in CVI. The majority of patients have normal numbers of T-cell subsets, but a subgroup of CVI patients have increased CD8+ T cells and decreased CD4+ T cells. Some patients show a functional T-cell defect with abnormal signal transduction after activation through the T-cell receptor (TCR)CD3 complex with decreased production or expression of second messengers, [57] intracellular protein tyrosine kinases, [58] cytokines IL-2, IL-4, IL-5, -interferon (IFN), [37] [48] [59] [60] and cell surface CD40 ligand.[61] [62] These defects obviously may contribute to defective interactions between T and B cells. An in vivo correlate of these in vitro findings is the presence, in CVI patients, of a profound decrease in the number of antigen-specific T cells that are induced in vivo after immunization.[63] Rarely, circulating antilymphocyte antibodies, inhibitors or cytokines, or suppressor lymphocytes that block in vivo B-cell activation and differentiation have been demonstrated in CVI patients. [64]

Clinical Manifestations

Common variable immunodeficiency occurs in both sexes, with a variable clinical spectrum and an age of onset varying from childhood to late adulthood. Recurrent pyogenic infections with otitis media, sinusitis, bronchitis, and pneumonia, often from pneumococci, staphylococci, *H. influenzae*, or *Mycoplasma pneumoniae*, occur.[34] [35] [36] [37] [65] [66] [67] Bronchiectasis is a frequent complication and may even be the presenting abnormality. Cor pulmonale and respiratory failure on the basis of chronic lung disease are common causes of death. In contrast to XLA, lymphoid tissue is normal or enlarged as a result of B-cell proliferation and prominent germinal center formation. Splenomegaly, secondary to reactive follicular hyperplasia, occurs in approximately 25% of patients and may be associated with neutropenia and thrombocytopenia from hypersplenism. Noncaseating granulomas, resembling sarcoidosis, in the spleen, liver, or lung may be found. Arthritis and arthralgia are common. Gastrointestinal abnormalities with chronic gastritis and achlorhydria, diarrhea, a spruelike syndrome with malabsorption, lactose deficiency, or protein-losing enteropathy may complicate the clinical course. [68] [69] *G. lamblia* or *Campylobacter* infection of the gastrointestinal tract may contribute to these symptoms. Pernicious anemia develops in some patients because of atrophic gastritis and absence of intrinsic factor. Hepatitis may develop. Reactivation of varicella zoster and recurrent herpes simplex infection occur commonly. Autoimmune diseases, including autoimmune hemolytic anemia, thrombocytopenia or neutropenia, rheumatoid arthritis, SLE, chronic active hepatitis, or thyroiditis, are seen frequently.

There is overall a 5-fold increase in the incidence of cancer, with a 47-fold increase in gastric cancer and a 30-fold increase in lymphoma. [7] [8] [9] [70] [71] This increased incidence of gastric cancer may be related to the high frequency of achlorhydria and atrophic gastritis in CVI. [72] A nonmalignant lymphoproliferation with follicular hyperplasia of lymph nodes or nodular lymphoid hyperplasia of the intestine and splenomegaly may also occur and must be distinguished from a malignancy. [73]

Laboratory Evaluation

Serum immunoglobulins, especially IgG and IgA, are decreased and antibody responses are impaired. The peripheral blood B-cell number is normal in about 75% of patients, but the surface phenotype may be immature, and the cells of some patients fail to differentiate to antibody-secreting cells after appropriate stimuli. If a low number of B cells are detected in a male with a presumed diagnosis of CVI, atypical XLA must be excluded. T-cell numbers may be decreased and a reversal of the helper/suppressor (CD4/CD8) T-cell ratio may be present, usually secondary to an increase in CD8+ T cells. Some patients have an expanded number of activated CD8+CD57+CD25+HLA-DR+ circulating T cells.[48] [74] Depressed T-cell function and absent delayed hypersensitivity skin test reactivity may be found, especially in older patients and patients with lymphopenia. As described above, CD40 ligand expression of T cells activated in vitro may be abnormal.

Therapy

Immunoglobulin replacement and aggressive antibiotic therapy are indicated. [29] [30] [31] After Ig replacement therapy is instituted, pulmonary radiographic and functional changes may stabilize. Serial pulmonary function tests need to be performed. Acute infections, however, may still occur with Ig replacement therapy and necessitate the prolonged use of antibiotics to prevent deterioration of lung function. In the face of chronic lung disease and bronchitis, some patients require doses of Ig replacement higher (600 mg/kg or higher) than commonly used to prevent acute lung exacerbations. [75] Parenteral therapy with vitamin B_{12} is required for pernicious anemia. Therapy with IL-2 has been used experimentally in a limited number of patients, with some improvement of in vitro lymphocyte function. [76]

Selective IgA Deficiency

Etiology and Pathogenesis

Deficiency of serum IgA, defined as a serum IgA concentration <5 mg/dl, is found in approximately 1 in 600 individuals of northern European ancestry. [77] [78] Most of these individuals are asymptomatic, but some patients suffer recurrent sinopulmonary infections, autoimmunity, or allergies. [79] [80] [81] [82] The latter two symptoms may occur in some patients on the basis of either absorption of environmental and dietary antigens into the systemic circulation due to the lack of secretary IgA at mucosal surfaces, or an underlying broader immunologic defect with poor regulation of autoantibody production due to common susceptibility genes in IgA deficiency and autoimmunity.

IgA deficiency may be primary or secondary and can occur in a sporadic form or with an autosomal dominant or recessive pattern of inheritance. [77] [78] [83] IgA deficiency or CVI may be found in extended families with evidence of linkage or nonlinkage to the MHC locus. [39] [40] [41] [42] As described under CVI, an autosomal dominant inheritance pattern has been observed in families. [40] An increased frequency of particular fixed MHC haplotypes, so-called supratypes reflecting conserved ancestral haplotypes, has been associated with IgA deficiency. An important locus for regulation of IgA production may exist in the MHC class III gene cluster, [41] [42] an 1,100 kb region of DNA located between the class I and II gene clusters that encodes heat shock proteins, tumor necrosis factor (TNF)-, lymphotoxin and , complement proteins C2, C4, and factor B, and the 21-hydroxylase steroid-biosynthesis gene. An MHC class II susceptibility gene for IgA deficiency has also been suggested.[84] These putative MHC gene(s) may be necessary, but not sufficient, for the development of the immunodeficiency, and may also increase susceptibility to the autoimmune diseases observed in IgA deficiency.

Congenital infections with rubella, cytomegalovirus, or *Toxoplasma gondii* and acquired EBV infection have been associated with IgA deficiency. IgA deficiency may also occur after use of drugs such as penicillamine, phenytoins, sodium aurothiomalate, captopril, sulfasalazine, or antimalarial agents. [77] [78] IgA deficiency can be transitory in children, especially when the serum

IgA is only moderately decreased, but in adults the deficiency is usually permanent. [85] [86]

Most patients lack both serum and secretory IgA and have a deficiency of both the IgA1 and IgA2 subclasses. Patients with IgA deficiency who lack serum IgA but express IgA2 secretory plasma cells in the gastrointestinal tract have been described. Rarely, IgA deficiency is associated with a deficiency of the secretory component, the protein that is required for secretion of IgA into the mucosa. The susceptibility to infection in IgA deficiency may be secondary, not to IgA deficiency per se, but to a concomitant deficiency of IgG2 and IgG4 subclass immunoglobulins. [81] [87] [88] [89] Thus, IgA deficiency, in some patients, may represent a manifestation of a more widely based immunologic defect with similarities to CVI, which also is an arrest in B-cell differentiation. As noted previously, CVI also occurs in family members and shares MHC haplotypes with IgA deficiency. IgA deficiency may in fact evolve into CVI, and is also frequently found in ataxia-telangiectasia, which is also associated with T-cell abnormalities and IgG subclass deficiency.

IgA deficiency is usually associated with intact structural IgA immunoglobulin heavy chain genes. Surface IgA-bearing B cells are present in normal or reduced numbers, and the cells may display an immature phenotype with coexpression of surface IgM in some patients. [90] The primary defect appears to be failure of B cells to differentiate to mature isotype-switched surface IgA-positive B cells and IgA-secreting plasma cells with appropriate stimuli. [77] [78] [91] The basis for the defect is not

known. B cells from patients with IgA deficiency activated via CD40 and IL-10 are capable of synthesizing and secreting IgA. [92] Defective in vitro T-helper cells and excessive suppressor T-cell activity for IgA production are rarely present. The possibility that cytokines that favor isotype switching to IgA are abnormal has been explored. Normal levels of transforming growth factor (TGF)-1 in RNA were detected in mononuclear cells, [93] although decreased serum levels of TGF-1 were observed in some patients.[94]

Clinical Manifestations

The majority of individuals with IgA deficiency are healthy, but many have variable clinical symptoms, and IgA deficiency patients, asymptomatic when young, may develop symptoms in later life.[77] [78] [79] [80] [81] [82] An increased frequency of upper and lower respiratory tract infections occurs. Infections occur more commonly when low levels of IgA occur with a deficiency in IgG subclasses and with decreased levels of antipneumococcal polysaccharide antibodies. [87] [88] [89] Some patients have recurrent bacterial infections with recurrent sinusitis, otitis media, bronchitis, and even bronchiectasis. There is an increased incidence of autoimmune diseases, especially rheumatoid arthritis and SLE.[95] Other autoimmune diseases may be observed, and autoantibodies may be present without symptoms. Allergic diseases, including rhinitis, urticaria, eczema, and asthma, occur with increased frequency. Gastrointestinal and hepatic complications associated with IgA deficiency include malabsorption, celiac disease, giardiasis, nodular lymphoid hyperplasia, pernicious anemia, atrophic gastritis, primary biliary cirrhosis, and chronic active hepatitis. Gastric and colon carcinoma are found with increased frequency. Production of antibodies to food antigens, such as cows milk, may cause immune complexes that may be pathogenic.

Laboratory Evaluation

The serum concentration of IgA is <5mg/dl, and the levels of IgG and IgM are normal. Secretory IgA is also decreased. Approximately 25% of patients have decreased levels of IgE. Some patients, especially those with recurrent sinopulmonary infections, have a concomitant deficiency of IgG2 and IgG4 subclasses or defective antibody responses to polysaccharides. IgA and IgG levels should be repeated after a period of time to determine whether the deficiency of IgA is persistent, or whether the immunologic disorder has become more extensive and has begun to resemble CVI. An increased incidence of autoantibodies directed against nuclear proteins; immunoglobulins; thyroglobulin; or adrenal, parietal, smooth muscle, or pancreatic cells; or antibodies to food antigens, such as cows milk protein, is found. Serum antibodies to IgA occur in >20% of patients with IgA deficiency, especially those with severe IgA deficiency, but are not predictive of adverse reactions to blood products containing IgA. Exposure to blood products with IgA may, however, induce IgE anti-IgA antibodies that can cause anaphylactic reactions. [96]

Therapy

Treatment should be directed at the associated infections and allergic, autoimmune, and gastrointestinal diseases. Precipitating drugs should be discontinued. Antibiotic therapy is indicated for bacterial infections. If infections are severe or cause significant sequelae, prophylactic antibiotics should be considered. Ig replacement therapy is not usually indicated for IgA deficiency. Severe or fatal anaphylactic reactions can occur with the intravenous administration of IgA-containing blood products to IgA-deficient patients. [96] This may be caused by anti-IgA antibody of the IgE isotype, which may increase after Ig replacement therapy. Some patients with anaphylactic reactions to IgA and with severe, recurrent infections and concomitant deficiency of IgG subclasses and antibody production, however, benefit clinically from treatment with intravenous replacement therapy with Ig preparations containing low levels of IgA. [97] Blood products should be washed prior to transfusing IgA-deficient individuals or obtained from IgA-deficient donors, including the recipient, prior to surgery. Plasma for transfusion should be collected from IgA-deficient individuals. Oral administration of gamma globulin has improved chronic diarrhea in some patients.

Selective Deficiency of Immunoglobulin Isotypes

Selective deficiency of an immunoglobulin isotype is not usually associated with structural gene abnormalities. Rarely, deletions of the Ig constant region genes have been described and may involve several or single constant region gene segments. [98] Most of the patients with these partial deletions, however, are usually healthy.

Selective IgM deficiency is a rare immunodeficiency disorder characterized by a low level of serum IgM and severe or life-threatening, recurrent infections. [99] Pneumococcal and meningococcal sepsis or meningitis may occur. IgM antibodies are not produced and IgG antibody responses are usually decreased, although the IgG level is normal. Defects of T-cell helper and excessive suppressor T-cell activity have been described. Some of these patients subsequently develop CVI.

Absence of either or light chains associated with hypogammaglobulinemia or IgA deficiency and recurrent respiratory tract infections, pernicious anemia, diarrhea, or malabsorption have rarely been reported. In one patient with light chain deficiency, there was a point mutation in the Ck gene involving amino acid residues that are required for the formation of intrachain disulfide bonds and protein folding. [100]

IgG Subclass Deficiency and Selective Antibody Deficiency with Normal Immunoglobulins

Recurrent upper and lower respiratory tract bacterial infections, sinusitis, and otitis media may occur in patients with low levels of an IgG subclass. [101] The most common symptomatic IgG subclass

IMMUNOGLOBULIN REPLACEMENT THERAPY

Replacement therapy with gammaglobulins or immunoglobulins is indicated for patients with recurrent infections who have defects in antibody production. It can be life-saving, and prevent recurrent infections and progressive lung damage. The cost of immunoglobulin replacement therapy is high, however, so it should not be used indiscriminately. Ideally, antibody titers before and after immunization with protein and polysaccharide antigens should be evaluated to assess the need for replacement therapy (see Table 42-1). Usual indications include X-linked agammaglobulinemia, common variable immunodeficiency, severe combined immunodeficiency disease, hyper-IgM syndrome, and the Wiskott-Aldrich syndrome. Replacement therapy should not be given to patients with IgG subclass deficiency unless they have been shown to have a deficiency of antibody production and have failed antibiotic prophylaxis. IgG replacement therapy is not usually indicated for isolated IgA deficiency, but some patients with IgA deficiency with concomitant deficiency of IgG antibody production and severe, recurrent infections have benefitted from replacement therapy. Patients with IgA deficiency may develop anti-IgA antibodies of the IgE isotype that can cause severe or fatal anaphylactic reactions with the intravenous administration of IgA-containing blood products. Immunoglobulin replacement therapy with immunoglobulin preparations containing low levels of IgA that are administered by the intravenous or subcutaneous route have been used successfully in these patients.

Development of safe intravenous gammaglobulin formulations allows high doses to be given without the pain, side effects, and tissue loss experienced with intramuscular gammaglobulin replacement. There are multiple intravenous immunoglobulin (IVIG) preparations approved by the Food and Drug Administration (FDA). All preparations are safe, contain predominantly IgG1 and IgG2, have roughly comparable and consistent IgG antibody titers, and are generally therapeutically equivalent. The FDA-licensed immunoglobulin preparations do not carry a risk of transmitting the human immunodeficiency virus. Patients vary in their half-life of administered IgG. The optimal amount and frequency of infusion of immunoglobulin and the serum level of IgG to be achieved need to be individualized to render the patient asymptomatic from recurrent infections, but it is recommended that a trough serum level be attained before the next injection at least 200400 mg/dl higher than the pretherapy level or >500 mg/dl. An initial IgG dose of 400 mg/kg is usually given, followed by administration of a similar dose 37 days later, and then monthly. Trough levels should be checked bimonthly for the first 8 months, and then every 6 months. This dose will result in a steady-state level after 48 months with trough levels >500 mg/dl. In general, a dose of 100 mg/kg of IVIG will increase the IgG peak by 200300 mg/dl and trough levels by 100 mg/dl (28 days postinfusion). Higher doses (600 mg/kg or greater) of IVIG may be required to prevent infections in some patients, and may prove better at preventing acute infections or exacerbations in patients with chronic pulmonary disease. If patients complain of fatigue, upper respiratory infections, or conjunctivitis during the week before the next infusion, or if intercurrent infections occur, the frequency of infusion or the dose should be increased to raise the trough serum level to >500 mg/dl. During intercurrent infections, extra infusions are required because of increases in the catabolic rate of the passively provided immunoglobulin. Home infusion therapy is now being practiced widely. Subcutaneous infusion of immunoglobulins is an alternative to intravenous infusions, with a low frequency of systemic adverse reactions.

Intramuscular gammaglobulin (16% solution of Cohn fraction II) may still be useful in small infants in whom chronic complications of infections have not occurred, and there is difficulty with access to a center where IVIG can be administered. Contraindications to intramuscular gammaglobulin include thrombocytopenia, inadequate muscle mass, and poor control of infections. A dose of 100 mg/kg/month is given after loading the patient with the same dose administered three times over a 1- to 2-week period. Intramuscular preparations can never be administered intravenously.

Adverse reactions to IVIG administration include flushing, chest tightness, flank or abdominal pain, nausea and vomiting, chills, fever, headache, myalgias, dyspnea, diaphoresis, or hypotension. These reactions can usually be controlled by decreasing the infusion rate. In general, reactions are more common with the first infused dose, and in the presence of an intercurrent infection. In these situations, infusions should be slower. Repeated, severe reactions not responding to a decrease in the rate or volume of the infusion may, in some individuals, be averted by pretreatment 1 hour before IVIG with aspirin or acetaminophen, antihistamines, or steroids. Rarely, anaphylactic reactions may occur. Anaphylactic reactions require stopping the infusion and immediate administration of epinephrine, steroids, and antihistamines, together with respiratory and circulatory support. Anaphylactic reactions are rarely caused by production of IgE antibodies to IgA in patients with IgA deficiency or those with common variable immunodeficiency with absent IgA. Anti-IgA antibodies should be quantitated following an anaphylactic reaction and, if present, IVIG preparations with low levels of IgA should be used and the subcutaneous route considered.

deficiency in childhood is IgG2 subclass deficiency, a subclass that represents approximately 2025% of the total IgG. IgG2 subclass deficiency may occur alone or with concomitant IgG4 subclass or IgA deficiency[101] [102] and can be associated with the inability to generate antibodies to polysaccharides. [88] [89] [103] The clinical picture of IgG2 subclass deficiency, IgA deficiency, and poor antibody responses reflects an underlying broad immunoregulatory defect in these patients, and it should not be thought that the IgG2 deficiency represents the primary etiology for the poor antibody responses to polysaccharide antigens. Recurrent infections with decreased antibody responses to polysaccharide antigens may, in fact, occur with either normal or low IgG subclass levels. [104] The poor antibody responses to polysaccharide antigens may contribute, however, to a low IgG2 subclass level, which contributes disproportionately to antibody responses to polysaccharides. In most patients, the structural IgG heavy chain genes on chromosome 14 are normal. Antibody deficiency, and not isolated IgG subclass deficiency per se, is therefore the cause of recurrent infections. The etiology of IgG subclass deficiency or poor antibody responses to polysaccharides is not known. Patients who lack one or more IgG subclasses on the basis of a homozygous deletion of heavy chain IgG constant region genes have been described, [98] but such patients are usually asymptomatic. A defect in production of IFN- by

LABORATORY EVALUATION FOR SUSPECTED IMMUNODEFICIENCY

GENERAL

Complete blood cell count

Chest radiograph

Serology, culture, and polymerase chain reaction or antigen detection for human immunodeficiency virus

ANTIBODY DEFICIENCY
Initial

Quantitative immunoglobulins

Antibody titers before and after immunization: tetanus toxoid, diphtheria toxoid, *Hemophilus influenzae* b or pneumococcal capsular polysaccharides

Isohemagglutinin titer

Later

Quantitation of B cells

IgG subclasses

In vitro Ig isotype switching and synthesis

Btk gene mutation

T-cell CD40 ligand expression

T-LYMPHOCYTE DEFICIENCY
Initial

Absolute lymphocyte count

Quantitation of T cells and T-cell subsets

Delayed-type hypersensitivity skin tests

Later

Lymphocyte proliferative response to mitogens, antigens, and allogeneic cells

Quantitation of enzyme activity: adenosine deaminase, nucleoside phosphorylase

IL-2 receptor chain expression; mutations

MHC class I and II expression

In vitro T-cell activation studies

COMPLEMENT DEFICIENCY
Initial

Total hemolytic complement (CH_{50})

Later

Quantitation and functional analysis of individual components

POLYMORPHONUCLEAR LEUKOCYTE AND MACROPHAGE DEFICIENCY
Initial

Polymorphonuclear count and morphology

Howell-Jolly bodies

IgE level

Nitroblue tetrazolium reduction test, chemiluminescence or flow cytometric respiratory burst assay

IgE level

Nitroblue tetrazolium reduction test, chemiluminescence or flow cytometric respiratory burst assay

Later

Rebuck skin window

Assays for chemotaxis, chemokinesis, random migration, phagocytosis, killing, oxidative metabolism, enzyme activity

CD11/CD18 expression

Spleen scan

mononuclear cells of patients with IgG2 subclass deficiency, with restoration of IgG2 production by addition of IFN- to in vitro cultured cells has been observed. [105] Isolated IgG subclass deficiency may be associated with IgA deficiency, as noted previously, or with ataxia-telangiectasia. Although IgG2 subclass deficiency predominates in children, symptomatic isolated IgG3 deficiency more commonly is detected in adults. [101]

Interpretation of IgG subclass values must be approached with attention to the wide range of normal age-related values. A low IgG subclass level should be followed by quantifying the antibody response to a polysaccharide vaccine, such as the *H. influenzae* b or pneumococcal capsular polysaccharide vaccine. [106] In children with recurrent sinopulmonary infections, quantitation of antibody responses to protein and polysaccharide antigens is preferable to quantitating IgG subclass levels. Immunization with polysaccharide-protein conjugate vaccines can overcome the unresponsiveness to polysaccharide antigens in IgG2 subclass deficiency [107] and may be of therapeutic value. If the frequency of infections in patients with symptomatic IgG subclass deficiency is not decreased with prophylactic antibiotics, Ig replacement therapy may be used, but caution must be exercised if there is concomitant IgA deficiency. In some children, the IgG subclass deficiency disappears spontaneously in later childhood.

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COMBINED IMMUNODEFICIENCIES

Etiology and Pathophysiology

Severe combined immunodeficiency (SCID) is a heterogeneous disorder characterized by profound T- and B-lymphocyte deficiency that may be autosomal recessive or X-linked inherited.[108] [109] [110] About 80% of affected patients are boys, and only about one third of SCID patients have a positive family history. In SCID, there is a defect in T- or T- and B-cell development. SCID with preservation of B-lymphocyte numbers (T B + SCID) occurs in X-linked SCID (X-SCID) and autosomal recessive Janus-associated kinase (JAK)3 deficiency. The other autosomal forms of SCID are usually associated with decreased numbers of both T and B lymphocytes (T B SCID). Because bone marrow transplantation can completely correct the defect, a primary thymic defect is not the underlying basis of the disease, although the thymus is secondarily affected, with defective epithelial cell differentiation and absence of lymphoid cells and Hassalls corpuscles.

T B + Severe Combined Immunodeficiency

X-Linked Severe Combined Immunodeficiency

X-linked SCID, thought to be the most common form of SCID with estimates ranging from 2842% of cases, occurs in an estimated 1 per 50,000 births. [108] [109] [110] X-SCID maps to Xq13.113.3 and is characterized by absence of mature T cells in the presence of B cells. The molecular basis of X-SCID is a mutation in the $_{c}$ chain that is common to and indispensable for signaling through the high- and intermediate-affinity interleukin-2 (IL-2) receptors, and the receptors for IL-4, IL-7, IL-9, and IL-15 (Fig. 42-3). [5] [111] [112] [113] [114] [115] Mutation of this common cytokine receptor chain interferes with T-cell development in the thymus through lack of IL-7 signaling, and with signal transduction through lack of IL-2 signaling. B cells are present in the periphery, but the late stage of B-cell differentiation is impaired through decreased IL-2 and possibly IL-4 and IL-15 signaling. Although the number of B cells in X-SCID may be normal, the B cells have an immature phenotype, are not functional, fail to differentiate or produce IgG after in vitro stimulation, and are defective in IL-2 and IL-4 signaling. Natural killer (NK) cells are absent because of their impaired development from abnormal IL-15 signaling. In female carriers of X-SCID, there is nonrandom lyonization of

PERIPHERAL BLOOD ABNORMALITIES IN IMMUNODEFICIENCY

ANEMIA

Hemolytic: antibody deficiency

Hypoplastic: PNP deficiency, thymoma, X-linked lymphoproliferative syndrome

Megaloblastic: transcobalamin II deficiency, chronic mucotaneous candidiasis, CVI, IgA deficiency, PNP deficiency

Blood loss: Wiskott-Aldrich syndrome, malabsorption and diarrheal diseases with antibody deficiency diseases

LYMPHOPENIA

T-cell and combined T- and B-cell deficiency

CD8 T-cell lymphopeniaZAP-70 deficiency

T-cell lymphopenia with preserved B-cell numbersX-SCID, JAK3 deficiency, ZAP-70 deficiency, PNP deficiency, MHC class II deficiency

NEUTROPENIA

White blood cell deficiency; antibody deficiency

EOSINOPENIA

Thymoma

NEUTROPHILIA

Qualitative white blood cell disorders (e.g., LAD)

EOSINOPHILIA

SCID, GVHD, Omenn syndrome, T-cell deficiency, Wiskott-Aldrich syndrome, ataxia-telangiectasia

MONOCYTOSIS

Antibody, T-cell, and white blood cell deficiency

THROMBOCYTOPENIA

Wiskott-Aldrich syndrome (small platelets), Evans syndrome, antibody deficiency with autoantibodies

OTHER

Howell-Jolly bodiessplenic absence or dysfunction

Giant lysosomal cytoplasmic granulesChediak-Higashi syndrome

Abbreviations: PNP, purine nucleoside phosphorylase; CVI, common variable immunodeficiency; LAD, leukocyte adhesion deficiency; SCID, severe combined immunodeficiency; X-SCID, X-linked severe combined immunodeficiency; GVHD, graft-versus-host disease.

the X chromosome bearing the X-SCID mutation in T cells, mature differentiated B cells, and NK cells. Milder forms of the disease have been described in which $_{c}$ is present but fails to associate with its downstream signaling molecule, the tyrosine kinase JAK3. Up to one third of cases arise spontaneously and, therefore, lack a family history.

JAK3 Deficiency

Another form of B-cell-positive SCID, one that is phenotypically indistinguishable from X-SCID, is autosomal recessive inherited

Figure 42-3 IL-2 receptor activation. IL-2 binds to the multimeric IL-2 receptor (IL-2R) composed of three transmembrane proteins IL-2R, which regulates receptor complex affinity for IL-2, and the intracellular signaling proteins IL-2R and $_c$ that heterodimerize after binding IL-2. IL-2R is constitutively associated with JAK1 and $_c$ binds JAK3. With IL-2 binding, JAK1 and JAK3 are activated, and in turn lead to tyrosine phosphorylation of various signal transducers and activation factors of transcriptionSTAT1, STAT3, STAT5. Phosphorylation of STATs leads to their dimerization through reciprocal phosphotyrosineSH_2 domain interactions and subsequent translocation to the nucleus, where they mediate transcriptional activation. SCID arises from mutation of the $_c$ chain (X-SCID) or the JAK3 gene (autosomal recessive SCID). Immunodeficiency also occurs with mutations of IL-2R.

and arises from mutations in the Janus family protein tyrosine kinase JAK3, which is essential for signaling through the $_c$ common cytokine receptor chain.[116] [117] After cytokine binding, the cytoplasmic tail of the cytokine receptor dimerizes and brings JAKs into close proximity, promoting their tyrosine phosphorylation. JAK3 phosphorylation leads to the phosphorylation of cytokine receptor chains and the creation of docking sites for *Src* homology (SH_2)-containing signal transducer and activator of transcripts (STAT) proteins (Fig. 42-3). STAT proteins become phosphorylated, dimerize, and translocate to the nucleus to activate gene transcription. Activation of JAKs also leads to recruitment and activation of the SHC-Grb2-SOS-Ras-Raf1-MAPK signaling pathway, which leads to transcription of early-immediate genes (*c-fos, c-jun*) and of phosphatidylinositol 3-kinase, a signal for cell growth. The phenotypic picture of low T-cell numbers, absence of NK cells, and defective B-cell signaling in JAK3 deficiency is identical to X-SCID.

T B Severe Combined Immunodeficiency

Adenosine Deaminase Deficiency

Approximately 15% of patients with SCID and 3040% of patients with autosomal recessive inherited SCID have deficiencies of the enzyme adenosine deaminase (ADA), an enzyme of the purine salvage pathway that deaminates adenosine and deoxyadenosine

to inosine and deoxyinosine.[118] [119] [120] [121] Although ADA is expressed by all cell types, ADA deficiency affects preferentially the lymphoid system and interferes with lymphoid development in the thymus, which contains the highest level of ADA activity in the body. In ADA deficiency, there is accumulation of the toxic metabolites of purine metabolism, deoxyadenosine triphosphate (dATP) and 2-deoxyadenosine. Deoxyadenosine is generated in high amounts in the thymus from the extensive lymphocyte death that occurs with lymphoid development at that site. Deoxyadenosine is freely transported out of cells but is converted intracellularly by deoxyadenosine kinase into dATP that is trapped in the cell. These metabolites preferentially accumulate in lymphocytes because of the high expression of deoxyadenosine kinase. dATP inhibits T-cell proliferation by affecting DNA synthesis through inhibition of ribonucleotide reductase. Also contributing to the lymphocyte defect is the accumulation of *S*-adenosylhomocysteine, which occurs from inactivation of the enzyme *S*-adenosylhomocysteine hydrolase by 2-deoxyadenosine. The accumulation of these metabolites may also block endogenous DNA repair, leading to chromosomal breaks.

ADA deficiency, which may be complete or partial, is usually due to point mutations of the ADA gene, located on the long arm of chromosome 20, giving rise to a nonfunctional protein because of alteration in enzyme activity, stability, or structure. Splicing defects and chromosomal deletions of the ADA gene have also been described. The immunodeficiency is progressive and variable in age of presentation, depending on the amount of residual normal ADA activity, and may even present in adulthood. In contrast to other forms of SCID, ADA deficiency may be associated with thymus development and production of Hassalls corpuscles.

Lymphocyte Receptor Gene Rearrangement Defects

In another autosomal recessive form of the disease, ADA activity is normal, with failure of maturation of T and B lymphocytes resulting in lymphopenia. NK cells are present and functional. The possibility that this form of SCID is due to defective DNA repair, which is required during the normal rearrangement of immunoglobulin and T-cell receptor (TCR) genes, was suggested by the finding of aberrant D-J $_H$ joining in bone marrow pre-B cells from patients with SCID.[122] This suggestion was confirmed with the demonstration that in almost half of autosomal recessive B, ADA+ SCID patients, nonsense, missense, or deletional mutations in RAG1 and/or RAG2 occur with loss of functional V(D)J recombinase activity that results in an inability to generate antigen receptors in both T and B lymphocytes. [123] [124] Other patients with ADA+ autosomal recessive SCID have normal RAG1 and RAG2 activity but show increased radiosensitivity of granulocyte-macrophage colony-forming units and skin fibroblasts, suggesting a DNA repair defect. [125] [126] The possibility is under investigation that the defects in those autosomal recessive SCID patients with normal RAG1 and RAG2 arise from mutations in the enzyme DNA-dependent protein kinase p350, or associated proteins (e.g., KU70 and KU80, XRCC-4) that mediate DNA double strand break repair and are required for Ig and TCR gene rearrangements.

Omenn Syndrome

Omenn syndrome is an autosomal recessive combined immunodeficiency with scaly erythroderma, generalized lymphadenopathy, hepatosplenomegaly, failure to thrive, eosinophilia, and increased IgE levels. [127] The syndrome clinically resembles graft-versus-host disease, (GVHD), but the T cells infiltrating the skin and organs are autologous, activated (HLA-DR+ CD45R0+CD29+CD30+) self-reactive cells with restricted heterogeneity and spontaneously produce high levels of IL-4 and IL-5, thereby explaining the eosinophilia and increased IgE levels. [128] [129] [130] [131] Families in which one offspring had Omenn syndrome and another had typical lymphopenic SCID have been described, and cells from patients with Omenn syndrome also have displayed increased radiosensitivity, as described above. Both findings suggest that Omenn syndrome may be a leaky type of autosomal recessive SCID. Treatment with either cyclosporin [128] or IFN-[132] has improved the clinical condition and even the eosinophilia, but bone marrow transplantation is required to correct the syndrome permanently. [133]

Reticular Dysgenesis

Another rare autosomal recessive form of SCID, reticular dysgenesis, is characterized by the absence of both B and T lymphocytes and granulocytes in the peripheral blood and bone marrow.[134] [135] [136] [137] There is defective maturation of both lymphoid and myeloid lineage cells in the bone marrow. The molecular basis of this defect is unknown. Overwhelming infections ensue unless the defect is corrected by bone marrow transplantation. [137]

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OTHER COMBINED IMMUNODEFICIENCIES

Major Histocompatibility Complex Class II Deficiency

Major histocompatibility complex class II deficiency, or bare lymphocyte syndrome, is an autosomal recessive disorder characterized by failure of expression of MHC or HLA class II surface molecules on B lymphocytes, monocytes, activated T lymphocytes, dendritic cells, Langerhans cells in the skin, and epithelial cells in the thymus and intestine.[138] [139] [140] [141] The immunodeficiency arises from mutations of regulatory factors essential for MHC class II gene transcription that are not linked to the MHC locus. MHC class II recognition is required for positive selection of T cells in the thymus and for interaction and activation of T cells by antigen presented to the T-cell receptor as a peptideMHC complex on the surface of antigen-presenting cells. There is heterogeneity in the gene defects in MHC class II deficiency, with four distinct genetic defects of transactivating factors that regulate MHC class II gene expression. [140] [141] In most patients, there are mutations of the multimeric protein transactivating regulatory factor X (RFX) complex, including RFX5 (complementation group C) and RFXAP (complementation group D), that cause failure of transcription factor activity or binding to the X box in the 5 promoter common to HLA class II molecules and prevent transcription of HLA class II molecules. In another form of the disease (complementation group A), there is a defect of the MHC class II transactivator (CIITA), a master switch that regulates constitutive expression of MHC class II genes, as well as genes involved in antigen processing and presentation (e.g., invariant chain and DM molecules) and induction of transcription by IFN-. CIITA is thought to be a non-DNA-binding coactivator that binds to RFX and other transcription factors after their binding to the upstream promoter.

In MHC class II deficiency, there is inconsistent reduction of HLA class I expression in fresh cells of some patients, which is not well understood since CIITA and RFX do not regulate class I expression. MHC class II deficiency differs from another subtype of the bare lymphocyte syndrome (termed type 1), which is characterized by inconstant and incomplete expression of HLA class I antigens. [142] HLA class I deficiency may arise from mutations of the TAP2 gene that encodes the TAP2 peptide transporter, which is required for transport of peptides into the endoplasmic reticulum for binding to MHC class I molecules. [142] HLA class I molecules remain peptide-free and fail to reach the cell surface. TAP2 deficiency is associated with chronic bacterial lung inflammation, decreased CD8 T-cell counts, but without

severe viral infections. The latter may be due to TAP-independent HLA class I restricted viral antigen recognition and preserved NK cell activity.

Normal numbers of T and B lymphocytes are found in MHC class II deficiency, but CD4+ T lymphocytes are commonly decreased and CD8+ T lymphocytes increased.[138] [139] It is unclear why CD4+ T cells are detected in these patients in the absence of MHC class II expression, which is required for their selection and development in the thymus. One possible explanation is the presence of residual MHC class II expression in the thymus. Lymphocyte function is abnormal in MHC class II deficiency, with absent in vitro and in vivo T-cell responses to specific antigens. Mitogen and allogeneic in vitro responses may be preserved, although class II molecules are not expressed after T-cell activation. Macrophages fail to present antigens to normal T cells. Although immunoglobulin levels may be normal, antibody responses after immunization are usually absent. Diagnosis is made by demonstrating absence of MHC class II expression on B lymphocytes, monocytes, or activated T cells.

T-Cell Activation Defects

T-cell defects that impair T-cell activation through the TCR and CD3 complex may lead to SCID in the presence of normal numbers of T lymphocytes. [143] [144] [145] Clinically, an increased incidence of infections, autoimmune hemolytic anemia, neutropenia or thrombocytopenia, allergies, cancer, and chronic diarrhea are observed. Different defects can lead to impairment of early events in T-cell signaling and signal transduction. In some of these defects, the TCR/CD3 complex is poorly expressed on T cells because of mutations in the genes encoding one of the protein constituents of the CD3 receptor. Mutations in the genes encoding the CD3- or epsilon chains may lead to defective expression of and signaling through the TCRT-cell complex. [146] [147] Rare cases have been associated with abnormal transmembrane calcium influxes after anti-CD3 antibody binding. Calcium entry is required for calcineurin activation and nuclear translocation of cytoplasmic nuclear factor of activated T cells (NF-AT),[148] [149] a T-cell-specific transactivating factor that binds to a response element in the regulatory region of the IL-2 gene.

Occasional instances of combined immunodeficiency with a normal number of T cells have been attributed to defective production of IL-2 or from failure of IL-2 gene transcription after T-cell activation. [150] [151] [152] The defects causing IL-2 deficiency may be heterogeneous. In vitro proliferative responses to mitogens are augmented by exogenous IL-2. In defects associated with defective in vitro production of IL-2, in vivo trials with IL-2 or polyethylene glycol-modified IL-2 have been attempted. [150] [153] IL-2 deficiency may be accompanied by deficiency of other cytokines (IL-4, IL-5, and IFN-). [151] In some patients, there is a defect in activation or binding activity of the T lymphocyte NF-AT.[149] [154] Abnormal responses to IL-1 and IL-2 have also been associated with combined immunodeficiency. Mutation of the chain of the IL-2 receptor gene can give rise to a form of SCID with decreased numbers of circulating T cells and abnormal T-cell infiltration of tissues secondary to failure of down-regulation of the antiapoptotic protein bcl-2 during thymocyte development. [155] [156]

ZAP-70 Deficiency (CD8 Deficiency)

This rare immunodeficiency is characterized by decreased CD8+ T lymphocytes with normal to increased numbers of CD4+ T cells, which fail to function. [157] [158] [159] The disorder arises from mutations of the ZAP-70 chain, a zeta (TCR component) associated protein kinase gene, that encodes a 70 kDa, non- *Src* protein tyrosine kinase of the Syk family. ZAP-70 kinase associates with the TCR after T-cell activation to transduce signals from the TCR (Fig. 42-4 (Figure Not Available)). [160] Cross-linking of the TCRCD3 complex with antigen leads to recruitment of CD4 or CD8, which is associated with the *Src* protein tyrosine kinase Lck. The recruited *Lck* or protein tyrosine kinase Fyn phosphorylates the various immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCRCD3 complex, providing binding sites with SH2 domains that are capable of binding phosphotyrosines in the proper context. ZAP-70, a protein tyrosine kinase with tandem SH2 domains, binds to the phosphotyrosines of individual ITAMs and is phosphorylated by Lck or Fyn to induce its activation. Subsequently, other signaling and adaptor molecules (e.g., phospholipase C1, Vav, Cbl, SLP-76, pp36) are recruited to the activated TCR and ZAP-70, and in turn are activated to form a multicomponent complex that signals downstream activation events. In the absence of a functional ZAP-70, TCR-mediated signaling is lost.

Figure 42-4 (Figure Not Available) T-cell signal transduction pathway. The TCR is associated with CD3 and , CD4 or CD8, CD28, and CD45 on the plasma membrane. Immediately after antigen binds to the TCR, cytoplasmic protein tyrosine kinases (PTK) (Lck, Fyn, ZAP-70, and Syk) associated with the TCR become activated. PTK activation results in the phosphorylation of PLC1 and the activation of other kinases. Distal signaling events, including PKC activation and calcium mobilization, result in the transcription of genes encoding IL-2 and other molecules required for T-cell activation and proliferation. ZAP-70 deficiency prevents TCR signaling. *(From Elder ME: Investigation of signal transduction defects. In Rose NR, Conway de Macario E, Folds JD et al [eds]: Manual of Clinical Laboratory Immunology, ASM Press, Washington, DC, 1997, p. 830.)*

The paucity of CD8+ T cells is due to the failure of maturation and selection in the thymus. [157] Although there is an accumulation of double-positive thymocytes in the cortex in the thymus medulla in this disorder, only single-positive CD4+ T cells and not CD8+ T cells are observed, and only mature single-positive CD4+ T cells egress to the periphery. The CD4+ T cells fail to proliferate, however, when incubated in vitro with phytohemagglutinin, concanavalin A, or antigens that activate through the TCR, but are normally activated by stimulation with phorbol esters and calcium ionophore, which bypass the requirements of TCR-associated protein tyrosine kinases. NK cells are normal in number and function.

Purine Nucleoside Phosphorylase Deficiency

Purine nucleoside phosphorylase deficiency is a rare, autosomal recessive disease of the purine metabolic pathway. [161] The immunodeficiency is progressive, and symptoms, which are variable, begin commonly in early infancy with enhanced susceptibility to opportunistic viral and fungal infections and failure to thrive. Death may result from an overwhelming viral infection from herpes zoster, measles, or cytomegalovirus. The immunodeficiency is thought to be due to failure of conversion of deoxyguanosine to guanine and its consequent phosphorylation to deoxyguanylic acid (dGMP) in lymphoid tissue. The toxic metabolite deoxyguanosine triphosphate (dGTP) accumulates in cells and inhibits ribonucleotide reductase, required for DNA synthesis and proliferation of thymocytes. T-cell numbers are markedly decreased. In vitro T-cell responses are abnormal, with absent delayed-type cutaneous hypersensitivity. There is an attrition in T-cell function over time. In contrast, antibody and Ig production in some patients is preserved, and at times the antibody response is exaggerated. Autoantibodies, including rheumatoid factors, antinuclear antibodies, and antibodies to blood cells, may be produced, and autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, and systemic lupus erythematosus may ensue. The reasons for preferential toxicity for T cells and the clinical and laboratory differences from ADA deficiency have not been completely elucidated. Megaloblastic anemia, neurologic abnormalities with spastic paresis of the trunk and extremities, developmental delay, or mental retardation may be observed. Diagnosis is made by quantitating purine nucleoside phosphorylase activity in erythrocytes, lymphocytes, or fibroblasts and levels of deoxyguanosine and dGTP in blood. A low serum uric acid level is a useful screening assay for this enzyme deficiency. Prenatal diagnosis is possible. Attempts at enzyme replacement therapy have not altered the clinical course, and bone marrow transplantation is the preferred form of therapy. Gene therapy will probably be tried in the future to correct the enzymatic abnormality.

Clinical Manifestations

The different forms of SCID are generally clinically indistinguishable. [108] [109] [110] SCID usually presents in early infancy with recurrent severe infections, or persistent infections with low-virulence, opportunistic organisms, such as *Candida albicans* or *Pneumocystis carinii*, giving rise to chronic thrush or interstitial pneumonitis, respectively. Chronic diarrhea and failure to thrive usually occur. Recurrent otitis media, skin infections, and chronic severe viral infections are frequently present. The diagnosis of SCID should be considered a pediatric emergency, because correction of the defect becomes more difficult if serious infection ensues before bone marrow transplantation.

In contrast to other forms of SCID, patients with partial ADA deficiency may have symptoms delayed until the latter half of the first or second year of life, or even at older ages (38 years). [118] [119] [120] With partial ADA deficiency, there is slower progression of the deterioration of immunity because of the time required to accumulate the toxic metabolites. About half of the children with ADA deficiency have radiologic anomalies of the rib cage, with flared costochondral junctions, scapula, and skeleton, but these abnormalities are not specific for ADA deficiency. Neurologic and hepatic abnormalities have been observed with ADA deficiency.

Patients with MHC class II deficiency clinically present in the first year of life with a picture typical of SCID with recurrent, severe bacterial, viral, or fungal infections causing recurrent bronchopulmonary disease, chronic protracted diarrhea, malabsorption, liver disease, or failure to thrive. [138] [139] Death is often caused by severe viral infections that occur in the first years of life.

Graft-versus-host disease may arise from transfusion of nonirradiated blood products or from maternal/fetal transfer of lymphocytes in utero or at delivery. It may be associated with fever, eczematoid rash, diarrhea, hepatosplenomegaly, and eosinophilia. [162] Although engraftment with maternal lymphocytes occurs in up to 4050% of cases of SCID, symptomatic GVHD often does not ensue, probably because the maternal lymphocyte cells are immature, represent a limited repertoire, and have suboptimal responses to activation. [163] [164]

Laboratory Evaluation

The diagnosis of SCID should be established at the earliest age possible so that optimal therapy can be offered. The total lymphocyte count is almost invariably low in SCID, but normal lymphocyte counts do not exclude the diagnosis. The lymphocyte counts must be compared to age-matched healthy controls. T-cell numbers, especially CD4+ T cells, are usually markedly decreased, and those present may display an immature phenotype. If substantial numbers of T cells are present, they may be derived from engraftment of maternal lymphocytes, or reflect the presence of a T-cell activation defect or MHC class II deficiency. With the latter, an increase in CD8+ T cells masks a decrease in CD4+ T cells. ZAP-70 deficiency is associated with a normal number of dysfunctional CD4+ T cells with a paucity of CD8+ T cells. In X-SCID and JAK3 deficiency, the B cells are normal or increased in number but fail to mature and function. ADA deficiency and deficiency of RAG1 or RAG2 are associated with a decrease in both T and B lymphocytes.

In vitro T-cell responses to mitogens and allogeneic cells are absent or very poor, and responses to antigens are absent in SCID. Cutaneous delayed-type hypersensitivity reactions are absent. Immunoglobulin levels are usually decreased, and antibody responses are usually absent, except in some patients with partial ADA deficiency. NK cells are decreased in $_{c}$ -deficient, JAK3-deficient, and ADA-deficient SCID, although in ADA-deficient SCID, NK cell function may be preserved in the face of low numbers of NK cells. The thymus in SCID is small, weighing <1 g, and lacks thymocytes, Hassalls corpuscles, and a corticomedullary junction.

Special studies that may be necessary include assays for the $_{c}$ chain, JAK3, ADA enzyme activity, dATP, and deoxyadenosine, purine nucleoside phosphorylase activity, dGTP and deoxyguanosine, ZAP-70 kinase, signal transduction in T cells, class I and class II MHC surface expression, and skin biopsy of rashes to establish a diagnosis of GVHD.

For many of these diseases, prenatal diagnoses by genetic analysis or immunologic evaluation of fetal blood can be performed. ADA-deficient SCID, X-SCID, and JAK3 deficiency can be diagnosed prenatally and the carrier stage can be identified. The prenatal diagnosis of ADA deficiency is performed by assaying ADA activity or dATP levels in cultured amniotic cells, fetal blood obtained by fetoscopy, or chorionic villi samples. However, some children have small amounts of residual ADA activity, which may make diagnosis difficult. The prenatal diagnosis of SCID can be performed by molecular approaches

once the $_{c}$ or JAK3 gene defect in family members has been identified.

Therapy

If the immunodeficiency is not corrected, SCID is invariably fatal. Transplantation of bone marrow cells from HLA genotypically identical family donors completely restores the immunologic function of patients with different forms (X-linked, JAK3 deficiency, autosomal recessive ADA/+, reticular dysgenesis) of SCID, and is successful in correcting MHC class II deficiency, purine nucleoside phosphorylase deficiency, Wiskott-Aldrich syndrome, and X-linked hyper IgM. [165] [166] Pretransplant preparative cytoablation is not required for haploidentical transplants. The success rate of HLA-identical bone marrow transplantation now approaches 100%, with engraftment of both donor T and B lymphocytes. Lymphoid and hematopoietic stem cell engraftment is required to correct SCID from reticular dysgenesis, MHC class II deficiency, and IL-1 deficiency. In the absence of matched related donors, T-cell-depleted haploidentical parental, [165] [166] [167] [168] matched unrelated donor bone marrow transplantation, [169] and umbilical cord blood stem cell transplantation [170] have been successful with relatively low frequencies of GVHD. Mature T cells are depleted from the bone marrow by physical or immunologic approaches to prevent GVHD from transferred mature donor T cells. Donor stem cells develop into T cells that undergo clonal deletion and anergy in the host thymus to establish a state of tolerance in the host. Although T cells can engraft in the absence of GVHD with these purged bone marrows, immunologic reconstitution is slower than that observed after HLA-identical bone marrow transplantation. This is probably because T cells are developing solely from donor lymphoid stem cells with recapitulation of immunologic ontogeny. B-cell engraftment may be extremely delayed or fail to occur

with failure of cooperation between donor haploidentical T-helper cells and host B cells. The success rate of haploidentical bone marrow transplants now exceeds 70% and is approaching the success rate of genotypically identical bone marrow transplants. The presence of pre-existing infection or GVHD hinders the success rate of transplantation. In utero transplantation of parenteral CD34+ hematopoietic progenitor cells has corrected X-SCID diagnosed in the fetus. [171] [172]

All blood products should be irradiated before transfusion of patients with SCID to prevent GVHD. Ig replacement therapy, nutritional support, and prophylaxis against *P. carinii* infection are usually required prior to bone marrow transplantation. Family members should be immunized with killed poliovirus vaccine to prevent infection in patients.

In the absence of an HLA-identical bone marrow donor for correction of ADA-deficient SCID, and as an alternative to HLA-haploidentical bone marrow transplantation, patients may be treated with ADA enzyme replacement therapy by intramuscular injection of polyethylene glycol (PEG) modified bovine ADA. [173] Modification of ADA with PEG prolongs the circulating half-life of the enzyme and decreases its immunogenicity. PEG-ADA can improve the immunologic and clinical status of patients with ADA deficiency. The exogenous ADA lowers the level of the toxic metabolites in ADA-deficient lymphocytes by deaminating adenosine and 2-deoxyadenosine. HLA-haploidentical bone marrow transplantation is an alternative to ADA replacement therapy favored by some for treatment of ADA-SCID in the absence of an HLA-identical donor. Use of PEG-ADA before bone marrow transplantation, however, may restore host immunologic function and predispose to graft rejection. Similarly, ADA activity in the transplanted marrow may stimulate immunologic function of the recipients residual T cells and prevent engraftment, which can be avoided by the use of pretransplant cytotoxic agents.

Gene replacement therapy to correct ADA deficiency permanently is under investigation. [174] [175] [176] [177] Older children have been treated with autologous IL-2 activated peripheral blood T cells and neonates with autologous CD34+ pluripotent umbilical cord blood stem cells that were transduced with retroviral vectors containing the gene encoding human ADA. The long-term success of somatic ADA gene therapy is not defined at this time.

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IMMUNOGLOBULIN DEFICIENCY WITH NORMAL OR INCREASED IgM (HYPER-IgM SYNDROME)

Etiology and Pathophysiology

Immunoglobulin deficiency with normal or increased IgM is associated with recurrent infections and normal or increased serum levels of polyclonal IgM and, at times, IgD, with low levels of other immunoglobulin isotypes.[12] [178] [179] Both pyogenic and opportunistic infections, such as *P. carinii, Candida, Crytosporidium, Cytomegalovirus,* and *Mycobacteria,* occur. Although B cells are normal in number, the only isotypes expressed on their surface are usually IgM and IgD. When B cells are stimulated in vitro or immortalized with EBV, they secrete only IgM. Plasmacytoid cells spontaneously secreting IgM may be present in the circulation and in lymphoid tissues. Rarely, cells expressing surface IgM and IgG can be detected, but this may result from alternative splicing of a long germline encoded C_H region sequence RNA transcript that has not undergone isotype switch recombination.

The disease may be inherited in an X-linked (X-HIM) or autosomal recessive manner. X-HIM, which maps to Xq26, is due to failure of activated T cells to express a functional CD40 ligand, a type II transmembrane glycoprotein that binds CD40 on B cells to signal the B cell to proliferate, undergo deletional isotype switching, and develop into memory B cells.[5] [12] [180] [181] CD40 ligandCD40 interactions are required for induction of germinal center formation in lymphoid follicles and generation of memory B cells, which occurs in germinal centers.[181] CD40 ligandCD40 interactions induce the expression or up-regulation of costimulatory molecules (e.g., B7.1 and B7.2) on B cells that are required for effective T cellB cell interactions. Defective CD40 ligand expression may therefore lead to decreased full activation of responding T cells from the poor expression of costimulatory molecules and cytokine production. The opportunistic infections observed in X-HIM may reflect this T-cell defect as well as decreased activation of CD40-expressing macrophages. Missense and nonsense mutations and deletions in the CD40 ligand gene have been described.[180] Cross-linking of CD40 on B cells from patients with X-HIM with antibody or functional CD40 ligand and stimulation with the appropriate cytokines can bypass the defect and activate normal isotype switching and immunoglobulin secretion.

A disease similar to X-HIM is autosomal recessive inherited and arises from defective CD40 expression or signal transduction in B cells.[182] In some of these patients, no mutations have been detected in CD40 or CD40 binding proteins, and the syndrome arises secondary to a defect in the CD40-triggered signaling cascade. An acquired form of this syndrome has been described associated with congenital rubella infection or anticonvulsant (phenytoin) therapy, but usually the etiology of the acquired form is unknown.

Clinical Manifestations

Pyogenic bacterial infections begin during the first or second year of life, as observed in XLA, with recurrent otitis media, upper respiratory tract infections, and pneumonia.[178] [179] In contrast to other primary humoral immunodeficiencies, the hyper IgM syndrome is associated with an unusual susceptibility to *P. carinii* pneumonia, *Candida, Cryptosporidium, Cytomegalovirus,* and *Mycobacteria.* In fact, *P. carinii* pneumonia is a common form

of presentation in the first year of life.[179] This susceptibility may be explained by failure of CD40 ligand on activated T cells to activate CD40-expressing macrophages or dendritic cells to secrete IL-12, which is required for T_H 1-type immune responses. Thus, there is failure to develop the ability to kill microbes or form complete T-cell activation due to lack of costimulation or cytokine activation. Diarrhea and sclerosing cholangitis occur secondary to *Cryptosporidium* infection. Lymphoid hyperplasia with lymphadenopathy, tonsillar enlargement, and hepatosplenomegaly occur. Chronic neutropenia occurs in about half of the patients and arises from defective myeloid differentiation at the promyelocyte or myelocyte stage. The neutropenia may be cyclic or episodic, where it is associated with infection. Stomatitis and chronic recurrent oral or rectal ulcers occur as a complication of the neutropenia. Autoimmune manifestations include arthritis or arthralgia, nephritis, and hemolytic anemia. Surface IgM-positive non-Hodgkins and Hodgkins lymphoma with gastrointestinal tract, liver, or gallbladder involvement may also be observed.

Laboratory Evaluation

Serum levels of IgM and, at times, IgD are markedly elevated, and the other isotypes are low or absent. At an early age, however, the IgM level may be normal. In a recent study, only half the patients with X-HIM had increased serum IgM levels at the time of diagnosis, and one third of patients failed to develop increased levels of IgM with follow-up.[179] The IgM is polyclonal, and antibody responses, when they occur, are restricted to the IgM isotype. In addition to the normally occurring 19S IgM molecules, a 7S IgM fraction may be produced. Peripheral blood B cells are normal in number and express IgM and IgD, but cells with surface IgG or IgA usually, but not invariably, are absent. In vitro stimulation of peripheral blood B lymphocytes with anti-CD40 in the presence of IL-10 or IL-4 will activate isotype switching and secretion of IgG or IgE, respectively, in X-HIM. Definitive diagnosis of X-HIM is made by demonstrating the lack of binding of soluble forms of CD40 to CD40 ligand on activated T cells. Some patients generate a defective CD40 ligand protein that binds anti-CD40 ligand antibody but fails to bind CD40. In vitro lymphocyte proliferative responses to soluble antigens may be decreased. Lymph nodes lack secondary follicles with germinal centers, and show a severe depletion of follicular dendritic cells. Female carriers of X-HIM can be diagnosed by demonstrating expression of functional CD40 ligand on only half of their activated T cells.

Therapy

Bone marrow transplantation should be considered because of the high morbidity and mortality of the syndrome. Ig replacement therapy is indicated. With Ig replacement therapy, increased IgM levels decrease in about half of the patients,[179] presumably on the basis of feedback inhibition and prevention of infection. Lymphoid hyperplasia and neutropenia may also be reversed with Ig replacement therapy. The neutropenia may also respond to therapy with granulocyte colony-stimulating factor. Prophylaxis to prevent *P. carinii* pneumonia is required. Identification of the genetic basis of X-HIM has improved diagnosis, prenatal detection, and counseling, and will lead to gene therapy for this disease.

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WISKOTT-ALDRICH SYNDROME

Etiology and Pathophysiology

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive syndrome characterized by the triad of eczema, thrombocytopenia, and immunodeficiency. [183] [184] The cell lineages affected by the defect include both lymphoid and hematopoietic lines. The Wiskott-Aldrich gene maps to position Xp11.22-p11.3 on the X chromosome and encodes a 502 amino acid protein termed WASP (Wiskott-Aldrich syndrome protein). [185] [186] WASP localizes in the cytoplasm with polymerized actin, where it is thought to link the actin cytoskeleton to signaling from surface receptors. [187] There is a GTPase binding site on WASP that binds to GTP carrying Cdc42, a member of the Rho family of GTPases. This results in remodeling of the actin cytoskeleton to alter cell shape and motility. Actin remodeling leads to formation of filopodia, finger-like projections on the cell surface that aid cell movement in response to external stimuli; filopodia are critical for polarization and orientation of T cells toward antigen-presenting cells. [188] An additional site where WASP acts is in signal transduction pathways by binding to *Src* homology 3 (SH3)-containing proteins, such as Nck, and nonreceptor tyrosine kinases, such as Fyn and Itk.

The exact contributions of the WASP defect to the hematologic and immunologic abnormalities in WAS are under investigation. [189] An increased percentage of peripheral blood lymphocytes or T-cell lines with decreased surface microvilli, giving a bald appearance, are observed by scanning electron microscopy. The T cells in WAS display an unusual morphology, with disorganized submembranous actin cytoskeleton cells and decreased surface microvilli, that have actin at their core. The cell surface glycoprotein CD43, termed leukosialin or sialophorin, a major component of the plasma membrane that contributes heavily to the surface charge on lymphocytes and colocalizes with the cell surface microvilli, has been reported to be absent, decreased, or structurally altered in lymphocytes of patients with WAS. [190] The defect in polarization and orientation of T cells toward antigen-presenting cells described above may explain the poor antigen-specific T-cell responses in WAS.

The etiology of the thrombocytopenia, which is the most consistent finding of the triad, has not been completely explained. [189] [191] Abnormal thrombocytopoiesis makes a major contribution to the thrombocytopenia, which may be explained by a tyrosine kinase signaling pathway that signals through WASP and controls assembly of actin, and has been shown to be essential for megakaryocyte differentiation. [192] Increased platelet destruction due to an intrinsic platelet defect also contributes to the thrombocytopenia. The mean platelet volume is reduced, but there is a broad overlap with the normal range. The platelets in WAS express decreased surface glycoprotein IIb, IIIa, and IV and display defective thrombin-induced expression of CD62P and CD63 and decreased actin. After splenectomy, the platelet count usually increases, and the mean distribution of the platelet volume returns to normal, indicating that platelet circulation through the splenic microarchitecture leads to reduced platelet size. [193] [194] Platelet-associated IgG is frequently present; it disappears after splenectomy. Relapse of thrombocytopenia after splenectomy may be accompanied by autoimmune thrombocytopenia and redevelopment of platelet-associated IgG. With relapse, the platelet volume does not decrease to the presplenectomy size, but fails to increase to the size observed in immune-mediated platelet destruction.

Hereditary X-linked thrombocytopenia is characterized by thrombocytopenia and small platelets, similar to WAS, without the immunodeficiency or eczema. [195] This disorder is an allelic variant of WAS arising from mutations of the WASP gene that are usually point or missense mutations instead of the frameshifts, splice, or nonsense mutations that may be observed in WAS. [196] [197]

Carrier females are asymptomatic because of selection against cells that have the X chromosome carrying the mutant Wiskott-Aldrich gene as their active X chromosome. Nonrandom X chromosome inactivation patterns are observed in obligate carriers in T and B lymphocytes, monocytes, polymorphonuclear leukocytes, and platelets, but not in fibroblasts. [5]

Clinical Manifestations

The initial symptoms are usually petechiae or bleeding in the skin, mouth, nose, the gastrointestinal or urinary tract, or from the umbilical cord, beginning in the first several months of life. Thrombocytopenia is present at birth. [198] [199] A life-threatening bleed (gastrointestinal, intracranial, or severe oral) is observed in up to one third of patients before a diagnosis is made. Early in life, an eczematoid rash appears, most prominent in the antecubital and popliteal fossae; it tends to become petechial. At the time of diagnosis, only about 25% of patients with WAS have the classic triad of thrombocytopenia, eczema, and recurrent otitis media. [199] Infections with bacteria, fungi, or viruses cause recurrent otitis media with chronic purulent drainage and perforated tympanic membranes, recurrent pneumonia and sinusitis, and recurrent herpes infections. The thrombocytopenia and bleeding tendency are often worsened with infection. Chronic or recurrent herpes simplex or disseminated varicella may occur. Autoimmune disorders develop in up to 40% of patients with arthritis, vasculitis, uveitis, inflammatory bowel disease, or autoimmune hemolytic anemia.

The median survival in WAS patients is 6.5 years, with the cause of death being infection, usually from pneumonia or sepsis, in 59%, bleeding in 27%, and malignancy in 5% of patients. The risk of malignancy in WAS is 100-fold higher than in normals. A high incidence of lymphoreticular tumors that initially present at extranodal sites, especially non-Hodgkins lymphomas with B-cell-immunoblastic sarcomas, is observed in those who survive to later childhood. [7] [8] [9]

Laboratory Evaluation

The serum IgM level is decreased in older children and adults with WAS but may be normal early in life. IgA and IgE levels are often increased. IgG paraproteins may be present. Poor to virtually absent antibody responses to polysaccharide antigens, and defective low titer with antibody responses to protein antigens, lack of immunologic memory, and isotype switching occur. Titers of isohemagglutinins to the blood group polysaccharide antigens are usually low. T-cell responses to nonspecific mitogens are normal to decreased, but are usually absent to anti-CD3 antibody and antigens. Delayed-type hypersensitivity skin reactions are also usually absent. Lymphocyte counts are usually normal at diagnosis, and then decrease with age as a result of a progressive decline in T-cell numbers, especially CD8+ T cells. Peripheral lymphoid tissues gradually become depleted of T cells. Eosinophilia may be present. The lymphocytes have a characteristic bald appearance on scanning electron microscopy. The platelet count is decreased, ranging from 10,000 to 100,000/mm ^{3} , and the platelet size is characteristically small, a feature relatively specific for WAS or X-linked thrombocytopenia. The platelet count may vary considerably in a patient, with a decrease occurring with viral infections. The number and morphology of megakaryocytes in the bone marrow are normal. The bleeding time is more prolonged than would be predicted from the platelet count. The carrier state can be diagnosed by demonstrating nonrandom X-chromosome inactivation patterns in T cells, B cells, or polymorphonuclear leukocytes. Prenatal diagnosis can be made by detecting polymorphisms flanking the WASP gene or WASP mutations and by decreased fetal platelet count and size.

Therapy

Bone marrow transplantation can correct all of the defects of this otherwise fatal disorder, including the susceptibility to malignancy. [200] Successful engraftment requires preparative ablation or cytoreduction of the recipients bone marrow. While the success rate with HLA-identical marrow is high, the success rate of haploidentical parental bone marrow transplantation has been disappointing, complicated by severe viral infections, lymphomas, EBV-induced B-cell lymphoproliferative disease, or chronic GVH in the post-transplantation period. Matched unrelated donor bone marrow transplantation for WAS has met with some success, especially when performed at an early age. [169]

Splenectomy combined with regularly administered prophylactic antibiotics has been used to manage some patients with severe bleeding problems who could not undergo transplantation. [193] [194] Splenectomy increases the platelet count and decreases the bleeding tendency, but it may increase susceptibility to overwhelming sepsis, even with the use of prophylactic antibiotics. The majority of splenectomized patients have a sustained increase of platelet counts by at least 20,000/mm ^{3}, and in over half of patients by at least 100,000/mm ^{3}. Recurrences of thrombocytopenia with an acute ITP-like episode, however, can occur after splenectomy. Corticosteroids and intravenous Ig have been used to treat such patients. Intravenous Ig replacement therapy is indicated to decrease the risk of infections but is not invariably efficacious in decreasing the frequency of infections.

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ATAXIA-TELANGIECTASIA

Etiology and Pathophysiology

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder characterized by progressive cerebellar ataxia, telangiectasia, recurrent sinopulmonary infections, combined immunodeficiency, an increased incidence of malignancies, and premature aging. [201] Although the frequency of A-T is estimated to be only 1 per 40100,000 live births, the carrier state is relatively frequent (1%) in the population, and heterozygotes, as well as homozygotes, appear to be at increased risk for malignancy. [202] The underlying defect in A-T is due to mutations of the ATM (A-T mutated) gene that encodes a 350 kDa protein kinase with a phosphatidylinositol (PI) 3-kinase domain involved in multiple signaling pathways of cellular responses to DNA damage, and in control of the cell cycle and DNA replication, recombination, and repair.[203] [204] [205] [206] A-T cells display chromosomal instability and hypersensitivity to agents that cause DNA strand breaks, including -rays and x-rays, ionizing radiation, and radiomimetic chemicals such as bleomycin. [201] [202] [203] [204] Cells of heterozygous carriers of A-T show a sensitivity intermediate between those of normal individuals and homozygotes. A-T cells have an increased number of spontaneous and radiation-induced chromosome breaks, which are not rejoined as efficiently as normal cells.

Cells in A-T do not reduce their rate of DNA synthesis after irradiation as do normal cells because of dysfunction or absence of the ATM protein. In normal cells, irradiation-induced double-strand breaks in DNA lead to an arrest at the G_1-S transition, S, or G_2-M transition phases of the cell cycle. These cell cycle checkpoints ensure that damaged DNA is not replicated and cell division does not occur until DNA strand breaks have been repaired. The A-T gene product functions in several irradiation-induced signal transduction pathways (which operate through p53, p21, and cyclinE-cyclin-dependent kinase [cdk] complexes or through the *abl* proto-oncogene, or *Chk1,* Cdc25, and Cdc2, cyclin B) that recognize DNA damage and transduce a signal from damaged DNA to arrest the cell cycle and repair damaged DNA.[205] [206]

A high spontaneous rate of intrachromosomal recombination is observed in A-T, and there is a high frequency of lymphocytes with chromosome translocations and aberrant interlocus TCR gene rearrangements.[207] [208] The defective checkpoint response to DNA strand breaks may lead to an accumulation of aberrant recombinations of immunoglobulin and T-cell receptor genes, and result in the high frequency of lymphoid tumors observed in A-T. Chromosomal breaks, inversions, and translocations cluster at bands 7p13-p14, 7q32-q35, 14q11, and 14q32 in

510% of peripheral T lymphocytes in A-T. [209] [210] It is at these sites that the T-cell and immunoglobulin receptor genes normally undergo DNA rearrangement to generate a functional gene. This may account for the T- and B-cell abnormalities and low number of / compared to / bearing TCR in A-T. [211] In approximately 10% of A-T patients, expanded clonal populations of nonmalignant T cells with translocations involving chromosomes 7 or 14 at 14q32.1, centromeric to the immunoglobulin heavy chain locus, and possibly the site of a putative oncogene, appear in the blood. [209] [210] These clonal cells have a proliferative advantage, and may increase in a quiescent fashion in the blood over a period of years. In some instances, they evolve into a T-cell leukemia. It is possible that the nonrandom translocation confers a preactivated, premalignant state that progresses to malignant transformation after a second genetic event.

In A-T there are also defects in tissue differentiation, with failure of organ maturation. The immunologic defect is characterized by absence of or production of only a small thymus that has an embryonic histologic appearance, with lack of Hassalls corpuscles and sparse population with lymphoid cells. [201] Other evidence for tissue immaturity includes the presence of elevated levels of serum -fetoprotein and carcinoembryonic antigen, produced normally by fetal organs, ovarian agenesis, and hypogonadism. In the absence of cell cycle arrest with ATM gene dysfunction, even low levels of DNA damage in A-T cells may activate an apoptotic cell death, which in normal cells would be activated only by irreparable DNA damage. This increased apoptosis may explain the thymic and other organ atrophy in A-T.

Clinical Manifestations

The most common initial presentation of A-T is a progressive cerebellar ataxia that is observed in the second year of life. [212] Other neurologic symptoms occurring later are choreoathetosis, nystagmus, strabismus, dysarthric speech, and decreased deep tendon reflexes. Telangiectasias appear later in childhood, and are most prominent on the bulbar conjunctivae, exposed areas of the skin, external ear, eyelids, face, flexor folds of the neck, extremities, and dorsa of hands and feet. Recurrent sinopulmonary infections occur in the majority of patients and may become chronic, resulting in bronchiectasis and respiratory insufficiency. The overall risk of cancer is 60180 times the normal rate, with up to 10% of patients developing malignancies. [7] [8] [9] [202] [206] [213] Black patients with A-T have even higher risks of malignancy, especially lymphomas and leukemia. The majority of neoplasms occur before age 15. Non-Hodgkins lymphomas with histologic subtypes that are observed with chromosome 14 translocations, and Hodgkins lymphoma, mostly of the lymphocytic depletion type, T-cell leukemia, and epithelial carcinomas predominate. Heterozygotes for the A-T gene have an approximately threefold to fourfold increased risk of malignancy. It is suggested that heterozygous females, who have an approximately fivefold increased risk of breast cancer, account for up to 8% of all cases of breast cancer in the United States. Caution must be exercised in the treatment of neoplasms because of the increased sensitivity of patients with A-T to radiation therapy. Endocrinologic abnormalities occur commonly, including delayed somatic growth, insulin resistance, delayed development of secondary sexual characteristics in females (secondary to absent or hypoplastic ovaries), hirsutism in females, and hypogonadism in males. Premature aging of the skin and diffuse graying of the hair usually occur.

Laboratory Evaluation

Absence or deficiency of serum IgA, secretory IgA, and IgE are found in about 75% of patients. Low levels of IgG2 and IgG4 and decreased antibody responses to immunization are also found. Anergy to delayed-type hypersensitivity skin testing, decreased T-cell numbers, and abnormal in vitro lymphocyte proliferative responses commonly occur. Serum levels of -fetoprotein are increased in over 95% of patients, and levels of carcinoembryonic antigen may be elevated. Nonrandom chromosomal breaks and chromosome 14 translocations are frequently detected.

Therapy

No therapy can halt the disease, and treatment is symptomatic. Antibiotics are indicated for recurrent pulmonary infections, and Ig replacement therapy may be indicated. Exposure to excessive sunlight should be minimized to prevent skin changes. Malignancies in A-T patients may require the use of lower doses of radiotherapy and chemotherapy.

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DIGEORGE/VELOCARDIOFACIAL ANOMALY (THIRD AND FOURTH PHARYNGEAL POUCH SYNDROME)

Etiology and Pathophysiology

The DiGeorge or velocardiofacial syndrome arises from either monosomy of 22q11.2, which occurs from a deletion, most frequently of maternal origin, or an unbalanced translocation of the chromosome.[214] [215] [216] Up to 25% of cases are inherited as a 22q11.2 deletion. This genetic defect results in an abnormality of, or an insult to development of, the third and fourth pharyngeal pouches, occurring during the fourth to seventh week of fetal development. This contributes to the abnormal development of the thymus, parathyroid glands, conotruncal components of the heart, ear, and facial structures. [214] [217] The acronym CATCH22*c*ardiac abnormalities, *a*bnormal facies, *t*hymic hypoplasia, *c*left palate, *h*ypocalcemiahas been applied to the syndrome to designate the involved organs. The genetic basis by which the 22q11 deletion gives rise to the DiGeorge anomaly is not known, and will require identification of the minimal critical genetic region. It is suggested that the embryologic reactive unit affected is the cephalic neural-crest mesenchymal population of cells. [217] The failure of the neural crest to contribute to the mesenchymal derivatives of the pharyngeal pouches may explain the heart lesions typically found in the syndromeinterrupted aortic arch type B and truncus arteriosus. The anomaly may arise from an environmental insult and has been associated with maternal alcoholism and diabetes or exposure to retinoids. [214] [218]

Development of the thymus, which is formed from the endodermal epithelium of the third and fourth pharyngeal pouches, is interrupted. [214] The thymus may be completely absent in the most severe form of this developmental field defect, but more frequently it is small and hypoplastic and ectopic in location, because of lack of descent into the mediastinum. This variability in thymic development produces variable T-cell deficiency. Most patients have a partial T-cell deficiency with decreased T-cell number and function at birth that corrects itself with time. More rarely, complete thymic aplasia (complete DiGeorge anomaly) is present with profound deficiency of T-cell number and function. When present, it is associated with a marked susceptibility to viral and fungal infections. Parathyroid aplasia is present at birth and produces hypocalcemia.

Clinical Manifestations

A broad and variable spectrum of malformations may occur.[214] [219] [220] Affected infants usually present with hypocalcemic tetany in the newborn period. Cardiac malformations include interrupted, right-sided, or double aortic arch, truncus arteriosus

, patent ductus arteriosus, or ventricular septal defect, and give rise to cyanosis or cardiac murmurs. Type B interrupted aortic arch or truncus arteriosus occurs frequently and is suggestive of the diagnosis. Facial dysmorphic features include hypertelorism, antimongoloid slant of the eyes, midline facial clefts, micrognathia, shortened philtrum of the lip, cleft palate, choanal atresia, low-set posteriorly rotated ears, and notched ear pinnae. If the T-cell deficiency is severe, which occurs in less than 25% of patients,[214] [221] [222] the infant may develop chronic or recurrent rhinitis, pneumonia, candidal infections, diarrhea, and failure to thrive. Moderate to severe mental retardation and developmental delay are common, and hearing loss may be present.

Laboratory Evaluation

Total T cells are low, and B-cell numbers and total lymphocyte counts are usually normal. At times, B-cell numbers are increased. In vitro T-cell responses to nonspecific mitogens or allogeneic lymphocytes may be normal or absent. Unless there is a severe T-cell defect, immunoglobulin levels of IgG, IgA, and IgM are usually normal, or at times increased. Patients with CD4+ T-cell counts of <400/l and decreased in vitro T-cell responses to mitogens may have increased susceptibility to infection and a persistent immunodeficiency. [221] Profound T-cell deficiency results in defective antibody responses. Decreased serum calcium, increased phosphorus, and decreased parathyroid hormone levels are present. Cytogenetics with fluorescence in situ hybridization should be used to identify chromosome 22q11.2 abnormalities in the patient and parents.

Therapy

Hypocalcemia should be treated with intravenous calcium replacement; it may prove difficult to control. Long-term treatment with oral calcium replacement, vitamin D, and a low-phosphate formula is usually required. The cardiac abnormalities, which may be severe, must be evaluated completely. Therapy is not usually required for partial T-cell deficiency, since there is spontaneous improvement in T-cell function with increasing age. In patients with severe, persistent T-cell deficiency with marked susceptibility to infections and failure to thrive, transplantation of fetal thymus, postnatal thymus, or bone marrow may be indicated, but the efficacy of thymic transplantation for complete DiGeorge anomaly has not been critically evaluated. [214] [223] Blood products should be used from cytomegalovirus-negative donors and be irradiated before administration to prevent GVHD, a potential complication with severe T-cell deficiency. Antibiotic prophylaxis to prevent *P. carinii* pneumonia is also indicated in the face of T-cell deficiency. Prenatal diagnosis is possible, and may be indicated if molecular evaluation is informative in a parent of an affected child.

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Chapter 43 - Histiocytic Disorders

Jeffrey M. Lipton

INTRODUCTION

The histiocytic disorders comprise a broad grouping of hematologic diseases united only by the observation that a dendritic cell or monocyte/macrophage appears to be the principal pathologic protagonist. The ubiquitous nature of these histiocytes, their extraordinary metabolic capabilities, their role as regulators of hematopoiesis, and their prominence in the immune and inflammatory response (as well as uncertainty regarding dendritic cell and monocyte/macrophage ontogeny) all have contributed to the persistent confusion regarding the nosology of these disorders. To help clarify the nomenclature, Favara et al. refer to histiocytes as a group of immune cells that include[s] macrophages and dendritic cells. [1] Thus, the term histiocyte is analogous to lymphocyte in that both denote groups of immune cells that are phenotypically and functionally diverse. Based on this definition, a nosology derived from new information regarding cell lineage and biologic behavior has been developed,[1] replacing the previous classification system. [2] In doing so the World Health Organizations Committee on Histiocytic/Reticulum Cell Proliferations and the Reclassification Working Group of the Histiocyte Society have

TABLE 43-1 -- A Contemporary Classification of Histiocytic Disorders

(Not Available)

From Favara BE, Feller AC, Pauli M et al: Contemporary classification of histiocytic disorders. Med Pediatr Oncol 29:157, 1997. Copyright 1997. Reprinted by permission of Wiley-Liss, a division of John Wiley & Sons, Inc.

created a paradigm for the continued reassessment of the classification of these disorders as new laboratory and clinical science comes to light.

Currently, two broad categories of disease, disorders of varied biological behavior and malignant disorders, are each subdivided into dendritic (antigen-presenting) cell- or macrophage/monocyte (antigen processing cell)-related disorders. Other disorders in which histiocytes are implicated such as storage diseases, hyperlipidemic xanthomas, well-defined chronic infections such as tuberculosis and leprosy and granulomatous reactions to foreign materials are excluded. [1]

The clinical syndromes making up the major dendritic cell-related disorders of varied biologic behavior termed Langerhans cell histiocytosis, [3] [4] previously known as histiocytosis X, are the main focus of this chapter. The macrophage-related histiocytoses, also covered, include familial primary hematophagocytic lymphohistiocytosis, still referred to as familial hematophagocytic lymphohistiocytosis (FHL) or previously as familial erythrophagocytic lymphohistiocytosis (FEL), sporadic primary hematophagocytic lymphohistiocytosis, and sinus histiocytosis with massive lymphadenopathy. The malignant histiocytic disorders, which include the malignant proliferations of the monocyte/macrophage lineage, acute monocytic leukemia (FAB-M5), acute myelomonocytic leukemia (FAB-M4), chronic myelomonocytic leukemia, macrophage-related histiocytic sarcoma, and the more recently described dendritic cell-related neoplasms, are discussed in other chapters. Table 43-1 (Table Not Available) presents the currently accepted classification of the histiocytic syndromes.

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EPIDEMIOLOGY

The syndromes constituting Langerhans cell histiocytosis (LCH) were originally considered by many to be malignant neoplasms. That they have been treated with aggressive chemotherapy and radiation therapy, and more recently that they have been found to be clonally derived, have not resolved the difficulties in properly categorizing this class of disorders. Still, there are clear differences between the clinical behavior observed in most instances of LCH and true malignant disease. These differences suggest conservative management for many patients.

The clinical course of malignant neoplasia is generally relentlessly progressive, with virtually no survival in untreated patients (stage IVS neuroblastoma being the notable exception). In LCH, however, even with multiple site involvement, spontaneous remissions have been described. [5] In addition, varying morbidity and mortality have been reported in untreated patients with LCH, depending on the extent of disease. Pathologically, the lesions of LCH appear as reactive infiltrates, the hallmark being immature Langerhans cells that have little of the cellular atypically and homogeneity characteristic of most malignancies. Using flow cytometry, most investigators have found no evidence of DNA aneuploidy. [6] [7] [8] Although the cause of these phenomena is unknown, LCH is thought by some, as previously proposed, [3] [9] [10] to represent a reactive immune disorder triggered by unknown stimuli. There is no evidence

implicating viruses in this process, either directly or as immune triggers. [11]

Using X-chromosome-linked DNA probes to detect clonal X-chromosome inactivation, it was found that LCH is characterized by a clonal proliferation of CD1a+ cells.[12] All tissues derived from either localized or disseminated LCH were found to be clonally derived. [13] However, the identification of clonality does not necessarily indicate malignancy. More information on the biology of dendritic cell differentiation and activation is needed to interpret these data, and ultimately to develop new therapeutic strategies.[14]

Historically, the classic LCH syndromes are (1) monostotic or solitary eosinophilic granuloma (SEG) or multifocal eosinophilic granuloma (MEG)localized lesion(s) confined to bone;[15] [16] (2) Hand-Schüller-Christian diseaseprotracted multiple-site involvement with the classic but rare triad of skull defects, diabetes insipidus, and exophthalmos;[17] [18] [19] [20] and (3) Letterer-Siwe diseasedeeper visceral lesions involving skin, liver, lungs, bone marrow, lymph nodes, spleen, and other organs. [21] [22] The prevalence of these syndromes is estimated to be 1:50,000, with an incidence of 1.08 per 200,000 per year in children <15 years old. [23] The syndromes predominantly affect infants and young children, although disease in adults, even the elderly, [24] is well described. A male predominance has been widely reported. [25] [26] [27] [28] [29] [30] Since disease occurs on a continuum and frequently does not fit these rigid and arbitrary designations, a prognostic grouping system has been developed. Classification is best accomplished according to the criteria originally developed by Lahey [31] and refined by Komp and co-workers[32] and Osband et al. [10] Although the Histiocytosis Society has simplified its prognostic groups for the stratification of systemic treatment protocols into a low-risk group (patients >2 years old with no involvement of the hematopoietic system, liver, lungs, or spleen) and an at-risk group (patients <2 years old with involvement of these organs), the system described in Table

TABLE 43-2 -- Grouping System for Langerhans Cell Histiocytosis

Factor	**Points**
Age	
<2	0
2	1
Extent of disease	
<4 organs	0
>4 organs	1
Dysfunction[a] (1, 2, or 3)	
No	0
Yes	1
Group	**Total Points**[b]
0	Monostotic eosinophilic granuloma
I	0
II	1
III	2
IV	3

Modified from Osband et al.,[10] with permission.

[a] Dysfunction includes the following:

1. Hepatic dysfunction: one or more of the followinghypoproteinemia (total protein <5.5 mg/dl or albumin <2.5 mg/dl), hyperbilirubinemia (>1.5 mg/dl), edmerna, ascites.
2. Pulmonary dysfunction: one or more of the followingtachpnea, dyspnea, cyanosis, cough, pneumothrorax, pleural effusion.
3. Hematopoietic dysfunction: one or more of the followinganemia in the absence of iron deficiency or significant infection (<10 g/dl hemoglobin), leukopenia (<4,000/ml), thrombocytopenia (<100,000/ml).

[b] By arbitrarily assigning either 0 or 1 point for the absence or presence of one of the three important prognostic variables, a number of total points is obtained and a patient is assigned a group.

43-2 may be helpful in defining prognosis and treatment options over a broad spectrum of patient types.

Patients in groups 0 to I and some in group II (Table 43-2), particularly those with any single system disease, do well, with some morbidity but virtually no disease-related mortality. They frequently need little or no systemic therapy. Other patients in group II and most in group III need systemic therapy and generally do well, while patients in group IV have significant morbidity and mortality regardless of therapy. [10]

The yet to be determined optimal management for the patient with LCH would be one that balances therapeutic intervention structured to minimize both short- and long-term disease-related morbidity with appropriate systemic therapy designed to reduce treatment-associated morbidity. Although recurrences are common, almost all patients who respond to therapy will be able to discontinue treatment completely. This philosophy applies to those patients (generally in groups IIII) with relatively favorable prognoses. Another goal thus remains to find effective therapy for those patients whose disease does not respond to therapy (predominantly group IV patients).[2] These patients should have HLA typing performed at diagnosis and should be considered for bone marrow transplantation or other experimental therapies.[2]

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BIOLOGIC ASPECTS

In order to understand the histiocytic disorders, in particular Langerhans cell histiocytosis, it is important to review monocyte/macrophage and dendritic cell differentiation, characterized by the developmental sequence shown in Figure 43-1 .[33] [34] [35] [36] [37] [38] [39]

In the bone marrow this process begins with the pluripotent hematopoietic stem cell, which may give rise either to a lymphoid progenitor capable of T- or B-lymphocyte lineage commitment or to a multipotent myeloid progenitor capable of differentiation to either erythroid, megakaryocytic, eosinophil, basophil/mast cell, or granulocyte/macrophage cell lines. The granulocyte and the macrophage share a common progenitor, the granulocyte/macrophage colony-forming unit (GM-CFU). The GM-CFU, capable of granulocyte or monocyte differentiation, gives rise to the monocyte/macrophage progenitor (M-CFU). M-CFU gives rise to the monoblast, then to the bone marrow promonocyte (the first morphologically identifiable macrophage precursor), and finally to the monocyte, a process that takes approximately 6 days.[34] The promonocyte ranges from 1018 m in diameter and has a well-developed Golgi apparatus as well as peroxidase-positive granules. The typical monocyte has a folded nucleus, lightly basophilic cytoplasm, and faintly azurophilic granules. [36]

Bone marrow monocytes enter the circulation and migrate, apparently in a random fashion, into tissues, where they transform into tissue-specific macrophages under the influence of the local environment and become the cells of the mononuclear phagocyte system (MPS). [34] Dendritic cells likewise have their origin in the bone marrow[37] and can be cultured from progenitors residing in both the bone marrow and peripheral blood. [38] A CD34+ progenitor can, in single cell culture, give rise to either dendritic cells, via the dendritic Langerhans cell colony-forming unit (DL-CFU), or macrophages confirming that dendritic cells and macrophages share a common progenitor. Proliferative precursors of the dendritic cell enter the circulation from the bone marrow in a fashion somewhat analogous to the monocyte. They then enter the tissues,[39] where proliferation and differentiation to functional, antigen-presenting dendritic cells ensue. The morphology of the dendritic cell may vary depending on its functional (veiled versus dendritic) state. It is a large cell with multiple cytoplasmic extensions that is essentially devoid of phagolysosomes and other cytoplasmic organelles. The cytoplasm of this mobile cell is rich in mitochondria. [40] [41] The nucleus

Figure 43-1 Development of cells of the monocyte/macrophage lineage. CFU-GM, colony-forming unitgranulocyte/macrophage; CFU-M, colony-forming unitmacrophage; CFU-DL, colony-forming unitdendritic Langerhans cell.

is eccentric with small nucleoli.[41] Langerhans cells are characterized by Birbeck granules, [42] which appear ultrastructurally as tennis-racquet shaped pentalaminar structures.[33]

Functionally the MPS, also known as the reticuloendothelial system, consists of a multitude of ordinary tissue histiocytes (fixed macrophages) that act to provide host defense by disposing of senescent or damaged blood cells, ingesting invading organisms, processing antigens for the immune system, and stimulating the inflammatory process through the production of a number of cytokines.[34] [35] [42] [43] The most important of these cytokines are interleukin-1 (IL-1) and tumor necrosis factor (TNF), which activate T lymphocytes as well as other macrophages.

The MPS system is a component of many organs. These cells include splenic sinusoidal macrophages, hepatic Kupffer cells, [44] pulmonary alveolar macrophages,[45] bone osteoclasts, [46] pleural, peritoneal, and synovial macrophages, and microglial cells of the brain. [47] [48]

As phagocytic cells, the ordinary tissue histiocytes have IgG Fc receptors, whereas tissue-based dendritic cells, which make up the dendritic cell system (DCS), lack phagocytic capacity, peroxidase or esterase expression, or Fc receptors and are predominantly antigen-presenting cells. [33] [49] Found in virtually all tissues except the brain,[33] the Langerhans cell is the major immunologic cellular component of the integument and mucosa. Normal Langerhans cells reside within the epidermis and regional lymph nodes as well as the thymic epithelium and bronchial mucosa.[50] Functionally, cells of the DCS interact with and process antigen, then migrate (most likely as veiled cells) to lymphoid organs, where they participate as effector cells with T cells in generating a cellular and, indirectly, a humoral immune response. [33] The types of dendritic cells are summarized in Table 43-3 . As with the cells of the MPS, dendritic cells are capable of elaborating a wide array of cytokines, including IL-1 and TNF.[51]

Hematopoietic differentiation is regulated by a family of glycoproteins termed colony-stimulating factors and interleukins. A large family of these growth factors has been purified, cloned, and functionally characterized. GM-CSF, IL-3 or multi-CSF, M-CSF, and G-CSF [52] [53] [54] [55] exert a major influence on monocyte/macrophage differentiation.[52] [56] [57] [58] GM-CSF and IL-3 have a broader range of activity, influencing differentiation of primitive as well as committed progenitors. [56] [58] In addition, GM-CSF increases the functional activity of mature granulocytes, eosinophils, and monocytes. [59] [60] [61] M-CSF and G-CSF, restricted in their activity, influence the discrete monocyte/macrophage and granulocyte pathways, respectively, and enhance granulocyte and monocyte/macrophage function. [62] [63] Early work implicated T cells as a source of stimulating activity. [64] Since then, the monocyte/macrophage, the fibroblast, and other cells have also been shown to be involved in factor production.[65] [66] In addition, the production of these factors by cells outside the bone marrow supports the finding that some factors have the dual functions of stimulating marrow progenitor differentiation and proliferation and of enhancing the function of mature myeloid cells. [67]

Dendritic cells can be generated from progenitors in vitro using GM-CSF plus IL-4 or TNF [39] as well as other cytokines. GM-CSF appears to serve as a survival rather than a differentiation factor, while TNF induces maturation of these cells. [68] Thus, there exists a complex regulatory network, activated by a wide variety of stimuli, that controls the proliferation and function of the MPS and the DCS as well as associated myeloid cells, whose presence characterizes the lesions of the histiocytic syndromes. The complexity of activation/inhibition, cellular transformation, proliferation, and phagocytosis, and the multitude of factors that modulate the DCS and MPS, contribute to the difficulty in understanding the histiocytic disorders described in this chapter. The ability of dendritic cells and macrophages to produce cytokines and activate T lymphocytes and other inflammatory cells also contributes to the varied, sometimes

TABLE 43-3 -- Types of Dendritic Cells

Cell Type	Location	Function
Peripheral blood dendritic cell	Peripheral blood	Migratory

Langerhans cell[a]	Skin, epidermis, cervix, vagina, stomach, esophagus	Antigen uptake and processing(?), transport to lymph nodes
Veiled cells[a]	Afferent lymphatics	Antigen transfer
Interdigitating dendritic cell	Lymph node, paracortex (T area), periarteriolar lymphatic sheath of the spleen	Antigen presentation for the T cells
Thymic dendritic cell[a]	Thymic medulla	Induction of immune tolerance
Interstitial dendritic cell	Parenchymal organs (excluding brain and central cornea)	Antigen uptake and processing(?)
Indeterminate cells of the skin (dermal dendrocytes)	Dermis	Unknown
Follicular dendritic cell[b]	Germinal center of lymph nodes	Antigen presentation in B cells and maintenance of long-term immunologic memory

From Wright-Browne et al.,[33] with permission.

[a] Contains Birbeck granules.
[b] Derived from the lymphoid lineage.

protean clinical manifestations of the histiocytic disorders. In particular, since Nezelof et al. [69] first concluded that histiocytosis X was characterized by the proliferation of Langerhans cells, hematopoietic cell culture, immunopathology, electron microscopy, and immunophenotyping have led to a greater (yet still incomplete) understanding of the pathobiology of LCH in the context of the developmental scheme described in Figure 43-1 .

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CLINICAL MANIFESTATIONS

The signs and symptoms of LCH vary considerably, depending on which organs are infiltrated by the Langerhans and accompanying immunoreactive cells. Bone, skin, teeth, gingival tissue, ear, endocrine organs, lung, liver, spleen, lymph nodes, and bone marrow can all become involved, and exhibit dysfunction secondary to cellular infiltration. [70] Although patients rarely have disease that falls into the discrete classic categories of eosinophilic granuloma, Hand-Schüller-Christian disease, and Letterer-Siwe disease, this nomenclature remains valuable, if only to catalogue the clinical manifestations of LCH. In addition, these eponyms preserve an important historical perspective on this enigmatic group of disorders.

The difficulty in diagnosis and the historically complex nomenclature have made the true prevalence and incidence of these disorders difficult to ascertain. [71] Malones review reveals that most cases do occur between 1 and 15 years of age. [72] A 1993 study reported from Denmark placed the prevalence at 150,000 and the incidence at 1.08/200,000 per year in children <15 years old. [23]

Solitary or multifocal eosinophilic granuloma (SEG or MEG) is found predominantly in older children as well as in young adults, usually within the first three decades of life. The incidence peaks between the ages of 5 and 10 years. [73] SEG and MEG account for 6080% of all instances of LCH. [73] Patients with systemic involvement frequently have similar bony lesions in addition to other manifestations of disease. [70] There is often an inability to bear weight and a tender (sometimes warm) swelling that is due to tissue infiltrates overlying the bony lesions. [70] [74] Radiographically, the lesions are sharply marginated, round, or oval, with a beveled edge that gives the appearance of depth. [70] [75]

Hand-Schüller-Christian disease is described in younger children aged 25 years and represents 1540% of cases of LCH. [73] Signs and symptoms include bony defects with exophthalmos due to a tumor mass in the orbital cavity. This usually occurs because of involvement of the roof and lateral wall of the orbital bones (although bony involvement is not necessary). [76] In addition, teeth are often lost consequent on gum infiltration and mandibular involvement. The most frequent sites of bony involvement are the flat bones of the skull, ribs, pelvis, and scapula. [70] There may be extensive involvement of the skull with irregularly shaped lucent lesions, giving rise to the so-called geographic skull. [77] Somewhat less frequently, long bones and lumbosacral vertebrae (usually the anterior portion of the vertebral body) are involved. In one recent series the majority of children with vertebral involvement had involvement of only a single vertebra, with nine of the 15 having other skeletal lesions. [78] In long bones, growth of lesions in the medullary cavity leads to pressure that may result in erosion through the cortex, stimulating the formation of periosteal new bone, accompanied by soft tissue involvement. [79] A differential diagnosis that includes Ewing sarcoma and osteogenic sarcoma or osteomyelitis must be considered in these instances. Only rarely are the wrists, hands, knees, feet, or cervical vertebrae involved. [70] Orbital involvement may result in loss of vision or strabismus as a result of optic nerve or orbital muscle involvement, respectively. Oral involvement may begin in the gums or in the periapical regions of the teeth. Erosion of the lamina dura gives rise to the characteristic floating tooth seen on dental radiographs. [75] The entire mandible may be involved, with loss of bone leading to diminished height of the mandibular rami. Erosion of gingival tissue causes premature eruption, decay, and tooth loss. [80] Parents of affected children, particularly infants, frequently report precocious eruption of teeth when in fact the gums are receding to expose immature dentition. Chronic otitis media due to involvement of the mastoid and petrous portion of the temporal bone and otitis externa are not uncommon.

Diabetes insipidus (DI) affects 550% of patients with LCH. [81] Most instances of DI occur in children, who present with systemic disease and involvement of the orbit and skull. [81] Less than one-third of children who ultimately develop DI have polydypsia and polyuria as presenting symptoms of LCH. [81] In most children DI develops within 4 years of diagnosis. DI is due to infiltration by Langerhans cells of the hypothalamus, with or without involvement of the posterior pituitary gland. [82] Local tissue

damage may be a consequence of IL-1 and prostaglandin E_2 production. [81] Polydypsia and polyuria may be present at presentation or may develop during active disease (even when there is improvement in other areas) or after therapy is discontinued and there is no other apparent active disease.

Short stature has been found in up to 40% of children with systemic LCH. [82] [83] Chronic illness and steroid therapy play an important role in this phenomenon. Short stature may also be a consequence of anterior pituitary involvement and growth hormone deficiency. [83] Other endocrine manifestations include hyperprolactinemia and hypogonadism due to hypothalamic infiltration. [84] Pancreatic and thyroid involvement have also been reported. [84] [85]

Gastrointestinal tract disease has been identified. [86] [87] In one retrospective series only 2% of 348 children had biopsy-proved gastrointestinal involvement, [88] but in a prospective trial, 13% of 78 patients had digestive tract symptoms. [89]

The rarest (10% of cases) and most severe form of LCH is Letterer-Siwe disease. [73] Typically, an infant <2 years old presents with a scaly seborrheic, eczematoid, sometimes purpuric rash involving the scalp, ear canals, abdomen, and intertriginous areas of the neck and face. The rash may be maculopapular or nodulopapular. Ulceration may result, especially in intertriginous areas. Ulcerated and denuded skin may serve as a portal of entry for microorganisms, leading to sepsis. Draining ears, lymphadenopathy, hepatosplenomegaly, and, in severe cases, hepatic dysfunction with hypoproteinemia and diminished synthesis of clotting factors can occur. Anorexia, irritability, failure to thrive, and significant pulmonary symptoms such as cough, tachypnea, or pneumothorax may occur as well. One of the most clinically important areas of involvement is the hematopoietic system, where involvement manifests as anemia or thrombocytopenia. The pathophysiology of hematopoietic dysfunction is not well understood, since direct bone marrow involvement is only occasionally noted and significant marrow infiltrates are rare. Thrombocytopenia most frequently portends a fatal outcome. [25] Because of their physical appearance, these patients are frequently diagnosed as victims of abuse or neglect.

A relatively recently described and severe manifestation of LCH is non-hypothalamic-pituitary involvement of the central nervous system (CNS). [90] The majority of patients with CNS involvement had associated skull, orbit, or mastoid disease. Magnetic resonance imaging (MRI) with contrast agent enhancement is the best way to identify lesions of the CNS. As expected, the hypothalamic-pituitary region, characterized by DI, was the most common site of involvement, followed by the cerebellum, pons, and cerebral hemispheres. Lesions of the basal ganglia, spinal cord, and optic nerves were also seen. Involvement of the CNS was frequently observed years after the initial diagnosis. Extraxial lesions of the dura, meninges, or choroid plexus usually responded well to treatment. However, lesions of the cerebellum or pons frequently resulted in severe, even fatal sequelae. Pathologically, CNS lesions, other than for those of the hypothalamic-pituitary axis, almost uniformly were not CD1a, Birbeck granule-positive. The etiology and pathophysiology of this gliotic and demyelinating process, [91] which often leads to fatal degeneration of the CNS, are poorly understood.

Three other so-called presentations are described. A nodal presentation (not to be confused with sinus histiocytosis with massive lymphadenopathy) is characterized by significant enlargement of multiple lymph node groups, with little or no other signs of disease. In the pulmonary syndrome there is almost exclusive involvement of the lungs. This condition is usually seen in men in their third or fourth decade (and occasionally in adolescents) and may follow an indolent, severe, and chronic debilitating course, or may manifest acutely with a pneumothorax. [92] Cigarette smoking has been strongly implicated. [92] In contrast, the pulmonary involvement in younger patients with systemic disease is frequently mild, although fulminant pulmonary disease may occur in this age group. [93] [94] The chest radiograph may vary

from a diffuse infiltrate consistent with bilateral interstitial pneumonia, to a honeycomb lung appearance due to pulmonary fibrosis. [94] [95] Finally, pure cutaneous disease with no evidence of dissemination has been described in children and adults. [96] Rarely, patients present with deep subcutaneous skin nodules only (formerly described as Hashimoto-Pritzker syndrome). [71]

With such a vast spectrum of disease manifestations, adherence to strict labels such as eosinophilic granuloma, Hand-Schüller-Christian disease, or Letterer-Siwe disease, Hashimoto-Pritzker syndrome, and other syndromes or presentations cannot adequately describe a particular patient, and the development of a classification scheme predictive of prognosis becomes necessary (Table 43-2).

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NATURAL HISTORY AND PROGNOSIS

As originally proposed by Lahey, [31] the prognosis for patients with LCH depends on three factors: age at onset, number of organs involved, and degree of dysfunction of specific organs. An adolescent or adult who presents with solitary bone eosinophilic granuloma has the best prognosis, [97] whereas an infant with multiple affected organs has the worst.[25] [26] [27] [28] [31] [32] A prognosis-based classification (Table 43-2) allows one to discard the labels of eosinophilic granuloma, Hand-Schüller-Christian disease, Letterer-Siwe disease, and other syndromes (or presentations) and to group patients according to age at onset, localization, and severity of disease. Specifically, age 2 years is associated with a good prognosis, whereas age <2 years is associated with a worse prognosis (Table 43-4 (Table Not Available)). When LCH develops in individuals >65 years old, regardless of site and extent of disease, the prognosis is usually poor. [72] Involvement of fewer than four organ systems is a relatively good prognostic sign, whereas involvement of four or more organ systems predicts a less favorable outcome (Table 43-5), and dysfunction of three specific organ systems (hepatic, pulmonary, and hematopoietic) is an extremely important adverse predictor of outcome (Table 43-4 (Table Not Available)). In a review covering a 25-year experience at a single institution, [96] 7 of 28 patients with multisystem disease died, 5 of 14 had single disease recurrences, and 9 of 14 had multiple disease recurrences. Of interest, the recurrence rate in those patients with polyostotic bone involvement was higher (8 of 12) than in those with multisystem disease. However, there were no deaths in this group.

Organ dysfunction, as described in Table 43-2 , must be distinguished from involvement (e.g., hypoproteinemia or hyperbilirubinemia versus hepatomegaly), since involvement alone is not as adverse a prognostic sign as dysfunction. By arbitrarily assigning either 0 or 1 point for absence or presence of one of the three important prognostic variables, the grouping system described in Table 43-2 is defined. This classification is a reliable prognostic tool and also indicates which patients need systemic therapy. A review of 62 patients ages 0 (birth) to 14 years [30] confirms these observations, stressing the importance of two factorsage and localized disease. All but one of the 15 patients who died were <2 years old at diagnosis. With regard to local involvement, the presence of bony involvement was a good prognostic sign, and skin involvement that was localized to the scalp was associated with a considerably better outcome than generalized cutaneous involvement.

TABLE 43-4 -- Survival Experience by Age and Organ Dysfunction in Langerhans Cell Histiocytosis

(Not Available)

From Komp DM, Herson J, Starling KA et al: A staging system for histiocytosis X: a Southwest Oncology Group study. Cancer 47:798, 1981. Copyright 1981 American Cancer Society. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

TABLE 43-5 -- Organ System Involvement as a Prognostic Indicator in Langerhans Cell Histiocytosis

Organ Systems Involved	No. of Patients	Response to Therapy (CR + PR)[a]	No. Dead
12	13	8	0
34	20	15	4
56	22	12	9
7+	18	7	11

From Lahey, [31] with permission.

[a] Complete (CR) plus partial (PR) response.

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HISTOLOGIC DIAGNOSIS

The typical histologic appearance of LCH varies with the age of the lesion examined. The Langerhans cell is the prominent diagnostic feature in the histology of Langerhans cell histiocytosis.[99] Early lesions are cellular and locally destructive, with a proliferation of essentially normal Langerhans cells. [72] LCH cells, however, differ somewhat from normal Langerhans cells in that they are functionally defective, are frequently found in tissues where Langerhans cells do not normally reside, and are arrested at an early stage of cell activation. [50] Mitoses are usually not present in great numbers, but when found are of no prognostic significance. [100] Multinucleated histiocytes are often present. Other inflammatory cells, such as granulocytes, eosinophils, macrophages, and occasionally lymphocytes and plasma cells, are present as well. The histiocytes and macrophages may be phagocytic, but Langerhans cells are not. [100] With time these cells may accumulate cholesterol. Occasionally there is necrosis present, with only rare Langerhans cells. In addition, as lesions mature, there are fewer Langerhans cells. Pathologically, a presumptive diagnosis of LCH is made on the basis of a biopsy specimen demonstrating the characteristic histopathology. [72] [95] [100] [101] [102] The Langerhans cell is 1525 m in diameter, with a central to slightly eccentric ovoid to uniform-shaped nucleus with a delicate chromatin network and inconspicuous nucleoli. An indentation or groove across the face of the nucleus is a feature of many cells.[101] Additional diagnostic criteria beyond standard histopathology, immunochemical staining with ATPase, S-100 protein, -mannosidase, peanut lectin, vimentin, and other markers [100] [103] [104] are necessary to establish the diagnosis. An extensive immunophenotype of normal and LCH Langerhans cells (Table 43-6) has been recently reviewed.[33] However, the definitive diagnosis of LCH relies most specifically on the immunohistochemical identification of the presence of Langerhans cells by cell-surface CD1a or of Birbeck granules [42] [65] by electron microscopy. Thus, pathologic criteria for the diagnosis of LCH have been established, [72] [100] [103] and in 1987 the Histiocyte Society formalized these criteria (Table 43-7).[2] Favara et al. now suggest that with the availability of new antibodies to CD1a for use in routinely processed paraffin-embedded specimens, the category of diagnosis can be omitted as redundant.[1] Clinicians

TABLE 43-6 -- Surface Markers of Langerhans Cells (LC), Activated Langerhans Cells, and LCH Cells

Marker	LC	Activated LC	LCH cells
Surface ATPase	+	+	+
MHC II	+	+	+
MHC I	+	+	+
Fc IgG receptor	+	+	+
Fc IgE receptor	+	+	+
C3bi receptor	+	+	+
CD1a and c	+	+	+
CD4	±	+	+
CD45	+	+	+
CD14	+	+	?
CDw29	+	+	?
IL-2R		+	+
B7		+(cultured)	?
CD11b and c	+	+	+
S-100	+	+	+
PLAP		+(transient)	+
PNA			+
IFN-R			+

From Chu and Jaffe,[50] with permission.

must keep in mind that standard histology still permits only a presumptive diagnosis and must insist, in all cases, on a definitive immunohistochemical or ultrastructural diagnosis.

The differential diagnosis of LCH is limited and depends on the clinical presentation. It includes immunodeficiency syndromes with graft-versus-host disease, viral infections, infiltrative diseases such as leukemia or lymphoma, reticuloendothelial storage diseases, congenital infections, benign and malignant bone tumors and cysts, and papular xanthomas.

A careful evaluation of biopsy material will generally result in a diagnosis. However, one of the historic dilemmas in the evaluation of LCH is that the histopathology of lesions from patients with single-site eosinophilic granuloma (group 0) is similar, if not identical, to that of classic Letterer-Siwe disease (group III or IV). [70] [102] Indeed, this is consistent with the recent finding that tissue in lesions from patients with localized or systemic disease cannot be distinguished on the basis of clonality. [12] [13] As expected on the basis of these observations, efforts to distinguish favorable from unfavorable histologic types [105] have been shown not to be of any value.[106] Although patients are grouped in large part based on the extent of disease, untreated patients generally do not progress to a higher stage, as occurs with lymphoma patients. In fact, progression from a limited syndrome to diffuse systemic disease is extremely rare. [70] Within a few months after presentation it will become apparent that the bony lesions seen initially are limited to the skeleton or were the heralding lesion(s) of diffuse systemic involvement. [97]

TABLE 43-7 -- Criteria of the Writing Group of the Histiocyte Society (1987) for the Diagnosis of Langerhans Cell Histiocytosis (Formerly Histiocytosis X)

I. Presumptive diagnosis: study of conventionally stained biopsy material with findings that are consistent with those defined in the literature.
II. Diagnosis: an increased degree of diagnostic confidence with presumptive diagnosis plus presence of two or more features: positive stain for ATPase, S-100 protein, -mannosidase, or peanut lectin.
III. Definitive diagnosis: requires the demonstration of Birbeck granules by electron microscopy or CD1 antigenic determinants (T6 positivity) on cryostat sections in the context of presumptive diagnosis or diagnosis.

From Hall et al.,[103] with permission from Blackwell Science, Ltd.

When cutaneous involvement is the only obvious presenting sign, 612 months may be required to determine the ultimate extent of disease. [7C]

These observations strengthen the need for a complete clinical and laboratory evaluation with proper imaging studies once a definitive diagnosis of LCH is made. In general, the baseline evaluation of a newly diagnosed patient requires a reasonable search for all sites of disease. The laboratory and imaging evaluation must be guided by a complete history and thorough physical examination, including a dental examination. All patients should be evaluated with a complete blood cell count, liver function tests and liver enzyme assays, a coagulation workup, urine osmolality, chest radiography, and a skeletal survey, including views of the skull. In a recent series of children with LCH in whom both radiographic and radionucleotide skeletal evaluations were performed, the radiologic evaluation detected virtually all significant skeletal lesions.[107] Other tests such as panoramic dental radiography, pulmonary function tests, MRI of the brain and middle ear, and bone marrow aspirates/biopsies and other tissue biopsies should be done based on findings on the initial evaluation.

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THERAPY

The philosophy for treatment of LCH is to use an appropriate amount of the least toxic therapy to treat the disease. In patients with potentially morbid or life-threatening disease at presentation, or in those in whom morbid or life-threatening disease develops during the course of treatment, more aggressive therapy should be implemented. This approach emphasizes the need for treatment protocols based on careful prognosis-based risk stratification. Whether more intense front-line therapy in lower-risk patients with systemic disease can reduce disease sequelae such as DI, CNS degeneration, sclerosing cholangitis, or disease recurrence, and whether a reduction in these problems outweighs the risk of more intense therapy, are under evaluation.

The primary specialist should coordinate the care of these patients. Subspecialty consultation should be sought from dentists, dermatologists, endocrinologists, gastroenterologists, neurologists, orthopedic surgeons, otolaryngologists, and others experienced in managing LCH. The goal of therapy for the majority of patients with localized or limited systemic disease must be to minimize loss of function and prevent cosmetic deformity.

Severely ill patients are hospitalized and given antibiotic, ventilatory, nutritional (including hyperalimentation), blood product, skin care, physical therapy, medical, and nursing support as required in addition to definitive treatment. Scrupulous hygiene is quite effective in limiting auditory canal, cutaneous, and dental lesions. Debridement, and even resection of severely affected gingival tissue, is done to limit oral involvement. The seborrhea-like dermatitis of the scalp may improve with the use of a selenium-based shampoo twice a week. Topical steroids are occasionally effective but should be used sparingly, and only for short-term control of small areas.

Surgery and Radiation Therapy

After complete evaluation, those patients with disease involving a single bone, and some patients with disease involving multiple lesions and multiple bones, are managed with local therapy. This involves surgical curettage for patients whose lesions are in easily accessible, noncritical locations. Complete cancer operation resections are not necessary. Surgical restraint must be exercised to avoid drastic cosmetic and orthopedic deformities and loss of function. Local soft tissue disease (e.g., of the scalp, thymus, or lymph nodes) generally recurs despite surgery; thus, systemic treatment or low-dose radiation in emergency situations is recommended for these types of lesions. Localized radiation therapy (usually 600900 cGy, with 450 cGy for small lesions and 1,500 cGy for large lesions) in 200-cGy fractions, utilizing only megavoltage equipment, is currently employed. Older patients may need slightly higher doses (2,000 cGy for large lesions). If possible, structures such as the lens of the eye and the thyroid gland should be spared. Patients at risk for skeletal deformity, visual loss secondary to exophthalmos, pathologic fractures, vertebral collapse, or spinal cord injury should receive radiation therapy. Patients with severe pain, symptomatic adenopathy, or multiple lesions may also warrant low-dose radiation therapy to affected areas if systemic therapy is not rapidly effective. Lesions in poorly accessible sites, such as the orbit, or lesions recurring after curettage should also be irradiated.[108] [109] DI may occur at any time during the course of LCH. Patients should be instructed to report signs of DI as soon as they develop, since dehydration and electrolyte imbalance may be quite serious. The results of hypothalamic and pituitary radiation therapy, even instituted early in the course of DI, have been poor, and the use of this modality is not recommended. [109] [110] [111]

A review of 40 patients receiving radiation therapy between 1970 and 1984 indicates that patients with unifocal disease have a higher rate of response for individual lesions than patients with multifocal disease (two or more soft tissue sites). Thus, patients who appear to have an isolated lesion that does not respond to appropriate radiation therapy should be carefully re-evaluated for additional sites of disease. In this review, the complete plus partial response rate for solitary bone lesions was 100% (35 of 35), with 24 complete responses. Soft tissue lesions adjacent to involved bone healed better than isolated soft tissue lesions. There was no response to irradiation for liver, spleen, or lung lesions, and none of eight patients with DI responded. [110]

Chemotherapy

Historically, drugs used in the therapy of classic malignant diseases have been used systemically for LCH. Nitrogen mustard, vincristine, vinblastine, cyclophosphamide, procarbazine, chlorambucil, etoposide, methotrexate, corticosteroids, and 6-mercaptopurine (6-MP) have been used, singly or in combination, with variable success. Response to chemotherapy varies from 25% to 90%. An early study did show that treatment improves survival in patients <2 years old (Table 43-8).[25] Table 43-9 summarizes selected best-designed early chemotherapeutic trials utilizing well-characterized patients. [112] [113] [114] [115] [116] [117] [118] [119] [120] [121] [122] A reasonable therapeutic approach to systemic therapy is to observe patients in group I (some in group II) who respond to local (i.e., surgery, radiation therapy) or nonsystemic (i.e., topical steroids) therapy and look for signs of continuing disease resolution. If there are symptomatic lesions or signs of failure to thrive, systemic treatment should be pursued. The erythrocyte sedimentation rate is sometimes a useful indicator of disease activity. Patients in groups II and III will benefit dramatically from chemotherapy, while there are poor responses and death in many group IV patients. Because of the rarity of LCH and the large number of therapeutic questions for which answers must be sought, all patients with disseminated disease should be enrolled in well-designed collaborative studies. Based on data from several small studies demonstrating the efficacy of etoposide in previously untreated patients [121] [123] and those with resistant disease,[124] the Histiocyte Society in April 1991 sponsored its first international randomized study, LCH-I, for patients <18 years old with disseminated disease. This study compared the efficacy of vinblastine with that of etoposide for previously untreated disseminated LCH. [125] Treatment in each arm consisted of a single pulse of high-dose methylprednisolone for induction, followed by weekly vinblastine (6 mg/m^2 by intravenous bolus) versus 3-consecutive-day courses of etoposide (150 mg/m^2 by intravenous infusion over 2 hours) every 3 weeks. Each treatment continued for 24 weeks. An interim

TABLE 43-8 -- Treatment versus No Treatment[a]

No. of Patients	Treatment	No. Dead	No. Alive	Mean Age (mo)
27	No	27	0	10.5
64	Yes	40	24	15.0

Data from Lahey.[25]

[a] Treatment consisted of variable chemotherapy (steroids, antimetabolites, and alkylating agents); no treatment entailed supportive care.

evaluation of LCH-I showed that the rapidity of the initial response seemed to correlate with prognosis and that those patients with a poor initial response in either arm did not do well in a crossover to the alternative arm. [125] [126] With no difference in survival between the two arms, combined with the potential risk of leukemogenesis

with etoposide, the study has given rise to LCH-II. LCH-II uses the framework of the DAL HX-83 study, [126] in which all patients receive a 6-week induction with prednisone, vinblastine, and etoposide, followed by 1 year of therapy with only oral 6-MP, vinblastine, and prednisone (a), or 6-MP, vinblastine, and prednisone plus either etoposide (b) or etoposide and methotrexate (c) for patients with multifocal bone involvement (a), soft tissue involvement without organ dysfunction (b), and organ dysfunction (c), respectively. [126] In LCH-II, patients are stratified into low risk (patients >2 years old with no involvement of the hematopoietic system, liver, lungs, or spleen) and at risk (patients <2 years old with involvement of these organs). Patients with multisystem disease in the low-risk group receive initial treatment with a 6-week course of prednisone and weekly vinblastine, followed by continuation treatment with oral 6-MP, prednisone pulses, and weekly vinblastine for 24 weeks. Patients in the at-risk group are randomized to the low-risk arm treatment versus that treatment with the addition of etoposide to both initial and continuation treatment. [126] Low-risk patients whose disease does not respond are switched to the etoposide arm. Those patients whose disease does not respond to conventional chemotherapy containing etoposide should be evaluated for salvage therapy.

A variety of experimental modalities, such as hemibody irradiation, [127] thymic hormone therapy, [10] [128] and IFN-, [129] have been explored at some centers for advanced or refractory disease, but these approaches have been largely abandoned. A number of chemotherapeutic approaches have had limited success, and need to be evaluated in prospective studies. [130] [131] [132] The relatively good results reported to date with bone marrow transplantation [133] [134] [135] for high-risk patients or those with refractory disease warrant further evaluation of this modality in a controlled clinical trial.

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LONG-TERM FOLLOW-UP

A careful retrospective analysis of 71 patients from a single institution who were followed up for a median of 8.1 years from diagnosis revealed significant late sequelae in 64% of patients followed up for more than 3 years, despite a relatively good survival rate of 88%, 88%, and 77% at 5, 15, and 20 years, respectively. [98] Skeletal defects were found in 42%, dental problems in 30%, DI in 25%, growth failure in 20%, sex hormone deficiency in 16%, hypothyroidism in 14%, hearing loss in 16%, and CNS dysfunction in 14%. Indeed, these data emphasize the importance of appropriate therapeutic choices, as sequelae such as bony malformations and scoliosis seemed to be a consequence of treatment with radiation. Although previous studies [89] [126] found a lower incidence of DI and fewer recurrences when aggressive initial chemotherapy that included etoposide was used in patients with multifocal bone and disseminated disease, the study discussed above included 28 patients with multisystem disease, many of whom received aggressive chemotherapy and still had recurrences and late sequelae. [98] These contradictory results emphasize the importance of a controlled clinical study (LCH-II) to determine the effect of more aggressive chemotherapy on the outcome and toxic effects in patients with LCH. In addition, three of 51 patients followed up for more than 3 years developed a malignancy. All three were <3 years old at diagnosis. Two tumors were in a radiation field, but one was a leukemia in a patient treated with an etoposide-containing chemotherapy regimen.

TABLE 43-9 -- Comparison of Selected Chemotherapy Trials for Langerhans Cell Histiocytosis

Drug(s)[a]	Study Group		No. of Patients	Response (CR + PR)[b]
Single agents				
Vincristine (VCR)	SWCCG	1972	6	50
Vinblastine (VBL)	SWCCG	1972	20	55%
VBL	CCSG	1975	18	56%
VBL	Mayo	1980	18	77%
Cyclophosphamide (CTX)	SWCCG	1972	22	63%
Procarbazine (PCB)	SWOG	1974	10	50%
Chlorambucil (CMB)	SWOG	1980	32	56%
CMB	CCSG	1980	26	27%
Etoposide (VP-16)	Italy	1988	18	83%
VP-16	AIEOP (Italy)	1993	27	70%
Combination therapy				
Methotrexate (MTX) + prednisone (PRED)	ALGB	1974	17	53%
VCR + PRED	ALGB	1974	11	64%
VBL + PRED	CCSG	1975	18	67%
6-Mercaptopurine (6-MP)	CCSG	1975	25	48%
CTX + VCR + PRED + PCB	SWOG	1977	21	38%
CTX + VBL + PRED	SWOG	1977	25	25% (<1 yr)
MTX + CMB + VBL + PRED	Mexico	1988	68	75%

Data from references[113] [114] [115] [116] [117] [118] [119] [120] [121] [122] .

[a] Pre-1972: anecdotal responses reported with antibiotics, corticosteroids, vincristine, vinblastine, methotrexate, cyclophosphamide, nitrogen mustard, 6-mercaptopurine, daunorubicin.
[b] CR, complete response; PR, partial response in which there is resolution of >50% of the lesions.

THERAPY FOR LANGERHANS CELL HISTIOCYTOSIS PHILOSOPHY

PHILOSOPHY

The optimal management, yet to be determined, for the patient with LCH would be one that balances therapeutic intervention structured to minimize both short- and long-term disease-related morbidity with appropriate systemic therapy designed to reduce treatment-associated morbidity. Currently almost all patients who respond to therapy will be able to discontinue treatment completely, although recurrences are common. This philosophy applies to those patients (generally in groups IIII) with relatively favorable prognoses. Another goal thus remains, to find effective therapy for those patients whose disease does not respond to therapy (predominantly group IV). These patients should have HLA typing performed at diagnosis and should be considered for bone marrow transplantation or other experimental therapies.

PROGNOSTIC GROUPING

When patients are grouped according to three prognostic variablesage, extent of disease, and presence of organ dysfunctionthe prognosis and the need for and extent of treatment can be evaluated. By assigning either 0 to 1 point for the absence or presence of one of the three important prognostic variables, a patient may have from 0 points (group I) to 3 points (group IV) (Table 43-2). Patients in groups 0 to I and some in group II, particularly those with any single system disease, do well, with some morbidity but virtually no disease-related mortality. They frequently need little or no systemic therapy. Other patients in group II and most in group III need systemic therapy and generally do well, while patients in group IV experience significant morbidity and mortality regardless of therapy. All patients should be considered for well-designed collaborative trials. Group IV patients as well as those with disease refractory to chemotherapy should undergo HLA typing and be considered for bone marrow transplantation, if a suitable donor exists. If not, these patients should be considered for experimental therapy at an appropriate center.

SUPPORTIVE CARE

Severely ill patients are hospitalized and given antibiotic, ventilatory, nutritional (including hyperalimentation), blood product, skin care, physical therapy, medical, and nursing support as required, in addition to definitive treatment. Scrupulous hygiene is quite effective in limiting auditory canal, cutaneous, and dental lesions. Debridement, and even resection of severely affected gingival tissue, is done to limit oral involvement. The seborrhea-like dermatitis of the scalp may improve with the use of a selenium-based shampoo twice a week. Topical steroids are occasionally effective but should be used sparingly, and only for short-term control of small areas. Diabetes insipidus may occur at any time during the course of LCH. Patients should be instructed to report signs of DI as soon as they develop, since dehydration and electrolyte imbalance may be quite serious. The results of hypothalamic and pituitary radiation therapy instituted early in the course of DI have been poor, and this modality is not recommended.

LOCAL THERAPY (SURGERY AND RADIATION THERAPY)

After complete evaluation, patients with disease involving a single bone and some patients with disease involving multiple lesions and multiple bones are managed with local therapy. This entails surgical curettage for patients whose lesions are in easily accessible noncritical locations. Complete cancer operation resections are not necessary. Surgi- cal restraint must be exercised to avoid drastic cosmetic and orthopedic deformities and loss of function. Local soft tissue disease (e.g., of the scalp, thymus, or lymph nodes) generally recurs despite surgery; thus, systemic treatment or low-dose radiation in emergency situations is recommended for these types of lesions. Localized radiation therapy (usually 600900 cGy, with 450 cGy for small lesions and 1,500 cGy for large lesions) in 200-cGy fractions, utilizing only megavoltage equipment, is currently employed. Older patients may need slightly higher doses (2,000 cGy for large lesions). Since individual lesions in patients with unifocal disease have a higher rate of response than those in patients with multifocal disease, patients who appear to have an isolated lesion and who fail to respond to appropriate radiation therapy should be carefully re-evaluated for additional sites of disease. Structures such as the lens of the eye and the thyroid gland should be spared if at all possible. Patients at risk of skeletal deformity, visual loss secondary to exophthalmos, pathologic fractures, vertebral collapse, and spinal cord injury should receive radiation therapy. Patients with severe pain, symptomatic adenopathy, or multiple lesions may also warrant low-dose radiation therapy to affected areas if systemic therapy is not rapidly effective. Lesions in poorly accessible sites, such as the orbit or lesions, that recur after curettage should also be irradiated.

CHEMOTHERAPY

Patients in group I and some in group II can be observed for signs of spontaneous improvement. If symptomatic lesions or failure to thrive are evident, treatment should be pursued. Patients in groups II and III will benefit dramatically from chemotherapy, while those in group IV will usually die despite chemotherapy. Experimental modalities such as bone marrow transplantation are being pursued at specialized centers for group IV patients whose disease is nonresponsive to conventional chemotherapy.

The philosophy for treatment of LCH is to use an appropriate amount of the least toxic therapy to treat the disease. In patients with potentially morbid or life-threatening disease at presentation, or in those in whom morbid or life-threatening disease develops during the course of treatment, more aggressive therapy should be implemented. This approach emphasizes the need for treatment protocols based upon careful prognosis-based risk stratification. Whether more intense front-line therapy in lower-risk patients with systemic disease can reduce disease sequelae such as DI, CNS degeneration, sclerosing cholangitis, or disease recurrence and whether a reduction in these problems outweighs the risk of more intense therapy are under evaluation. Patients with this rare disease are best served by treatment according to collaborative protocols such as those of the Histiocytosis Society. Bactrim prophylaxis in patients for whom long-term immunosuppressive therapy is anticipated should be instituted.

LONG-TERM FOLLOW-UP

All patients with LCH must receive long-term follow-up. In addition to late malignancies, patients should be monitored for signs of long-term disabilities, including cosmetic and functional orthopedic and cutaneous deformities that may lead to loss of function and severe emotional disorders, loss of permanent dentition, endocrine disorders and growth failure, hearing impairment, CNS abnormalities, and sclerosing cholangitis with biliary cirrhosis, as well as pulmonary fibrosis and cor pulmonale. Patients with chronic disabilities as sequelae of LCH should be followed up with the help of appropriate subspecialists.

TABLE 43-10 -- Late Malignancy Following Treatment of Langerhans Cell Histiocytosis

Patient	Treatment	Malignancy	Interval Between Histiocytosis and Malignancy (yr)
1	CMB	Hepatocellular carcinoma	14
2	CMB + RT	Thyroid carcinoma	16
3	CMB (CT) + RT	Acute leukemia	2
4	CMB, NM (CT) + RT	Acute leukemia	6.5
5	CMB, NM (CT) + RT	Acute leukemia	5
6	RT	Thyroid carcinoma	28

Abbreviations: CMB, chlorambucil; RT, radiation therapy; CT, combination therapy; NM, nitrogen mustard.

Data from Greenberger et al.[28]

This risk of malignancy in patients with LCH undergoing radiation and chemotherapy is well documented. Of 127 patients with LCH, [28] 84 received chemotherapy, and

six of these developed late malignancies. Two patients who received radiation therapy developed an in-field tumor, and one of these received no chemotherapy. Table 43-10 briefly describes those patients. Late malignancy developed in five of 54 patients who received chlorambucil as a single agent or in combination chemotherapy and in two of 29 patients given nitrogen mustard as part of combination therapy that included chlorambucil, vincristine, procarbazine, and prednisone. Thus, the judicious application of radiation therapy using modern techniques, avoidance of potentially carcinogenic chemotherapeutic agents, and good supportive care are highly recommended as a treatment philosophy. Although patients with LCH could have an inherently increased risk of malignancy, [136] the experience cited suggests an excess of malignancy associated with chlorambucil use. Also, the relatively poor outcome with this drug (Table 43-9) has argued effectively against its use in this disease. The issue of etoposide use is more difficult, since this is a very active agent in LCH (Table 43-9). Early reports of leukemia in LCH patients treated with etoposide as a single agent [122] [137] and in combination with other agents [98] [138] [139] have appeared. Depending on the true incidence of leukemia, [126] [140] to be determined in LCH-II, the use of this drug as a front-line agent in LCH may be modified.

Another serious sequela of LCH, sclerosing cholangitis, has been reported. [141] This complication may lead to secondary biliary cirrhosis and liver failure. The only successful treatment has been liver transplantation. [141] Sclerosing cholangitis joins severe CNS dysfunction as the most devastating sequelae of LCH. Thus, all patients with LCH must receive long-term follow-up. In addition to late malignancies, patients should be monitored for signs of long-term disabilities, including cosmetic and functional orthopedic and cutaneous deformities that may lead to loss of function and severe emotional disorders, loss of permanent dentition, endocrinologic disorders and growth failure, hearing impairment, CNS abnormalities, and sclerosing cholangitis with biliary cirrhosis, as well as pulmonary fibrosis and cor pulmonale. The hope is that newer approaches to treatment that reduce the incidence of these sequelae will become available as a consequence of a better understanding of the pathophysiology of LCH.

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HEMATOPHAGOCYTIC SYNDROMES

Primary Hematophagocytic Lymphohistiocytosis (Familial and Sporadic)

Familial hematophagocytic lymphohistiocytosis, or FHL (formerly known as familial erythrophagocytic lymphohistiocytosis, or FEL), is a rapidly fatal, inherited disorder characterized by peripheral pancytopenia with bone marrow hyperplasia, systemic lymphadenopathy, intermittent fevers, and severe liver dysfunction accompanied by hepatosplenomegaly and increased liver enzyme levels.[142] [143] Patients have a characteristic severe hypofibrinogenemia not attributable to disseminated intravascular coagulation, since plasma levels of all other clotting factors are normal and no evidence of fibrinolysis is observed. [144] Hypertriglyceridemia is characteristic. Most affected children are infants (ages 2 weeks to 7 years), and two-thirds of them present within the first 3 months of life. [145] Patients usually present with pallor, irritability, anorexia, diarrhea, and failure to thrive. Occasionally there is a nonspecific, sometimes sunburned-appearing or maculopapular skin rash. [142] [145] Pulmonary effusions are present in one-third of patients. The Histiocyte Societys diagnostic criteria consist of fever of unknown etiology, splenomegaly, hematopoietic cytopenias, hypofibrinogenemia or hypertriglyceridemia, and hematophagocytosis in the bone marrow, spleen, lymph node, or other tissue. [146] The clinical course is usually progressive, lasting only an average of 6 weeks. [145] Most patients die as a consequence of sepsis, bleeding, or a lymphocytic meningitis accompanied by refractory seizures. Approximately 75% of patients have CNS involvement at diagnosis. [145] Disease essentially confined to the CNS, leading to seizures, has been reported.[147] There is no sex predilection, [145] and the presence of the disorder in siblings and cousins [145] and parental consanguinity[144] support an autosomal recessive mode of inheritance.

There are no specific biologic markers of FHL, but laboratory evaluation consistently demonstrates hypofibrinogenemia, hyperlipidemia, [148] pancytopenia, hyperbilirubinemia, and increased liver enzyme levels. [142]

Pathologic study of the liver shows focal fatty changes, necrosis, and infiltration by lymphocytes and histiocytes. The infiltrate is periportal but extends into adjacent lobules. Erythrophagocytosis and hematophagocytosis are prominent, and essential to the diagnosis. The spleen demonstrates similar focal necrosis and foci of lymphocytes and histiocytes with erythrophagocytosis. Involvement of the thymus and lymph nodes is similar, but later in the course of disease, lymph nodes show significant lymphocytic depletion. Hematophagocytosis is also present. The lungs are infiltrated with lymphocytes and histiocytes, again with some erythrophagocytosis. The bone marrow is hyperplastic, with increased numbers of hematophagocytic histiocytes. [142] [145] [149] The cellular infiltrate lacks features of malignant cells. The characteristic pathologic findings and clinical presentation clearly distinguish FHL from LCH and other histiocytic disorders.

As there are no diagnostic tests and a gene defect has not been identified, FHL may be difficult to distinguish from sporadic or secondary hematophagocytic lymphohistiocytosis, formally called infection-associated hemophagocytic syndrome (IAHS), which was originally described in immunocompromised hosts as a consequence of underlying viral infections [150] as well as bacterial and protozoal infections. [151] [152] In addition, the syndrome is now known to be associated with T-cell

lymphomas, Chédiak-Higashi disease, and, rarely, rheumatoid arthritis or even LCH. [1] The clinical course of secondary hematophagocytic lymphohistiocytosis is variable, but a 40% mortality has been reported, [150] and some patients may benefit from systemic treatment.[1] In addition, treatment of the underlying disorder is essential.

Although the etiology remains obscure, immunodeficiency is a consistent finding in FHL. Evidence strongly suggests that the disease is mediated by hypercytokinemia with elevated levels of IFN-, TNF, and in some instances IL-6 as a consequence of the uncontrolled activation of macrophages and perhaps T cells. [153] [154] [155] The authors propose a genetic defect in cytokine regulation. There is depressed antigen-induced lymphocyte proliferation and decreased monocyte antibody-dependent cytotoxicity.[156] Removal of plasma by plasma exchange transiently corrects these abnormalities but does not improve the prognosis. [156] Whether the disorder is due to a primary immunodeficiency or the immune abnormalities are secondary to another underlying defect is unknown.

Steroids and cytotoxic agents have generally been ineffective in the treatment of FHL. Etoposide has induced clinical remissions accompanied by resolution of the characteristic hyperlipidemia.[157] Although virtually all patients experience relapse, this agent, usually given in combination with prednisone and intrathecal methotrexate,[145] has resulted in complete to very good responses in patients with FHL and has been used prior to more definitive therapy with bone marrow transplantation in this otherwise lethal disorder. [145] [158] [159]

Sinus Histiocytosis with Massive Lymphadenopathy

First described in 1969 and more recently reviewed in 1990, [160] [161] sinus histiocytosis with massive lymphadenopathy (SHML), or Rosai-Dorfman disease, is characterized clinically as a benign, frequently chronic, painless massive lymphadenopathy that usually involves the cervical lymph nodes and, less frequently, the axillary, hilar, peritracheal, and inguinal nodes. [162] Extranodal disease is present in 28% of patients. [163] The upper respiratory mucosa is involved in 20%, [160] bone in 25%,[164] [165] and the orbit or eyelid in 10% of patients. [166] [167] Occasionally there is skin involvement [168] as well as CNS, lung, liver, and kidney involvement. [161]

Although SHML is a benign and polyclonal disorder, [169] as opposed to LCH, significant morbidity and even death have been associated with massive tissue invasion of the liver, kidney, lung, and other critical structures. [161] [170] In these instances, the disease has a rapid downhill course. Respiratory distress due to tracheal obstruction[171] and paraplegia secondary to epidural involvement have been described. [172] Death in SHML has also occurred as a consequence of severe hemolytic anemia.[170]

Eighty percent of patients are diagnosed in their first or second decade, [173] [174] [175] but the disorder can also affect the elderly. [173] [175] Typically, patients are of African descent. [173] [174] Males and females are equally affected. Worldwide, the incidence of SHML is highest in Africa and the West Indies. [174]

Laboratory evaluation frequently reveals an elevated erythrocyte sedimentation rate, moderate polyclonal and (rarely) benign monoclonal hypergammaglobulinemia, anemia, and granulocytosis.[168] [171]

Involved lymph nodes show marked sinusoidal dilation and follicular hyperplasia with proliferation of foamy histiocytes and multinucleated giant cells within the sinuses. There are no eosinophils, and abundant plasma cells are present. [160] [162] [163] [164] Lymphocytes are often found in the cytoplasm of histiocyte-like cells. This characteristic finding is referred to as emperipolesis. [171] The proliferating histiocytes share properties of macrophages and interdigitating cells. These large pale cells are S-100 positive[176] but are morphologically distinguished from Langerhans cells found in the lymph nodes of patients with classic LCH by the absence of Birbeck

granules[164] as well as by the surface phenotype, determined by monoclonal antibodies, [168] [177] and by the presence of $_1$ -antichymotrypsin.[171]

As with LCH, the etiology of this disorder is unknown, and as in LCH, disordered immune regulation has been proposed. [168] It was originally thought that SHML represented an unusual response to a *Klebsiella* antigen or Epstein-Barr virus,[175] but this has not been confirmed.[168] Although patients are frequently febrile, infectious agents have not been implicated, and the fever is presumed to be a manifestation of systemic disease.

Treatment is usually both unnecessary and ineffective. Disease manifestations usually subside over several months to years. Of 215 cases in a patient registry, 21% had complete resolution of disease. However, 14 patients died. Five died from immunologic causes, such as severe hemolysis, three died from infections, and six probably died as a direct consequence of disease infiltration. [178] As the disease resolves, extranodal disease regresses prior to nodal disease. Radiation, corticosteroids, vinblastine, and low-dose cyclophosphamide are sometimes effective; however, the results with these agents have been inconsistent. [163] [172] [179] [180] Attempts at treatment should be reserved for special circumstances, such as tracheal or epidural compression or invasion of other vital structures. Local excision may also be useful in selected patients. [164]

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Chapter 44 - Lysosomal Storage Diseases: Perspective and Principles

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THE CONCEPT OF THE LYSOSOME

The lysosome was first described by deDuve as an acid phosphatase-containing unit membrane-bounded organelle within the cytoplasm of eukaryotic cells. [1] As the name implies, lysosomes participate in the lysis or breakdown of cellular material, that is, the digestive tracts of cells or of suicide packages. The containment of hydrolytic enzymes within these organelles prevents autodigestion of cellular contents. Since this description, the concept of the lysosome has expanded to a dynamic heterogeneous subcellular organelle with tissue and cellular-specific differentiated functions and contents. Several tissues have specialized lysosomal mechanisms that extrude hydrolytic enzymes into the extracellular microenvironment. Such cells and their lysosomal enzymes are critical to host defense, wound healing, and bone remodeling (e.g., neutrophils and osteoclasts). Implicit in DeDuves description was the need for systems to sort and specifically localize proteins to lysosomes and the potential of replacing deficient enzymes. [1]

The concepts of lysosomes as hydrolytic sacs and the need for specific targeting of enzymes to the lysosome have provided a fertile milieu for research during the past three decades. Hers was the first to delineate engorged lysosomes in patients with -glucosidase deficiency [2] and other lysosomal storage diseases.[3] This description led to a pathophysiologic view that related the malfunction of these organelles or cells principally to the resultant architectural distortion produced by stored materials in the lysosome. Recently, this passive process has been enhanced by postulating a more active role for toxic degradative products (e.g., lysosphingolipids) in altering normal cellular function. [4] Goldstein et al., [5] Neufeld,[6] and others, developed the concept of receptor-mediated endocytosis and emphasized directed targeting and sorting of proteins from the extracellular space. This culminated in the Brown and Goldstein model of receptormediated endocytosis for the low-density lipoprotein (LDL) particle [5] and its general implications for the uptake and targeting of proteins to cells. Neufeld described the phenomena of cross-correction during coculture of skin fibroblasts from patients with two different types of mucopolysaccharidoses (MPS). [7] The subsequent loss of accumulated cellular deposits in each cell type suggested the presence of specific corrective factors that were secreted by one MPS cell type and internalized by the other cell type.[7] These factors were later identified as enzymes. Cross-correction required the secretion of a functional enzyme that contained a signal for receptor-mediated endocytosis by other cells. Because only one lysosomal hydrolase is usually defective in MPS variants, all other secretable lysosomal hydrolases are normal and, potentially, would be able to correct a specific deficiency in other cells. These investigations culminated in the characterization of the mannose-6-phosphate receptor system by the groups of Sly,[8] Kornfeld,[9] [10] von Figura,[11] and others. This ligand-mediated targeting system is central to selective sorting of most soluble lysosomal proteins within many cells, [10] their uptake into cells, and delivery to the lysosome. In comparison, membrane-bound lysosomal proteins use different, incompletely defined, amino acid-based, targeting systems. [12] These concepts of storage, cross-correction, and targeting have provided the foundation for the development of therapeutic interventions for lysosomal storage diseases including enzyme reconstitution by transplanted tissue or enzyme replacement. Both approaches may stabilize or reverse the pathophysiologic process in selected lysosomal storage diseases. These findings implied a dynamic and interactive communication of the lysosome with intra- and extracellular compartments. Comprehensive reviews of individual lysosomal diseases are available (Table 44-1).

TABLE 44-1 -- Selected Lysosomal Storage Diseases

Disease (Major Review)	Common Name	Enzyme Defect	Major Organs InvolvedPhenotype Variation	Stored Substrate
Mucopolysaccharidoses (MPS)[124]				
MPS IH	Hurler	-L-Iduronidase	Liver, spleen, brain, heart, cornea, bonesmild and severe variants	Dermatan and heparan sulfate
MPS II	Hunter	Iduronidate sulfatase	Liver, spleen, brain, heart, bones	Dermatan and heparan sulfate
MPS III	Sanfilippo A	Heparan *N*-sulfatase	Brain, liver, spleen, heart, bones	Heparan sulfate
	Sanfilippo B	*N*-Acetyl-glucosaminidase	Brain, liver, spleen, heart, bones	Heparan sulfate
MPS IV	Morquio A	*N*-Acetyl-galactosamine 6-sulfatase	Bone, cornea	Keratan sulfate, chondroitin-6-sulfate
	Morquio B	-Galactosidase	Bone, cornea	Keratan sulfate
MPS VI	Maroteaux-Lamy	*N*-Acetyl-galactosamine 4-sulfatase	Bone, cornea, liver, spleen, heartmoderate and severe variants	Dermatan sulfate
MPS VII	Sly	-Glucuronidase	Brain, liver, spleen, bone, coronary arteries	Dermatan sulfate, heparan sulfate, chondroitin-4 and -6 sulfate
Glycoproteinoses[125]				
Mannosidosis		Lysosomal -mannosidase	Brain, liver, spleen, boneseveral variants	-Mannose-rich oligosaccharides

Fucosidosis		Glycoprotein -fucosidase	Brain, liver, spleen, heart, skinseveral variants	Fucose-containing oligosaccharides
Aspartylglucosaminuria		Aspartyl-glucosaminidase	Brain, liver, spleen, bone, heart	Aspartylglucosamine-containing peptides
Sialidosis		Glycoprotein -neuraminidase	Brain, liver, spleen, bone, retinaseveral variants	Sialylated glycopeptides
Galactosialidosis		Protector proteincombined -neuraminidase/-galactosidase deficiency	Brain, liver, spleen, boneseveral variants	G_{M1} -Ganglioside and sialyated glycopeptides
Mucolipidosis II[126]	I-Cell disease	*N*-Acetylglucosamine-1-phosphotransferase	Brain, bones, connective tissue	Glycoproteins, glycolipids
Mucolipidosis III	Pseudohurler polydystrophy	*N*-Acetylglucosamine-1-phosphotransferase	Brain, bones, connective tissue	Glycoproteins, glycolipids
Sphingolipidoses				
Gaucher disease[53]	Gaucher disease type 1 (non-neuronopathic)	Acid -glucosidase; glucocerebrosidase	Liver, spleen, bone, bone marrowhighly variable phenotype	Glucosylceramide
	Gaucher disease type 2 (acute neuronopathic)	Acid -glucosidase; glucocerebrosidase	Brain, brainstem, liver, spleen, bone marrow, lungs	Glucosylceramide; glucosyl-sphingosine
	Gaucher disease type 3 (subacute neuronopathic)	Acid -glucosidase; glucocerebrosidase	Brain, liver, spleen, bone marrow, lungsvariable phenotype	Glucosylceramide; glucosyl-sphingosine
Metachromatic leukodystrophy (MLD)[127]	Infantile MLD	Arylsulfatase A	Brain, peripheral nerves	Sulfatide
	Juvenile MLD	Arylsulfatase A	Brain, peripheral nerves	Sulfatide
	Adult MLD	Arylsulfatase A	Brain, peripheral nerves	Sulfatide
		Saposin B deficiency	Brain, peripheral nerves	Sulfatide
	Pseudodeficiency	Partial arylsulfatase A	Normal	None
Multiple sulfatase deficiency[127]		Cysteine modification enzyme	Brain, liver, spleen, bones	Sulfatide, dermatan, and heparan sulfate
Gangliosidoses				
G_{M2} -Gangliosidoses[65]	Infantile Tay-Sachs (TSD)	-Hexosaminidase A (-chain)	Brain	G_{M2} -Ganglioside
	Juvenile TSD	-Hexosaminidase A (-chain)	Brain	G_{M2} -Ganglioside
	Adult TSD	-Hexosaminidase A (-chain)	Brain	G_{M2} -Ganglioside
	Activator deficiency	G_{M2} -Activator	Brain	G_{M2} -Ganglioside
	Sandhoff disease	-Hexosaminidase B and A (chain)	Brain, liver, spleen, bone	G_{M2} -Ganglioside, globoside
G_{M1} -Gangliosidoses[55]	Landing disease	-Galactosidase	Brain, liver, spleen, bone	G_{M1} -Ganglioside, keratan sulfate
Neutral Sphingolipidoses				
Fabry disease[39]		-Galactosidase A	Kidney, vascular endothelial system, heart, central nervous system vessels	Globotriaosylceramide
Schindler disease[39]		-*N*-Acetyl-galactosamidase	Brainprobable several variants	*N*-Acetylgalactose-linked oligosaccharides and ?
Krabbe disease[128]		Galactocerebrosidase	Brain	Galactocerebroside
Niemann-Pick[129]	Niemann-Pick A (infantile)	Sphingomyelinase	Brain, liver, spleen, lungs	Sphingomyelin
	Niemann-Pick B (late onset)	Sphingomyelinase	Liver, spleen, lungs	Sphingomyelin
Neutral Lipid Storage Diseases				
Wolman disease[40]		Lysosomal acid lipase	Liver, spleen, adrenal glands, bone marrow	Cholesteryl esters, triglycerides
Cholesterol ester storage disease[40]	CESD	Lysosomal acid lipase	Liver, spleen, blood vessels	Cholesteryl esters
Farber disease[130]		Ceramide	Brain, joints, tendons, skin, liver	Ceramide

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PHYSIOLOGY OF LYSOSOMES

Unlike mitochondria, lysosomes do not contain DNA and, therefore, cannot self-replicate. Consequently, lysosomal biogenesis is a continuous process that requires synthesis of lysosomal hydrolases, membrane constitutive proteins, and membrane. This process includes the fusion of vesicles from the *trans*-Golgi network (TGN) with late endosomes (multivesicular bodies)[13] [14] (Fig. 44-1). The TGN vesicles contain lysosomal hydrolases. The maturation of TGN vesicular bodies to lysosomes and of endosomes from early to late compartments accompanies a progressive internal vesicular acidification. A gradient is established so that early endosomes have an internal pH 6.06.2, whereas late endosomes and lysosomes have a pH of 5.56.0 and 5, respectively. This acidification is needed for the pH-dependent dissociation of receptors and ligands (e.g., the mannose-6-phosphate receptor and mannose-6-phosphate-containing

Figure 44-1 Schematic diagram of lysosomal development from the endoplasmic reticulum (ER), through the Golgi apparatus (Golgi), and the endosomal compartments (EN). EN develop from the plasma membrane (PM)-coated pits. The EN and TGN pathways converge in vesicles containing material from newly synthesized lysosomal proteins, and ER or multivesicular body (MVB) contents. Between the MVB, PL-EN, and return to the PM exists the CURL, a virtual compartment for the uncoupling of receptors and ligands. The various EN and lysosomal compartments are distinguished by the progressive acidification of the vesicles from early to late endosomes and the loss of the mannose-6-phosphate receptors in the CURL. The MVB is an ultrastructural compartment that contains internal small vesicles derived from the PM. Vesicles that bud from the TGN fuse with late EN or MVB to form the lysosomal compartment. Mannose-6-phosphate receptors are recycled to the PM and to the mid-Golgi. Secretion indicates a direct route from the TGN to EN for delivery outside of the cell. Auto and Phago refer to autophagy and phagocytosis, respectively, by which internal and external materials are delivered to preexisting lysosomes. Larger arrows indicate a major common pathway through the mature lysosomes. The double-headed arrows depict a necessary interaction between various types of lysosomes to account for the major similarities of lysosomes in cells. Lysosomes are inherently heterogeneous. This indicates a dynamic compartment that communicates, probably bidirectionally, with other components of the endosomal/lysosomal system. In addition to what is shown, vesicular transport is needed between the ER and Golgi.

oligosaccharides) and the optimal functioning of lysosomal hydrolases. [15] Dissociation occurs in a virtual compartment termed CURL (compartment of uncoupling of receptors and ligands). Formation and segregation of lysosomes with their constitutive integral and associated membrane proteins are less well understood. [16] These proteins are essential for lysosomal integrity and function. In particular, the lysosomal membrane contains a proton pump needed for developing and maintaining the acidic internal environment required for lysosomal hydrolase function. The signals for the fusion of TGN vesicles with endosomal vesicles requires the interaction of several membrane components, the sorting of proteins destined for the lysosome, the loss of the mannose-6-phosphate receptors, the recycling of these receptors to the mid-Golgi bodies and plasma membrane,[15] and segregation of endosomal membrane and contents. Consequently, the view of the lysosome as an end-stage compartment has been replaced by one of a dynamic communicating organelle with continuously changing contents, that, at least in part, exchanges material with other lysosomes in the cell. This leads to a lysosomal compartment that is composed of heterogeneous populations of similar organelles whose individual contents differ significantly in time and space.

Post-translational modifications of lysosomal hydrolases are important for specific sorting and targeting of enzymes and for their enzymatic activity. Lysosomal enzymes are glycoproteins that are synthesized on ribosomes of the rough endoplasmic reticulum (RER). As with all secretory proteins, lysosomal hydrolases are synthesized with an N-terminal hydrophobic leader or signal peptide that is required for penetration through the ER membrane and into the lumen of the ER. During penetration through the ER membrane, the lysosomal proteins are co-translationally glycosylated on selected N-glycosylation consensus sequences (asparagine-X-serine or threonine). This occurs by the en bloc transfer of antennerary mannosyl oligosaccharide chains from a dolicholphosphate (Fig. 44-2). It is unclear how N-glycosylation sequences are selected for occupancy [17] except that asparagine-proline-serine or threonine sites are not used and the surrounding protein sequences appear important for transfer of the dolicholphosphate-oligosaccharide. Following complete synthesis and glycosylation, the lysosomal enzymes may undergo additional proteolytic processing or assembly and are incorporated into transport vesicles for further processing in the *cis*-Golgi apparatus. At this stage all proteins destined for the lysosomes contain only branched mannosyl chains that terminate with short chain -glucosyl moieties. Most enzymes destined for the lysosome acquire complex oligosaccharide modifications during transport through the Golgi apparatus (Fig. 44-2). A series of glycosyl hydrolases and glycosyltransferases within specific regions of the *cis*-, mid-, or *trans*-Golgi participate in these sequential modifications. In the *cis*-Golgi network, -glucosidases and -mannosidases remove terminal glucose and mannose residues to produce mannose-terminated core oligosaccharides. For each branch point of the mannosyl chain there are specific -mannosidases for cleavage. Following mannosyl trimming, additional sugars including - *N*-acetylglucosamine and -galactoside are added to the short mannosyl core by the mid-Golgi system. The addition of terminal sialic acid residues occurs in the *trans*-Golgi. Each of these sugars is added by glycosyl transferases that have been retained in their respective Golgi compartment by specific signals. Thus, the carbohydrate composition of the lysosomal proteins is indicative of its passage through regions of the Golgi during the synthetic process. Prior to, or coincident with, ER or *cis*-Golgi modifications, is the attachment of an *N*-acetylglucosamine-1-phosphate to the sixth position on mannosyl residues of the mannosyl core oligosaccharide. This modification can be uni- or multivalent and is selective for various branches of the oligosaccharides on a specific lysosomal protein. An *N*-acetylglucosylaminyl-1-phosphotransferase [9] [11]

Figure 44-2 Compartmentalized *N*-linked oligosaccharide modifications of lysosomal proteins. Attachment of the core mannosyl-glucose to selected Asn-X-Ser/Thr consensus sequences occurs co-translationally in the ER lumen via en bloc transfer of the shown core oligosaccharide structure from a dolichophosphate. Initial removal of terminal glucosyl residues and a mannosyl residue are removed prior to exit from the ER to the Golgi. Additional -mannosyl residues are removed by specific mannosidases in the cis-Golgi to a pentamannosyl core. *N*-acetyl-glucosamine-1-phosphate is transferred by glucoaminylphosphotransferases for the creation of a mannose-6-phosphate. In the mid- and *trans*-Golgi -galactosyl and sialyl (NeuNAc) residues are added by transferases localized to those compartments. The final oligosaccharide structures can contain mostly mannose (high mannose), short mannose cores, *N*-acetyl-glucosamines, -galactosyl and sialyl residues on the core structure (complex), or several combinations of the above on different branches of the structure.

is essential for this process, and its deficiency leads to a severe condition, I-cell disease, in which many soluble lysosomal enzymes are lost by default secretion out of the cell.[9] Shortly after the attachment of the phosphosugar group, a specific hydrolase cleaves the protecting *N*-acetylglucosamine from the phosphate to expose the mannose-6-phosphate residue, the targeting signal for soluble lysosomal proteins. These modification processes culminate in most lysosomal enzymes having several oligosaccharide chains with nonidentical antennerary modifications. As a result, the lysosomal enzymes can have portions of the attached oligosaccharide tree having only mannose residues (high mannose modification), mannose residues, *N*-acetylglucosylaminyl, -galactosyl- and sialyl-containing oligosaccharides (mixed type modification), or only terminal sialyl acid residues (complex type modification). [18] [19] Importantly, many soluble enzymes use ill-defined M-6-P independent intracellular trafficking signals alone or in combination with the mannose-6-phosphate system.

Defective mannose-6-phosphate targeting of soluble lysosomal proteins, as occurs in I-cell disease, has no effect on the integrity of the lysosome per se or its membrane. Lysosomal integral or associated membrane proteins (LIMPS or LAMPS) are sorted to the lysosomal membrane or to the interior of the lysosome via mannose-6-phosphate-independent trafficking systems. Signals for such trafficking have been identified as strategically located tyrosine residues near the carboxyl terminal end of some LIMPS and LAMPS.[12] It is apparent that additional signals will be required for targeting of other lysosomal membrane components. Importantly, for theoretical diseases involving LIMPS and LAMPS, cross-correction of cocultured cells would not occur; this has significance for therapeutic strategies of such

diseases.

In addition to glycosylation, some lysosomal proteins require proteolytic clipping, phosphorylation, or macromolecular assembly for the development of full function within the lysosomal environment. Proteolytic processing can occur at the NH_2 or carboxyl termini or by clipping of single peptide precursors into mature subunits or active peptides. For example, prosaposin is cleaved into four biologically active saposins A, B, C, and D that are functional in the lysosomal compartment [20] [21] [22] [23] [24] (Fig. 44-3). A protector protein precursor is involved in galactosialidosis and is clipped from its zymogen form into two, disulfide-linked, subunits within the Golgi apparatus prior to its achieving proteolytic activity. [25] [26] Some proteins also may acquire phosphorylation of serines or threonines or sulfation of tyrosines. Macromolecular assembly is required for several heteromeric lysosomal proteins. The association of the -hexosaminidase - and -chains in the mid-Golgi is necessary for synthesis of active hexosaminidase A (- and -heteromers) or hexosaminidase B (-homomers). Macroassembly of the -ga- lactosidase/neuraminidase/protector protein complex probably occurs within the Golgi apparatus coincident with other processing required for the assembly of the active catalytic complex. [25]

Although the control of tissue expression of many lysosomal proteins has not been characterized, tissue- and cell-specific expressions have been described for prosaposin and lysosomal acid lipase [27] [28] and probably exist for other lysosomal proteins. These expression patterns may have import for the variability of the phenotype of lysosomal diseases. Most tissues contain similar lysosomal hydrolases for degradation of macromolecules (e.g., mucopolysaccharides, glycoproteins, and glycosphingolipids) because these are essential to cell function. Thus, lysosomal storage disease involvement is apparent in most tissues at the histologic or biochemical levels. However, concordant levels of lysosomal hydrolase activities are not found in all tissues. Lysosomes of neutrophils contain myeloperoxidases that are not found in fibroblast lysosomes. [29] Also, -L-iduronidase, prosaposin, and other lysosomal proteins are at similarly high levels in seminal fluid, but not in plasma or cerebrospinal fluid. -L-iduronidase activity is easily detected in amniocytes and placental tissue but is nearly absent in early chorionic villi. [30] Acid -glucosidase (deficient in Gaucher disease) is present at nearly 10-fold lower levels in myeloid-derived cells compared to fibroblastic cells. [31]

Such differential expression allows for extension of the Conzelmann and Sandhoff threshold hypothesis for the development

Figure 44-3 Diagram of the processing of prosaposin from the chromosomal gene to mRNA to mature saposin proteins that interact with lysosomal enzymes. The chromosomal gene is over 30 kb in length, the first intron is >20 kb, and is located on chromosome 10p. Introns are shown as the white rectangles and exons are filled and matched to the domains for each saposin. The mRNA contains four highly homologous, but not identical, domains that code for the saposins A, B, C, and D. The mature mRNA encodes the saposins and intersaposin peptide links in the same reading frame. The intersaposin peptides are removed by proteolysis. The individual saposins are produced by proteolytic cleavage mostly within the lysosomes where they interact with their respective lysosomal hydrolases.

of manifestations of specific lysosomal hydrolase deficiency diseases. [32] [33] This working hypothesis implies that a set level of residual enzymatic activity, or substrate flux, is necessary to prevent lysosomal disease manifestations. Below this threshold, disease manifestations (in tissues or specific cell type) will be expressed, whereas above this threshold there will be no phenotype. In the most simple form, this formalizes the notion that less enzyme activity leads to more severe disease and more enzyme activity results in less severe or delayed disease manifestations. This formalism is conceptually important because it incorporates the potential for regional and cell-type localization of disease manifestations. The hypothesis also implies that particular levels of enzyme activity will be necessary to normalize tissues and that each tissue or cellular type may require specific levels of activity. The implications for therapeutic strategies are clear because only about 110% of normal lysosomal enzyme levels may be sufficient to correct metabolism in many tissues and prevent, reverse, or significantly delay localized disease manifestations. Also, very small incremental changes in enzyme activity or substrate flux could have profound effects on disease development and severity. Expansion of this hypothesis incorporates the potential need for threshold levels of enzymatic activity at critical times during development. If adequate levels of activity are not present at these developmental stages, disease manifestations or irreversible damage may occur and may not be amenable to later therapeutic interventions. For example, after a progression to a pathologic stage, dysostosis multiplex of bones, cardiac valvular involvement, or neurodegeneration in the MPS may not be reversed by bone marrow transplantation (BMT) or enzyme replacement therapies.

This threshold hypothesis then leads directly to a model for the pathogenesis of lysosomal disease phenotypes, that is, necessary and sufficient conditions can be identified for the occurrence of the disease state. The presence of deleterious mutations that lead to the deficiency of specific proteins is a necessary prelude to the phenotype. However, the relationships of the residual mutant activity to the level of substrate flux through the lysosome, to the temporal and spatial metabolic needs of a particular tissue, and to the phenotype are poorly understood. Conzelmann and Sandhoff studied G_{M2} -ganglioside and sulfatide degradation in fibroblasts from patients with Tay-Sachs disease (TSD) and metachromatic leukodystrophy (MLD) respectively. These data support the contention that the age of onset (and therefore disease severity) of a lysosomal disease phenotype correlates with a threshold level of residual activity within lysosomes of cells. [33] However, additional factors can dramatically affect substrate flux and must be defined to provide a larger conceptual background as a model for pathogenesis.

As shown in Equation 1 and the accompanying box, the total body turnover of a particular substrate represents the total rate of change of substrate concentrations, $(v_T) = S_T/t$, in a variety of cell types. The flux of substrate in individual cells or organs, $v_i(t) = S_i/t$, varies substantially throughout the body and potentially with developmental stage. For our purposes, the subscript i incorporates the organ- or cell-specific property at different development stages. Thus, the sum of all $[v_i(t)]$s would represent the total body substrate flux. v_T also may depend on developmental stage and the level of substrate in the cell, that is, $v(S_i)$. Four major factors impinge on S_i attained in each cell at each developmental stage (scheme 2) including: S_{exo}, S_{end}, L_{ex}, and L_{en}. These represent, respectively, the exogenous (exo) and endogenous (end) source(s) of substrate(s) that alter intracellular concentration (S_i). Also included are the pathways that lead to decreases in substrate levels (L) through excretion/secretion (ex) and enzymatic degradation (en) to end products within the lysosome. L_{en} represents decreases in substrate concentration due to the residual activity. Although such an equation is a heuristic device, it provides a framework to qualitatively, and eventually quantitatively, examine each of the factors that influence intracellular substrate concentration. This could provide an overall conceptual frame for the dissection of the various phenotypes in lysosomal storage disease and potential targets for therapeutic intervention that may have the greatest impact on the phenotype.

Equation representing flux (v_T) of substrate (S_i) through various cell types (i). The sum of various rates of substrate change in n tissues (S_i/t) equals the total flux of substrate (S_T/t) through the body of an affected individual. $v(S_i)$ represents the developmental stage-specific

rate of substrateconcentration changes in a particular cell type and its potential dependence on the concentration of substrate (S_i) in that tissue. The subscripts of v represent substrates from various sources (i.e., x_i = exogenous, phagocytosis, n_i = endogenous sources from catabolism of more complex macromolecules, L_i = losses due to excretion/secretion, en_i = decreases due to enzymatic degradation).

As shown in scheme 3, the level of S_{exo} is influenced by several factors including: (1) genetic factors, that is, genetic determinants of cellular turnover or substrate delivery; (2) exogenous (environmental) agents that lead to increased substrate availability; and (3) the developmental stage that could include increased cellular and substrate turnover. This last aspect may be of importance for changes in the progression of various lysosomal diseases with age. Thus, different developmental stage-specific genes and substrate turnovers could affect an age-dependent threshold for the balance of v_{in} v_{out}. It is implicit in this discussion that disease progression is likely to be nonuniform, and there could be exacerbations and remissions of symptoms depending on exogenous and other genetic factors. Also, the balance of v_{Sex} and $v_{Ser.}$ between cells and organs may differ substantially. This will depend greatly on the pathophysiology of various organs. Gaucher disease is a prime example. In many

Scheme representing major factors influencing flux of substrate through the cells. S_i indicates the different substrate concentrations within various cell types. Each of the parameters indicated can vary with developmental stage and tissue type; exo, end, exi, and eni refer to exogenous (phagocytosis), endogenous (degradation from other glycosphingolipids), excretion/secretion out of the cell, and enzymatic degradation of the substrate, respectively. The other terms are defined in equation 1.

Factors influencing the substrate concentration (S) in phagocytic (S_{exc}) and nonphagocytic (S_{end}) cells. These reflect the major, but not exclusive, dependence of S on these pathways in these different cell types.

visceral tissues (hepatocytes, skin cells, lymphoid cells), v_{Sex} for glucosylceramide is much less than v_{Sen} . v_{Ser} is probably very low because, even in the most severe variants, storage of glucosylceramide cannot be detected ultrastructurally in such cells. In comparison, in phagocytic cells of the viscera, v_{Sex} v_{Sen} . This implies that phagocytic cells will have a much greater variation in degree of involvement because of the inherent greater dependence on the exogenous supply of substrate, that is, greater variation in S_{ex} and v_{xi} (scheme 1). In contrast, involved central nervous system (CNS) cells are primarily dependent on the S_{en} for glucosylceramide and other substrates that accumulate in the brain. The contribution of S_{ex} is small. Detailed histology of the brains from Gaucher disease type 2 consistently show significant regional variation in neuronal loss, neuronophagia, and gliosis. There is a rostral-caudal gradient of involvement with greatest severity in the basal ganglia and the dentate nucleus. [34] [35] Thus, in Gaucher disease, not only are the pathophysiologies in the viscera and the brain fundamentally different, but within the CNS regional variation exists. The greater dependence of the visceral pathology on S_{ex} implies a potentially greater number of steps that could be altered to influence the rate and degree of disease progression.

This discussion indicates that a necessary condition for the development of disease is the *relative* enzymatic deficiency, that is, is there sufficient hydrolytic power to cleave the substrate presented at any given time? The enzyme deficiency reduces the ability of cells, and, therefore, the individual, to adapt to changes in v_T . Thus, the variation in v_T , either endogenous or exogenous, determines, to a great extent, the eventual phenotypic severity. Because v_T can vary, the progression of the specific disease manifestations may be variable at different stages of the disease. The most maladaptive mutations, such as a homozygous null mutation, frequently may be associated with the most severe phenotypes, but modifier factors (genes) could lessen disease severity. The converse also applies. Currently, little is understood about the essential factors that influence substrate flux in the tissues of patients with lysosomal storage diseases.

Definition of these thresholds of activity also may provide clues to dose-response relationships for treatment of various storage diseases and the evaluation of exogenous enzyme replacement therapy. Once delivered to the tissue and cellular sites of pathology, exogenous enzymes will have finite life spans. Assuming appropriate stoichiometric relationships for components required for intracellular activity, the initial, high hydrolytic rates will decrease to background levels over several half-lives of the supplied enzyme. Thus, large amounts of enzyme, potentially several-fold above threshold levels, may be needed to eliminate the extreme excess tissue burden of substrate. Normal metabolism could then be maintained with pulses or intermittent dosing of lesser amounts of enzyme, presumably at levels slightly above the disease threshold, because only catabolism of new substrate would be required. [33] This pulse model of enzyme therapy assumes that small amounts of reaccumulating substrates are not irreversibly toxic. Otherwise, a continuous supply of enzyme would be required to avoid such toxic effects. This model also assumes metabolic interaction between lysosomal compartments in cells and that the exogenous enzyme is delivered to the cells that are causal to the disease manifestations.

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PATHOPHYSIOLOGY OF LYSOSOMAL STORAGE DISEASES

The majority of lysosomal enzyme deficiencies result from point mutations or genetic rearrangements at a locus encoding a single protein required for the activity of the lysosomal hydrolase (Tables 44-1 and 44-2). Some mutations at a locus can result in the deficiencies of several lysosomal hydrolases due to abnormalities in macromolecular association or trafficking. The final

TABLE 44-2 -- Gene Mapping of Lysosomal Storage Diseases

Disease	Regional Mapping	cDNA Cloned
Mucopolysaccharidoses (MPS)/Enzyme		
MPS IH/-L-iduronidase	4p16.3	yes[131]
MPS II/Iduronidate sulfatase	Xq28.1	yes[132]
MPS IIIA/Heparan *N*-sulfatase	17q25.3	yes[133]
MPS IIIB/-*N*-acetyl-glucosaminidase	17q21	yes[134]
MPS IIIC/acetyl Co A:-glucosaminidase *N*-acetyl transferase		
MPS IIID/*N*-acetylglucosamine-6-sulfate sulfatase	12q14	yes[135]
MPS IVA/*N*-acetyl-galactosamine 6-sulfatase	16q24.3	yes[136]
MPS VI/*N*-acetyl-galactosamine 4-sulfatase	5q11q13	yes [137]
MPS VII/-glucuronidase	7q21.11	yes[138]
Glycoproteinoses		
Mannosidosis/-mannosidase	19 cent -q12	yes[139]
Fucosidosis/-L-fucosidase	1p34	yes[140]
Aspartylglucosaminuria/aspartylglucosaminidase	4q23q27	yes[141]
Sialidosis/-neuraminidase	6p21.3	yes[142]
Galactosialidosis/protector protein	20q13.1	yes[143]
Mucolipidosis II/*N*-acetyl glucosamine 1-phosphotransferase	4q21q23	
Mucolipidosis III	4q21q23	
Sphingolipidoses		
Gaucher disease/acid -glucosidase	1q2123	yes[144]
Metachromatic leukodystrophy (MLD)/arylsulfatase A	22q13.31 -qter	yes[145]
Metachromatic leukodystrophy (MLD)/saposin B deficiency	10q22.1	yes[21] [146]
G_{M2} -Gangliosidosis/-hexosaminidase -chain	15q2324	yes [147]
G_{M2} -Gangliosidoses/-hexosaminidase -chain	5q13	yes[148]
G_{M2} -Gangliosidoses/GM_2 -activator	5q31.333.1	yes[149]
G_{M1} -Gangliosidoses/-galactosidase	3p21p14.2	yes[150]
Fabry disease/-galactosidase	Xq21.33q22	yes[151]
Schindler disease/*N*-acetyl--galactosaminidase	22q11	yes[152]
Krabbe disease/galactocerebrosidase	14q24.3q32.1	yes[153]
Niemann-Pick/sphingomyelinase	11p15.115.4	yes[154]
Neutral Lipid Storage Diseases		
Wolman disease/lysosomal acid lipase	10q23q23.3	yes[155]
Cholesterol ester storage disease/lysosomal acid lipase	10q23q23.3	yes[155]
Farber disease/ceramidase		yes[156]

common pathway for lysosomal storage diseases is the accumulation of macromolecules within tissues and cells that synthesize or ingest particular substrates or toxic metabolites in substantial amounts. For many lysosomal diseases, the clinical manifestations derive from the accumulation of substrates that are synthesized endogenously within particular tissue sites of pathology. The degree of involvement of any particular organ or organ system reflects the rate of endogenous synthesis or degradation of those specific compounds in particular tissues. The stored substrates are macromoleculesmucopolysaccharides, glycoproteins, or glycosphingolipidsthat require degradation through a pathway with the sequential removal of single components of the substrate molecules at each step in a catabolic cascade (Fig. 44-4). Most of the enzymes are exohydrolases. Table 44-1 lists selected lysosomal storage diseases categorized according to substrate groups and indicates some of the tissues involved by the various diseases. Specific organ systems are involved by particular diseases to a greater or lesser extent and reflect the balance of endogenous and exogenous substrate presentation. For example, in Hurler disease (MPS IH), dermatan and heparan sulphate accumulate in connective tissue leading to joint contractures and abnormal skin consistency. Tissue-specific pathology also is present in several other diseases. Galactocerebroside is found in

greatest concentration within the myelin sheaths of the central and peripheral nervous systems. Although the deficiency of galactocerebrosidase in Krabbe disease occurs in all tissues, the synthesis of galactocerebroside is much greater in the central and peripheral nervous systems. [36] [37] Consequently, the manifestations of the disease are localized to the nervous system. Similarly, in TSD or Sandhoff disease, the deficiency of -hexosaminidase A or -hexosaminidase A and B, respectively, results in either primarily CNS disease or in combined CNS and visceral diseases due to the different accumulated substrates. [38] -Hexosaminidase A cleaves the specific substrate, G_{M2} -ganglioside, whereas -hexosaminidase B cleaves primarily sialylgangliosides and globosides. G_{M2} -ganglioside is synthesized in large amounts only in brain or other nervous system tissues, and globoside is synthesized primarily in visceral tissues.

Additional mechanisms, other than endogenous substrate synthesis, must be involved in pathologic substrate deposition in Fabry disease, Gaucher disease, and cholesterol ester storage disease (CESD). Fabry disease (-galactosidase-A deficiency [39]) and CESD[40] (lysosomal acid lipase deficiency) result from an inability to catabolize globotriaosylceramide and cholesterol esters, respectively. Both of these compounds are found in plasma associated with LDL particles. These LDL particles are internalized via receptor-mediated endocytosis and presented to the lysosome for degradation. In these two disorders, the major pathophysiology, endothelial cell involvement, is due to the LDL uptake of these substrates and the inability to degrade them within the lysosomes. Endogenous synthesis of globotriaosylceramide as well as cholesterol esters is relatively low within most tissues. The vast majority of accumulated substrate is derived from distant sources (i.e., hepatocytes). In comparison, the visceral manifestations of Gaucher disease (acid - glucosidase deficiency) derive from the storage of glucosylceramide primarily in cells of monocyte/macrophage origin[41] (Fig.44-5).

Figure 44-4 Pathway of glycosphingolipid degradation and the diseases that result from specific enzyme deficiencies. The numbers refer to the following lysosomal hydrolases: (1) -galactosidase. (2) -hexosaminidase A, (3) ganglioside neuraminidase, (4) -hexosaminidase B, (5) -galactosidase A, (6) -galactosidase for lactosylceramide, (7) acid -glucosidase (glucocerebrosidase), (8) sphingomyelinase, (9) arylsulfatase A, (10) -galactocerebrosidase, (11) ceramidase. The deficiency of each of the respective enzymes leads to the accumulations of the substrate preceding the hydrolytic step. Diseases have yet to be described due to the deficiencies of either enzyme 3 or 6.

The vast majority of the viscerally stored substrate probably derives from leukocyte and erythrocyte membrane turnover via phagocytosis of aging blood cells. [42] The inability to degrade all the glucosylceramide from the membranes of these cells results in storage in macrophage lysosomes. Thus, the major pathophysiology of the visceral disease involves imported glycosphingolipid substrate, rather than endogenously synthesized substrate within cells of the monocyte/macrophage system. In comparison, Gaucher disease type 2 is a severe neurodegenerative disorder of infancy. The massive visceromegaly, particularly of the spleen and liver, and bone marrow infiltration by Gaucher cells in this disease result from the phagocytic pathway. However, the severe neurodegenerative disease appears related to the presence of toxic by-products derived from degradation of endogenously synthesized glucosylceramide within CNS neurons of affected patients. These endogenous toxic metabolites, and the importation into the brain of glucosylceramide from peripheral sources, jointly contribute to the severe neurodegenerative disease. The contribution of transported and imported substrates to the pathophysiology of other diseasesMLD, MPS, and the glycoproteinosesare not fully understood and certainly would have an impact on therapeutic approaches to these diseases. Thus, several mechanismsthresholds of enzyme activity, tissue or developmental specificity, substrate localization, and substrate importationplay roles in the pathophysiology of lysosomal storage diseases. The relative importance of each process varies among diseases and may explain differential organ involvement within a specific disease category.

The direct distortion of the lysosomal architecture (i.e., engorgement of lysosomes) probably has significant pathologic

Figure 44-5 Diagram of the cellular pathophysiology of Gaucher disease. Monocytes are produced in the bone marrow and mature to macrophages in the marrow or in specific sites of distribution as liver Kupffer cells, bone osteoclasts, and lung and tissue macrophages. Once resident, they accumulate glucosylceramide by phagocytosis and become end-stage Gaucher cells. The resultant tissue fibrosis, ischemia, osteoporosis, and other pathologic features have not been adequately explained.

consequences, but the pharmacologic effects of the accumulated substrates or by-products are increasingly recognized as major components of the disease pathophysiology.[4] [43] [44] In the glycosphingolipidoses, the toxic metabolites of these complex lipids derive from their incomplete or improper degradation in the lysosome. The deacylated analogs of glycosphingolipids are known as sphingoid bases. These compounds are potent inhibitors of a variety of cellular enzymes including protein kinase C isozymes and other membrane proteins. [44] [45] Sphingoid bases (lyso-glycosphingolipids) accumulate in TSD, Sandhoff disease, [46] Krabbe disease,[47] and Gaucher disease types 2 and 3.[48] The toxicity of sphingoid bases is most evident in Krabbe disease and Gaucher disease type 2. The excessive accumulation of galactosylsphingosine and glucosylsphingosine in these diseases, respectively, leads to the destruction of neurons within the brains of affected individuals. Consequently, the CNS disease manifestations likely are the result of neuronal loss and death rather than the storage of galactosyl- or glucosyl-ceramide per se. In Gaucher disease type 1, small amounts of glucosylsphingosine accumulate in visceral organs and may be related directly or indirectly via cytokines [49] [50] to the fibrosis in the liver, spleen, and bone marrow of affected patients. [51] This would provide a unifying pathophysiologic basis (i.e., cell death and scarring) for the manifestations of Gaucher disease. In TSD G_{M2} -ganglioside and lyso-G_{M2} -ganglioside accumulate.[46] The formation of meganeurites in TSD indicates that at least one of these stored substrates may alter the growth and development of neurons, as well as accumulating in lysosomes. [52] Additional toxic products would be expected in visceral tissues and in a variety of the lysosomal storage diseases, but direct evidence is lacking. In some categories of lysosomal diseases characteristic pathologic patterns are present. In MPS I, II, IV, and VII, dysostosis multiplex is the pattern of bony abnormalities. These bony manifestations must result from an as yet unknown common pathophysiology of stored mucopolysaccharides that lead to similar altered pathways in bony development.

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MOLECULAR GENETIC MECHANISMS OF LYSOSOMAL STORAGE DISEASES

The molecular mechanisms that result in the lysosomal storage diseases are as varied as the diseases. The mechanistic abnormalities include:

1. Deficiency of a single hydrolase, predisposing to multiple phenotypes
2. Deficiencies of multiple lysosomal proteins mediated by a single gene defect
3. Specific in vivo substrate accumulation with intact in vitro hydrolase activity
4. Absence of functional lysosomal hydrolysis with intact hydrolase activities

Single Gene, Multiple Phenotypes

The vast majority of the lysosomal storage diseases result from mutations at single loci that involve a single gene product and are either point mutations or deletions or insertions in genes encoding subunits of a particular protein. Missense or nonsense mutations result in the production of proteins with abnormal catalytic function, stability, or processing. For many of these mutations an abnormal protein with low enzymatic activity is synthesized. For example, >100 mutations have been described in Gaucher disease or TSD at the respective loci. These mutations have differential effects on enzyme stability, catalytic activity, or effector interactions. [53] Because these two diseases are found at high frequencies in the Ashkenazi Jewish population, a few common mutations would be expected, due to either founder effect or selective pressures. The phenotypic variation in these two diseases represents a continuum of degrees of involvement or age at onset of symptoms. For Gaucher disease, this continuum extends to the asymptomatic population. The majority of affected Ashkenazi Jews with the N370S/N370S genotype are asymptomatic and never come to medical attention. [54]

In comparison, two completely different disease phenotypes appear to result from the same enzymatic deficiency due to mutations at the -galactosidase locus: the G_{M1} -gangliosidoses and Morquio type B (MPS IVB). In the former, G_{M1} -ganglioside accumulates preferentially in the CNS and mucopolysaccharide metabolites in the visceral organs. [55] In Morquio type B disease, keratan sulphate accumulates in visceral organs leading to skeletal dysplasia and abnormal connective tissue properties (e.g., hypermobility). The CNS is not directly involved. These very different phenotypes result from mutations in the structural locus for -galactosidase affecting different protein domains of -galactosidase. [55] [56] Thus, mutations in single lysosomal polypeptides can result in multiple disease variants, but their molecular etiologies differ. The severe infantile variant (G_{M2} , gangliosidosis) can be associated with a protein that poorly interacts with its protector protein and is rapidly degraded. The later onset variant has a protein peptide that can be improperly targeted with little enzyme routed to the lysosome. The MPS IVB protein binds to protector protein and is properly routed to the lysosome, but exhibits little catalytic activity toward keratan sulfate. [57]

Several lysosomal hydrolases are multimeric or occur as multienzyme complexes. A mutation in a common subunit or a component of the multienzyme structure could result in selective deficiencies of individual enzyme components or in all the components of the complex. TSD and Sandhoff disease are typical examples of heteromeric proteins in which distinct phenotypes result from mutations in different subunits. -Hexosaminidase A is composed of - and -chains, whereas -hexosaminidase B is composed of only -chains. Mutations at the -chain locus result in TSD (-hexosaminidase A deficiency), whereas those in the -chain result in Sandhoff disease (-hexosaminidase A and B deficiency). [58] The substrates for -hexosaminidase B occur in the viscera and those for -hexosaminidase A, G_{M2} -ganglioside, occur predominantly in the CNS. Therefore, Sandhoff disease has manifestations in the CNS and in the viscera.

The -galactosidase/neuraminidase/protector protein complex is more complicated. Isolated deficiencies of each of these enzymatic components have been described. [55] Neuraminidase deficiency results in sialidosis, a glycoprotein storage disease, and protector protein defects result in deficiencies of both -galactosidase and neuraminidase. The protector protein is a protease that attaches to the neuraminidase/-galactosidase complex sometime during synthesis and protects this complex from inactivation or degradation. [25] Thus, point mutations in the protector protein can lead to galactosialidosis, a triple enzymatic deficiency producing variable phenotypic effects. [26]

A unique and important example of a mutation in a nonlysosomal enzyme causing multiple enzyme deficiencies is multiple sulfatase deficiency (MSD). In this disease, hydrolysis of sulfated mucopolysaccharides and glycolipids is impaired and a phenotype is produced that resembles a composite of MLD (arylsulfatase A deficiency), MPS II (iduronate sulfatase deficiency), and ichthyosis (microsomal steroid sulfatase deficiency). Because the structural genes for the sulfatases are not involved, a co- or post-translational modification defect common to cellular sulfatases was suspected. By direct analyses of cellular sulfatases, it was shown that active sulfatases require a specific post-translational modification of a conserved cysteine. An enzyme is required to modify this cysteine to 2-amino-3-oxo-propionic acid that acts as a carrier of the sulfite group

at the active site of most (maybe all) sulfatases. MSD cells are deficient in this post-translational process. [59]

Normal In Vitro, Deficient In Vivo, Substrate Cleavage

Mutations in protein activators or cofactors necessary for the catalytic activity of a variety of glycosphingolipid hydrolases result in diseases that are phenocopies of particular enzyme deficiencies. For glycosphingolipid hydrolysis, five such protein activators have been described. Four of these, termed saposins A, B, C, and D, are encoded by a single locus on chromosome 10 (Fig. 44-3). The G_{M2} -activator required for catabolism of G_{M2} -ganglioside by -hexosaminidase A is encoded by a locus on chromosome 5. [60] The hallmark of the saposin deficiencies is the accumulation of specific lipid substrates for a suspected deficient enzyme activity, with normal in vitro activity of the associated enzyme. Gaucher-like diseases have been described due to the deficiency of saposin C and prosaposin. [61] [62] Metachromatic leukodystrophy-like diseases result from saposin B deficiency, [63] [64] and Tay Sachs-like diseases are due to G_{M2} -activator deficiencies. [65] Of these saposin deficiencies, the defects in the prosaposin locus are particularly interesting. Each of the saposins, A, B, C, and D, participates in the hydrolysis of a variety of glycosphingolipids (Fig. 44-3). These saposins are thought to participate in specific interactions with the cognate enzyme in glycosphingolipid catalysis, [66] in ganglioside transport, [67] and in substrate presentation. As expected, the deficiency of prosaposin leads to a complex disorder mimicking the deficiency of several glycosphingolipid hydrolases. [68] Importantly, the in vitro activities of the corresponding enzymes are nearly normal in tissues from patients with the saposin defects. It is anticipated that several other activator or cofactor molecules, required for the assistance in hydrolysis of complex macromolecules, will be discovered in the future.

Intracellular Deficiencies/Extracellular Excess

I-cell disease exemplifies the impact on lysosomal enzymes of mutations at loci for nonlysosomal proteins. The enzyme, *N*-acetylglucosamine-1-phosphotransferase is required for the attachment to many lysosomal proteins of the mannose-6-phosphate ligand sorting signal. [9] [11] Mutations at the loci for subunits encoding this

enzyme result in the absence of the mannose-6-phosphate ligand and the secretion into the media of most soluble lysosomal enzyme proteins. The lysosomes are nearly devoid of mannose-6-phosphate receptor targeted enzymes. Consequently, the phenotypes resemble a mixture of various mucopolysaccharide, glycoprotein, and glycosphingolipid degradation defects, due to the loss of many enzymes and proteins involved in the respective catabolic pathways. Because some of the membrane-bound proteins of the lysosome are targeted via mannose-6-phosphate independent pathways, they are retained within the lysosomes. However, their catalytic activity may be affected by the loss of activator proteins and other lysosomal components that require mannose-6-phosphate receptor-mediated sorting.

Mucopolysaccharidoses II is associated with multiple de novo micro- and macro-deletions in the iduronate sulfatase gene. [69] A pseudogene adjacent to the iduronate sulfatase gene promotes unequal homologous recombination events as a major cause of new mutations in this condition. [70] At least one case of contiguous gene deletion including both iduronate sulfatase and the fragile X gene has been reported related to the proximity of these loci. [71] In comparison, the gene for -L-iduronidase (MPS I) overlaps with a gene for a sulfate transporter. This latter gene is encoded on the opposite strand within the structural gene for - L-iduronidase.[72] This raises the possibility that mutations could affect both genes and lead to phenotypic variation in MPS IH. Several new mutational mechanisms have been found and may be important in phenotypic variation of lysosomal storage diseases.

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GENOTYPE/PHENOTYPE CORRELATIONS

Implicit in the threshold hypothesis is a relationship between the level of enzymatic activity in cells and the phenotype expressed in the patients tissues. Consequently, for lysosomal storage diseases, and other inherited defects, investigators have attempted to relate genetic mutations to phenotypic manifestations. For three lysosomal storage diseases good, albeit imperfect, correlations have been obtained: MLD, TSD, and Gaucher disease. In each of these disorders, major variants include rapidly to slowly progressive onset types. In MLD combinations of two mutant arylsulfatase A alleles account for the majority of infantile-, juvenile-, and adult-onset variants of this disease.[73] [74] [75] The presence of two copies of a null allele (splicing defect in intron 2) results in a severe infantile disease. In comparison, homozygotes for a P426L substitution develop an adult-onset disease. The combination of a null and P426L mutant alleles can lead to a juvenile-onset variant. These results correlate well with the measured in situ lysosomal cleavage of exogenously supplied sulfatide substrate. [33] It is noteworthy that a very common pseudodeficiency allele at the arylsulfatase A locus results in deficient activity toward artificial substrates as measured in vitro, whereas the in vivo cleavage of sulfatide is low-normal in these cells. This mutant allele contains an altered polyadenylation signal in the 3 untranslated region, leading to inefficient polyadenylation and a decrease in available mRNA for protein synthesis. In combination with the glycosylation or null alleles for MLD, individuals with a pseudodeficient allele appear to be deficient for arylsulfatase A activity and would be expected to develop MLD symptoms. However, these individuals are asymptomatic. Because this pseudoallele is present in 1015% of the population, it is important to exclude its presence in suspected MLD families, particularly before the institution of therapies. [74] Disease-causing mutations can occur on the background of the pseudodeficient allele. Thus, the detection of the pseudodeficient mutation does not exclude a disease mutation on the same allele. In situ measurement of sulfatide cleavage is required to ensure proper diagnosis, and interpretation can be difficult. [76] The pseudodeficient alleles for -hexosaminidase A [77] and -L-iduronidase [78] raise similar concerns.

In TSD, various combinations of null and missense mutations produce different onset forms of -hexosaminidase A deficiency. The most common allele in the Ashkenazi Jewish population is a four-base pair insertion that results in the production of a nonfunctional mRNA, [58] [79] that is, a null allele resulting in the total lack of -chains for -hexosaminidase A synthesis. In comparison, the missense mutation leading to a glycine to serine (G269S) substitution produces residual -hexosaminidase A activity and a more slowly progressive disease. The onset of neurodegeneration and dementia occurs in adolescence to adulthood. [80] Similar to MLD, the combinations of null mutations with those that lead to partially functional enzymes lead to the different ages of disease onset. In these disease variants, neuropathic manifestations are progressive, and the rate of deterioration correlates inversely with the in situ level of enzyme activity from 05% of normal. In comparison, nearly all Gaucher disease types are associated with residual enzymatic activity within cells. Although good correlations have not been obtained between the level of in vitro enzymatic activity of acid -glucosidase and the disease phenotype, [81] improved correlations have been found when in situ lysosomal glucosylceramide cleavage is estimated.[82] Within this more physiologic environment, lower levels of residual activity were found in the more severe types 2 and 3 variants as compared to the type 1 non-neuronopathic variants. Unlike the TSD and MLD variants, the

genotype/phenotype correlations in Gaucher disease relate to the presence or absence of neuronopathic disease, not the delayed onset of CNS involvement (for a review see reference [53]).

Gaucher disease is the prototype for lysosomal storage diseases where several thresholds of substrate flux are apparent. The first threshold is for the preservation of normal glucosylceramide metabolism. This threshold (50% of normal) is apparent in carriers because they do not develop disease manifestations or have Gaucher cells in their tissues. The next level is that in affected homozygotes who are enzyme deficient (<20% normal) and are relatively mild to asymptomatic. These patients have enzyme deficiency and Gaucher cells in their bone marrow but minimal to no disease involvement. The next threshold is for children, adolescents, or young adults with significant to severe visceral manifestations and shortened life span. Within this group of type 1 individuals, the presence of the N370S and other disease alleles modifies the disease expression. Although the degree of involvement is highly variable, homozygotes for N370S are less severely involved by the disease, and some are asymptomatic. Indeed, about 6070% of N370S homozygotes in the Ashkenazi Jewish population are minimally symptomatic to asymptomatic.[54] In comparison, the N370S/null allele genotype usually leads to a very severe, but non-neuronopathic, course of disease. The next threshold is for the development of neuronopathic disease. Within this group are patients with types 2 and 3 disease. Type 3 patients have later onset of neuronopathic manifestations. Among type 3 variants there is significant heterogeneity with two different subtypes of neuronopathic disease, designated 3a and 3b. [83] From studies of in situ glucosylceramide metabolism, as well as genotype analysis among homozygotes for the L444P mutation, the type 3 patients appear to have significantly greater flux of glucosylceramide through their fibroblasts than do type 2 patients. The most severe type 2 patients may have one L444P mutation and an additional allele, which is null. These thresholds are relative because even in genetically normal individuals, the massive substrate load presented by the turnover of cells in diseases such as acute myelogenous leukemia, leads to the presence in bone marrow of Gaucher-like cells in patients with completely normal enzymatic activity. Thus, not unexpectedly, other factors or genes can modify the disease expression in patients with the same genotype. These factors or genes have yet to be identified, and the role of ethnic or racial background on disease expression requires definition.

From a practical view, the level of residual enzymatic activity could determine the eventual need for enzyme or gene therapy. In patients with substantial amounts of residual enzymatic activity (e.g., symptomatic N370S homozygotes), less total enzyme may be needed than in patients who have the genotype N370S/null or other severely compromised alleles. The former patients can supply significant amounts of endogenous enzymatic activity for the cleavage of glucosylceramide and may have decreased need for exogenous enzyme. For other lysosomal diseases, calculations based on in situ activity of enzymes indicate that very small differences in the quantity of enzyme in cells have major impacts on the phenotype.[33] Exogenously supplied enzyme might also influence the onset of specific organ, tissue, or cellular disease manifestations. The findings in TSD, MLD, and Gaucher disease variants anticipate similar findings in other lysosomal storage diseases. Clearly, mutation analysis in the defective gene alone will not provide for accurate prognostication. Considerable intrafamilial variation exists between affected members with these diseases. This makes it difficult to counsel and prognosticate from historical family data alone. [84]

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THERAPEUTIC APPROACHES AND IMPLICATIONS

The threshold hypothesis implies that small amounts of enzymatic activity may be sufficient for prevention of various disease manifestations in the lysosomal diseases. During the past two decades, two major approaches have been exploited to overcome these thresholds and correct the underlying metabolic defects: (1) direct enzyme therapy by delivering a specific, pure, lysosomal enzyme that is missing and (2) supplying the missing enzyme by transplantation of normal organs or cells. For the transplantation strategy, bone marrow, liver, spleen, or skin cells could act as metabolic or enzyme pumps for delivery of enzyme to the plasma for uptake at distant sites to contiguous host cells or direct replacement and correction of specific cell types. Both of these approaches require receptor-mediated endocytosis through specific recognition in the target sites of pathology. This implies the need to correct only specific cell types, for example, those using the specific receptor system or being derived from bone marrow precursor cells. The normal enzyme could be supplied to deficient cells via partially corrected cell populations (chimerism) or by the administered enzyme. This applies to enzymes that are soluble or those that can be solubilized in plasma or interstitial spaces. For membrane-bound proteins, cell-cell contact (i.e., metabolic cooperation) may be required for the direct transfer of the enzyme with subsequent targeting to the lysosome. This mechanism may constrain delivery of the enzyme only to those cells in direct proximity to the normal cell and could lead to nonuniform correction. In diseases where the target sites of pathology may be distant from the sites of synthesis of a membrane-bound protein, delivery of enzymes via a secretory pathway will not work, and alternative strategies are required for delivery of enzyme. Thus, the approach with enzyme therapy or cellular transplantation strategies is disease dependent, based on knowledge of enzyme distribution and intra- and intercellular transport.

Substantial amounts of accumulated substrates in various tissues may be of exogenous origin, that is, transported from distant sites and deposited in lysosomes within particular sites of pathology. Consequently, depletion of the plasma pool of these accumulating substrates may lead to a re-equilibration of previously stored material from tissue sources into the plasma. Although this is hypothetical for most diseases, in Fabry disease previously stored substrate can be mobilized from tissue storage sites.[85] Such a depletion mechanism has obvious therapeutic implications and implies an interaction of several organs and cell types in phenotypic development. In the neuronopathic Gaucher phenotypes, some accumulating substrate in adventitial layers of CNS blood vessels is derived from visceral sources. [86] Depletion of substrate from these visceral sources might lead to a modified progression of the phenotype, depending on the impact of this pathology on CNS function. This depletion approach has not been exploited in many of the lysosomal storage diseases because little is known about glycosphingolipid, mucopolysaccharide, and glycopeptide systemic trafficking. If such depletion and re-equilibration can occur, the delivery of particular enzymes for specific diseases to the target sites of pathology may not be essential, and transport of soluble disease metabolites from tissues might prove efficacious alone or in combination with enzyme reconstitution.

The implications for genetic therapeutic approaches to the treatment of lysosomal storage diseases are clear. If the deficient enzyme must be delivered directly to the target site of pathology, transplantation of the appropriate stem cell containing the appropriate genes is obtainable in selected visceral tissues. The macrophage/monocytes of liver, spleen, bone marrow, lungs, and other reticuloendothelial tissues can be replaced by BMT with normal cells. Thus, irrespective of the complex practical and theoretical issues surrounding gene therapy approaches, gene transfer into bone marrow progenitor stem cells of the affected individuals should prove efficacious. However, metabolites might not re-equilibrate out of brain rapidly enough to prevent the manifestations of the disease in the CNS. If so, the enzyme or cells would need to be delivered to the CNS cell

types or areas that are pathologically involved. The delivery of macromolecules, cells, and recombinant viruses to the brain has proven too difficult to date, and continues as a significant barrier to the development of gene delivery for neurologic diseases, as it has been for their enzyme therapy.

Animal models of human lysosomal storage diseases are essential to the continuing development of therapies and pathophysiology studies. Although numerous naturally occurring models have been identified in mice, rats, dogs, cats, cattle, and other species, targeted gene disruption techniques provide for the generation of null and missense mutations in any desired gene, particularly in mice. Although critical to applied and basic advances, caution is needed in extrapolating data from such mice directly to humans. There are several examples of knock-out mice that have either no or lethal phenotypes and do not correspond to the human phenotypes. For the lysosomal storage diseases, the generated phenotypes have been similar to those in humans when similar mutations are compared (e.g., Gaucher disease, TSD, Niemann-Pick A). However, the presence or absence of alternative metabolic pathways in different species can have major impact on the phenotype or the efficacy of a therapeutic intervention. For example, *N*-butyl-deoxynojirimycin inhibits the synthesis of G_{M2} -ganglioside. When given to mice homozygous for null mutations for -hexosaminidase A, dramatic reductions in storage in G_{M2} -ganglioside storage were observed, suggesting a major therapeutic effect.[87] However, the observed effects rely on an alternative pathway for G_{M2} -ganglioside degradation in mice; G_{M2} -ganglioside asialo G_{M2} -ganglioside degradative products, in which the last step is not dependent on -hexosaminidase A. This provides an alternative disposal of limited quantities of G_{M2} -ganglioside. This pathway is not present in humans and similar dramatic effects might not be anticipated.

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RESULTS AND LESSONS FROM THERAPY

The principal objective of cellular transplantation and enzyme therapy approaches has been to supply enzyme to various organs in the body, either via cells or by direct enzyme administration. The results and interpretations of these experiences are critically important to the development of future gene-based strategies for the lysosomal storage diseases. Based on the pluripotency of bone marrow stem cells, most transplantation studies have focused on these cells. The isolation and characterization of stem cells from other organs, including neuronal stem cells, have been encouraging and may provide pluripotent sources of enzyme in the future.[88] [89] As indicated in Table 44-1 , various lysosomal storage diseases have preferential sites of pathology. However, all cells in the body are involved with the enzyme deficiency, and most tissues can manifest symptoms and signs of these diseases. For BMT or other organ transplantations to be effective, the cells containing enzymes must be able to catabolize supplied substrate. This ultimately depends on the mass of cells transplanted because each cell can synthesize only limited amounts of enzyme for supply to the body. Such considerations are particularly relevant for organs like kidneys and liver where donor cells do not migrate from the organ.

Transplantation Approaches

Animal and human studies identified a critical limitation to transplantation strategies for lysosomal storage diseases: the inability to get enzyme or sufficient bone marrow-derived cells into brain substance. The delivery of the enzyme directly into the brain may not be necessary for CNS removal of cytotoxic substrates. However, it is theoretically desirable to deliver the enzyme to the involved cells and organs. Currently, insufficient data are available from autopsy or clinical material of transplanted humans to conclusively show the extent of biochemical or clinical improvements in the CNS of patients with most of the lysosomal storage diseases, making data obtained from animal models important in design of therapies.

The MPS I dog results from a point mutation in the -L-iduronidase gene leading to a truncated protein. [90] The visceral phenotype is similar to the moderately severe MPS I HS in humans, although the CNS storage resembles the more severe MPS IH. BMT from unaffected litter mates leads to functional and pathologic improvements in bony deformities, cardiac abnormalities, corneal clouding, and hepatosplenomegaly of affected dogs. [91] The host hepatocytes were cleared of storage material, consistent with uptake of -L-iduronidase from donor Kupffer cells. Vertebral and joint pathology continue to progress, although at a slower rate than in untransplanted affected dogs. The CNS effects were difficult to evaluate functionally, but lysosomal distention was decreased in glial cells, and there were varying improvements in neurons.

The efficacy of BMT, enzyme infusions, and gene therapy [92] [93] [94] [95] have been compared in MPS VII mice. A single base pair deletion in exon 10 of the *gus* gene (e.g., -glucuronidase) causes the disease. BMT from syngenetic unaffected mice into irradiated MPS VII affected mice increased life span and cleared storage material from liver, spleen, kidney, and cornea. Early BMT resulted in greater improvements in the skeleton and in auditory function, but neurologic and behavioral improvements were not noted. The pre-BMT irradiation regimen was shown to directly affect CNS development in young mice, producing a CNS dysplasia. [92] In comparison, infusion of recombinant -glucuronidase had no effect on the cornea and limited ability to slow the skeletal disease when initiated at 6 weeks of age. Combination of neonatal enzyme infusions, followed by later BMT, showed the synergistic effects even though brain enzyme levels were restored to only 0.75% of normal, compared to 20% in spleen, in treated mice. Poor entry of enzyme into the brain, either by infusion or BMT, is a barrier, but it is possible that very low levels of reconstitution may be sufficient to prevent severe disease. This was shown in the Twitcher mouse (a model for Krabbe disease) rescued by a -galactocerebrosidase transgene driven by a myelin basic protein promoter (K. Suzuki, personal communication). Expression was detectable only by reverse transcriptase-polymerase chain reaction, but substantial improvement in the phenotype was shown. These results indicate that reconstitution toward normal substrate flux may require very small amounts of enzyme, but the threshold amount will be disease, and probably, tissue dependent.

Continued efforts to evaluate BMT in affected animals are important because conclusive outcomes in human lysosomal storage diseases accrue only over many years. Also, the relatively small numbers of affected patients affect study design and appropriate controls. Although mouse analogs can supplement the human data on potential effects, the neurologic issues can only be addressed in humans. The greatest experience with human BMT has been in MPS IH. For this disease, early transplantation improves the clinical course and longevity in matched related and unrelated donors. The skeletal disease continues to be a major progressive clinical problem. Also, the risks of death, graft-versus-host disease (GVHD), and graft failure are substantial in those receiving matched unrelated donor marrow. The effects on developmental and neurologic function are less clear. In a study of 40 unrelated donor BMT with MPS IH, the developmental outcome was evaluable in only 11 patients. Although the trajectory of development was said to improve in unrelated donor BMT patients with initial mean developmental index (MDI) >70, the absolute MDI values decreased in 50%,[96] and if the initial MDI was <70, the results were poor. Very poor CNS results have been obtained in MPS II and III, where severe behavioral disorders appear to progress even in those patients stably engrafted. [97] In MPS III, GVHD may play a role in the

poor outcome. The older age of patients undergoing BMT for MPS II and III is thought to influence the poor outcome. But even young MPS III patients do poorly. [97] In MLD and Krabbe disease, the experience has been less extensive and varied. In both disorders, better CNS outcomes were obtained in later-onset phenotypes.

The effects of cytoreductive therapy and GVHD, and their interaction with the underlying disease, make the analysis of effectiveness even more complicated in humans where controlled trials are difficult. Also, the therapeutic goals should be carefully delineated. In humans, the natural evolution of tissue lesions in many of the lysosomal storage diseases and, particularly in the brain, are poorly documented. However, with time, these lesions probably become irreversible in a tissue-dependent manner, and a window of opportunity may exist for achieving reversal of disease manifestations in each disease. Once this disease-specific developmental threshold is passed, the damage to the brain or other organs may progress despite therapy. Progressive CNS involvement in Krabbe disease (human and murine) and Sanfilippo syndrome, and the worsening skeletal disease of MPS IH following BMT, support this notion. In addition, the varying levels of reconstitution of metabolic flux in each tissue may lead to differing results in patients. For example, variable reconstitution in the CNS following BMT may convert infantile to juvenile MLD, that is, from an earlier-onset, severe abnormality to a later-onset progressive disease. BMT will have its greatest potential beneficial effects in diseases that are pathophysiologically derived from a single cell line that can be replaced by BMT. Gaucher disease is the prime example where BMT cures the visceral lesions if instituted before irreversible damage in liver, spleen, lung, or bone marrow. [98] [99] [100] [101] The visceral components of this disease derive from monocyte/macrophage-lineage cells. The effects of BMT on the resolution or progression of signs and symptoms have been disease variant and stage specific. BMT is probably not warranted in patients with severe irreversible pathology. Careful baseline evaluation, selection, and follow-up of patients will be required to assess the therapeutic impact of BMT in the individual lysosomal storage diseases.

The results of transplantation of other organs, such as liver, kidney, and spleen, in these diseases have been limited. In diseases that result from soluble enzyme defects, hepatic, splenic, or other solid organ transplantation may be beneficial if sufficient amounts of enzyme can be synthesized and secreted for delivery to distant sites. In comparison, in diseases such as Gaucher disease, where the enzyme is not normally secreted, transplantation of liver has resulted in the accumulation of Gaucher cells and development of pathology in transplanted livers. [102] These results indicate that hepatic transplantation alone is insufficient for preventing

redevelopment of symptoms in such severely involved patients. Blood chimerism may develop following hepatic transplantation because the donor liver may supply small amounts of enzyme and bone morrow cells to recipient organs. [103] Patients receiving hepatic transplantation and small amounts of donor bone marrow may become chimeric and develop two populations of bone marrow-derived cells in their bodythose that are normal and those that are not. At a low level of chimerism, the storage cells would be expected to accumulate in tissues and in the absence of cross-correction eventually result in recurrence of disease manifestations. Greater degrees of monoctye/macrophage chimerism could lead to improvement if the normal cells can prevent accumulation of end-stage Gaucher cells in tissues. [104]

Enzyme Therapy Approaches

Enzyme therapy has been evaluated in several MPS animal models and in human Gaucher disease. The results in MPS animals were similar to the human diseases, with histologic improvements in hepatocytes, Kupffer cells, myocardium, heart valves, cartilage, and cornea. These results vary with the disease, but in general, few improvements are observed in the brain. [105] [106] [107] The skeletal disease slows but is not reversed. These results also vary somewhat depending on the source of the enzyme, that is, type of recombinant cell type, and, therefore, oligosaccharide modification.

A significant concern for the use of enzyme therapy is the development of antibodies to exogenous protein. This has been assessed in only a few studies. MPS I dogs given recombinant canine or human -L-iduronidase developed antibodies. Similarly in dogs implanted with retrovirally transduced myoblasts expressing canine -L-iduronidase, anti-iduronidase antibodies were induced, with eventual loss of the transduced cells in these animals. [108] The molecular lesion in the MPS I dog leads to premature termination of translation after the first exon, that is, these animals are null for - L-iduronidase protein. Mutations causing premature termination of -L-iduronidase are frequent in human patients with MPS IH, and similar problems might be anticipated. MPS VI cats had repeated episodes of anaphylaxis during enzyme infusions, but the IgG antibodies were not directed against the recombinant human enzyme itself. These difficulties cannot be ascribed only to the presence or absence of endogenous enzyme protein because 15% of treated Gaucher disease patients develop antibodies to the administered enzyme, and all have residual enzyme activity.

Unlike many other therapeutic modalities, enzyme therapy has been studied extensively only in human Gaucher disease. Enzyme therapy in this disease exploits the receptor for -mannosyl-terminated oligosaccharides on the surface of cells of monocyte/macrophage origin. The membrane-associated human acid -glucosidase is extracted from placental tissue or from over-expressing Chinese hamster ovary cells, purified to near homogeneity, treated with exoglycosidases to expose -mannosyl groups, and intravenously administered to affected patients. Several studies have demonstrated the utility of this approach, and many patients have excellent responses. [109] [110] [111] [112] [113] [114] [115] The population of reported patients is varied, and insight can be gained into responses for individual patients during treatment. There is substantial variation in the response to therapy, but after 1 year of enzyme therapy responses can include: (1) 2030% decrease in liver volume if the liver is initially enlarged, (2) 3050% decrease in splenic volume, (3) a 1.5 gm% hemoglobin increase if initially anemic with 3350% of such patients achieving normal hemoglobin levels, and (4) an increase in platelet counts. In many patients with massive or heavily scarred spleens, thrombocytopenia may persist for several years. Patients on higher doses have somewhat better responses in splenic volume than those on smaller doses, but the dose-response relationships are highly variable. Patients with massive splenic enlargement have poor hematologic responses until the spleen decreases by 5070%. This suggests that there may be a threshold size of spleen for adequate hematologic response, secondary to intrasplenic hemolysis and platelet consumption. There is a large variation in response to enzyme therapy among patients and at different times during therapy in one patient. Consequently, patients continue to require follow-up and monitoring to evaluate responses and to initiate treatment changes.

Hepatic and splenic changes are more rapid than those in bones or lungs. One cannot exclude the potential for each tissue having a different dose-response threshold. This is a particular concern in light of the inability to discern bony or pulmonary changes during a period of rapid dose reduction while maintaining hematologic, hepatic, and splenic improvement rates. However, little data are available on the tissue, cellular, and subcellular distribution or the half-lives of the administered enzyme. Data in mice suggest that the enzyme has relatively low uptake by monocyte/macrophage cells [116] and very different uptake and survival patterns in bone marrow, liver, and lung. [110] [117]

In addition, the cellular distribution of exogenous enzyme in affected patients is unknown, but the slowness of response to enzyme suggests that the pre-Gaucher cell compartment may be the major target for enzyme uptake. These corrected cells would then ingest Gaucher cells and degrade the contained glucosylceramide.

In view of the high cost of enzyme therapy, the timing of the initiation of therapy must be carefully evaluated. Highly symptomatic patients should begin therapy immediately. In addition, other patients with major hematologic and visceral organ involvement should receive high priority for therapy. Although bone disease appears to be the slowest to respond, [118] patients with substantial bone involvement are candidates for treatment to recover bone integrity and to prevent irreversibility of some bony damage. More difficult questions are posed by presymptomatic patients whose susceptibility to major disease manifestations can be anticipated by genotype analysis. [54] Clearly, the issue of the timing and use of an apparently universally effective therapy must be carefully considered and evaluated as a potential preventive intervention.

Using Gaucher disease as a prototype, several difficult scientific issues need to be resolved to promote optimal use of therapeutic strategies in other lysosomal storage diseases. There is extensive interpatient variability in dose response. What are the determinants of this variability? Will the effects of more easily accessed tissues be preferentially evaluated rather than those in more difficult, potentially more relevant, tissues? Is there a diminution in the need for intense therapy for continued improvement as the tissues are healed? Most importantly, what is the window of opportunity for effective therapy? Is it more efficacious to prevent the disease manifestations than to attempt to return a sick individual, with potentially irreversible tissue damage, to health? This is particularly relevant for those disorders which involve the CNS.

Gene Transfer

Lysosomal diseases, which can be treated successfully by BMT, should be amenable to permanent correction by gene therapy using hematopoietic stem cells carrying a copy of the normal gene. Following autologous transplantation with these corrected cells, somatic genetic reconstitution could be achieved by the repopulation of tissues with monocyte/macrophage derived cells. For diseases due to defects of soluble enzymes, only a portion of stem cells would need to be corrected because the corrected cells could supply enzyme to other tissues. For diseases that result from membrane-associated enzymes (e.g., Gaucher disease), all or most of the cells would need to be corrected because the enzyme cannot cross-correct other cells, and tissue macrophage chimerism could have pathophysiologic effects. For either of these types of enzyme defects, the amount of enzyme supplied to tissues directly or via cellular replacement would be mass dependent, and insufficient amounts of enzyme or cells would lead to the redevelopment or just delay of onset of symptoms and signs. Stem cells that produce active lysosomal enzymes do not have any obvious proliferative advantage over those that do not. Thus, therapeutic effects would be expected only if the patients untransformed cells were ablated by chemotherapy or irradiation. Rational strategy for the treatment of these diseases might include marrow ablation followed by autologous transplantation with transformed hematopoietic stem cells. [94] Considerable effort has been expended to develop the required efficient gene transfer technology and hematopoietic stem cell culture systems. Significant long-term expression of transgene has been accomplished with several of these enzymes. [119] [120] [121] [122] Protocols for somatic gene therapy of lysosomal diseases will likely be evaluated during the next several years.

Gene therapy studies in animal analogs have attempted to exploit targeted enzyme delivery and cell transplantation strategies. The objective of the former is to implant a continuous source of enzyme for delivery to distant sites. The latter approach attempts to replace the primary cells of pathologic involvement with gene-corrected cells. The major motivation for cellular replacement approaches to gene therapy is to avoid the real and potential immunologic barriers to tissue transplantation by using autologous transplants of corrected cells. Overexpression of -glucuronidase or - L-iduronidase from retrovirally transduced fibroblasts, implanted as neo-organs, results in secretion of sufficient enzyme for detection in distant organs. The levels achieved in these enzyme-deficient organs are modest, up to 2% of control in liver. This can be compared to those levels achieved by enzyme infusion or BMT 518%. [123] Immunohistochemical studies show detection of the secreted enzyme only in periadventitial macrophages in the brain and not in brain substance, despite in vitro studies showing enzyme uptake into neuronal and glial cells. The use of neuronal stem cell transplants appears to have promise for neuronopathic diseases. Ideally, these cells would be pluripotent neuronal cells that when transplanted into neonatal or adult mice would migrate to specific brain regions and differentiate into appropriate neurons. These cells, allogenic or immunotolerized, could then replace diseased neurons or supply enzyme to neighboring diseased cells for metabolic correction. Initial animal studies are promising, but clinical trials are in the future.

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DIAGNOSIS OF LYSOSOMAL STORAGE DISEASES

The elucidation of the molecular lesions causing the lysosomal storage diseases has provided insight into the great heterogeneity within each of these diseases. In addition, the proliferation of tests for the specific genetic lesions has led many to substitute molecular testing for enzyme-based diagnoses. The great specificity of molecular testing is its downfall, whereas the more generic nature of enzyme testing is a major strength. For any of the disease categories listed in Table 44-1 , the diagnosis is established by detection of the specific enzyme defect. In experienced laboratories many of these activities are accurately determined and will establish the specific diagnosis. Once the diagnosis is established, additional molecular testing can provide supplementary information and, in some cases, prognostic insight. Because of the presence of pseudodeficient alleles in some lysosomal diseases, molecular testing and other adjunct investigations (e.g., sulfatide loading studies) are necessary to fully establish a phenotype and prognosis. Because of the therapeutic and basic import, every effort should be made to thoroughly and completely characterize these patients at the clinical, biochemical, and molecular levels. Detailed neurologic and physical assessments by clinical and imaging examinations should be made longitudinally in all patients, so that the effects of intervention or relationship of specific mutations to phenotype can be evaluated.

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Chapter 45 - Infectious Mononucleosis and Other Epstein-Barr Virus-Associated Diseases

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INTRODUCTION AND HISTORY

The association of Epstein-Barr virus (EBV) with diseases of lymphoreticular and hematopoietic tissue began in 1964 with the discovery of the virus. [1] Clinically apparent EBV infection was probably first described in 1885 by the Russian pediatrician Filatov as an idiopathic lymphadenopathy in childhood. [2] In 1885, Pfeiffer described glandular fever,[3] and the term *infectious mononucleosis* (IM) was first used in 1923 by Sprunt and Evans.[4] Paul and Bunnell described the appearance of heterophil antibodies during the course of IM, [5] and Davidsohn and Walker devised the differential absorption test, which rendered the heterophil antibody test highly specific for IM.[6]

A major advance in our understanding of EBV infection occurred in 1966, when Gertrude and Werner Henle developed indirect immunofluorescent antibody assays that detected EBV-specific viral antigens. [7] [8]

In 1968, the Henles made a critical observation when they noted that seroconversion to EBV occurred during the course of acute IM in a female laboratory technician.[9] Furthermore, an immortalized EBV-carrying lymphoblastoid cell line was established from a peripheral blood leukocyte culture taken during the acute phase of this womans illness. The Henles followed up this observation with a serologic study of a collection of sera provided by Niederman and McCollum of Yale University. This collection consisted of sera obtained routinely from incoming freshmen, and later from individuals who had had acute IM. This study, as well as others, revealed EBV-specific antibodies in the sera of all students in whom IM developed, thus confirming the etiologic association between EBV and IM. [10] [11]

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EPIDEMIOLOGY

Transmission and Persistence of EBV

Epstein-Barr virus was reported in the early 1970s to be transmitted primarily through contact with oropharyngeal secretions. [12] [13] Observations of EBV in the uterine cervix, reported by Sixbey et al. in 1986, suggest a possible sexual mode of transmission as well. [14] Until recently, it was thought that EBV infection was initiated after infection of oropharyngeal epithelial cells with subsequent infection and dissemination by B lymphocytes. Recent studies, however, have demonstrated that B lymphocytes can support lytic EBV replication in vivo, and it now appears that B lymphocytes may initiate, disseminate, and maintain persistent infection with EBV. [15]

Distribution and Prevalence

Seroepidemiologic studies have demonstrated a wide variation in the age at which EBV infection may be acquired. [16] Differences in geographic and socioeconomic factors account for the differences in prevalence of EBV infection. In the New Hebrides,

where large extended family units exist and the custom of mastication of food for infants by family members is practiced, nearly all infants are infected with EBV by the age of 1 year. [17] In the West Nile district in Africa, where Burkitts lymphoma is endemic, prospective studies have demonstrated that primary infection occurs in most infants between the ages of 4 months and 1 year. [18] [19] By 2 years of age, 81% have experienced primary EBV infection. Most of these infections are asymptomatic.

These studies suggest that early widespread seroconversions to EBV and other herpesviruses during infancy are associated with intense interpersonal contact with many different people. EBV infections have been considered to be of low contagiousness, and true epidemics have not occurred. Spread of EBV within families has been studied by Fleisher et al., who demonstrated serologic evidence of transmission in 7 of 35 families. [20] Each year in the college-age population in the United States, approximately 1015% of susceptible students become infected, and 5070% of the infections are associated with a mononucleosis syndrome.

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BIOLOGIC AND MOLECULAR ASPECTS OF THE DISORDER

Characteristics of EBV Infection

Epstein-Barr virus is a ubiquitous virus infecting most (>90%) of the worlds population. It is associated with both benign disorders (IM) and malignant diseases (African Burkitts lymphoma and nasopharyngeal carcinoma).[8] [21] [22] EBV is a 172kilobase-pair, guanine-cytosinerich (60%), double-stranded DNA virus. [23] [24] EBV is a member of the herpesvirus family, a family that also includes the human viruses herpes simplex, herpes zoster (varicella), and cytomegalovirus. The herpesvirus family, which contains over 80 members, is clustered into 3 subfamilies, , , and herpesviridae, based on host cell range, site of latent infection, cytopathology, and duration of the replicative cycle.[25] EBV is the prototype of the herpesviridae, a subfamily characterized by tropism for B and T lymphocytes. Other members of the subfamily include Kaposis sarcoma-associated herpesvirus, herpesvirus saimiri, a monkey T-lymphotropic virus, and Mareks disease herpesvirus, a chicken lymphotropic virus.[25]

Structurally, the EBV virion is composed of four elements: (1) a dense central genetic core composed of DNA coiled around protein; (2) a nucleocapsid with 162 capsomeres in an icosahedral shape; (3) a protein integument; and (4) an outer membrane or envelope containing four major virus-encoded proteins, three glycosylated proteins (gp) of size 350, 220, and 85 kd (gp350, gp220, and gp85, respectively), and a nonglycosylated 140-kd protein (p140). [21] [25] These proteins are incorporated into the host plasma membrane during virus assembly, as evidenced by their identification on the plasma membrane of cells replicating virus. [26] [27] These proteins are acquired by the virion as it buds through the host cell membrane.

Virion Infectivity

The receptor for EBV is CD21,[28] the 140-kd complement receptor type 2 (CR2), specific for the C3d/C3dg component of the complement cascade. The gp350 and gp220 components mediate EBV virion absorption by binding CD21.[29]

Epstein-Barr virus is internalized into normal B cells by endocytosis into large, thin-walled vesicles, which are distinct from the clathrin-receptosome-lysomal pathway used by other viruses.[30] These vesicles are distinct from clathrin-coated vesicles both in size (larger) and morphologic appearance (no electron-dense, thickened appearance); they do not contain lysosomal enzymes and are not sensitive to weak bases, such as chloroquine, methylamine, or substituted ammonium chlorides. [30]

The mechanism of release of EBV nucleocapsids from vesicles into the cytoplasm is poorly understood. However, it is presumed to occur through virusvesicle membrane fusion. The major glycoprotein mediating this fusion is believed to be gp85, on the basis of its amino acid sequence being similar to that of the glycoprotein of herpes simplex virus (HSV-1), which is implicated in HSV-1 virusvesicle membrane fusion.[31] Antibodies to gp85 do not inhibit virus adsorption or internalization, but do inhibit virusvesicle fusion and thereby inhibit release of EBV into the cytoplasm. [32]

Host Cell Range of EBV

Epstein-Barr virus has traditionally been thought to have a very narrow host cell range, confined primarily to B lymphocytes and certain epithelial cells. [33] [34] Recent descriptions of EBV infection of T cells in patients with peripheral T-cell lymphoma, [35] [36] Reed-Sternberg cells in patients with Hodgkin disease, [37] [38] and smooth muscle cells in human immunodeficiency virus (HIV)-infected people [39] [40] suggest a broader host cell range for EBV infection.

Expression of CD21 antigen is a specific feature of mature B lymphocytes, and either is not present, or is present at very low density, on pre-B and immature B cells. However, mature B cells circulating in the peripheral blood express significant levels of CD21. [41] Activated B cells but not plasma cells also uniformly express CD21. [41] Although CD21 is necessary for infection, it is not sufficient. When B cells are fractionated by size only, the low-density, or resting B cells are susceptible to infection.[42] These results suggest that prior activation of B cells may render cells refractory to EBV infection. [42] Susceptibility of B cells to EBV apparently depends on a number of factors, including CD21 expression and the physiologic state of the B cell.

It has long been recognized that a strict tropism for B cells is inconsistent with many experimental observations, including (1) the association of EBV with nasopharyngeal carcinoma, an epithelial cell malignancy; (2) a study demonstrating EBV viral genome in epithelial cells from a nasopharyngeal tumor; [43] and (3) a large body of evidence demonstrating the presence of CD21 or a related structure on T lymphocytes. [44] Cytohybridization studies with oropharyngeal epithelial cells, reported in 1977, demonstrated the presence of EBV nucleic acid, [45] and subsequent work demonstrated EBV infection and replication in epithelial cells. [34]

Initial studies focused on the mechanism of infection were unable to detect CD21 on the epithelial cell surface, which led to suggestions that infection resulted from infected lymphocytes.[46] The anti-CD21 monoclonal antibodies HB5 and anti-B2 have been shown to bind a 200-kd epithelial protein, which could be a homologue of CD21 and a receptor for EBV.[47] Epithelial cell expression of the CD21 homologue is concentrated on the microvilli, and a less dense expression of the CD21 homologue is present on epithelial cells compared with lymphoid cells. [47] Studies by Sixbey and Yao have shown that polymeric IgA specific to the EBV membrane glycoprotein gp350 can block immortalization of B lymphocytes while concurrently mediating virus entry into epithelium by the polymeric immunoglobulin receptor (secretory component).[48]

Thymocytes and peripheral T cells express CD21 or CD21-like molecules. T-cell binding of anti-CD21 antibodies differs from that of B cells, suggesting structural differences between T- and B-cell CD21 molecules. Human T-lymphotropic virus type 1transformed T-cell lines also express low levels of the CD21 receptor. [49]

Latent Infection

Epstein-Barr virus is associated with both latent and lytic infections. Infection of primary B lymphocytes is primarily a latent,

non-virus-producing infection, whereas infection of epithelial cells is believed to be primarily a lytic, virus-producing infection. Latent infection of B lymphocytes by EBV causes activation and transformation, as evidenced by establishment of continuously growing B-lymphoblastoid cell lines. [50]

A limited number of viral genes are expressed during latent infection. At present, 10 genes are known to be expressed: (1) six code for EBV nuclear protein, *EBNA-1, EBNA-2, EBNA-3A, -3B, -3C,* and EBNA leader protein (*LP*); (2) two code for latent membrane proteins (LMP), *LMP-1* and *LMP-2*; and (3) two code for small, nonpolyadenylated nuclear RNAs (EB-encoded RNAs, *EBER*s). The *EBER*s are primarily transcribed by cellular RNA polymerase III and the latent genes by cellular

RNA polymerase II.[51] After infection, *EBNA2* and *LP* are expressed the earliest with maximal levels obtained by 24 hours.[52] [53] By 48 hours, the remainder of the genes have reached maximal expression.[52] [53] Expression of at least five of the latency genes is estimated for the transformation process: *EBNA-1, EBNA-2, EBNA-3A, EBNA-3C,* and *LMP-1*.[54]

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PATHOGENESIS

Epstein-Barr virus replicates in the lymphoreticular system and provokes an intense immunologic response, resulting in the immunopathology and clinical symptomatology that follows the initial infection. The virus remains latent in the lymphoreticular system after host recovery, and can be reactivated during periods of immunosuppression, which leads to further disease manifestations. EBV is acquired by intimate contact of a susceptible person with a virus-containing secretion from a previously infected person. Saliva appears to be the most common vehicle of viral spread; [43] [55] however, the recent demonstration of cell-free virus in female genital secretions raises the possibility of sexual transmission. [14] Initiation of the life cycle of EBV may occur with the infection of oropharyngeal epithelial cells [56] or intraepithelial B lymphocytes. [57] EBV-infected B lymphocytes are disseminated throughout the lymphoid system, with EBV-infected B cells appearing in the circulation. Persistence of EBV infection in B lymphocytes has been demonstrated for the lifetime of an infected person. The incubation period from initial contact with a virus excreter to the appearance of large numbers of EBV-infected B cells in the circulation is approximately 3050 days. [58]

Infectious mononucleosis is a self-limiting lymphoproliferative disease characterized by lymphadenopathy, splenomegaly, and lymphocytosis. A hallmark of IM is the appearance of atypical lymphocytes in the peripheral blood, which account for 6070% percent of the total white cell count, averaging 12,00018,000/mm ^{3} . The atypical lymphocytes are primarily activated CD8 T lymphocytes and natural killer cells and are believed to be the principal mediators of clinical symptoms during acute IM. This intense immune response to the virus results in a marked reduction in the numbers of latently infected B cells in the peripheral blood, which decrease to approximately 1 in 10^5 10^6 B cells during the subsequent months, and remain at such levels for life. This small pool of latently infected B cells serves as a reservoir for viral persistence or reactivation and, when grown in vitro, these latently infected B cells transform and can be propagated as B-lymphoblastoid cell lines (BLCL). The efficient in vitro expansion of latently infected B cells into BLCL is accomplished by the removal of EBV-specific CD8+ cytotoxic T cells or their inhibition by cyclosporine A. The virus-specific cellular immune response is long-lived, such that EBV-specific cytotoxic T cells persist at a relatively high frequency in the peripheral blood for life, likely a result of continued restimulation by EBV antigen-bearing B cells. [59] [60]

Stimulation of both the humoral and cellular immune responses is evident during acute IM. The humoral response during acute IM is characterized by nonEBV-specific (e.g., heterophil antibody and autoantibody responses) and EBV-specific responses directed against viral capsid antigen (VCA), early antigen (EA), membrane antigen, and Epstein-Barr nuclear antigen (EBNA). [61] [62] In addition to IgM and IgG isotypes, polymeric IgA specific to EBV membrane antigen is transiently present in the serum during acute infection. [48]

The production of irrelevant antibodies is likely the product of polyclonal stimulation of B cells by EBV, a potent B-cell stimulator. [63]

Although there exists a strong humoral response to EBV, as demonstrated by the generation of many EBV-specific antibodies, it is the cellular immune response that is believed to be primarily responsible for controlling and preventing EBV-induced lymphoproliferative disorders. [64]

The specific cellular immune responses responsible for controlling EBV are among the best studied in humans. The in vivo activated T cells originally found during acute IM have recently been shown predominantly to comprise EBV-specific, major histocompatibility complex class I-restricted cytotoxic T lymphocytes that recognize EBV lytic as well as latent gene products. [59] [65]

The pathogenesis of acute EBV infection is summarized in Figure 45-1 . Infection is initiated in the oropharynx after contact with infected secretions. Viral replication occurs first in epithelial cells or B lymphocytes, which disseminate virus throughout the lymphoreticular system. The cellular immune responses that occur after EBV infection are complex, and it is likely that these cellular events result in the immunopathologic process that accompanies acute infection. Although humoral immune responses appear to be important in preventing recurrent infections, current evidence suggests that cellular mechanisms are responsible for the control of acute and reactivation infections with herpesviruses. Perturbations of these cellular immune responses may result in poorly controlled EBV infections or in immunologic disease clinically manifested as a lymphoproliferative disorder.

Figure 45-1 Immunopathogenesis of acute EBV-induced infectious mononucleosis.

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CLINICAL MANIFESTATIONS

Congenital and Neonatal Infections

Intrauterine infection with EBV appears to be a very rare event. A few case reports suggest that an embryopathy may occasionally occur as a result of congenital EBV infection.[66] Chang and Seto evaluated over 2,000 cord blood samples for evidence of in utero EBV infection and found only 1 to be positive. [67] The EBV-positive infant demonstrated normal growth and development through the first 2 years of life. It is possible that EBV infection during pregnancy is associated with a high frequency of fetal wastage, and this could explain the difficulty in finding infants with congenital EBV infection.

Relatively little is known about the importance of EBV as a cause of perinatal infections. The description of a postnatal cytomegalovirus syndrome associated with blood transfusions in premature infants[68] highlights the importance of conducting similar studies to determine if transfusion-acquired EBV infections pose a risk to the premature infant.

Primary EBV Infection in Infants and Children

Primary EBV infections in infants and children are frequently asymptomatic. When symptoms do occur, a variety of syndromes have been observed, including otitis media, diarrhea, abdominal complaints, upper respiratory infection, and IM. [69] It is now appreciated that these syndromes may occur without the production of heterophil antibodies, and EBV-specific serologic studies are necessary to establish the diagnosis. Several studies have focused on the clinical and serologic findings that are associated with IM in infants and children. Horwitz et al. have reported 32 patients younger than 4 years of age with IM. [69] Their patients were selected from a population of 200 children by review of blood smears. Those children with >50% mononuclear cells and >10% atypical lymphocytes (a total of 34 patients) were evaluated by EBV-specific serology; primary EBV infection was documented in 32 of the 34. Most of the patients had clinical evidence compatible with IM (significant cervical adenopathy and tonsillar pharyngitis). Respiratory symptoms were frequently prominent, especially in young infants. Only 25% of infants 1024 months of age demonstrated heterophil antibody responses, whereas 75% percent of children ages 2428 months were heterophil antibody positive. Only 60% of infants demonstrated VCA-IgM antibodies, compared with 100% of older children and young adults. Peak titers of VCA antibody and the development of antibodies to EA were also decreased in infants compared with older children and young adults.

This study suggests that the IM syndrome is not uncommon among young children. The presence of atypical lymphocytes and lymphadenopathy in the absence of heterophil antibodies further documents the age-related differences in host responses to EBV. Tamaki et al. have demonstrated that young infants can mount an EBV-specific cytotoxic T-lymphocyte response during acute EBV infection. These cytotoxic T-lymphocyte responses in young infants appear to be directed against the same epitopes recognized by young adults.

Infectious Mononucleosis in the Young Adult

The IM syndrome appearing in young adults is characterized by fever, anterior and posterior cervical lymphadenopathy, exudative pharyngitis, and fatigue. The syndrome is self-limited, lasting on the average 23 weeks. It is estimated that 3050% of students entering college in the United States are susceptible (seronegative) to EBV.[71] Approximately 1015% of seronegative people become infected each year, and most of those infected show signs and symptoms of classic IM. [71] Studies conducted at West Point have demonstrated an association of clinically apparent IM with the likelihood of being under stress. [71]

In people experiencing acute IM, morbilliform rashes may develop when they are treated with ampicillin or penicillin during the acute phase of the disease. [71] Hepatosplenomegaly is commonly present and severe, but only rarely results in splenic rupture, after trauma and fulminant hepatitis secondary to periportal necrosis. One of the most common causes of hospitalization during acute IM is severe pharyngitis, with concern for airway obstruction. This complication, which usually resolves in 2472 hours, may be severe enough to warrant empiric treatment with intravenous steroids, although the efficacy of such treatment is not proven. [72]

Chronic Active or Persistent EBV Infection

The use of the terms *chronic mononucleosis*,[73] *active EBV*,[74] and *persistent EBV*[75] has caused confusion and frustration for physicians investigating clinical disorders caused by EBV, as well as for those involved in day-to-day patient care. The major problem with these terms is the complete lack of proven objective criteria for applying them to a patient or group of patients. Their use in describing a syndrome characterized by chronic fatigue, fever, pharyngitis, myalgias, headaches, arthralgias, paresthesias, depression, and cognitive deficits is misleading because many of the people presenting with the syndrome have not had an identified acute EBV infection at the onset of clinical symptoms.[73] [75] These patients have been diagnosed on the basis of certain clinical symptoms associated with EBV-induced IM (i.e., fatigue, fever, pharyngitis) and an unusual or elevated pattern of EBV antibodies (especially those directed against early antigens). [73] [75]

In a study of 500 unselected patients, 1750 years of age, seeking primary care for any reason, 21% were found to be suffering from a chronic fatigue syndrome suggestive of chronic EBV.[76] They had been experiencing severe fatigue, usually cyclic, for a median of 16 months (range, 6458 months), with associated sore throat, myalgias, or headaches. Of these patients, 45% were periodically bedridden, and 2573% reported recurrent low-grade fevers, cervical adenopathy, paresthesia, arthralgias, difficulty in concentrating, and difficulty in sleeping. The patients had no recognized chronic physical illness and were not receiving psychiatric care. Although antibody titers to several EBV-specific antigens were higher in these patients than in age- and sex-matched control subjects, the differences were not significant. The available data do not support a causal role for EBV in the pathogenesis of a chronic fatigue syndrome.

By contrast, there seems little doubt that there are rare patients with extraordinarily high titers of EBV antibodies (VCA-IgG, EA-IgG) who experience clinical symptoms in the face of chronic or persistently active EBV infection. These patients may have pancytopenia, chronic lymphadenopathy, hepatosplenomegaly, interstitial pneumonitis, and chronic liver dysfunction. [76] Preliminary data suggest that an abnormal humoral immune response to EBV-EBNA antigens or infection with a variant EBV strain may be associated with chronic or persistent infection. [77] A relationship between these clinical syndromes and replication of EBV is suggested by apparent clinical responses to acyclovir therapy. [78]

Effects of EBV Infection on Hematopoietic Elements

The effects of EBV infection on the hematopoietic system have been well described. [79] [80] Patients with acute EBV infection present with lymphocytosis and not uncommonly have an associated thrombocytopenia. Infrequently, patients experience more serious hematologic complications that may involve the erythroid, myeloid, or megakaryocytic lineages.

Neutropenia is seen in many cases of IM during the first month of disease; [81] however, severe neutropenia is seen relatively

infrequently. A study by Sumaya and Ench[82] showed that 8% of patients had an absolute neutrophil count of 5001,000 cells/mm ^{3} , and 8% had a neutrophil count <500/mm^3 . In most instances, the neutropenia resolved within 2 weeks. Fatal infections attributed to severe neutropenia have been reported. [83] Examination of the bone marrow may show left-shifted myelopoiesis with a paucity of mature myeloid forms.[83] Neutrophil autoantibodies, which may be seen in a high proportion of patients with acute IM, may contribute to the neutropenia.[84] It has also been shown that T cells from patients with IM may decrease colony-forming unitsgranulocyte-macrophage (CFU-GM), suggesting that these T cells down-regulate myelopoiesis.

Epstein-Barr virus infections in infants may rarely result, at presentation, in a clinical syndrome similar to juvenile chronic myeloid leukemia. [85] These patients present with hepatosplenomegaly, leukocytosis, and thrombocytopenia and have increased levels of fetal hemoglobin. This syndrome may be distinguished from juvenile chronic myeloid leukemia by evidence of acute or recent EBV infection. Clinical symptoms may persist for months or years, but usually resolve spontaneously. Immunoregulatory abnormalities have been described, but whether these are of primary or secondary importance in pathogenesis remains uncertain.

Erythropoiesis

Mild hemolysis is common in patients experiencing acute EBV infection, but clinically significant anemia is uncommon, occurring in 13% of patients. [86] When hemolysis develops, it is usually seen within the first 2 weeks of illness. Patients usually have a positive direct Coombs test. Most frequently the autoantibody has anti-i specificity.[87] Antibodies with anti-N[88] and anti-I specificity, [89] as well as Donath-Landsteiner antibodies, [90] have also been described. In addition, patients with hereditary red cell defects may have an exaggerated rate of red blood cell destruction during acute EBV infection. [91]

Pure red cell aplasia has been described after acute EBV infection, and has been associated with persistently abnormal EBV serology over a 2-year period. [92]

Thrombopoiesis

Mild thrombocytopenia with platelet counts ranging from 50,000 to 150,000/mm ^{3} is commonly seen in patients with acute EBV infection.[93] [94] The thrombocytopenia usually occurs in the first 2 weeks after the onset of clinical symptoms. Severe thrombocytopenia may be seen in 13.5% of patients. The normalization of platelet counts may take up to 2 months.[94] Bone marrow examination shows normal or increased numbers of megakaryocytes, compatible with peripheral platelet destruction. It is thought that anti-platelet antibodies and sequestration with destruction in the spleen are responsible for the thrombocytopenia. Platelet counts usually return to normal within 2 months, and even the more chronic cases tend to resolve spontaneously. [94]

Bone Marrow Progenitors

Acute EBV infection is rarely complicated by pancytopenia and marrow hypoplasia compatible with aplastic anemia. This bone marrow complication is similar to that seen in patients with infection-associated hemophagocytic syndrome (IAHS) with pancytopenia. In a case report and review of the literature, Lazarus and Baehner described six patients with aplastic anemia in whom a bone marrow examination was performed; in four patients, the findings met the criteria for severe aplastic anemia.[95] The mean time from the onset of symptoms of IM to the development of pancytopenia was 21 days, with recovery occurring by the end of the first week of bone marrow aplasia. Several mechanisms have been proposed for EBV-associated marrow aplasia, with experimental data supporting some of them. [96] Suppressor T lymphocytes, which inhibit the growth of colony-forming unitsculture (CFU-C) in vitro, have been described in some patients. A marked increase in T cells bearing the morphologic markers for the T-cytotoxic suppressor cell population occurs during acute EBV infection and could have a role in marrow aplasia. It has also been postulated that cytotoxic cells may lyse hematopoietic stem cells. Autologous lymphocytotoxicity has been reported in patients with aplasia anemia. [96]

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FATAL INFECTIOUS MONONUCLEOSIS

Infection-Associated Hemophagocytic Lymphohistiocytosis

The term *lymphohistiocytosis* was adopted by the Histiocyte Society in 1987 to include IAHS as well as other hemophagocytic disorders. [97] Virus-associated hemophagocytic syndrome is a term introduced by Risdall and co-workers in 1979 to describe a disorder characterized by a benign, generalized histiocytic proliferation with marked hemophagocytosis associated with a systemic virus infection. Since this first description, the agents associated with the induction of a hemophagocytic syndrome have expanded beyond viruses to include virtually any infectious agent, carcinomas, and hematologic malignancies. Table 45-1 lists some of the infections associated with hemophagocytic syndromes.

The syndrome is characterized by fever and by generalized constitutional symptoms with myalgias and malaise. Physical examination reveals an enlarged liver and spleen with generalized lymphadenopathy. Laboratory studies commonly demonstrate abnormal liver function tests with a coagulopathy that is more severe than that expected on the basis of the abnormal liver function. The patient is usually pancytopenic and may appear very toxic. This syndrome has been most frequently observed in people with underlying immunosuppression, including allograft recipients, patients with leukemia, and patients

TABLE 45-1 -- Infection-Associated Hemophagocytic Syndrome

Viral
Epstein-Barr virus
Cytomegalovirus
Herpes simplex virus
Varicella-zoster virus
Adenovirus
Parvovirus B19
Bacterial
Enteric gram-negative rods
Hemophilus influenzae
Streptococcus pneumoniae
Staphylococcus aureus
Brucella abortus
Mycoplasma pneumoniae
Fungal
Histoplasma capsulatum
Candida albicans
Cryptococcus neoformans
Mycobacterial
Mycobacterium tuberculosis
Rickettsial
Coxiella burnetii
Parasitic
Babesia microti
Leishmania donovani

with severe collagen vascular disease receiving high-dose corticosteroids. The mortality rate in patients experiencing infection-associated hemophagocytic lymphohistiocytosis (HLH) has been high; however, it is likely that the use of immunosuppressive agents in patients experiencing infection-associated HLH has contributed to the high mortality rate.

There has been some controversy regarding the pathologic differentiation between secondary HLH and malignant histiocytosis. The pathologic features of secondary HLH vary with the time that biopsies are performed. Early in the disease, the bone marrow may be hypercellular with few infiltrating histiocytes. Erythrophagocytosis is usually best demonstrated in aspirate smears. Later in the disease, the bone marrow is hypocellular and shows varying numbers of infiltrating histiocytes. Lymph nodes early in the disease may exhibit an intense immunoblastic proliferative response with partial effacement of the lymph node architecture. The number of histiocytes early in the disease may be low. Later in the disease, lymphoid depletion supervenes, and there may be a massive sinusoidal infiltration by benign-appearing histiocytes, many of them exhibiting erythrophagocytosis. Liver biopsy reveals large portal infiltrates of lymphocytes, immunoblasts, and histiocytes. Histiocytes, many of which exhibit erythrophagocytosis, are seen in liver sinusoids. Mild fatty metamorphosis may also be present. Immunologic studies have been reported in only a few patients. In most patients studied, EBV was the associated infectious agent. Atypical lymphocytes, which are the hallmark of acute EBV infection, are absent or diminished, reflecting a decrease in activated CD8 T cells normally seen in response to EBV. We have speculated that immunodeficiency or underlying immunosuppression may play a role in the pathogenesis of HLH. Many of the infectious agents associated with HLH are potent stimulators of the immune system and require complex interactions of immunoregulatory cells for host recovery. Underlying immunoregulatory disturbances may allow an inappropriate antiviral

response. As this response progresses, cytokines secreted by activated T lymphocytes may elicit the proliferation and activation of histiocytes.

X-Linked Lymphoproliferative Syndrome

The X-linked lymphoproliferative (XLP) syndrome is characterized by a selective immunodeficiency to EBV manifested by severe or fatal IM and acquired immunodeficiency.[98] Prospective studies in male patients before EBV infection have demonstrated normal cellular and humoral immunity. [99] During acute EBV infection, male patients with XLP demonstrate vigorous cytotoxic cellular responses that predominantly involve polyclonally activated alloreactive cytotoxic T cells, but cytotoxic T cells that recognize EBV-infected autologous B cells have been demonstrated. Fatal EBV infections in male patients with XLP usually result from extensive liver necrosis, and those who survive acute EBV infection demonstrate global cellular immune defects with deficient T-cell, B-cell, and natural killer cell responses. It is hypothesized that uncontrolled alloreactive T-cell responses triggered by EBV-transformed B cells result in the immunopathologic process of XLP. The gene for XLP is localized in the 9q region of the X chromosome,[100] and has recently been identified as the Duncans disease SH2 protein (DSHP). [101] The identification of the XLP gene will enable diagnosis of sporadic cases of XLP, and when DSHP function is understood, new therapeutic interventions during acute EBV infection may be realized.

EBV and Post-transplantation Lymphomas

Allograft recipients are at increased risk for the development of EBV-induced lymphoreticular malignancies. [102] The incidence of post-transplantation lymphomas is in the range of 115%, depending on the allograft and the immunosuppressive regimen. Most of the lymphomas are categorized as diffuse large cell lymphomas, with most having immunoblastic features. Molecular probes specific for EBV have streamlined the diagnosis of EBV-associated post-transplantation lymphoma. Studies using molecular probes to analyze clonality of post-transplantation lymphomas have demonstrated that most patients with lymphomas have monoclonal tumors, but often the disease is multiclonal, with biopsy material from different anatomic sites showing different proliferating clones of tumor cells. A recent study suggests that LMP-1 plays a critical role in the pathogenesis of EBV-induced lymphoproliferative disorders in immunosuppressed patients. [103] The specific immune defect responsible for these lymphoproliferative disorders is unknown, but is thought to be related to the loss of memory cytotoxic T lymphocytes specific for EBV-transformed B lymphocytes.

EBV- and HIV-Associated Non-Hodgkin Lymphoma

The incidence of non-Hodgkin lymphomas is 60100 times that expected in HIV-infected people. [104] The incidence of lymphomas increases with the duration of HIV infection. It has been reported that lymphoma developed in 19% of patients with symptomatic HIV infection receiving antiretroviral therapy and surviving 3 years. [105]

For unknown reasons, most EBV-associated non-Hodgkin lymphomas in HIV-infected patients have presented as primary central nervous system lymphomas. [106] It appears that the combination of chronic B-lymphocyte stimulation in the setting of severe T-cell immunodeficiency in a person with previous EBV infection is a set-up for the development of non-Hodgkin lymphoma.[107]

Peripheral T-Cell Lymphoma and Hodgkin Disease

Recent studies suggest that peripheral T-cell non-Hodgkin lymphomas, which occur in people without global immune defects, are associated with EBV in 1035% of cases.[35] [36] Angioimmunoblastic lymphadenopathy and angiocentric polymorphous lymphoreticular infiltrates known clinically as lethal midline granuloma are T-cell lymphomas frequently associated with EBV. Both the A and B strains of EBV have been associated with EBV-associated T-cell lymphomas.

Epstein-Barr virus is detectable in Reed-Sternberg cells in approximately 4050% of cases of Hodgkin disease, particularly of the mixed cellulosity subtype. [37] [38] [40] EBV is detectable in most cases of Hodgkin disease developing in HIV-infected people. The Reed-Sternberg cell has been shown to contain monoclonal EBV genomes with restricted expression (*LMP-1, EBER-1, EBER-2*) of latent gene products. Recently it was demonstrated that EBV replicates in Reed-Sternberg cells of Hodgkin disease.[38]

Recent studies also suggest mechanisms that may play a role in EBV-induced oncogenesis. [103]

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LABORATORY DIAGNOSIS OF IM

Serology

In 9095% of young adults with clinical EBV-induced IM, atypical lymphocytosis and heterophil antibodies are present. [108] However, in infants and children with primary EBV infection (regardless of whether associated with IM), heterophil antibody responses are frequently absent. [69] Primary EBV infection is usually suggested by the presence of atypical lymphocytes, but these may also be absent or present in small numbers. [109] Diagnosis of primary infection in childhood frequently requires EBV-specific serologic tests. Our approach to a patient suspected of having a primary EBV infection is first to perform a rapid slide test for heterophil antibodies; if this is positive (differential absorption

with guinea pig kidney and beef erythrocyte antigens should be performed), EBV-specific serology is unnecessary, but if the rapid slide test is negative, the serum sample should be tested for EBV-specific antibodies.

In people with negative Paul-Bunnell heterophil antibody tests (especially infants and children) and patients with atypical primary EBV infections, specific antibodies for EBV should be determined. Antibodies to three specific EBV antigens have been thoroughly studied and found to be of diagnostic importance: (1) VCA; (2) EAs (both of which may be expressed by certain EBV-infected B-lymphoblastoid cell lines); and (3) EBNAs, which are expressed by all cell lines. [110] These antigens have been detected by the indirect immunofluorescence test in serum samples from patients with EBV-associated diseases. Patients experiencing acute EBV-induced IM and the EBV-associated malignancies (Burkitts lymphoma and nasopharyngeal carcinoma) have been thoroughly studied, as well as normal control subjects, and certain characteristic antibody patterns have been described.

Figure 45-2 shows the characteristic antibody patterns observed in young adults experiencing EBV-induced IM. [110] Before EBV infection, all three antibodies are absent, but during the acute phase of infection, high titers of IgM and IgG antibodies to VCA are seen. IgM antibodies are transient and disappear after a few months. Most people develop transient IgG antibodies against EAs; these antibodies disappear after a few months. In 1985, it was reported that between 12 and 39% of normal people maintain moderate (1:20 to 1:40) antibody titers to EAs for years after primary infection. [111] Antibodies directed against the EBNA proteins are produced early in infection; however, detectable titers by indirect immunofluorescence are not usually found until 12 months after acute infection. This pattern of late appearance is probably a reflection of the insensitivity of the complement-dependent indirect immunofluorescent antibody test, because sensitive enzyme-linked immunosorbent assay-based methods detect antibodies to the EBNAs early after infection. Healthy people who have had past infection with EBV show VCA-IgG antibodies. In infants, VCA-IgM responses may be seen in only 60% and EA responses in approximately 50% of patients during acute EBV infection. [67]

In general, acute or recent primary infection is indicated by the following: (1) the presence of VCA-specific IgM antibodies, (2) high titers of VCA-specific IgG antibodies (1:320 or greater), (3) detection of anti-EA antibodies (1:10 or greater), and (4) the absence of anti-EBNA by the immunofluorescent antibody test. [110] Convalescent serum samples should be obtained to demonstrate the disappearance of VCA-IgM and appearance of EBNA antibodies. EBV antibody titers should not be used to make a diagnosis of chronic IM or other EBV-associated syndromes on the basis of mild to moderate elevation of VCA (1:160 to 1:320) or EA (1:20 to 1:40) antibodies because normal people may show such titers years after uncomplicated infection. [111] Elevated EBV titers are also seen in patients with EBV-associated malignancies and in those with virtually any condition associated with suppression of cellular immune function (e.g., allograft recipients, patients receiving chemotherapy, patients with HIV infection). Past infection is indicated by the presence of VCA-IgG and EBNA-IgG antibodies.

Isolation of EBV

Epstein-Barr virus cannot be cultured by routine virologic culture methods that use standard animal cell cultures; it grows only in primate B lymphocytes. The presence of virus can be demonstrated by inoculating human umbilical cord blood lymphocytes (EBV-nonimmune B lymphocytes) with throat washings or by establishing an EBNA-positive, spontaneous B-lymphoblastoid cell line from the peripheral blood of the infected person. However, both methods detect virus in people with past infection because EBV is a latent virus, and most healthy, previously infected people chronically shed virus in the oropharynx. [55] Because of the difficulties in making a serologic diagnosis in patients with unusual or rare manifestations of EBV infection, molecular diagnostic techniques must be used.

Molecular Diagnostic Techniques

Molecular diagnostic techniques have contributed greatly to the identification of EBV in pathologic materials (see Pagano [112]). In situ hybridization (ISH), Southern blot analysis, and the polymerase

Figure 45-2 Characteristic EBV-specific antibody responses observed in young adults with acute IM. EA, early antigen; EBNA, Epstein-Barr nuclear antigen; VCA, viral capsid antigen. *(Adapted from Henle W, Henle GE, Horwitz CA: Epstein-Barr virus specific diagnostic tests in infectious mononucleosis. Hum Pathol 5:551, 1974, with permission.)*

chain reaction (PCR) are useful techniques used alone and in combination.

Southern blot hybridization of DNA extracted from affected tissue is used when it is not necessary morphologically to identify infected cells or when sufficient cells containing EBV are present in the lesion. Such cases include entities such as HIV-associated lymphoma or Burkitts lymphoma. Southern blots are probed with a Bam W probe, which detects an internal repeat in the EBV genome.

The EBV genome contains terminal repeats that hybridize when the genome is in the circular or episomal form. When a clonal cell population is present, only one form of the genome with a single terminal repeat is present. When a Southern blot is probed with a terminal repeat probe such as Bam NJ, a single band is obtained.[108] Such results are found in Burkitts lymphoma or nasopharyngeal carcinoma. In a polyclonal infection, a heterogeneous collection of bands is found. This technique also discriminates replicating from nonreplicating (latent) EBV infection. [108]

In situ hybridization using oligonucleotide probes complementary to the *EBER* genes has provided an extremely powerful technique for the identification of EBV-infected cells in tissue sections and smears.[113] The advantages of ISH for the detection of *EBER*-encoded RNA is that it is a relatively simple technique, uses cheap, stable probes, can be used on archival material, does not require radioactivity, and provides a permanent morphologic endpoint. Because latently infected cells may express millions of copies of *EBER*-RNA, ISH provides an extremely sensitive technique for the identification of even a few infected cells in a tissue. Not only does this technique provide morphologic information, it is more sensitive than Southern blotting and approaches the sensitivity of PCR. ISH is used when it is important to obtain morphologic information, when only access to paraffin-embedded archival material is possible, when it is desired to obtain information about what portion of cells may be infected, or when high sensitivity is needed. This technique is now widely applied and has been extremely useful in identifying EBV in entities such as Hodgkin disease, T-cell lymphoma, IAHS, and nasopharyngeal carcinoma.

A third modality useful for the detection of EBV is PCR.[114] [115] [116] The advantages of PCR are its ability to detect rare infected cells (on the order of 1 in 1×10^6) and its rapidity. DNA may be extracted from fresh material or from paraffin-embedded archival material. However, PCR is a relatively complex technique, and great care must be taken to ensure that positive signals are true positives. Currently, a technique described by Tamaki et al. that amplifies the *EBNA-3C* region of EBV is commonly used.[117] This technique is useful in screening materials that require high sensitivity, such as in determining whether EBV is present in novel lymph node diseases or carcinomas. Another utility of the PCR technique is its ability to subtype EBV strains. EBV strains are classifiable into two subtypes based on differences in genomic sequences, especially the *EBNA-2* and *EBNA-3* genes. The A subtype is prevalent in Western countries and Japan and has an enhanced ability to transform B lymphocytes in vitro. The B subtype is more prevalent in Africa, although it has been shown that in oropharyngeal fluid obtained from people in the United States, both strains are equally distributed.

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THERAPY AND FUTURE DIRECTIONS

The use of corticosteroids in severe cases of EBV infection and IM is recommended in many standard textbooks and review articles. [93] Controlled studies have demonstrated a shortened duration of fever in corticosteroid-treated IM. [72] However, controlled studies have not been made of the efficacy of corticosteroids in the treatment of severe or life-threatening EBV infections, including those associated with severe pharyngeal involvement, neurologic involvement, thrombocytopenia, and myocarditis. Immunologic interactions appear to be so important for normal host recovery that the use of corticosteroids in nonlife-threatening EBV infections should be discouraged. It is possible that steroid-related effects on the hostvirus relationship may take months or years to develop, and long-term epidemiologic studies of steroid-treated patients have not been reported.

Acyclovir has been shown to inhibit EBV replication in vitro. [118] In a placebo-controlled trial of intravenous acyclovir in patients with acute IM who required hospitalization, no significant differences in outcome were observed between the placebo- and acyclovir-treated groups. [119] A placebo-controlled trial of oral acyclovir in ambulatory college students experiencing acute IM also demonstrated no significant effects of acyclovir on clinical outcome. [120]

Various therapeutic agents have been tried unsuccessfully in XLP patients experiencing acute EBV infection. It is clear that high-dose immunosuppressive therapy predisposes these patients to fatal B-cell lymphoproliferative disorders. This effect has been observed with the use of high-dose corticosteroids and antithymocyte globulin. Acyclovir therapy of patients with the XLP syndrome has been reported. [121] In no instance was objective evidence of clinical improvement apparent. Patients have died with disseminated EBV-infected B lymphocytes throughout the lymphoreticular organs despite a 2-week course of acyclovir (1,500 mg/m ^{2} /day). Virologic studies have revealed that the virus in infected B lymphocytes was in a nonreplicating state, and mature virus particles were not being produced. In view of these results, it is likely that acyclovir will prove to be efficacious only in those patients with syndromes associated with replicating EBV infection.

A recent study of acyclovir and ganciclovir prophylaxis in pediatric liver transplant recipients demonstrated no apparent benefit toward the prevention of EBV disease.[122] It is thought that male patients with XLP experiencing primary EBV infection should be treated with broad-spectrum cytotoxic chemotherapy in an effort to eliminate the activated CD8 T lymphocytes and, at the same time, control the proliferation of the EBV-transformed B cells. Such therapy remains unproven but appears worthy of trial.

Correction of XLP by transplantation of bone marrow[123] and cord blood stem cells[124] has been reported. The advent of genetic diagnosis of presymptomatic (EBV-seronegative) XLP male patients now makes it possible to correct the defect by transplantation before primary EBV infection has occurred. In the absence of a marrow or stem cell donor, high-dose (600 mg/kg/month) intravenous gamma globulin containing high titers of EBV antibodies is being used in an attempt to prevent primary infection. It is hoped that the discovery of the XLP gene defect will lead to new therapeutic modalities as well as prevention of EBV-induced immunodeficiencies.

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Chapter 46 - The Spleen and Its Disorders

Susan B. Shurin

INTRODUCTION

Galen described the spleen as the organ of mystery, with functions related to mood and good or ill humors. It was not until the 18th century that the spleens relationship to the immune and hematologic systems was appreciated. The complexities of splenic function continue to be the focus of research and observation. The spleen is not necessary to life because its many functions are redundant, overlap with the functions of other organs, and can be assumed by other organs. Nonetheless, the spleen is an important part of many disease processes as well as of normal function of both the immune and hematologic systems. An appreciation for the subtleties of its anatomy and

function is important for the physician evaluating patients with many hematologic, immunologic, and hepatic diseases.

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NORMAL SPLENIC ANATOMY AND FUNCTION

Embryology

The spleen arises from the mesoderm. By the ninth week of gestation, layers of the left dorsal mesogastrium condense, and blood vessels appear. Sheaths are formed around arterioles by reticular cells and fibers. Macrophages are present and phagocytic by the end of the first trimester. Lymphocytes appear during the fourth month. Red and white pulp can be identified by the sixth month. Germinal centers do not develop during fetal life, but primitive inactive follicles are evident at birth. [1] In mice, a homeobox gene, *Hox11*, controls the genesis of the spleen;[2] a human counterpart has yet to be described. The spleen is capable of supporting hematopoiesis both during fetal life and, in a variety of pathologic states, postnatally. The circulation of primitive hematopoietic stem cells in peripheral blood during prenatal life through birth makes it difficult to distinguish between hematopoiesis dependent on tissues in the organs and the incidental presence of hematopoietic cells within the circulation of those organs. [3] [4]

Anatomy

The spleen is covered with a fibrous capsule with blood vessels, lymphatics, and nerves and is coated by peritoneal mesothelium (Fig. 46-1). The splenic artery comes from the celiac axis, enters the capsule at the hilum, and branches into trabecular arteries. The trabecular arteries then branch into central arteries and enter the white pulp. On the cut surface of the normal spleen, white pulp is visible as white nodules 12 mm in diameter. The periarterial lymphoid sheath consists of a cuff of T lymphocytes, plasma cells, and macrophages around the central arteries. As the arteries branch, the sheath narrows. B-lymphocyte clusters appear in follicles along the periarterial lymphoid sheath at arterial branch points. The components of the white pulp are connected by a reticular network and supporting stromal cells. On the cut surface of the normal spleen, white pulp is visible as white nodules 12 mm in diameter. Their size varies with age and antigenic stimulation. Normally developed at birth, the size of the nodules increases in childhood, especially with immunizations and infections, peaks at puberty, and involutes

Figure 46-1 Anatomy of the spleen. *(From Weiss and Tavassoli,[10] with permission.)*

in adulthood. Immunologically normal, uninfected adults normally have no evident germinal centers. A mantle zone of B lymphocytes surrounds the follicle, or secondary germinal center. Antigen trapping and processing take place in the marginal zone of the white pulp.

The red pulp of the spleen consists of vascular sinuses, the cords of Billroth, and the terminal branches of the penicilliary arteries. [5] [6] Vascular sinuses are lined with endothelial cells with long processes and a basement membrane with ring fibers, which attach to macrophage dendritic processes. No tight junctions or interdigitations connect the cytoplasmic processes. Intact leukocytes, erythrocytes, and platelets are able to squeeze through the potential spaces between these cells and between the ring fibers. Processes of the reticular cells of the cords of Billroth are outside the sinus walls. Endothelial cells line the sinuses and pulp veins, but not the cords. Circulation of blood through red pulp lined with endothelium, or closed circulation, is rapid. Circulation into the cords, or open circulation, is slower and permits the macrophages lining the cord to remove damaged or aged cells.

Functions

The functions of the spleen are summarized in Table 46-1 .

Hematopoiesis

Active hematopoiesis can be seen in the fetal spleen throughout the second trimester, and decreasing in the third trimester. [7] Erythropoiesis and megakaryocytopoiesis predominate, with myelopoieis present to a lesser extent. As hematopoiesis moves from the hepatic phase into the bone marrow, it is less evident within the fetal spleen. The spleen is not normally a site of hematopoiesis in postnatal life but may resume that function in a number of pathologic states. [3] [4] Extramedullary hematopoiesis is a significant cause of splenomegaly in bone marrow diseases (e.g., myelofibrosis, osteopetrosis) and chronic hemolytic anemias (e.g., thalassemia). The stromal cells of the spleen appear to be capable of supporting hematopoiesis and may produce c- *kit* ligand,[8] [9] as do marrow stromal cells. When splenic hematopoiesis occurs, the erythrocytes and platelets that circulate tend to be less mature than when hematopoiesis occurs in the bone marrow, which suggests that egress from the spleen is easier than from the marrow.

Removal of Damaged and Aged Formed Elements

As erythrocytes and platelets age, they tolerate the hostile splenic environment less well than when they are healthy with metabolic reserve. [10] The spleen is acidotic (pH 6.87.2), hypoxic (P_{O_2} 54 mm Hg), and hypoglycemic (glucose concentration 60% of that in venous blood). Damage to aged anucleate cells includes changes in their membrane complex carbohydrates, which facilitate recognition by splenic macrophages and removal from the circulation. [11] [12] *Culling* describes the destruction of erythrocytes, either the normal removal of aging cells or the removal of damaged cells in pathologic states. Most platelets and leukocytes are not removed by the spleen as they age but adhere to vessel walls and migrate into tissues, where they die. *Pitting* refers to the removal of inclusions from within erythrocytes, which are then released back into the circulation. Erythrocytes must pass between or through the cordal macrophages, through the fenestrations of the basement membrane of the sinus, and then between the endothelial cells. Poorly deformable cells are unable to enter the sinuses. The erythrocyte membrane is in close apposition to the macrophage membrane, so that aged or damaged glycoproteins, antibody, or complement on the surface of the cell are easily recognized and activate

TABLE 46-1 -- Functions of the Spleen

Category	Function	Effector Cells/Areas
Clearance/filtration	Antibody-mediated clearance	Macrophages
	Culling and pitting	Reticular meshwork
Immune response		

Marginal zone	Interaction of antigens with effector cells	Monocytes, lymphocytes
	Antigen processing	Macrophages
White pulp	Immune recognition	T cells (periarteriolar sheath)
	Immunoregulation, antibody production	B cells (lymphoid follicles)
Red pulp	Antigen processing, preservation	Macrophages
	Phagocytosis	Macrophages, monocytes, neutrophils
Hematologic	Hematopoiesis	Cords and sinuses
	Storage of erythrocytes, platelets, leukocytes	Reticular meshwork, red pulp
	Finishing/polishing of erythrocytes	Reticular meshwork, macrophages
Hemostasis	Production of factor VIII, von Willebrand factor	Endothelial cells

phagocytosis. Particulate matterHowell-Jolly bodies, Heinz bodies, Pappenheimer bodies, and malarial and other parasitescan be removed and the cell returned to the bloodstream. Membrane-containing adsorbed antibody can be removed, and the remainder of the cell released with changes in shape and volume. Internal vesicles near the erythrocyte membrane appear as if they were on the surface of the cell, and are termed *pits* or *pocks*. [13] [14] [15] [16] The spleen also normally removes these, with the polished erythrocyte returned to the circulation. The lack of this polishing function can be used to assess splenic function.

Immunologic Function

The spleen is important in the recognition of antigens, especially when administered intravenously; in the production of antibody; and in the clearance of antibody-coated particles from the bloodstream (Table 46-1).[17] [18] [19] The white pulp of the spleen is the largest mass of lymphoid tissue in the body. Trapping and processing of antigens is a major function of the marginal zone, with its abundant blood supply (2 L/min) and large number of macrophages. Antigens penetrate the germinal center, where T lymphocytes predominate. Processing of carbohydrate antigens is a special function of splenic marginal zone lymphocytes. [20] The B lymphocytes, which ultimately produce antibodies, predominate in the nearby germinal centers and mantle zones. These areas all increase in size and activity with immunization or infection. Splenic macrophages are more sensitive to small amounts of opsonic antibody or complement on the surface of particles than are macrophages in the liver, lung, or bone marrow.[21] In the absence of a spleen, nonimmune individuals may fail to remove bacteria with little opsonic coating. [22] [23] The production of antigen-specific IgM is impaired in the absence of a spleen. [20] [24] Thus, an asplenic individual has defective recognition of carbohydrate antigens, defective production of IgM early in infection, and defective removal of lightly opsonized particlesall crucial components of response to invasive infection with encapsulated organisms.

Storage of Cells

The normal spleen contains 2040 ml of blood in an adult, and does not serve as a reservoir for either blood volume or erythrocytes. This changes in many conditions associated with splenomegaly, especially if portal hypertension is also present. The widening of the pulp cords creates an organ with more storage volume. Vascular pooling of both blood and formed elements occurs, regardless of the underlying cause of the splenomegaly. [25] Platelets and granulocytes, however, are normally stored in the red pulp of the spleen. As much as one-third of the total platelet mass may be stored in the spleen and released when cytokines affecting platelet adhesiveness are released.[26] The lack of musculature in the human splenic capsule prevents the distention and contraction that occur in many animals, such as dogs. After splenectomy, both platelet counts and granulocyte counts rise significantly, then fall. [27] [28] However, the circulating masses of both of these cell pools are chronically increased, which may contribute to an increased incidence of atherosclerosis years after splenectomy. [29] [30]

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EXAMINATION OF THE SPLEEN

For adequate examination of the spleen, the examinee must be fully supine, relaxed, with arms adjacent to the trunk, and the examiner must be on the examinees right side.[31] Beginning in the right iliac fossa, the examiners hand gently advances toward the left upper quadrant. This minimizes the likelihood that a grossly enlarged spleen will be unappreciated, and the lower pole and medial border should be easily appreciated. If the spleen cannot be felt with this approach, the left hand of the examiner is placed on the left flank, lifting the lower part of the rib cage to displace the spleen medially toward the examiners right hand. The splenic notch should be felt in the inferior medial border. Rotating the subject into the right lateral position while still recumbent may make it easier to palpate the spleen. The spleen should move with deep inspiration. It is dull to percussion. The degree of enlargement is usually measured in centimeters below the costal margin. It may be difficult to appreciate even significantly enlarged spleens, depending on the position of the spleen within the abdomen. Normal splenic size in an adult is up to 250 g, and up to 13 cm in its long axis. Up to half of spleens weighing 600750 g are not palpable. [32] Greater degrees of splenomegaly are less difficult to appreciate on physical examination.

Up to 15% of normal children and 3% of young adults have palpable spleens, up to 2 cm below the left costal margin, without evidence of illness. [33] The spleen involutes with age, and hence a spleen that is palpable in an older person is less likely to be a normal variant and more likely to be associated with clinical disease.

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IMAGING OF THE SPLEEN

Radionuclide scintigraphy assesses both anatomic and functional aspects of the spleen. [34] The most common procedure is a liver-spleen scan in which ^{99m}Tc sulfur colloid is injected intravenously and taken up by hepatic and splenic macrophages.

Thus, it is the phagocytic activity of macrophages, rather than the presence of the spleen itself or any aspects of lymphoid function, which is assessed. A dynamic ^{99m}Tc scan can also assess the distribution of blood within the portal system, and suggest the presence of portal hypertension.

Ultrasonography readily shows the size, shape, and several aspects of splenic anatomy, including the presence of cysts and abscesses. [35] The procedure is noninvasive, painless, low cost, and avoids radiation exposure for patients, and thus is a good screening study when the spleen is thought to be enlarged. Accessory spleens tend not to be well visualized on ultrasonography.

Computed tomography (CT) has the advantage of showing both the anatomy and some aspects of splenic function. [36] [37] [38] Contrast material in the stomach, small bowel, and colon, given enterally, helps delineate splenic tissue when it impinges on these organs. Intravenous contrast material is required to delineate splenic lesions whose density is the same as normal splenic tissue. Abscesses have a rim of contrast agent enhancement. [39]

CT can be used to estimate the volume of the spleen. Accessory spleens have the same attenuation as a normal spleen, which is somewhat less than that of liver. [40] Accessory spleens are usually located in the gastrosplenic ligament near the hilum. Subcapsular and intrasplenic hematomas and splenic lacerations are clearly seen on CT. Leukemias and many inflammatory diseases produce diffuse splenomegaly. Granulomas and infarcted areas eventually calcify. Cysts, abscesses, and some malignancies have inhomogeneous patterns on CT scan. [41] [42] [43]

In addition to imaging the spleen, CT can be used for diagnostic and even therapeutic studies. Abscesses, hematomas, and cysts can be drained. As long as portal hypertension is not present and vascular lesions have been excluded, thin needle aspiration under CT guidance is generally a safe intervention.

Magnetic resonance imaging (MRI) is primarily useful for identifying vascular lesions that would otherwise require angiography. It is more difficult to image the contents of the upper abdomen with MRI than with CT because of respiratory motion. Some of these limitations are likely to be overcome with the newer, more rapid MRI technology.

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TESTS OF SPLENIC FUNCTION

The peripheral blood smear may be the most sensitive tool for identification of functional or anatomic hyposplenia. The presence of Howell-Jolly bodies, which are nuclear remnants normally removed by the spleen, is an excellent indicator of hyposplenism. [44] These are rarely seen until the spleen is largely nonfunctional or overwhelmed by other phagocytic functions, such as extravascular hemolysis. Newborn infants commonly have visible Howell-Jolly bodies, and splenic function appears to be at least somewhat impaired in the first week of life. [14] [15] [45] Pappenheimer bodies (siderotic granules normally removed by the spleen) are often seen in hyposplenic states, particularly when a component of hemolysis exists. [46] Erythrocyte morphology reflects the lack of membrane polishing by the spleen, with the presence of acanthocytes and target cells. [11] [13] [27] Both granulocyte and platelet numbers are increased during asplenic states, including splenic infarction and surgical splenectomy. [27] [28]

To confirm suspected hyposplenism, the simplest test is a count of pitted or pocked erythrocytes. Fixation in 0.51.0% glutaraldehyde and examination under interference optics for surface pits reveals endocytic vesicles containing hemoglobin, ferritin, and remnants of mitochondria. [13] These form in mature erythrocytes and are normally removed by the functioning spleen. The number of pitted cells (not the number of pits per cell) is inversely proportional to splenic function, with normal persons having <2% pitted cells.

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ASPLENIA AND HYPOSPLENIA

Congenital asplenia may be an isolated lesion or associated with severe cyanotic congenital hearing disease and bilateral right-sidedness. [47] Life-threatening cardiac lesions, including transposition of the great vessels, pulmonary artery atresia or stenosis, septal defects, anomalous venous drainage, and single atrioventricular valve, are components of this condition. The liver is central, and both lungs have three lobes. The peripheral blood smear shows Howell-Jolly bodies and other signs of hyposplenism. Children who survive the cardiac difficulties in the neonatal period have a significant incidence of sepsis due to a variety of organisms. [48]

Polysplenia is associated with bilateral left-sidedness. Dextrocardia, bilateral superior venae cava, septal defects, and anomalous pulmonary venous return are associated cardiac lesions. Both lungs have two lobes, the liver is midline, and bowel malrotation is common. The splenic tissue is divided into two to nine masses. [49] The peripheral blood smear usually does not suggest hyposplenism, and no clear association with an increased risk of infection has been documented.

Asplenia occurring without heart disease is less likely to be detected before an infection develops than when associated cardiac lesions bring the patient to medical attention.[50] [51] Some of these patients also have situs inversus. Isolated cases of asplenia discovered after death of an otherwise healthy adult or child from overwhelming sepsis with encapsulated organisms have been reported. Imaging to determine whether a spleen is present should follow identification of Howell-Jolly bodies on the blood smear of an otherwise healthy person. Immunization with polysaccharide vaccines and early intervention with antibiotics for apparent infection may prove lifesaving for these individuals. [48]

Acquired Hyposplenism

Infarction in Sickle Cell Disease

The course of sickle cell disease is marked by progressive dysfunction of many organs over many years. [52] [53] One of the earliest organs to be affected is the spleen. Serial measurement of pitted erythrocytes in sickle syndromes demonstrates that splenic dysfunction develops progressively over the first few years of life in the major sickle syndromes (Figs. 46-2 (Figure Not Available) and 46-3). The hypoxic, acidotic, hypoglycemic environment of the spleen creates optimal conditions for tactoid formation and for sickling of the poorly deformable erythrocytes, which then block splenic blood vessels and infarct the tissues. The hyposplenism is reversible with transfusion for at least the first few years of life, but becomes irreversible by age 6 in patients with hemoglobin SS disease, with progressive damage to blood vessels.[54] [55] [56]

Splenic sequestration is a manifestation of the infarctive process of sickling in the spleen that extends to involve larger veins. [57] [58] The unimpeded flow of well-oxygenated blood in the splenic arterioles into the distensible splenic tissues results in pooling of a large amount of blood, while the venous drainage is occluded by sickled hypoxic erythrocytes. Unlike the chronic process of smaller vessel infarction, the acute splenic sequestration crisis can be life-threatening because a large amount of blood can collect in the spleen. The tendency to recurrence and the potential for fatal outcome lead to the common recommendation for splenectomy in a patient who has had one severe splenic sequestration crisis, or more than one less severe crisis. Occlusion of venous drainage also occurs in the liver, but the less distensible capsule of the liver and the options for venous drainage through the portal system make this less likely to threaten life.

Figure 46-2 (Figure Not Available) Development of functional asplenia in sickle cell disorders. *(From Pearson HA, Gallagher D et al: Developmental patterns of splenic dysfunction in sickle cell disorders. Pediatrics 76:392, 1985. Reproduced by permission of Pediatrics in Review.)*

Immunologic and Autoimmune Diseases

Poor phagocytic function of the spleen is associated with impaired function of the Fc receptors on splenic macrophages in a wide variety of immunologic, rheumatic, and inflammatory disorders. Among those in which hyposplenism have been clearly defined and associated with a risk of infection are systemic lupus erythematosus, rheumatoid arthritis, sarcoidosis,[59] systemic vasculitis, ulcerative colitis, celiac disease, [60] amyloidosis,[61] chronic graft-versus-host disease, [62] [63] mastocytosis, [64]

Figure 46-3 Pocked erythrocytes in the sickle hemoglobinopathies. *(From Sills and Oski,[995] with permission. Copyright 1979, American Medical Association.)*

and combined immunodeficiency.[65] Both the diseases themselves and the immunosuppressive therapies that are used in their management may contribute to the risk of infection in these conditions. Immunization to polysaccharide antigens and recognition of the risk of bacterial infection are both important to minimizing the risks of splenic hypofunction.[66]

Splenomegaly and the production of autoantibodies (such as to platelets, in idiopathic thrombocytopenic purpura) are features of the acquired immunodeficiency syndrome (AIDS). In the late stages of AIDS, atrophy of lymphoid follicles and depletion of T-cell-dependent areas are common. It is not clear that impairment of phagocytic function is a component of this splenic atrophy in AIDS.

Therapy-Induced Splenic Hypofunction

Radiation therapy affects splenic function, depending on the dose administered. In general, phagocytic cells are little affected by irradiation, but lymphoid cells are extremely sensitive. B-cell function is nearly fully ablated with as little as 500 cGy. T-cell lymphoblastogenesis is eliminated by administration of 3,000 cGy. With doses of 2,000 cGy, splenic hypofunction is usually transient, as the macrophages and splenic stroma are little affected, and circulating B and T lymphocytes can repopulate the splenic follicles. Thus, patients receiving irradiation to the bed of a left Wilms tumor are not hyposplenic. However, permanent splenic hypofunction develops with doses of 4,000 cGy, which are standard doses for Hodgkin disease. [67] The risk of infection is significant after such therapy.

Corticosteroid therapy impairs the affinity of the Fc receptors of splenic macrophages for opsonic IgG, and decreases the adhesiveness of granulocytes and monocytes.[68] [69] This creates a pharmacologic splenectomy acutely with administration of corticosteroids at common therapeutic dosages. The function of the Fc receptors on hepatic, pulmonary, and bone marrow macrophages is far less affected than that of splenic macrophages. The acute rise in platelet count or hemoglobin values seen with corticosteroid therapy in idiopathic thrombocytopenic purpura or autoimmune hemolytic anemia is due to decreased clearance of sensitized cells.

With longer therapy, the production of antibodies by splenic lymphocytes is also affected, and splenic function continues to be impaired.

Intravenous Immune Globulin G appears to decrease the phagocytic function of the spleen by binding to Fc receptors and impeding their recognition of opsonized particles.[70] This is a transient effect, as the Fc receptors are internalized and recycled, and opsonic function returns to normal within 23 weeks. [71] Occupancy and impairment of function of Fc receptors is also seen with administration of anti-D immunoglobulin to Rh-positive patients, which induces a transient hemolytic anemia. Reticuloendothelial blockade, or occupancy of these receptors, refers to the impaired ability of the spleen to recognize and remove other IgG-coated particles, including bacteria, in the presence of these agents. [72]

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SPLENOMEGALY AND HYPERSPLENISM

An enlarged spleen is not a disease state in itself but usually indicates some underlying pathology. The processes run the gamut from minor to life-threatening, from congenital disorders to those at the end of life, and few organ systems are not represented in the underlying conditions. It is useful to approach splenomegaly by considering the processes that may cause splenic enlargement, and then focus on the specific diagnoses within those categories.

The spleen may be enlarged as a result of infiltration, hypertrophy of normal elements (macrophages and lymphoid components), extramedullary hematopoiesis, inflammatory/immunologic processes, and systemic or portal congestion. Rarely,

TABLE 46-2 -- Causes of Splenomegaly

Primary Process	Pathogenesis	Examples
Anatomic	Developmental anomalies	Cysts, pseudocysts, hamartomas, peliosis, hemangiomas
Hematologic	Hemolysis	Intrinsic (membrane, enzyme, hemoglobin disorders); extrinsic (immune)
	Extramedullary hematopoiesis	Myeloproliferative diseases/myelodysplasias, myelofibrosis, osteopetrosis
Infectious	Bacteria	Acute and chronic systemic infection, abscesses, SBE
	Mycobacteria	Miliary tuberculosis
	Spirochetes	Syphilis, Lyme disease, leptospirosis
	Viruses	EBV, CMV, HIV, hepatitis A,B,C
	Rickettsia	Rocky Mountain spotted fever, Q fever, typhus
	Fungi	Disseminated candidiasis, histoplasmosis, South American blastomycosis
	Parasites	Malaria, babesiosis, toxoplasmosis, *Toxocara canis/catis*, leishmaniasis, schistosomiasis, trypanosomiasis
Immunologic	Collagen vascular diseases	Felty syndrome, SLE, mixed connective tissue disorder, systemic vasculitis, Sjögrens syndrome, systemic mastocytosis
	Immunodeficiency	Common variable immunodeficiency
	Immune/inflammatory	Graft-versus-host disease, serum sickness, LGL lymphocytosis, Weber Christian histiocytoses
Neoplastic	Primary malignancies	Lymphomas, leukemias
	Metastatic malignancies	Breast, lung, skin, colon
Infiltrative	Storage diseases	Gaucher disease, Niemann-Pick disease, GM-1 gangliosidosis, glycogen storage disease type IV, Tangier disease, Wolman disease, mucopolysaccharidoses, hyperchylomicronemia types I and IV
Congestive	Portal hypertension	Intrahepatic cirrhosis, extrahepatic cirrhosis (Budd-Chiari syndrome)
	Systemic	Congestive heart failure

Abbreviations: SBE, subacute bacterial endocarditis; EBV, Epstein-Barr virus; CMV, cytomegalovirus; HIV, human immunodeficiency virus; SLE, systemic lupus erythematosus; LGL, large granular lymphocyte.

anatomic abnormalities will cause splenomegaly. Splenomegaly is important to investigate because it is frequently the presenting finding of a serious disorder whose earlier recognition and treatment may prevent significant long-term morbidity and mortality. The diseases associated with splenomegaly are detailed in Table 46-2 .

Hypersplenism refers to nonimmune, indiscriminate destruction of the formed elements of the blood by a spleen that is enlarged, affected by portal hypertension, or both. The bone marrow is hyperplastic, and the peripheral blood cell counts are decreased as a consequence of destruction of mature formed elements. Splenectomy corrects the cytopenia. Any of the formed elements of the blooderythrocytes, neutrophils, or plateletscan be affected, alone or in combination. Splenic hypertrophy results in both pooling of formed elements and premature destruction of cells by splenic macrophages. Because a normal function of splenic macrophages is to remove senescent cells, this is an exaggeration of a process that is physiologic for splenic macrophages.

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SPLENECTOMY

Indications and Timing

Splenectomy should be performed for clinical indications rather than for specific diagnoses. In many instances, removal of the spleen will improve the condition of patients with hemolytic anemia due to intrinsic disorders of erythrocyte membranes and enzyme disorders, and of those with chronic conditions such as storage diseases and portal hypertension. Specific clinical indices that require intervention should be identified, and parameters that can be used to identify clinical improvement (usually an increase in peripheral blood cell counts, growth, or energy level) determined before the procedure is performed. Thus, for patients with inherited erythrocyte membrane or enzyme disorders such as hereditary spherocytosis or pyruvate kinase deficiency, marked reticulocytosis indicating significant metabolic energy required for erythropoiesis, somatic growth failure, or lack of exercise tolerance would be potential indications for splenectomy. Avoidance of formation of gallstones, formerly often considered an indication for splenectomy when a diagnosis of hereditary spherocytosis was made, is less important today, as minimally invasive surgical procedures have improved the management of cholelithiasis. Patients with storage disorders such as Gaucher disease develop splenomegaly and hypersplenism. Cytopenias requiring intervention should be present before splenectomy is performed. The clinical benefit to be obtained from splenectomy should at least balance, and preferably outweigh, the potential long-term risks of postsplenectomy septicemia, [73] [74] [75] [76] [77] [78] [79] [80] [81] [82] [83] the possibly increased incidence of arteriosclerotic cardiovascular disease, [30] and the shift of storage cells from the spleen to other organs such as bone

marrow, where they may do more harm in the absence of the spleen.[84] [85] [86] [87] Not all patients with hemolytic anemias such as hereditary spherocytosis need splenectomy. If they develop an aplastic crisis, as with parvovirus B-19 infection, while their spleens are intact, they may need transfusion. Many patients with mild chronic hemolysis and well-compensated anemia may be better off with their spleens remaining in situ. Patients with immune thrombocytopenic purpura should undergo surgical splenectomy when the risks of bleeding or of medical therapies (such as long-term corticosteroids) are such that the benefits of splenectomy exceed the risks. These indications for splenectomy are different in adults and in children, and are affected by the presence of underlying disorders such as systemic lupus erythematosus and HIV infection.

The timing of splenectomywhen it is to be performedshould again be chosen to minimize risks and maximize benefits. Immunity to carbohydrate antigens, such as those in the cell walls of encapsulated organisms such as *Streptococcus pneumoniae, Neisseria*, and *Hemophilus influenzae* type b, develops over the first 23 years of life. When splenectomy can be delayed until the patient is at least 2 and preferably >5 years of age, specific immunity and response to administered polysaccharide vaccines will improve host defenses and lessen the risk of postsplenectomy sepsis. [73] [81] When patients have storage diseases such as Gaucher disease, or hemolytic anemia predisposing to iron overload, such as thalassemia, the presence of the spleen as a preferential site for storage of harmful products may protect other organs from damage.[84] [85] [86] [87] Delaying splenectomy until a clear clinical indication is present will balance risks optimally.

Relative Contraindications

Extramedullary hematopoiesis is a relative contraindication; in some conditions, splenomegaly reflects the fact that the bone marrow is not the primary site of hematopoiesis, as intrinsic bone marrow pathology such as myelofibrosis renders the marrow cavity incapable of supporting hematopoiesis. Myeloid metaplasia may reflect the fact that the spleen has become a predominant site of hematopoiesis. Splenectomy under these circumstances may render the patient pancytopenic and produce massive hepatomegaly.[85] The major conditions leading to this circumstance are malignant osteopetrosis in young children and myelofibrosis in adults. It is important to determine before splenectomy that the patient is not dependent on splenic hematopoiesis for survival.

Portal hypertension is not a contraindication to splenectomy, but it affects the surgical approach. It is helpful to determine before surgery whether the patient is likely to need a shunt to reduce portal hypertension and manage esophageal varices.

Surgical Options

When splenectomy is clearly indicated, acute complications are rarely a consideration in the decision to perform surgery. Nevertheless, advances in surgical procedures have minimized the short-term risks of the procedure itself and of postoperative complications such as intestinal obstruction from adhesions. Subtotal splenectomy can be performed when total splenectomy is not desirable, such as for the removal of a cyst, a pseudocyst, or tumors; after trauma; or for Gaucher disease.[78] [86] Wedge resection with mattress sutures and cyanoacrylate adhesions and microfibrillar collagen omental packs have greatly improved partial splenectomy procedures in the past decade. These procedures require a laparotomy, as do any procedures in which extensive peritoneal adhesions are present; the staging for Hodgkin disease;[88] [89] and the removal of most massively enlarged spleens.[90] [91] A sufficiently large incision to permit full visualization and mobilization is essential when the spleen is very large or when inspection is a major part of the surgical procedure. A retroperitoneal approach is useful when the spleen is not massively enlarged but needs to be fully removed, such as when cytopenias are the indication for the procedure. This approach shortens the postoperative recovery time and avoids induction of peritoneal adhesions.

Laparoscopy is now the procedure of choice for splenectomy when possible. Although the operative time is significantly greater than for laparotomy, the postoperative recovery time, the risk of damage to the pancreas, the likelihood of development of subphrenic abscess and peritoneal adhesions postoperatively, and the nutritional and metabolic challenges to the patient are considerably reduced.

Appreciation of the risk of postsplenectomy septicemia[92] [93] and refinements in noninvasive, accurate radiologic monitoring techniques have led to more conservative approaches to splenic injury.[78] [94] After traumatic rupture, splenic tissue may regenerate minisplenules in the peritoneal cavity, a process termed splenosis. [94] Although splenosis is partially protective against postsplenectomy infection in animals, [95] its protective value in humans is not known, and probably depends on the adequacy of perfusion of splenic tissue. It is generally valuable to attempt preservation of splenic function when possible after traumatic rupture.

Complications After Splenectomy

Acute complications in the perioperative period include rupture of the spleen, the development of subphrenic abscess, and injury to the pancreas with the operative procedure. In a healthy patient, the acute risk of splenectomy is low. The degree of splenomegaly greatly affects the risk of rupture and pancreatic injury. The technical difficulty of splenectomy is much greater when the spleen is massively enlarged than when the spleen is small. Rupture of the spleen once the arterial supply has been ligated is rarely a problem. The splenic hilum is retroperitoneal, so that when the spleen is very large, mobilization to access the splenic artery and

vein can be difficult.

After recovery from surgery, intestinal obstruction due to formation of peritoneal adhesions is a complication that usually occurs within the first few months, if it is to occur at all.

Two late complications of splenectomy give the greatest concern: overwhelming postsplenectomy septicemia and atherosclerotic heart disease. Postsplenectomy septicemia is rare but may be rapidly lethal. It is due to the inability of hepatic and pulmonary macrophages to effectively clear organisms from the bloodstream in the absence of protective levels of opsonic (IgG) antibody. Organisms that enter the bloodstream and would ordinarily be removed by splenic macrophages are able to evade recognition by macrophages whose Fc C3b receptors appear to be less avid than those of splenic macrophages. Circulation of the blood through the liver and lung is more rapid than through the spleen, and there is little opportunity for macrophages to recognize organisms whose surfaces contain no IgG and only small amounts of C3bi. Generation of cytokines, including tumor necrosis factor-, and bacterial endotoxins leads to cardiovascular collapse and shock. It is rare to revive an asplenic patient once the patient is in shock, even with effective antibiotic therapy. The risk that this will happen varies with the indication for splenectomy (Table 46-3) and the patients medical condition. Factors that impair host defenses significantly increase the risk of infection. These include deficient opsonins (hypogammaglobulinemia, specific antibody production deficiency); reticuloendothelial blockade related to increased phagocytic activity of macrophages in other organs; impaired antigen processing or recognition (AIDS, lymphoma, other malignancies,

TABLE 46-3 -- Incidence of Postsplenectomy Sepsis

Indication for Splenectomy	Cumulative Incidence of Bacterial Sepsis (%)
Trauma	1.5
Hematologic disorders	3.4
Portal hypertension	8.2
Hodgkin disease	10
Sickle cell disease	15
Thalassemia	25

From Gorse,[96] with permission.

some collagen vascular diseases); neutropenia; and high iron load (thalassemia). Patients at the lowest risk are those in whom splenectomy cures the underlying problem, such as isolated ITP, hereditary spherocytosis, and trauma. Whereas patients undergoing splenectomy for trauma have a 50-fold greater risk of septic death than those with spleens intact, the risk in patients with sickle cell disease is increased 350-fold over that of the general population.

Atherosclerosis developing many years after splenectomy may be related to the thrombocytosis that occurs, with enhancement of plaque formation. Statistically, the increased risk of atherosclerosis is not great, [30] but for individuals with other risk factors such as high levels of cholesterol or homocysteine, heterozygous protein C or S deficiency, or factor V Leiden, splenectomy may pose a more significant risk. No data are currently available in this area.

Prevention of Complications

Postsplenectomy Septicemia

The major risk of postsplenectomy sepsis is infection with encapsulated organisms such as *S. pneumoniae, H. influenzae* type b, and *Neisseria meningitidis*, which require opsonization for effective phagocytosis. Purified polysaccharide vaccines are available for all three groups: 23-valent pneumococcal, quadrivalent meningococcal, and Hib. These vaccines are highly effective when administered to persons >2 years old and are rarely associated with adverse effects. However, they are more effective in the presence of the spleen than in its absence. [18] [20] [83] [96] Thus, it is recommended that immunization be done 3 weeks before the anticipated splenectomy to optimize antigen recognition and processing and induce more effective immunity. [97] Adults are presumed to be immune to *H. influenzae* type b, and thus may receive the pneumococcal and meningococcal vaccines alone. Re-immunization for children is recommended 25 years after initial immunization. Repeat administration of pneumococcal vaccine is recommended every 10 years. [98] Because these recommendations change with experience and vaccine usage, it is wise to consult current guidelines for individual patients.

There is less data on the efficacy of prophylactic antibiotics for asplenic patients, except for young children with sickle cell disease, in whom antibiotics should be initiated as soon as the diagnosis is established. Present recommendations are that twice daily oral penicillin or amoxacillin be continued in sickle cell patients until 6 years of age. High-risk patients with surgical or functional asplenia, including those with diagnoses of thalassemia, Hodgkin disease, other malignancies, immunodeficiency disease, or chronic graft-versus-host disease, should receive prophylactic antibiotics unless specific contraindications exist. Whether or not such patients receive prophylactic antibiotics, and regardless of their immunization status, they may develop overwhelming infections with other organisms, including gram-negative organisms and *S. aureus*.

The single most important measure to prevent postsplenectomy septicemia is education of patient, family, and physician. Recognition of the risk, the institution of appropriate preventative measures, and the rapid administration of antibiotics to a patient who is showing signs of infection (fever, chills) can be lifesaving.

Atherosclerosis

The magnitude of the risk of development of atherosclerosis is not clear. Appropriate preventative measures include identifying and minimizing concurrent risk factors, such as obesity, dietary patterns, a sedentary lifestyle, and the presence of other hereditary factors that would predispose to thrombosis. Low-dose aspirin could be considered, but no data exist regarding its efficacy in this situation.

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Chapter 47 - Basophils, Mast Cells, and Systemic Mastocytosis

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INTRODUCTION

Mast cells and basophils share common, although not identical, functions in the immune response, express on their surface the receptor that binds with high affinity the Fc portion of the IgE antibody (Fc RI), and exhibit metachromatic staining granules in their cytoplasm. [1] [2] [3] [4] Consequently, they have in the past been thought to represent tissue-based (mast cell) and blood-borne (basophil) forms of the same cell. Indeed, we know that basophils share a common committed stem cell with eosinophils.[5] [6] More recent studies have demonstrated that despite their similarity, mast cells and basophils represent distinct terminally differentiated cells derived from bone marrow CD34+ myeloid progenitors.[7] [8] [9] Whereas basophils mature in the marrow and circulate in the blood fully mature, mast cells migrate to the tissues when immature and complete maturation at these sites.[1] Major biologic differences between basophils and mast cells are listed in Table 47-1 . Differences in surface immunophenotypes between mature mast cells and basophils are shown in Table 47-2 . Both basophils and mast cells are major repositories of histamine and other chemo- and vasoactive mediators, and have been implicated in the initiation and propagation of a variety of allergic and inflammatory processes. These include atopic disorders such as allergic rhinitis, asthma, and anaphylaxis. [2] [4] [10] However, there are distinct differences in cytokine/mediator responses and content of mast cells and basophils (Table 47-3). [1] [2] In addition to the direct actions mediating allergic responses exhibited by both cell types, mast cells and basophils have also been shown to contribute to host defenses against parasites in a cooperative manner. [11] [12] [13]

TABLE 47-1 -- Natural History, Major Mediators, and Surface-Membrane Structures of Human Mast Cells and Basophils

Characteristic	Basophils	Mast Cells
Biology		
Origin of precursor cells	Bone marrow	Bone marrow
Site of maturation	Bone marrow	Connective tissue (a few in bone marrow)
Mature cells in circulation	Yes (usually <1% of blood leukocytes)	No
Mature cells recruited into tissues from circulation	Yes (during immunologic inflammatory responses)	No
Proliferative ability of morphologically mature cells	None reported	Yes (under certain circumstances)
Life span	Days (like other granulocytes)	Weeks to months (according to studies in rodents)
Mediators		
Major mediators stored in preformed cytoplasmic granules	Histamine, chondroitin sulfates, neutral protease with bradykinin-generating activity, -glucuronidase, elastase, cathespin G-like enzyme, major basic protein, Chercot-Leyden crystal protein	Histamine, heparine, or chondroitin sulfates: neutral proteases (tryptase with or without chymase); many acid hydrolases; cathespin G, carboxypeptidase
Major lipid mediators produced on appropriate activation	Leukotriene C_4	Prostaglandin D_2 , leukotriene C_4 , platelet-activating factor
Cytokines released on appropriate activation	IL-4	TNF-, IL-4, IL-5, IL-6
Surface structures		
Immunoglobulin receptors	Fc RI, FcRIII (CDw32)	Fc RI, FcRII (CDW32) c-*kit* receptor
Cytokine or growth-factor receptors	Receptors for IL-2 (CD25), -3, -4, -5, and -8 and for SCF (some basophils express low numbers of c-*kit* receptors)	
Cell-adhesion structures	LFA-1 -chain (CD11a), C43bi receptor (CD11b), gp 150, 95 (CD11c), LFA-1 chain (CD18), IL-2R (CD25), ICAM-1 (CD54), and CD44	CD11c, CD18, ICAM-1 (CD54), CD44, VLA-4

TABLE 47-2 -- Immunophenotype of Mature Basophils and Mast Cellsa[a]

Marker	Basophils	Mast Cells
BSP-1	+	
YB5B8 (c-kit product)		+
CD9 (p24)	+	+
CD14		+

CD25	+	
CDW32 (Fc RII)	+	+
CD68		+
Fc RI	+	+
Cytokine receptors		
IL-1RII (CD121b)	+	
IL-2R (CD25)	+	
IL-3R (CD123)	+	
IL-4R	+	+
IL-5R	+[a]	?
IL-8R (CD128)	±[b]	
IFN-R (CD19)	±	±
GM-CSFR (CD116)	±	
SCFR/c-kit	±	+
CD40	+	
Cell adhesion structures		
LFA-1	+	
CD11a	+	
CD11b/18 (C43biR)	+	
gp150/95 (CD11c)	+	
LFA-1	+	
LFA-3 (CD58)	+	+
ICAM-1 (CD54)	±	+
ICAM-2 (CD102)	+	±
CD44	+	+
CD51/CD61	?	+[c]
VCAM-1		
VLA-4 (CD49d/CD29)	+	+
VLA-5 (CD49c)	+	+
VLA-6 (CD49f)		
MAC-1 (CD11b/CD18)	+	
PECAM (CD31)	+	

BSP, basophil specific protein; IL, interleukin; IFN, interferon; R, receptor; GM-CSF, granulocyte monocyte colony stimulating factor; SCFR, stem cell factor receptor; LFA, leukocyte function antigen; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; gp, glycoprotein; VLA, very late antigen; PECAM, platelet endothelial cell adhesion molecule.

After Galli, [1] *Denberg,* [7] *and Agis.* [9]

[a]Using leukemia HL-60 cells.
[b]Variable expression and/or conflicting results.
[c]Uterine mast cells.

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BASOPHILS

Basophils are the least common of the human granulocytes, comprising <0.5% of nucleated bone marrow and peripheral blood leukocytes. In contrast to mast cells which generally have oval, unilobed nuclei, basophils have a bilobed nucleus and cytoplasm that contains fewer but larger metachromatic granules than do mast cells.[10] Using tritium-labeled thymidine techniques, basophils are shown to mature in the marrow over 2.5 to 7 days before release into the peripheral circulation, and then to survive for up to 2 weeks thereafter. Basophils arise from a common basophil-eosinophil progenitor cell which can be isolated from marrow, peripheral blood, and cord blood.[5] [6] [14] Differentiation of the eosinophils and basophils derived from these colonies is regulated in a reciprocal fashion. [14] Further maturation of basophils does not occur in the circulation. Upon stimulation, or initiation of an allergic response, basophils may be recruited out of the vascular space to affect allergy or inflammation in tissue sites.[1] [2] [3] [4]

Basophils are key mediators of immediate hypersensitivity

TABLE 47-3 -- Biochemical Comparison of Basophils and Mast Cells

Mediators	Basophils	Mast Cells
Mediators stored in granules		
Histamine	+	+
Heparin		+
Chondroitan sulfate	+	+
Neutral proteases (tryptase ± chymase)	+	+
Acid hydrolases		+
Cathepsin G	+	+
Carboxypeptidase		+
-glucuronidase	+	
Elastase	+	
Major basic protein	+	
Lysophospholipase (Charcot-Leyden crystal protein)	+	
Kallekrein	+	
Lipid mediators produced on activation		
Prostaglandin-D_2		+
Leukotriene C_4	+	+
Platelet-activating factor		+
Cytokines[a]		
IL-4	+	+
IL-5		+
IL-6		+
IL-8		+
IL-13		+
TNF-		+
MIP-1		+
bFGF		+

IL, interleukin; TNF-, tumor necrosis factor ; MIP-1, macrophage inflammatory protein-1; bFGF, basic fibroblast growth factor.

[a]Based on immunohistochemical staining detecting cytokine in some or all of cell type.

reactions such as asthma, urticaria, allergic rhinitis, and anaphylaxis. In addition, they have also been implicated in the pathophysiology of delayed cutaneous hypersensitivity and in the late-phase responses of anaphylaxis. [4] IgE is the primary stimulator of basophils, although other mediators such as IL-3, C5a, GM-CSF, and morphine have been shown to stimulate histamine release from these cells.[10] Under normal conditions, the ultimate effect of basophil activation is to produce a localized inflammatory and allergic reaction. However, under pathologic conditions in which basophils are increased in number, either systemically or locally, or in which basophil responses are not appropriately modulated, systemic, severe, allergic/inflammatory reactions may result from basophil activation.

Basophil Structure

Granules

The cytoplasmic granules of basophils and mast cells contain sulfated glycosoaminoglycans. In normal basophils, these are predominantly heparin, whereas in leukemic basophils, chondroitan sulfate is more prevalent. There is little systemic anticoagulant effect of these glycosoaminoglycans although localized anticoagulation with bleeding may occur. These sulfated glycosoaminoglycans are responsible for the intense metachromatic staining of the mast cell and basophil granules. Basophil granules are the major source of circulating histamine, which is synthesized and stored in these granules. Release of histamine accounts for many

of the elements of the immediate hypersensitivity reaction (so called allergic or Type 1 hypersensitivity reaction). Histamine reacts with tissue-based receptors increasing

vascular permeability and tissue edema and is also a potent eosinophil chemoattractant, recruiting eosinophils to the area of basophil activation. Trypsin and chymotrypsin released from basophil granules are responsible for some of the tissue damage and functional disruption of the vascular membrane (e.g., capillary leak) that occurs during chronic inflammation. Basophil granules also contain kallekrein, which, when released, acts directly to affect tissue and vascular integrity and complement and coagulation activation.[10]

Plasma Membrane

Basophils and mast cells express a high-affinity receptor for the Fc portion of IgE (Fc RI) in large numbers on their surface. These receptors allow basophils and mast cells to bind and cross-link specific IgE on the cell surface resulting in the generation of a transmembrane signal and ultimately the release of granule contents. In addition, the plasma membrane lipids serve as a substrate for the metabolism of arachidonic acid. Several metabolites of arachidonic acid are released from basophils upon activation. These include leukotriene C4 (LTC4), which can further enhance the inflammatory reaction and result in tissue damage. [3] [15] [16] Basophils express plasma membrane receptors for adhesion molecules that enhance the selective recruitment of basophils to areas of inflammation (Table 47-2).[2] The expression of these receptors on the basophil surface is markedly upregulated by a number of inflammatory stimuli including endotoxin (LPS), IL-1, IL-3, IL-4, TNF-, and INF-. The upregulation is maximal at 2448 hours following stimulation. The pattern of adhesion receptor expression following exposure to IL-3 greatly favors basophil recruitment and adhesion over that of neutrophils [17] thereby selectively promoting the sticking and diapedesis of basophils into areas of inflammation. Basophils are recruited to areas of inflammation by the chemoattractant action of IL-5. IL-4 upregulates endothelial cell expression of VCAM-1 further enhancing basophil adhesion to endothelial cells.

Basophil Function

Interaction between IgE and basophil IgE receptors, with multivalent antigens bridging the receptors, leads to an anaphylactic secretion of basophil granule contents.[18] In addition to IgE, other stimuli, including C5a, C3, neutrophil lysosomal proteins, insect venoms, hormones, and some drugs can also affect basophil degranulation (Table 47-4).[1] [10] [19] [20] [21] [22] [23] Clinically significant symptoms may also result from the release of other mediators from stimulated basophils. These include kallekrein, the slow-reacting substance of anaphylaxis (SRS-A, leukotriene D4 [LTD4]), platelet-activating factor (PAF-acether), and eosinophilotactic factors (ECFAs). Eosinophilotactic factors recruit eosinophils to areas of inflammation, where they can downregulate the allergic reaction by inactivation of SRS-A and oxidation of histamine.[24]

Basophils in Health and Disease

Few patients have been described to have a deficiency of either basophils or mast cells. Consequently, the role these cells play in host defenses is difficult to discern. However, it appears that both cell types play a central role in immediate hypersensitivity reactions and play supporting roles in chronic inflammatory conditions of both allergic and non-allergic etiologies, and in host responses to parasitic infections. In all of these conditions, basophils and mast cells appear to serve as localizers of the allergic reaction. A role for basophils in primary hematologic disorders is not at all clear, although the finding of increased numbers of basophils heralding a terminal blast crisis in CML, and the demonstration that basophilia is associated with a poorer

TABLE 47-4 -- Stimuli That Directly Induce Histamine Secretion from Human Basophil and Mast Cells

Stimuli	Basophil	Mast Cell
Interleukins		
IL-1	±[a]	±
IL-3	+	
IL-5		
GM-CSF	+	
Stem cell factor	+	+
MIP-1	+	+
MCAF	+	ND
RANTES	+	ND
NAP-1	+	ND
CTAP-III	+	ND
NGF	+	ND
C3a	+	
C5a	+	+[b]
Substance P		+[b]
fMet-Leu-Phe	+	+[b]
Gastrin	ND	+
Drugs		
Morphine	+	+[b]
Mannitol (hyperosmolar)	+	+
Radio contrast	+	ND
Muscle relaxants	+	ND

ND, not determined.

[a]Conflicting results.
[b]Primary cutaneous mast cells.

prognosis in CML,[5] [25] [26] may give some clues to the cellular and molecular abberrations present in this disease.

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MAST CELLS AND SYSTEMIC MASTOCYTOSIS

Mastocytosis has been confounding both clinicians and scientists for more than a century. In 1869, Nettleship and Tay [27] described what we now know as urticaria pigmentosa in a 2-year-old girl. In 1877, Paul Ehrlich [28] was the first to describe mast cells in his study of granulated connective tissue cells. He believed that these cells represented overnourished or overfed connective tissue cells and termed them mastzellen. At the same time, Unna [29] demonstrated mast cells in the skin lesions of urticaria pigmentosa, the term suggested by Sangster[30] the following year. Nearly 50 years later, uriticaria pigmentosa was recognized as the characteristic skin lesion of mastocytosis;[31] finally, in 1949 the systemic nature of mastocytosis was recognized by Ellis [32] in his report of the autopsy findings of a 1-year old infant with diffuse organ infiltration by mast cells. This systemic organ infiltration by mast cells often results in protean clinical manifestations that frequently obscure the underlying disease process. Although the most commonly involved organ in systemic mastocytosis is the skin, a significant number of cases eventually show mast cell infiltration in multiple organs. It is in this clinical setting that the term disseminated or systemic mastocytosis has generally been used. The classification schema proposed by Travis et al.[33] and modified by Friedman and Metcalfe[34] to include lymphadenopathic mastocytosis with eosinophilia, points out the difficulty clinicians and researchers have in classifying this disorder (Table 47-5). Some researchers suggest that mastocytosis can be considered primarily a biochemical disorder that results from the uncontrolled release of

TABLE 47-5 -- Mayo Clinic Classification of Mastocytosis

Indolent mastocytosis
Skin only
Urticaria pigmentosa
Diffuse cutaneous mastocytosis
Systemic
Marrow
Gastrointestinal (with or without urticaria pigmentosa)
Mastocytosis with an associated hematologic disorder (with or without urticaria pigmentosa)
Myelodysplastic disorders
Myeloproliferative disorders
Acute myeloid leukemia
Malignant lymphoma
Chronic neutropenia
Mast cell leukemia
Lymphadenopathic mastocytosis with eosinophilia (with or without urticaria mastocytosis)
Aggressive mastocytosis

mediators contained within the mast cell or induced by mast cell activation (Table 47-6).[35] However, this approach understates the significance of the pathologic effects of the organ infiltration by mast cells and does not adequately address the spectrum of hematologic disorders and mutations in c-kit [36] observed in this disease. A consensus classification schema suggested in 1991 [37] is widely employed as a reasonable approach to the classification and diagnosis of this disease (Table 47-7).

TABLE 47-6 -- Mediators in Mastocytosis

Skin specimens
Histamine
Leukotriene B_4
5-hydroxyeicosatetraenoic acid
Tryptase
Chymotryptase
Heparin
Cutaneous blister fluid
Histamine
Prostaglandin D_2
Platelet-activating factor
Plasma
Histamine
Tryptase
Histamine metabolites
N-methyhistamine

N-methymidazole acetic acid
PGD_2 metabolites
9-Hydroxy-11,15-dioxo-2,3,4,5-tetra-norprostane-1,20-dioic acid
9-Hydroxy-11,15-dioxo-2,3,18,19-tetra-norprost-5 ene-1,20-dioic acid
Cytokines
TNF-
IL-4
IL-5
IL-6
IL-7
IL-16
Chemokines
I-309
MCP-1
MIP-1 &
Lymphotactin
Chondroitin sulfate B

TABLE 47-7 -- Consensus Revised Classification of Systemic Mastocytosis

Indolent
Syncope
Cutaneous disease
Ulcer disease
Malabsorption
Bone marrow mast cell aggregates
Skeletal disease
Hepatosplenomegaly
Lymphadenopathy
Hematologic disorder
Myeloproliferative
Myelodysplastic
Aggressive lymphadenopathic mastocytosis with eosinophilia
Mastocytic leukemia

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BIOLOGY AND PATHOGENESIS OF MAST CELL DISEASE

Mast cells and basophils are recognized as the effector cells of the immediate allergic reaction by virtue of their high-affinity receptors for IgE. The wide distribution of mast cells throughout the human body, especially in proximity to blood vessels in atopic and nonatopic individuals alike, and the recognition of mast cell and basophil activation by nonimmunologic means, suggest that these cells may also be involved in nonatopic conditions. Recent evidence that these cells synthesize and release cytokines in response to IgE and non-IgE mediated stimuli adds further support to the hypothesis that these cells play a role not only in the pathobiology of several disease processes, but also in maintaining homeostasis. Mast cell- and basophil-mediated events have been postulated to play a role in host defense against parasitic diseases, in wound healing, in tumor angiogenesis, and in immunoregulation. Furthermore, the early appearance of Fc RI during cell differentiation may be important for these cells to respond to IgE-mediated stimuli before degranulation, possibly by mediating cytokine production.

Although basophils and mast cells are similar in their ability to bind IgE to their surface and to be stimulated after antigen-mediated cross-linking of surface bound IgE, these cells do not represent blood and tissue forms of the same cell type. [38] [39] They differ in significant ways with regard to their morphology, biology, and cell-surface structures (Table 47-1). [39] [40] [41] [42] [43] These cells also exhibit significant differences in response to cytokines and the complement system. [39] [42] [43] [44]

It is now accepted that mast cells are derived from pluripotential hematopoietic cells. [45] This was first demonstrated by Kitamura with in vivo experiments using genetically mast cell-deficient mutant mice and their co-geneic normal littermates. One of the mutants, the WBB6 $_{F1}$ -*W/W^{V}* mouse, is ordinarily devoid of mast cells but can develop mast cells if it receives a bone marrow graft from its normal littermates (WBB6 $_{F1}$ +/+ mice) or from semisyngeneic C57BL/6-bg/bg ("beige") mice. These experiments established that mouse mast cells develop from bone marrow precursors. Mast cells originate from a cell more primitive than those precursors committed either to the neutrophil/macrophage or erythroid cell lineages. [46]

In contrast to the WBB6$_{F1}$ -*W/W^{V}* mouse, the genetically mast cell-deficient WBB6$_{F1}$ -*Sl/Sl^{d}* mouse fails to develop mature mast cells after either systemic or local injection of WCB6$_{F1}$ -+/+ cell populations containing mast cell precursors. [47] *Sl/Sl^{d}* mouse bone marrow cells can, however, differentiate into tissue mast cells after intravenous injection into *W/W^{V}* mice. Taken together, these findings indicate that the mast cell deficiency of the

W/W^{V} mouse reflects the abnormality of the mast cell precursors themselves, whereas the mast cell deficiency of the *Sl/Sl^{d}* mouse reflects an abnormality of tissue microenvironmental factors regulating mast cell differentiation. Products of the *W* or *Sl* loci that influence mast cell development have been identified. The *W* locus encodes the c-kit tyrosine kinase receptor, [48] whereas Sl encodes the c-kit ligand often referred to as a stem cell factor (SCF). [49] [50] [51]

Several molecules are known to promote or augment murine mast cell proliferation: interleukin (IL)-3, IL-4, SCF, IL-9, and IL-10. [52] [53] [54] Bone marrow cells cloned in a collagen gel in the presence of IL-3 produced either pure or mixed mast cell colonies. [55] Mouse bone marrow cultured in IL-3 for 3 weeks gives rise to cultures that consist of >85% mast cells. In contrast, colony-stimulating factor-granulocyte/macrophage (CSF-GM) or transforming growth factor- (TGF-) inhibit the differentiation of IL-3-dependent mast cells. [56] [57] IL-4 has little or no ability to sustain the proliferation of mouse mast cells in the absence of IL-3. [53] In the presence of IL-3, IL-4 promotes mast cell proliferation and maturation in vivo. Administration of anti-IL-4 antibodies to mice on days 0 and 7 of infection with *Nippostrongylus brasiliensis* results in a 50% reduction in mucosal mastocytosis. [58] SCF or c-kit ligand can induce the proliferation in mast cells both in vitro [49] [50] [51] and in vivo. [59] Although SCF alone can increase colony-forming unit-spleen (CFU-S) number in vivo, its capacity to do so in vitro is dependent on its interaction with other growth factors. [60] IL-10, when used in combination with IL-3 or IL-4, also enhances mast cell proliferation. These data suggest that IL-3 is both necessary and sufficient for murine mast cell growth from bone marrow, but SCF and other cytokines are necessary for long-term mast cell survival and maturation. In fact, the removal of IL-3-dependent murine mast cell cultures leads to apoptosis. [61] These mast cells are rescued from apoptosis by the addition of SCF. [38]

The proliferation and differentiation of mast cells may also be influenced by adhesive interactions with components of the marrow matrix. Mast cells adhere spontaneously to vitronectin. This interaction increases the proliferative rate of mast cells maximally stimulated by IL-3. [62] Human mast cells have now been cultured from blood or bone marrow in sufficient numbers to permit the study of mast cell growth and differentiation. Human bone marrow CD34+ cells maintained in IL-3 containing liquid suspension cultures produce basophils and small numbers of immature mast cells after 23 weeks of incubation. [63] Similarly, single-lineage and mixed CFUs containing basophils are observed when mononuclear cells are assayed in semisolid media in the presence of recombinant human IL-3. [64]

As in rodents, c-kit and its ligand SCF are involved in the growth and differentiation of human mast cells. Human SCF acts synergistically with human IL-3 to produce a three- to fivefold increase in total mast cell and basophil numbers over human IL-3 alone. [65] The percentage of cell types in the cultures grown in human IL-3 with or without SCF remain the same, with basophils constituting 1835%, and mast cells 3% at 3 weeks of culture. In the presence of human IL-3 followed by SCF alone, the percentage of mast cells increases over 6 weeks. Mast cells cultured in the presence of IL-3 plus SCF, but not IL-3 alone, are berberine sulfate-positive, suggesting the presence of heparin proteoglycans within granules, a sign of mast cell differentiation. Electron microscopic examination of cultures supplemented with IL-3 and SCF, but not IL-3 alone, also revealed maturational changes in which mast cell granules contain tryptase and exhibited scroll, reticular, and homogenous patterns as seen in CD34+ hematopoietic progenitor cells cultured on 3T3 fibroblasts. [65] Thus, to produce mast cells from human bone marrow, the optimal conditions require at least a brief exposure of CD34+ cells to IL-3, followed by SCF. In contrast, mast cells may be produced from human fetal liver [66] or from human peripheral blood following the addition of SCF only.

Although mast cells originate in the bone marrow, they migrate to connective tissue sites and mature. Under normal conditions, mast cells are found throughout connective tissues and are particularly numerous in the epithelial surface of the skin and respiratory tract, in the gastrointestinal and genitourinary tracts, and in the perivascular areas. [37] [38] [39] [40] [41] [67] [68] At these sites, mast cells exhibit one of two predominant phenotypes, as determined by staining characteristics and granule enzyme content. Mast cells within the skin and other connective tissue sites stain intensively with dyes, probably due to their heparin content. [36] Historically, these mast cells have been referred to as connective tissue mast cells. Mast cells at mucosal locations, such as the lamina propria of gastrointestinal tract, stain less intensively and are referred to as mucosal mast cells. IL-3-dependent murine bone marrow-derived mast cells exposed to IL-4 and IL-10 contain more mouse mast cell protease (MMCP)-2 mRNA, which is more characteristic of the mucosal phenotype. [69] In contrast, murine mast cells exposed to SCF stain more intensely and synthesize heparin, [59] which is more characteristic of the connective tissue mast cell. In humans, the mucosal phenotype contains a specific tryptase, whereas the connective tissue phenotype contains both this tryptase and a chymotryptase. [70] Both human mast cell phenotypes appear to contain heparin. [71] The human mucosal mast cell is sometimes referred to as the T mast cell for its tryptase content, and the human connective tissue mast cell as a CT mast cell for its content of both tryptase and chymotryptase.

Mast cells express adhesion molecules that are involved in their attachment to connective tissue matrix or to other cell types. Co-culture of murine mast cells and activated murine T cells results in formation of heterotypic aggregates. Both the size and number of aggregates are reduced by the addition of antibodies directed

against ICAM-1 and leukocyte function-associated antigen-1 (LFA-1). [42] [72] Human lung and uterine mast cells express surface antigens for ICAM-1. [73]

Mast cell adherence to laminin appears to explain, in part, the distribution of mast cells in tissues. Mast cells accumulate on the under surface of the endothelial cell basement membrane, which is known to be rich in laminin. Exposure to laminin and its degradation products may promote the migration of mast cells into a wound site with subsequent attachment. [74] [75] [76] In addition, mouse bone marrow cells, cultured for 1 week in the presence of IL-3 and sorted on the basis of IgE receptors, adhere to laminin after activation. [77] The ability of such mast cell precursors to adhere to laminin before their expression of mature phenotypic characteristics provides a mechanism by which cells can be localized. The mast cell precursors may then develop their terminal phenotype, dependent on the local tissue environment. Mast cell adhesion receptors that have been defined include a RGD-sensitive fibronectin receptor, [78] vitronectin receptor, [62] laminin receptor, [79] and Peyers patch homing lymphocyte and adhesion molecule (LPAM-1). [80]

The demonstration of adhesion surface antigens on both human lung and uterine mast cells by immunofluorescence and flow cytometry has provided further insight into possible interactions between mast cells and extracellular matrix that may permit mast cell migration. Mast cell migration can be stimulated by IL-3 or SCF and further potentiated by the combination of IL-3 and SCF. [81] Similarly, mast cell adhesion is promoted by SCF or aggregation of Fc RI or Fc RIII. [82] [83]

The pathophysiology of systemic mast cell disease is largely a reflection of increased mast cell numbers in abnormal and normal sites. In at least some instances, the focal accumulation of mast cells is associated with increased local production of soluble mast cell growth factor (c-kit ligand) [84] and may be a reversible event. Although the tissue accumulation of mast cells per se does not induce mast cell activation or degranulation, their presence in large numbers may facilitate dysregulated degranulation.

More recently, an activating mutation in c-kit that may increase mast cell numbers has been reported in patients with

mastocytosis. [36] The original observation was suggested by the report that CD34+ cells from the peripheral blood of patients with mastocytosis, when cultured in vitro in the presence of SCF, gave rise to higher numbers of mast cells per CD34+ cell compared with cells from normal subjects. [85] A search for activating mutations in c-kit based on this observation led to the identification of the somatic mutation Asp816 to Val in c-kit in peripheral blood mononuclear cells (PBMC) from patients with mastocytosis. [36] This mutation has now also been identified in the skin and tissues of mastocytosis patients, [86] and is identical to one of two mutations identified in a human mast cell line. [87] Other polymorphisms of c-kit have also been noted, but are to date of uncertain significance. [88] Expression of c-kit in PBMC of patients with mastocytosis may correlate with disease category. [89] Such observations offer the possibility of improved diagnosis and prognosis based on molecular assays.

The granules of human mast cells are known to contain histamine, heparin, neutral proteases, and IL-4. Upon activation, mast cells release these substances to the surrounding environment. It is believed that many of the manifestations of systemic mastocytosis are the consequence of mast cell degranulation. Although early work focused on histamine and other primary mediators released by mast cells, the recognition that mast cells are a potential source of cytokines has suggested new mechanisms by which mast cell activation may result in pathologic responses. It has now been clearly shown that stimulation of mast cells by the Fc $_{epsilor.}$ RI (the high-affinity for the Fc portion of IgE) or by other mechanisms, induces mouse mast cells to synthesize increased levels of mRNA or to secrete products for multifunctional proinflammatory or mitogenic cytokines such as IL-1, -3, -4, -5, and -6. [90] In addition, high local levels of CSF-GM, interferon- (IFN-), tumor necrosis factor (TNF-), and macrophage inflammatory proteins 1 and 1, T-cell activation antigen-3, and JE are produced in mouse mast cells. [38] [90] Human mast cells have been shown to synthesize and release TNF-, IL-4, IL-5, and IL-6. [38] [91] [92] [93] These cytokines play a critical role in the regulation of not only inflammation, but also hemostasis, hematopoiesis, angiogenesis, and tissue remodeling, and potentially have critical roles in tumor development or resistance. [38] The recognition that mast cell stimulation results in the initiation of both an acute and a sustained inflammatory response has brought us closer to understanding the true nature of the tissue pathology noted in mastocytosis.

PATHOPHYSIOLOGY AND PATHOLOGY

The pathophysiology of mast cell disease can be divided into systemic and local effects. The systemic effects of this disorder arise from the release of significant amounts of mediators into the systemic circulation and can result in either flushing or syncope. The local manifestations of this disease result largely from the effects of local collections of mast cells. However, other systemic effects of mast cell disease may result largely from the consequences of severe end-organ dysfunction (e.g., cardiomyopathy, bone marrow dysfunction).

The classic pathologic lesion of cutaneous mast cell disease is the lesion of urticaria pigmentosa. However, in nearly one-half of patients with systemic mastocytosis, cutaneous lesions are lacking, and other organs must be biopsied to make the diagnosis. The most frequently involved organs in systemic mastocytosis are the bone marrow, skin, lymph nodes, spleen, liver, and gastrointestinal tract; the lungs and kidneys are almost never involved. Because of the high frequency of bone marrow involvement and the relatively high frequency of bone marrow biopsy in the diagnosis of this disorder, a description of the mast cell lesion most frequently found in the bone marrow is presented.

Bone Marrow

Focal mast cell lesions of the bone marrow, identified on bone marrow biopsy specimens, have been reported to be present in about 90% of adults with systemic mast cell disease (Table 47-8). [33] [94] [95] [96] [97] [98] The typical bone marrow lesions observed in a bone marrow biopsy are foci of spindle-shaped mast cells in a fibrotic background (Figs. 47-1 (Figure Not Available) and 47-2). Usually there is an abundant mixture of eosinophils and lymphocytes, creating the so-called MEL lesion. These lesions may be located in perivascular, peritrabecular, or intertrabecular sites. They usually are characterized by increased reticulin staining; occasionally, Masson trichome staining reveals collagen deposition. Occasional mast cell lesions are infiltrated by normal-appearing small noncleaved lymphocytes, or are ringed by a cuff of mature small noncleaved lymphocytes (Fig. 47-3). Frequently, in bone marrow extensively involved by mast cell lesions, the bony trabeculae are moderately to markedly thickened.

Although some studies of patients with systemic mast cell disease have reported increased marrow mast cells in the absence of focal mast cell lesions, [94] [95] [96] most patient marrows reviewed contain focal mast cell lesions. On hematoxyolin and eosin staining, the dominant granulated cell in these lesions is often the eosinophil. Eosinophils can be distinguished from mast cells by the presence of round, often bilobed, nuclei and abundant deeply granulated cytoplasm. In contrast, the typical mast cell has a spindle-shaped or oval nucleus and fine eosinophilic granules that are generally apparent only at high power magnification. Mast cells with bilobed nuclei are occasionally seen in these lesions; some investigators consider them an indicator of a poor prognosis. [33] Wright-Giemsa and toluidine blue stains of the biopsy are often necessary for a definitive demonstration of mast cells (Plate 47-1AC). Frequently these stains are negative on EDTA-decalcified paraffin-embedded material as a consequence of the decalcification process. Marrow biopsies, rather than aspirate smears or clot sections, are most likely to demonstrate mast cell disease. When histochemical stains are performed on the plastic-embedded marrow biopsy and in

TABLE 47-8 -- Distribution of Mast Cells in Bone Marrow of Patients with Systemic Mastocytosis

Series	Patients (N)	Age Range (yrs)	Median Age (yrs)	Number (%) with Focal Lesions	Number (%) with Focal Lesions or Increased Mast Cells
Webb et al. [94]	26	3278	61	21 (84)	25 (100)
Brunning et al. [95]	14	2588	71	9 (84)	11 (100)
Horny et al. [96]	38	4672	49	35 (92)	38 (100)
Ridell et al. [98]	18	2172	49	10 (56)	9 (50)
Travis et al. [33]	58	1780	60	NA	NA
Lawrence et al. [97]	46	576	39	32 (74)	32 (74)

NA, not available.

Figure 47-1 (Figure Not Available) **(A)** Low-power (×20) of a small bone marrow mast cell lesion. **(B)** High-power (× 156) of a marrow mast cell lesion. Note the presence of eosinophils and lymphocytes along with mast cell lesion. *(From Parker RI: Hematologic aspects of mastocytosis I: bone marrow pathology in adult and pediatric systematic mast cell disease. J invest Dermatol 96: 47S, 1991. Reprinted by permission of Blackwell Science, Inc.)*

Figure 47-2 Low power (×20) of a marrow mast cell lesion with a fibrotic matrix. *(Reprinted from Travis et al.,[187] with permission.)*

Figure 47-3 Low power (×20) of marrow mast cell lesion demonstrating infiltration and cuffing by normal small non-cleaved lymphocytes.

blood smears, the mast cells stain positively for chloracetate esterase and aminocaproate esterase (Table 47-9).

Increased numbers of mast cells are frequently present on the marrow aspirate smears of patients with systemic mast cell disease; however, other non-mast cell disorders may be associated with the finding of increased marrow mast cells as well. In systemic mast cell disease, a more specific finding is the presence of clusters of confluent mast cells (20) in which individual cells frequently cannot be discerned. [99] [100] In diseases producing a reactive increase in marrow mast cells, the mast cells are usually found singly or in small clusters. In comparison to adults with systemic mast cell disease, marrow involvement in children appears to be much less common.[100] In a study of 19 children with cutaneous or disseminated mast cell disease, only 10 patients demonstrated small focal mast cell lesions in the marrow biopsy and only 5 demonstrated increased mast cells on aspirate

TABLE 47-9 -- Histochemical Stains Useful in Distinguishing Mast Cells and Basophils

Stain	Mast Cells	Basophils	AML-M3[a]
Giemsa	+	+	
Toluidine blue	+	+	
Chloracetate esterase	+		
Aminocaproate esterase	+		
Tartrate-resistant acid phosphate	+		
Peroxidase			+

[a] Acute myeloid leukemia, FAB, acute promyelocytic subclassification.

smear. In contrast to the focal lesions found in the marrow of adults with systemic mast cell disease, the lesions seen in children were uniformly small and subtle, and were most frequently found in perivascular sites. Aside from the small size, however, these lesions had the other characteristics of the larger lesions seen in adult marrow.[100]

Although the typical mast cell lesions in adults with systemic mast cell disease are highly specific for that disorder, they are not necessarily diagnostic; they should only be considered diagnostic of systemic mast cell disease when other clinical and laboratory parameters indicative of a mast cell disorder are present. The finding of clusters of confluent mast cells on aspirate smear appears to be highly specific for systemic mast cell disease, but only some patients (<30%) manifest this finding. This number may, however, represent a significant underestimation, since the ability to obtain adequate marrow spicules on aspiration in regions of marrow fibrosis is quite variable. The progression of marrow involvement in systemic mast cell disease is unknown. Although many adults appear to have stable, or possibly decreasing, marrow involvement over time,[97] the clinical significance of the extent of marrow involvement by mast cells remains elusive. Studies of children with systemic mast cell disease have not included large enough numbers, or extended over a long enough time period, to justify any statements regarding either the significance of marrow mast cell lesions at initial presentation or the incidence or significance of the progression of mast cell involvement of the marrow.

Lymph Node

Although lymph node, liver, and spleen are rarely biopsied in patients with mast cell disease, pathologic studies have documented significant involvement in these organs in patients with systemic involvement.[96] [100] [101] [102] [103] Travis and Li[101] demonstrated peripheral lymphadenopathy in 26% and central lymphadenopathy in 19% of patients at diagnosis. Lymphadenopathy was more pronounced in patients with associated hematologic malignancies and aggressive nonleukemic disease. Within the lymph nodes, mast cell infiltrates were most common in the paracortex, followed by the follicles, the medullary cords, and the sinuses. Early infiltrates were exemplified by clusters of mast cells. Eosinophils accompanied mast cell infiltrates in lymph node tissues in approximately one-half of lymph nodes studied. Blood vessel proliferation in the paracortical areas infiltrated by mast cells and extramedullary hematopoiesis were demonstrated in a few patients. Mast cell infiltrates in lymph node tissues can resemble T-cell lymphomas in their pericortical distribution, clear cytoplasm of the mast cells in some cases, and an associated vascular proliferation and eosinophilia. When mast cells replaced the lymphoid follicles, the pattern often bore a resemblance to follicular hyperplasia or lymphoma.

Spleen

Splenic involvement is also common in mastocytosis, seen in 4050% of patients with systemic disease at presentation. [97] [101]

Travis and Li[101] reviewed the pathologic features of 16 spleens; 14 were involved with mast cell disease. All but one spleen showed a paratrabecular distribution of mast cell infiltrates; 10 (64%) perifollicular, 2 (14%) follicular, and 1 (7%) diffuse. Various degrees of trabecular and capsular fibrosis and eosinophilic infiltration were present in biopsies; 71% of biopsies revealed extramedullary hematopoiesis. Cross-sections of the parenchyma showed multiple small nodular areas attributed to fibrosis or infiltrations. Mast cell infiltrations in the spleen produce a lesion that could be confused with T-cell lymphoma, follicular hyperplasia, follicular lymphoma, Kaposis sarcoma, or a granulomatous process. Mast cell infiltrates can also resemble a myeloproliferative disorder or hairy cell leukemia. Although splenomegaly has been demonstrated in up to three-quarters of patients with systemic mast cell disease, only one-half of patients have been shown to have splenic infiltration by mast cells.[104] Markedly increased splenic weights (>700g) generally occurred in patients who fit into unfavorable categories of mastocytosis such as aggressive mastocytosis or mastocytosis associated with a hematologic disorder.

Liver

Liver disease with mast cell infiltration is a common finding in patients with mastocytosis. [102] However, severe liver disease was uncommon, except in patients with aggressive splenomegaly (those with an associated hematologic disorder or lymphadenopathic mastocytosis with eosinophilia). Alkaline phosphatase directly correlated with hepatomegaly, liver mast cell infiltrations, and fibrosis. Elevations in alkaline phosphatase are observed more frequently in patients with categories of aggressive disease. Some patients with aggressive disease develop ascites or portal hypertension and die of complications of mastocytosis. Mast cell infiltrations are more severe in patients with aggressive forms of mastocytosis. Portal fibrosis is observed in two-thirds of patients and correlates with inflammation and mast cell infiltrates.

Organ Fibrosis

The lymph nodes, liver, spleen, and bone marrow are common sites of involvement in mastocytosis. Involvement of these organs is most common in patients in whom the disease is aggressive or associated with a hematologic disorder. Fibrosis associated with mast cell proliferation and eosinophilic infiltrations are common accompaniments of the disease process. Fibrosis accompanies mast cell infiltrates, particularly in lymphoid tissue and in the marrow. Mast cells themselves may produce certain connective tissue components, and mast cells synthesize TGF- and other agents that may promote fibrosis. Histamine has been shown to promote fibroblast proliferation both in vivo and in vitro through an H_2 -receptor-mediated process.[105] [106] Other mast cell constituents have also been implicated in radiation, chemotherapy, inflammation, and injury-induced pulmonary fibrosis.[107] [108] [109] [110] Ultimately, fibrosis of the liver may lead to ascites, requiring aggressive therapy.

CLINICAL MANIFESTATIONS

The consensus revised classification schema [34] for systemic mast cell disease is clinically useful in categorizing the various forms of the disease (Table 47-7). This classification schema divides mast cell disease into four categories of increasing clinical aggressiveness and is adapted from the work of Travis et al. [33] The first category is termed indolent mastocytosis. By a large margin, most mastocytosis patients will fall into this category. Their disease eventually involves the target organs listed and results in a pathophysiologic process that can be managed successfully for decades and does not appear to shorten life span. Eight manifestations of indolent mastocytosis were proposed and are listed in Table 47-7: (1) hemodynamic instability manifested as repeated episodes of flushing and syncope; (2) cutaneous mast cell disease with clear dermatopathology resulting from an increase in dermal mast cells; (3) ulcers of the stomach and duodenum associated with increased gastric acid; (4) malabsorption due to systemic mediators and mast cell infiltration of the intestine; (5) mast cell infiltration of the bone marrow; (6) skeletal disease due to the activity of mast cells on bony surfaces; (7) hepatosplenomegaly; and (8) lymphadenopathy resulting from mast cell infiltration. These manifestations do not define subgroups, since a particular patient may have more than one manifestation. The second category consists of mastocytosis associated with a hematologic disorder. Affected patients have increased mast cells in one or more target organs plus a demonstrable bone marrow abnormality such as a myeloproliferative or myelodysplastic disorder; the skin is variably involved. In this category, the prognosis is determined primarily by the associated hematologic disorder. The third category, aggressive mastocytosis, was previously called lymphadenopathic mastocytosis with eosinophilia. It is typically a rapidly progressive disease involving first the bone marrow and then the gastrointestinal tract, liver, spleen, and lymph nodes. The prognosis is much more guarded. The last category of systemic mastocytosis is mast cell leukemia, a primary leukemic process with increased mast cell burdens in both bone marrow and blood. The prognosis for patients with mast cell leukemia is extremely poor and duration of survival is typically less than 1 year, even with aggressive chemotherapy. [111]

The typical clinical presentation for patients with a systemic mast cell disorder is hard to define, as any patient may have more than one organ involved with mast cells. In addition, many patients exhibit vague or nonspecific constitutional symptoms such as weakness, fatigue, night sweats, and weight loss that cannot be attributed to any particular organ dysfunction. Some patients present with neurologic or neuropsychiatric manifestations, including alterations in cognitive abilities, or depression that may be related to the underlying disease process or, in the case of neuropsychiatric abnormalities, to the inability of the medical community to reach a diagnosis. The etiology of these symptoms is unclear, although mediator-induced hypotensive effects on the brain, a mixed organic brain syndrome, or both, have been hypothesized. [112] [113]

Syncope/Flushing Disorders

Typically, a patient will describe attacks characterized by flushing or sensations of warmth, usually accompanied by palpitations, shortness of breath, chest discomfort, nausea and diarrhea, headache, lightheadedness, and occasionally overt syncope. A unique characteristic of this syndrome is that after the episodes, patients may experience fatigue lasting several hours. Syncopal episodes may be precipitated by heat exposure, emotional stress, or physical exertion, or may occur premenstrually. Some patients may have a variant of the syncopal disorder manifested solely as hemodynamic abnormalities. During episodes of flushing, many patients experience a significant decrease in blood pressure accompanied by tachycardia, whereas other patients may exhibit tachycardia accompanied by an elevation in blood pressure. In this second group of patients, pheochromocytoma must be ruled out. These hemodynamic variants of the mast cell syncope/flushing disorder may be related to mast cell mediator release involving prostaglandin D_2 (PGD_2). [35] [114] PGD_2 is a potent vasodilator metabolized in vivo by 11-ketoreductase to the metabolite 9, 11-PGF_2, a potent pressor substance. [115] It is attractive to speculate that the hemodynamic instability noted in some patients, and the variation noted among patients, is a consequence of differences in prostaglandin metabolism resulting

in marked differences in acute levels of PGD_2 relative to its metabolite 9, 11-PGF_2.

Cutaneous Disease

Cutaneous manifestations of systemic mast cell disease may appear early in life. In a series of 112 patients, [116] solitary mastocytomas, when observed, were present at birth or developed within the first week of life. Multiple lesions of urticaria pigmentosa may also be found at birth, or may appear during early childhood. The onset of urticaria pigmentosa follows a biphasic curve with one peak at 2.5 months of age and the second peak at 26.5 years. [117] Lesions are present in 80% of affected children by 6 months of age. Skin lesions are reported in 50100% of patients with systemic mastocytosis. [33] [94] [95] [118] [119] [120] There appears to be no sex predilection, and a familial association is generally not seen. In fewer than 50 families, mastocytosis of one form or another has affected more than one member, including several instances of affected twins. [121] [122] [123] [124]

Mast cell disease has four cutaneous manifestations: urticaria pigmentosa, mastocytoma, diffuse/erythrodermic disease, and telangiectasia macularis eruptiva perstans. The most common cutaneous mast cell lesion is urticaria pigmentosa, which appears as red-brown macules, papules, and plaques (Fig. 47-4 (Figure Not Available)). Lesions occur in a generalized and random distribution, and may form clusters with a cobblestone appearance. Erythema, edema, and blister formation with subsequent crusting of the lesions has been reported, particularly in young children. Gross hemorrhage into bullous lesions can occur, presumably secondary to high local levels of heparin released from mast cells. After the age of 10, vesicles generally do not occur, and the lesions tend to be smaller and more numerous. Approximately one-half of patients in whom urticaria appears in infancy or childhood experience resolution by adolescence; in

Figure 47-4 (Figure Not Available) Urticaria pigmentosa. *(From Soter NA: The skin in mastocytosis. J Invest Dermatol 96:32S, 1991. Reprinted by permission of Blackwell Science, Inc.)*

the remainder of patients, only lightly pigmented macules remain. [116] [125] Lesions that appear after age 10 tend to persist and remain symptomatic. The lesions of urticaria pigmentosa are usually most dense on the trunk, although they may affect all skin areas, including mucous membranes. The palms, soles, face, and scalp are often free of disease or only lightly affected. Telangiectasias, petechiae, or ecchymosis may occur in the lesions or in adjacent clinically normal skin. The most common clinical manifestations include pruritis, dermatographism, and the presence of a Darier sign (wheal and erythema occurring after a brisk stroke to a lesion). A positive Darier sign may result in blister formation with hemorrhage, particularly in infants. Flushing has been reported to occur in up to one-third of patients with urticaria pigmentosa. In various studies, 1070% of patients with urticaria pigmentosa have been shown to have systemic disease, with bone marrow the most frequently involved site. However, the absence of urticaria pigmentosa and the absence of skin symptoms are poor prognostic features in patients with systemic disease. [33]

Mastocytomas may be present at birth, although most appear within the first 3 months of life and are rarely described in adults. [95] [126] [127] Lesions are generally few in number and may be solitary or show focal clustering. The most common presentation is as a macule, plaque, or nodule, but bullous lesions have been described. Most mastocytomas form on the extremities and rarely involve palms or soles. In most cases, the lesions are thought to involute spontaneously; however, this has not

invariably been reported. Rare patients have had systemic manifestations such as flushing. [128]

Diffuse cutaneous mastocytosis is a rare disorder that generally presents before the age of 3 and involves the entire cutaneous integument. The skin may appear normal; more commonly, it has a yellow-red-brown color with an orange-peel appearance. Yellow to cream-colored papules that resemble xanthomas and pseudoxanthoma elasticum have also been described. [129] A generalized erythroderma form of diffuse cutaneous mastocytosis is manifested by severe edema and a doughy thickening of the skin. [130] Dermatographism with the formation of hemorrhagic blisters is a common finding in this disorder. Extensive bullae with rupture and crusting may be the first presentation in an infant who later develops diffuse cutaneous mastocytosis. Thus, bullous erythrodermic mastocytosis must be included in the differential diagnosis of neonatal blister disorders. [131] Diffuse cutaneous mastocytosis reportedly may resolve spontaneously by age 515 months; in other patients the disease persists; however, when present, these children are at risk for complications such as flushing, hypotension, shock, and occasionally death. Diarrhea and other gastrointestinal manifestations are common in this disorder and occasional patients have significant gastrointestinal bleeding. [132]

Telangiectasia macularis eruptiva perstans is a rare form of mastocytosis traditionally thought to be limited to the skin. In isolated instances, splenomegaly, increased numbers of mast cells in the bone marrow, and abnormal skeletal radiographs suggest that this form of mastocytosis may have systemic features. [133] Many of the original patients reported with this disorder were obese middle-aged women; however, one report involves a 10-year-old girl. [134] The lesions in this disorder are generalized, red, telangiectatic macules on a tan to brown background. Individual lesions are 26 mm in diameter and are without sharply defined borders. Sites become edematous when rubbed. Pruritis, purpura, and blister formation are generally not associated with this disorder. In occasional patients, the lesions may coexist with those of urticaria pigmentosa. [135]

Gastrointestinal Hyperacidity

Gastrointestinal symptoms have commonly been reported in patients with systemic mastocytosis (Table 47-10). Both retrospective

TABLE 47-10 -- Gastrointestinal Manifestations of Systemic Mastocytosis

Abdominal pain
Diarrhea
Nausea
Peptic ulcer disease
Malabsorption

and prospective studies [94] [95] [136] [137] have documented abdominal pain, diarrhea, nausea, vomiting, and peptic ulcer disease in one-fourth to one-half of individuals with systemic mastocytosis. Studies with the mast cell-deficient mouse [138] suggest that mast cells may be essential for normal gastric acid secretion. Cherner et al. [139] have demonstrated a broad range of basal acid secretion values in a prospective study of 16 patients with systemic mastocytosis. Six of these patients were shown to have clinically significant acid hypersecretion. The importance of histamine in inducing the acid hypersecretion in these patients was borne out by the finding of low gastrin levels in many of the patients.

Gastrointestinal Malabsorption

Up to one-third of patients with systemic mastocytosis have been demonstrated to have some laboratory evidence of fat malabsorption. [139] However, the degree of malabsorption is generally not of clinical importance. Although the etiology of this mild malabsorption is unclear, potential explanations include acid hypersecretion and mucosal dysfunction. Shortened intestinal transit time does not appear to contribute to the malabsorption syndrome. [139] Studies have indicated that mast cells may be increased in the small intestine of some patients with systemic mastocytosis, although a clear correlation between mucosal mast cell numbers and gastrointestinal symptoms has not been noted. Mucosal biopsies from some patients do demonstrate a variation in mast cell number, depending upon the site measured. [140]

Skeletal Disease

Occasional patients with marked bony infiltration with mast cells have been noted. The most common radiographic abnormality is diffuse ostopenia, which is frequently an incidental finding. [139] [141] [142] [143] [144] In some patients, osteoporosis may be the sole initial manifestation of systemic mast cell disease. [145] Both lytic and sclerotic lesions have been described. Bone scans may be normal or may show focal or diffuse abnormalities. [146] In patients with aggressive disease, diffuse bone pain as well as pathologic fractures have been reported. [147]

Visceral/Hepatosplenomegaly

Hepatic involvement with mast cell lesions is a common finding in systemic mastocytosis, although a significant degree of hepatic dysfunction is generally not observed. [102] Alkaline phosphatase is frequently elevated, but this elevation is largely attributable to bone disease. Webb et al. [94] demonstrated hepatomegaly in 45% and splenomegaly in 50% of 26 patients they studied. Rarely, patients with systemic mastocytosis have been demonstrated to have portal hypertension, [102] and significant splenomegaly may be a contributing factor to the hematologic abnormalities noted in many patients.

Lymphadenopathy

Lymphadenopathy has been demonstrated in about 60% of patients with systemic mast cell disease. [101] The presence of lymphadenopathy, by itself, does not signal aggressive disease, and no specific symptoms are referable to lymphadenopathy. However, patients who present with lymphadenopathy and significant hepatosplenomegaly should be followed closely for possible evolution into a more aggressive systemic disorder.

Bone Marrow Mast Cell Aggregates and Hematologic Abnormalities

In 90% of patients with systemic mastocytosis, either a focal or diffuse increase of mast cells in the bone marrow will be demonstrated. This likely represents an overestimation of the incidence of bone marrow involvement, since most early studies used bone marrow involvement as a necessary criterion for the diagnosis of systemic mast cell disease. Although marrow involvement may not be necessary for the diagnosis, the bone marrow is the extracutaneous organ most commonly documented to be involved in systemic mast cell disorders.

A number of hematologic abnormalities are reported in patients with systemic mast cell disease. Table 47-11 lists the hematologic

TABLE 47-11 -- Hematologic Abnormalities in Systemic Mast Cell Disease

	Travis et al. [33]	Webb et al. [94]	Brunning et al. [95]	Lawrence et al. [97]
Patients (N)	58	26	14	32
Cytopenias				
Anemia	47%	37%	36%	50%
Thrombocytopenia	16	22	14	19
Leukopenia	16	15	21	9

Lymphopenia				
Increased white blood elements				
Leukocytosis	19%	29%	21%	31%
Eosinophilia	19	17	43	21
Basophilia	7	0	0	
Monocytosis	16	17	0	18
Lymphocytosis	16			
Thrombocytosis	9			
Increased mast cells[a]				
<10%	2	2	0	
>10%	2	2	0	

, data not reported.

[a] In peripheral blood.

TABLE 47-12 -- Systemic Cell Disease Malignant/Premalignant Manifestations

Myeloproliferative disorder
Myelodysplastic syndrome
Lymphadenopathic syndrome
Non-Hodgkins lymphoma
Mast cell leukemia
Acute leukemia (secondary)

abnormalities noted in several large studies of patients with systemic mast cell disease. Anemia is the most common finding, occurring in one-third to one-half of all patients. Thrombocytopenia and leukopenia have been demonstrated in roughly 1520% of patients. Leukocytosis has been demonstrated in 2030% of patients, and eosinophilia in up to 40% of patients with systemic mast cell disease. Lymphocytosis and thrombocytosis are unusual findings.

Although a study conducted by Travis and colleagues demonstrated that up to 7% of patients with systemic mastocytosis have a basophilia, and up to 4% have circulating mast cells,[33] these findings have not been universally demonstrated in other similarly large studies. Although mast cells in the bone marrow are densely granulated cells, when seen in the peripheral blood they frequently have the appearance of atypical monocytes containing scattered large basophilic granules. They also frequently appear somewhat dysplastic, making identification on Wright stain difficult. Frequently, stains specific for mast cells must be performed to identify the cells in the peripheral blood conclusively and to distinguish them from either basophils or hypergranulated monocytes. Table 47-9 lists several histochemical stains used to differentiate mast cells from basophils.

According to the revised consensus classification, the second category of systemic mastocytosis includes patients who present with systemic disease and a hematologic syndrome resembling a myeloproliferative or myelodysplastic process. Several premalignant or overtly malignant syndromes have been described with systemic mast cell disease (Table 47-12). A relatively small subgroup of adult patients with systemic mast cell disease has been documented to have either a myeloproliferative or myelodysplastic process.[33] [94] [97] [119] [148] [149] The peripheral blood picture in these patients may be consistent with that of either chronic myeloid leukemia (CML) or chronic myelomonocytic leukemia (CMML). The presence of either of these disorders is associated with a poor prognosis. [97] [98] As with primary myeloproliferative and myelodysplastic syndromes, a secondary acute leukemia may develop in patients with systemic mast cell disease. Many patients with systemic mast cell disease who have significant cytopenias have overtly dysplastic myeloid or erythroid maturation on bone marrow examination. In addition, a small number of patients have developed diffuse fibrosis of the bone marrow with marked hypocellularity. Patients with systemic mast cell disease with a CML-like picture are Philadelphia chromosome-negative. Surprisingly, some patients may have decreasing marrow mast cell involvement, in spite of increasing systemic symptomatology.[97]

Lymphadenopathic Mastocytosis with Eosinophilia

A subset of patients with systemic mastocytosis who present with, or subsequently develop, significant lymphadenopathy, hepatosplenomegaly, and peripheral eosinophilia has been described. [33] [34] [96] Lymph node biopsies in these patients frequently show a hyperplastic picture suggestive of a malignant lymphoproliferative disorder. However, tissue pathology does not support a diagnosis of lymphoma. Clinically, these patients have an aggressive form of the disease with marked visceral and bony involvement and dramatically shortened survival. Many of these patients display a marked eosinophilia on peripheral blood. Frequently, the eosinophils are dysplastic and hypogranular, similar to those seen in the idiopathic hypereosinophilic syndrome.

Mast Cell Leukemia

These patients are characterized by having a large number of atypical-appearing mast cells in the peripheral blood, a leukocytosis and granulocytosis, and a compressed clinical course.[111] Survival with aggressive chemotherapy is 29 months, with a mean of <6 months. This syndrome appears to represent the most aggressive form of mast cell disorder and appears to be a de novo malignant proliferation of mast cells.

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LABORATORY EVALUATION

A suggested diagnostic evaluation for systemic mast cell disease is presented in Table 47-13 . If mastocytosis is suspected on clinical grounds, the evaluation should consist of examination of the skin, both grossly and microscopically; a bone marrow biopsy and aspirate; serum for tryptase levels; and a 24-hour urine for mediators (particularly histamine and/or its metabolites). Additional studies, as suggested by symptomatology or findings during the routine evaluation, may include a bone scan or skeletal survey, or both; a gastrointestinal evaluation involving radiographic studies of the upper gastrointestinal tract and small intestines, computed tomography scan, and endoscopy; and a neuropsychiatric workup. In all patients, the fundamental requirement for the diagnosis of mastocytosis remains the presence of significant increases in mast cell numbers in one or more target tissues. The classic lesions of mastocytosis, namely, mast cell aggregates, are required for the diagnosis of bone marrow involvement. However, a dramatic increase in the mast cells within the lamina propria of the gastrointestinal tract may establish the diagnosis of systemic mast cell disease without evidence of cutaneous, marrow, or other visceral involvement. Although it is clear that symptoms of mast cell disease can reflect local increases of mast cells, slight increases in mast cell numbers in target tissues, such as the skin, gastrointestinal tract, or bone marrow, are not diagnostic because they may only reflect a normal inflammatory or reactive process. Likewise, while plasma or urinary levels of histamine are frequently increased in systemic mastocytosis,[150] the solitary finding of increased levels of histamine or histamine metabolites may reflect other situations, such as anaphylaxis or response to unusual immunologic stimuli. Similarly, serum tryptase may be elevated after anaphylaxis, and may sometimes be normal despite marrow involvement. Thus, no single laboratory test can establish the diagnosis of mastocytosis. Rather, the demonstration of mast cell mediators in blood or urine prompts the clinician to investigate further for the presence of abnormal collections of mast cells.

TABLE 47-13 -- Consensus Diagnostic Workup

If mastocytosis is suspected on clinical grounds
Routine
Examine skingross and microscopic
Bone marrow biopsy and aspiration
Serum for mast cell tryptase
24-hr urine for mediators
Additional studies
Bone scan/skeletal survey
Gastrointestinal evaluation[a] upper gastrointestinal series, small bowel radiography, CT scan, endoscopy
Electroencephalography, neuropsychiatry workup

[a]May be adequate criteria for primary diagnosis.

TABLE 47-14 -- Differential Diagnosis of Systemic Mastocytosis

Clinical syndrome
Hyper-IgE syndrome
Hereditary/acquired angioneurotic edema
Complement disorders
Parasitic infestations
Idiopathic anaphylaxis
Idiopathic capillary leak syndrome
Pheochromocytoma
Zollinger-Ellison syndrome/gastrinoma
Marrow histopathology
Primary myelofibrosis
Angioimmunoblastic lymphadenopathy
Eosinophilic fibrohistocytoma of the bone

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DIFFERENTIAL DIAGNOSIS

The differential diagnosis of systemic mast cell disease must be considered on two levels (Table 47-14). The first is that of clinical grounds, where other disease processes that may produce symptoms similar to those seen in systemic mast cell disorders must be excluded. These disorders include diseases such as hyper-IgE syndrome, parasitic infestations of the gastrointestinal tract or viscera, hereditary or acquired angioneurotic edema, idiopathic anaphylaxis, complement disorders, or idiopathic capillary leak syndrome. When episodic hypertension is a major symptom component, pheochromocytoma must be considered; when significant unexplained ulcer disease is present, a Zollinger-Ellison/gastrinoma syndrome must be ruled out. *Helicobacter pyelori* infection should be considered in patients with ulcer disease.

The second level at which one must consider a differential diagnosis is at the level of histopathology. In this regard, three particular disorders may present with bone marrow lesions similar to those seen in systemic mastocytosis. These disorders are primary myelofibrosis, angioimmunoblastic lymphadenopathy, and eosinophilic fibrohistiocytoma. The distinction of mastocytosis from these three disorders is generally accomplished by close review of the histopathologic material, and correlation with other clinical and laboratory features. When the marrow is diffusely infiltrated with an increased number of mast cells, there is frequently a background of fibrosis to these lesions. In this setting, differentiation from primary myelofibrosis is often difficult. [95] [151] In areas of fibrosis, the mast cells are often elongated and relatively agranular on routine staining. Toluidine blue stain or other stains to bring out the metachromatic granules of the mast cell may be particularly useful in these cases. The differentiation of systemic mastocytosis from angioimmunoblastic lymphadenopathy generally relies on the lack of plasma cells and immunoblasts and the absence of neovascularity in the lesions of mastocytosis. Differentiation of systemic mastocytosis from the bone marrow lesions of fibrohistiocytoma is much more problematic. Large histiocytic cells described in eosinophilic fibrohistiocytic lesions have a striking resemblance to the large mast cells seen in many mastocytic lesions.[96] [152] [153] It may be that eosinophilic fibrohistiocytic lesions of the bone represent an indolent form of systemic mast cell disease. [113]

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THERAPY

No cure has been found for mastocytosis, and little documentation suggests that symptomatic therapy substantially alters the course of disease. A primary concern in therapy for both cutaneous and systemic mast cell disease is the avoidance of triggering factors (which vary from patient to patient) such as temperature extremes, physical exertion, or in some instances, the ingestion of agents such as ethanol, nonsteroidal anti-inflammatory drugs, or opiate analgesics. Physical trauma to lesions or environmental factors may also trigger acute episodes, with reports of anaphylaxis after *Hymenoptera* stings and exposure to iodinated contrast materials in patients with systemic mast cell disease.[154] Epinephrine remains the drug of choice in the treatment of anaphylaxis, either idiopathic or that induced by environmental factors. Patients with mast cell disease and a history of anaphylaxis should be advised to carry epinephrine-filled syringes and taught to self-medicate. These patients may also benefit from the concurrent use of H_1 and H_2 antihistamines prophylactically. Tables 47-15 and 47-16 list some of the more common therapeutic modalities used in both cutaneous and systemic mast cell disease.

TABLE 47-15 -- Therapy for Cutaneous Mastocytosis

Mastocytoma
Observation
Excision (if highly symptomatic)
Urticaria pigmentosa and diffuse and erythrodermic cutaneous mastocytosis
H_1 antihistamines
H_1 and H_2 antihistamines
Ketotifen
Disodium cromoglycate
Topical corticosteroids with occlusion
PUVA
Telangiectasia macularis eruptive perstans
PUVA

Classic as well as newer nonsedative H_1 antihistamines are used to decrease irritability of the skin and mitigate symptoms

TABLE 47-16 -- Therapy for Systemic Mastocytosis

Antihistamine[a]
H_1 receptor blockade
Hydroxyzine
Fexofenadine
Doxepin
H_2 receptor blockade
Ranitidine
Famotidine
Epinephrine
Steroids
Cromolyn sodium
Aspirin (if patient is not sensitive)
Anticholinergics
Omeprazole
PUVA
Chemotherapy[b]
Radiotherapy[b]
Splenectomy[b]
Diphosphonate[b]
Interferons, growth factors[b]
Cyclosporins[b]

[a] Representative drugs only.
[b] Use restricted to treatment of aggressive mastocytosis (third major category), Table 47-7 or mastocytosis with an associated hematologic disorder (second major category), Table 47-7 .

of pruritis. Amelioration can be achieved, but total ablation of signs and symptoms rarely occurs. Hydroxyzine and doxepin are two potent H_1 antihistamines found quite useful. Doxepin is particularly useful for patients who have central nervous system manifestations of mast cell disease. Frequently the dose-limiting side effect of antihistamine therapy is sedation. Patients sensitive to the sedative effects of antihistamines may benefit from the use of newer nonsedative antihistamines. For patients who continue to have significant disease symptoms while on H_1 antihistamines, the combination of H_1 and H_2 antagonists has been shown to be occasionally effective in relieving pruritis and wheal formation. [155] H_2 antihistamines, such as ranitidine and famotidine, have been quite useful in the treatment of gastritis and peptic ulcer disease associated with mastocytosis. Doses of these H_2 antihistamines can be titrated on the basis of symptom control, or to a particular level of gastric acid secretion. Proton pump inhibitors (omeprazole) are also useful in the management of gastric acid hypersecretion.

Oral administration of disodium cromoglycolate has been reported to reduce pruritis and wheal formation in urticaria pigmentosa with or without systemic disease. [153] [156] [157] [158] [159] This agent has also been reported to be of benefit in cutaneous mast call disease in children and infants. [155] [156] It has also proved useful for the control of gastrointestinal complaints often seen with systemic disease. [140] Sometimes other symptoms, such as headache and bone pain, have also been reported to improve with the use of cromolyn sodium.

Ketotifen has been widely used in Europe. It has been reported to prevent mast cell degranulation and to be effective in the relief of pruritis and wheal formation in urticaria pigmentosa and other forms of diffuse cutaneous mastocytosis. [160] [161] [162] One patient has been reported in whom findings of osteoporosis improved after therapy with ketotifen.[163] In contrast, one pediatric study found ketotifen no more effective than hydroxyzine. [164] Similarly, the drug azelastine, an antihistamine with mast cell-stabilizing properties, offered little benefit over chlorpheniramine. [165] Diphosphonates have also been reported useful in the treatment of mastocytosis-associated ostopenia.[143]

Although systemic corticosteroids have not been shown to be effective in the treatment of cutaneous mastocytosis, topical administration or intralesional injections of corticosteroids have resulted in symptomatic and cosmetic improvement. Caution must be exercised with repeated or extensive application of corticosteroids, as this may result in cutaneous atrophy or adrenocortical suppression. [166] The oral administration of 8-methoxypsoralen plus ultraviolet A (PUVA) photochemotherapy has resulted in a decrease in pruritis and wheal formation in patients with urticaria pigmentosa with or without systemic disease. [167] [168] [169] [170] Frequently, prolonged control of disease results after a single course of PUVA therapy, but relapses occurring 36 months after cessation of therapy are common. PUVA therapy has been associated with decreased levels of urine and blood mediators. [167] [168] [171] Occasional patients experience a decrease in cutaneous lesions after exposure to natural sunlight; however, there are no controlled studies using ultraviolet B phototherapy. [117]

Significant malabsorption, hepatic fibrosis, and ascites have been noted in patients with severe mastocytosis. [137] [172] [173] Systemic corticosteroids have been useful in decreasing the malabsorption and ascites in some of these patients. In adults, oral prednisone (4060 mg/day) usually results in a decrease in symptoms over a 23 week period.[174] After initial improvement, steroids can frequently be tapered to an alternate-day regimen. However, with time, the ascites frequently recurs; it has been suggested that these patients may benefit from a portacaval shunt. [173]

Aspirin and nonsteroidal anti-inflammatory agents have been useful in some patients whose primary manifestation is recurrent episodes of flushing or syncope, or both. The use of these agents may be problematic in patients with significant ulcer disease. Calcium channel blockers and platelet-activating factor inhibitors have been effective anecdotally. [175] [176]

A small percentage of patients with systemic mast cell disease may have a syndrome mimicking non-Hodgkins lymphoma, an aggressive myeloproliferative disease, or, rarely, an overt nonlymphocytic leukemia. [34] [97] [148] [177] Two patients have been reported with systemic mast cell disease associated with primary mediastinal germ cell tumor. [178] [179] In these patients, traditional chemotherapy directed toward their neoplastic process may be appropriate. Chemotherapy with cyclophosphamide, vincristine, and prednisone has been used in some patients whose clinical picture is that of a non-Hodgkins lymphoma, although the response to chemotherapy is variable.[178] Radiotherapy has been used in some patients to control local disease. [180]

Splenectomy has been performed on a number of patients with severe aggressive mastocytosis, in an attempt to improve their limiting cytopenias. [181] With splenectomy, survival increased by an average of 12 months. Patients who had undergone splenectomy appeared better able to tolerate chemotherapy. Although splenectomy is of no value in the management of indolent mast cell disease, it should be considered in selected patients with more aggressive forms of mastocytosis.

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PROGNOSIS

The prognosis for patients with mast cell disorders is clearly related to the extent of the disease. Patients who present with cutaneous disease, flushing disorders, or limited extracutaneous organ involvement frequently have an indolent course requiring chronic medical management. Few, if any, of these patients have been documented to progress into a more advanced form of the disease. [97] [146] In contrast to the clinical course of patients with limited disease, a prospective analysis of 46 patients identified elevated lactate dehydrogenase levels, late age of onset, and the presence of a significant hematologic abnormality (such as myeloproliferative, myelodysplastic, or overt leukemic picture) as indicators of a poor prognosis and shortened survival. [97] Of the parameters studied by multivariate analysis, only late age of onset of symptoms and elevated serum lactate dehydrogenase levels were found to be predictive of a poor prognosis. Some groups have also identified the presence of a myeloproliferative or myelodysplastic blood picture as conferring a poorer prognosis.

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FUTURE DIRECTIONS

In addition to the therapeutic modalities already mentioned, some innovative therapies for the future are worth discussing separately. Mastocytosis involves both mast cell hyperplasia and systemic mediator release. Mast cells release primary mediators that induce the production and secretion of secondary mediators, but these mediators also effect mast cell proliferation and degranulation. An approach using an antiproliferative mediator to treat mastocytosis is attractive and may warrant investigation. CSF-GM has been shown to inhibit the growth of IL-3-dependent mast cells in murine bone marrow [182] as does IFN-.[183] Clinical responses to IFN-2B have been mixed.[184] [185] Immunotherapy using an anti-IgE antibody may have potential to decrease mast cell stimulation and proliferation. [186] Much clinical work is necessary before any such therapies can be brought into the clinical arena. However, given our inability to significantly affect survival in individuals afflicted with the more aggressive forms of mastocytosis, it may be reasonable to consider these therapies on a selected basis.

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Chapter 48 - Cytogenetics and Neoplasia

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INTRODUCTION

The malignant cells in many patients who have leukemia, lymphoma, or another hematologic neoplasm have acquired clonal chromosomal abnormalities. A number of specific cytogenetic abnormalities have been recognized that are very closely, and sometimes uniquely, associated with morphologically and clinically distinct subsets of leukemia or lymphoma.[1] [2] [3] The detection of one of these recurring abnormalities can help in establishing the correct diagnosis and can add information of prognostic importance. The appearance of new abnormalities in the karyotype of a patient under observation often signals a change in the pace of the disease, usually to a more aggressive disorder. The detection of a cytogenetic abnormality clearly distinguishes between benign reactive lymphoid or myeloid hyperplasia and a monoclonal malignant proliferation.

This chapter focuses on the genetics of the leukemias and lymphomas from primarily a cytogenetic perspective. Although these disorders do occasionally exhibit familial aggregation, this is for the most part uncommon, poorly defined, and has yielded few biologic insights or clinical recommendations. In contrast, the delineation of recurring cytogenetic abnormalities correlated with specific hematologic malignancies has helped to delineate disorders with distinct prognoses and etiologies and, in combination with molecular biologic studies, has led to important insights into their pathogenesis. This understanding of the relationship between cytogenetic abnormalities and the pathogenesis and natural history of cancer is much farther advanced for the leukemias than for other malignancies. In large part, this is the result of the relative ease of obtaining and processing bone marrow or peripheral blood samples from patients with leukemia. Nonetheless, recent improvements in cell culture and processing techniques have resulted in the identification of a number of recurring abnormalities in solid tumors, and the analysis of these neoplasms is an exciting area of cancer research. [2]

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GENETIC CONSEQUENCES OF CHROMOSOMAL REARRANGEMENTS

During the past few years, the genes that are located at the breakpoints of a number of the recurring chromosomal translocations

TABLE 48-1 -- Functional Classification of Transforming Genes at Translocation Junctions

Gene	Location	Translocation	Consequence	Disease
SRC family (TYR protein kinases)				
ABL	9q34	t(9;22)	Fusion protein	CML/ALL
LCK	1p34	t(1;7)	Deregulated expression	T-ALL
Serine protein kinase				
BCR[a]	22q11	t(9;22)	Fusion protein	CML/ALL
Cell-surface receptor				
TAN1[a]	9q34	t(7;9)	Deregulated expression	T-ALL
Growth factor				
IL3	5q31	t(5;14)	Deregulated expression	PreB-ALL
Inner mitochondrial membrane				
protein *BCL2*[a]	18q21	t(14;18)	Deregulated expression	NHL
Transcriptional regulating factors[b]				
BCL3[a]	19q13	t(14;19)	Deregulated expression	B-CLL
LYT10	10q24	t(10;14)	Deregulated expression	B-NHL
PBX1[a]	1q23	t(1;19)	Fusion protein	PreB-ALL
E2A	19p13	t(1;19)	Fusion protein	PreB-ALL
CAN[a]	9q34	t(6;9)	Fusion protein	AML
HOX11[a]	10q24	t(10;14)/t(7;10)	Deregulated expression	T-ALL
LYL1[a]	19p13	t(7;19)	Deregulated expression	T-ALL
MYC	8q24	t(8;14)	Deregulated expression	B-ALL/T-ALL
PML[a]	15q22	t(15;17)	Fusion protein	APL
RARA	17q11.2	t(15;17)	Fusion protein	APL
AML1[a]	21q22	t(8;21)/t(3;21)	Fusion protein	AML
		t(12;21)	Fusion protein	ALL
TEL[a]	12p12	t(12;21)	Fusion protein	ALL
TAL1(SCL)[a]	1p32	t(1;14)	Deregulated expression	T-ALL
TAL2[a]	9q32	t(7;9)	Deregulated expression	T-ALL
RBTN1(TTG1)[a]	11p15	t(11;14)	Deregulated expression	T-ALL
RBTN2[a]	11p13	t(11;14)	Deregulated expression	T-ALL

Abbreviations: CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; T-ALL, T-cell ALL; pre-B-ALL, precursor B-cell ALL; NHL, non-Hodgkin lymphoma; APL, acute promyelocytic leukemia; AML, acute myeloid leukemia.

[a] Gene first identified by cloning breakpoints.
[b] Partial list of transcription factors.

have been identified (Table 48-1). Molecular analysis has revealed that alterations in the expression of the genes or in the properties of the encoded proteins resulting from the rearrangement play an integral role in the process of malignant transformation. [3] [4] The transforming genes that are involved in chromosomal translocations fall into several functional classes, including tyrosine protein kinases, serine protein kinases, cell-surface receptors, and growth factors (Table 48-1). However, the largest class of genes involved in translocations encodes transcriptional regulatory factors. [4] Transcription factors are proteins that are involved in the initiation of gene transcription; they recognize and bind to target sequences located in the regulatory elements of genes or to other DNA-binding proteins, often functioning in a tissue-specific fashion. In this way, they play critical roles in differentiation and development as well as in maintaining the function of differentiated cells. These genes have been implicated in the pathogenesis of T-cell and B-cell neoplasms as well as of some myeloid leukemias (Table 48-1). Many of these genes were first identified as a result of the molecular characterization of translocations in tumor cells.

There are several mechanisms by which chromosomal translocations result in altered gene function. The first is deregulation of gene expression. This mechanism is characteristic of the translocations in the lymphoid leukemias and lymphomas that involve the immunoglobulin genes in B-lineage tumors and the T-cell receptor genes in T-lineage tumors. These rearrangements result in inappropriate expression (either overexpression or aberrant expression in a tissue that does not normally

express the gene) of the partner gene involved in the translocation, with no alteration in its protein structure.

The second mechanism is the expression of a novel fusion protein as a result of the juxtaposition of coding sequences from two genes that are normally located on different chromosomes. Such fusion proteins are tumor specific in that the fusion gene does not exist in nonmalignant cells; thus, the detection of such a fusion gene/protein can be important in diagnosis and in the detection of residual disease or early relapse. Examples of chimeric proteins include the BCR/ABL protein or the PML/RARA protein, resulting from t(9;22) in chronic myelogenous leukemia or t(15;17) in acute myeloid leukemia (AML)-M3, respectively. Of note, all of the translocations cloned to date in the myeloid leukemias result in a fusion mRNA and a chimeric protein (Tables 48-1 and 48-2).

Chromosomal translocations result in the activation of genes in a dominant fashion. A number of human tumors, including retinoblastoma, Wilms tumor, and colon carcinoma, are believed to result from recessive mutations that, when present in a homozygous state, lead to tumor formation. [5] These mutations lead to the *absence* of the protein product, suggesting that these genes function as suppressor genes whose normal role is to limit cellular proliferation. The hallmark of tumor suppressor genes is the loss of genetic material in malignant cells. Such a loss may result from chromosomal loss or deletion or may occur through other genetic mechanisms, such as mitotic recombination. The identification of recurring chromosomal losses or deletions in the leukemias and lymphomas suggests that, as for a number of solid tumors, tumor suppressor genes may be involved in the pathogenesis of some hematologic malignant diseases. [3] [5]

METHODS

Cytogenetic analyses of malignant diseases must be based on the study of the tumor cells themselves. In leukemia, the specimen is usually obtained by bone marrow aspiration and is either processed immediately (direct preparation) or cultured for

TABLE 48-2 -- Recurring Chromosome Abnormalities in Malignant Myeloid Diseases

Disease	Chromosome Abnormality	Frequency[a] (%)	Involved Genes[b]	
CML	t(9;22)(q34;q11)	98 (100)[c]	*ABL*	*BCR*
CML blast phase	t(9;22) with +8, +Ph, +19, or i(17q)	70	*ABL*	*BCR*
AML-M2	t(8;21)(q22;q22)	18 (30)	*ETO*	*AML1*
AML-M3, M3V	t(15;17)(q22;q1112)	14 (98)	*PML*	*RARA*
AMMoL-M4Eo	inv(16)(p13q22) or t(16;16)(p13;q22)	6 (100)	*MYH11*	*CBFB*
AMMoL-M4, AMoL-M5	t(9;11)(p22;q23)	11 (30) for all t(11q23)	*AF9*	*MLL*
	t(10;11)(p11p15;q23)		*AF10*	*MLL*
	t(11;17)(q23;q25)		*MLL*	*AF17*
	t(11;19)(q23;p13.3)		*MLL*	*ENL*
	t(11;19)(q23;p13.1)		*MLL*	*ELL*
	t(6;11)(q27;q23)		*AF6*	*MLL*
	Other t(11q23)		*MLL*	
	del(11)(q23)			
AML	+8	13		
	7 or del(7q)	9		
	5 or del (5q)	10		
	t(6;9)(p23;q34)	1	*DEK*	*CAN*
	t(3;3)(q21;q26) or inv (3)(q21q26)	2	*EVI1*	
	del (20q)	5		
	t(12p) or del(12p)	2		
Therapy related AML	7 or del(7q) and/or 5 or del(5q)	75		
	der(1;7)(q10;p10)	2		
	t(9;11)(p22;q23)/t(11)(q23)	3	*MLL*	
	t(21q22)	2	*AML1*	
CMMoL	t(5;12)(q33;p12)	25	*PDGFRB*	*TEL*

Abbreviations: CML, chronic myelogenous leukemia; AML-M2, acute myeloblastic leukemia with maturation; AML-M3, M3V, hypergranular (M3) and microgranular (M3V) acute promyelocytic leukemia; AMMoL, acute myelomonocytic leukemia; AMMoL-M4Eo, acute myelomonocytic leukemia with abnormal eosinophils; AMoL, acute monoblastic leukemia; AML, acute myeloid leukemia; CMMoL, chronic myelomonocytic leukemia.

[a]The percentage refers to the frequency within the disease overall. The numbers within the parentheses (also percentages) refer to the frequency within the morphologic or immunologic subtype of the disease.[2]

[b]Genes are listed in order of citation in the karyotype; e.g., for CML, *ABL* is at 9q34 and *BCR* is at 22q11.

[c]Some patients with CML have an insertion of *ABL* adjacent to *BCR* in a normal-appearing chromosome 22.

2448 hours. When a bone marrow aspirate cannot be obtained, a bone marrow biopsy specimen (bone core specimen) can often be processed successfully. Alternatively, for patients who have a white blood cell (WBC) count of >10,000/l with more than 10% immature myeloid or lymphoid cells, a sample of peripheral blood can be cultured without adding phytohemagglutinin (PHA). The karyotype of the dividing cells will be similar to that obtained from the bone marrow. Mitogens such as PHA are not routinely added to peripheral blood cultures in acute leukemia, because stimulation of division of normal lymphocytes may interfere with the analysis of spontaneously dividing malignant cells. An involved lymph node or tumor mass specimen or malignant effusion may be processed similarly for the analysis of lymphomas. The use of amethopterin or fluorodeoxyuridine to synchronize dividing cells, combined with brief exposures to mitotic inhibitors such as colchicine or DNA-binding agents (ethidium bromide), is practiced by some laboratories to obtain elongated chromosomes that have an increased number of bands.

Cytogenetic studies are feasible only for specimens that contain viable dividing cells. For this reason, it is critical that the specimen be transported to the cytogenetics laboratory without delay. In some cases analyses may be performed on specimens that have been transported by overnight delivery services; however, the shipment of specimens frequently results in loss of cell viability, and most laboratories experience a high proportion of inadequate analyses when using such specimens. For optimally handled specimens, 9598% of all cases should be adequate for cytogenetic analysis.

Chromosomal abnormalities are described according to the International System for Human Cytogenetic Nomenclature (Appendixes 48-1 and 48-2).[6] The

observation of at least two cells with the same structural rearrangement (e.g., translocations, deletions, or inversions) or gain of the same chromosome, or of three hypodiploid cells, each showing loss of the same chromosome, is considered evidence of the presence of an abnormal clone. However, one cell with a normal karyotype is considered evidence of the presence of a normal cell line. Patients whose cells show no alteration or nonclonal (single cell) abnormalities are considered to be normal. That is, one abnormal cell is generally not considered evidence of the presence of an abnormal clone because occasional abnormalities (primarily numerical abnormalities) may result from technical artifact. An exception is a single cell characterized by a recurring abnormality. It is likely that such a cell represents the karyotype of the malignant cells in that particular patient.

Many laboratories have incorporated the technique of fluorescence in situ hybridization (FISH) into their diagnostic services. In FISH, labeled probes are hybridized to either metaphase chromosomes or interphase nuclei, and the hybridized probe is detected with fluorochromes. This technique is a rapid and sensitive means of detecting recurring numerical and structural abnormalities.

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CHRONIC MYELOPROLIFERATIVE DISORDERS

The chronic myeloproliferative disorders are a group of diseases characterized by the neoplastic proliferation of hematopoietic stem cells and their differentiated progeny within the bone marrow and in extramedullary sites. Although each of these disorders has distinctive clinical and laboratory features, there is a great deal of overlap in clinical and pathologic characteristics and natural history. The clonal nature of each of these diseases has been demonstrated by cytogenetic and isoenzyme techniques. In each disorder, multiple cell lineages are quantitatively and qualitatively abnormal, providing evidence that the neoplastic transformation occurs in a multipotential hematopoietic stem cell.

Chronic Myelogenous Leukemia

Chronic Phase

Chronic myelogenous leukemia (CML) is a particularly important subtype of leukemia because it was in this disease that the first consistent chromosomal abnormality in a malignant disease was noted (Table 48-2). This abnormality, the Philadelphia or Ph chromosome, was first described in 1960 by Nowell and Hungerford as a deletion of part of the long arm of a G-group chromosome, and later, with the use of quinacrine fluorescence banding techniques, as a 22q. [7] The nature of the chromosomal aberration was clarified in 1973, when Rowley reported that the Ph chromosome resulted from a reciprocal translocation involving chromosomes 9 (break at band q34) and 22 (break at band q11) rather than from a terminal deletion, as many investigators had previously assumed (Fig. 48-1).[8]

Historically, 85% of patients diagnosed as having CML were found to have the Ph chromosome (Ph+). [9] Identification of patients as Ph+ or Ph negative (Ph neg) was found to be clinically significant in that Ph+ patients had a better prognosis than patients with Ph neg CML (42 vs. 15 months survival). Patients with a Ph chromosome and additional chromosomal abnormalities (130% of patients examined at initial diagnosis during the chronic phase) have not had a substantially poorer survival rate in some series than patients with only a Ph chromosome. [9] [10] A change in the karyotype, however, is considered to be a grave prognostic sign indicating progression to the acute blast phase; the usual duration of survival after such a change is 25 months. [9]

Evidence has accumulated which suggests that CML should be defined by the presence of the Ph chromosome or its molecular consequence, the *BCR*/*ABL* fusion gene (see later), and that patients without this abnormality should be considered to have a different myeloproliferative disorder. Pugh et al. reviewed 25 patients initially diagnosed as having CML but whose cells lacked the Ph chromosome and showed that most of these patients had some type of myelodysplastic syndrome (MDS), most commonly chronic myelomonocytic leukemia or refractory anemia with excess blasts.[11] The most common abnormality in these patients was trisomy 8. These observations, which have been confirmed by others, suggest that, with very few exceptions, Ph neg CML does not exist. The absence of the Ph chromosome thus raises the suspicion that the patient actually has a myelodysplastic syndrome or a myeloproliferative disorder

Figure 48-1 Schematic diagram of a specific chromosomal abnormality, namely, the reciprocal translocation involving chromosomes 9 and 22, t(9;22)(q34;q11), which gives rise to the Philadelphia (Ph) chromosome in the malignant cells of patients with chronic myelogenous leukemia. Chromosomal breaks occur in bands q34 and q11 of chromosomes 9 and 22, respectively, followed by a reciprocal exchange of chromosomal material. This rearrangement results in the translocation of the *ABL* oncogene, normally located at 9q34, adjacent to the *BCR* gene on chromosome 22, giving rise to a chimeric *BCR*/*ABL* gene, whose protein product plays a role in the transformation of myeloid cells.

other than CML. It is notable, however, that the leukemia cells in occasional individual cases that lack the Ph chromosome appear to contain a DNA rearrangement in which the molecular consequences are identical to those of t(9;22) (described following).

About 92% of Ph-positive patients with CML have the standard 9;22 translocation. The remaining patients have variant translocations, such as three-way translocations involving chromosomes 9, 22, and a third chromosome. [12] [13] Molecular analysis of the DNA sequences that are located at the chromosomal breakpoints of both the standard and variant translocations in CML has revealed that the oncogene *ABL* is consistently translocated adjacent to the *BCR* gene on chromosome 22 (Fig. 48-1).[14] [15] Analysis of leukemia cells from rare patients with CML who lack the Ph chromosome has revealed a rearrangement involving *ABL* and *BCR* that is detectable only at the molecular level. [16] However, this rearrangement is not present in most patients who have been diagnosed as having Ph neg CML, providing further evidence that t(9;22) and subsequent fusion of the *ABL* and *BCR* genes is characteristic of all CML cases.

On chromosome 22, most breaks cluster in a small, 5.8-kilobase (kb) region known as the breakpoint cluster region (bcr). [14] [15] In contrast, the breaks on chromosome 9 occur over >200 kb, usually upstream (5) of exon II, and the *ABL* coding sequences are translocated to chromosome 22 and fused in-frame with the *BCR* gene. A novel 8.5-kb mRNA is found in all CML patients, because the *BCR* exons are spliced to *ABL* exon II, resulting in a chimeric mRNA that is translated into a chimeric protein ($p210^{BCR\text{-}ABL}$).[15] Experiments in the mouse, either with myeloid cell lines transfected with the fusion gene or in transgenic mice, have clearly shown that the fusion gene leads to leukemia.[17] The ABL protein is normally a nuclear protein and may function as a transcription factor that is regulated by the retinoblastoma protein, RB1. The BCR/ABL protein is located on the cytoplasmic surface of the cell membrane and acquires a novel function in transmitting growth-regulatory signals from cell-surface receptors to the nucleus via the RAS signal transduction pathway. [18] [19] The *BCR*/*ABL* fusion gene can be detected with standard Southern blot analysis of DNA or by polymerase chain reaction (PCR) analysis of mRNA by reverse transcription (RT-PCR) [20] for the diagnosis and detection of residual disease. FISH can also be used to detect the Ph chromosome in both metaphase and interphase cells. [21]

When patients with CML are treated with busulfan or hydroxyurea and a hematologic remission is achieved, the bone marrow morphology and leukocyte alkaline phosphatase (LAP) score may return to normal. However, when such a remission bone marrow is analyzed cytogenetically, the Ph clone persists, and the percentage of Ph+ cells in the bone marrow usually remains unchanged (the Ph chromosome is usually found in 100% of cells examined). Attempts have been made to eradicate the Ph+ clone with aggressive cytotoxic therapy, but only rarely can this be achieved, and even then, the benefit is transient. Studies using glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme techniques together with cytogenetic studies have shown that all three hematopoietic cell lines (myeloid, erythroid, and megakaryocytic) are involved in the malignant process, as well as lymphocytes of B-cell origin. [22] This suggests that the cell of origin of CML is a multipotential stem cell. Intensive chemoradiotherapy followed by allogeneic bone marrow transplantation has been successful in eradicating the Ph+ cell line and restoring normal hematopoiesis of donor origin. Of great importance is the evidence that daily use of interferon- (IFN-) can induce complete cytogenetic remission in approximately 20% of patients with CML in the chronic phase.[23] [24] The reappearance of cytogenetically normal marrow cells requires several months of treatment. In most cases, molecular methods can still detect cells with the *BCR*/*ABL* fusion transcript. However, patients who have a cytogenetically complete response to interferon therapy have a longer survival than nonresponders or those treated only with hydroxyurea.

Acute Phase

As they enter the terminal acute phase (blast crisis of CML), most patients (80%) show karyotypic evolution, with the appearance of new chromosomal abnormalities in very distinct patterns in addition to the Ph chromosome. For this reason, cytogenetic studies may be useful in confirming the clinical impression of the accelerated or acute phase of the disease. A change in the karyotype is considered to be a grave prognostic sign. [9] The available data suggest that with the exception of an isochromosome of the long arm of chromosome 17 (i(17)(q10)), which is usually associated with a myeloid type of blast transformation, there is no association of a particular karyotype with the lymphoid or the myeloid type of blast transformation, and that these additional abnormalities are not correlated with the response to therapy during the acute phase. [12] [25] The most common changes, a gain of chromosomes 8 or 19, or a second Ph (by gain of the first), or an i(17q), frequently occur in combination to produce modal chromosome numbers of 4750. [12]

As described later, the t(9;22) can be observed in acute lymphoblastic leukemia (ALL); patients who present with the lymphoblastoid blast phase but who have no prior history suggestive of CML can be difficult to differentiate from those with Ph+ ALL. In CML patients, the Ph chromosome is present in granulocytic, erythroid, and megakaryocytic cells, in some B cells, and probably in a few T cells. Recent studies have shown that Ph+ ALL is a lymphoblast-restricted disease and usually involves a different breakpoint in *BCR*, whereas the lymphoid blast phase of CML is a multilineage disease involving a break in the bcr of the *BCR* gene. [26] [27] The overall survival of patients with multilineage disease is significantly better than that of patients with lymphoblast-restricted disease. [26] [27]

Polycythemia Vera

Polycythemia vera is the second most extensively studied chronic myeloproliferative disorder (for review, see Rowley and Testa [12]). Abnormalities are less common (14%) in untreated patients than in those who have been treated with cytotoxic agents prior to the first cytogenetic examination (39%). An abnormal karyotype, frequently with multiple abnormalities, is detected in 85% of patients studied after they have developed leukemia. In polycythemia vera, the presence of cytogenetic abnormalities at diagnosis does not necessarily predict a short survival or the development of leukemia. [12] [28] An evolutionary change in the karyotype during the disease course, however, may be an ominous sign.

In the polycythemic phase, a gain of chromosomes, which usually involves chromosomes 8 (15%) or 9 (20%), is frequently observed. It is notable that a number of patients with polycythemia vera show gains of both chromosomes 8 and 9; clones containing both +8 and +9 are seldom observed in other hematologic diseases and thus may be unique to polycythemia vera. Structural rearrangements most often involve a deletion of chromosome 20 (q11.2q13.3) (30%) or a duplication of 1q (20%), especially bands 1q2532. A deletion of 20q is not specific for polycythemia vera and has been noted in other malignant myeloid diseases; a duplication of 1q has been observed in other hematologic diseases as well as in solid tumors. [1] [2] [3]

The cytogenetic pattern of the malignant cells in polycythemia vera patients who have developed AML show some similarities to the patterns observed in the polycythemic phase (e.g., +8, +9, del(20q)), but there are certain striking differences as well. For example, loss of chromosome 7 is rarely observed in the polycythemic phase but is seen in 20% of patients in the leukemic phase. Rearrangements of chromosome 5, particularly

a del(5q), are the most frequent changes noted in advanced disease (40% of patients). [1] [29] [30] As discussed later, abnormalities of chromosomes 5 or 7 (or both) are the most common abnormalities noted in therapy-related leukemia, suggesting that the leukemia in some patients with polycythemia vera may have a similar cause. It has been shown that the chronic use of oral alkylating agents such as chlorambucil or the use of phosphorous-32 during the chronic phase of polycythemia vera significantly increases the rate of transformation to leukemia.

Essential Thrombocythemia and Myeloid Metaplasia with Myelofibrosis

One hundred seventy cases of essential thrombocythemia were analyzed during the Third International Workshop on Chromosomes in Leukemia, and only 5% of patients had a definite chromosomal abnormality that was accepted by all of the workshop participants. [31] Moreover, no recurring abnormality could be identified in these patients.

Cytogenetic analysis of bone marrow cells of patients with myeloid metaplasia with myelofibrosis (MMM) has revealed the presence of clonal abnormalities in 35% of patients. In general, these abnormalities are similar to those noted in other malignant myeloid disorders. The most common anomalies are +8, 7 or a del(7q), and del(11q) or del(20q). [1] [32] [33] A recurring deletion of the long arm of chromosome 13 has been reported in patients with MMM as well as with other malignant myeloid disorders. [2] [3] [34] In these rearrangements, the consistently deleted band is 13q14. As for CML and polycythemia vera, a change in the karyotype in MMM may signal evolution to acute leukemia.

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PRIMARY MYELODYSPLASTIC SYNDROMES

The myelodysplastic syndromes are a heterogeneous group of hematopoietic stem cell disorders characterized by anemia, neutropenia, or thrombocytopenia in various combinations.[35] [36] Several laboratories have reported that clonal chromosomal abnormalities can be detected in bone marrow cells in 4070% of patients with primary MDS at the time of diagnosis.[37] [38] [39] [40] [41] [42] This contrasts with the 7095% incidence of cytogenetic abnormalities detected in patients with AML de novo. Although +8,5/del(5q), 7/del(7q), and del(20q) are common in both disorders, the specific structural rearrangements that are closely associated with distinct morphologic subsets of AML de novo (Table 48-2) are almost never seen in MDS (Table 48-3). With several exceptions (such as the 5q syndrome), chromosomal abnormalities in MDS have not correlated with specific clinical or morphologic subsets when the criteria of the French-American-British (FAB) group were applied.

Patients with MDS may have single or multiple chromosomal changes (Table 48-3). A single chromosome change involves

TABLE 48-3 -- Single Chromosome Changes in Primary MDS

7 or del(7q)
+8
del(5)(q13q33) and translocations involving 5q [a]
del(11q)[b]
del(12)(p11p13)
del(13q)[c]
del(20)(q11q13)
t(1;3)(p36;q21.2)
del(1;7)(q10;p10)
t(2;11)(p21;q23)
t(6;9)(p23;q34)

[a]Chromosomal breakpoints of the interstitial deletions of 5q are variable, but band q31 is invariably deleted; proximal breakpoints frequently occur in bands 5q1315 and distal breakpoints frequently occur in bands q3335.
[b]q23.1 is always involved in either interstitial or terminal deletions.
[c]q14 is always involved in interstitial deletions of variable size.

a single numerical change or a structural abnormality involving only one chromosome or a balanced translocation involving only two chromosomes. Occasionally, several unrelated abnormal clones may be detected (5% of cases); the frequency of such unrelated clones is higher than that observed in AML de novo (<1%). Additional chromosomal aberrations may evolve during the course of MDS or an abnormal clone may emerge in a patient with a previously normal karyotype; these changes appear to portend transformation to leukemia.

The ability of cytogenetic analysis to predict the outcome of any individual patient with MDS is made more difficult because MDS is a life-threatening disorder as a result of persistent and profound pancytopenia (marrow failure), regardless of whether transformation to acute leukemia occurs. Nonetheless, several studies have demonstrated the prognostic significance of cytogenetic abnormalities in MDS in relation to survival and progression to AML. Patients with abnormal karyotypes (particularly those with complex abnormalities) were found to have a shorter survival time and a higher incidence of progression to AML than patients with normal karyotypes.[37] [38] [39] [40] [41] Patients with a del(5q) or del(20q) alone appear to have a favorable outcome, patients with other single abnormalities have an intermediate outcome, and patients with complex karyotypes (most have abnormalities of chromosome 5 or 7, or both) have a poor outcome.[40] In a recent study, the International MDS Risk Analysis Workshop combined cytogenetic, morphologic, and clinical data for 816 patients from seven large, previously reported risk-based studies. [42] Univariate analysis indicated that the major variables predictive of evolution to AML were cytogenetic abnormalities, percentage of bone marrow blasts, and number of cytopenias; those variables predictive of survival included the above as well as age and sex. Patients with a good outcome had normal karyotypes, Y alone, del(5q) alone, or del(20q) alone; those with an intermediate outcome had other abnormalities; and those with a poor outcome had complex karyotypes (three or more abnormalities, typically with abnormalities of chromosome 5 or 7, or both) or chromosome 7 abnormalities. The median survival of patients within these three groups was 3.8, 2.4, and 0.8 years, respectively, and the median survival for 25% of the patients to undergo evolution to AML was 5.6, 1.6, and 0.9 years. These cytogenetic subgroups were also significant independent variables for predicting outcome in multivariate analysis.

The 5q syndrome is a distinctive hematologic disorder that occurs primarily in older women. [29] [30] [43] In contrast to other MDS, where males predominate, the male:female ratio in the 5q syndrome is 0.5. Eighty percent of patients with the 5q syndrome are >50 years old. Patients present with a refractory macrocytic anemia and normal or elevated platelet counts. The marrow is characterized by the presence of micromegakaryocytes with monolobulated and bilobulated nuclei. Approximately two-thirds of patients have less than 5% blasts in the marrow (refractory anemia [RA] or RA with ringed sideroblasts [RARS]) and the remainder have RA with excess blasts (RAEB). Although 75% of cases have a del(5)(q13q33), other interstitial deletions (del(5)(q15q33) or del(5)(q22q33)) may be present. These patients may experience a relatively benign disease course that extends over several years.

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ACUTE MYELOID LEUKEMIA DE NOVO

The acute leukemias are generally classified as either lymphoblastic or myeloid. In either case, they result from neoplastic transformations of uncommitted or partially committed hematopoietic stem cells. Traditionally, classification of the acute leukemias has relied on morphology, reflecting the predominant cell type and relating that cell to its presumed normal counterpart.[44] In large part, the correlation of cytogenetic abnormalities with the morphologic features of the leukemia was made possible

by the development of a classification system for the acute leukemias by the FAB Cooperative Group. [44] [45]

Numerous reports have now described the cytogenetic findings in relatively large series of unselected patients with AML as well as in individual patients. [12] [46] [47] [48] In earlier series, abnormal karyotypes were reported in approximately 50% of all patients with AML de novo whose bone marrow cells were examined with banding techniques. At the Fourth International Workshop on Chromosomes in Leukemia, held in Chicago in September 1982, 54% (354 of 660) of patients had chromosomal abnormalities.[49] The detection of cytogenetic abnormalities increases markedly when techniques for culturing leukemia cells and for obtaining prophase and prometaphase chromosomes are used. Currently, at our institution we are detecting an abnormal clone in 85% of AML patients. Initially, it appeared that AML patients with a normal karyotype had a significantly longer survival time than those with any detectable chromosomal abnormality. More recently, it has become clear that the prognostic importance resides in specific chromosomal changes, several of which are associated with higher response rates and longer survival times than the medians observed in AML patients who have no detectable abnormality. [46] [47] [48] [49] [50] [51] [52] The following discussion emphasizes some specific aberrations that occur frequently and also appear to be of exceptional biologic interest (Table 48-2).

Chromosomal Gain and Loss

Although the karyotypes of patients with AML may be variable, both a recurring gain and loss of chromosomes and an involvement in structural rearrangements are evident. The number of chromosomes gained or lost in 354 patients with a clonal abnormality was examined at the Fourth International Workshop on Chromosomes in Leukemia.[49] With the exception of chromosome 16, which was never observed as a gain, and chromosome 1, which was never lost, each of the autosomes and sex chromosomes contributed to the numerical changes. Some chromosomes were clearly overrepresented as gains or losses, while others were underrepresented. Thus, a gain of chromosome 8, the most frequent abnormality seen in AML, was found in 13% (47 of 354) of patients. Loss of chromosome 7, another frequent numerical change, was observed in 9% (30 of 354) of patients, and loss of chromosome 5 was noted in 6% (20 of 354) of cases. A gain of either of these chromosomes is rarely observed. These abnormalities are seen in most subtypes of AML, although there are some interesting differences in frequency. [46] [49] [53] [54]

For example, in an analysis of 26 patients with acute erythroblastic leukemia (M6) who were seen at the University of Chicago, 77% had clonal abnormalities, and in 85% of these cases there was a loss of all or part of chromosomes 5 and/or 7. [55] In addition, the karyotypes were often complex, with multiple abnormalities and subclones. These patients were older than patients with M6 disease with normal karyotypes or simple chromosomal changes, and they had a shorter survival time. In addition, they showed striking similarities, biologically and clinically, to patients with therapy-related AML (discussed following).

Loss of the Y chromosome, the second most frequent numerical change in the patients examined at the workshop, or loss of the X chromosome often occurred in association with an 8;21 translocation (8090% of these patients). [49] Loss of a Y chromosome as the sole abnormality is also observed, but the significance of this abnormality is uncertain because a missing Y chromosome has been reported in bone marrow cells of hematologically normal males, particularly those >60 years old.[2] [12] In contrast, the t(8;21) in acute myeloblastic leukemia (AML-M2) is usually observed in younger adults. [12] [45] [46] [49] [50]

Specific Structural Rearrangements

t(8;21) in Acute Myeloblastic Leukemia (M2)

In 1973, Rowley first described a balanced translocation between chromosomes 8 and 21 (t(8;21)(q22;q22), Fig. 48-2A). The

Figure 48-2 Schematic diagram of four recurring chromosomal abnormalities characteristic of acute myeloid leukemia. **(A)** t(8;21) (q22;q22), AML-M2. **(B)** t(15;17)(q22;q1112), AMLM3. **(C)** inv(16) (p13q22), AMMoL-M4Eo. **(D)** del(5)(q13q33), observed in MDS and AML. A del(5q) is frequently observed in t-MDS/t-AML.

t(8;21) is common, being observed in 18% of all AML cases with an abnormal karyotype and in 30% of patients with M2 leukemia. This translocation is the most frequent abnormality in children with AML, being reported in 17% (10 of 60) of karyotypically abnormal cases. This abnormality initially appeared to be restricted to patients with a diagnosis of M2 leukemia (acute myeloblastic leukemia with maturation) according to the FAB classification. [44] However, 7% (3 of 44) of patients analyzed at the Fourth International Workshop who had a t(8;21) and had adequate bone marrow material available for morphologic review had a diagnosis of acute myelomonocytic leukemia (M4).[49]

In the t(8;21), the *AML1* gene on chromosome 21 is fused with the *ETO* gene on chromosome 8 to form an AML1/ETO chimeric product (Fig. 48-3).[56] The AML1 protein (also known as core-binding factor-, CBFA2) is a member of a family of transcription factors with homology to the pair-rule *Drosophila* gene, *Runt*. AML1 heterodimerizes with another protein, CBFB, to form a transcription factor. Of note is that the *CBFB* gene is located at 16q22, as the breakpoint in the inv(16)/t(16;16) associated with AML-M4 with abnormal eosinophils. The AML1/CBFB transcription factor binds directly to an enhancer core motif that is present in the transcriptional regulatory regions of a number of genes that are critical to myeloid cell growth, differentiation, and function, and thereby regulates expression of the genes. [57] The target genes include the genes encoding interleukin-3 (IL3), granulocyte-macrophage colony-stimulating factor (GM-CSF), the CSF-1 receptor, myeloperoxidase, and neutrophil elastase.[57] Mutant mice with loss of *AML1* or *CBFB* gene function are deficient in hematopoiesis and die during embryogenesis, indicating that the AML1/CBFB-regulated target genes are essential for hematopoiesis of all lineages. [58]

AML1/ETO retains the ability to interact with the enhancer core motif, but it interferes with normal activation of gene expression by the normal AML1/CBFB transcription factor.[57] [58] Moreover, AML1/ETO has gained a new function within the cell, and is capable of activating expression of the *BCL2* gene, an anti-apoptosis

gene, and possibly other genes.[59] Thus, transformation by AML1/ETO likely results from altered transcriptional regulation of normal AML1 target genes, combined with the activation of new target genes that prevent programmed cell death.

Although the M2 type of AML is heterogeneous, the presence of t(8;21) identifies a morphologically and clinically distinct subset. In this disorder, blasts tend to have indented nuclei, and the cytoplasm is generally basophilic with a prominent paranuclear hof that may contain a few azurophilic granules. [60] Promyelocytes, myelocytes, and metamyelocytes are often quite prominent and may be large. Their cytoplasm has a waxy, orange appearance and lacks a granular texture in Romanowsky-stained specimens. Auer rods are easily identified, and several may be seen in a single cell. Bone marrow eosinophilia is also common. [60] Malignant cells in some of these patients may appear cytogenetically normal or to contain only a Y or del(9q). However, FISH or RT-PCR methods will demonstrate the *AML1*/*ETO* rearrangement.[61]

AML-M2 with t(8;21) appears to have a favorable prognosis in adults. The median age of these patients is 2530 years, significantly younger than that of patients with AML overall.[46] [49] [50] Some patients have <30% myeloblasts in the marrow at diagnosis and are therefore inappropriately classified as having MDS rather than AML. Some patients have granulocytic sarcomas. The complete remission rate is uniformly high, and with intensive postremission consolidation chemotherapy, the expected disease-free survival exceeds 2 years, after which time relapses are uncommon.[50] [62] Remarkably, however, some patients in continuous remission for as long as 8 years have *AML1*/*ETO* mRNA detectable in circulating leukocytes.[63] In contrast, children with a t(8;21) have a poor outcome. [64]

Figure 48-3 Schematic diagram of the normal AML1B, ETO, and TEL proteins, and the AML1/ETO and TEL/AML1 fusion proteins. **(A)** Diagram of the heterodimeric AML1/CBFB transcription factor. The transcription factor binds via the DNA-binding domain of AML1 (runt homology domain, or RHD) directly to a specific core motif present in the regulatory region of a number of genes that are involved in myeloid cell growth, and thereby regulates their expression. **(B)** Schematic diagram of the normal AML1B and ETO proteins and the AML1/ETO fusion protein; schematic model of the role of AML1/ETO in the pathogenesis of myeloid leukemias. For AML1, RHD refers to the runt homology domain and TA refers to the trans-activation domain. The ETO protein contains three domains characteristic of transcription factors: TAF110 refers to a domain with homology to the *Drosophila* TAF110 transcriptional co-activator, HHR refers to a leucine zipper-like hydrophobic heptad repeat, and ZF refers to a zinc finger domain. Nervy refers to a domain homologous to the *Drosophila* gene, nervy. **(C)** Schematic diagram of the normal AML1B and TEL proteins and the TEL/AML1 fusion protein. For TEL, HLH refers to a helix-loop-helix domain and ETS refers to an ETS DNA-binding domain.

t(15;17) in Acute Promyelocytic Leukemia (M3)

A structural rearrangement involving the long arms of chromosomes 15 and 17 in acute promyelocytic leukemia (APL; AML-M3) was first recognized by Rowley et al. in 1977. For many years, the precise breakpoints involved in this translocation were controversial; however, the rearrangement is defined as

t(15;17)(q22;q1112)(Fig. 48-2B). At the Fourth International Workshop only 25% (15 of 61) of patients with APL analyzed had a normal karyotype. Seventy percent (43 of 61) of patients had a t(15;17), and three had other abnormalities.[49] In our laboratory, each of more than 150 patients with APL whom we have examined have had a t(15;17) (M.M. Le Beau, unpublished data).[65] This rearrangement is highly specific for APL and has not been found in patients with any other type of leukemia or with a solid tumor.

The breakpoint on chromosome 17 occurs within the first intron of the -retinoic acid receptor gene (*RARA*) in most patients, whereas the break on chromosome 15 occurs within the *PML* gene.[66] *RARA* is a member of the steroid/thyroid hormone receptor superfamily. Retinoic acid is a ligand that binds to the nuclear receptor homodimer complex; this complex acts as a transcription factor (activates transcription of other genes) and thereby induces cellular differentiation. The translocation results in a fusion *PML*/*RARA* gene that contains most of the *PML* coding sequences and the DNA-binding and ligand-binding domains of the *RARA* gene. The PML protein complexes with other proteins to form a novel nuclear domain of unknown function.[67] [68] On exposure to all-trans retinoic acid (ATRA), the PML/RARA fusion protein is released from the complexes, and may then activate retinoid-responsive gene transcription, resulting in differentiation of APL cells. [67]

APL is widely recognized as a unique clinicopathologic entity characterized by infiltration of the bone marrow by promyelocytes in association with a hemorrhagic diathesis. The characteristic folded, reniform (kidney-shaped), or bilobed nucleus is invariably found in some of the promyelocytes. Coarse azurophilic granules and multiple Auer rods are common.[60] [65] The microgranular variant of APL differs from the more frequent hypergranular type only in that the cytoplasmic granules of the leukemia cells are smaller and sometimes beyond the limit of resolution of the light microscope. [60] [65] Auer rods may also be fewer in number and the peripheral WBC count is higher in the M3 microgranular variant, but the t(15;17) is similarly present.

Patients with APL and t(15;17) are younger than other individuals who have AML. [46] [49] [65] Clinical or laboratory evidence of disseminated intravascular coagulation (DIC) is almost invariably present at diagnosis and may worsen during the initial cytolytic response to chemotherapy. APL cells are exquisitely sensitive to the differentiating effect of ATRA, and evidence of DIC rapidly resolves after this treatment is begun. [69] Cases that lack the t(15;17) do not respond to ATRA. Patients with APL may enter complete remission without experiencing a period of marrow hypoplasia; the malignant promyelocytes are often slow to clear from the marrow even as normal hematopoiesis recovers.[50] The optimal use of ATRA therapy has not yet been determined, but it is clearly a mandatory component of treatment. Having achieved a remission, many patients remain disease-free after intensive consolidation chemotherapy.

inv(16) and t(16;16) in Acute Myelomonocytic Leukemia with Abnormal Eosinophils (M4Eo)

In 1983, Arthur and Bloomfield described five cases (three with AML-M2 and two with AMMoL-M4 leukemia) in which the bone marrow contained an excess of eosinophils (854%); all five patients were reported to have a deleted chromosome 16 (del(16q)). [70] Le Beau et al. reported on a related entity in 18 patients, all of whom had M4 leukemia with eosinophils that showed alterations of morphology, cytochemical reactions, and ultrastructure; these alterations included the presence of large and irregular basophilic granules and positive reactions with periodic acidSchiff and chloroacetate esterase. [71] [72] Many of these patients did not have an increased percentage of marrow eosinophils; one-third had <5% eosinophils. Fifteen patients had a pericentric inversion of chromosome 16, inv(16)(p13q22) (Fig. 48-2C), and in three patients a reciprocal translocation involving both chromosome 16 homologs (t(16;16)(p13;q22)) was noted. [71] [72] [73] [74] Among M4 patients in the University of Chicago series, 23% have had an inv(16) or t(16;16). This correlation between abnormal eosinophils and structural rearrangements of chromosome 16 was confirmed at the Fourth International Workshop. [49] Of 25 patients with M4 and >5% marrow eosinophils, ten had either a del(16) or an inv(16). Our experience suggests that breakpoints at both 16p13 and 16q22 are required for manifestation of the complete M4Eo syndrome, as patients with only del(16)(q22) have had different morphologic and clinical features. [74] Patients with inv(16) or t(16;16) have a good response to intensive chemotherapy. [62] In our updated series, 78% (25 of 32) of treated patients entered a complete remission.[74] The median survival for all 32 treated patients was >66 weeks, and the median survival for those 25 patients who had a complete remission was >104 weeks. This survival markedly exceeded the median of 29 weeks attained by 58 treated AMMoL patients who did not have this chromosomal rearrangement.

The inversion breakpoint at 16q22 occurs near the end of the coding region of the *CBFB* gene (also known as *PEBP2B*), [75] which encodes one subunit of a novel heterodimeric AML1/CBFB transcription factor. A smooth muscle myosin heavy chain gene (*MYH11*) is interrupted by the breakpoint on 16p. A fusion protein containing the 5 region of *CBFB* (165 of 182 amino acids), including the domain that heterodimerizes with *AML1*, fused to the 3 portion of *MYH11* is produced. This portion of MYH11 contains a repeated -helical structure involved in myosin filament interactions and thus may be important in dimerization of the fusion protein in M4 leukemia cells. The CBFB/MYH11 fusion protein presumably disrupts the function of the AML1/CBFB transcription factor.

Rearrangements of the Long Arm of Chromosome 11 in Acute Monoblastic Leukemia (M5)

In 1980, Berger and co-workers first reported a higher than expected frequency of abnormalities of the long arm of chromosome 11 (11q) in 10 patients with acute monoblastic leukemia (AMoL). In an expanded series of cases, rearrangements of 11q were observed in 35% (12 of 34) patients with M5, and the investigators emphasized an especially strong association between abnormalities of 11q and the poorly differentiated form of AMoL (M5a). [76] Rowley in 1983 noted that the association between 11q abnormalities and M5a was particularly strong in children. [77] At the Fourth International Workshop on Chromosomes in Leukemia, the associations between 11q abnormalities, M5, and young age were confirmed. [49] Abnormalities of 11q23 are seen in 35% of M5 patients and in slightly less than one-half of patients with M5a.[76] [78] [79] [80]

Recurring translocations involving chromosome 11, band q23, are of great interest in human acute leukemia for at least three reasons. First, there are >30 different recurring rearrangements that involve 11q23 and, thus, along with band 14q32, 11q23 is one of the bands most frequently involved in rearrangements in human tumor

cells.[1] [3] [79] [80] The breakpoints in the 11q23 translocation partners include 1p32, 4q21, and 19p13.3 in ALL, and 1q21, 2q21, 6q27, 9p22, 10p11, 17q25, 19p13.3, and 19p13.1 in AML, especially the monoblastic and myelomonocytic subtypes. Second, these translocations occur in both lymphoid and myeloid leukemias. One common translocation in infants, t(4;11)(q21;q23), usually has a lymphoblastic phenotype, although the leukemia cells may express some myeloid surface markers; in some cases, variable numbers of monocytoid blast cells have been identified. [60] Other translocations, such as t(9;11) and t(11;19)(q23;p13.1), are common in monoblastic leukemias. These data suggest that a gene at 11q23 may be involved in determining the differentiation of primitive hematopoietic stem cells into lymphoblasts or monoblasts, or

that it may be a gene that is active in both cell lineages. Finally, translocations involving 11q23 have a very unusual age distribution; they account for about three-fourths of the chromosomal abnormalities in leukemia cells of children <1 year old. [78] [79] [80]

Translocations of 11q23 involve the *MLL* gene (also called *ALL1* or *HRX*). The *MLL* gene is a very large gene (>100 kb) with multiple large-sized transcripts in the 1215 kb range.[81] [82] All breakpoints fall within an 8.3-kb breakpoint cluster region of the gene encompassing exons 511; thus, *MLL* translocations can be detected by Southern blot analysis of DNA using a small cDNA probe containing these exons. [83] The MLL protein contains two potential DNA-binding motifs (zinc fingers and AT hooks), a transcriptional activation domain in the COOH-terminus, and a repression domain in the N-terminal portion. [84] This protein has homology to the *Drosophila* trithorax gene product, a transcription factor that regulates embryonic development and tissue differentiation in this organism. Thus, by homology to other proteins, it is likely that MLL can bind DNA and regulate the expression of downstream genes, presumed to be important in hematopoietic and lymphoid cell development.

Translocations of *MLL* result in the formation of a chimeric gene on the derivative 11 chromosome, consisting of the 5 region of *MLL* and the 3 region of the partner gene from the other chromosome, with subsequent expression of fusion mRNAs. The AT hook and repression domain are retained in the fusion protein; however, the strong activation domain is lost.[84] The splitting of these domains may alter the function of MLL and contribute to leukemogenesis. Chimeric mice containing an *MLL*/*AF9* fusion gene from the t(9;11) develop AML, whereas leukemia is not observed in mice with a disrupted *MLL* gene.[85] These results suggest that the *MLL*/*AF9* fusion gene plays a role in leukemogenesis and that the partner gene is required for this process. Whether the genes on the partner chromosomes interact with *MLL* to affect the myeloid versus lymphoid phenotype of the corresponding leukemias or the cell lineage and stage at which the translocation occurs dictate the phenotype is unknown.

t(3;3) and inv(3) in AML with Thrombocytosis

Several groups of investigators have identified an association of certain cytogenetic abnormalities of 3q with thrombocytosis in AML patients. [86] [87] The specific cytogenetic abnormalities associated with thrombocytosis in these patients involve bands 3q21 and 3q26 simultaneously, and they include inv(3)(q21q26), t(3;3)(q21;q26), and ins(5;3)(q14;q21q26) (insertion of chromosomal material from 3q into 5q). The t(3;3) and inv(3) account for 34% of AML cases in our series. Seven of the eight patients in our series with these abnormalities had platelet counts of >100,000/l before the initiation of cytoreductive therapy. Four patients had significant thrombocytosis with platelet counts of up to 1,731,000/l. This finding is quite striking when compared with the low incidence of thrombocytosis in a large group of patients with AML who were studied at the Fourth International Workshop on Chromosomes in Leukemia. [49] At that workshop, only eight (including two with inv(3)) of 716 patients had elevated platelet counts (unpublished data).

As might be expected, the most consistent bone marrow finding in these patients is an increase in the number of megakaryocytes, many of which are morphologically abnormal. In some of the patients, nearly all of the identifiable megakaryocytes are micromegakaryocytes. A few circulating megakaryoblasts were identified by the presence of platelet peroxidase activity in one of two patients whom the authors studied with this reaction. [87] These histopathologic findings alone are not indicative of acute megakaryoblastic leukemia.

The *EVI1* gene, located at 3q26, is not normally expressed in hematopoietic cells. This gene is activated by chromosomal rearrangements either 5 of the gene in t(3;3) or 3 of the gene in inv(3) by juxtaposition of the gene to enhancer elements of the ribophorin gene located at 3q21. [88] Activation of *EVI1* can also occur in t(3;21)(q26;q22) as part of the fusion mRNA, *AML1*/*EVI1*, that is transcribed from the der(3) chromosome. Abnormal expression of *EVI1* has also been detected in patients with myeloid leukemia and a normal karyotype,[89] suggesting that inappropriate activation of this gene occurs through various mechanisms. EVI1 is a transcription factor and contains a seven-zinc finger domain at the N-terminal end, a three-finger domain in the central part of the molecule, and an acidic domain distal to the second group of zinc fingers. Inappropriate gene expression induced by this transcription factor may mediate malignant transformation.

Environmental Associations with Acute Myeloid Leukemia

Mitelman et al. reported on a retrospective study of 162 Swedish patients with AML de novo; 52 of these patients gave a history suggesting occupational exposure to chemical solvents, insecticides, or petroleum products, whereas 110 patients had no such exposure. [90] Seventy-five percent of the exposed group had clonal chromosomal abnormalities, compared with 32% of the nonexposed group. There was a distinctly nonrandom pattern of changes in the exposed group, with 79% of the chromosomally abnormal cases (60% of all patients) having at least one of four specific abnormalities: 5/del(5q), 7/del(7q), +8, or +21. Other investigators have found similar associations.[91] [92] More recently, the Cancer and Leukemia Group B (CALGB) has reported on the association between smoking, irradiation, and exposure to solvents and hair dyes, and specific cytogenetic abnormalities and *RAS* oncogene activation in a prospective study of patients with AML or ALL. [93]

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THERAPY-RELATED MYELODYSPLASTIC SYNDROME AND ACUTE MYELOID LEUKEMIA

Characteristic chromosomal abnormalities have been found in patients who develop a therapy-related MDS or AML (t-MDS or t-AML) after chemotherapy or radiation therapy (or both) for an earlier disorder such as Hodgkin disease, non-Hodgkin lymphoma (NHL), carcinoma, rheumatoid arthritis, or renal transplantation. [94] [95] Several clinical and biologic subsets of t-MDS or t-AML have been recognized; distinct subsets are correlated with the specific therapy administered for the primary disease (Table 48-4). The most common type typically presents after a latency period of 5 years in patients who received alkylating agents. Therapy-related disease in two-thirds of these patients is first recognized from evidence of myelodysplasia (usually trilineage dysplasia), marrow failure, and pancytopenia. Not uncommonly, the initial disease is still present at the time of secondary bone marrow dysfunction. Half of cases diagnosed as t-MDS (<30% marrow blasts) will evolve to t-AML within a median of 6 months, but the other half of patients will die of infectious or hemorrhagic complications of pancytopenia first. Survival times are usually short (median, 8 months). Abnormalities of chromosomes 5 or 7 (or both) are characteristic of this subtype of t-MDS/t-AML. [94] [95] [96] [97] Chromosomal abnormalities are found in most patients with t-MDS prior to evolution to overt leukemia, and these changes are often multiple and complex. Thus, the detection of a clonal abnormality in a pancytopenic patient is convincing evidence of the existence of a malignant secondary neoplasm even though the percentage of blasts in the marrow is not yet elevated.

A second subtype of t-AML has been identified that is distinctly different from the more common leukemia that follows the use of alkylating agents or irradiation. This type of t-AML

TABLE 48-4 -- Contrasting Features of Therapy-Related Myeloid Leukemia Secondary to Either Alkylating Agents or Topoisomerase II Inhibitors

	Chromosome Abnormality	Preleukemia Phase	French-American-British Classification	Age	Latency	Response to Induction Chemotherapy	Long-term Survival	Chemotherapy Drugs
Alkylating agents	5/del(5q) 7/del(7q)	Myelodysplastic syndrome	Not classifiable by current criteria	Typically older patients	57 yr	Poor	Poor	Melphalan, mechlorethamine, chlorambucil, cyclophosphamide, carmustine, lomustine, semustine, procarbazine, dacarba-zine, mitolactol
Topoisomerase II inhibitor	t(11q23) t(21q22)	None	Usually M4, M5; some M1, M2, and ALL-L1	Younger patients	6 mo5 yr	Good	Poor	Etoposide, teniposide, actinomycin D, doxorubicin, 4 epi-doxorubicin, mitoxantrone
Various agents	t(15;17) inv(16)	None None	M3 M4Eo	Younger patients	23 yr <3 yr	Good Good	Good Good	Bimolane

Adapted from Thirman and Larson,[94] with permission.

was first observed among patients receiving extremely high cumulative doses of etoposide for lung cancer, but it has also been seen in patients receiving other drugs known to inhibit topoisomerase II (e.g., teniposide, doxorubicin). [94] [97] [98] Clinically, the secondary disease has a shorter latency period (12 years), manifests with overt leukemia, usually with monocytic features (patients rarely present with MDS), and responds to intensive remission induction therapy (Table 48-4). Balanced translocations involving the *MLL* gene at 11q23 and the *AML1* gene at 21q22 are common in this subgroup. [94] [97] [98] [95] A third subtype of t-AML is therapy-related APL characterized by t(15;17) and prior treatment for psoriasis with bimolane, a dioxopiperazine derivative that also interacts with topoisomerase II. [94]

Findings in the updated University of Chicago series of 270 patients with t-MDS/t-AML are summarized here (Le Beau et al. [96] and unpublished data). Seventy-two had Hodgkin disease, 61 had NHL, 22 had multiple myeloma or another hematologic malignant disease, 101 had various solid tumors, and 14 had nonmalignant disease, primarily an autoimmune disease or organ transplantation. One hundred eighteen of the patients had received both radiation therapy and chemotherapy prior to the development of the myeloid malignancy and 112 patients had received only chemotherapy. Thirty-eight patients had undergone only radiation therapy and, in the majority of these, major areas containing active marrow had been irradiated. The median time between the original diagnosis and the diagnosis of secondary bone marrow dysfunction was 62 months. The median latency period was shorter for patients who had been treated for solid tumors (56 months) and shorter for those who had received only chemotherapy (54 months).

Ninety-three percent (252 of 270) of our patients with therapy-related leukemia were chromosomally abnormal (Le Beau et al. [96] and unpublished data). More important, one or both of two consistent changes were noted in 70% (191 of 270) of patients. Among these 191 patients, 35 had loss of chromosome 5, 55 had a del(5q) (Fig. 48-2D), 25 had loss of 5q following unbalanced translocations, 96 had loss of chromosome 7, 26 had a del(7q), 15 had loss of 7q as a result of an unbalanced translocation, and 1 patient had a balanced translocation of 7q22. Sixty-two patients had abnormalities of both chromosomes 5 and 7. Overall, 115 patients (43%) had loss of 5q and 138 (51%) had loss of 7q. A del(5q) was the most common structural aberration in our series.

By analogy to retinoblastoma and Wilms tumor, there may be certain as yet unidentified critical genes located on 5q that are related to leukemogenesis. By cytogenetic analysis of 177 patients with malignant myeloid diseases and a del(5q), we identified a small segment of 5q, consisting of band 5q31, that was deleted in each patient.[100] [101] This segment has been termed the commonly deleted segment. Distal 5q contains a number of genes encoding growth factors, hormone receptors, and proteins involved in signal transduction or transcriptional regulation. [100] [101] These genes include several genes that are good candidates for a tumor suppressor gene as well as the genes encoding five hematopoietic growth factors (GM-CSF [CSF-2], IL-3, IL-4, IL-5, and IL-9). By FISH analysis of probes to metaphase cells with overlapping deletions involving 5q31, we have narrowed the commonly deleted segment to a region of approximately 1 Mb, flanked by the D5S479 and D5S500 markers and containing the *EGR1* and *CDC25C* genes.[100] [101] The five hematopoietic growth factor genes and seven other genes are excluded from this region. A similar commonly detected segment has been identified by other investigators. [102] To date, we have detected no mutations of the remaining *EGR1* or *CDC25C* alleles, suggesting that a novel tumor suppressor gene in 5q31 is involved in the pathogenesis of t-AML characterized by a del(5q). [101]

Boultwood et al. examined three patients with the 5q syndrome who had small deletions extending from q31q33, using gene dosage and in situ hybridization

analysis.[103] The commonly deleted segment was an 3 Mb region between *ADRB2* and *NKSF1* and includes the gene encoding the receptor for CSF-1. This region is distal to that identified in other studies, [100] [101] [102] suggesting that there is likely to be more than one region and hence more than one gene involved in the pathogenesis of myeloid disorders associated with abnormalities of chromosome 5. Whether the putative tumor suppressor gene in 5q33 is involved in myeloid leukemias or is restricted to the 5q syndrome is unknown.

To determine the location of genes on 7q that may be involved in myeloid leukemogenesis, Le Beau et al. examined the breakpoints and the extent of the del(7q) in 81 patients with MDS or de novo AML (55 patients) or t-MDS/t-AML (26 patients).[104] This analysis suggested that the deletions were interstitial, similar to the deletions of chromosome 5, and there may be two distinct deleted segments of chromosome 7. The majority of patients (65 of 81) had proximal breakpoints in q1122 and distal breakpoints in q2236; the smallest overlapping deleted segment was within q22. A subset of 16 patients had interstitial deletions involving the distal q arm (q3136), with a commonly deleted segment consisting of q3233. To define the deleted segment at 7q22 using molecular probes, the investigators used FISH with a panel of probes from 7q to examine 15 patients with deletion breakpoints in 7q22. The commonly deleted segment was 23 Mb and was flanked by the markers

D7S1503 and D7S1841.[104] Using similar techniques, Fischer et al. identified a slightly more distal, but overlapping, commonly deleted segment on 7q. [105] There are no known candidate tumor suppressor genes in this interval; thus, 7q22 may contain a novel tumor suppressor gene involved in t-AML.

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ACUTE LYMPHOBLASTIC LEUKEMIA

The most useful prognostic indicators in ALLthe most frequent leukemia in childrenare age, WBC count, immunophenotype, and karyotype. Children who are between 2 and 10 years old with a WBC count of <10,000/l and whose leukemia cells express the common ALL antigen (CALLA, CD10) have the best prognosis. It was rigorously demonstrated for the first time at the Third International Workshop on Chromosomes in Leukemia that the karyotype is an important independent prognostic factor in ALL.[31] A number of recurring cytogenetic abnormalities are associated with distinct immunologic phenotypes of ALL (Table 48-5), with distinct outcomes.[31] [106] [107] [108]

This review includes data on the chromosomal patterns of 330 patients evaluated at the Third International Workshop, [31] [109] as well as chromosomal patterns in four other large series of 443 adults[110] and 547 children.[111] [112] [113] The study of 173 adults and 157 children at the Third International Workshop revealed that a high proportion (65%) of the patients had clonal abnormalities. [31] [109] Also, of the 213 aneuploid patients, 51% were pseudodiploid, 37% were hyperdiploid, and only 12% were hypodiploid. Notable differences in the frequency of recurring abnormalities between children and adults were observed for t(9;22) and hyperdiploidy (>50 chromosomes). The t(9;22) was observed in 5% of children but in 30% of adults with ALL, whereas a hyperdiploid karyotype was found in 30% of children but rarely in adults (25%). The presence of the specific translocations, t(4;11) or t(9;22), was associated with treatment failure even when intensive therapy was used, whereas t(12;21) and hyperdiploidy (5060 chromosomes) were associated with a favorable outcome.

Specific Abnormalities in Acute Lymphoblastic Leukemia

t(8;14)

A reciprocal translocation involving the long arms of chromosomes 8 and 14 (t(8;14)(q24;q32)) has been detected in a high proportion of patients with Burkitt tumors of both African and non-African origin. An identical translocation is observed in ALL patients with L3-type leukemia cells, indicating that Burkitt lymphoma and most B-cell ALL of the L3 type are probably different manifestations of the same disease (Table 48-5). Sixteen patients with this rearrangement were studied at the Third International Workshop (7% of all cases with abnormalities). [31] In this group, there was an excess of males over females and of adults over children. Most notable was the finding that, with one exception, all patients tested had B-cell markers and in all but one case the disease was FAB type L3. In the exceptional patient, the leukemia cells had a pre-B-cell phenotype. This group of patients had a high incidence of central nervous system involvement at diagnosis and a poorer prognosis (complete remission rate: children, 83%; adults, 44%; median survival: 5 months) than any other group of patients classified according to chromosomal patterns. A poor outcome was also noted in a recent series of 21 adult patients with a t(8;14), all of whom had B-cell ALL (62% complete remissions; median event-free survival [EFS], 2 months). [110] Most recently, however, the use of short-duration, high-intensity chemotherapy programs has markedly improved the outcome of patients with B-cell ALL.[114]

Variant translocations have been reported in Burkitt lymphoma and B-cell ALL (t(2;8)(p12;q24) and t(8;22)(q24;q11)). The first chromosomal abnormalities to be analyzed at the molecular level were the three translocations characteristic of L3 leukemia and Burkitt lymphoma; the outcome of these studies is described later in the section on lymphomas.

t(4;11)

Translocations involving 11q23 are observed in 57% of ALL patients. [31] [110] [111] Of these, the most common is t(4;11)(q21;q23). The t(11;19)(q23;p13.3) is second in frequency; however, this rearrangement is not limited to ALL in that 50% of these patients have AML, usually AML-M5. Patients with t(4;11) have high leukocyte counts (median WBC count, 183,000/l), L1 or L2 morphology, an immature immunophenotype (CD10, CD19+), B-cell lineage leukemia, and a poor outcome. [31] [110] [111] [112] [113] [114] [115] [116] [117] [118] [119] In one series, adults with t(4;11) had a remission rate of 75% but a median EFS of only 7 months. [110] Children with t(4;11) have a similar poor outcome (88% complete remissions, median survival of 10 months).[31] [111] [112] [113]

A notable finding is that acute leukemia with t(4;11) may express myeloid antigens (50%). In most cases of t(4;11) acute leukemia, blasts have been described as lymphoid in appearance and have been classified by light microscopy as L1 or L2 according to the FAB system. [31] [110] In some cases that otherwise appear to be lymphoid, occasional blasts may appear monocytic, and in some, populations of lymphoid and monocytoid blasts may occur in approximately equal proportion. [115] By ultrastructural examination, Parkin and associates confirmed the presence of a myeloid component in the leukemia of many of their patients. In addition to noting monocytoid blasts in some patients, they observed dysplasia of myeloid cells. Positive staining for myeloperoxidase or with Sudan black B (myeloid cytochemical markers) may be present in some cases, and the nonspecific esterase reaction (granulocyte-monocyte cytochemical marker) may be positive in variable numbers of cells.[60] [115] These leukemia cells are generally terminal deoxynucleotidyl transferase (TdT) positive, and they have expressed pan-B-cell antigens in the majority of cases studied.[60] [115] CD10 positivity has been observed in some instances. T-cell markers have consistently been negative.

Of note is the association of translocations involving 11q23 in infant ALL (6080%); this association is particularly interesting in view of the low incidence of ALL in this age group (acute leukemias in the very young are usually of the myeloid type). [31] [117] As described earlier, the breakpoint on 11q23 involves the *MLL* gene. Several recent studies have examined the frequency of *MLL* rearrangements in infant ALL using Southern blot analysis, [117] [118] or RT-PCR[119] to detect the t(4;11). These studies revealed *MLL* rearrangements in 70% and 81%, respectively, including some cases that were normal or inadequate by conventional cytogenetic analysis. [117] [118] [119] Abnormalities of *MLL* were associated with early treatment failure and a very poor outcome; in one series, the estimated EFS for patients with *MLL* rearrangements was 19% at 3 years, compared with 46% for patients with germline *MLL*.[118] Similar results were observed in a second series (EFS at 24 months: 24% for patients with rearranged *MLL* and 100% for patients with germline *MLL*).[119] Among patients with *MLL* rearrangements, t(4;11) may confer a particularly poor prognosis, and some authors have recommended intensified therapy, including bone marrow transplantation, for this group of patients. [119] Thus, rearrangements affecting *MLL* represent a major class of mutations in acute leukemia and identify patients with a poor outcome.

t(9;22)

Ph+ leukemia occurs in two major forms, CML (described earlier) and ALL. Thirty-nine Ph+ ALL patients (18% of patients with abnormalities) were evaluated at the Third International

TABLE 48-5 -- Cytogenetic-Immunophenotypic Correlations in Malignant Lymphoid Diseases

Phenotype	Chromosome Abnormality	Frequency [a] (%)	Involved Genes [b]	
Acute lymphoblastic leukemia				
Precursor B	t(12;21)(p12;q22)	25	*TEL*	*AML1*
	t(17;19)(q2122;p13)	1	*HLF*	*E2A*
Pre-B, B	t(9;22)(q34;q11)	10[c]	*ABL*	*BCR*
	t(1;19)(q23;p13)	6 (30)	*PBX1*	*TCF3* (*E2A*)
B(SIg+)	t(8;14)(q24;q32)	5 (95)	*MYC*	*IGH*
	t(2;8)(p12;q24)	<1 (1)	*IGK*	*MYC*
	t(8;22)(q24;q1)	<1 (4)	*MYC*	*IGL*
	dic(9;12)(p11;p12)	1		
B or B-myeloid	t(4;11)(q21;q23)	5	*AF4*	*MLL*
Other	hyperdiploidy (5060 chromosomes)	10		
	del(9p),t(9p)	10		
	del(12p),t(12p)	10		
T	t(11;14)(p15;q11)	1	*RBTN1*	*TCRA*
	t(11;14)(p13;q11)	1	*RBTN2*	*TCRA*
	t(8;14)(q24;q11)	<1	*MYC*	*TCRA*
	inv(14)(q11q32)	<1	*TCRA*	*IGH*
	inv(14)(q11q32)	<1	*TCRA*	*TCL1*
	t(10;14)(q24;q11)	1	*HOX11*	*TCRA*
	t(1;14)(p34;q11)	<1	*LCK*	*TCRD*
	t(1;14)(p32;q11)	1	*TALI*	*TCRD*
	t(7;9)(q3435;q32)			
	t(7;9)(q3435;q34)	<1	*TCRB*	*TAL2*
	t(7;7)(p15;q11)		*TCRB*	*TAN1*
	t(14;14)(q11;q32)	<1	*TCRG*	
	t(7;14)(q3435;q11)	<1	*TCRA*	*IGH*
	t(7;14)(p15;q11)	<1	*TCRB*	*TCRD*
	t(7;19)(q3435;p13)	<1		
	del(9p),t(9p)	<1 (10)	*CDKN2*	
Non-Hodgkin lymphoma				
B-cell NHL				
Burkitt	t(8;14)(q24;q32)	95	*MYC*	*IGH*
	t(2;8)(p12;q24)	1	*IGK*	*MYC*
	t(8;22)(q24;q11)	4	*MYC*	*IGL*
Follicular DLCL	t(14;18)(q32;q21)	80 20	*IGH*	*BCL2*
DLCL	t(3;22)(q27;q11)	45 for all	*BCL6*	*IGL*
	t(3;14)(q27;q32)	t(3q27)	*BCL6*	*IGH*
	t(3q27)		*BCL6*	
MCL	t(11;14)(q13;q32)		*CCND1*	*IGH*
LPL	t(9;14)(p13;q32)		*PAX5*	*IGH*
SLL	t(14;19)(q32;q13.3)		*IGH*	*BCL3*
MALT	t(11;18)(q21;q21)			
T-cell NHL				
(Ki-1+)NHL ALCL	t(2;5)(p23;q35)	75	*ALK*	*NPM*
Other	See T-cell ALL			
	t(4;16)(q26;p13.1)	>1	*IL2*	*BCM*
CTCL	t(10q24)		*LYT10*	
Chronic lymphocytic leukemia				
B	t(11;14)(q13;q32)	10	*CCND1*	*IGH*
	t(14;19)(q32;q13)	10	*IGH*	*BCL3*
	t(2;14)(p13;q32)	5		*IGH*
	t(14q32)	20		
	del(13q)	30		
	+12	30		
T	t(8;14)(q24;q11)	5	*MYC*	*TCRA*
	inv(14)(q11q32)	5	*TCRA/D*	*IGH*
	inv(14)(q11q32)	5	*TCRA/D*	*TCL1*
Multiple myeloma				
B	t(11;14)(q13;q32)	10	*CCND1*	*IGH*
	t(14q32)			

Adult T-cell leukemia/lymphoma				
T	t(14;14)(q11;q32)		*TCRA*	*IGH*
	inv(14)(q11q32)		*TCRA/D*	*IGH*
	+3			

Abbreviations: SIg, surface immunoglobulin; DLCL, diffuse large cell lymphoma; MCL, mantle cell lymphoma; LPL, lymphoplasmacytoid lymphoma; SLL, small lymphocytic lymphoma; MALT, mucosa-associated lymphoid tumor; Ki-1, anti-CD30 antibody; ALCL, anaplastic large cell lymphoma; CTCL, cutaneous T-cell lymphoma.

[a] The percentage refers to the frequency within the disease overall. The number within parentheses (also a percentage) refers to the frequency within the morphologic or immunologic subtype of the disease.

[b] Genes are listed in order of citation in karyotype; e.g., for precursor B-cell ALL, *TEL* is at 12p12 and *AML1* is at 21q22.

[c] By cytogenetic analysis, the frequency in children is 5% and in adults is 25%; using molecular probes, this frequency is 30% in adults.

Workshop on Chromosomes in Leukemia; 30 were adults and nine were children. [31] [109] The incidence of Ph+ patients with ALL was 17% for adults and 6% for children. Thus, the Ph chromosome is the most frequent rearrangement in adult ALL. Thirty-six patients had the typical t(9;22) and the remaining three had variant translocations. The incidence of the variant form was 8%, which is similar to that observed in CML patients. About one-half of the patients showed abnormalities in addition to the Ph chromosome, a frequency that is substantially higher than that observed in CML in the chronic phase. With the exception of trisomy 8, which is seen occasionally, these abnormalities differ from those observed in the acute phase of CML. Monosomy 7 is a common secondary abnormality in Ph+ ALL and is associated with a poorer outcome. [120] A chromosomally normal cell line is frequently noted in the bone marrow of Ph+ ALL patients (70%), whereas normal cells are rarely observed in untreated CML patients.

The results of more recent studies using newer immunophenotyping techniques have suggested that Ph+ ALL is of pre-B-cell lineage; however, some cases have had both B-cell and myeloid markers. [121] [122] In a prospective study of ALL patients conducted by CALGB, 23% of adult ALL patients had t(9;22); this frequency was 30% when molecular techniques were used to detect the *BCR/ABL* fusion. [122] Approximately half of patients with B-cell lineage ALL were Ph positive. These prospectively identified patients were no older than Ph negative patients (median age, 39 vs. 37 years), nor was their complete remission rate significantly lower (71% vs. 77%). However, median remission duration (10 vs. 18 months) and survival (11 vs. 22 months) were considerably shorter for Ph+ ALL patients.

Molecular studies of Ph+ ALL have revealed two distinct subgroups of patients. In the first group (about 30% of Ph+ adult cases), the molecular rearrangement is identical to that observed in CML, in that the breaks occur within the *ABL* gene and within the bcr region of the *BCR* gene, giving rise to a chimeric gene and the production of an 8.5-kb message and a 210-kd fusion protein. In the remaining patients, the breakpoint occurs upstream (5) of bcr but still within the *BCR* gene, giving rise to smaller fusion messages (6.57.4 kb) and smaller proteins (185190 kd). [123] [124] The clinical outcome for both groups of patients appears to be similar. At present, we do not know whether the structural difference in the 210-kd and 185-kd proteins corresponds to functional differences in the leukemia cells. Both proteins are involved in constitutive signaling via the RAS pathway of signal transduction. In a mouse model, animals given transplants of cells transfected with $p185^{BCR/ABL}$ develop tumors more rapidly than those given transplants of $p210^{BCR/ABL}$ -containing bone marrow cells. Such a functional difference might account for the biologic and clinical differences of Ph+ ALL, which, although characterized by the same chromosomal abnormality, is a disease entity distinct from CML. Patients in whom the Ph chromosome is restricted to lymphoblasts have shorter survivals than those with multilineage involvement. [26] [27]

t(1;19)

In 1978, pre-B-cell ALL was recognized as a distinct immunologic subtype of ALL that can be distinguished from null cell and B-cell ALL by the presence of cytoplasmic immunoglobulin -chain (C) expression. Subsequently it was recognized that pre-B-cell leukemias have rearrangements of the immunoglobulin heavy chain genes, and occasionally of the light chain genes, and that patients with this form of leukemia have a less favorable response to therapy than do patients who have common ALL (CALLA positive). In 1984, Williams et al. described the association of a recurring chromosomal abnormality, namely, a reciprocal translocation involving chromosomes 1 and 19 (t(1;19)(q23;p13)), with pre-B-cell ALL. The t(1;19) is observed in 30% of patients with pre-B-cell ALL and in 6% of all ALL cases. This association was subsequently confirmed by other investigators, who also noted that children with pre-B-cell ALL and a t(1;19) had low WBC counts and were CD10+. [125] A characteristic surface antigen profile is CD19+, CD10+, CD22+, CD34, and CD20+/. Patients with the t(1;19) experience early treatment failure, suggesting that this translocation may distinguish a subgroup of patients with pre-B-cell ALL who have a poor prognosis. [126] [127] In one large study, the adverse outcome of patients with the t(1;19) remained significant even after adjustment for recognized adverse clinical features, indicating that it is an independent risk factor. [127] The t(1;19) occurs in two forms: a reciprocal translocation, t(1;19)(q23;p13), noted in 25% of cases, and an unbalanced form characterized by two normal chromosome 1 homologs and a rearranged chromosome 19, der(19)t(1;19)(q23;p13) (75%). Patients with the balanced, reciprocal translocations have a poorer outcome than do patients with the unbalanced form.

The t(1;19) involves the *E2A* gene at 19p13, which encodes two transcription factors (E12 and E47), which bind to enhancer elements in the *IGK* gene, as well as the regulatory elements of other genes. [128] [129] The *E2A* gene is juxtaposed with *PBX1*, a homeobox (HOX) gene on chromosome 1. *HOX* genes encode DNA-binding transcription factors that regulate developmental processes. *E2A/PBX1* fusion mRNAs code for chimeric proteins that consist of the transcriptional activating domain of E12/E47 and the DNA-binding and protein dimerization domains of PBX1. [128] [129] *E2A* is expressed in all tissues, but the *PBX1* gene is not expressed in lymphoid cells, although it is expressed in many other fetal and adult tissues.

PBX1 normally forms complexes with select HOX proteins, and these heterocomplexes then regulate transcription of target genes in a positive or negative manner. [128] [129] The E2A/PBX1 fusion protein is a potent transactivator and thus may directly activate gene transcription of PBX1-HOX complex target genes. A second possibility is that E2A/PBX1 may bind DNA cooperatively with a specific HOX protein, and the potent transactivation domain of E2A may activate gene transcription. Alternatively, PBX-HOX complexes formed with other ubiquitously expressed PBX family members, PBX2 and PBX3, might normally inhibit transcription, whereas E2A/PBX1/HOX complexes may induce transcription of genes normally repressed in lymphoid cells. In each of these scenarios, the presence of the E2A/PBX1 fusion protein may result in the transactivation of a cadre of genes that are not normally expressed in lymphoid tissues.

t(12;21) in Precursor B-Cell Acute Lymphoblastic Leukemia

Recently, a new recurring translocation, t(12;21)(p12;q22), has been identified in a high proportion (25%) of childhood B-lineage ALL, specifically, precursor B-cell leukemia. [130] [131] The translocation is not easily detected by cytogenetic analysis because of the similarity in size and banding patterns of 12p and 21q. However, the rearrangement can be reliably detected using RT-PCR or FISH analysis. [131] The t(12;21) defines a distinct subgroup of patients characterized by age between 1 and 10 years, B-lineage immunophenotype (CD10+, CD19+), and a favorable outcome. [132] [133] It is not seen in T-cell ALL and is uncommon in adults (4% of ALL cases). In a recent series, patients with the t(12;21) had a 5-year EFS of 91%, compared with 65% for patients without this rearrangement. [132] One-half of these patients would have fallen into a high-risk group according to standard risk factors; thus, the presence of this rearrangement may identify a subset of patients within the high-risk group who would benefit from less toxic and less intensive therapy.

The t(12;21) fuses the *TEL* gene at 12p12 with the *AML1* gene at 21q22 and results in the production of a fusion protein (Fig. 48-3). [134] TEL is a ubiquitously expressed 453-amino acid protein

that contains two domains found in other ETS family transcription factors: a helix-loop-helix (HLH) protein dimerization domain and an ETS DNA-binding domain. The consequence of the translocation is fusion of the 5 HLH domain of TEL with the 3 DNA-binding and transactivation domain of the AML1 transcription factor. [134] Via the HLH domain, TEL/AML1 can form homodimers as well as heterodimers with the TEL protein. [133] Moreover, the TEL/AML1 complex inhibits transactivation of gene

expression by the normal AML1 protein, and the HLH domain of TEL is required for repression. Recent studies have shown that in the majority of ALL cases with the t(12;21), the *TEL* allele on the other chromosome 12 homolog is deleted.[135] Alteration of both *TEL* alleles in leukemia cells suggests that TEL may be a negative regulator of cell proliferation, i.e., a tumor suppressor gene. The *TEL* gene is also involved in several other recurring translocationst(5;12)(q33;p12) in chronic myelomonocytic leukemia (CMMoL), t(9;12)(q34;p12) in AML, ALL, and CML, and t(12;22)(p12;q11) in myeloproliferative disorderssuggesting that interference with the regulation of TEL-targeted genes plays a significant role in leukemogenesis.

Hyperdiploidy with 5060 Chromosomes

The leukemia cells of some patients with ALL are characterized by a gain of many chromosomes and relatively few structural abnormalities. Two distinct subgroups are recognized: a group with 14 extra chromosomes (4750), and the more common group with >50 chromosomes. Chromosome numbers usually range from 51 to 60, and a few patients may have a clone with up to 65 chromosomes. Certain additional chromosomes are commonly seen, typically the X chromosome and chromosomes 4, 6, 10, 14, 17, 18, and 21.[136] [137] Chromosome 21 is gained most frequently (97100% of cases), and multiple additional copies of this chromosome are also frequent.[31] [136] [137]

Patients who have hyperdiploidy with >50 chromosomes have all of the previously recognized clinical factors that indicate a good prognosis, including age between 1 and 9 years, low WBC count (median, 6,700/l), and favorable immunophenotype (early pre-B or pre-B). [31] In a recent analysis of 186 children with hyperdiploid ALL, Raimondi et al. suggested that ALL defined by 5155 chromosomes may be a distinct clinical entity, different from that characterized by 5665 chromosomes. [138] The 105 patients in the first group (5155 chromosomes) had an EFS at 5 years of 72%, compared with 86% (*P* = 0.04) for those patients with >56 chromosomes (63 patients). Structural rearrangements occur in hyperdiploid ALL cells more frequently than was previously recognized, perhaps in as many as 50% of cases. [138] The most common structural abnormalities are duplication of 1q (15%) and an i(17q) (5%). [138] The t(9;22) and t(1;19) have also been observed. Overall, the presence of structural abnormalities does not appear to influence EFS; however, specific recurring abnormalities, such as t(9;22), have a poor prognosis. [138]

T-Cell Acute Lymphoblastic Leukemia

A distinct pattern of recurring karyotypic abnormalities in T-cell neoplasms has emerged. [1] [2] Rearrangements involving the proximal bands of chromosome 14 (14q11) and two regions of chromosome 7 (7q3435 and 7p15) are particularly frequent in T-cell malignancies (Table 48-5). The majority of the T-cellspecific abnormalities involve 14q11. The first recurring abnormality to be defined was the reciprocal translocation involving chromosomes 8 and 14, t(8;14)(q24;q11). Subsequently other abnormalities involving 14q11 were recognized. Chromosome 7 is involved in multiple recurring abnormalities, several of which involve 7p15 (t(7;7)(p15;q11), t(7;14)(p15;q11)), and others which involve the distal long arm (t(7;14)(q3435;q11), t(7;9) (q3435;q34), t(7;9)(q3435;q32), and t(7;19)(q3435;p13)). [1] In addition to their occurrence in T-cell leukemia, these T-cell-specific abnormalities have also been observed in lymphomas of T-cell origin. The genes that are located at the breakpoints of a number of these abnormalities have been identified and are described in a later section (Table 48-5). Patients with T-ALL are most often young males and often have a mediastinal tumor mass, high WBC count, and leukemia cells in the cerebrospinal fluid. These same clinical characteristics are associated with lymphoblastic lymphoma, another T-cell malignancy.

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MALIGNANT LYMPHOPROLIFERATIVE DISORDERS

Cytogenetic analyses of NHL have been reported in several large series. [139] [140] [141] [142] [143] [144] [145] These investigations have demonstrated that a high proportion of cases (>90%) are characterized by clonal chromosomal abnormalities and, more important, that many of the recurring abnormalities correlate with histology and immunophenotype (Table 48-5). For example, t(14;18) is observed in a high proportion of follicular small cleaved cell lymphomas (7090%), most patients with t(3;22)(q27;q11) or t(3;14)(q27;q32) have B-cell diffuse large cell lymphomas (DLCL), and patients with t(8;14)(q24;q32) have either small noncleaved cell or diffuse large cell lymphomas. Band 14q32, the location of the Ig heavy chain gene (*IGH*), is frequently involved in translocations in neoplasms of B-cell lineage (70%) (Table 48-5). In contrast, a large proportion of neoplasms of T-cell origin are characterized by rearrangements that involve 14q11, 7q3435 or 7p15, the locations of the T-cell-receptor genes, *TCRA/D, TCRB*, and *TCRG*, respectively (Table 48-5).

A number of karyotypic parameters have been reported to influence survival in NHL. [142] [146] [147] [148] [149] [150] [151] [152] [153] [154] [155] [156] [157] Adverse factors include the complexity of the karyotype,[141] [144] the number of rearranged chromosomes,[144] and the presence of specific chromosomal abnormalities, such as t(8;14), +7, breaks at 1q2123, and breaks at 6q2125. [143] [144] [146] [147] [148] In contrast, t(14;18) and rearrangements of *BCL6* are associated with a favorable prognosis. [142] [144] [147] Histology remains the most important prognostic factor in predicting response to therapy and survival in lymphoma. Chromosomal abnormalities correlate with histology in NHL, albeit incompletely, and they may be independent prognostic factors. Within histologic subtypes, they may identify patients who are likely to be long-term survivors. Thus, a major focus of future studies will be to determine the prognostic significance of recurring chromosomal abnormalities in NHL, including that of the less frequent abnormalities and secondary changes.

Burkitt Lymphoma

In 1972 Manolov and Manolova identified a consistent abnormality (14q+) in the cells of fresh Burkitt lymphomas and in cultured cell lines. Several years later Zech and associates suggested that the rearrangement was a reciprocal translocation involving chromosomes 8 and 14, t(8;14)(q24;q32). The t(8;14) has also been observed in nonendemic Burkitt tumors in patients from America, Europe, and Japan; thus, this rearrangement is a highly characteristic anomaly in Burkitt tumors. This translocation was identified in Burkitt tumors that lacked any markers for the Epstein-Barr virus (EBV) as well as in EBV-positive tumors. It should be noted that the t(8;14) has also been observed in other lymphomas, particularly small noncleaved cell (non-Burkitt) and large cell immunoblastic lymphomas, AIDS-associated Burkitt lymphoma (100%), and AIDS-related DLCL (30%), as well as in B-cell ALL. [139] [149] [152] [153] [154]

As additional Burkitt tumors were examined, it became apparent that at least two other related translocations occur. All three translocations involved chromosome 8 with a break in the same band, 8q24. One variant translocation involved chromosome 2 with a break in the short arm (t(2;8)(p12;q24)), and

the other involved chromosome 22 with a break in band q11 (t(8;22)(q24;q11)). As discussed earlier, these same translocations have been seen in some patients with B-cell ALL.

The t(8;14) involves a break within the *IGH* locus on chromosome 14 and either 5 or within *MYC* on chromosome 8 in a head-to-head orientation. The translocations correlate roughly with the two forms of Burkitt lymphoma: endemic Burkitt lymphoma usually contains breaks upstream of *MYC*, whereas sporadic or AIDS-related Burkitt lymphoma usually contains breaks within *MYC*.[153] [154] MYC is a ubiquitous nuclear protein whose expression is normally tightly controlled, with an extremely short half-life. MYC plays a role in a number of cellular processes, including proliferation and apoptosis, and its oncogenic properties are due to its constitutive expression. It is an immediate early response gene and is rapidly up-regulated during mitogenic stimulation in lymphocytes. Furthermore, *MYC* antisense oligonucleotides prevent activated lymphocytes from entering S phase, cause growth arrest of hematopoietic cells, and induce differentiation of lymphoid cells. Both in vitro and in vivo studies have demonstrated its transforming abilities: expression of *MYC* results in the transformation of EBV-immortalized B-cell lines, [155] while E-*Myc* transgenic mice frequently develop lymphoblastic lymphoma. [156] The biologic function of MYC resides in two regions. The amino-terminus is important in activating transcription, whereas the carboxy-terminus contains a basic-helix-loop-helix-leucine zipper domain that promotes interaction with a related protein, MAX. [157] Binding to MAX appears to be essential for the transforming activity of MYC. MAX has been shown to have two other partners that are important in regulating its function: MAD and MXI1.[157] Both of these proteins are thought to influence MYC-mediated activation of target genes, either by binding to MAX and sequestering it from forming complexes with MYC or by competing with MYC-MAX heterodimers for binding to common target sites.[157]

t(14;18)

Between 70% and 90% of follicular lymphomas and 20% of diffuse B-cell lymphomas have the t(14;18) in which the *BCL2* gene at 18q21 is juxtaposed to the *IGH* J segment, [141] [158] leading to the deregulated expression of *BCL2*.[153] [154] [159] Other lymphocytic malignancies that overexpress *BCL2* but do not harbor t(14;18) include hairy cell leukemia and chronic lymphocytic leukemia (CLL). The cloning of this gene led to the discovery of a new class of oncogenes that, instead of promoting proliferation, contribute to development of a neoplastic state by preventing programmed cell death. [160]

The gene encodes a 26-kd protein that is localized to the mitochondrial outer membrane, as well as the smooth endoplasmic reticulum and perinuclear membrane, [161] and functions to increase cell survival. For example, BCL2 protects lymphoid cells against a number of apoptotic stimuli, including -irradiation, glucocorticoids, and the cross-linking of cell surface receptors. [161] Targeted gene disruption studies have shown that BCL2 is required for survival beyond the first few weeks of life. *Bcl2/* mice developed polycystic kidney disease, hair hypopigmentation, and immunodeficiency as a result of massive B- and T-lymphocyte death. [161] Transgenic mice containing a *Bcl2-Ig* minigene developed polyclonal follicular hyperplasia, and some, after a long latency, developed high-grade lymphomas, many of which possessed translocations involving *Myc*.[162] Of note, many normal individuals harbor the t(14;18) in tonsils and peripheral blood lymphocytes. [163] These findings suggest that the t(14;18) occurs as an early event in the multistep process of lymphomagenesis. Moreover, *BCL2* overexpression is not transforming per se, but requires the presence of other transforming events, such as *MYC* or *TP53* mutations.

BCL2 interactions with other proteins, specifically BAX [164] and BAD,[165] regulate its function. BAX is capable of dimerizing with itself and with BCL2. In cells in which BAX is overexpressed, apoptosis is accelerated in response to apoptotic stimuli. In cells in which BCL2 is overexpressed, heterodimers with BAX predominate, and cell death is repressed. [164] Thus, the ratio of BCL2 to BAX determines the likelihood of a cell undergoing apoptosis on exposure to a given stimulus. BAD, another partner of BCL2, promotes cell death by dimerizing with either BCL-XL or BCL2, decreasing the amount of BAX bound to either BCL-XL or BCL2 and thereby increasing the amount of BAX homodimers present.[161]

The prognostic significance of having the t(14;18) or of overexpressing *BCL2* is unclear, and studies have been conflicting. One study suggested that rearrangements in the major breakpoint region of the *BCL2* gene were associated with a shorter disease-free period in patients with extranodal lymphomas, but this proposition

remains to be confirmed.[166] Another study examining diffuse large B-cell lymphoma[167] showed that high levels of *BCL2* expression predicted a shorter disease-free survival. The biologic basis for the poorer prognosis may be that such cells have increased resistance to chemotherapeutic drugs. [168]

t(11;14)

The t(11;14)(q13;q32) is most commonly seen in a relatively new pathologic entity known as mantle cell lymphoma. [169] In the Kiel classification, these tumors were classified as centrocytic lymphomas and in the Working Formulation as small cleaved cell, diffuse or nodular, or, rarely, diffuse mixed or large cleaved cell lymphomas. In most series, the reported percentage of cases with the t(11;14) by molecular analysis has varied between 30% and 55%, but these figures may be underestimates, as only the major translocation cluster regions have been looked for. Besides mantle cell lymphomas, the t(11;14) has been reported in 3% of multiple myelomas and in up to 20% of prolymphocytic leukemias.[169] Mantle cell lymphomas are currently associated with a poor prognosis, with a median survival from diagnosis of 3 years.

This translocation results in the juxtaposition of the *IGH* gene, specifically the JH segment of the *Ig* gene locus, to a putative oncogene, *BCL1*. However, the gene itself was not identified until 1991, after the discovery of the parathyroid adenomatosis 1 (*PRAD1*) gene, identified at 11q13 by virtue of its clonal rearrangement with the parathyroid hormone locus, in a subset of benign parathyroid adenomas. *PRAD1* was overexpressed in lymphoid malignancies with the t(11;14)(q13;q32) and was found to be 100130 kb away from the *BCL1* locus. Subsequently, *PRAD1*(*BCL1*) was found to have significant sequence homology with the known cyclins, specifically cyclin D1 (*CCND1*).[170] The D-type cyclins appear to act as growth factor sensors, causing cells to go through the restriction start point of the cell cycle at G_1 and committing them to divide. CCND1 together with activated cyclin-dependent kinase 4, or CDK4, phosphorylates and inactivates retinoblastoma protein, releasing from it E2F, a transcription factor responsible for activating a series of genes required for cell division. [171] The ectopic expression of *CCND1* under the control of the E promoter in mice is not transforming per se, as E-*Bcl1* mice do not develop lymphoma. In lymphoid cells, the oncogenic ability of *CCND1* appears to depend on synergy with other oncogenes, specifically *MYC*.[172] Circumstantial evidence of its involvement in other tumors includes the frequent amplifications of 11q13 and overexpression of *CCND1* in solid tumors, including head and neck, esophageal, bladder, breast, small cell lung, and hepatocellular carcinomas. [173]

Translocations of 3q27

The *BCL6* oncogene was cloned from the recurring breakpoint at 3q27 in cells characterized by a t(3;22)(q27;q11), t(3;14)(q27;q32), or, rarely, t(2;3)(p12;q27). [174] [175] *BCL6* rearrangements occur in 40% of diffuse large cell lymphomas and, in some series, up to 10% of follicular lymphomas. [176] The translocations lead to truncation of the gene within the first exon or the first intron, substitution of its promoter sequences with an *Ig* promoter, and deregulated expression.

The *BCL6* gene product is a 96-kd nuclear protein that is predominantly expressed in the B-cell lineage, particularly in mature B cells, but not in immature bone marrow precursors or the more mature plasma cell. The protein contains six zinc finger motifs and an N-terminal POZ domain, which in related proteins have been shown to regulate the DNA binding of zinc finger proteins. [177] *BCL6* has been shown to be a potent transcriptional repressor. [178] A role in germinal center formation is suggested by topographic restriction of *BCL6* expression to germinal centers in normal human lymphoid tissue, and by the observation that mice with targeted disruptions of *BCL6* are incapable of forming germinal centers. Such mice also develop an inflammatory disorder that appears to be Th2-like lymphokine dependent, implicating a role for *BCL6* in the control of inflammatory responses. [179]

Two retrospective studies have assessed the clinical significance of *BCL6* rearrangements. One found a more favorable outcome in terms of survival and freedom from progression among diffuse lymphomas with a large cell component,[180] whereas the other failed to confirm these findings.[181] At present, the prognostic relevance of *BCL6* rearrangements or expression is unclear.

T-Cell Non-Hodgkin Lymphoma

A number of recurring chromosomal abnormalities have been recognized in lymphomas of T-cell origin (Table 48-5). These abnormalities are also observed in T-cell leukemias. Similar to B-cell neoplasms, in which rearrangements frequently involve the chromosomal bands containing the immunoglobulin gene loci, T-cell neoplasms often have rearrangements involving band q11 of chromosome 14, the site of the T-cell receptor -chain and -chain genes (*TCRA, TCRD*),[182] [183] or, less often, one of two regions of chromosome 7 (7q3435 and 7p15) to which the T-cell receptor -chain (*TCRB*) and -chain (*TCRG*) genes have been localized, respectively (Table 48-5).[1] With few exceptions, the involved gene on the partner chromosome encodes a transcription factor whose expression is deregulated or activated in an aberrant tissue as a result of the rearrangement (Table 48-5).[3] [4] These studies indicate that, perhaps as a result of their capacity to be specifically rearranged, transcribed, and mutated in B cells or in T cells, the immunoglobulin and T-cell receptor gene loci are appropriate DNA sequences to mediate the activation of cellular oncogenes. A chromosomal rearrangement that brings an oncogene under the controlling influence of promoters or enhancers that are active for immunoglobulin synthesis in B-cells or for T-cell receptor synthesis in T cells may as a consequence impart a proliferative advantage to that cell and result in malignant clonal expansion.

Ki-1 Anaplastic Large Cell Lymphomas

A distinctive subtype of NHL, namely, Ki-1-positive anaplastic large cell lymphoma (Ki-1+ ALCL), has been characterized during the past few years. The Ki-1 antigen (CD30) is observed in nearly all cases of Hodgkin disease; however, this antigen is also expressed by a variable proportion of lymphoma cells in a variety of non-Hodgkin lymphoma subtypes. A subset of non-Hodgkin lymphoma with distinctive clinical and morphologic features is strongly Ki-1 positive. These patients tend to be young and present with skin or lymph node infiltration (or both) by large, often bizarre lymphoma cells that preferentially involve the paracortical areas and lymph node sinuses.[184] [185] The majority of such tumors express one or more T-cell-associated antigens, and a minority express B-cell antigens. However, a significant number express both T- and B-cell antigens (the null phenotype).

A reciprocal translocation, t(2;5)(p23;q35), appears to be restricted to ALCL of either T-cell or null phenotype and is present in a high percentage of these cases. [186] [187] Since its initial description in ALCL, this translocation has also been found in the CD30-positive primary cutaneous lymphomas and the related entity of lymphomatoid papulosis, suggesting a common pathogenesis of these cutaneous disorders and ALCL. The initial report that the fusion transcript resulting from t(2;5) was also present in the Reed-Sternberg (RS) cells of some Hodgkin disease cases has not been confirmed, and the consensus is that, at the cytogenetic and molecular level, both t(2;5) and the resultant fusion gene are absent in RS cells. [188] The molecular consequence of the translocation is the production of a unique fusion protein consisting of the nucleophosmin (*NPM*) gene on 5q35 fused to a tyrosine protein kinase gene (*ALK*) on 2p23. ALK encodes a tyrosine kinase that is normally expressed in intestine, testis, and brain but not in normal lymphoid cells. [189] The NPM/ALK fusion protein is localized to the cytoplasm and is able to transform NIH 3T3 cells, suggesting that the translocation is the primary transforming event in ALCL. [190] The intracellular targets for phosphorylation by normal ALK and for the fusion protein have yet to be elucidated.

Post-transplant Lymphomas

Post-transplant lymphoproliferative disease (PTLD) denotes a heterogeneous group of lymphoproliferations occurring in patients who are immunosuppressed after organ transplantation. There have been relatively few cytogenetic studies of this disease, but trisomy 11 and translocations of *MYC* have been reported.[191] [192] In a recent series, cytogenetic analysis of 16 cases (five cases polymorphic, 11 monomorphic) revealed six distinct subgroups with recurring abnormalities: (1) six patients (38%) had nonclonal abnormalities or normal karyotypes, (2) three patients (19%) had *MYC* translocations, (3) three patients (19%) had +9, (4) three patients (19%) had +11, (5) two patients (13%) had *BCL6* translocations, and (6) one patient (6%) had a translocation involving *IGH* at 14q32.[193] All cases in group 1 were classified histologically as polymorphic and diffuse large cell-centroblastic, all cases in group 2 were Burkitt lymphoma, and all cases in groups 36 were diffuse large cell-immunoblastic. Thus, the majority of monomorphic cases (10 of 11) had abnormal karyotypes, and the different recurring abnormalities segregated with the histologic subtype of the tumor.

Other Lymphoproliferative Disorders

Less is known about the chromosomal abnormalities in malignant lymphoproliferative disorders other than those already discussed. In part, this lack of knowledge is due to the low proliferative rate and mitotic index in these diseases and to the inability to stimulate mitoses in the malignant lymphoid cells without also stimulating cell division in the residual normal T or B lymphocytes. Thus, karyotypes have most often been reported to be normal in patients with CLL, multiple myeloma, or hairy cell

leukemia, for example.

Trisomy 12 is the most common cytogenetic abnormality reported in patients with B-cell chronic lymphocytic leukemia (B-CLL); it is found in 2060% of those with a cytogenetic abnormality.[194] Abnormalities involving band 14q32 are also common,

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e.g., t(14;19)(q32;q13) (Table 48-5).[195] Unfortunately, only half of patients with B-CLL will have an adequate number of metaphase cells in unstimulated cultures for thorough evaluation. Several groups have shown that FISH is a simple and sensitive method for detecting trisomy 12 in interphase CLL cells; 30% of patients had trisomy 12, and this abnormality was associated with a poorer survival. [196] Similarly, a del(13q) can be detected in 30% of CLL cases by FISH technology.

Chronic T-cell leukemia (T-CLL) and large granular lymphocytic leukemia are uncommon disorders in which the malignant mature lymphocytes have a T-cell immunophenotype. Rearrangements involving bands 14q11 with or without an accompanying break in 14q32 have been reported in T-CLL as well as other T-cell lymphomas (Table 48-5). [1] [197] Cutaneous T-cell lymphoma, a malignant proliferation of CD4-positive (helper/inducer) cells that involves the skin (mycosis fungoides), blood (Sézary syndrome), and lymph nodes, has been reported to have a high incidence of random heteroploidy; less often, complex clonal abnormalities are present, sometimes involving chromosome 6.

Hairy cell leukemia is an uncommon B-cell disorder with a low proliferative rate. Recurring chromosomal abnormalities have been difficult to identify in hairy cell leukemia because of the low yield of mitotic cells. Del(6q), trisomy 3 and 12, and translocations involving 14q32 have been reported in several patients each. [1] Unrelated structural abnormalities may also be present in individual patients.

Angioimmunoblastic lymphadenopathy with dysproteinemia is another rare lymphoproliferative disorder. Although it has been linked to drug hypersensitivity reactions, progression to a high-grade immunoblastic lymphoma of T-cell origin occurs in some cases. Clonal abnormalities involving trisomy 3 or 5 have been reported. [198] Although better understanding of these recurring abnormalities will no doubt increase our knowledge of malignant transformation, the greatest clinical importance of cytogenetic analyses in these disorders currently is to differentiate a reactive (polyclonal) lymphoid proliferation from a malignant (monoclonal) one. The identification of a clonal chromosomal abnormality in an enlarged lymph node or in peripheral blood cells provides convincing evidence of a neoplasm.

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NEW TECHNIQUES TO DETECT CHROMOSOMAL ABNORMALITIES

Cytogenetic analysis of human tumors is often technically difficult owing to the presence of multiple abnormal cell lines and the complexity of the chromosomal pattern. Moreover, it requires highly skilled personnel. These factors have led investigators to seek alternative methods for identifying chromosomal abnormalities, such as Southern blot analysis of DNA or RT-PCR analysis of RNA from tumor cells (described following), or FISH. [199]

The technique of FISH is based on the same principle as Southern blot analysis, namely, the ability of single-stranded DNA to anneal to complementary DNA. In the case of FISH, the target DNA is the nuclear DNA of interphase cells, or the DNA of metaphase chromosomes that are affixed to a glass microscope slide. FISH can also be accomplished with bone marrow or peripheral blood smears, or fixed and sectioned tissue. The test probe is labeled with biotin- or digoxigenin-labeled nucleotides, and detected with fluorescein isothiocyanate (FITC)-conjugated avidin or rhodamine-labeled antidigoxigenin antibodies. Probes that are directly labeled with fluorochrome are also available for hybridization, thereby simplifying the technique by eliminating the probe detection steps. With the development of dual- and triple-pass filters, most laboratories now have the capacity to hybridize and detect two to three probes simultaneously.

Several types of probes can be used to detect chromosomal abnormalities by FISH. Hybridization of centromere-specific probes has been used to detect monosomy, trisomy, and other aneuploidies in both leukemias and solid tumors (Fig. 48-4). Chromosome-specific libraries, which paint the chromosomes, are particularly useful in identifying marker chromosomes (rearranged chromosomes of unidentified origin) or structural rearrangements such as translocations. Chromosomal translocations can also be identified in interphase or metaphase cells by using probes (phage, cosmid, or yeast artificial chromosome probes) that are derived from the breakpoints of recurring translocations. Recently, investigators have demonstrated the use of FISH to detect allele loss in tumor cells, specifically the loss of the *RB1* gene in CLL.

FISH techniques have a number of applications (Table 48-6). In some cases, FISH analysis provides more sensitivity, in that cytogenetic abnormalities have been identified by FISH in samples that appeared to be normal by morphologic and conventional cytogenetic analyses. FISH is most powerful when the

Figure 48-4 (A) Schematic diagram of the hybridization pattern observed in metaphase cells or interphase cells hybridized with a centromere-specific repetitive probe. Centromere-specific probes hybridize to the repetitive DNA sequences that are present at the centromeres of human chromosomes. These probes result in two intense signals at the centromeres of the target chromosome in diploid metaphase cells or interphase nuclei. **(B)** Photomicrographs of metaphase cells and interphase cells following FISH. Hybridization of a chromosome-specific centromere probe for chromosome 8 to metaphase and interphase cells with trisomy 8 from a bone marrow sample of a patient with acute myeloid leukemia. The chromosome 8 homologs are identified with arrows.

TABLE 48-6 -- Applications and Advantages of FISH

Applications
Detection of numerical and structural chromosomal abnormalities
Identification of marker chromosomes (rearranged chromosomes of uncertain origin)
Monitoring the effects of therapy, and detection of minimal residual disease or early relapse
Identification of the origin of bone marrow cells after bone marrow transplantation
Identification of the lineage of neoplastic cells
Examination of the karyotypic pattern of nondividing or interphase cells
Detection of gene amplification
Advantages
Rapid technique
Efficiency of hybridization and detection is high
Sensitivity and specificity are very high
Large numbers of cells can be analyzed in a short time
Cytogenetic data can be obtained from nondividing or terminally differentiated cells, from tumors with a low mitotic index (e.g., CLL), or from poor samples that contain too few cells for routine cytogenetic studies
Permits direct correlation of cytogenetic and cytologic/morphologic features, which enables pathologists to differentiate malignant from benign conditions in equivocal cases
Automated systems for analysis of hybridized slides are available

analysis is targeted toward those abnormalities that are known to be associated with a particular tumor or disease. An example of how FISH could be used in a clinical setting is as follows. Cytogenetic analysis could be performed at the time of diagnosis to identify the chromosomal abnormalities in an individual patients malignant cells. Thereafter, FISH with the appropriate probes could be used to detect residual disease or early relapse, and to assess the efficacy of therapeutic regimens.

Our new sophistication regarding the genetic changes in hematologic malignant diseases provides us with some very critical new diagnostic tools. Standard Southern blot analysis of tumor DNA can reveal clonal rearrangements of genes (e.g., immunoglobulin or T-cell receptor genes) using the appropriate probes, as well as a number of recurring translocations. PCR can increase the sensitivity of detection of these aberrations; sometimes the sensitivity is too great to be clinically applicable. Translocations that result in fusion genes are especially suited for RT-PCR, a technique in which the fusion mRNA is copied into cDNA and then, with appropriate primers from each gene, the fusion gene is amplified by PCR. Based on the position of the primers, the size(s) of the expected fusion product is known and can be compared with that actually obtained. The correctness of the amplified product can be confirmed using a smaller probe that contains the expected portion of the fusion gene. We and others have used this strategy to detect the rearranged genes in t(8;21). [63] Using probes from *AML1* and *ETO* on standard Southern blot analysis, rearrangements can usually be detected in DNA from about 80% of patients known to have a t(8;21). With RT-PCR, the detection rate is 100%. We found positive signals of fusion mRNA in patients in complete remission who were negative on cytogenetic analysis and standard Southern blotting. More recently, we have detected

the translocation in peripheral blood cells from three patients in unmaintained remission for 58 years. [63] This indicates that these patients have circulating t(8;21)-positive cells even though they appear to be cured of their leukemia. The biologic significance of these observations remains to be determined; it seems clear, however, that decisions on whether to continue therapy cannot be based solely on a positive signal with RT-PCR methods. Other translocations that can be detected routinely by RT-PCR include t(9;22) and t(15;17).

This increasing precision in identifying the genetic changes in the malignant cells comes at a most opportune time, because physicians may soon be in a position to use targeted therapy aimed at the specific genetic defect in the malignant cells. To use this targeted therapy effectively requires a precise genotype of the malignant cells. Although a number of genes will be involved, with various genetic changes, those reflected in chromosomal changes may be amongst the easiest to monitor.

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CLINICAL IMPLICATIONS OF CYTOGENETICS

Cytogenetic analysis provides clinicians with a powerful tool for the diagnosis and classification of hematopoietic malignant diseases. The detection of an acquired somatic mutation establishes the diagnosis of a neoplastic disorder and rules out a reactive hyperplasia or morphologic changes due to toxic injury or vitamin deficiency. When the pathologic diagnosis is equivocal, the detection of a clonal chromosomal abnormality in a bone marrow specimen or in lymph node tissue is sufficient reason to institute cytotoxic treatment with radiation therapy or chemotherapy.

Specific cytogenetic abnormalities identify homogeneous subsets of various malignant diseases and enable clinicians to predict their clinical course and their likelihood of responding to particular treatments. In many cases, the prognostic information derived from cytogenetic analysis is independent of that provided by other clinical features. Patients with favorable prognostic features benefit from standard therapies with well-known spectra of toxicities, whereas those with less favorable clinical or cytogenetic characteristics may be better treated with more intensive or investigational therapies. The disappearance of a chromosomal abnormality that is present at diagnosis is an important concomitant of complete remission following treatment, and its reappearance invariably signals relapse of the disease. More recently, molecular methods have provided evidence of clonal remissions, suggesting that cytogenetic changes may be a late step in malignant transformation and not always present in the neoplastic stem cell. FISH analyses allow differentiated or nondividing cells to be studied, and molecular methods allow a whole population rather than just individual cells to be analyzed.

The presence of the Ph chromosome differentiates CML from other myeloproliferative disorders or MDS and also serves as an important marker of persistent disease during interferon therapy or following allogeneic bone marrow transplantation. Karyotypic evolution in a patient with CML portends transformation to the acute phase and provides a useful signal to proceed, if possible, with bone marrow transplantation in higher-risk groups, such as older patients or those without HLA-identical sibling donors.

Currently, there is controversy concerning the optimal timing of marrow transplantation in the treatment of AML and ALL. Pretreatment cytogenetic analysis can be useful in choosing

among post-remission therapies that differ widely in cost, acute and chronic morbid effects, and effectiveness.

Our own treatment results suggest that the majority of patients with AML and favorable cytogenetic featuresi.e., t(8;21), t(15;17), inv(16) or t(16;16), and perhaps t(9;11)can be cured with intensive consolidation chemotherapy and therefore should not undergo allogeneic transplantation when in first remission. [50] Those in whom disease relapses can generally be salvaged with bone marrow transplantation in early relapse or second remission. Alternatively, AML patients with loss or deletion of chromosome 5, or ALL patients with t(9;22) or t(4;11) have relatively drug-resistant disease and are seldom cured with conventional chemotherapy alone. Thus, the use of investigational agents or transplantation in first remission is warranted.

As molecular probes for the genes involved in recurring translocations become more readily available, it will be possible to evaluate marrow or blood samples of patients in clinical remission to determine the persistence of minimal residual disease at the level of 1 in 10^5 cells. As yet, however, it is too soon to know whether patients who are persistently positive by PCR methods are always destined to experience disease relapse. However, it seems likely that patients who are negative by current PCR methods are most likely cured of their disease.

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APPENDIX: Glossary of Cytogenetic Terminology*

Appendix Not Available.

* Modified from Rowley JD; Chromosome abnormalities in human cancer. In DeVita VT, Hellman S, Rosenberg S (eds): Principles and Practice of Ocology, 3rd ed. JB Lippincott, Philadelphia, 1991, with permission.

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APPENDIX: Examples of Cytogenetic Nomenclature

46,XX or 46,XY: Nomenclature description of a normal female or male karyotype, respectively.

47,XX,+8,t(9;22)(q34;q11): Female with an extra chromosome 8 as well as a translocation affecting chromosome 9 with a break in the long arm, band q34, and chromosome 22 with a break in the long arm, band q11. The latter is the usual translocation seen in chronic myelogenous leukemia. The abnormal chromosome 22 is known as the Philadelphia (Ph) chromosome.

46,XX,t(9;22;10)(q34;q11;q24): Female with a variant (complex) Philadelphia translocation affecting chromosome 9 with a break in band q34, chromosome 22 with a break in band q11, and chromosome 10 with a break in the long arm at band q24. Chromosomal material from 9q is translocated to 22q, material from 22q is translocated to 10q, and material from 10q is translocated to 9q.

46,XY,inv(16)(p13q22): Male with a pericentric inversion of chromosome 16 resulting from breaks in the short arm in band p13, and in the long arm in band q22. The inv(16) is a recurring chromosomal abnormality that is associated with acute myelomonocytic leukemia with abnormal eosinophils.

46,XY,del(5)(q13q33): Deletion of part of the long arm of chromosome 5, including bands q1333.

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Chapter 49 - The Molecular Basis of Neoplasia

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INTRODUCTION

Malignant transformation involves both somatic and heritable changes in some of the genes that control normal cell growth and development. These genes are of two general types: oncogenes, genes whose products induce oncogenic growth when expressed in appropriate cell types, and tumor suppressor genes, genes whose products negatively regulate cell growth. Many oncogenes were initially discovered because of their presence in a variety of retroviruses that induce tumors in animals. Others were identified because their expression is directly affected by retrovirus infection, leading to tumor development. Although they are all involved in malignant transformation, oncogenes are a diverse group, encoding proteins involved in many aspects of cellular growth and metabolism. Some function by mimicking the action of growth factors, growth factor receptors, and signal transducers, while others are transcription factors that directly affect gene expression. Tumor suppressor genes were largely discovered through genetic analyses of familial cancers. Like oncogenes, the products of tumor suppressor genes are functionally diverse. However, many influence progression

through the cell cycle, providing cues for either cell cycle arrest or apoptosis; others influence growth by sensing environmental signals. This chapter reviews some of the historical experiments that defined the molecular basis of neoplasia and set the stage for current basic and translational research on the pathogenesis and management of hematologic malignancies.

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RETROVIRUSES AND CANCER: HISTORICAL PERSPECTIVE

Except for human T-cell leukemia virus 1 (HTLV-1), retroviruses are not involved in human malignancies. However, two seminal discoveries focused attention on oncogenic retroviruses as tools to study human malignancy. First, the finding that retroviral oncogenes are derived from highly conserved normal cellular genes demonstrated that these transforming genes are not unique to retroviruses. Second, the discovery that many of the same oncogenes are altered in non-retrovirus-induced tumors cemented the relationship of these sequences to oncogenesis. More recent work has demonstrated that retroviral and cellular oncogenes can cooperate to influence tumor incidence and latency and that inactivation of at least some tumor suppressor genes can play an important role in retrovirus-induced tumors. In addition, the simple genetic structure of retroviruses and the ability to study many of them in the genetically defined background provided by inbred strains of mice have given investigators important tools that are not available to researchers studying human tumors.

RetrovirusesA Brief Introduction

Oncogenes originally came to light in experiments that used retroviruses as model systems to understand tumorigenesis. Retroviruses are RNA viruses that contain two copies of their genome in each virion.[1] These viruses have the unique ability to transcribe these sequences into a double-stranded DNA copy, which is then integrated into the genome of the infected cell (Fig. 49-1). This occurs early in the infectious process within a capsid-like viral structure and is catalyzed by an enzyme called reverse transcriptase.[2] This enzyme uses both copies of the viral genome in a complex jumping process to generate a complete DNA copy called a provirus that, once integrated, behaves as any other cellular gene. It is expressed from a viral promoter by the cellular transcription machinery and is passed to daughter cells as are other cellular sequences. RNA transcribed from the provirus can function either as mRNA, directing the synthesis of viral gene products, or as new retroviral genomes that can be packaged and released from the cell as virus particles.

Retroviruses that induce tumors in chickens were discovered in the early 1900s. One of these viruses, Rous sarcoma virus (RSV), became the subject of intense research in succeeding years. These efforts demonstrated that induction of fibrosarcomas in chickens and transformation of chicken embryo fibroblasts in tissue culture required a viral gene[3] named *src* (pronounced sark). What has revolutionized the field since the mid-1970s has been finding that the *src* gene is homologous to a normal cellular gene present not only in birds but also in many other eukaryotes, including *Drosophila.*[4] [5] Studies soon revealed that virtually all retroviruses that transform tissue culture cells or induce tumors with a short latency in vivo contain transforming genes that are distinct from *src* but are also derived from normal cellular genes.[6] Indeed, by the early 1980s more than ten different oncogenes had been identified. The term proto-oncogene was introduced to distinguish the normal cellular form of these genes from their viral counterparts. Also, a three-letter designation was introduced to standardize the nomenclature of individual oncogenes; the three-letter code is often preceded by a c- or a v- to denote the cellular or viral form, respectively. Today, investigators have identified more than 30 different viral oncogenes.

Origin of Retroviral Oncogenes

A large body of work has shown that oncogenes found in rapidly transforming retroviruses are not only related to normal cellular proto-oncogenes but also are actually derived from the cellular genes by recombination between the viral and cellular genomes. Although the actual steps in the recombination process have not been duplicated in a reconstructed system, analysis of the structure of retroviruses that contain oncogenes and proto-oncogenes has suggested that recombination between the

Figure 49-1 Replication of retroviruses. Retroviruses interact with cells via specific receptors, a process called adsorption (1). Following binding to the receptor, the virus/receptor complex is taken up by the cell in a process of penetration (2). The virus core is released into the cytoplasm, and the RNA genomes are copied into a double-stranded DNA form (3). The core structure migrates to the nucleus, and the viral genome integrates into the cellular DNA (4). Cellular RNA polymerase transcribes the viral genes (5), and the RNA is processed and transported to the cytoplasm, where it is translated to give rise to viral proteins (6). Other transcripts serve as genomes for new virions, which are assembled (7) and bud from the cell (8).

Figure 49-2 Incorporation of oncogenes into viruses. **(A)** In the first step, a proviral form integrates into a cellular gene between two exons, represented by the open boxes. Following integration, two possible paths can be followed. On the left, a DNA deletion involving viral and cellular sequence occurs (*) and a fused virus and cell transcript is produced. On the right, a fraction of the normal viral transcripts fail to undergo normal processing and continue beyond the virus into flanking cellular sequences. **(B)** Either pathway can give rise to some virions that contain both a normal viral transcript and a hybrid transcript generated from viral and cellular sequences **(C).** When such a virion infects a new cell, recombination occurs during reverse transcription **(D)** and generates a recombinant virus containing the cellular sequences.

sequences occurs in a minimum of two steps.[2] The first step in the process occurs at the DNA level when a provirus integrates near or into a proto-oncogene. Next, one of two events is thought to occur. According to one scenario, a deletion occurs and the viral and cellular sequences are fused in the DNA. The second possibility predicts that some of the viral transcripts that would normally terminate in the 3 end of the long terminal repeat (LTR) extend beyond this point into the proto-oncogene sequence, generating a hybrid transcript (Fig. 49-2). In either case, RNAs containing both viral and cellular sequences are generated. Because retroviruses can package RNAs that differ in structure from the usual genome, some of the virions released from the cell will contain one conventional genome and one hybrid RNA. When such a virion infects a new cell, the second step in oncogene capture occurs. Reverse transcription of the two different RNA molecules results in generation of a proviral form in which the cellular sequences are now completely incorporated into a viral genome.

Differences Between Oncogenes and Proto-oncogenes

Proto-oncogenes are highly conserved genes, which suggests that their proteins play key roles in cellular metabolism and growth. This feature raises a fundamental question: How does a gene involved in a normal cellular process become a gene that induces malignant growth? A single answer, beyond the obvious unifying feature of incorporation into retroviral genomes, does not exist. Indeed, several types of alterations can occur as a consequence of the capture of these genes. [6] First, the cellular genes are removed from the elements that normally control their expression. In a retrovirus, expression of the gene is controlled by the viral promoter and enhancer elements located at the 5 end of the integrated retrovirus, in the LTR. Polyadenylation signals are contributed by LTR sequences located at the 3 end of the viral genome. Second, because the gene is contained in an infectious element, it can be introduced into cell types that would not normally express the sequences. Finally, in most cases the coding sequence of the cellular gene is altered as a consequence of recombination with the viral genome. These alterations can result in expression of truncated proteins or of fusion proteins containing a portion specified by the cellular oncogene and a portion specified by a viral gene. Proteins that

contain more subtle mutational changes may also occur.

Insertional Mutagenesis and Oncogene Activation

Most oncogenic retroviruses do not carry oncogenes. Indeed, although incorporation of proto-oncogene sequences into retroviruses can create highly oncogenic viruses, such viruses are generated rarely and are involved in a minority of naturally occurring retrovirus-induced tumors. The majority of oncogenic retroviruses induce tumors by proviral insertional mutagenesis. [6] The key event in this process, integration of the provirus in the vicinity of a proto-oncogene, is similar to the first event in oncogene capture. The introduction of the strong promoter and enhancer elements located in the retroviral LTR, and the disruption of proto-oncogene structure caused by proviral insertion, can have drastic effects on proto-oncogene expression in the absence of incorporation into a virus. For example, hybrid RNAs similar to those formed during oncogene capture can be translated, leading to production of an altered proto-oncogene product. The presence of strong promoter and enhancer elements in viral LTRs can also activate the expression of proto-oncogenes if the provirus has integrated in the vicinity of the cellular gene. Although most integrations are within 1020 kb of the proto-oncogene, in some instances these effects appear to be mediated when the provirus is >300 kb away. Integration can also disrupt sequences within the proto-oncogene that control turnover of the mRNA, resulting in production of an abnormally

stable RNA and elevated levels of protein. Less commonly, integration of a provirus can inactivate gene expression; in certain types of retrovirus-induced tumors, proviral integration into the *TP53* and *NF1* tumor suppressor genes plays a role in tumor development. [7] [8]

An important aspect of proviral insertional mutagenesis is the flexibility provided; insertion can alter gene expression in several different ways (Fig. 49-3). Thus, integration into a precise location relative to the proto-oncogene is not necessary. In addition, generation of an RNA with the size and structure required for incorporation into a virion is not necessary. As long as the integration event leads to aberrant proto-oncogene expression and confers a distinct growth advantage, a tumor can develop without production of a retrovirus that carries an oncogene. Although some of the proto-oncogenes that are affected by insertional mutagenesis are identical to those that have been captured by retroviruses, many have never been recovered in an infectious form. Indeed, >70 different loci have been identified as targets for proviral insertion. [6] This large number probably reflects both the flexibility inherent in proviral insertional mutagenesis and the fact that altered expression that induces even subtle changes in growth can contribute to tumor development. Tumors induced by proviral insertional mutagenesis develop over a long period of time and often involve activation of more than one proto-oncogene. As might be expected, many

Figure 49-3 Three common mechanisms by which retroviral integration affects gene expression. **(A)** A typical eukaryotic gene with four exons represented by the open boxes. RNA is transcribed as indicated by the wavy line, with transcription proceeding in the direction indicated by the arrow. The protein product is represented below the RNA as the fused products of each of the exons. **(B)** Consequences of promoter insertion. Here a retrovirus integrates between exons 1 and 2 of the gene, and promoter sequences at the 3 end of the virus are used to direct synthesis of a truncated protein. **(C)** Consequences of enhancer insertion. Here a retrovirus integrates near a cellular gene in the opposite transcriptional orientation. Enhancer sequences located in the retroviral LTR affect transcription from the normal promoter of the gene and elevate the level of expression. **(D)** Consequences of leader insertion. Here the retrovirus integrates within the gene as in B, but promoter sequences at the 5 end of the virus in concert with viral leader sequences lead to the translation of an altered form of the cellular gene product.

of the proto-oncogenes that have been identified as targets for proviral insertion are altered by other types of mutations in spontaneous, carcinogen-induced, or genetically influenced tumors in animals, including humans.

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TYPES OF RETROVIRUS ONCOGENES

The current list of known oncogenes involved in retrovirus-induced tumors contains nearly 100 entries (Table 49-1), and new oncogenes or candidate oncogenes are still being identified. Although some of these genes are clearly capable of inducing tumors and transforming cells in tissue culture, others have been identified as genes expressed in spontaneous tumors that are related by nucleic acid homology to known oncogenes. Still others are loci known to be targets of proviral insertion for which no gene has yet been identified. In these latter cases, the actual role of the gene in the tumor and its potential to induce malignant transformation have not always been proved. This chapter does not attempt to cover all oncogenes but focuses on the major groups involved in retrovirus-induced tumors, using examples that illustrate general mechanisms.

Oncogenes can encode secreted, membrane-associated, cytoplasmic, or nuclear proteins that appear to affect virtually every aspect of cellular metabolism (Fig. 49-4). Indeed, it is possible that any gene playing a key role in cellular growth can become an oncogene if mutated in the appropriate way. Most of the known oncogenes can be divided into groups based on their apparent mechanism of action and functional similarities. Oncogenes encoding secreted proteins include several that encode growth factors. Those that encode membrane-associated proteins include tyrosine kinase growth factor receptors and membrane-bound intracellular tyrosine kinases that interact with these receptors. Other proteins that are sometimes associated with membrane complexes include adaptor molecules, which transmit signals from receptors to downstream signaling intermediates, members of the G protein superfamily, and serine/threonine kinases that transmit signals to the nucleus. Oncogenes that encode nuclear proteins that regulate transcription and cell cycle progression have also been identified. Indeed, it is difficult to find an aspect of cellular growth control that cannot be disrupted in some retrovirus-induced tumor.

Protein Tyrosine Kinase Oncogenes

The first oncogene protein to be assigned an enzymatic activity was *src,* a protein tyrosine kinase (PTK). [9] [10] Prior to this discovery a role for tyrosine phosphorylation in cellular growth had not even been proposed. Today, not only do PTKs constitute a major group of oncoproteins, but tyrosine phosphorylation and dephosphorylation are recognized as one of the principal signaling mechanisms in higher eukaryotic cells. [11] Proteins with PTK activity include many growth factor receptors and intracellular, membrane-associated molecules. Most of these latter proteins interact with transmembrane receptors, many of which are PTKs themselves. The kinase activity of these proteins is required for transmission of signals. Normally, the kinase activity of the receptors is activated when ligand binds the receptor; the activity of the intracellular PTKs is also activated in response to a specific signal, which is usually transmitted as a consequence of a receptor-ligand interaction. In both cases, the kinase activity initiates a signal by phosphorylating other proteins, which transmit the signal downstream through multiple pathways, leading to altered gene expression.

Unlike their normal counterparts, which are tightly regulated, the kinase activity of oncogenic forms of these proteins is not dependent on interaction with ligand, allowing these proteins to transmit growth signals constitutively. Several different types of alterations can lead to constitutive activation. For

TABLE 49-1 -- Retrovirus Oncogenes

Function of Gene Product	Name of Gene	Species of Origin	Disease(s) Associated	Comments
Nonreceptor tyrosine kinase	*src*	Chicken	Sarcoma	
	fps	Chicken	Sarcoma	*fes* homolog
	yes	Chicken	Sarcoma	
	fgr	Chicken	Sarcoma	
	fes	Cat	Sarcoma	*fps* homolog
	abl	Mouse, cat	Lymphoma, sarcoma	
Receptor tyrosine kinase	*ros*	Chicken	Sarcoma	Related to insulin receptor
	*erb*B	Chicken	Erythroblastosis	EGF receptor
	fms	Chicken	Sarcoma	CSF-1 receptor
	kit	Chicken	Sarcoma	SCL receptor
	sea	Chicken	Sarcoma	*HGFR* family member
	eyk	Chicken	Sarcoma	
G-protein	*ras*	Chicken, rat, cat, mouse	Sarcoma	Some isolates induce several types of tumors
Serine/threonine kinase	*raf*	Mouse	Sarcoma	*mil* homolog
	mil	Chicken	Sarcoma, carcinoma, myelocytoma	*raf* homolog
	akt	Mouse	T-cell lymphoma	
	mos	Mouse	Sarcoma	
Growth factors	*sis*	Monkey	Sarcoma	PDGF- chain
Growth factor receptor	*mpl*	Mouse	Myeloproliferative disease	Thrombopoietin receptor
Adaptor protein	*crk*	Chicken	Sarcoma	
	cbl	Mouse	Pre-B lymphoma, myeloid leukemia	

Transcription factor	*ski*	Chicken	None	
	*erb*A	Chicken	Erythroblastosis	Thyroid hormone receptor
	jun	Chicken	Sarcoma	AP-1 component
	fos	Mouse, chicken	Sarcoma	AP-1 component
	myc	Chicken	Sarcoma, myelocytoma	Complexes with Max
	myb	Chicken	Myeloblastosis	
	rel	Chicken	Reticuloendotheliosis	NF-B family member
	ets	Chicken	Erythroblastosis, myeloblastosis	
	maf	Chicken	Sarcoma	bZip protein interacting with Fos and Jun
	qin	Chicken	Sarcoma	Winged helix family member

example, *erb*-b, an oncogene related to the epidermal growth factor receptor (EGFR), expresses an altered protein that no longer interacts appropriately with its normal ligand.[12] Similar changes have occurred in the v-*kit,* v-*sea,* v-*eyk,* and v-*ros* oncoproteins.[13] [14] [15] [16] In some cases, alterations affecting the sequence of the kinase domain and other portions of the molecule are also present. Mutations altering the sequence of the extracellular portion of the *fms* protein outside the ligand-binding domain, as well as changes in the carboxyl-terminus of the molecule, play a role in activating the oncogenic potential of this gene. [17]

Receptor PTK oncogenes have been captured by several different retroviruses associated with tumors in chickens and cats, and one proto-oncogene in this group is a common target of proviral insertional mutagenesis. Although the receptors have specific functions in normal cells, the types of tumors associated with the oncogenic forms do not always have an obvious association with the normal expression pattern. For example, a retrovirus expressing an altered form of the colony-stimulating factor-1 receptor (CSF-1), a molecule involved in myeloid cell differentiation, induces sarcomas in cats; [18] the *sea* oncogene, related to the hepatic growth factor receptor, is associated with both erythroblastosis and sarcoma in chickens. [15] The *erb*-b gene, related to the EGFR, has been captured by several different chicken retroviruses that induce erythroblastosis; the gene can also be activated by proviral insertional mutagenesis, leading to erythroblastosis. [19] These examples reveal two features common to many oncogenic systems: activation of different oncogenes can induce similar types of tumors, and the cell type involved in the tumor is not always the same as that in which the cellular form of the oncoprotein normally functions.

A number of oncogenes encode PTKs that bind to the inner surface of the plasma membrane. These proteins share essential features with the membrane-associated PTKs in the region of the molecule that is responsible for enzyme activity but do not contain transmembrane or extracellular domains. [20] Many of these oncoproteins contain two regions, called SH2 and SH3. These regions are not found in receptor PTKs and mediate interactions with other signaling molecules. [21] The specificity of these interactions is influenced by the exact sequences present in the SH2 and SH3 domains. Oncogenes in this group include v-*src,* v-*fgr,* v-*yes,* v-*abl,* and v-*fes.* All these genes were originally identified in retroviruses. The *src, fps,* and *yes* genes were obtained from independent isolates of chicken sarcoma virus, [22] [23] the *fgr* and *fes* genes (a gene identical to *fps*) were isolated from cat sarcoma viruses, [24] [25] [26] and the *abl* oncogene was isolated from a murine virus that induces pre-B-cell lymphoma and a cat retrovirus that induces

Figure 49-4 Some signaling pathways important in oncogene-mediated transformation. The scheme depicts some of the signaling pathways that are activated when a growth factor interacts with its receptor. In transformation, one or more of these signaling molecules can be constitutively active. In many cases the receptor is a receptor protein tyrosine kinase. Binding of ligand triggers signaling events that eventually lead to changes in gene expression. Molecules involved include nonreceptor protein tyrosine kinases (NR PTKs); adaptor proteins such as Grb-2; GTP-binding proteins such as Ras; and other protein kinases such as Raf, MEK, ERK, MEKK, and Jnk. Phosphatases (PP2A) and GAPs can downregulate these signals as indicated by the lines with flat tips.

sarcomas.[27] [28] The *lck* oncogene can be activated by proviral insertion, an event that can be associated with induction of thymic tumors in rats. [29] Most of these oncoproteins lack small regions of sequence that normally regulate the enzymatic activity of the molecule. [30] [31] [32] However, overexpression of these proteins caused by the strong retrovirus promoter and enhancer elements can also activate the oncogenic potential of these proteins.

Oncoproteins Related to G-Proteins

The proteins encoded by the different *ras* oncogenes are the classic examples of oncoproteins of the G protein superfamily. The first members of this family of oncogenes were initially discovered in two murine retroviruses that induce sarcomas in rodents. Members of this group are all called *ras* but are distinguished by prefixes that denote the cellular gene from which they were derived or the names of the retroviruses in which the genes were first discovered. For example, Ki-*ras,* homologous with the viral oncogene found in the Kirsten strain of murine sarcoma virus, denotes one cellular gene locus of the *ras* family,[33] while Ha-*ras,* homologous with the viral oncogene isolated from the Harvey murine sarcoma virus, refers to another, distinct locus. [33] [34] The *ras* oncogenes have also been recovered in several other retroviruses, and activation of *ras* by proviral insertion has been noted rarely. [35] [36] [37] [38] [39] [40] [41] In contrast, altered *ras* genes are relatively common in several types of human tumors.

Although each *ras* gene encodes a distinct product, all of these proteins are found on the inner surface of the plasma, where they interact with guanosine di- and triphosphate (GDP and GTP).[42] [43] When GTP is bound to Ras, the protein transmits signals downstream; the molecule is inactive in the GDP-bound state. The amount of Ras-GTP and Ras-GDP is controlled by signals received from several interacting proteins, including Grb-2/Sos, a complex that activates Ras, and GAP, a protein that inactivates Ras. All oncogenic forms of Ras display a reduced ability to be converted to the inactive state, and a disproportionately high level of Ras-GTP is found in the cells. Thus, as with the PTK oncoproteins, transformation by *ras* oncogenes is a consequence

of constitutive expression of the active or signal-transducing state of the molecule. In most cases, oncogenic Ras proteins have one of several amino acid substitutions that alter the ability of the molecule to interact with GTP, GDP, and GAP. [44] [45] [46] [47] [48] [49]

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PROTEIN SERINE/THREONINE KINASE ONCOGENES

Two retrovirual oncogenes, v-*mos* and v-*raf/mil,* encode proteins that are protein serine/threonine kinases. [50] [51] [52] Two other serine/threonine kinases, encoded by the related *pim*-1 and *pim*-2 oncogenes, can be targeted by proviral insertional mutagenesis in murine hematopoietic tumors. [53] [54] The v-*mos* gene was first identified in a murine sarcoma virus,[55] while v-*raf* was first isolated from a murine virus that induces fibrosarcomas.[56] The v-*mil* gene, the avian homolog of the *raf* gene,[57] was isolated in an avian virus that induces myeloid leukemia. As with the PTK group, the enzyme activity of these oncoproteins is essential for transformation. [50] [51] The Raf/Mil proteins interact with Ras and can transmit Ras-mediated signals downstream to components of the MAP kinase pathway. [58] [59] Oncogenic forms of Raf lack amino-terminal sequences that are involved in regulating the activity of the protein. [60] [61] The Mos protein normally plays a key role in oocyte maturation by controlling meiosis at several points;[62] most somatic cells do not express the protein, and simple overexpression of the molecule in cells that do not normally express Mos is sufficient for transformation.[63] [64] This is an example of the general principle that inappropriate expression of an oncogenetoo much product, wrong cell type, or wrong time of the cell cycleis often sufficient to result in transformation.

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GROWTH FACTOR ONCOGENES

Growth factor oncogenes include one retroviral oncogene, v-*sis,* and several targets of proviral insertional mutagenesis. Among the latter are several members of the gene family encoding fibroblast growth factor (*fgf*), the gene encoding granulocyte-macrophage colony-stimulating factor, and genes of the *Wnt/Int* family of growth factors. The v-*sis* gene that encodes an altered form of the platelet-derived growth factor chain was discovered in a primate sarcoma virus. [65] [66] [67] The *fgf* and *Wnt* genes are targets of common proviral insertion in murine mammary carcinoma. [68] [69] [70] [71] [72] Oncogenes of this type appear to transform cells by direct autocrine stimulation; that is, their products are produced by the very cells they induce to grow. [72] [73] [74]

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TRANSCRIPTION FACTOR ONCOGENES

Unlike all the oncogene products previously discussed, these genes encode proteins that are primarily localized to the nucleus; many of these products are directly involved in regulating gene expression. Among these are the products of the v- *erb*-a, v-*jun,* v-*fos,* v-*myc,* v-*ski,* v-*ets,* v-*rel,* v-*maf,* v-*qin,* and v-*myb* genes.[75] [76] [77] [78] [79] [80] [81] [82] [83] [84] [85] [86] [87] [88] [89] [90] Many of these and a variety of other genes encoding transcriptional regulatory proteins are also targets of proviral insertional mutagenesis.[6] As might be expected for a diverse group of oncogenes that control expression of an equally diverse group of genes, the members of this group are associated with many different types of tumors. For example, v-*myc* has been isolated in retroviruses that induce a form of myeloid leukemia and carcinomas in chickens, and a T-cell lymphoma in cats. [91] [92] [93] [94] [95] Proviral insertional activation of *myc* has been found in both B- and T-cell lymphoma, erythroleukemia, and, rarely, in plasmacytoma.[96] [97] [98] [99] [100] The v-*erb*-a oncogene was discovered in a virus that induces erythroleukemia in chickens, [101] [102] and the v-*fos* oncogene was found in two different viruses that induce osteosarcoma in mice and in an avian virus that induces fibrosarcoma and nephroblastoma. [103] [104] [105]

Several types of structural changes are involved in the activation of these types of oncogenes. For some of these, particularly those that are regulated by pairing with other cellular factors, increased levels of expression are particularly important in activating their oncogenic potential. The Myc protein, which is normally tightly regulated at the transcriptional level and is highly active only when bound to the Max protein, is a classic example of this type of activation. [106] [107] [108] Other members of this group, including v-*myb* and v-*jun,* express proteins that are lacking important regulatory regions that would normally down-modulate transcriptional activation. The v-*erb*-a gene encodes an altered form of the thyroid hormone receptor, which can no longer interact with its ligand. [73] [74] Under normal circumstances, binding of thyroid hormone activates expression of genes regulated by the factor. In the absence of ligand, the normal receptor represses expression of these genes; [75] the oncogenic form of *erb*-a appears to act by constitutive repression of genes normally up-regulated by ligand-receptor interaction.

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OTHER NUCLEAR PROTEINS

Several genes that encode proteins involved in cell cycle regulation have been identified as targets of proviral insertional mutagenesis. Among these are the genes encoding cyclin D1 and cyclin D2 and the *TRP53* gene (the mouse version of *TP53*) encoding the p53 tumor suppressor protein.[6] The D cyclins are normally expressed during the G_1 phase of the cell cycle, where they complex with cyclin-dependent kinases, forming a complex that phosphorylates the retinoblastoma protein (pRb).[109] This modification causes release of E2F transcription factors and stimulates progression from the G_1 phase of the cell cycle to the S phase. Targeting of the cyclin D genes results in overexpression of these proteins, leading to aberrant cell cycle regulation during the G_1 phase.

Unlike genes whose expression is activated by proviral insertion, consistent with its role as a tumor suppressor gene, integration into the *TRP53* locus causes loss of p53 expression.[7] The p53 protein is a multifunctional protein involved in the regulation of G_1 progression and cellular responses to DNA damage and hypoxia; its absence facilitates tumor formation by allowing damaged cells to proceed through G_1.[110] [111] Integrations into *TRP53* have been documented in retrovirus-induced erythroleukemias and more rarely in several other types of retrovirus-induced tumors. [6] [7] As might be expected based on the frequent targeting of *TRP53* in spontaneous tumors, mutations in this tumor suppressor gene have also been documented in several retrovirus tumor systems.

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CELLULAR PROTO-ONCOGENE ACTIVATION

Proto-oncogenes normally play a role in controlling the cellular response to mitogenic signals as well as passage through various compartments of the cell cycle. The initiation of DNA replication, cell division, and the commitment to cellular differentiation are all considered to be levels at which proto-oncogenes and their products may exert a regulatory influence. Consequently, proto-oncogenes represent obvious targets for processes that damage the growth control apparatus of the cell and lead to malignant transformation. Such damage is often referred to as the activation of a proto-oncogene, resulting in the creation of an oncogene. In both leukemias and solid tumors, these processes have two basic effects. The first is to alter the protein product of the proto-oncogene so that a qualitative change in function occurs. Mutation may lead to a protein with an enhanced catalytic activity or an activity no longer subject to the control of cognate regulatory factors. The second principal effect is to alter the regulation of proto-oncogene expression so that a quantitative effect on function results. The overproduction of an otherwise normal oncogene product is an example of this type of activation.

Damage to cellular proto-oncogenes leading to their activation occurs by one of three mechanisms: genetic mutation, genetic rearrangement, or gene amplification. The first of these is usually associated with qualitative changes in function and the last with quantitative changes. Genetic rearrangement can have quite spectacular consequences, leading either to changes in the regulation of proto-oncogene transcription or to the creation of entirely new oncogene products.

Oncogene Mutation

The first defined alteration of a proto-oncogene to be discovered in a human tumor was the single-base mutation of a *ras* family member, Ha-*ras*-1.[112] [113] Point mutations affecting other *ras* genes (Ki-*ras*-2 and N-*ras*) have subsequently been described in a wide variety of human leukemias and solid tumors. [114] The activating point mutations occur in quite specific locationsthe bases of codons 12, 13, or 61and yield single amino acid substitutions in what is now known to be the GTP-binding site of the *ras* gene product, p21. The biochemical phenotype of such mutations is the most prominent example of a qualitative change arising out of oncogene activation, namely, the p21-associated reduction in GTPase activity. [114] Since the GTP-bound form of p21 is considered to be the active one, the net effect of *ras* mutations is to increase the proportion of activated p21 molecules in the cell. Given the previously described role of p21 as G protein in a signaling apparatus, the consequence of this shift toward the active form presumably is enhanced or constitutive transduction of mitogenic signals.

Although mutation was first detected in the Ha-*ras*-1 gene, Ki-*ras*-2 and N-*ras* are far more frequently the targets of somatic mutation in human tumors; Ha-*ras*-1 mutations are, in fact, rarely observed. Interestingly, a pattern of tissue-specific mutation has emerged. For example, Ha- *ras*-1 mutations are apparently limited largely to transitional epithelial tumors. Codon 13 mutations of the N- *ras* gene were found in 2570% of acute myelocytic leukemia (AML) samples examined.[115] Observations such as these have several important implications. First, the path traversed to produce, say, a specific leukemia subtype must be quite similar in most patients. This validates observations on the nature of the pathogenetic lesions and gives hope that the complexity of carcinogenesis will be resolved into a manageable number of combinatorial events. Second, despite this strong pattern of similarities, a significant fraction of tumors within a given class arise by a different pathdifferent at least in the choice of *ras* substitutes. Finally, the specificity of these mutations hints at underlying functional attributes peculiar to each member of the *ras* gene family. In some experimental systems, for example, the introduction of a mutant Ha-*ras*-1 gene may actually lead to differentiation rather than to unregulated proliferation. [116] Taking these disparate observations into account, we may speculate that each *ras* family member plays a role in signal transduction pathways with potentially distinct outcomes. Furthermore, the action of a given *ras* gene may be quite different from cell type to cell type.

Evidently, *ras* mutations can occur before the transformation to frank malignancy takes place. There is abundant indirect evidence for this assertion in humans and direct evidence in animal tumor models. First, *ras* mutations may be detected in a significant proportion of myeloid cells present in a myelodysplastic bone marrow. [117] The mutations occur principally in patients destined to progress to AML but can occur in patients who remain free of leukemia. Second, the mutations can be demonstrated in a significant fraction of the precursor lesions to colon carcinoma (i.e., adenomatous polyps). [118] In the murine skin carcinoma model, benign epitheliomas generated by the application of chemical carcinogens uniformly contain Ha- *ras*-1 mutations.[119] The epitheliomas may progress to invasive squamous carcinoma, or they may regress. Finally, transgenic mice bearing mutant *ras* genes are viable and develop normally but show a marked predisposition to the development of cancer.[120]

Although mutational activation in human tumors principally affects members of the *ras* family, several other candidate oncogenes have recently been described whose somatic mutations are involved, rarely, in pathogenesis. In addition, six distinct oncogenes bearing mutations have been isolated from animal tumors. Each of these oncogenes has a human counterpart, although human mutations have not yet been observed. The most prominent of this set is the *neu* oncogene (lately *her* or *erb*-b2), representing a transmembrane protein highly related to the EGF receptor. [121] This oncogene has been implicated in breast cancer.

Oncogene Amplification

The expression of a given gene can be augmented in a variety of ways. Under normal circumstances, the complex interaction of control sequences located near genes with DNA-binding proteins that function as transcriptional regulatory factors leads to properly timed production of mRNA in a tissue- or cell-cycle-specific manner. Any disorganization of the plan of expression in genes responsible for the regulation of cell proliferation can result in loss of growth control. The first described occurrence of inappropriately elevated proto-oncogene expression in humans revealed activation by the mechanism of gene amplification. In this process, errors of DNA replication, followed by large-scale somatic recombination, result in the appearance of a large tandem array of DNA sequences not previously present in the normal cell genome. The array represents an increase in the number of copies of genes that happen to be present in the amplified DNA segment of the affected cell. This increase can be as large as several thousand-fold.

Gene amplification in somatic cells was originally described for the dihydrofolate reductase gene, in which amplification led to methotrexate resistance. [122] In human tumors the consequence of proto-oncogene amplification is usually a large increase in the level of transcribed mRNA and a corresponding increase in proto-oncogene protein product. Unlike dihydrofolate reductase amplification, in which a mutant enzyme is often the target of amplification, the product of an amplified proto-oncogene is usually qualitatively unchanged. Amplification most often involves genes whose transcripts have short half-lives. Therefore, the net result of amplification is the prolonged presence of mRNA end product, especially at inappropriate points in the cell cycle. This leads to a disruption of growth control, since the product of the amplified gene, which would ordinarily be absent after a specified segment of the cell cycle passes, continues to provide the stimulus for cell DNA synthesis and/or cell division.

The discovery of proto-oncogene (and drug resistance gene) amplification provides an explanation for two of the most prominent changes observed in tumor cell karyotypes. Double minutes (bipolar chromatin bodies without centrioles) commonly occur in malignant cells. The other chromosome abnormality, known as the homogeneously staining region, appears when variable lengths of pale-staining chromatin are inserted into chromosomes. It is now recognized that these structures,

which are apparently interconvertible within a given cell, represent the locations of gene amplification. Therefore the amplification process can be so extensive that it results in grossly visible changes in chromosome structure.

The most prominent proto-oncogenes that undergo amplification in human tumors are members of the *myc* family. First observed in promyelocytic leukemia,[123] *myc* amplification has subsequently been documented in a broad range of cancers, including carcinomas of the lung, colon, and breast.[124] A consistent pattern of *myc* amplification in a number of tumor subtypes has come to be associated with a poor prognosis. For example, a particularly aggressive subset of small cell carcinoma of the lung that responds poorly to chemotherapy consistently demonstrates

amplification of one or another of the three *myc* genes.[125] Amplification of N-*myc* is a marker for poor prognosis in neuroblastoma.[126]

Another example of oncogene amplification with prognostic significance is c-*erb*-b2. Overexpression of the c-*erb*-b2 (*her/neu*) oncogene in breast cancer has been associated with a poor prognosis.[127] The overexpression occasionally results from gene amplification, although excessive transcription of the gene can be seen in the absence of such amplification. This observation is potentially of great prognostic value, since *erb*-b2 identifies individuals who are considered to be at low risk for recurrence on the basis of other criteria, such as node status.

We now know that the process of oncogene amplification requires abrogation of cellular functions that guard the integrity of genetic information. For example, amplification does not occur in normal cells challenged by drug selection. However, when the *TP53* gene is knocked out, amplification of several drug resistance genes can be observed following addition of the drugs to culture medium. The initial stages of the process involve double strand breaks that are not properly repaired as a consequence of p53 inactivation (see following discussion). This leads to multiple cycles of bridge-breakage-fusion that advance the amplification process.[128]

Oncogene Rearrangement

The most dramatic examples of proto-oncogene activation occur by genetic rearrangement, usually as a result of reciprocal chromosome translocations. These events are of particular pathogenetic significance in leukemias and lymphomas, for which specific translocations have been identified and analyzed in detail (see Chap. 50).

The significance of chromosome translocations, although long suspected to reflect the recruitment of genes important in carcinogenesis, was first demonstrated conclusively in human undifferentiated lymphoma and murine plasmacytoma.[129] [130] [131] In both instances the cellular proto-oncogene, *myc,* was translocated from its normal position (in humans, chromosome 8) to sites adjacent to or within the immunoglobulin heavy chain or Ig or Ig light chain constant region genes. The resulting human translocations were t(8;14), t(2;8), and t(8;22), respectively. The mechanisms responsible for abnormal *myc* rearrangements are poorly understood; perhaps they represent errors in the normal rearrangement process that results in functional immunoglobulin genes.

The c-*myc* gene consists of three exons. The first exon does not encode protein and is thought to be one of the elements involved in regulating the level and timing of c-*myc* expression. In most translocations this exon is truncated from the remaining two, which are then transferred to, say, the switch region of the heavy chain constant region genes. In the minority of translocations in which all three exons of the gene are translocated to an immunoglobulin gene locus, somatic mutations are often observed to occur in exon 1. However, the DNA sequence of the protein product, which is encoded in exons 2 and 3, is nearly always unaffected.[132] Therefore, the c-*myc* protein produced by a translocated c-*myc* gene is usually identical to that produced by the normal gene. Finally, it has been observed that c-*myc* transcription from the translocated gene is constitutive, if not always elevated. Transcription from the normal gene ceases in a tumor with a c-*myc* translocation (Fig. 49-5).

The picture that emerges from this set of observations, although not yet verified in significant details, is compelling. Moving a proto-oncogene away from resident influences that control the level and timing of transcription and into a locus that is actively expressed in a given state of differentiation (e.g., immunoglobulin genes in B lymphocytes) can result in oncogene activation and subsequent tumorigenesis. In the specific instance of c-*myc,* removal or mutational alteration of exon 1 seems critical to deregulation of transcription. Under circumstances in which the normal c-*myc* allele is silent, the translocated one continues to be expressed. This is an example of quantitative alteration of oncogene function.

Another prominent example of this type of activation is the

Figure 49-5 Translocation of the c-*myc* oncogene. **(A)** Many c-*myc* translocations result from breakage of c-*myc* within intron 1, in which a cryptic promoter (P) is located. The c-*myc* chromosome 8 fragment is then joined to a C_H gene switch region. This creates the 14q+ karyotypic anomaly noted in Burkitt lymphoma. **(B)** Translocation of C . Translocation to distal (rightward) C_H genes also occurs. **(C)** Transcription of the translocated c-*myc* gene ensues from the cryptic promoter (now without parentheses) in the direction of the arrow. The boxes indicate c-*myc* exons.

t(14;18) translocation of human follicular lymphoma.[133] Once again, the immunoglobulin heavy chain gene is the recipient of a chromosome fragment bearing an oncogene from another chromosome, this time the *bcl*-2 oncogene from the long arm of chromosome 18. As with c-*myc,* transcription of *bcl*-2 is inappropriately constitutive in tumor cells as a result of the translocation.

In addition to affecting the control of oncogene expression, translocations can alter the form of the oncogene product. In chronic myelogenous leukemia the c-*abl* oncogene on chromosome 9 is moved into the *bcr* gene on chromosome 22 by t(9;22), the classic Philadelphia translocation.[134] Each translocation accomplishes the same result, truncating the N-terminus of c-*abl.* The coding sequences responsible for the N-terminus of the c-*abl* protein are replaced with coding sequences from *bcr.* Thus, an entirely new fusion protein is derived from two genes on two different chromosomes. Functionally, the translocation results in one certain and another possible outcome. The normal c-*abl* product, as discussed above, is an intracellular tyrosine kinase. The activity of the enzyme is tightly regulated in vivo, however. Following translocation, the fusion product, which retains the tyrosine kinase domain of the original c-*abl* oncogene, has constitutively high kinase activity.[135] The second likely outcome of the translocation, although not yet proved, must be to alter some aspect of c-*abl* effector specificity or domain of action. This is inferred from the regularity of the translocation: only *bcr* is involved, and the same region of the c-*abl* protein is affected.

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TUMOR SUPPRESSOR GENES

We have shown that proto-oncogenes comprise a set of cellular genes that are involved at all levels of control of cell division and differentiation. In each of the genes discussed thus far, a somatic activating event results in a dominant change. A mutant *ras* gene has a dominant phenotype in the presence of the wild-type allele. A translocated c-*myc* gene is dominant over its unrearranged counterpart. In principle, however, genes that regulate growth and differentiation could demonstrate the opposite pattern: regulatory function preserving a normal pattern of growth would only be observed in the wild-type form. Any mutation or rearrangement would tend to inactivate such genes, leading to loss of growth control. Since the wild-type allele could suppress these mutations, they would be recessive at the cellular level.

Genes that display this property have now been discovered. Originally called recessive oncogenes or anti-oncogenes, they have come to be known as tumor suppressor genes because of several additional properties described below. Although tumor suppressor gene products may participate in many different regulatory pathways, it is generally true that inhibition of cell division, often combined with control of repair of DNA damage, is the principal action of this class of regulators. Thus, cell cycle control at checkpoints that determine passage of the cell from G_1 to S or from G_2 to M is a common means by which tumor suppressor genes influence cell division. As discussed later, the proteins encoded by the *RB1* and *TP53* genes are prominent examples of such regulation. More recently, a host of small proteins that function as cyclin-dependent kinase inhibitors have also been characterized as growth inhibitors. At least one has been implicated in familial melanoma. Others have been placed in the pathways influenced by such genes as *TP53* (see following sections).

Retinoblastoma Model

The childhood tumor retinoblastoma is currently the best-characterized model for action of a recessive oncogene. In this cancer, which occurs in both inherited and sporadic forms, a retinoblastoma gene was proposed as the recessive oncogene, which normally suppresses the malignant transformation of retinoblasts. Individuals at risk for the familial form of the disease, according to a two-hit hypothesis advanced by Knudson, [136] inherit a damaged, nonfunctional gene. The second hit in such individuals would then be a somatic mutation that inactivated the remaining normal allele (Fig. 49-6). Individuals with sporadic retinoblastoma would suffer two independent somatic mutations damaging both alleles. More recent investigations have advanced this set of conjectures a considerable distance, culminating in the molecular cloning and characterization of the retinoblastoma gene, *RB1.*

Cytogenetic abnormalities in the form of interstitial deletions and a fortuitously observed linkage to the enzyme esterase D established the long arm of chromosome 13 as the probable site of a retinoblastoma gene. [137] Shortly thereafter investigators were able to exploit the newly developed technique of restriction fragment length polymorphism (RFLP) to confirm an important prediction of the above-described model, namely, that loss of genetic material in the region of the putative *RB1* gene routinely occurred in tumors. [138] DNA probes identifying several polymorphic regions on chromosome 13 were used to compare patients tumor DNA with their normal, or constitutional, DNA. In this approach the polymorphic probes identify both maternal and paternal chromosomes on Southern blots; loss of genetic material is evident as a loss of heterozygosity. The results indicated a specific loss of heterozygosity at 13q. Furthermore, the chromosome (*RB1* allele) retained in each tumor of a patient with familial retinoblastoma was from the affected parent. This was additional evidence for the supposition that somatic mutation (in this instance, gene loss) was required to advance the pathogenetic process in an individual inheriting one bad copy of the retinoblastoma gene. Subsequent work with DNA probes specific to 13q has demonstrated that chromosome loss and duplication, as well as mitotic recombination, are the somatic mechanisms by which loss of genetic material occurs.

Several groups have now reported molecular cloning of the human *RB1* gene. [139] [140] The locus is quite large, extending over 200 kb of DNA and consisting of 27 exons. mRNA transcribed from this gene encodes a 110-kd phosphoprotein, described in more detail later. All cell types examined thus far express the *RB1* gene, and knowledge of its structure has enabled workers in the field to complete the molecular characterization of pathogenetic lesions in familial and sporadic retinoblastoma. First, *RB1* mRNA is either absent or of aberrant size in almost all tumors examined. In those tumors with grossly undetectable changes in mRNA, small deletions or mutations are observed that either alter the reading frame of the mRNA or lead to abnormal splicing. Interestingly, similar lesions of the *RB1* locus occur in the osteosarcomas that arise frequently during adolescence in those patients surviving childhood retinoblastoma. The ability of the *RB1* gene to suppress tumorigenesis has been demonstrated directly by transferring a copy of the wild-type gene into cells of a retinoblastoma cell line. [141] Those cells incorporating the normal *RB1* gene assume a slower growth rate in vitro and are no longer tumorigenic when injected into nude mice. Thus, the anti-oncogene or tumor suppressor gene phenotype of *RB1* is evident.

The *RB1* product, $pp110^{RB}$, is a zinc finger protein that displays interesting behavior during the course of the cell cycle. In G_1 , most molecules of $pp110^{RB}$ are relatively underphosphorylated. [142] [143] During S and G_2 , all become hyperphosphorylated at multiple sites. Several DNA tumor viruses encode protein products that bind only the underphosphorylated form of $pp110^{RB}$.[144] [145] It is probably through this binding of the cellular *RB1* gene product that transformation by these viruses is mediated. Thus far, this set of observations suggests that the underphosphorylated form of the *RB1* protein is the active one and that $pp110^{RB}$ probably functions as part of the barrier to entry into S phase. The mechanism by which this occurs involves the binding of underphosphorylated *RB1* protein through its

Figure 49-6 (Left) Tumor progression in familial retinoblastoma. One damaged allele is inherited from an affected parent. (The allele could also represent a new mutation in the parents germ cells.) Cells with one functioning (wild-type) copy of the *RB1* gene remain normal. As a result of somatic genetic damage (mutation, loss, mitotic recombination), the second allele may be inactivated, with malignant conversion resulting. This scenario is depicted for retinoblastoma but holds for most dominantly inherited familial cancer syndromes. In sporadic retinoblastoma, damage to the first allele occurs as a somatic event. **(Right)** Tumor loss of heterozygosity. If informative (heterozygous) polymorphic markers are used to analyze patients normal and tumor DNA, a common accompaniment of damage to recessively acting oncogenes is loss of heterozygosity. Generally, one band (allele) on a Southern blot will disappear if the marker employed is close enough to the recessive oncogene locus, in this case, *RB1.*

pocket to the E2F family of transcription factors. [146] [147] Phosphorylation of $pp110^{RB}$ by cyclin-dependent kinases releases E2F to activate transcription at a host of critical gene promoters (see Chap. 6). *RB1* protein also interacts with histone deacetylase, [148] [149] an enzyme thought to have a major role in remodeling chromatin structure. Presumably, the *RB1* protein may act through this enzyme to rearrange inhibitory nucleosome arrays at critical gene promoters. Several other pocket proteins related to $pp110^{RB}$ have subsequently been discovered, although how each functions in relation to cell cycle control is not yet known.

TP53, Guardian of the Genome

Although *RB1* was the first well-characterized example of a human tumor suppressor gene, *TP53* has proved more important as a gene central to human tumorigenesis. First discovered as a 53-kd nuclear protein associated with the SV40 large T antigen,

TP53 has subsequently been shown to be mutated in the overwhelming majority of nearly all human tumor types.[150] Inactivation of the gene takes one of two forms: missense mutations resulting in dominant-negative forms of the protein that are capable of binding to and inactivating wild-type protein produced by the other, normal allele, and nonsense and deletion mutations that completely inactivate the gene.

The p53 protein operates at the intersection of three important cell processes: DNA damage recognition, control of cell cycle progression, and programmed cell death (apoptosis). It connects these processes in such a way that DNA damage produces cell cycle arrest at the G_1/S boundary.[151] This checkpoint is active until DNA repair occurs and the cell re-enters the cell cycle. If repair does not occur within a certain period of time, programmed cell death is initiated. Several cyclin-dependent kinase inhibitors, most prominently CDKN1A ($p21^{cip/waf}$), are the downstream targets of p53 that are responsible for this blockade.[152]

Thus, from the point of view of tumorigenesis, cells lacking a functional p53 protein tolerate genetic damage and genetic instability that would produce arrest and repair or death in normal cells. From the point of view of anticancer therapy, cells lacking a functional p53 are often incapable of initiating certain forms of programmed cell death. Since most cytotoxic therapies, including irradiation, kill cells through apoptotic pathways, cells without p53 are relatively resistant to such agents.

Tumor Suppressor Genes in Other Familial Tumors

A pattern of similarity has emerged between familial retinoblastoma and nearly all the dominantly inherited cancer syndromes. In the past several years many of the disease loci for these familial syndromes have been mapped and cloned, including loci for Wilms tumor,[153] several forms of multiple endocrine neoplasia,[154] [155] polyposis coli,[156] and neurofibromatosis types I[157] and II.[158] In each instance examined, tumor DNAs from such patients show loss of heterozygosity, with markers near the appropriate family cancer gene. Each disease locus maps to a unique chromosome position, which strongly suggests the existence of multiple genes with the properties of *RB1.* Finally, mutations of the *TP53* gene itself have been shown to be responsible for the Li-Fraumeni syndrome.[159]

In recent years, genes responsible for several familial forms of more common tumors have been isolated and characterized. The *BRCA1* and *BRCA2* genes are now known to account for approximately 50% of cases of familial breast cancer.[160] [161] The proteins encoded by these genes are large, with no previously known domains or functions. Recently, both products have been detected in nuclear macromolecular complexes with the Rad51 protein, a component of DNA repair and recombination.[162] [163] Finally, mutations in at least a half-dozen genes responsible for DNA mismatch repair, including *MLH1, MSH2, PMS1,* and *PMS2,* have been implicated in hereditary nonpolyposis colon cancer.[164] [165] [166] The implication of these results, coupled with previous findings concerning *TP53,* is that compromise of genomic stability from defects in any one of several possible pathways results in cancer.

Tumor Suppressor Genes in Sporadic Cancer

Although patients with dominantly inherited cancer syndromes do not form a large fraction of all cancer patients, the molecular genetics of these syndromes is highly relevant to sporadic cancer. First, specific patterns of loss of heterozygosity are observed in cancers of the breast, lung, colon, stomach, and kidney.[167] [168] [169] [170] [171] As we have shown, this molecular lesion is the hallmark of a recessive oncogene. Since each of these tumors displays different chromosomal sites for loss of heterozygosity, the existence of a large set of anti-oncogenes is likely. Furthermore, in tumors such as colon cancer, sporadic cases sometimes show loss of heterozygosity of the same chromosome region (in this instance 5q) as the familial form of the same cancer.[172] This implies that, like retinoblastoma, sporadic cancer can involve molecular lesions of the same genes that cause the familial form of the disease. *BRCA1* and *BRCA2* are, thus far, interesting exceptions to this pattern.

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STAGES OF CARCINOGENESIS

One of the seminal achievements of research in molecular oncology has been the development of a paradigm for stages of carcinogenesis. [173] Early studies established that cell lines that are nontumorigenic and that display relatively normal growth and morphology in vitro often require only one activated *ras* gene to become tumorigenic. This probably reflects genetic damage already undergone simply to obtain the immortalized state that cell lines by definition possess. Cells that have not been established permanently in culture and that have normal karyotypes are not significantly altered by the introduction of one activated oncogene. In this case, two activated oncogenes are required, one with a product located in the cytoplasm and one with a nuclear product. [174] [175] [176] Thus, the introduction of a mutant *ras* gene and a constitutively expressed *myc* gene (but not either alone) will transform a normal diploid fibroblast. Interestingly, cells transformed in this fashion will produce tumors when injected into recipient animals, but the tumors are generally small and often regress. Therefore, at least one other event must occur for the appearance of the fully malignant phenotype.

If tumorigenic cells bearing activated oncogenes are fused to normal cells, the tumorigenic phenotype is usually suppressed. [177] Such hybrid cells, injected into an animal host, will differentiate normally and remain quiescent. With prolonged passage in culture, however, tumorigenic segregants do arise rarely. This subset of the original population of hybrids maintains the activated oncogenes but now demonstrates specific chromosome loss and/or loss of heterozygosity. The loss of heterozygosity is often specific to the cell types employed in the original fusions. Thus, somatic cell genetic experiments with activated oncogenes provide additional evidence for the class of tumor suppressor genes or anti-oncogenes discussed above and suggest that loss of such genes is required for progression of tumorigenesis. Furthermore, wild-type tumor suppressor genes appear dominant over mutant (activated) proto-oncogenes of the *ras* and *myc* variety.

The model system that has subsequently advanced this earlier work is colon cancer. This is because histopathologic progression from benign adenoma to frankly invasive carcinoma may be observed microscopically and characterized at the molecular level. A series of studies have revealed that adenoma formation is frequently coincident with *ras* gene mutation, followed sequentially by loss of genetic material at several locations on chromosomes 5, 17, and 18. [118] Eventually, genes at each of these locations were identified: *APC* on chromosome 5,[156] *TP53* on chromosome 17, and *DCC* on chromosome 18.[178]

Another finding from these studies, representing a new and potentially fruitful area of investigation, is the change in DNA methylation that occurs in colorectal tumor progression. Relatively early in tumor development, DNA becomes hypomethylated. Because DNA methylation is inversely correlated with gene expression, this strongly implicates such epigenetic changes in carcinogenesis. Although a comprehensive discussion of epigenetic changes is beyond the scope of this chapter, we now know that specific sites of methylation, rather than global changes, are more important to the expression patterns

of specific genes. Tumor suppressor genes may potentially be inactivated by inappropriate methylation of their expression control regions. [179]

Finally, although malignant transformation apparently requires the complex interplay of oncogenes and tumor suppressor genes, the evolution of cancer cells with metastatic potential may require the participation of further classes of genes not ordinarily included in the oncogene category. These classes include adhesion molecules and homing receptors expressed on the tumor cell surface, as well as specific proteases secreted by these same cells. Such products presumably enable a cell in which genetic changes are altering the mitogenic signal apparatus and program for cell division to invade local blood vessels and lymphatics, travel to distant tissue targets, and begin anew the expansion of a malignant cell population.

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GENETIC INSTABILITY IN THE EVOLUTION OF CANCER AND LEUKEMIA

It is evident from recent work that the preservation of genomic integrity is a central feature of the protection of cells from malignant transformation. Abrogation of checkpoints and/or repair and recombination pathways, as indicated by studies of *TP53, BRCA1*, and *BRCA2,* mismatch repair, radiation sensitivity, and gene amplification, is now recognized as a major, and perhaps the most significant, contribution to carcinogenesis. This work complements an extensive series of earlier observations coupling chromosome instability and UV- and x-irradiation sensitivity to risk of cancer and leukemia. Recently, genes responsible for several of these syndromes, including xeroderma pigmentosa group G (XP-G) and ataxia-telangiectasia (AT), have been cloned. The AT gene, *ATM,* is now placed in the pathway linking DNA strand break detection to p53 activation.[180] It is related to another protein involved in double strand break repair, the DNA-dependent protein kinase. The XP-G product is implicated not only in DNA excision repair but also in transcriptional repair, by participating as a component of the basal transcription apparatus.[181]

These cumulative results bring us closer to elucidating two long-standing speculations about the nature of cancer. First, tumor cell heterogeneity and the plasticity of tumor cell response to therapy had previously led to the predominant view that cancer cells are intrinsically genetically unstable and that this instability is directly related to the progression of the neoplastic state. We now know that this view is largely correct, and we know further that inherited states of genetic instability can be responsible for the initiation as well as the progression of cancer and leukemia.

The second area of speculation that is yielding to current research is the relationship during tumorigenesis between extrinsic and intrinsic insults to the genome. Although many somatic mutations (e.g., *TP53* and *ras* family members) are clearly important, it has not been possible to specify the meansenvironmental damage versus intrinsic mutation mechanismsby which these changes arise. Now, as the result of a better understanding of mechanisms, as well as the development of sensitive technology to detect DNA damage, we are positioned to characterize comprehensively the source and nature of DNA damage in tumors. Several examples serve to illustrate the range of possibilities. When mismatch repair is malfunctioning, genes possessing short tracts of repetitive nucleotides should be at risk of mutation. This is because replication slippage at such sites is not repaired. At least one important confirmation of this prediction has been published. The transforming growth factor- type II receptor, which contains just such a nucleotide tract, is frequently mutated in gastrointestinal tumors with mismatch repair defects.[182] Also, inherited gene variants can be intrinsically more mutable. A relatively frequent variant of the familial polyposis gene *APC* in Ashkenazim bears a mutation that converts a portion of the coding sequence to a short repetitive tract. The inherited mutation does not itself cause any change in the function of the gene. However, the region becomes a hotspot of somatic mutation because of increased replication slippage, leading to pathogenetic damage to the gene.[183] Finally, recent work has directly connected environmental mutagens to specific DNA targets within cancer-related genes. Adducts of benzo(a)pyrene, one of the principal mutagens of cigarette smoke, have been detected at precisely the nucleotides of *TP53* that are frequently mutated in lung tumors of smokers.[184] We may expect future studies to elucidate further the interplay between host and environmental determinants of carcinogenesis.

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Chapter 50 - Pharmacology and Molecular Mechanism of Action or Resistance of Antineoplastic Agents: Current Status and Future Potential

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INTRODUCTION

The past few decades represent the modern era of chemotherapy against cancer. During these decades, the activities of diverse anticancer agents, alone or in combination, have been explored through clinical trials. The development of antimetabolites (e.g., cytosine arabinoside, methotrexate, and hydroxyurea), alkylating agents (e.g., cyclophosphamide, melphalan, and busulfan), anthracyclines (e.g., doxorubicin, daunomycin, and idarubicin), topoisomerase I or II inhibitors (e.g., etoposide, mitoxantrone, and topotecan), the platinum compounds (e.g., cisplatin and carboplatin), as well as the antimicrotubule drugs (e.g., vincristine, vinblastine, and taxol), has gradually expanded our armamentarium of chemotherapeutic agents. Impressive gains have been made in our understanding of the pharmacology, toxicity profile, and antitumor activity of these and other classes of drugs. Combination chemotherapy incorporating drugs with different mechanisms of action and toxicity profiles has produced a long-term disease-free state in some patients with leukemia, lymphoma, and epithelial cancers. However, in the majority of patients, these attempts have resulted in considerable morbidity and eventual tumor progression. Collectively, this clinical trial experience in cancer has highlighted the importance of understanding the unique features of tumor biology that may either confer drug resistance or explain the antitumor selectivity of the chemotherapeutic drugs. A rapidly expanding knowledge of the functions of proteins encoded by oncogenes, tumor suppressor genes, and the DNA mismatch repair genes has greatly contributed toward a better understanding of tumor cell genomic instability and biology. The role of some of these proteins in growth factor receptor signaling, cell-cycle progression, and programmed cell death (apoptosis) has elucidated potential determinants of drug sensitivity and mechanisms of resistance in tumor cells.

This chapter reviews the mechanism of action and pharmacology of antineoplastic drugs, with special emphasis on drugs used in the treatment of hematologic malignancies. In addition, it provides an overview of the recent advances in our understanding of tumor biology as it relates to anticancer drug sensitivity and strategies to overcome drug resistance.

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CELL KINETICS, THE CELL CYCLE, AND TUMOR GROWTH

The cell cycle consists of a series of stages through which normal and neoplastic cells proceed during the course of cellular

replication (Fig. 50-1). It is divided into G_1 (pre-DNA synthetic phase), S (in which DNA replication takes place), G_2 (post-DNA synthetic phase), and mitosis (M), during which chromosomal division and segregation occur.[1] In addition, nonproliferating, resting cells are said to reside in G_o, a phase that may theoretically last for an indefinite period. Such cells remain in G_o until they are induced to enter the cell cycle (at G_1) by specific triggers, such as hematopoietic growth factors. The *growth fraction* of a tumor represents the percentage of cycling cells relative to the total cell population and is given by the formula $(G_1 + S + G_2 + M)/(G_o + G_1 + S + G_2 + M)$. The *noncycling fraction* is given by the formula $(G_o)/(G_1 + S + G_2 + M)$. Growth fractions of various neoplasms range from <10% in the case of certain slowly growing solid tumors to >90% in the case of certain hematologic malignancies (e.g., Burkitts lymphoma). The *generation time* represents the time required for a cell to proceed through a single cell cycle (generally 2436 hours for hematopoietic tissues). Surprisingly, in the case of acute myeloid leukemia (AML), the generation time of leukemic blasts is not

Figure 50-1 Progression through the cell cycle is controlled through complex interactions between cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKIs). Progression across the G_1 -S interface and through S phase involves the E2F transcription factor, which activates numerous enzymes (e.g., thymidylate synthase, dihydrofolate reductase) required for DNA replication. The retinoblastoma protein (pRb) in its dephosphorylated state binds to and inactivates E2F in conjunction with DP proteins, thereby inhibiting S-phase progression. Conversely, phosphorylation of pRb antagonizes binding to E2F, allowing S-phase events to proceed. Phosphorylation of pRb results from activation of (a) cyclin-dependent kinase-2 (CDK2):cyclin A/E and (b) CDK4/6:cyclin D complexes. The former complexes are inhibited by the CDKIs p21, p27, and p57, whereas the latter are inhibited by the low-molecular-weight CDKIs (p1418), but also by p21 and p27. The complex formed by CDK1 ($p34^{cdc2}$) and cyclins A and B regulates G_2 M progression and is inhibited by the universal CDKI, p21. Moreover, its phosphorylation status, which plays a major role in determining activity, is regulated by the phosphatase, cdc25. Proteins such as pRb, E2F, p21, and p27 can influence the response of cells to chemotherapeutic agents by controlling cell-cycle progression, and possibly via cell cycle-unrelated actions.

significantly shorter than that of normal hematopoietic progenitors, and may in fact be longer. The proliferative advantage of malignant hematopoietic cells (and of many nonhematopoietic tumors) stems, at least in part, from the fact that a higher percentage is in cycle at any one point in time (i.e., the growth fraction is higher). The *doubling time* represents the period required for a tumor to double in mass, and is in general inversely related to the tumors growth fraction. Thus, tumor doubling times range from >120 days in the case of some solid tumors (e.g., lung and colon) to <2 weeks (in the case of some leukemias and lymphomas). Tumor doubling times are influenced by multiple other factors, including the rate of spontaneous cell death (or apoptosis) and the availability of appropriate nutrients. The importance of these considerations stems from the fact that tumors with high growth fractions and short doubling times tend to be more sensitive to chemotherapy than slowly growing neoplasms with low growth fractions and long doubling times. It is unlikely to be a coincidence that many advanced neoplasms that are potentially curable by chemotherapy (e.g., hematologic malignancies such as leukemia, lymphoma; testicular cancer) fall into the former group; conversely, most incurable advanced malignancies (e.g., non-small cell lung cancer, colon cancer) fall into the latter.

The traverse of neoplastic cells through the cell cycle, as in the case of their normal counterparts, is governed by a complex network of proteins consisting of *cyclins*, *cyclin-dependent kinases* (CDKs), and *CDK inhibitors* (CDKIs).[2] S-phase traverse is regulated primarily by CDK2 in association with cyclins A and E; G_2 M traverse is regulated by CDK1 ($p34^{cdc2}$) and cyclin B; and progression through G_1 involves CDKs 4/6 in conjunction with cyclin D. CDKIs fall into two major categories: the low-molecular-weight inhibitors (pINK14, 15, 16, 17, and 18), which primarily inhibit cyclin D (and to some extent CDK2) complexes, and the higher-molecular-weight inhibitors (p21, p27, and p57), which are more universal in their actions and inhibit most or all CDKs.[3] Signals for progression of cells through G_1 -S are clearly essential to maintenance of the neoplastic phenotype. In the commonly accepted model of G_1 -S progression, inactivation of the retinoblastoma protein (pRB) is required. In quiescent cells, pRB is in an active dephosphorylated state and bound to the transcription factor E2F. Phosphorylation of pRB by CDK4/6 and CDK2 leads to release of E2F, which is then free to activate diverse genes essential for S-phase progression, such as c-Myc, thymidylate synthetase, and dihydrofolate reductase (DHFR). Conversely, induction of CDKIs (e.g., by TGF or differentiation-inducing agents) results in inactivation of CDK 4/6 and CDK2, dephosphorylation of pRB, inactivation of E2F, and inhibition of S-phase traverse. Aberrant expression prediction induction of cyclins and CDKIs is commonly encountered in human malignancies, including those of hematopoietic origin, and it has been postulated that the resultant cell-cycle dysregulation contributes to the neoplastic phenotype.

In addition to cellular growth control, cell-cycle proteins are intimately involved in the regulation of programmed cell death (apoptosis) and checkpoint control mechanisms.[4] Consequently, cell-cycle regulatory proteins can exert a major influence on the response of neoplastic cells to various cytotoxic agents. For example, when cells undergo DNA damage, they may arrest in G_2 M or G_1, during which repair occurs; if the damage is too severe, the cells undergo apoptosis. In particular, the tumor suppressor gene, *p53*, and its downstream inducible target, p21, have been implicated in the G_1 arrest process following genotoxic insult.[5]

During the past several years, it has been shown that dysregulation of various cell-cycle regulatory proteins can have a major impact on the sensitivity of neoplastic cells to various chemotherapeutic agents. Thus, loss of the *p53* gene renders cells resistant to diverse chemotherapeutic agents, presumably by preventing cells from undergoing repair in G_1. Conversely,

transfection of *p53*-negative cells with wild-type *p53* restores responsiveness to most drugs.[6] More recently, it has been reported that dysregulation of the CDKIs p21 (a downstream target of *p53*) and p27 increases the sensitivity of neoplastic cells to various cytotoxic agents, possibly by uncoupling S-phase progression and mitosis.[7] [8] In this model, loss of the CDKI p21 or p27 prevents cells that have sustained DNA damage from arresting in G_1 and allows them to progress inappropriately through S and ultimately G_2 M, eventually leading to cell death. Mutations in the E2F protein have been shown to lengthen the S phase and to increase the sensitivity of malignant cells to S-phase-specific agents.[9] Further, cells lacking functional pRB have been shown to be significantly less sensitive to the actions of antimetabolites, including methotrexate.[10] Thus, aside from regulating cell-cycle status and the susceptibility of neoplastic cells to cell-cycle-specific agents, cell-cycle regulatory proteins can actively contribute to the cell death response.

The growth of tumors is best described by *Gompertzian*, rather than classic exponential kinetics. For neoplasms that grow exponentially, the tumor growth rate remains constant as the number of cells increases. However, in the in vivo setting, the growth of tumors is limited by various factors such as vascular supply, nutritional requirements, and possibly physical restraints. Consequently, the rate of tumor growth declines as the number of cells increases (i.e., *Gompertzian* growth

kinetics). To the extent that tumor doubling times are inversely correlated with drug responsiveness, large, late-stage tumors would be predicted to be less susceptible to cytotoxic drugs than their earlier, higher-growth-fraction counterparts. Most chemotherapeutic drugs kill by *first-order kinetics:* this indicates that a given drug dose kills a constant fraction, rather than number, of tumor cells. The implication of this phenomenon is that it requires the same drug dose to reduce the number of tumor cells from 10^4 to 10^1 cells as it does to reduce the tumor burden from 10^{10} to 10^7 cells.

Cytotoxic agents may be divided into several categories with respect to their effects on the cell cycle and/or the cell-cycle specificity of their actions:

1. Non-cycle-active drugs kill both cycling and noncycling cells in all phases of the cell cycle. Examples include steroids and antitumor antibiotics (except bleomycin).
2. Cycle-active, non-phase-specific drugs are more active against cycling cells and can kill cells in each phase of the cell cycle. However, such drugs may preferentially kill cells in a particular phase of the cell cycle. Examples include alkylating agents, cisplatinum, and 5-fluorouracil.
3. Cycle-active, phase-specific drugs kill cells primarily in a specific phase of the cell cycle. Examples include most antimetabolites, which are active against cells engaged in DNA synthesis (S-phase cells), and microtubule-active drugs (e.g., vinca alkaloids, taxanes), which kill cells in G_2 M.

An example of a cytokinetically rational approach to chemotherapy involves the combination of a non-cycle-active agent (e.g., daunorubicin) with a cycle- and phase-specific agent (e.g., Ara-C). From a theoretical standpoint, administration of a non-cycle-active agent may reduce tumor mass, leading in turn to an increase in the growth fraction via recruitment of cells into cycle. Such cells would then be more susceptible to a cycle- and phase-specific agent, particularly one administered over a prolonged interval. In the case of hematopoietic malignancies, attempts have been made to recruit neoplastic cells into the more susceptible S phase of the cell cycle through the use of *hematopoietic growth factors*. The success of such a strategy may be limited by several factors, including the inability of growth factors to increase the S-phase fraction significantly, the lack of selectivity of this strategy, and the theoretical possibility that growth factors may protect neoplastic cells from apoptosis.

Unfortunately, cytokinetic differences between normal and neoplastic tissues have been difficult to identify and exploit. Consequently, rapidly dividing normal tissues such as gastrointestinal epithelium and normal hematopoietic progenitors tend to be very sensitive to most chemotherapeutic agents. As a result, mucositis and myelosuppression represent frequent dose-limiting toxicities for many cytotoxic drugs.

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TUMOR CELL HETEROGENEITY

Although there is considerable evidence that most if not all hematopoietic malignancies are of *clonal* origin (i.e., they are derived from a single transformed cell), individual neoplastic cells may exhibit a great deal of phenotypic diversity with respect to their behavior. Such differences may involve growth rates and cytokinetic features, growth factor responsiveness and dependence, and chemosensitivity, among many other features. The diversity in neoplastic cell behavior is referred to as *tumor cell heterogeneity.* There is general agreement that tumor cell heterogeneity arises as a consequence of spontaneous mutational events that provide a survival advantage for the affected cells and their progeny. [11] For example, a mutation that renders a hematopoietic cell clone autonomous or growth-factor-independent would be expected to render such cells less susceptible to adverse environmental conditions (e.g., growth factor withdrawal). Similarly, one would also predict that a genetic change facilitating cell-cycle entry or disruption of cellular maturation would ultimately lead to overgrowth of affected clones. For obvious reasons, mutations that interfere with drug metabolism or the cell death pathway itself would provide a net survival advantage, particularly under the selection pressure of cytotoxic drug treatment. In view of the multiple mechanisms by which cellular growth or survival may be enhanced, it is not surprising that tumor cells exhibit considerable phenotypic diversity despite their putative derivation from a common ancestor.

Common mechanisms may be involved in events associated with malignant transformation and the development of mutations that result in tumor heterogeneity. For example, the cell-cycle checkpoint and tumor suppressor gene, *p53*, is induced during DNA damage, leading to G_1 arrest and, if the damage is too severe to repair, cell death by apoptosis. The presumed goal of this process is to eliminate cells that develop deleterious mutations as a result of damage to the genome. Thus, loss of *p53* may not only increase cellular survival by inhibiting the cell death process, but may also promote the transmission of mutations in cells that would otherwise be deleted. In this manner, a defect of the cell death pathway can have multiple consequences: (1) selection of cells exhibiting a growth advantage over their normal counterparts; (2) development of drug resistance; and (3) promotion of mutations that result in either (1) or (2) as well as neoplastic cell heterogeneity.

A model for understanding the relation among tumor growth rate, the occurrence of spontaneous mutations, and the development of drug resistance was first described by Goldie and Coldman, and is referred to as the *Goldie and Coldman hypothesis*.[12] In this model, the size of a tumor depends on a complex interaction between tumor growth rate and cell loss, the latter stemming from the status of the cell death process, exhaustion of available nutrients, and outstripping of the blood supply. As tumors increase in size, the cell death rate tends to increase. Thus, for larger tumors, further increases in tumor mass require a progressively greater number of cell doublings. If one assumes a constant mutational rate, the cells of which a large tumor is composed (and which have undergone a considerably greater number of cell divisions than smaller tumors) would have had a much greater opportunity to acquire mutations conferring a survival advantage. The heterogeneous nature of such mutations makes it likely that multiple mechanisms of resistance will develop as well. Such a model may explain

the common observation that advanced, late-stage tumors are frequently resistant to all available classes of antineoplastic agents.

From an operational standpoint, this model has clear implications for the rational design of therapeutic strategies and provides a basis for early and intensive therapy, as proposed by Hyrniuk and Bush.[13] Thus, treatment of tumors with high-dose, multiagent therapy early in their history (i.e., when their growth fraction is high and the number of cell divisions has been relatively low) is less likely to fail due to the prior development of tumor resistance. Conversely, delay of therapy until the tumor mass is high (and under circumstances in which many cell divisions have occurred) or treatment with suboptimal drug doses is more likely to fail due to the greater opportunity that cells have had to undergo mutations conferring drug resistance.

The successful implementation of this strategy is exemplified by the administration of dose-intensive multidrug regimens (i.e., MOPP in Hodgkins disease, PROMACE-CYTOBOM in non-Hodgkins lymphoma), which are potentially curative when given early in the course of the disease. However, as predicted by the model, administration of these or other intensive regimens in patients with relapsed or late-stage disease generally fails due to a generalized resistance of tumor cells to all classes of chemotherapeutic agents.

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APOPTOSIS AND DRUG SENSITIVITY

All normal and cancer cells possess the requisite molecular machinery to execute apoptosis. This includes a family of cysteine proteases recently designated as caspases because they cleave or degrade a growing list of protein substrates after an aspartic acid residue (Fig. 50-2).[14] This degradation of several protein substrates collectively results in the morphologic and biochemical features of apoptosis. [15] Stimuli that activate the intracellular

Figure 50-2 Overview of the molecular signaling leading to apoptosis.

caspases and trigger apoptosis include anticancer drugs, radiation, and growth factor withdrawal. [15] It is now well established that following interaction with their specific intracellular targets, cytotoxic agents ultimately produce DNA damage and/or cell-cycle perturbation. With most agents, this is somehow translated into a signal that triggers specific preapoptotic mitochondrial events that culminate in the cleavage and activity of the key caspases involved in apoptosis. [16] The preapoptotic mitochondrial perturbations include the release of the electron transfer protein, cytochrome c (cyt c), into the cytosol and the mitochondrial permeability transition (m) associated with a fall in the inner membrane potential (MMP) and an increase in the generation of reactive oxygen species (ROS). The cytosolic cyt c binds to an adaptor protein, APAF-1 (apoptotic protease activation factor-1), which in the presence of dATP binds, cleaves, and activates caspase-9. This in turn cleaves and activates the key executioner caspase, caspase-3. Caspase-3 and other executioner caspases (caspases 6 and 7) cleave the various protein substrates that result in apoptosis.

Among a growing list of genes that regulate apoptosis is the *bcl-2* family of genes.[17] The prototype *bcl-2* gene encodes for p26Bcl-2 protein, which inhibits apoptosis. *bcl-x*$_L$, a homolog of *bcl-2*, encodes for p29Bcl-x$_L$, which is also an antiapoptotic protein. Both Bcl-2 and Bcl-x $_L$ are located in the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope. Bcl-2 and Bcl-x $_L$ exert their antiapoptotic effect by inhibiting the mitochondrial m and blocking the release of cyt c into the cytosol.[16] Bax, Bak, and Bad are the key proapoptotic members of the Bcl-2 family of proteins. Bax, Bak, or Bad can form heterodimers with Bcl-2 or Bcl-x$_L$. A low ratio of Bcl-2 to Bax by increasing free Bax may promote cell death due to anticancer drugs. [19] [20] The loss or low levels of Bax have been correlated with poor response to chemotherapy.[21] The phosphorylation of Bad by Akt kinase, which is activated by growth factor receptor tyrosine kinase signaling, forces disassociation of Bad from Bcl-x$_L$ and Bcl-2.[22] The latter can then exert their antiapoptotic effect.

The three-dimensional structure of Bcl-2 or Bcl-x$_L$ is strikingly similar to the pore-forming domains of certain bacterial toxins. [17] This indicates that Bcl-2 and Bcl-x $_L$ may be channel proteins regulating the traffic of ions or small peptides such as cyt c across mitochondrial membranes, thereby regulating m and apoptosis. The channel function of these proteins may be affected by their levels, oligomerization status, or post-translational modification such as phosphorylation. [17] For example, studies have demonstrated that antimicrotubule drugs such as taxol or vinca alkaloids induce mitotic arrest and the phosphorylation of Bcl-2, which is associated with an increase in free Bax levels and apoptosis of human leukemia cells. [19]

Recently, various cytotoxic drugs, irrespective of their intracellular targets, have also been shown to trigger apoptosis through the death receptor/death ligand-mediated signaling for apoptosis. [23] Cytotoxic drugs may induce Fas death receptor (FasR, CD95)/Fas ligand (FasL) expression. [24] CD95 is a transmembrane cell surface receptor belonging to the TNF receptor family. Following oligomerization, FasR triggers apoptosis through interaction with adaptor molecules (FADD/MORT-1) and caspase-8 (FLICE/Mach-1). This results in the autocleavage and activity of the upstream (e.g., caspase-8 and caspase-10) followed by downstream (e.g., caspase-3 and caspase-6) caspases. Recruitment of this apoptotic pathway by cytotoxic drugs has been shown to be blocked by the inhibitors of CD95-mediated signaling for apoptosis, although this mechanism may not be induced by all cytotoxic agents or operative in all cell types. FasR-mediated caspase activation can also result in the mitochondrial release of cyt c. [16] In addition, the induction of FasR-mediated signaling has been shown to generate intracellular ceramide through the activation of neutral sphingomyelinase

(SMase) and hydrolysis of sphingomyelin (SM) in the cell membrane. [25] Increase in the intracellular ceramide levels triggers the mitochondrial release of cyt c and the molecular cascade of apoptosis. This has been shown to be inhibited by sphingosine phosphate (SPP) or the activation of protein kinase C (PKC). [26] [27]

The normal (wild-type) gene product of the tumor suppressor gene *p53*^{wt} is a sequence-specific transcription factor that exists as a mutated protein in almost 50% of cancers.[5] In response to DNA damage induced by cytotoxic drugs, p53^{wt} induces the expression of p21 protein, which inhibits cell-cycle progression in the G $_1$ /S phase. As noted above, this cell-cycle checkpoint provides an opportunity for DNA repair and genomic integrity. In addition to mediating G $_1$ arrest, p53^{wt} can also induce apoptosis. p53^{wt} has been shown to transcriptionally upregulate Bax and decrease Bcl-2 levels, which may promote apoptosis. [28] These observations have spurred gene therapy trials of adenoviral vectors containing p53 ^{wt} to target malignancies with mutant p53.

Various steps in this sequence of molecular events culminating in the activity of cytosolic caspases can potentially be blocked by any of several mechanisms. These mechanisms would inhibit drug-induced apoptosis and confer drug resistance on cancer cells. Therefore, resistance to drug-induced apoptosis may result from a proximal or upstream mechanism based on the reduced intracellular uptake of drugs, drug target interaction, or drug-induced DNA damage or cell-cycle effect. Alternatively, a mechanism that blocks the preapoptotic mitochondrial events or inhibits cytosolic caspase activity would represent a more distal or downstream mechanism of resistance to drug-induced apoptosis.

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DEVELOPMENT OF CHEMOTHERAPEUTIC AGENTS

Following the initial description of sulfur mustard gas as a biologic warfare agent in World War I, the nitrogen mustard compounds were developed as chemotherapeutic agents in the 1940s. Since that time, the National Cancer Institute (NCI) and the pharmaceutical industry have developed complex techniques for drug development, screening, and evaluation. [29] Initial screening consisted of toxicity assessment against murine tumor cell lines. [30] Current screening is directed toward numerous cell targets known to influence cell death pathways, interrupt cell cycle, induce apoptosis, block mitosis, cause DNA damage, and interrupt DNA repair. Killing cells remains the backbone of chemotherapeutic approaches to human malignancies, although a number of differentiating agents have now entered the chemotherapy armamentarium. With cytotoxic alkylating agents, the most common dose-limiting toxicity is toward hematopoietic progenitor cells, leading to neutropenia, thrombocytopenia, and anemia. [31] Thus, these agents are often used to treat hematologic malignancies. Certain chemotherapeutic agents induce cumulative myelosuppression by killing early hematopoietic progenitor cells, whereas others attack only late progenitors. Secondary leukemias have arisen in patients receiving alkylating agents, [32] topoisomerase inhibitors, [33] [34] and combination chemotherapy and radiation. [35] Thus, chemotherapeutic agents have an important role in both the treatment and etiology of hematologic malignancies.

Screening for Antitumor Activity Among Chemotherapeutic Agents

In the 1950s, the NCI initiated a drug-screening program centered on antitumor activity of the murine leukemia cell lines L1210 and P388, as well as murine tumor panels, which included the Lewis lung carcinoma and the B16 melanoma. [30] [36] Since 1985, a new program for drug screening has been developed within the NCI Division of Cancer Treatment that has resulted in the ability to evaluate >60,000 compounds for cytotoxic and growth inhibitory activity. The NCI in vitro human tumor cell line screen consists of 60 cell lines. [37] [38] These lines include major adult solid tumors such as lung, colon, renal, ovary, melanoma, and brain tumors, as well as rhabdomyosarcomas, medulloblastomas, and neuroblastomas affecting children. Among the leukemia cell lines, K562, MOLT-4, and HL-60 are used, but there are few B-cell lymphomas. [39] Initial screening is performed using the stain sulforhodamine-b, which binds to basic immuno acids and is stable after cell fixation. The screen was validated against known anticancer drugs and standard cytotoxicity assays before formal implementation. [40] Incubation of the various tumor cells at routine drug dilutions (10^{-4} 10^{-8} molar) of the various test compounds is performed followed by an assessment of the compounds effects on the growth of the tumor cell lines. [38]

Following an initial in vitro assay screen, human tumor cell activity is evaluated with a series of athymic mouse xenograft studies targeting tumors from tissues that show promise in in vitro assays. [41] The NCI Decision Network Panel meets regularly to evaluate the progress of drugs through the development pathway. Drugs with promising efficacy and novel mechanisms of action then go on to formulation and toxicology testing and ultimately are developed for phase I clinical testing [38] [42] through the NCI Cancer Therapy Evaluation Program. A number of novel observations have been made regarding these chemotherapeutic agents from the comprehensive database of toxicity assessment. As recently reported by Weinstein, [38] the database has been used to identify clustered cytotoxic activity among agents based on similar cytotoxicity profiles against the 60-cell line panel. Among many observed clustered responses, agents with clustered activity against p53 wild-type or mutant cells, multiple drug resistance, gene-expressing cells, and agents that cause G_1 checkpoint arrest have been identified. With this information, a better understanding of the mechanisms of drug effect and disease-specific responses can be proposed. This has profound implications for future drug development and for use of the NCI database for further assessment of existing compounds.

Human tumor xenografts have been used as a requisite screen in preclinical testing of new chemotherapeutic agents. All tumors are grown either subcutaneously or as ascites in athymic nude mice. In the subcutaneous models, tumor volume is assessed three times weekly and anticancer efficacy is documented by *tumor growth delay*, a measure of the time required to reach a certain volume based on a multiple of the tumor volume at the time of treatment, expressed as (T C)/C × 100, where T is the treatment time and C is the untreated time in days. [41] Other critical parameters and assessments of drug efficacy include drug-related deaths and maximum percent relative mean body weight loss. Other investigators measure net log cell kill, an indication of the number of cells killed at the end of treatment, defined by the formula [(T C) duration of treatment × 0.3 ÷ median doubling time], where the doubling time is the time required for the tumor to enlarge from 200 to 400 mm^3 regardless of tumor regression. [43] [44] [45] [46] Finally, tumor regressions are noted as partial if the tumor increases volume by 50% and as complete if the residual tumor size is below measurable limits of approximately 15 mm. [31]

For leukemia and lymphomas, increase in life span is used as an endpoint for ascites tumors or after intravenous injection. This is measured as the percent increased life span (T C)/c × 100, where T is the life span after drug treatment and C is untreated. [43] Recently, severe combined immunodeficiency (SCID) mice, which carry a nonsense mutation in the DNA-dependent protein kinase disabling the production of normal T and B cells, have been used as hosts for leukemia, lymphoma, and myeloma cell lines. [47] Zhang et al. [48] established a human acute promyelocytic leukemia ascites model in the SCID mouse and found responses to all-transretinoic acid. A number of other

investigators have used the SCID/leukemia model to test antisense oligonucleotide strategies [49] for such tumor-specific genes as bcr-abl [50] for chronic myelogenous leukemia. In both instances, infusion of antisense constructs increased the life span significantly and led to clinical studies with these agents. Others have used the SCID model for B-cell lymphomas to study either chemotherapy or lymphotoxins. [51] While these models have not entered the NCI drug screen, they represent potentially useful systems with which to test new agents, in particular novel gene therapy and immunotoxin approaches.

Phase I Clinical Trial Design

New anticancer agents are assessed through a series of clinical trials termed phase I, phase II, and phase III (Table 50-1). The purpose of phase I clinical trials is to establish the safe and optimal biochemically active dose of the compound in question with acceptable toxicity that can be used in disease-targeted phase II testing. During phase I development, pharmacokinetics and pharmacodynamic measures are studied in detail so that appreciable information can be forthcoming from the very first set of patients targeted for treatment and so that these observations can be confirmed in larger phase II disease-focused trials. Dose, schedule and route of administration are key considerations in early phase I development for some agents, such as those with cell-cycle-dependent toxicity (e.g., cytosine arabinoside), radiolabeled antibodies, photodynamic therapy, and agents with particularly long half-lives. When these issues are resolved early in development, phase II trials proceed more quickly.

A number of considerations have guided dose-escalation strategies that accompany phase I trial development. The starting dose is typically approximately 10% of the lethal dose (LD_{10}) in animals. In classic phase I development, a modified Fibonacci dose schedule is used. [52] Groups of 3 patients are treated at each of the following doses until the maximum tolerated dose (MTD) is observed: 1N (the starting dose), 2N, 5N, 7N, 9N, 12N, and 16N. Typically, the MTD is defined as the maximum tolerated dose not causing irreversible toxicity of any type and causing less than grade 4 toxicity in any organ. For some myeloablative strategies used in high-dose therapy, grade 4 myelosuppression, mucositis, and diarrhea are allowed, but only with careful consideration of supportive care, including the use of hematopoietic

growth factors, antidiarrheal agents, narcotics, and intravenous alimentation to manage patients through severe mucositis. Typically, if 1 dose-limiting toxicity is observed, the patient cohort is expanded to 6 patients; if 2 patients develop dose-limiting toxicity, typically defined as grade 4 toxicity except as noted above, then further entry at this dose is not pursued and the next lower dose level is used to establish the MTD with a total of 6 patients accrued at that dose level.

Alternative strategies of drug escalation have included the use of toxicity grades to enhance dose escalation in early drug development; in other words, if no toxicity is observed, fewer

TABLE 50-1 -- Clinical Trial Design

Phase 1
Evaluate safety by dose escalation, multiple dose schedules.
Establish maximum tolerated dose and dose-limiting toxicity.
Consider use of hematopoietic support if myelosuppression is dose-limiting.
Phase 2
Establish response (complete, partial, objective) in specific diseases.
Phase 3
Compare new treatment with established regimen for the disease in randomized trials.

patients may be accrued to each dose.[53] Recently, the NCI has finished an evaluation of phase I trials conducted under the auspices of the Cancer Therapy Evaluation Program.[54] [55] A modified dose-escalation strategy has been proposed. Using the modified Fibonacci scheme, 1 patient is entered at each dose level until grade 2 toxicity is observed, at which point cohorts of 3 patients are entered at each level. Early in drug development, level-skipping may take place if no toxicity is observed. The overall impact of this is to reduce the number of patients treated at suboptimal doses of therapy and to enhance the ultimate number of patients evaluated at biologically active doses.

With the rapid development of new anticancer agents in preclinical and early clinical testing, emphasis has shifted toward mechanism-based therapeutics. Recent targets of interest include receptor tyrosine kinases, [56] [57] DNA repair enzymes,[58] drug resistance proteins,[59] drug detoxifying enzymes,[60] protein kinase C,[61] topoisomerase,[62] chromosomal translocationally activated protein products such as Bcr-abl and Bcl-2, [49] [50] and cell-cycle-regulating proteins. Many agents are now being evaluated in the phase I setting for evidence of biochemical efficacy using either tumor tissue or surrogate cells to define the drug effect. These will clearly have increased emphasis in the near future as new agents are developed that target specific malignant cell phenotypes; examples include the multidrug resistance in MDR-1,[59] O^6 -alkylguanine-DNA alkyltransferase, [63] glutathione synthesis,[64] and topoisomerase I and II. Analysis of inhibition of these enzyme systems in tumor cells is currently the focus of a number of phase I clinical trials and has led to a new concept in drug development: using a biochemical rather than a toxic endpoint in drug development.

Phase II and III Drug Development

Phase II drug development uses the established phase I dose to define therapeutic efficacy, typically in a two-stage design. An expectant response rate >20% is deemed clinically significant; 15 patients are accrued, and if 2 or more responses are seen, accrual is continued to a total of 26 patients to establish the definite response rate.[65] At this point, combination therapies are instituted in an effort to optimize therapeutic efficacy. For instance, topotecan was found in phase I testing to have efficacy against refractory leukemias, leading to combinations of topotecan with cytosine arabinoside and other agents. [66] [67] [68] Likewise, fludarabine was initially evaluated as a single agent for activity against low-grade lymphomas and both acute and chronic leukemias, and is now used in combination with Ara-C for the treatment of acute lymphocytic leukemia and with chlorambucil for the treatment of chronic lymphocytic leukemias. [69] [70] [71] In the past, strategies for combination chemotherapeutic agents included the use of non-cross-resistant agents with nonoverlapping toxicities. More recently, however, mechanism-based therapeutics have become the focus of drug development. For instance, with the combination of fludarabine and cisplatin, the proposal has been made that fludarabine will inhibit nucleotide excision repair and enhance persistence of cisplatin-induced DNA adducts, resulting in increased antitumor efficacy in chronic lymphocytic leukemia (CLL).[72]

Most phase III trials randomize between an established standard therapy and a new therapy that appeared promising in the phase II setting. These studies are usually multi-institutional, and many involve large national and international cooperative groups. The endpoint of these studies is disease response, survival, and patient tolerance. Linking multiple centers removes the vagaries of reports from single centers, which remain the focus of phase II trials. Phase III trials are important for both positive and negative results. Statistically significant differences between outcomes may cause early cessation of the trial, such as the Eastern Cooperative Oncology Group trial comparing the hybrid of MOPP/ABV with sequential MOPP-ABVD, which found superiority of the MOPP/ABV hybrid with better patient tolerance, a higher complete response rate, and a lower incidence of secondary leukemias. [73] Likewise, a trial in intermediate-grade non-Hodgkins lymphomas comparing PROMACE-CYTOBOM and CHOP therapy found no difference in survival and better tolerance with CHOP. [74]

PHARMACOLOGY OF CHEMOTHERAPEUTIC AGENTS

As noted above, although cytotoxic anticancer agents may be divided into several categories based on the cell-cycle specificity of their action, in keeping with traditional classification they are more appropriately divided into alkylating agents, antimicrotubule agents, antimetabolites, inhibitors of topoisomerases, platinum analogs, and miscellaneous agents. The pharmacology and cellular mechanisms of action of these agents are shown in Figure 50-3 .

Alkylating Agents

Drug treatment for cancer began with the use of the mustard class of alkylating agentsinitially mechlorethamine (nitrogen mustard), which entered into clinical use in the mid-1940s.[75] Alkylating agents remain a mainstay of treatment for numerous hematologic malignancies and are the backbone of high-dose chemotherapeutic agents used to eradicate cancer in the setting of bone marrow or peripheral blood progenitor cell reinfusion (Table 50-2). The use of mechlorethamine in combination with vincristine, procarbazine, and prednisone in the MOPP regimen for the treatment of advanced stage IIIIV Hodgkins disease represented the first example of an empiric combination chemotherapy approach, which led to a dramatic improvement in cure rates for this disease. [76] This model has been replicated in numerous other settings for the systematic treatment of numerous hematologic and solid tumor malignancies.

Nitrogen Mustard

The nitrogen mustard class includes mechlorethamine, cyclophosphamide, 4-hydroperoxy cyclophosphamide (Fig. 50-4), ifosfamide, chlorambucil, and melphalan. These all share a common bis-chloroethyl group attached to nitrogen and a substituted R group, which provides drug specificity (Fig. 50-5). All nitrogen mustards react with DNA in an SN2 reaction, a bimolecular nucleophilic displacement reaction, also called a second-order reaction. [77] While numerous sites are targeted for alkylation, including protein, membranes, RNA, and DNA, the latter appears to be most critical for cytotoxic effects. Nucleophiles in DNA, including nitrogen (N), oxygen (O), and phosphate (P), attract the chloroethyl moiety attached to the R-N backbone, and the chlorine is displaced by the nucleophilic atom to form an aziridinium moiety.[78] The remaining

Figure 50-3 Overview of sites and mechanism of action of the most useful chemotherapeutic agents. [a]Investigational agents.

TABLE 50-2 -- Alkylating Agents

Nitrogen mustards
Mechlorethamine
Cyclophosphamide
Ifosfamide
Chlorambucil
Melphalan
Ethylenimines
Thiotepa
Hexamethylmelamine
Alkylsulphonates
Busulfan
Nitrosoureas
Carmustine (BCNU)
Lomustine (CCNU)
Chlorozotocin
Streptozotocin
Triazines
Procarbazine
Dacarbazine (DTIC)
Tetrazines
Temozolomide

chloroethyl group is then attracted to a second nucleophilic atom, forming a second aziridinium intermediate leading to a second alkylation, forming a cross-link. Both intrastrand and interstrand cross-links are formed. [79] The cytotoxic cross-link formed by the mustard class of alkylating agents appears to be a displaced one or two bases on the opposite strand yielding diguanin-7-ethylene, [80] which appears to provide the needed structural apposition based on base stacking and the helical structure of DNA.[81] [82] This displaced cross-link has been isolated from reaction mixtures between mechlorethamine and DNA. While the nitrogen mustards also attack DNA at other nitrogens, including N1 and N3 of the adenosine and N3 of thymidine, it appears that the N7 guanine position is critical for cytotoxic cross-link formation.[80]

Clinical use of mechlorethamine is now limited to MOPP chemotherapy in Hodgkins disease [73] [83] and local use in a dermatologic suspension for the treatment of

cutaneous T-cell lymphomas.[84] Mechlorethamine is a highly reactive compound, with a half-life <15 minutes, and is a highly reactive vesicant.

Cyclophosphamide and ifosfamide are similar agents with ringed structures off the end-chloroethyl backbone that stabilize the compounds and make them much less reactive. Enzymatic activation is required through multifunction P450 enzymes in the liver, which is why a number of agents, including phenobarbital and prednisone, may alter activation. Cyclophosphamide is an effective oral as well as intravenous agent used in numerous protocols for solid tumors, as well as in regimens for non-Hodgkins lymphomas and high-dose therapy regimens designed to eradicate tumor and bone marrow in patients with solid tumors, lymphomas, and leukemias and those undergoing bone marrow transplantation. Cyclophosphamide is a potent immunosuppressive as well as immunomodulating agent that is toxic to lymphocytes.[85] It has also been identified as an immunomodulatory agent capable of inducing tolerance. [86] While the immunosuppression is useful for patients undergoing allogeneic bone marrow transplantation and for patients with immune thrombocytopenic purpura, as well as other nonmalignant autoimmune diseases such as rheumatoid arthritis[87] and nephritis,[88] it also has been used in settings in which tolerance to allogeneic infused cells may be preferred, such as for tumor vaccine therapy.[89]

Cyclophosphamide is metabolically activated by cytochrome P450 mixed-function oxidases in the liver to 4-hydroxycyclophosphamide (Fig. 50-4).[90] 4-hydroxycyclophosphamide is further converted to aldophosphamide and then to phosphoramide mustard, the alkylated species, and acrolein. Acrolein is a highly reactive aldehyde and the cause of hemorrhagic cystitis. Initially, N-acetylcistein [91] and, more recently, mercaptoethane sulfanate (mesna)[92] have been used to prevent hemorrhagic cystitis secondary to the use of cyclophosphamide and ifosfamide. Use of mesna as a protective agent is now standard for doses of cyclophosphamide and ifosfamide >1,000 mg/m^2 . Mesna is given in divided doses every 4 hours or as a continuous infusion for 1824 hours in a dose equivalent to either cyclophosphamide or ifosfamide.[92]

Figure 50-4 Metabolism of cyclophosphamide.

Figure 50-5 Structure of common alkylating agents.

Bioavailability of cyclophosphamide is quite similar whether given orally or intravenously, although the drug is generally given by bolus intravenous injection. Cyclophosphamide is detoxified through oxidation to 4-keto-cyclophosphamide and carboxyphosphamide by aldehyde dehydrogenase. [77] Cyclophosphamide is used in doses as little as 50100 mg/day orally and in bolus doses of 400750 mg/m ^{2} for solid tumors and 700 mg/m^2 in combination with doxorubicin and vincristine and prednisone as part of the CHOP regimen for non-Hodgkins lymphomas.[74] It is also used at doses of up to 60 mg/kg daily for 4 days in autologous and allogeneic bone marrow transplantation protocols.[93] [94] [95] Other than hemorrhagic cystitis, bone marrow suppression is dose-limiting. Patients receiving such high doses of cyclophosphamide can be rescued by reinfusion of autologous or allogeneic hematopoietic progenitor cells. [96] [97] Other toxicities observed include alopecia and cardiac toxicity,[98] which are unusual and most often seen after high-dose therapy.

4-Hydroperoxycyclophosphamide is a chemically stable form of the reactive intermediate of cyclophosphamide, 4-hydroxycyclophosphamide. It has been used to purge leukemic cells from the bone marrow of patients with minimal residual disease [99] and is currently under evaluation as an effective method to remove leukemic cells from peripheral blood progenitor cell collections in patients with both acute myelogenous and lymphoid malignancies undergoing progenitor cell infusions after high-dose therapy.[100] [101] Remarkably, both cyclophosphamide and 4-hydroperoxycyclophosphamide are much more toxic to committed hematopoietic progenitors such as CFU-GM, BFU-E, and CFU-E, but spare early progenitors, presumably on the basis of their expression of the detoxifying enzyme, aldehyde dehydrogenase.[102] [103] The difficulty in proving efficacy in previous leukemia-purging studies has been the toxicity associated with delayed engraftment following depletion of these committed progenitor cells in the ex vivo graft. [101]

Ifosfamide also undergoes hepatic activation and yields a similar alkylating species. [104] However, hydroxylation appears to occur at a slower rate, yielding a longer terminal half-life of the parent compound in the alkylating species. Chloroacetate acetaldehyde, a metabolite of ifosfamide, reacts with proteins and may be responsible for its urinary toxicity.[105] Biphosphamide is used mainly in the treatment of solid tumors such as renal cell carcinoma, sarcomas, and non-small cell lung cancer.[106] Recently, high-dose ifosfamide regimens have been described in combination with carboplatin and etoposide. [107] High-dose therapy protocols with peripheral blood progenitor cell and/or bone marrow reinfusion have been used to treat patients with testicular and ovarian carcinomas.

Melphalan, or phenylalanine mustard, has an amino acid side chain that stabilizes its structure, allowing oral administration. It is available in both oral and intravenous dosing and has a similar intracellular pathway to DNA cross-linking as cyclophosphamide and the other nitrogen mustards. Melphalan uptake into cells is by means of a neutral amino acid transporter.[108] [109] Its rate of cross-link formation is much slower than that of mechlorethamine, presumably due to delayed metabolism. Melphalan is used orally predominantly in the treatment of multiple myeloma and intravenously in high-dose regimens for multiple myeloma. [108] Its unpredictable oral high availability has led investigators to suggest dose adjustment in the treatment of myeloma based on evidence of myelosuppression rather than simply using a standard oral dose of 0.2 mg/kg.

Chlorambucil is used in the treatment of chronic lymphocytic leukemia [110] and in some instances for low-grade non-Hodgkins lymphomas. Chlorambucil is the phenylbutyric acid derivative of nitrogen mustard and is very stable, entering the cell by diffusion rather than by a specific uptake mechanism. It is typically administered orally on a daily basis or intermittently for 47 days per month. [109] It appears to have greater bioavailability than melphalan and to have a more consistent half-life of approximately 2 hours.

Busulfan

Busulfan is an alkylsulfonate, unique among alkylating agents because of its two sulfur groups and the lack of a chloroethyl moiety (Fig. 50-5). Busulfan, like the nitrogen mustards, reacts predominantly at the N7 position of guanine and produces an N7-N7 biguanyl DNA cross-link, although the precise nature of this cross-link appears different than that of the nitrogen mustards.[81] This may explain the different clinical utility of busulfan in comparison with the nitrogen mustards. The pharmacokinetics of busulfan are important for its use in high-dose therapy for ablation of the bone marrow in patients undergoing autologous transplantation for acute leukemia or allogeneic transplantation. Busulfan clearance does not appear to be predicted accurately by creatinine clearance; thus, children are typically dosed at much higher levels per kilogram than adults to achieve the same peak concentrations and area under the curve values. [111] [112] [113] Recently, it has been shown that the incidence of

veno-occlusive disease is lower in patients receiving high-dose busulfan based on predosing pharmacokinetics. [114] Busulfan is a potent stem cell toxin, killing both early and late hematopoietic progenitor cells and damaging the bone marrow stroma.[115] [116] In this regard, busulfan is quite different from cyclophosphamide but similar to the nitrosoureas in their ability to cause cumulative and severe myelosuppression. Other toxicities of busulfan include nausea and vomiting and pulmonary interstitial and intra-alveolar edema, leading to fibrosis. [117] [118] The pulmonary fibrosis is distinct from the interstitial pneumonitis that accompanies allogeneic bone marrow transplantation and is not related to cytomegaloviral or other viral infections.

In the past, busulfan was recommended for the management of chronic myelogenous leukemia.[119] However, it has been supplanted by the use of hydroxyurea and interferon-.[120] [121] It has excellent bioavailability and is given orally, even in high-dose regimens, where it has an uptake within 12 hours. [111] [112] [113] [114] In high-dose regimens, a standard dose of 1 mg/kg every 6 hours for 4 days is used to target an area under the curve of 6,0007,000 ng/ml. [113]

Nitrosoureas

There are four chloroethyl-nitrosoureas and one methyl-nitrosourea in clinical use. The chloroethyl nitrosoureas were derived from methylnitrosourea by

Montgomery.[122] These agents are different from the nitrogen mustards in that they alkylate through an SN1 reaction, forming a highly reactive intermediate in the presence of N, O, and P nucleophiles in DNA, leading to decomposition of the parent compound and chloroethylation. The commonly used clinical agent is (2-chloroethyl)-N-nitro-sourea (BCNU, carmustine) (Fig. 50-5).[123] [124] [125] N-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-N-(2-chloroethyl)-N-nitrosourea (ACNU) is commonly used in Japan.[126] A third agent, N-(2-chloroethyl)-N-cyclohexyl-N-nitrosourea (CCNU, lomustine), is used predominantly as an oral nitrosourea in children with brain tumors.[126] All of these compounds have a high hydrophobicity, actively penetrate the bloodbrain barrier, and are useful for the treatment of gliomas and other central nervous system malignancies.[126]

The key chemical activation process is decomposition of the parent nitrosourea in the presence of a nucleophile, forming a chloroethyl-diazonium hydroxide that becomes the chloroethyl donor group. The DNA alkylation sites [124] [126] include N7 and O^6 of guanine. Chloroethylation at the O^6 position of guanine appears critical to cytotoxicity. In addition, Ludlum and Tong[124] identified hydroxyethyl derivatives at N7 and O^6 guanine that appear to have arisen either from hydrolysis of the chloroethyl group or an ill-defined decomposition product. DNA cross-linking by chloroethyl nitrosoureas was identified by Erickson et al., [127] and two types of cross-links were found, at 1-(3-cytosinyl), 2-(1-guanyl) ethane and 1-2-bis(7-guanyl) ethane (Fig. 50-6). [124] [126] The former appears to be responsible for much of the cytotoxicity observed with the chloroethyl nitrosoureas. It is formed after alkylation at the O^6 position of guanine. This adduct undergoes intramolecular rearrangement to a circular intermediate, N1; O^6 ethanoguanine is formed, which can then rearrange by attack at the opposite hydrogen-bonded base N3 of cystine, forming the interstrand cross-link.[128] This is a unique DNA cross-link and is poorly recognized by DNA repair processes, leading to the marked cytotoxic potency of this cross-link.

The pharmacokinetics of the chloroethyl-nitrosoureas has been evaluated in both conventional and high-dose schedules. A very short half-life has been detected, which is quite variable, but an association between the area under the curve of BCNU and pulmonary toxicity has been observed by Jones et al. [129] [130] BCNU is predominantly used for the treatment of gliomas and malignant melanoma.[131] [132] [133] [134] At high doses it is part of the STAMP 1 and 5 regimen for the treatment of advanced breast cancer[129] [135] and the BEP and related regimens for the treatment of recurrent lymphomas.[136] [137] In both settings, it is an effective form of therapy

Figure 50-6 Cross-links formed by nitrogen mustards **(top)** and chloroethyl nitrosoureas such as carmustine (BCNU) **(bottom)**.

with sustained complete remission rates of approximately 40%. Doses of 6001,000 mg/m^2 have been safely administered.

The chloroethyl-nitrosoureas cause profound and cumulative bone marrow suppression at conventional doses of 120150 mg/m^2, limiting treatment to 35 cycles at 6-week intervals.[129] [135] The 6-week administration interval is needed because the nitrosoureas are most toxic to hematopoietic progenitor cells and myelosuppression nadirs are typically observed 35 weeks after treatment, becoming intensified with additional cycles of treatment. [136] [137] [138] [139] [140]

Complications of high-dose BCNU therapy include pulmonary toxicity and renal toxicity at doses >6001,200 mg/m^2. Pulmonary toxicity, as evidenced by a decrease in the DLCO, occurs in up to 40% of patients and is symptomatic in 1015%.[129] [130] [136] It can be managed with steroids during the inflammatory but not the fibrotic phase and is lethal in some cases.[141] Chronic pulmonary damage leading to fibrosis is particularly common at cumulative doses >1,000 mg/m^2 or following single doses of 9001500 mg/m^2. However, symptoms of pulmonary dysfunction are found in only 1015% of patients receiving 450600 mg/m^2.[136] Fibrosis is associated with an early interstitial infiltrate followed by evidence of hyaline membrane formation and replacement of the chronic inflammatory cells by fibrosis over a 4- to 6-week period. [142] Renal dysfunction associated with nitrosourea therapy occurs less frequently than severe cumulative myelosuppression and pulmonary fibrosis and is typically seen at doses >1,000 mg/m^2. Interstitial nephritis with glomerulosclerosis, interstitial fibrosis, and dropout of tubules has been reported [143] [144] with BCNU or CCNU. This can progress to renal failure but is reversible in many instances. Finally, a new nitrosourea derivative, 2-chloroethyl-3-sarcosinamide-1-nitrosourea, [145] will soon enter clinical trials and in preclinical models has improved activity against gliomas and colon cancers. [146]

Methylating Agents

Four methylating chemotherapeutic agents are available for clinical use: procarbazine, dacarbazine, streptozotocin, and temozolomide. Procarbazine and dacarbazine are triazines.[147] Streptozotocin is a monofunctional methylnitrosourea derivative with an attached sugar moiety. [31] [148] Temozolomide is an imidazotetrazinone synthesized in England in the 1960s, reaching clinical trials in the late 1980s. [149] All react with DNA by undergoing SN1 reactions forming a methyldiazonium ion, resulting in methylation of N^7 guanine, O^6 guanine, O^4 and O^2 thymine,

and N^3 adenine.[150] [151] [152] None form DNA cross-links, but all induce high levels of DNA methylation. N^7 methylguanine is unstable, leading to ring opening and both spontaneous and enzymatically mediated depurination. Repair proceeds through the base excision repair system. [153] [154] Recognition by the N-methylpurine glycosylase results in removal of the adducted base with formation of an abasic site recognized by the apurinic endonuclease, which then cleaves the backbone at the apurinic site.[115] Subsequently, the free 5 sugar is released by DNA lyase, with repair initiated by polymerase and DNA ligase. [153] Base excision repair effectively removes N7 methylguanine and N3 methyladenine and restores DNA to normal. These lesions appear to contribute little to cytotoxicity and appear to be up to 500-fold less potent as cytotoxic adducts than O^6 methylguanine.[152]

O^6 methylguanine mispairs with thymine during DNA synthesis, resulting in a lesion recognized by the mismatch repair system. [152] [156] This system consists of two sets of enzyme homologs of mismatch repair in prokaryotes, mutS and mutL.[156] Mispair recognition proteins are GT binding protein or MSH6 and MSH2, homologs of the mutS system in bacteria. MSH2 and MSH6 form a stable heterodimer.[156] Recognition of the mispair recruits additional proteins to the complex, including MLH1 and PMS1/PMS2, which are homologs of mutL- in bacteria. These proteins initiate exonuclease cleavage of a long patch in the newly synthesized strand of DNA. This is then repaired by polymerases and . Unfortunately, if the O^6 methylguanine adduct is not removed before repair synthesis, a thymine is again inserted opposite the O^6 methylguanine and the repair process begins again, resulting in an aberrant episode of repair and multiple single-strand breaks. [152] [156] [157] [158] [159] Cell protection from methylating agents arises either by removal of O^6 methylguanine or inactivation of the mismatch repair system.[152] Cells expressing high levels of the DNA repair protein for O^6 methylguanine, O^6 alkylguanine DNA alkyltransferase are approximately 10-fold more resistant to methylating agents than alkyltransferase-negative cells.[152] [160] Mismatch repair-mediated, single-strand patches promote chromosomal aberrations, homologous and nonhomologous recombination, and induction of apoptosis in a p53-dependent and -independent manner.[161] Absence of one or more protein components of mismatch repair, leading to the phenotype of replication error repair, is commonly observed in hereditary colon cancer as well as endometrial cancer, gastric cancer, [156] [162] [163] [164] and about 15% of lymphomas.[165] Cells lacking mismatch repair are up to 100-fold more resistant to methylating agents than are cells with mismatch repair, regardless of alkyltransferase activity. [152] Acquisition of mismatch repair defects is associated with acquired resistance to methylating agents and cisplatin, which is also recognized by this protein complex. [152] [156] [157] [158] [159]

Procarbazine was originally synthesized as a monoamine oxidase inhibitor but has been used since the 1950s for the treatment of both Hodgkins and non-Hodgkins lymphomas, as well as in combination therapies for gliomas.[166] [167] [168] [169] It is used with mechlorethamine, prednisone, and vincristine in the MOPP regimen; with BCNU, vinblastine, cyclophosphamide, and prednisone (BVCPP) for Hodgkins disease; [170] and in combination with vincristine and BCNU for the treatment of high-grade gliomas.[132] Procarbazine is metabolically activated in the liver by P450 multifunction oxidase. [171] A methyldiazonium derivative formed by each of the methylating agents is the penultimate compound leading to DNA methylation by an SN1 reaction.[172] Recent evidence has shown that patients receiving procarbazine form O^6 methylguanine DNA adducts with depletion of the alkyltransferase enzyme, a suicide protein that removes the methyl group in an irreversible stoichiometric reaction.[46] Chromosomal breaks, single-strand breaks, and chromosomal aberrations and sister chromosome exchanges observed following procarbazine treatment support its association with treatment-related myelodysplasia and leukemia. [173] [174] All methylating agents are more cytotoxic to cells in the second round of DNA synthesis following DNA methylation with a G_2 blocked cell cycle. This is due to processing of the methyl adducts by both base excision repair and mismatched repair, leading to apoptosis and cell death. [58] [175] Inhibition of DNA synthesis, RNA synthesis, and protein synthesis is delayed until the second round of DNA synthesis, again suggesting that disruption of replication by processing of the methyl adducts leads to the cytotoxic effect rather than the direct effect of the adducts themselves. Procarbazine is administered orally with moderate nausea and vomiting. During the 14-day course, induction of microsomal cytochrome P450 oxidoreductase alters the enzymatic conversion and may increase formation of the methyldiazonium ion, leading to increased methylation. [145]

Dacarbazine (DTIC) is metabolically activated by cytochrome P450 microsomal oxidoreductases, ultimately leading to formation of the methyldiazonium ion and DNA methylation.[150] Dacarbazine is used in combination with doxorubicin, vinblastine, and bleomycin for the treatment of Hodgkins disease [75] and is also used for the treatment of metastatic malignant melanoma in combination with BCNU, cisplatin, and tamoxifen.[176] Activation of dacarbazine requires hydroxylation of one terminal

methyl group caused by demethylation, forming 5(3-methyltriazeno) imidazole-4-carboxamide (MTIC), with spontaneous decomposition to the methyldiazonium ion, which alkylates the DNA as noted previously.[61] [177] [178] Like procarbazine, dacarbazine has been shown to form N^7 methylguanine and O^6 methylguanine DNA adducts in human peripheral blood lymphocytes and to deplete alkyltransferase through repair of O^6 methylguanine DNA adducts. A correlation has been found between tumor response and formation of O^6 methylguanine DNA adducts,[179] indicating the importance of this lesion in clinical antitumor effects. Maximum tolerated doses of dacarbazine are approximately 1,000 mg/m^2 , with myelosuppression and gastrointestinal toxicity (including severe watery diarrhea) as the most common side effects.[180] [181] As with all methylating agents, cumulative myelosuppression has not been observed, unlike with the chloroethyl-nitrosoureas, suggesting preservation of primitive quiescent hematopoietic progenitor cells, which may take longer to recognize and repair O^6 methylguanine DNA adducts, such that they contribute less to cytotoxicity than do DNA cross-links.

Temozolomide represents a new class of imidazotetrazinones synthesized at the Medical Research Council of Great Britain in the 1960s. [149] [182] [183] It differs from DTIC in that it is chemically degraded to the monomethyl triazine, MTIC, at neutral pH and does not require P450 enzymatic demethylation. [184] Thus, in phase I and II trials, it has much more consistent pharmacokinetic parameters, including peak serum concentrations, volume of distribution, and clearance, as well as conversion to MTIC, compared to dacarbazine.[149] [182] [183] It continues to undergo phase II and III clinical testing in Europe and the United States and has shown particular effectiveness against high-grade gliomas, anaplastic astrocytomas, [149] [183] and malignant melanomas.[185] Dose-limiting toxicity is thrombocytopenia and, less frequently, neutropenia, with maximum tolerated doses of 1,000 mg/m^2 given over 5 days on a daily or twice-daily regimen. [149] [182] [183] Nausea and vomiting, the other common side effects, are easily controlled with antiemetics. Formation of O^6 methylguanine has been documented in clinical trials with temozolomide with much more rapid depletion of the alkyltransferase, suggesting that temozolomide may be a more effective methylating agent because of its ability to form higher levels of toxic O^6 methylguanine DNA adducts. It may eventually replace the other methylating agents in combination regimens.

Alkylating Agent-Induced Leukemias

Alkylating agents induce dose-limiting myelosuppression, and thus it is not surprising that these agents also cause sublethal

DNA damage to hematopoietic progenitors, causing mutational events leading to both myelodysplastic syndromes and malignant transformation to preleukemic and leukemic states. A concern has been the use of hematopoietic growth factors after exposure to alkylating agents. There is evidence of increased cytotoxicity to hematopoietic progenitors during simultaneous exposure to these agents and growth factors.[186] Treatment-related acute nonlymphocytic leukemia (T-ANLL) accounts for approximately 15% of all cases of adult ANLL. About 50% of T-ANLL patients have a preleukemic phase, compared to only 10% of patients with de novo ANLL. [35] Complete remission rates are 1530% in patients with T-ANLL.[187] In addition, remission duration is a mean of only 2 months. Chromosomal abnormalities are common in T-ANLL, with >90% of cases expressing a chromosomal rearrangement, loss, or addition.[35]

Historically, patients with Hodgkins disease treated with mechlorethamine and procarbazine in the MOPP regimen or with CCNU were at highest risk if exposed to radiation as well as an alkylating agent combination.[188] Patients with polycythemia vera treated with chlorambucil were at a much higher risk than patients treated with phlebotomy alone, which contributed to the shift in treatment strategy.[189] Patients with myeloma and ovarian cancer also developed T-ANLL, especially following prolonged exposure to alkylating agents.[190] Patients treated with alkylating agents for benign diseases also have an increased risk of T-ANLL, such as patients with nephritis, lupus, psoriasis, rheumatoid arthritis, [191] and Wegeners granulomatosis. [192] The mean latency between exposure and T-ANLL from alkylating agents is 45 years,[32] [35] [189] [190] [193] in contrast to T-ANLL from etoposide, which has a latency as short as 1 year.[194] The cumulative risk for developing T-ANLL is 1017% at 46 years for myeloma treated with nitrogen mustards and 210% at 710 years for Hodgkins disease.[188] More recently, alkylating agent-associated T-ANLL has been recognized in patients with breast [195] [196] or colon[197] cancer. A series of nonrandom chromosomal aberrations associated with T-ANLL have been identified.[193] Loss or deletion of all or part of the long arm [q] of chromosomes 5 or 7 [190] is common, as are trisomy chromosome 8, deletions of the short arm of chromosomes 12 and 17, and 21.[35]

The etiology of alkylating agent-induced leukemias has been established in murine models of nitrosourea-induced T-cell lymphoblastic lymphoma/leukemia and ANLL.[198] [199] The mouse lymphoma/leukemia is often associated with nitrosourea-mediated activating mutations of K-*ras*.[200] In addition, transgenic mice carrying mutant p53 or pim-1 are much more susceptible to nitrosourea induction of lymphoblastic lymphoma than their nontransgenic counterparts. [200] [201] A reasonable hypothesis is that hematopoietic cells are susceptible to nitrosourea-induced carcinogenesis due to the low capacity for DNA repair. Because mouse thymus and human bone marrow contain very low levels of O^6 -alkylguanine-DNA alkyltransferase,[202] they accumulate mutagenic O^6 -alkylguanine-DNA adducts. Proof of the association between lack of DNA repair and nitrosourea susceptibility was established by Dumenco et al., [203] who generated transgenic mice expressing high levels of the human alkyltransferase DNA repair protein in the thymus. These mice had significant protection from nitrosourea-induced lymphomas.

Antimicrotubule Agents

The antimicrotubule drugs include vinca alkaloids (e.g., vincristine, vinblastine, and vinorelbine), taxanes (e.g., paclitaxel and docetaxel), and a unique synthetic compound, estramustine (Table 50-3).

The vinca alkaloids are naturally occurring (vincristine and vinblastine) or semisynthetic (vinorelbine) nitrogenous bases derived from the pink periwinkle plant, *Catharanthus roseus*.[204] Paclitaxel was originally isolated from the bark of the Pacific yew, *Taxus brevifolia*.[205] Paclitaxel can also be isolated from other

TABLE 50-3 -- Antimicrotubule Agents

Vinca alkaloids
Vincristine
Vinblastine
Vinorelbine
Taxanes
Paclitaxel
Docetaxel
Estramustine

members of the *Taxus* gene and from a fungal endophyte that grows on the Pacific yew.[206] Docetaxel is derived semisynthetically from 10-deacetyl-baccatin III, which is obtained from the needles of the European yew, *Taxus baccata*. [207]

Both vincristine and vinblastine bind to the protein tubulin at a site distinct from the taxanes and, at low concentrations, inhibit microtubule dynamics. At higher concentrations these vinca alkaloids disrupt microtubules and the mitotic spindle, resulting in cell-cycle mitotic arrest and apoptosis of cells (Fig. 50-2). In contrast, after binding to -tubulin, taxanes kinetically stabilize microtubule dynamics at their plus ends, as well as shift the equilibrium toward tubulin polymerization into microtubule bundles. This also causes mitotic arrest and apoptosis of cells.[19] The mitotic arrest caused by antimicrotubule drugs is associated with phosphorylation of Bcl-2 protein and increased intracellular levels of the free Bax protein, which promote apoptosis. [19]

Antimetabolites

The antimetabolites consist of low-molecular-weight compounds that interfere with micromolecular synthesis. As a group, they may be contrasted with agents such as the anthracycline antibiotics, which interfere with macromolecular synthesis. The nucleoside analogs exhibit structural similarities to naturally occurring nucleosides and are incorporated into either DNA or RNA with lethal consequences. Alternatively, they block key enzymes in de novo purine or pyrimidine biosynthesis. The antimetabolites in use in hematologic disorders can be divided into the following broad categories:

1. Inhibitors of de novo purine or pyrimidine synthesis (e.g., hydroxyurea)
2. Folic acid analogs (e.g., methotrexate)
3. Pyrimidine analogs (e.g., Ara-C, 5-azacytidine, gemcitabine)
4. Purine analogs (e.g., 6-thioguanine, 6-mercaptopurine, fludarabine, chlorodeoxyadenosine, deoxycoformycin)

These categories are not mutually exclusive; for example, some nucleoside analogs (e.g., Ara-C and 6-thioguanine) also inhibit enzymes involved in DNA or deoxyribonucleotide biosynthesis. As noted previously, the antimetabolites are always cycle-active agents and in most cases are phase-specific, being primarily active against cells in S phase. Because the growth fraction of hematologic malignancies tends to be higher than that of nonhematologic malignancies, antimetabolites are particularly useful in the former disorders. In contrast to alkylating agents, antimetabolites have limited carcinogenic and leukemogenic potential. The fluorinated pyrimidines (e.g., 5-fluorouracil) are generally not employed in hematologic disorders and are not discussed here.

Inhibitors of DNA Topoisomerase I and II

The inhibitors of DNA topoisomerase I and II, some of the most commonly used antineoplastic agents in the treatment of hematologic malignancies, include such drugs as doxorubicin, daunorubicin, m-AMSA, mitoxantrone, VP-16, and topotecan. Before

TABLE 50-4 -- Characteristics of Mammalian DNA Topoisomerases

	Topo I	Topo II	Topo II
Size of monomer	100 kDa	170 kDa	180 kDa
mRNA	4.2 kb	6.2 kb	6.5 kb
Chromosome	20q1213.2	17q2122	3p24
DNA cleavage	Single-strand breaks	Double-strand breaks	Double-strand breaks
Covalent intermediate	$3PO_4$ -Tyr^{723}	$5PO_4$ -Tyr^{804}	$5PO_4$ -Tyr^{821}
ATP requirement	No	Yes	Yes
Nuclear location	Nucleoli, diffuse	Nuclear matrix and scaffold, nucleoli	Nucleoli? Nuclear matrix?
Cell-cycle dependence	None	Yes, maximum in G_2 /M	None
Nuclear localization signal	NH_2 -end	COOH-end	COOH-end
Phosphorylation	By CK II and PKC (increases activity)	By CK II, PKC, $p34^{cdc2}$, MAP kinase	Increases mass to 190 kDa in mitosis
Role	In replication, transcription, and recombination	In replication, transcription, chromosome condensation/ segregation, and recombination (See Table 50-2)	rRNA transcription?
Inhibitors	Camptothecins		

describing the specific inhibitors, a brief review of the drug targets (topoisomerase enzymes) will be presented (Table 50-4).

DNA Topoisomerase I

Several excellent recent reviews describe the structure, enzymology, and biologic function of mammalian topoisomerase I. [208] [209] [210] [211] [212] Topoisomerase I is a ubiquitous enzyme whose function in vivo is to relieve the torsional strain in DNA, specifically to remove positive supercoils generated in front of the replication fork and to relieve negative supercoils occurring downstream of RNA polymerase during transcription. Topoisomerase I is catalytically active as a 100 kDa monomer and is concentrated in nucleoli, although lower amounts are found in a diffuse nuclear distribution. The gene for this enzyme is located on human chromosome 20q1213.2. Topoisomerase I, in contrast to the type II enzymes, does not require ATP for catalytic activity. Its mechanism of action involves binding double-strand DNA over 1525 bp (with a preference for supercoiled or bent DNA), followed by cleavage of one DNA strand and the formation of a transient covalent phosphotyrosyl bond at the 3-end of DNA. DNA torsional strain is then relieved by a controlled rotation mechanism (Plate 50-1), subsequent to which the cleaved DNA is religated. The three-dimensional crystal structure of human topoisomerase I, both in covalent and noncovalent complexes with DNA, has defined the structural elements of the enzyme that contact DNA.[213] [214] The association between topoisomerase I and the 3-end of cleaved DNA has been termed the cleavable complex, and it is this complex that is stabilized by topoisomerase I inhibitors.

DNA Topoisomerase II

The biochemistry and enzymology of type II enzymes have been recently reviewed by several authors. [208] [210] [212] [215] Two isoforms of human topoisomerase II exist, and . The and isoforms are similar in that they are active as homodimers, their activity results in cleavage of double-strand DNA with a transient covalent phosphotyrosyl bond at the 5-end of DNA, they both require ATP for full activity, their role in vivo is to relieve torsional strain in DNA, and their cellular distribution is determined by nuclear localization signals contained in the C-terminal domain. [216] [217] These isoforms are distinct in that they have different-size monomers (Table 50-1), their genes are on separate chromosomes, their nuclear distribution is different, and only the isoform shows cell-cycle variations in amount and activity (with maximal activity in G_2 /M). The mechanism of action of topoisomerase II involves several steps (Fig. 50-7 (Figure Not Available)): DNA recognition and binding (curved and supercoiled DNA,

Figure 50-7 (Figure Not Available) DNA topoisomerase II catalytic cycle. (1) Noncovalent binding of DNA by the topoisomerase II homodimer. (2) DNA recognition and preferential binding to crossovers by topoisomerase II. (3) Binding of ATP promotes the formation of a topologic complex. (4) DNA cleavage with covalent linkage of each topoisomerase II monomer to the 5-DNA terminus of the break. (5) Poststrand passage cleavable complex. (6) Religation of the cleaved DNA is followed by ATP hydrolysis and enzyme turnover. DNA topoisomerase II inhibitors generally increase cleavable complexes by inhibiting the religation activity. *(Reproduced with permission from Pommier et al.[234])*

Figure 50-8 Structure of camptothecin analogs.

as well as DNA crossovers, are preferred); the sequential cleavage of the two strands of DNA with covalent attachment of a monomer to each 5-end of the cleaved DNA; passage of another DNA duplex through the break site (e.g., to relieve DNA torsional strain or decatenate daughter chromosomes at the end of replication); religation of the cleaved DNA; and ATP hydrolysis-dependent enzyme turnover. The binding of ATP by topoisomerase II is required for the strand passage reaction. Again, the association between topoisomerase II monomers and the 5-end of the cleaved DNA has been termed the cleavable complex, the stabilization of which generally correlates with the cytotoxic activity of specific topoisomerase II inhibitors.

Because topoisomerase I and II inhibitors convert their respective enzymes into DNA-damaging agents, it is usually true that the more enzyme target a cell contains

(provided it is in the nucleus), the more cytotoxic the specific inhibitor. An exception to this generalization is found in CLL cells, which have abundant topoisomerase I but are not very sensitive to topoisomerase I inhibitors. This is because topoisomerase I inhibitors are S phase-specific and CLL cells have very few cells in S phase.

Finally, in addition to topoisomerase I and II, a mammalian DNA topoisomerase III has recently been described and found to be essential for early embryogenesis in the mouse.[218] Thus, in addition to the presumed lethality of a homozygous deletion of the topoisomerase II gene, topoisomerase I and III appear to be essential for cell growth and division in mammals.[218] [219] The specific role of topoisomerase III in humans is not known at present.

DNA Topoisomerase I Inhibitors

Camptothecin is a plant alkaloid first identified in 1966 from the tree *Camptotheca acuminata.* Early clinical studies with camptothecin were stopped primarily because of hemorrhagic cystitis, resulting from conversion of the sodium salt form given to the active lactone form by the acidic pH in the bladder. Renewed interest in camptothecin occurred in 1985 when topoisomerase I was identified as the target of this drug, [220] and as new, more water-soluble analogs became available. At present, there are two topoisomerase I inhibitors approved by the FDA as second-line agents for the treatment of ovarian carcinoma and colorectal cancer; these are topotecan and CPT-11 (irinotecan), respectively. Other analogs, including 9-aminocamptothecin, are in clinical trials (Fig. 50-8). With respect to hematologic malignancies, topotecan has been shown to be active in the treatment of myelodysplastic syndromes [221] and inactive in the treatment of CLL.[222] Responses to topotecan have also been seen in refractory multiple myeloma [223] and refractory acute leukemia.[224] [225]

There are several recent reviews of topoisomerase I inhibitors and the clinical trials using them. [226] [227] [228] [229] The lactone forms of topotecan and SN-38 (the active form of CPT-11 generated in vivo by the action of a carboxylesterase) are as much as 1,000-fold

Figure 50-9 (Figure Not Available) Action of camptothecin to stabilize the DNAtopoisomerase I cleavable complex. Cytotoxicity is believed to result from the collision of the DNA replication fork with the cleavable complex, which results in a lethal double-strand DNA break. *(Reproduced with permission from Takimoto and Arbuck.[225])*

TABLE 50-5 -- Mechanism(s) of Action of Antineoplastic Agents That Are Primarily Topoisomerase II Inhibitors

Drug	Topo II Inhibition		DNA Intercalation	Free Radical Formation
	Poison	Suppressor		
Epipodophyllotoxins	+++			+
VP-16				
VM-26				
Anthracyclines				
Doxorubicin				
Daunorubicin	++	++	++	+
Idarubicin				
Epirubicin				
Anthracenedione				
Mitoxantrone	++	++	++	+
Acridine				
m-AMSA	+++	+	+	
Catalytic inhibitors				
Aclarubicin		+++	+	
Others (merbarone, fostriecin,bis-2,6-dioxopiperazines)		+++		

more active inhibitors of DNA topoisomerase I than their carboxylate forms. The lactone form predominates at an acidic pH. Topoisomerase I inhibitors stabilize the DNAenzyme cleavable complex and thus inhibit DNA religation, but it is the production of DNA double-strand breaks that results from a collision of the DNA replication fork with the ternary drugenzymeDNA complex that is the lethal event (Fig. 50-9 (Figure Not Available)). [230] [231] [232] Topoisomerase I inhibitors are considered S phase-specific agents because they require ongoing DNA synthesis to exert their cytotoxic effect.

DNA Topoisomerase II Inhibitors

Inhibitors of DNA topoisomerase II are some of the most commonly used agents in the treatment of hematopoietic malignancies, and their biochemical and pharmacologic properties have recently been reviewed by several investigators. [233] [234] [235] [236] There are three general types of topoisomerase II inhibitors (Table 50-5). The first group are the topoisomerase II *poisons*, typified by VP-16 and m-AMSA, whose activity results in the stabilization of cleavable complexes. The second group are the *catalytic inhibitors*, represented by aclarubicin, merbarone, and the bis-2,6-dioxopiperazine derivatives (ICRF-193, ICRF-159, ICRF-187). These drugs, except for aclarubicin, do not bind DNA and do not stabilize cleavable complexes; rather, they interfere with some aspect of topoisomerase II catalytic activity (e.g., ICRF-187 inhibits topoisomerase II ATPase activity). The final class includes drugs that can inhibit both DNA topoisomerase I and II, and is represented by intoplicine[237] and saintopin.[238] These dual inhibitors are in clinical trials and will not be discussed further. These first two classes of inhibitors are not mutually exclusive; that is, anthracyclines at low doses behave more like topoisomerase II poisons, but at higher concentrations they suppress the formation of cleavable complexes. Some of these antineoplastic agents, especially the anthracyclines and mitoxantrone, exert their cytotoxic effects (at least in part) by also intercalating in DNA and by forming free radicals.

DNA topoisomerase II poisons, the most frequently used drugs in the clinical setting (Fig. 50-10), are most likely cytotoxic because they trap DNAtopoisomerase II complexes on nascent DNA in the nuclear matrix.[239] [240] Recent evidence suggests that the topoisomerase II poison-stabilized enzymeDNA complex acts as a replication fork barrier and leads to the generation of irreversible DNA damage and cell death in proliferating cells, [241] while other experiments in yeast show that although DNA synthesis is a major determinant for cell killing by topoisomerase I inhibitors, topoisomerase II poisons may be cytotoxic during other phases of the cell cycle as well.[242] [243]

Platinum Analogs

During a study of the effects of electric current on growing bacteria, the antibacterial and, later, the antitumor activities of the platinum compounds were fortuitously discovered. The antitumor agent, cisplatin, and its cis-carboxylester analog, carboplatin, are heavy-metal platinum complexes. Both are activated by displacement of their ligands (cisplatin: chloride; carboplatin: carboxylester) by water to form positively charged aquated platinum complexes. This allows platinum to stably bind DNA, RNA, proteins, or other critical biomacromolecules. With DNA, platinum complexes form covalent links to the N7 position of guanine and adenine. The N7 adducts at d(GpG) or d(ApG) result in intra- or interstrand DNA cross-links, which bend the DNA helix and inhibit DNA synthesis. The cytotoxicity of platinum analogs correlates with the total platinum binding to DNA, as well as with the intra- or interstrand cross-links. This results in DNA damage, which triggers apoptosis of sensitive cells.

Miscellaneous Agents

Among the agents in this category, only plicamycin, bleomycin, procarbazine, L-asparaginase, gallium nitrate, and glucocorticoids are of current interest to the

hematologist; these are discussed in Appendix 50-6 .

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DRUG RESISTANCE TO ALKYLATING AGENTS

Tumors and some normal tissues express high levels of some detoxification enzymes and DNA repair enzymes that reduce the cytotoxic effect of alkylating agents (Table 50-6). For the most part, however, normal hematopoietic progenitors have lower levels of these enzymes, and are thus more sensitive to these agents, than do malignant cells.[186] The exception to this is the high level of aldehyde dehydrogenase found in early CD34+ and even earlier CD34 hematopoietic progenitors, [102] which converts cyclophosphamide to 4 keto-cyclophosphamide

Figure 50-10 Structure of DNA topoisomerase II inhibitors. For epirubicin and idarubicin, arrows point to the sites where these new drugs differ, respectively, from doxorubicin and daunomycin.

and carboxyphosphamide and prevents formation of phosphoramide mustard, the cytotoxic alkylating species. For this reason, early progenitors are resistant to cyclophosphamide, cumulative hematopoietic toxicity is usually not observed with this class of agents, [244] and purging of leukemic cells from bone marrow preparations with 4-hydroperoxycyclophosphamide [100] preserves sufficient normal progenitors to allow engraftment after autologous transplantation. Since multidrug resistance-1 (MDR-1) protein is not involved in the resistance to alkylating agents, expression of MDR-1 is not a factor either in progenitor cell sensitivity to alkylating agents or in tumor responses to these agents. However, the high level of this aldehyde dehydrogenase, as well as the MDR-1 protein, in early progenitors protects [245] them from combination chemotherapy involving alkylating agents and other agents such as paclitaxel and etoposide, [59] and may explain the efficacy of combination therapy against tumors expressing MDR-1.

Glutathione and Glutathione S-Transferase

The enzymes involved in glutathione conjugation of drugs are important in resistance to the nitrogen mustard class of alkylating

TABLE 50-6 -- Mechanisms of Chemotherapy Drug Resistance

Natural compounds
Multidrug resistance-1 (MDR-1)
Multidrug-resistance protein (MRP)
Alkylating agents
Defects in mismatch repair
O^6 -alkylguanine-DNA alkyltransferase
Base excision repair
Nucleotide excision repair
Glutathione
Glutathione-S transferase

agents, including melphalan, and to a lesser extent the nitrosoureas, where direct reaction with glutathione S-transferase (GST) has been noted, associated with a reduction in DNA cross-links.[246] Many of the 12 enzymes in the GST superfamily have been shown to detoxify alkylating agents, but their activity varies considerably. The members of the GST family bind directly to alkylating agents and conjugate them to glutathione (GSH), and they also bind and reduce organic peroxides formed during the initial uptake of drugs into cells even before decomposition to DNA-damaging agents. [247] For instance, human GST enzyme catalyzes the reaction of the aziridinium ion of the phosphoramide mustard derivative of cyclophosphamide with glutathione [248] and also reacts with melphalan. [249] High expression of these enzymes in the NCI tumor bank is associated with resistance to alkylating agents. When the patterns for the GST enzymes and mRNA levels were correlated with drug sensitivity patterns using the COMPARE program, alkylating agents had the greatest correlation between GST expression and drug resistance. [250] In contrast, none of the other enzymes or GSH had patterns of expression that resulted in an obvious correlation with the pattern of alkylating agent resistance. [250] In this study, -glutamylcysteine synthase, the synthetic enzyme for glutathione, also showed a high degree of correlation with alkylating agent resistance in the COMPARE program, suggesting that the ability to generate glutathione, the substrate for GST conjugation, contributed to drug resistance.

Overexpression of multiple enzymes in the GST family confers resistance to alkylating agents to variable degrees, [251] [252] although little effect is seen in cell lines that express the enzymes constitutively. Increased expression of GSTs is associated with alkylating agent resistance in tumor cell lines and in human tumors treated with alkylating agents.[44] [60] [253] [254] Whether this represents selection of pre-existing, high-expressing cells or selection of cells mutant in GST expression remains unclear and the focus of current study. However, GST expression has been used as a predictor of clinical responses to alkylating agents. [255]

Among hematologic malignancies, non-Hodgkins lymphomas were found to display high levels of GST , whereas the expression of and GST varied from undetectable to levels as high as those observed in drug-resistant cell lines. [256] In a group of nine of the patients treated with bifunctional alkylating agents, a significant correlation was observed between complete response to chemotherapy and low expression of GST but not GST .[256] In CLL, Marie et al.[257] found similar levels of GST , , and in normal and CLL cells, and suggested that low GST levels may explain the sensitivity of CLL cells to nitrogen mustards such as chlorambucil observed clinically.

A number of studies have found that depletion of glutathione by buthionine sulfoximine (L-BSO) sensitizes cells and xenografts to melphalan, other nitrogen mustards, and cisplatin.[44] [258] [259] This led to clinical trials with L-BSO designed to achieve the biochemical endpoint of >90% depletion of blood mononuclear cell GSH levels; however, very large doses of L-BSO were required because it was found that glutamylcysteine synthase was induced in vivo during exposure to L-BSO. [260] [261] A number of dosing strategies have been used, and early phase II studies are underway. Despite the suggestion that GST binds BCNU and decreases cross-links, [246] the effect of L-BSO on nitrosourea resistance in breast cancer cells expressing GST was modest, much less than the effect of inhibition of alkyltransferase, suggesting that L-BSO and the GST conjugation pathway play a modest role in nitrosourea resistance. [262] For this reason, there are no current plans to pursue the

use of the L-BSO and nitrosourea combination.

DNA Repair Pathway Mechanisms of Drug Resistance

O^6 -Alkylguanine-DNA Alkyltransferase

As noted above, the nitrosoureas and methylating agents are cytotoxic in large part due to formation of DNA adducts at the O^6 position of guanine. [263] The most efficient means of protection from the cytotoxicity of adducts at O^6 position of guanine is rapid repair by the O^6 -alkylguanine-DNA alkyltransferase (AGT). [263] This protein serves as the stoichiometric acceptor protein for O^6 -alkylguanine DNA monoadducts, transferring the alkyl group from DNA to the active site of the protein, inactivating the protein, and restoring DNA to normal. [263] [264] However, the N^1 G-N^3 C DNA cross-link that follows chloroethylation is not a substrate for AGT. There is a striking correlation between drug resistance and alkyltransferase activity. [45] [46] High levels of AGT are found in many leukemias, [265] whereas low levels are observed in normal human CD34+ cells, [186] perhaps explaining why nitrosoureas are not used in leukemia management and why nitrosoureas are effective myeloablative agents used in high-dose chemotherapy regimens. Recently, a novel inhibitor of AGT, O^6 -benzylguanine (BG), has been used to sensitize human tumors to BCNU. [45] [266] [267] On the basis of these studies, BG passed the NCI decision network review and entered clinical trials as a biochemical modulator of AGT. The endpoint of these trials is to establish the dose of BG that inhibits AGT in tumor tissues, thereby sensitizing these tumors to BCNU and, in future studies, to methylating agents such as procarbazine or temozolomide. Results indicate that biochemical modulation can be achieved at nontoxic doses of BG. [268]

Mismatch Repair

The spectrum of drug resistance and sensitivity to methylating agents does not end with AGT. Recent evidence suggests that methylating agent-induced cell death involves an aborted effort at mismatch repair. Karran et al. [157] and others, in mammalian systems, have shown that the replicative DNA polymerase and the repair polymerase pauses at O^6 -mG and preferentially inserts a thymine (T) at the site. [157] [269] The O^6 -mG:T base pair is recognized by the mismatch repair (MMR) system that initiates base excision repair. In human cells, the MMR complex consists of at least six proteins involved in the recognition and repair of mismatch lesions: hMLH1, hMSH2, hMSH3, hPMS1, hPMS2, and GTBP (GT binding protein, also called MSH6), all of which appear to be homologs of MMR proteins found in *E. coli* and in yeast. [156] [270] [271] Following binding recognition, an endonuclease removes a patch of 1001000 bp containing the T mismatch, DNA polymerase or fills in the patch, with reinsertion of a T opposite the O^6 -mG, and a DNA ligase closes the strand break. [156] Since the O^6 -mG:T is reformed, cytotoxicity ensues due to repetitive efforts at DNA repair and induction of chromosomal breakage, rearrangements, energy depletion, and apoptosis. [272] The mutator phenotype was originally described in cells with acquired resistance to methylnitrosourea (MNU), methylmethanesulfonate, or N-methyl-N-nitroso-N-nitrosogua-nidine, which were tolerant to G A point mutations based on the inability to repair O^6 -mG. [273] Parallel studies identifying mutations in the MMR pathway suggested that these cell lines carry an MMR mutation, rendering them unable to recognize or initiate repair at the O^6 -mG:T mismatch. [274] Cells resistant to the abortive MMR process had a survival advantage in the face of persistent mutations. In addition, Karran et al. [157] noted that MNU-resistant Chinese hamster ovary (CHO) cells replicated DNA past O^6 -mG or 6-thioguanine lesions without cell-cycle arrest. These MNU- and methylmethanesulfonate-resistant cells were cross-resistant to 6-thioguanine, which forms the 6-thioguanine:T mismatch, but were sensitive to chloroethylating agents. [158]

MMR Mutations and Methylating Agent Resistance

Mismatch repair defects in humans were initially described in hereditary nonpolyposis colon cancer, which represents 15% of all colon cancer cases, [156] [164] [271] and more recently in some lymphomas. [165] The genetic defect results in a high rate of spontaneous mutations within microsatellite DNA, resulting in the replication error phenotype arising as the expansion or contraction of mono-, di-, or tri-nucleotide repeats within the microsatellites. [156] In affected individuals, a heterozygous mutation is inherited and a homozygous defect occurs in the tumor. Tumor cell lines defective in MMR are remarkably resistant to temozolomide, regardless of AGT activity or its inhibition by BG, confirming the importance of MMR in regulating sensitivity to methylating agents. [152] Of interest, MMR mutant cell lines are also two- to three-fold more resistant to cisplatin, [275] perhaps because the cisplatin DNA adduct is bound by the MMR complex, slowing its recognition and repair by the nucleotide repair pathway and increasing its cytotoxicity. [276]

Base Excision Repair

Methylating agents such as procarbazine and temozolomide form large numbers of N^3 -A and N^7 -G adducts in addition to O^6 -mG (with temozolomide, the relative amounts are 72 N^7 mG, 8 O^6 mG, 5 N^3 mA). Thus, under normal circumstances, cells process many more N^7 mG and N^3 mA lesions than O^6 mG lesions, even though the latter appear much more cytotoxic, except in MMR-defective cells. Repair of N^3 -A and N^7 -G adducts through base excision repair is efficient and normally leads to cell survival rather than cell death. Adducts are recognized by the methylpurine glycosylase with removal of the base, generating an abasic (or AP) site. The AP site is then cleaved by the class II hydrolytic endonuclease (or apurinic endonuclease), generating a single-strand break with a 5PO_4 that becomes the substrate for DNA polymerase and to a lesser extent polymerases and , followed by DNA ligase (reviewed by Sancar [153]). In addition, MAG mediates the repair of CNU lesions, either by repair of the pre-crosslink N^7 G adduct or by initiating repair of

the cross-link. [277] [278] Thus, the base excision repair pathway appears to have a role in both repair of temozolomide- and BCNU-induced lesions and reduction in cytotoxicity. Recently, it has been proposed that defects in base excision repair may lead to drug sensitivity. The clearest experiments come from studies in homozygous knockout ES cell lines. Knockout of methylpurine glycosylase increased sensitivity to methylmethanesulfonate and BCNU. [277] [278] Knockout of polymerase - [154] produced sensitivity to methylating agents.

Another component of alkylating agent resistance is poly (ADP)ribose polymerase (PARP), which binds single-strand breaks formed during repair of alkylating agents and cisplatin-induced DNA adducts, resulting in autoribosylation of PARP. The negative charge induced by this process unfolds the DNA, exposing the site to repair enzymes. Ultimately, PARP disassociates from the break, allowing repair to proceed. [279] [280] Alternatively, through p53-dependent and -independent means, PARP is cleaved by the caspases activated during apoptosis, preventing DNA repair and inducing DNA strand breaks and cell death. [62] [281] Inhibitors of PARP result in increased sensitivity to alkylating agents [279] [280] and are a potential therapeutic intervention for drug resistance.

Transfer of Drug Resistance Genes into Hematopoietic Progenitors to Confer Protection from Chemotherapy-Induced Myelosuppression

Any discussion of chemotherapy-induced myelosuppression would be incomplete without mention of the progress being made in the delivery of drug resistance genes to hematopoietic progenitor cells to increase the resistance of these cells during chemotherapy exposure.

Transfer of a number of drug resistance genes, including dihydrofolate reductase (DHFR), [282] [283] MDR-1, [284] [285] and ALDH-1, [286] into hematopoietic progenitors has been proposed as a method to protect the bone marrow from the toxic effects of chemotherapeutic agents, improving tolerance to anticancer agents and perhaps providing a means for dominant selection of a second therapeutic gene. [287] Studies using mutant forms of the murine or human DHFR have shown the ability to select for drug-resistant cells after methotrexate treatment in transplanted mice. [288] Clearly, MDR-1 provides the broadest spectrum of drug resistance and may be very useful in the clinical setting. Two clinical trials have been reported showing evidence of gene transfer, but no therapeutic benefit has yet been observed. [289] [290] Nonetheless, the observation that expression of endogenous MDR is relatively high in CD34+ cells suggests that early hematopoietic progenitors may be more resistant than later committed cells. [291] [292]

The methyl guanine methyl transferase (MGMT) or AGT gene has also been retrovirally transduced into mouse and human hematopoietic progenitor cells, resulting in resistance to BCNU in vitro and in mice [291] [292] for at least 37 weeks after bone marrow transplantation. In addition, recent studies have shown that this vector can be used as a dominant selectable marker because treatment of mice with BCNU increases the proportion of hematopoietic progenitors carrying the provirus and increases expression of MGMT as well. [293] Animals receiving mock-transduced cells suffered severe pancytopenia, whereas animals receiving MGMT-transduced bone marrow progenitors had significantly higher hematocrits and leukocyte and platelet counts. [294] Recently, a series of AGT point mutations that retain the functional activity of DNA repair but are remarkably resistant to inactivation by BG have been described. [295] One of these, the glycine to alanine mutation at position 156 in the human protein, results in a 240-fold resistance to BG in cell-free systems. Based on these data, a retrovirus containing this mutant MGMT was introduced into human CD34 cells and mouse bone marrow cells, both of which became resistant to the combination of BG and BCNU. [296] [297] Further, the degree of resistance was much greater for marrow progenitors transduced with the mutant MGMT and treated with the BCNUBG combination than in cells transduced with the wild-type

gene and treated with BCNU alone. Cumulatively, these studies suggest that gene transfer strategies may protect the marrow progenitor pool from the myelosuppression of a wide range of chemotherapeutic agents. Whether this improves tolerance to these agents or has application for selection of cells carrying other genes remains to be experimentally determined.

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DRUG RESISTANCE TO PLATINUM ANALOGS

Several mechanisms of resistance to platinum analogs have been described, including altered transport across the cell membrane, which results in decreased intracellular drug accumulation. Increased intracellular levels of glutathione (GSH) or sulfhydryl conjugating enzymes (e.g., GST) may also confer in vitro resistance to platinum analogs. As noted previously, MMR mutations also confer resistance to cisplatin. Finally, platinum resistance may also be due to an enhanced ability to repair DNAplatinum adducts. Decreased intracellular accumulation of platinum analogs is a consistent finding in platinum-resistant cells. Platinum analogs may form complexes with two GSH molecules in a nonenzymatic reaction, and this complex may be exported out of the cell by an organic ion pump referred to as the GS-X pump. However, the nature of this pump has not been established conclusively.

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DRUG RESISTANCE TO ANTIMICROTUBULE AGENTS

Two main mechanisms of in vitro resistance to antimicrotubule drugs have been elucidated. Cells resistant to antimicrotubule agents often display an MDR phenotype. This results in decreased drug accumulation and retention and confers varying degrees of cross-resistance to other natural products, including anthracyclines, epipodophyllotoxins, and actinomycin D. As discussed previously, the MDR phenotype is due to the overexpression of a membrane transporter, P-glycoprotein, encoded by the *mdr-1* gene. This is discussed below in the section on MDR. The second mechanism of acquired drug resistance to antimicrotubule agents results from alterations in - and/or -tubulin proteins. These alterations may cause either decreased drug binding or increased resistance to microtubule assembly or disassembly. This would impair the cell-cycle mitotic arrest due to antimicrotubule drugs.

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DRUG RESISTANCE TO ANTIMETABOLITES

Although overlap exists, antimetabolites can be classified into nucleoside analogs that are incorporated into RNA and/or DNA, and agents that inhibit de novo purine and pyrimidine biosynthetic pathways. Mechanisms of resistance to these agents fall into several broad categories. For example, many antimetabolites are prodrugs in that they must be converted intracellularly into active nucleotide forms to exert their cytotoxic actions. Consequently, events that interfere with cellular accumulation of drug and/or nucleotide formation will reduce activity. Examples include decreased transport of methotrexate, or decreased nucleotide formation of Ara-C and 6-thioguanine by reductions in activity of deoxycytidine kinase or hypoxanthine guanine phosphoribosyl transferase (HGPRT), respectively. Alternatively, enhanced drug catabolism reduces cytotoxicity. Examples include the deamination of Ara-C (to inactive Ara-U) by cytidine deaminase or catabolism of 6-thioguanine by thiopurine methyltransferase. A third mechanism of resistance stems from the presence of increased intracellular levels of a competing metabolite (e.g., dCTP in the case of Ara-C, or

hypoxanthine or guanine in the case of 6-thioguanine). Fourth, alterations in the level of activity of a target enzyme or the presence of a mutant form that is less inhibitable will also confer resistance. Examples include increased activity or a mutant form of DHFR (in the case of methotrexate), an altered DNA polymerase (in the case of Ara-C), or increased activity of ribonucleotide reductase (through overexpression of either subunit). Finally, cytokinetic factors represent a common theme in the case of most (but not all) antimetabolites in that a reduction in the S-phase fraction generally leads to reduced drug sensitivity. These resistance mechanisms are agent-specific and are distinct from the more general modes of resistance (e.g., increased expression of Bcl-2) associated with defects in the distal cell death pathway.

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DRUG RESISTANCE RESULTING FROM ALTERATIONS IN DNA TOPOISOMERASE I OR DNA TOPOISOMERASE II

Resistance to several antineoplastic agents may result from a perturbation in either DNA topoisomerase I or II; for example, decreased expression of topoisomerase II may result in MDR to VP-16, doxorubicin, mitoxantrone, actinomycin D, and m-AMSA (Fig. 50-11). The mechanisms of drug resistance to either topoisomerase I or II inhibitors have been defined at a molecular level by detailed in vitro studies with cell lines; only recently have studies attempted to define the role of these putative resistance mechanisms in clinical samples. Since more is known about the mechanisms of resistance to topoisomerase II inhibitors, these will be discussed first, followed by a brief review of the ways cells become resistant to topoisomerase I inhibitors. The basic biochemistry and pharmacology of the topoisomerase enzymes and their inhibitors were described previously.

Drug Resistance to Topoisomerase II Inhibitors

In vitro studies have demonstrated that cells can become resistant to topoisomerase II inhibitors by four general mechanisms. [298] [299] [300] [301] First, altered transport of the antitumor agent, either decreased uptake or increased efflux, can certainly result in drug resistance. Essentially all of the topoisomerase II poisons (Table 50-7) are substrates for the drug efflux pump Pgp, and many are substrates for MDR-associated protein (MRP) and lung resistance-related protein (LRP). Second, several point mutations and gene deletions have been defined in the gene for topoisomerase II resulting in the production of an enzyme with altered catalytic and/or cleavage activity.[301] [302] The third mechanism of drug resistance to topoisomerase II poisons, and probably the most common, is a decrease in expression of the enzyme such that there is less target for the inhibitor to convert to a DNA-damaging agent. This can result from a proliferation-dependent or cell cycle-dependent decrease in topoisomerase II,[303] from a specific attenuation of either topoisomerase II [304] or topoisomerase II,[305] or from an intrinsic absence of topoisomerase II (e.g., CLL cells have virtually no topoisomerase II protein). The fourth and final mechanism of resistance to

Figure 50-11 Mitoxantrone.

topoisomerase II inhibitors involves alterations in the subcellular distribution of the enzyme. Truncation of the COOH-end of topoisomerase II has resulted in the cytoplasmic distribution of enzyme due to a loss of nuclear localization signals; thus, the enzyme cannot interact with DNA in the presence of an inhibitor, and the cell is thus resistant.[306] [307] [308] An altered subcellular distribution of topoisomerase II, such that the quantity of enzyme associated with the nuclear matrix is reduced (thought to be a critical location for interacting with nascent DNA transcripts), has also been found to result in drug resistance to topoisomerase II inhibitors. [309]

The possible roles of these four general mechanisms in clinical drug resistance and clinical outcomes for patients with hematologic malignancies are under investigation. Mutations in the gene for topoisomerase II do not appear to be a common clinical event, as only a single patient with AML has been found to have a point mutation. The available data indicate that the content of topoisomerase II in blast does not correlate with clinical outcome in AML, [310] and this may also be true for acute lymphocytic leukemia (ALL). CLL cells are resistant to topoisomerase II inhibitors because they express very low levels of the protein. Further studies using sequential biopsy samples and assays that focus on topoisomerase II activity should establish the role that changes in topoisomerase II play in the development of drug resistance in hematologic malignancies.

Drug Resistance to Topoisomerase I Inhibitors

Defining the molecular mechanisms of resistance to topoisomerase I inhibitors has lagged behind topoisomerase II inhibitors, but in general the four mechanisms described above have been found with the camptothecins. Although alterations in drug transport of topoisomerase I inhibitors may result in drug resistance, it is unlikely that either Pgp or MRP will have a major role in clinical drug resistance. [311] [312] Similar to the previous discussion, drug resistance to topoisomerase I inhibitors has been found in vitro to result from point mutations in the gene for topoisomerase I, [298] [313] [314] [315] an attenuation of nuclear topoisomerase I content, [315] [316] and a subcellular redistribution of topoisomerase I in cells treated with topotecan. [316] The role of these mechanisms of resistance to topoisomerase I inhibitors in the development of drug resistance in clinical hematologic malignancies is unknown.

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MULTIDRUG RESISTANCE

One of the major reasons for the failure of antineoplastic agents in the treatment of both solid tumors and hematologic malignancies is the presence of drug resistance mechanisms that efficiently neutralize the cytotoxic effects of the antitumor drugs. Drug resistance may be either an intrinsic (de novo) property of the tumor or an acquired characteristic. Examples of malignancies that are intrinsically drug-resistant include malignant melanoma and primary refractory non-Hodgkins lymphoma, while relapsed small cell lung cancer, AML, and multiple myeloma commonly have acquired drug-resistance mechanisms. With very few exceptions, it is not possible to attribute a specific mechanism(s) of drug resistance (discussed later) to a particular tumor type to account for either de novo or acquired drug resistance. This is for several reasons. First, and most importantly, large-scale prospective studies of clinical tumor biopsies obtained before and after drug exposure and at the time of disease relapse, which assess the relevant gene product function, simply have not been done. In addition, by virtue of having been exposed to multiagent chemotherapy, and because the same drug can evoke different resistance mechanisms, it is very likely that the majority of human tumors have multiple concurrent mechanisms, either those already defined or one not yet described, for circumventing the cytotoxic effects of antineoplastic drugs. Finally, the discrete biochemical mechanisms defined in

TABLE 50-7 -- Characteristics of Three Mechanisms of Multidrug Resistance That Result from Overexpression of P-Glycoprotein (PGP), Multidrug Resistance-Associated Protein (MRP), or Lung Resistance-Related Protein (LRP)

	PGP	MRP	LRP
Gene on chromosome	7q21.1	16p13.1	16p11.2
Protein			
Molecular mass	170 kDa	190 kDa	110 kDa
Cellular location	Plasma membrane	Plasma membrane	Cytoplasm>>nuclear membrane
Function	Efflux pump, chloride channel	Drug transporter	Major vault protein (nucleocytoplasmic transport?)
Energy source	ATP	ATP	?
Post-translational modifications	N-glycosylation, phosphorylation	N-glycosylation, phosphorylation	*No* N- or O-glycosylation
Analogs	Member ABC superfamily	Member ABC superfamily; GS-X pump, MOAT, LTC_4 transport	?
Drug resistance phenotype			
Antitumor agents	Act-D, AMSA, dauno, dox, epi, ida, mito-C (low), mtz, nav, tax, txtr, tpt (low), vbl, vcr, VM-26, VP-16	Act-D, chlor, CDDP-GSH, dauno, dox, epi, mel, tax (low), vbl (low), vcr, VM-26, VP-16	Carbo, CDDP, dox, mel, vcr, VP-16
Other drugs	Colch, rhod	As, Cd, colch (low), GSH conjugates, GSSG, LT_4 , Sb	?
Reversing agents	CSA, FK506, nifed, PSC833, quin, rap, verap	CSA, gnstn, indo, nicard, prbn, PSC833, verap, VX-710	?
Normal hematopoietic tissues with increased expression	NK (CD56+) T cells, suppressor T cells (CD8+), B cells, CD34+ stem cells	PBMNs (esp. T cells), rbc membranes; liver and spleen low level	Macrophages
Prognostic significance	AML, MM, NHL	AML (inv 16)	AML, ALL?

ABC, ATP-binding cassette; act-D, actinomycin D; As, arsenicals; carbo, carboplatin; Cd, cadmium; CDDP-GSH; cisplatin glutathione conjugate; chlor, chlorambucil; colch, colchicine; CSA, cyclosporin A; dauno, daunomycin; dox, doxorubicin; epi, epirubicin; gnstn, genistein; GS-X, glutathione conjugate; GSSG, oxidized glutathione; ida, idarubicin; indo, indomethacin; LTC_4 , cysteinyl leukotriene; mel, melphalan; mito-C, mitomycin C; mtz, mitoxantrone; MOAT, multispecific organic anion transporter; NK, natural killer; nav, navelbine; nicard, nicardipine; nifed, nifedipine; prbn, probenecid; quin, quinidine; rap, rapamycin; rhod, rhodamine; Sb, antimonials; tax, taxol; txtr, taxotere; tpt, topotecan; vbl, vinblastine; vcr, vincristine; verap, verapamil; VM-26, teniposide; VP-16, etoposide.

vitro are only one potential contributor to the overall resistance of the tumor in vivo; consideration must also be given to pharmacologic sanctuaries (testes, central nervous system), the activation (phosphorylation) and metabolism of certain drugs, the pH and blood supply of the tumor, and possible kinetic resistance of noncycling cells residing in G_0 .

The Goldie-Coldman hypothesis predicts that drug-resistant tumor cell clones survive because of a favorable spontaneous mutation that occurs in about one in a million cells. Since 1 g of tumor contains 1×10^9 cells, it becomes obvious that states of high tumor burden have a tremendous number of mutations that can contribute to drug resistance. This is the rationale for using combination chemotherapy at specific dose intervals to maximize dose intensity.

Drug-resistance mechanisms have been discovered and subsequently defined at the molecular level by investigators working in vitro with tumor cell lines selected in the presence of specific antitumor agents. These studies have shown that cellular drug resistance may be to a specific drug (e.g., methotrexate resistance resulting from amplification of the *DHFR* gene), to a class of antineoplastic agents (e.g., resistance to all topoisomerase I inhibitors resulting from a downregulation of nuclear topoisomerase I), or to several different classes of structurally dissimilar drugs (e.g., cross-resistance to taxanes, vinca alkaloids, and anthracyclines resulting from Pgp overexpression). Again, the role of the majority of these in vitro mechanisms in clinical drug resistance will await the completion of translational studies now underway. The cross-resistance to several different classes of antitumor agents that results from exposure to a single drug is termed multidrug resistance and classically refers to the MDR associated with P-glycoprotein overexpression. Here, however, we will use MDR to refer to drug resistance that results from Pgp overexpression, MRP overexpression, lung resistance-related protein overexpression, or alterations in DNA topoisomerase I or II. The reader is referred to two comprehensive reviews of drug resistance in hematologic malignancies recently published. [317] [318]

P-Glycoprotein

Structure and Function

P-glycoprotein has been the subject of intense biochemical and clinical studies since it was discovered in drug-resistant cell lines >20 years ago. [319] The biochemistry of Pgp has been reviewed in detail by several investigators. [320] [321] [322] This phosphorylated glycoprotein has a molecular mass of about 170 kDa and is localized to the plasma membrane, where it functions as a

drug efflux pump (Table 50-3). The ATP-dependent extrusion of antineoplastic agents confers a relative level of resistance to the cell that overexpresses Pgp. The observation of double minute chromosomes and homogeneously staining regions in several MDR cell lines suggested that gene amplification is involved in Pgp-mediated MDR. This has been shown to be true for several mammalian cell lines, [323] [324] but is not a requirement for the involvement of Pgp in MDR. The human *MDR1* gene that codes for Pgp and is involved in antitumor drug resistance, [325] [326] and the human *MDR2* gene, the product of which is expressed by hepatocytes, are located very near each other on human chromosome 7q21.1. The human *MDR1* gene has 28 exons and codes for a protein of 1280 amino acids. The Pgp molecule has homologous halves, each with a hydrophobic region containing six transmembrane domains and a hydrophilic region containing an ATP binding site. The N-linked glycosylation occurs on the extracellular side. Pgp is a member of the ATP-binding cassette superfamily, which includes among more than 100 others MRP; the pf*mdr* pump in *Plasmodium falciparum* that results in chloroquine resistance; STE6, the transporter of the a peptide mating factor in yeast; the cystic fibrosis transmembrane conductance regulator; and the TAP-1 and TAP-2 proteins, which transport antigenic peptides for association with class I molecules and surface antigen presentation. [321] [322] MRP has been demonstrated in several MDR mammalian cell lines and in human tumors (see below), while a recent study [327] in human tumor cell lines has shown TAP overexpression associated with MRP, as well as drug resistance resulting from transfection of the *TAP* genes.

P-glycoprotein has a broad specificity for hydrophobic compounds and can both reduce the influx of drugs into the cytosol and increase efflux from the cytosol. To accomplish the former, this hydrophobic vacuum cleaner must detect drugs and expel them while they are still in the plasma membrane. Drugs are thought to be effluxed from the cytosol through a single barrel of the Pgp transporter, although an exact mechanism has been lacking. A recent study used electron microscopy to generate an initial structure of Pgp to 2.5-nm resolution. [328] The structure was further refined by three-dimensional reconstructions from single particle image analysis of detergent-solubilized Pgp and by Fourier projection maps of small crystalline arrays of Pgp. This is the first structural data for any ATP-binding cassette transporter and demonstrates that Pgp is monomeric with the shape of a cylinder 10 nm in diameter with a maximum height (in the plane of the membrane) of 8 nm (Fig. 50-12). This suggests that about half of the Pgp molecule is within the membrane, as the lipid bilayer is about 4 nm in depth. When viewed from the extracellular surface of the membrane, Pgp is toroidal, with a large central pore 5 nm in diameter. This large aqueous chamber in the membrane, which is open to the extracellular space, is closed on the cytoplasmic side, presumably by the two 3-nm intracellular lobes (putative nucleotide binding domains) and the hydrophilic cytoplasmic loops between the transmembrane domains. Thus, this large pore has a gate on the cytoplasmic side of the membrane that can regulate the transport of different-sized substrates. Finally, this study demonstrated that the transmembrane aqueous chamber is open to the lipid phase within the plane of the membrane, consistent with the role of Pgp as a hydrophobic vacuum cleaner whereby substrates can gain access to the pore from the membrane lipid phase.

Substrates

Cross-resistance phenotypes have been determined after in vitro selection to a primary agent for several mammalian drug-resistant tumor cell lines that overexpress Pgp. These studies have shown that the antineoplastic agents that are substrates

Figure 50-12 Structure of P-glycoprotein determined by electron microscopy; a computer graphic representation of the three-dimensional reconstruction is shown as a shaded surface representation of the structure. The straight arrow shows the putative ATP binding domains. P represents the aqueous pore open at the extracellular face of the membrane. TMD, two thumbs, each of which probably corresponds to one of the two transmembrane domains. NBD, 3-nm lobes projecting from the structure at the cytoplasmic face of the membrane, probably corresponding to the two nucleotide binding domains. **(A)** Shows a view perpendicular to the extracellular surface of the lipid bilayer, and **(B)** shows a side view of P-glycoprotein in which the approximate position of the lipid bilayer is indicated by the two horizontal dashed lines. Arrow, asymmetric opening providing access from the lipid phase to the aqueous core of the protein. *(Reproduced with permission from Rosenberg et al.[326])*

for Pgp and are actively effluxed in an ATP-dependent manner (Table 50-7) include anthracyclines (doxorubicin, daunorubicin, epirubicin, and idarubicin), anthracenediones (mitoxantrone), aminoacridines (*m*-AMSA), taxanes (taxol and taxotere), epipodophyllotoxins (VP-16 and VM-26), vinca alkaloids (vincristine, vinblastine, and vinorelbine), and actinomycin D (Fig. 50-13). Mitomycin C and one of the topoisomerase I inhibitors (topotecan) are both weak substrates for Pgp. [321] [327] Definitive proof that Pgp is responsible for MDR has come from transfection experiments where, for example, a full-length cDNA for the human *MDR1* gene was transfected into both NIH 3T3 and human KB carcinoma cells and found to result in resistance to doxorubicin, vinblastine, and colchicine. [329] [330]

Several drugs have been found to reverse the resistance mediated by Pgp overexpression and sensitize cells to the cytotoxic effects of antineoplastic agents. These drugs compete with antitumor agents for efflux from the cell, effectively increasing the intracellular concentration of the cytotoxic drug. They include immunosuppressants (cyclosporin A, FK 506, rapamycin, PSC 833), calcium channel blockers (verapamil, nifedipine), antiarrhythmics (quinidine), and other miscellaneous agents. [321] [322] [329] [331] Several of these MDR-modulating agents have been used in clinical trials in an effort to sensitize resistant tumor cells.

Methods of Detection

When defining the role of any particular molecular mechanism (e.g., Pgp, MRP, LRP) in the drug resistance of a tumor, several considerations should be kept in mind. First, one should assess the functional moiety of the putative mechanism in the tumor; for example, it is more germane to measure Pgp itself or rhodamine efflux rather than the mRNA for Pgp. The amount and activity of DNA topoisomerase II determine cellular sensitivity to topoisomerase II inhibitors, not the mRNA level. Second, the heterogeneity of the tumor sample may give misleading results due to the contribution of nontumor cells to gross measurements of specific RNA and protein. This is not as significant a concern with the relatively homogeneous hematologic tumors (compared to solid tumors) and can be overcome by evaluating individual cells by immunocytochemistry. Third, the investigator needs to know whether the monoclonal or polyclonal antibody being used detects an external or internal epitope (for ATP-binding cassette proteins), and if the antibody cross-reacts with nonfunctional isoforms or analogs. For example, the C219 monoclonal antibody detects both the *MDR1* and *MDR2* gene products; however, the *MDR1* gene product (Pgp) alone is involved in antineoplastic drug resistance, although the *MDR2* gene product is detected in human liver. [332] Finally, the most informative studies will sample the same patient tumor before and after chemotherapy and at relapse for the presence and amount of resistance protein. Only then is it possible to correlate, for example, the attenuation of topoisomerase II amount with drug resistance at relapse following treatment with VP-16.

Several monoclonal antibodies that recognize Pgp and are commercially available for routine analyses have been described. [321] [322] [333] Monoclonal antibodies C219[334] and JSB-1[335] recognize internal epitopes of Pgp, while antibodies MRK16 [336] and UIC2[337] detect external antigens and are more suited for flow-cytometric analysis.

Pgp Expression in Normal Human Tissue

High levels of expression of *MDR1*/Pgp have been found in the epithelia of several human tissues with excretory function, suggesting that Pgp is normally involved in transporting both exogenous toxic compounds and endogenous metabolites. [321] These tissues include the adrenal cortex, renal proximal tubule epithelium, biliary hepatocytes, small and large intestinal mucosa, pancreas, and endothelial cells of the brain and testis. High levels of Pgp expression have also been found in the placenta and pregnant endometrium. Normal human hematopoietic tissues

Figure 50-13 Chemical structure of epipodophyllotoxins, *m*-AMSA, CI921, and elliptinium.

with high levels of *MDR1*/Pgp include $CD34^+$ progenitor cells, $CD56^+$ (natural killer) cells, and $CD8^+$ (T-suppressor) cells.[332] [336] Lower levels of expression have also been observed in $CD4^+$ (T-helper) cells, $CD19^+$ B cells, and $CD14^+$ cells (monocytes). In these specific lineages, the expression of Pgp measured with the MRK16 monoclonal antibody correlated with the *MDR1* mRNA analysis. $CD15^+$ cells (granulocytes) have no functional Pgp, are negative by MRK16 analysis, but do have a level of *MDR1* mRNA expression similar to T-helper cells and do express Pgp by analysis with the monoclonal antibodies C219 and JSB1. Perhaps the Pgp in granulocytes is not in the membrane.

Pgp Expression in Human Malignancies

Increased expression of Pgp has been observed in several human tumors, especially those malignancies that arise in tissues that normally have high levels of Pgp expression. Thus, the presence of Pgp in a tumor at diagnosis may contribute to de novo drug resistance, while its overexpression at relapse following chemotherapy may result in acquired drug resistance. The drug resistance of a particular tumor is unlikely to depend on a single mechanism (e.g., overexpression of Pgp or MRP, attenuation of topoisomerase II levels). It is more likely to result from the involvement of more than one mechanism. A recent analysis of 61 human tumor cell lines (from leukemia, central nervous system tumors, melanoma, breast cancer, ovarian cancer, colon cancer, lung cancer, and kidney cancer), which were not selected for resistance to antitumor agents, demonstrated coexpression of 2 or 3 of the MDR proteins (Pgp, LRP, or MRP) in 64% of the cell lines. [339] Pgp and LRP were overexpressed in 3% of the tumors, MRP and LRP in 43%, and Pgp, LRP, and MRP in 18%. The cell lines with the highest levels of drug resistance were found to overexpress all three proteins. Whether this is true in primary human tumors awaits further investigations.

Solid Tumors

MDR1/Pgp expression has been determined in several solid human tumors[321] [322] [333] [340] [341] and has been found to be elevated in untreated tumors whose tissues of origin normally have increased expression of Pgp, including renal cell cancer, colon cancer, hepatomas, adrenocortical tumors, islet cell tumors of the pancreas, carcinoid tumors, and pheochromocytomas. Untreated tumors with increased Pgp expression arising in tissues that normally have low levels of expression include neuroblastomas, sarcomas, astrocytomas, and non-small cell lung cancer with neuroendocrine features. Human malignancies that often demonstrate increased levels of Pgp after exposure to antitumor agents include breast cancer, ovarian cancer, neuroblastoma, pheochromocytoma, and rhabdomyosarcoma. An association between Pgp level and poor clinical outcome has been found in neuroblastoma in children [342] and in high-grade osteosarcoma. [343]

MDR1/Pgp has been found to have increased expression (with significant variation between studies for a specific tumor type) in essentially all hematologic malignancies.[319] [322] [332] [344] [345] The wide variation in the number of positive tumor samples for a particular malignancy results from the different assays used to detect Pgp or *MDR1* mRNA (which involved different monoclonal antibodies and nucleic acid probes), as well as from the different criteria used to define positivity. At present, the hematologic malignancies in which Pgp has significant prognostic value are AML and multiple myeloma, and perhaps non-Hodgkins lymphoma.

Acute Myeloid Leukemia

A meta-analysis of the first 12 studies that examined, by various techniques, the expression of *MDR1*/Pgp in blasts of patients with AML, at different stages of disease with different chemotherapy regimens, was reported in 1994.[346] When 2 studies (360 patients total) were excluded due to lack of detail in the abstract form reported, the MDR remission relative risk was found to be 0.50. Forty percent (105/261) of the patients who were Pgp-positive achieved a complete response, while 81% (192/238) of the Pgp-negative patients obtained a complete response. When the other 360 patients were included in the analysis, the relative risk was 0.68. This meta-analysis suggests that there are significant differences in remission rates between patients whose AML blasts do or do not overexpress Pgp. Thus, as suggested by early investigators,[347] [348] Pgp is a contributor to drug resistance in AML, and this should be considered when treatment regimens are designed.

Subsequent studies have expanded on these findings. An analysis of 96 untreated patients with AML showed that Pgp expression predicted induction failure ($p = 0.0001$) and decreased overall survival ($p = 0.001$), as did unfavorable cytogenetics. [349] Pgp expression was not detected in patients with favorable cytogenetic abnormalities [t(15;17), inv(16), t(8;21)], was found in 29% of those samples with a normal karyotype, and was present in 62% of patients with an unfavorable cytogenetic abnormality. Pgp was also detected in 63% of those with secondary AML, compared to 25% of those with de novo disease. Another recent study of 110 newly diagnosed untreated patients with AML detected *MDR1* mRNA in 40% at diagnosis and confirmed that this is associated with both a higher rate of resistance to first induction ($p = 0.001$) and decreased overall survival ($p = 0.001$).[350] Pgp analysis with the MRK16 antibody in 211 elderly patients (>55 years old) with untreated AML once again showed that Pgp expression is significantly associated with a decreased complete response rate and resistant disease. [351] Patients in this report with de novo Pgp-negative AML with favorable cytogenetics had a complete response rate of 81%, compared with 12% for those with secondary AML who were Pgp-positive and had unfavorable cytogenetics.

In summary, Pgp expression is increased in secondary AML, in patients with unfavorable cytogenetics, and at the time of relapse; it is associated with chemotherapy induction failure and a decrease in overall survival. The presence of the Pgp drug efflux pump in AML blasts certainly contributes to drug resistance in this disease.

Acute Lymphocytic Leukemia

The role of Pgp in the drug resistance of ALL is not nearly as well established as in AML. This is due to the wide variation in techniques used to detect *MDR1*/Pgp, the variation in criteria used to define positivity, the contamination of samples with nonleukemic cells, and the fact that few large studies have been performed. Finally, and most importantly, the data to date do not support a significant role for Pgp in the drug resistance in ALL, as they have in AML. Of the smaller studies (2435 patients), only one has suggested a relation between Pgp drug transport and clinical outcome. [352] [353] [354] [355] An early study[352] showed Pgp mRNA and protein overexpression in only 14% of 28 patients, while Pgp function (efflux of rhodamine 123) was found in 8% of 24 patients in a more recent study (compared with increased rhodamine 123 efflux in 54% of patients with AML).[353] A study of 35 newly diagnosed patients reported Pgp positivity (defined as >1% positive cells with the JSB1 antibody) in 37% of patients; however, no statistical difference in the Pgp-dependent complete response rate was found ($p = 0.095$), nor did Pgp expression influence the relapse rate.[355] In contrast, a study of 33 children with ALL (19 untreated and 14 relapsed) in which Pgp function (rhodamine 123 efflux) and protein were measured demonstrated a significant increase in rhodamine efflux and Pgp expression in relapsed ALL, as well as a decrease in efflux in those achieving a complete response ($p < 0.001$).[354]

A higher incidence of Pgp positivity has been observed in adult T-cell leukemia, where 40% of 20 patients at diagnosis overexpressed Pgp and all 6 patients who were initially negative at presentation were Pgp-positive at relapse. [356]

Larger studies (50104 patients) in ALL have shown conflicting results. The first large study of 59 cases of de novo ALL (36 children and 23 adults) demonstrated Pgp overexpression in 38%, but only in adults did Pgp expression significantly affect the complete response rate ($p = 0.05$).[357] Relapses were seen in 81% and 37% of those Pgp-positive and -negative, respectively ($p = 0.008$), and survival was significantly increased in Pgp-negative patients. No other study has demonstrated as strong a correlation between Pgp expression and clinical outcome. A retrospective analysis of 104 newly diagnosed children with ALL showed Pgp positivity in 35%, with no significant association between Pgp expression and relapse ($p = 0.099$), but a decreased probability of remaining in first continuous complete remission if Pgp-positive ($p = 0.025$). [358] [359] A recent report of 83 adult ALL patients, which used a higher cutoff of >10% Pgp positive blasts to assess 39 patients at diagnosis and 44 at relapse, demonstrated 10% and 27% positivity, respectively ($p < 0.05$).[360] Pgp expression was found to be significantly less than that in AML at diagnosis (22%), and did not predict the clinical outcome of those newly diagnosed versus relapsed patients. *MDR1* mRNA alone was assayed by polymerase chain reaction in 60 patients and was found to be increased 3- to 4-fold in only the 10 recurrent relapses (not first relapses); however, an association with survival was not examined.[361] Finally, 64% of 50 newly diagnosed adult cases of ALL were found to be Pgp-positive; however, no correlation was found between Pgp expression and pretreatment characteristics, complete response rate, actuarial disease-free survival, or overall survival. [362] In summary, the studies reported to date do not support a major role for Pgp overexpression in the drug resistance of ALL.

Multiple Myeloma

The role of Pgp in the drug resistance of multiple myeloma has been reviewed by several investigators. [322] [344] [363] These studies have demonstrated overexpression of Pgp in human myeloma cell lines selected for resistance to doxorubicin as well as in clinical samples. *MDR1* mRNA was measured in myeloma cells obtained from the bone marrow of 34 patients who were subsequently treated with chemotherapy (7 had prior chemotherapy and 27 were newly diagnosed). [364] The newly diagnosed patients who failed to respond to chemotherapy had a significantly increased level of *MDR1* mRNA ($p = 0.011$), as did those who failed chemotherapy and had received prior treatment ($p = 0.025$). This study showed a correlation between the *MDR1* mRNA level and response to chemotherapy, but no correlation between the mRNA level and survival. [365] [366] Other studies have shown that before chemotherapy exposure, myeloma patients have a low level of Pgp overexpression in their plasma cells (about 5%), which increases to 75% when they become VAD-resistant (VAD: vincristine, doxorubicin, dexamethasone), or to 100% if they have received both a total vincristine dose of >20 mg and a total doxorubicin dose of >340 mg. However, a correlation between Pgp overexpression and survival has not been seen. [366] [367]

The overexpression of Pgp in human myeloma cells certainly contributes to the drug resistance of these cells (see chemosensitization section below as well); however, this is unlikely to be the only mechanism of drug resistance in myeloma. A recent paradigm proposes that the Pgp expressed in plasma cells in the bone marrow of myeloma patients is not functional and the cells are thus chemosensitive; the drug-resistant reservoir may be a CD34- and CD38-positive monoclonal B lineage in the peripheral blood mononuclear cell fraction that upregulates functional Pgp after drug exposure. [368]

Non-Hodgkins Lymphoma

The role of Pgp in the drug resistance of non-Hodgkins lymphoma (NHL) has recently been reviewed [369] and shown to be less well defined than the hematologic malignancies already discussed. The expression of Pgp in NHL depends on the prior exposure to chemotherapeutic agents known to induce Pgp overexpression, as well as on the method used to detect *MDR1*/Pgp and the cut-off to define positivity. The expression of Pgp in untreated intermediate- and high-grade lymphoma is about 10%, which increases to about 40% after treatment. A retrospective study of 60 NHL specimens demonstrated that 25 (42%) overexpressed Pgp; 19 of these patients had drug-resistant disease, while the remaining 6 achieved a complete response. [370] Thus, resistance to chemotherapy was found to be a function of Pgp expression ($p < 0.001$). A later study of 57 untreated large cell and immunoblastic lymphomas showed high (>50% staining) de novo Pgp expression in 23% of the patients, intermediate staining (1150%) in 26% of the patients, and equivocal or negative Pgp expression in the remaining 51%. [371] These investigators found no correlation between the level of Pgp expression and either response to chemotherapy or median survival. No correlation between *MDR1* mRNA expression and either previous exposure to drugs or survival was found in studies of 41 patients [372] and 32 patients [373] with NHL. Clearly, further studies of Pgp expression (and function) need to be performed prospectively in fresh biopsies obtained from all types of NHL at the time of diagnosis and at the time of relapse.

Chronic Myeloid and Lymphocytic Leukemias

The expression of *MDR1*/Pgp in CML and CLL has been examined by several investigators. A low level of Pgp expression has been demonstrated in chronic-phase CML, [374] [375] which appears to increase in accelerated-phase and blast crisis CML. [375] [376] However, a role for Pgp in the drug resistance of CML has not yet been demonstrated. In the treatment of CLL, few Pgp-related antineoplastic agents are used. The expression of *MDR1*/Pgp in B-CLL is highly variable and depends on the assay used and the criteria for positivity. Several studies have shown high levels of *MDR1* mRNA and Pgp expression in CLL cells from both treated and untreated patients, [377] [378] [379] [380] while other studies have shown very limited Pgp expression [381] and function. [382] A single study has shown a correlation between *MDR1* expression and survival, in which the 10 B-CLL patients who were *MDR1*-positive had a median survival of 19 months compared with 46 months for the 17 *MDR1*-negative patients ($p < 0.01$). [380] Normal B lymphocytes from peripheral blood (CD5+/CD19+) [378] and from human tonsils (CD5+/CD19+/CD10) [383] have been shown to have a low level of Pgp expression, to contain *MDR1* mRNA transcripts, and to efflux rhodamine 123. Perhaps these normal Pgp-expressing B lymphocytes give rise to B-CLL cells that overexpress Pgp, thus accounting for the de novo drug resistance of CLL cells. It is unlikely that the overexpression of Pgp plays a major role in the drug resistance of chemotherapy-naive or -exposed CML or CLL cells.

Clinical Studies with Modulators of Pgp

The clinical trials that have used various modulators of Pgp have been reviewed by several investigators. [329] [331] [384] An early study in VAD-refractory multiple myeloma resulted in short-lived partial responses to VAD plus racemic verapamil in 5/22 patients. [385] [386] Four of the five responders overexpressed Pgp, but cardiac side effects precluded further dose escalation of intravenous R,S-verapamil. Continuous intravenous infusion of cyclosporin A with VAD in VAD-resistant myeloma patients resulted in 7/15 responses, which were more common in patients who overexpressed Pgp. [365] A more recent randomized

Southwest Oncology Group (SWOG) phase III study of VAD with or without oral Verapamil in 120 patients with refractory myeloma demonstrated a 41% and 36% response in the VAD and VAD/verapamil arms, respectively, with median survivals of 10 and 13 months. [387] The lack of a beneficial effect of oral verapamil in this study is likely due to suboptimal serum levels of verapamil. A final dose-escalation study of the cyclosporin A (CSA) analog PSC 833 (which is 5- to 10-fold more potent an inhibitor of Pgp than CSA), in VAD-refractory myeloma, resulted in responses in 8/22 patients. [388] This study demonstrated that the dose of doxorubicin had to be decreased in the presence of PSC 833 as the plasma area under the curve of this drug increased twofold.

Modulators of Pgp have also been used in phase I and II studies of poor-risk AML. Continuous-infusion CSA has been dose-escalated in combination with daunorubicin and cytosine arabinoside. [389] A transient hyperbilirubinemia was seen in 62% of the patients; these same patients had increased serum daunorubicin levels and a higher response rate. A complete response was seen in 26/42 patients; however, the MDR phenotype was not found to influence the response. A recent phase I study of continuous-infusion PSC 833 with mitoxantrone and VP-16 in patients with AML demonstrated that the doses of both antineoplastic agents had to be attenuated 66% in the presence of PSC 833 due to severe mucositis. [390] No complete responses were seen with this regimen, and again a transient increase in bilirubin was common. Dexverapamil, the less cardiotoxic enantiomer, has been dose-escalated in oral form in patients with either Hodgkins disease or NHL who are refractory to EPOCH chemotherapy (etoposide, prednisone, vincristine, cytoxan, doxorubicin). [391] Responses were few: 2 partial responses in 10 patients with Hodgkins disease, and 3 complete responses and 2 partial responses in 41 patients with NHL.

The studies discussed above, as well as several others, suggest that modulators of Pgp have a role in reversing MDR in leukemia and lymphoma. However, MDR mediated by Pgp is seldom the only mechanism of resistance in tumors previously exposed to antineoplastic agents; thus, the modulation of Pgp will likely have a minor role in sensitizing cells in this setting. Treating patients upfront at the time of diagnosis with antitumor agents plus a Pgp modulator that achieves optimal serum levels may preclude the emergence of Pgp-mediated drug resistance, an observation reported for myeloma cells selected in vitro to doxorubicin and verapamil. [392]

Multidrug Resistance-Associated Protein

Structure and Function

The multidrug resistance-associated protein was first described in 1992 in the doxorubicin-selected small cell lung cancer cell line, [393] and its biochemical characteristics and biologic properties have recently been reviewed. [394] This N-glycosylated plasma membrane phosphoprotein has a molecular mass of 190 kDa (1531 amino acids) and is a member of the ATP-binding cassette transporter superfamily (Table 50-7 and Fig. 50-14). This transporter has 18 transmembrane domains (12 in the amino end and 6 in the carboxyl end), and its gene is on human chromosome 16p13.1. Several isoforms of the MRP glycoprotein exist, including the GSH S-conjugate export carrier (GS-X pump), the multispecific organic anion transporter, and the leukotriene C_4 (LTC_4) transporter. [395] [396] [397] [398] To confirm that the overexpression of MRP results in an MDR phenotype, HeLa cells have been transfected with vectors containing MRP cDNA and found to have 5- to 15-fold levels of resistance to doxorubicin, daunomycin, epirubicin, vincristine, and VP-16. [399] [400] Low levels of resistance were also found to taxol, vinblastine, and colchicine, as well as to the heavy metals arsenite, arsenate, and antimonials; no cross-resistance to mitoxantrone, CDDP, or cadmium was observed.

Figure 50-14 Models of multidrug resistance protein (MRP) membrane topology. MRP possesses features common to all members of the ATP-binding cassette transporter superfamily, in that each half of the protein is predicted to consist of several transmembrane domains followed by a cytosolic nucleotide binding domain (NBD). The first model **(A)** is based on computer-assisted hydropathy

analyses of the human MRP amino acid sequence and predicts that MRP is composed of 12 transmembrane domains (solid bars), eight of which are within the NH-2 proximal half of the protein. The second model **(B)**, based on a comparison of human and murine MRP with other ATP-binding cassette transporters, suggests that there are up to four additional transmembrane domains in the NH2-proximal half of the protein. *(Reproduced from Loe et al.,[394] with permission from Elsevier Science.)*

Chemosensitization by either verapamil or cyclosporin A was no different in MRP-transfected cells than in cells transfected with vector alone. Human non-small cell lung cancer SW-1573 cells have also been stably transfected with MRP cDNA and been found to be 2.7- to 5.3-fold resistant to doxorubicin, daunomycin, vincristine, VP-16, colchicine, and rhodamine, but not resistant to m-AMSA or taxol.[401] A study of drug sensitivity in single and double knockouts of the MRP gene in W9.5 murine embryonic stem cells, in which no MRP mRNA or MRP protein expression was seen in the double knockout, demonstrates three- to fourfold hypersensitivity to VP-16, VM-26, and Na arsenite, with twofold levels of collateral sensitivity to vincristine, doxorubicin, and daunomycin.[402] Thus, MRP is an ATP-dependent plasma membrane drug transporter that when overexpressed can result in an MDR phenotype that has several substrates in common with Pgp.

Substrates and Modulators of MRP

The overexpression of MRP has been shown in vitro to result in different levels of drug resistance to several classes of antineoplastic agents,[394] [395] [397] [398] [400] [402] [403] represented by actinomycin D, chlorambucil, melphalan, CDDP, daunomycin, doxorubicin, epirubicin, VM-26, VP-16, and vincristine. Low levels of resistance have also been reported to taxol, vinblastine, and colchicine. In addition to antitumor agents, MRP and its isoforms are capable of transporting heavy metals (arsenicals, cadmium, and antimonials), as well as glutathione conjugates and cysteinyl leukotriene.

Compounds reported to modulate MRP-mediated drug resistance in vitro include the calcium channel blocker verapamil, nicardipine, the protein kinase C inhibitor GF109203X, the cyclosporin analog PSC 833, the tyrosine kinase inhibitor genistein, the gyrase-inhibiting antibiotic difloxacin, and amiodarone.[394] More recently, VX-710, a nonmacrocyclic ligand of the

FK506-binding protein FKBP12 and a potent modulator of Pgp-mediated MDR, has been found to restore sensitivity of MRP-expressing HL60/ADR cells to the cytotoxic action of doxorubicin, VP-16, and vincristine.[165] The nonsteroidal anti-inflammatory agent indomethacin has also been shown to increase significantly the sensitivity of HL60/ADR cells to doxorubicin and vincristine, and it may be a specific inhibitor of MRP.[404] Finally, the organic anion transport inhibitor probenecid has been found to reverse the MRP-mediated resistance of HL60/AR cells to daunomycin and vincristine by increasing the accumulation of these drugs.[405] The in vitro work to date suggests that there may be specific inhibitors of MRP-mediated drug resistance, and that these modulators may not overlap with Pgp-reversing agents. Clinical studies using these agents have not yet been reported.

MRP Expression in Normal Human Tissues

Expression of MRP mRNA has been measured in several normal human tissues by reverse transcriptionpolymerase chain reaction (RT-PCR),[406] an RNase protection assay,[407] and by filter hybridization of polyA$^+$ RNA.[408] These studies have demonstrated that most normal peripheral blood cells and bone marrow cells express a basal level of MRP mRNA, which is moderately increased in granulocytes and T cells. High levels of expression were found in skeletal muscle, lung, kidney, pancreas, testis, ovary, prostate, thymus, and pancreas, with low levels of expression in brain, liver, intestine, heart, and spleen. Protein levels of MRP (a more relevant measure of this transporter) have also been determined in normal tissues by both Western blotting and immunohistochemistry with three monoclonal antibodies.[409] Western blotting showed high levels of MRP in the adrenal gland, lung, heart, and skeletal muscle, with lower levels in liver, spleen, kidney, and red blood cell membranes. Immunohistochemistry confirmed the high expression of MRP in epithelial, endocrine, and muscle tissues (i.e., in skin, epithelium of the esophagus and intestine, bronchioles and alveolar macrophages of the lung, testis, stromal cells of the ovary, heart, adrenal cortex, and pancreas).

MRP Expression in Human Malignancies

Solid Tumors

Expression of MRP has been determined in human solid tumors and in cell lines derived from human tumors by measuring either MRP mRNA or the protein expression itself.[408] [409] [410] Cell lines derived from cancers of the lung (non-small cell and small cell), colon, stomach, breast, and ovary, as well as human sarcomas, astrocytomas, and melanomas, were found to have a basal level of MRP expression in 54/55 tumors.[408] When 3 monoclonal anti-MRP antibodies were used to determine MRP expression in 119 solid tumor frozen biopsies, it became apparent that staining was dependent on the specific antibody used.[409] Nonetheless, strong MRP staining was seen in 20% of untreated solid human tumors and was especially prominent in non-small cell lung cancer and esophageal cancer biopsies.

Hematologic Malignancies

The expression of MRP mRNA (measured by a RNase protection assay) and/or the level of MRP protein (by immunohistochemistry) has been assessed in 148 patients with hematopoietic malignancies.[407] [411] [412] MRP mRNA expression was found to be significantly increased in 84% of patients with CLL and in 30% of those with AML. The vast majority of patients with ALL, CML, multiple myeloma, hairy cell leukemia, and NHL were found to have low levels of MRP mRNA expression in the malignant cells. MRP protein was assayed using monoclonal antibody MRPr1, and the results generally followed those of the message, with increased expression in most patients with CLL. Southern analyses of a cohort of these patients showed that the increased expression of MRP was not due to gene amplification, and that the lack of coexpression of Pgp suggested that these two genes are independently expressed. Other investigators, using RT-PCR, have confirmed low levels of expression of MRP mRNA in AML blasts and in bone marrow samples of multiple myeloma.[406] A recent study of 40 patients with refractory lymphoma and 16 with newly diagnosed lymphoma suggests a limited role for MRP mRNA expression in drug resistance in NHL: 15 paired samples in the refractory group showed no difference in MRP expression before and after EPOCH treatment.[413] In addition, the untreated patients MRP mRNA levels were no different than those of the treated patients, before or after EPOCH.

Other studies using RT-PCR to determine MRP mRNA expression have found increased levels in acute leukemic blasts. A study of 49 patients with AML and 29 with ALL demonstrated significantly higher expression of MRP in ALL ($p = 0.007$) and in secondary AML ($p = 0.016$), but not in de novo AML.[414] MRP mRNA expression was found to be significantly ($p < 0.05$) elevated at second and later relapses (but not before treatment or at first relapse) in a group of 60 patients (52 children and 8 adults) with ALL.[415] Finally, 14 patients with relapsed AML were found to have a 2-fold increase in blast MRP mRNA relative to 29 patients with newly diagnosed AML ($p< 0.01$).[416] Paired blast samples (obtained at diagnosis and at relapse) from 13 AML and 4 ALL patients showed a 2-fold increase in 80% of the patients at relapse, suggesting that the expression of the MRP transporter at relapse may be involved in drug resistance.

An interesting association between MRP expression and clinical outcome has been found in patients with inversion 16 AML.[417] [418] Inv(16)(p13q22) is most often associated with acute myelomonocytic leukemia with abnormal eosinophils (M4Eo) and has a favorable prognosis. In a series of 22 patients with inversion 16, 7 were found to have deleted the MRP gene at the short arm breakpoint, 14 had retained the gene, and 1 was indeterminate. The deletion of the MRP gene was associated with an increase in time from diagnosis to relapse or death ($p = 0.007$), possibly due to a loss of this particular mechanism of drug resistance.

The role of the overexpression of the MRP transporter in the drug resistance of hematologic malignancies is not yet defined. Most of the studies to date have not assayed for the level of the effector molecule (protein) or its transport activity, but rather have focused on MRP mRNA expression. Highly concentrated populations of blasts (nearer 100%) need to be assayed both at diagnosis and at relapse, using activity assays and antibodies that are well controlled and validated, to define the role of MRP in drug resistance. The data collected thus far suggest that MRP may be significantly overexpressed in CLL and may be increased in relapsed ALL and AML. The deletion of the MRP gene in inversion 16 AML appears to be a favorable prognostic indicator, and may have a biologic role in the drug resistance of this disease.

Lung Resistance-Related Protein

Structure and Function

The lung resistance-related protein (LRP) or major vault protein (MVP) was first described using murine monoclonal antibody LRP-56 in the doxorubicin-resistant non-small cell lung cancer cell line SW1573/2R120 in 1993.[419] The biochemical and physical characteristics of this putative transporter have subsequently been

described (Table 50-7). [420] [421] [422] LRP has a molecular mass of 110 kDa. Unlike Pgp and MRP, it is not a member of the ATP-binding cassette family, and its gene is located on chromosome 16p11.2 (MRP is at 16p13.1). While the majority of LRP is located

in the cytoplasm of the cell, 5% is associated with the nuclear membranepore complex, where it is thought to be involved in nucleocytoplasmic transport. The human-deduced LRP amino acid sequence has 88% identity with the 104 kDa rat MVP. [420] Vaults are hollow, barrel-like structures approximately 57 × 32 nm with a total mass of 13 MDa. In the cytoplasm they are associated with vesicular structures (Fig. 50-15). The rat vault is a ribonucleoprotein particle containing 4 proteins of molecular mass 210 kDa, 192 kDa, 104 kDa, and 54 kDa and a species of small RNA of approximately 140 bases, in a relative molar ratio of 3:1:55:7:9. Thus, the MVP (or LRP homolog) is the most abundant component of this organelle.

While co-overexpression of LRP and MRP is common in non-Pgp cell lines, LRP and Pgp are rarely overexpressed together. [422] LRP and MRP are rarely coamplified, and gene amplification is unlikely to account for the overexpression of LRP. [421] An increased expression of LRP has been reported in several Pgp-negative human drug-resistant cell lines, including a small cell lung cancer cell line (GLC4/ADR), a fibrosarcoma cell line (HT1080/DR4), breast cancer cell lines (MCF 7/Mitox and MCF 7/MR), and a myeloma cell line (8226/MR40). [419] The expression of LRP mRNA has recently been examined in 8 of the NCI panel of 60 human cancer cell lines used for in vitro anticancer drug screening. [423] These non-drug-selected cell lines were from lung, leukemia, central nervous system, colon, ovarian, and renal cancers. The expression of LRP mRNA, determined by an RNase protection assay, varied widely among the 8 cell lines but correlated closely with in vitro drug resistance to melphalan, CDDP, doxorubicin, and m-AMSA (p = 0.010.0001); the most resistant cell line had the highest LRP mRNA level. Similar to mRNA levels, LRP protein expression in these cell lines was also found to be a better indicator of drug sensitivity than either MRP or Pgp protein expression. The human myeloma 8226/DOXint5 cell line was selected to doxorubicin on a schedule designed to mimic the administration of VAD chemotherapy, and was found to have three- to fourfold levels of resistance to doxorubicin, VP-16, and m-AMSA. [424] DOXint5 cells do not overexpress Pgp, have a minimal increase in MRP levels and a fourfold attenuation of topoisomerase II, and overexpress LRP (detected with the LRP-56 antibody) in 70% of the cells. These findings suggest that LRP overexpression may have a role in drug resistance in multiple myeloma.

The in vitro studies to date suggest that overexpression of LRP may result in an MDR phenotype with cross-resistance to various combinations of doxorubicin, VP-16, m-AMSA, carboplatin, CDDP, melphalan, and vincristine. Transfecting the LRP gene into LRP-negative cells to define the drug resistance phenotype and to help define the role of LRP in cellular drug resistance will be difficult because vaults are composed of four distinct proteins and RNA.

LRP Expression in Normal Human Tissues and Human Malignancies

The expression of LRP in acetone-fixed, frozen normal human tissue has been comprehensively examined by immunohistochemistry using the monoclonal antibody LRP-56. [425] High levels of expression were found in keratinocytes, bronchial cells and alveolar macrophages of the lung, the epithelium of the digestive tract, adrenal cortex, and macrophages, with moderately high levels of LRP in melanocytes, ductal cells of the pancreas, the urothelium, germinal cells of the testes, and proximal tubules of the kidneys. LRP expression was detected in immunoprecipitates of normal peripheral blood and bone marrow

In this same study, the expression of LRP in 174 tumor samples of 28 types of human cancer (mostly untreated tumors) was determined by using the LRP-56 antibody. A low frequency of LRP expression was seen in germ cell tumors, Wilms tumor,

Figure 50-15 Hypothetical role of LRP in drug resistance. LRP, or vaults, may mediate nucleocytoplasmic and vesicular transport of drugs. Through exocytotic vesicles, the drugs would be transported out of the cell. *(Reproduced from Izquierdo et al.,[422] with permission from Elsevier Science.)*

rhabdomyosarcoma, neuroblastoma, Ewings sarcoma, squamous cell carcinoma of the lung, and AML. The majority of the other solid tumors were LRP-positive; colorectal, pancreatic, renal, adrenal, pheochromocytoma, gastric, and endometrial cancers expressed LRP in all cases examined.

An attempt to address the relevance of LRP expression in the drug resistance of human tumors has been made for ovarian cancer [426] and acute leukemias. [427] [428] [429] Fifty-seven frozen ovarian carcinoma samples from patients with stage III and IV disease were assessed for Pgp, MRP, and LRP expression using monoclonal antibodies MRK16/JSB-1, MRPr1, and LRP-56, respectively. [426] Sixteen percent of the tumors were found to stain positively for Pgp, 68% positive for MRP, and 77% positive for LRP. Patients whose tumors stained positive for LRP had a poorer response to chemotherapy (p = 0.004), a shorter progression-free survival (p= 0.003), and a decreased overall survival (p = 0.007) relative to LRP-negative patients. The median overall survival was 5 months for LRP-positive and 42 months for LRP-negative patients. LRP expression appears to have prognostic significance in ovarian cancer, although this needs to be confirmed in a prospective study.

Immunohistochemical analyses of 87 consecutive bone marrow samples for LRP expression from 21 patients with de novo AML, 27 patients with secondary AML, 29 patients with relapsed AML, and 10 patients with blast-phase CML demonstrated significant LRP expression in 33%, 48%, 38%, and 10% of these patient groups. [427] LRP overexpression was found to be associated with an inferior response to induction chemotherapy (p = 0.0017), as complete plus partial responses were seen in 68% of those who were LRP-negative and in only 35% of those who were LRP-positive. LRP expression was found to have independent prognostic significance, to develop after exposure to the Pgp modulator cyclosporin A, and to be associated with prior mitoxantrone exposure. An evaluation of Pgp, MRP, and LRP expression by flow cytometry in 65 patients (38 AML, 8 ALL, 19 blast CML) has shown Pgp expression alone in 24.5%, LRP alone in 11%, MRP alone in 1.5%, Pgp and LRP concurrent expression in 24.5%, and Pgp and MRP concurrent expression in 4.5%. [428] These results suggest that LRP (and not MRP) is frequently overexpressed, and often with Pgp, in acute leukemia. A study of blasts from 67 patients with AML by RT-PCR for MDR1, MRP, and LRP mRNA expression demonstrated significantly higher (p = 0.033) LRP mRNA levels in the 8 patients who did not achieve a complete response compared to the 25 who did. [429] While a significant difference in MDR1 mRNA levels was found between presentation and relapse samples (p = 0.031), no difference in LRP mRNA levels was seen in these groups or in eight paired samples studied sequentially.

In conclusion, the studies presented to date suggest that the overexpression of LRP may indeed be involved in the MDR of ovarian carcinoma and AML, and that its expression may be a significant prognostic indicator for these two diseases. The role of LRP in the drug resistance of other hematopoietic malignancies and the possible circumvention of this mechanism by modulating drugs await further investigation.

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FUTURE THERAPEUTIC IMPLICATIONS OF CANCER BIOLOGY

Future treatment strategies against cancer are likely to be directed against the cellular targets responsible for the altered biology and transformed phenotype of cancer versus normal cells. This altered biology results from the accumulation of genetic mutations and alterations secondary to the unique genomic instability of cancer cells. As noted above, defects in the DNA repair pathway and in cell-cycle checkpoints that cause inappropriate progression through the cell cycle facilitate the genomic instability of cancer cells. Promising novel small molecular drugs and other treatments are being developed and investigated that target the altered macromolecular gene products in the cancer cells. Table 50-8 lists some of the potential targets and evolving therapeutic strategies for cancer.

Since dysregulated cell-cycle progression is a common feature of neoplastic cells, several strategies are being developed and tested to exploit this as a therapeutic target. These strategies include the use of drugs that inhibit the function of the mitotic kinase $p34^{cdc-2}$ (CDK1), such as flavopiridol, or inhibitors of the dual specific phosphatase cdc25, which dephosphorylates and activates $p34^{cdc-2}$. Flavopiridol also inhibits CDK2 and CDK4 by binding to the hydrophobic, adenine-binding pocket of the ATP site of these G_1 -S kinases, thereby inducing G_1 arrest. An additional strategy is to inhibit mitogenic signaling mediated by oncogene products such as activated Ras, Raf, and Bcr-Abl. Antisense oligonucleotides to C-Raf, bcr-Abl, C-myb, Ras, or bcl-2 are being tested for their antitumor effects alone or in combination with other cytotoxic drugs. Inhibitors of Ras farnesylation exert their antitumor effects by as yet unclear mechanisms.

Adenoviral vector-mediated transfer of $p53^{wl}$ into cancers that harbor mutant p53 is being investigated in clinical trials against a variety of tumor types. An additional promising approach to eradicate tumor cells expressing a mutant p53 may be to treat with a mutant adenovirus that can selectively replicate and lyse $p53^{wt}$ -deficient tumor cells. Preclinical or clinical trials are also underway to use antibodies or drugs to inhibit HER-2-neu or EGFR-mediated mitogenic signaling in breast or other epithelial cancers.

The relentless growth of tumors and leukemia requires the delivery of nutrients and growth factors through new blood microvessels (angiogenesis). Inhibitors of angiogenesis have exciting potential in cancer and leukemia therapy. These agents target the vascular endothelial growth factors (e.g., VEGF, bFGF, and PDGF) or their receptor-mediated signaling (e.g., SU 5416). Novel angiogenesis inhibitors are also being investigated (e.g., TNP-470, angiostatin, and endostatin) in preclinical or phase I studies. Relative genomic stability of the tumor vascular endothelial cells suggests that these would be far less prone to develop drug-resistance mechanisms than tumor cells. Preclinical in vivo studies have demonstrated that intermittent administration of endostatin for a few courses completely inhibits the regrowth

TABLE 50-8 -- Potential New Targets to Aim for Future Cancer Therapy

Target	Potential Inhibitor(s)
Cell-cycle regulatory genes/protein	$cdc25/p34^{cdc2}$: flavopiridol
Oncogenes	Anti-Raf, -Bcr-Abl, -myb, -Bcl-2 antisense oligonucleotides; Ras: farnesylation inhibitors
Tumor suppressor genes	Adenoviral vector with $p53^{wl}$, adenovirus targeted to $p53^{mut}$
Growth factor receptors	Suramin,[a] pentosanpolysulfate, monoclonal antibodies: anti-HER2-neu, anti-EGFR; PDGFR: SU-101
Signal transduction proteins	Suramin,[a] anti-Raf and anti-Ras monoclonal antibody, SH2 peptide inhibitors, bryostatin-1, anti-PKC: 7- OH-staurosporine
DNA topoisomerases	Camptothecin analogs[a]
Microtubules	Taxotere,[a] dolostatin 10[a]
Mitochondria	Dianylsulfonylureas
Microvessels	TNP-470,[a] angiostatin, endostatin, SU-5416
Immune system	Vaccines,[a] adoptive immunotherapy[a]
Immunotoxins	Rituximab[a]

[a]Currently in clinical trials.

of human tumor xenograft in mice. Clinical studies with the furnigillin (antiangiogenesis fungal product) analog TNP-470 are being conducted in AIDS-related Kaposis sarcoma and hormone-refractory prostate cancer.

The promise of cancer vaccines in the treatment of epithelial cancers and hematologic malignancies resides in the generation of active tumor-specific immune response in the host that would reject metastatic foci and induce long-term immunologic memory against relapse. Autologous tumor cells ex vivo engineered to express immune modulatory cytokines (IL-2, IL-4, IL-7, IL-12, GM-CSF, -interferon) or costimulatory molecules (B7-1 and B7-2) have been used as a source of antigen in preclinical animal studies. However, it is not essential for the autologous tumor to be the source of the cytokine, as long as there is paracrine production of cytokines. Impaired immunoresponsiveness to tumor vaccines has also been linked to high tumor burden, which may induce tumor antigen-specific tolerance and global immunosuppression. To overcome this, tumor vaccines may have to be used in the minimal residual disease setting and in drugvaccine combinations. In hematologic malignancies, the administration of a tumor vaccine during the immunoreconstitution period after bone marrow transplantation is also an attractive strategy. This may force the developing T-cell repertoire toward the recognition of tumor antigens. The adoptive transfer of antitumor immunity by primed T cells from the donor to the recipient may allow transfer of systemic tumor-specific immunity. Because of the large component of lymphocytes in the graft used in peripheral blood stem cell autologous transplantation, this may facilitate adoptive transfer of donor antitumor immunity. The promise of adoptive immunotherapy remains to be investigated in hematologic malignancies.

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APPENDIX: Clinical Pharmacology of Alkylating Agents

Mechlorethamine (Mustargen)

Chemistry: Mechlorethamine, also called nitrogen mustard, is a water- and alcohol-soluble analog of sulfur mustard gas. It is a bifunctional chloroethylating agent that alkylates DNA, RNA, and protein.

Absorption, Fate, and Excretion: The parent compound is highly reactive and has a biologic half-life of approximately 15 minutes. The principal route of degradation is spontaneous hydrolysis, but some enzymatic demethylation also occurs.

Preparation and Administration: Mechlorethamine is supplied in vials of 10 mg with 100 mg of sodium chloride, and is reconstituted with 10 ml sterile water to yield a 1-mg/ml solution, ideally prepared immediately before use. However, the manufacturer considers the drug expired 1 hour after reconstitution. The drug is injected over a few minutes through a tubing as a freely running intravenous infusion. For topical application (e.g., in mycosis fungoides), 10 mg of drug is dissolved in 60 ml of tap water. Alternatively, a 10% ointment has been used by dissolving the drug in 95% ethyl alcohol and petrolatum (Aquaphor). Mechlorethamine is a powerful vesicant. In the event of extravasation, vigorous irrigation followed by 0.25% sodium thiosulfate injection at the site of extravasation should be attempted. Ice packs may be placed for 612 hours to minimize the local reaction.

Toxic Effects: Myelosuppression is the dose-limiting systemic side effect. This worsens with each additive cycle. Severe nausea and vomiting, infertility, alopecia, and pain at the site of injection, which can sometimes spread to involve the venous system (tracking), are also common. Occasionally, a macular papular rash is observed, but this does not appear to be allergic in nature and does not contraindicate continuation of therapy. Infertility is common, but may be reversible. Infrequent adverse effects include alopecia, anorexia, weakness, and diarrhea. The drug has also been shown to induce chromosomal abnormalities and may contribute to the development of secondary leukemias, as seen in patients treated with this agent as part of the MOPP regimen.

Potential Drug Interactions: None reported.

Therapeutic Indications in Hematology: Mechlorethamine is incorporated in many chemotherapy combinations used in the treatment of Hodgkin disease (MOPP [mechlorethamine, vincristine, procarbazine, prednisone] and MOPP/ABV [doxorubicin [Adriamycin], bleomycin, and vinblastine] hybrid) and in some non-Hodgkin lymphomas (prednisone, etoposide, methotrexate, doxorubicin, cyclophosphamide, leucovorin [PROMACE]/MOPP). However, its use has largely been supplanted by other agents.

Cyclophosphamide (Cytoxan)

Chemistry: Cyclophosphamide is a cyclic phosphamide ester of mechlorethamine. Once metabolically activated, it alkylates DNA, forming cross-links.

Absorption, Fate, and Excretion: The drug is relatively well absorbed orally, with approximately 75% oral bioavailability. The parent compound is not active. The drug is metabolized by the hepatic cytochrome P-450 system, which ultimately generates at least two active compounds: phosphoramide mustard and acrolein. The latter appears to be responsible for cyclophosphamides bladder toxicities. The plasma half-life of cyclophosphamide varies from 4 to 6.5 hours. Approximately 15% of the drug is excreted unchanged in the urine. Dose reduction should be considered in patients with severe renal failure.

Preparation and Administration: Cyclophosphamide is supplied as 25- and 50-mg tablets and as a powder for parenteral administration in 100-, 200-, and 500-mg and 1- and 2-g vials. It is dissolved by adding 5 ml of preservative-free sterile water for every 100 mg of drug. Cyclophosphamide is chemically stable for 24 hours at room temperature and for 6 days if refrigerated.

Toxic Effects: Marrow suppression is the major side effect. The myeloid series is primarily affected, although thrombocytopenia also occurs at high doses and alopecia is common. Nausea and vomiting can be severe and are usually delayed, occurring 68 hours after administration. Hemorrhagic cystitis occurs in 10% of patients receiving nontransplant doses and is apparently due to the formation of the urotoxin acrolein. Because of this potential side effect, patients should be well hydrated. Mesna disulfide (sodium 2-mercaptoethanesulfonate disulfide) has also been used on a weight equivalent basis to ameliorate cyclophosphamide-induced

bladder toxicity. Other potential toxic effects include stomatitis, skin and nail hyperpigmentation, interstitial pulmonary fibrosis, and the syndrome of inappropriate secretion of antidiuretic hormone. Rare episodes of acute congestive heart failure have been reported. After bone marrow transplant doses, hemorrhagic cystitis is common and cardiac toxicity (cardiomyopathy) may be seen. Late sequelae include bladder fibrosis (more common with daily [oral] therapy), bladder cancer, leukemogenesis, and infertility.

Potential Drug Interactions: Corticosteroids may increase P-450 enzyme-induced metabolism and is often avoided in high-dose therapy. When combined with doxorubicin, it may increase cardiac toxicity. This may be prevented by amifostine. In animal studies, conflicting results were reported when the P-450 enzyme inducer phenobarbital was given with cyclophosphamide. Most investigators, however, have observed a reduction in the amounts of active metabolites. Conversely, when cimetidine (but not ranitidine) was administered in leukemia-bearing mice before treatment with cyclophosphamide, a significant prolongation of their survival and higher plasma concentrations of alkylating metabolites were observed. Although one should remain alert for these potential drug interactions, none has been demonstrated in humans. Cyclophosphamide reduces serum pseudocholinesterase levels, which may prolong the neuromuscular blocking effects if given simultaneously. Caution must be exercised when administering high doses of these two drugs to critically ill patients. Life-threatening hyponatremia may develop when used in conjunction with indomethacin, although the precise incidence is unknown.

Therapeutic Indications in Hematology: Cyclophosphamide is a key drug in the treatment of lymphomas and myeloma. It is incorporated in many chemotherapy regimens, including CHOP, MACOP-B, PROMACE/CYTABOM, CVP, and VMCP (see chapter for details). In addition, cyclophosphamide is the drug most commonly used in preparatory regimens for bone marrow transplantation. It is also used in solid tumors and as an immunosuppressant in nonmalignant conditions such as glomerulonephritis and systemic lupus erythematosus.

Ifosfamide (Ifex)

Chemistry: Ifosfamide is an oxazaphosphine nitrogen mustard that differs from cyclophosphamide by the placement of chloroethyl groups.

Absorption, Fate, and Excretion: As in the case of cyclophosphamide, the parent compound is inactive and is metabolized by the cytochrome P-450 system in the liver. The metabolism of ifosfamide is influenced by the dose and schedule of administration. When administered as a single bolus, 60% is eliminated into the urine, 53% as unchanged inactive drug. When administered daily for 5 consecutive days, 56% is excreted into the urine, 15% as the inactive parent compound. The half-life is 7 hours when administered daily for 5 consecutive days and 15 hours when given as a single bolus dose. There is poor penetration across the bloodbrain barrier. Its longer half-life and slower metabolic activation allows higher doses to be given.

Preparation and Administration: The drug is provided in 1-g vials and should be reconstituted in sterile water or bacteriostatic water to a final concentration of 50 mg/ml. Ifosfamide can be diluted further in 5% dextrose, normal saline, or Ringers solution for injection to achieve concentrations of 0.620 mg/ml. The solution should be infused over 30 min. To prevent hemorrhagic cystitis, patients must receive mesna disulfide for protection against urotoxicity and must be kept well hydrated (2 L/day). Mesna is a thiol compound that is rapidly oxidized to dimesna in vivo. Both mesna and dimesna are filtered by the glomeruli, reabsorbed in the proximal tubule, and finally secreted back into the tubular lumen of the kidney. In the tubules, approximately one third of the filtered dimesna is readily converted back to mesna. The free sulfhydryl group of this compound reacts with the urotoxic metabolite, acrolein, produced by both ifosfamide and cyclophosphamide (Fig. 50-6). This reaction creates a nontoxic acrolein/mesna thioether that is safely eliminated in the urine. Mesna has also been shown to inhibit the degradation of ifosfamide or cyclophosphamide to acrolein.

Mesna has been given in combination with ifosfamide in different doses and schedules. One recommended schedule employs intravenous bolus injection in a dosage equal to 20% of the ifosfamide dose (on a milligram-to-milligram basis) at the time of ifosfamide administration and 4 and 8 hours after each dose of ifosfamide. Mesna has also been given by continuous infusion with excellent results. The two agents may be mixed together in the same intravenous solution; however, mesna is not compatible with cisplatin.

Toxic Effects: With the use of mesna to protect against urotoxicity, myelosuppressionespecially leukopenia and, to a lesser extent, thrombocytopeniais the dose-limiting side effect. Renal tubular acidosis can occur. Central nervous system effects, observed in approximately 10% of patients treated, include somnolence, confusion, depressive psychosis, and hallucinations. Less commonly, dizziness, disorientation, and cranial nerve dysfunction occur. Nausea and vomiting are common. Low serum albumin and elevated serum creatinine may enhance central nervous system toxicity. As with cyclophosphamide, such side effects as alopecia, leukemogenesis, and infertility also occur. Cardiac toxicity is rare.

Potential Drug Interactions: Since ifosfamide is also metabolized by the P-450 system, one should remain alert for the same type of potential drug interactions that have been reported with cyclophosphamide. A recent report advises close monitoring of warfarin anticoagulant control in patients receiving ifosfamide/mesna.

Therapeutic Indications in Hematology: Ifosfamide was recently approved for treatment of patients with refractory testicular cancer. In hematologic malignancies its major indication is in the treatment of refractory lymphomas.

Melphalan (Melphalan)

Chemistry: Melphalan is synthesized from nitrogen mustard and phenylalanine. It is a bifunctional chloroethylating agent that forms DNA cross-links.

Absorption, Fate, and Excretion: The oral bioavailability of melphalan is quite variable, 2050% of the drug being excreted in the stool. Some patients show virtually no oral absorption. This fact is particularly pertinent in the treatment of myeloma patients, in whom a lack of response to melphal may be due simply to poor oral absorption. Melphalan has a half-life of approximately 90 minutes. It is extensively metabolized, with only about 1015% of an administered dose excreted unchanged in the urine.

Preparation and Administration: Melphalan is available in 2-mg tablets and in intravenous formulation for high-dose therapy.

Toxic Effects: The dose-limiting toxicity is myelosuppression, manifested by leukopenia and thrombocytopenia and generally occurring 23 weeks after therapy. Recovery may take 6 weeks, however, in patients who have been heavily pretreated

with chemotherapy drugs or radiotherapy, or both. Nausea, vomiting, and alopecia are uncommon side effects and are usually mild. Occasionally, amenorrhea and azoospermia, pulmonary fibrosis, dermatitis, and secondary malignancies (e.g., leukemia) occur, especially in patients receiving the drug over the long term. At cumulative doses of <600 mg, the incidence of second hematologic malignancy is probably <2%, but may be 15% at higher doses. Higher doses used in transplant patients result in gastrointestinal toxicity that is dose-limiting. At these doses, the syndrome of inappropriate secretion of antidiuretic hormone, pneumonitis, and hepatic veno-occlusive disease have been observed.

Potential Drug Interactions: Administration of high-dose intravenous melphalan with cyclosporine increases the risk of cyclosporine nephrotoxicity.

Therapeutic Indications: The major use of melphalan is for the treatment of multiple myeloma, either as a single agent or in combination with other alkylating agents and prednisone (e.g., the MP and VMCP regimens [CP, cyclophosphamide]). The intravenous formulation has been approved for isolated limb perfusion in melanoma. It is used in high-dose protocols for myeloma and solid tumors at doses of 140200 mg/m^2 .

Chlorambucil (Chlorambucil)

Chemistry: Chlorambucil is an aromatic derivative of mechlorethamine.

Absorption, Fate, and Excretion: Chlorambucil is well absorbed after oral administration. It is extensively metabolized in the liver to its major metabolite, phenylacetic acid mustard (PAAM), which also has bifunctional alkylating activity. The half-lives of chlorambucil and PAAM are 1.5 and 2.5 hours, respectively; <1% of either chlorambucil or PAAM is excreted in the urine.

Preparation and Administration: Chlorambucil is available as 2-mg tablets.

Toxic Effects: Treatment is usually well tolerated, with myelosuppression the dose-limiting toxic effect. Patients on a daily oral schedule should have biweekly CBCs. Nausea and vomiting are uncommon, but mild alopecia and skin rashes occasionally occur. As with the other alkylating agents, azoospermia (especially above cumulative dose of 400 mg), amenorrhea, and secondary leukemia are potential risks of prolonged therapy. Rare cases of pulmonary fibrosis have also been reported.

Potential Drug Interactions: None reported.

Therapeutic Indications in Hematology: The major uses are in the treatment of Waldenstroms macroglobulinemia, low-grade lymphomas, CLL, and Hodgkins disease. Except for CLL, its use has been supplanted by newer agents.

Busulfan (Myleran)

Chemistry: Busulfan is an alkylsulfonate bifunctional alkylating agent not chemically related to mechlorethamine. It forms DNA intrastrand and interstrand cross-links.

Absorption, Fate, and Excretion: Busulfan is well absorbed after oral administration. When given by the intravenous route, >90% is cleared from the plasma after 3

minutes. The drug is extensively metabolized to inactive compounds, which are excreted renally. The major metabolite is methane sulfonic acid, although >10 other not fully identified metabolites exist. Virtually no intact busulfan is found in the urine. The biologic half-life of busulfan is approximately 2.5 hours.

Preparation and Administration: The drug is commercially available as 2-mg tablets.

Toxic Effects: Although at low doses the major effect of busulfan is on the granulocytic series, at high doses all three hematologic series are affected. Compared with the other alkylating agents, its nadir of myelosuppression may be relatively late, in a range of 1130 days. Hematologic recovery is also prolonged and may take 54 days. A relatively common side effect is an addisonian-like syndrome characterized by skin hyperpigmentation and weakness, but without abnormalities in adrenal function. Cumulative pulmonary toxicity has been well described and consists of a mixed alveolar and interstitial pneumonitis. As with the other alkylating agents, infertility and leukemogenesis can occur. Nausea and vomiting are rare. At high doses it is associated with hepatic veno-occlusive disease in up to 19% of patients. Seizures may also occur and are controlled by diphenylhydantoin.

Potential Drug Interactions: A metabolic interaction may take place between busulfan and various anticonvulsant medications; however, further description of the specific effects is awaited.

Therapeutic Indications in Hematology: Busulfan is used mainly in the treatment of CML. More recently, high-dose busulfan has been incorporated into preparatory regimens for bone marrow transplantation. Blood level monitoring with adjustment for higher dose levels improved therapeutic outcome and reduced toxicity.

Carmustine (BCNU)

Chemistry: Carmustine, also called BCNU (1,3[bis]2-chloroethyl-nitrosourea), decomposes spontaneously into a chloroethyl hydroxide that can alkylate the DNA, and into an isocyanide molecule, which may produce carbamylation of proteins. Cytotoxicity is due to DNA cross-links.

Absorption, Fate, and Excretion: Intravenously administered carmustine is rapidly metabolized, with a half-life of 70 minutes. Approximately 3080% of metabolites are eliminated in the urine within 24 hours. The drug and/or its metabolites readily cross the bloodbrain barrier, resulting in cerebrospinal fluid concentrations within the range of 1570% of plasma levels. Peak serum levels vary widely in patients treated at 200600 mg/m ^{2} .

Preparation and Administration: Carmustine is available in 100-mg vials as a white lyophilized powder. The drug is reconstituted with 3 ml of absolute alcohol provided by the manufacturer and 27 ml of sterile water, and can be further diluted with normal saline or 5% dextrose in water. It should be used immediately after reconstitution and can be infused over 12 hours. Carmustine is chemically stable for 3 hours at room temperature and for 24 hours when refrigerated.

Toxic Effects: Myelosuppression is the dose-limiting toxic effect and tends to increase with successive cycles of therapy. Leukopenia and thrombocytopenia are characteristically delayed and reach their maximum between the third and sixth weeks after drug administration. Nausea and vomiting can be severe. Abnormal liver function tests may be found in 25% of patients, but the abnormalities are usually mild and reversible. Two rare but serious toxic effects include cumulative pulmonary/interstitial pneumonitis progressing to fibrosis and progressive renal damage, which are dose-related. Secondary leukemias can also occur 510 years after treatment. Patients who receive >1,100 mg/m^2 are at increased

risk of pulmonary fibrosis. Carmustine is not a vesicant, but rapid infusion often produces a burning sensation at the injection site.

Potential Drug Interactions: Cimetidine may enhance the myelosuppressive effect of carmustine. Carmustine may decrease the pharmacologic effects of phenytoin. In rats with intracerebrally implanted tumors, pretreatment with phenobarbital eliminated the antitumor activity of carmustine. The reduction in carmustine antitumor activity correlated with increased carmustine metabolism, which is apparently the result of hepatic microsomal enzyme induction.

Therapeutic Indications in Hematology: Carmustine in combination with other cytotoxic agents may be used in the initial treatment of Hodgkins disease (BCVPP regimen) and multiple myeloma (VBAP regimen). In high-dose therapy, it appears in BEP, for relapsed lymphomas.

Lomustine (CCNU)

Chemistry: Lomustine, also called CCNU, is a nitrosourea derivative with choloroethyl and cyclohexyl side chains.

Absorption, Fate, and Excretion: The drug is rapidly absorbed from the gastrointestinal tract and is rapidly and completely metabolized. Its active metabolites have prolonged plasma half-lives, within a range of 1648 hours. Approximately 50% of an administered dose is detectable (as metabolites) in the urine within 24 hours, and 75% is detectable within 4 days. Active metabolites cross the bloodbrain barrier and can be detected in significant concentrations in the cerebrospinal fluid.

Preparation and Administration: The drug is available in 10-, 40-, and 100-mg capsules.

Toxic Effects: The toxicity profile of lomustine is similar to that of carmustine. Since lomustine can produce vomiting, and the drug is given orally, special attention should be directed to emesis control. If the patient vomits soon after ingestion, the vomitus should be inspected for the presence of intact capsules. The drug should be given again if capsules are identified with certainty. Secondary leukemias are reported 310 years after use.

Potential Drug Interactions: Similar to those of carmustine.

Therapeutic Indications in Hematology: Lomustine is occasionally used as second-line treatment for patients with Hodgkins disease and non-Hodgkins lymphoma, and for childhood gliomas.

Streptozocin (Zanosar)

Chemistry: Streptozocin is a naturally occurring nitrosourea derived from *Streptomyces acromogenes.* The drug is a glucosamine-1-methyl-nitrosourea, which, unlike the other nitrosoureas, methylates DNA and is cytotoxic due to induced mismatch repair.

Absorption, Fate, and Excretion: After intravenous administration, the drug is rapidly metabolized, with no intact drug detectable in the plasma after 3 hours. Its half-life is 40 hours. Within the first 24 hours after administration, approximately 10% of the parent compound is excreted in the urine.

Preparation and Administration: The drug is available in 1-g vials and is reconstituted with either 9.5 ml of normal saline or 5% dextrose in water for injection to form a 100-mg/ml solution. Intravenous infusion of the drug over 3045 minutes usually prevents discomfort at the injection site. Patients should be kept well hydrated to preclude renal tubular toxicity.

Toxic Effects: Although nausea and vomiting have been considered by some investigators to be the limiting toxic effects, in most phase I trials nephrotoxicity was the principal dose-limiting effect. Nausea and vomiting are severe and require aggressive antiemetic support. Streptozocin may also aggravate duodenal ulcers. Renal toxicity frequently occurs and includes mild proteinuria, glycosuria, hypophosphatemia, renal tubular acidosis, and occasionally irreversible azotemia. Although the myelosuppressive effect of streptozocin is mild, it can potentiate the bone marrow suppression of other cytotoxic drugs. Slight increases in hepatic enzymes can also occur. Occasionally, patients (primarily those with insulinomas) may experience transient alterations in glucose metabolism.

Potential Drug Interactions: Streptozocin can potentiate the hyperglycemic effect of glucocorticosteroids. Phenytoin therapy decreases the cytotoxic effect of streptozocin on the pancreatic cells, leading to potential interference with its therapeutic effect in patients with pancreatic islet cell tumors. Steptozocin is a potent

renal toxin, and every effort should be made to avoid concomitant administration of other nephrotoxins.

Therapeutic Indications in Hematology: Streptozocin has been used in the initial treatment of Hodgkins disease and, less commonly, in non-Hodgins lymphomas.

Dacarbazine (DTIC)

Chemistry: Dacarbazine is also called DTIC [5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide]. After undergoing metabolic activation by microsomal enzymes in the liver, it acts primarily as an alkylating agent.

Absorption, Fate, and Excretion: After intravenous administration, the drug is extensively metabolized. Activated dacarbazine has an elimination half-life of 57 hours. Approximately 4050% of the parent drug is found in the urine within the first 24 hours after administration.

Preparation and Administration: Dacarbazine is available in 100- and 200-mg vials, which must be protected from light and stored at 28°C. The drug is reconstituted with normal saline or sterile water to produce a 10-mg/ml solution. It can be administered as a slow intravenous push or by infusion over 1530 minutes.

Toxic Effects: Myelosuppression, primarily represented by leukopenia, is the dose-limiting toxic effect. Use of the drug leads to considerable problems with emesis and requires aggressive antiemetic support. A flulike syndrome consisting of fever, malaise, and myalgias may occur. Direct sunlight during the first 2 days after drug administration may result in facial flushing, facial paresthesias, and light-headedness. Hepatotoxicity and diarrhea have also been reported. Pain along the injection site can occur if the drug is rapidly infused, but can usually be lessened by prolonging the infusion rate. Secondary leukemias are reported 310 years after use.

Potential Drug Interactions: Dacarbazine activation may be enhanced by phenytoin or phenobarbital, although the clinical significance of this potential interaction remains uncertain. There may be a potential, as yet poorly characterized drug interaction with levodopa whereby the response to levodopa is diminished.

Therapeutic Indications in Hematology: Dacarbazine is used primarily in the treatment of Hodgkins disease as part of the ABVD (doxorubicin [Adriamycin], bleomycin, vinblastine, and dacarbazine) regimen, and for melanoma.

APPENDIX: Clinical Pharmacology of Antimicrotubule Agents

Vincristine (Oncovin) and Vinblastine (Velban)

Chemistry and Mechanism of Action: Both vincristine and vinblastine are asymmetric dimeric compounds that bind to the protein tubulin at a site distinct from that for the taxanes. At low concentrations, vincristine and vinblastine inhibit microtubule dynamics. At higher concentrations, they disrupt microtubules that constitute the mitotic spindle, resulting in metaphase arrest. They are relatively M phase-specific. Due to their lipophilicity, vinca alkaloids are rapidly taken into cells and achieve several hundred-fold higher intracellular than extracellular concentrations. Overexpression of the multidrug resistance transporters P-glycoprotein or MRP can reduce the intracellular accumulation, while alterations in the or tubulins can affect drugtarget interaction for vinca alkaloids.

Absorption, Fate, and Excretion: After intravenous injection, both drugs are rapidly distributed to the body tissues, especially the red blood cells and platelets. Their elimination follows a triphasic pattern: the elimination half-lives are < 5 minutes; , 50155 minutes; and , 2085 hours. Both vinca alkaloids are primarily eliminated through the liver into the bile and feces, making patients with obstructive liver disease more susceptible to toxic effects. A 50% reduction in the dose is recommended for serum bilirubin concentrations of 1.53.5 mg/dl. Dose modification for renal dysfunction is not indicated. After brief intravenous bolus administration, peak plasma vincristine concentrations of 100400 M are achieved, which decline to <10 M in 24 hours. Continuous infusion doses of 1.0 mg/m ^{2} /day produce vincristine plasma concentrations ranging from 1 to 10 nM.

Preparation and Administration: Vincristine is available in 1-, 2-, and 5-mg vials. Each milliliter contains 1 mg vincristine sulfate, 100 mg mannitol, 1.3 mg methylparaben, and 0.2 mg propylparaben. Vincristine is a powerful vesicant that should be administered only intravenously into a freely running infusion of normal saline or dextrous solution. If the drug is given by continuous infusion, it must be infused through a central intravenous line. In case of extravasation, infusion should be discontinued and any residual drug aspirated through the line. The manufacturer also recommends infiltrating the area with 12 ml of hyaluronidase, 150 U/ml, and then applying warm compresses for 72 hours to facilitate dispersion of the drug. Vinblastine is commercially available as a lyophilized powder and a 1 mg/ml solution in 10-mg vials. The lyophilized drug is reconstituted by adding sodium chloride for injection (which may be preserved with either phenol or benzyl alcohol) to the 10-mg vial. Administration of vinblastine should follow the same guidelines described for vincristine.

Toxic Effects: Vincristines dose-limiting toxic effect is neurotoxicity, which appears to be related to its relative polarity. Peripheral neurotoxicity is usually manifested by sensory impairment, decreased deep tendon reflexes, and paresthesias. Less commonly, severe painful dysesthesias, ataxia, foot drop, and cranial nerve palsy (e.g., affecting the extraocular and laryngeal muscles) can occur. Autonomic neurotoxicities include constipation, abdominal cramps, and ileus, which may be prevented by use of mild laxatives. Alopecia occurs frequently, but myelosuppressive effects are minimal. Rare side effects include inappropriate secretion of antidiuretic hormone and ischemic cardiac toxicity. Vinblastines dose-limiting toxic effect is myelosuppression, with leukopenia more pronounced than thrombocytopenia. Anemia is uncommon. Neurotoxicity can also occur but is significantly less common than with vincristine. Vinblastine is also a vesicant.

Potential Drug Interactions: Both vinca alkaloids have been reported to increase the accumulation of methotrexate and etoposide in tumor cells. Acute shortness of breath and bronchospasm can occur when vincristine or vinblastine is given in conjunction with mitomycin C. Since asparaginase may impair the hepatic clearance of vincristine, it is preferable to give the latter 1224 hours before L-asparaginase administration. Vincristine may decrease the absorption and plasma levels of orally administered drugs such as digoxin. Dilantin may increase the cytotoxicity of vincristine in multidrug-resistant tumor cells; however, this remains to be demonstrated in the clinic. When concurrently administered, erythromycin may increase the toxicity of vinca alkaloids, especially vinblastine.

Therapeutic Indications in Hematology: The vinca alkaloids are among the most important drugs in the treatment of hematologic malignancies. They have a broad spectrum of activity and are often incorporated into many chemotherapy regimens used in the treatment of ALL, Hodgkins disease, non-Hodgkins lymphomas, CLL, and multiple myeloma.

Vinorelbine (Navelbine)

Chemistry and Mechanism of Action: Vinorelbine is a semisynthetic derivative of vinblastine (5-nor-hydrovinblastine) with an eight-member catharanthine ring. Similar to other vinca alkaloids, it also binds to tubulin, inhibits microtubule assembly, and produces a mitotic arrest of cells. This occurs at concentrations that relatively spare axonal microtubules, which may reduce neurotoxicity.

Absorption, Fate, and Excretion: Short (610 minutes) intravenous infusions of 30 mg/m^{2} produce peak plasma concentrations of approximately 1.0 g/ml with a triphase decay. Rapid (<5 minutes) and (49168 minutes) half-lives result in a rapid decline in the plasma concentration in the first hour post-treatment, followed by a prolonged terminal half-life of 1849 hours, reflecting slow efflux from the peripheral compartment. The volume of distribution at steady state is 2075.6 L/kg. The drug is extensively bound to platelets, lymphocytes, and plasma proteins. The major site of metabolism is the liver, with 3380% of the drug excretion in feces and approximately 20% in urine.

Preparation and Administration: Vinorelbine is available for injection in single use as 10 mg/ml in 1-ml or 5-ml vials without preservatives. The calculated dose is diluted to 1.53.5 mg/ml for a slow injection (610 minutes) by a syringe with 5% dextrose or 0.9% saline, or between 0.5 and 2.0 mg/ml in an intravenous bag. Since vinorelbine is a strong vesicant, it should be administered through a freely flowing intravenous access, avoiding all extravasation.

Toxic Effects: Vinorelbine shares many of the principal toxicities of vinblastine. Myelosuppression is dose-limiting but not cumulative, with nadirs occurring 710 days after administration. Anemia and thrombocytopenia occur infrequently. Because of lower affinity for axonal versus spindle microtubules, neurotoxicity is less prominent with vinorelbine.

Mild to moderate peripheral neuropathy and constipation occur in approximately 30% of patients, and the incidence of neuropathy increases with the duration of treatment. Mild to moderate nausea and vomiting is seen in one third of patients. Stomatitis and diarrhea are less frequent. Transient elevations of transaminases have been reported. Among the miscellaneous side effects noted are chest pain with or without electrocardiographic changes (6%; most with underlying cardiac disease), as well as bronchospasm and dyspnea (5%). Alopecia is seen in 10% of patients.

Therapeutic Indications in Hematology: Objective responses have been observed in approximately one third of patients with Hodgkins or non-Hodgkins

lymphomas.

Paclitaxel (Taxol) and Docetaxel (Taxotere)

Chemistry and Mechanism of Action: Both paclitaxel and docetaxel are complex diterpene alkaloid esters consisting of a taxane system linked to an oxetane ring and a C-13 side chain that is necessary for their cytotoxic effects in mammalian cells. After binding to the N-terminal 31 amino acids of the tubulin subunit in the tubulin oligomers or polymers, these taxanes kinetically stabilize microtubule dynamics at plus ends. They also decrease the lag time and shift the equilibrium toward tubulin polymerization into microtubule bundles. The disequilibrium of tubulin-microtubule polymerization results in mitotic arrest and apoptosis of cells. Taxane-induced mitotic arrest is associated with phosphorylation of Bcl-2 protein and increased intracellular levels of free Bax protein, which promote apoptosis. Compared to paclitaxel, docetaxel demonstrates 1.9-fold greater affinity for tubulin binding sites and greater potency in mediating Bcl-2 phosphorylation.

Absorption, Fate, and Excretion: Taxanes generally are administered by intravenous infusion lasting over 3, 24, or 96 hours (paclitaxel) or 1 hour (docetaxel). Depending on the dose and schedule, peak plasma concentrations of paclitaxel range between 0.0515.0 M. Its steady state volume of distribution ranges between 48 and 182 L/m^2 , with rapid uptake in almost all tissues except the central nervous system and 98% plasma protein binding. Plasma decay for paclitaxel is biphasic, with and half-lives of 0.34 and 5.8 hours, respectively. Saturable distribution and elimination appear to be responsible for paclitaxels nonlinear pharmacokinetics. This means that paclitaxel dose escalation in shorter schedules may result in disproportionate increases in area under the concentration time curve and peak plasma concentration. It is metabolized to 6 hydroxy paclitaxel by the CYP3A isoform of the PU50 mixed function oxidases in the hepatic microsomes. Total fecal and urinary excretion of paclitaxel and its metabolites is approximately 70% and 10%, respectively. While dose modification is not necessary for renal insufficiency, a 50% reduction in dose is recommended even for moderate hyperbilirubinemia or significant elevations in hepatocellular enzymes. When administered as a 1-hour intravenous infusion, docetaxel has linear pharmacokinetics that fit a three-compartment model. Similar to paclitaxel, docetaxel also has a high clearance rate (0.36 L/h), steady state volume of distribution (67.3 L/m^2), and terminal half-life of 12 hours. Docetaxel also has high protein binding (97%) and extensive tissue distribution. The drug or its metabolites also have high fecal (80%) and low urinary elimination (5%). Metabolism of docetaxel also primarily occurs in hepatic microsomal P450 mixed function oxidases, CYP3A, CYP2B, and CYP1A.

Preparation and Administration: Paclitaxel is available as a 30-mg/5-ml single-dose vial in polyoxyethylated castor oil (Cremophor EL) 50% and dehydrated alcohol, USP 50%. The contents of the vial must be diluted before use. Docetaxel for injection is available as a concentrate in polysorbate 80 in two vial contents (23.6 mg/0.59 ml or 94.4 mg/2.36 ml), along with the appropriate diluent (1.83 ml or 7.33 ml) in separate vials. Adding diluent that is 13% (w/w) ethanol in water for injection to the concentrate produces a final premix concentration of 10 mg docetaxel/ml. The required amount of premix is transferred by a calibrated syringe into 0.9% saline or 5% dextrose to produce a final concentration of 0.3 to 0.9 mg/ml. The intravenous infusion is administered over 1 hour.

Toxic Effects: Hypersensitivity reaction (HSR) was noted in up to 30% of patients in the early phase I studies. HSR occurs early in the first or second infusion and may be caused by vehicle, Cremophor EL, or paclitaxel itself. HSR consists of dyspnea, bronchospasm, urticaria, and hypotension. Most HSRs regress completely after stopping the infusion and treatment with antihistamines, fluids, and vasopressors. Prolonged infusion (3 hours) and premedication (dexamethasone 20 mg orally, 12 and 6 hours diphenhydramine 30 mg, and ranitidine 150 mg IV 30 minutes before treatment) have reduced the incidence of major HSRs to <3%. Patients with a history of HSR may be rechallenged with paclitaxel at a markedly slower infusion rate, 20 mg dexamethasone IV every 6 hours for 4 doses before treatment. Although not formulated in Cremophor EL, HSRs can occur in up to 25% of patients receiving docetaxel. Most HSRs are minor, consisting of flushing, chest tightness, and low back pain. Premedication with dexamethasone 8 mg PO BID for 3 days, starting a day before treatment with docetaxel, considerably reduces the incidence of HSRs and fluid retention.

Neutropenia is the main toxicity of paclitaxel and docetaxel, but it is not cumulative. With higher doses of paclitaxel (250 mg/m^2 over 24 hours), this can be ameliorated with subsequent administration of G-CSF. Severe thrombocytopenia and anemia are rare. Symmetric, distal, peripheral sensory neuropathy is usually seen with higher doses or multiple doses of paclitaxel. This often limits chronic use of paclitaxel. Diffuse areflexia and neuronopathy are less commonly seen. Higher doses can also cause motor and autonomic neuropathy as well as myalgias, especially in patients with pre-existing neuropathy or when paclitaxel is used with cisplatin. Severe peripheral neuropathy or myalgias are less common after repetitive docetaxel at 100 mg/m^2 . Cardiac rhythm abnormalities, especially bradyarrhythmias and (rarely) heart blocks, have been reported secondary to paclitaxel treatment. A direct causal link between paclitaxel and myocardial ischemic episodes and tachyarrhythmias has not been established. Although noted, a direct link has also not been established between the occurrence of cardiac conductance abnormalities or ischemia and docetaxel treatment. Nausea, vomiting, diarrhea, and stomatitis are uncommon and generally mild to moderate. Alopecia is universal with both drugs. Skin toxicity is more severe and common with docetaxel. It is characterized by an erythematous pruritic maculopapular rash affecting forearms and hands. Onychodystrophy with discoloration, ridging, and brittleness of fingernails also occurs. Docetaxel can cause cumulative fluid retention resulting in peripheral edema, third space fluid collection, and weight gain, which usually resolves slowly after stopping docetaxel. Concurrent treatment with dexamethasone, as noted above, delays the onset and decreases the incidence of these side effects.

Potential Drug Interactions: When paclitaxel infusion (24-hour) is administered following cisplatin, there is a 33% reduction in the clearance rate of paclitaxel. This produces suboptimal antitumor cytotoxicity and more profound neutropenia. Hence, the sequence of paclitaxel followed by

cisplatin is commonly recommended. The use of carboplatin following paclitaxel has been reported to cause less thrombocytopenia than carboplatin alone. Mucositis is more pronounced when paclitaxel is used before doxorubicin, a sequence that reduces the clearance of doxorubicin. Hematologic toxicity is more prominent with the sequence of cyclophosphamide followed by paclitaxel, as compared to the reverse sequence of administration. Anticonvulsants such as phenytoin and phenobarbital induce the metabolism of paclitaxel and docetaxel by the p450 mixed function oxidases. Conversely, in vitro studies have shown that inhibitors of the p450 system can interfere with the metabolism of both drugs. These inhibitors include erythromycin, testosterone, ketoconazole, and fluconazole.

Therapeutic Indications in Hematology: Both paclitaxel and docetaxel have significant activity against previously treated patients with non-Hodgkins lymphomas. Paclitaxel is also very active against HIV-associated Kaposis sarcoma.

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APPENDIX: Clinical Pharmacology of Antimetabolites

Cytosine Arabinoside

Chemistry and Mechanism of Action: Cytosine arabinoside (1--D-arabinofuranosylcytosine; Ara-C) is a nucleoside analog that differs from its naturally occurring counterpart (2-deoxycytidine) by virtue of the presence of a hydryoxyl group in the 2- configuration. The altered reactivity of the resulting arabinosyl sugar moiety confers on Ara-C its cytotoxic activity. Ara-C enters the cell by a facilitated nucleoside diffusion mechanism, and is converted to its nucleoside monophosphate form, Ara-CMP, by the pyrimdine salvage pathway enzyme, deoxycytidine kinase. This represents the rate-limiting step in Ara-C metabolism. Ara-C may also be catabolized intracellularly to an inactive form, Ara-U, by the enzyme cytidine deaminase. Ara-C is ultimately converted to its lethal triphosphate derivative, Ara-CTP, by a mono- and diphosphate kinase. Ara-CTP is an inhibitor of DNA polymerases , , and , and is also incorporated into replicating DNA strands, leading to inhibition of chain initiation and elongation, and premature chain termination. The extent of incorporation of Ara-C into DNA closely correlates with lethality in leukemic cells. Although Ara-C is generally thought of as a prototypical S-phase-specific agent, its ability to interfere with DNA repair polymerases (e.g., and) as well as lipid biosynthetic enzymes may account for lethal effects in noncycling cells.

Absorption, Fate, and Excretion: Following intravenous administration, Ara-C is rapidly deaminated to an inactive form, Ara-U, by cytidine deaminase. This enzyme is present in the plasma, liver, and kidney, but is present at very low levels in the central nervous system. The initial plasma half-life of Ara-C has been estimated to be 1012 minutes. Approximately 90% of the administered Ara-C dose is excreted by the kidney as Ara-U or other inactive metabolites. The terminal half-life of Ara-C is 23 hours. Central nervous system Ara-C levels following a 2-hour infusion approximate 50% of plasma concentrations. Steady-state plasma concentrations following standard dose therapy (e.g., 100200 mg/m^2 /d as a continuous infusion) approximate 10^{-7} 10^{-6} M. When Ara-C is given as a highdose bolus infusion (e.g., 13 gm/m^2 over 13 hours), plasma levels as high as 100 M can be achieved.

Preparation and Administration: Ara-C is provided as a sterile, lyophilized powder for reconstitution in vials containing 100 mg, 200 mg, 1 g, or 2 g of material. The powder is reconstituted with sterile bacteriostatic water for injection with benzyl alcohol (0.945%) added as a preservative. When reconstituted in this way, solutions are stable for up to 48 hours under controlled temperatures (e.g., 1530°C). Material reconstituted without preservative should be used immediately. For intrathecal injection, Ara-C should be reconstituted in a diluent that does not contain preservative (e.g., preservative-free 0.9% sodium chloride, USP) and used immediately.

Toxic Effects: Ara-C is primarily toxic to rapidly dividing tissues; consequently, myelosuppression and gastrointestinal toxicity represent the major side effects of this agent. Patients receiving Ara-C regularly experience leukopenia, anemia, and thrombocytopenia, with nadirs appearing 714 days after drug administration. Gastrointestinal toxicity includes nausea and vomiting, abdominal pain, mucositis, and a chemical hepatitis characterized by elevation of liver function enzymes. The latter is generally reversible. Patients receiving Ara-C as a high-dose bolus infusion (e.g., 13 g/m^2 /d q12h × 612 doses) experience, in addition to the standard toxicities, several unique ones. These include alopecia, an exfoliative dermatitis, a chemical conjunctivitis (generally ameliorated by the prophylactic administration of a steroid or saline ophthalmic solution), a respiratory distress-like syndrome (characterized by the appearance of rales, an abnormal radiogram, and pulmonary insufficiency), and cerebellar toxicity. The latter, which is characterized by nystagmus, ataxia, and other cerebellar signs, may be irreversible, and its appearance mandates discontinuation of therapy. Intrathecal administration of Ara-C has been rarely associated with toxicities described for methotrexate.

Potential Drug Interactions: None reported.

Therapeutic Indications in Hematology: Ara-C represents a mainstay in the treatment of acute myelogenous leukemia (e.g., as part of the 7 and 3 regimen in which it is given in conjunction with daunorubicin). It is also incorporated into some induction regimens for acute lymphoblastic leukemia. High-dose Ara-C (HIDAC), either alone or in combination with anthracycline antibiotics, is frequently employed in the treatment of refractory or relapsed AML or ALL. HIDAC has also been used in some salvage regimens for non-Hodgkins lymphoma (e.g., ESHAP). Chronic low-dose Ara-C has been used in the treatment of myelodysplastic syndrome.

Methotrexate

Chemistry and Mechanism of Action: Methotrexate (N-4-[[2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic acid) represents a member of a class of compounds referred to as antifolates. Methotrexate is a potent inhibitor of dihydrofolate reductase, an enzyme responsible for the reduction of dihydrofolates to tetrahydrofolates. The latter are required in 1-carbon transfer reactions involved in

de novo purine and pyrimidine biosynthesis, including conversion of deoxyuridylate (dUMP) to thymidylate (dTMP) by thymidylate synthase. As in the case of most antimetabolites, methotrexate is primarily active against S-phase cells. Methotrexate is transported across cell membranes by an energy-dependent, temperature-sensitive, concentration-dependent process involving folate binding proteins, after which it is polyglutamylated by the enzyme folylpolyglutamyl synthetase (FPGS). Polyglutamylation of methotrexate enhances its intracellular retention and in some studies has been shown to correlate with the sensitivity of leukemia cells to this agent. The mechanism by which methotrexate kills cells may stem from interference with DNA synthesis (leading to a thymine-less death) secondary to DHFR inhibition; disruption of purine biosynthesis; or a combination of these actions. The lethal actions of methotrexate may be reversed by reduced folates such as 5-formyltetrahydrofolate (leucovorin). The possibility that tumor cells may exhibit impaired transport of such reduced folates serves as the basis for strategies involving administration of high-dose methotrexate in conjunction with leucovorin rescue.

Absorption, Fate, and Excretion: In adults, oral absorption is dose-dependent, with mean bioavailability approximating 60% at doses 30 mg/m^2 . At higher doses (e.g., 80 mg/m^2), bioavailability is less. Peak plasma concentrations occur 12 hours following oral administration. Methotrexate bioavailability approximates 100% for parenteral routes of administration; with these routes, peak plasma methotrexate levels are achieved 3060 minutes after administration. For each route, the steady state volume of distribution ranges from 40% to 80% of body weight. Methotrexate tends to accumulate in third space fluids (e.g., ascites, pleural effusions) and can result in prolonged release and accompanying toxicity. Consequently, it is generally not advisable for patients with fluid accumulations to receive methotrexate. Methotrexate competes with reduced folates for transport across cell membranes; however, at high doses (e.g., 100 mg/m^2), passive diffusion is the primary mechanism through which intracellular accumulation occurs. Methotrexate is approximately 50% protein bound, and does not penetrate the CNS barrier when administered orally or parenterally at conventional doses. However, when given by the intrathecal route, high CNS levels are achieved. Administration of high-dose methotrexate with leucovorin rescue can also result in therapeutic CNS levels.

The primary route of excretion is renal, with 8090% of the drug appearing unchanged in the urine within 24 hours following intravenous administration. The terminal half-life of methotrexate is 410 hours for patients receiving low-dose therapy and 815 hours for those receiving high-dose therapy. Because of the primary renal rate

of excretion and the possibility of nephrotoxicity, methotrexate should be withheld or administered at reduced doses in patients with impaired renal function. Patients receiving high-dose methotrexate therapy should be hydrated and their urine alkalinized before administration to reduce the risks of toxicity.

Preparation and Administration: Methotrexate is available in multiple formulations: (a) tablets, containing 2.5 mg methotrexate and inactive ingredients (lactose, magnesium stearate, and pregelatinized starch); (b) methotrexate sodium injection, available in 25-, 50-, and 250-mg vials, containing benzyl alcohol as a preservative, sodium chloride, and water for injection (preservative-containing solutions should not be used for intrathecal or high-dose administration); (c) methotrexate sodium injection without preservative, which can be used for intravenous, intra-arterial, intrathecal, and high-dose administration; (d) lyophilized powder, which is provided in 20-mg vials and is reconstituted with preservative-free sodium chloride or 5% dextrose in water to a final concentration <25 mg/ml.

For intrathecal administration, solutions of 11.5 mg/ml should be prepared using preservative-free 0.9% sodium chloride as the diluent. For high-dose therapy, leucovorin rescue is required to prevent significant toxicity. Leucovorin is administered 1224 hours after the methotrexate at a dose of 1525 mg IV, IM, or PO q6h until the methotrexate dose declines to levels $<5 \times 10^{7}$ M. For patients receiving intermediate- or high-dose methotrexate (e.g., 500 mg/m^2), serum methotrexate and creatinine levels should be monitored at 24-hour intervals. If, after 48 hours, serum methotrexate levels are $>5 \times 10^{7}$ M but $<1 \times 10^{6}$ M, leucovorin is continued at a dose of 25 mg/m^2 q6h × 8 doses until methotrexate levels decline to $<5 \times 10^{7}$ M. If levels are $>1 \times 10^{6}$ M but $<2 \times 10^{6}$ M at 48 hours, the dose of leucovorin is increased to 100 mg/m^2 q6h × 8 doses. For methotrexate levels 2×10^{6} M at 48 hours, the dose of leucovorin is 200 mg/m^2 q6h × 8 doses.

Toxic Effects: Methotrexate primarily exhibits its toxic effects toward proliferating tissues. Consequently, dose-limiting toxicities include bone marrow suppression (leukopenia, thrombocytopenia, anemia), mucositis, and diarrhea. High-dose therapy is occasionally accompanied by transient elevations in liver function tests, whereas chronic low-dose therapy is more often associated with hepatic fibrosis. Standard-dose therapy is rarely associated with nephrotoxicity, but acute renal failure can be seen with high-dose therapy secondary to deposition of 7-OH methotrexate in the renal tubules. The risk of methotrexate nephrotoxicity is significantly reduced by ensuring adequate hydration and alkalinization of the urine. Other reported toxicities include a maculopapular rash, and an idiosyncratic pulmonary toxicity characterized by cough, fever, dyspnea, hypoxia, and interstitial infiltrates.

A necrotizing leukoencephalopathy has been reported in patients receiving methotrexate who have had prior cranial irradiation. Intrathecal methotrexate has been associated with several toxicities, including: (a) chemical arachnoiditis; (b) motor paralysis accompanied by cranial nerve dysfunction, seizures, and coma; (c) chronic demyelinating syndrome. Each of these may be exacerbated by prior craniospinal irradiation.

Potential Drug Interactions: Methotrexate exhibits many potential drug interactions related to plasma protein binding. For example, many compounds are known to displace methotrexate from serum albumin, potentially increasing its bioavailability. These agents include sulfonamides, salicylates, tetracyclines, chloramphenicol, and phenytoin. However, the clinical implications of such interactions are not clear. Nonsteroidal anti-inflammatory drugs should not be administered in conjunction with methotrexate when the latter is given at intermediate/high doses, due to the potential for elevation and prolongation of methotrexate plasma concentrations. Penicillins can reduce renal clearance for methotrexate and should be used with caution in this setting. Probenecid may also reduce renal transport of methotrexate. Administration of methotrexate can also reduce the clearance of theophyllines, and concomitant use of these agents requires careful monitoring. Increases in methotrexate toxicity have been observed in some patients receiving trimethoprim/sulfamethoxazole, possibly as a consequence of enhanced antifolate effects. Administration of folates in vitamin preparations may reduce the efficacy of methotrexate by bypassing DHFR inhibition. Methotrexate may increase the toxicity (and potentially the activity) of various antineoplastic agents in a schedule-dependent manner (e.g., when given prior to 5-FU).

Therapeutic Indications in Hematology: Methotrexate is widely employed in the treatment of acute lymphoblastic leukemia, particularly in the maintenance phase. Methotrexate is frequently administered intrathecally in patients with CNS leukemia, and prophylactically in certain patients with ALL. It also represents a component of various multidrug regimens used in the treatment of non-Hodgkin lymphoma (e.g., M-BACOD, PROMACE-CYTABOM).

Hydroxyurea

Chemistry and Mechanism of Action: Hydroxyurea is an inhibitor of the ribonucleotide reductase system that catalyzes the rate-limiting step in the de novo biosynthesis of purine and pyrimidine deoxyribonucleotides (i.e., the conversion of ribonucleotide diphosphates to their deoxyribonucleoside diphosphate derivatives). Ribonucleotide reductase consists of two subunits: a binding and allosteric effector component and an iron-binding catalytic component. Hydroxyurea binds to and inactivates the catalytic subunit of the enzyme. Like most antimetabolites, hydroxyurea is an S-phase-specific agent and block cells n the G_1/S phase of the cell cycle. Exposure of cells to hydroxyurea leads to a depletion of deoxyribonucleotide triphosphate (dNTP) pools, the extent of which correlates with DNA synthesis inhibition and cell death. Two consequences of hydroxyurea administration include potentiation of the metabolism/cytotoxicity of nucleoside analog (e.g., Ara-C) as a result of dNTP pool depletion, and elimination of amplified genes present extrasomally in double minute chromosomes.

Absorption, Fate, and Excretion: Hydroxyurea is generally administered orally, although intravenous regimens are being investigated. The drug is readily absorbed from the gastrointestinal tract, with peak plasma levels as high as 2.0 mM occurring approximately 2 hours after oral administration. Serum concentrations decline to undetectable levels after 24 hours. The drug is primarily excreted via the renal route, with 7580% of the drug appearing in the urine at 12 hours. The drug penetrates the cerebrospinal fluid, although it has not been established that therapeutic levels are achieved following standard oral administration.

Preparation and Administration: Hydroxyurea is provided as 500-mg capsules. Drug is stored at room temperature in tightly capped containers and protected from heat.

Toxic Effects: The most common adverse reactions include myelosuppression (leukopenia, thrombocytopenia, anemia), gastrointestinal symptoms (e.g., nausea and vomiting, stomatitis, anorexia, appetite disturbances), and dermatologic toxicity, such as rashes, skin ulcerations, and facial erythema. Rarer toxicities, generally seen at high doses, include neurologic disturbances, such as drowsiness, dizziness, headache, and convulsions, altered renal function, and alopecia. The mutagenic potential of hydroxyurea is unknown, and the drug should be avoided when possible in pregnant women.

Potential Drug Interactions: As noted previously, hydroxyurea may increase the toxicity of certain nucleoside analogs. Hydroxyurea may also serve as a radiosensitizing agent; consequently, patients receiving concurrent radiation therapy may experience enhanced toxicity.

Therapeutic Indications in Hematology: Hydroxyurea has become, along with interferon , the mainstay of therapy in patients with chronic or accelerated phase CML. It is currently recommended for initial therapy of CML over busulfan, and may prolong the chronic phase of this disease compared to the latter agent. Hydroxyurea has also been successfully used in the treatment of other myeloproliferative disorders, including myeloid metaplasia and myelofibrosis, polycythemia vera, and essential thrombocytosis. Its leukemogenic potential is uncertain, however, and it should be used with caution, particularly in younger patients. Hydroxyurea has also been shown to reduce the incidence of painful crises in individuals with sickle cell anemia in a subset of patients, a phenomenon that may result from increases in red blood cell fetal hemoglobin levels.

Fludarabine

Chemistry and Mechanism of Action: Fludarabine phosphate is a fluorinated derivative of the nucleotide analog 9--D-arabinofuranosyladenine (Ara-A) that is resistant to deamination by the degradative enzyme cytidine deaminase. It is converted intracellularly to its triphosphate derivative, which inhibits ribonucleotide reductase, as well as DNA polymerase and DNA primase. Fluoro-Ara-ATP is also incorporated into DNA, a process that appears to be essential for the induction of apoptosis in leukemic cells. Fludarabine is toxic to S-phase cells, but its ability to interfere with DNA repair may contribute to lethality in their noncycling counterparts.

Absorption, Fate, and Excretion: Following intravenous injection, fludarabine phosphate is rapidly deaminated (i.e., within minutes) in the plasma to its nucleoside derivative, 2-fluoro-Ara-A, which is then converted intracellularly to its nucleotide form, 2-fluoro-Ara-AMP by the pyrimidine salvage pathway enzyme deoxycytidine kinase. The half-life of 2-fluoro-Ara-A is approximately 10 hours; the primary mode of elimination is renal, with 25% of the total dose appearing in the urine as unchanged 2-fluoro-Ara-A. Total body clearance of fludarabine is inversely correlated with serum creatinine.

Preparation and Administration: Fludarabine is supplied as a sterile powder in 50-mg vials containing 50 mg of mannitol and sodium hydroxide to adjust the pH to

7.7. Material is reconstituted in 2 ml of sterile water to yield a 25 mg/ml solution for injection. The material may be stored atg 4°C; because the reconstituted solution contains no antimicrobial preservative, drug should be administered within 8 hours of formulation.

Toxic Effects: The most common dose-limiting toxicity is myelosuppression (neutropenia, thrombocytopenia, and anemia). Other toxicities include fever, chills, infection, nausea, and vomiting. Rarer toxicities include malaise, fatigue, anorexia, and weakness. Patients with CLL receiving fludarabine have experienced serious opportunistic infections and tumor lysis syndrome. The most serious toxicity of fludarabine when administered at high doses (>40 mg/m ^{2} /d × 5 days) is irreversible neurotoxicity, including cortical blindness, necrotizing leukoencephalopathy, and death. This phenomenon has rarely if ever been seen in patients receiving conventional doses (e.g., 25 mg/m^2 /d × 5 days). Rare reports of interstitial pneumonitis have appeared.

Potential Drug Interactions: Fludarabine has been shown to potentiate the intracellular metabolism and activity of Ara-C, although the toxicity of this combination may also be enhanced. No other interactions have been reported.

Therapeutic Indications in Hematology: Fludarabine has shown marked activity in both untreated CLL and in disease refractory to standard alkylating agent therapy. Fludarabine has also shown activity as a single agent, and particularly in combination with others (e.g., mitoxantrone, cytoxan) in indolent non-Hodgkin lymphoma.

2-Chlorodeoxyadenosine

Chemistry and Mechanism of Action: 2-Chlorodeoxyadenosine (CdA; cladribine) is a derivative of deoxyadenosine that differs from its parent compound by the presence of a chlorine moiety at the 2-position of the purine ring. It is transported intracellularly by facilitated nucleoside diffusion and phosphorylated by the pyrimidine salvage pathway enzyme deoxycytidine kinase. CdA is relatively resistant to deamination by cytidine deaminase. CdA is readily converted to its triphosphate derivative, 2-chlorodeoxyadenosine-5-triphosphate, particularly in cells of lymphoid origin, and is incorporated into tumor cell DNA by DNA polymerase . CdATP is also an effective inhibitor of ribonucleotide reductase, which may contribute to lethal effects. CdA induces cell death (apoptosis) in both cycling and noncycling cells, possibly by promoting DNA fragmentation and by depleting cells of ATP and/or NAD.

Absorption, Fate, and Excretion: Relatively little pharmacokinetic information concerning 2-chlorodeoxyadenosine is available. The drug is most commonly administered as a 7-day continuous infusion or as a 2-hour infusion over 5 days. Bioavailability of CdA following subcutaneous administration approximates that of the intravenous route but is less than that after oral administration. Renal excretion appears to be the major route of elimination. When given as a 2-hour infusion, CdA has a relatively long terminal half-life (e.g., 6 hours), and plasma concentrations following such a schedule may approximate those associated with the continuous infusion. Spinal fluid levels are approximately 25% of plamsa concentrations.

Preparation and Administration: For daily infusions, CdA is diluted under sterile conditions in bags containing 500 ml of 0.9% sodium chloride injection, USP. The use of 5% dextrose solutions is not recommended due to enhanced degradation of the drug. For preparation of longer infusions (e.g., 7 days) the use of bacteriostatic sodium chloride injection, USP, is recommended. Once prepared, solutions should be stored refrigerated at 48°C for 8 hours before administration.

Toxic Effects: The major toxicity is myleosuppression, which is primarily observed following intermittent rather than continuous infusion. Other toxicities include fever, generally beginning several days after initiation of therapy, and increased susceptibility to opportunistic infections. Rare side effects include nausea and hepatic and renal toxicity.

Potential Drug Interactions: None reported.

Therapeutic Indications in Hematology: CdA has shown significant activity in CLL and hairy cell leukemia. However, response rates in the former disorder appear to be somewhat less than those obtained with fludarabine; moreover, patients who have progressed on fludarabine therapy infrequently respond to CdA. Other diseases in which CdA has shown activity include non-Hodgkin lymphoma and Waldenstrom macroglobulinemia.

2-Deoxycoformycin (pentostatin; dCF)

Chemistry and Mechanism of Action: dCF is an adenosine analog that is a highly effective inhibitor of the purine biosynthetic enzyme adenosine deaminase (ADA). It is transported across cell membranes by facilitated nucleoside diffusion, where it binds tightly to ADA. Inhibition of ADA results in accumulation of deoxyadenosine metabolites, most notably dATP. dATP exerts its toxic effects through inhibition of ribonucleotide reductase and induction of global imbalances in deoxyribonucleotide triphosphate pools. These result in interference with DNA synthesis and repair. dCF is particularly toxic to certain lymphoid cells with low levels of ADA activity. It is also toxic to both cycling and resting cells; the mechanism underlying its cytotoxicity toward quiescent cells is unknown.

Absorption, Fate, and Excretion: After intravenous injection of dCF, the plasma clearance follows a biphasic pattern with a terminal elimination half-life of 315 hours. Protein binding is limited. The drug is only partially metabolized, with approximately 6080% of the drug appearing unchanged in the urine after 24 hours. The total body clearance correlates well with creatine clearance. Patients with impaired renal function may require reductions in the dCF dose.

Preparation and Administration: dCF is unstable when reconstituted in solutions of pH <5.0. Consequently, it is customarily reconstituted in normal saline. dCF is provided in vials containing 10 mg of drug, 50 mg of mannitol, and sodium hydroxide to adjust the pH to 7.0. It is administered as an intravenous infusion over 2030 minutes. Hydration is recommended before and after administration.

Toxic Effects: The major toxicities include myelosuppression, nausea and vomiting, immunosuppression, acute renal failure, keratoconjunctivitis, fever, and elevations of liver function enzymes. At high doses, neurologic toxicity, including somnolence, seizures, and coma, has been reported, although these are seen infrequently in patients receiving standard-dose therapy. When administered at such doses (e.g., 4 mg/m ^{2} biweekly), side effects are relatively minor.

Potential Drug Interactions: dCF may augment the toxicity of Ara-A (vidarabine) as a consequence of inhibition of ADA.

Therapeutic Indications in Hematology: dCF is primarily used in the treatment of hairy cell leukemia, where response rates of up to 90% have been reported, even in patients refractory to other therapy, including interferon. Activity has also been reported in other lymphoid malignancies, such as T-cell lymphoma, CLL, prolymphocytic leukemia, and Waldenstrom macroglobulinemia, although its precise role in the treatment of these disorders remains to be fully evaluated.

6-Thioguanine (6-TG)

Chemistry and Mechanism of Action: 6-TG is a guanine analog in which the 6-hydroxyl group is replaced by a sulfhydryl group. It interferes with de novo purine biosynthesis at multiple levels. Following transport across the cell membrane by facilitated diffusion, 6-TG competes with hypoxanthine and guanine for phosphorylation by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and is converted to its nucleotide form, 6-thioguanylic acid (TGMP), which accumulates within cells. TGMP inhibits several purine biosynthetic enzymes, including glutamine-5-phosphoribosylpyrophosphate amidotransferase and IMP dehydrogenase. 6-TG nucleotides are also incorporated in DNA and RNA, where they function as fraudulent bases. It is unknown which of these actions (interference with purine interconversions, blockade of de novo purine biosynthesis, or nucleic acid incorporation) is primarily responsible for 6-TG cytotoxicity, although DNA incorporation appears to play a significant role. 6-TG is considered to be an S-phase-specific agent.

Absorption, Fate, and Excretion: Following oral administration, the bioavailability of 6-TG is variable, ranging from 14 to 46% of the administered dose (mean = 30%). Peak plasma levels are achieved 8 hours after administration and decline slowly thereafter. The average plasma disappearance of 6-TG is 80 minutes, with a range of 25240 minutes. Relatively little unchanged material appears in the urine; the major

excreted product is the methylated derivative, 2-amino-6-methyl thiopurine. Spinal fluid penetrance after parenteral administration is minimal.

Preparation and Administration: 6-TG is available in tablet form for oral administration. Each tablet contains 40 mg of 6-TG and inactive ingredients including gum acacia, lactose, magnesium stearate, potato starch, and stearic acid. Intravenous preparations are available only in experimental settings.

Toxic Effects: The major dose-limiting toxicity of 6-TG is myelosuppression. Other less common toxicities include gastrointestinal disturbances (nausea and vomiting, anorexia, diarrhea), jaundice, and elevated liver function tests.

Potential Drug Interactions: In contrast to 6-mercaptopurine, the metabolism of 6-TG is not modified by allopurinol, and consequently dose adjustments do not have to be made when these agents are administered concurrently.

Therapeutic Indications in Hematology: The primary indication for 6-TG is in the treatment of AML, generally in conjunction with other agents (e.g., daunorubicin and Ara-C). However, it has not been firmly established that addition of 6-TG to such regimens improves therapeutic efficacy. 6-TG also has activity in CML, although it has been supplanted by other agents (e.g., hydroxyurea, interferon) in this disorder.

6-Mercaptopurine (6-MP)

Chemistry and Mechanism of Action: 6-MP (1,7-dihydro-6*H*-purine 6-thione monohydrate; purinethol) is an analog of the purine bases adenine and hypoxanthine. It is both an antineoplastic and immunosuppressive agent. Like 6-TG, 6-MP and its metabolites act at multiple levels to interfere with purine biosynthesis and interconversions. It competes with hypoxanthine and guanine for HGPRTase, and, following conversion to thioinosinic acid (TIMP), blocks conversion of IMP to xanthylic acid and IMP to AMP. Both TIMP and another metabolite, 6-methylthioinosinate (MTIMP), inhibit glutamine-5-phosphoribosylpyrophosphate aminotransferase. 6-MP is also incorporated into RNA and DNA, thereby functioning as a fraudulent base. It is unknown which of these actions is primarily responsible for the lethal actions of 6-MP, although available evidence points to DNA incorporation as a prime determinant of cytotoxicity.

Fate, Absorption, and Excretion: Following oral administration, the bioavailability of 6-MP is highly variable, presumably due to interpatient differences in gastrointestinal absorption, which averages 50% of the administered dose. Extensive catabolism by hepatic xanthine oxidase also contributes to drug elimination. Approximately 50% of the administered 6-MP or its metabolites is recovered in the urine. The volume of distribution generally exceeds the total body water. Following intravenous administration, the plasma disappearance half-life was 47 minutes in adults. Plasma protein binding is modest (19%), and spinal fluid penetrance is minimal.

Preparation and Administration: 6-MP is supplied as tablets for oral administration. Each tablet contains 50 mg of 6-MP and the inactive ingredients corn and potato starch, lactose, magnesium stearate, and stearic acid. An intravenous preparation containing 500 mg of 6-MP per vial is available for investigational use.

Toxic Effects: The major dose-limiting toxicity of 6-MP is myelosuppression. This is dose-related and is manifested by leukopenia, thrombocytopenia, and anemia. The hematologic effects of 6-MP may be delayed, so it is important to withdraw the medication temporarily at the first sign of unusual hematologic toxicity. Individuals with an inherited disorder of thiopurine methyltransferase deficiency may be particularly susceptible to 6-MP-mediated hematopoietic suppression. Other toxicities include hepatotoxicity (elevated liver function tests, cholestasis, hepatic necrosis, ascites), nausea, vomiting, mucositis, fever, rash, and diarrhea. The hepatotoxicity, which occurs in 1040% of patients, requires close monitoring and discontinuation of therapy until recovery occurs. Patients receiving 6-MP uniformly experience immunosuppression.

Potential Drug Interactions: Allopurinol, an inhibitor of xanthine oxidase, significantly reduces the catabolism of 6-MP when the latter is given by the oral route, leading to major increases in plasma concentrations. Allopurinol does not alter the pharmacokinetics of intravenous 6-MP, presumably because of the absence of first-pass metabolism of 6-MP when administered by this route. When administered in conjunction with allopurinol, the dose of 6-MP should be reduced by 1/3 to 1/4. Increased toxicity has been reported in patients receiving concurrent 6-MP and trimethoprim-sulfamethoxazole. 6-MP may also modify the effects of warfarin.

Therapeutic Indications in Hematology: The major use for 6-MP is in the maintenance phase of treatment for ALL. 6-MP has also been used in the treatment of patients with immune thrombocytopenic purpura (ITP) or autoimmune hemolytic anemia refractory to all other forms of therapy.

5-Azacytidine

Chemistry and Mechanism of Action: 5-Azacytidine is an analog of the nucleoside cytidine, differing from the parent compound by virtue of the presence of a nitrogen at the 5 position of the heterocyclic ring. 5-Azacytidine is transported across the cell membrane by facilitated nucleoside diffusion and is converted to its nucleotide monophosphate form, 5-aza-CMP, by the pyrimidine salvage pathway enzyme uridine-cytidine kinase. 5-Azacytidine is also a substrate for the degradative enzyme cytidine deaminase. It is ultimately converted to its lethal derivative, 5-aza-CTP, which is incorporated into RNA and to a lesser extent DNA. The lethal actions of 5-azacytidine are believed to result from its ability to interfere with protein synthesis through disruption of RNA processing. The chemical instability of the 5-azacytidine ring structure is also thought to contribute to the cytotoxicity of this compound.

Absorption, Fate, and Excretion: The drug distributes into a volume corresponding to the total body water after intravenous administration, and is also well absorbed following subcutaneous injection. It is extensively deaminated in the plasma and liver and displays minimal plasma binding. Peak plasma concentrations following intravenous injection approximate 1.0 mM. The initial half-life of 5-azacytidine (and/or its metabolites) is approximately 4 hours, although the drug is rapidly converted to various derivatives within minutes of administration. There is minimal spinal fluid penetrance.

Preparation and Administration: 5-Azacytidine is an investigational agent provided by the National Cancer Institute as a group C drug for patients with refractory AML. It is supplied in 100-mg vials and is reconstituted in 19.9 ml of sterile water for injection. When further diluted to a concentration of 0.22 mg/ml in normal saline, 5% dextrose in water, or lactated Ringers solution, 10% of the drug undergoes decomposition within 3 hours. Consequently, the drug should be administered immediately after reconstitution and not stored. When administered as a continuous infusion, fresh solutions should be prepared every 34 hours.

Toxic Effects: The major toxicities of 5-azacytidine have been leukopenia and to a lesser extent thrombocytopenia. Nausea and vomiting, often refractory to standard antiemetic therapy, have also been encountered, most frequently in patients receiving bolus infusions. Gastrointestinal toxicity is ameliorated by administering 5-azacytidine as a continuous infusion. Other potential side effects include diarrhea, fever, hepatotoxicity (most frequently in patients with pre-existing hepatic disease), neuromuscular toxicity, rash, and hypotension.

Potential Drug Interactions: None reported.

Therapeutic Indications in Hematology: 5-Azacytidine is primarily used in the treatment of refractory AML, with response rates ranging from 17 to 30% when used as a single agent. 5-Azacytidine has also yielded clinical responses in a subset of patients with the myelodysplastic syndrome when administered as a low-dose continuous infusion. In early trials, low-dose 5-azacytidine has increased fetal hemoglobin levels in some patients with sickle cell anemia and thalassemia.

Gemcitabine

Chemistry and Mechanism of Action: Gemcitabine (2,2-difluorocytidine monohydrochloride) is a nucleoside analog that differs from 2-deoxycytidine by virtue of the presence of fluorine atoms in the 2 and 2 positions of the cytidine ring. It is transported across the cell membrane by facilitated nucleoside diffusion, phosphorylated by deoxycytine kinase, and ultimately converted to its lethal metabolites, dFdCDP and dFdCTP. The diphosphate form (dFdCDP) inhibits ribonucleotide reductase,

leading to disruption of dNTP pools and resultant interference with DNA synthesis and repair. The triphosphate form (dFdCTP) competes with dCTP for incorporation into DNA. Reductions in dCTP pools (secondary to ribonucleotide reductase inhibition) result in self-potentiation of gemcitabine action. Incorporation of gemcitabine into DNA in S phase inhibits elongation of the replicating strand, leading to DNA chain termination. The lethal actions of gemcitabine in leukemia cells have been related to the induction of apoptosis and are not restricted to cells actively engaged in DNA synthesis. Gemcitabine has been shown to be considerably more potent in inducing apoptosis in cultured human leukemia cells than Ara-C.

Absorption, Fate, and Excretion: In studies involving intravenously administered labeled gemcitabine, up to 98% of the drug was recovered in the urine after 1 week. The excreted dose was composed of a minor fraction (gemcitabine; <10%) and inactive metabolites (e.g., 2-deoxy-2,2-difluoruridine). Plasma protein binding was minimal. In studies involving both short and long gemcitabine infusions, pharmacokinetics were found to be linear and best described by a two-compartment model. Plasma half-life and clearance are influenced by both age and gender. For short infusions, half-lives varied from 3294 minutes; for longer infusions, half-lives varied from 245638 minutes. Volume of distribution was 50 L/m^2 for short infusions and 370 L/m^2 for long infusions.

Preparation and Administration: Vials of gemcitabine contain 200 mg or 1 g of the HCl derivative formulated with mannitol (200 mg or 1 g) and sodium acetate (12.5 or 62.5 mg) as a sterile lyophilized powder. HCl or NaOH has been used for pH adjustment.

Toxic Effects: The major dose-limiting toxicity of gemcitabine is myelosuppression, although anemia and thrombocytopenia have also been encountered. Other toxicities include nausea and vomiting, transient elevations in liver function tests, mild hematuria, proteinuria (and in rare cases hemolytic uremia syndrome), fever, rash, dyspnea, edema, and a flulike syndrome. Other infrequent toxicities include alopecia, paresthesias, and bronchospasm.

Potential Drug Interactions: Gemcitabine may function as a radiosensitizing agent and can increase the toxicity of ionizing radiation. No other interactions are known.

Indications in Hematology: The primary indication for gemcitabine is in the treatment of pancreatic carcinoma. However, as an experimental agent, gemcitabine is being evaluated for the treatment of acute leukemia and chronic lymphocytic leukemia.

Arabinofuranosylguanine (Ara-G)

Ara-G is an experimental purine analog that was initially the object of interest due to its resistance to phosphorylysis by purine nucleoside phosphorylase. Its lethal actions stem from formation of Ara-GTP, incorporation of this metabolite into DNA, and interference with DNA synthesis. For reasons that are not yet clear, T cells appear to be particularly sensitive to this agent, a phenomenon that may result from preferential accumulation of the lethal metabolite, Ara-GTP. In a recent clinical trial involving a water-soluble pro-drug (GW506U) in patients with leukemia, cells from patients with T-cell ALL preferentially accumulated Ara-GTP. Phase II studies of Ara-G are ongoing in patients with T-cell ALL and other T-cell malignancies.

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APPENDIX: Topoisomerase I and II Inhibitors

TOPOISOMERASE II INHIBITORS
Etoposide (Vepesid), Etoposide Phosphate (Etopophos), Teniposide (Vumon)

Chemistry and Mechanism of Action: *Etoposide* (VP-16), etoposide phosphate, and teniposide (VM-26) are semisynthetic derivatives of epipodophyllotoxin. The mechanism of action of these drugs appears to be related to their ability to stabilize a topoisomerase II/DNA cleavable complex, which acts as a replication fork barrier and leads to the generation of irreversible DNA damage and cell death in proliferating cells.

Absorption, Fate, and Excretion: Etoposide has an oral bioavailability of 2575%. Its terminal half-life is 68 hours, with approximately 3040% excreted in the urine, two thirds as unchanged drug. There is no accumulation with consecutive daily administration, but cytotoxicity has strict schedule dependency. Clinical studies suggest that in patients with a plasma creatinine >130 mol/L, the etoposide dose should be reduced by 25%.

Etoposide phosphate is rapidly and completely converted in vivo to VP-16 by the activity of phosphatase and has been shown to have the same pharmacokinetics as VP-16. Due to

its increased water solubility, etoposide phosphate can be given intravenously in much less volume. In addition, the metabolic acidosis and hypotension seen with the infusion of VP-16 are not seen with this pro-drug.

Teniposide has a multiphasic pattern of clearance from plasma with a terminal half-life of 9.521 hours. Unlike those of etoposide, metabolites of teniposide account for 80% of the drug excreted in the urine. Like etoposide, there is significant interpatient and intrapatient variation in clinical pharmacokinetics. There are currently no formal recommendations for dose modification in patients with renal insufficiency.

Preparation and Administration: *Etoposide* is available as 50-mg capsules and in vials of 50 and 100 mg at a concentration of 20 mg/ml. When the drug is diluted with normal saline or 5% dextrose in water to a concentration of 0.2 or 0.4 mg/ml, it is stable for 96 or 48 hours, respectively. Etoposide must be administered slowly over 30 minutes to prevent hypotension.

Etoposide phosphate is available in single-dose vials containing etoposide phosphate equivalent to 100 mg of etoposide. When it is diluted with water, 5% dextrose, or normal saline to a concentration of 1020 mg/ml, it can be administered without dilution over 510 minutes. Etoposide phosphate when reconstituted is stable for 24 hours at room temperature or under refrigeration.

Teniposide is supplied in 50-mg vials for intravenous use only. The intravenous solution may be taken orally but is unpalatable. Currently, no oral preparation is available on the market; however, for investigational purposes each 50-mg vial may be dissolved in 50100 ml of syrup or juice. A single oral dose of 60 mg/m ^{2} is advised to achieve optimal absorption. It may be repeated at 6-hour intervals. As with etoposide, rapid infusion can produce hypotension.

Toxic Side Effects: Myelosuppression, especially leukopenia, is the dose-limiting toxic effect of *etoposide* and *teniposide*. Nausea and vomiting are usually mild and easily prevented with antiemetics. Rapid infusion of etoposide (<30 minutes) may cause hypotension. Anaphylactoid reactions (e.g., bronchospasm) occur in 2% of patients and may be related to the cremaphor vehicle. Alopecia occurs in approximately 20% of patients treated with etoposide; this side effect is more common with teniposide. When the drug is given in high doses as a component of a preparative regimen for bone marrow transplantation, mucositis and diarrhea are prominent and may be dose-limiting.

Potential Drug Interactions: Theoretically, any drug that increases the S-phase fraction will increase the cytotoxicity of epipodophyllotoxins and other topoisomerase inhibitors. Conversely, drugs that inhibit DNA synthesis antagonize the effect of *etoposide* and *teniposide* (e.g., 5-fluoro-2-deoxyuridine given before etoposide in some human cancer cell lines decreases the cytotoxicity of the latter). More recent in vitro data suggest that synergistic cytotoxic effects are seen when VP-16 is given after a topoisomerase I inhibitor, which appears to upregulate the amount of topoisomerase II enzyme. Antagonistic effects have been reported when a topoisomerase II inhibitor is given before a topoisomerase I inhibitor. In hematology, etoposide and teniposide may inhibit the intracellular Ara-CTP formation, leading to reduced cytosine arabinoside cytotoxicity. Potentiation of teniposide activity has been seen with methotrexate and dipyridamoles. There is at least a twofold increase in the clearance of teniposide with concomitant administration of phenobarbital or phenytoin. Cyclosporine and other P-glycoprotein antagonists (PSC 833) potentiate the cytotoxic effects of etoposide.

Therapeutic Indications:*Etoposide* is employed in the treatment of non-Hodgkin lymphomas and as a second-line treatment for Hodgkin disease. It is also incorporated in the preparatory regimens for bone marrow transplantation of refractory lymphomas (CBV) and acute leukemia. *Teniposide* has been approved as a front-line agent with combination chemotherapy for childhood ALL. Combination chemotherapy with teniposide has been used successfully in some cases of refractory adult ALL and acute monocytic leukemia, but duration of remission is not significantly different from that with other standard salvage regimens. In non-Hodgkin lymphoma, teniposide has shown comparable activity to vincristine. *Etoposide phosphate* has been given in both standard-dose and high-dose (as a single agent) chemotherapy regimens and appears to have the same pharmacokinetics and antitumor activity as VP-16.

Daunorubicin

Chemistry and Mechanism of Action: Daunorubicin is an anthracycline that inhibits DNA topoisomerase II, acting as a poison at lower concentrations and a suppressor of cleavable complex formation at higher doses. Daunorubicin is also a DNA intercalator and generates reactive oxygen intermediates.

Absorption, Fate, and Excretion: After intravenous injection, daunorubicin undergoes rapid tissue uptake and concentration. It is rapidly metabolized in the liver, where approximately 25% of the drug concentrates and has a half-life of 2050 hours. The principal metabolite is daunorubicinol, which also displays antineoplastic activity. Biliary excretion accounts for approximately 75% of the drug and metabolite elimination. Patients with significant hepatic dysfunction should receive an attenuated dose of daunorubicin.

Preparation and Administration: Daunorubicin is supplied with 100 mg of mannitol in 20-mg vials, from which it is reconstituted with 4 ml of sterile water for injection. The vial should be protected from sunlight. Daunorubicin is a powerful vesicant that should be administered into the tubing of a freely flowing intravenous infusion of

either 5% dextrose in water or normal saline. In the event of extravasation, as much infiltrated drug as possible should be aspirated from the tissue, and cold compresses should be maintained on the site for several hours. Despite these measures, skin grafting may be necessary. Daunorubicin is not physically compatible with heparin, and the two drugs should not be coadministered in the same intravenous tubing. The patient should be informed that daunorubicin may impart a red color to the urine for up to 72 hours after administration.

Toxic Effects: Myelosuppression, predominantly leukopenia, is the dose-limiting toxic effect. Mucositis, nausea and vomiting, and alopecia are common. Facial flushing, conjunctivitis, and lacrimation may occur in rare cases. Erythematous streaking near the site of injection occurs as a benign local allergic reaction and should not be confused with extravasation. The drug can produce a severe local reaction (e.g., pneumonitis, esophagitis) in previously irradiated areas, even when both therapies are not administered concomitantly (radiation recall).

Cardiac toxicity is a unique characteristic of the anthracycline antibiotics and can be acute or chronic. In the acute form, abnormal EKG changes such as ST-T wave elevation and arrhythmias may be seen. Transient reduction in the ejection fraction can also occur acutely and is often associated with pericarditis (pericarditis/myocarditis syndrome). The chronic form of anthracycline cardiac toxicity is related to the cumulative dose. The dose limit of doxorubicin is generally considered to be 450500 mg/m^2 , where the risk of clinical cardiotoxicity is 110%. The corresponding cumulative dose limit for daunorubicin is 9001000 mg/m ^{2} . The cardiac

toxicity is clinically characterized by congestive heart failure, usually refractory to medical therapy. Cardiac irradiation or the administration of cyclophosphamide may increase the risk of cardiotoxicity. The cardiotoxic effects appear to be related to the formation of free radicals and not to the inhibition of DNA topoisomerase II. The cardioprotective agent dexrazoxane (Zinecard) is now available and recommended to be started at a doxorubicin cumulative dose >350 mg/m ^{2} .

Potential Drug Interactions: Daunorubicin is not physically compatible with heparin or dexamethasone. The drug interactions described for doxorubicin (see below) probably occur with daunorubicin as well.

Therapeutic Indications in Hematology: Daunorubicin is used in combination with other drugs in the treatment of AML and ALL.

Doxorubicin (Adriamycin)

Chemistry and Mechanism of Action: Doxorubicin is also an anthracycline glycoside antibiotic. It differs from daunorubicin at C-8, where a hydroxyacetyl group replaces an acetyl group. Because of this, doxorubicin is also called hydroxyl daunorubicin. Its mechanisms of action also involve stabilizing DNAtopoisomerase II complexes, DNA intercalation, and free radical formation.

Absorption, Fate, and Excretion: Doxorubicin has a triphasic plasma clearance with a half-life of approximately 30 hours. The drug is extensively metabolized in the liver to yield an active metabolite (doxorubicinol) and a number of inactive metabolites (aglycones). Within 7 days, 50% of an injected dose is excreted in the bile, but only 510% of the drug is excreted in the urine. Penetration into the cerebrospinal fluid is poor.

Preparation and Administration: Doxorubicin is available in 10-, 20-, 50-, 150-, and 200-mg vials. The lyophilized powder is reconstituted with either normal saline or sterile water for injection to yield a 2-mg/ml solution. The reconstituted solution must be protected from sunlight. The drug should be injected slowly into the tubing of freely running intravenous infusion of normal saline or 5% dextrose in water. Erythematous streaking along the vein is often an indication that the administration rate is too rapid. The drug is a powerful vesicant, and in case of extravasation the measures described for daunorubicin should be followed.

Toxic Effects: The toxic effects are similar to those of daunorubicin. Weekly low-dose regimens or administration by continuous infusion can decrease the risk of cardiotoxicity with doxorubicin.

Potential Drug Interactions: When used in combination with other drugs as treatment for leukemia or lymphoma, doxorubicin may decrease the oral bioavailability of digoxin. It is not physically compatible with heparin or 5-fluorouracil. Barbiturates may increase the plasma clearance of doxorubicin and decrease its cytotoxic effect. Doxorubicin is compatible with vincristine, and the two drugs can be administered together in the same intravenous solution.

Therapeutic Indications in Hematology: Doxorubicin is one of the most important drugs in the treatment of hematologic malignancies. It is used in the treatment of Hodgkin disease (ABVD regimen), non-Hodgkin lymphomas (CHOP, MACOP-B), and multiple myeloma (VBAP, VAD).

Idarubicin (Idamycin)

Chemistry and Mechanism of Action: Idarubicin, also called 4-demethoxydaunorubicin (4-DMDR), is an analogue of daunorubicin in which the methoxy group from the aglycone has been replaced with hydrogen. Idarubicin is also a topoisomerase II inhibitor and generates free radicals.

Absorption, Fate, and Excretion: The elimination of half-life of the parent compound is 11.3 hours, and that of the primary metabolite, 13-epirubicinol, is 4060 hours. The major metabolite is as active as idarubicin. The oral bioavailability of this drug is approximately 30%. Eighty percent of the drug is excreted in the urine as 13-epirubicinol.

Preparation and Administration: Idarubicin is supplied in 5- and 10-mg vials, from which it is reconstituted with sterile water or normal saline to obtain a 1-mg/ml solution. The drug should be infused over 1015 minutes through the tubing of a freely running intravenous infusion. Extravasation precautions should be instituted during administration. The oral formulation remains investigational.

Toxic Effects: The side effects of idarubicin are similar to those of daunorubicin and doxorubicin but are of lesser intensity at equal myelosuppressive doses.

Potential Drug Interactions: None reported.

Therapeutic Indications in Hematology: Idarubicin in combination with cytosine arabinoside is equivalent if not superior to combination chemotherapy with daunorubicin in the treatment of adult AML and myelodysplastic syndromes. Idarubicin has been approved for use in combination therapy for adult AML.

Mitoxantrone (Novantrone)

Chemistry and Mechanism of Action: Mitoxantrone is a synthetic anthracenedione. Its mechanism of action appears to involve primarily the inhibition of DNA topoisomerase II. Its reduced potential for free radical formation may explain the decreased cardiotoxicity of this drug.

Absorption, Fate, and Excretion: Mitoxantrone is excreted via the renal and hepatobiliary systems, but hepatobiliary elimination accounts for approximately 30% of active drug elimination and appears to be of greater importance. The half-life is quite variable, with a range of 2342 hours. Patients with severe hepatic dysfunction have been shown to eliminate the drug more slowly.

Preparation and Administration: Mitoxantrone is available as a 2-mg/ml solution in 10-, 12.5-, and 15-ml vials (20, 25, and 30 mg per vial, respectively). The drug is further diluted in normal saline or 5% dextrose in water for injection and is administered over 1530 minutes into the tubing of a freely running intravenous infusion. As with the anthracyclines, erythema or streaking along the vein of infusion indicates that the drug is being infused too rapidly. Although mitoxantrone is not a vesicant, there have been rare reports of tissue necrosis following extravasation.

Toxic Effects: Myelosuppression, principally leukopenia, is the dose-limiting toxic effect. Thrombocytopenia is relatively mild. Nausea, vomiting, and alopecia are usually mild and occur in <30% of patients treated. Rarely, mucositis and elevation of liver enzymes occur. The drug imparts a blue color to the urine of patients

treated. One of the primary advantages of mitoxantrone, in comparison with doxorubicin, is its much-reduced incidence of cardiac toxicity. Occasionally patients develop congestive heart failure after treatment with mitoxantrone in the absence of prior anthracycline exposure, although the incidence appears to be <5%.

Potential Drug Interactions: None reported.

Therapeutic Indications in Hematology: Mitoxantrone is approved for induction therapy of AML in adults.

Amsacrine (AMSA)

Chemistry and Mechanism of Action: Amsacrine, or 4-(9-acridinylamino) methanesulfon-m-anisidide (AMSA), is a synthetic aminoacridine derivative. Amsacrine is a DNA intercalator and also inhibits the activity of DNA topoisomerase II.

Absorption, Fate, and Excretion: When given intravenously, this drug has an initial half-life of 30 minutes and a terminal half-life of 7.9 hours. It is 50% protein bound after 2 hours. It is metabolized by conjugation with glutathione, and approximately 50% of the drug is eliminated in the bile. The remainder of the drug is eliminated via the urinary route as metabolites and parent drug.

Preparation and Administration: Amsacrine is an investigational agent supplied by the National Cancer Institute as a group C drug. It is provided in a dual pack containing two sterile liquids that must be combined prior to use. One vial contains 1.5 ml of 50 mg/ml of AMSA in anhydrous N, N-dimethylactamide, and the other contains 13.5 ml of 0.0353 ml lactic acid diluent. When these are combined, the resulting orange-red solution contains 5 mg/ml of AMSA. Because of the N, N-dimethylactamide solvent, plastic syringes should not be used with the undiluted AMSA solution.

Toxic Effects: The dose-limiting toxic effect is myelosuppression, predominantly affecting granulocytes. Alopecia is common, and nausea, vomiting, and mucositis can occur. Cardiotoxicity, manifested as a decrease in ejection fraction, acute arrhythmias, or EKG changes, was reported in 2.3% of 3,200 patients, but most of these patients had been heavily pretreated with anthracyclines. Hypokalemia seems to enhance amsacrine cardiotoxicity and if present should be corrected prior to administration of the drug.

Potential Drug Interactions: The reconstituted solution is physically incompatible with chloride-containing solutions.

Therapeutic Indications in Hematology: Amsacrine is a group C investigational drug approved for the treatment of refractory AML, although it is being evaluated in combination with other cytotoxic agents in the initial treatment of this disease. As a group C drug, amsacrine must be administered as a single agent.

TOPOISOMERASE I INHIBITORS
Topotecan (Hycamtin)

Chemistry and Mechanism of Action: Topotecan is a semisynthetic derivative of camptothecin that stabilizes a complex between DNA topoisomerase I and DNA. The cytotoxic effect of this drug is believed to result from the collision of DNA replication forks with a ternary complex of topoisomerase I, DNA, and topotecan. The resulting double-strand DNA breaks are lethal. The lactone form of topotecan, which predominates at an acidic pH, is a much more potent inhibitor of DNA topoisomerase I.

Absorption, Fate, and Excretion: At neutral or physiologic pH, the carboxylate form of topotecan is favored, and at a pH <7 the lactone form is favored. Topotecan has been given as a bolus or by continuous infusion. In <1 hour after an infusion, most of the circulating drug in the plasma is in the carboxylate form due to the physiologic pH. The terminal half-life of the lactone form of this S-phase-specific agent is 2.6 hours; the terminal half-life of the total drug is 3.3 hours. Thirty-six percent of an intravenous dose is excreted unchanged in the urine, and there is a 1.5-fold concentration of the drug in bile. CSF levels of topotecan lactone reach approximately 32% of plasma levels. Dose adjustment is required for a creatinine clearance <60 ml/min, but no adjustment is necessary for a bilirubin up to 10 mg/dl.

Preparation and Administration: Topotecan is available in 4-mg vials reconstituted with 4 ml of sterile water. This solution can be further diluted in normal saline or 5% dextrose in water and should be used immediately.

Toxic Effects: The dose-limiting toxicity for topotecan for all schedules is neutropenia. Thrombocytopenia and anemia are less common, although there is an increase in thrombocytopenia with continuous infusion schedules. Other less common and mild toxicities include nausea, vomiting, diarrhea, fever, fatigue, alopecia, skin rash, and increased liver function tests. Mucositis has been seen with prolonged infusion schedules over 5 days or when topotecan is given in higher doses.

Potential Drug Interactions: In vitro data suggest that there may be some synergism if a topoisomerase I inhibitor is given before a topoisomerase II inhibitor. In vitro data also suggest that synergism may be seen if a topoisomerase I inhibitor (topotecan) is given after an alkylating agent, suggesting that topoisomerase I may be involved in the repair of alkylator-induced DNA damage.

Therapeutic Indications in Hematology: Phase II studies suggest that topotecan has activity in myelodysplastic syndromes, AML, and multiple myeloma.

Irinotecan (Camptosar or CPT-11)

Chemistry and Mechanism of Action: CPT-11 is a pro-drug that has a bulky piperidino side chain at C-10 that is cleaved in vivo by carboxylesterase converting enzyme to generate SN-38. SN-38 is approximately 1,000-fold more potent a topoisomerase I inhibitor than CPT-11. The lactone forms of both SN-38 and CPT-11 are more potent inhibitors of topoisomerase I than the carboxylate forms, which is thought to be the mechanism of action of these drugs as described for topotecan.

Absorption, Fate, and Excretion: The terminal half-life of the lactone form of CPT-11 is 7 hours and of the total drug 10.5 hours. The terminal half-life of SN-38 lactone is 8.7 hours and that of the total drug is 14.7 hours. Twenty-two percent of a dose of irinotecan is excreted unchanged in the urine. SN-38 is excreted into the bile and can undergo glucuronidation. The plasma protein binding of CPT-11 is reported to be 3068% and that of SN-38 is 95%.

Preparation and Administration: Irinotecan is available as a 100-mg single-dose vial with 20 mg/ml irinotecan. This preparation also contains 45 mg of sorbitol and 0.9 mg lactic acid per ml with the pH adjusted to 3.5. This solution can be diluted with 5% dextrose in water (preferred) or in normal saline to a final concentration of 0.11.2 mg/ml. The solution is stable for up to 24 hours at room temperature or 48 hours when refrigerated. The dose should be modified for severe diarrhea.

Toxic Effects: The major toxic effect of irinotecan is diarrhea. This can be early-onset diarrhea occurring within hours of administration or during the infusion that can be associated with cramping, vomiting, flushing, and diaphoresis. These side effects are due to the cholinergic effects of CPT-11 and can be managed with atropine. Severe later-onset diarrhea can be treated with high-dose loperamide, which has been found to decrease the incidence of grade 4 diarrhea from 20% to 2%. Diarrhea has been found to be the dose-limiting toxicity when irinotecan is given on a weekly schedule, and neutropenia when the drug is given every 3 weeks. Also seen are alopecia, nausea, vomiting, mucositis, fatigue, increased liver function tests, and rare cases of pulmonary toxicity.

Potential Drug Interactions: As described for topotecan, in vitro data suggest some synergism when topoisomerase I inhibitors precede topoisomerase II inhibitors or follow alkylating agent administration.

Therapeutic Indications in Hematology: Phase I and II studies have shown responses in refractory leukemia and lymphoma.

APPENDIX: Clinical Pharmacology of Platinum Analogs

Cisplatin (Platinol)

Chemistry and Mechanism of Action: Cisplatin [cisdiamminedichloroplatinum(II)] is an inorganic heavy metal complex. This complex can have cis- and trans-isomers; the cis-isomer is the active antitumor drug. In the relatively higher chloride concentrations of plasma, cisplatin is uncharged in the dichloro form and passes through plamsa membranes. Intracellularly, the low chloride concentrations allow the displacement of the chloride ligands by water to form the positively charged aquated complex. This forms covalent cross-links between two nucleophilic atoms of macromolecules such as the N7 positions of guanine and adenine in DNA. The cytotoxicity of cisplatin correlates closely with total platinum binding to DNA, to interstrand cross-links, and to the formation of intrastrand bidentate N7 adducts at d(GpG) and d(ApG), resulting in intrastrand cross-links that bend the DNA helix and inhibit DNA synthesis. Cisplatin damage to DNA induces apoptosis of sensitive cells.

Absorption, Fate, and Excretion: Following its intravenous injection, the drug concentrates in the liver, kidneys, and bowel. Plasma levels of cisplatin decay in a biphasic manner, with an initial half-life of 2549 minutes and a terminal half-life of 5873 hours. Although 15% of the administered cisplatin is excreted unchanged in the urine, up to 90% of the administered dose of the drug can be recovered from urine.

Preparation and Administration: Cisplatin is available as a lyophilized powder, supplied in 10- and 50-mg vials also containing mannitol, sodium chloride, and hydrochloric acid, and as an aqueous solution in 50- and 100-mg vials. Reconstitution of the powder for injection is achieved by adding sterile water to make a 1-mg/ml solution. The reconstituted solution should be further diluted in normal saline (usually 500 ml to 1 L) and administered over 13 hours. To prevent nephrotoxic effects, 2550 g of mannitol is often added to the saline solution, and patients are aggressively hydrated before and after cisplatin infusion. Magnesium sulfate (1224 mEq) is commonly added to the saline solution to preclude the development of hypomagnesemia.

Toxic Effects: Nephrotoxicity is the dose-limiting toxic effect. Cisplatin produces a dose-dependent impairment of renal tubular function, manifested by an increase in serum creatinine as well as potassium and magnesium wasting. The renal dysfunction is usually reversible, but repeated treatments may produce a cumulative and permanent mild to moderate impairment of renal function. Administration of other nephrotoxic agents such as aminoglycosides, even between courses, can potentiate its toxicity. Nausea and vomiting are usually severe and require aggressive antiemetic support. When doses >70 mg/m ^{2} are used, it is also important to protect against delayed nausea and vomiting by administering antiemetic agents (e.g., prochlorperazine plus dexamethasone) for 3 days following therapy. Myelosuppression is usually mild. High-frequency hearing loss, tinnitus, and frank deafness may occur. Peripheral neurotoxicity, characterized by paresthesias or sensory loss in a glove-and-stocking distribution or as muscular weakness, is relatively common in patients who receive total cumulative doses of >500 mg/m ^{2} . The peripheral neuropathy may take many months to resolve, if it does at all. Vestibular toxicity and anaphylactic reactions occur rarely.

Potential Drug Interactions: Aminoglycosides and amphotericin may enhance cisplatin nephrotoxicity. Caution should be exercised when cisplatin is administered with bleomycin and methotrexate, as cisplatin-induced renal damage may delay the excretion and thus increase the toxicity of these agents.

Therapeutic Indications in Hematology: Cisplatin is used in the treatment of refractory lymphomas, usually in combination with cytosine arabinoside and high-dose dexamethasone.

Carboplatin (Paraplatin)

Chemistry and Mechanism of Action: Carboplatin is a second-generation platinum (II) complex. Its mechanism of action is very similar to that of cisplatin. However, the carboxyl ester groups in this platinum complex are less easily displaced and less chemically reactive. The peak level of DNA cross-linking also occurs 612 hours later for carboplatin than for cisplatin.

Absorption, Fate, and Excretion: Carboplatin is eliminated primarily through the kidneys. Its elimination is slower than cisplatin, with a terminal half-life of 26 hours. Following intravenous injection, approximately 60% of the total drug is excreted within 24 hours.

Preparation and Administration: Carboplatin is available as a lyophilized powder in 50- and 150-mg vials containing carboplatin and mannitol. It is reconstituted with sterile water to a final concentration of 10 mg/ml. For injection further dilution with 5% dextrose and water or normal saline to a concentration of 0.5 or 2 mg/ml, it is stable for 8 hours at room temperature. Carboplatin is often administered by intravenous injection over 1530 minutes. Patients with reduced renal function (creatinine clearance <60 ml/min) should have the dose of carboplatin decreased according to the formula described by M.J. Egorin.

For previously untreated patients:
Dosage (mg/m^2)
= (0.091) (creatinine clearance/body surface area)
× ([(pretreatment platelet count
platelet nadir desired)/pretreatment platelet count]
× 100) + 86

For heavily pretreated patients:
Dosage (mg/m^2)
= (0.091) (creatinine clearance/body surface area)
× ([(pretreatment platelet count
platelet nadir desired/pretreatment platelet count)
× 100] 17) + 86

A formula developed by A.H. Calvert also takes into account the patients pretreatment renal function, as follows:
Dose (mg) = target AUC (mg/ml × min)
× [GFR (ml/min) + 25]
where the dose in mg (not mg/m^2 body surface area)

equals target AUC × GFR + 25
where AUC is the area under the plasma clearance curve and GFR is the glomerular filtration rate. In previously untreated adults, the AUC can be estimated at 7 when carboplatin is used alone and 4.5 when used in combination. If the AUC is set lower, less toxicity is expected.

Toxic Effects: The dose-limiting toxic effect is myelosuppression; thrombocytopenia is more significant than leukopenia. Carboplatin leads to less emesis than cisplatin. Although nausea and vomiting are common, they can be easily controlled with antiemetics. At high doses such as those used for bone marrow transplantation, hepatotoxicity, renal dysfunction, and moderate to severe cytotoxicity can occur.

Potential Drug Interactions: None reported.

Clinical Indications in Hematology: Carboplatin has been recently approved for the treatment of ovarian cancer. It is also used to treat small cell lung, testicular, head and neck, and genitourinary cancers. High-dose carboplatin is under evaluation in acute leukemias and lymphomas.

APPENDIX: Clinical Pharmacology of Miscellaneous Agents

Plicamycin

Chemistry and Mechanism of Action: Plicamycin, also called mithramycin, forms complexes with DNA and inhibits DNA-directed synthesis of RNA. Plicamycin also inhibits the effect of parathyrin on osteoclasts. The hypocalcemic effect is independent of the antitumor effect.

Absorption, Fate, and Excretion: The pharmacology of plicamycin has been poorly described. Within 15 hours of intravenous administration, 40% is excreted in the urine. The elimination half-life has been estimated to be approximately 2 hours.

Preparation and Administration: Plicamycin is available as a lyophilized powder in 2.5-mg vials, from which it is reconstituted with 4.9 ml of sterile water for injection. This dose should be further diluted in 1 L of 5% dextrose in water or normal saline and infused over 46 hours.

Toxic Effects: When plicamycin is employed as an antitumor agent, its most common side effects include myelosuppression, elevated liver enzymes, increased serum creatinine and proteinuria, and coagulopathy due to decreased clotting factors II, V, VII, and X. The drug also causes nausea and vomiting, diarrhea, stomatitis, headache, and irritability. Cutaneous toxicity may occur in up to one third of patients, manifested by progressive blushing of the face and thickening and coarsening of the skinfolds. The drug can produce severe local irritation if extravasation occurs.

Potential Drug Interactions: None reported.

Therapeutic Indications in Hematology: Plicamycin is used primarily to treat hypercalcemia of malignancy. Claims of antitumor activity in the blast phase of CML have been made but not confirmed.

Bleomycin

Chemistry and Mechanism of Action: Bleomycin is a glycopeptide. Its antitumor effect correlates with its ability to cause scission of both double- and single-stranded DNA via activated oxygen formed by the iron/bleomycin complex. Bleomycin also affects DNA repair by inhibiting DNA ligase.

Absorption, Fate, and Excretion: Bleomycin is rapidly distributed throughout the body and concentrates in the skin, lung, kidney, peritoneum, and lymph nodes. Its plasma half-life is 24 hours. Within 24 hours of injection, approximately 50% of an administered dose is excreted unchanged in the urine. Bleomycin elimination correlates well with creatinine clearance; accordingly, patients with renal failure should receive a reduced dose. In the tissues, bleomycin is inactivated by bleomycin hydrolase. Tissues lacking this enzyme, such as lung and skin, are more susceptible to the drugs toxic effects.

Preparation and Administration: Bleomycin is available in vials containing 15 U (approximately equivalent to 15 mg), from which it is reconstituted for injection with 35 ml of sterile water, normal saline, 5% dextrose in water, or bacteriostatic water. For intravenous infusion, the reconstituted solution can be further diluted with either normal saline or 5% dextrose in water and administered over 5 minutes. Bleomycin can also be administered by the subcutaneous, intravenous, intramuscular, intracavitary, and intra-arterial routes. Because patients with lymphomas are at an increased risk of anaphylactoid reactions, which may not occur until 12 hours after administration, the first two doses should be intramuscular test doses of 12 mg. If no reactions occur, full doses may be given.

Toxic Effects: The most serious toxic effect is interstitial pneumonitis, which is dose-related and occurs in approximately 10% of patients treated with cumulative doses of >350400 U. The interstitial pneumonitis may evolve into life-threatening pulmonary fibrosis. Pulmonary toxicity is more common in patients >70 years, in those receiving a total dose of >400 U, and in those who received prior radiotherapy to the lung. However, the pulmonary toxicity is unpredictable; it has been reported in patients who had none of these risk factors and has occurred in a patient after administration of only 20 U. Some reports suggest that an increased concentration of

inspired oxygen acts synergistically with bleomycin to produce pulmonary fibrosis. During critical illness and perioperatively, therefore, an attempt should be made to maintain the inspired oxygen concentration at 21%. The early phases of the pulmonary toxicity are clinically manifested by dyspnea and fine rales. Although corticosteroids are often employed in this setting, it is not clear that they are of benefit.

Mucocutaneous toxicity occurs in 50% of patients treated and is manifested by hyperpigmentation, pruritic erythema, mucositis, desquamation of the plantar surface skin of the hands and/or feet, ridging of the nails, and alopecia. The mucositis can be severe and is the acute dose-limiting toxic effect. Febrile reactions, which occur a few hours after bleomycin administration and may last 412 hours, are also common. Fever becomes less frequent with continued use of the drug and can usually be prevented by concurrent administration of glucocorticosteroids (e.g., 100 mg of hydrocortisone). Bleomycin has virtually no myelosuppressive effect. Anaphylactoid reactions are observed in approximately 1% (8% in some series) of patients with lymphomas treated with bleomycin.

Potential Drug Interactions: Bleomycin, administered with other drugs for the treatment of lymphorra, can decrease the oral bioavailability of digoxin and the pharmacologic effect of phenytoin and certain anesthetic drugs.

Therapeutic Indications in Hematology: Bleomycin is often incorporated in the chemotherapy regimens of Hodgkin disease (ABVD and MOPP/ABV hybrid regimens) and non-Hodgkins lymphomas (MACOP-B, PROMACE/ CYTABOM, M-BACOD, and CHOP-Bleo).

Procarbazine

Chemistry and Mechanism of Action: Procarbazine is a substituted hydrazine derivative with a chemical structure similar to that of the monoamine oxidase inhibitors (MAOI). Accordingly, procarbazine exhibits weak MAOI effects. Procarbazine itself is inert and must undergo metabolic activation to generate cytotoxic reactants, the mode of action of which is not clear. They may inhibit transmethylation of methyl groups of methionine into tRNA or may also directly damage DNA. Hydrogen peroxide, formed during the auto-oxidation of procarbazine, may attack protein sulfhydryl groups contained in residual proteins tightly bound to DNA.

Absorption, Fate, and Excretion: Procarbazine is rapidly and completely absorbed by the oral route, peak plasma levels occurring within 60 minutes. It penetrates well into the cerebrospinal fluid. The drug is readily metabolized in the liver and has a plasma half-life of 10 minutes after intravenous injection. The major sites of elimination are the kidneys, where approximately 70% of the drug is excreted as N-isopropylterephthalamic acid and <5% is excreted unchanged.

Preparation and Administration: Procarbazine is available in 50-mg capsules.

Toxic Effects: The usual dose-limiting toxic effect is myelosuppression. Occasionally nausea and vomiting may be dose-limiting, although tolerance to those effects may develop during continued administration. Other less common side effects include paresthesias, headache, dizziness, depression, apprehension, insomnia, nightmares, hallucinations, drowsiness, ataxia, foot drop, decreased reflexes, tremors, coma, confusion, convulsions, skin rash, alopecia, myalgia, and arthralgia. Procarbazine may possibly be leukemogenic.

Potential Drug Interactions: Combination chemotherapy that includes procarbazine may result in a decrease in digoxin plasma levels. Because procarbazine is a weak MAOI, hypertensive reactions could theoretically occur following concurrent ingestion of sympathomimetics, levodopa, tricyclic antidepressants, or foods with high tyramine content (e.g., dark beer, yogurt, cheeses, and red wines). However, such reactions have not been reported. Concomitant use of narcotics or other strong sedatives may result in exaggerated depressant effects, leading to coma and possibly death. Procarbazine also interacts with alcohol, causing a disulfiram-like reaction.

Therapeutic Indications in Hematology: Procarbazine is often used in combination with other cytotoxic agents in the treatment of Hodgkins disease (MOPP and MOPP derivatives) and to a lesser extent in the treatment of non-Hodgkin lymphomas (PROMACE/MOPP).

Asparaginase

Chemistry and Mechanism of Action: Asparaginase contains the high-molecular-weight enzyme L-asparaginase amidohydrolase, type EC-2, derived from *Escherichia coli*. Asparaginase hydrolyzes serum asparagine to nonfunctional asparatic acid and ammonia, depriving tumor cells of a required amino acid; thus, tumor cell proliferation is blocked by the interruption of asparagine-dependent protein synthesis. The drug appears to be most active in the G_1 phase.

Absorption, Fate, and Excretion: Asparaginase is not absorbed orally. Its plasma half-life varies from 8 to 30 hours and is not influenced by dosage, age, sex, surface area, or renal or hepatic function.

Preparation and Administration: Asparaginase is available in vials containing 10,000 IU of asparaginase in 80 mg of mannitol. For intravenous use, the drug should be reconstituted with 5 ml of either sterile water or sodium chloride for injection and injected in the tubing of a freely running infusion of either normal saline or 5% dextrose in water over 30 minutes. For intramuscular or subcutaneous use, each vial should be reconstituted with 2 ml of sodium chloride for injection to obtain a 5,000-U/ml solution. For dosages >2 ml, use of two injection sites is recommended. For both intravenous and intramuscular administration, the drug must be used within 8 hours of reconstitution, and only if it is clear. Because of the possibility of hypersensitivity reactions (particularly in patients with lymphomas), an intradermal skin test is recommended before initial administration of asparaginase or when 1 week has elapsed between doses. For this test, 2 IU should be injected intradermally and observed for a wheal or erythema for 1 hour. A negative skin test, however, does not preclude possible development of a hypersensitivity reaction. It is recommended that oxygen, epinephrine, and corticosteroids be available at the bedside during administration of the drug. For allergic patients, the *E. coli* form of asparaginase should be replaced by the asparaginase derived from *Erwinia carotovora*, provided by the National Cancer Institute as an investigational group C agent.

Toxic Effects: The toxicity of asparaginase is reported to be greater in adults than in children. Anorexia, nausea, or vomiting occurs in approximately one third of patients. Most of the other side effects can be divided into two main groups, those related to hypersensitivity reactions to the foreign protein and those resulting from decreased protein synthesis. The hypersensitivity reaction is characterized by urticaria, laryngeal edema, bronchospasm, or hypotension and may occur with the initial dose of the drug, even if the skin test is negative. More commonly, however, allergic phenomena are observed after multiple courses of treatment. Adverse effects related to the inhibition of protein synthesis include hypoalbuminemia and decreases in serum fibrinogen, prothrombin,

antithrombin III, and other coagulation factors, which may lead to both clotting and hemorrhagic complications; decreased serum insulin with hyperglycemia; and decreased serum lipoproteins. In 25% of patients cerebral dysfunction, characterized by confusion, stupor, and frank coma, can occur. Although the neurotoxic effects resemble those of ammonia toxicity, they are apparently due to low concentrations of either L-asparagine or L-glutamine in the brain. Acute pancreatitis, which may progress to severe hemorrhagic pancreatitis, may occur in 15% of patients. Elevation of liver enzymes and serum bilirubin is almost universal and is histologically represented by fatty metamorphosis. Liver toxicity, although usually not clinically significant, has resulted in occasional fatalities. Asparaginase occasionally produces renal functional impairment with oliguric renal failure.

Potential Drug Interactions: When asparaginase is administered immediately before or with methotrexate, it decreases the cytotoxic effect of the latter. When administered to patients with acute leukemia 910 days before or shortly after methotrexate, however, asparaginase appears to enhance the cytotoxic effect of methotrexate. Concurrent administration of asparaginase with vincristine may increase vincristines neurotoxic effects, but this effect appears to be less pronounced when asparaginase is given after vincristine. The effects of asparaginase on liver function may interfere with the activation or metabolism of other cytotoxic agents.

Therapeutic Indications in Hematology: Asparaginase is used in combination therapy for remission induction of patients with ALL.

Gallium Nitrate

Chemistry and Mechanism of Action: Gallium nitrate is a group IIA metal salt; the mechanism of action is not completely understood. Its selective cytotoxic effects in humans are probably related to its predominant concentration in certain malignant tumors. Gallium nitrate binds to intracellular calcium and magnesium sites. Transferrin binding appears to be necessary to produce the cytotoxic effect.

Absorption, Fate, and Metabolism: Gallium nitrate is primarily eliminated by renal excretion and follows a biphasic pharmacokinetic pattern. The reported half-life is 636 hours.

Preparation and Administration: Gallium nitrate is an investigational agent supplied as a 25-mg/ml solution in 20-ml vials (500 mg per vial), also containing trisodium citrate and sodium hydroxide to adjust the pH. Gallium nitrate can be administered by slow intravenous infusion, although in the treatment of hypercalcemia it is usually given by continuous infusion over 57 days.

Toxic Effects: When given as a single bolus injection, the dose-limiting toxicity is renal impairment. The renal toxicity is significantly minimized when the drug is given by continuous infusion. Other toxic effects include mild myelosuppression, hypocalcemia (when not desired), nausea, vomiting, diarrhea, and mucositis. Rarely, gallium nitrate produces neurotoxic effects such as hearing loss, visual disturbances, paresthesias, and mental status changes.

Potential Drug Interactions: None reported.

Clinical Indications in Hematology: Gallium nitrate has shown promising results in the treatment of hypercalcemia associated with malignancy, in prevention of bone loss in patients with multiple myeloma, and in the treatment of refractory lymphomas.

Glucocorticoids

Chemistry and Mechanism of Action: Glucocorticoids are synthetic compounds derived from the natural adrenal hormone cortisol. Glucocorticoids mediate their biologic actions predominantly by binding to their cytosolic receptor, which then translocates to the nucleus. There, as a homodimer, it binds to specific DNA

sequences located in the regulatory regions of a number of genes. Gene transcription can be up- or downregulated by glucocorticoids. They can also inhibit binding of the AP-1 transcription factor to its DNA consensus sequence site. Lymphocytes treated with glucocorticoids undergo apoptosis mediated by glucocorticoid receptors. An early cytostatic phase is marked by growth inhibition and cessation of proliferation due to inhibition of cellular uptake of glucose, amino acids, and nucleosides as well as inhibition of macromolecular synthesis. This is followed by a cytolytic phase characterized by chromatin condensation and internucleosomal DNA cleavage.

Absorption, Fate, and Excretion: Many synthetic glucocorticoids are available; the three most commonly used in hematology are prednisone, dexamethasone, and methylprednisolone. The glucocorticoids are well absorbed orally and are metabolized primarily in the liver. Unlike the other two glucocorticoids, the activity of prednisone is dependent on hepatic conversion to the 11-hydroxy form (prednisolone). The biologic half-lives of prednisone and methylprednisolone are approximately 1236 hours, whereas dexamethasone has a biologic half-life of 3672 hours. Plasma half-lives for all three drugs are 34 hours. Compared with cortisol, the relative anti-inflammatory potencies of dexamethasone, methylprednisolone, and prednisone are 25, 5, and 4, respectively, for equivalent doses.

Preparation and Administration: Prednisone is available only for oral administration; methylprednisolone and dexamethasone are available in oral and parenteral dosage forms.

Toxic Effects: When glucocorticoids are used for <14 days, as is often done when they are employed in combination with other cytotoxic agents, the most common side effects include euphoria, insomnia, psychosis, hyperglycemia, hypokalemia, increased appetite, metabolic alkalosis, proximal muscular weakness, and fluid retention with edema formation and hypertension. When used on a chronic basis, glucocorticoids also may induce a cushingoid appearance, easy bruisability, peptic ulcers, osteoporosis, subcapsular cataracts, and increased susceptibility to infections related to impaired cellular immunity. Because of this, H_2 blockers, antifungal agents (e.g., ketoconazole), and/or sulfamethoxazole-trimethoprim have been employed in certain glucocorticoid chemotherapy combinations.

Potential Drug Interactions: Glucocorticoids interact with a variety of drugs, including barbiturates, oral contraceptives, erythromycin, hydantoins, rifampin, isoniazid, and salicylates. Given the wide range of doses of glucocorticoids used, however, these interactions are of no major clinical relevance.

Clinical Indications in Hematology: Glucocorticoids have direct anticancer activity in many hematologic malignancies, including ALL and CLL, Hodgkins and non-Hodgkins lymphomas, and plasma cell neoplasms. Because of their efficacy and toxic profiles, which do not overlap with the toxic effects of the other cytotoxic agents, glucocorticoids are employed in many chemotherapy regimens. In addition, they are useful in the management of hypercalcemia secondary to myeloma and lymphomas and are of paramount importance in the treatment of autoimmune hematologic disorders.

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Chapter 51 - Clinical Application of Cytokines and Biologic Response Modifiers

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INTRODUCTION

Cytokines and biologic response modifiers (BRMs) represent a broad class of therapeutic agents that modify the hosts response to cancer or cancer therapy. Table 51-1 summarizes the biologic therapies currently in clinical use or undergoing clinical evaluation (see also Chap. 14). Many of the biologic agents evaluated to date activate natural host immune defense mechanisms. However, the term biologic response modifier is not equivalent to immunotherapy of cancer. Many of these agents exhibit direct biologic effects on malignant cells and may act as

TABLE 51-1 -- Cytokines/Biologic Response Modifiers

Recombinant Cytokine	Molecular Weight (kd)	Principle Cell Source	Biologic Activities	References
IL-1 and IL-1	1417	Monocyte/macrophages DC, B, T, NK cells, fibroblasts, epithelial cells, endothelial cells	Stimulates primitive lymphohematopoietic progenitors; induces secondary cytokine release	[134] [135] [136] [137] [138] [139] [140] [141] [142] , [144]
IL-2	15	T cells	Activates T-cell proliferation; induces cytokine production from NK and T cells; induces proliferation of NK and B cells; induces cytotoxicity in T and NK cells; activates monocyte cytotoxicity	[229] [239] [240]
IL-3 (multi-CSF)	28	T cells, myelomonocytic cells	Induces proliferation of myeloid (including eosinophilic), erythroid, and megakaryocytic progenitors; stimulates early lymphoid development	[143] [144] [145] [146] [147] [148] [149] [150] [151] [152] [153] [154] , [156] [157] [158] [159] [160] [161] [162] [163] [164]
IL-4	1520	T cells, mast cells	Induces T-cell proliferation and cytotoxicity; enhances antigen-specific T-cell responses; enhances growth of mast cells and stimulates monocyte cytotoxicity; stimulates Th2 helper responses; inhibits Th1 helper responses; enhances expression of MHC and adhesion molecules	[284] [285] [286] [287] [288] [289] [290]
IL-5	1218	T cells	Induces proliferation of eosinophils; enhances B-cell antibody production	[11] [12] [13] [14]
IL-6	2129	T cells, macrophages, fibroblasts	Stimulates megakaryocytic maturation and induces thrombopoiesis; stimulates B-cell proliferation and immunoglobulin production; synergistic with early-acting factors for primitive progenitor cell proliferation; induces hepatocyte protein synthesis	[155] , [165] [166] [167] [168] [169] [170] [171] [172] [173] [174] [175] [176] [177] [178] [179] [180] [181] [182] [183] [184] [185] [186] [187] [188] [189] [190] [191]
IL-7	25	Stromal cells (BM)	Co-stimulates T-cell proliferation and cytotoxicity; induces monocyte cytotoxicity; supports growth of B-cell precursors	
IL-8, RANTES, etc.	816	Monocytes/macrophages T cells, fibroblasts	Induces neutrophil chemotaxis; removes proliferating hematopoietic cells from S-phase (similar to other chemokines)	
IL-9	3239	T cells (helper)	Stimulates specific T-cell growth; stimulates mast cells; supports IgE production from B cells with IL-4	
IL-10	1721	T cells, B cells	Inhibits production of cytokines from Th1 cells (IFN; inhibits monocyte activation and cytokine release; inhibits NK cell production of IFN; co-stimulates T-cell proliferation and cytotoxicity	
IL-11	24	Fibroblasts, adipocytes, endothelial cells, monocytes	Induces megakaryocyte maturation and thrombopoiesis; synergistic with early-acting factors for primitive lymphohematopoietic proliferation; induces B-cell Ig production via accessory T cells, inhibits adipogenesis; induces hepatocyte protein synthesis	[192] [193] [194] [195] [196] [197] [198] [199] [200] [201] [202]
IL-12	75 (3540)	B-lymphoblastoid cell lines, activated T cells, monocytes, keratinocytes, mast cells, and DC	Induces TH1 responses; potent induction of IFN from NK and T cells; antimetastatic, anti-angiogenic effects	[307] [308] [309] [310] [311] [312] , [313] [314] [315] [316] [317] [318] [319] [320]
IL-13	132aa	T cells	Induces B-cell activation; induces expression of MHC and CD23 on B cells	
IL-14	468aa	T cells	Induces B-cell proliferation and inhibits B-cell immunoglobulin production	

IL-15	114aa	Monocytes, fibroblasts, epithelial cells, and stromal cells (bone marrow and thymus)	Activation of NK cell proliferation and cytotoxicity; activates T-cell proliferation	[322] [323] [324] [325] [326] [327]
IL-16	56 (tetrameric)	Activated T (CD8+) cells; eosinophils	Chemoattractant for CD4+ lymphocytes; induces proliferation of CD4+ T cells; chemoattractant for eosinophils and monocytes; ligand for CD4; inhibits T-cell receptor activation; inhibits HIV promoter	
IL-17	155aa	CD4+ T cells	Co-stimulation of T cells; induces IL-6 and IL-8 from fibroblasts; induces stromal cells (endothelial cells, epithelial cells, etc.) to secrete G-CSF, IL-6, IL-8, and PGE2	
IL-18	18	Activated macrophages and Kupffer cells	Induces IFN from NK and T and B cells especially with IL-12; inhibits osteoclast activity; augments NK activity; enhances GM-CSF and inhibits IL-10 from activated PBMC	
IFN or IFN	1627	Leukocytes/fibroblasts	Antiproliferative; enhances antigen and MHC class I expression; augments NK activation; anti-viral effects; anti-angiogenic effect; effects expression of adhesion receptors; induces differentiation of tumor cell lines	[338] [339]
IFN	15.525	T cells, NK cells, and monocytes	Induces monocyte activation; induces MHC class II and I and tumor antigen expression; activates CTL; induces B-cell antibody production	
TNF	17/26	Monocytes/macrophages, T cells	Stimulates T-cell proliferation, activates NK cells; activates monocyte cytotoxicity; activates neutrophils; has direct effects on vascular endothelial cells; disrupts lipid metabolism; induces acute phase reactants	[61] [62] [63] [64] [65] [66] [67] [68] [69]
G-CSF	2025	Stromal cells, endothelial cells, and monocytes/macrophages	Induces proliferation and maturation of neutrophilic precursors and progenitors; mobilizes myeloid progenitors into peripheral blood; activates neutrophil function	[12] [15] [16] [17] [18] [19]
GM-CSF	1830	Fibroblasts, stromal cells, endothelial cells, and T cells	Induces proliferation and maturation of neutrophilic, monocytic precursors and progenitors; mobilizes myeloid progenitors into peripheral blood; activates neutrophil/monocyte function; induces eosinophilia; weak activator of NK cell activity	[41] [42] [43] [44] [45] [46] [47] [48] [49] [50] [51] [52] [53] [54] [55] [56] [57] [58] [59] [60] [61] [62] [63] [64] [65] [66]
M-CSF	45	Monocytes, endothelial cells, and fibroblasts	Monocyte differentiation and proliferation; activates monocyte cytotoxicity and ADCC; enhances expression of secondary cytokines and costimulatory molecules on monocytes	[407] [408] [409] [410] [411] [412] [413] [414] [415] [416] [417] [418] [419] [420] [421] [422]
SCF/kit ligand	3035		Induces primitive and committed progenitor cell proliferation; synergistic with other factors for multiple lineage effects; induces activation and proliferation of mast cells	[104] [105] [106] [107] [108] [109] [110] [111] [112] [113] [114] [115] [116] [117] [118] [119] [120] [121] [122] [123] [124]
Thrombopoietin	153332aa	Hepatocytes, kidney, bone marrow stromal cells	Stimulates broad range of megakaryocytic (MK) progenitors and precursors; induces increases in number and maturation of MKs; promotes thrombopoiesis; has indirect effects on myelopoiesis and erythropoiesis	[203] [204] [205] [206] [207] [208] [209] [210] [211] [212] [213] [214] [215] [216] [218] [219] [220] [221]
Flt3 ligand	1829	Stromal cells and stem cells	Stimulates hematopoietic progenitor cells; costimulates colony formation with lineage-specific growth factors; induces proliferation of DC cells and their maturation with other cytokines	[328] [329] [330] [331] [332] [333] [334]
PIXY321	IL-3/GM-CSF fusion protein	Fibroblasts, stromal cells, endothelial cells and T cells; myelomonocytic cells	Enhances in vitro activity on myelomonocytic, erythroid, and megakaryocytic lineages	[125] [126] [127] [128] [129] [130] [131] [132] [133]
Erythropoietin	35	Renal peri-tubular cells	Stimulates erythroid progenitor proliferation and erythroid maturation	[67] [68] [69] [70] [71] [72] [73] [74] [75] [76] [77] [78] [79] [80] [81] [82] [83] [84] [85] [86] [87] [88] [89] [90] [91] [92] [93] [94] [95] [96] [97] [98] [99] [100] [101] [102] [103]

MCH, major histocompatibility complex; PBMC, peripheral blood mononuclear cell.

differentiating, antiproliferative, and/or cytotoxic agents. These effects are independent of their immunomodulatory activities. There has been a considerable focus on cytokines and BRMs in the treatment of cancer patients over the past 12 decades. Advances in cellular and molecular biology have permitted the isolation, identification, genetic cloning, and pharmacologic production of purified recombinant proteins that were previously available only in scant quantities. In addition, significant progress has been made in understanding the regulation of the immune system and the molecular nature of an immunologic response to cell-surface antigens within the context of the major histocompatibility complex (MHC). This has had its greatest effect on the development of vaccine strategies directed at cancer.

Immune cells secrete two major classes of soluble proteins. One is the class of immunoglobulin (Ig) molecules (antibodies) that bind soluble antigens and activate B cells through Ig surface receptors. The other class of soluble molecules secreted by lymphocytes and monocytes are true hormones that act upon many different target cells both within and outside the immune system. The term interleukin denotes the activity between leukocytes. Cytokines represent the class of glycoproteins, which includes interleukins (ILs), interferons (IFNs), and colony-stimulating factors (CSFs). A system for naming cytokines has been developed by the International Congress of Immunology. A cytokine is initially described based on biologic properties, but once the amino acid sequence is defined, an interleukin number is assigned. The system has some exceptions and inconsistencies; for historical reasons, some cytokines retain their original names (i.e., IFNs and CSFs). To date, 18 ILs have been identified.

Over the past 10 years, cytokine therapy has in many ways revolutionized the fields of hematology and oncology. The use of CSFs and hematopoietic growth factors (HGFs) to support patients receiving chemotherapy has permitted the implementation of higher chemotherapy doses with a significant reduction in toxicity. [1] In addition, cytokine therapy has expanded treatment options for patients with various malignancies, particularly hematologic malignancies.

When considering the use of cytokines during the treatment of malignancies, one must be aware of the increasing evidence that some cytokines are aberrantly expressed by malignant hematopoietic cells and may participate pathogenetically through autocrine or paracrine regulatory loops in the expansion of the tumor. [2] IL-1 and tumor necrosis factor- (TNF-) have been found to be constitutively expressed by virtually all leukemias, both myeloid and lymphoid. [3] [4] Receptors for TNF have been detected on the surface of hairy cell leukemia (HCL) and B-cell chronic lymphocytic leukemia (CLL) cells; in vitro studies have shown that TNF promotes survival and induces proliferation of these malignant cells. [5] The mRNA for TNF, IL-1, and IL-6 have been detected in some malignant lymphoid cell lines. [6] Contradictory data have been reported concerning the expression of other cytokines by CLL cells and the consequences of this cytokine production on stimulation of leukemia cell proliferation. [7] With the exception of acute myeloid leukemias (AMLs), cytokines have not been found to have an in vivo proliferative effect on malignant tumors.

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GENERAL PRINCIPLES OF THERAPY

Cytokines are low-molecular-weight (1540,000 dalton) glycoproteins that act primarily in a paracrine or autocrine fashion and are therefore short-range mediators. The cytokines in general bind to their cell surface receptors which are part of a family called the hematopoietin (cytokine) receptor superfamily. The receptors that are part of this family are all transmembrane (TM) glycoproteins with four positionally conserved cysteines, a conserved WSXWS motif and Ig, fibronectin type III domains. Intracellular portions of these molecules include two box regions, one with proline and hydrophobic regions, and a second with two charged amino acids and hydrophobic regions. These domains are critical for binding to associated intracellular molecules such as the JAK kinases which lead to signaling mostly through a family of intracytoplasmic molecules called STAT molecules, which then translocate into the nucleus and activate gene transcription. Ultimately, changes in protein synthesis lead to alterations in cellular proliferation, growth inhibition, enhanced cytotoxicity, production of secondary cytokines, and/or modulation of the biologic effects of other cytokines. Many of the cytokines have multiple and overlapping functions. The action of a cytokine may depend on the local concentration of the cytokine, target cell type, available effector cells, and presence of other signal molecules.

Several principles of cytokine therapy are distinct from those used during the administration of traditional chemotherapy. First, for most of the cytokines, a simple dose-response relationship does not exist. It is not clear in many cases whether the optimal biologic dose is the maximum tolerated dose and whether either correlates with antitumor responses. Second, treatment with chemotherapy, if effective, is associated with a prompt response. By contrast, the therapeutic response to cytokines may develop slowly and take months to document. In addition, cytokine therapy is associated with unique toxicities, including fever, rigors, flu-like symptoms, and, for many agents, a capillary leak syndrome at higher doses. The side effects occur acutely, are generally short-lived, and abate with discontinuation of therapy. Long-term chronic toxicities are rare but can occur, and include autoimmune phenomena and cardiac toxicity. [8]

The cytokines approved by the Food and Drug Administration (FDA) for clinical use are still few in number and include granulocyte-CSF (G-CSF) and granulocyte/macrophage-CSF (GM-CSF) which are utilized primarily to accelerate recovery from granulocytopenia following myelosuppressive chemotherapy or bone marrow transplantation (BMT), respectively; erythropoietin (EPO), for the treatment of chemotherapy-associated anemia, human immunodeficiency virus (HIV)-associated anemia and the anemia associated with renal failure; IL-11 for the prevention of severe chemotherapy-induced thrombocytopenia; IFN- for the treatment of chronic myelogenous leukemia (CML), low grade non-Hodgkin lymphoma, hairy cell leukemia (HCL), Kaposis sarcoma in patients with acquired immunodeficiency syndrome (AIDS), and adjuvant therapy for high-risk melanoma and for certain viral syndromes, IFN- for the treatment of multiple sclerosis, IFN- for the treatment of chronic granulomatous disease, and IL-2 for the treatment of metastatic renal cell cancer and metastatic melanoma.

This chapter covers each of the HGFs and BRMs currently in clinical use, or undergoing clinical evaluation with the prospect of finding a significant role in cancer therapy. For a review of the biology of these agents, the reader should refer to Chapter 14 .

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HEMATOPOIETIC GROWTH FACTORS

The HGFs are a family of glycoproteins primarily responsible for the proliferation, differentiation, and maturation of the hematopoietic system. Many of these agents have effects not only on blood-forming cells, but also on bone marrow stroma, stimulating the secondary release of cytokines, or enhancing the microenvironment to favor cellular growth. These factors can broadly be divided into early-acting (predominantly proliferative) and late-acting (primarily maturational) agents. The early-acting HGFs act primarily at the level of noncommitted progenitors, resulting in proliferation of these often pluripotential cells. The late-acting factors exert their effects at the level

of committed progenitors. They are responsible for the differentiation of these more mature cells, as well as having effects on terminally differentiated effector cells. The potential clinical effects of HGFs are summarized in Table 51-1 .

The clinical use of the HGFs has concentrated mainly on their ability to stimulate the production of peripheral blood cells. The use of HGFs in the treatment of various diseases, both malignant and nonmalignant, has been studied. HGFs have found their greatest potential application with their use following the administration of intensive chemotherapy with or without progenitor cell support. The primary concern regarding the use of HGFs in patients with cancer is whether cytokines can stimulate malignant cell growth. While in vitro studies have provided some evidence that some HGFs promote tumor cell growth, with the exception of AML, no malignancy has been shown in vivo to be stimulated by HGF therapy. [9] The following sections discuss the clinical development of the clinically applicable cytokines.

Granulocyte-Colony-Stimulating Factor

G-CSF was the first of the myeloid growth factors to be approved by the FDA. Its primary use is in the post-chemotherapy setting, reducing the depth and duration of chemotherapy-induced neutropenia as well as decreasing the incidence of episodes of febrile neutropenia. G-CSF was initially identified and purified by investigators at Memorial Sloan-Kettering Cancer Center during the mid-1980s. [10]

The biologic properties of G-CSF have been well studied, and it appears to be a lineage-restricted HGF, having its principle effect on the neutrophilic granulocyte series. [11] It is present in serum during normal conditions and in disease states. [12] G-CSF is capable not only of reducing the time required for maturation of a committed progenitor pool, but of prolonging the life span of mature effector cells (neutrophilic granulocytes) in the circulation. In addition, G-CSF is an activator of neutrophil function and has been demonstrated to have the ability, both in vitro and in vivo, to prime neutrophils for the activation of the respiratory burst, enhance chemotaxis, and increase phagocytosis. These effects are seen not only on neutrophils of normal subjects, but also in those with hematologic disorders, such as the myelodysplastic syndromes, which are characterized by distinct abnormalities of polymorphonuclear leukocyte function. [13]

Clinical Applications

Phase I trials with G-CSF demonstrated minimal toxicity over a wide range of doses tested. With administration of the agent either intravenously or preferably subcutaneously, dose-related increases in peripheral blood neutrophils were observed, but effects on other nonmyeloid lineages were not evident. Increased bone marrow cellularity with enhanced numbers of committed bone marrow progenitors as well as mobilization of these cells into the circulation was observed following treatment. Side effects of G-CSF include bone pain (due to medullary expansion), elevation of certain serum enzymes representative of increased cellular turnover (leukocyte alkaline phosphatase and lactate dehydrogenase), potential exacerbation of dermatologic conditions such as psoriasis or Sweets syndrome (neutrophilic dermatitis), and with chronic therapy, splenomegaly and hair loss. Fever is not a characteristic side effect of G-CSF.

After Chemotherapy

Following the recognition by Bodey et al. [14] that both the depth and duration of neutropenia correlate with the incidence of infection in myelosuppressed hosts, the need for an agent that could reduce this risk was evident. Because of its ability to promote increases in peripheral blood neutrophil numbers, G-CSF was judged to be an ideal agent to alter the incidence and severity of chemotherapy-induced myelosuppression. Initial studies performed in the post-chemotherapy setting demonstrated an apparent benefit associated with G-CSF therapy. In a phase I trial reported by Gabrilove et al., [15] treatment with G-CSF resulted in a reduction in the severity of neutropenia and the ability to deliver chemotherapy in a more timely fashion in patients with bladder cancer receiving MVAC chemotherapy. These investigators also noted an apparent reduction in the incidence of mucositis in courses of therapy in which G-CSF was administered. Bronchud et al. [16] demonstrated that with the use of G-CSF, doxorubicin chemotherapy for women with breast and ovarian cancer could be intensified from doses of 75 mg/m ^{2} to doses of 150 mg/m^2 administered every 2 weeks. Morstyn et al. [17] studied the relationship between the time of G-CSF administration and the administration of chemotherapy. Although a delay in the initiation of G-CSF therapy was associated with a deeper neutrophil nadir, the neutrophil recovery was essentially the same as that seen in patients for whom G-CSF therapy was initiated 1 day after chemotherapy. It is important to recognize that delayed therapy may be associated with a reduced benefit with regard to eliminating the incidence of febrile neutropenia.

The greatest benefit from the administration of G-CSF occurs after the use of chemotherapy regimens commonly associated with a high risk of developing neutropenic fever (defined as fever occurring during prolonged, severe neutropenia). Neidhart et al. demonstrated that repeated cycles of high-dose chemotherapy can be safely administered without progenitor cell reinfusions with the adjunctive use of G-CSF. [1] Recovery of granulocytes was found to be significantly faster in cycles containing G-CSF than in those without. The benefit of the administration of G-CSF after chemotherapy was tested in two large phase III trials conducted in the United States and Europe. [18] [19] These trials compared the use of G-CSF versus placebo in patients with small cell lung cancer receiving chemotherapy composed of cyclophosphamide, doxorubicin, and etoposide. Although these studies have been criticized for the use of overly intensive doses of chemotherapy, they clearly demonstrate that with adequate myelosuppression, a benefit of G-CSF can be identified. The study by Crawford et al., [18] conducted in the United States, allowed crossover to the G-CSF arm for any patients who received placebo therapy and experienced a neutropenic fever during any course of therapy. For this reason, the maximum data derived from this study are from the first cycle of chemotherapy when all patient therapy was double-blinded. These data demonstrate that the use of G-CSF results in a reduction of the incidence of neutropenic fever by approximately 2530% when the overall incidence of the event approaches 5060%. In addition, the number of days of antibiotics required, as well as the number of days of hospitalization, was reduced by the administration of G-CSF. In the European study, which did not allow crossover and therefore maintained the double-blind format, similar results were observed with regard to the incidence of neutropenic fever. No survival benefit for the patients treated on the G-CSF arm has been seen. Although there is little doubt that G-CSF has the ability to reduce neutropenia following chemotherapy, it is critical to determine which regimens are myelosuppressive enough to warrant routine use of this agent. The American Society of Clinical Oncology has established guidelines stating that the use of a CSF to prevent febrile neutropenia is warranted when the expected risk of such an event is predicted to exceed 40%. [20] To date, no controlled randomized trial has demonstrated a survival benefit for the adjunctive use of any CSF.

Most clinical studies have focused on the use of G-CSF to prevent episodes of neutropenic fever. Two studies have evaluated the use of G-CSF in patients with an established neutropenic fever. [21] [22] In the largest study, 216 patients with solid

tumors, lymphoma, or acute lymphocytic leukemia (ALL) were randomized on admission to receive either G-CSF or placebo by continuous subcutaneous infusion. Although patients treated with G-CSF generally had fewer days of hospitalization, fewer days of neutropenia, and fewer febrile days, the true benefit appeared to be experienced by patients with solid tumors or those with clinically documented or culture-positive infections. Similar results were seen in the second study. Although these two studies appear to define a subset of patients who may benefit from the use of G-CSF in this setting, they also demonstrate the lack of necessity for every patient to be treated in this manner. Timing of G-CSF was studied in a randomized trial conducted by the North Central Cancer Group (NCCG). In this trial, patients were followed and randomized between G-CSF and placebo once their absolute neutrophil count actually fell below 1,000 cells/l. The authors sought to determine whether the initiation of therapy once neutropenia had developed but before a febrile episode had occurred was worthwhile in preventing the latter. In this randomized trial, there was no statistically significant difference in the incidence of febrile neutropenia. Neither the duration of hospitalization or antibiotic therapy though recovery to an ANC >500 cells/l was accelerated by G-CSF (2 days vs. 4 days). [23] Hence, the early initiation of G-CSF is crucial for maximal activity. Late initiation, in general, is no better than no initiation.

The use of G-CSF in patients with acute leukemias has been extensively studied. Ohno et al. [24] published their initial observations in patients with both myeloid and lymphoid leukemias treated with G-CSF following intensive induction chemotherapy. There appeared to be a reduction of chemotherapy-related toxicity in the patients treated on the G-CSF arm. No evidence of increased relapse rate or poorer outcome with G-CSF therapy was seen. The use of G-CSF following induction chemotherapy in elderly patients with myeloid malignancies has been studied. G-CSF resulted in an apparent reduction in therapy-related morbidity and mortality characterized by fewer severe infections compared with historical controls. No negative impact on remission induction was seen but the effect on survival remains unknown. Randomized, placebo-controlled trials in patients with AML undergoing induction and consolidation therapy have demonstrated an apparent improvement in infection rates and a reduction in the number of days of neutropenia. Although several studies have been neutral with regard to the survival benefit of adjunctive G-CSF, no evidence of worsened outcome has been seen. [25] [26] As a result, G-CSF has received an expanded approval covering its use in AML patients.

Following Autologous and Allogeneic Bone Marrow Transplantation

The use of G-CSF following high-dose chemotherapy with progenitor cell support has been shown to reduce the time to engraftment and recovery from neutropenia in patients receiving progenitors derived from either bone marrow or peripheral blood. [27] G-CSF as a single agent, however, does not appear to affect the time for engraftment of either platelets or red cells. The most frequently studied doses of G-CSF in this setting are in a range of 510 g/kg/day, administered predominantly by subcutaneous injection. The benefits of G-CSF in this setting have been seen in patients with solid tumors as well as those with lymphomas. No large randomized trials have been performed using the myeloid growth factors in patients with AML undergoing autologous BMT, though no prospective trial has shown a worsened outcome as a result of G-CSF use in this patient population.

Nonrandomized data regarding the use of G-CSF following allogeneic BMT are limited. Several small series of patients have been reported, demonstrating reduced time to myeloid engraftment, and no increase in the incidence of acute graft-versus-host disease (GVHD). [28] These studies, however, are preliminary and therefore cannot address the larger issue of risk of leukemic relapse in the AML or ALL patients treated. For this reason, it is prudent to consider the use of G-CSF in these patients as investigational.

G-CSF has long been known to have the ability to mobilize progenitor cells into the peripheral blood. The use of these mobilized peripheral blood progenitor cells (PBPC) after chemotherapy has resulted in rapid multilineage engraftment. G-CSF is administered for 710 days, generally by subcutaneous injection at a dose of 10 g/kg/day. This results in mobilization of significant numbers of PBPC which are then collected by leukopheresis on days 57. [29] With the increased use of PBPC for allogeneic BMT, G-CSF has become the preferred mobilizing agent for normal donors. The use of this agent is associated with few short-term and no expected long-term toxicities and allows donors to avoid the inconvenience of a bone marrow harvest and provides an accelerated hematologic recovery. [30]

Bone Marrow Failure States

Negrin et al. [31] [32] evaluated the use of G-CSF in patients with myelodysplastic syndromes (MDSs). Serial dose escalation of G-CSF in patients with <20% blasts resulted in significant increases in white blood cell (WBC) counts and absolute neutrophil counts (ANC). In 18 patients with MDS treated for 68 weeks with subcutaneously administered G-CSF, 16 of the 18 patients demonstrated an increase in both leukocyte and granulocyte counts. There were no changes in platelet counts, while 3 of 12 red-cell-transfusiondependent patients experienced a decrease in their transfusion requirement. All peripheral counts returned to pretreatment values after discontinuation of the agent. Eleven of the patients received chronic administration of G-CSF for >11 months, with persistence of the leukocyte effects. No patients experienced transformation of their MDS to AML while on G-CSF. Studies are focusing on the combination of G-CSF and erythropoietin (EPO) in this patient population. A study performed by Negrin et al. [33] demonstrated that the combination of G-CSF with EPO therapy resulted in neutrophilic responses in all evaluable patients, and erythroid responses in 42% of those treated. Responding patients generally had a lower endogenous serum EPO level than that of nonresponders. Similar responses to G-CSF therapy alone have been seen in patients with other bone marrow failure states, such as aplastic anemia.

Patients with Primary or Secondary Neutropenia

The neutropenic states represent a category of diseases for which G-CSF has become the therapy of choice. Most adults presenting with mild to moderate neutropenia (for whom increased neutrophil margination and drug-induced neutropenia have been ruled out) are categorized as having idiopathic neutropenia. This disorder is characterized by a neutrophil count of <500 cells/l and an increase in infectious complications. Cyclic neutropenia is characterized by cyclical hematopoiesis affecting multiple lineages. Neutrophil counts in these patients oscillate with a periodicity of approximately 21 days, often dropping to levels of near zero and rarely reaching normal levels. During these periods of neutropenia, patients are at increased risk of infectious complications and often develop painful ulcerations of their mucosal surfaces. Finally, congenital forms of neutropenia, such as Kostmann or Schwachmann-Diamond syndrome, are associated with severe neutropenia and infectious complications that are usually manifested in the first year of life and can be life-threatening. The use of G-CSF in each of these states has been studied; therapy results in an increase in the number of circulating neutrophils and reduction in the number of infectious complications. [34] [35] [36] G-CSF should be the initial line of therapy once the appropriate diagnostic

workup has been completed. Although no distinct evidence indicates that chronic G-CSF therapy will result in an increased risk of the emergence of leukemic disorders, such concern exists because it is known that some chronic bone marrow failure disorders (such as aplastic anemia or Fanconis anemia) can evolve into clonal hematologic disorders. Pilot studies in this latter disorder have demonstrated small numbers of multi-lineage responses to G-CSF. [37] Neutropenia that complicates hematologic diseases such as HCL or CLL has been shown to be responsive to G-CSF therapy. [38] Neutropenia of an autoimmune etiology, such as occurs in Feltys syndrome or that associated with systemic lupus erythematosus, tends to respond less well to G-CSF, probably due to the presence of antineutrophil antibodies.

Infectious Disease

Whether G-CSF will be of benefit in patients suffering from severe infections not associated with neutropenia is the focus of a randomized trial in patients with pneumonia. In that study, patients with community-acquired pneumonia are randomized to G-CSF or placebo as an adjunct to antibiotic therapy. No survival advantage was seen for the incorporation of G-CSF, though earlier discharge from the hospital was noted. Overall, the use of G-CSF in patients with community-acquired pneumonia is not yet indicated. It is likely that there are subsets of patients, such as those with severe burns, who may benefit from G-CSF therapy in this setting. The beneficial effect of G-CSF may be a result of its ability to enhance effector cell function and to reduce the mortality due to superinfection in these patients.

The use of G-CSF in patients with AIDS has been studied. G-CSF has been shown to reduce neutropenia related to retroviral therapy, permitting the administration of high doses of antiviral drugs, and is approved for this indication. [39] [40]

Granulocyte/Macrophage-Colony-Stimulating Factor

GM-CSF has been approved by the FDA for its use in patients undergoing high-dose chemotherapy and BMT, as well as for the treatment of neutropenia following induction therapy in elderly patients with AML. GM-CSF is a glycosylated molecule with a molecular weight of 14.532 kD. It is encoded by a gene on the short arm of chromosome 5.

Unlike G-CSF, GM-CSF has effects on multiple stages of hematopoiesis. In vitro, it appears to act synergistically with other factors (e.g., IL-3) to stimulate the production of erythroid and multipotential colonies. [41] In vivo, GM-CSF stimulates the proliferation and maturation of myeloid cells, including neutrophils and eosinophils. In addition, at variable doses, GM-CSF has potent monocyte/macrophage potentiating ability, as well as effects on dendritic cells. Similar to G-CSF, GM-CSF can activate effector cells and enhance their function. GM-CSF primes neutrophils for the respiratory burst and enhances phagocytosis. Unlike G-CSF, GM-CSF reduces chemotaxis. GM-CSF is a locally active factor at the site of inflammation, felt to be responsible for inhibiting the ability of effector cells to migrate away from this site.

Clinical Applications

Phase I trials with GM-CSF demonstrated several important aspects of its use. While it is well-tolerated at clinically effective doses for the treatment of neutropenia, its toxicity at higher doses is composed of arthralgias, myalgias, fever, and serositis (pleuropericarditis). At doses of approximately 5 g/kg/day (250 /m ^{2} /day). GM-CSF appears to have a primarily myeloid effect, promoting increased production of neutrophils and eosinophils. At higher doses, macrophage production and activation become apparent, and it is possible that the toxicity related to GM-CSF is primarily associated with this monocyte macrophage activation (possibly related to a secondary cytokine cascade). Toxicity due to GM-CSF appears to be altered depending on the route of administration. Lieschke et al. [42] reported on the first-dose effect seen in their phase I clinical trial. This toxicity occurred much more frequently in patients treated with intravenous bolus than in those treated with subcutaneous therapy. The first-dose effect is characterized by a constellation of symptoms, including dyspnea, flushing, tachycardia, hypotension, and musculoskeletal pain. It typically occurs several hours after the first dose of GM-CSF in a given cycle and may occur with the first dose of each cycle that a patient receives. It is self-limiting, and patients need only be treated with supportive care. This phenomenon, which may be related to sequestration of neutrophils in the pulmonary microcirculation, appears to be more prevalent with the nonglycosylated bacterially derived formulation of GM-CSF as compared with the glycosylated yeast-derived agent (the FDA-approved formulation).

After Chemotherapy

As with G-CSF, GM-CSF has been studied for its ability to reduce the depth and duration of neutropenia following the administration of chemotherapy. While broad chemotherapy-induced neutropenia is not the primary indication for this agent, several studies address its use in this clinical setting. In a randomized trial of lymphoma patients receiving cyclophosphamide, vincristine, prednisone-bleomycin, leucovorin, doxorubicin, methotrexate (COP-BLAM), GM-CSF reduced chemotherapy-induced neutropenia as well as days of fever and hospitalization. [43] Other randomized trials have been less impressive. Bunn et al. [44] demonstrated an inferior response rate to primary therapy in patients with limited-stage small-cell lung cancer receiving GM-CSF treated with chemotherapy and radiotherapy. Patients treated with GM-CSF, while having a reduction in granulocytopenia, experienced more infections and febrile days as well as a greater degree of thrombocytopenia. The presence of fevers as a possible side effect of GM-CSF confounds the interpretation of randomized trials. For this reason, the ability of GM-CSF to have an impact on chemotherapy-induced neutropenia remains to be proven in a large-scale randomized trial.

A major development in the use of GM-CSF was the demonstration of its ability to reduce the mortality related to induction chemotherapy in patients with AML, Rowe et al. studied GM-CSF administered from the time of documentation of bone marrow aplasia compared to placebo controls. In these patients (>55 years old), the addition of GM-CSF not only accelerated neutrophil recovery but was also associated with a statistically significant improvement in overall survival (GM-CSF 10.6 months vs. placebo 4.8 months, $P = 0.048$).[45] Other randomized trials have failed to show a survival advantage from the use of GM-CSF although the benefit of accelerated neutrophil counts has generally been confirmed. [46]

After Autologous and Allogeneic Bone Marrow Transplantation

GM-CSF has been studied extensively in patients undergoing high-dose chemotherapy with autologous progenitor cell support. Nemunaitis et al. [47] [48] reported on the use of this agent in patients undergoing autologous bone marrow transplantation for lymphoid malignancies. Therapy with GM-CSF following reinfusion of marrow resulted in a shorter time to myeloid recovery, fewer infections, and shorter hospitalizations. This effect has been confirmed by other investigators in similar patient populations.[49] [50] Similar results have been seen in patients with solid tumors undergoing high-dose chemotherapy with autologous BMT. [51] In these settings, the dose of GM-CSF is 250 g/m^2 /day administered intravenously.

GM-CSF was evaluated for its effects in 37 patients with marrow graft failure following allogeneic (15 patients), autologous (21 patients), or syngeneic (1 patient) BMT.[52] GM-CSF was administered as a 2-hour infusion for 1421 days at doses of 601,000 g/m^2 /day. Nine of 15 allogeneic BMT patients, 11 of 21 autologous BMT patients, and 1 syngeneic BMT patient responded with increases of granulocytes to 1500 cells/l. Most patients maintained their granulocyte counts at >500 cells/l after discontinuation of GM-CSF. GM-CSF had no effect on platelet or red blood cell (RBC) counts. Fevers resolved in all responding patients. Toxicities were typical for GM-CSF and included primarily constitutional complaints.

Similar to G-CSF, the use of GM-CSF in the allogeneic BMT setting is under continued investigation. Studies reported to date suggest that GM-CSF is capable of reducing the time to myeloid engraftment without a significant impact on the development of GVHD. [53] Long-term follow-up will be required to determine whether the use of these myeloid agents will affect long-term disease-free survival.

Gianni et al.[54] were the first group to demonstrate that the use of GM-CSF after intensive chemotherapy resulted in the mobilization of PBPCs. Such grafts are highly effective following

WHICH PATIENTS SHOULD RECEIVE HGF THERAPY?

Although it is clear that both G-CSF and GM-CSF can reduce the depth and duration of neutropenia following myelosuppressive therapy, not all patients receiving chemotherapy are candidates for the use of these agents. In general, the use of hematopoietic growth factors benefits patients who are at a high risk of developing neutropenic febrile complications. Most standard regimens used in hematology/oncology do not promote sufficient neutropenia to warrant the routine use of these agents in cancer patients. Regimens associated with an incidence of febrile neutropenia <30% probably will not be affected by the addition of HGFs. In light of this, many cooperative groups as well as the National Cancer Institute are developing guidelines for the routine use of HGFs in clinical medicine.

Patients at high risk for the development of febrile neutropenic complications will generally benefit from the use of HGFs. Such patients include but are not limited to the following groups, and this list is meant to serve as a rough guide for the initiation of HGF therapy.

Patients with prior episode of febrile neutropenia in whom a dose reduction is not planned or patients with febrile neutropenia following dose reduction

Patients receiving intensive chemotherapy, either front line or salvage, with an increased incidence of febrile neutropenia complications (i.e., vinblastine, ifosfamide, cisplatin (VIP) salvage in germ cell tumors with 70% febrile neutropenia incidence)

Elderly or debilitated patients who in the judgment of the clinician will not tolerate a febrile neutropenia episode

Patients who are predisposed to severe myelosuppression (i.e., post-BMT or patients with bone marrow involvement)

For these patients, therapy with G-CSF or GM-CSF can be instituted after completion of chemotherapy.

With regard to the use of thrombopoietic growth factors, clinical judgment must be exercised since an alternative (platelet transfusions) exists for the clinical management of these patients.

GUIDELINES FOR THE USE OF CSFs

Therapy with both G-CSF and GM-CSF should be instituted approximately 24 hours after the last dose of chemotherapy. The use of G-CSF or GM-CSF concomitant with chemotherapy should still be considered investigational pending adequate data regarding toxicity of this approach.

Both G-CSF and GM-CSF can be administered by daily subcutaneous injection.

Both G-CSF and GM-CSF should be administered at a dosage of 5 g/kg/day.

The last dose of G-CSF or GM-CSF or IL-11 should precede chemotherapy by >24 hours.

Since premature discontinuation of G-CSF or GM-CSF can result in a drop in absolute neutrophil count, therapy should be continued until the absolute neutrophil count is >10,000 cells/l.

Monitor CBC and differential counts twice weekly while on therapy.

Interleukin-11 (Neumega) is FDA-approved for the prevention of chemotherapy-induced thrombocytopenia. It may be initiated within 624 hours following the completion of chemotherapy and should be continued until the post-nadir platelet count has exceeded 50,000/l.

high-dose chemotherapy. Since that time, various investigators have used GM-CSF for this purpose. [55] [56] Timing of pheresis has generally been on a schedule similar to that of G-CSF. When the growth factor is administered following chemotherapy administration, pheresis is often initiated when the ANC reaches a minimum of 1,000 cells/l, although some physicians use CD34 counts as an indication of when to harvest. [57]

Bone Marrow Failure States

GM-CSF has been studied in a series of phase I and II trials in patients with MDS. In three studies, GM-CSF was administered intravenously over a variety of doses.[58] [59] [60] Of the 26 patients studied in this manner, 23 demonstrated increases in leukocyte and granulocyte counts. Only 3 displayed any evidence of effect on other hematopoietic lineages. As in the responses seen with G-CSF, peripheral blood counts returned to pretreatment values after cessation of the administration of GM-CSF. Thompson[61] studied the effect of subcutaneously administered GM-CSF on 16 patients with MDS. Of 13 patients treated at doses of 1.0 g/kg/day, 11 demonstrated a significant increase in granulocytes that returned to pretreatment values after completion of therapy. Effects on other hematopoietic lineages were seen in only 2 patients. Progression to acute leukemia occurred in a total of 7 patients and was directly related to the pretreatment bone marrow blast percentage. The results of a randomized trial demonstrate a twofold or greater rise in granulocyte counts in 27 of 28 patients at a dose of 3.0 g/kg/day. [62] [63] No benefit on platelet counts or hemoglobin was detected, and only 1 patient on therapy progressed to acute leukemia.

The use of GM-CSF in patients with aplastic anemia has demonstrated its ability to transiently improve peripheral blood neutrophils but lacks the multilineage effect that was initially sought.[58] In addition, as in patients with MDS, responses in aplastic anemia patients are generally not durable, and counts quickly drop to pretreatment levels following cessation of therapy.

Patients with AIDS or HIV Infection

Patients with HIV infection or AIDS have received GM-CSF either to reduce neutropenia resulting from retroviral therapy or

as a component of a treatment plan in those with Kaposis sarcoma.[64] Levine et al.[65] reported on a small randomized trial in which patients who were hematologically intolerant of zidovudine (AZT) received GM-CSF either before the AZT was restarted or with continued AZT therapy. GM-CSF was administered subcutaneously with intra-patient dose escalation. All patients started at a dose of 1.0 g/kg/day and underwent dose escalation every 2 weeks to 3, 5, and 10 g/kg/day to maintain the ANC >1,000 cells/l. All patients were able to continue AZT therapy without significant neutropenia while GM-CSF was administered. No evidence of HIV proliferation (assayed by p24 antigen levels) was seen, and the regimen did not alter the ability to culture the virus from the peripheral blood mononuclear cells.

GM-CSF has not had demonstrable benefit in patients with congenital neutropenic disorders. It has been studied with some success both alone and in combination with IL-3 in children with amegakaryocytic thrombocytopenia.[66]

Erythropoietin

Erythropoietin (EPO) is a 34-kd glycoprotein whose primary function is the regulation of erythrocyte production. It is essentially lineage-specific in its effects on red blood cells. The regulation of EPO production is under the control of a simple feedback mechanism. EPO production is increased in hypoxemic conditions, and suppressed during times of hyperoxemia.[67] [68] Under normal conditions, the EPO level is maintained at approximately 1020 U/L of plasma. This level can rise exponentially when anemia develops.

The gene for EPO was initially cloned by two separate groups. Depending on the expression vector used, one can obtain either a glycosylated (Chinese hamster ovary cells) or nonglycosylated (*Escherichia coli*) form of the drug. Because native EPO is glycosylated, the mammalian cell-derived product is nearer to the natural product than the latter.[69] [70] In addition, the product derived from *E. coli* appears to have reduced in vivo activity, probably related to the lack of glycosylation.[71]

It was evident very early in the evaluation of the clinical value of EPO that it may have a significant impact on the treatment of anemia. The availability of sensitive radioimmunoassays permitted identification of disorders of EPO deficiency.[72]

Anemia of Renal Failure

Among the patients who were initially designated for the study of EPO were those with chronic renal failure (CRF). The use of EPO in this patient population is one of the most important advances in the field of clinical hematology. As one would expect, patients with nonfunctioning kidneys fail to produce EPO in sufficient amounts to support RBC production. The theory that the anemia related to CRF was at least partially due to EPO deficiency was confirmed by investigators in the United States and United Kingdom.[73] [74] Doses of 25500 U/kg three times per week were administered intravenously and resulted in a dose-dependent increase in hematocrits, while significantly reducing and even eliminating the need for RBC transfusion. In the large multicenter phase III trial reported by Eschbach et al.,[75] patients treated with EPO experienced increases in hematocrits. Those who were treated at initial dose of 300 U/kg three times per week achieved their target hematocrit within 68 weeks of therapy. Patients treated with a dose of 150 U/kg (same schedule) achieved their target hematocrit in approximately 10 weeks. The 333 patients enrolled in this study required 1,030 erythrocyte transfusions over the 6 months before study initiation. Within 2 months of EPO therapy, all patients were erythrocyte-transfusion independent and remained as such with continued EPO maintenance. The only significant toxicities related to EPO therapy were the development of iron deficiency (related to utilization), hypertension, and seizures. The hypertension related to EPO therapy is thought to be secondary to a reversal of the vasodilatory effects of anemia. In the large study by Eschbach et al.,[75] blood pressure increases were noted in 35% of previously hypertensive patients. In addition, 44% of the patients who were not hypertensive before EPO therapy experienced increases in blood pressure, with 32% requiring the institution of antihypertensive therapy. There is no specific etiology for the seizures, although the investigators note that the incidence is not significantly higher than that of an untreated group of CRF patients (EPO 5.4%, untreated 8%). The recommended starting dose of EPO for dialysis-dependent CRF patients is 50100 U/kg body weight IV three times per week. A phase IV study by Nissenson et al.[76] evaluated the treatment practices for patients receiving EPO therapy for CRF. They found that, in general, relatively low doses of EPO were being used with resulting marginal increases in hematocrit levels. This suggests that uniformity is lacking regarding the maintenance of patients with CRF on EPO. Although EPO therapy has produced improvements in hematocrit levels in patients with CRF not requiring dialysis, the degree of anemia in these patients, hence the benefit from therapy, is not great enough to warrant a universal recommendation for this group. Studies have suggested that the subcutaneous route of administration is more efficacious than the intravenous route due to the better pharmacokinetics and length of exposure.[77] Patients treated on this study were able to reduce their dose of EPO by 3050% when the drug was administered subcutaneously compared with their intravenous EPO dose.

Patients with AIDS or HIV Infection

Because of the beneficial effects of EPO therapy in patients with CRF, EPO therapy has been evaluated in other diseases. In addition to the presence of anemia related to chronic illness and opportunistic infections, therapies developed to treat HIV and its related infections are associated with hematologic toxicity. Retroviral therapy, particularly AZT, is associated with the development of significant anemia, which in many patients is the dose-limiting toxicity.[78] Henry et al.[79] reported on four randomized placebo-controlled trials evaluating the effect of EPO on the anemia of HIV infection and AZT therapy. In these studies, patients with endogenous EPO levels of <500 U/L experienced a statistically significant increase in hematocrit and a reduction in RBC transfusion requirements compared with placebo-treated controls. This benefit was associated with an overall improved sense of well-being. Patients with endogenous EPO levels of >500 U/L obtained no benefit. No significant effect of EPO therapy on the incidence of opportunistic infections was found. EPO has subsequently been further studied and has been approved by the FDA for use in this patient population.

Cancer Patients Receiving Chemotherapy

Use of EPO is expanding in patients with malignancies who are receiving chemotherapy. The anemia that develops in these patients is very similar to that of the anemia of chronic disease (i.e., an anemia in the presence of increased circulating levels of EPO). Many cancer patients have elevated levels of circulating cytokines such as TNF, which are negative regulators of hematopoiesis. In addition, chemotherapy, particularly with platinum analogues, is often associated with the development of progressive anemia.[80] [81] Miller et al.[82] previously demonstrated that an impaired EPO response is at least partially responsible for the anemia of cancer and is worsened with subsequent chemotherapy. EPO administration has been studied in patients with malignancies in various settings. Randomized trials have been performed studying EPO in patients receiving chemotherapy. They have demonstrated a reduction in the transfusion requirements,

particularly in platinum-treated patients.[83] [84] This has translated not only into an improvement regarding the ability to reduce red blood cell transfusion requirements but also into an improvement in the overall quality of life of cancer patients.[85] An important issue regarding patients receiving EPO for chemotherapy-induced anemia centers around the timing of initiation of therapy. Early initiation is critical to achieve maximal efficacy. Henry and Glasby have studied prognostic factors in patients with chemotherapy-induced anemia.[86] Early initiation (prior to the need for transfusions), a low observed/predicted endogenous EPO level (<0.825) and evidence for early response (>1 gram rise in hemoglobin within the first 4 weeks) were all associated with increased likelihood of response. Patients meeting all three of these criteria demonstrated an 85% chance of major (>2 gram rise in hemoglobin) response. The use of EPO for the treatment of anemia in patients not receiving chemotherapy has also been studied. In both multiple myeloma and other malignancies with bone marrow infiltration, EPO therapy has resulted in increases in hematocrit levels and reduction in the need for RBC transfusions.[87] [88] As in the anemia of malignancy, the etiology of the anemia associated with diseases such as rheumatoid arthritis is multifactorial. Because one component of the development of anemia in this patient population is a blunted EPO response, the use of EPO has become a therapeutic option in these patients.[89]

Bone Marrow Failure States

Primary bone marrow failure states are often associated with anemia, requiring frequent transfusions and therefore presenting the risk of developing iron overload. Among these, the MDSs have been most aggressively studied with regard to the role of EPO therapy. Circulating EPO levels have been studied in patients with MDS.

Jacobs and coworkers[90] found an inverse relationship between EPO level and degree of anemia. They noted, however, that among patients with the same hemoglobin concentrations, a wide variation existed in EPO levels. Many investigators have studied EPO therapy in patients with MDS. Overall, the results have been disappointing.[91] [92] Responding patients tend to be those who have extremely low EPO levels (<100 U/L). Even at high doses of EPO, although responses can be seen, they are often not durable.[93] [94] EPO alone or with G-CSF[95] has been studied in patients with aplastic anemia with some improvement in RBC transfusion requirements noted. A randomized phase II trial conducted by Hellstrom-Lindberg et al. studied sequential vs. simultaneous administration of EPO and G-CSF. [96] The overall erythroid response was similar, though half of the responding patients in the sequential arm responded to initial EPO administration. The additional responding patients did so following addition of G-CSF therapy. Finally, Hellstrom-Lindberg et al. have performed a meta-analysis of EPO trials in MDS. Patients initiated on therapy prior to requiring RBC transfusions, those with low blast percentages (<5%), and those with low endogenous EPO levels responded best. [97]

Autologous Blood Donation

The use of EPO to prime for autologous RBC donation has been perceived as a major step toward reducing the risks associated with blood transfusions. While the actual risk of contracting a viral infection from a unit of RBC has dropped significantly with improved testing, it still remains a concern. The use of EPO preoperatively has been demonstrated to promote an increase in the number of autologous units of blood donated. In a randomized, placebo-controlled, double-blind trial reported by Goodnough et al.,[98] the mean number of units collected from the EPO-treated group (600 U/kg twice weekly) was 5.4 ± 0.2 compared with 4.1 ± 0.2 in the placebo arm. In addition, the preoperative hematocrits in the EPO-treated patients were 3.4% higher (38.6 versus 35.2) than those of the placebo-treated patients. In a second study, the administration of EPO preoperatively has been shown to increase the amount of blood donated by patients by about 40%. [99] Results of a randomized trial conducted by the Canadian Orthopedic Perioperative Erythropoietin Study Group demonstrated that patients who received EPO preoperatively had a statistically significantly lower incidence of requiring any blood transfusions or of having a hemoglobin level <8 g/dl following hip replacement surgery. [100] Preoperative EPO therapy reduces the need for RBC transfusions and provides a method for autologous red cell donation. EPO is approved for priming for autologous donation as well as for preoperative administration in patients undergoing orthopedic surgical procedures.

Anemia of Prematurity

The use of EPO for the treatment of the anemia of prematurity has been widely studied. In this disorder, EPO levels are low and are not appropriately elevated in response to the anemia. This disorder is usually self-limiting and resolves spontaneously after 12 months. A pilot study with EPO appeared to demonstrate promise for the treatment of this disorder.[101] In a randomized double-blind study, however, Shannon et al.[102] showed no significant benefit from EPO treatment. Although treated patients experienced a more rapid rise in reticulocytes, untreated patients spontaneously recovered, precluding a clinical benefit. This topic has been reviewed elsewhere.[103]

Stem Cell Factor

Stem cell factor (SCF) is the ligand for the proto-oncogene receptor c-kit. [104] [105] [106] Also known as the c-kit ligand, mast cell growth factor, and steel factor (SLF) SCF has the ability to stimulate the proliferation of cells that bear its receptor. Cells that bear the c-kit receptor are ubiquitous, with expression on most normal tissue including melanocytes and gonadal cells. In particular, c-kit is present on the hematopoietic stem cell, and acts to stimulate the proliferation and, in concert with other HGFs, the differentiation of these early cells. The recombinant protein is 165 amino acids long and has a molecular weight of 38 kD. SCF as a single agent appears to have only modest effects on the in vitro growth of hematopoietic progenitors (see Chap. 14). However, when combined with other agents, such as IL-1 or IL-3, or with more lineage-specific factors, such as G-CSF, GM-CSF, IL-6, and EPO, SCF has potent synergistic effects on the proliferative capacity of these cells. [107] [108] [109] [110] Although the receptor for SCF is present on malignant cells, no clear evidence shows that SCF actually stimulates the growth of tumor cells, with the exception of myeloid leukemia cells.[111] [112] SCF may be able to induce apoptosis in some leukemic blasts.[113]

SCF has been studied in several preclinical animal models. The ability of SCF to stimulate the mobilization of hematopoietic progenitors into the peripheral blood is of primary importance. Initial studies by Andrews et al.[114] demonstrated that PBPCs mobilized by SCF at a dose of 200 g/kg/day for 11 days have the ability to engraft and rescue lethally irradiated baboons. This was compared with nonprimed mononuclear cells that failed to engraft in control animals, resulting in their death. Subsequent work by the same investigator has demonstrated that a significantly lower dose of SCF is required (20 g/kg/day) if a standard dose of G-CSF is co-administered to baboons before pheresis.[115] The apparent synergistic interaction between these two agents has led to the investigation of the cytokine combination for use as a stem cell mobilizing regimen.

Clinical Applications

The initial phase I trials of SCF were performed in patients with metastatic breast cancer and nonsmall-cell lung cancer respectively. [116] [117]

In each of the studies, patients received a trial of SCF administration before chemotherapy as part of the study of effects of SCFs on unperturbed hematopoiesis.

In each trial, doses of 10, 25, and 50 g/kg/day were studied. SCF alone demonstrated a modest dose-related increase in peripheral blood neutrophils. Less substantial increases at the higher doses were seen in platelets and reticulocytes, suggesting a possible multilineage effect. At the doses studied, no significant increases in bone marrow cellularity were found. SCF, however, did have substantial effects on bone marrow hematopoietic elements. Immunohistochemical stains of bone marrow biopsies demonstrated significant increases in numbers of cells expressing CD34 as well as Ki-67 and proliferating cell nuclear antigen (PCNA), a proliferation-associated antigen.[118] These results suggest that even at low doses SCF has the ability to stimulate the proliferation of hematopoietic stem cells. This effect was further investigated by Tong et al.,[119] who performed flow cytometric phenotyping of bone marrow and demonstrated statistically significant increases in various subsets of CD34+ cells, including CD34+DR+ and CD34+DRCD15 cells; the latter are believed to include the human hematopoietic stem cell. Additional assays demonstrated significant increases in the number of both primitive (high proliferative potential-colony-forming cells [HPP-CFC], burst-forming unit-megakaryocytes [BFU-MK], and long-term culture-initiating cells [LTC-IC]), as well as committed (colony-forming unit [CFU]-GM, CFU-GEMM, BFU-erythroid [E], and CFU-Mk) progenitors following therapy with SCF. Studies evaluating the ability of SCF to mobilize progenitors into the peripheral blood demonstrated mild increases in the number of BFU-E and CFU-GM at the highest dose levels studied. [119]

Toxicities in these studies were composed primarily of allergic-like reactions related to the ability of SCF to prime mast cells for degranulation. Dose escalation was limited in each study by respiratory (throat tightness, laryngospasm) and/or cutaneous toxicity (systemic urticaria) to 50 g/kg/day. Treatment was delivered by subcutaneous injection daily for 14 days. The effects of SCF following chemotherapy have only been preliminarily reported in the lung cancer study. While a trend toward earlier recovery of neutrophils and platelets was seen at the highest dose, it is unlikely that this agent will have a major impact on postchemotherapy recovery.

Two large phase III trials evaluating the ability of the SCF/G-CSF combination compared to G-CSF alone to mobilize PBPC have been completed and reported. [120] [121] Initial phase I trials demonstrated the ability of the combination to mobilize adequate numbers of progenitors such that a single standard apheresis was adequate to obtain enough PBPC for a course of high-dose chemotherapy. Unlike the phase I trials, severe allergic reactions were not seen in these trials. This was either due to the well-developed prophylactic anti-allergy regimen (H_1 and H_2 blocks with a bronchodilator) or a synergistic interaction between the two HGFs. The phase II/III doses were G-CSF 10 g/kg/day with or without SCF 20 g/kg/day. Both drugs were administered subcutaneously with apheresis performed starting on day +5 and continuing until the target yield of CD34+ PBPC had been obtained. The combination of SCF/G-CSF resulted in a significant increase in the number of patients who achieved the target yield following five aphereses (67% vs. 48%) and the median number of aphereses needed was statistically significantly fewer (3 vs. 6, $P < 0.01$) for the combination. In the second study, heavily pretreated lymphoma patients were mobilized either with the combination or G-CSF alone. Again, the combination of SCF/G-CSF resulted in a statistically significant increase in the number of successfully pheresed patients. Engraftment of the SCF/G-CSF mobilized progenitor cells appears to be at least as good as that of those mobilized by other more standard methods.

The use of SCF to prime bone marrow in vivo before bone marrow harvest is another interesting potential use of this agent. [119] Unquestionably, the two other major areas of interest for this factor are its use in ex vivo expansion of hematopoietic progenitor and stem cells and in patients with bone marrow failure states. Ex vivo expansion of bone marrow could theoretically replace the need to harvest large numbers of PBPCs or bone marrow progenitor cells. Multiple studies of this technique have demonstrated substantial increases in progenitor cells affording the opportunity to generate transplantable quantities of progenitors in the laboratory from a small volume of bone marrow.[122] Initial clinical trials using this approach have been reported. [122] All these studies have demonstrated the importance of SCF as a component of the cytokine cocktail needed for maximal expansion. In addition, SCF has been shown to increase progenitor numbers in vitro from patients with various

bone marrow failure syndromes.[123] A phase I trial of SCF in patients with aplastic anemia demonstrated that in patients treated at doses ranging from 530 g/kg/day, SCF was capable of generating modest trilineage responses in approximately one-third of patients treated with refractory immunosuppressive therapy. [124]

PIXY321

PIXY321 is a genetically engineered fusion protein that links the genes encoding IL-3 and GM-CSF by a flexible amino acid linker. [125] In vitro, PIXY321 appears to have equal, if not superior, effects on hematopoiesis compared with the combination of IL-3 and GM-CSF as individual agents. [125] [126] Preclinical animal studies have demonstrated that PIXY321 has the ability to effect multilineage responses following the administration of radiotherapy. [127]

After Chemotherapy

Several phase I clinical trials with PIXY321 were intially conducted and demonstrated a favorable safety profile for the agent. Vadhan-Raj et al. [128] studied effects of PIXY321 on patients with normal hematopoiesis in patients with sarcoma where PIXY321 was administered subcutaneously in two divided doses (251000 g/m ^{2} /day) for 14 days. PIXY321 treatment was associated with modest increases in peripheral blood WBC count, neutrophils, and platelets. Bone marrow biopsy specimens demonstrated a significant increase in cellularity. In addition, treatment with PIXY321 resulted in increases in assayable committed progenitors, including CFU-GM, CFU-GEMM, and BFU-E, as well as the number of circulating CD34+ cells. At the doses studied, toxicity in this study consisted primarily of local erythema at the injection site.

The effect of PIXY321 on hematologic recovery after the administration of chemotherapy has been studied by a variety of investigators. As a part of their phase I trial in sarcoma patients, Vadhan-Raj et al.[129] examined the effects of PIXY321 following cyclophosphamide, doxorubicin, dacarbazine (CyADIC) chemotherapy. These investigators found that the administration of PIXY321 at doses of >500 g/m ^{2} /day resulted in a reduction in mean nadir neutrophil counts following chemotherapy cycles in which PIXY321 was administered compared with those without PIXY321 therapy (90 cells/l vs. 380 cells/l). The duration of neutropenia was also reduced from 6.7 to 3.1 days by PIXY321. Mean nadir platelet counts were also improved with PIXY321 therapy at doses of >500 g/m ^{2} /day with a reduction in the development of cumulative thrombocytopenia after two cycles of chemotherapy. Small numbers of patients preclude drawing statistical conclusions, but comparison to historical controls treated with GM-CSF reportedly demonstrates trends toward reduced myelosuppression. Additional phase I trials demonstrated less impressive results. Two randomized, placebo-controlled phase II or III trials have been completed. The

first, in relapsed/refractory lymphoma patients, failed to demonstrate a benefit of PIXY321 compared to G-CSF. [130] In the second study, a trial of breast cancer patients receiving 5-fluorouracil, leucovorin, doxorubicin, cyclophosphamide (FLAC) chemotherapy, a superior outcome was seen for patients receiving GM-CSF and not PIXY321.[131]

After Autologous Bone Marrow Transplantation

Initial phase I results in lymphoma patients receiving PIXY321 after transplant suggested a superior outcome compared to historical controls treated with GM-CSF. A subsequent phase III trial comparing GM-CSF to PIXY321 failed to demonstrate a benefit for the investigational agent. [132] A phase I/II trial assessing the mobilization activity of PIXY321 demonstrated only modest activity and this indication was not pursued. [133]

Given the failure of PIXY321 to produce any positive phase II/III data in randomized trials, clinical development with this agent was discontinued.

Interleukin-1

Interleukin-1 consists of two forms, IL-1 and IL-1, that exert their effects via a common receptor. In vitro studies with both agents have demonstrated their ability to stimulate the proliferation of early hematopoietic progenitors and produce a multilineage effect. In addition, their administration in preclinical models results in enhanced hematopoietic recovery following both chemotherapy and radiotherapy-induced myelosuppression. [134] [135] [136]

Clinical Applications

Clinical studies have been performed with both IL-1 and IL-1. The initial phase I trial with IL-1 by Crown et al. [137] demonstrated that a short infusion of IL-1 resulted in a dose-dependent increase in WBC counts (primarily neutrophils) within the first hours of administration. A delayed thrombopoietic effect of IL-1 was seen approximately 34 weeks after administration. Whether this increase in platelet count is a direct effect of IL-1, or indirect through the induction of secondary cytokines, remains unanswered. The major toxic events encountered primarily constitutional symptoms at the lower doses; however, hypotension became the dose-limiting toxicity at a dose of 0.1 g/kg/day. The administration of IL-1 following 5-fluorouracil chemotherapy in subsequent cycles of treatment demonstrated no significant impact on altering chemotherapy-induced leukopenia or thrombocytopenia at the dose levels studied.

IL-1 has been evaluated in various clinical trials as an antitumor agent. In a phase I trial performed by Smith et al. [138] doses of >0.3 g/kg/day were administered by rapid intravenous infusion. Therapy was tolerated, but the principal side effect at these doses was hypotension requiring vasopressor support. Nonetheless, a dose-related increase in WBCs (primarily neutrophils) during therapy, as well as increments in platelet counts occurring 12 weeks after completion of therapy were seen. Serum levels of IL-6, a factor with known thrombopoietic effects, were increased following treatment with IL-1, raising the question of whether the thrombopoietic effects of IL-1 are actually mediated through the induction of IL-6. Regardless, because of the hematologic effects seen in this initial trial with IL-1, a subsequent study was performed in which IL-1 was administered following intensive carboplatin chemotherapy in patients with advanced cancer. [139] In this study, 43 patients received carboplatin at a dose of 800 mg/m^2 . Patients were treated with one of three doses of intravenous IL-1, ranging from 0.030.3 g/kg/day for 3 days. Various schedules of IL-1 administration were used. These patients were compared with a control group who received chemotherapy alone. Treatment with IL-1 resulted in significant blunting of the platelet and WBC nadirs after chemotherapy. The optimal schedule was the administration of IL-1 after the completion of chemotherapy, in an optimal dosage of 0.10.3 g/kg/day. Toxicity was significant, consisting of reversible hypotension (requiring intensive care unit monitoring and vasopressor support), supraventricular tachycardias, and capillary leak syndrome.

After High-Dose Chemotherapy and Progenitor Cell Support

IL-1 has been investigated regarding its potential to accelerate engraftment following high-dose chemotherapy and autologous progenitor cell support. An initial dose escalation study conducted by Vredenburgh et al.[140] examined the effect of 7 days of subcutaneously administered IL-1 following reinfusion of progenitor cells. Doses of IL-1 of 432 ng/kg/day were studied. Of 14 patients, 7 experienced local pain at the injection site; 1 patient, at the highest dose studied, had severe hypotension. No benefit regarding accelerated hematologic recovery was noted, although IL-1-treated patients were found to have greater numbers of bone marrow CFU-GM progenitors on day +21. Similar results and toxicities were seen by Nemunaitis et al. [141] when IL-1 was administered by short intravenous infusion.

Weisdorf et al.[142] studied IL-1 following high-dose chemotherapy and autologous BMT. Patients received IL-1 for 14 days after reinfusion of autologous progenitor cells at doses of 0.110 g/m^2 /day by 6-hour intravenous infusion. Dose-limiting toxicity was hypotension, seen at the 10-g/m ^{2} dose level. Engraftment of neutrophils (ANC >500 cells/l) occurred at a median of 24 days for historical controls, and patients receiving IL-1 at 0.11.0 g/m ^{2} /day compared with 13 days for patients treated with IL-1 at 3.0 g/m^2 /day. Similar trends for platelet engraftment were seen.

In a small study by Walsh et al. [143] IL-1 was administered intravenously in 5-day courses to four patients with severe refractory aplastic anemia. No significant changes occurred in peripheral blood counts or bone marrow morphology associated with the IL-1 therapy. Similar results were seen by Nemunaitis et al. [144]

Although both IL-1 and IL-1 are both potentially capable of reducing the myelosuppressive effects of chemotherapy, neither appears to exert these effects at doses that have been well tolerated. Attempts at using agents such as indomethacin to reduce the hypotension of IL-1 have proved ineffective. [138] Because of its unacceptable toxicity, both forms of IL-1 have been removed from clinical development.

Interleukin-3

IL-3 is the first of the early-acting hematopoietic growth factors to be studied broadly in various clinical trials. IL-3 has its action on early uncommitted progenitor cells and has been shown to act synergistically with various other HGFs, such as GM-CSF, G-CSF, and EPO to support in vitro colony formation. [145] [146] In vivo, IL-3 has been shown to increase WBCs, RBCs, and platelets in normal animals, and to enhance the hematologic recovery of all lineages following chemotherapy or radiotherapy. IL-3 has a molecular weight of 1428 kd and is encoded for by a gene on chromosome 5q23-q31.

Patients with Refractory Malignancy and After Chemotherapy

The multilineage effects of IL-3 in patients with malignancy and following chemotherapy have been the focus of clinical investigation. As a single agent in patients with normal bone marrow, IL-3 results in a dose-dependent increase in neutrophil and

platelet numbers over doses of 0.2510 g/kg/day.[147] Lindemann et al. treated 30 patients with refractory malignancies.[148] IL-3 was administered as a single intravenous bolus on day 1, followed by 14 days of subcutaneously administered IL-3 (days 215). Doses studied were in the range of 60500 g/m ^{2} /day. Dose-dependent increases in WBC and neutrophil counts were seen. Platelets were mildly increased (twofold). Interestingly, significant increases in circulating numbers of basophils and eosinophils were seen. Toxicities were primarily constitutional in nature and consisted of mild fever and headache that were clinically significant. Therefore, Bhatia et al. added propranolol to the IL-3 therapy to ameliorate the headache associated with IL-3. [149] Doses of 604,000 g/kg were evaluated. Hematologic effects were similar to the previously noted trial. No grade III or greater toxicity (including headache) or antitumor activity was reported in either of these studies. The effects of IL-3 on bone marrow and PBPCs were reported by Ottmann et al.[150] These investigators studied the effect of IL-3 administered for 15 days in various patients with normal and abnormal hematopoiesis. Patients were treated with subcutaneous doses of 60500 g/m ^{2} /day. Significant increases in peripheral blood CFU-GM and CFU-GEMM over baseline values were seen after 7 days of therapy (100% and 72% increased respectively). These effects were not seen after the second week of therapy (25% and 28%, respectively). Peripheral blood BFU-E were reduced in nearly all patients with normal hematopoiesis. Cycling rates of progenitors in the bone marrow were increased with therapy, as was bone marrow cellularity. Again, dose-dependent increases in peripheral blood WBC, neutrophil, and eosinophil counts were seen, with peak values at or about day 13 of treatment.

Several studies have been performed using IL-3 as a single agent after chemotherapy. [151] [152] [153] In phase I studies, IL-3 has tolerable side effects below doses of approximately 15 g/kg/day. Above this level, headache and other constitutional complaints have been dose-limiting. Other toxicities seen at lower dose levels include fever, malaise, fatigue, arthralgias, and myalgias. Overall these studies demonstrate that IL-3 does not appear to alter the degree of neutropenia or thrombocytopenia but may, at higher doses, hasten the recovery of these nadirs. At least in one study by Biesma et al., [154] this effect appeared to be associated with a reduction of platelet transfusions. The impact of IL-3 as a single agent, however, appears to be minimal, since equivalent or greater neutrophil effects can be obtained with either G-CSF or GM-CSF, and dose escalation of IL-3 to doses that might affect platelet numbers appears to be limited due to toxicity.

Combinations of HGFs such as G-CSF or GM-CSF with IL-3 have been pursued. Preclinical data suggest that sequential, as opposed to simultaneous, administration of IL-3 with GM-CSF results in increased platelet production via enhanced megakaryocyte maturational effect. [155] Combination HGF therapy, including IL-3, in humans has been studied after chemotherapy. Brugger et al.[152] reported the clinical results of sequential treatment, with 5 days of IL-3 (days 15) and 10 days of GM-CSF (days 515) following intensive VIP chemotherapy. This combination and schedule resulted in neutrophil effects similar to those of GM-CSF alone with additional platelet effects seen only in those patients who were previously heavily pretreated. Other combination trials of IL-3 with G-CSF have been reported with similar results.[156]

After High-Dose Chemotherapy

The use of IL-3 after high-dose chemotherapy and autologous progenitor cell transplants has been investigated. Nemunaitis et al. administered IL-3 as a 2-hour infusion following reinfusion of bone marrow. [157] Doses of 110 g/kg/day were studied; however, doses of >5 g/kg/day were not well tolerated due to constitutional side effects. Compared to historical controls treated with GM-CSF, IL-3 appeared to produce neutrophil recovery at a rate similar to that of GM-CSF. Combinations of IL-3 with later-acting factors have been studied in the autologous BMT setting. Fay et al. performed an initial phase I trial of sequential IL-3 and GM-CSF in patients undergoing autologous BMT for lymphoma.[158] Patients received IL-3 at a dose of 2.5 g/kg/day as a 2-hour intravenous infusion, or subcutaneously. This was followed by GM-CSF at 250 g/m^2 /day as a 2-hour infusion until hematologic recovery. Rapid engraftment was seen in the few patients treated with a median ANC recovery to >1,000 cells/l in 12.5 days. The phase III trial of sequential IL-3/GM-CSF performed by Fay and colleagues failed to demonstrate an advantage for the combination over standard therapy alone.[159]

Other

The multilineage effects of IL-3 make it a potential agent to study alone and in combination with other cytokines in patients with pancytopenia. Ganser et al. studied the effects of subcutaneously administered IL-3 in doses of 250500 g/m ^{2} /day in nine patients with the myelodysplastic syndromes (six refractory anemia, three refractory anemia with excess blasts).[160] Patients were treated for 15 days. Increases in leukocyte counts (consisting of neutrophils, eosinophils, basophils, lymphocytes, and monocytes) were seen in all patients. Increments in platelet numbers occurred in two severely thrombocytopenic patients, while RBC transfusion requirements decreased in one other patient. Therapy was tolerated with acceptable constitutional symptoms. Only one patient experienced an increase in the number of blasts. Other trials with IL-3 in patients with MDS have demonstrated similar findings, with some patients experiencing short-lasting multilineage responses.[161]

While patients with MDS appear to obtain multilineage responses to IL-3, patients with aplastic anemia tend to have less significant hematologic responses. Ganser et al. treated nine patients with aplastic anemia with subcutaneous IL-3 administered for 15 days at doses of 250500 g/kg/day. [162] A platelet response was seen in one patient, and transient leukocyte responses were seen in several others. Two patients were found to have significant increases in bone marrow cellularity without significant increases in peripheral blood counts. This suggests that although IL-3 is capable of stimulating increases in early bone marrow cells, additional factors may be required to produce maturation and peripheral mobilization of these cells. Kurzrock et al. [163] demonstrated similar findings in their phase I trial, which included eight patients with aplastic anemia.

Other uses of IL-3 that have been explored include its use as a single agent in patients with HIV infection. In a phase I trial, Scadden et al. reported on 11 HIV-positive patients who were treated with 14 days of subcutaneous IL-3.[164] Although no significant hematologic improvements were seen, treatment with IL-3 was not associated with increases in HIV replication as measured by HIV p24 antigen levels of quantitative virus cultures.

The failure of IL-3 to demonstrate any positive data from controlled randomized trials resulted in its removal from clinical investigation.

Interleukin-6

IL-6 is a 2130-kd protein that was initially characterized and independently identified by multiple investigators. Investigators have identified this protein as B-cell-stimulating factor-2,[165] IFN-2,[166] hepatocyte-stimulating factor, [167] hybridoma growth factor,[168] T-cell replacement factor,[169] and monocyte granulocyte inducer-2.[170] By international convention, all these factors are now referred to as IL-6.

IL-6 expression and production is increased by various other cytokines, such as IL-1 and TNF, as well as in response to a large number of conditions, including chronic inflammation, autoimmune diseases, and sepsis.[171] [172]

The hematopoietic effects of IL-6 have been well studied in vitro, where its primary effect is on megakaryocyte maturation. IL-6 is a potent megakaryoctye

maturational factor that has demonstrated the ability to increase the number of megakaryocyte progenitors (CFU-Mk) in vitro. [173] Mei et al. demonstrated that IL-6 bioactivity in long-term bone marrow cultures correlated directly with megakaryocyte size and ploidy. [174] However, there was no correlation between IL-6 bioactivity and megakaryocyte number, suggesting that IL-6 acts not to increase megakaryocyte numbers, but rather to influence the maturation of these cells.

IL-6 also has been shown to have the ability to stimulate the generation of granulocyte, granulocyte/macrophage, and megakaryocyte progenitor colonies. [175] These effects, although seen with IL-6 alone, are significantly enhanced by the addition of other cytokines such as IL-3 or GM-CSF. [176] In addition, IL-6 appears to stimulate the proliferation of both murine and human stem cells. These data suggest that in addition to a lineage-specific effect on megakaryocytes, IL-6 may also be an early-acting factor with the ability to activate progenitor cells. [177] [178] [179]

Preclinical studies in both mice and nonhuman primates demonstrated that treatment with IL-6 results in significant increases in peripheral blood platelet counts. [180] [181] [182] The effects of IL-6 on thrombopoiesis appear to be mediated by its action on megakaryocyte maturation. This conclusion is based on the observations that IL-6 increases megakaryocyte ploidy and that the maximal elevation in platelet counts is delayed, occurring primarily after completion of IL-6 therapy. Unlike the effects of G-CSF and GM-CSF on neutrophils, IL-6 does not appear to function as a platelet-releasing factor. IL-6 therapy results in the induction of acute-phase proteins, including C-reactive protein, fibrinogen, and haptoglobin. Following chemotherapy or irradiation, IL-6 enhances the hematologic recovery, thereby reducing the duration of myelosuppression.

Clinical Applications

Thrombopoietic Activity

Clinical studies with IL-6 have been completed and have generally been disappointing with regard to its thrombopoietic activity. In initial phase I trials, IL-6 was studied in patients receiving chemotherapy for sarcoma or advanced malignancies. [183] In the first study, patients were treated with IL-6 before the initiation of chemotherapy to study the effects of this agent on normal hematopoiesis. Patients received 10 days of IL-6 by daily subcutaneous injections at doses of 1.025.0 g/kg/day. IL-6 therapy resulted in significant dose-dependent increases in platelet counts that peaked following completion of the IL-6 therapy. Toxicities of IL-6 included fevers, chills, malaise, fatigue, and a transient therapy-related anemia. This anemia characteristically developed early after the initiation of IL-6 therapy and rapidly resolved after completion of treatment. Plasma volume and chromium-labeled red cell mass studies have confirmed that these effects are related to plasma volume expansion.[184] Dose-related increases in C-reactive protein and fibrinogen were consistent with the known effects of IL-6 on acute-phase proteins; elevations in these parameters can serve as an indicator of IL-6 activity.

The complicating issue regarding the clinical development of IL-6 is related to its poor safety profile. At doses >5 g/kg/day, severe constitutional toxicites result in intolerance to the agent. Unfortunately, it is at these doses that IL-6 appears to be most effective in inducing thrombopoiesis. While single-arm studies at doses of 10 g/kg/day suggested some potentially clinically relevant activity, [185] randomized phase III trials in patients with breast cancer, performed at much lower doses (1 g/kg/day), failed to demonstrate any meaningful thrombopoietic activity. [186] A similar lack of activity was seen for IL-6 whether derived from *E. coli* or mammalian cells. The lack of clinically significant thrombopoietic activity, resulted in IL-6 being abandoned for this indication.

IL-6 was also studied in patients with myelodysplasia and thrombocytopenia. In a phase I trial, 22 patients with refractory anemia, refractory anemia with ringed sideroblasts, or chronic myelomonocytic leukemia with <5% bone marrow blasts were treated with doses of IL-6 ranging from 1.05.0 g/kg/day. [187] Dose-limiting toxicity of malaise and fatigue was reached at 5.0 g/kg/day, and the maximum tolerated dose in this population was 3.75 g/kg/day. Of 22 patients, 5 had transient (3 patients) or lasting (2 patients) responses to IL-6 therapy. Only 1 patient demonstrated progression of MDS to acute leukemia.

Anti-tumor Activity

In addition to its hematopoietic effects, IL-6 has potential anti-tumor activity. IL-6 is synergistic with IL-2 in the generation of cytotoxic T lymphocytes. [188] This action is possible mediated by the ability of IL-6 to up-regulate the expression of the IL-2R on thymocytes. [189] Mulé et al. demonstrated that IL-6 therapy results in the regression of established pulmonary metastases in mice that appears to be mediated via both CD4+ and CD8+ lymphocytes. [155]

Weber [190] treated 11 patients with advanced malignancies with daily subcutaneous injections of IL-6. Patients were treated on an every-other-week schedule for 3 weeks at doses of 3, 10, and 30 g/kg/day. Dose-limiting toxicity of hepatotoxicity and cardiac arrhythmias precluded further dose-escalation by this route of administration. Other side effects included fevers, chills, and malaise. Although no anti-tumor responses were reported in this study, peripheral blood immunophenotyping did demonstrate an up-regulation in the expression of the low affinity IL-2R on CD3+ cells, suggesting the activation of peripheral blood lymphocytes. The Cytokine Working Group performed a phase II trial of IL-6 in patients with metastatic renal cancer that failed to demonstrate any clinically significant anti-tumor activity.[191]

Interleukin-11

IL-11 is a 199-amino-acid polypeptide that was initially cloned from an immortalized and IL-1induced primate bone marrow stromal cell line, PU-34. The human gene was subsequently cloned from a fetal pulmonary fibroblast cell line. [192] IL-11 has effects similar to those of IL-6 and is capable of stimulating the proliferation of the IL-6-dependent plasmacytoma cell line, T1165. IL-11 has the ability in vitro to synergize with other early-acting hematopoietic factors, including IL-3. Studies performed by Yin and Yang demonstrated that IL-11 uses the same signal transduction pathway (GP-130) as IL-6, leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF) but appears to operate through an independent receptor. [193] [194] In preclinical models, IL-11 results in enhanced recovery of platelets as well as neutrophils after both chemotherapy and lethal irradiation following the infusion of progenitor cell transplants. [195] The preclinical data have been extensively reviewed.[195]

IL-11 has a variety of other interesting activities defined in preclinical models, such as its ability to inhibit adipogenesis and prevent the development of chemoradiotherapy-induced mucositis.[195] Keller et al. demonstrated that IL-11 has the ability to promote myelopoiesis in long-term bone marrow cultures. [196] This effect may be mediated by the inhibition of adipogenesis and promotion of stromal cell and macrophage development by IL-11. These findings could be relevant for the treatment of various bone marrow failure states in which bone marrow microenvironmental

defects could play a role in the pathogenesis of the disorders. The prevention of mucositis extends to IL-11s ability to prevent and/or decrease the degree of inflammatory bowel disease in a variety of models.[197] At low doses, IL-11 has anti-inflammatory activity that may be related to these activities.

Clinical Applications

The phase I trial of IL-11 has been performed in women with locally advanced or metastatic breast cancer. [198] [199] Patients received IL-11 at doses of 10100 g/kg/day. Therapy was administered initially by daily subcutaneous injection for 14 consecutive days during a 28-day prechemotherapy period. Following completion of the prechemotherapy portion of the study, all patients received IL-11 at their assigned dose level after each of four cycles of chemotherapy. Chemotherapy consisted of cyclophosphamide (1,500 mg/m^2 /day) and doxorubicin (60 mg/m^2 /day) given on day 1 of each 28-day cycle. IL-11 was administered for 12 consecutive days on days 314.

Toxicity of IL-11 primarily included reversible grade 2 fatigue, myalgias, arthralgias, and edema of the lower extremities, seen in all patients treated at 75 g/kg/day. In addition, a transient therapy-related anemia which is similar in character to that described for IL-6 and is associated with a similar plasma volume expansion was seen at all doses studied. IL-11 therapy was associated with a dose-related increase in mean peak platelet counts over baseline of 76%, 93%, 108%, 185% (for doses of 10, 25, 50, and 75 g/kg/day, respectively). For all doses studied, maximum platelet counts occurred after the completion of IL-11 therapy. No effect was seen on the WBC count. Acute-phase proteins, including C-reactive protein, haptoglobin, and fibrinogen, were increased at all doses. Platelet aggregation studies performed pretreatment and after 14 days of IL-11 demonstrated no therapy-related changes.

IL-11 administered after chemotherapy was associated with a similar toxicity profile. Compared to patients at the 10-g/kg/day dose level, patients treated at doses of

25 g/kg/day experienced less thrombocytopenia. In addition, cumulative thrombocytopenia appeared to be attenuated by IL-11 therapy. No effect of IL-11 on neutrophil nadirs was seen. A similar therapy-related anemia was seen during chemotherapy cycles. Subsequently, two randomized, placebo-controlled phase II trials have been conducted.

IL-11 was subsequently studied in two randomized, placebo-controlled phase II trials. [200] [201] In the first of these trials, a secondary prophylaxis study, IL-11 at either 25 or 50 g/kg/day or placebo was administered to patients with a history of severe thrombocytopenia. [200] Patients were required to receive the identical doses of chemotherapy they received in the previous nonstudy cycle. For all patients evaluable, only one patient (4%) of the placebo groups did not require a platelet transfusion compared to 30% of the IL-11 patients treated at the 50 g/kg dose level ($p < 0.05$). A dose-related effect was suggested in that the 25 g/kg dose resulted in 18% of patients not requiring a platelet transfusion (p = NS compared to placebo). In addition to the improvement in the number of patients in whom severe thrombocytopenia developed, there was a trend toward fewer transfusion episodes in the IL-11-treated patients compared to placebo, though this difference was not statistically significant.

In the second phase II trial, women with breast cancer (stages 2, 3, and 4) received dose-intensive chemotherapy (cyclophosphamide 3,200 mg/m ^{2} and doxorubicin 75 mg/m^2) with placebo or IL-11 (50 g/kg) following each of two blinded cycles of therapy. [201] Only 15 of 37 patients (41%) in the placebo arm did not develop severe thrombocytopenia compared to 68% of the IL-11-treated patients ($p < 0.02$). This benefit was maximal in the second cycle of therapy. IL-11 also had the ability to reduce the number of episodes of platelet transfusions compared to the placebo arm. Overall, patients receiving IL-11 also tended to recover their platelets faster than the placebo group.

The side effect profile of IL-11 in these studies has been similar to those seen in the phase I trial and the side effects are primarily grade 1 or 2 in nature. In a small number of patients, plasma volume expansion may be associated with edema, dyspnea, and infrequently atrial arrhythmias that are usually asymptomatic and self-limiting. Phase I and II trials of IL-11 in the bone marrow transplant setting have demonstrated a similar safety profile. Data regarding the thrombopoietic activity of IL-11 in that setting is less well-defined, though there is some suggestion of enhanced platelet recovery in a placebo-controlled, randomized, phase II trial. [202] A randomized, placebo-controlled phase III trial using the secondary prophylaxis design is ongoing. On the basis of the preceding randomized phase II data, IL-11 was approved by the FDA in 1997 for the prevention of severe chemotherapy-induced thrombocytopenia. The role of IL-11 in other thrombocytopenic disorders, such as the myelodysplastic syndromes, aplastic anemia, and immune thrombocytopenia, is being considered for investigation.

Other potential applications for IL-11 include the prevention of chemotherapy-induced mucositis and the treatment of inflammatory conditions such as inflammatory bowel diseases and psoriasis. The latter has been studied in early phase I trials in which low doses of IL-11 appear to have some effect on the severity of inflammatory bowel disease. Doses under investigation for these indications are typically lower than those for the thrombopoietic effect.

Thrombopoietin

Thrombopoietin is the ligand for the c-mpl receptor, a proto-oncogene which is the human correlate for the avian myeloproliferative leukemia (v-mpl) receptor. The ligand has been cloned by a variety of investigators. [203] [204] The native molecule is recognized as thrombopoietin (TPO) whereas a truncated, PEGylated form is known as megakaryocyte growth and development factor (MGDF). Both molecules have a similar range of activity. The PEGylation of the latter molecule allows for protein stability and maintenance of half-life. Thrombopoietin has been extensively studied and appears to be a lineage-dominant factor for megakaryocytic progenitor cell proliferation and megakaryocyte maturation and platelet production. Thrombopoietin has also been shown to affect erythropoiesis as well as the proliferation of the pluripotent stem cell. Thrombopoietin has an approximately 20% sequence homology to erythropoietin and is primarily produced by the liver and to a lesser degree, the kidney. The gene for thrombopoietin is located on chromosome 3q27-28. [205] Thrombopoietin levels in hematologic malignancies vary and are related to the presence of the megakaryocyte mass. [206]

When administered to animals in preclinical models, thrombopoietin and MGDF induce rapid platelet production associated with significant increments in both the number of megakaryocyte progenitors as well as the overall number of megakaryocytes. [207] [208] [209] [210] They exert not only a proliferative effect but also a maturational effect on bone marrow megakaryocytes. Following the administration of chemotherapy or radiotherapy, both TPO and MGDF have the ability to accelerate platelet recovery in a wide variety of preclinical animal models. In addition, both agents are potent inducers of peripheral blood progenitor cell mobilization.

Clinical Applications

Phase I studies with both TPO and MGDF have been completed. The former has been studied in patients with soft tissue sarcomas

who received a single dose of TPO intravenously at doses ranging from 0.32.4 g/kg 3 weeks prior to chemotherapy. [211] A single dose of TPO was associated with a dose-related increase in peripheral blood (PB) platelet counts. Rises in platelet counts were noted as early as day 4 though, like many other thrombopoietic agents, the peak platelet count was seen in the second week following therapy. Unlike many other HGFs, TPO has a prolonged half-life following a single IV injection, measured at between 2030 hours. Peak platelet counts exceeded 1,000,000/l at the 2.4 g/kg dose level. Additional biologic effects of TPO administration were seen in the bone marrow, where increases in the absolute number of megakaryocytes (MKs), the number of MK progenitors, and the mobilization of a broad range of PB progenitors was seen. Data following the administration of chemotherapy is scant but it appears that the post-chemotherapy thrombopoietic response is somewhat dependent on the degree of chemotherapy intensity. Hence, multiple-day dosing, scheduled every third day due to the prolonged half-life, is being tested though no definitive data regarding the ability of TPO to reduce the degree of chemotherapy-induced severe thrombocytopenia has been shown. Safety of the single-dose TPO formulation was excellent with mild headache as the only therapy-related adverse event.

MGDF has been studied in two placebo-controlled phase I trials as well as at least one reported randomized, placebo-controlled phase II trial. The initial phase I trial was conducted in patients with refractory cancer by Basser and colleagues in Australia. [212] This trial assessed the effect of MGDF administered subcutaneously at doses ranging from 0.035.0 g/kg/day for 10 days during a prechemotherapy phase. Reproducible increases in PB platelet counts were seen at all doses though these were noted to occur earlier at higher doses of 0.3 and 1.0 g/kg/day. At these doses, a median threefold increase in platelets was seen. Peak platelet counts of 0.8, 1.3, and 1.8 million were seen at the highest dose level. These platelets were not hyperaggregable and appeared to function normally. [213] Following the administration of chemotherapy (cyclophosphamide 1,200 mg/m^2 and carboplatin 600 mg/m^2) MGDF was administered for between 7 and 20 days. Although severe thrombocytopenia was not routinely seen with this regimen, the nadir platelet count occurred earlier in the MGDF patients compared to placebo controls although there was no difference in the depth of the thrombocytopenia. In addition, platelet count recovery to prechemotherapy levels occurred faster in the MGDF patients compared to those in the placebo group.

In the second phase I trial, Fannuchi and colleagues administered MGDF following carboplatin (AUC 9) and paclitaxel (225 mg/m ^{2}) chemotherapy at doses ranging from 0.035.0 g/kg/day. [214] MGDF was given for as few as 3 days at the highest doses due to excessive thrombocytosis with platelet counts exceeded 1.52.0 million in some patients. No significant increase in thromboembolic events was identified. Nadir platelet counts in the MGDF patients were 189,000/l compared to 111,000/l in the placebo group. This difference was statistically significantly improved. In addition, recovery to baseline platelet counts occurred earlier among the MGDF patients.

In a randomized phase II trial, Archimbaud et al. studied the effect of MGDF compared to placebo in reducing the duration of severe thrombocytopenia in patients with AML undergoing induction chemotherapy. [215] In this study, all patients received a standard cytarabine/anthracycline/etoposide-containing induction regimen followed by either placebo or MGDF at either 2.5 or 5.0 g/kg/day for up to 21 days (starting on day 8). The median time to recovery of platelet count of 20,000/l was 21 days in each group with excessive thrombocytosis seen in approximately 50% of the MGDF patients compared to a minority of the placebo patients. No untoward effects were seen in this trial.

Overall, both TPO and MGDF continue to be investigated in the setting of chemotherapy-induced thrombocytopenia and further results are expected.

Other Applications

Results of phase I and phase II trials of MGDF in the autologous transplant setting appear to be dependent on the type of progenitor cell reinfused. Preliminary data suggest that if bone marrow is used as the source of progenitor cells, a modest improvement in platelet count recovery may be seen. [216] In contrast, when PBPC are

used, MGDF was unable to accelerate recovery regardless of being given prior to or following PBPC reinfusion. [217]

As was noted in the phase I trials, both TPO and MGDF have potent PBPC mobilization activity. Studies exploring this as a potential approval indication are underway.[218] [219] [220] Finally, the use of MGDF to mobilize increasing numbers of platelets into the PB for plateletpheresis has been reported by Kuter et al. [221] The administration of a single dose of MGDF at doses of 1.0 and 3.0 g/kg was associated with a statistically significant increase in the percentage of donors who succeeded in donating adequate platelet volumes. In addition, preliminary results suggest that these super-plateletpheresis packs promoted greater increases in platelet counts in their recipients. Larger studies assessing their safety in a nonselected group of donors will be necessary since this has the potential to increase the number of available plateletpheresis donors.

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BIOLOGIC RESPONSE MODIFIERS (BRMs)

The rationale for using BRMs for the treatment of malignancies is based primarily on preclinical data and indirect clinical evidence that suggests an important relationship between the immune system and the neoplastic process. These clinical and histopathologic observations include favorable clinical prognosis associated with brisk lymphocytic infiltrate in solid tumors, increased risk of malignancies in the setting of immunodeficiency states or immunosuppression, rare spontaneous regression of metastases after resection of primary tumor, and the presence of antitumor antibodies in sera and specific cytotoxic T lymphocytes in the peripheral blood and at tumor sites of cancer patients.[222] [223] [224] [225] [226] [227] More recently, compelling preclinical animal data have further supported the use of BRMs for the treatment of cancer.[228] Table 51-1 summarizes some of the biologic effects of many of these agents that may be relevant to their potential role in cancer therapy.

Interleukin-2

Interleukin-2 (IL-2) is a 15,000 dalton glycoprotein encoded by a single gene on chromosome 4 which acts in a monomeric form as an autocrine growth factor. [229] IL-2 is critical in allowing T lymphocytes to proceed from G_1 into cell cycle (S phase) following activation of their unique T-cell receptor complex. [229] It also induces the secondary secretion of proinflammatory cytokines from NK-and T lymphocytes and induces the proliferation of NK-and B lymphocytes. [229] [230] [231] It activates the cytotoxic machinery in T cells, NK cells, and monocytes. IL-2 activation of effector cells results in the release of secondary cytokines, including IL-1, TNF-, IFN-, and GM-CSF, which further recruits and activates monocytes.[231] This secondary release of cytokines may account for the therapeutic activity of IL-2 and likely contributes to the toxicity associated with IL-2 treatment.[232] As a biologic agent IL-2 appears to function primarily through host immunomodulation.

IL-2 binds to a heterodimeric receptor composed of three component molecules, , and subunits.[233] IL-2 effects are

mediated through interaction with a specific high-affinity membrane receptor.[233] The IL-2R is composed of three IL-2R peptide chains; the -chain (or Tac molecule), a 55-kd peptide; the -chain, a 75-kd peptide; and the -chain, a 64-kd protein. [233] [234] When all three chains are present in close approximation on the T-cell surface, a high-affinity IL-2R is formed which has 1,000-fold greater binding affinity compared to cells that express only the 55-kd -chain. The high-affinity receptor responds to IL-2 levels 100 times lower than the intermediate affinity receptor (receptor). T lymphocytes have only a high-affinity form of the receptor when activated, whereas NK cells have both an intermediate and high-affinity receptor and monocytes have only the intermediate receptors. [235] [236] [237] The subunit is shared by a number of cytokine receptors and it is critical to effective signal transduction for IL-2, as well as IL-4, IL-7, IL-9, and IL-15. [236] [237] The specificity, duration, and magnitude of a T-cell response is in part regulated by IL-2. [229] [238] IL-2Rs have a fairly restricted pattern of expression (NK cells, T cells, activated B cells, activated macrophages, and on some cells of malignant lymphoproliferative disorders). In addition, IL-2 stimulates a resting population of lymphocytes that, when activated, have cytotoxic activity against isolated tumor cells and tumor cell lines. [229] [230] [238] [239] [240] This cytotoxic activity is not MHC-restricted.[230] These cells have been termed lymphokine activated killer (LAK) cells; although no single effector cell is responsible for LAK activity, cell-surface phenotyping studies suggest that LAK activity is primarily mediated by CD3, CD56+ NK cells.[230] A minor contribution of LAK activity can be derived from cytotoxic T cells under some in vitro conditions.

In addition to the expression of the IL-2R by activated T cells, a fully soluble form of the subunit of this trimeric structure has been described and referred to as soluble p55 IL-2R (sIL-2R). Low levels of sIL-2R have been detected in the sera of healthy individuals and markedly increased levels have been detected in patients with hematologic malignancies.[241] [242] This has been most extensively evaluated in patients with human T-cell leukemia/lymphoma virus-1 (HTLV-1)-associated T-cell leukemia.

Clinical Application

IL-2 has undergone extensive clinical evaluation as a single agent, in combination with effector cells (including LAK cells and tumor-infiltrating lymphocytes) and, more recently, in combination with other cytokines and chemotherapy. [228] [243] [244] [245] Early studies, based on extensive animal experiments, used IL-2 combined with ex vivo IL-2-activated LAK cells.[228] The LAK cells were obtained by daily leukopheresis, and were then cultured in vitro with IL-2. Initial clinical studies, reported in 1986, revealed objective regressions in traditionally unresponsive tumors and therefore generated considerable interest. [243] [245] Since then, numerous studies have been performed to define both the optimal dose and schedule of IL-2 therapy, as well as the overall antitumor activity. IL-2 has shown activity as a single agent and in combination with other cytokines (IFN-) or with chemotherapy in solid tumors, most clearly demonstrated in renal cell cancer and metastatic melanoma. [246] [247] [248] [249] Most importantly, in both of these chemotherapy-resistant diseases, IL-2 induces significant durable complete or near complete remissions in 1020% of patients. These patients can have very durable unmaintained remissions lasting over 5 years. The mechanism of the antitumor effects, and the reason only a small percentage of patients experience these impressive tumor responses is unknown. The most convincing data for durable tumor responses have been reported in patients receiving the high dose bolus IL-2 regimens initially developed at the NCI-Surgical Branch. [242] Lower dose regimens and even outpatient subcutaneous regimens of IL-2 alone or with IFN- have shown similar response rates (1020%), but have failed to demonstrate the durability of their responses. [247] [248] Adoptive cellular therapy with ex vivo activated LAK cells or TIL cells has been too cumbersome and expensive to warrants its continued use. Results with LAK cells or TIL cells were not obviously superior to IL-2 alone.

Therapy of Hematologic Malignancies

Numerous trials have been conducted with IL-2 in hematologic malignancies. These have included trials in both acute and chronic leukemias, non-Hodgkins lymphomas, and multiple myeloma.[250] [255] Trials have been initiated utilizing a variety of schedules and doses of IL-2 and administered in a number of clinical settings including early relapse of acute leukemia, following remission as maintenance therapy, and following hematopoietic stem cell transplantation with autologous or even allogeneic stem cell donors (see Table 51-2). While many of these trials have provided some promising leads, they have largely been difficult to interpret and failed to determine a definitive role of IL-2 in the treatment of hematologic malignancies. Early studies with high-dose IL-2 regimens suggested activity against lymphoma. Weber and colleagues treated 19 patients with either low grade (follicular) lymphomas (8 patients) or intermediate grade lymphomas (11 patients) with high-dose bolus IL-2.[256] There were three partial responses and one complete response among the eight patients with follicular lymphoma and no responses among those with intermediate grade lymphoma. Duggan et al. reported a phase II study of recombinant IL-2 with or without IFN- in patients with relapsed or refractory non-Hodgkins lymphoma.[251] Although designed as an outpatient regimen, toxicity was severe and the response rate was only 17%. A relatively large phase II experience with continuous infusion IL-2 at inpatient dose intensity was reported from France and included 61 patients with lymphoma. [252] Of 23 patients with intermediate- and high-grade NHL, there were 3 CRs and 4 PRS; of 24 patients with low-grade NHL, there was a single CR; and of seven patients with mycoses fungoides, there was 1 CR and 4 PRs. The CR durations were 4, 12, 17, 20, and 23 months.

Several uncontrolled phase II trials in small cohorts of patients suggest antileukemic activity of IL-2. [257] [258] [259] [260] In acute myeloid leukemia (AML) patients with refractory or relapsed disease with between 530% bone marrow myeloblasts, IL-2 administered through continuous intravenous infusion followed by lower dose

subcutaneous IL-2 maintenance led to CRs in 11 of 20 patients with 6 continuing in CR at median follow-up of 50 months (9, 33, 49, 51, 52, and 87 months). [257] These remission durations were all longer than the patients previous remission durations. Although toxicity was significant, there were no deaths and side effects were completely reversible. Intensive bolus IL-2 at 68 MIU/m^2 every 8 hours was administered to 10 AML patients with relapsed disease following prior chemotherapy or autologous stem cell transplant. [258] Two patients had complete remissions of 3 and 4 months in duration. IL-2 has also been administered to AML patients at high risk for recurrence following induction of second remission with chemotherapy or stem cell transplantation. IL-2 has been administered as a bolus infusion, or via polyethylene glycol (PEG) delivery as a means of slow release to simulate continuous infusion. [259] [260] Prolongation of second remissions was suggested, but not proven by the results. IL-2 has potential to enhance the immune state of patients with myelodysplastic syndromes and may be a treatment worth exploring, especially in low-risk MDS patients. [261]

A potentially more promising approach has been the incorporation of IL-2 into BMT regimens to augment immunologic recovery after BMT and, possibly, to stimulate graft-versus-leukemia activity. Defective IL-2 production and defective T-cell proliferation have been reported following BMT. Higuchi et al. reported that IL-2 responsive LAK precursor cells are available in the peripheral circulation as early as 3 weeks after

TABLE 51-2 -- Clinical Trials with Interleukin-2 in Hematologic Malignancies

Patient Population	IL-2 Regimen	Significant Toxicity	Outcome/Findings	References
Lymphoma				
Non-Hodgkin lymphoma Hodgkin disease	Moderate IL-2 IV CI + LAK		15 treated: 1 PR in NHL 1 PR in HD	[114]
Non-Hodgkin lymphoma Hodgkin disease	High dose bolus IL-2 IV + LAK	Hypotension, renal insufficiency, fever	0/15 responses in NHL 2/12 PR in HD	[115]
Non-Hodgkin lymphoma	High dose bolus IL-2 IV + LAK	Hypotension, renal insufficiency, fever	3 PR and 1 CR in 19 NHL (8 low grade and 11 intermediate grade)	[116]
Non-Hodgkin lymphoma Hodgkin disease	IV CI Moderate dose IL-2		1 CR, 5PR in 10 low grade NHL, 0 responses in 7 intermediate NHL and 4 HD	[108]
Non-Hodgkin lymphoma Hodgkin disease Cutaneous T-lymphoma	IV CI Moderate to high dose IL-2	Cardiac, renal VLS, platelets	1 CR-24 low-grade NHL 3 CR, 2PR-23 Int grade NHL 0/7 responses in HD 1 CR, 4 PR in 7 CTCL	[110]
Acute Leukemia				
Early relapsed/refractory AML (<30% BM blasts)	IV CI Moderate dose IL-2	Fever, renal, N/V, platelets, hypotension, sepsis	11/20 CR, 1 SD 6 CCR for 9+ to 87+ mos 1 SD for 18+ mos	[117]
Relapsed (overt) AML	IV bolus high dose IL-2	Fever, hypotension, platelets, renal, liver	2 CR among 10 pts Duration of 3 and 4 mos	117a
2nd remission AML as consolidation	IV 1 hr IL-2	Fever, rash, sepsis, liver	5/21 2nd CR longer than 1st CR 7/21 ongoing 2nd CR Others 2nd CR < 1st CR	[118]
2nd remission IL-2	PEG IL-2 weekly IV	Fever, chills, fatigue, VLS (mild)	3/7 2nd CR longer than 1st CR	[119]
Adult ALL: phase III of autologous BMT ± IL-2 in 1st CR versus allogeneic BMT	IL-2 CI IV moderate dose after engraftment		30 IL-2 vs. 30 with no IL-2: median CR, 3 yr CCR no different and inferior to allogeneic BMT	[120]
Hematologic malignancies: CML, AML, ALL, NHL, MDS following T-depleted allogeneic-BMT	Low dose CI IL-2 after engraftment	Fatigue, fever, N/V, infection, rash, diarrhea	In 25 patients: relapse of IL-2 treated similar to controls with no-IL-2 with GvHD and superior to no IL-2 without GvHD	[121]
Relapsed AML post autologous BMT	CI IV IL-2 + LAK moderate dose post engraftment	Fever, rash, platelets	10/14 in CCR for 15+ to 50+ mos (median 36+ mos) 71% 4 yr DFS actuarial	[122]
High-risk AML ex vivo IL-2 activated BMT + IL-2	Ex vivo IL-2 BM activation + sc IL-2 low to moderate dose	Fever, malaise, hypotension, delayed engraftment	5/10 in CCR for 18 to 32+ mos	[123]
Multiple Myeloma				
Multiple myeloma	Low dose sc IL-2 (1.8 MIU/m2/d)	Fever, splenomegaly, herpes zoster, eosinophilia	2/17 objective responses 4/17 stable disease	[124]
Multiple myeloma	SC IL-2 (4.5 MIU) + IFN (4.5 MU) TIW	Headache, anorexia, nausea	0/17 objective responses	[125]

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BM, bone marrow; BMT, bone marrow transplant; CI, continuous infusion; CML, chronic myeloid leukemia; CR, complete remission; CCR, continuous complete remission; CTCL, cutaneous T-cell lymphoma; DFS, disease-free survival; GvHD, graft-versus-host disease; HD, Hodgkin disease; IV, intravenous; LAK, lymphokine-activated killer cells; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; N/V, nausea and vomiting; PEG, polyethylene glycol; PR, partial remission; SC, subcutaneous; SD, stable disease; TIW, thrice weekly; VLS, vascular leak syndrome.

autologous BMT. [262] [263] Fefer and colleagues hypothesized that IL-2 may be most effective as a consolidative immunotherapy for lymphoma and AML early after ABMT. [264] [265] This was based on the in vitro and in vivo activity of IL-2 in NHL and AML and the minimal disease state following ABMT; treatment of clonogenic malignant cells within the graft with IL-2; activation of the recovering immune system with IL-2; and the ability of IL-2 to induce a cutaneous graft-versus-host disease (histologically and clinically) which has been associated with a graft versus tumor effect in allogeneic transplants. [253] [264] Two preliminary trials of IL-2 with and without LAK cells administered shortly after hematologic recovery (1491 days after BMT) have appeared promising, with a majority of AML patients remaining in a prolonged disease-free-remission far longer than historical controls. [253] [265] These preliminary trials have led to ongoing randomized phase III trials in cooperative groups for NHL with consolidative immunotherapy of IL-2 alone following ABMT. In a small randomized trial in adults (1555 years) with ALL, 135 previously untreated patients, 126 who went into a complete remission with BFM induction chemotherapy either received an allogeneic BMT if they had an HLA-matched sibling or underwent an autologous stem cell transplant with or without post-transplant IL-2. [266] Only 60 patients were randomized between IL-2 or observation following their autologous transplants. The patients undergoing allogeneic BMT (68% vs. 26%) had a far better DFS, whereas IL-2 did not appear to improve the results in patients receiving autologous transplant (29% vs. 27%). The dose of IL-2 was defined by a prior phase I trial. IL-2 was administered a median of 80 days post-autologous transplant, which may have had a relationship to the high early relapse rate and the few patients randomized to IL-2 or observation. Earlier administration of IL-2 may be critical to the

treatments efficacy. Others have cultured stem cells ex vivo with IL-2 as a means of purging residual leukemic cells and to induce immune effectors within the graft. [261] [262] [263] [264] [267] [268] [269] In these trials IL-2 was administered to patients immediately following stem cell infusion in the hope of activating these immune cells within the graft. Engraftment of platelets and neutrophils appeared to be significantly delayed by this approach and a number of manageable toxicities were observed. Slavin and colleagues have reported on the use of both IL-2 and IFN following autologous stem cell transplantation for both NHL and Hodgkins disease. [270] [271] Results of post-transplant immunotherapy in 56 patients again appeared superior to historical controls in terms of disease-free survival. The regimen was self-administered,

outpatient, and subcutaneous.

Others have explored the feasibility of administering IL-2 following allogeneic BMT as a means of enhancing the existing graft-versus-tumor effect. [254] [272] [273] Soiffer and colleagues at the Dana Farber Cancer Institute have treated patients with hematologic malignancies who underwent a T cell (CD6) depleted HLA-matched sibling allogeneic BMT with a low dose regimen of continuous IL-2 following recovery from the BMT for a period of 3 months. [272] T-cell depleted allogeneic BMT has been an effective approach at limiting GvHD, but increases the risk for disease relapse. The schedule of low dose continuous IL-2 was able to activate and expand endogenous NK cells, with minimal toxicity. Results from the 25 patients who received IL-2 for greater than 30 days suggest a better outcome (relapse rate) compared to historical controls who did not develop GvHD. Robinson and colleagues have administered IL-2 following HLA-matched sibling allogeneic BMT in children with acute leukemia.[273] Patients without GvHD off immunosuppressive agents received IL-2 by continuous infusion. At higher doses of IL-2, pulmonary toxicity and chronic GvHD was observed. Severe acute GvHD was not observed. This may set the stage for further trials of IL-2 in unmodified allogeneic BMT where GvHD is not observed. Another modification of this approach has been explored by Slavin and colleagues, who treated relapses post-allogeneic BMT with donor lymphocytes activated in vitro and/or in vivo with IL-2. [270] The allogeneic cell therapy may be enhanced by further activation with IL-2 in these studies.

Finally, IL-2 has shown promise in patients with HIV disease as an immunostimulant capable of increasing CD4 cell counts, without changing HIV viral load. [274] Bernstein and colleagues have also treated patients with HIV-associated malignancies and demonstrated large in vivo expansion of NK cells without increases in HIV viral levels.[275] Most striking was the absence of opportunistic infections during the 50 months of IL-2 therapy, far fewer than would be expected for this highly immunosuppressed population.

IL-2 Associated Clinical Toxicity

Most of the toxicities associated with IL-2 have been reported in patients receiving the high dose intravenous bolus (600,000720,000 IU/m ^{2}) or continuous infusion regimens.[8] [276] The adverse effects of high dose IL-2 resemble sepsis and include fever, rigors, oliguria, and hypotension that frequently requires vasopressor support.[276] The vascular leak syndrome is believed to be due to the induction of proinflammatory cytokines such as IL-1, TNF-, IFN-, and the induction of nitric oxide which acts to mediate the vascular and organ toxicities. [231] [232] [277] A variety of hematologic, hepatic, neurologic, renal, cutaneous, and gastrointestinal toxicities have also been well described. [276] [279] [280] [281] All patients experience a reversible malaise and flu-like syndrome with fever, chills, and gastrointestinal symptoms. Other end-organ toxicities have included hepatic dysfunction manifested as elevated bilirubin due to intrahepatic cholestasis. Several autoimmune processes have been reported including thyroiditis, vitiligo, and inflammatory bowel changes. [8] [276] Finally, infectious complications such as catheter-associated bacteremias have been associated with skin changes due to IL-2. [8] Most of the deaths occurring in patients while receiving IL-2 were due to sepsis, prior to institution of prophylactic antibiotics in all patients with central venous catheters. Cardiopulmonary effects have been most prominent among the multiple sequelae of IL-2. Initially, mortality associated with myocardial infarctions, cardiac arrythmias, and pulmonary edema requiring intubation and mechanical ventilation were reported in 210% of patients receiving high dose IL-2 with or without LAK cells. [276] [278] This initial experience led to more rigid selection criteria of patients, thereby excluding those at risk for cardiac problems. In addition, management changes were instituted that led to discontinuation of IL-2 if any of the following occurred; excessive vasopressor support, neurologic toxicity, significant cardiac arrhythmias, or respiratory distress requiring significant oxygen support. [276] In more recent series, there were no deaths from treatment and no myocardial infarctions. [278] Hypotension requiring vasopressors occurred in 53% of courses and intubation occurred in four patients (1.2% of courses). Only one patient required intubation for respiratory distress; the other three patients required protection of the airway because of obtundation. Atrial arrhythmias occurred in 5.2% of courses, but were associated with hypotension in only two courses (0.6%). This decreased toxicity was associated with a large reduction in doses to approximately 15 doses administered of a maximum total of 28 for a course (two cycles) with 720,000 IU/kg per dose. Several patients also demonstrated a transient rise in CPK-MB without definitive findings of myocardial infarctions. In addition, the use of prophylactic antibiotics and aggressive therapy of infection may have led to a decline in cardiopulmonary complications. Renal toxicity including azotemia, oliguria, and weight gain due to fluid retention was frequent, but completely reversible without long-term renal dysfunction. [279] It was more prevalent in association with prior renal compromise from a nephrectomy. Finally, hematologic toxicities including anemia, severe thrombocytopenia and lymphocytopenia, rebound lymphocytosis, and eosinophilia have all been reported. [280] [281] The lymphopenia occurs early in therapy and may be the result of margination. Thrombocytopenia has been relatively rare, requiring transfusions or resulting in hemorrhage in very few cases. Virtually all the side effects of IL-2 are rapidly reversible with discontinuation of therapy. Several attempts to limit IL-2 toxicities with inhibitors of secondary cytokine induction such as pentoxyfilline, lysofilline, and ciprofloxacillin, or inhibitors of TNF function have shown only marginal benefit in limiting toxicity or allowing greater dose intensity. [282] [283]

Interleukin-4

Interleukin-4 (IL-4) is a 20,000 dalton glycoprotein produced by activated T cells, first described as a B-cell stimulatory factor. [284] More recent studies have demonstrated a broad range of regulatory activities for IL-4. [285] [286] [287] [288] [289] [290] IL-4 induces T-cell proliferation and enhances cytotoxicity of T cells. It enhances the generation of antigen-specific T lymphocytes from tumor-infiltrating lymphocytes. [289] IL-4 enhances the growth of mast cells and stimulates macrophage antitumor cytotoxicity.[284] Interleukin-4 is a pluripotent cytokine that has the ability to activate T cells in combination with IL-2 or by itself; to stimulate T-helper type 2 (Th2) responses; enhance expression of MHC class II, CD23, CD40, LFA1/LFA-3; and enhance antibody-dependent cellular cytotoxicity (ADCC) responses of human mononuclear cells.[285] [286] [287] [288] It is composed of a single 129-amino-acid chain and predominantly produced by the Th2 CD4+ subset of T cells. Preclinical studies of IL-2 and IL-4 in combination resulted in inhibition of IL-2-induced LAK activity. In addition, IL-4 inhibits cytokine production and certain effector functions in human monocytes.[291] [292]

IL-4 can antagonize some of the responses induced by IFN- and TNF-. [291] Therefore, IL-4 appears to play an important role in the down-regulation and resolution of an inflammatory response. IL-4 has also been shown to inhibit megakaryocyte colony formation in a dose-dependent fashion and to suppress CFU-GM proliferations.[293] The high-affinity IL-4 receptor can be found on T cells, mast cells, macrophages, and hematopoietic progenitors, as well as fibroblasts, brain cells, and most epithelial cells. The IL-4 gene is found on human chromosome 5. IL-4 has been shown to modulate tumors directly by changing surface phenotypes by modifying MHC class II expression and tumor-associated ganglioside expression, inhibiting proliferation, and inhibiting secretion of autocrine growth factors (IL-6) for some cancers (e.g., myeloma); and, in combination with GM-CSF, may enhance the ex vivo induction of potent antigen presenting cells (dendritic cells). [294] [295] IL-4 transfected murine cancers have been shown to protect hosts from subsequent tumor challenge from parental untransfected tumor cells through activation of host immune cells and eosinophils.[296] [297]

Phase I and II trials with IL-4 have been performed in patients with solid tumors. [298] [299] [300] These trials have demonstrated no significant anti-tumor activity in patients with renal cancer or melanoma. IL-4 has the unique ability to inhibit the release of proinflammatory cytokines and induce inhibitors to IL-1 (IL-1ra). These anti-inflammatory effects differ greatly from IL-2. Toxicity of IL-4 has differed from IL-2 in terms of frequent development of gastritis, nasal congestion, and myocardial inflammation and dysfunction in IL-4 treated patients, while still exhibiting common cytokine toxicities including fever, rash, myalgias, and headaches. [300] The in vitro effects of IL-4 on human lymphoid and plasma cell malignancies have been investigated. [294] IL-4 inhibits the in vitro growth of most tumor specimens, with <10% of specimens studied demonstrating a proliferative response. The addition of IL-4 to chronic myeloid leukemia (CML) cell cultures has resulted in divergent effects with suppression of CFU-GM in some patients and stimulation in other samples. [295] IL-4 has also been shown to inhibit the growth of Philadelphia-chromosome positive ALL cells in vitro and is being explored in the treatment of relapsed ALL. [301] [302] More recently, IL-4 has demonstrated promising preclinical effects in tumor models of Burkitts lymphoma, B-cell precursor acute leukemia, and high risk acute lymphoblastic leukemia. [303] [304] These effects may be due to direct antiproliferative effects or induction of programmed cell death (apoptosis) of the leukemic cell or via indirect effects on the host effector cells. Trials in myeloma and low grade lymphoma are anxiously being awaited. Finally, IL-4 has been used in combination with GM-CSF in clinical trials to ex vivo expand dendritic cells as a means to enhance tumor vaccines with these potent antigen-presenting cells. [305] [306]

Interleukin-12

Interleukin-12 (IL-12) was initially known as cytotoxic lymphocyte maturation factor (CLMF) or natural killer cell stimulatory factor (NKSF). [307] [308] Once characterized and cloned, its profound effects on the proliferation, cytolytic activity, and cytokine induction of both T and NK cells became apparent. [309] [310] It plays a profound role in regulating the induction of the T-helper (CD4+) type 1 (Th1) subset and is an important component of the immune response to foreign antigens. [309] [310] [311] [312] [313] IL-12 is unique among cytokines in that it is composed of two different disulfide-linked protein subunits: p40 with sequence homology to the cytokine receptors of the gp130 family; and p35 which is structurally similar to the IL-6 family of cytokines. [314] IL-12 is postulated to have arisen from a disulfide linkage between a receptor and its ligand. The disulfide-linked heterodimer is required for optimal biologic activity. IL-12 can be produced from a variety of cells including EBV-transformed B-cell lines (from which it was first isolated), B lymphocytes, monocytes, macrophages, mast cells, keratinocytes, dendritic cells, and thymic stromal cells. Its surface receptor is

still poorly characterized, but at least one of its subunits is a member of the cytokine receptor superfamily and is associated with members of the JAK kinase family (JAK2 and TYK2).[314] IL-12 can augment both T-cell and NK-cell cytolytic activity, but most impressively induces the activation of T- and NK-cell secretion of Th1 type cytokines, especially IFN.[315] This shift to a Th1 functional phenotype may be important in the induction of cell-mediated responses against tumors. IL-12 has been shown to have potent antitumor effects in animal tumor models. It also has antimetastatic activity in various tumor models. [315] These activities appear more dependent upon T cells than on NK cells or monocytes/macrophages. It is active in mediating antitumor effects when delivered directly to tumor sites. [315] In some tumor models, IL-12 appears more active when combined with IL-2.[316] IL-12 has been shown to be capable of suppressing experimental tumors through an indirect anti-angiogenic effect through its effect on IFN- and IP-10, an early response gene induced by IFN-. [317] Additional studies have demonstrated that peripheral blood lymphocytes from patients with metastatic cancer or following allogeneic BMT are activated by IL-12. [318] Future clinical studies with IL-12 will focus the potent immune-enhancing properties of this cytokine both alone and in combination with IL-2. Phase I trials of IL-12 in cancer and HIV patients have been completed. [319] The major toxicities have been oral stomatitis and transient hepatic dysfunction. Serum IFN- is markedly increased in these patients. Interestingly, when IL-12 proceeded into phase II trials, a single test dose was removed from the treatment schedule.[320] Without the single test dose of IL-12, toxicity of the 5-day course of IL-12 was much more severe with life-threatening systemic toxicities including gastrointestinal hemorrhage, acidosis, dyspnea, and severe fatigue. The single dose of IL-12 abrogated the toxicity, as well as the IFN- induction. This unique biologic activity was found to be reproducible in animals, as well. [320] Phase II trials are now underway with the added understanding of these unique biologic effects of IL-12 and its scheduling dependence. These trials are anticipated with great promise in solid tumors, hematologic malignancies, and in the stem cell transplant setting.

Interleukin-15

Interleukin-15 (IL-15) has been isolated based on the T-cell stimulatory activity of supernatant from an epithelial cell line. [321] It shares a number of biologic activities with and has structural similarities to IL-2 in its four-helix bundle structure, but virtually no sequence homology exists between the two cytokines. IL-15, unlike IL-2, is not predominantly produced by activated T lymphocytes, but abundant levels are produced by monocytes, fibroblasts, epithelial cells, and stromal cell lines derived from the bone marrow and thymus.[322] IL-15 is abundantly secreted from monocytes and macrophages and can then induce the proliferation of activated and antigen-dependent T cells, induce the proliferation of NK cells, co-stimulate the secretion of cytokines from NK cells in combination with IL-12, and induce cytolytic effector cell activity from T and NK/LAK cells.[321] [323] [324] IL-15 may act to recruit T lymphocytes to sites of inflammation by changing their polarity and enhancing expression of adhesion molecules. IL-15 also appears to induce the proliferation and cytokine release from peripheral blood mononuclear cells (PBMC) and CD4+ T cells from HIV-infected individuals.[325] IL-15 appears critical to the survival of resting NK cells. [323] Its absence leads to apoptotic cell death of NK cells. The abundant release of this cytokine by stromal and antigen-presenting cells (APC) suggest that its presence maintains NK cells to a much greater degree than IL-2. The IL-15 receptor shares the and subunits with the IL-2 receptor, but has a different and unique

receptor.[321] It has been shown that IL-15 mediates proliferation of both CD3+ and CD3 granular lymphocytes from patients with lymphoproliferative disorders of granular lymphocytes.[326] The presence of mRNA for IL-15 and its receptor in the peripheral blood mononuclear cells of these patients suggests a role in the expansion of granular lymphocytes.[326] Similar findings have been described for B-cell malignancies such as hairy cell leukemia and B-cell CLL. [327] IL-15 has not yet entered clinical trial development, but may ultimately be a component in therapy for immunodeficiency states or cancer. Antagonists to IL-15 may have a role in T- and B-cell chronic leukemic conditions.

Flt3 Ligand

Flt3 ligand (flt3L) is a novel hematopoietic growth factor and the ligand for the flt3 tyrosine kinase receptor, a member of the tyrosine kinase (TK) receptor family involved in hematopoiesis (i.e., c-kit and c-fms). [328] [329] Primitive CD34+ cells from bone marrow, cord blood, and fetal liver in human and mouse respond to flt3L to form colonies and flt3L synergizes with other more lineage-specific colony-stimulating factors promoting in vitro colony formation. [328] [329] In vitro colony assays show that flt3L has activity in regulating both primitive multipotential and lineage-committed hematopoietic progenitor cells. Studies also suggest that the presence of flt3L may enhance the ability to genetically transduce human and murine hematopoietic progenitor cells. [329] Normal expression of flt3L is limited to CD34+ stem/progenitor cells. Overexpression of flt3L and its receptor appears common in many cases of AML, ALL, and CML blast crisis, regardless of whether CD34 is expressed. [328] [330] Flt3L alone or with other cytokines (IL-7) can induce the in vitro proliferation of ALL and AML cells. [330] This finding suggests a potential role for flt3L in the pathophysiology of acute leukemia. Patients with aplastic anemia, and patients undergoing intensive marrow ablative chemotherapy have large increases in flt3L levels, which return to baseline following hematologic recovery or remission. [331] Flt3L appears to be a critical regulator of early hematopoiesis. Daily subcutaneous administration of flt3L has been shown to induce large numbers of dendritic cells in lymph nodes, spleen, and other tissue. [332] These dendritic cells are antigen-presenting cells capable of stimulating primary T-cell responses to antigen. The dendritic cells expanded in vivo or ex vivo by flt3L are functionally active and capable of stimulating antigen-specific T-cell responses. Based on this dendritic cell activation, flt3L was examined as an anti-tumor agent in murine tumors. These experiments revealed that in vivo tumor rejection and specific immunologic memory can be induced. [332] Therefore, administration of flt3L alone or in combination with other colony-stimulating factors may not only lead to mobilization of hematopoietic progenitor cells, but also to the generation and mobilization of dendritic cells. These effects may enhance hematologic recovery and immune recovery, and potentially mediate a direct or indirect anti-tumor effect. Flt3L-stimulated cells could also be utilized for the induction of more optimal protein- or peptide-based vaccines. These concepts will soon be tested in various clinical trials. [333] [334]

Interferons (IFN- and - and -)

Interferons are produced in response to various stimuli, including viral infection, double-stranded RNA, mitogens, and antigens. [335] The IFNs are a family of glycoproteins that were initially discovered in 1957 and named for their ability to interfere with viral replication. It has since become clear that IFNs have pleotropic biologic effects, including immunostimulatory effects, direct antiproliferative effects, and antiviral properties. [335] The three major types are IFN-, IFN-, and IFN-. IFN- and IFN- bind to the same type I receptor and are stable at pH 2. IFN- binds to a type II receptor and is labile at pH 2. IFN- is produced by a variety of cells including macrophages and lymphocytes. Virtually every nucleated cell produces IFN- in response to a variety of stimuli, including viruses, other pathogens, and double-stranded RNA. IFN- is produced by fibroblasts and epithelial cells and IFN- is produced by T lymphocytes and NK cells. IFN- is a potent activator of monocytes and macrophages, enhances NK-cell cytotoxicity, and results in the up-regulation and expression of both class I and class II MHC antigens. [336] [337] The other biologic activities of IFNs include antiviral activities; ability to interfere with cell proliferation; inhibition of angiogenesis; and regulation of differentiation and enhancement of expression of cell surface antigens, including tumor-associated antigens, MHC antigens, or adhesion molecules. [335] In part, IFNs mediate their action by enhancing the expression of IFN-inducible genes. [338] The biologic role for most of the proteins activated by IFNs is unknown, but two of the best-characterized molecules regulate protein translation. [338]

Both IFN- and IFN- are derived from a single gene, whereas 23 genes exist for IFN-, all located on the short arm of chromosome 9. The IFN- gene is also located on chromosome 9, and the gene for IFN- is located on chromosome 12.[339] Interestingly, some malignant hematopoietic cell lines have been shown to have homozygous deletions of type I IFN genes on chromosome 9, suggesting that these genes may function as tumor suppressor genes; therefore, their loss may be associated with tumor progression.[339] The interferons have activity in a variety of malignancies. Anti-tumor activity has been demonstrated in hairy cell leukemia (HCL), chronic myeloid leukemia, cutaneous T-cell lymphomas, multiple myeloma, and other indolent lymphomas.

Clinical Application

The IFNs were the first of the cytokines to undergo extensive clinical evaluation, and most of the trials have studied IFN-. Initial clinical studies began in the 1970s with partially purified IFN preparations. Over the previous decade, numerous phase II studies of IFN- have been completed, with antitumor activity primarily being demonstrated against hematologic malignancies. Limited studies have been conducted with IFN-, and results to date demonstrate little role for this cytokine as a therapeutic anticancer agent. IFN- is administered at a wide range of doses and schedules depending on the clinical setting. The toxicities associated with IFN- are dose-related. The most common side effects include fever, chills, fatigue, headache, myalgias, arthralgias, and a flu-like syndrome. [335] Patients generally develop tolerance to the fever and chills after repetitive administration. However, there is no tolerance to the other constitutional symptoms, which are usually dose-limiting. Higher doses of IFN are associated with neurologic toxicity (depression, confusion, seizures); cardiac abnormalities (atrial arrhythmias), and laboratory abnormalities, including transaminase elevation, leukopenia, thrombocytopenia, and anemia. [340] Bone marrow suppression must be monitored closely in patients with compromised marrow reserve due to underlying malignancy, concomitant drug administration (AZT), and/or chemotherapy. The salient features of IFN therapy in hematologic malignancies are discussed in the following sections.

Chronic Myelogenous Leukemia (CML)

IFN- produces hematologic responses in 5080% of patients with chronic phase CML. [341] [342] A major difference from chemotherapy is the induction of cytogenetic responses (suppression of the Ph+ cells) by IFN therapy. [341] [342] Cytogenetic responses can be observed in up to 50% of patients with major cytogenetic

responses (<35% Ph+ cells) in 1525% of patients. In 1994, the Italian Study Group randomized 218 patients between IFN- and hydroxyurea and found that IFN-treated patients had a significantly longer survival (>72 vs. 52 months), time to transformation (>72 vs. 45 months), and increased incidence of major cytogenetic responses (19% vs. 1%). [342A] This was achieved with regimens administering IFN daily at doses of 5 MIU/m^2 or 9 MIU daily. Based upon the experience at M.D. Anderson, the development of a major cytogenetic response was associated with improved survival even with landmark analyses performed 12 months into therapy. [341] Most studies suggest the benefit of IFN- is limited to patients who develop a cytogenetic response. A medical research council trial reported improved survival compared to chemotherapy alone in the IFN--treated cohort of patients who did not demonstrate cytogenetic response. [343] The presence of splenomegaly and bone marrow basophilia were poor prognostic factors for outcome. Most studies suggest that higher doses of IFN- are required for this beneficial effect. However, a trial performed at the University of Colorado among 41 patients with newly diagnosed CML reported a 70% complete hematologic remission rate with 22% major cytogenetic responses using IFN- at 2 MIU subcutaneously three times a week. [344] This activity appears similar to IFN activity with higher, more frequent dosing, but with less toxicity and much lower overall cost. These results will require confirmation and even randomized trials to define the role of lower dosing. Most patients treated with IFN do not eliminate their minimal disease state defined by positive RT-PCR for bcr-abl mRNA. [345] [346] [347] [348] However, recently patients in complete cytogenetic remission on IFN for many years have been found to be RT-PCR negative for the transcript. The minimal disease state is generally only eliminated in patients following an allogeneic BMT. Toxicity of the higher-dose standard regimen of IFN is significant in patients over 60 years of age with up to 65% requiring dose modification due to toxicity (frequently neurotoxicity and fatigue). [349] Response rates appear similar to younger patients with 26% experiencing major cytogenetic responses and a median survival of 64 months and 5-year survival of 62%. Finally, a study from France combined IFN- with cytarabine (Ara-C) and compared it to IFN- alone in newly diagnosed CML patients. Ara-C was administered at 20mg/m^2 /day for 10 days while IFN- was administered in both cohorts at 5 MIU/m^2 /day daily. [350] Those receiving the combined IFN plus Ara-C had significantly greater cytogenetic responses (41% vs. 24%); higher hematologic remission rate ($P = 0.003$), and at 3 years a significantly better survival (85.7% vs. 79.1%). After a short follow-up, the combined arm appears superior in terms of overall survival. This appears to be a step forward in the treatment of chronic phase CML.

IFN is most effective when used to treat patients with early-stage CML, with very little activity in accelerated phase disease, and no benefit when used in blast crisis. [351] Many patients experience significant side effects from IFN, as relatively high doses are generally utilized to induce responses. IFN- has also been evaluated in patients with CML, but the available data suggest that IFN- has greater activity in CML, and the incidence of toxic events has been greater with IFN-. [352] [353] Additional approaches currently being explored include combining IFN with chemotherapy and the use of maintenance IFN after allogeneic BMT for CML.

In limited pilot studies, IFN- has been used to treat patients with polycythemia vera, agnogenic myeloid metaplasia, and primary thrombocythemia with activity in subsets of patients. [354] [355] [356] [357] [358] Similar to the effects seen in CML, IFN- has the ability to suppress hematopoiesis of affected lineages, thereby reducing peripheral blood counts. Clearly, IFN- has potential clinical application for the treatment of myeloproliferative disorders, in addition to CML.

Hairy Cell Leukemia (HCL)

Since the initial report in 1984 by Quesada that IFN--induced improvement in the hematologic parameters of patients with HCL, it has become well established that IFN- is an active agent in its treatment. [359] [360] HCL is extremely responsive to IFN- therapy, with an overall response rate of 8090%. IFN- has only a 1015% complete remission rate. These results can be obtained with relatively low doses of IFN, 23 MIU subcutaneously three times a week, but require continued therapy to maintain the hematologic remission. Recovery of the platelet count can occur within 2 weeks; reduction in spleen size and improvement in peripheral blood counts usually occur within 23 months of therapy. Responses to IFN have been reported in splenectomized and previously untreated patients. [361] Progression of HCL has been associated with the development of neutralizing anti-IFN antibodies. [242] Survival in patients treated with IFN- is excellent. However, the development of newer agents, pentostatin and 2-chlorodeoxyadenosine, have replaced IFN as front line therapy because of their durable and frequently complete remissions. [361]

The precise mechanism of IFN activity against HCL is not entirely clear, but its antitumor activity may involve direct effects, modification of oncogene expression, modulation of cytokine expression, or specific alterations in protein synthesis leading to partial differentiation of the malignant leukemia cells. [3] [7] [335] Interesting preclinical and clinical data suggest that TNF may act as an autocrine growth factor for some cases of CLL and HCL. [3] Digel et al. [7] found increased circulating levels of soluble TNF-binding proteins (TNF-BP) in the sera of patients with B-CLL and HCL compared with human sera from healthy controls. Levels of TNF-BP have been reported to be decreased after effective therapy with IFN-. [362] [363] In addition, preclinical studies have shown that IFN- can inhibit TNF-dependent HCL growth with decreases in TNF mRNA occurring after IFN treatment. [362] [363]

Multiple Myeloma

IFN- has been known to bind to myeloma cells and cell lines and to mediate anti-proliferative effects. [364] Despite years of evaluation, the role of IFN in the treatment of multiple myeloma remains unclear. Early studies explored the activity of IFN- as a single agent and resulted in response rates of only 2025%. [364A] [364B] [364C] This was considerably less than that achieved with chemotherapy. Clinical effects in end stage refractory myeloma patients have been observed, but only in few patients for limited periods of time. Many more clinical trials have utilized IFN- either in combination with chemotherapy during the induction phase of treatment or following achievement of a maximal treatment effect. [365] [366] [367] [368] [369] [370] [371] Treatment during the plateau phase with maintenance IFN to prevent tumor cell cycling has been explored. Results of these studies have been contradictory. The Nordic Myeloma Group randomized 583 patients to chemotherapy (melphalan plus prednisone) with or without IFN during all phases of treatment including induction, plateau phase, and even relapse. [366] The IFN had no effect on overall survival or response rate, but it did increase the duration of response and plateau phase. On the other hand, a Canadian study randomized 176 patients following a response to melphalan and prednisone to either observation or IFN- at 2 MU/m^2 /d three times a week. [371] This trial showed a benefit in overall median survival (4335 months) that was significant only when adjusted for baseline characteristics. Progression-free survival greatly favored those randomized to IFN maintenance. Other trials, including the Swedish and Southwest Oncology Group trials, showed no survival benefit from maintenance treatment with IFN. [368] [370] The continuation of IFN at relapse has been proposed

as a reason for the different outcomes between some of the trials. [372] Several small randomized trials showed that IFN can maintain remission following autologous BMT and even improve overall survival in post-transplant patients. [373]

Non-Hodgkin Lymphoma (NHL) (Including Cutaneous T-Cell Lymphoma and HTLV-Associated T-Cell Lymphoma)

IFN- studies in patients with non-Hodgkin lymphoma have also examined the single-agent activity, effects of maintenance therapy after induction chemotherapy, and use of IFN- in combination with chemotherapy. [374] [375] [376] [377] [378] [379] [380] [381] For the most part, these studies have been limited to indolent lymphomas in which the overall response rate is approximately 2540%. [374] [375] [376] Patients with intermediate-grade non-Hodgkin lymphoma tend to have fewer objective responses. [374] IFN has also been studied in combination with cytotoxic chemotherapy in patients with low-grade lymphomas. The results of a prospective randomized trial designed to evaluate the role of IFN- with a multidrug cytotoxic therapy (COPA [cyclophosphamide, vincristine, prednisone, doxorubicin]) have been reported. [377] Two hundred ninety-one patients with low-grade lymphoma were randomized. The regimens produced comparable objective response rates, but the addition of IFN resulted in prolongation in the time to treatment failure, in duration of complete response, and in overall survival. [377] A follow-up report at 5 years failed to confirm the overall survival advantage. [380] A trial reported by a French cooperative multicenter group enrolled 242 eligible patients with advanced low-grade follicular NHL and randomized them between cyclophosphamide, doxorubicin, vinblastine, prednisone (CHVP) with or without IFN-. [381] Those receiving CHVP plus IFN- had a better response rate, event-free survival, and overall survival. CHVP and IFN- were administered simultaneously and for prolonged periods of time. In contrast, the preliminary results of a large prospective trial in which patients with follicular low-grade lymphoma were randomized to chemotherapy alone or with IFN- suggests no difference in complete response rates and overall survival between the two groups. [378] The results of ongoing randomized trials should address whether the combination of IFN and chemotherapy increase response rates, disease-free time, or overall survival time in patients with low-grade lymphoma. IFN has also been

added following both autologous and allogeneic stem cell transplants for both non-Hodgkin lymphoma and Hodgkin disease. [382] The treatment is associated with an increase in cytopenia thereby limiting doses of IFN to 23 MIU/m ^{2} three times a week. Whether it improved the remission duration or overall survival is unclear and randomized trials will be required to definitively answer this question.

Mycosis fungoides and Sezary syndrome are categories of cutaneous T-cell lymphomas that can be controlled by a variety of modalities including psoralen ultraviolet A (PUVA), electron-beam whole skin irradiation, and topical chemotherapy. [383] Responses are best achieved while the disease is confined to the skin. Nodal and systemic disease is poorly controlled by these measures and systemic chemotherapy has only transient palliative effects. The use of IFN- in cutaneous T-cell lymphomas (CTCL) was first reported by Bunn in 1984. [383A] Initial reports utilized the maximal tolerated doses of IFN administered intravenously and showed a 45% response rate. [383] As further trials were performed and reported, it became clear that the high doses were not necessary for responses and lower more tolerable doses (510 MIU/m^2 three times a week) could induce responses. [383] Response duration generally lasts 35 months for partial responses and longer for complete responses. Response rates appear superior in patients with stage IAIIB and lower; those with stage IV and those with large cell histology or visceral disease are less likely to respond to IFN-based therapy. IFN has been combined with various chemotherapy agents with generally unimpressive results. Some promising results have been observed when IFN was combined with PUVA phototherapy. [384] A high percentage of complete responses were seen in previously treated patients and these responses were quite durable. The responding patients had stage IB to IVA, whereas no responses were seen in patients with stage IVB disease. In this trial IFN was administered with PUVA at 12 MIU/m^2 three times a week.

HTLV-1-associated adult T-cell leukemia/lymphoma can be chronic and indolent with >2 year median survival, or very aggressive with median survivals of <6 months. Therapy with systemic combination chemotherapy has been quite unsatisfactory. Recent reports have combined IFN with the antiretroviral nucleoside inhibitor AZT and shown remarkable effects. [385] [386] Some of the 19 patients reported by Gill et al. had failed prior combination chemotherapy. [386] There were 11 major responses out of 19 patients with 5 complete responses. Six of the patients had survived longer than 12 months. Another report described clinical responses to IFN and AZT in all five patients treated with high-dose AZT and IFN at 9 MIU/m ^{2} /d three times a week. [386]

Chronic Lymphocytic Leukemia

IFN- produces responses in early stage CLL and immunomodulatory activity has been documented in patients with CLL who were treated with IFN-. [387] Attempts have been made to use IFN to maintain remissions in patients with CLL who have been induced into a remission with fludarabine. [388] The hypothesis to be tested was that patients with low CD4 counts are frequently anergic and have low IgG levels, and that uniform disease progression may be halted by either the direct antitumor effects or immunomodulatory effects of IFNs. In a trial of 22 patients, no beneficial immune effects were observed with IFN at 3 MIU subcutaneously three times a week. [388] Furthermore, the time to progression did not appear any different from that in a historical control group not maintained with IFN. It is possible that the immune defects within the T-cell compartment following fludarabine may limit the effectiveness of IFN in CLL.

Solid Tumors

Therapy of melanoma has been drastically changed by the results of a randomized trial of high dose IFN- as adjuvant therapy of high-risk patients with either deep melanoma primary lesions (>4.0 mm) or regional lymph node disease. [389] Patients treated with 4 weeks of intravenous IFN at 20 MIU/m^2 /day, 5 days a week, followed by 11 months of 10 MIU/m^2 /day three times a week had a significant improvement in progression-free survival and overall survival. While toxicity was significant, with many constitutional symptoms, most patients completed over 80% of the intended treatment and there were only 2 early deaths of 143 patients treated with IFN. The deaths were due to liver failure, which was not observed later in the trial when liver function tests were more rigorously monitored. Other solid tumors including renal cancer and colon cancer have been studied with IFN in combination with other cytokines, or chemotherapy. The actual role and benefit achieved by the addition of IFN is still poorly defined in most solid tumors.

Interferon-

IFN- has not found a definite role in the treatment of either hematologic malignancies or solid tumors. Based on its many immunomodulatory effects compared to type I IFNs, there was much hope for this agent as a component in the immune-based therapy of cancer. [336] [364] [390] [391] IFN- has antiproliferative effects in myeloma cell lines and may inhibit IL-6 mediated growth of these same myeloma cells. [392] In addition, it has been utilized in

combination with cyclosporin A to induce an autologous graft-versus-host-like syndrome in patients with breast cancer undergoing autologous stem cell transplantation. [393] It may help induce this syndrome through its ability to enhance MHC class I and II expression on tissues. The induction of a graft-versus-host syndrome is one of many attempts to induce a concomittant antitumor response (graft-versus-tumor). Attempts have been made to activate host antitumor immunity with IL-2 and IFN- in acute leukemic patients at high risk for relapse following autologous stem cell transplantation. [394] The addition of IFN- to IL-2 may allow a lower dose of IL-2 to stimulate NK cells and also stimulate MHC expression by target leukemic cells. IFN- can also induce the production of a number of other cytokines, some of which appear to mediate antitumor effects by themselves. [336] [337] IP-10 is an IFN- inducible protein with anti-angiogenic properties capable of inducing tumor necrosis. Mig is a chemokine induced in monocytes by IFN- that also mediates antitumor effects through vasculature damage and necrosis. [336] IL-18 is a recently described cytokine produced by monocytes in response to IFN- that can co-stimulate T and NK cells and stimulate Fas-ligand-mediated tumor cell cytotoxicity by NK cells. IL-18, like Mig and IP-10, has antitumor effects on its own, through the activation of NK cells and CD4+T cells. [337]

Tumor Necrosis Factor- (TNF-)

TNF- and lymphotoxin (TNF-, LT) are closely related cytokines that share common receptors and have similar biologic effects with a central role in immune and inflammatory responses. [395] [396] Release of TNF- and LT is stimulated by endotoxin, and other cytokines, including IFN-, IL-1, and GM-CSF. [397] Monocytes/macrophages are the primary producers of TNF-, and activated T cells are the primary source of TNF-. In humans, the genes for TNF- and LT are closely linked on the short arm of chromosome 6. [395]

Interest in the use of TNF for the treatment of human cancers stems from intriguing animal studies in which TNF resulted in dramatic regression and hemorrhagic necrosis of tumors. [398] In addition, the in vitro antitumor effects of TNF are very specific for malignant cells, with very few cytotoxic effects observed against normal cells. [395] The biologic effects of TNF include profound effects on immune effector cells and hematopoietic cells, inhibition of enzymes involved in adipocyte differentiation and lipid metabolism, and stimulation of both collagen production and collagenase activity. [397] TNF specifically induces a number of proteins in vascular endothelial cells, which may be closely linked to its hemostatic properties. Although mature neutrophil activity is stimulated by TNF, it suppresses the colony growth of CFU-GM and CFU-E. The inhibitory effects of TNF on hematopoietic progenitor cells depend on prolonged exposure and high concentrations. In addition, TNF activates macrophages and is an important inducer of acute-phase reactants. [399] TNF- is identical to the protein associated with cachexia, hence the alternative name, cachetin. Importantly, TNF also induces secretion of a number of cytokines, including GM-CSF, IL-1, and IL-6, as well as other mediators of inflammation. [395] The precise mechanism whereby TNF mediates its antitumor activity is unclear, but evidence suggests that direct cytotoxicity against malignant cells is one important mechanism that may be due to free radical formation. TNF- has indirect effects on the vascular supply of tumors with platelet aggregation, erythrocytosis, and endothelial and vascular destruction. The tumor vascular bed may be the most critical target for TNFs antitumor effects.

Phase I and II studies with TNF have been completed with limited single-agent activity demonstrated against hematologic malignancies or solid tumors. [400] [401] [402] However, in studies to date, only modest doses of TNF have been administered due to considerable toxicity, including fever and hypotension. [400] [401] [402] Systemic toxicity (hypotension) in humans allows only 1/201/50 the effective dose administered in the murine tumor models. Thus, therapeutically inadequate doses of TNF have been administered due to the significant toxicity associated with the systemic administration of TNF. Strategies designed to overcome this limitation have included the use of agents to reduce hypotension associated with TNF, such as nitric oxide inhibitors and the use of effector cells (tumor-infiltrating lymphocytes) transfected with the gene for TNF augmenting the local delivery of TNF to tumors and limiting systemic exposure. [403] [404] TNF has been combined with chemotherapy and IFN- in isolated limb perfusion (ILP) therapy of extremity sarcomas and melanoma. [405] [406] The isolated perfusion of an extremity with this combination has allowed very high regional levels of TNF with little exposure for the systemic circulation. In a series of 55 patients with sarcomas (30 primary and 25 recurrent), all with bulky disease and many with multiple sites, there were 36% pathologic complete remission and 51% pathologic partial remission with limb salvage feasible in >80% of patients. [406] Systemic toxicity was mild to moderate with some leakage of TNF- into the central circulation. Compared to prior studies of melphalan alone, these results appear far superior. A similar approach has shown promising effects for in-transit melanoma with a number of complete responses. [405] Higher doses of TNF were more toxic and not more effective. Trials are now underway to more definitively demonstrate the benefit of addition of TNF- to the ILP chemotherapy in melanomas

with regional lesions.

Monocyte/Macrophage-Colony-Stimulating Factor (M-CSF)

M-CSF, or CSF-1, is a homodimer composed of two proteins of approximately 45 kd molecular weight, linked by a disulfide bond. Produced primarily by monocytes, fibroblasts, and endothelial cells, M-CSF stimulates the proliferation, differentiation, and activation of the monocyte/macrophage lineage. [407] [408] The receptor for M-CSF is the product of the proto-oncogene c-*fms*, a member of the tyrosine kinase family of receptors. M-CSF is detectable in the serum at low levels in normal healthy adults.

When administered to mice, M-CSF results in an initial increase in cycling rates of bone marrow and splenic committed progenitors. Further treatment with M-CSF (>2 doses) leads to a reduction in cycling rates. [409] The precise mechanism of this effect is unclear. M-CSF is a potent activator of monocyte/macrophage function. [410] The mechanism by which macrophages promote their antitumor activity following exposure to M-CSF may involve the elaboration of secondary cytokines such as IL-1 or TNF or the promotion of oxygen free radicals. Activation of monocytes/macrophages by M-CSF significantly enhances their antibody-dependent cellular cytotoxicity (ADCC).[410]

M-CSF stimulates the differentiation of hematopoietic progenitor cells into mature monocytes and enhances their proliferation, survival, the production and release of other cytokines (colony-stimulating factors, IL-1 IFNs) from mature monocytes, and the enhanced surface expression of proteins which act as important costimulatory molecules.[411] M-CSF can also act to enhance the differentiation of cytotrophoblasts into syncytotrophoblasts which produce human chorionic gonadotropin and can enhance LDL-receptor clearance of cholesterol from the serum by other cell types. The op/op osteopetrotic mouse has a mutation in the structural gene for M-CSF making it inactive.[412] M-CSF appears to enhance antibody-independent lysis of tumors through activation of reactive nitrogen intermediates. [410] [413] These effects on mature monocytes may be relevant to both infectious and tumor targets. In vitro experiments comparing M-CSF to GM-CSF and to G-CSF have demonstrated that lysis of various human tumor cell lines (glioma, lymphoma, colonic, and melanoma) by either peripheral mononuclear cells or granulocytes were enhanced by M-CSF and GM-CSF.[413] Only M-CSF enhanced the spontaneous lysis of tumors, while GM-CSF and

M-CSF enhanced ADCC-mediated lysis. M-CSF transfection of murine melanoma tumor cell lines (B16) induced a protective antitumor response and prolonged the mouses survival.[414] Both monocyte and T-cell activation occur secondary to the transfected tumor vaccines and may be involved in tumor rejection. Therefore, M-CSF is a candidate cytokine for gene therapy, as well.

Munn et al. studied the effects of parenteral M-CSF on the ADCC of monocytes of nonhuman primates. [408] M-CSF administered by continuous intravenous infusion or subcutaneous injection daily for 14 days at doses of 50100 g/kg/day resulted in significant increases in peripheral blood monocyte number. ADCC of monocytes from treated animals incubated for 3 days with M-CSF demonstrated significantly enhanced tumoricidal activity against a melanoma cell line (SKMel-1) in the presence of the monoclonal antibody 3F8. M-CSF therapy was associated with a mild decrease in peripheral blood platelet counts. No other significant toxicity was noted.

Anti-tumor Agent

Several phase I and phase II trials of M-CSF have been completed and reported. [410] [414] [415] [416] [417] The first, performed by Sanda et al., evaluated intravenous bolus M-CSF administered every 8 hours for 7 days over a range of doses from 10100,000 g/m^2 /day with ocular toxicity (iritis), periorbital inflammation, and malaise as the dose-limiting toxicities at the 30,000 and 100,000 g/m^2 dose levels.[410] Pharmacokinetics revealed levels up to 1,000 baseline of M-CSF with a $t_{½}$ of 16 hours. Monocyte counts were increased in those patients who received >1000/g/m^2 /day and platelets declined during M-CSF administration, but platelet counts remained above 100,000/l. One patient with renal cell carcinoma had regression of his disease for a prolonged period of time. M-CSF was also administered in a phase I trial by continuous intravenous infusion for 14 days at doses of 50150 g/kg/24 hours. [417] Dose-limiting toxicities were both iritis and thrombocytopenia. The maximum tolerated dose was 1,000 g/kg/day for 14 days and 7-day infusions were recommended as more tolerable. Monocyte counts increased and peaked at days 79 of the 14-day course. A subcutaneous dosing schedule was evaluated in a phase I trial by Bukowski and his colleagues in 42 cancer patients. [416] These investigators explored a dose range from 10025,600 g/m^2 /day of M-CSF on days 15 and 812 every 4 weeks. Dose-limiting toxicities at the highest dose tested were thrombocytopenia and iritis as observed in the previous trials. Monocytes in the blood were increased in number and appeared stimulated (primed) based on their in vitro release of TNF and IL-1 following LPS exposure (in vitro). Pharmacokinetics revealed increased clearance of M-CSF following the initial week of therapy.

Much of the interest surrounding M-CSF has centered on its ability to enhance monocyte function in mediating ADCC. Adoptive immunotherapy in the SCID mouse with a human melanoma xenograft (M-14 melanoma cell line) combined R24, an anti-melanoma (anti-GD2) antibody with M-CSF. [418] These studies revealed that activated T cells could be targeted to infiltrate human tumors and mediate anti-tumor effects when exposed ex vivo to antibody and M-CSF. Other cytokines such as GM-CSF and/or RANTES chemokine were unable to target tumors and mediate antitumor effects. A phase II trial of M-CSF was performed with the agent administered at 80 g/kg/day by continuous intravenous infusion for 14 days with monoclonal antibody D612, a murine IgG2a antibody directed at a glycoprotein expressed on cancer of the gastrointestinal tract. [419] Biologic activity was observed, characterized by monocytosis and elevated serum neopterin (a product of activated monocytes). No anti-tumor activity was observed in 14 patients with metastatic gastrointestinal malignancies. Eleven of 14 patients developed low to moderate levels of anti-D612 antibodies. Finally, a phase I trial by Weiner et al. combined M-CSF with IFN- in an attempt to optimally expand and activate tumoricidal mononuclear phagocytes.[420] M-CSF could be combined with IFN- without increasing the toxicity of M-CSF. However, there was no significant increase in peripheral blood monocytosis. Although monocyte phenotype changes suggested activation superior to M-CSF alone, there was no other evidence for superior effects with the combination of agents as compared to either agent alone.

Infectious Diseases

Because of its ability to prime and activate monocytes and macrophages, M-CSF is a potentially attractive agent in diseases in which enhanced effector-cell function may improve therapy. M-CSF has been shown to enhance antifungal activity of its target effector cells, making invasive fungal infections ideal targets on which to test its clinical potential. [409] [411] In phase I and II trials conducted at the Fred Hutchinson Research Cancer Center after the documentation of progressive fungal infection M-CSF was administered at 1002,000 g/m^2 daily for 28 days in 46 patients. [421] [422] The initial 24 patients were reported in a phase I trial of M-CSF and the subsequent 22 treated at the maximally tolerated dose of 2,000 g/m^2 /day. [422] These were all BMT patients with a historical morbidity of 80% due to their invasive fungal infection. These 46 patients, compared to a historical matched group of 58 patients, had a better survival of 27% versus 5% in the control patients. Those with candidal infection and performance status greater than a Karnofsky score of 20 benefited from the therapy (50% survival), although those with aspergillus infection and poor PS (20%) had a dismal outcome with or without M-CSF. There was some thrombocytopenia that complicated the 2,000 g/m^2 /day dosing. Determining whether M-CSF definitively enhances monocyte function, thereby improving therapy of fungal disease, will require randomized trials.

Osteopetrosis

Another particularly interesting application for M-CSF is in osteopetrosis. Osteopetrosis is a heterogeneous family of disorders characterized by the inability of osteoclasts to resorb bone.[423] This results in failure of the remodeling of bone, and obliteration of the medullary cavity that leads to extramedullary hematopoiesis. Since osteoclasts and macrophages are thought to be derived from a common hematopoietic precursor, M-CSF could possibly enhance osteoclast function or recruit new osteoclasts into bone with resultant normalization of bone resorption. [424] [425] Although Orchard et al. demonstrated that serum levels of biologically active M-CSF are normal in patients with osteopetrosis, other investigators have shown absence of bioactive M-CSF in *op/op* mice (an animal osteopetrosis model).[412] [426] For this reason, a phase I trial of intravenous M-CSF has been performed in this disease setting. [427] Although doses of M-CSF were low (1002,000 g/m^2 /day), some clinical benefit, defined as reduced bone density, eruption of new teeth, and improved bone architecture, was seen in several of the patients suggesting that more prolonged therapy beginning at an earlier age may be of considerable benefit.

Monoclonal Antibodies

The successful use of monoclonal antibodies as a therapeutic approach in the treatment of cancer depends on two basic principles; that cancer cells are immunologically distinct from normal cells and that recognition of these differences can result in an effective antitumor response through specific binding to target antigens expressed on tumor cells. Ideally, one can identify tumor-specific antigens (i.e., an antigen expressed solely on the tumor cell, but not on normal cells) as a

antibody. Unfortunately, truly tumor-specific antigens are rare and limited to B-cell and T-cell malignancies in which the variable region of the immunoglobulin molecule or T-cell receptor serves as a unique marker referred to as the idiotype determinant. [428] [429] Most currently identified tumor antigens represent differentiation antigens and are therefore expressed on both malignant cells and normal cells, albeit the expression of these antigens may be at lower levels on nonmalignant cells.[222] [223] [430] In addition, the products of cellular oncogenes have been defined as cell-surface receptors (i.e., platelet-derived growth factor, epidermal growth factor) and represent potential targets for monoclonal antibodies. [431] [432] [433]

Because technologic developments allowed for the production of monoclonal antibodies specific for antigens of choice, these monoclonal antibodies promised a revolution in human diagnostics and therapy. The antibodies could be used unmodified or naked, bound to radioisotopes or toxins, or modified to provide two different specificities (bispecific). Their potential to home in on tumor cells specifically led to them being identified as magic bullets. New second- and third-generation antibodies have been created that are now humanized to reduce immune reactions in patients and simplified into a single variable chain construct. The distinct problems associated with murine antibodies (immunogenicity) and human antibodies (limited availability) have led to the construct of these so-called chimeric antibodies, which retain the murine antigen-binding sites of the murine antibody, but closely resemble the constant region of the human immunoglobulin. [434] Chimeric antibodies have been shown to be less immunogenic, with longer half-lives than murine antibodies. Future directions in research include the production of more effective antibodies with single-chain antibody-derived components (Fsv). In addition, researchers are attempting to identify better targets in tumors or associated cells and even to use the monoclonal antibodies to disrupt growth signals. Although the initial expectations for monoclonal antibodies were unrealistic and many of the initial claims overstated, the perspective now appears to be more realistic and balanced. Now a variety of antibodies including those which are unmodified, humanized, and linked to radioisotopes, and toxins that inhibit specific steps in protein synthesis are demonstrating efficacy in phase II and III trials. These may be close to becoming a component of standard therapy for malignancies. New targets for monoclonal antibodies may boost their efficacy by binding to an antigen that is a growth factor receptor on the surface of the cancer cell, or by blocking the signal that induces the formation of blood vessels in tumors, or antibodies directed at the stroma of tumor cells.[434] [435] These alternate targets may lead to more sustained tumor regression and may avoid resistance by the cancer cells through their ability to constantly mutate and escape detection.

Considerable obstacles to the therapeutic use of monoclonal antibodies have been identified and include the short half-life of administered antibody due to the presence of free circulating antigen and the development of neutralizing antibodies to the murine antibody (human anti-mouse antibody). Tumor cells can escape the monoclonal antibody through the phenomenon of antigenic modulation, in which binding of the antibody to the antigen results in the transient disappearance of the antigen. In addition, only a proportion of the cancer cells express the relevant antigen due to antigenic heterogeneity, again limiting efficacy. Other barriers to this approach include poor affinity of the antibody to the tumor target antigen and impaired delivery of the antibody due to poor tumor vascularity and necrotic centers. [436]

Clinical Application

Naked Antibodies

Numerous phase I and II studies of monoclonal antibodies have been completed in patients with solid tumors and hematologic malignancies. [432] [435] [437] [438] [439] [440] [441] Initial studies focused on the safety and pharmacology of unconjugated murine antibodies in patients with leukemia and lymphoma. The tumor-associated antigens identified were differentiation surface markers expressed by both malignant and normal lymphoid cells. Generally, the side effects of unconjugated monoclonal antibodies have been mild and include fever, chills, urticaria, bronchospasm, and pain. Rarely patients have developed symptoms of anaphylaxis and serum sickness, which have been reversible in all reported cases.

One strategy for testing immune intervention in patients with hematologic malignancies is to target cytokine receptors, such as the IL-2R. [438] [442] Waldmann et al. developed a murine monoclonal antibody, anti-Tac, which binds to IL-2R and prevents interaction of IL-2 with this subunit. [442] The rationale for this approach is that resting normal T cells do not express the high-affinity IL-2R, whereas expression of this receptor is found on a proportion of lymphoid neoplasms, including HCL, large and mixed-cell lymphomas, T-cell and B-cell monocytic leukemias, T-cell lymphoma, and some Reed-Sternberg cells in Hodgkin disease. [442] In addition, virtually all patients with HTLV-1 associated adult T-cell leukemia express the IL-2R. Therefore, initial clinical studies with the anti-Tac antibody have been directed at patients with HTLV-1 associated T-cell leukemia.[442] Preliminary results with 19 patients have shown that therapy is well-tolerated. Seven patients had partial or complete remission, lasting 9 weeks to >3 years. The elimination of clonal malignant cells was confirmed by molecular genetic analysis of HTLV-1 proviral integration and T-cell receptor rearrangements. The mechanism of action of anti-Tac is not clear, but it does not appear to be mediated by ADCC.

One very intriguing and labor-intensive approach has involved the use of anti-idiotype monoclonal antibodies for the treatment of indolent lymphomas. [435] Levy and his colleagues[437] generated monoclonal antibodies that recognize the specific idiotype of surface immunoglobulin on the patients lymphoma cells. Initial clinical trials reported a 5070% reduction in the tumor size with some patients obtaining complete responses. [437] [443] The mechanism of antitumor effects remains unknown. Treatment with anti-idiotypic murine antibodies either alone or in combination with other treatment modalities such as IFN- or chlorambucil have produced up to 70% partial or complete responses in patients with B-cell NHL. It has been generally accepted that host effector systems, such as complement or antibody-dependent cellular cytotoxicity, that interact with the Fc-portion of the antibody are required for the generation of an antitumor effect. Studies have shown that the degree of in vitro stimulation of tyrosine phosphorylation of intracellular proteins correlated with direct antiproliferative effects and very strongly with tumor regression in vivo. [443] Expression of bcl-2 did not correlate with tumor regression.

Anti-CD20 monoclonal antibody IDEC-C2B8 has shown significant activity in recurrent or refractory B-cell lymphomas leading to its approval by the FDA. [440] [444] This is a nonmodulated antigen that mediates complement and antibody-dependent cellular cytotoxicity in vitro. Toxicity due to a single dose of therapy includes fever, chills, nausea, and mild hypotension. These were all very tolerable and easily managed. The $t_{½}$ was quite long, with persistent levels after 14 days following a single dose of treatment. CD20+ B cells were rapidly depleted from the blood and chimeric antibody could be found within the biopsied lymph node cells. Significant tumor regression was observed after a single dose. No significant infections were observed. The pivotal trial in relapsed low grade lymphomas included 166 patients and showed a response rate of 48% with a median duration of responses greater than 9 months (not reached yet). This humanized chimeric antibody has also shown promise in combination with chemotherapy such as cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP). T-cell

lymphomas such as mycoses fungoides have been treated with chimeric humanized anti-CD4. Clinical responses to anti-CD4 were induced with low-level immunogenicity, some immunosuppression, but no tolerance to foreign antigens. [441]

Although encouraging results have been described with antibodies against leukemia and lymphoma, little success has been reported with naked monoclonal antibodies against solid tumors. One apparent exception has been a report on Dukes C colon cancer treated with a murine monoclonal antibody. [445] This antibody, 17-1A, is directed at a surface glycoprotein present on epithelial carcinoma cells, which can induce antibody dependent cellular cytotoxicity and prevent colorectal cancer growth in human xenografts in nude mice. Patients with Dukes C colorectal cancer were randomized to observation (before the proven benefit of 5-FU based regimens) or 17-1A administered intravenously postoperatively (2 weeks after surgery) and then monthly for 4 months. [445] Patients receiving the antibody in their minimal disease state had a significantly better relapse-free and overall survival. The improvement was in the range of 2530% and most of the benefit appeared to be based on the decrease in distant metastases. Toxicity was minimal with only four controllable allergic reactions to the antibody infusion. These results are being confirmed in other trials and would certainly be a major step forward for antibody-based therapy of cancer.

Her-2/neu, a proto-oncogene, encodes a transmembrane protein that is a receptor for a growth factor peptide and is amplified and overexpressed in 2040% of breast cancer.[446] It appears to be associated with a poor prognosis. A murine monoclonal antibody directed at the extracellular domain of p185-Her-2/neu is a potent inhibitor of growth of human breast cancer in vitro and in vivo in animal xenografts. [432] A humanized recombinant anti-p185her2 monoclonal antibody was produced for further phase I and II trials. [432] [447] When the anti-p185her2/neu antibody was administered by intravenous injection weekly, 5 of 43 patients with advanced, chemotherapy-resistant breast cancer experienced objective tumor responses. The one complete and four partial responses lasted from 1 to >24 months. Toxicity was minimal with mild fever and chills and only one grade 3 toxicity. More than 90% of patients had trough antibody levels above the targeted 10 g/ml level. An additional 16 (37%) patients had minor responses or stable disease at 11 weeks. Serum levels of extracellular domain (ECD) of Her2 appeared to correlate with poor

pharmacokinetics and a short $t_{½}$ for the antibody. Combination of anti-p185her2/neu with chemotherapeutic agents may more effectively induce tumor responses. This has been reported in abstract form for the combination of anti-p185 her 2 antibody (Herceptin) and chemotherapy (paditaxol or Adriamycin plus cyclophosphamide) in advanced breast cancer.[447A]

Angiogenesis is critical for the growth of tumors and is mediated by substances produced by tumors, such as VEGF (vascular endothelial growth factor), which is a key regulatory paracrine growth factor for endothelial cells. [448] A neutralizing monoclonal antibody specific for human VEGF has the capacity to inhibit growth and angiogenesis of human tumors in SCID mice.[448] The neovascularization of microscopic tumors is inhibited by anti-VEGF leading to suppression of tumor growth at less than 1 mm^3 . A rapidly proliferating human rhabdomyosarcoma cell line, A673 changes its growth characteristics and enters an apparent dormant state simply by exposure to anti-VEGF, thereby inhibiting tumor angiogenesis. This has potential application in a variety of solid tumors, especially renal cell cancer, in which VEGF levels are increased in association with a common genetic alteration of the VHL gene.

Antibodies against CD44 (the major receptor for hyaluronic acid), a surface molecule involved in the adhesion of tumor cells and their implantation, appears to effect the in vitro binding of ovarian cancer cells to peritoneal mesothelial layers. [449] Animal studies with human ovarian cancer cells exposed to anti-CD44 suggest that in vivo implantation in the murine peritoneal cavity is greatly impaired, significantly decreasing the number of tumor implants.

Antibodies may also be used as cytokine antagonists or cytokine binding proteins. Anti-IL-6 can neutralize the activity of IL-6, which has been postulated as a growth factor in multiple myeloma, HIV-associated lymphomas, and possibly in renal cell cancer. [450] In multiple myeloma, elevated serum levels of IL-6 have been associated with a high proliferative phase index for the malignant plasma cells and a short survival. Initial trials with anti-IL-6 (murine antibody) in 13 patients with multiple myeloma and renal cell cancer were able to demonstrate a decrease in the fever and hypercalcemia associated with the disease states. [451] However, patients who had very high production of IL-6 were not effectively inhibited with the doses of anti-IL-6 administered. The reduction of C-reactive protein (CRP) was a good indicator of effective IL-6 inhibition.[451] While the proliferative index of some patients plasma cells could be reduced, this was not of significant clinical benefit to most patients with advanced disease. Therapy with antibodies to cytokines may not be a practical way to effectively inhibit tumor-associated cytokines, and better approaches that can overcome the high levels of cytokines are needed.

Bispecific antibodies/ligands have been used as a strategy to target molecules on the tumor cell surface, while the other antibody specificity/ligand binds to an immune cell and is capable of activating it (anti-CD3, anti-CD16, anti-CD64, or SEA [staphylococcus enterotoxin-superantigens] or is a cytokine (IL-2), itself. [452] [453] [454] [455] [456] [457] [458] Bispecific molecules bring targeted cells or cytokines directly to the tumor site and overcome the systemic toxicity by linking them to an antibody that has specificity for tumor cells. An example of such a bispecific molecule is SEA (staphylococcal enterotoxin A)-C242 (Fab), which shows promise in animal studies. [459] The SEA binds and activates large numbers of T lymphocytes through their V T-cell receptor in an MHC class II-independent manner. The attachment of antibodies to the superantigens allows the tumor-infiltrating lymphocytes to be activated and release cytokines at the tumor sites alone. This approach has been used in conjunction with antibodies directed at epithelial surface antigens (C242) and at B-lymphocyte lineage markers such as CD19. These approaches have been effective in animal trials and initial clinical trials have revealed tolerable toxicities with fever and hypotension, cytokine release, and decrease in peripheral monocyte counts. [453] Another approach taken by several investigators with antibodies to the Her2/neu surface receptor, is linking them to antibodies directed at Fc-receptors (CD16), thereby activating NK cells, monocytes, and neutrophils.[455] These trials have also been initiated with similar toxicities including thrombocytopenia in heavily pretreated patients.[455] [456] Bispecific antibodies with either CD3 or CD16 as the activating moiety and CD19 (for B-cell lymphomas) and CD30 (for Hodgkins disease) for tumor targeting have undergone preclinical and some clinical testing. [457] [458] Anti-CD30 monoclonal antibodies react with the CD30 surface antigen on Reed-Sternberg cells of Hodgkins disease and the malignant cells of anaplastic large cell lymphoma (ALCL). [460] [461] CD30 is a member of the nerve growth factor receptor family including CD27, CD40, tumor necrosis factor receptor, and Fas, all of which are involved in cellular activation or apoptosis. [461] Activation of T or NK cells with IL-2 ex vivo appears to generate more effective immune cells and allows for better subsequent activation by antibody. A phase I trial of anti-CD16/CD30 administered every 34 days with dose escalation of antibody from 1 mg/m^2 to 64 mg/m^2 resulted in only mild, nondose limiting side effects.[457] A number of patients developed human anti-mouse antibody (HAMA) responses and several had allergic reactions when rechallenged. In this phase I trial, one complete remission, one partial remission, and three minor

responses were observed at various dose levels with durations of 111 months. Finally, bispecific ligands may be promising compounds in delivering cytokines directly to tumors. IL-2 bound to antibodies against epidermal growth factor (EGF) or gangliosides appears to allow release and activation of cytokine at the tumor site that expresses the targeted antigens.[459] This has proven effective in animal studies and against human cancers in SCID-human chimeras. These effects are superior to antibodies and cytokines administered together, but not linked covalently. This approach may avoid the systemic toxicity of IL-2 (hypotension, fevers, etc.), while increasing the dose of IL-2 at the tumor site similar to cytokine-transfected tumor vaccines.

Attempts have been made to combine antibody-based therapy with cytokines that activate host immune effector cells. [452] [462] [463] [464] [465] Based on murine experiments utilizing anti-CD19 or anti-CD20 with IL-2 or GM-CSF, it was apparent that the combination of IL-2 with anti-CD20 was superior to all other combinations of cytokine and antibody in inducing remission of human B-cell lymphomas.[462] Clinical trials have combined IL-2 or GM-CSF with antibodies such as R24 (anti-GD2 murine antibody).[463] These attempts to activate immune effector cells with a cytokine and then add an antibody capable of triggering ADCC have successfully activated immune effector cells in vivo. Whether this approach enhances the clinical benefit of the cytokine or the antibody is not clear. Additionally, bispecific antibodies that bind to immune effector cells, such as T cells (anti-CD3) or NK cells (CD16), may be expanded and activated in vivo with cytokines such as IL-2. A bispecific antibody directed at CD3 and EGP-2 (a 40 kD pancarcinoma antigen) has been combined with subcutaneous IL-2 in renal cancer patients with some evidence that T cells were more effective at tumor cell killing in the presence of the bispecific antibody following in vivo IL-2. [452]

Monoclonal antibodies have also been utilized to deplete neoplastic cells from the bone marrow grafts of patients with leukemia and lymphoma undergoing autologous BMT.[464] Early trials focused on non-T-cell ALL and B-cell non-Hodgkin lymphoma and confirmed the value of antibodies and complement for purging bone marrow with very little toxicity against hematopoietic progenitor cells. [464] Thus, hematologic engraftment has not been affected; however, relapse of leukemia and lymphoma remains a significant problem. Monoclonal antibodies have also been used to prevent GVHD in patients undergoing allogeneic transplants for hematologic malignancies.[465] However, the use of anti-T-cell monoclonal antibodies for the treatment of donor marrow to decrease GVHD has been associated with reduced frequency of bone marrow engraftment and a higher incidence of leukemia recurrence. [466]

Radioisotope-Conjugated Antibodies

For a number of years, antibodies have been sought as targeting vehicles to deliver radioactivity specifically to tumor sites. [436] Advances in the chemistry and conjugation of isotopes to proteins and the development of monoclonal antibodies with relative tumor specificity have been critical to advancing the field. Radioisotopes have two advantages over conjugation to toxins or chemotherapy agents; beta-emitting compounds can kill adjacent targets and radioisotopes are not subject to multidrug resistance. Iodine [131] , yttrium [90] , a pure beta emitter, and Cu [67] are the most widely used isotopes in cancer therapy. Dosimetry of radioimmunotherapy is dependent on the uptake of the antibody and its clearance; the distribution of the antibodies; and the radioisotope used. Imaging of tumors can be improved by use of positron-emission tomography (PET) scanning. More than 100 radioimmunotherapy trials have been reported. The most promising results have been in hematologic malignancies.

Numerous pilot trials have demonstrated the efficacy of radioimmunotherapy in chronic lymphocytic leukemia, T-cell leukemia/lymphoma, and acute myelogenous leukemia (see Table 51-3).[467] [468] [469] [470] [471] [472] [473] [474] M195, a monoclonal antibody directed at CD33 (a myeloid antigen expressed by many AML cells) has been labeled with I [131] and used in the treatment of AML in combination with high-dose chemotherapy and allogeneic bone marrow transplantation and also in relapsed acute promyelocytic leukemia (APL) patients following an ATRA-induced remission. [473] [474] Results in both populations appear promising, but are early and other complicating issues in the transplant patients make the findings difficult to interpret. A few of the APL patients no longer had the PML/RAR transcript while on therapy with radiolabeled antibody. The investigators at the Fred Hutchinson Cancer Center have studied another antibody, BC8, that binds to CD45, a common leukocyte antigen present on most cells of the lymphoid and hematopoietic lineages in 8590% of AML and ALL cases. [475] BC8 was labeled with I [131] and administered to patients undergoing cyclophosphamide with total body irradiation (TBI) and allogeneic or autologous transplants. [475] Twenty patients were treated with no obvious increase in toxicity above what was expected with cyclophosphamide plus TBI. Eleven of the twenty patients were alive and in remission at 8 to 41 months. This may be a way to enhance the delivery of radioactivity as part of a transplant therapy without dose-limiting systemic exposure. The major issues continue to be tumor relapse and the development of HAMA.

The experience with radioimmunotherapy in B-cell lymphoma is even more promising. B-cell non-Hodgkin lymphoma is an especially attractive target for this therapy

since it is relatively radioresponsive. Patients have a depressed humoral immune response which makes them mount a diminished, if any, human anti-mouse immune response, and lymphoma cells may be more accessible than many solid tumors to antibody delivery.[436] This has been elegantly demonstrated by the work of Press and Kaminski utilizing anti-CD20 radiolabeled I[131] antibody.[467] [468] Press utilized this antibody at myeloablative doses with stem cell support.[467] These patients generally had minimal disease and a favorable dosimetry for tumor site accumulation. The results show a very high response rate with durable CRs in over 50% of the patients. Of 25 patients, 22 had favorable antibody biodistribution (delivery of higher doses of radioactivity to tumor compared to normal tissue). Twenty-one patients received therapeutic treatment with the anti-CD20-I[131] . Those with bulky disease or splenomegaly did not initially have favorable biodistribution. Eighteen of 21 had objective responses to the treatment, with 16 complete responses and overall progression-free survival at 2 years of 62% and survival at 2 years of 93%.[467] All patients received 2.5 mg/kg of radiolabeled antibody and tumor sites received an estimated 2792 Gy of radioactivity; all patients received stem cell support. Kaminski also observed a very high response rate with a lower nonbone marrow ablative dose of antibody, which followed a trace dose of labeled antibody given to assess radiolabeled antibody distribution.[468] The therapy was limited by hematologic toxicity and of 28 patients, there were 14 CRs and 8 PRs. The median duration of the complete responders was over 16 months. Low-grade, intermediate-grade, and transformed lymphomas all showed evidence of a response. A number of patients had large tumor burdens (>500 gm). Additionally, studies of cutaneous T-cell lymphomas with an anti-CD5 (T101) I[131] labeled radioimmunoconjugate have shown activity with partial and minor clinical responses.[471] These patients have developed HAMA that have interfered with treatment. Finally, patients with refractory Hodgkin disease have also shown promising results when treated with [90] Y-anti-ferritin polyclonal antibodies.[472] Both partial and complete responses were observed in over 50% of patients. Bone marrow toxicity did require autologous stem cell support in several patients.

Radioimmunotherapy of solid tumors has not been nearly as promising as that used to treat hematologic malignancies,

TABLE 51-3 -- Clinical Studies of Radioimmunoconjugates in the Treatment of Hematologic Malignancies

Patient Population	Radioimmunoconjugate	Target Antigen	Toxicity	Results	References
B-cell non-Hodgkin lymphoma	I[131] IgG2a anti-B1	CD20	Severe myelosuppression, stem cell rescue, infections, nausea, mild mucositis	18/21 with objective responses, 16 CRs, 15 progression-free at 3+ to 23+ mos	[291]
B-cell non-Hodgkin lymphoma	I[131] IgG2a anti-B1; trace labeled Ab, unlabeled AB, then full dose	CD20	Fever, rash, hematologic toxicity grade 34 WBC, plat	22/28 with objective responses, 14 CRs, CR median duration >16 mos, 6 DFS 16+ to 31+ mos	[292]
B-cell non-Hodgkin lymphoma	Y[90] anti-idiotype	Ig idiotype specific to B-cell NHL	NV, mucosal congestion, arthralgias, pain Fever, arthralgias	2CR, 1PR, 1 MR, 2 SD of 9 pts TTP 112 mos	[293]
B-cell non-Hodgkin lymphoma	I[131] Lym-1	HLA-DR variant		8 CRs, 5PRs of 24 patients	[294]
Cutaneous T-cell lymphoma	I[131] T101	CD5	Fever, pruritis, dyspnea, cytopenia	2PRs, 4MRs of 7 patients	[295]
Hodgkin disease	Y[90] anti-ferritin polyclonal	Ferritin receptor	BM toxicity, stem cell support required in some patients	10CRs, 10 PRs of 39 patients median survival 6 mos	[296]
Acute myelogenous leukemia	I[131] Hu M195 +Bu/CY+ allogeneic BMT	CD33 early myeloid Ag	BMT toxicity, GvHD, urticaria	1st BMT-14/15 CR, 3 CCR-18+ to 29+ mos 2nd BMTall died of transplant complications	[297]
Acute promyelocytic leukemia	I[131] Hu M195 following ATRA in relapsed disease	CD33 early myeloid Ag	Myelosuppression	2/7 molecular remission, 8 mos median PFS	[298]
Acute myelogenous leukemia	I[131] BC8 antibody + Cytoxan and TBI + BMT (allogeneic or auto-4HC)	CD45 pan-leukocyte marker	Cyt/TBI toxicity, infection, cardiac	9/13 AML/MDS and 2/7 ALL DFS at 841 mos (median 17 mos)	[299]

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ATRA, all-*trans*-retinoic acid; BM, bone marrow; BMT, bone marrow transplant; CR, complete remission; CCR, continuous complete remission; DFS, disease-free survival; MDS, myelodysplastic syndrome; MR, molecular remission; NHL, non-Hodgkin lymphoma; NV, nausea and vomiting; PFS, progression-free survival; TBI, total body irradiation; WBC, white blood cells.

excluding thyroid cancer. Therapy of breast cancer, brain tumors, and intraperitoneal therapy for ovarian cancer has limited clinical activity with occasional durable responses.[476] [477] [478] [479] Trials in human breast cancer with anti-mucin antibodies labeled with Y[90] has already been initiated. Therapy directed at the carcinoembryonic antigen (CEA) expressed on colorectal cancers, as well as normal colonic and small bowel mucosa with radiolabled I[131] antibody, A33 was escalated to sufficient levels to lyse colon cancer in vitro without significant gastrointestinal toxicity.[476] In some trials the level of serum CEA (antigen) and the degree of tumor bulk led to more rapid antibody clearance and a shorter $T_{½}$ for anti-CEA labeled with I[131] .[479] Intraperitoneal administration of a Lu[77] -labeled murine antibody, CC49, directed at TAG-72 tumor-associated glycoprotein expressed by the majority of common epithelial cancers, has been used to treat ovarian cancer.[478] Among 27 patients who had failed prior chemotherapy, 13 had measurable disease, 9 had disease <1 cm^2 , and 5 had microscopic disease only. Of those with microscopic disease only 1 of 5 relapsed with four being progression-free at >6 to 35 months. Regional therapy in patients with small-volume disease may be a more effective route of radioimmunotherapy.

Overall toxicity issues include allergic reactions, myelosuppression, and more rarely with extremely high doses of radioisotope, pulmonary, cardiac, or hepatic toxicity. Future directions include the production of more effective antibodieschimeric, CDR-grafted, or single-chain antibody-derived components.[436] Use of cytokines to enhance antigen expression (interferons) on tumor cells, or to enhance immune effector cell function (IL-2) is also being explored with radioimmunotherapy. Finally, the use of agents to improve radiotherapy, such as hypoxic cytotoxins (radiosensitizers) and local hyperthermia, could be incorporated into therapy.

Toxin-Conjugated Antibodies

Immunotoxins allow for the targeting of a variety of highly cytotoxic molecules to cancer cells.[480] [481] Additional modifications are made to prevent nonspecific binding and normal cell killing. These toxins include ricin, modified with a chemically blocked -chain or only a single -chain that has been deglycosylated; gelonin toxin, which is naturally without the chain and therefore associated with less nonspecific binding; saporin or PAP-S, a potent type I ribosome-inactivating protein (RIP); pseudomonas exotoxin modified to remove domain I (binding domain); or diphtheria toxin without receptor binding domain. The toxins may be linked either to antibodies (murine or humanized) or ligands (cytokines or growth factors) that will both target the delivery to the site of the antigen or cytokine receptor on the cancer cell.

Patients with advanced, refractory Hodgkins disease were treated with anti-CD25 (IL-2 R) bound to ricin chain and toxicities included a vascular leak syndrome, mild allergic reactions, some dyspnea, and relatively frequent induction of both anti-mouse and anti-ricin antibodies.[482] Three of 15 patients had signs of clinical tumor regression. Both anti-CD25 and anti-CD30 have been bound to ricin chain and utilized for targeting Hodgkins disease (Reed-Sternberg cells). In a phase I trial of pseudomonas exotoxin conjugated with an antibody (B3) directed at the carbohydrate antigen (LeY) present on many

solid tumors, 38 patients were treated every other day intravenously with doses from 10 to 100 g/kg. [483] Dose-limiting toxicities observed above 75 g/kg were a vascular leak syndrome, hypoalbuminemia, fluid retention, and peripheral edema. Five patients with breast and colon cancer had objective tumor regressions. Neutralizing antibodies to the immunotoxin limited therapy developed in almost all patients. Small-cell lung cancer (SCLC) has been treated with N901, an immunotoxin composed of an antibody directed at CD56 (N-CAM) linked to ricin with a blocked chain. [483] A continuous intravenous infusion for 7 days defined toxicity as a vascular leak syndrome. Cardiac and neurologic toxicity was acceptable and one clinical response was observed among 21 refractory or relapsed patients with SCLC. Again, most patients developed either human anti-ricin antibodies or human anti-mouse antibodies. Both anti-CD33 (humanized M195) and anti-CD30 have been linked to immunotoxins (gelonin toxin and PAP-S) and shown impressive activity in human leukemias and lymphomas engrafted in mice. [484] [485]

Another strategy involves the use of chimeric toxins, in which bacterial toxin genes are fused with cytokine genes or growth factors. The first such construct for clinical use involved the gene for IL-2 fused to a gene for diphtheria or pseudomonas exotoxin. [486] [487] These fused proteins recognize cells bearing the IL-2R, and are subsequently internalized by receptor-mediated endocytosis, and result in cell lysis mediated by the toxin. Clinical studies of malignancies are now underway in which the malignant cells express the IL-2Rs, including HTLV-1-associated leukemia, T-cell leukemia and lymphoma, and Hodgkins disease. [487] IL-2-diphtheria A chain toxin (DAB) has shown the most promise with documented clinical remissions in Hodgkins disease and cutaneous T-cell lymphomas. [487] Again, toxicities, including hepatic enzyme elevation, were observed. DAB EGF is being targeted for therapy of breast cancer where EGF-R is overexpressed. [433] The fact that these receptors are linked to critical signaling pathways with tyrosine kinases may make them especially critical to the clonogenic cancer cell growth. Finally, IL-4-pseudomonas exotoxin may have efficacy in cancers such as renal, lung, gastric, colon, as well as Burkitts lymphoma, T-cell leukemia, glioblastoma, and melanoma, which all express IL-4R. [488]

Tumor Vaccines

Numerous vaccine-based strategies aimed at cancer have entered the clinic over the past several years. The concept of active specific immunotherapy has been of interest in the treatment of both hematologic malignancies and solid tumors for a long period of time. The use of autologous tumor cells, allogeneic tumor cell lines, or tumor cell lysates in combination with a nonspecific immune-stimulating substance (adjuvant) have been attempted in numerous clinical trials with suggested, but still questionable benefit. Only with the enhanced understanding of the process of immune recognition and T-cell activation have more defined and potentially effective approaches been identified. Two major advances have moved the field of tumor immunology and tumor vaccines forward. First, antigens recognized by T lymphocytes are processed from endogenous or exogenous peptides, which are then presented on the antigen-presenting cell surface to a T cell. [489] This critical interaction can take place if the antigen is appropriately processed and binds to the MHC antigens and the T-cell repertoire includes receptors that bind to this complex. [490] Therefore, tumor antigens may include a variety of surface and intracellular molecules including viral-induced genes (HPV, EBV), mutated (ras) or nonmutated oncoproteins (her-2/neu), fusion proteins derived from translocations (bcr-abl), differentiation antigens from the tissue-specific proteins (gp-100, gp-75 tyrosinase), and other normal or abnormally expressed proteins. [489] [490] Work on human melanoma has led to the isolation of a variety of genes that express tumor-regression antigens and are recognized by tumor-infiltrating lymphocytes (TIL) cells which can lyse tumor targets and lead to in vivo tumor regression. [489] These tumor regression antigens have been primarily found to be tissue differentiation antigens that are normally expressed by the melanocyte lineage, including MART-1, gp-100, and tyrosinase. [489] Peptides derived from these proteins can bind to MHC molecules and subsequently be recognized by T cells from melanoma patients. The second finding critical to improved vaccines is the need for presentation of the antigen in a setting which will break tolerance, enhance recognition, and lead to the danger response. [491] This may be achieved by the release of certain cytokines at the site of a vaccine, either through genetic delivery or local injection. The delivery of local cytokines such as IL-2, IL-12, GM-CSF, lymphotactin, and IL-4 may allow the enhanced recruitment and activation of T lymphocytes or the activation and recruitment of dendritic cells to the tumor site where antigen can be presented with the appropriate second signal to the T cell. [492] [493] [494] [495] In addition, the presentation of peptide antigen by activated dendritic cells may allow resting T cells in the repertoire to be activated and expanded in response to either foreign or self antigens. It is clear that these maneuvers can enhance the stimulation of T lymphocytes and allow a relatively weak tumor antigen to be a more effective target. Therefore, many of the present vaccine trials are aimed at utilizing defined proteins or peptides with the simultaneous delivery of cytokines to better locally stimulate the host. Use of autologous tumor or allogeneic tumor cell lines that have been genetically modified to secrete critical cytokines such as GM-CSF or IL-2 is actively being pursued. [496] These trials can incorporate biologic intermediate endpoints, but in the end will require randomized trials to prove their efficacy.

Cell Products-Proteins

Serologically defined gangliosides are a major cell surface component of various neoplasms, especially melanoma, sarcoma, and small-cell lung cancers. [495] [497] The gangliosides include GM2, GD2, and GD3, which are specific to the malignant phenotype and have been isolated by monoclonal antibody purification. Vaccine-induced or natural antibodies directed at GM2 appear to confer an advantage in survival as well as relapse-free survival in patients with melanoma. [498] Human antibodies induced by vaccination can be directly cytotoxic for melanoma tumor cells that express GM2. A trial in patients with stage III melanoma (involved, resected regional lymph nodes) showed that patients vaccinated with GM2 plus an adjuvant BCG with cyclophosphamide had a marginal improvement in relapse-free survival compared to patients receiving BCG and cyclophosphamide alone ($P = 0.09$). This benefit was much more apparent and reached statistical significance if all patients with natural GM2 antibodies were excluded from the analysis ($P = 0.02$). Further modifications of the vaccine protocol with linkage of GM2 to a protein carrier KLH and the use of a superior adjuvant, QS21, has led to much better induction of both IgM and IgG antibodies with higher titer levels. A trial is now ongoing to evaluate the efficacy of this vaccine compared to IFN- in high-risk melanoma patients.

Another protein antigen that has been the target of a number of clinical efforts is the Her-2/neu molecule, which is overexpressed in a large number of patients with both breast and ovarian cancer. [446] A growing literature indicates that patients with Her-2/neu overexpressing cancers have an endogenous antibody, helper, and cytolytic T-cell response directed at Her-2/neu protein as a whole or to component peptide fragments. [446] The presence of natural immunity to Her-2/neu heightens the expectations that vaccine strategies can increase the host immune response. Data is only now being collected to determine the significance of endogenous host responses to Her-2/neu

and whether its presence is associated with a better or a worse outcome. Several investigators have now demonstrated the presence of cytolytic T cells in the blood and at tumor sites of patients with breast and ovarian cancer and have shown that the T cells specifically recognize a peptide derived from Her-2/neu and can lyse tumor cells expressing the appropriate HLA molecules and Her-2/neu molecules. [446] This has led to several vaccine protocols enrolling patients at various stages of breast and ovarian cancer with Her-2/neu protein plus adjuvant, Her-2/neu peptides plus GM-CSF, Her-2/neu peptides presented by autologous dendritic cells, or Her-2/neu expressing viral or vaccinia vectors to deliver the protein.

The unique portions of the surface immunoglobulin on each B-cell lymphoma determine idiotypes that are unique tumor-specific antigens. Active immunotherapy in patients with their idiotypes coupled to a protein carrier such as KLH, in combination with an immunologic adjuvant or with autologous antigen-presenting cells such as dendritic cells (DC) isolated from peripheral blood have been utilized in clinical trials. [499] [500] Specific T-cell responses against idiotypes were induced in numerous patients, especially when DC were used in the vaccination. [499] Several patients experienced objective tumor regressions, and many patients had prolonged progression-free periods post-vaccination. Patients who developed idiotype-specific immune responses had superior progression-free periods compared to those patients who were unable to develop specific immunity. [500] Although this is not a practical strategy for all lymphoma patients because of the cost and time required to produce the specific protein for immunization, it is a model for effective tumor-specific vaccination. Finally, others have utilized anti-idiotype antibodies themselves as antigens. [501] The anti-antibodies are evidently somehow viewed by the immune system as similar to the initial antigen used to generate them. This concept of mirror image may allow for better recognition and stimulation than the original antigen itself. [501] [502] This approach has been used in melanoma (GD3), colon cancer (CEA), and small-cell lung cancer.

Peptide Vaccineswith or Without Adjuvants

Numerous peptide components of defined protein antigens expressed by malignant cells have now been defined in terms of their HLA-binding capacity and their ability to induce T-lymphocyte responses (cytolytic [class I] and helper [class II]). [489] [490] Peptides from the melanoma differentiation antigens (MART-1, gp-100, tyrosinase, TRP-1), melanoma fetal-embryonic antigens (MAGE), Her-2/neu, PSA, CEA, p53 (mutant and wild-type), and ras have all been shown to stimulate T cells [489] [490] However, in many cases the endogenous protein expressed in the malignant cell is not processed in a manner that allows the peptide to be presented on its surface and therefore, the cancer cell will not be a target for the peptide-induced T cell. Nevertheless, examples of peptide-induced T-cell responses to endogenously expressed proteins have been the basis for numerous trials with MART-1, gp-100, tryosinase, PSA, CEA, Her-2/neu peptides in their native form or

modified to enhance their MHC-binding capacity (gp-100 peptide 209-2M). These trials have utilized peptides with adjuvants, cytokines (e.g., GM-CSF, IFN, IL-2, IL-12), helper peptides, or antigen-presenting cells (APC) such as dendritic cells. Other approaches have included DNA expression of the peptide and direct transfection of cells via liposomal transfer, gene gun, virus, or vaccinia. [502] Finally, approaches may include peptide vaccine administered with systemic cytokine or use of ex vivo-stimulated T cells expanded with peptide, cytokines, and APC.

Genetically Modified VaccinesCytokine Modified

Attempting to modify either autologous tumor cells or allogeneic tumor cell lines to allow a high level of cytokine expression and release at the site of the tumor cell vaccine has been a popular strategy. [296] [492] [493] [494] [496] [503] These studies have been based on the evidence that cytokine expression at sites of tumor antigens may allow the migration and activation of immune effector cells, such as T lymphocytes or natural killer cells, to the tumor site. [503] This may be accomplished by genetically transfecting tumor cells with IL-2, IL-4, or lymphotactin. In addition, the transfection of tumor cells with GM-CSF appears especially effective in promoting the recruitment and activation of the premiere antigen-presenting cell, the dendritic cell. [305] [496] Once at the tumor vaccine site, these dendritic cells can capture antigens and present them to the host T cells. Work with GM-CSF has been attempted in renal cancer and melanoma using autologous tumor cells transfected ex vivo. [496] Tumor responses have been observed and tumor deposits have exhibited marked inflammatory responses characterized by massive numbers of infiltrating eosinophils. Finally, preclinical studies suggest that expression of more than a single cytokine can lead to more effective immune activation and antitumor effects. This is exemplified by studies in A20 lymphoma with IL-2 (activation of T and NK cells) with lymphotactin (Lptn) (recruitment of T lymphocytes) or in B16 melanoma intracerebrally with IL-4 and GM-CSF (dendritic cell activation). [493] These studies have numerous obstacles, including inefficient transfection of autologous tumor cells to obtain effective cytokine level and the frequent difficulty in obtaining fresh autologous tumor to allow for transfection.

Cellular Therapy

It has been known through experimental models of leukemia and analysis of allogeneic transplant data that cells within the donor graft are capable of mediating a graft-versus-leukemia effect.[504] Recent data have demonstrated conclusively that the infusion of donor leukocytes can induce prolonged, complete remission in patients with CML who relapse following an allogeneic BMT. [505] [506] This effect of donor leukocytes can be observed in patients with cytogenetic and hematologic relapse of CML, as well as in select patients with relapse of their acute leukemia. Patients with multiple myeloma also may respond to donor leukocyte infusion if they are relapsing after an allogeneic BMT. [506] Several variations of this approach have included the addition, either in vivo or in vitro, of cytokines such as IL-2 or IL-2 and IFN to activate these donor leukocytes further. [507] [508] [509] Responses have been seen among patients with myelodysplastic syndromes, ALL, and AML. A modification of this form of cellular therapy has involved the genetic modification of the allogeneic donor leukocytes via transduction with herpes simplex thymidine kinase (HSV-TK) suicide gene.[510] Therefore, patients who developed graft-versus-host disease following infusion of donor leukocytes had their graft-versus-host disease controlled by treatment with ganciclovir which was cytotoxic to cells transduced with the HSV-TK gene. [510] This type of gene therapy may increase the safety and efficacy of donor leukocyte infusions and ultimately may be applied to allogeneic BMT in general.

Another type of cellular therapy being incorporated into cancer therapy involves dendritic cells that provide effective antigen presentation to naive T cells in situ and are critical for effectively stimulating T-cell activation to a variety of antigens, including many tumor antigens. [305] [306] The development of a better understanding of DC progenitors and how to induce their maturation and proliferation has allowed investigators to expand their numbers for use in clinical trials. Furthermore, the use in vivo of cytokines to mobilize hematopoietic progenitor cells and newer cytokines such as Flt3L has allowed the isolation from peripheral blood of DC in larger numbers capable of stimulating immune responses.[333] [334] Dendritic cells are now being applied to vaccine therapy of cancer by pulsing them ex vivo with the proteins or peptides that are being targeted. Idiotype vaccines for B-cell lymphomas have already been used as

a paradigm for this type of cancer vaccine and both immune and clinical responses have been observed. [499] Other approaches, including the acid stripping of peptides from fresh tumors or the use of known specific peptides or proteins (such as mutant oncogenes [ras, p53, etc]), viral proteins, or tissue-restricted peptides expressed by cancer cells, could all be pulsed onto DC, which are derived and expanded from the autologous cancer patients and then used as active immunotherapy.

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APPENDIX: Approved Agents

Trade name: Proleukin

Generic name: Interleukin-2

Other names: Aldesleukin

FDA status: Approved

Current approved indications: Indicated for adult patients with metastatic renal cell cancer and metastatic melanoma

Investigational uses: Bone marrow transplantation, hematologic malignancies

Dosing: 600,000 IU/kg administered IV over 15 minutes, every 8 hours, for two 5-day treatment cycles (days 15 and 1519)

Toxicity: Fever, chills, hypotension, capillary leak syndrome, oliguria, pulmonary edema, anemia, elevated liver function studies, atrial and ventricular cardiac arrhythmias

Trade name: Actimmune

Generic name: Interferon-

Other names: None

FDA status: Approved, limited indication

Current approved indications: Reducing the frequency and severity of serious infections associated with chronic granulomatous disease

Investigational uses: Anticancer agent, immunomodulator

Dosing: 50 g/m^2 for patients whose body surface area is >0.5 m^2 and 1.5 g/kg/dose for patients whose body surface area is 0.5 m^2 ; injections should be administered subcutaneously three times weekly

Toxicity: Flu-like or constitutional symptoms such as headache, fever, chills, myalgias

Trade name: Roferon-A, Intron-A, Alferon N

Generic name: Interferon-$_{2a}$, interferon-$_{2b}$, human leukocyte-derived (interferon-$_{n3}$)

Other names: None

FDA status: Approved

Current approved indications: Indicated for use in hairy cell leukemia and AIDS-related Kaposi sarcoma; non-Hodgkins lymphoma; chronic myeloid leukemia; Alferon N approved for refractory or recurrent external condylomata accuminata.

Investigational uses: Malignant melanoma, carcinoid

Dosing: Hairy cell leukemiainduction dose is 3 million IU/day for 1624 weeks, administered as a subcutaneous or intramuscular injection; recommended maintenance dose is 3 million IU, three times per week; dose reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur; use of doses >3 million IU is not recommended in hairy cell leukemia.

Kaposis sarcomarecommended induction dose is 36 million IU/day for 1012 weeks, administered as an intramuscular or subcutaneous injection; recommended maintenance dose is 36 million IU, three times per week; dose reduction by one-half or withholding of individual doses may be required when severe adverse reactions occur

Condylomata accuminatarecommended doses of Alferon N injection (interferon-$_{n3}$) for treatment is 0.05 ml (250,000 IU) per wart; Alferon N injection should be administered twice weekly for 8 weeks

Toxicity: Flu-like syndrome, fever, fatigue, chills, arthralgia, anorexia, and headache

Trade name: Neupogen

Generic name: Filgrastim

Other name: G-CSF

FDA status: Approved

Current approved indications: Chemotherapy-induced neutropenia, idiopathic/cyclic neutropenia, PBPC mobilization, HIV infection

Investigational uses: Bone marrow failure

Dosing: 5 g/kg/day by subcutaneous injection or intravenous infusion; check CBC twice per week; discontinue Neupogen when absolute neutrophil count >10,000 cells/l

Toxicity: Elevation of serum chemistries (lactate dehydrogenase, leukocyte alkaline phosphatase), hair thinning, and splenomegaly (with chronic use), exacerbation of pre-existing vasculitis, bone pain

Trade name: Leukine, Prokine

Generic name: Sargramostim

Other names: GM-CSF

FDA status: Approved

Current approved indications: High-dose chemotherapy with autologous bone marrow transplantation; post-chemotherapy for AML

Investigational uses: Chemotherapy-induced myelosuppression, bone marrow failure states, HIV infection

Dosing: 5 g/kg/day administered by subcutaneous injection or intravenous infusion; continue therapy until neutrophil count has recovered; decrease dose by 50% if absolute neutrophil count >20,000 cells/l; check CBC twice per week

Toxicity: Fever, chills, arthralgias, myalgias, pericarditis, pleuritis, first-dose effect, bone pain, fatigue, malaise

Trade name: Procrit, Epogen

Generic name: Erythropoietin

Other names: Not applicable

FDA status: Approved

Current approved indications: Anemia associated with chronic renal failure, chemotherapy-induced anemia, anemia associated with HIV infection, and pre-operative orthopedic surgeries

Investigational uses: Anemia of prematurity, bone marrow failure states

Dosing: Chronic renal failurestarting doses 5100 U/kg three times per week; dose is usually administered by intravenous bolus to patients on dialysis; dose may be increased at intervals of 25 U/kg three times per week until target hematocrit level is reached (36%); maintenance dosing can be either at a lower dose, or increased interval of therapy

HIVbefore initiating therapy, check endogenous erythropoietin level, since most patients with erythropoietin levels >500 mU/ml will not respond to erythropoietin therapy; recommended starting dose for patients with erythropoietin levels <500 mU/ml and AZT doses <4200 mg/wk is 100 U/kg three times per week for 8 weeks; dose can be increased by 50100 U/kg three times per week every 48 weeks and response monitored; erythropoietin therapy should be held when the hematocrit is >40% and resumed at 25% dose reduction when the hematocrit is <36%

Toxicity: Iron deficiency, hypertension, seizures, thrombotic events, headache, allergic reaction

Trade name: Neumega

Generic name: Oprelvekin

Other names: Interleukin-11

FDA status: Approved

Current approved indications: prevention of severe chemotherapy-induced thrombocytopenia

Investigational uses: Bone marrow failure states, BMT

Dosing: 50 g/kg/day SC starting within one day of last dose of chemotherapy

Toxicity: Plasma volume expansion, anemia, edema, constitutional symptoms

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APPENDIX: Investigational Agents

Name: Stem cell factor

Other names: c-kit ligand, mast cell growth factor, steel factor

FDA status: Investigational

Current approved indications: None

Investigational uses: Peripheral blood progenitor cell mobilization, bone marrow failure states, bone marrow priming

Dosing: To be established; 530 mg/kg/day, subcutaneous administration only

Toxicity: Allergic-like reactions, including urticaria, shortness of breath, laryngospasm

Name: Interleukin-12

Other names: None

FDA status: Investigational

Current approved indications: None

Investigational uses: Immunotherapy of renal cell carcinoma, melanoma

Dosing: To be established

Toxicity: Constitutional toxicity, liver function abnormalities, mucositis

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Chapter 52 - Pathobiology of Acute Myeloid Leukemia

Wendy Stock
Michael J. Thirman

INTRODUCTION

The development of acute myeloid leukemia (AML) is the consequence of a multistep process resulting from a series of genetic changes in a hematopoietic precursor cell. These genetic changes produce an uncoupling of normal hematopoietic growth and differentiation, with an accumulation in the bone marrow and blood of large numbers of abnormal immature myeloid cells that retain the capacity to divide and proliferate but have lost the ability to differentiate terminally into mature hematopoietic cells. As in other malignancies, the genetic alterations in AML include the loss of tumor suppressor genes and mutation of oncogenes. In contrast to most solid tumors, many hematologic malignancies can be categorized on the basis of a single characteristic cytogenetic abnormality. The identification of specific recurring chromosomal abnormalities, including translocations, has provided great insight into the pathobiology of AML and its heterogeneous nature. This chapter discusses the molecular genetic events involved in the pathogenesis of AML.

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HETEROGENEITY OF AML

The classification of AML has relied to a large extent on the morphologic and cytochemical criteria of the French-American-British (FAB) classification, which attempts to assign a single lineage to each leukemia (divided into eight subgroups, M0 through M7). [1] Use of the FAB classification has improved the reproducibility of AML diagnosis and classification and has allowed AML to be separated from acute lymphoblastic leukemia (ALL) on the basis of morphology and cytochemical analysis, supplemented by immunophenotyping, which is fundamental to the treatment of acute leukemia. [2] [3] In addition, some FAB AML subgroups correlate with selected disease features. As examples, megakaryoblastic differentiation with marrow fibrosis (M7), monocytic differentiation with gingival infiltrates (M4, M5), and the morphologic subgroup acute promyelocytic leukemia (M3) each correlate closely with a specific genotype and specific clinical characteristics. [1] [4] However, the morphologic features of the large population of leukemic cells typically give few insights into the pathobiology of AML and do not necessarily correlate with the treatment outcome, biologic features, or genetic markers of these diseases.

Several modifications have been proposed to improve the classification of AML and make it more relevant to clinical decision making through incorporation of biologic and genetic data, including sophisticated immunophenotyping data and the recurring chromosomal abnormalities that characterize subgroups of AML. A comprehensive classification system that has been recently proposed subdivides AML into two groups not recognized by traditional classificationAML evolving from prior myelodysplastic syndromes, and true de novo AMLand incorporates pathogenetic mechanisms and treatment responses, in addition to morphology and cytogenetics. [5]

Establishment of a correlation between specific morphologic subtypes and recurring chromosome translocations that result in the creation of novel fusion genes in AML has provided insight

TABLE 52-1 -- Correlation Between Morphologic Subtypes of AML and Recurring Chromosomal Translocations

FAB Subtype	Cytogenetic Aberration	Molecular Aberration
M0, M1, and ALL	t(10:11)	*CALM-AF10*
M1	Trisomy (11)	Partial duplication of *MLL*
M2	t(8;21)	*AML1-ETO*
M3	t(15;17), t(11;17), t(5;17)	*PML-RAR*, *PLZF-RAR*, *NPM-RAR*
M4Eo	inv(16)	*CBF-MYH11*
M4 with erythrophagocytosis	t(8;16)	*MOZ-CBP*
M5	11q23 translocations	*MLL* fused to one of its partner genes
M6	t(3;5)	*NPM-MLF1*
M1, M2, M4, and MDS	t(6;9), ?t(9;9)	*DEK-CAN, SET-CAN*
Multiple FAB subtypes, MDS, and CML	t(16;21)	*TLS/FUS-ERG*
M2, M4, CML	t(7;11)	*NUP98-HOXA9*
MDS, de novo/t-AML	inv(11)	*NUP98-DDX10*
MDS, t-AML, CML	t(3;21)	*AML1-EAP/MDS1/EVI1*

into the potential mechanisms of leukemogenesis and has led to important clinical observations (Table 52-1). Examples of this correlation include the association of FAB-M3 morphology with acute promyelocytic leukemia with a t(15;17)(q22;q1112) and the *PML-RAR* fusion gene involving the retinoic acid receptor- gene on chromosome 17 and the *PML* gene on chromosome 15. This observation provides a rationale for the highly effective treatment of acute promyelocytic leukemia with all-trans-retinoic acid. [6] [7] [8] [9] A subgroup of FAB-M2 leukemias associated with prominent Auer rods and salmon-colored granules in the myeloblasts have a t(8;21)(q22;q22) and the *AML1-ETO* fusion gene, and a subgroup of M4 leukemias with atypical eosinophilic cytoplasmic granules in the blasts (M4Eo) have the inv(16)(p13q22) and the *CBF-MYH11* fusion gene. Both of these leukemias have a very favorable prognosis when patients receive consolidation treatment that includes high doses of cytarabine. [10] Interestingly, the fusion genes created as a result of these translocations both contain components of the core-binding factor complex (*AML1* and *CBF-*), which may render these leukemia cells more sensitive to the effect of high-dose cytarabine. Efforts are under way to elucidate the mechanism of drug sensitivity in these leukemias. These fusion genes, and other AML-associated chromosomal translocations, are reviewed in detail below. Because these and other specific recurring chromosomal abnormalities in AML have prognostic relevance, [3] [11] [12] current AML trials are attempting to stratify treatment based on the presenting cytogenetic abnormalities and known response to treatment of a specific subtype.

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CLONAL ORIGIN OF AML

AML cells exhibit characteristic maturation defects that correspond to various stages in hematopoietic differentiation; therefore, to begin to understand the pathogenesis of this heterogeneous group of diseases, it is useful to review the stages of normal hematopoiesis. Mature blood cells of multiple lineages, including erythrocytes, platelets, neutrophils, monocytes, and lymphocytes, are derived from a pluripotent hematopoietic stem cell. [13] To sustain hematopoiesis, stem cells are part of a developmental hierarchy capable of three basic functions: (1) maintenance in a noncycling state; (2) self-renewal capacity, to allow the production of additional stem cells; and (3) the production of progenitor cells with more limited development potential. These progenitor cells commit to subsets of myeloid and lymphoid lineages and ultimately to single developmental pathways, resulting in the expression of the terminally differentiated stage of each cell type. [14]

Normal hematopoiesis is a dynamic yet highly regulated process controlled by the combined effects of growth factors, which permit cellular proliferation, and nuclear transcription factors, which activate specific genetic programs resulting in lineage commitment and terminal differentiation. In the past few years many of the regulatory growth factors and a number of specific transcription factors have been identified that play critical roles in lineage commitment and in the subsequent development of the mature lymphoid and myeloid (erythroid, granulocytic/monocytic, and megakaryocytic) lineages (Fig. 52-1).[5] [16] Some of the genes encoding these transcription factors

Figure 52-1 Transcription factors essential for normal hematopoietic development as defined by gene targeting studies in murine embryonic stem cells. Transcription factors known to be involved in human leukemias are highlighted in red, whereas transcription factors involved in mouse leukemia models are highlighted in pink. Transcription factors essential for normal hematopoiesis but not known to be involved in leukemogenesis are shown in gray. The primitive hematopoietic stem cell gives rise to nucleated erythroid cells at the yolk sac stage of development. Subsequently, multilineage hematopoiesis is thought to be derived from the definitive hematopoietic stem cell. The origins and relationships of the primitive and definitive hematopoietic stem cells remain controversial. Experimental evidence suggests that a common precursor to the primitive and definitive hematopoietic stem cell may exist (blast cell colony-forming cell). The precise hematopoietic pathways remain to be defined. (*From Kennedy et al.*[275] *Reprinted by permission Nature, copyright 1997, Macmillan Magazines Ltd.*)

are involved in the recurring chromosomal translocations of AML, leading to the concept that these leukemias occur because of alterations in master regulatory proteins that control hematopoietic growth and differentiation programs. [17] The novel fusion genes created by these translocations are reviewed in detail later in the chapter.

A large body of evidence supports the development of acute leukemia from a single transformed hematopoietic progenitor cell. Initial studies of glucose-6-phosphate dehydrogenase (G6PD) isoenzyme restriction in the leukemic myeloblasts of heterozygous females demonstrated the clonal origin of AML. [18] [19] In these studies, X-chromosome inactivation mosaicism was used to determine clonality. The G6PD locus is on a portion of the X chromosome that undergoes inactivation in XX somatic cells (Lyonization). Females who are heterozygous at this locus produce two isoforms of the enzyme (A and B) and, because their normal tissues are composed of mixtures of cells, these tissues manifest both forms of the enzyme. Approximately one-half of the cells in normal somatic tissue will have randomly inactivated one of the X chromosomes, allowing expression of the A isoform, and the other half of the cells will have inactivated the other X chromosome, resulting in expression of the B isoform. Cells of clonal origin, however, express only one type of G6PD. In each of the G6PD heterozygous females with AML who were studied, both types of enzyme were found in normal tissues, but only a single type was observed in the leukemic myeloblasts. [18] [19] [20] Similar conclusions about the clonal origin of AML have been reached using X-linked recombinant DNA probes, standard cytogenetics, and fluorescence in situ hybridization (FISH). [21] [22] [23]

Leukemic Transformation: Cell of Origin

The precise cell of origin of leukemic transformation in AML has been the subject of intense investigation. Two models have been proposed to explain the heterogeneity of the disease that is observed at the molecular, cytogenetic, phenotypic, and clinical levels (Fig. 52-2A). One model proposes that *many cell types in the stem cell/progenitor cell hierarchy are susceptible to leukemic transformation*, resulting in the expansion of abnormal cells that are blocked at a particular stage of differentiation. According to this model, the transforming event(s) in AML may occur during different stages of hematopoiesis, from the stage of primitive multipotent stem cells to lineage-committed progenitor cells. The level of commitment of the transformed target cell defines the characteristics of the resulting leukemic blast cells; therefore, this model predicts that the phenotype of the leukemic stem cells from patients with AML restricted to the granulocytic-monocytic series differs from the phenotype of the leukemic stem cells from AML patients with involvement of erythroid, megakaryocytic, and granulocytic-monocytic lineages.

The correlation of specific cytogenetic and molecular genetic aberrations with the morphologic appearance of leukemic cells suggests that the transforming event occurs at different stages of myeloid differentiation. This hypothesis is underscored by the FAB criteria, which distinguish different subtypes of AML based on the stage of apparent differentiation. [1] Evidence in support of this model also comes from a variety of studies of clonal hematopoiesis in AML. [21] [22] [23] [24] Although these clonality studies demonstrate that the majority of cells derived from the leukemic clone undergo variable degrees of differentiation to cells of the granulocytic-monocytic lineage, clonally derived cells from some AML patients also may differentiate into cells of the erythroid and/or megakaryocytic pathways. Multilineage involvement has been noted more frequently in elderly patients with AML, in patients in whom AML arose after a documented prior preleukemic (myelodysplastic) phase, or in patients in whom AML developed after treatment for another malignancy; [23] [24] multilineage involvement was noted to a lesser degree in younger adults and children with AML. [22] [24]

A second model for leukemogenesis challenges this concept and proposes that mutations responsible for *leukemic transformation and progression occur only in primitive multipotent cells* (Fig. 52-2B).[25] According to this model, the heterogeneity seen in AML results from the variable ability of these primitive stem cells to differentiate and acquire specific phenotypic lineage markers. The heterogeneity noted is a direct consequence of the transforming gene itself. Depending on the nature of the transforming gene(s), in some patients, the pluripotent stem cells have lost the capacity for erythroid and megakaryocytic differentiation, whereas in other patients the pluripotent stem cells retain the capacity for multilineage differentiation.

A number of studies support this model of a hierarchical development of AML originating from a primitive, multipotent stem cell. Hematopoietic stem cells express a characteristic cell-surface antigen, classified as CD34, and can be further subdivided by the expression of additional cell surface antigens, including CD38 and HLA-DR.[26] [27] CD34+/38/HLA-DR cells are multipotent hematopoietic stem cells, whereas CD34+/CD38+/HLA-DR+ cells define a committed population of myeloid progenitor cells.[28] Using fluorescence-activated cell sorting techniques, it has been possible to specifically sort these subpopulations of hematopoietic cells from bone marrow samples of patients with different phenotypic and cytogenetic subtypes of AML. CD34+/CD38 and CD34+/CD38+ cells have been obtained from patients with AML that evolved from a prior myelodysplastic syndrome and from patients with AML without any antecedent hematologic disorder (de novo AML). Cytogenetic and FISH studies of sorted stem cell compartments from these patients have shown that the characteristic cytogenetic abnormality in both leukemia sample groups was present in the CD34+/CD38 multipotent stem cell compartment.[29] [30] These data support the idea that the target cell for leukemic transformation in phenotypically

diverse AML cases arises from a similar primitive, multipotent stem cell in all cases studied by these investigators.

More compelling evidence in support of the CD34+/CD38 (multipotent) stem cell as the target for leukemic transformation in most, but not all, cases of AML comes from recent studies in which purified AML subpopulations were transplanted into mice with severe combined immunodeficiency disease (SCID). [31] These experiments have defined a SCID mouse leukemia-initiating cell (SL-IC) in the bone marrow of patients with AML. SL-ICs compose approximately 1 in 10^5 AML cells and can repopulate immune deficient mice with leukemia cells that are phenotypically identical to those of the AML patient from which they were derived. [31] In several AML patient samples studied, the SL-ICs resided in the most primitive compartment of cellsthose that are CD34+/CD38. CD34+/CD38 cells purified from normal marrow give rise to mixed-lineage granulocytic-erythroid-megakaryocytic colonies in culture and can repopulate immune deficient mice with normal cells. Therefore, these experiments indicate that the SL-ICs share an immunophenotype expressed by normal multilineage stem cells and that the target for leukemic transformation resides in this primitive pluripotent population of cells rather than in a lineage-restricted cell.

Using nonobese diabetic mice with SCID (NOD/SCID), a modified SCID mouse,[32] it has been shown recently that in AML patient samples, the SL-ICs reside only in the CD34+/CD38 fraction, and not in the CD34+/CD38+ fraction. [33] The SL-IC phenotype was consistent regardless of the FAB subtype (M1, M2, M4, M5), lineage markers, or percentage of leukemic blast cells expressing the CD34 antigen. The SL-ICs also demonstrated self-renewal capacity, a requirement for maintenance of the leukemic clone, assessed by their ability to be serially transplanted into secondary recipient mice. The uniformity of the

Figure 52-2 Two models to explain the heterogeneity of AML. Hatched lines

represent the leukemic transformation event for the different AML subtypes. **(A)** This model proposes that the transforming event(s) occurs at any one of several stages of differentiation in the stem cell / progenitor cell hierarchy. **(B)** In this model, the transforming event almost always occurs in a primitive, pluripotent progenitor cell. The heterogeneity of the disease is then determined by the specific molecular transforming event(s). Acute promyelocytic leukemia (AML M3) is an exception to the model shown in **B** as it appears to arise from a more differentiated myeloid progenitor cell.

leukemic stem cell phenotype, together with the observation that normal stem cell repopulating cells are also found in the CD34+/CD38 fraction, strongly suggests that the leukemia-initiating transformation- and progression-associated genetic events always occur in primitive cells and not in committed progenitors. As a result of the specific transforming event(s) itself, the leukemic multipotent stem cells in some patients may have lost the capacity for erythroid and platelet differentiation. This would explain the conflicting results of clonality studies, which demonstrate multilineage (erythroid, megakaryocytic, granulocytic-monocytic) involvement in some AML cases and lineage-restricted disease (granulocytic-monocytic) in other cases. Interestingly, an exception to these findings may be acute promyelocytic leukemia (APL). In the NOD/SCID mice, peripheral blood leukocytes from patients with APL did not engraft. In addition, flow cytometric/molecular analysis of APL patient samples suggests that the leukemic stem cell arises in a committed lineage-restricted, CD34+/CD38+ progenitor cell. [34] This contrasts with the CD34+/CD38 fraction observed in the other subtypes of AML.

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MULTISTEP PATHOGENESIS

Progression to acute leukemia may require a series of genetic events that begins with clonal expansion of a transformed leukemic stem cell. [35] The specific mutational events required for leukemic progression are not well defined; however, insights into the multistep pathogenesis of human AML have been obtained from mouse models of leukemia and from several lines of evidence in human leukemias. For example, in chronic myeloid leukemia (CML), the leukemia cells contain the t(9;22) transposition, resulting in the *BCR-ABL* fusion gene at the time of the clinical diagnosis of chronic phase disease. [36] This fusion gene product has been demonstrated to be critical in the pathogenesis of CML. [37] As the disease progresses, additional cytogenetic abnormalities [38] are acquired that include loss of important tumor suppressor genes such as *p53*.[39]

Additional evidence for a multistage pathogenesis comes from study of clonal remissions after treatment for AML. A variety of clonality studies using X-linked polymorphisms, identical to those described above that demonstrate a clonal origin for AML, have shown that patients in clinical remission may still have clonal rather than polyclonal hematopoiesis. [21] [40] [41] [42] This finding supports a multistep process, since the clonal remission marrow may represent the preleukemic stem cell that has undergone an initial transforming event but has not acquired the additional mutation(s) essential to progression to overt leukemia. In these cases, it is presumed that the transformed, overt leukemic cell responded to chemotherapy and probably represented a subclone of the original preleukemic stem cell that had acquired the genetic mutations required for the definitive block in differentiation and manifestation of the frank leukemic phenotype. Although some recent studies suggest that these clonal remissions may actually be the result of skewed Lyonization (preferential inactivation of one X chromosome over another), [43] [44] more carefully controlled studies suggest that clonal remissions do occur after treatment for AML. [45] [46] Bone marrow samples from patients in remission from de novo AML (FAB-M2) with a t(8;21)(q22;q22) transposition and the *AML-ETO* fusion transcript (described below) have been found to harbor the aberrant fusion transcript as long as 8 years after cessation of all chemotherapy. [47] Recently, the *AML-ETO* fusion transcript has also been detected in remission bone marrow samples from patients who underwent allogeneic bone marrow transplantation for AML and have remained in clinical remission. [48] These data suggest that expression of the chimeric protein is only one of the genetic modifications necessary for the development of cancer and leukemia, and that the affected clone would require additional mutation(s) to express the transformed phenotype.

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THERAPY-RELATED AML

The development of AML after chemotherapy administered for a variety of other malignancies, ranging from Hodgkin's disease to breast cancer, is an unfortunate complication of curative treatment strategies.[49] Leukemia develops after a latency period of varying length, which suggests that the initial mutations resulting in clonal hematopoiesis may occur without any obvious hematologic change (no dysplasia or cytopenia). The subsequent acquisition of a variety of additional genetic lesions may be essential for the development of a preleukemia (myelodysplasia) or overt leukemia.[35] Clonal chromosomal abnormalities have been reported in the majority of cases of therapy-related AML (see Chap. 48). The most frequently reported abnormalities involve complete loss or interstitial deletions of the long arm of chromosome 5 or 7 (or both). Typically these leukemias develop after alkylating agent-induced damage a median of 35 years after therapy for the primary malignancy and are associated with an antecedent myelodysplastic disorder.[50] A second group of therapy-related leukemias is associated with rearrangements of the *MLL* gene in chromosome band 11q23.[51] 11q23-associated AML often develops after treatment with drugs that target DNA-topoisomerase II (e.g., epipodophyllotoxins, anthracyclines), with a very short latency of 1218 months after treatment, and is not typically associated with an antecedent myelodysplastic syndrome.[52] [53] [54] Of concern are recent reports of the development of myelodysplastic syndrome and AML after dose-intensive therapy with (and without) autologous stem cell and hematopoietic growth factor support for the treatment of Hodgkin's and non-Hodgkin's lymphoma and breast cancer.[55] [56] [57] [58] The identification of an increasing incidence of therapy-related AML consequent on attempts to improve cure rates for other malignancies emphasizes the importance of understanding the underlying pathogenetic mechanisms.

The genetic damage induced by chemotherapeutic agents can result in clonal proliferation, which may be an essential early step in leukemogenesis, occurring before the development of clinical abnormalities. Clonal hematopoiesis has been demonstrated in clinically asymptomatic patients who received prior cytotoxic chemotherapy for a variety of non-Hodgkin's lymphomas.[59] [60] [61] Recently it has also been shown that sequential X-linked clonality assays may help predict subsequent evolution to frank myelodysplasia or AML.[62] The specific genetic events induced by chemotherapeutic agents remain unknown; nevertheless, the resultant clonal proliferation and observed cytogenetic abnormalities provide a valuable model for multistep leukemogenesis.

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ETIOLOGY

In addition to certain chemotherapeutic agents that are associated with the development of therapy-related leukemia, exposure to a variety of environmental agents has been implicated in the pathogenesis of AML. Ionizing radiation shares with the alkylating agents the ability to damage DNA, usually by inducing double strand breaks that may cause critical mutations, genomic deletions, or the chromosomal translocations required for hematopoietic stem cell transformation. [49] [63] An increased incidence of AML has been noted in atomic bomb explosion survivors. [64] This risk may be directly proportional to the radiation exposure. [65]

Ionizing radiation used in the treatment of malignancies such as Hodgkin's disease has also been linked to the development of AML, although this risk appears to be quite low when radiation alone is used as treatment and is associated with age of the patient and doses of more than 2,000 cGy. [66] [67] Whether irradiation adds to the risk of therapy-related AML associated with chemotherapy remains controversial. Although some studies suggest that the risk of development of AML is significantly

increased when the two modalities are combined, other studies have demonstrated that the combination of high-dose radiation therapy confined to small volumes and chemotherapy did not significantly increase the leukemogenic risk. [68] [69] [70] [71]

Chemical exposure to organic solvents such as benzene and occupational exposure to petroleum products have been associated with a higher risk of developing AML;[72] [73] however, case-control studies of leukemia have demonstrated only a slight increase in risk of disease for persons with occupational or chemical exposures. Except for special groups exposed to high levels of benzene or radiation, the reported risks associated with occupational and chemical exposure have generally been less than twofold, making these exposures of questionable pathogenetic significance. [74] The presence of *ras* mutations in patients with AML has been associated with specific occupational exposure to chemicals, suggesting that these exposures may induce genetic damage that may culminate in acute leukemia. [75] In a case-control study, cigarette smoking was associated with only a modest increase in risk for leukemia; however, a twofold increase in risk for AML was noted in participants over the age of 60.[76]

In a number of animal models, retroviruses have been demonstrated to play an important role in leukemogenesis, and these are reviewed later in the chapter. The human T-cell leukemia retrovirus (HTLV-I) is associated with acute T-cell leukemia in adults. [77] In human AML, however, despite extensive investigation, there has been no clear association of a retrovirus with leukemogenesis. [78]

Familial leukemia occurs rarely, but, as with other cancer family syndromes, study may lead to the identification of genes involved in the pathogenesis of the far more common sporadic cases of AML. Familial leukemia can occur in the context of a medical syndrome where it is one component of the overall disease, or it can occur as isolated leukemia not specifically associated with co-morbid conditions. [79] Several illnesses have leukemia as a component of the syndrome. These include a rare constitutional trisomy 8 syndrome associated with a characteristic facial and skeletal muscle dysmorphism; the development of hematologic disorders, including aplastic anemia, myelodysplasia, and acute and chronic myeloid leukemia; [80] and Down's syndrome, trisomy 21, in which there is a 10- to 18-fold increased risk for leukemia.[81] Potential chromosome 8 genes associated with the development of AML (reviewed later) include the *ETO* gene, involved in the t(8;21)(q22;q22) translocation, and the *MOZ* gene, involved in FAB M4/M5 leukemias with a t(8;16)(p11;p13) translocation. [82] [83] In individuals with Down's syndrome who are less than 3 years old, the leukemia is most frequently AML, of the FAB M7 subtype; after age 3, the development of ALL is more common. [84] [85] Interestingly, from the study of atypical Down's karyotypes, it has been possible to narrow the critical region for leukemia, and this region includes the *AML1* locus on 21q22.3, which encodes the -subunit of core-binding factor (CBF) and is the site of recurrent t(8;21)(q22;q22) translocations in AML. [82] [86]

Defective DNA repair syndromes have also been associated with a high incidence of hematologic malignancies, including AML. These familial disorders include Bloom's syndrome, in which AML, ALL, lymphoma, or other malignancies occur in about 25% of affected individuals, [87] [88] Fanconi's anemia, in which approximately 52% of patients develop AML or myelodysplasia by the age of 40; [89] neurofibromatosis, which results from mutations in the neurofibromin tumor suppressor gene on chromosome 17q11.2 and which is associated with the development of juvenile CML, ALL, lymphomas, and a disproportionately high rate of myelodysplastic syndrome evolving into AML in young patients; [90] [91] Li-Fraumeni syndrome, which occurs as a consequence of dominantly inherited germline mutations of the *p53* tumor suppressor genes and is associated with the development of multiple types of tumors, occasionally including leukemia; and Wiskott-Aldrich syndrome, an X-linked immunodeficiency syndrome associated with the occasional development of lymphomas, AML, and ALL. [92] Other syndromes associated with high rates of AML include Kostmann's infantile genetic agranulocytosis, an autosomal recessive disorder that has been associated with mutations in the G-CSF receptor on chromosome 1p35p34.3, [93] [94] and the Blackfan Diamond syndrome, consisting of congenital hypoplastic anemia and growth retardation. [95]

There have also been reported cases of familial leukemias not associated with any defined syndrome. Several families have been reported in which multiple siblings have developed childhood-onset myelodysplasia with bone marrow monosomy for chromosome 7 with evolution to AML.[96] The inheritance pattern is autosomal recessive. Autosomal dominant patterns of inheritance of leukemia have also been reported, with a variety of morphologic and cytogenetic subtypes. [97] [98] [99] [100] Some are associated with an antecedent myelodysplastic syndrome, while others appear to arise as de novo AML.

Although the environmental and hereditary conditions described above serve as excellent models for obtaining insights into the molecular pathogenesis of AML, it must be emphasized that the vast majority of patients with de novo AML show no evidence of any of these risk factors, and the etiologic factors contributing to the development of AML remain unknown.

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HEMATOPOIETIC GROWTH FACTORS

The role of hematopoietic growth factors in promoting the growth and differentiation of leukemic blood cells has been studied extensively. With in vitro clonogenic assays it has been possible to demonstrate that leukemic cells proliferate in response to many of the endogenous hematopoietic growth factors critical for normal hematopoiesis, including granulocyte, granulocyte-monocyte, macrophage, stem cell colony-stimulating factors (G-CSF, GM-CSF, M-CSF, SCF), interleukin-3 (IL-3), and Flt3 ligand (Flt3-L). [101] [102] [103] [104] [105] It appears that a combination of these factors can produce a synergistic growth response in clonogenic assays.

Disruption of normal hematopoietic growth factor signal transduction pathways could also play a role in leukemogenesis. Mutations in the G-CSF receptor gene have been described in some children with Kostmann's syndrome and with nonfamilial severe congenital neutropenia (see previous discussion). [94] [106] The high incidence of AML in these patients supports the hypothesis that defective signaling function by the aberrant receptor increases susceptibility to AML. These data are intriguing; however, outside of these cases, mutations in critical hematopoietic growth factor receptors have not been reported as a general pathogenetic mechanism for AML.

In a substantial number of patients with AML, autonomous growth has been reported to occur as a result of autocrine or paracrine stimulation of a number of factors, including G-CSF, GM-CSF, IL-1, and IL-6. [107] [108] Several investigators have noted that the presence of autonomous growth characteristics in AML cells grown in vitro correlates with lower remission rates and poor survival. [109] [110] Recently it was demonstrated in a multivariate analysis that expression by leukemic blasts of c-*mpl*, the receptor for thrombopoietin, correlates with a significantly decreased duration of remission in patients with AML. [111] Several explanations for these observations have been proposed. One possibility is that the acquisition of autonomous growth capability allows AML cells to become more aggressive by making them independent of stromal cell production of essential growth factors. [110] Others have suggested that autonomous production of growth factors such as GM-CSF may reduce the cytotoxicity of chemotherapy agents by altering intracellular drug metabolism. [112] Some data suggest that exogenously administered

hematopoietic growth factors not only stimulate in vitro growth and proliferation but also inhibit apoptosis of cultured AML cells. [113] [114] It is possible that the autonomous production of hematopoietic growth factors by leukemia cells may also inhibit apoptosis.

As a result of these in vitro data demonstrating the growth-promoting effects of a variety of cytokines on AML cells, one of the recent controversies in the treatment of AML has centered on the use of hematopoietic growth factors during or after induction chemotherapy. The desired goal of reducing the toxicity of treatment and the duration of cytopenia with exogenous administration of G-CSF or GM-CSF was tempered by concerns about the leukemogenic potential of these growth factors. A number of large randomized clinical trials in AML demonstrate variable clinical efficacy of these growth factors with respect to significant decreases in morbidity and mortality. [115] Although different conclusions have been reached regarding clinical efficacy, there is consensus about the safety and lack of increased leukemogenic potential of these growth factors when they are administered after induction or consolidation chemotherapy.

Two early trials demonstrated that either G-CSF [116] or GM-CSF [117] could be administered safely after induction chemotherapy without increased toxicity or an increased rate of relapse. In addition, these early trials demonstrated that cytokine support was efficacious, with a reduction in neutropenia and early death rate and a trend toward an improvement in the rate of complete remission. Since that time, a number of phase III trials testing the role of GM-CSF or G-CSF in patients with newly diagnosed AML have been concluded. [118] [119] [120] [121] [122] [123] A primary objective of these randomized trials was to evaluate the potential role that these growth factors might play in decreasing the neutropenic phase; however, these studies are difficult to evaluate as a whole since the trial designs varied considerably with respect to the time of administration of growth factor. Only two of these trials required documentation of marrow hypoplasia before CSF use. [118] [120] Despite these differences, administration of either GM-CSF or G-CSF was found to be safe, without an increase in acute toxicity or an increase in relapse rate. Most of the studies demonstrated a significant improvement in neutrophil recovery, and two demonstrated an improvement in complete remission or overall survival rates. [118] [122]

In contrast, the clinical use of cytokines prior to induction chemotherapy in an attempt to recruit leukemic cells into the S phase of the cell cycle to enhance chemotherapeutic effect remains investigational. Several clinical trials in patients with relapsed or refractory AML have attempted to evaluate this concept with a variety of cytokines, including GM-CSF, G-CSF, IL-3, and PIXY321. [124] [125] [126] [127] [128] Most studies incorporated either 24 or 48 hours of cytokine priming before chemotherapy, and some were able to demonstrate the ability to recruit leukemia blast cells into the cell cycle before chemotherapy administration. [125] [126] All but one of these trials demonstrated that cytokine administration prior to chemotherapy was safe; however, one trial [124] found that prolonged infusion of GM-CSF before chemotherapy was associated with severe toxicity, including the development of leukostasis.

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MOLECULAR GENETICS OF AML

Tumor Suppressor Genes

Several of the known tumor suppressor genes have been well characterized in myeloid leukemias, such as the association of *p53* mutations with progression to blast crisis in CML.[129] [130] [131] In AML, *p53* mutations occur less frequently. Only about 7% of cases have known *p53* mutations at the time of diagnosis. The incidence of *p53* abnormalities may be slightly higher in patients with AML evolving from a prior myelodysplastic syndrome [132] and in cases with 11q23 translocations.[133] [134] The *p16INK4* gene is another critical cell cycle regulatory gene that has been shown to be inactivated by a variety of mechanisms in a number of tumors, including frequent deletion in ALL but only very rarely in AML. [135] Similarly, overexpression of the *BCL-2* gene occurs in a wide variety of lymphoid malignancies as a result of the t(14;18) translocation, which juxtaposes *BCL-2* with the immunoglobin (Ig) heavy chain locus. In AML, *BCL-2* overexpression in AML has been reported only rarely; however, in one study of patients with newly diagnosed AML, a multivariate analysis showed that *BCL-2* overexpression was associated with lower complete remission rates and a significantly shorter survival. [136] As noted above, patients with neurofibromatosis and mutations of the *NF1* tumor suppressor gene have a high rate of developing neoplasms, including progression of myelodysplastic syndrome to AML. [90] [91] The Wilms' tumor suppressor gene, *WT1*, has been found to be overexpressed in the leukemic blasts of approximately 75% of cases of newly diagnosed AML. [137] [138] The *WT1* gene, located in the 11p13 region, encodes a transcription factor normally expressed in a time- and tissue-dependent manner, mainly in the kidneys and gonads, [139] [140] although it is also expressed in normal CD34+/CD38 hematopoietic progenitor cells.[141] In AML, high *WT1* levels at diagnosis correlate with lower remission rates and overall survival. [137] [138]

Certain genetic alterations are specific to AML. These include loss of the long arms of chromosomes 5, 7, and 20, which occurs commonly in therapy-related AML and AML associated with prior myelodysplastic syndrome, and is associated with a poor prognosis. [50] [53] [67] These deletion events are discussed in detail in Chapter 48 . The involved genes have not yet been cloned, although novel tumor genes are likely to be identified at these loci.

Oncogene Mutations

Mutations of three closely related *ras*(H-*ras*, K-*ras*, N-*ras*) proto-oncogenes occur in human malignancies.[142] Each of these genes encodes a structurally similar 21-kd protein that localizes to the inner plasma membrane and plays an important role in signal transduction. [143] Specific point mutations occur characteristically in each of the three genes, although no consistent karyotypic abnormalities have been associated with *ras* mutations and result in its activation. [144] Mutations of *ras* have been identified in approximately 25% of cases of AML [144] [145] and are observed with higher frequency in myelodysplastic syndrome (35%). [146] [147] The majority of the mutations in AML and myelodysplastic syndrome are in the N-*ras* gene; K-*ras* and H-*ras* mutations occur less frequently. In both AML and myelodysplastic syndrome, *ras* mutations have been reported more frequently in cases with a monocytic morphology, FAB M4 and chronic myelomonocytic leukemia (CMML) respectively. [148] An association between cases of AML in patients with a variety of occupational exposures and *ras* mutations has been observed.[75]

The pathophysiologic and clinical significance of these mutations in AML remains unclear. Mutations in *ras* have been reported at diagnosis but not at relapse in some AML cases.[149] Conversely, cases have been reported in which *ras* mutations were acquired at the time of relapse or disease progression. [148] [150] It is therefore possible that activation of *ras* may be important in the initiation but not in progression of disease in some cases, whereas *ras* mutation may play an important role in disease progression in other cases of AML and myelodysplastic syndrome. The presence of *ras* mutations has been associated with improved survival in patients undergoing treatment for AML.[145] This improvement in outcome may be related to the fact that these mutations were also associated with a lower leukemia burden in the bone marrow at presentation of AML, thus rendering the cells more susceptible to chemotherapy. Alternatively, it has been suggested that cases of AML with *ras* mutations are more sensitive to chemotherapy with cytarabine,

one of the most active drugs employed in chemotherapy regimens for AML.[151]

CHROMOSOME TRANSLOCATIONS

Chromosome translocations can affect the cell by one of two main mechanisms: by juxtaposition of an intact transcription unit from one chromosome to an enhancer element from a gene on another chromosome, or by the formation of chimeric fusion proteins (Fig. 52-3). [17] For example, in the t(14;18) translocation in non-Hodgkin's lymphoma, the *BCL-2* gene translocates into the *IgH* locus, leading to the inappropriate expression of *BCL-2*. However, the *BCL-2* gene product is identical to the wild-type protein. Alternatively, chromosome translocations can disrupt two different genes within their coding sequences, leading to the creation of a chimeric protein. For example, t(9;22) in CML results in the formation of a chimeric *BCR-ABL* fusion protein. In AML, the vast majority of chromosome translocations result in the generation of chimeric fusion genes that are never expressed in wild-type cells (Table 52-1). With the development of positional cloning techniques, identification of the genes involved in these chromosomal breakpoints has occurred rapidly. In addition, the application of sophisticated techniques such as the polymerase chain reaction (PCR) and FISH has revealed an expanding number of molecular rearrangements in leukemia.

Characterization of the genes involved in these translocations has led to the elucidation of essential transcriptional regulatory pathways in hematopoiesis. [16] [152] [153] For example, the - and -subunits of core-binding factor have been found to be required for definitive hematopoiesis, and both of these subunits are involved in chromosome translocations in AML. The involvement in chromosome translocations of several other classes of transcription factors has been recognized, including those that contain zinc fingers, ring fingers, leucine zippers, basic helix-loop-helix (HLH) motifs, and ETS domains. [154] Often, homologs of these transcription factors have been well characterized in model organisms such as *Drosophila*, including the *AML1* homolog, *runt*, and the *MLL* homolog, *trithorax*. Several other proteins with well-defined functions in normal hematopoiesis have been recognized at chromosome translocation breakpoints, including retinoic acid receptors and Hox genes. However, structural proteins that do not appear to be specific to myeloid cells have also been identified, including members of the nuclear pore complex and nucleolar phosphoproteins. The functions of many other genes involved in chromosome translocations remain to be elucidated. The heterogeneity of the classes of genes involved in different translocations underscores the complexity of normal hematopoietic differentiation and provides insight into the disrupted regulation of these pathways that result from the formation of chromosome translocations.

With the intensive investigation of molecular genetic alterations in leukemia cells, it has become clear that many genes are capable of participating in translocations that involve multiple alternative partner genes (Fig. 52-4). For example, the *MLL* gene has been found to form chimeric fusion genes with at least 14 known partners and an additional 1015 that have not yet been cloned. Moreover, although the *RAR* gene classically fuses to *PML* in acute promyelocytic leukemia, its fusion to other partner genes, including *PLZF* and *NPM*, has recently been identified. These alternative fusions provide an opportunity to contrast the contributions of each component of the fusion gene and thus dissect the motifs within these genes that are critical to leukemogenesis. The cloning of new genes at translocation breakpoints often leads to the identification of family members of genes previously identified in other translocations. Examples include the *AF10* and *AF17* genes as well as the *TLS/FUS* and *EWS* genes. In addition, certain classes of genes have been identified in different translocations such as the nucleoporins, *CAN* and *NUP98*, each of which is involved with two other fusion partner genes. In the majority of cases, the contribution to different fusion genes from members of the same family is usually similar. However, in the t(12;21), the contribution of *AML1* to the *TEL-AML1* fusion differs from the pattern observed in the t(8;21) and the t(3;21). The involvement of the same genes in multiple different translocations leads to great difficulties in the development of classification schemes for the genes involved in leukemia. For example, the fusion of *NPM* to *ALK* is a frequently recurring abnormality in anaplastic large cell lymphoma. However, *NPM* also fuses to *RAR* in APL and to *MLF1* in other subtypes of AML. In addition, rearrangements of *AML1* and *MLL* occur in both ALL and AML. We will discuss specific fusion genes in the context of other similar families of fusions that share common features. The recurring involvement of the same genes in multiple different translocations has two important implications. First, it suggests that the genomic structure of these genes may contain hot spots for chromosomal breakage. Second, it indicates that disruption of the normal pathways regulated by these genes results in malignant transformation rather than death of the hematopoietic cell in which that chromosome translocation first arises.

Figure 52-3 Two potential consequences of chromosome translocations. In (I), the chromosomal breakpoints occur within the transcription units, leading to the formation of chimeric genes and thus to the expression of fusion proteins. In (II), an intact transcription unit is juxtaposed to regulatory elements from a gene on another chromosome, leading to its inappropriate expression.

Figure 52-4 Branching networks of genes involved in chromosome translocations. Each line represents a pair of genes that form chimeric fusion proteins. The myeloid-specific fusion genes are highlighted in black and the lymphoid-specific fusions in red. The fusion genes highlighted in pink are involved in chromosome translocations in sarcomas and are shown because of their overlap with fusion genes involved in the myeloid leukemias.

CBF Family of Leukemias

Chromosome aberrations in AML commonly involve subunits of the core-binding factor (CBF) group of transcription factors. Characterization of the functions of this family of transcription factors was pioneered by study of their roles in leukemogenesis. [82] The members of the CBF group function as heterodimeric complexes to regulate diverse target genes involved in differentiation in many tissues (Fig. 52-5).[155] CBF factors consist of an -subunit that binds DNA, and a -subunit that does not bind DNA directly but enhances binding by the -subunit. Because of the discovery of the various subunits in different contexts, their nomenclature is somewhat confusing. Three -subunits have been identified: *AML1/CBFA2/PEBP2B, AML2/CBFA3/PEBP2C,* and *AML3/CBFA1/PEBP2A.* In view of its pivotal role in both normal hematopoiesis and leukemia, we will refer to *AML1/CBFA2/PEBP2B* as *AML1*. At their N-terminus, the -subunits of CBF contain a DNA-binding domain that is highly homologous to the *Drosophila melanogaster runt* gene.[156] In *Drosophila, runt* functions as a pair-rule segmentation gene involved in body pattern development and the regulation of other segmentation genes. The consensus binding site for the -subunit, TGPyGGT, was identified initially in enhancers of polyoma and murine leukemia viruses.[157]

Figure 52-5 Core-binding factor (CBF) consists of a heterodimer that contains an DNA-binding subunit and a -subunit that does not bind DNA directly but enhances DNA binding by the -subunit. Each of the components of the CBF heterodimer is involved in chromosome translocations in AML. These fusion genes are highlighted in red. Formation of these fusions may disrupt the normal regulation of target genes that contain CBF regulatory sites.

In hematopoietic cells, CBF-binding sites have been observed in genes specific to both the lymphoid lineage (T-cell receptor enhancers, CD3epsilon, and LCK proximal promoter) and genes specific to the myeloid lineage (M-CSF receptor, IL-3, GM-CSF, myeloperoxidase, granzyme B, and neutrophil elastase). [16] [158] The -subunit of the CBF complex appears to contribute to the tissue specificity of CBF gene regulation. For example, the *AML3/CBFA1/PEBP2A* gene has been found to

be a critical factor in the regulation of osteoblast differentiation, and disruption of this gene in mice has demonstrated it to be essential for the development of bone. [155] The *AML1* subunit has been studied extensively in hematopoiesis. Multiple splice variants have been identified; *AML1B* contains the *runt* domain and a trans-activation domain, whereas *AML1A* lacks the trans-activation domain.[159] Disruption of *AML1* by gene targeting in embryonic stem cells results in embryonic lethality at embryonic day 12.5 due to an absence of fetal liver hematopoiesis and to central nervous system hemorrhage. [160] Strikingly, the knockout of *CBF* results in a very similar phenotype, indicating that both the - and -subunits of CBF are essential for definitive but not primitive hematopoiesis. [161] [162] Because these knockouts affect the earliest stages of hematopoiesis, the role of CBF in later stages of hematopoietic differentiation is not yet clear.

AML1-ETO in the t(8;21)(q22;q22)

The t(8;21)(q22;q22) translocation is associated with the M2 subtype of AML and was the first recurring chromosome translocation in AML to be identified. [82] The clinical and morphologic correlations of this subtype of AML include a high incidence of development of extramedullary collections of leukemic blasts known as granulocytic sarcomas. The blasts have a characteristic morphology, including prominent Auer rods, salmon-colored granules, and large cytoplasmic granules and vacuoles. When the characteristic histologic appearance of these cells is identified in AML blasts that do not contain a t(8;21), the *AML-ETO* fusion can be identified by PCR, indicating that formation of the molecular fusion is responsible for the morphologic and clinical features of this subtype of AML. [163]

The *AML1* gene was first identified by cloning the chromosome 21q22 breakpoint junction in the t(8;21)(q22;q22) in the M2 subtype of AML. [164] Although no function could be ascribed to *AML1* at the time of its initial cloning, the identification of the homology to the *Drosophila runt* gene has enabled a series of researchers to determine the normal functions of CBF transcription

factors in hematopoiesis. In t(8;21), *AML1* fuses to the *ETO* gene on the der(8) chromosome to form a chimeric fusion protein.[165] The normal functions of the *ETO* gene are not yet known; however, it contains regions of homology to the *Drosophila nervy* gene.[166] The chimeric protein fuses the N-terminus of *AML1*, including the *runt* homology domain but excluding the trans-activation domain to nearly all of *ETO* except for a few N-terminal amino acids. The *AML1-ETO* fusion gene has pleiotropic effects in reporter gene assays as it appears to repress several *AML1* target genes but to activate the M-CSF receptor promoter.[167] By engineering a knock-in of the *ETO* cDNA into the murine *AML1* locus, a mouse model of the t(8;21) has been generated. These mice exhibit a midgestation embryonic lethality with an absence of fetal liver hematopoiesis and central nervous system hemorrhage that is quite similar to what is seen in the *AML1* and *CBF* knockout mice.[168] This result suggests that the *AML1-ETO* fusion gene can act as a dominant negative of normal *AML1* function. The embryonic stem cells containing the *AML1-ETO* knock-in do not contribute significantly to hematopoiesis in the chimeric mice; thus these mice do not develop leukemia. Thus, a mouse model that would recapitulate the leukemia awaits the development of an inducible expression system that can bypass the early block in hematopoietic development caused by *AML1-ETO* in the knock-in mice.

AML1-EAP, MDS1, EVI1 in the t(3;21)(q26;q22)

The t(3;21)(q26;q22) translocation has been observed in the blast crisis of CML and also in therapy-related MDS and AML. The *AML1* gene has been found to be rearranged in these cases, leading to the formation of alternative fusion transcripts involving the *EAP, MDS1*, and *EVI1* genes.[169] [170] The fusions contain the N-terminus of *AML1*, including its *runt* homology domain fused to alternative C-terminal sequences derived from its partner genes on 3q26. A complex pattern of these fusion transcripts has been identified. The fusion to *EAP* is out of frame, and thus the chimeric transcript would encode a truncated form of *AML1* that contains only a few additional amino acids from *EAP*. By PCR analysis of cases with this translocation, independent fusions of *AML1* to *MDS1* and *EVI1*, as well as a combined *AML1/MDS1/EVI1* fusion transcript, have been identified.[171] *EVI1* is also involved in the inv(3)(q21q26) and the t(3;3)(q21;q26) in AML. [172] However, the consequence of these chromosome aberrations is the inappropriate expression of the *EVI1* transcription unit, whereas in the t(3;21), *EVI1* participates in the formation of a fusion gene with *AML1*. Sequences from another transcription unit, *MDS1*, are also observed in the t(3;21). Although mapping data indicate that the exons of *MDS1* and *EVI1* are separated by several hundred kilobases, they have been found to be spliced together not only in cases with the t(3;21), but also in normal tissues. A mouse model of the t(3;21) has not yet been generated. However, the *AML1-MDS1* fusion has been shown to transform the Rat1A fibroblast cell line. [173]

TEL(ETV6) and *AML1* in the t(12;21)(p13;q22)

AML1 is involved in the t(12;21)(p13;q22), resulting in the generation of a chimeric protein with the *TEL(ETV6)* gene.[174] The t(12;21) fusion is observed commonly in pre-B-cell ALL. The *TEL(ETV6)* gene was originally cloned from a t(5;12)(q33; p13) in CMML, where it fuses to the *PDGFR* gene, and it has also been found to fuse to the *ABL* gene in a t(12;22) in AML. [175] [176] *TEL(ETV6)* is a member of the ETS family of transcription factors that contain an HLH motif and an ETS DNA-binding domain. In the t(12;21), the structure of the fusion protein differs from that of other fusion genes involving *AML1*. The HLH domain of *TEL(ETV6)* comprises the N-terminus of the fusion, whereas sequences from *AML1*, including both the *runt* homology domain and the trans-activation domain, are located at the C-terminus of the chimeric protein.[177] *TEL(ETV6)* has also been found to fuse to *MDS1/EVI1* in the t(3;12)(q26;p13) (Fig. 52-4).

CBF-MYH11 in the inv(16)(p13q22) and the t(16;16)(p13;q22)

The aberrations of the inv(16)(p13q22) and the t(16;16)(p13;q22) are observed primarily in the M4Eo subtype of AML. These patients, similar to those with the t(8;21), have a relatively good prognosis and a high likelihood of responding to high-dose cytarabine-containing chemotherapy regimens. [10] In contrast to the t(8;21), the inv(16) is not typically associated with the development of granulocytic sarcomas. The -subunit of CBF, located at 16q22, is disrupted as a result of either this chromosomal inversion or a translocation leading to the formation of a fusion transcript with the gene for smooth muscle myosin heavy chain, *MYH11*, located at 16p13.[178] Thus both the - and -subunits of CBF are disrupted in common subtypes of AML and form chimeric fusion transcripts with genes not normally expressed in hematopoietic cells. The CBF-MYH11 chimeric protein contains nearly all of *CBF*, including the heterodimerization domain for *AML1*, fused to the C-terminal sequences of *MYH11*, including its coiled-coil dimerization motif. The chimeric CBF-MYH11 protein thus retains its ability to bind to *AML1* and is also capable of forming high-molecular-weight multimers through the coiled-coil domain of *MYH11*. The *CBF-MYH11* fusion has been shown to interfere with the trans-activation capacity of the normal CBF heterodimer.

By inserting the *MYH11* cDNA into the murine *CBF* locus, a mouse model for inv(16) leukemia has been generated through a knock-in approach. [179] These mice exhibit a midgestation embryonic lethality, an absence of fetal liver hematopoiesis, and central nervous system hemorrhage. The chimeric mice generated from embryonic stem cells containing *CBF-MYH11* did not develop leukemia. However, there was no contribution by the embryonic stem cells to hematopoiesis. Thus, a striking similarity has been observed for the knockouts of *AML1* and *CBF*, as well as the *AML1-ETO* and *CBF-MYH11* knock-in mice. This convergence in phenotypes from seemingly disparate gene-targeting experiments indicates that *AML1* and *CBF* are both essential for definitive hematopoiesis and are both required for the function of the CBF heterodimer. In addition, although there are two other -subunits of CBF, they cannot compensate for *AML1* in hematopoiesis. Further, the fusion genes formed as a result of chromosomal aberrations involving either the - or the -subunits of CBF appear to act as dominant negative inhibitors of CBF function.

RAR Family of Leukemias

The cytogenetic hallmark of acute promyelocytic leukemia (APL), the M3 subtype of AML in the FAB classification, is a translocation involving the *RAR* locus on chromosome 17.[180] The vast majority of these cases contain a t(15;17)(q22;q1112), although several variant translocations involving *RAR* have been identified. These include *PLZF* in a t(11;17)(q23;q1112) and *NPM* in a t(5;17)(q35;q1112). *NPM* is also involved in the t(2;5)(p23;q35) in anaplastic large cell lymphoma (Fig. 52-4).[249] These translocations lead to the formation of chimeric fusion transcripts from each of the derivative chromosomes. Retinoic acid is a critical ligand in the differentiation pathway of multiple tissues. The diverse effects of retinoids are mediated through binding to a retinoic acid receptor (RAR). RARs belong to the nuclear steroid/thyroid hormone receptor superfamily and possess a modular structure with discrete ligand-binding and DNA-binding domains. Of the three isoforms of RARs, *RAR* is expressed primarily in hematopoietic cells. *RAR* heterodimerizes with retinoid X receptor (RXR) and binds to retinoic

Figure 52-6 Retinoic acid receptor- (RAR-) and retinoic acid X receptor (R × R) bind as heterodimers to retinoic acid receptor response elements (RARE). The fusions of RAR- to its partner genes in leukemia are highlighted in red. These RAR- fusion proteins disrupt the regulation of genes that contain RARE regulatory elements.

acid-responsive elements to regulate transcription of target genes (Fig. 52-6).[181]

The capacity of retinoids to induce myeloid differentiation was recognized prior to the identification of the involvement of *RAR* in APL. Retinoic acid had been shown to enhance the growth of normal myeloid progenitors, to induce differentiation of the HL-60 promyelocytic cell line, and to induce terminal differentiation of primary human APL cells cultured in vitro.[182] [183] [184] Subsequently, the use of all-*trans*-retinoic acid (ATRA) was found to induce complete remissions in patients with APL. Rather than inducing cell death from cytotoxicity, ATRA induces differentiation of the malignant promyelocytic clone, an effect that can be observed by serial morphologic examination of the peripheral blood of patients undergoing treatment. [185] Although patients with APL can achieve a complete remission with ATRA alone, it has become clear that without additional cytotoxic chemotherapy, most patients will ultimately relapse. The basis for the development of ATRA resistance remains unclear but it appears that additional genetic events might occur in APL cells that confer resistance. The use of arsenic trioxide has also been shown to induce remissions in APL, possibly by inducing the degradation of PML-RAR-.

PML-RAR in the t(15;17)(q22;q1112)

The *PML* gene was first identified by its fusion to *RAR* in the t(15;17).[186] [187] It is expressed ubiquitously, and multiple alternative splice variants have been isolated. *PML* contains a cysteine-rich ring finger motif and an -helical coiled-coil region. The normal function of *PML* is not yet known, but overexpression of *PML* inhibits growth in cell lines. As a result of the t(15;17), a reciprocal fusion transcript is formed from each derivative chromosome. The *PML-RAR* fusion contains the N-terminus of *PML*, including its ring finger and part of the coiled-coil domain fused to the DNA-binding and ligand-binding motifs of *RAR*. The reciprocal *RAR-PML* fusion is much smaller and contains the N-terminal activation domain of *RAR* fused to the C-terminus of the coiled-coil motif of *PML*. In addition, a truncated version of *PML* is also formed as a result of the fusion of N-terminal sequences of *PML* to an exon of *RAR* that is out of frame. The contribution of each of these transcripts has been the focus of intense investigation. Whereas the *PML-RAR* fusion is found consistently in all cases of the t(15;17), the reciprocal *RAR-PML* fusion can be detected in approximately 80% of cases.[188] Although this suggests that the *RAR-PML* fusion may not be essential, its expression has been postulated to represent a potential second genetic event that contributes to the leukemic phenotype.

The PML-RAR- protein has been shown to function as an aberrant retinoid receptor that possesses altered DNA-binding and transcriptional regulatory properties compared to wild-type RAR-.[189] PML-RAR- can heterodimize with RXR and bind to retinoic acid-responsive elements in target genes. In addition, PML-RAR- can form homodimers and is also capable of forming heterodimers with PML. Expression of *PML-RAR* has been shown to block retinoic acid-induced myeloid differentiation. [190] In addition, PML-RAR- can block RAR--mediated trans-activation in a dominant negative manner. In line with the hypothesis that translocations affecting the *RAR* locus act as dominant negative inhibitors of normal RAR--mediated functions, a dominant negative mutation of RAR- has been observed to affect myeloid differentiation. When this mutation was introduced into a murine FDCP multipotent hematopoietic cell line by retroviral infection, a block in differentiation along the neutrophil and monocytic lineages was observed and a switch to the development of mast cells and basophils occurred. [191] Because PML-RAR- contains most of PML, the fusion protein may also be acting as a dominant negative inhibitor of potential PML target genes that have not yet been defined.

The PML protein is a component of a novel nuclear structure referred to by several names, including PODs (PML oncogenic domains), NBs (nuclear bodies), or ND10 (nuclear domain 10).[192] [193] This nuclear structure was initially discovered by immunohistochemical analysis of the targets of autoantibodies obtained from patients with primary biliary cirrhosis. The Sp100 protein was the first component of this structure to be identified. Type I and II interferons induce the expression of both PML and Sp100. Infection of cells with adenovirus or herpesvirus leads to the delocalization of Sp100 and PML. In APL cells, the integrity of the nuclear body is disrupted and a microspeckled distribution of PML-RAR- is observed (Fig. 52-7). Treatment of APL cells with ATRA causes the nuclear bodies to regenerate, with proper relocalization of PML. Recently, arsenic trioxide has also been found to target PML and PML-RAR- to nuclear bodies and to induce degradation of these proteins. [194] Thus, the restoration of nuclear body structures by either ATRA or arsenic trioxide correlates with the ability of these agents to induce remission of APL.

Several groups have generated transgenic mice that express *PML-RAR* under the control of various myeloid-specific promoters. The phenotype in these mice depended on the stage of myeloid differentiation when the transgene was expressed. In mice with the transgene expressed under the control of the human MRP8 promoter, which drives expression in early myeloid progenitors as well as in mature neutrophils and monocytes, neutrophil numbers were normal but differentiation was impaired. Approximately 30% developed acute leukemia, with a latency period of 39 months, and remission of the leukemia could be induced by ATRA. [195] Two groups have generated transgenic mice expressing *PML-RAR* under the control of the human cathepsin G promoter, which drives expression in promonocytes and promyelocytes. In contrast to the MRP8 transgenics, these mice develop elevated numbers of immature myeloid cells in the bone marrow and peripheral blood. Approximately 1030% of the cathepsin G transgenic mice develop leukemia, with a latency period of 1214 months. [196] [197] Treatment of these mice with ATRA caused apoptosis of myeloid precursors rather than differentiation. Transgenic mice expressing *PML-RAR* under the control of the CD11b promoter have also been generated.[198] This promoter drives expression in later stages of myeloid differentiation. The CD11b transgenic mice do not develop leukemia and have normal numbers as well as normal maturation of myeloid cells. However, the ability of these mice to recover granulocytes following sublethal irradiation was impaired. Comparison of the phenotypes observed in the different transgenic mice reveals that the timing of *PML-RAR* expression during myeloid differentiation is a critical determinant in the development of leukemia. In addition, the relatively low frequency and long latency period to the development of leukemia imply that other genetic events, in addition to the expression of *PML-RAR*, are necessary for APL to occur.

Figure 52-7 In normal cells, PML and Sp 100 are localized in PODs (PML oncogenic domains), whereas RAR- is in the nucleoplasm. As a result of t(15;17), PML-RAR- is delocalized from the PODs. Treatment with all-trans-retinoic acid (ATRA) or arsenic trioxide restores proper subnuclear localization.

PLZF-RAR in the t(11;17)(q23;q1112)

The *PLZF* (promyelocytic zinc finger) gene fuses to *RAR* in APL with the variant (11;17)(q23;q21) translocation. [199] *PLZF* is a member of the *Drosophila Kruppel* family of transcription factors that contains nine zinc fingers. *PLZF* is expressed in myeloid but not lymphoid lineages, and its expression has been found to be down-regulated during differentiation. Unlike *PML, PLZF* is not a component of nuclear bodies, but it is localized in smaller, more numerous nuclear subdomains. Reciprocal fusion transcripts are expressed from each of the derivative chromosomes that are similar to the structure of the t(15;17) fusions. The *PLZF-RAR* fusion transcript consists of the first two zinc fingers of *PLZF* fused to the DNA-binding and ligand-binding domains of *RAR*, and the reciprocal *RAR-PLZF* fusion transcript contains the activation domain of *RAR* fused to the last seven zinc fingers of *PLZF*. PLZF-RAR- can either homodimerize with itself or heterodimerize with RXR. Similar to PML-RAR-, PLZF-RAR- can inhibit the activity of wild-type RAR- in a dominant negative manner. [200] In contrast to APL with the t(15;17), patients with the t(11;17) appear to be resistant to treatment with ATRA.[201]

NPM-RAR in the t(5;17)(q35;q1112)

Recently, another variant translocation in APL has been described. In the t(5;17)(q35;q21), the *NPM* gene has been found to fuse to *RAR*.[202] *NPM* is a nucleolar phosphoprotein that is involved in ribosomal ribonucleoprotein processing and transport. Two alternatively spliced *NPM-RAR* fusion transcripts have been isolated that differ by 129 bases in the contribution of *NPM* sequences to the fusion. The sequences in the fusion transcript derived from *RAR* include its DNA-binding and ligand-binding domains, similar to what is observed in the *PML-RAR* and *PLZF-RAR* fusions. In transcriptional assays, NPM-RAR- has been shown to trans-activate retinoic acid response element reporter constructs at similar levels to wild-type RAR-. The localization of the NPM-RAR- fusion protein has not yet been determined. *NPM* is also involved in the t(2;5)(p23;q35) in anaplastic large cell lymphoma, where it fuses to the *ALK* gene. In addition, *NPM* has been found to fuse to the *MLF1* gene in the t(3:5)(q25.1;q35) in AML.

MLL Family of Leukemias

Translocations involving chromosome band 11q23 occur frequently in both AML and ALL and have several unique features in comparison with other subtypes of leukemia. There are at least 30 different partner chromosomes involved in recurring reciprocal 11q23 translocations. [51] This exceeds the number of known translocations affecting the immunoglobulin loci, suggesting that the 11q23 breakpoint region may contain genomically unstable sequences that lead to chromosomal

recombination events. Translocations involving 11q23 are the single most common cytogenetic abnormality in infants with acute leukemia, regardless of phenotype, occurring in approximately 7080% of these cases. 11q23 translocations are also observed frequently in therapy-related leukemias in patients who have previously been treated with chemotherapy drugs that inhibit topoisomerase II, especially the epipodophyllotoxins.

The chromosomal partners in 11q23 translocations are usually lineage specific. In AML, the t(9;11)(p22;q23), the t(11;19)(q23;p13.1), and the t(6;11)(q27;q23) are the most common, and in ALL, the t(4;11)(q21;q23) and the t(11;19)(q23;p13.3) occur predominantly. The *MLL* gene was isolated from the 11q23 breakpoint by several groups and is referred to by other names, including *HRX, ALL-1*, and *Htrx*.[203] [204] [205] [206] 11q23 translocations have been observed in several FAB subtypes but occur most commonly in the M4 myelomonocytic and M5 monoblastic leukemias. Myeloid leukemias with 11q23 translocations often coexpress lymphoid markers, whereas 11q23 lymphoid leukemias often express myeloid or monocytoid markers in addition to B-cell markers. These observations suggest that rearrangement of *MLL* may affect a pluripotent stem cell or, alternatively, that disruption of *MLL* may affect a common differentiation pathway shared by lymphoid and myeloid progenitor cells.

MLL encodes a large protein, with a predicted molecular weight of 430 kd, that contains two regions of extensive homology to the *Drosophila trithorax* gene.[207] The regions that are conserved with *trithorax* include a series of Cys4-His-Cys3 zinc fingers in the middle portion of the protein that have been referred to as PHD or LAP domains.[208] [209] The C-terminus of MLL contains a conserved motif, named the SET domain, because it is present in several proteins, including Su(var)3-9, enhancer of zeste, and trithorax.[210] At the N-terminus of *MLL*, there are three AT-hook motifs similar to those present in the HMG-I(Y) proteins that bind to AT-rich sequences in the minor groove of DNA.[211] There is also a region of homology to mammalian DNA methyltransferases. Neither the AT hooks nor the DNA methyltransferase domain are present in trithorax. The MLL protein also contains transcriptional activation and repression domains.[212] In *Drosophila*, trithorax is essential to maintain the proper expression of homeotic genes but not to initiate their expression.[213] Targeted disruption of *Mll* in mice causes lethality at embryonic day 10.5.[214] *Mll* heterozygous mice also exhibited a phenotype with growth retardation, bidirectional homoeotic transformations of the axial skeleton, and sternal malformations. In the *Mll* homozygous deficient mice, expression of *Hoxa-7* and *Hoxc-9* was abolished, whereas in *Mll* heterozygous

Figure 52-8 Potential consequences of 11q23 translocations. As an example, t(11;19)(q23;p13.1) results in the formation of a fusion gene involving the *MLL* gene from chromosome band 11q23 and the *ELL* gene from chromosome band 19p13.1. Motifs within *MLL* are shown on the upper line and those in *ELL* on the bottom line. Two potential fusion genes encoded by each of the derivative chromosomes can be formed and are shown in the middle. In 11q23 translocations, the fusion transcript encoded by the derivative (11) chromosome has been implicated in leukemogenesis.

mice, the anterior boundaries of expression of these genes were shifted posteriorly. These findings indicate that MLL is a positive regulator of *Hox* gene expression.

In 11q23 translocations, the chromosomal breakpoints of both de novo and therapy-related leukemias cluster within an 8.3-kb genomic *Bam*HI fragment within *MLL*. As a result, all *MLL* gene rearrangements can be identified on Southern blot analysis with a 0.74-kb *Bam*HI cDNA fragment that contains the exons that span the breakpoint cluster region.[51] [215] 11q23 translocations result in the formation of two derivative chromosomes that each encode chimeric fusion transcripts. The fusion transcript encoded by the derivative 11 chromosome contains the N-terminal sequences of *MLL*, including its AT hooks, DNA methyltransferase motif, and repression domain, fused in frame to C-terminal sequences from the partner gene (Fig. 52-8). The reciprocal fusion contains the N-terminal sequences of the partner gene fused to the C-terminal sequences of *MLL*, including the zinc fingers, activation domain, and the SET domain. However, the C-terminal sequences of *MLL* that would be contained in the reciprocal fusion transcript are deleted in the formation of the translocation in approximately 2530% of cases, suggesting that its expression is not essential to leukemogenesis.

Fourteen genes at 11q23 partner chromosomal breakpoints have been cloned. These are *AF4* in the t(4;11)(q21;q23),[204] *ENL* in the t(11;19)(q23;p13.3),[205] *ELL* in the t(11;19)(q23;p13.1),[216] *AF9* in the t(9;11)(p22;q23),[217] *AF6* in the t(6;11)(q27;q23),[218] *AF1p* in the t(1;11)(p32;q23),[219] *AF1q* in the t(1;11)(q21;q23),[220] *AF-X* in the t(X;11)(q13;q23),[221] *AF10* in the t(10;11)(p12;q23),[222] *AF17* in the t(11;17)(q23;q21),[223] *EEN* in the t(11;19)(q23;p13),[224] and *CBP* in the t(11;16)(q23;p13.3).[225] [226] [227] Two *MLL* partner genes are involved in other chromosome translocations. *AF10* has been shown to fuse to the *CALM* gene in the t(10;11)(p13;q14), and *CBP* fuses to the *MOZ* gene in the t(8;16)(p11;p13). The functions of many *MLL* partner genes are not yet known. Although no consistent homologies or motifs among the partner gene sequences have been identified that might explain how their fusion to *MLL* results in leukemia, certain partner genes have similar features (Fig. 52-9). *ENL* and *AF9* contain transcriptional

Figure 52-9 Motifs in *MLL* partner genes. The partner genes can be grouped into several classes that share common motifs.

activation domains and share extensive homology at their C-termini. *AF10* and *AF17* contain leucine zipper and zinc finger motifs and also share extensive homology with each other. In addition, *AFX* and *AF6q21* contain forkhead domains and also share other regions of homology. Three *MLL* partner genes have well-characterized functions. *ELL* has been shown to function as an RNA polymerase II transcriptional elongation factor. It serves to increase the catalytic rate of RNA polymerase II by suppressing transient pausing by the polymerase along the DNA template.[228] *CBP* and *p300* are transcriptional co-activators that mediate interactions between multiple transcription factors and the basal transcriptional machinery. The pattern of protein-protein interactions of *MLL* partner genes may provide insight into the nature of the aberrant functions of MLL fusion proteins in leukemia.

In a subset of patients with AML and either trisomy 11 or a normal karyotype, a unique pattern of rearrangement of the *MLL* gene has been observed. As in the translocations that affect *MLL*, a fusion occurs involving one of the exons in its breakpoint cluster region. However, rather than fusing to a partner gene, the fusion is to 5 sequences from *MLL* itself.[229] [230] For example, the structure of the partial duplication might contain *MLL* exons 17 fused to exon 2 through the 3 end of the gene. The largest duplication identified contains exons 29, and the smallest, exons 46. The region of the duplication contains the AT hooks, methyltransferase, and repression domains of *MLL*. In several cases, the 5 and 3 genomic breaks within *MLL* occur at similar points within *Alu* repetitive sequences, suggesting that partial duplications may be generated as a result of homologous recombination. In contrast to the reciprocal translocations involving *MLL*, the partial duplications of *MLL* appear to occur primarily in older patients and are infrequent in childhood and therapy-related leukemias. The morphology of the leukemias also differs in that the partial duplication patients usually are classified as FAB-M1 or -M2, rather than the M4 or M5 typically observed in cases with *MLL* translocations. The basis for the correlation of the partial duplication event with the trisomy for chromosome 11 remains unknown.

Two different strategies have been used to generate mouse models of 11q23 leukemias. To create an *Mll-AF9* knock-in mouse, the *AF9* cDNA was targeted into the murine *Mll* genomic locus by homologous recombination in embryonic stem cells.[231] The chimeric mice generated from these embryonic stem cells all developed AML within 69 months. Chimeric mice that expressed only the N-terminal sequences of *Mll* did not develop leukemia. In addition, retroviral bone marrow infection has been used to express another 11q23 fusion gene, *MLL-ENL*, in hematopoietic cells.[232] In serial replating assays on methylcellulose, the *MLL-ENL*-transduced cells could be immortalized, whereas constructs containing either N-terminal *MLL, ENL* alone, or vector controls could not maintain proliferative capacity beyond three passages. After transplantation of *MLL-ENL*-transduced bone marrow, syngeneic recipient mice developed AML. These models have established proof of the principles that (1) the expression of *MLL* fusion genes contributes to leukemogenesis, and (2) the *MLL* partner genes are essential to this process.

Other Fusion Genes in AML

DEK-CAN in the t(6;9)(p23;q34) and *SET-CAN*

The t(6;9)(p23;q34) translocation is usually observed in the M2 and M4 subtypes of AML but has also been identified in patients with the M1 subtype and in myelodysplastic syndrome. This translocation is associated with a younger age and a poor prognosis. The 9q34 chromosome breakpoint junction was cloned by chromosomal walking from the nearby *ABL* gene, resulting in the identification of the *CAN* gene at this breakpoint.[233] [234] Subsequently, a chimeric transcript was isolated that contained 3 *CAN* sequences fused to 5 sequences of a gene named *DEK* at 6p23. The *CAN* gene was also found to be rearranged in a patient with acute undifferentiated leukemia and a normal karyotype. A chimeric transcript containing an alternative *CAN* partner gene, named *SET*, was isolated in this case.[235] The

SET gene was also localized to 9q34, suggesting that this fusion results from an inversion of chromosome 9 or a cryptic translocation.

DEK and *SET* are both nuclear proteins with little homology to each other, except that both contain highly acidic regions. The structure of the *DEK-CAN* and *SET-CAN* fusions are similar, with nearly all of either *DEK* or *SET* fused to the C-terminal two-thirds of *CAN*.[236] The *CAN* gene has been localized to the nuclear envelope at both its nuclear and cytoplasmic faces. *CAN* contains a coiled-coil domain and a leucine zipper N-terminal to the breakpoint in the t(6;9), and a second coiled-coil domain that flanks the breakpoint. In addition, *CAN* contains a number of repeat sequences, including 35 FG repeats (repeats of the amino acids phenylalanine and glycine) at its C-terminus, which are retained in the formation of the fusions to *DEK* and *SET. CAN* has been found to belong to a family of nucleoporins.[237] These are nuclear pore complex-associated proteins that are involved in the transport of RNA and proteins across the nuclear membrane. The fusion of *CAN* to *DEK* or *SET* disrupts its normal nuclear envelope localization, as both the *DEK-CAN* and *SET-CAN* fusions are located exclusively in the nucleus.

Knockout of the *CAN* gene results in embryonic lethality at embryonic day 4 to 4.5. The embryo is capable of survival until that stage with maternally derived *CAN*. Day 3.5 embryos exhibit impaired nuclear protein import and mRNA export, consistent with the function of *CAN* as a nucleoporin. Recently, *CAN* has been found to interact with two proteins, NUP88 and hCRM1. NUP88 is another member of the nuclear pore complex.[238] In yeast, CRM1 is required for maintenance of correct chromosome structure. Although NUP88 does not interact with the DEK-CAN fusion protein, hCRM1 has been found to associate with DEK-CAN. Thus, aberrant protein-protein interactions may contribute to the leukemogenic properties of the *CAN* fusion proteins.

TLS/FUS-ERG and the t(16;21)(p11;q22)

The t(16;21)(p11;q22) translocation has been observed in several FAB subtypes of AML, in blast crisis of CML, and in myelodysplastic syndrome.[239] [240] In this translocation, the *TLS/FUS* gene at 16p11 fuses to the *ERG* gene at 21q22 to generate a chimeric protein. Both *TLS/FUS* and *ERG* are involved in chromosome translocations with other partner genes in sarcomas. *TLS/FUS* fuses to the *CHOP* gene in the t(12;16)(q13;p11) in myxoid liposarcoma, and *ERG* fuses to the *EWS* gene in the t(21;22)(q22;q12) in Ewing's sarcoma and PNET tumors.[241] [242] *EWS* has also been shown to fuse to the *CHOP* gene in myxoid liposarcoma.[243] In addition, *EWS* has been identified in chimeric fusion proteins generated as a result of several different chromosomal translocations in other distinct types of sarcomas (see Fig. 52-4). These include fusions to the *ETS* family members *FL11, ETV1, FEV*, and *E1AF*, and fusions to the *WT1, CHN/TEC*, and *ATF1* genes. *TLS/FUS* and *EWS* share extensive homology with each other.[244] [245] [246] Both contain a polypeptide repeat SYGQQS and have the capacity to bind to RNA. *ERG* is a member of the *ETS* family of transcription factors that is homologous to *FLI1* and contains an N-terminal activation domain and a C-terminal DNA-binding domain. In the t(16;21), the N-terminal sequences of *TLS/FUS*, including the SYGQQS motif, are fused to the C-terminal sequences of *ERG*, including the ETS DNA-binding domain. This is quite similar to the structure of the other fusion proteins involving *TLS/FUS* and *EWS* and their myriad partner genes in leukemia and sarcomas. The TLS/FUS-ERG fusion

protein exhibits alterations in both DNA binding and transcriptional activation when compared with the wild-type proteins.[247] The striking similarity of the *TLS/FUS-ERG* fusion gene in AML to the sarcoma-associated fusion genes suggests that disruption of a common differentiation pathway may lead to transformation in multiple cell types.

NPM-MLF1 in the t(3;5)(q25.1;q35)

The t(3;5)(q25.1;q35) translocation has been observed in several FAB subtypes of AML, especially in M6 or erythroleukemia, and also in myelodysplastic syndrome.[248] The *NPM* gene at 5q35 was found to be rearranged in patients with this translocation, resulting in the formation of a fusion gene with the *MLF1* (myelodysplasia-myeloid leukemia factor 1) gene at 3q25.1. Previously, the involvement of *NPM* was identified in the t(2;5) (p23;q35) in anaplastic large cell lymphoma, in which it fuses to the *ALK* gene.[249] NPM is also rearranged in the t(5;17)(q35;q21) in APL, in which it fuses to *RAR*, as discussed above. *MLF1* contains no known structural motifs and its functions are not yet known. The *NPM-MLF1* fusion transcript contains 58 more amino acids of *NPM* than the *NPM-ALK* and *NPM-RAR* fusions. The metal-binding motif at the N-terminus of *NPM* is retained in all three types of *NPM* fusions. However, in the *NPM-MLF1* fusion, the additional contribution from *NPM* includes an acidic cluster and a nuclear localization signal. The MLF1 protein localizes to the cytoplasm, whereas in leukemia cells with the t(3;5), the NPM-MLF1 fusion protein is observed primarily in nucleoli. Thus, the normal subcellular localization of MLF1 is altered as a result of its fusion to NPM. The involvement of *NPM* in chromosome translocations involving three different partner genes, resulting in three discrete types of hematologic malignancies, suggests that *NPM* plays an important role in normal hematopoietic differentiation.

EVI1 in the inv(3)(q21q26) and the t(3;3)(q21;q26)

EVI1 (ecotropic virus integration site 1) was initially cloned as the gene at a common site of retroviral integration in murine myeloid leukemia. The *EVI1* gene is normally expressed only in the kidney and ovary, and not in hematopoietic cells. In mice, retroviral integration at the murine *Evi-1* locus leads to its inappropriate expression. A similar phenomenon occurs in human AML with the inv(3)(q21q26) and the t(3;3)(q21;q26).[172] As a result of these cytogenetic aberrations, the *EVI1* gene is juxtaposed to enhancer elements of the ribophorin gene, leading to inappropriate activation of the *EVI1* transcription unit. In contrast to the majority of chromosome translocations in AML, the inv(3)(q21q26) and the t(3;3)(q21;q26) do not result in the formation of a fusion gene. However, in the t(3;21)(q26;q22), *EVI1* is a component of the complex fusion transcripts that involve the *AML1* gene.

MOZ-CBP in the t(8;16)(p11;p13)

The t(8;16)(p11;p13) translocation has been recognized in the M4 and M5 subtypes of AML that exhibit a characteristic erythrophagocytosis. This translocation has been observed in both de novo and therapy-related leukemias. The *CBP* (CREB-binding protein) gene was identified at the 16p13 breakpoint, and a novel gene, *MOZ* (monocytic zinc finger), was cloned at the 8p11 breakpoint.[250] *CBP* is a transcriptional co-activator that coordinates signals between sequence-specific transcription factors and the basal transcription machinery. It interacts with multiple proteins, including CREB, Fos, Jun, ATF1, YY1, E1A, TFIIB, p53, and myb. CBP can function as a histone acetyltransferase and thus modulate transcription. *MOZ* contains two Cys4-His-Cys3 and one Cys2-His-Cys zinc fingers. *MOZ* also contains a putative acetyltransferase domain. The *MOZ-CBP* fusion contains the zinc fingers and acetyltransferase domain of *MOZ* fused in frame to nearly all of *CBP*. Several variant translocations involving 8p11 have been recognized in monocytic leukemias with erythrophagocytosis, but the genes involved in these translocations have not yet been cloned. *CBP* is also involved with a fusion to *MLL* in the t(11;16)(q23;p13).[225]

CALM-AF10 in the t(10;11)(p13;q14)

The t(10;11)(p13;q14) translocation has been recognized in both ALL and AML. In AML, the cases are primarily M0 and M1. This translocation has also been observed in the U937 cell line.[251] The *AF10* gene was identified at the 10p13 breakpoint, and a novel gene, *CALM*, was cloned from the 11q14 breakpoint.[252] *AF10* is also one of the partner genes of MLL in translocations and insertions with 10p12 and 11q23 breakpoints. *AF10* is highly homologous to another MLL partner gene, *AF17. AF10* and *AF17* both contain a zinc finger and a leucine zipper motif. The *CALM* (clathrin assembly lymphoid myeloid leukemia) gene has a high homology to the murine *ap-3* gene, a clathrin assembly protein. The CALM-AF10 fusion protein contains virtually all of the coding sequence of the involved genes.

NUP98-HOXA9 in the t(7;11)(p15;p15), *NUP98-DDX10* in the inv(11)(p15q22)

The t(7;11)(p15;p15) translocation is observed in the M2 and M4 subtypes of AML and also in CML. The *HOXA9* gene was identified at the 7p15 breakpoint and the *NUP98* gene at the 11p15 breakpoint.[253] In BXH-2 mice, the *Hoxa9* gene has also been implicated in myeloid leukemia as a result of its activation by proviral insertion. In addition, other homeobox proteins, such as *PBX1* in the t(1;19) and *HOX11* in the t(10;14), have been identified in chromosome translocations in lymphoblastic leukemia. *NUP98* is a member of the FG repeat nucleoporin family. Another nucleoporin, the *CAN* gene, is involved in the fusion to *DEK* in the t(6;9). The *NUP98-HOXA9* fusion contains the FG repeats of *NUP98* fused to the homeobox domain of *HOXA9. NUP98* has also recently been found to be involved in the inv(11)(p15q22) in AML. This cytogenetic aberration has been recognized in both de novo and therapy-related MDS and AML. The *DDX10* gene, which is a putative RNA helicase gene, was identified at the 11q22 breakpoint.[254] The *NUP98-DDX10* fusion contains the FG repeats of *NUP98* fused to the RNA helicase domain of *DDX10*.

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ANIMAL MODELS OF AML

There are several examples of naturally occurring animal models of AML that are caused by retroviruses. There are two mechanisms of retroviral transformation: a retrovirus can encode for an oncogene that leads to transformation, or, alternatively, the retrovirus can inappropriately activate the expression of a gene adjacent to its integration site. The characterization of these processes has revealed a number of genes that are critical to both normal hematopoiesis and to human myeloid leukemias. In addition, these animal models have demonstrated the multistage nature of the evolution of leukemia and have provided reagents for analyzing the transformation of hematopoietic cells. Moreover, the naturally occurring models of leukemia have provided the basis for generating mouse models of human acute leukemia.

MYB Leukemias

The v-*MYB* gene is involved in two different avian retroviruses that induce leukemia in chickens. The v- *MYB* gene was originally identified as the transforming element in the avian myeloblastosis virus (AMV). In the E26 retrovirus, a fusion gene

consisting of *gag*, v-*MYB*, and v-*ets* has been identified.[255] The formation of a fusion gene containing sequences of two transcription factors is reminiscent of the fusions that result from chromosome translocations in human acute leukemia. In chickens, AMV causes monoblastic leukemias and transforms only myelomonocytic cells in vitro, whereas E26 induces erythroleukemias but is capable of transforming myeloid, erythroid, and megakaryocytic lineages in vitro. [256] C-*MYB*, the normal cellular counterpart of v-*MYB*, has been shown to be essential for definitive hematopoiesis in gene targeting studies. [257] Activation of the c-*MYB* gene has also been identified in murine myeloid leukemias. Promonocytic leukemias have been induced by priming mice with intraperitoneal injections of Pristane followed by infection with the Moloney murine leukemia virus.[258] The majority of the mice that develop leukemia have undergone insertional mutagenesis at the c- *MYB* locus.[259] The role of Pristane is unknown but appears to promote leukemogenesis by mediating an inflammatory response. At this time, there is no evidence for the rearrangement of c-*MYB* in human leukemias.

Friend Virus/SFFV Erythroleukemia

The erythroleukemia induced by infection with the Friend viral complex is the culmination of a three-step process that has become a paradigm for multistage neoplastic transformation.[260] Infection of susceptible mice with the Friend murine leukemia virus/spleen focus-forming virus (SFFV) complex induces, within 48 hours, a polyclonal proliferation of erythroid progenitors. This expansion of erythroid precursors has been shown to be secondary to the expression of the SFFV *env* gene product, the GP55 glycoprotein, which has the capacity to bind and activate the erythropoietin receptor. These erythroid cells are not transformed and retain the ability to undergo terminal differentiation. After approximately 2 weeks, however, a clonal population of transformed erythroblasts emerges that can be transplanted into syngeneic recipients. The evolution from a polyclonal proliferation of normal erythroid cells to a clonal population of transformed erythroblasts has been shown to depend on two additional independent genetic events. One of these is the inactivation of the *p53* tumor suppressor gene either by deletion, mutation, or proviral insertion.[261] [262] In addition, activation of either the *PU.1* gene, also referred to as *Spi-1* (SFFV proviral integration 1), or of the *Fli-1* gene occurs as a result of proviral integration adjacent to these loci. [263] [264] *PU.1* and *Fli-1* are members of the ETS family of transcription factors. In gene-targeting studies, *PU.1* has been shown to be essential for both myeloid and lymphoid development. [265] Thus the Friend/SFFV erythroleukemia model depends on three events: (1) polyclonal proliferation induced by a cytokine, (2) inactivation of a tumor suppressor gene, and (3) oncogenic activation of a gene involved in normal hematopoiesis.

BXH-2 Leukemias

BXH-2 mice are an inbred strain of mice derived from a cross of C57BL/6J and C3H/He mice. Although these parental strains have a low incidence of leukemia, more than 95% of BXH-2 mice develop myeloid leukemia by 1 year of age, one of the highest rates of spontaneous leukemia in any inbred strain of mice. This high incidence of leukemia has been shown to be caused by the expression of an ecotropic murine leukemia virus that is horizontally transmitted in this strain. [266] These retroviruses induce leukemia by insertional activation or mutation of proto-oncogenes adjacent to the viral integration site. One of the most frequent viral integration sites to be characterized is EVI2 (ecotropic viral integration site 2). The EVI2 was localized to a large intron within the *NF1* tumor suppressor gene.[267] The consequence of this integration event is disruption of the normal expression of *NF1* in the affected mice. In humans, the neurofibromatosis type I autosomal dominant disorder is caused by mutation of the *NF1* gene.[268] In addition to neurofibromas, patients with neurofibromatosis have an increased risk of developing several types of solid tumors and also malignant myeloid disorders, especially juvenile chronic myelogenous leukemia (JCML) and the monosomy 7 syndrome. [269] Gene-targeting studies of *NF1* revealed that mice heterozygous for the *NF1* allele developed myeloid leukemia with loss of the wild-type allele, indicating that *NF1* acts as a tumor suppressor gene.[270] The *NF1* gene product, neurofibromin, has been shown to accelerate GTP hydrolysis on ras proteins. In JCML patients without neurofibromatosis, activating *ras* mutations are frequently identified. The consequence of either the disruption of *NF1* or the activation of *ras* has been shown to be an inability to negatively regulate GM-CSF signaling in hematopoietic cells. [271] [272]

Because viral integration within the *NF1* gene has been observed in only 15% of BXH-2 mice, the identification of additional mutations within other genes associated with myeloid leukemias has also been pursued. A proviral tagging approach has been used to identify additional viral integration sites that alter the expression of three genes, *Hoxa7, Hoxa9*, and *Meis-1*. [273] [274] The *HOXA9* gene is involved in the t(7;11)(p15;p15) in AML, resulting in the formation of a fusion gene with *NUP98. Meis-1* is a member of the *PBX1* family of homeobox proteins that bind to DNA cooperatively with Hox proteins. *PBX1* is involved in the t(1;19) in ALL, where it fuses to the *E2A* gene. This underscores a recurring theme: the involvement of similar families of genes in both human and spontaneous animal models of AML.

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Chapter 53 - Clinical Manifestations of Acute Myeloid Leukemia

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INTRODUCTION

Acute myeloid leukemia (AML) is not a single disease but a group of neoplastic disorders characterized by the proliferation and accumulation of immature hematopoietic cells in the bone marrow and blood. These malignant cells gradually replace and inhibit the growth and maturation of normal erythroid, myeloid, and megakaryocytic precursors. If untreated, AML is usually fatal within weeks to months from the time of diagnosis.

The clinical evaluation, therapy, and prognosis of patients with AML has changed dramatically over the last two decades. The initial evaluation of a patient with acute leukemia should be directed at defining the factors that are important in planning therapy and assessing the long term prognosis. Molecular, cytogenetic, and immunologic studies have contributed to our understanding of the pathogenesis and prognosis of the acute myeloid leukemias. Knowledge of environmental or occupational exposures to known or suspected leukemogenic agents or prior illnesses that predisposes an individual to develop AML must be considered in planning treatment and evaluating the response to therapy.

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HISTORY

Velpeau in 1827 reported the first accurate description of a case of leukemia. [1] The patient was a 63-year-old florist who developed an illness characterized by fever, weakness, urinary stones, and massive hepatosplenomegaly. Velpeau reported that the blood of this patient was like gruel. He found no infectious cause for this disorder and speculated that the elevated number of white corpuscles and the unique appearance of the blood may not have been the result of infection.

In 1845, Bennett, a brilliant and controversial pathologist from Edinburgh, published a report of a series of patients who died with enlarged spleens and changes in the color and consistency of their blood. [2] While he also found no infectious etiology for the peculiar appearance of the blood, he attributed these changes to the presence of purulent material in the blood and introduced the term *leucocythemia*. Virchow, the noted German pathologist, reported a similar case. [3] Virchow commented on the reversal of the normal ratio of pigmented, red, to colorless, white cells, in his patient but did not attribute these changes to an infection. Unsure of the etiology of his finding, he was content to simply describe his observations and used the descriptive name white blood. Later, in a monograph entitled *Die Leukemia*, Virchow introduced the word *leukemia*, which he derived from the Greek meaning white blood. [4]

The name and the interpretation of this new pathologic condition were acrimoniously debated by Virchow and Bennett, the two leading pathologists of the day. Virchow noted that the leukemias were not one disorder but a heterogeneous group of disorders, and he attempted to subdivide them into a splenic, probably myeloid, and a lymphatic type. In addition, he noted that some patients had a chronic or indolent course, while others presented with a rapidly progressive fatal illness. Virchows and Bennetts observations and interpretations were remarkable in view that neither had the tools to stain blood smears and that little was known about the mechanisms of hematopoiesis or the fate, origin, or function of the various cellular elements.

Neumann and Konigberg in the 1870s were the first to suggest that the colorless white cells were made in the bone marrow independently of red cells. [5] Ehrlich in 1877 developed the first aniline-based stains that permitted the clear definition of cellular detail in air-dried films of blood. He was then able to

accurately describe and differentiate the various types of normal and abnormal white blood cells. Ebstein in 1889 introduced the term acute leukemia to describe a rapidly fatal illness that failed to respond to available therapy. [6] Neumann in 1869 first suggested that the white blood cells were made in the bone marrow and not the spleen. This concept gave rise to the term myeloid, meaning marrow-derived. Mosler in 1879 described the aspiration of bone marrow as a means to diagnose leukemia. [7] Naegeli in 1900 described the myeloblast and divided the leukemias into a myelocytic and a lymphocytic type. [8] Hirschfeld, DiGuglielmo, and Schilling described the granulocytic, erythroid, and monocytic leukemic variants, respectively. [9] [10] [11]

The development of histochemical stains and cytogenetic, immunologic, molecular, and biochemical markers have helped to further define the lineage of the leukemic cell and to classify the leukemias. Effective therapy and advances in supportive care have resulted in dramatic changes in the approach to the patient with AML. The treatment and prognosis of patients with AML has changed AML from a disease that formerly was uniformly fatal to one that responds to chemotherapy and is potentially curable.

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ETIOLOGY

AML causes approximately 1.2% of all cancer deaths in the United States, an annual death rate of 2.2 per 100,000. There are approximately 9,200 new cases of AML each year. The incidence increases with age and has remained remarkably steady since the late 1960s. In adults AML represents approximately 90% of all acute leukemias[12] (Fig. 53-1).

Genetic predisposition, drugs and environmental exposures, and occupational factors have been implicated as possible leukemogenic agents in children and adults.[13] [14] Leukemogenesis is a multistep process that requires the susceptibility of a hematopoietic progenitor cell to inductive agents at multiple stages. The different subtypes of AML may have distinct causal mechanisms, suggesting a functional link between a particular molecular abnormality or mutation and the causal agent.[15] No one factor has been shown to cause leukemia in all individuals exposed.

Evidence supporting a genetic predisposition for human leukemia has come from epidemiologic and family studies. [16] The highest incidence of AML in adults is in North America, Western Europe, and Oceania, and the lowest in Asia and Latin America. [17] [18] In contrast, the highest rate of childhood AML is in Asia and the lowest is in North America and India.[18] [19] There are numerous reports of multiple cases of both acute lymphocytic leukemia (ALL) and AML occurring within the same family.[20] [21]

Figure 53-1 Relative incidence of ALL versus AML (ANLL) in different age groups.

The concordance of each of the leukemia subtypes in families is also more common than could be expected on the basis of chance alone. [22] [23] Reports of familial leukemia suggest that dynamic mutations of unstable DNA sequence repeats could be a common mechanism of inherited AML.[24] For all types, there is a threefold increase in leukemia incidence among first-degree relatives of patients with acute leukemia. [24]

In monozygotic twins there is an increased concordance for childhood leukemia. If one twin develops leukemia before the age of 6 years the risk of leukemia occurring in the second twin is up to 25%.[25] [26] The clinical presentation of AML in monozygotic twins is atypical: the leukemia usually occurs before 2 years of age; it occurs in both twins in close succession, typically in the same year; and it is of the same morphologic and cytogenetic subtype. [27] [28] [29] The twin studies are compatible with a genetic or a nongenetic intrauterine postzygotic event, which affects both twins simultaneously. [30] [31] [32] Moreover, no similar excess of leukemia has been observed in nonidentical twins of children with leukemia, and adult twin studies do not demonstrate such a high concordance for acute leukemia. [33] A possible explanation for these observations is that a single leukemogenic event occurring in utero leads to involvement of both twins as a result of the shared placental fetal circulation of monozygotic twins.[34]

The existence of a genetic predisposition to develop AML is suggested by the increased leukemia incidence associated with a number of congenital disorders, including Downs syndrome and Klinefelter syndrome.[35] [36] In Downs syndrome the incidence of acute leukemia is 10 times that of the general population. [37] [38] [39] The incidence of megakaryoblastic leukemia (M7) is 500 times higher than expected in children with Downs syndrome. [40] Moreover, families that have a Downs syndrome child may have an overall higher incidence of acute leukemias in the genetically normal family members. [41] Parents of children with Downs syndrome may also have an increased incidence of AML. [41] [42] [43] [44] These reports of familial clustering of Downs syndrome and leukemia have led to the speculations that a familial tendency to meiotic nondisjuction may be a risk factor for leukemia.[37] However, the familial association of Downs syndrome and acute leukemia remains controversial. [45]

In addition, the risk of childhood AML appears to increase with increasing maternal age, independently of the presence of Downs syndrome. [46] [47] [48] [49] Disorders associated with chromosomal instability and increased chromosome breakage, including Fanconis anemia, ataxia telangiectasia, and Blooms syndrome, are associated with an increased incidence of AML.[50] [51] [52] The development of leukemia in these disorders appears to be a multistep process and not a single transforming event. The genetic disorders give rise to a cellular environment that results in chromosomal instability, a hypersensitivity to DNA damage, and an increased susceptibility to mutations.[53] Von Recklinghausens disease, congenital neurofibromatosis, is associated with an increased incidence of childhood AML. [54] In case-controlled studies a maternal history of fetal loss was associated with an increased risk for childhood AML. Mothers of children with AML diagnosed before 2 years of age had a five-fold increase in previous fetal loss and a 12-fold increase in two or more fetal losses. [49]

Exposure to ionizing radiation and to a number of chemicals has been linked to the development of acute leukemia. The evidence linking radiation exposure and leukemia comes in part from the long-term follow-up of survivors of the atomic bomb explosions in Hiroshima and Nagasaki. [55] [56] [57] [58] The latency time from exposure to the development of leukemia was between 5 and 21 years, and the risk was related to age at the time of exposure and to radiation dose. [56] The development of leukemia was predictable and dose-related. In Hiroshima there was a 30-fold increase in the incidence of both AML and chronic myelogenous leukemia (CML). The highest rates were observed in persons younger than 10 or older than 50 years at the time of exposure.

In Nagasaki, where victims were exposed to a higher amount of gamma radiation, the incidence of AML was even greater. [57] All variants of AML except the M3, acute promyelocytic leukemia subtype, were observed. [59]

Exposure to even moderate doses of radiation therapy appears to be associated with an increased risk for developing AML. Workers at radium plants and military personnel exposed to ionizing radiation during nuclear test explosions have a higher than expected incidence of AML. [60] [61] Patients who received low doses of radiation for benign disorders such as ankylosing spondylitis, [62] [63] menorrhagia,[64] tinea capitis,[65] benign thymic enlargement,[65] and rheumatoid arthritis,[66] develop AML at a greater than expected rate. Exposure to thorotrast, a colloidal suspension of thorium dioxide used widely in the 1940s as a radiographic contrast medium, has been associated with an increased risk of AML, specifically the acute erythroleukemia subtype. [67] [68] The principal thorium isotope is ^{232}Th which, on decay, exposes the individual to chronic low dose alpha particles. The thorotrast-associated leukemia occurred 10 to 30 years after exposure. [67] The incidence of alpha particle-induced AML after thorotrast exposure was related to the combined effects of the amount of thorotrast administered, the exposure time, and the attained age of the individual.[67] Exposure to extremely low frequency electromagnetic fields generated by high voltage power lines has been reported to be associated with an increased incidence of acute leukemia, but this association remains controversial and is not clearly proven. [69] [70] The incidence of AML is increased in workers with chronic magnetic and electric field exposures, including telegraph, telephone, and other communication equipment operators. [71] [72] There appears to be an association between the duration of electric field exposure and the risk for the subsequent development of AML. [73] Workers exposed to extremely low frequency magnetic fields also have an increased incidence of AML. [74] Childhood leukemia has been linked to exposure to electromagnetic radiation associated with certain wiring configurations of a childs home.[75] These observations remain controversial and it is unclear from the reported case-controlled studies if the exposure to

electromagnetic radiation generated from wiring and power lines is physiologically, clinically, or epidemiologically important. [76] [77]

Chronic exposure to a number of chemicals has been associated with the development of acute leukemia.[78] Benzene is the best studied and has been the most widely used chemical leukemogenic agent.[78] [79] [80] [81] [82] Leather and rubber industry workers chronically exposed to benzene and benzene derivatives have a significantly increased incidence of AML.[83] Case-controlled studies have found that truck drivers, filling station attendants, workers in petroleum and gas plants, and painters have an increased incidence of AML, which is perhaps related to their chronic exposure to benzene and other hydrocarbons. [84] [85] [86] [87] [88] Persons exposed to embalming fluid, ethylene oxides, and herbicides also appear to be at an increased risk for acute leukemia. [89] [90] [91] Workers exposed to organic solvents used in the processing of medical radiographs and the manufacturing of electrical wiring may have an increased incidence of AML. [92] [93] Cigarette smokers and those chronically exposed to cigarette smoke appear to be at an increased risk of developing AML. [94] [95] [96] [97] Heavy cigarette smoking is associated with the development of clonal, nonrandom, cytogenetic abnormalities.[98] Metabolites of benzene are found in the urine of chronic cigarette smokers. [99] [100] Moreover, cigarette smoke contains measurable quantities of other known and suspected leukemogenic agents and mutagens. [100] [101]

An increased incidence of acute leukemia has been reported in patients who have received chemotherapy for a number of malignant and nonmalignant disorders. The nitrosoureas, the alkylating agents, and procarbazine appear to have the highest leukemogenic potential. [102] [103] [104] The development of AML following exposure to chemotherapy is usually preceded by a myelodysplastic syndrome.[105] [106] All the commonly used alkylating agents including cyclophosphamide, mechlorethamine, chlorambucil, busulfan, lomustine, and semustine have been associated with an increased risk for AML. The combination of chemotherapy and radiation therapy further increases the risk of developing leukemia. [107] [108]

The risk of developing AML is proportional to the age of the patient and the cumulative dose of the administered alkylating agent. In Hodgkins disease the cumulative risk of developing AML after treatment with alkylating agents increases steadily from 1 year after the start of treatment and reaches a peak of 13% at 7 years. [108] [109] The post-treatment incidence of AML increases most markedly in the first 2 years and plateaus after 8 to 10 years; [108] [109] this increase is related to the administration of a chemotherapy regimen that contains an alkylating agent. The incidence of AML in patients treated with the mechlorethamine-Oncovin-procarbazine-prednisone (MOPP) regimen containing the alkylating agent mechlorethamine, is 3, 4, and 7% at 3, 5, and 7 years, respectively. This is in contrast to a less than 1% incidence of treatment-related AML in patients treated with a regimen that does not include alkylating agents, or with radiation therapy alone. AML occurs after autologous bone marrow and stem cell transplants. Patients who are greater than 40 years of age at the time of transplant and receive total body irradiation are at highest risk for AML.[110] [111] The actuarial risk for secondary AML following chemoradiation therapy and autologous transplantation for Hodgkins disease is between 9 and 18%. [111]

An increased incidence of leukemia has been reported in patients receiving alkylating agents for small-cell lung cancer, [112] ovarian cancer,[113] germ cell tumors,[114] [115] [116] breast cancer,[117] non-Hodgkins lymphoma, [118] chronic lymphocytic leukemia,[119] multiple myeloma,[120] [121] polycythemia vera,[122] [123] and other malignancies.[102] [124] [125] Patients with non-neoplastic disorders such as nephritis, rheumatoid arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus, and Wegeners granulomatosis who have received alkylating agents also have a increased incidence of AML. [125] [126] [127] [128]

Therapy-related leukemias now represent between 10 and 20% of all cases presenting with AML. [129] These treatment-related, or so-called secondary leukemias are clinically and prognostically different from AML that occurs de novo. The secondary leukemias are frequently preceded by a variable period of anemia, neutropenia, or thrombocytopenia. Dysplastic changes are common and can be found in all cell lines. Circulating platelets may be large, with abnormal granulation; neutrophils may be hypogranular or agranular with pseudo-Pelger-Huet nuclei; and the red cells may demonstrate coarse basophilic stippling with prominent anisocytosis. The bone marrow early in the course may demonstrate dysplastic changes characterized by megaloblastic erythroblasts, micromegakaryocytes, ringed sideroblasts, and an increased number of myeloblasts (5 to 25%) with abnormal maturation. These morphologic abnormalities may also be accompanied by functional defects. Platelet and neutrophil function may be markedly impaired, which results in excess bleeding and recurrent infections despite an adequate number of circulating platelets and granulocytes. These findings in the peripheral blood and bone marrow may precede the development of overt AML by many months. [129] [130] Clonal, nonrandom, cytogenetic abnormalities involving chromosomes 7, 5, and 8 occur in 50 to 90% of patients with therapy-related leukemia. [129] [131] [132] These leukemias are associated with an overall poor prognosis. Although some patients may attain a complete hematologic remission with intensive chemotherapy, these responses are usually of short duration.[129] [133] [134]

A clinically and cytogenetically distinct group of secondary leukemias have been reported in individuals who have received one of the topoisomerase II inhibitors. [116] This group includes the epipodophyllotoxins, etoposide and teniposide, and the anthracyclines,

daunomycin and doxorubicin. The topoisomerase II inhibitors cause DNA damage via the intranuclear enzyme topo II. In contrast to alkylating agentrelated AML, the topoisomerase II therapyrelated leukemias typically lack a preceding myelodysplastic phase and are characterized by a shorter latency of onset, generally less than 23 years.[135] [136] [137] Most of the patients reported have received combination therapy that has included alkylating agents and/or radiation therapy. [138] [139] These topoisomerase IIrelated secondary leukemias are associated with chromosomal rearrangement involving chromosomes 3, 11, and 21, characterized by a balanced translocation involving bands 11q23 and 21q22. The MLL gene (mixed lineage or myeloid-lymphoid leukemia) located on chromosome 11 band q23 is the most frequent site of the leukemogenic translocation induced by topoisomerase II inhibitors. Most of the patients develop morphological M4 or M5 leukemias and present with a rapidly progressive disease with an elevated blast count. The treatment outcome for these patients is much worse than patients with de novo AML with similar clinical and cytogenetic abnormalities. In children with ALL the incidence of secondary AML after exposure to one of the epipodophyllotoxins is approximately 5%. [138] However, in children treated for a T cell ALL the incidence of secondary AML approaches 19% after exposure to one of the topoisomerase II inhibitors. [138] Cytogenetic studies suggest that the second leukemogenic event occurs in a normal hematopoietic progenitor and not in the initial leukemic clone. The outcome for these children with standard treatment remains poor. Topoisomerase II inhibitors have also been associated with a secondary acute promyelocytic leukemia with the characteristic 15;17 translocation.[139] It appears that the topoisomerase II inhibitors are leukemogenic by themselves and the effect may be amplified by the addition of other agents, such as prior or concomitant administration of alkylating agents or radiation therapy.

Drugs other than the cytotoxic agents that have been reported to be associated with the development of acute leukemia include chloramphenicol, [140] phenylbutazone,[141] chloroquine,[142] methoxypsoralen,[143] and LSD.[144] The strength of such associations, however, remains unclear.

Certain acquired diseases are associated with transformation to AML. Patients with the myeloproliferative disorders, including polycythemia vera, primary thrombocythemia, and agnogenic myeloid metaplasia, have an increased incidence of leukemic transformation. [123] [145] [146] [147] In polycythemia vera the incidence of AML is approximately 1% for patients treated only with periodic phlebotomies; the addition of chemotherapy or radiation therapy significantly increases this incidence.[148] [149] Moreover, the incidence of acute leukemia is related to the intensity and duration of alkylating agent therapy. The relative risk for AML was 410 times as great for those patients treated with daily continuous doses of an alkylating agent as for those treated by intermittent pulse therapy. [123] A similar phenomenon has been observed in patients with multiple myeloma and Waldenstroms macroglobulinemia. [124] Aplastic anemia is associated with late development of acute leukemia.[147] In patients with aplastic anemia treated successfully with antithymocyte globulin, 26% developed AML or one of the myelodysplastic syndromes after 8 years. In patients with aplastic anemia who were treated successfully with cyclosporin and recombinant granulocyte colony stimulating factor, 22% developed AML or a myelodysplastic syndrome.[147] The risk for AML appears higher in patients with aplastic anemia following irradiation or chemical exposure. [150] AML occurs in patients with paroxysmal nocturnal hemoglobinuria, and appears to involve the same clone from which the abnormal erythrocytes are derived. [151] Multiple myeloma is associated with the development of AML.[152] The association between AML, multiple myeloma, and administration of multiple alkylating drugs is well documented, but AML can occur in patients with myeloma who have not received prior chemotherapy or radiation therapy. [153]

Primary nonseminomatous mediastinal germ cell tumors are associated with the development of AML.[154] The acute megakaryocytic leukemia (M7) subtype is a frequent type of AML following these germ cell tumors. This secondary leukemia appears associated with the primary disease and is unrelated to prior treatment for the germ cell neoplasm.

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CLINICAL MANIFESTATIONS

The presenting signs and symptoms of AML are usually nonspecific and are related to the decreased production of normal hematopoietic cells and invasion of other organs by the leukemic cells. Patients usually complain of a brief viral-like illness characterized by fatigue and malaise or may present with a progressive skin infection after a minor abrasion. While anorexia is common, weight loss is unusual, generally reflecting the acute onset of the disease. Diffuse bone tenderness involving the long bones, ribs, and sternum is the initial clinical manifestation in 25% of patients. Joint pain and swelling, localized to large joints, may antedate other symptoms by weeks. The bone pain, which can be severe, is caused by the expansion of the intramedullary space or direct involvement of the periosteum by the leukemic cells.

The findings on physical examination relate to the interference with normal hematopoiesis by the leukemic cells. Typically, all three cell lines are affected. Anemia results in pallor and the onset of cardiovascular symptoms. Thrombocytopenia produces hemostatic defects, which result in petechiae and ecchymosis. Oozing from the gums, epistaxis, and excess bleeding after dental procedures or minor trauma are common initial manifestations. Petechiae are most prominent in the lower extremities and may appear suddenly after minor physical activity or standing for prolonged periods of time. Splenomegaly occurs in up to 50% of patients with AML, but the splenic enlargement is usually modest and rarely extends more than 5 cm below the left costal margin. A very large spleen suggests that the leukemia has evolved from an underlying prior myeloproliferative disorder. Lymphadenopathy is rare in AML, in contrast to ALL in which peripheral lymphadenopathy may be a prominent presenting finding. Involvement of the thymus or hilar nodes is very uncommon in AML. Skin involvement, leukemia cutis, occurs in about 10% of patients and usually presents as violaceous, raised, nontender plaques or nodules, which on biopsy are found to be infiltrated with myeloblasts (Plate 53-1). There may also be diffuse involvement of the skin. On biopsy there may be a prominent diffuse infiltrate involving deep dermal surface but with preservation of the superficial dermis.[155] Skin involvement is more common with the monocytic subtypes, including the acute monocytic and myelomonocytic leukemias.[156] Sweets syndrome, acute neutrophilic dermatosis, is a cutaneous paraneoplastic syndrome that is associated with AML and other hematologic disorders. [157] It is characterized by tender red plaques and nodules usually on the upper or lower extremities and may precede the diagnosis of AML by several months (Plate 53-2). The histological finding in Sweets syndrome is a dense infiltrate primarily composed of mature neutrophils located predominantly in the mid- and upper dermis. [158] The pathogenesis of Sweets syndrome in AML is unknown. It has been postulated that leukemia-related growth substances or antigens may directly or indirectly stimulate epidermal or dermal cells.[159] Sweets syndrome may occur in patients with normal, low, or elevated white blood counts. Similar lesion have been noted in patients with inflammatory disease and associated with the use of granulocyte colony stimulating factors. [160] [161] The use of topical or systemic steroids is the treatment of choice for Sweets syndrome.[162]

Chloromas, local collections of blasts, may present as isolated subcutaneous masses and may therefore be confused with a primary or metastatic carcinoma. [163]

Chloromas, granulocytic

sarcomas, are extramedullary solid aggregates of blast forms. The term chloromas is related to the greenish appearance on sectioning, which is secondary to the presence of myeloperoxidase granules in the myeloblasts. Granulocytic sarcomas are more common in M2-AML with the 8;21 translocation and leukocytosis. [156] [164] The presence of extramedullary leukemia is associated with a generally poorer response to treatment and shorter overall survival. [165] Gingival hyperplasia caused by leukemic infiltration is more frequent in the monocytic leukemias (Plate 53-3) but may occur in all the leukemic subtypes. The patient may present initially to a dentist complaining of painful gums, rapidly progressive gingival disease, and gum bleeding after dental brushing.

Central nervous system (CNS) involvement in AML is an uncommon presenting finding, but 5 to 7% of all patients have asymptomatic central nervous system involvement, as determined by positive cerebrospinal fluid cytology. [166] Magnetic resonance imaging (MRI) also appears to be a more sensitive and specific imaging modality than the computed tomography (CT) scan to detect dural involvement in suspected cases of CNS leukemia. [167] The finding of asymptomatic central nervous system disease does not alone appear to predict a poor prognosis. [168] [169] [170] Patients at highest risk for developing central nervous system leukemia include those with a high circulating blast count, elevated lactate dehydrogenase activity, and one of the monocytic leukemic subtypes. [166] [171] Of note is the very high frequency of central nervous system leukemia, up to 35%, in AML with increased eosinophils, the M4EO variant, which is associated with an inversion of chromosome 16. Leptomeningeal leukemia and intracerebral myeloblastomas are common in this otherwise prognostically favorable subtype. [172] Unless there are signs of overt central nervous system involvement that requires therapy, it is preferable to defer a lumbar puncture until the peripheral blasts have been cleared with chemotherapy in order to prevent the possibility of accidentally contaminating the spinal fluid with circulating leukemic cells. [173]

The majority of patients with leukemic involvement of the central nervous system are asymptomatic.[174] Some patients, however, present with meningeal signs and symptoms caused by increased intracranial pressure. In these patients a lumbar puncture is required and typically reveals an elevated opening pressure with an increased protein and a low glucose concentration in the cerebrospinal fluid. The cell count may be low, and therefore a Millipore filter technique or cytocentrifuge preparation of the cerebrospinal fluid is required to detect the presence of leukemic cells. Cranial nerve palsies secondary to leukemic infiltration of the nerve sheath are rare in AML. When they do occur, the Vth and VIIth cranial nerves are most frequently involved. [175] [176] Patients may present with the sudden onset of facial muscle weakness, which rapidly progresses to paralysis. Optic nerve infiltration can result in papilledema, eye pain, blurred vision, or the sudden onset of unilateral blindness. Retinopathy can occur and result in progressive impairment of vision. [177] Cranial nerve involvement can occur in the absence of overt central nervous system disease, and therefore the cerebrospinal fluid can be negative for leukemic cells in these cases. [178] A CT scan or preferably MRI of the affected nerve root may demonstrate thickening of the nerve sheath, which is suggestive of leukemic involvement. To prevent permanent loss of cranial nerve function, the affected cranial nerve roots should be irradiated as soon as possible after the onset of symptoms to prevent permanent loss of function. [176] Patients presenting with neurologic findings and a greater than 50,000/mm^3 concentration of circulating leukemic cells are at high risk for a major central nervous system event and require emergency intervention to rapidly lower the blast count. [179] [180] The high number of circulating blasts increases the blood viscosity and is associated with small-vessel leukoblastic emboli, resulting in leukostasis in the cerebral vessels. The leukemic blasts can infiltrate the arteriolar endothelial

Figure 53-2 Head CT scan of a patient with a presenting WBC count of 100,000/mm ^{3} and a massive intracranial hemorrhage secondary to leukostasis.

walls and cause a secondary hemorrhage (Fig. 53-2). Suspected or developing central nervous system leukostasis requires emergency efforts to rapidly lower the blast count. Patients may complain of diffuse headaches and fatigue, which rapidly progress to confusion and coma. Leukostatic hemorrhage clinically resembles a major cerebrovascular accident. The adhesion and aggregation of leukemic cells as well as local anatomical factors appear to play a role in development of CNS leukostasis.[180] Leukostasis is more intensive in the white matter and leptomeninges with involvement of medium-sized vessels. The risk of CNS leukostasis rapidly increases when the blast count is greater than 50,000/mm^3 . Despite most efforts to rapidly lower the white blood count and the blood viscosity, the prognosis for patients with central nervous system leukostasis remains very poor. [180]

Metabolic and electrolyte derangements are common in patients with AML.[181] Hyperuricemia is the most frequent leukemia-related biochemical abnormality and results from the increased turnover of the proliferating leukemic clone and subsequent purine catabolism. Hyperuricemia and hyperuricuria can develop before therapy is started. Typically, however, the uric acid level rises rapidly once therapy is initiated as a result of release of intracellular nucleic acids by the lysis of large numbers of cells. Urate crystals can precipitate in the renal tubules and ureters, causing acute renal failure. To prevent the development of urate nephropathy, all patients should receive intravenous hydration and should be started on allopurinol before beginning chemotherapy. Allopurinol, by inhibiting xanthine oxidase, causes an increase in the urine xanthine and can produce xanthine crystalluria and calculi.[182] Therefore, it is important to maintain adequate hydration in addition to administering allopurinol before and during induction chemotherapy. Rarely, leukemic cells produce an obstructive uropathy as a result

of direct infiltration of the prostate gland.[183] Direct bilateral cortical, medullary, and interstitial kidney involvement with myeloblasts may result in renal failure.[184] [185] However, in AML, in contrast to ALL, kidney involvement is uncommon. Hyperkalemia may occur as the result of rapid cell breakdown.[186] Hypokalemia, however, is a more common presentation in patients with AML.[187] The hypokalemia, which can be profound and require large doses of intravenous potassium, is most pronounced in the myelomonocytic and monocytic leukemias, in which intracellular levels of the lysozyme muramidase, which is toxic to renal tubular cells, are high.[188] [189] [190] Ineffective myelopoiesis or destruction of the leukemic cells with therapy causes the release of large amounts of this enzyme, which produces a proximal renal tubular dysfunction and leads to renal potassium wasting. In most cases, however, muramidase-induced tubular damage is not the sole mechanism of the hypokalemia.[190] Attempts to directly correlate the elevated serum and urinary muramidase lysozyme level with the leukemic subtype and the development of hypokalemia and the renal tubular defect have produced conflicting results.[191] [192] Leukemic cells can synthesize other factors with renin-like activity that may contribute to the development hypokalemia.[193] Antibiotics and chemotherapy-induced nephropathy, diarrhea, vomiting, and the development of hypomagnesemia all contribute to the development of potentially life-threatening hypokalemia during and before induction therapy for the AML.[192] [194]

Hypercalcemia has been reported in association with AML.[195] The mechanism is unclear, but may be related to the release of parathyroid hormone or parathyroid hormone-like fragments produced by the leukemic cells.[196] [197] The hypercalcemia may be caused by direct bone involvement or stimulation of osteoclasts and the resultant lytic bone lesions.[198] In these instances the blood calcium level parallels the activity of the disease. Hypocalcemia presumably a result of release by the leukemic cells of factors that result in accelerated bone formation has been reported. The hypocalcemia can be profound, and patients can present with tetany and potentially fatal cardiac arrhythmias.[198] The hyperphosphatemia and hyperphosphaturia associated with underlying renal insufficiency can also contribute to the hypocalcemia.

The rapid lysis of leukemic cells can acutely precipitate a number of serious metabolic problems as a result of the release of intracellular phosphate, potassium, and urate.[199] This so-called tumor lysis syndrome is characterized by the rapid development of hyperuricemia, hyperkalemia, hyperphosphatemia, and hypocalcemia.[200] The consequences of tumor lysis syndrome are directly related to the metabolic abnormalities: the hyperuricemia produces a urate nephropathy and acute renal failure; the hyperkalemia is associated with potentially lethal cardiac arrhythmias; and the hyperphosphatemia causes a reciprocal depression of the serum calcium and progressive renal insufficiency, with further reduction of the excretion of potassium and phosphate. The hypocalcemia, a result of the hyperphosphatemia, can cause tetany, cardiac arrhythmias, and muscle cramps. The tumor lysis syndrome occurs in patients with a rapidly rising or very high blast count.[201] [202] [203] It is important to recognize those patients at risk for this syndrome and to address and correct metabolic and electrolyte abnormalities before starting therapy. Allopurinol and intravenous hydration should be started early, before beginning chemotherapy. Serum electrolytes, including potassium, calcium, phosphate, and uric acid, and renal function should be carefully monitored. Renal function can rapidly deteriorate, and patients should be monitored daily to correct developing electrolyte abnormalities. Dialysis may be required and should be considered early in the course to prevent the complications of rapidly rising serum potassium, phosphate, and uric acid.[204] [205] In many patients recovery of renal function after tumor lysis occurs early and patients can be supported through the relatively brief period of renal insufficiency.[206] [207] With careful attention to electrolytes and the use of intensive hydration, the tumor lysis syndrome remains a complication in the treatment of AML. In the majority of patients with AML the development of renal insufficiency is associated with acute tubular necrosis, sepsis, and the use of nephrotoxic drugs.[205] The use of nephrotoxic antibiotics and other medications are frequent contributing factors for the development of renal failure during induction therapy. Many of the renal complications of AML and its treatment can be prevented with close attention to the medications given and the fluid status of the patient.

Lactic acidosis has been associated with AML.[208] The etiology of this metabolic acidosis is unclear but may result from anaerobic glycolysis by the leukemia cells. When lactic acidosis occurs, it is usually associated with a very high blast count, extramedullary disease, and leukostasis. The lactic acidosis parallels the disease activity in these cases.[209]

Hypocholesteremia and a reduction in total low-density lipoproteins in the plasma are frequently noted in untreated patients with AML.[210] The mechanism of the reduced lipoproteins is unclear but may involve the leukemic cells use of cholesterol for membrane synthesis. Alternatively, the leukemic cells may secrete growth factors that are associated with changes in the concentration of cholesterol.[211]

A spuriously low serum glucose level and arterial oxygen saturation can occur in the presence of high numbers of circulating blasts.[212] These spuriously low values reflect use by the leukemic blasts of the glucose and oxygen and typically reflect a delay in processing the test sample. A spurious elevation of blood potassium levels may occur with hyperleukocytosis as a result of the release of potassium from leukocytes undergoing lysis during clotting or as a result of prolonged storage of the sample before analysis.[186] This phenomenon is more common in patients with a high blast count. If a spuriously elevated potassium is suspected, the serum electrolyte studies should be repeated with an anticoagulated blood sample that is rapidly analyzed to prevent the in vitro lysis of leukemic blasts.[213]

Ophthalmic problems occur in patients with AML.[214] All portions of the eye may be involved including the optic nerve, choroid, and retina.[177] Chloromas, which are collection of blasts, can occur anywhere in the eye. Leukemia can involve the optic disc and optic nerve, resulting in the sudden onset of blurred vision, which can rapidly progress to total blindness.[215] This diagnosis should be suspected when the funduscopic examination reveals papilledema and disc pallor. Iritis may present with photophobia, excess lacrimation, and orbital pain. Ophthalmic involvement is very suggestive of meningeal leukemia. Ophthalmic involvement can occur in all the leukemic subtypes and is associated with a higher relapse rate and shorter survival as compared to patients without eye involvement.[177] [216]

Cardiorespiratory symptoms are common in patients with acute leukemia, pneumonias being the most common pulmonary problems. At presentation gram-positive and gram-negative bacteria are the major pathogens. However, patients who have a history of prolonged neutropenia or abnormal neutrophil function or who are receiving broad-spectrum antibiotics are at an increased risk for pulmonary infection with fungi or other opportunistic organisms. Pulmonary leukostasis is a serious potential problem for patients who present with a blast count greater than 50,000/mm ^{3} .[217] [218] In this setting leukocyte thrombi and plugging of pulmonary microvascular channels lead to vascular rupture and infiltration of the lung parenchyma. Patients may note the sudden onset of shortness of breath and progressive dyspnea, and on physical examination they are tachypneic, with diffuse bilateral rales. The chest radiograph usually demonstrates a diffuse interstitial infiltrate (Figure 53-3). Fever is common. Hypercapnia, hypoxemia, and progressive respiratory acidosis are signs of a very poor prognosis despite intensive efforts to rapidly lower the blast count

Figure 53-3 Pulmonary leukostasis in a patient with monoblastic leukemia and a presenting WBC count of 150,000/mm ^{3} .

and institution of ventilatory support. Pulmonary leukostasis and the hyperleukocytosis syndrome are more common in patients with one of the monocytic subtypes and the microgranular variant of acute promyelocytic leukemia.[218]

Pulmonary hemorrhage and leukostasis may mimic the signs and symptoms of a bacterial or fungal pneumonia. Pulmonary hemorrhage may be diffuse, involving both lungs, or localized to a single segment or lobe. Patients typically complain of the sudden onset of shortness of breath. Hemoptysis may occur, and hypoxemia and hypercapnia are common. The presentation may be similar to other forms of noncardiogenic pulmonary edema.[219] On physical examination there may be few objective pulmonary findings, although the chest radiograph typically reveals an interstitial pattern in the area of the hemorrhage. The use of high-resolution CT scan may help in differentiating fungal infections from other causes of respiratory failure.[220] The prognosis of patients with AML and respiratory failure is poor.[221] Progressive pulmonary infiltrates caused by either leukostasis or pulmonary hemorrhage are associated with a poor prognosis.[222] The treatment of suspected

pulmonary hemorrhage should be directed at correcting the underlying coagulopathy.

Cardiovascular abnormalities are usually a result of derangements in metabolic, electrolyte, and pulmonary function. Leukemic infiltration of the heart or great vessels is rare, but there are reports of leukemic involvement of the conduction system, pericardium, and myocardium, as well as involvement of the arterial endothelial wall with monoblasts and subsequent formation and rupture of an aortic aneurysm.[223] [224] Chemotherapy-related toxicities produce the majority of the cardiovascular problems in patients with AML. The cardiotoxicity of the anthracyclines is dose-related, and these agents act synergistically with other cardiotoxic agents. Cardiac function should be assessed before beginning therapy. The left and right ventricle ejection fractions should be measured in all patients who have received a known cardiotoxic drug or who have a history of cardiac disease before they are given an anthracycline. An echocardiogram may be more helpful in assessing wall motion abnormalities. A pretherapy baseline ejection fraction or echocardiogram is not, however, warranted for all patients before starting induction therapy. Unless the patient has a known or suspected cardiac disease that would alter the chemotherapy regimen, treatment need not be delayed pending the result of ejection fraction.

Gastrointestinal abnormalities are frequent in AML patients. Dysphagia is common and is usually a result of oral or pharyngeal infections, mucosal involvement with leukemia, or chemotherapy-induced mucositis. Oral candidiasis is a common presenting finding and may involve the tongue and/or the soft or hard palate. Esophageal candidiasis can produce substernal pain and a midepigastric burning sensation, and dysphagia is common with oral and esophageal candidiasis. These symptoms may be severe and interfere with the patients ability to eat and to take oral medications. A barium swallow is usually sufficient to confirm the diagnosis of candida esophagitis. For patients who fail treatment, atypical cases, or cases in which the barium swallow is not diagnostic, an upper endoscopy is helpful. Obtaining a culture at the time of endoscopy is important. The clinical appearance of the lesions may be deceptive, and mixed fungal and viral infections are common. [225] [226] Candida infections can obscure an underlying herpes simplex or cytomegalovirus infection, and herpes simplex virus reactivation frequently occurs during therapy for AML. Viral lesions may be atypical, and superinfection with bacteria or fungi is common. Viral cultures for herpes simplex should be obtained on all atypical lesions in and around the oral cavity. Serologic viral studies are usually not helpful, since the majority (>80%) of patients will have had prior exposure to the herpes virus and have a positive serology. Patients with microbiologically documented or clinically suspected herpes simplex virus infection should be treated with acyclovir while undergoing induction therapy. Gingival involvement can occur in any of the subtypes of AML. Gingival hypertrophy is most frequent in the well-differentiated monocytic types. Gastrointestinal involvement with AML is rare, but chloromas or granulocytic sarcomas can form in the esophagus and small intestine and produce obstructive symptoms.[227] [228] [229]

In patients with leukemia the anal and perirectal areas are important potential sources for infection, the first signs of which may be induration and tenderness in the perirectal area without other signs of inflammation or infection. Patients may initially only complain of pain on defecation and diffuse anal tenderness. It is important to recognize and treat these potential sources of infection early. Perirectal abscesses are usually due to gram-negative bacteria and in the setting of granulocytopenia can rapidly progress to perirectal cellulitis and septicemia. While digital rectal examinations are generally avoided in patients who are granulocytopenic, the perirectal area should be carefully and gently examined.

Patients should be instructed on the importance of perirectal hygiene. Constipation should be avoided in order to prevent small mucosal tears. Diarrhea and drugs or agents that cause diarrhea should be carefully monitored and the use of harsh laxatives or agents that cause prolonged diarrhea should be avoided. The use of contrast agents that are cathartics must be critically evaluated before their use in patients with AML. The perirectal and oral area are two important portals for infection, and patients should be to instructed on how to perform daily oral and perirectal care.

The use of more intensive chemotherapy regimens has resulted in an increase in the incidence of gastrointestinal complications. [230] The use of broad-spectrum antibiotics and mucosal damage from chemotherapy has increased the incidence of fungal infections originating in the gastrointestinal tract. [231]

Typhlitis, a fulminant necrotizing colitis related to granulocytopenia and cytotoxic therapy, occurs in up to 10% of patients with leukemia who are undergoing intensive therapy.[232] This entity may present a diagnostic and therapeutic dilemma. Patients present with the sudden onset of abdominal pain, fever, and a distended and tense abdomen. Bowel sounds are decreased, and abdominal radiographs are nonspecific, usually revealing an incomplete small bowel obstruction, a questionable right lower quadrant mass, pneumatosis, or no appreciable abnormality. CT scanning frequently demonstrates an edematous

right colon with spiculation of the pericolic fat and subcutaneous edema. The clinical presentation frequently mimics that of acute appendicitis. The diagnosis of typhlitis is usually made on clinical findings. The CT scan may be helpful in differentiating appendicitis from other causes of abdominal pain and fever. [233]

Treatment of typhlitis is controversial, and for many patients the prognosis is poor. [234] [235] [236] [237] Medical management includes bowel rest, broad-spectrum antibiotics directed at bowel pathogens including anaerobes and fungi, intravenous fluid replacement, total parental nutrition, and transfusion support. [230] Surgical intervention, usually a hemicolectomy, should be reserved for patients with localizing peritoneal signs suggesting an abscess or clear evidence of perforation, as well as for those who do not respond to medical therapy. Patients who are neutropenic and thrombocytopenic can tolerate an exploratory laparotomy, but their postoperative course is more complicated.[236] The morbidity and mortality of surgery must be carefully weighed against the risk of medical therapy and support therapy in the individual patient. A majority of the patients with typhlitis can be managed medically with intensive supportive therapy and antibiotics, with only a minority of patients requiring surgical intervention.

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DIAGNOSIS

The presumptive diagnosis of acute leukemia is usually apparent after examining the patient and reviewing the blood smear. Most patients present with pancytopenia and circulating blast forms, which are apparent on the peripheral blood smear. The total white blood count may range from less than 1,000/mm ^{3} to more than 200,000/mm^3 , with the majority of patients having a total white blood count between 5,000 and 30,000/mm ^{3} . The blood smear is usually sufficient to make the presumptive diagnosis of AML, but in the 10% of patients who may present with only a modest thrombocytopenia, low-grade anemia, and a normal WBC without circulating blast forms, a bone marrow examination is required to make the diagnosis. The bone marrow aspiration and biopsy should be performed at a time when the necessary pretherapy studies can be obtained. While many patients and physicians feel compelled to start treatment for AML immediately, in most instances emergency therapy is not needed. Treatment can usually be delayed until the necessary clinical and laboratory evaluations are available. Patients may also need time to accept the diagnosis and address personal, financial, and family needs. As part of the initial evaluation the psychological and emotional needs and concerns of patients and their families must be considered. Moreover, prior medical and dental problems and intercurrent illnesses need to be evaluated or treated before starting induction chemotherapy.

Even in the severely neutropenic and thrombocytopenic patient, a bone marrow biopsy and aspiration can be safely performed. Local bleeding or infection at the site of the procedure is very rare. The posterior iliac crest is the preferred site unless the patient has received prior radiation therapy to the pelvis or has evidence of an active infection at the site. The sternum is an alternate site for performing a bone marrow aspirate. A bone marrow biopsy cannot be performed by the sternal route.

Bone marrow smears from the aspirate and touch preparations from the biopsy should be prepared at the bedside for best results. Slides should be made for Wright/Giemsa and cytochemical stains. Cytogenetic studies are important prognostic indicators and should be performed at the time of the initial bone marrow aspiration and biopsy. Cell surface markers, molecular studies, and enzyme studies are helpful if there is a possibility that a lymphoid or a biphenotypic leukemia may be present. The bone marrow aspirate allows the qualitative assessment of bone marrow cell morphology. The bone marrow biopsy allows quantitative assessment of bone marrow cellularity, megakaryocyte number, and reticulin fibrosis and should be performed on all patients. [238] When insufficient aspirate material is available, circulating blasts in the blood may be used for cytogenetic studies, surface markers, and cytochemical stains. Immunohistochemical stains may be performed on the biopsy specimen to aid in the diagnosis, especially when the marrow smear or imprint material is scant or absent.

The bone marrow aspirate should be evaluated for cellularity, number and morphology of megakaryocytes, myeloid to erythroid ratio (M:E), cellular maturation, and the presence of dysplasia or asynchronous maturation. The blast percentage should be determined on at least a 200-cell differential of the bone marrow aspirate. Iron stores should be assessed with the Prussian blue stain, and the presence or absence of ringed sideroblasts specifically noted. Evaluation of the cellularity of a bone marrow aspirate is a qualitative assessment and may reflect the number of spicules obtained, slide preparation technique, and volume of diluting blood. Despite these limitations, a number of criteria have been adopted by cooperative groups to evaluate the cellularity of a bone marrow aspirate. Marrow aspirate cellularity is grouped into five broad categories, from 0 to +4: aplastic, hypocellular, normal cellular, hypercellular, and intensely hypercellular.

The initial bone marrow in a patient with AML is typically hypercellular, with absent or decreased megakaryocytes. However, elderly patients or those with secondary or treatment-related AML may have a normal cellular or hypocellular bone marrow. [239] Dysplastic myeloid and erythroid maturation may be noted. Myeloid precursors may be morphologically bizarre with asynchronous granulation. The prognostic significance of dysplasia in de novo AML is controversial. [240] [241] Trilineage dysplasia is associated with other poor prognostic features including unfavorable chromosomal abnormalities. Trilineage dysplasis appear to be a marker for a high-risk goup of patients and is associated with a less-favorable response to treatment. [242] [243] In addition, prominent dysplasia may suggest prior exposure to hematotoxins or that the patients leukemia evolved from a prior myelodysplastic syndrome or is secondary to prior cytotoxic chemotherapy. Features of dysplasia at presentation are not a negative prognostic feature unless there are other features to suggest a prior myelodysplastic syndrome. [244] Auer bodies or rods, which are reddish rodlike filaments of aggregated primary granules, may be present in the leukemic cells. These bodies, or rods, first described by Joseph Auer, are derived from incorporation of primary azurophilic granules into autophagic vacuoles. The term *phi bodies* has also been used to describe small spindle-shaped Auer bodies, and single cells containing multiple Auer bodies are sometimes referred to as *faggot cells* (from the term meaning bundle of sticks). Auer rods are faintly birefringent in polarized light. Ultrastructurally they have a defined three-dimensional crystal structure with a characteristic 6-nm to 13-nm periodicity, which is different for each of the leukemic subtypes.[245] Auer rods have been considered virtually pathognomonic of AML. The French-American-British Cooperative Group, however, does not place this degree of importance on the Auer rods and in fact includes the presence of Auer rods as part of the definition of one of the myelodysplastic syndromes, which it terms refractory anemia with excess blasts in transformation. [246] [247] Auer rods should therefore not be considered as unequivocal evidence of AML but as a manifestation of a myeloid malignant disorder with abnormal maturation. Auer rods are found in approximately 50% of newly diagnosed patients with AML and are found most frequently in the M1, M2, M3, and M4 subtypes.[247] The M2 subtype has characteristically thin and elongated Auer rods which may help in the diagnosis of this generally favorable subtype. The remission rate and duration of remission may be higher in patients whose leukemic cells demonstrate Auer rods. [248]

The number of blasts in the blood and bone marrow is the defining criterion to distinguish myelodysplastic syndromes

Figure 53-4 Definition of acute myeloid leukemia. TNC, total nucleated cells; NEC, nonerythroid cells.

from acute leukemia as well as subclassifying the acute leukemias. The diagnosis of acute leukemia requires that at least 30% of either total nucleated cells or nonerythroid cells in the bone marrow are blast forms[247] (Fig. 53-4). Blast cells must be distinguished from promyelocytes in the bone marrow count because the latter are included with differentiated granulocytes. The determination of the number of blasts in the bone marrow is crucial and the distinction between a blast and a promyelocyte can be difficult. The French, American, British Group (FAB) has recognized three types of myeloblasts [249] (Table 53-1). The type I blasts lack granules, have open chromatin, a high nucleocytoplasmic ratio, and prominent nucleoli. The type II blasts have a granulated basophilic cytoplasm with few (up to 20) azurophilic granules and nuclear features similar to the type I blasts except for the presence of azurophilic granules and a lower nucleocytoplasmic ratio. These type II blasts can be confused with early promyelocytes. Type III blasts have a heavily granulated basophilic cytoplasm with numerous azurophilic granules and nuclear features similar to Type I myeloblasts. Myeloid precursors are classified as promyelocytes when they have moderately basophilic cytoplasm with numerous azurophilic granules and an eccentric nucleus. The promyelocytes has a defined perinuclear region of pallor or clear Golgi zone, nucleoli, and a low nuclear-cytoplasmic ratio. [247] In a patient with acute leukemia the most important initial morphologic evaluation is to distinguish between AML, ALL, or one of the myelodysplastic syndromes. The prognosis and therapeutic strategies remain very different for adults with these disorders. In most cases the morphologic evaluation and cytochemical stains will define the appropriate lineage. It may, however, be difficult to differentiate between one of the myelodysplastic syndromes, refractory anemia with excess blasts in transformation, and AML. The myelodysplastic syndromes are most frequently confused with the M6 variant, acute erythroleukemia, or a hypoplastic AML. [250] In

TABLE 53-1 -- Blast Types in Acute Leukemia

Type	Description
Type I myeloblast	Agranular basophilic cytoplasm, fine structured chromatin, and two to four distinct nucleoli.
Type II myeloblast	Granulated basophilic cytoplasm with few (20) azurophilic granules, and nuclear features similar to type I blasts.
Type III myeloblast	Heavily granulated basophilic cytoplasm with numerous (>20) azurophilic granules, and nuclear features similar to type I blasts.
Promyelocyte[a]	Large with a lower nuclear-cytoplasmic ratio than myeloblasts, moderately basophilic cytoplasm with numerous azurophilic granules, an eccentric nucleus with some chromatin clumping, distinct nucleoli, and a perinuclear region of pallor or Golgi region.

[a]For the purposes of diagnosing acute leukemia, promyelocytes are included in the count of differentiating granulocytes. [247] [253]

TABLE 53-2 -- Laboratory Differential Diagnosis of Subtypes of AML

Subtype	Differential Diagnosis
AML-M0	ALL-L2, AML-M5A, AML-M7
AML-M1	ALL-L2, AML-M5A, AML-M7
AML-M2	AML-M1, AML-M4, AML-M6, RAEB-T, agranulocytosis, leukemoid reaction
AML-M3	AML-M5B, AML-M4, AML-M2, agranulocytosis
AML-M4	AML-M2, AML-M5B, CMML in transformation
AML-M5A	AML-M3V, AML-M4
AML-M5B	AML-M3v, AML-M4
AML-M6	AML-M2, AML-M5A and AML-M1, AML-M7, ALL; L2
AML-M7	AML-M1, AML-M5A, AML-M0, ALL-L1, ALL-L2, myelofibrosis, agranulocytosis

cases in which the nucleated erythroid cells constitute greater than 50% of all bone marrow nucleated marrow cells the diagnosis may be difficult. In these cases, if the combined total of all blasts, type I, II, and III is less than 30% the case is classified as a myelodysplastic syndrome. [247] If the nucleated red cells comprise less than 50% of bone marrow cells the percent of blasts is calculated as the percent of all nucleated cells. In many cases the difference between one of the myelodysplastic syndromes and AML can represent a difficult diagnostic problem (Table 53-2; Fig. 53-4). Cytogenetic studies may help define specific abnormalities associated with AML or MDS. In some cases, the morphologic distinction between an undifferentiated myeloblastic leukemia and a lymphoblastic leukemia can be difficult. The acute myeloblastic leukemias with no or minimal differentiation, the monoblastic leukemias without differentiation, and some of the acute megakaryocytic leukemias can be difficult to differentiate from ALL by morphology or cytochemical stains alone. In these instances the use of monoclonal antibody markers for lineage-associated markers is important. When the blasts demonstrate less than 3% positivity with either myeloperoxidase or Sudan black stain, immunologic markers are needed to define the lineage of the leukemia. In the majority of cases of acute leukemia, morphology and cytochemistry are sufficient to assign the correct lineage. In approximately 15% of cases, however, the distinction between an immature AML variant and ALL can not be made morphologically. The use of monoclonal antibodies that identify myeloid and lymphoid associated antigens is very useful in these cases [251] [252] (Table 53-3). A number of monoclonal antibodies have been generated that react with specific antigens expressed on the surface of normal and leukemic myeloid and lymphoid cells. To identify AML the percentage of positive reacting blasts should be greater than 20%, with one or more of the myeloid-associated antigens, CD33, or CD14. [253] [254] The determination of cellular terminal deoxynucleotidyl transferase (TdT) activity, in combination with other markers of lymphoid differentiation may be useful

TABLE 53-3 -- Monoclonal Antibodies Commonly Used to Distinguish AML from ALL

AML	ALL
CD11 (Anti Mo1)	CD10 (CALLA)
CD13 (MY7)	CD2 (T11, Leu 5)
CD14 (Anti MY4)	CD4 (T4, Leu 3)
CD15 (VIM-D5)	CD5 (Leu 1)
CD33 (MY 9)	CD3 (T8, Leu 2)
CD41	CD19 (Anti B4)
CD61	CD20 (Anti B1)

CD, cluster designation.

in selected cases.[255] However, TdT activity is not lineage-specific and can be found in 1550% of myeloblasts. [256]

In ALL the immunologic markers are important in assigning cell lineage, defining leukemic specific subsets, and assessing prognosis. In AML biochemical and immunologic markers have been less widely applied. The expression of these antibodies corresponds to the normal stages of myeloid and monocytic differentiation. None of the currently available myeloid monoclonal antibodies identifies leukemia-specific determinants. The monoclonal antibodies have been useful tools for defining the maturation and differentiation of normal myeloid and monocytic precursors. [257] However, leukemic cells frequently express markers of multiple levels of maturation and different lineages. Therefore, unlike morphologic classifications that attempt to place the predominant cell type within a specific defined group, immunophenotyping marker studies have demonstrated that AML cells are antigenically and morphologically heterogeneous. [258] [259] Immunophenotyping has been most useful in distinguishing between AML and lymphoid leukemias and in defining hybrid or biphenotypic leukemias. In ALL the immunophenotyping studies have defined functionally and prognostically relevant subgroups that are unrelated to both morphology and cytochemistry. Although a number of the individual antibodies correlate well with the morphologic classification, as in the expression of CD34 with M0 and M1, but not M3; CD14 (MY4) in the M4 and M5 subtypes; and the absence of HLA DR in the M3 subtype, the overall expression of surface antigens on myeloblasts does not entirely agree with either morphology or cytochemical staining.[260] [261] [262] The use of multiple monoclonal antibodies has identified certain phenotypic groups that may be clinically important, such as the association of the M2 subtype a t(8;21) cytogenetic abnormality, the expression of CD34 and the B-cellassociated cell surface antigen CD19 the expression of CD33, but not CD34, in M3 leukemias[263] [264] (Table 53-4). The use of immunophenotyping markers has demonstrated that the majority of myeloid blast cells express differentiation markers asynchronously and that the unusual coexpression of normal differentiation antigens is common. Moreover myeloid blasts frequently express lymphoid associated antigens. A meaningful proportion, 2045%, of myeloid blasts express lymphoid-associated antigens, most frequently CD2, CD7, and CD19. [265] The CD2 antigen, known as the sheep erythrocyte receptor, which is characteristic of mature T cells, is expressed on approximately 30% of defined myeloid leukemias. CD7, a T-cell marker, is found in up to 32% of AML blasts.[263] [265] [266] The CD19, found on B cells, is frequently noted in association with the 8;21 translocation in M2-AML, and is found in up to 34% of newly diagnosed myeloid leukemias.[263] [265] [266] The myeloid blast cells frequently express both myeloid and lymphoid differentiation antigens. The prognostic significance of this phenotypic heterogeneity is unclear, but is not associated with a uniformly adverse prognosis. [258] [259] [260] [267] [268] The use of immunophenotyping is particularly important for the identification of AML with minimal differentiation (M0), erythroleukemia (M6) and megakaryoblastic leukemia (M7).[269] [270] In addition, immunophenotyping may be helpful for detecting minimal residual disease following intensive chemotherapy. [267] The role of immunophenotyping to identify high-risk patients remains controversial. [262] [265] [271] Immunophenotyping may help in identifying subsets of patients who are at risk for shorter remission durations and resistant disease such as CD34+ AML, but the role of immunophenotyping as a independent prognostic indicator is unclear. At the present time the use of immunophenotyping in the diagnosis of AML is most appropriate for the evaluation of morphologically atypical or undifferentiated leukemias or

suspected hybrid leukemias. CD13 and CD33 are the most useful markers to identify myeloid leukemias. [271] Immunophenotyping has demonstrated the phenotypic heterogeneity and mixed lineage differentiation of many myeloid leukemias. [272]

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USE OF CYTOCHEMICAL STAINS IN THE EVALUATION OF AML

The acute leukemias are classified according to the predominant neoplastic cell type. The FAB Cooperative Group in 1976 introduced a classification system for the AML subtypes.[273]

The initially proposed classification system was based solely on morphology and cytochemical stains and did not include the use of cytogenetics, molecular markers, or immunophenotyping markers. The FAB has periodically updated its proposed classification system to incorporate these additional studies. The recommendations of the FAB group have gained wide acceptance and are now used to classify AML in most cooperative groups, cancer centers, and international workshops involved in clinical trials and studies on AML.

The FAB group divides the myeloid leukemias into eight broad categories based upon the morphology, cytochemical staining, and immunological phenotype of the predominant cell type (Table 53-5 and Plates 53-4 , 53-5 , 53-6 , 53-7 , 53-8 , 53-9 , 53-10 , 53-11 , 53-12 , 53-13 , and 53-14). The classification system was initially proposed solely to define morphologically the subtypes of the acute leukemias. However, it has subsequently been expanded to include ultrastructural morphology, cytogenetics, immunophenotyping, and immunohistochemistry markers. This classification system has been shown to be both clinically and prognostically useful. The FAB criteria are based on a Wright-Giemsa stained blood smear and the bone marrow aspirate or biopsy. Four basic histochemical stains are employed, including periodic acid-Schiff (PAS), reagent Sudan black, peroxidase, and esterase (specific and nonspecific) (Table 53-6).

PAS stains carbohydrates, including monosaccharides, polysaccharides, mucoproteins, and phosphorylated sugars. Myeloblasts

TABLE 53-4 -- Immunophenotypic Markers in AML

FAB Subtype									
Marker	**M0**	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**ALL**
HLA-DR	++	++	++		++	++	+	+	+
CD 11b	+	+	+		++	++			
CD13	+	++	++	++	++	++			
CD14		+	+		++	++			
CD15			+	+	+	±			
CD33	+	++	+++	+++	+++	+++	+	+	
CD41, CD61								+++	
Glycophorin A							++		
TDT	++	+							++
CD34	++	+						+	+

, <25% cells positive; +, 2550% cells positive; ++, 5075% cells positive; +++, >75% cells positive; ALL, acute lymphoblastic leukemia.

TABLE 53-5 -- Classification and Incidence of Subtypes of AML

Cell Line	Classification		Subtype	Incidence
Myeloid	AML	M0	Acute myeloblastic leukemia, minimal differentiated	35%
		M1	Acute myeloblastic leukemia, without maturation	1520%
		M2	Acute myeloblastic leukemia, with maturation	2530%
	APL	M3	Acute promyelocytic leukemia, hypergranular	510%
		M3v	Acute promyelocytic leukemia, variant, microgranular	20%
Myeloid and monocytic	AMML	M4	Acute myelomonocytic leukemia	2030%
		M4e0	Acute myelomonocytic leukemia with eosinophilia	1530%
Monocytic	AML	M5a	Acute monoblastic leukemia, poorly differentiated	29%
		M5b	Acute monocytic leukemia, differentiated	
Erythroid and myeloid	AEL	M6	Acute erythroleukemia	35%
Megakaryocytic		M7	Acute megakaryoblastic leukemia	312%

are PAS-negative, while a majority of lymphoblasts are PAS-positive with a distinctive pattern. PAS-positive lymphoblasts usually demonstrate a concentric ring of coarse granules or heavy blocks against a negative cytoplasmic background. Because not all lymphoblasts on a bone marrow sample will stain with PAS, it is the pattern of the staining rather than the number of positive cells that suggests the diagnosis of ALL. Promyelocytes show a faint diffuse PAS-positive tinge, often with fine cytoplasmic granules; Auer rods are usually negative; and monoblasts stain variably with PAS, resulting in a diffuse and granular pattern. Immature monoblasts are usually PAS-negative, while more differentiated monoblasts and monocytes may react as do normal mature monocytes with a diffuse reddish background superimposed on fine or coarse granules. Erythroblasts generally demonstrate strong PAS-positivity, with a diffusely coarse granular reaction. Maturing erythrocytes may also show heavy PAS-positivity, with concentric annular rings of moderately coarse cytoplasmic granules. Megakaryoblasts stain variably with PAS; the mature megakaryocyte is positive but the megakaryoblast may be negative, and therefore the PAS stain is not a reliable marker for this subtype. The PAS stain is most useful for differentiating a lymphoid from a nonlymphoid leukemia and less helpful in defining the AML subtype.

Sudan black B is a lipophilic dye, which stains phospholipids and lipoprotein complexes. The Sudan black staining pattern is similar to the profile of the peroxidase reactivity, but the reaction is usually more strongly positive in myeloblasts and shows a different distribution from the peroxidase reaction in monocytes and monoblasts. Myeloblasts, promyelocytes, and myelomonocytes

TABLE 53-6 -- Cytochemical Stains in Acute Leukemia

Leukemia	Myeloperoxidase or Sudan Black B	Nonspecific Esterase (NSE)
ALL		±[a]
AML		
M0	±	
M1	+(>3% blasts)	
M2	++	
M3	+++	±[b]
M4	+(granulocytes)	+(monocytes)
M5		++[c]
M6	+(myeloblasts)	
M7		±[d]

[a]ALL may show granular staining that differs from the diffuse staining of monoblasts and fails to inhibit with sodium fluoride.
[b]In 1520% of cases of APL, some atypical promyelocytes stain weakly for NSE.
[c]In 2025% of cases of monoblastic leukemia, monoblasts are weakly reactive or nonreactive for NSE.
[d]Megakaryocytes/megakaryoblasts may show weak focal positivity for NSE.

are positive while erythroid, megakaryocyte, and lymphoid precursors are negative. Myeloblasts are usually positive, with coarse heavy granules. Myeloblasts without azurophilic or specific granules on Wright-Giemsa stain may stain with Sudan black, and the myelomonocytic leukemias demonstrate both coarse and fine granules. Pure monoblasts are usually Sudan blacknegative. Auer rods stain prominently with Sudan black even when not apparent on the standard Wrights preparation. In general, the Sudan black B stain represents a sensitive marker of myeloblasts and myelomonocytes. It is most helpful in differentiating immature cells that appear undifferentiated on smear and give a negative peroxidase reaction.

The esterase stains are biochemically complex and based on the reaction of a specific substrate with a cellular enzyme. The nomenclature is confusing and depends on the substrate used. In hematopoietic cells nine esterase isoenzymes have been identified, all of which cleave the alpha-naphthyl acetate or alpha-naphthyl butyrate substrate; this activity is referred to as nonspecific esterase activity. The substrate in the chloroacetate esterase stains is the chloroacetate of alpha-naphthol or naphthol AS; which react with enzymes peculiar to the myeloid series at all stages of maturation, and the reaction is therefore referred to as a specific esterase reaction.

The chloroacetate esterase reaction gives sharply localized granular staining in the cytoplasm of nearly all granulocytes and mast cells. The reactivity of this stain increases with cell maturity, and myeloblasts without granules are specific esterase-negative. Thus, this reaction is less sensitive than the peroxidase or Sudan black stain for the diagnosis of acute myeloid leukemia without maturation AML (M1). The chloroacetate stain is intensively positive in promyelocytic leukemia (M3), but monoblasts, lymphoblasts, erythroblasts, megakaryocytes, eosinophils, basophils, and monocytes are chloroacetate esterase negative.

The nonspecific esterase stain has a markedly different spectrum of reactivity; the naphthyl acetate and butyrate reactions are largely confined to cells of the monocytic lineage, including monoblasts and monocytes. Monocytes contain two isoenzymes that react strongly with the nonspecific esterase substrate but are dramatically inhibited by addition of sodium fluoride. Therefore, a positive esterase activity that is inhibited by sodium fluoride is characteristic of monocytic differentiation. The two subclasses of the monocytic leukemias, the M5a undifferentiated and the M5b differentiated types, react with the nonspecific esterase stain. Neutrophil precursors and B lymphocytes are nonspecific esterase-negative, and other cells may show varying patterns of positivity that are not inhibited by sodium fluoride. Lymphoblastic leukemias may show a granular nonspecific esterase activity that is resistant to fluoride inhibition. The nonspecific esterase reaction aids in recognizing a monocytic component to the leukemia.

THE USE OF MORPHOLOGY, CYTOCHEMISTRY, AND IMMUNOPHENOTYPING TO DEFINE ACUTE LEUKEMIA

Morphology, cytochemistry, and immunophenotyping are used to define the lineage of acute leukemias. The blast cells in a Wright-Giemsa stained bone marrow aspirate demonstrate either no definable myeloid differentiation or features of myeloid differentiation. The blast cells of the undifferentiated myeloid leukemia (M0), minimally differentiated myeloid leukemia (M1), undifferentiated monocytic leukemia (M5a) and megakaryocytic leukemia (M7) cannot be differentiated from an acute lymphoid leukemia by morphology alone. Immunophenotyping and additional cytochemical stains are required to define the AML subtype. Immunophenotyping is most useful for differentiating a myeloid versus a lymphoid leukemia. Myeloblasts are further characterized by myeloperoxidase (MPO) or Sudan black (SBB) cytochemical staining. The nonspecific esterase reactions are helpful in defining the myeloid leukemias with monocytic features. Ultrastructural analysis and ultrastructural cytochemistry are helpful in defining the M7 and M0 AML subtypes. The M7 subtype is also CD41 positive and stains with Factor VIII ag.

The differentiated myeloid leukemias (M3M6) can usually be defined on the basis of morphology and cytochemistry. The M3 and M3 variant subtype must be confirmed with cytogenetic analysis demonstrating the 15;17 translocation. Sudan black B and myeloperoxidase stains are positive in M2, M6, and M3 subtypes and are differentiated from the M4 and M5b leukemic subtypes by the NSE reaction. The esterase reactions are helpful in defining one of the monocytic subtypes, including M4, M5a, and M5b.

FVIIIag, factor VIII antigen; NSE, nonspecific esterase; PAS, periodic acid-Schiff stain; PPO, platelet peroxidase; CD41, platelet glycoprotein IIb/IIIA.

In the myeloblastic leukemias the specific esterase stain is positive and the nonspecific esterase is negative. Auer rods are positive with the specific esterase. In myelomonocytic leukemia at least 20% of the cells must show monocytic differentiation that is nonspecific esterase-positive and is inhibited by sodium fluoride. In this type of leukemia both activities may be present in the same cell. In the pure monocytic leukemias, 80100% of the blasts are nonspecific esterase-positive and specific esterase-negative. Therefore, the esterase stains are particularly useful in distinguishing an undifferentiated monocytic leukemia (M5b) from undifferentiated myeloblastic (M1,M0) or undifferentiated lymphoid leukemia.

SUBTYPES OF ACUTE MYELOBLASTIC LEUKEMIA

The FAB group has defined eight subtypes of acute nonlymphocytic leukemia (see Tables 53-5 and 53-7 and Plates 53-4 , 53-5 , 53-6 , 53-7 , 53-8 , 53-9 , 53-10 , 53-11 , 53-12 , 53-13 , and 53-14) on the basis of morphologic, immunophenotyping, and cytochemical criteria. The FAB groups purpose was to subdivide the myeloid leukemias according to their predominant cell type and to define the leukemic cells position in the maturation sequence of that specific lineage (Table 53-5). The classification attempts to assign a single lineage to each leukemia, however, cases demonstrate some admixture of cell lines and express features of multiple lineages. Some cases of AML cannot be classified according to the FAB criteria. These unclassifible cases are more frequent among patients with secondary leukemia and represent approximately 25% of all cases. [274] [275] The FAB classification

TABLE 53-7 -- Summary of Diagnostic Features of AMLs

FAB Type	Diagnostic Features
AML-M0	30% blasts; >3% blasts reactive to MPO, SBB, or NSE; immunophenotyping CD33+, CD13+, may be CD34+, TdT+
AML-M1	30% blasts; 3% blasts reactive to MPO or SBB; <10% of marrow nucleated cells are promyelocytes or more mature neutrophils
AML-M2	30% blasts; 3% blasts reactive for MPO or SBB; 10% of marrow nucleated cells are promyelocytes or more mature neutrophils; t(8:21) chromosome abnormality
AML-M3	30% blasts and abnormal promyelocytes; intense MPO and SBB reactivity; promyelocytes and blasts with multiple Auer rods (faggot cells); t(15:17) cytogenetic abnormality
AML-M4	30% myeloblasts, monoblasts, and promonocytes; 20% monocytic cells in marrow; 5×10^9 /L monocytic cells in blood: 20% neutrophils and precursors in marrow; monocytic cells reactive for NSE; abnormal eosinophils in M4 with associated Inv(16) chromosome abnormality
AML-M5a	80% monocytic cells; monoblasts 80% of monocytic cells; monoblasts and promonocytes NSE positive monoblasts usually MPO and SBB negative
AML-M5b	80% monocytic cells; monoblasts 80% of monocytic cells; promonocytes predominate; monoblasts and promonocytes NSE positive; promonocytes may have scattered MPO- and SBB-positive granules
AML-M6	50% erythroid precursors: 30% of nonerythroid precursors are myeloblasts; Auer rods may be present in myeloblasts: dysplastic erythroid precursors frequently PAS positive
AML-M7	30% blasts; 50% cells megakaryoblasts by morphology or electron microscopy; immunophenotyping CD41+, CD61+

CD, cluster designation; MPO, myeloperoxidase; NSE, nonspecific esterase; PAS, periodic acid-Schiff; SBB, sudan black B; TdT, terminal deoxynucleotidyl transferase.

does not include hybrid or biphenotypic leukemias that cannot be classified on the basis of morphology or cytochemistry alone. The FAB classification did not consider clinical characteristics, cytogenetic patterns, response to therapy, or prognosis in its formulation. [276] Other classification systems have been proposed in an attempt to address these issues, but despite its limitations, the FAB classification has been widely adopted and is used almost universally. The classification system does try to take into account the heterogenous nature of AML and recognizes that it is not a single disease but a group of disorders affecting the hematopoietic precursors in the bone marrow.[277]

The FAB group defines five basic classes of AML based on the predominant cell type: (1) myeloid, (2) myeloidmonocytic, (3) monocytic, (4) erythroid, and (5) megakaryocytic (Table 53-5). In each of these classes a number of subtypes are defined that reflect the degree of differentiation or unique morphologic findings. In the myeloid cell type four subsets of myeloblastic leukemia are recognized, based on the percentage of maturing cells beyond the myeloblast stage. These range from the myeloblastic leukemia without morphologic evidence of maturation (M0-AML with minimal differentiation) to the promyelocytic leukemia (M3) where the dominant cell is an abnormal promyelocyte. The division between each of the myeloblast subtypes M0, M1, and M2 is somewhat arbitrary and there is a spectrum of myeloid differentiation. Immunophenotyping and cytogenetics are helpful in defining the subtypes of myeloid leukemia (Table 53-7).

AML of the M0 Subtype (AML Without Differentiation or Maturation)

Myeloblastic leukemia without differentiation (M0) (Plate 53-4) constitutes approximately 3% of all cases of AML. Morphologically, this group is the most difficult to differentiate from the L2 variant of ALL (Plate 57-1). The blasts appear to be very immature and less than 3% of the blast cells are positive with myeloperoxidase or Sudan black B. Lymphoid markers are generally negative except for the enzyme terminal deoxynucleotidyl terminal transferase (TdT) which is expressed in over 60% of the cases.[278] [279] The bone marrow has greater than 30% blasts of all nucleated cells. The blasts are usually large, with fine structured chromatin and distinct, usually multiple nucleoli. The nuclear/cytoplasmic ratio is variable and cells may resemble the L2 lymphoblasts. Some evidence of trilineage dysplasia may be noted but is frequently absent as a result of the high blast count in the bone marrow. Azurophilic granules and Auer rods are absent. The blasts are identified as myeloid either by ultrastructural myeloperoxidase or immunophenotypic markers. [280] [281] The use of monoclonal antibodies to identify the myeloid lineage is essential in this subtype. The lymphoid markers characteristic of B- and T-lineage ALL, including CD10, CD19, CD24, CD3, and CD5, are negative. In about 30% of cases the blasts mark with the T-cell markers CD4 and CD7. CD4 is a marker of mature T cells that is expressed on some myeloid cells. The CD7 antigen is one of the early T-cell antigens and is usually expressed on immature T cells and prothymocytes. This subtype may mark with CD7, usually in association with TdT expression. [282] [283] Myeloid-specific monoclonal antibodies are positive, including CD13 and CD33. Other myeloid antigens may be positive but are less helpful in establishing the diagnosis. The stem cell antigen is strongly associated with AML-M0, with over 90% of M0 cases expressing CD34. [270] M0 blasts may also resemble megakaryoblasts (M7) or undifferentiated monoblasts (M5a). The use of a monoclonal antibody, anti-myeloperoxidase (anti-MPO), is useful in detecting the M0 subtype. [278] The diagnosis of the M0 subtype cannot be made on morphologic criteria alone and requires confirmation with phenotypic markers demonstrating myeloid antigens or ultrastructural cytochemistry.

AML-M0 has a high incidence of complex karyotypes and frequent involvement of chromosomes 5, 7, 8, and 13.[270] The subtype of leukemia expresses the immunophenotypic and karyotypic features of a stem cell leukemia. The M0 subtype is also generally associated with a poor prognosis. [284]

AML of the M1 Subtype (AML Without Maturation)

The M1 subtype accounts for 1520% of AML cases. The bone marrow is infiltrated with poorly differentiated blasts with rare azurophilic granules, Auer rods are rare or absent (Plate 53-5). If granules are absent, the myeloblasts resemble the lymphoblasts of the L2 variant of ALL, and cytochemical stains are necessary to distinguish these two leukemias. The peroxidase reaction is positive in more than 3% of the blast cells, and the PAS stain is negative. The bone marrow contains less than 3% promyelocytes and less than 10% maturing granulocytes. Morphologically the cells may appear pleomorphic, with irregular nuclei with an open chromatin. Some of the cells express CD7, an antigen present on early T cells. [282] [283]

AML of the M2 Subtype (AML with Maturation)

Myeloblastic leukemia with maturation (M2) (Plate 53-6) demonstrates clear evidence of maturation to and beyond the promyelocyte. It occurs in approximately 2530% of all AML patients and represents the most common subtype in most reported series. Morphologically typical promyelocytes account for between 320% of all myeloid cells. There is evidence of maturation in the myeloid series with occasional maturation to eosinophils and basophils. The bone marrow monocytic component represents less than 20% of nonerythroid cells. Auer rods are usually found, and the myeloblasts contain prominent azurophilic granules. The peroxidase and Sudan black stain are both strongly positive; the chloroacetate esterase (specific esterase) stain is positive, reflecting maturation beyond the myeloblast; and the alpha-naphthyl acetate and butyrate esterase (nonspecific esterase) stains are negative. On Wright-Giemsa stain the blasts have a higher nuclear/cytoplasmic ratio than in the M1 variant, and the nuclear chromatin is condensed with less distinct nucleoli. [285]

About half of the patients in this subtype will have a translocation involving chromosomes 8 and 21 [t(8;21)]. [286] [287] The (8;21) (q22;q22) translocation juxtaposes the AML1 gene on chromosome 21 to the ETO (for 8;21) gene on chromosome 8 which results in the production of the AML1ETO fusion protein. [288] [289] In these patients about 25% will present with splenomegaly and 20% will develop extramedullary disease during their course. [290] [291] Morphologically the 8;21 AML cells characteristically contain thin and elongated Auer rods, a basophilic cytoplasm, and indented nuclei. The marrow may have an increased number of eosinophil precursors, some of which may contain Auer rods. In a majority of the patients there is abnormal granulocytic maturation, with dysplastic granulocytes and a pseudo-Pelger-Huet abnormality. There may be dysplastic erythroid precursors but rarely dysplastic megakaryocytes. The dysplastic features are not associated with a poorer response to therapy or a change in the otherwise favorable prognosis of this subtype. [292] [293] Histochemical staining with Sudan black and peroxidase demonstrates localized densely staining clumps, usually on one side of the nucleus or in a cleft of the nucleus, in contrast to the diffuse staining seen in patients without the 8;21 translocation. This variant has a high remission induction rate after standard chemotherapy. However, the remission duration is very variable. Patients with the AMLM2 subtype with a translocation of 8;21 who present with leukocytosis have a higher frequency of extramedullary involvement and a less favorable prognosis than patients who present with this subtype without leukocytosis. [290] [293] The overall survival of patients with the 8;21 translocation, however, is generally better than the other subtypes of AML without this translocation. [287] [292]

AML of the M3 Subtype (Acute Promyelocytic Leukemia)

Acute promyelocytic leukemia (APL-M3) (Plate 53-7) is characterized by the presence of atypical promyelocytes in the bone marrow and peripheral blood. The M3 subtype accounts for 510% of all cases of AML. APL is distinguished from other subtypes of AML by its distinctive morphology, younger patient age at presentation, specific chromosomal abnormality, associated coagulopathy, and unique response to treatment with retinoic acid. There are two morphologic variants of acute promyelocytic leukemia, a hypergranular (M3) and a variant microgranular from (M3v) Plate 53-8 .[294] [295] [296] These two variants differ in their clinical presentation, prognosis, and morphologic appearance. The hypergranular form is the more common of APL, representing 75% of APL cases. In this form the cytoplasm of the promyelocyte typically demonstrates bright pink or dark purplish coarse granules with abundant Auer rods. The promyelocytes are larger than nonmalignant promyelocytes with pleomorphic nucleoli. The Sudan black and peroxidase stains are strongly positive; the PAS stain is usually negative but in some cases may show a diffuse positive tinge with fine cytoplasmic granules; the chloroacetate esterase stain is usually strongly positive. In 1520% of cases some atypical promyelocytes stain weakly for nonspecific esterase. Atypical promyelocytes account for 30% or more of the myeloid cells. Auer rods may be so numerous as to form Auer bundles.

Patients are typically younger, with a median age of 31 years, and present with a lower white blood cell count (WBC) usually in the range of 3,000 to 15,000/mm ^{3} . A majority of the patients present with a WBC less than 5,000/mm ^{3} . Approximately 25% of patients with APL present with the microgranular variant (M3v). The microgranular variant presents with a higher WBC (range 50,000200,000/mm ^{3}) and morphologically atypical promyelocytes. [295] [296] [297] [298]

Cytogenetic studies of the M3 and M3 variant demonstrate the characteristic 15;17 translocation, which is a balanced translocation from the long arm of chromosome 17 to the long arm of chromosome 15 [t(15q+;17q)]. This cytogenetic finding is diagnostic of APL. [299] The chromosome break point on chromosome 17 has been mapped to the site of the retinoic acid alpha receptor (RAR). [300] The retinoic acid receptor is a member of a family of steroid hormone nuclear receptors that are important in the regulation and control of both normal and malignant cellular differentiation and proliferation. [301] In APL the cytogenetic translocation results in a fusion of the retinoic acid receptor alpha gene on chromosome 17 and a region from chromosome 15 referred to as PML (for promyelocytic leukemia). [302] The translocation encodes for a novel DNAbinding protein, which results in the expression of an abnormal mRNA transcript for the RAR and confers a unique therapeutic sensitivity to one of its ligands, all trans-retinoic acid. Myeloid differentiation appears to be blocked by the abnormal PML-RAR fusion protein. This abnormal receptor is the target of all trans-retinoic acid treatment. [303]

Treatment of patients with APL with all trans-retinoic acid, a vitamin A derivative, results in differentiation of the leukemic cells with approximately 7085% of patients attaining a complete remission. [304] Moreover, unlike standard induction therapy, treatment with oral all trans-retinoic acid is not associated with bone marrow hypoplasia and the usual complications of cytotoxic chemotherapy. [305] All trans-retinoic acid induces leukemic cells to replicate and differentiate into cells capable of undergoing normal senescence and cell death.

Patients with APL often present with thrombocytopenia, prolongation of the thromboplastin and thrombin time, increased

levels of fibrin degradation products, and hypofibrinogenemia. The coagulation disorder in APL results from at least three distinct mechanisms: disseminated intravascular coagulation, fibrinolysis, and proteolysis. [304] [306] The disseminated intravascular coagulation (DIC) is attributed to the spontaneous or chemotherapy-associated release of a tissue factor with procoagulant activity present in the granules of the leukemic promyelocytes. [304] [307] [308] The bleeding disorder, however, cannot always be related solely to DIC and in many patients there are signs of primary fibrinolysis. [309] [310] The promyelocytic leukemic cell has both strong procoagulant activity on its cell membrane and proteolytic activity in the cytoplasmic granules, a combination that is unique to the leukemic promyelocyte and may explain the severe coagulopathy seen in some patients. [311] [312] [313] Enhanced fibrinolysis is suggested by the increase in fibrin-fibrinogen degradation products, reduced alpha 2-antiplasmin levels, and normal antithrombin III and protein C levels present in all patients at diagnosis and up to initiation of therapy. [308] [311] Plasma from patients with APL has a plasminogen activator of tissue origin. [311] The proteolysis is due to release from the leukemic cells of lysosomal neutrophilic enzymes, human leukocyte elastase, cathepsin G, and proteinase 3, which are all able to cleave fibrinogen. [295] The mechanism of the coagulopathy therefore remains unclear. [307] The coagulopathy present at diagnosis in 7085% of cases is exacerbated with treatment with cytotoxic chemotherapy. Treatment of the bleeding disorders in patients with acute promyelocytic leukemia following cytotoxic chemotherapy is also controversial with some groups using either heparin, antifibrinolytic therapy, or supportive therapy alone. [307] [308] [309] [314] [315] However, the coagulopathy rapidly improves following treatment with all trans-retinoic acid, and anticoagulation or antifibrinolytic therapy is rarely needed. [302] The prognosis for patients of this subtype is usually favorable. The use of all trans-retinoic acid with or without chemotherapy has changed the prognosis, course, and management of this subtype. The bleeding and clotting problems generally respond promptly to treatment with retinoic acid. The prognosis of patients with APL following treatment with all trans-retinoic acid and chemotherapy is very favorable. The prognostic features associated with treatment with chemotherapy alone include age >50 years, underlying renal function, pretreatment fibrinogen, and presenting WBC. [294] [295]

The microgranular variant typically presents with a very elevated WBC, frequently in excess of 100,000/mm ^{3} , and the cells have minimal rather than excessive cytoplasmic granulation. [316] [317] Auer rods are rare. The nucleus is typically irregular, folded, or bilobed and resembles the nucleus of a monocytic precursor (Plate 53-8). The cells may appear devoid of granules or may contain many or a few fine, dust-like azurophilic granules, often concentrated in one area of the cytoplasm. Cytochemical stains reveal that the cells are intensely peroxidase-positive, Sudan black-Bpositive, and chloroacetate esterasepositive. Ultrastructural examination has demonstrated that the majority of the cells contain numerous microgranules, which are smaller than 250 nm and thus below the limit of resolution of light microscopy. These patients have the typical 15;17 translocation of the standard hypergranular promyelocytic leukemia. The microgranular form can be confused morphologically with a monocytic leukemia. A minority of patients with M3v present with hyperbasophilic cytoplasm and cytoplasmic projections that may resemble megakaryoblasts. [317]

In the microgranular variant the appearance of the blasts in the peripheral blood and bone marrow may be very different. In the bone marrow some of the cells are frequently closer in appearance to those of the typical M3 hypergranular variant. [311] The very high presenting blast count and the coagulopathy associated with this variant affect the outcome of remission induction. Leukostasis and fatal central nervous system and pulmonary hemorrhage are more common in this subtype.

The immunophenotyping of promyelocytic leukemic cells is distinctive as compared to other subtypes of AML. The APL cells are usually CD34, CD14, CD11b, HLA DR, and CD33+.[318]

AML of the M4 Subtype (Acute Myelomonocytic Leukemia)

In the M4 subtype (Plate 53-9), cells have differentiating characteristics of both neutrophilic and monocytes. Patients often present with extramedullary disease, gingival hypertrophy, leukemia cutis, and meningeal leukemia are more common in this subtype than in the myeloid leukemias (M0M3). Morphologically this subtype is similar to AML-M2, but the blast count includes myeloblasts, monoblasts, and promonocytes, which together must exceed 30%. In the marrow, more than 20% of the nonerythroid cells must be of the monocytic lineage at different stages of maturation, as defined by morphological or cytochemical findings. The number of monocytic cells in the blood (including monoblasts, promonocytes, and monocytes) is greater than 5×10^9 /L. This subtype represents 2030% of all AML patients. Cytochemical stains are helpful in identifying the monocytes in the peripheral blood and bone marrow: the bone marrow typically stains with the cytochemical markers for both neutrophilic and monocytic precursors, and the leukemic blasts stain positive with peroxidase, Sudan black, and chloroacetate esterase as well as the nonspecific esterase that is inhibited by addition of sodium fluoride. Serum and urinary lysozyme levels, reflecting the monocytic component, are frequently elevated.

A variant of this subtype, called acute myelomonocytic leukemia with abnormal eosinophils (M4EO) (Plate 53-10), is characterized by the presence of myelomonocytic blasts and 530% of morphologically and cytochemically abnormal eosinophils. [313] This variant represents approximately 510% of all patients with AML or about one-third of all patients with the M4 subtype. Patients with the M4EO subtype often present with a high peripheral WBC (range 30,000100,000/mm^3) and hepatosplenomegaly. The eosinophils have monocytic nuclei with abnormal, often basophilic, granules and lack the typical eosinophil crystals. The eosinophilic granules stain positive with PAS, Sudan black, chloroacetate esterase, and nonspecific esterase, which is typical of granulocytic, but not of eosinophilic, precursors. Presumably the hybrid morphologic and histochemical features of monocytes, eosinophils, and granulocytes in these cells result from abnormal differentiation of a primitive uncommitted leukemic cell before the divergence of the three cell lines. The abnormal eosinophils are part of the leukemic cell population. [314] These cells also express the T-cell antigen, CD2. The myeloblasts are typically negative for other T-cell markers. [315] [316] The peripheral blood typically has myeloblasts and increased monocytes. The abnormal-appearing eosinophils are usually rare in the peripheral blood. Central nervous system involvement is very common in this variant. In one study, 35% of patients relapsed with leptomeningeal disease. [172]

The M4EO variant has a unique karyotypic abnormality involving the long arm of chromosome 16. All patients have an inversion of chromosome 16 between the long and short arm or a balanced translocation between two homologous chromosomes 16. The cytogenetic feature in both of these translocations is the break in the long arm of chromosome 16 at band q22.[319] [320] [321]

The overall complete response rate for patients with myelomonocytic leukemia is between 50 and 65%. However, patients with the M4EO variant have a complete response rate between 70 and 80%, which is significantly higher than the standard M4 subtype. [319] The median duration of the remission and overall survival is also significantly prolonged in the M4EO variant, compared with that in other acute myelomonocytic leukemia

cases.[320] [322] [323] It is therefore important to recognize patients with the M4EO phenotype because they appear to have a very responsive disease and may have a very favorable prognosis with currently available therapies. [322] [324]

AML of the M5 Subtype (Acute Monocytic Leukemia)

The acute monocytic subtype (Plates 53-11 and 53-12) represents 29% of all cases of AML.[325] [326] The monocytic leukemias are divided into two variants, a poorly differentiated monoblastic leukemia (M5a), and a differentiated monocytic leukemia (M5b). The FAB criteria for the diagnosis of M5 (AMoL) requires that 80% or more of the nonerythroid cells in the bone marrow be monoblasts, promonocytes, or monocytes. The blasts in the M5a variant (Plate 53-11) are poorly differentiated monoblasts, with rare granules and occasional cytoplasmic vacuoles. The poorly differentiated monoblasts are large cells with plentiful cytoplasm that may contain vacuoles and are usually basophilic. Auer rods are not usually seen. Morphologically, these cells may resemble lymphoblasts of the L2 variant. The M5a subtype classification requires that greater than 80% of the bone marrow monocytic component are monoblasts. The M5b variant (Plate 53-12) consists of more differentiated monocytes, which have the typical lobulated monocytic nucleus. Nucleoli are not always present. The cytoplasm typically is grayish blue, and Auer rods are rare. Monoblasts comprise less than 80% of the monocytic component. The diagnosis of AMoL is confirmed by the positive alpha naphthyl acetate esterase reaction. The peroxidase, Sudan black, and chloroacetate esterase stains are usually negative in the blasts, but Sudan black can be positive in the more mature monocytic precursors. The PAS reaction is variable: while most of the monocytic leukemias are PAS negative, some will stain with coarse positive granules on a background of diffuse positivity.

There are meaningful clinical differences between the two monocytic leukemias. [327] Patients with the M5a variant are younger, present with a higher blast count in the peripheral blood and bone marrow, and have an overall poorer prognosis. [325] The M5b variant has an increased incidence of gingival hypertrophy and a higher percentage of circulating monocytes. The peripheral blood may show cells of the monocytic lineage which appear to be more mature and differentiated than those found in the bone marrow. The diagnosis of this subtype is determined by the bone marrow findings. Immunocytochemical staining may help in differentiating the two monocytic leukemias. The more immature (M5a) variant is characterized by focal lysozyme staining and a negative CD68 phenotype, whereas the more differentiated monoblastic leukemia (M5b) expresses a diffusely positive lysozyme staining pattern and is positive for CD68. [328]

Extramedullary disease involving the liver, spleen, and lymph nodes is more common in the monocytic than in other AMLs occurring in up to 54% of patients. [327] [328] An elevated white blood count is seen in the majority of cases, up to 30% of patients present with a white blood count higher than 100,000/mm^3 .[328] [329] Laboratory evidence of DIC is common at presentation. Central nervous system involvement occurs in up to 22% of all patients. [329] However, prophylactic central nervous system therapy has not improved survival or prolonged remissions. [328] Gingival hyperplasia and skin involvement are frequent prominent features of the monocytic leukemias and in many patients may be the presenting complaint (Plate 53-3).[330] [331]

The response of patients with the monocytic leukemias is generally poor. The complete remission rate is between 5070%, which is comparable with that in the other types of AML. However, the duration of the complete responses and the overall survival are significantly shorter in this leukemic subtype. [329] [332] [333] This resistance to standard chemotherapy is also reflected in the higher relapse rate for patients with both variants of the monocytic leukemia following autologous and allogeneic bone marrow transplantation.[327] [334]

AML of the M6 Subtype (Acute Erythroleukemia)

Acute erythroleukemia (Plate 53-13) represents between 35% of all cases of AML. This subtype (M6) was initially described by Di Guglielmo and is still often referred to as acute Di Guglielmo syndrome. The FAB defines this subtype differently than other subtypes of AML (Fig. 53-4). The approach to counting blasts depends on the proportion of all nucleated cells that are erythroblasts. The defining criteria for acute leukemia is the same, i.e., greater than 30% blasts. This classification recognizes the continuous spectrum of the mixed proportion of cells in this leukemic subtype and parallel involvement of myeloid and monocytic precursors.

The erythroblasts are morphologically abnormal, with multilobed nuclei, multiple nucleoli, nuclear fragments, and giant pronormoblasts with megaloblastic features. The PAS stain is usually positive, with a granular or diffuse cytoplasmic pattern. The intensity of the stain and the percentage of positive-staining erythroblasts varies considerably in this subtype. Typically the cells with the most abnormal cytologic appearance demonstrate the strongest PAS reaction. However, the PAS reaction is variable, and a negative reaction does not exclude the diagnosis of erythroleukemia if the other morphologic features are present. Erythroblasts are Sudan black Bnegative, peroxidasenegative, and generally esterase-negative. However, considering the usual involvement of the myeloid line, myeloblasts with Auer rods are often present. The granulocytic component demonstrates the typical positive cytochemical staining with Sudan black, peroxidase, and chloroacetate esterase. Iron stains, such as Prussian blue, are useful in this subtype and may reveal prominent ringed sideroblasts. A monoclonal antibody that binds to glycophorin A on the

cytoplasmic membrane appears to be specific for the erythroblast.[335] Trilineage dysplasia is typical in this subtype and differentiating this subtype from an evolving myelodysplastic syndrome can be difficult.[250] In the classic cases of acute erythroleukemia a dominant fraction of erythroblasts is required. Differentiating erythroblasts (proerythroblasts and later) must comprise over 50% of all nucleated cells. Of the nonerythroid cells, 30% must be blasts and typically are myeloblasts frequently containing Auer rods.[249] The erythroid component shows varying degrees of dysplasia, which may be marked in the granulocytes and megakaryocytic lineages. Some cases of erythroleukemia may demonstrate minimal morphological erythroid differentiation and are difficult to differentiate from a undifferentiated leukemia or M0 leukemia or a myelodysplastic syndrome.[336] In these cases the bone marrow has a high fraction of proerythroblasts without a myeloblastic component or dysplastic erythroid hyperplasia. Mature erythroid precursors are absent and a majority of the cells are proerythroblasts, less than 3% of the cells are myeloperoxidase-positive blasts, and Auer rods are usually not present. The proerythroblast cells may be positive for glycophorin A and B, antigens of the AB blood group system, or CD36, which is strongly expressed on erythroid progenitors, erythroblasts, and fetal erythrocytes. In bone marrow biopsy sections immunohistochemical staining for hemoglobin A may be helpful to identify these cells.

In other rare cases dysplastic differentiating erythroblasts may account for more than 90% of the marrow cells without an increase in myeloblasts. This variant has been termed erythremic myelosis but should be considered as an erythroleukemia with a course similar to other cases of M6. [249]

Patients with erythroleukemia tend to be older at the time of diagnosis, with a mean age of over 50 in most studies. [337] [338] The presenting complaints are usually associated with the development

of anemia. The peripheral smear may have only rare circulating blast forms. Hepatomegaly and splenomegaly occur in less that 25% of patients. Some patients present with peculiar rheumatic and immunologic findings, and up to one-third will complain of diffuse joint pain and abdominal, back, and chest pain. [339] Many patients will have a positive rheumatoid factor, increased polyclonal immunoglobulins, a positive antinuclear antibody test, and a positive Coombs test. [339] The erythroleukemias, which are frequently preceded by a myelodysplastic syndrome, represent 1020% of the secondary leukemias and 35% of all de novo AML cases.[340] The preceding myelodysplastic or erythremic myelosis stage is characterized by progressive anemia often associated with intense erythroid hyperplasia in the bone marrow. The peripheral blood may demonstrate prominent basophilic stippling, with abnormal red blood cells with rare circulating blast forms. The bone marrow frequently demonstrates dysplasia of all cell lines. Patients with this subtype generally respond to therapy poorly with a short remission duration. [340] The response to treatment may reflect the increased incidence of secondary leukemias and patients with a prior MDS with this subtype.

AML of the M7 Subtype (Acute Megakaryoblastic Leukemia)

Acute megakaryocytic leukemia (M7) (Plate 53-14) is a recent addition to the FAB classification.[341] However, this is not a new entity; previous cases have been described, and many, if not all, of the previously reported cases of so-called acute myelofibrosis or malignant myelosclerosis probably represented acute megakaryocytic leukemia.[342] Acute megakaryocytic leukemia represents 312% of all cases of AML but its incidence may be higher in patients who transform from a prior myeloproliferative disorder.[343] [344] The incidence of acute megakaryocytic leukemia in patients with a prior myeloproliferative disorder, myelofibrosis, or chronic myelogenous leukemia is between 2451%.[345] [346] Megakaryoblasts are morphologically heterogeneous and vary from small round cells, resembling cells found in the L2 variant of ALL or in an undifferentiated M0 or M1 leukemia, to large atypical megakaryocytes with or without cytoplasmic granules. Binuclear or multinuclear blasts with deeply basophilic cytoplasm, cytoplasmic projections, and vacuoles are common. Undifferentiated blasts may be surrounded by shed platelets and recognizable micromegakaryocytes. In the peripheral blood megakaryocytic fragments are seen, along with large atypical cells with prominent cytoplasmic blebs representing megakaryoblasts. The bone marrow aspirate typically yields a dry tap, and the biopsy shows increased reticulin and fibrosis, the latter a result of the stimulation of the normal fibroblasts in the bone marrow by the local secretion of platelet-derived growth factor by the leukemic cells. [347] [348]

The megakaryoblast is Sudan black-, peroxidase-, and chloroacetate esterase-negative. The nonspecific esterase reaction is difficult to interpret in this leukemic subtype. The alpha-naphthyl acetate esterase reaction may be positive and is inhibited by the addition of fluoride, but the other nonspecific esterase stain, the alpha-naphthyl butyrate esterase, is usually negative. This differential staining distinguishes the megakaryoblast from the monocyte or monoblast. The PAS reaction is often, but not universally, positive. The FAB group recognized the difficulty of using routine morphology and cytochemistry to diagnose this subtype and included ultrastructural analysis and immunologic and cytogenetic criteria. In many cases megakaryocytic features may not be recognized by light or electron microscopy, so that use of monoclonal antibodies to specific platelet glycoproteins is necessary to define this subtype. [349] [350] Immunophenotyping with monoclonal antibodies to platelet glycoproteins 11b/111a, CD42b, CD61, CD41 or to factor VIII-related antigen may be needed to identify the megakaryoblasts. The ultrastructural platelet peroxidase reaction is technically difficult to perform and has been largely replaced by immunologic techniques. When flow cytometric techniques are used to determine surface antigen profile of leukemic cells, platelets adhere to the leukemic cells and may cause a false positive result with antibodies to platelet glycoproteins.[351] Cytospin immunofluorescence techniques are needed to distinguish adherent platelets versus a true positive cytoplasmic and membrane activity.

Cytogenetic abnormalities of chromosomes 3 and 21 have been associated with the M7 subtype.[352] [353] For the diagnosis of the M7 subtype at least 30% of the blast cells must be megakaryoblasts as defined by use of one of the above methods.

The clinical and hematologic features of the M7 subtype are varied and reflect the fact that in many patients the disease evolved from a prior myeloproliferative disorder. These patients frequently present with hepatomegaly and splenomegaly, a finding uncommon in patients with de novo acute megakaryocytic leukemia. [354] The presenting WBC is usually low, less than 5,000/mm ^{3} , and the platelet count is normal or increased in over one-third of cases. Anemia is usually present. The bone marrow aspirate is frequently a dry tap, and the bone marrow biopsy is fibrotic in over 90% of patients. The response to conventional induction chemotherapy is generally poor, with a complete response rate of less than 40%. A complete remission is frequently associated with reversal of the bone marrow fibrosis. [355] The clinical course can be very variable and atypical for an acute leukemia. Some patients whose disease has evolved from a preexisting myeloproliferative disorder may have a slowly progressive indolent disease that extends over a number of months to years. [356]

Patients with Downs syndrome have an increased incidence of acute megakaryoblastic leukemia.[357] In some of these patients the M7 leukemia appears to originate in early progenitor cells and expresses markers of mixed lineages.

Biphenotypic, Hybrid, and Bilineage Leukemias

The classification of AML is based on the predominant cell type and is an arbitrary system that does not attempt to address the biology of the disease or the leukemogenic events. In many cases it is impossible to define morphologically, cytochemically, or with the use of phenotypic markers a single lineage or cell type of a leukemic cell. In an attempt to explain this phenomenon, a number of different terms have been used, including *lineage infidelity, mixed lineage leukemias, biphenotypic or bilineage leukemias, hybrid or biclonal leukemias, and lineage switches*.[358] [359] [360] [361] [362] This confusing and at times arbitrary terminology reflects the heterogeneous nature of these disorders and the lack of specificity of currently available markers. *Lineage infidelity* refers to the expression of markers of more than one cell type by the same leukemic cell.[360] While many of the reported cases reflect the lack of specificity of the phenotypic markers, there are clear examples of blasts that express markers of more than one lineage. The monoclonal antibodies that are used to characterize lymphoid or myeloid leukemias recognize hematopoietic differentiation antigens. These antigens, which are expressed on a number of epithelial cells and overlapping subsets of hematopoietic cells, have important roles in the biology of normal and malignant hematopoiesis.[363] Immunophenotyping is of particular importance in recognizing the major immunologic subclasses of acute lymphoblastic leukemia and identifying the subtypes of AML that cannot be differentiated by morphology and cytochemistry alone, such as subtypes M0 and M7.[364]

Leukemic cells can demonstrate cytochemical and phenotypic markers of both myeloid and lymphoid precursors. [363] [365] [366] This phenomenon may reflect a fundamental abnormality of gene expression that is specific for the malignant clone. [362] [365] The clinical significance of lymphoid antigen expression in myeloid

leukemias is unclear. Lymphoid antigens may be positive in up to 48% of myeloid leukemias.[366] The most common lymphoid antigens expressed in myeloid leukemias are CD2 and CD7, which are expressed in 34% and 42% of patients with AML respectively.[366] The presence of lymphoid-associated antigens does not appear to be associated with a uniformly poorer prognosis. Alternatively, the leukemic cells may express markers of more than one lineage, reflecting the abnormal maturation of an earlier uncommitted stem cell.[367] [365] Many of the widely used monoclonal antibodies are not lineage-specific and are markers of differentiation. While many of the

monoclonal antibodies are considered lineage-nonspecific the two myeloid-associated monoclonals, CD13 and CD33, remain myeloid-specific.

Use of monoclonal antibodies and other molecular probes has shown that leukemic cells can demonstrate characteristics of more than one hematopoietic lineage. [364] [369] These cells may demonstrate Auer rods, stain with peroxidase and/or Sudan black, and react with monoclonal antibodies typical of both myeloid precursors and mature T cells.[370] A number of classification systems have attempted to address the biphenotypic leukemias but none have been widely accepted. [368] [370] Many cases express myeloid and lymphoid antigens and these cases do not represent evidence of biphenotypia, but reflect the inappropriate expression of lymphoid antigens on immature myeloblasts.[282] [366] A minority of acute leukemias have features of both myeloid and lymphoid lineages and are characterized as biphenotypic leukemias. [368] Biphenotypic leukemias can be suspected on morphological grounds when two distinct population of blast cells are noted. The incidence of acute biphenotypic leukemia represents approximately 7% of all adult acute leukemias.[361] In this setting two distinct leukemic cell populations are noted on immunophenotyping. The most common immunophenotype is co-expression of B-lymphoid and myeloid markers and less frequently T-lymphoid and myeloid markers. Biphenotypic leukemias have a high incidence of clonal chromosome abnormalities the most frequent being the t(9;22) (q34;q11), the Philadelphia chromosome, and structural abnormalities involving 11q23. In these cases the malignant transformation presumably occurred in a progenitor cell capable of differentiating into two distinct lines. This is in contrast to mixed lineage or hybrid leukemias, in which the leukemic cell expresses characteristics of more than one lineage. [371]

Lineage switch is the expression of markers of one lineage at diagnosis but markers of a different phenotype or lineage at the time of leukemic relapse. [371] [372] [373] This transformation, which usually occurs following a treatment interval of 1 year or more from the time of initial diagnosis, may reflect the selection of a clone from a bilineage leukemia or modulation of antigens expressed on leukemic cells. This phenomenon has been frequently reported in leukemias of the T-cell subtype in which relapse occurred as acute myeloid or myelomonocytic leukemia.[374] [375]

Hybrid leukemias do not fit into a morphologically or cytochemically defined group. Hybrid leukemias demonstrate malignant transformation of both lymphoid and myeloid cells.[376] The hybrid leukemias are morphologically heterogeneous and can present with undifferentiated or differentiated blasts. The undifferentiated myeloblastic leukemias present with agranular blasts with a high nuclear/cytoplasmic ratio, which are morphologically similar to those found in the lymphocytic leukemias.[377] [378] [379] [380] Some of these undifferentiated leukemias express the CD34 antigen, a marker expressed by hematopoietic stem cells, and early progenitor cells. Some of these disorders may in fact represent true stem cell leukemias and generally have a poor prognosis. [381] More typically the hybrid leukemias demonstrate commitment toward myeloid differentiation with morphologically identifiable myeloblasts. The myeloblasts may contain rare granules but a majority of the blasts do not demonstrate evidence of maturation or reaction with standard histochemical stains that would indicate a myeloid phenotype. The blasts may, however, be peroxidase-positive on electron microscopy or immunocytochemistry.[380] Use of monoclonal antibodies against lymphoid and myeloid surface antigens is necessary to define these hybrid leukemias.

Clinically the biphenotypic and hybrid leukemias present with the usual clinical findings of AML. However, they may present with features of both myeloid and lymphoid leukemias, including prominent diffuse lymphadenopathy and higher circulating blast and platelet counts. [382] [383] Otherwise the clinical presentations are indistinguishable from those of the other forms of AML.

The complete remission rates for the hybrid and biphenotypic leukemias are variable, as are their clinical courses. The undifferentiated and minimally differentiated hybrid leukemias do poorly with standard induction chemotherapy. [381] [384] Myeloid leukemias that are TdT-positive and express T-lymphoid markers are a more heterogeneous population and show variable response to induction therapy, with some patients having a better prognosis than patients with other AMLs. [382] Patients who fail standard AML treatment and demonstrate lymphoid markers may respond to the addition of vincristine, prednisone, and L-asparaginase to their induction regimen.[366] [381] Monoclonal antibodies to cell surface markers are necessary to define these morphologically atypical leukemias, and should be part of the initial evaluation in selected patients who present with an atypical morphological or clinical pattern.

Current models of hematopoietic differentiation are based on the evidence that normal pluripotential precursors give rise to committed precursors of a single-cell lineage and then undergo a series of discrete developmental steps. [377] The acute myeloid leukemias are believed to arise from a single clone that is arrested at a normal stage of committed differentiation. The currently used classification system is based on the premise that leukemic cells adhere morphologically and immunologically to a single lineage. The cases of biphenotypic leukemias, hybrid leukemias, lineage infidelity, mixed lineage, or lineage switch leukemias demonstrate the heterogeneity of these neoplastic disorders and support the concept that in at least some acute leukemias the transforming event occurs at the level of the pluripotential stem cell. Moreover, these data suggest that leukemic cells can differentiate, although aberrantly, and express differentiation markers.

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PREDICTORS OF RESPONSE

A number of clinical characteristics have been defined that are important prognostic factors (Table 53-8). Age is the most consistent prognostic variable for induction therapy. While some single-institution studies have treated elderly patients with intensive therapy, most patients over the age of 60 years tolerate intensive treatment poorly.[384] [385] [386] In addition patients over the age of 60 have an increased incidence of unfavorable prognostic variables, such as cytogenetic patterns including abnormalities of chromosomes 5, 7, and 8, and poor-prognosis AML subtypes.[387] [388] [389] [390] Older patients with AML have an increased incidence of multilineage features, suggesting a more primitive stem cell origin. Elderly patients have an increased incidence of dysplastic morphology in the bone marrow and peripheral blood.[388] Moreover, the generally favorable cytogenetic and FAB types are less common in elderly patients. Cytotoxic chemotherapy in elderly patients is associated with a higher morbidity and mortality because of the presence of comorbid diseases, poor tolerance of prolonged pancytopenia, and perhaps impaired drug metabolism and excretion.[391] [392] The use of colony-stimulating factors to improve the neutrophil recovery and outcome of treatment in elderly patients with AML remains controversial. Colony-stimulating factors have been shown to reduce the duration of neutropenia in elderly patients undergoing induction

TABLE 53-8 -- Prognostic Factors in ANLL

Factor	Favorable	Unfavorable
	Clinical	
Age	<50 years	>60 years
Leukemia	De novo	Secondary
WBC	<25,000/mm^3	>100,000/mm^3
FAB type	M3, M4Eo	M0, M5a, M5b, M6, M7
Cytogenetics	t(15;17), inv16 normal cytogenetics	Abnormalities 5, 7, 8
		Philadelphia chromosome
Extramedullary disease	Absent	Present
Auer rods	Present	Absent
Phenotype	CD34+	CD34
	Mdr1	Mdr1+
	In Vitro	
Clonogenic assay	Normal growth	Abnormal growth
Ara-CTP retention	High	Low
Labeling index	High	Low
	Bone Marrow	
Fibrosis	Absent	Present
Cytoreduction	Rapid	Slow
Courses to CR	Single	Multiple
Abnormal pronormoblasts	Rare	Many
Dysphasia	None	Trilineage

chemotherapy but overall effect on the morbidity or mortality is unclear.[391] [392] Less intensive chemotherapy has also been administered to elderly patients in an attempt to decrease the toxicity with variable results.[393] The duration of remission in elderly patients who attain a complete remission is also shorter and the role of post induction chemotherapy in this group is unclear.[387] [389] Patients with secondary AML or a prior myelodysplastic syndrome or myeloproliferative disorder respond poorly to standard chemotherapy regimens.[391] [392] [393] Patients presenting with blast cell counts greater than 100,000/mm^3 and signs of leukostasis also respond poorly to induction therapy. The pretreatment serum albumin, serum lactic dehydrogenase (LDH) level and performance status are important predictors of response.[390] [392] The lower the serum albumin, the less likely the patient will attain a complete remission and the higher the likelihood of having the morbidity and mortality associated with intensive chemotherapy.[394] [395] A poor performance status or elevated serum LDH is also associated with a lower complete response rate and shorter remission duration.[390]

A number of in vitro studies have attempted to correlate leukemic progenitor cell growth characteristics, drug sensitivity, and drug metabolism, retention, and incorporation with outcome of therapy. In vitro growth characteristics may be predictive of the outcome of remission induction therapy and remission duration, but results from such studies remain conflicting.[396] [397] [398] [399] [400] [401] The in vitro growth patterns and the presence of autonomous blast cell colony formation may be predictive of the response to treatment.[397] [402] [403] Additional in vitro studies that correlate with the probability of attaining a complete remission include the proliferative characteristics of leukemic cells, including the percent of cells in S phase, the cell cycle time and the expression of the multidrug resistance gene. [404] [405] [406] [407] Prospective studies are needed before therapy can be altered on the basis of labeling indices or in vitro growth characteristics. The expression of multidrug resistance gene has been implicated as a poor prognostic factor.[407] The best-characterized therapeutic resistance mechanism in AML is that mediated by the multidrug-resistance gene-1 (MDR-1).[406] MDR-1 encodes an ATP-binding transmembrane protein, which extrudes a variety of antineoplastic compounds from the cells including anthracyclines. Twenty percent of de novo AML and 75% of secondary AML cases express MDR-1.[408] MDR-1 expression is linked to the expression of the CD34 antigen.[407] [409] The MDR-1 phenotype is linked to other indicators of adverse outcome in AML including expression of the CD34+ antigen on blast cells, older age, poor prognostic cytogenetics, and immature phenotype and FAB group. The MDR-1 phenotype identifies a group of high-risk, poor-prognosis leukemias, but its role as an independent prognostic indicator remains unclear.[409]

The major determinant of the outcome of remission induction therapy is the capacity of the patient to tolerate intensive therapy. A majority of the patients who fail to

attain a complete remission do so because of the complications of the therapy. Resistant disease accounts for approximately 20% of all induction failures. The majority of patients who die during their initial treatment for AML succumb to complications of treatment including infection or hemorrhage. Therefore, prior medical problems and performance status are important predictors of response. Underlying renal insufficiency and impaired cardiac or hepatic function all limit the amount of chemotherapy that can be administered. Factors that predispose to serious infections decrease the likelihood of a patient attaining complete remission. Patients with concomittant diseases that impair their immune response or who are on medications that predispose them to fungal or other opportunistic infections have increased infectious complications and a higher mortality rate during induction therapy. [410] It remains unclear whether the introduction of hematopoietic growth factors will decrease the incidence of serious infections in patients receiving induction chemotherapy.

In most studies 6070% of patients under the age of 60 will attain a complete remission with induction therapy, and 4070% will relapse in the first 1824 months (Fig. 53-5). Those factors that determine remission duration are still very controversial and dependent in part on the type of post induction chemotherapy employed. [387] [411] [412] Cytogenetic abnormalities are present in the leukemic cells of the majority of patients with AML. [413] Cytogenetics are important prognosticators of response to treatment and remission duration. Cytogenetic abnormalities including t(8;21) and inv(16) are associated with significantly longer remission and survival, while abnormalities of chromosomes 5, 7, and 11q are associated with a poor response to therapy and shorter overall survival. These and other studies have suggested that chromosomal abnormalities constitute an important independent prognostic factor for remission duration but not for remission induction. [414] Other factors that are predictors of remission duration include the previously noted FAB subtypes M0, M4, M5, M6, and M7 having a poorer prognosis, and M2 and M3 and M4E having a better prognosis. The absolute percentage of erythroblasts appears to correlate inversely with remission duration. [415] Auer rods and an increase in the bone marrow eosinophils may also be associated with longer remission and survival. [415] The in vivo sensitivity of the leukemic cell as determined by the number of courses needed to attain a complete remission and the rate of cytoreduction in bone marrow cellularity, appear to be independent predictors of remission duration. [412] Patients who require two courses of induction chemotherapy to attain a complete remission appear to have a shorter remission duration. The morphologic appearance of the bone marrow biopsy at the time of complete remission may also be an important predictor of remission duration. The presence of morphologic dysplasia involving more than one cell line may be associated with a shorter remission duration. [242] [412] However, chemotherapy can induce dysplastic features in the bone marrow, and findings of dysplastic erythroid and myeloid precursors following high-dose induction and consolidation therapy must be interpreted with caution. All these factors, however, are

Figure 53-5 Overall survival for patients with AML (ANLL) can be divided into three phases. Phase 1 is induction therapy. The overall survival during this phase reflects the complete remission rate and the ability of the patient to tolerate chemotherapy and prolonged neutropenia and thrombocytopenia. Most patients who fail to respond die of infections or hemorrhage. True resistant disease accounts for <15% of induction failures. The complete response rate is approximately 6080%. Phase 2 is the first 2 years after attainment of complete remission. Most patients in complete remission will relapse and die of their leukemia in these first 2 years. While much of the attention in therapy for acute leukemia has focused on the first phase surviving induction therapy and attaining complete remissionas this curve suggests, most patients fail after attaining complete remission. This reflects occult disease not recognized at the time of complete remission. In an effort to prolong this phase, multiple studies are addressing the use of post-induction consolidation and maintenance therapy. Treatment of minimal residual disease remains a controversial area of investigation. Phase 3 begins 2 years after complete remission is attained. Patients in remission for >2 years have a markedly increased chance of prolonged survival; 7580% of patients in complete remission at 2 years will have a prolonged disease-free survival. However, the curve of this phase is not flat, and patients continue to relapse 310 years after attaining complete remission. The biologic factors responsible for these late relapses are unknown and may reflect one or more of the etiologic events in the development of AML.

determined from retrospective studies. None of these variables have been critically analyzed in controlled prospective trials.

The clinical events for patients with AML can be divided into three phases (Fig. 53-5). The induction therapy is the first phase. The outcome during this phase reflects the ability of the patient to tolerate chemotherapy and prolonged neutropenia and thrombocytopenia and attain a complete remission. A majority of patients who fail to respond to induction chemotherapy die of infection or bleeding. True resistant leukemia accounts for less than 20% of induction failures. The complete response rate with combination intensive induction chemotherapy is between 60 and 80%. Phase 2 encompasses the first 2 years after attainment of complete remission. The majority of patients in complete remission will relapse and die of their leukemia during the first 2 years. Improvements in supportive therapy have resulted in an increase in the percentage of patients attaining a complete remission as a result of receiving consolidation therapy. However, the majority of patients still relapse after attaining a complete remission. The high relapse rate reflects occult disease not recognized at the time of complete remission. In an effort to prolong this phase, multiple studies are addressing the use of postinduction consolidation and maintenance therapy. The treatment of minimal residual disease remains a controversial area of investigation. Phase 3 begins 2 years after complete remission is attained. Patients in remission for more than 3 years have a markedly increased chance of prolonged survival; 7580% of patients in complete remission at 3 years will have a prolonged disease-free survival. [425] However, patients continue to relapse 310 years after attaining complete remission. The biologic factors responsible for these late relapses are unknown and may reflect one or more of the etiologic events in the development of AML. In large cooperative trials, age < 55 years, a white blood count at presentation of <10,000/mm ^{3} , and favorable cytogenetics were associated with long-term survival. [426] [427] [428] [429]

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Chapter 54 - Therapy for Acute Myeloid Leukemia

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The goal of the treatment of acute myeloid leukemia (AML) is to produce and maintain a complete remission (CR). Criteria for CR are achievement after chemotherapy of a platelet count >100,000/L, a neutrophil count >1,0001,500/L, and a bone marrow that has <5% blasts. [1] In the 1960s, Freireich et al. demonstrated that patients who achieved a CR, thus defined, lived longer than patients who did not, although the CRs were relatively brief. When not in CR, the two groups had similar survival rates; thus, the difference in survival time was entirely attributable to the duration of time spent in CR, suggesting a correlation between achievement of CR and survival time.[2] These findings have been confirmed for patients treated in the 1980s and 1990s. Patients who achieve a CR on any given day after beginning therapy have longer survival subsequent to that day than patients who are resistant to therapy on the day in question.

Two types of events can interfere with the achievement and maintenance of a CR.[3] First, patients can die as a result of chemotherapy administered to eradicate the AML. This event is classified as a supportive care failure. Second, and more frequent, chemotherapy can be ineffective. Such resistance to therapy is usually manifested when disease recurs after a period in CR, but can also manifest as a failure to enter CR (primary refractory AML). These two types of resistance correspond to the two principal phases of chemotherapy, remission induction and therapy in remission. This chapter considers both phases of treatment, beginning with standard approaches.

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STANDARD REMISSION INDUCTION THERAPY

Modern remission induction therapy for AML usually consists of a combination of cytosine arabinoside (ara-C) and an anthracycline. Ara-C, an antimetabolite that is an analogue of the normal nucleoside cytidine, is converted to ara-C triphosphate (ara-CTP). The enzyme deoxycytidine kinase catalyzes the rate-limiting step in the process. Once converted to ara-CTP, ara-C is incorporated into DNA and causes cell death. Mechanisms of cytotoxicity that do not require ara-CTP formation may also exist. When originally used in the 1960s and 1970s, [4] ara-C was administered to patients with leukemia at daily doses of 100200 mg/m ^{2} (standard dose ara-C) either by twice-daily intravenous (IV) infusions or by a continuous IV infusion, the latter because of the drugs very short half-life (15 minutes). When given by continuous infusion, results were schedule dependent: at similar total doses, CR rates were higher when ara-C was given over 5 days rather than 2 days. Given at 200 mg/m^2 daily for 5 days by continuous IV infusion, ara-C produces CR rates of approximately 40% At the standard doses described previously, ara-Cs principal adverse affects are myelosuppression and gastrointestinal toxicities. Anthracyclines such as daunorubicin, doxorubicin, and rubidazone were introduced into the treatment of AML around the same time as ara-C. Daunorubicin, probably the most frequently used anthracycline , when given at 60 mg/m ^{2} daily for 37 days, produces CR rates similar to those seen with ara-C. [5] Anthracyclines and ara-C have similar toxicities, but anthracyclines also cause alopecia and a cardiomyopathy that can be prevented by limiting the total dose administered. Anthracyclines belong to a class of drugs, which also includes etoposide, that are believed to exert cytotoxicity by stabilizing the normally occurring complex between DNA and the enzyme topoisomerase II; stabilization of the complex leads to cell death.

Probably the most commonly used combination therapy for AML is the so-called 3 + 7 regimen, the name reflecting the typical use of 3 days of daunorubicin at 4560 mg/m^2 daily and 7 days of ara-C at 100200 mg/m^2 daily. CR rates are usually approximately 6070% Randomized trials have concluded that daily ara-C doses of 100 and 200 mg/m^2 are therapeutically equivalent in the 3 + 7 regimen. In addition, the 3 + 7 and 3 + 10 (3 days of anthracycline + 10 days of ara-C) regimens give identical CR rates, either regimen producing higher CR rates and quicker remissions, and thus less morbidity, than the less intense 2 + 5 and 1 + 5 regimens. [6] [7] [8] [9] Approximately half the patients who fail to enter CR die during induction, usually of multiorgan, particularly pulmonary, failure with infection, especially fungal infection, often the proximate cause. Hemorrhage, chiefly pulmonary, often contributes, especially in patients with acute promyelocytic leukemia (APL). [3] [10] The other half of the group of patients who fail to enter CR survive induction therapy but nonetheless do not achieve CR. These patients are divided into those in whom marrow hypoplasia is never observed and those in whom hypoplasia develops but is followed by reappearance of AML rather than achievement of CR. In clinical practice, a bone marrow biopsy is usually obtained 23 weeks after beginning therapy. If the marrow continues to show blasts and is cellular, a second course of the same therapy is usually given, sometimes at reduced total dose (e.g., 2 + 5). If the day 14 or 21 marrow is hypoplastic, therapy is usually delayed until it is clear that leukemia has reappeared, at which time the second course begins. A second repeated course of therapy can certainly produce remissions, but these are usually shorter in duration than remissions produced after one course of therapy, [11] illustrating the connection between results of induction therapy and results of postremission therapy. It is important to recognize that the initial marrow aspirate and biopsy obtained after a period of hypoplasia may demonstrate up to 3050% blasts as a reflection of the regeneration of normal, not leukemic, marrow. In the former circumstance, follow-up marrow biopsies show reduction in percentage blasts concomitant with a rise in neutrophils and platelets.

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STANDARD POSTREMISSION THERAPY

Three types of postremission therapy can be distinguished. *Maintenance* therapy is usually defined as therapy less myelosuppressive than that used to produce remission. The doses used in *consolidation* therapy usually approach, whereas those in *intensification* therapy may surpass, the doses used during induction. Traditionally, once in remission after treatment with the 3 + 7 regimen, patients receive maintenance therapy with the same drugs given during induction, or only ara-C, administered at approximately monthly intervals for 6 months to 1 year. With this approach, the median remission duration is approximately 11.5 years. [12] One study found that the risk of relapse

begins to decline sharply to a relatively low level once 3 years have been spent in CR. [13] It is thus reasonable to consider patients who are given traditional regimens and who are in CR at this time as potentially cured. Only 1020% of unselected patients fall into this category. [12] [13]

Much attention has been devoted to the need for maintenance therapy. The Eastern Cooperative Oncology Group (ECOG) randomized patients achieving CR either to receive no therapy or to receive a regimen consisting of 4 days of 6-thioguanine (6-TG, probably a minimally active agent, as described later) plus one subcutaneous injection of a relatively low dose of ara-C (60 mg/m^2) weekly for 2 years.[14] Remission duration was superior among patients assigned to receive maintenance. The likelihood of achieving a second remission was the same in both groups, whereas survival was significantly ($P < 0.05$) longer in the maintenance group.

The ECOG study clearly shows that some postremission therapy is needed. Several randomized studies have addressed the issue of how long such therapy should last. As seen in Table 54-1 , 3 studies have found some benefit to maintenance therapy. The Southwestern Oncology Group (SWOG) studied randomized patients who had received 3 + 7 induction therapy followed by two courses of maintenance and three courses of late intensification (consisting of POMP; see later) either with or without additional maintenance.[15] The relative risk of relapse or death was 1.63-fold higher in the no-maintenance arm (95% confidence interval 1.072.56), but there was no difference in survival. The Japanese Acute Leukemia Study Group (JALSG) reported that patients given 12 rather than 4 months of maintenance therapy had superior disease-free survival rates ($P = 0.004$) with probabilities of 3-year disease-free survival (dated from time of CR) of 48% versus 34%. [16] No overall survival data were presented. Büchner et al. reported median remission durations of 13 and 8 months in their maintenance and no-maintenance groups, with probabilities of 3-year disease-free survival of 30% versus 17% dated from time of CR. [17] Again, no survival data were given. In contrast, the Swiss study found no benefit from further maintenance after two courses of consolidation and one maintenance course had been given, [18] and the Southeastern Cancer Study Group found that 3 months of consolidation therapy and 7 months of maintenance therapy gave identical results. [19] In addition, several nonrandomized studies [20] [21] have found no differences between patients given or not given longer durations of consolidation or maintenance therapy.

Why the discrepancies in results illustrated in Table 54-1 ? It might be reasonable to expect that maintenance would primarily benefit patients who had previously received relatively little therapy. Such a hypothesis would explain the German data, because patients in the no-maintenance group were given only one prior course of consolidation. However, it would not explain the positive results in the SWOG and JALSG studies because the therapy given patients before randomization in these studies seemed equally intense as that administered to the patients in the studies finding no effect of maintenance therapy. Another explanation is that the maintenance therapy itself might have been more intense in the positive studies. The JALSG report noted their maintenance therapy was sufficiently myelosuppressive that patients required platelet transfusions after each course. Unfortunately, this type information usually is not available in the other studies. Two of the positive studies (JALSG, German) used drugs (etoposide or cyclophosphamide) that are potentially non-cross-resistant with ara-C or daunorubicin, but the SWOG study did not (Table 54-1). Another possible explanation is that the patients treated in the positive and negative studies differed with respect to variables that predict outcome. Although this does not appear obviously to be the case, information on cytogenetics, perhaps the factor most predictive of length of remission, is included in none of the studies.

Despite the discrepancies in the studies noted in Table 54-1 , it seems clear that any benefit from traditional maintenance after the administration of 34 months of consolidation is relatively small, and perhaps nonexistent with respect to survival. It may well be that this conclusion is not true for all prognostic subgroups of AML. However, it seems realistic to acknowledge that traditional maintenance therapy is on the whole unsatisfactory regardless of the length of maintenance. Therefore, future studies should address more pressing issues.

The poor prognosis of most patients given standard induction and postremission chemotherapy has led to trials investigating modification of such therapy. These have examined choice of anthracycline, use of high-dose ara-C (HDAC), hematopoietic growth factors, drugs other than anthracycline or ara-C, and allogeneic or autologous transplantation.

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CHOICE OF ANTHRACYCLINE

Idarubicin has been the most widely investigated alternative anthracycline. Three large U.S. randomized studies [22] [23] [24] compared

TABLE 54-1 -- Randomized Comparisons of Maintenance Versus No Maintenance in Patients in First Remission

Study	No. of Patients	Intensity of Therapy Before Randomization	Months in CR when Randomized	Months of Further Maintenance in Study Groups	Drugs Used in Maintenance	Positive Effect of Maintenance[a]
SWOG (Hewlett et al., 1995)[15]	150	3 + 7 then 2 courses M	23	0 vs. 22	Ara-C, 6-thioguanine, vincristine, prednisone	Yes
JALSG (Ohno et al., 1993)[16]	131	3 + 7 then 3 courses C	34	4 vs. 12	Ara-C, anthracyclines, etoposide, 6-mercaptopurine, prednisone, vindesine	Yes
German (Büchner et al., 1985)[17]	145	3 + 7 then 1 course C	1	0 vs. 36	Ara-C, daunorubicin, 6-thioguanine, cyclophosphamide	Yes
Swiss (Sauter et al., 1985)[18]	74	3 + 7 then 1 course C, 1 course M, 1 course C	34	0 vs. 24	Ara-C, 6-thioguanine, vincristine, prednisone	No
SEG (Vogler et al., 1995)[19]	124	3 + 7	0	3 mo C vs. 7 mo M	Ara-C, daunorubicin	No

SEG, Southern Cancer Group; SWOG, Southwestern Oncology Group; JALSG, Japan Acute Leukemia Study Group; 3 + 7, denotes induction regimen containing 3 days anthracycline + 710 days ara-C; C, consolidation (i.e. therapy equally intensive as 3 + 7); M, maintenance (i.e. therapy less intensive than 3 + 7).

[a] At $P < 0.05$.

standard-dose ara-C plus either daunorubicin (4550 mg/m^2 daily × 3) or idarubicin (1213 mg/m^2 daily × 3) as induction therapy followed in CR by two to three courses of the induction regimen at reduced dose. The studies found CR rates of 5560% with daunorubicin versus 6980% with idarubicin, and the difference was significant ($P < 0.05$) in two of the studies. Median survival ranged from 8.7 to 13.5 months in the daunorubicin groups versus 9.9 to 19.7 months in the idarubicin groups, these differences again being significant in two of the studies. Results seemed similar in both younger and older patients, and extramedullary toxicity was also similar, although duration of myelosuppression was greater in the idarubicin patients in two of the studies. The AML Collaborative Group conducted a meta-analysis of randomized trials comparing idarubicin and daunorubicin. [25] Data came not only from the three U.S. trials but from trials conducted by the Italian national cooperative group (GIMEMA), a French group, and a Mexican group. The meta-analysis indicated that idarubicin was associated with longer survival as well as higher CR rates. In contrast, a similar meta-analysis comparing mitoxantrone and daunorubicin found no differences in disease-free or overall survival rates. [25] These results seem to establish idarubicin, at the doses compared, as the standard anthracycline in newly diagnosed AML. Reasons for idarubicins superiority have focused on the long half-life of its active metabolite, idarubicinol, or the (disputed) possibility that idarubicin is a relatively poor substrate for the multidrug-resistance glycoprotein (MDR1) and hence less subject to efflux from blast cells. The probability of development of idarubicin-related clinical congestive heart failure was found to be 5% in patients receiving cumulative doses of 150290 mg/m[2] . [26] Cumulative doses of at least 150 mg/m^2 can probably be safely given to patients younger than age 70 years with no prior exposure to anthracyclines because the probability of congestive heart failure in these patients was zero, [26] compared with 1020% at the same dose level in patients older than age 70 years, or those with hypertension or prior exposure to anthracyclines.

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HIGH-DOSE ARA-C

High-dose ara-C (HDAC) in general denotes daily doses of 212 g/m ^{2} , whereas standard-dose ara-C (SDAC) denotes daily doses of 100 or 200 mg/m ^{2} . Use of HDAC for remission induction in newly diagnosed patients stems from observations that patients who relapse after maintenance with SDAC can again be induced into CR after HDAC.[27] Several toxicities also became apparent only when higher doses were administered. These included cerebellar and cerebral dysfunction, [28] noncardiogenic pulmonary edema,[29] pericardial effusion,[29] and conjunctivitis. The risk of central nervous system toxicity increases sharply with age and decreasing renal function.[28] The effectiveness of HDAC is presumed to be a result of higher intracellular concentrations of ara-CTP. Indeed, correlations exist between the ability of circulating leukemic blasts to form and retain ara-CTP and response of relapsed AML to HDAC. [30] Likewise, the superior outcome of children with Downs syndrome and AML relative to other children with AML may result from the increased ability of myeloblasts from the patients with Downs syndrome to form ara-CTP. [31] At any rate, the studies in relapsed disease led to clinical trials in patients with newly diagnosed disease comparing anthracycline + SDAC with anthracycline + HDAC.

Table 54-2 summarizes randomized trials of HDAC in newly diagnosed AML. The Australian Leukemia Study Group (ALSG) trial of Bishop et al. [32] found that although CR rates were similar in patients given anthracycline + etoposide with either SDAC or HDAC, the latter had longer remissions, with both groups receiving two courses of SDAC + daunorubicin + etoposide in remission. Survival rates were similar, but myelosuppression was greater in the HDAC group not only during induction but during postremission SDAC. This study was limited to patients 1760 years of age. The SWOG trial of Weick et al. [33] entered adults up to age 65 years and randomized patients to receive daunorubicin plus either HDAC or SDAC for induction; in CR, the SDAC induction group was further randomized to receive daunorubicin + SDAC (two courses) or + HDAC (one course). Patients given HDAC for induction received HDAC + daunorubicin (one course) as postremission therapy. Toxicity (neurologic, fatal infection, fatal central nervous system hemorrhage) was significantly more frequent during induction in the HDAC group, and although patients younger than 50 years of age were initially given 3 g/m ^{2} ara-C, this had to be reduced to 2 g/m ^{2} because of toxicity. Nonetheless, CR rates were the same in the SDAC and HDAC groups. Once in CR, disease-free survival was superior in patients given induction with HDAC rather than SDAC (*P* = 0.049, with 4-year estimates of disease-free survival being 21% (SDAC) versus 33% (HDAC) for patients younger than age 50 and 9% (SDAC) versus 21% (HDAC) for patients 5065 years of age. Among patients given SDAC induction, those who received HDAC in CR had similar outcomes, but more neurologic

TABLE 54-2 -- Randomized Comparison of Standard Dose ara-C with High-Dose ara-C in Newly Diagnosed Acute Myeloid Leukemia

Study	HDAC Given During	Patients	CR Rate		Statistically Significant($P < 0.05$) Effect on RD, EFS,EFS in CR, or Survival
			SDAC Regimen	HDAC Regimen	
ALSG (Bishop et al.)[32]	Induction only	279	74%	70%	RD longer with HDAC (median 36.9 vs. 12.7 mo)
SWOG(Weick et al.)[33]	Induction and/or intensification	723	56%	50%	EFS in CR better with HDAC induction, best with HDAC induction and intensification
ECOG (Cassileth et al.) [34]	Intensification only	170	NA	NA	EFS in CR superior with HDAC if age <60 y
CALGB (Mayer et al.)[35]	Intensification only	596	NA	NA	If age <60 y: EFS in CR superior with HDAC vs. IDAC and SDAC and survival in CR superior with HDAC vs. SDAC

SDAC, standard-dose ara-C; HDAC, high-dose ara-C; RD, remission duration; EFS, event-free survival; ALSG, Australian Leukemia Study Group; SWOG, Southwestern Oncology Group; ECOG, Eastern Cooperative Oncology Group; Cancer and Acute Leukemia Group B (CALGB); NA, not applicable.

THERAPY FOR ACUTE MYELOID LEUKEMIA INITIAL ASSESSMENT

INITIAL ASSESSMENT

The diagnosis of AML is made when the bone marrow contains >30% blasts, of which >3% stain positive for myeloperoxidase. Acute monocytic leukemia can be peroxidase negative but will usually show positivity after the performance of butyrate or nonspecific esterase stains. Surface markers and electron microscopy may be useful in diagnosing erythroleukemia and acute megakaryocytic leukemia. When the initial marrow is obtained, specimens are sent for cytogenetics. HLA typing of the patient and family members is also done at diagnosis. Unless symptoms warrant, there is no need to examine the cerebrospinal fluid at this time.

Once the diagnosis of AML is made, the need for emergency treatment should be assessed. Such treatment is required if the circulating blast count is >50,000/mm^3 or if the patient has APL, disseminated intravascular coagulation (DIC), or organ dysfunction attributed to leukemic infiltration. Patients most likely to have leukemic infiltration are those with circulating blast counts of >10,000/mm^3.

SUPPORTIVE CARE

Patients are given bicarbonate-containing IV fluid daily to prevent the development of a tumor lysis syndrome and attendant hyperuricemia. Allopurinol is given if the white blood cell (WBC) count is >10,000/mm^3. If the WBC is >10,00050,000, the patient, particularly if elderly or with a low platelet count, is at risk for development of pulmonary failure in the first week after the start of chemotherapy, characterized by hemorrhagic fluid being recovered after bronchoalveolar lavage. These patients are monitored for this complication in the intensive care unit and the amount of IV fluids is restricted. If the WBC count is >50,000/mm^3, leukapheresis is begun immediately, in conjunction with chemotherapy. By reducing tumor burden, pheresis may prevent the development of the tumor lysis syndrome or pulmonary failure syndrome. If renal function deteriorates or hyperphosphatemia or hyperkalemia develop, continuous hemodialysis is instituted.

Patients who are afebrile (<101°F) at presentation are begun prophylactically on trimethoprim-sulfamethoxazole one double-strength tablet bid, fluconazole 200 mg bid, and itraconazole 200 mg bid. If a temperature >101°F develops unrelated to blood product administration, patients are begun on intravenous imipenem. If pneumonia is documented, amphotericin is begun immediately. Otherwise, amphotericin is begun if the patient remains febrile with negative cultures after 3 days. We routinely use liposomal amphotericin preparations 35 mg/kg/day. Such preparations should certainly be used if the creatinine becomes elevated. If, despite use of amphotericin, there is evidence of progressive infection (development of pneumonia, persistently positive cultures) and persistent neutropenia, we begin administration of GM-CSF and consider granulocyte transfusions from family donors if the donors can be treated with GM-CSF to raise their granulocyte count.

Platelet transfusions are given routinely if the platelet count is <10,000. Exceptions may be made if the count has been below this level for weeks, if the patient is not bleeding, and if the patient does not have mucositis or DIC. If bleeding or DIC is present, platelet transfusions should be given if the platelet count is <50,000. If significant increments in the platelet count are not obtained after the administration of pooled platelet concentrates, family members are used as donors. Transfusions of cryoprecipitate are given to maintain the fibrinogen level at >200 in patients with APL. All blood products are filtered to reduce the possibility of alloimmunization.

REMISSION INDUCTION THERAPY

We treat our patients with APL with all-*trans* retinoic acid (ATRA) 45 mg/m^2/day until CR is achieved. Idarubicin (12 mg/m^2/day × 4 days) is begun 5 days after initiation of ATRA, or sooner if leukocytosis >10,000 develops. If ATRA syndrome develops, we discontinue ATRA and administer dexamethasone (10 mg IV bid) for 3 days, followed by a rapid taper. If the marrow contains persistent excess numbers of blasts, a second course of idarubicin is not begun until 5 weeks have elapsed after initial treatment, unless DIC occurs before then.

Patients with other types of AML are given idarubicin (12 mg/m^2/day on days 13) + ara-C (1.5 g/m^2/day on days 14 by continuous infusion, or 2 g/m^2 over 4 hours once daily on days 14) if they are younger than age 60 years and have a good performance status and no history of abnormal blood counts. If they have any of these features, they are offered investigational therapies. A second course of therapy is not begun before day 21 after the first course, unless the circulating blast count rises before then. Patients not in remission after two courses are first considered for allo-BMT; if this is not feasible, they are offered investigational chemotherapy.

An alternative approach would be to assign patients to treatment on the basis of cytogenetic results, provided these are available within 34 days of presentation. With this approach, patients with abnormalities of chromosome 5 or 7 would be immediately offered investigational therapies based on their low likelihood of achieving CR with conventional therapy or HDAC.

POSTREMISSION THERAPY

By the time remission is achieved, pretreatment cytogenetic information should be available and used to plan therapy. Patients with inv(16) or t(8;21) are given courses of HDAC (1.5 g/m^2/day × 2 days by continuous infusion) + idarubicin (8 mg/m^2/day on days 1 and 2) alternating with fludarabine 30 mg/m^2/day on days 12 + ara-C 1 g/m^2/day on days 12. Patients with APL receive three courses of idarubicin, the first two at 12 mg/m^2/day × 2. If PCR testing is negative, therapy is discontinued. If not, alternative therapies are offered. Patients with other karyotypes and an HLA-compatible donor are offered allo-BMT up to age 50 years and minitransplant up to age 70 years. Otherwise, they are considered for investigational therapy.

BONE MARROW TRANSPLANTATION

Aside from use in poor-prognosis patients in first remission, we recommend allo-BMT transplantation in first relapse if the patient is younger than 70 years of age and has an HLA-matched sibling donor, or is younger than 50 years of age and has an HLA-identical unrelated donor.

toxicity, than patients given SDAC in CR. Postremission outcomes (event-free survival, survival) were best in patients given HDAC for induction and in CR regardless of whether account is made for the more frequent inability of patients given HDAC induction to receive planned post-CR therapy. Both the Cassileth et al. (ECOG) study[34] and the Mayer et al. (Cancer and Acute Leukemia Group B) [CALGB]) study[35] randomized patients once in CR: HDAC versus SDAC (ECOG) or HDAC versus intermediate-dose ara-C (IDAC, 400 mg/m^2 daily × 5) versus SDAC (CALGB). In the ECOG study, one course of postremission therapy was given to the HDAC group (amsacrine + HDAC), whereas the SDAC group received 2 years of postremission therapy. In the CALGB study, patients were to receive four courses of their assigned postremission therapy and then received four courses of SDAC + daunorubicin. Both studies determined outcome to be better with HDAC, but only in patients younger than age 60 years (e.g., actuarial 4-year event-free survival rates of 28% vs. 15%; P= 0.049 [ECOG]; and 44% vs. 29% vs. 24%; P= 0.002 [CALGB], comparing HDAC, IDAC, and SDAC). In patients older than age 60 years, the ECOG noted a mortality rate of 57% (8/14) with a single course of HDAC (3 g/m^2 every 12 hours for 6 days) + amsacrine.

In summary, data from all four studies of HDAC in Table 54-2 note advantages for HDAC given during induction (Bishop et al.,[32] Weick et al.[33]) and/or during post-remission therapy (Weick et al.,[33] Cassileth et al.,[34] Mayer et al.[35]). It must be noted that three of these four studies did not include patients with a history of abnormal blood counts or secondary leukemia (important prognostic factors, as discussed later), that three were limited to patients younger than age 65 years, and that two explicitly noted that outcome was not better and toxicity worse in patients older than age 60 years. Furthermore, it appears that the beneficial effects of HDAC induction and intensification in patients younger than age 6065 years are limited to patients most likely to respond to SDAC, in particular those with favorable leukemia cell karyotypes, as discussed in the Prognostic Factors section.

Mitus et al. combined SDAC and HDAC in a regimen calling for daunorubicin 45 mg/m^2 on days 1, 2, and 3, SDAC 100 mg/m^2 daily on days 17, and HDAC 2 g/m^2 every 12 hours on days 8, 9, and 10.[36] They reported a CR rate of 89% in 94 consecutive patients younger than 65 years of age with de novo AML and no history of abnormal blood counts. Eighty-five percent of the patients with unfavorable cytogenetics achieved CR (95% confidence interval 6297%). Two patients had irreversible

cerebellar toxicity. In CR, patients received an allogeneic or autologous transplant. This so-called Mitus regimen is now being investigated by the SWOG and CALGB.

USE OF COLONY-STIMULATING FACTORS

In principle, administration of colony-stimulating factors (CSFs) in AML can affect either the rate of recovery of normal blood cells after, or the sensitivity of leukemic progenitors to, chemotherapy. The two most widely studied CSFs have been granulocyte-macrophage (GM)-CSF and granulocyte (G)-CSF. In most trials, GM- or G-CSF was begun only after completion of chemotherapy in order to hasten neutrophil recovery, thereby presumably decreasing infection rate; if leukemic cell recovery is not simultaneously enhanced, a higher CR rate should result. Although it was originally believed that sensitization of leukemia to chemotherapy could be achieved only if CSFs were given before or during chemotherapy, some evidence discussed later suggests that CSFs given only after completion of chemotherapy may also affect sensitivity of AML to chemotherapy.

Beginning with the question of neutrophil recovery, most studies have found that use of either GM- or G-CSF speeds this process by 25 days. This result seems to apply regardless of whether CSF is given before as well as during and after induction chemotherapy, or only after such chemotherapy. Less is known about the effects on blood count recovery of CSFs given in CR, although Moore et al. noted that G-CSF given after aziridnyl benzoquinone + mitoxantrone postremission chemotherapy shortened the time to neutrophil recovery compared with a control group of patients given the same chemotherapy. [37]

Accepting that GM- or G-CSF administered after induction or postremission therapy speeds recovery of neutrophils leads of course to the question, does this matter? Table 54-3 summarizes larger trials of GM- or G-CSF in AML according to whether they found significant reduction in morbidity or mortality rates in GM- or G-CSF-treated groups. The differences between G-CSF and placebo in the Godwin et al. [38] and Heil et al.[39] studies applied *only* to days of hospitalization or duration of fever and antibiotic usage, each of which were reduced from 1 to 5 days. The Moore et al. [37] study noted shorter hospitalizations in G-CSF groups receiving postremission therapy (as did the Heil et al. study), but no effect on remission duration or survival. Certainly, the Rowe et al. study [40] (conducted by the ECOG) is the only one in Table 54-3 that found that use of a CSF, GM-CSF, after induction chemotherapy decreased the incidence of *serious* infection and improved *survival*. Its results were directly contradicted by the CALGB study of Stone et al. [41] Possible explanations for the differences between the ECOG and CALGB studies include use of yeast-derived GM-CSF in the ECOG study but *Escherichia coli*-derived GM-CSF in the CALGB study, and the fact that ECOG patients only began GM-CSF or placebo if the marrow was aplastic on day 10, whereas all CALGB patients received GM-CSF or placebo. The most likely explanation, in the authors opinion, is the much smaller size of the ECOG study, making it likely that imbalances in prognostic factors made the placebo group unfavorable relative to the group receiving GM-CSF.

Regarding G-CSF, the SWOG study of Godwin et al. [38] found no difference in CR rate between placebo and G-CSF (49% vs. 42%), whereas the Dombret et al. [42] study found a significant improvement in the G-CSF groups (47% placebo vs. 70% G-CSF). This improvement was unrelated to a reduction in early, or infectious, mortality rates, but purportedly resulted from less resistance to chemotherapy in the G-CSF group. This was particularly noticeable in patients with residual blasts in the marrow on day 8 and in patients with unfavorable cytogenetics. The study is of interest because it is the first to suggest that administration of CSF after chemotherapy can influence sensitivity of leukemia cells to chemotherapy (e.g., by affecting rate of apoptosis). One commonly invoked explanation for the results of Dombret et al. is the possibility that G-CSF promoted differentiation and increased neutrophil numbers, thereby lowering the blast percentage in the marrow, that is, the G-CSF CRs were cosmetic in that only the percentage but not the number of blasts was reduced. Indeed, despite the higher CR rate in the G-CSF group, event-free survival from start of treatment was the same in G-CSF and placebo groups, reflecting short CR durations.

Given all of the aforementioned data, the authors do not favor routine administration of GM- or G-CSF after induction therapy in elderly (or other) patients. They believe that the yeast GM-CSF study (Rowe et al. [40]) should be repeated in a much larger group of patients for the reasons discussed previously. Although it could be argued that there is no harm in routine administration of GM-CSF after chemotherapy, Zittoun et al. [43] found that such use of bacteria-derived GM-CSF, regardless of whether GM-CSF was also given before and during chemotherapy, resulted in lowered CR ($P < 0.01$) and event-free survival ($P = 0.005$) rates compared with patients who received no GM-CSF or GM-CSF only before and during chemotherapy (a four-arm study; see Table 54-3). These results reflected increased resistance to chemotherapy in the GM-CSF after chemotherapy groups. On the other hand, a similar four-arm study by

TABLE 54-3 -- Does GM- or G-CSF Reduce Morbidity or Mortality After Induction Therapy?Results of Randomized Trials

Study	Patients (ages in y)	Answer to Question	What was Reduced? ($P < 0.05$)	Chemotherapy[a]	CSF[a]	Prophylactic Antibiotics
Godwin et al.[38] (1995)	193 (>55)	Yes	Days with fever, days on antibiotics	D45 × 3 A200 × 7	G 400 or placebo daily start day 11 if <5% marrow blasts on day 10	Not stated
Heil et al.[39] (1995)	521 (adults)	Yes	Days with fever, days on antibiotics, days in hospital	D45 × 3 A200 × 7 E100 × 5	G 5 or placebo daily start day 8	Oral quinolones
Rowe et al.[40] (1995)	117 (5570)	Yes	Major infections, deaths	D60 × 3 A200 × 7	Yeast GM 250 or placebo daily start day 11 if marrow aplastic without AML on day 10	Not stated
Stone et al.[41] (1995)	388 (60)	No	NA	D45 × 3 A200 × 7	Bacterial GM 200 or placebo daily start day 8	Not stated
Dombret et al.[42] (1995)	173 (>65)	No	NA	D45 × 4 A200 × 7	G 5 or placebo daily start day 9	Not stated
Zittoun et al.[43] (1996)	102 (1560)	No	NA	D45 × 3 A200 × 7	Bacterial GM 5 daily start either day 8 (arm 1), or day minus 1; latter pts. continue through day 7 (arm 2) or until neutrophil recovery (arm 3). Arm 4 = no GM	Not stated
Löwenberg et al.[44] (1997)	253 (1560)	No	NA	D45 × 3 A200 × 7	As in Zittoun et al.[43]	At discretion of local hospital
Löwenberg et al.[45] (1997)	318 (>60)	No	NA	D30 × 3 A200 × 7	Bacterial GM 5 or placebo daily start day before DA	At discretion of local hospital
Witz et al.[46] (1994)	163 (5575)	No	NA	I8 × 5 A100 × 7	Bacterial GM 5 daily or placebo start during induction therapy	Not stated

GM, granulocytemacrophage; G, granulocyte; CSF, colony-stimulating factor; NA, not applicable; D, daunorubicin; A, ara-C; E, etoposide; I, idarubicin.

[a] Numbers are doses in mg/m^2 per day × days administered for chemotherapy, and in g/m^2 (Rowe et al., Godwin et al., Stone et al.,) or g/kg (Heil et al., Löwenberg et al., Witz et al., Zittoun et al., and Dombret et al.) for CSFs.

Löwenberg et al.[44] found no difference between any of the four groups. The Zittoun et al. [43] study documented more fluid retention, weight gain, and hypotension in the GM-CSF postchemotherapy arms, whereas Löwenberg et al.[44] found a higher incidence of fever in these arms. Although the authors are not advocating routine use of GM-CSF after chemotherapy, it does appear reasonable to administer GM- or G-CSF if elderly patients contract fever or infection during remission induction.

As did Löwenberg et al.[44] [45] and Zittoun et al.,[43] others have given GM-CSF before or during chemotherapy in an attempt to sensitize blast cells to chemotherapy. [46] [47] [48] With the exception of a study by Büchner et al. [47] that found longer remission in younger patients given GM-CSF, these studies have either shown no difference in CR rate, remission duration, event-free survival, or survival, [43] [44] [45] [46] or a lower CR rate with no effect on remission duration or survival. [48] Pending results of other studies (e.g., a CALGB study of GM-CSF priming in relapsed AML), GM-CSF should not be used for priming purposes. G-CSF has been used less frequently for this approach, but it was not reported effective in a study at M. D. Anderson. [49] It remains to be seen if specific groups might benefit from this approach or whether pretreatment measurements of various biologic parameters will permit prediction of whether a given patient will benefit from priming. [50] Comprehensive reviews of CSFs in AML are available.[51] [52] [53] [54]

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USE OF DRUGS OTHER THAN ANTHRACYCLINE OR ARA-C

Randomized trials (Table 54-4) have addressed the usefulness of the purine analogue 6-TG, the mitotic poison vincristine, the topoisomerase II-reactive agent etoposide, and a four-drug combination (POMP) consisting of the purine analogue 6- mercaptopurine, vincristine, methotrexate, and prednisone when added to or integrated with the basic anthracycline + ara-C regimen. A CALGB study in which 427 patients were randomized to receive daunorubicin + ara-C with or without 6-TG found no difference in CR rate, remission duration, or survival between the regimens. [7] The JALSG reported that addition of vincristine to a daunorubicin, behenoyl ara-C, 6-mercaptopurine regimen produced an inferior CR rate in a 252-patient randomized trial. [16]

Results are more disparate with etoposide. Although this drug, like the anthracyclines, stabilizes the DNA-topoisomerase II complex, anthracyclines and etoposide may have different binding sites on the enzyme. The ALSG randomized 264 patients to receive either the standard 3 + 7 regimen or the same regimen plus etoposide (75 mg/m^2 /d on days 17).[55] In remission, patients received their induction regimen but at reduced dose. Although CR rates were statistically the same in both groups (56%, 59%), remission duration was significantly longer (P = 0.01) in the etoposide-treated patients, with median duration of remission of 12 versus 18 months. This finding reflected results in patients younger than age 55 years who also showed evidence (P = 0.03) of a survival advantage if given etoposide (medians of 9 vs. 17 months). The incidence of diarrhea during induction therapy was greater and the period of myelosuppression was longer in the etoposide-treated patients. At the time of publication in 1990, the median follow-up in the living patients ranged from 18 to 60 months; given that patients can be considered potentially cured once 3 years have elapsed from the CR

TABLE 54-4 -- Effect of Drugs Other than Anthracycline + ara-C When Added to or Integrated with Standard Therapy: Results of Randomized Trials

Drug/Regimen	Effect[a] When Added to or Intergrated with Anthracycline + ara-C		Survival	Patients	Reference
	CR Rate	Remission Duration			
Vincristine	Lower	No effect	Shorter event-free survival	252	[16]
6-Thioguanine	No effect	No effect	No effect	427	[7]
Etoposide	No effect	Longer	Longer	264	[55]
	No effect	No effect	No effect	1,874	[56]
Amsacrine + 5 azacytidine	NA	No effect	No effect	233	[5]
	NA	No effect	No effect	219	[19]
POMP	NA	Longer	Longer	131	[55]

NA, not applicable; POMP, 6-mercaptopurine, vincristine, methotrexate, and prednisone.

[a] P < 0.05.

date, it would be of interest to know if the studys results were the same after longer follow-up. In contrast to the ALSG, the Medical Research Council (MRC) in the United Kingdom reported no differences in remission duration or survival rates among 1,874 patients randomized to receive daunorubicin + ara-C + 6-TG or the same therapy but with etoposide (100 mg/m^2 daily × 5) replacing 6-TG.[56] Follow-up time appears longer than in the ALSG study, with 11% of the patients followed for 6 years. Other possible reasons for the discrepancies between the ALSG and MRC studies include different postremission therapies, and inclusion of children but not adults older than age 55 years, and of patients with a history of abnormal blood counts and secondary AML in the MRC but not the ALSG study. However, the MRC noted that results were the same in all age groups, whereas the ALSG noted that etoposides effects were most marked in patients younger than age 55 years; and that if there was any advantage for etoposide in the MRC study, it was in patients with secondary, not de novo AML. Given the much larger size of the MRC study, it is reasonable to conclude that any advantage gained by addition of etoposide to the 3 + 7 regimen is relatively small. Of note, neither the MRC nor ALSG found any benefit for etoposide in patients with French American British classification system (FAB) types M4 and M5, although this was once cited as a possibility. Bow et al. combined mitoxantrone + etoposide as initial induction therapy for patients aged 6080 years. [57] In CR, patients received one course of IDAC. The CR rate was 55% (95% confidence interval 4368%), with median remission duration and survivals of 89 months. It is not clear that these results are better than what could be obtained with 3 + 7 because no comparison group was provided.

The SWOG studied the effect of POMP by randomly assigning 131 patients who had been in CR for 10 months after conventional anthracycline + ara-C treatment to continue to receive ara-C for an additional 3 months or to receive three cycles of POMP. [58] With a median follow-up of 9 years, median survival from time of randomization was 34 months in the POMP-treated group versus 19 months in the ara-C-treated group (P = 0.03). Although trials incorporating amsacrine and 5-azacytidine into postremission therapy have not been successful, [19] [59] the SWOG POMP results suggest that other regimens that contain agents that are noncross-resistant with anthracycline + ara-C might prove effective.

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TIMING OF CHEMOTHERAPY

Another way in which therapy can be intensified is by repeating courses at more frequent intervals. Woods et al. treated 589 children and adolescents (up to age 21 years) with a five-drug induction regimen (daunorubicin, SDAC, etoposide, 6-TG, and dexamethasone [DCTER], given over 4 days). [60] Patients were randomized either to receive a second mandatory cycle of DCTER identical to cycle 1 beginning on day 10, or to receive the second course in standard fashionthat is, on day 14 if >40% blasts were present on that day but later if they were not. In CR, patients were allocated to receive an allogeneic transplant if they had a human leukocyte antigen (HLA)-matched family donor and otherwise were randomized between an autologous transplant and continued chemotherapy. Although CR rates were similar in the two arms, patients assigned to receive a second course on day 10 had superior event-free survival ($P < 0.001$), with actuarial rates of 42% versus 27% at 3 years, which corresponded to the median follow-up time. Furthermore, those results applied regardless of the type of postremission induction therapy given, illustrating again the connection between induction therapy and post-CR results. Castaigne et al. [61] examined this approach in adults (1560 years of age with de novo AML), randomizing 307 patients to receive either 3 + 7 standard induction or the same but with an obligatory second course (3 days of mitoxantrone + IDAC) beginning on either day 8 or day 20 (double induction), as in the Woods et al. study. Postremission outcome was better in patients given double induction ($P = 0.05$), but there was no effect on survival. Because the Woods et al. study included only a relatively small number (25%) of patients with unfavorable cytogenetic abnormalities, with no comparison of results by cytogenetic group in the two treatment arms, and because the Castaigne et al. study was limited to patients with de novo AML, it remains to be seen if the results of either study will apply to patients with poor prognostic features.

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ALLOGENEIC BONE MARROW TRANSPLANTATION

The use of allogeneic bone marrow transplantation (allo-BMT) was first investigated in patients in chemotherapy-resistant relapse. [62] Sustained remissions were observed in 1020% of patients. This led to trials of allo-BMT in first remission. Trials conducted in the early and late 1980s [63] [64] demonstrated that the relapse rate was invariably lower after allo-BMT than after chemotherapy in patients in first remission. This resulted to some extent from the effects of the high-dose chemotherapy, with or without total-body irradiation, used as the preparative regimen, but perhaps to a larger extent from an immunologic graft-versus-leukemia effect. This effect is presumed to exist because of the inverse relationship between the extent of graft-versus-host disease and relapse rate. [65] Despite the lower relapse rate, differences in survival were generally not observed because of

TABLE 54-5 -- Comparisons of Allogeneic Transplant Versus Chemotherapy in First CR

Study	Patients	Patients with Match Transplanted in First CR (%)	Patients in First CR Transplanted in First CR (%)	Actuarial LFS[a]		Actuarial Survival[a]		Statistically Significant Differences ($P < 0.05$)
				Chemotherapy (%)	Transplantation (%)	Chemotherapy (%)	Transplantation (%)	
Gale et al.[66] (1990)	901 AL	NA	NA	35	46	42	48	LFS
Zittoun et al.[67] (1995)	168 AL 126 C	63	23	30	55	46	59	LFS
Hewlett et al.[15] (1995)	53 AL 110 C	65	12	28	40	NA	NA	None
Cassileth et al.[68] (1997)	120 AL 118 C	88	23	35	42	54	46	None
Burnett et al.[69] (1994)	299 AL 357 C or AU[b]	58	23	NA	NA	51	58	None
Reiffers et al.[70] (1994)	36 AL 99 C or AU[b]	NA	NA	37	63	NA	NA	LFS
Archimbaud[71] (1994)	27 AL 31 C	74	34	27	41	46	41	None
Ravindranath et al.[72] (1996)	89 AL 117 C	89	14	36	52	40	55	None
Nesbit et al.[73] (1994)	89 AL 266 C	89	22	32	45	34	47	None (see text)
Schiller et al.[74] (1992)	28 AL 54 C	89	30	36	48	53	45	None

LFS, leukemia-free survival; CR, complete remission; AL, allogeneic transplant; C, chemotherapy; AU, autologous transplant; NA, not available.

[a] At 3 years in the studies of Reiffers et al. and Ravindranath et al., 4 years in the studies of Zittoun et al. and Cassileth et al., 5 years in the studies of Gale et al., Burnett et al., and Schiller et al., 7 years in the studies of Hewlett et al., and Archimbaud et al., and 8 years in the study of Nesbit et al.

[b] The comparisons are AL vs. C + AU in the Burnett et al. and Reiffers et al. studies and AL vs. C in the other studies.

a higher rate of death in CR in transplanted patients (from graft-versus-host disease, veno-occlusive disease, and infection) and perhaps a shorter survival after relapse in transplanted patients. [63] [64]

Table 54-5 examines studies, reported in the 1990s, comparing chemotherapy with allo-BMT. Chemotherapy consisted of one to two courses of HDAC or IDAC in the studies of Zittoun et al.,[67] Cassileth et al.,[68] Reiffers et al.,[70] Schiller et al.,[74] and Archimbaud et al.,[71] and long-term SDAC ± POMP in the studies of Gale et al.[66] and Hewlett et al.[15] However, in the study by Burnett et al.,[69] patients after completion of three courses of postremission therapy were assigned to allo-BMT or randomized between an autologous transplant or cessation of therapy (i.e., half of the control group received no further therapy). In all the studies, except those by Nesbit et al.[73] and Ravindranath et al.,[72] the chemotherapy and transplant patients were younger than 4155 years of age, whereas the latter studies were performed in children and adolescents (021 years of age). The Gale et al. study [66] compared patients who had been reported to the International Bone Marrow Transplant Registry (IBMTR) as having had an allo-BMT with patients given postremission chemotherapy in a German AML Cooperative Study. Although the Gale et al. study [66] used statistical techniques to adjust for the differences in age and cytogenetics (transplant patients younger and more likely to have the t[15;17] translocation) and for the fact that transplant patients had to remain in remission for a few months before they could be transplanted whereas chemotherapy patients did not, the study does not permit the reader to ascertain the total number of patients from whom the 971 transplanted patients were drawn. This is not a problem with the other studies in Table 54-5 . Each of these studies illustrates that as few as 60% of patients with a suitable match will actually receive a transplant. Because the patients with a match who are actually transplanted are usually presumed to have a better prognosis (e.g., are healthier) than patients who, despite the availability of a match, are not transplanted, the two groups are analyzed together in all the studies in Table 54-5 (except Gale et al.[66]) to avoid the bias that would result if the nontransplanted patients were analyzed with the chemotherapy group. Table 54-5 also illustrates that the percentage of patients younger than 4155 years of age who are actually transplanted in first CR is 1234%, with the latter figure being obtained in a single hospital trial. Further discussion of the relatively small impact that allo-BMT has, even in relatively young patients in CR, can be found in reports by Berman et al., [75] Gamberi et al.,[76] and Proctor et al.[77] Table 54-5 illustrates that an advantage in leukemia-free survival (LFS) was noted in three of the nine studies. Because some of the studies were relatively small and the trend is invariably in favor of improved LFS in the allo-BMT patients, it is possible that statistically significant differences would have been documented in more studies had larger numbers of patients been treated. Furthermore, as discussed by Nesbit et al., [73] the standard statistical comparison of the groups masks the poorer earlier but better later LFS in transplanted

patients. Differences between groups treated with allo-BMT or chemotherapy are least when survival becomes the study end point. Indeed, the study of Cassileth et al.[66] (median follow-up, 3 years) found a small, nonsignificant difference favoring chemotherapy, as did a 1992 study by Schiller et al. [74] Hence, the studies in the 1990s are similar to those in the 1980s in that both find that an advantage in LFS does not translate into an advantage in survival in the allo-BMT group. Furthermore, although approximately 90% of allo-BMT survivors are in good health years after the procedure, [78] they are clearly at increased risk for subsequent development of solid cancers.[79] [80] It is unclear if long-term survivors of chemotherapy have a similar risk for the development of secondary solitary tumors. [13] The authors believe that it might be more appropriate to defer further comparisons of chemotherapy versus allo-BMT in first CR until more time has been allowed for both techniques to improve, because both consistently cure only a minority of even younger patients. Some of these techniques are discussed in the section on Investigational Approaches.

The data in Table 54-5 indicate that either allo-BMT or

continued chemotherapy can currently be recommended for patients achieving CR. Might some patients do better after allo-BMT and others better after continued chemotherapy? Age has been the characteristic that best identifies such patients. It has generally been accepted that the risk of mortality after allo-BMT in patients older than age 5560 years is such that benefitrisk considerations favor chemotherapy in such patients, although, as discussed later, use of less intense preparative regimens may extend the upper age limit beyond that for which allo-BMT remains a realistic possibility. Conversely, it has generally been accepted that in patients younger than age 20 years, the risk of allo-BMT is sufficiently low that allo-BMT rather than continued chemotherapy is warranted. [72] [73] [81] Data indicating that LFS in patients transplanted only in second CR is influenced by length of the first chemotherapy-maintained CR suggest that, aside from the extremes of age noted, similar prognostic factors are operative for allo-BMT and chemotherapy. [82] Of particular interest is the prognostic significance of cytogenetics, which is discussed later in the section on Prognostic Factors.

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AUTOLOGOUS TRANSPLANTATION

Only 3060% of patients have an HLA-matched sibling who can serve as a donor for allo-BMT. The 60% figure comes from data reported from transplantation centers and probably reflects referral of patients already screened before referral. [83] There has been a recent surge of interest in allogeneic transplantation using matched but unrelated donors (discussed later). Still, donors will not be available for many patients (e.g., African Americans or other racial or ethnic groups who are underrepresented in transplantation registries), or the wait to identify and prepare an unrelated donor may be unrealistically long. As a result, interest in autologous bone marrow and blood transplantation (auto-trans) in first CR is likely to continue. In this procedure, high-dose chemotherapy regimens similar to those used in allo-BMT are administered. Hematopoietic rescue from the therapy is accomplished by infusion of the patients own marrow or, more recently, peripheral blood stem cells, collected and stored previously. In auto-trans, relapse can theoretically result not only from an inadequate preparative regimen but from the infusion of leukemia cells in the stored blood or marrow that serves as a graft. This possibility has led to use of techniques to purge such cells of residual leukemia cells. These purging strategies include in vitro treatment of the stored marrow with either chemotherapeutic agents [84] or leukemia cell-specific monoclonal antibodies. [85]

Most favorable reports with auto-trans in first CR with or without purging have been from Europe. [86] [87] [88] [89] [90] Frequently, patients appear to have been referred in CR to the auto-BMT center for the express purpose of undergoing the procedure. This raises the possibility of selection bias; that is, the referring physician may have sent a disproportionate number of patients considered likely to do either particularly well or particularly poorly. Because of these uncertainties, several trials comparing auto-trans in first CR with chemotherapy or, less directly, with allo-BMT have been undertaken. Table 54-6 shows results of the most recent of these. Both in the trials of Cassileth et al. (ECOG) [68] and Zittoun et al. (EORTC, GIMEMA), [67] patients without a suitable donor for an allogeneic transplant were randomized to one course of IDAC or HDAC or an autologous marrow transplant (auto-BMT). The trial reported by Ravindranath et al. (Pediatrics Oncology Group) [72] performed in children and adolescents (ages 021 years) was similar except that more HDAC-based chemotherapy was given than in the ECOG and EORTC/GIMEMA trials. In the trial reported by Burnett et al. (MRC), [69] patients in CR received two to three further courses of consolidation therapy and then, if no donor was available for an allo-BMT, patients were randomized to receive an auto-BMT or to stop therapy. Marrow purging was done in the Cassileth et al. [68] and Ravindranath et al. [72] studies. The data in Table 54-6 indicates that, as with allo-BMT, a significant number of patients did not receive auto-BMT even though randomized to do so. This again indicates the need to include all patients assigned to the treatment in the analysis, as was done in all the studies included in Table 54-6 . Furthermore, the MRC and Pediatrics Oncology Group studies demonstrate that 40% of patients eligible for the randomization do not participate in such a trial because of patient or physician choice. Obviously, such patients are excluded from the results in Table 54-6 . With this consideration in mind, the data in Table 54-6 indicate that only one trial [67] demonstrated a superiority in LFS, and no trial demonstrated a superiority in survival, for auto-BMT compared with chemotherapy (or no further therapy in the MRC study). In summary, there is currently no reason to prefer auto-BMT to chemotherapy in first CR.

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AML IN THE ELDERLY

The median age of patients with AML has been estimated to be 64 years.[92] The age-specific incidence rate increases until 75 years of age. [92] The poor prognosis of elderly patients with AML reflects in part the association of advanced age with poor performance status, unfavorable cytogenetics, MDR1 protein expression, and a history of abnormal blood counts. Thus, the management of an older patient cannot be considered independent

TABLE 54-6 -- Comparison of Autologous Marrow Transplant with Chemotherapy or Allogeneic Marrow Transplant in First CR

Study	Primary Comparison	Patients	Patients Assigned Auto Receiving Auto	LFS		Survival		Statistically Significant Differences ($P < 0.05$)
				Auto (%)	Chemo or Allo (%)	Auto (%)	Chemo or Allo (%)	
Zittoun et al.[67] (1995)	Auto vs. chemo	128 Auto 126 Chemo	74	48	30	56	46	LFS superior in auto
Cassileth et al.[68] (1997)	Auto vs. chemo	11 Auto 118 Chemo	60	37	35	47	54	None
Burnett et al.[69] (1994)	Auto vs. stop treatment	357 Randomized to auto or stop	72	NA	NA	54	52	None
Ravindranath et al.[72] (1996)	Auto vs. chemo	115 Auto 117 Chemo	62	38	36	40	44	None
Sierra et al.[91] (1996)	Auto vs. allo	68 Auto 47 Allo	69	50	31	50	32	None

Auto, autologous bone marrow transplant; chemo, continued chemotherapy; allo, allogeneic marrow transplant; LFS, leukemia-free survival; NA, not available.

of these features, although there is a clear-cut effect of age per se.

Therapeutic approaches in older patients with AML have included (1) no therapy, (2) reduced-dose therapy, and (3) use of GM- or G-CSF. Löwenberg et al. randomly assigned 60 otherwise healthy patients >65 years of age to receive the 3 + 7 regimen or supportive care, the latter continuing until leukocytosis of >50,000, symptomatic thrombocytopenia, organ infiltration, or clinical deterioration occurred, at which time ara-C was commenced. [93] The supportive care-only strategy shortened survival and did not decrease the frequency of hospital admission. These data argue against a supportive care-only approach, unless the patient refuses therapy or is bedridden. In an effort to reduce mortality during induction, investigators have administered attenuated doses of ara-C or daunorubicin, or both. Five randomized[8] [9] [94] [95] [96] and two nonrandomized studies[97] [98] have compared this strategy to administration of usual doses in patients aged >6070 years. The results suggest that although dose reduction may decrease the early death rate, at best it will produce an equivalent CR rate. As noted previously, use of GM- or G-CSF after or concomitant with initial therapy has not in general been successful in improving the outcome in older patients. Most newly diagnosed elderly patients should therefore be considered for investigational approaches in the setting of a formal clinical trial. Exceptions would be patients with favorable cytogenetic abnormalities who are younger than 70 years of age with good performance status and normal organ function. Because the same prognostic factors operate in older as in younger patients,[99] at least some of these patients can expect durable remissions after chemotherapy. The same investigational approaches taken to reduce mortality during induction or decrease resistance in elderly patients could be considered in younger high-risk patients, such as those with poor performance status or unfavorable cytogenetics. These approaches are discussed later.

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ACUTE PROMYELOCYTIC LEUKEMIA

Acute promyelocytic leukemia is distinguished morphologically by the presence of abnormal promyelocytes containing coarse, large granules and numerous Auer rods.[100] (See Plate 53-7 .) A microgranular variant exists that is identical clinically to typical APL but in which the granules can be resolved only with electron microscopy.[101] (See Plate 53-8 .) Ninety percent of patients with APL have a translocation between the long arms of chromosomes 15 and 17 (t [15q;17q]) that can be recognized by standard cytogenetic analysis. The t(15;17) is specific for APL. This translocation results from transposition of the gene for retinoic acid receptor (*RAR*) on chromosome 17 to an area adjacent to the promyelocytic leukemia (*PML*) gene on chromosome 15.[100] *PML* is a tumor suppressor gene and disruption of its function by the translocation may contribute to the pathogenesis of APL. Messenger RNA for the PMLRAR fusion protein, or the protein itself, can be recognized using polymerase chain reaction (PCR) technology.[100]

The chief clinical feature of APL is a bleeding diathesis that results both from plasmin-dependent primary fibrinolysis and disseminated intravascular coagulation. Until the early 1970s, most patients with APL died of central nervous system or pulmonary hemorrhage. This picture has changed drastically as a result of use of (1) frequent platelet and cryoprecipitate transfusions to maintain platelet counts above 30,000/L and fibrinogen above 200 mg/dL, and (2) introduction of daunorubicin-based chemotherapy that effectively shuts off the coagulopathy. With use of these measures, there is no need for routine use of heparin. [102] CR rates should be 7085%, with the higher rates obtained in patients presenting with low white blood cell counts and relatively higher platelet counts, both predicting a relatively low likelihood of fatal hemorrhage.[102] [103] The likelihood of primary resistance in APL is much lower than in other types of AML. The chemosensitivity of APL is also apparent from the lengthier remissions (median 2 years) seen in APL treated exclusively with anthracycline + ara-C than in similarly treated non-APL subtypes.[104]

The introduction of all-*trans* retinoic acid (ATRA) has further changed the management of APL. When used alone, ATRA produces CR rates of 6590%, but remissions are transient if chemotherapy is not added. Table 54-7 summarizes trials comparing ATRA + chemotherapy with chemotherapy alone. Comparisons were made with a group not receiving ATRA in the studies of Fenaux et al. [105] and Tallman et al., [106] and to a historical chemotherapy group in the other trials. In the Fenaux et

TABLE 54-7 -- Comparisons of ATRA + Chemotherapy with Chemotherapy in Newly Diagnosed Acute Promyelocytic Leukemia

Study	ATRA Given During	Patients	CR Rate		Actuarial EFS or Survival[a]		Statistically Significant($P < 0.05$) Effect
			Chemotherapy Only	ATRA± Chemotherapy	Chemotherapy (%)	Chemotherapy + ATRA (%)	
Fenaux et al.[105] (1997)	Induction only	101 (randomized)	81	91	21 EFS 52 Survival	64 EFS 76 Survival	EFS, survival (better with ATRA)
Tallman et al.[106] (1995)	Induction and/or post-CR	327 (randomized)	67	67	57 EFS	92 EFS	EFS (better with ATRA)
Kanamura et al.[107] (1995)	Induction only	173 (97 ATRA + chemo, 45 chemo)	70	89	48 EFS	75 EFS	CR, EFS (better with ATRA)
Frankel et al.[108] (1994)	Induction only	114 (34 ATRA + chemo, 80 chemo)	NA[b]	86	Median survival >31 mo	Median survival 17 mo	Survival (better with ATRA)
Estey et al.[109] (1997)	Induction only	100 (43 ATRA + chemo, 57 chemo)	68	77	EFS: 95% CI, 4369	EFS: 95% CI, 5282	EFS, survival (better with ATRA)

ATRA, all-trans retinoic acid; CR, complete remission; Chemo, chemotherapy; EFS, event-free survival; CI, confidence interval.

[a] Actuarial estimates at 3 years in study of Fenaux et al., 1 year in study of Tallman et al., 2 years in study of Kanamura et al., and beyond 1 year in study of Estey et al.
[b]Not given, but early death rates statistically similar in the two groups.

al.,[105] Tallman et al.,[106] Kanamuro et al.,[107] and Frankel et al.[108] studies, induction therapy was to consist solely of ATRA. However, to prevent the ATRA syndrome (see later), patients received chemotherapy when their white count was elevated. In the Estey et al. [109] study, all patients began idarubicin 15 days after ATRA, again depending on their leukocyte count. In all the studies, patients in CR received chemotherapy regardless of whether they had received ATRA during induction. Patient follow-up appears to be longest in the study of Fenaux et al. This study noted a striking difference in event-free survival rates (and, to a lesser extent, overall survival). This reflected a much lower recurrence rate in the ATRA group. This pattern of improvement in event-free survival in ATRA-treated patients out of proportion to an improvement in CR rate has been noted in four of the five studies listed in Table 54-7 . The results suggest that the major effect of ATRA in APL is to enhance sensitivity to chemotherapy.

Questions regarding ATRA in APL revolve around management of the ATRA syndrome and the optimum timing of ATRA administration. The potentially fatal ATRA syndrome develops in approximately 25% of patients and is characterized by fever, dyspnea, pleural and pericardial effusion, and hypotension. [108] There are two schools of thought regarding management. The first considers the leukocytosis often induced by ATRA a risk factor for development of the ATRA syndrome, and suggests adding chemotherapy whenever leukocytosis develops.[105] The second, popularized by the group at Memorial Sloan-Kettering in New York, does not regard leukocytosis as predictive of the ATRA syndrome,[108] [110] and adds dexamethasone 10 mg IV q6h, but not chemotherapy, at the first clinical sign of potential ATRA syndrome. Other toxicities of ATRA are rash, hearing loss, and headache, the latter perhaps reflecting pseudotumor cerebri possibly caused by ATRA-induced venous thromboses. Regarding timing, the usual approach has been to restrict use of ATRA to induction therapy. The Tallman et al. study [106] randomized patients in CR after two courses of consolidation therapy to receive ATRA or no further therapy. Hence, the study consisted of four groups: (1) ATRA induction, ATRA + chemotherapy post-CR; (2) chemotherapy induction, ATRA + chemotherapy post-CR; (3) ATRA induction, chemotherapy post-CR; and (4) chemotherapy induction, chemotherapy post-CR. Relapse rates were similar in groups 13 and highest in group 4 ($P < 0.00001$). This result clearly demonstrated the beneficial effects of ATRA but showed that it was irrelevant whether ATRA was used during induction or during remission.

The optimal chemotherapy for APL is also under discussion. A SWOG study [104] suggested that long-term outcome was better in patients who received more daunorubicin rather than more ara-C. Estey et al.[109] reported a trial in which ara-C was omitted, permitting use of more anthracycline. Results were better than those reported in historical control subjects, suggesting the feasibility of this approach. It is unclear whether chemotherapy should begin only when the patient is in

ATRA-induced CR, or together with ATRA during initial induction. A randomized study by Fenaux et al. is addressing this issue. To date, CR rates are over 90% in both groups but, as usual, lower in elderly patients. [105]

Use of molecular testing to detect the t(15;17) translocation is important in two phases of the management of APL. First, such testing is mandatory in cases in which a coagulopathy or morphologic appearance suggests APL but in which the t(15;17) cannot be demonstrated on standard analysis. [111] This is particularly important because APL is, together with inv(16) AML and t(8;21) AML, among the curable types of AML, particularly if treated with ATRA + anthracyclines. The second use of molecular testing is in follow-up of patients in remission. After ATRA followed by three courses of chemotherapy, >90% of patients should be PCR negative (i.e., no evidence of the t[15;17] on molecular testing done at a sensitivity of 10^{-4}).[112] [113] If not PCR negative or if a negative PCR becomes persistently positive, therapy should be changed (e.g., an allo-BMT). [112] [113] Likewise, once two negative PCRs are obtained several months apart, therapy can probably be stopped. [113]

Recurrence in APL, although likely to be relatively infrequent, remains difficult to treat. Second CRs after ATRA are common if the last exposure to ATRA occurred greater than 612 months before relapse, but long-term CRs are rare. [115] Three areas of research in this area are of interest. First is the use of new retinoids, particularly 9-*cis*-retinoic acid and liposomal ATRA. The former binds to retinoic acid receptors that remain unbound after standard oral ATRA, [116] whereas use of liposomal ATRA permits maintenance of serum tretinoin concentrations longer than possible with oral ATRA. [117] Second is the use of anti-CD33 monoclonal antibodies bound to toxins, with the antibodies intended selectively to target the APL cells. [118] Third is the use of arsenicals (e.g. arsenic trioxide, As_2O_3). A report from China[119] cited a CR rate of 9 of 10 in patients with relapsed APL refractory to ATRA + chemotherapy, although the definition of refractory is somewhat unclear.

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PROGNOSTIC FACTORS WITH STANDARD THERAPY

It is important to note that the results of standard therapy described in earlier sections, namely 6070% CR rates with <20% of all patients achieving long-term disease-free survival, are average results. The study of prognostic factors is important because it may permit recognition of groups of patients with outcomes that differ from the average. If these outcomes are sufficiently favorable, standard therapy might be sufficient. Knowledge of the likely prognosis of patients treated in an experimental study had they received standard therapy is also crucial in interpreting results of such trials.

Prognostic factors (Table 54-8) can be divided into those primarily associated with early death and those primarily associated with resistance to chemotherapy. The principal factors associated with early death are older age, poor pretreatment performance status, and abnormal organ function. [120] The effect of age is also mediated through the association of older age with unfavorable cytogenetics and a history of abnormal blood counts. The characteristic most associated with sensitivity or resistance to standard chemotherapy is pretreatment leukemia cell karyotype (cytogenetics). [121] [122] [123] [124] Excluding the translocation between the long arms of chromosomes 15 and 17 (t[15;17]), which is pathognomonic of APL, three cytogenetic groups can be distinguished. In the first group are patients with either a percentric inversion of chromosome 16 (inv[16] associated with FAB subtype M4EO) or a translocation between chromosomes 8 and 21 (t[8;21] associated with FAB subtype M2). These patients have CR rates of 90% and, more important, 4050% can be cured with standard chemotherapy. [121] [122] [123] [124] Given that these two groups share a better prognosis, the finding that the pathogenesis of these two types of AML likely involves the same core binding transcription factor (AML-1 protein family) is of interest. [125] Patients with a normal karyotype (about one third of patients, making this the most common cytogenetic finding) constitute an intermediate group, with a CR rate of 6070% but a long-term disease free survival rate of only 1025%. [121] [122] [123] [124] Patients with other cytogenetic findings, particularly with complex abnormalities involving chromosomes 5 or 7, have CR rates of 50% and are essentially incurable with standard therapy. [121] [122] [123] [124] The unfavorable prognosis of secondary AML (i.e., developing after chemotherapy for another malignancy) is largely due to its association with unfavorable cytogenetic abnormalities, [126] in particular abnormalities of chromosomes 5 or 7 in patients previously treated with alkylating

TABLE 54-8 -- Established Prognostic Factors for Response to Standard Chemotherapy in Newly Diagnosed Acute Myeloid Leukemia

Factor	Principal Effect on	Favorable if	Complete Remission Rate		Long-term Disease-Free Survival		Reference
			Favorable Group	Unfavorable Group	Favorable Group	Unfavorable Group	
Age	Early death	Younger (e.g., <60 y)	70%	50%	1520%	5%	[120]
Performance status	Early death	Zubrod <3	70%	30%	1520%	<5%	[120]
History of abnormal blood counts	Resistance to therapy	Absent	70%	50%	1520%	5%	[121]
Cytogenetics	Resistance to therapy	inv(16) or t(8;21)	90%	30%	4050%	<5%	[121] [122] [123] [124]
History of chemotherapy for prior malignancy	Resistance to therapy	Absent	6070%	4050%	10%	5%	[126]
MDR expression on blasts	Resistance to therapy	Absent	67%	34%	Same in both groups		[130]

MDR, multidrug resistance.

agents and a deletion of the long arm of chromosome 11 (11q) in patients treated with topoisomerase II-reactive drugs. In contrast, patients with t(15;17) appear to do equally well regardless of whether it develops de novo or as secondary AML. [127] Similarly, the poor prognosis of patients with the rare AML subtypes M6 (erythroleukemia) and M7 (megakaryocytic leukemia) probably largely reflects the association of M6 and M7 with unfavorable cytogenetic abnormalities. [128] [129] The most recently recognized, well established prognostic factor is expression of the multidrug resistance glycoprotein (MDR1) in patients older than age 55 years. [130] This effect is independent of cytogenetics. Although not predictive of survival or relapse-free survival once in CR, MDR1 expression was associated with resistance to, rather than early death after, chemotherapy. During the next few years, increasing numbers of prognostic factors that, like MDR1, are based on laboratory rather than clinical parameters, are likely to be identified. Examples of such prognostic factors awaiting further confirmation include expression of (1) the thrombopoietin receptor (reported unfavorable for CR duration), [131] (2) the immunoglobulin heavy chain gene rearrangement (unfavorable for CR and survival), [132] (3) expression of the transcription factor GATA-1 and the stem cell leukemia gene (unfavorable for CR and survival), [133] and (4) bcl-2 protein and WT-1, a transcription factor regulating bcl-2 (unfavorable for survival). [134] Assays measuring chemotherapy-induced apoptosis (mediated by bcl-2 and other similar proteins) are also likely to be tested for prognostic significance. [135] Finally, low levels of the retinoblastoma gene protein have also been associated with poorer prognosis. [136] It is of course important to consider the effect of prognostic factors when analyzing the therapeutic trials in AML listed in Table 54-2 when taken together. For example, elderly patients (>55 years) with de novo AML, with normal or favorable karyotypes, and whose blasts do not express MDR1, had an 81% CR rate, considerably higher than the average 50% CR rate for patients this age. [130] However, these patients with a favorable prognosis constituted only 25% of all elderly patients with de novo AML and only 20% of all elderly patients. Similarly, <10% of patients will have inv(16) or t(8;21).

The prognostic factors noted previously were largely identified in patients given standard induction and postremission therapy. An important question is whether these same prognostic factors are equally important regardless of which treatment is given. This question has been addressed for HDAC in two studies. [137] [138] Both found that HDACs biggest impact was in patients with t(8;21) or inv(16) in whom cure rates well in excess of 50% can be expected with HDAC-containing regimens. In the CALGB [137] but not the M. D. Anderson study, [138] there was evidence that both IDAC (400 mg/m^2 daily × 5) and HDAC improved outcome in patients with a normal karyotype. In these patients however, even with HDAC, the cure rate was estimated to be considerably less than 50%. Both the CALGB and M. D. Anderson studies found no beneficial effect of HDAC in patients with other karyotypes. Preliminary results from the SWOG [139] suggest that cytogenetics is prognostically significant after allo-BMT. However, data on the effect of cytogenetics on results of allo- or auto-BMT have come primarily from the British MRC. The MRC has reported that cytogenetics and marrow blast percentages after the first course of induction therapy are similarly associated with outcome regardless of whether patients subsequently receive chemotherapy, auto-BMT, or allo-BMT. [140] [141] Specifically, allo-BMT and chemotherapy do equally poorly in patients with unfavorable karyotypes. Any small advantage for allo-BMT is in patients with a normal karyotype, whereas patients with inv(16) or t(8;21) do worse after allo-BMT than after chemotherapy. Trials comparing auto-BMT versus stopping therapy after achievement of CR have indicated no differences in LFS in patients with unfavorable

karyotypes. In patients with favorable karyotypes, the lower relapse rate after auto-BMT was counterbalanced by a higher CR rate after salvage therapy in the stop group, whereas in the standard risk group the lower relapse rate with auto-BMT was counterbalanced by a higher death rate in CR. One can conclude from these studies that new therapies are needed for most patients with AML, except patients with inv(16), t(8;21), or APL. With these considerations in mind, Figure 54-1 illustrates a suggested approach to the management of newly diagnosed AML.

Prognostic factors must also be considered when assessing the results of all clinical trials. Specifically, many trials exclude

Figure 54-1 Suggested management of newly diagnosed acute myeloid leukemia. AHD, antecedent hematologic disorder (i.e., history of abnormal blood counts); AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATRA, all- *trans* retinoic acid; BMT, bone marrow transplant; CR, complete remission; HDAC, high-dose ara-C; MDR1, multidrug resistance protein; PCR, polymerase chain reaction; PS, performance status; Rx, treatment; SDAC, standard-dose ara-C.

patients older than age 70 years or with a history of abnormal blood counts, or secondary AML. The patients excluded can constitute up to 50% of patients with newly diagnosed AML. The invariable effect of such exclusions is to make results appear more favorable. [142]

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TREATMENT OF REFRACTORY/ RELAPSED AML

Recurrence of AML occurs in most newly diagnosed patients who attain CR. The most important predictor of outcome after recurrence is length of first CR. [143] At one extreme of the spectrum are patients whose initial CR lasted 2 years; at the other extreme are patients with first CRs lasting <1 year. If the regimen used initially is repeated, 5060% of the former but only 1030% of the latter patients achieve CR. More important, this approach may result in a 3-year disease-free survival rate of 2025% in patients with a long first CR but virtually a zero 3-year survival rate in patients with a short first CR. The prognosis of patients who fail initial induction therapy (with failure usually defined as no CR after two courses) is quantitatively similar to that of the patients with the short first CR. There is no indication that HDAC or the commonly used mitoxantrone + etoposide regimen improves outcome in patients with short or no first CRs, with the latter patients called primary refractory. [143] In contrast, data from the City of Hope[144] and the IBMTR[145] suggest that results are better in primary refractory patients given an allo-BMT. The general consensus is that patients younger than age 55 years whose AML is primary refractory, or recurs after a CR of <12 years after standard or HDAC-containing chemotherapy, should receive an allo-BMT if a suitable donor is available

Figure 54-2 Suggested management of relapsed acute myeloid leukemia. BMT, bone marrow transplant; CR, complete remission; HDAC, high-dose ara-C.

(matched or unmatched). Allo-BMT should not be delayed until second CR. [146] The IBMTR compared chemotherapy with allo-BMT as therapy in second CR. [147] Subject to the criticism that the transplanted patients were referred to IBMTR hospitals because they were thought to be relatively healthy, the results indicate that LFS was superior with transplantation if the patient was younger than 30 years of age with an initial CR 1 year (3-year actuarial LFS rates of 41% vs. 17%), or older than age 30 years with an initial CR <1 year (rates of 18% or 7%). Other patients had similar outcomes with allo-BMT and chemotherapy. Results from the GIMEMA[148] group also suggest a superiority for allo-BMT in second CR. The British MRC investigated the usefulness of auto-BMT in second CR. [149] Patients in first CR after four cycles of chemotherapy had storage of autologous marrow, with half the patients then randomized to receive an auto-BMT and the other half to receive the procedure only in a second CR. Thus, in second CR, patients would either receive or not receive auto-BMT, the latter patients having had an auto-BMT in first CR. There has been no difference in outcome in second CR among patients who did or did not receive an auto-BMT at this time.

Figure 54-2 illustrates an approach to management of refractory or relapsed AML. If an allo-BMT cannot be performed, patients with first CRs of >1 year should receive an induction regimen similar to that used initially. In CR, however, they can be offered investigational therapies. Patients with shorter first CRs or who are refractory to initial therapy should be offered investigational therapies at relapse if allo-BMT is not an option.

As with chemotherapy, the prognosis after relapse following allo-BMT depends heavily on duration of CR. [150] [151] Data suggest that remissions in some patients can be produced by the administration of G-CSF[152] or by donor lymphocyte infusions, [153] which presumably stimulate the graft-versus-leukemia effect. If such therapy is unsuccessful and the patient had a CR of >1 year after the previous allo-BMT, consideration should be given to a second allo-BMT. Otherwise, the patients should be referred for investigational therapy.

INVESTIGATIONAL APPROACHES

The ultimate role, if any, of the approaches described here awaits further patient entry and correlation of results with standard prognostic factors.

Chemotherapy

Topoisomerase I Inhibitors

These represent a new class of agents. Topotecan, the prototype of such agents, has been found to have single-agent activity [154] and is being combined with other active agents.[155] Toxicities of such therapy are myelosuppression and mucositis. Other drugs of this class are under investigation (e.g., 9-aminocamphothecin).

MDR Reversing Agents

Given the association of MDR1 expression with resistance to chemotherapy in elderly patients with AML, there has been interest in overcoming the MDR1-positive phenotype with drugs such as cyclosporine, quinine, and PSC-833.[156] List et al. reported that cyclosporine + daunorubicin + HDAC gave a CR rate of 67% in 15 patients with AML, most of whom had first remissions at <1 year or were primary refractory.[157] It was unclear if the responses reflected reversal of MDR or rather administration of effectively higher doses of daunorubicin consequent to cyclosporines blocking of the hepatic excretion of the drug. In a randomized study, Wattel et al.[158] noted that, provided their blasts expressed MDR, patients had a better CR rate and survival if they were given quinine in addition to mitoxantrone + IDAC. This result suggested a specific effect on MDR-mediated drug resistance. A qualitatively similar result was reported by Solary et al. [159] PSC-833, a cyclosporine analog with high anti-MDR1 and no immunosuppressive effects, was tested with mitoxantrone plus etoposide in a phase I trial. [160] Addition of PSC necessitated a 66% reduction in doses of these drugs. PSC-833 is undergoing phase II and III testing in cooperative group settings.

Toxins Bound to Antibodies

In this approach, a toxin is conjugated with a monoclonal antibody specific for AML blasts to target AML cells selectively. A frequently used antibody is anti-CD33. CD33 is expressed on the surface of most AML blasts, but not on the surface of normal stem cells. Remissions with this approach have been reported in relapsed/refractory cases.[161]

Fludarabine

The purine analog fludarabine can increase formation of ara-CTP by myeloblasts. [162] The combination of fludarabine + ara-C + G-CSF was shown equivalent to idarubicin + ara-C in one study.[133] As with HDAC itself, the fludarabine + ara-C combination may be most useful in patients with ara-Cresponsive disease.

Allogeneic Transplantation

Minitransplant

Giralt et al. demonstrated that engraftment of allogeneic hematopoietic progenitors could be accomplished with purine analog (fludarabine or 2-chlorodeoxyadenosine)-containing chemotherapy without need for the usual myeloablative preparative regimen. [163] The patients treated had a median age of 59 years and ranged up to age 71 years. This approach relies for its efficacy on graft-versus-leukemia effect and is being tested further particularly in older patients or patients with poor performance status, who would not otherwise be eligible for allo-BMT.

Immunologic Approaches

In 1993, Giralt et al.[152] demonstrated that G-CSF could produce remissions in AML that had recurred after allo-BMT. Although the remissions were brief, the results demonstrated the feasibility of manipulation of immune effector cells in the graft. Another approach has involved infusion of allogeneic lymphocytes. [153] Although to date most effective in chronic myeloid leukemia, donor lymphocyte infusions can produce remissions after recurrence of AML following allo-BMT and, more important, will lead to further efforts at stimulating the graft-versus-leukemia effect.

Unrelated Donor Transplants

Although only 3040% of patients have an HLA-compatible sibling, the development of a large computerized registry has increased the likelihood of finding at least an HLA-A, B, DR matched donor from 10% in 1987 to 64% in 1995.[164] The median time from initiation of the unrelated donor search to transplantation is 56 months. [165] The IBMTR[166] reported that compared with a transplant from an HLA-identical sibling donor, the relative risk of treatment failure among patients with acute leukemia in first CR or chronic myeloid leukemia was 2.43 (all *P*-values <.02) with a oneHLA antigen-mismatched related donor, 3.79 with a twoantigen-mismatched related donor, 2.11 with an HLA-matched unrelated donor, and 3.33 with a oneHLA antigen-mismatched unrelated donor. Among patients with more advanced leukemia, relative risks for a oneantigen-mismatched related donor were 1.18 (*P* = NS), and 1.39 (*P*= 0.002) for an HLA-matched unrelated donor, with of course overall results for advanced leukemia being worse than for leukemia in CR. Seattle investigators [165] noted actuarial LFS rates of 12% at 5 years for relapsed AML in patients receiving unrelated donor transplants, ranging from 30% for patients transplanted in relapse but with <30% blasts in marrow and none in blood, to 0% at 2 years for patients with blasts in blood. Patient outcome was improved if the size of the graft was increased.

Timing of Transplantation

Anderson et al.[167] demonstrated the feasibility of allo-BMT as initial therapy in patients with secondary AML with low blast counts. Such patients of course would be expected to have low CR rates with standard remission induction therapy.

Autologous Transplantation

Blood count recovery appears quicker when blood rather than marrow is the source of the stem cells. [168] The relative ease with which peripheral blood stem cells can be collected suggests the possibility of repeated stem cell collections to support multiple cycles of high-dose regimens, particularly in patients with chemotherapy-sensitive disease.

Supportive Care

Because fungal, particularly *Aspergillus*, infections are a leading cause of mortality in AML, [3] [10] it will be important to determine the value of liposomal amphotericin preparations. Finally, the availability of GM- and G-CSF may permit collection of sufficient granulocytes from normal donors [169] [170] to test the utility of prophylactic or therapeutic granulocyte transfusions, particularly in high-risk patients such as the elderly or those with impaired performance status.

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MONITORING OF MINIMAL RESIDUAL DISEASE

Approaches to detect minimal residual disease in patients in CR by more sensitive criteria are in progress. These include fluorescence in situ hybridization (FISH) [171] and PCR.[172] [173] Whereas the usual cytogenetic techniques are logistically limited to analysis of approximately 25 cells, FISH, and especially PCR, permit

examination of a several-log higher number of cells to detect residual cytogenetic abnormalities. Although use of PCR is established in APL, [112] [113] [114] in t(8;21) AML it has been demonstrated that despite positive PCRs patients can remain in CR for years. [174] Hence the value of these techniques may vary depending on the cytogenetic abnormality being monitored.

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Chapter 55 - Acute Myeloid Leukemia in Children

Howard J. Weinstein

The acute myeloid leukemias (AML) represent a clinically and biologically heterogeneous group of diseases caused by the malignant transformation of a myeloid stem cell or progenitor cell. [1] The proliferative advantage of the leukemic stem cell coupled with an impairment in differentiation leads to the accumulation of immature or blast cells in the bone marrow. The blasts eventually suppress normal hematopoiesis and infiltrate other organs and tissues. AML has been classified into eight subtypes (M0M7) by a French-American-British (FAB) cooperative group based primarily on blast cell morphology and reactivity with histochemical stains (see Chap. 53).

AML accounts for about 20% of acute leukemias in children and 80% of acute leukemias in adults. The biology of AML, and its response to chemotherapy, is quite similar in children and young adults (age <50 years). One difference is the more frequent extramedullary involvement of AML in infants and young children compared to adults.

In contrast to cure rates for childhood acute lymphoblastic leukemia (ALL), cure rates for AML have improved only modestly over the past two decades. [2] [3] Approximately 40% of children treated with chemotherapy alone appear to be cured of their leukemia. The outcome is somewhat better for children who receive bone marrow transplants from histocompatible sibling donors in first remission. [2] Greater understanding of the biologic and genetic heterogeneity of the acute myeloid leukemias is beginning to lead to better classification schemes and new therapeutic strategies. [4] [5] [6]

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EPIDEMIOLOGY

Approximately four hundred new cases of AML are diagnosed in children each year in the United States. [7] In contrast to the early age peak (34 years) in childhood ALL, the annual incidence of AML is quite constant from birth throughout the first 10 years with a slight peak in late adolescence. AML appears to account for the majority of the very rare cases of congenital leukemia. The predominance of AML in this age group may in part be due to the inclusion of infants with the transient myeloproliferative syndrome associated with Down syndrome (see discussion later in this chapter).

The incidence and subtypes of acute leukemia in children do not have significant geographic variation. In Japan and several African countries, however, there are more cases of childhood AML than ALL.[8] [9] Patterns of presentation also vary. For example, an unusually high percentage of children diagnosed with AML in Turkey and several African countries present with myeloblastomas or chloromas involving the orbital area. [10]

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ETIOLOGY

As in other forms of leukemia, the etiology of AML is unknown. The vast majority of children with AML have no obvious predisposing factors. Known risk factors include several congenital/genetic disorders, ionizing radiation, and certain drugs or toxic exposures (Table 55-1).[11] [12] [13] [14] [15] [16] [17] [18] [19] [20] [21] [22] [23] [24] For example, occupational exposure to benzene and treatment with alkylating agents or topoisomerase-2-inhibitors (especially the epipodophyllotoxins) for malignant or benign disorders are associated with an excess incidence of AML. [21] [24] In contrast to alkylating agent-induced AML, the secondary leukemias observed after treatment with etoposide or teniposide are usually of the FAB M4 or M5 subtypes, have a shorter latency period (24 years), and usually have chromosomal translocations involving band 11q23(MLL gene rearrangement). MLL gene rearrangements are also present in precursor B cell ALL (especially in infants), [25] [26] [27] and de novo AML of the FAB M4 and M5 subtypes. The likelihood of developing secondary AML from epipodophyllotoxin therapy appears to be schedule- and cumulative-dose-dependent. Increased risk has been noted with twice-weekly or weekly administration of etoposide/teniposide. [24]

TABLE 55-1 -- Congenital Disorders or Acquired Factors Predisposing to AML

Genetic factors
Down syndrome
Fanconi anemia
Blooms syndrome
Neurofibromatosis type I
Klinefelter syndrome
Turner syndrome
Congenital bone marrow failure syndromes
Kostmann syndrome
Diamond-Blackfan anemia
Drugs
Benzene
Alkylating agents
Epipodophyllotoxins
Ionizing radiation
Myelodysplastic syndromes

Children who were exposed to radiation from the atomic bombs in Japan had an increased incidence of leukemia, but no excess of leukemia was noted in Japanese children who were exposed prenatally to the atomic explosions. [20] Several studies have addressed whether exposure to low-frequency, nonionizing radiation (e.g., electromagnetic fields) is leukemogenic. [22] A recently reported study did not find an increased risk of acute lymphoblastic leukemia in children exposed to residential magnetic fields. [23]

Unusual susceptibility to AML has been observed in monozygotic twins and in children with certain genetic disorders. The increased concordance of acute leukemia observed in identical twins (approximately 15%), especially during the first year of life, results from transplacental passage of a single leukemic clone rather than a genetic predisposition in some cases. [11] Patients with diseases associated with chromosome fragility and impaired DNA repair mechanisms (e.g., Fanconis anemia and Blooms syndrome) are predisposed to develop AML. [9] Children with congenital disorders of granulopoiesis and erythropoiesis, such as Kostmanns syndrome and Diamond-Blackfan anemia, are also at increased risk for developing AML. [12] [13]

Children with Down syndrome (trisomy 21) have about a 15-fold increased risk of developing leukemia during the first 10 years of life. [14] From birth through 3 years of age, the incidence of AML (especially FAB M7) is much greater than ALL, and thereafter the ratio of ALL to AML follows the usual childhood distribution. [15]

Some neonates with trisomy 21 may manifest a transient myeloproliferative syndrome (TMS). This intriguing syndrome is diagnosed in neonates with trisomy 21 during the first week of life. [15] [16] [17] In most cases it cannot be readily distinguished from congenital AML. It is nearly always associated with complete clinical and hematologic recovery without therapy within weeks to two months. Infants with TMS may have very elevated white blood cell counts with circulating blasts (megakaryoblasts or erythroblasts), hepatosplenomegaly, and an increased percentage of blasts in the bone marrow. In several cases, the blasts have been shown to be clonal in origin. [15] [16] TMS has also been observed in phenotypically normal newborns who were mosaic for trisomy 21.

Data from retrospective surveys indicate that 2030% of infants who had TMS will eventually develop AML (mostly M7) before 4 years of age. [17] It is not known whether this is a recurrence of the original disease or the appearance of a new disease. Prior to the onset of AML, there is often a several-month prodrome characterized by thrombocytopenia and bone marrow myelofibrosis with dysplastic megakaryocytes. An unexpected finding has been the high cure rate of AML in children with Down syndrome, including those who had TMS as neonates. [28] [29] This may in part be due to an increased in vitro sensitivity of Down syndrome myeloblasts to cytarabine and daunorubicin and the greater generation of ara-C triphosphate (ara-CTP) compared to myeloblasts from children without Down syndrome. [30]

Children with neurofibromatosis (NF 1) are at increased risk for developing acute and chronic myeloid leukemias as well as certain benign and malignant tumors that primarily arise in cells derived from the embryonic neural crest. [18] In patients with NF 1 and leukemia, loss of both NF-1 alleles in bone marrow blasts has been detected in some cases. [19] These data are consistent with a tumor-suppressor function for the NF-1 gene. Inactivation of NF 1 may contribute to leukemogenesis by dysregulating the Ras signaling pathway.

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CELL OF ORIGIN AND BIOLOGIC PROPERTIES OF AML

Cytogenetic studies and assays using X-linked polymorphisms have confirmed the clonal nature of AML. [31] [32] [33] The chromosomal changes are varied but predominantly involve balanced translocations, deletions, and inversions. [33] These cytogenetic findings are restricted to the leukemic cells. The bone marrow karyotype returns to normal in complete remission. At relapse the original clone reappears (plus or minus additional genetic changes), suggesting that the leukemic clone was suppressed but not eliminated by treatment. In very rare situations, persistent clonal hematopoiesis has been observed in remission. [31] [34] The significance of a clonal remission is not known but in some cases may be due to skewed lyonization.

The precise target in hematopoiesis for the transforming event in AML has been the subject of investigation and controversy. Theoretically it could occur at any stage from the pluripotent stem cell to a committed precursor such as a myeloblast. [1] [35] Markers of clonality have been useful in addressing this issue. In several adult patients with AML, the clonal marker has been detected in both erythroid and granulocytic cells suggesting transformation of a less-committed progenitor (colony-forming unit granulocyte, erythroid, megakaryocyte, macrophage [CFU-GEMM]). [34] Recent data obtained from inoculating human adult AML cells in NOD/SCID mice suggests that a primitive normal stem cell rather than a committed progenitor is the target for leukemic transformation. [1] In the few studied pediatric cases of AML, the clonal marker has been noted only in cells of the granulocytic or monocytic lineage, suggesting involvement of a more committed progenitor (granulocyte-macrophage colony-forming unit [CFU-GM]). [34]

The principal defect in AML appears to be an arrest in the normal differentiation pathway of myeloid progenitors or precursors rather than abnormal growth kinetics. [4] [36] The molecular mechanism that underlies these blocks in differentiation is largely unknown. Most AML cells maintain their requirement for hematopoietic growth factors for sustained in vitro growth, although a minority exhibit spontaneous growth. [35] In the case of acute promyelocytic leukemia (FAB M3), characterized by the fusion of the PML and RAR alpha genes as a result of the translocation t(15;17), cells are arrested at the promyelocyte stage but the block in differentiation can be overcome (in vitro and in vivo) by pharmacologic doses of all-trans-retinoic acid. [37] Other attempts to treat patients with AML with other differentiating agents (e.g., vitamin D analogues and low-dose cytarabine) have been of limited success. [38]

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CLASSIFICATION

Careful examination of the bone marrow aspirate is required to establish the diagnosis of acute leukemia because as many as

TABLE 55-2 -- FAB Subtypes of Recurring Chromosome Abnormalities, and Clinical Features

FAB Subtype	% of Total	Chromosome Abnormality	Clinical or Laboratory Features
M0	2	5 or del (5), 7 or del (7)	Blasts often express CD34 and terminal deoxynucleotidyl transferase (TdT)
M1	1018		
M2	2729	t(8;21) (q22;q22) t(6;9) (p23;q34)	Myeloblastomas (especially orbital)
M3	510	t(15;17) (q22;q21)	Disseminated intravascular coagulation
M4eo		inv(16) (p13;q22) or t(16;16) (p13;q22)	CNS leukemia, eosinophilia
M4	1625	t(9;11) (p22;q23) t(11;19) (q23;p13.1) t(10;11) (p12;q23)	Congenital leukemia and young age (<2 yrs), extramedullary leukemia (especially leukemia cutis), hyperleukocytosis
M5	1322	t(9;11) (p22;q23) t(11;19) (q23;p13.1) t(10;11) (p12;q23)	Congenital leukemia and young age (<2 yrs), extramedullary leukemia (especially leukemia cutis), secondary leukemia after epipodophyllotoxins, hyperleukocytosis
M6	13		
M7	48	t(1;22) (p13;q13)	Infants <1 year with t(1;22), myelofibrosis, Down syndrome
All		+8	Prior myelodysplastic syndrome
All		5 or del (5) (q11-q35)	Older adults, toxic exposure, prior myelodysplastic syndrome
All		7 or del (7) (q22-q36)	Older adults, toxic exposure, prior myelodysplastic syndrome

20% of patients with acute leukemia do not have circulating blasts at diagnosis. Also, the morphology of leukemic cells in the peripheral blood may differ from that of cells in the bone marrow. Various methods are available for characterizing the blast cell population in patients with acute leukemia These include morphologic interpretation of Romanowsky-stained specimens in conjunction with cytochemistry, chromosome analysis, immunophenotype, and molecular genetics. Precise diagnosis and classification are essential to successful treatment and biologic investigation of the childhood leukemias. [3]

AML has been traditionally classified by the pattern of myeloid lineage differentiation noted in the bone marrow (e.g., myeloblastic or granulocytic, monocytic, erythroid, or megakaryocytic lineage). In 1976, the FAB Cooperative group proposed a classification system based primarily on morphologic and cytochemical features of the blast cells with subsequent revisions to include immunophenotypic or electron microscopic confirmation for the M0 and M7 subclasses. [34] [35] [36] [37] [38] [39] [40] [41] The FAB group recognizes eight subgroups of AML and also requires a minimum of 30% bone marrow blasts for the diagnosis of AML. This is an arbitrary cutoff that is sometimes problematic. For example, patients with a t(8;21) may have <30% bone marrow blasts but are as likely to achieve a durable remission as are patients with >30% bone marrow blasts and a t(8;21).[42] For treatment purposes, these patients should be considered to have AML rather than a myelodysplastic syndrome (MDS).

The distribution of FAB subtypes in most children is similar to that seen in young adults with AML (Table 55-2) except for children under 2 years of age, who tend to have M4 or M5.[43] [44] Infants and toddlers with M4 and M5 often present with hyperleukocytosis and extensive extramedullary leukemia, especially leukemia cutis and central nervous system (CNS) disease. Because of the rarity of the M0 subtype in children, no special clinical or biologic associations have been noted thus far. The M7 subtype is most common in children younger than 3 years of age, especially those with Down syndrome.[45]

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IMMUNOPHENOTYPE ANALYSIS IN AML

Normal hematopoietic cells undergo changes in expression of cell surface markers as they mature from stem cells into cells of a committed lineage. Monoclonal antibodies have been developed that react with lineage-specific and stage-specific lymphoid and myeloid activation and differentiation antigens. [46] By using a combination of monoclonal antibodies recognizing B-cell, T-cell, and myeloid antigens, it is possible to confirm the diagnosis of AML and differentiate AML from ALL if morphology and histochemistry are inconclusive (<15% of cases). Several classification schemes of AML based on immunophenotype have been proposed, but these do not provide additional prognostic or treatment-related information beyond that of the FAB system. [47] [48]

Several unusual immunophenotypes of AML blasts have been described that are FAB subtype-specific and also associated with unique chromosomal changes. For example, M2 AML with t(8;21) is characterized by expression of the myeloid and stem cell antigens CD13, CD15, CD34, HLA Dr, and expression of the B cell antigen CD19 and the neural adhesion molecule CD56. [49] In a recently published adult AML study, CD56-positive AML with t(8;21) was associated with shorter remissions and overall survival compared to other cases with t(8;21). [49]

It has been estimated from several studies that about 515% of patients with acute leukemia have morphologic, cytochemical, immunophenotypic, or genetic evidence of more than one hematopoietic lineage. Approximately 1025% of AML patients demonstrate lymphoid antigen expression on myeloid blasts and similarly 425% of ALL patients demonstrate expression of at least one myeloid antigen on the blast cell surface. [47] [48] [50] In most of these cases of hybrid or acute mixed-lineage leukemia there is co-expression of lymphoid and myeloid markers on the same blast, but in rare cases there have been two distinct populations of blasts. [50] The pathogenesis of these mixed-lineage leukemias remains poorly understood. They may represent a leukemic transformation in a primitive stem cell or the aberrant expression of a lymphoid gene in a myeloid leukemia.

In a pediatric study, 14 of 16 patients with lymphoid antigen-positive AML expressed a T-cell marker. [50] Interestingly, several of these patients had a poor response to AML induction therapy but subsequently responded to an ALL induction regimen. Mixed-lineage expression in pediatric AML, however, has not been shown to have prognostic significance. [47] [48] Children with AML whose blasts express lymphoid antigens should be treated on AML protocols.

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GENETICS OF AML

The chromosomal abnormalities detected in blasts from the majority of children with AML are important for diagnostic and prognostic purposes, and equally important for cloning genes involved in leukemogenesis. [33] [51] [52] Common repetitive cytogenetic abnormalities observed in cases of AML are listed in Table 55-2 in relation to the FAB classification. Monosomies or deletions of chromosomes 5 and/or 7 and trisomy 8 are not associated with a specific FAB subtype of AML, and are most commonly detected in older adults. In contrast, the balanced translocations and inversions are more frequently observed in children and young adults with AML. [33] Most of the described chromosome changes are not unique to a specific age group, except for the t(l;22)(pl3,ql3) that has only been reported in infants with M7 AML.[53] Newer cytogenetic techniques such as fluorescent in situ hybridization (FISH) can detect cryptic abnormalities that were not evident in banding studies. [54]

Many of the genes at the breakpoints of chromosomal translocations that have been cloned during the past 510 years are proving to be transcription factors. [4] [5] The translocation t(8;21) is the most frequently occurring translocation in AML. Three-way rearrangements may occur and the t(8;21) is frequently associated with loss of a sex chromosome. The t(8;21) produces a fusion protein involving the AML1 gene on chromosome 21 and the ETO gene on chromosome 8. [52] The AML1 gene encodes one of the DNA-binding subunits of the transcription factor complex core-binding factor (CBF). The fusion protein may act as a dominant negative inhibitor of the normal copy of AML1. The AML1/ETO gene has been detected by molecular methods in some patients with t(8;21) in long-term complete remission. [55] The significance of this finding is unknown.

As noted earlier in this chapter, chromosome band 11q23 translocations are commonly seen in infants and toddlers with the M4 and M5 subtypes and secondary leukemias after epipodophyllotoxin therapy. Many partner genes (e.g., t[9;11] and t[10;11]) are involved in this translocation. [25] [26] [27] The 11q23 translocation disrupts the MLL gene (mixed-lineage leukemia) that encodes a large protein with regions of homology to the *Drosophila* protein trithorax, a homeobox protein involved in pattern development in the fly.

The translocation t(15;17) is uniquely associated with both the hypergranular and hypogranular variants of M3 AML. [6] The cloning of the breakpoint revealed rearrangement of a retinoic acid receptor gene (RARalpha), with fusion to the PML gene. [37] The PML-RARalpha gene is expressed in leukemic cells from essentially all patients with the t(15;17) and is felt to be crucially involved in the pathogenesis of this disease. This molecular marker (PML-RARa transcript) provides a convenient assay for diagnosing acute promyelocytic leukemia (APL) and determining the presence of minimal residual disease. [56] Before the RARa gene was shown to be rearranged in t(15;17), observations were made that M3 AML cells differentiated in response to retinoic acid both in vitro and in vivo (see section on acute promyelocytic leukemia).[6]

RT-PCR assays for many of the common gene rearrangements in AML are now available for diagnostic purposes and for following minimal residual leukemia. Prospective studies are underway using these assays to determine the significance of detecting minimal numbers of leukemic cells in patients in hematologic remission.[57]

CLINICAL AND LABORATORY MANIFESTATIONS

The initial signs and symptoms in the majority of children with AML include some degree of pallor, fatigue, skin or mucosal bleeding, or fever/infection that has not responded to appropriate antibiotic therapy (Table 55-3).[58] These signs and symptoms

TABLE 55-3 -- Signs and Symptoms at Presentation in Acute Myeloid Leukemia

Sign or Symptom	Percentage of Patients
Fever	34
Pallor	25
Anorexia, weight loss	22
Weakness, fatigue	19
Sore throat	18
Other respiratory symptoms	23
Bleeding	
Cutaneous	18
Mucosal	10
Menorrhagia[a]	5
Bone or joint pain	18
Lymphadenopathy	14
Gastrointestinal symptoms	13
Neurologic signs or symptoms	10
Swollen gingiva	8
Chest pain	6
Recurrent infection	3

Data from Choi, S-I, Simone, JV: Acute nonlymphocytic leukemia in 171 children. Med Pediatr Oncol 1976;2:119.

[a]Thirty percent of postmenopausal females had menorrhagia.

reflect the anemia, thrombocytopenia, and neutropenia secondary to diminished production of normal blood cells due to bone marrow infiltration with leukemic blasts. AML, in contrast to chronic myelogenous leukemia, is rarely diagnosed as an incidental finding on a routine complete blood count (CBC) in an asymptomatic child. Bone pain and arthralgias are less-common presenting complaints in children with AML compared to those with ALL. Massive hepatosplenomegaly is uncommon except in infants with AML.[44]

Congenital Leukemia

The clinical manifestations of leukemia diagnosed in the first four weeks of life differ in varying degrees from the typical findings in infants over 6 months of age and older children with AML.[44] About one-half of newborns and infants under 2 months of age with AML have leukemia cutis.[59] These babies have been described as looking like a blueberry muffin (Fig. 55-1). The skin lesions are described in the following sections. It is important to note that they may precede bone marrow manifestations of leukemia. Transient spontaneous remissions of leukemia cutis may occur but are usually followed within weeks by their reappearance in association with overt bone marrow involvement.[59] Other signs and symptoms of congenital leukemia include hepatosplenomegaly, lethargy, pallor, and failure to thrive.

Extramedullary Leukemia

The most common sites of extramedullary leukemia in children with AML include skin, gingiva, CNS, and myeloblastomas in the head and neck area (Plate 55-1). [43] Extramedullary involvement with AML is more common in infants than in older children. In a review of 29 infants with AML at St. Jude Childrens Research Hospital, 13 were found to have leukemia cutis, and 11 presented with CNS leukemia.[44] The skin lesions are often widespread, range in size from several millimeters to several

Figure 55-1 Leukemia cutis as commonly seen in congenital leukemia.

centimeters, and are palpated as freely mobile subcutaneous nodules. The color of the overlying skin may be salmon, red-brown, or bluish to slate gray in color. Testicular involvement is extraordinarily rare in children with AML and, like CNS leukemia, is commonly associated with the M4 and M5 FAB subtypes.

The most common pathology of AML in the central nervous system is that of meningeal infiltration by blasts, but epidural or brain parenchymal myeloblastomas have also been described. The incidence of CNS (meningeal) leukemia at diagnosis in children with AML ranges from 515%, [60] [61] but most children are asymptomatic. The diagnosis of CNS leukemia is made by examining a cytocentrifuged specimen of CSF (Plate 55-2). The currently accepted definition of CNS leukemia is CSF with <5 white blood cells (WBCs)/l and definite blasts, or a cranial nerve palsy. The rare symptomatic patient with CNS leukemia may have a headache, vomiting, papilledema, and/or a cranial nerve palsy (VIIth is most common). In contrast, patients with cerebral leukostasis present with seizures, somnolence, and/or stroke

secondary to areas of hemorrhage and infarction in brain tissue.

Fewer than 5% of newly diagnosed patients with AML have myeloblastomas, also known as granulocytic sarcomas or chloromas. [62] These solid tumors of myeloid blasts may occur anywhere but are primarily detected in the bones and soft tissues of the head and neck (often orbits), and in intracranial or epidural locations. [62] [63] Myeloblastomas may herald overt bone marrow involvement with AML by weeks to months and are associated with FAB M2 and t(8;21) and infants with M4 and M5 subtypes.[43] [64]

Laboratory

The initial white blood cell count at presentation in children with AML is variable (1,000/l>500,000/l). About 1520% of children have WBC counts >100,000/l. [58] Marked leukocytosis is associated with the FAB M4 and M5 subtypes and congenital leukemia, whereas lower leukocyte counts are commonly seen in patients with M3 or acute promyelocytic leukemia. The leukocyte differential in most patients includes <1,000 neutrophils/l and a variable percentage of blasts. In about 10% of patients no peripheral blasts are detectable.

Most patients with AML have a normocytic anemia, with occasional teardrop forms and nucleated red blood cells noted on the peripheral smear. Initial hemoglobin levels ranged from 2.714.3 g/dl (median, 7 g/dl) in one large series of children with AML. [58] The rare hemolytic anemia associated with AML is usually a microangiopathic hemolytic anemia secondary to disseminated intravascular coagulation (DIC).

Thrombocytopenia is the usual cause of hemorrhage in patients with AML. Bleeding is usually not observed until the platelet count falls below 20,000/l unless there is an associated coagulopathy. Approximately 50% of children with AML present with platelet counts of <50,000/l. The other major cause of bleeding in children with AML is DIC. Patients with acute promyelocytic leukemia (FAB M3) are most at risk for DIC, but infants with M4 and M5 are also at increased risk. [6] [65] Prior to the use of all-trans-retinoic acid (ATRA) for patients with APL, prophylactic low-dose heparin in addition to transfusion of fresh frozen plasma and platelets was recommended by several groups of investigators. [66] ATRA appears to downregulate tissue factor expression and thereby reduces the severity and duration of DIC in these patients.

The definitive diagnosis of acute leukemia is made by examination of the bone marrow aspirate. [67] The bone marrow is hypercellular in most cases, with 3090% blasts. The bone marrow biopsy may be hypocellular, especially in patients with a history of prior MDS, Fanconis anemia, or paroxysmal nocturnal hemoglobinuria (PNH). Dysplastic changes have recently been reported in patients with de novo AML. The significance of these dysplastic changes remains to be determined.

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DIFFERENTIAL DIAGNOSIS

The diagnosis of AML is relatively straightforward after an examination of the peripheral blood and bone marrow. [67] Other diagnoses that can sometimes cause diagnostic difficulty include the myeloproliferative disorders (e.g., juvenile chronic myelogenous leukemia [JCML]), myelodysplastic (preleukemic) syndromes, and overwhelming infections that result in leukemoid reactions or neutropenia secondary to a bone marrow maturation arrest in granulocytic precursors. In the latter situation, the bone marrow may be confused with APL but with resolution of the sepsis, granulocytic maturation ensues.

The differential diagnosis of congenital leukemia is somewhat more challenging, in part because of the leuko-erythroblastic peripheral blood picture noted in neonates with hypoxia or sepsis. Congenital viral infections including cytomegalovirus, herpes simplex, and rubella need to be ruled out as well as the transient myeloproliferative syndrome associated with trisomy 21. [68]

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TREATMENT

The long-term survival (>5 years) for children with AML has increased from <10% to approximately 40% during the past 25 years. [69] [70] [71] [72] [73] [74] [75] This improved cure rate has resulted from more effective remission induction chemotherapy and better strategies to prevent relapse. Improvements in supportive care have been equally important, making it safe and feasible to treat patients with myelosuppressive doses of chemotherapy. [76] [77] [78] All children with AML

should be referred to a pediatric oncology center for treatment and are usually enrolled in a cooperative group clinical trial, such as Pediatric Oncology Group (POG), Childrens Cancer Group (CCG), Berlin-Frankfurt-Muenster (BFM) Group, and Medical Research Council (UKAML), or single-institution protocol.

Before chemotherapy is started, the infant or child should be stabilized with regard to renal, metabolic, hematologic (anemia, bleeding, DIC, leukostasis), and infectious disease issues. A double lumen indwelling central venous catheter should be placed early in the treatment course. The metabolic derangements associated with leukemic cell death (tumor lysis syndrome) include hyperuricemia, hyperkalemia, hyperphosphatemia, and hypocalcemia. [79] The tumor lysis syndrome is seen more often in children with ALL (especially T cell and mature B cell) than in those with AML. Uric acid nephropathy is avoided by prompt attention to hydration, alkalinization of the urine, and administration of allopurinol. Newer initiatives in supportive care include prophylactic fluconazole during prolonged periods of neutropenia and the use of hematopoietic growth factors (G- or GM-CSF) during induction and after cycles of intensification chemotherapy. Blood products should all be leukopoor and irradiated to prevent graft-vs.-host-disease (GVHD). Platelet transfusions are recommended prophylactically if the platelet count is <10,000/l.

One to two percent of children with AML still die within the first several days to a week after diagnosis. These early deaths usually result from intracranial hemorrhage from either a coagulopathy or CNS leukostasis. [65] [66] [80] [81] If the leukocyte count is >100,000/l, it is important to rapidly initiate measures to prevent leukostasis. Leukostasis refers to plugging of blasts in vessels with invasion of vessel walls leading to hemorrhagic infarction of the brain, lung, or other organs. Children with AML who have leukocyte counts >200,000/l are much more susceptible to leukostasis than patients with ALL and comparable WBC counts. [79] The most clinically relevant target organs for leukostasis are brain and lung with signs and symptoms including somnolence, seizures, stroke, tachypnea, and hypoxemia. Treatment should include immediate measures to lower the WBC, and exchange transfusion or leukapheresis if the patient is symptomatic. Oral hydroxyurea combined with leukapheresis is very effective for rapidly lowering the blast count in patients with AML and preventing the clinical manifestations of leukostasis. [80] [81]

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REMISSION INDUCTION

Because of the narrow therapeutic index of the active agents in AML, all induction regimens (except for FAB M3) are designed to achieve rapid bone marrow hypoplasia, an apparent prerequisite for successful remission induction. Children <3 years of age or <0.6 m^2 should have doses of chemotherapy calculated on a per-kilogram rather than per-m^2 basis.[71] The induction of a complete remission is necessary in order to prolong survival for patients with AML. For the past two decades, the gold standard remission induction regimen has included 3 days of an anthracycline, usually daunomycin, and 7 days of cytarabine with or without thioguanine. About 7585% of children with AML achieve a complete remission after this treatment, and about one-third of patients require two such courses to achieve a remission.[2] [71] Lower remission rates are seen in patients with secondary AML, a history of a prior MDS, and certain cytogenetic findings (see section Prognostic Factors). Because of the requisite period of bone marrow hypoplasia and the greater impairment of bone marrow reserve in patients with AML, the remission induction phase of therapy is more toxic than for patients with ALL.

Prospectively controlled pediatric and adult cooperative group studies have shown that daunorubicin is preferable to doxorubicin because it is associated with less oral mucositis and gastrointestinal toxicity.[71] The most disturbing gastrointestinal toxicity during induction is an enterocolitis involving the distal

PATIENT MANAGEMENT: AN APPROACH TO THERAPY FOR AML IN CHILDREN

Remission induction: the combination of cytarabine (seven days) and daunorubicin (three days) remains the cornerstone of induction chemotherapy and results in a complete remission rate of about 80%. Neither the substitution of idarubicin for daunorubicin nor the addition of thioguanine or etoposide to this regimen has been shown to significantly increase the complete remission rate. However, recent adult and pediatric cooperative group studies indicate that intensification of induction chemotherapy significantly prolongs disease-free survival and this is independent of the type of post-remission therapy (allogeneic BMT, autologous BMT, or intensive consolidation chemotherapy). One can intensify induction by either beginning the second cycle of induction on day 10 instead of the usual day 14 from the start of therapy or by using high-dose cytarabine. The author favors the former approach; this strategy also eliminates the need for a day 14 bone marrow exam, which has always been difficult to interpret. For patients with acute promyelocytic leukemia, the combined use of all-trans-retinoic acid and daunorubicin/cytarabine during induction appears to give the best long-term results in this unique subtype of AML.

CENTRAL NERVOUS SYSTEM PROPHYLAXIS

The termination of remission in children with AML by central nervous system relapse occurs in <5% of relapses. Most AML protocols administer periodic doses of intrathecal chemotherapy. No controlled trials support the use of preventive cranial irradiation in AML.

TREATMENT IN REMISSION

Allogeneic BMT early in first remission using an HLA matched family donor results in a significantly better disease-free survival compared with chemotherapy but is limited to about 20% of patients with a suitable donor. The best alternative to an allogeneic bone marrow transplant is 36 months of intensive combination chemotherapy featuring high-dose cytarabine. There is no proven role for maintenance or continuation chemotherapy if it follows several cycles of high-dose cytarabine. Autologous bone marrow transplants performed in first remission do not offer a survival advantage compared to chemotherapy. For those children with AML who have a relatively favorable prognosis after treatment with chemotherapy (e.g., t[8;21], inv 16, Down syndrome), it is reasonable to reserve BMT for early relapse or second remission. Transplants using a matched unrelated bone marrow or cord blood donor remain a high-risk procedure and are usually reserved for patients who have had a relapse.

The prognosis for children who relapse or fail to achieve an initial remission is poor. There is only a small possibility of a long second remission with chemotherapy alone, especially if the duration of the first remission was less than one year. The strategy in the relapsed patient should be to induce a second remission and proceed as rapidly as possible to an allogeneic or autologous bone marrow transplant. Published data is insufficient to recommend one type of transplant over another in this circumstance. The rare patient in relapse with stable clinical and hematologic parameters may be best served by an immediate bone marrow transplant without an attempt at reinduction chemotherapy.

ileum, cecum, and proximal colon. This syndrome is referred to as typhlitis or the right lower quadrant syndrome and is noted in <10% of patients. [82] The signs and symptoms of typhlitis develop about 1014 days after the start of chemotherapy. Treatment recommendations include bowel rest and broad-spectrum antibiotics with double coverage for gram-negative organisms. Surgery is rarely needed and is reserved for intestinal perforation, abdominal wall fasciitis, or massive bleeding.

Several adult and pediatric AML studies have explored the benefits of substituting either mitoxantrone or idarubicin for daunorubicin. [83] [84] Mitoxantrone is at least as effective as daunorubicin and idarubicin may be superior to daunorubicin for remission induction. The addition of thioguanine and/or etoposide to an anthracycline and cytarabine has not been shown to significantly increase the complete remission rate. [73] [74] Several recently completed clinical trials have intensified therapy during induction by using high-dose cytarabine in combination with daunorubicin (Australian Leukemia Study Group) or starting the second induction course at an earlier time (e.g., day 10 in-stead of days 1421) (CCG). [85] [86] Patients receiving intensified induction have had statistically significant increases in disease-free survival compared to those patients randomized to standard induction, but they have not had higher complete remission rates. These data are consistent with the hypothesis that more effective early leukemic cell kill reduces the likelihood of a subsequent relapse in children and adults with AML. [87]

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ACUTE PROMYELOCYTIC LEUKEMIA (FAB M3 or APL)

In the past, about 6575% of patients with M3 AML (APL) entered remission after a standard induction with daunorubicin and cytarabine. The remission induction period, however, was associated with significant early mortality secondary to hemorrhagic complications. [88] The use of ATRA alone during induction for patients with acute promyelocytic leukemia (PML/RARa-positive) results in a similar or even higher rate of complete remission with a different toxicity profile. [6] The ATRA-induced remissions are associated with differentiation of the blasts/promyelocytes and a peripheral blood leukocytosis during the first two weeks of therapy.

A significant leukocytosis (>15,000/l) during ATRA requires the addition of chemotherapy. ATRA is also associated with several life-threatening complications including a severe respiratory distress and capillary leak syndrome and pseudotumor cerebri (more common in pediatric patients). The retinoic acid syndrome (respiratory distress) has been successfully managed in most patients with temporary cessation of ATRA and administration of decadron. The symptoms of pseudotumor cerebri disappear within days after withdrawal of ATRA and the drug can usually be safely restarted at a reduced dose. Patients induced into remission with ATRA alone remain PCR-positive for PML/RARa after induction and all eventually relapse if no consolidation chemotherapy is added. The combined use of ATRA and chemotherapy during remission induction results in an improved disease-free survival compared to the sequential use of ATRA followed by chemotherapy.[89]

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CENTRAL NERVOUS SYSTEM LEUKEMIA

There is no evidence that CNS-directed therapy with either intrathecal chemotherapy alone or combined with cranial irradiation prolongs disease-free survival in children with AML.[2] [3] [72] Most pediatric AML studies, however, still include intrathecal chemotherapy because isolated CNS relapse occurred in about 20% of children with AML who did not receive any CNS-directed therapy. [60] [61] Using historical controls, the BFM AML group has made a case for using prophylactic cranial irradiation and plan to test their hypothesis in a prospectively controlled clinical trial. [90]

In contrast to ALL, the finding of blasts in the CSF at diagnosis in children with AML does not adversely impact on prognosis. An accepted approach for the treatment of CNS leukemia is weekly intrathecal methotrexate or ara-C until the cerebrospinal fluid (CSF) is clear of blasts. In some protocols this is followed by CNS irradiation at the end of systemic chemotherapy.[2]

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TREATMENT IN REMISSION

Without further treatment after standard induction chemotherapy, more than 90% of patients relapse within one year and the remainder relapse by two years. The intensity and duration of chemotherapy in remission, as well as the role of bone marrow transplantation in first remission, have been areas of active investigation and controversy during the past fifteen years.[74] [91] In the 1970s, pediatric AML studies tested whether modestly myelosuppressive combination chemotherapy given in remission would improve overall survival. These protocols led to a plateau in disease-free survival of 1520% at 5 years. [70] [71] [73] In an effort to improve upon these disappointing results, other approaches were explored, including early intensification or consolidation chemotherapy and bone marrow transplantation (BMT). Allogeneic BMT provided an opportunity for delivering very high doses of chemo-radiation and possibly stimulating a graft-versus-leukemia effect. [91] [92] [93] [94] Although the initial studies were not randomized, the results suggested a benefit for either the intensification of chemotherapy or BMT early in first remission.

Cytarabine, the single most active agent in AML, was the prototype drug for intensification because laboratory and clinical studies indicated that at least a log increase in its dose could overcome certain mechanisms of resistance.[95] [96] About 40% of patients with AML who are refractory to standard doses of cytarabine achieve a complete remission rate with high-dose cytarabine.[95] The superiority of high-dose compared to standard-dose cytarabine has also been confirmed in a prospective randomized clinical trial in adults with AML.[96] Most pediatric AML studies that were initiated in the late 1980s included several consolidation cycles of high-dose cytarabine. These protocols resulted in 5-year leukemia-free survival plateaus of 3560%. [74] [75] [94] Maintenance chemotherapy with oral thioguanine and standard doses of cytarabine given after consolidation chemotherapy has not been shown to further increase the proportion of patients in long-term remission. The optimal number of consolidation chemotherapy cycles has not been determined.

A recently completed multi-institutional trial has shown that ATRA, when combined with daunorubicin and ara-C for patients with M3 AML, significantly improves leukemia-free survival compared with chemotherapy alone.[97] Therefore, ATRA should be included in the induction regimens for all patients with acute promyelocytic leukemia. The role of maintenance ATRA is under active investigation.

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BONE MARROW TRANSPLANTATION IN FIRST REMISSION

Allogeneic BMT from a histocompatible family donor was first evaluated in children and young adults with AML in first remission in the mid-1970s. [91] [92] [93] It is an effective therapy, albeit with substantial up-front risks, that results in lower relapse rates compared to chemotherapy alone. Published data from many pediatric AML bone marrow transplant series show 5-year leukemia survival rates ranging from 5575%. [2] [93] [94]

Many of the early transplant studies, however, were not prospectively designed and therefore were biased in their selection criteria, not controlled for the timing of BMT in first remission, and excluded patients who were eligible for a transplant but who relapsed before the procedure. In an effort to avoid these biases, it was suggested that outcomes of studies comparing BMT to chemotherapy be analyzed according to the intention-to-treat

TABLE 55-4 -- Prospective Comparative Studies of BMT and Postremission Chemotherapy for Childhood AML

Study	Number of Patients	DFS or EFS from Remission or Randomization
POG 8821	89	allo BMT
	115	auto BMT
	117	chemo
CCG 2891	140	allo BMT
	150	auto BMT
	160	chemo
AIEOP/LAM 87	24	allo BMT
	35	auto BMT
	37	chemo
MRC AML 10	50	auto BMT
		chemo

analysis, which includes all eligible patients (i.e., those having a matched family donor) and not just individuals who actually received the transplant. [94] [98] When the data are analyzed in this way, there is still a statistically significant survival advantage for allogeneic BMT compared to chemotherapy in most studies. [86] [98]

A limited donor pool of histocompatible related individuals, and the late effects of BMT including chronic GVHD, continue to be major limitations in the more widespread application of this procedure in patients with AML. [99] [100] The late effects of BMT in young children may include growth problems, gonadal toxicity, secondary malignancy, and chronic GVHD.[100] [101] Because of these issues, some pediatric oncologists are beginning to recommend chemotherapy alone, especially for children with a relatively favorable prognosis, and reserving BMT for subsequent relapse. Thirty to forty percent of patients with AML transplanted (allogeneic or autologous) in early relapse or second remission appear to be long-term leukemia-free survivors. [102] [103] [104]

For patients who lack suitable donors (5 or 6 antigen-matched family member), autologous BMT became an attractive option in first remission after it was reported to be effective in some patients with AML in second remission (5-year survival rates of 3040%). [104] The results of three randomized trials in pediatric AML have concluded that autologous BMT is not superior to intensive chemotherapy in first remission [95] [105] [106] (Table 55-4). A fourth study indicated that the addition of autologous BMT to four courses of intensive chemotherapy substantially reduces the risk of relapse in patients with AML leading to an improvement in long-term survival.[107] Because of the good chance of salvage for children who relapse from chemotherapy and the morbidity, mortality, and late effects of autologous BMT, the authors suggest that delay of the autograft until second remission may be appropriate.

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PROGNOSTIC FACTORS

The prognostic significance of several clinical and biologic features in children with AML is summarized in Table 55-5 . In contrast to childhood ALL, very few clinical, laboratory, or treatment

TABLE 55-5 -- Patient Characteristics Relating to Duration of Remission

Characteristics	Favorable Value
Cytogenetics	t(15;17), t(8;21), inv 16
Leukocyte count	<100,000/l
Secondary AML	Not present
FAB subtype	M1 or M2 with Auer rods, M3 and M4eo
Courses to CR	1

associated factors have been consistently related to prognosis [2] [108] (Table 55-3). A white blood cell count >100,000/l at diagnosis, monosomy 7, and secondary AML are associated with lower remission rates. [109] [110] Favorable factors for predicting complete remission include FAB M1 with Auer rods, chromosome findings of inv 16 or t(8;21), and the combined use of ATRA and chemotherapy for acute promyelocytic leukemia(FAB M3). [89] [110] [111] [112]

In some studies, the M4 and M5 FAB subtypes, high initial leukocyte count, age under 2 years, extramedullary leukemia (other than CNS) at diagnosis, and longer time to enter remission (more than one induction cycle) were found to have an adverse impact on remission duration. [108] In the German BFM studies, two risk groups were identified. [110] The BFM favorable risk group includes children with FAB M1 or M2 with Auer rods, M3 and M4eo, rapid response to induction chemotherapy, and Down syndrome. Included in this favorable group are patients with t(8;21) and inv 16. Several groups of investigators have reported that children with AML and Down syndrome have a very good outcome after treatment with chemotherapy alone. [28] [29] The presence of acute mixed-lineage or biphenotypic leukemia (AML with lymphoid-associated antigen[s] does not influence prognosis. [48] [113]

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MANAGEMENT OF RELAPSE

The prognosis for children who relapse or fail to enter remission with front-line chemotherapy is very poor. [114] [115] Many different chemotherapy regimens have been evaluated in children and adults with refractory or relapsed AML. Relapse regimens that have been investigated include high-dose cytarabine with L-asparaginase or mitoxantrone, etoposide (VP-16) and amsacrine with or without azacytidine, fludarabine and cytarabine, and 2-chlorodeoxyadenosine (2-CDA). [116] The best predictor of response to chemotherapy after relapse is the duration of the first remission. Children who relapse within 1 year after achieving remission have a substantially lower likelihood of achieving a second remission compared to those patients who relapse later. [115] The complete remission rate for the former group is 3040% and 6070% for the latter group. Less than 20% of the complete responders are projected to remain in remission for more than two years unless they receive a bone marrow transplant.[102]

The projected 5 year survival rate after an allogeneic or autologous BMT in second remission is 3050%. [103] Because of the higher rate of relapse after an autologous compared to an allogeneic bone marrow transplant, there is a growing experience with matched unrelated transplants using bone marrow or cord blood. [117] [118] In selected patients (short duration of first remission or early relapse), it is reasonable to undertake a bone marrow transplant without an attempt at inducing a second remission. This is only feasible if a transplant option is immediately available and the patient is clinically stable. [102]

FUTURE DIRECTIONS

The major challenge for the future is to develop more effective therapies because the majority of children with AML continue to relapse and ultimately die from their disease.[2] [111] The primary cause of relapse is the development of drug resistance. Newer insights into the multiple mechanisms of drug resistance are beginning to be elucidated. For example, increased expression of the multidrug resistance gene (mdr 1) or its protein product P-glycoprotein has been demonstrated in blasts from 2040% of newly diagnosed patients with AML and increases approximately two-fold at the time of relapse. [119] [120] High in vitro expression of mdr is associated with resistance to natural product drugs such as etoposide, anthracyclines, and vincristine, by promoting their cellular efflux. A number of compounds including verapamil and cyclosporine are capable of reversing the mdr

phenotype in vitro via a direct interaction with P-glycoprotein. [120] Clinical trials are ongoing to determine whether such compounds can reverse drug resistance in vivo.

The increasing availability of alternative sources of hematopoietic stem cells from unrelated bone marrow or cord blood donors will greatly expand the use of BMT in all phases of therapy.[117] [118] Strategies based on preclinical models are also being developed that will hopefully achieve a more favorable balance of the graft-versus-host and graft-versus-leukemia reactions. Biologic response modifiers such as IL-2 and genetically engineered tumor vaccines are under study for their potential to enhance the patients immune response against the leukemia. [122] Eventually, it may be possible to target the genetic lesions of leukemic cells as exemplified by the use of all-trans-retinoic acid in acute promyelocytic leukemia. Finally, the increasingly sophisticated methods available for measuring minimal residual numbers of leukemic cells will allow us to better tailor our therapies.

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Chapter 56 - Pathobiology of Acute Lymphoblastic Leukemia

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INTRODUCTION

Normal lymphoid cell populations undergo diverse clonal rearrangements of their immunoglobulin or T-cell receptor (TCR) genes, followed by highly regulated proliferation of the cells that successfully complete these genetic changes. This developmental process generates B cells and T cells with the specificities needed to support a fully competent immune system. When a lymphoid progenitor cell becomes genetically altered through somatic changes, the result can be dysregulated proliferation and clonal expansion, eventually leading to acute lymphoblastic leukemia (ALL). In most cases, the pathobiology of transformed lymphoid cells reflects the altered expression of genes whose products contribute to the normal phenotypes of B- and T-cell progenitors, but it may also involve the aberrant expression of otherwise quiescent genes.

Because leukemic blasts represent the clonal expansion of hematopoietic progenitors that are blocked in differentiation at discrete stages of development, they provide large uniform populations for molecular and functional analyses. Leukemic cells duplicate most of the features of normal lymphoid progenitors and provide models for elucidating the regulatory cascades disrupted by specific genetic changes. For instance, molecular studies of chromosomal breakpoint regions in ALL cells have identified genes whose protein products are transcription factors that presumably control the expression of developmentally important genes. [1] [2] [3] [4] Indeed, most of the recent progress in understanding ALL pathobiology has come from the study of rearranged or mutated genes and their associated proteins.

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CLONAL ORIGIN OF LEUKEMIC LYMPHOID CELLS

Human ALL arises from a single progenitor cell that has undergone genetic damage leading to dysregulated growth and arrested differentiation. Evidence that each leukemic cell has descended from a single transformed progenitor comes from cytogenetic studies showing common numerical and structural chromosomal abnormalities within discrete leukemic cell populations. The clonal development of leukemic cell populations is further demonstrated by uniform rearrangements of immunoglobulin or TCR genes, as compared with the heterogeneous pattern of rearrangements observed in populations of normal T and B lymphocytes. [5] [6]

Perhaps the best evidence of clonality is provided by X chromosome-linked genes that are inactivated during embryogenesis. This process occurs before somatically acquired transformation events and thus is independent of changes induced by differentiation and stem line evolution. In accord with the Lyon principle, one or the other of the X chromosomes is randomly inactivated early in the embryogenesis of females, in large part through DNA methylation, leading to a heterozygous pattern of inactivation of either the paternally or maternally derived X chromosome in all tissues, including the hematopoietic system. By contrast, in clonally derived cell populations, as found in the leukemias and other malignancies, every cell is characterized by inactivation of the same X chromosome. Thus, the unicellular development of a leukemic cell population can be demonstrated by detecting a single type of glucose-6-phosphate dehydrogenasean enzyme encoded by a gene on the X chromosomein the neoplastic cells of heterozygous female patients who express a double-enzyme pattern in their normal tissues. [7] [8] In addition, the methylation patterns of restriction fragment length polymorphisms (RFLPs) in X-linked genes, detected by Southern blot analysis, have been used to show that even rare ALL cases with two completely different cytogenetic stem lines probably arise by clonal evolution from a single transformed progenitor. [9]

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LINEAGE-SPECIFIC FEATURES OF LEUKEMIC LYMPHOBLASTS

An important advance in the understanding and treatment of ALL was the realization that malignant lymphoblasts share many of the features of normal lymphoid progenitors.[10] [11] Thus, ALL cells rearrange their immunoglobulin and TCR genes and express components of antigen receptor molecules and other differentiation-linked cell-surface glycoproteins in ways that correspond to developing normal B and T lymphocytes. In many cases, leukemic cells appear to represent the clonal expansion of a lymphoid progenitor that is blocked or frozen in an early stage of B- or T-cell differentiation. [12] With better understanding of the normal patterns of antigen-independent lymphoid cell development, however, it has become clear that leukemic lymphoblasts can show asynchronous gene expression with subtle variations in phenotype.[13] Hence, it should not be surprising that in some cases of ALL, the blast cell phenotypes differ from those of normal lymphocyte progenitors, presumably because of aberrant regulation of gene expression. Still, the general concept that leukemic cells should be classified according to their normal developmental stage remains an important one, providing a basis for the study of immunophenotype-specific genetic changes and for the assignment of patients to phenotype-directed therapy.

B-Cell ALL

The diagnosis of B-cell leukemia depends on the detection of surface immunoglobulin on leukemic blasts. This rare phenotype accounts for only 23% of ALL cases, and the lymphoblasts generally have a distinctive morphology, with deeply basophilic cytoplasm containing prominent vacuoles; this morphologic pattern is designated L3 in the French-American-British (FAB) system. [14] [15] [16] Prominent clinical features include concomitant extramedullary lymphomatous masses in the abdomen or head and neck, frequent involvement of the central nervous system (CNS) and cranial nerves, and hyperuricemia with acute renal failure due to uric acid nephropathy. Most investigators believe that acute B-cell leukemia is a disseminated form of Burkitt lymphoma, as these conditions share common cytogenetic, molecular genetic, immunologic, cytologic, and clinical features. [17]

Acute B-cell leukemia does not respond well to chemotherapy traditionally used for childhood ALL. However, outstanding results, with event-free survival (EFS) rates of nearly 90%,

have been obtained with treatments designed for Burkitt lymphoma, which emphasize cyclophosphamide and the rapid rotation of antimetabolites in high dosages. [18] [19] [20] [21] [22] Thus, B-cell leukemia is the first form of ALL to be recognized as a distinct clinical entity based on immunophenotypic and cytogenetic features and the first to be treated by separate protocols designed specifically for the leukemias unique features.

Pre-B and Early Pre-B ALL

Approximately 80% of ALL patients have lymphoblasts with phenotypes corresponding to those of B-cell progenitors. [16] [23] These cases can be identified on the basis of cell-surface expression of CD19 and at least one other recognized B-lineage-associated antigen: CD20, CD24, CD22, CD21, or CD79. [16] [23] [24] [25] [26] Some 9095% of B-lineage ALL cases also express CD10 (CALLA, or common ALL antigen). The lymphoblasts may also express nuclear terminal deoxynucleotidyl transferase (TdT),[27] or CD34, an antigen of unknown function whose normal expression in hematopoiesis is restricted to lymphoid and hematopoietic precursors. [28] [29] About one-fourth of B-progenitor ALL cases express cytoplasmic immunoglobulin heavy chain proteins and are designated pre-B-cell ALL. Early pre-B ALL lacks the cytoplasmic heavy chain but express HLA-DR, TdT, and CD19 and have rearranged immunoglobulin genes. [30] [31] Pre-B cases were originally shown to have a worse long-term response to therapy than early pre-B cases, an observation that was later attributed to the presence of a specific t(1;19) in about one-fourth of the pre-B cases.[32] The adverse effect of this cytogenetic feature appears to have been nullified by the development of effective chemotherapy. [33] [34]

DNA rearrangement of immunoglobulin genes occurs before heavy chain gene expression in B-cell development, providing a genetic marker of B-lymphocyte ontogeny.[35] [36] The variable region of immunoglobulin heavy-chain genes is assembled from three classes of DNA segments on human chromosome 14, termed V_H (variable), D_H (diversity), and J_H (joining), which must recombine correctly during cell development to produce a functional protein. The initial attempt at rearrangement may be productive, resulting in immunoglobulin heavy chain synthesis, or it may be aberrant, with no production of heavy chain molecules, in which case rearrangement of the other heavy chain allele is attempted. Similar rearrangements occur between the V and J regions of the and light chain genes on chromosomes 2 and 22, with attempts to rearrange the alleles occurring first, followed by recombination of alleles if these attempts are nonproductive. Rearrangement of each of these genes results in deletion of introns containing restriction endonuclease cleavage sites. This in turn causes changes in the sizes of restriction fragments bearing the rearranged gene under study, which can be detected by Southern blot analysis. Because lymphocytic leukemias are clonal, the DNA from each cell contains identical rearrangements apparent as discrete bands that differ from germline bands.

Korsmeyer and co-workers pioneered the use of heavy and light chain gene rearrangements to support an early B-lineage origin of most ALL blasts. [35] [36] This work was extended to establish synchrony between immunoglobulin gene rearrangements and the expression of B-lineage-restricted cell-surface antigens. Immunoglobulin heavy chain gene rearrangements have also been documented in about 15% of T-cell ALL cases and a similar percentage of AML cases. [37] [38] [39] Thus, caution must be exercised when assigning cell lineage on the basis of studies of immunoglobulin gene rearrangement.

T-Cell ALL

Leukemias of T-cell precursors are identified and classified according to the sequence of expression of T-cell-associated surface antigens during normal thymocyte ontogeny.[40] [41] Thymocyte differentiation begins with the prothymocyte, which expresses CD7, TdT, and cytoplasmic T3 antigen. Next is the early thymocyte, which retains these markers and acquires CD5 and CD2, the receptor for sheep erythrocytes. Intermediate thymocytes co-express CD4 and CD8 as well as CD1. Mature thymocytes lose CD1 expression and produce functional TCRs, leading to expression of CD3 on the cell surface. Using a battery of monoclonal antibodies specific for these cell-surface glycoproteins, one can identify patterns of expression that correspond to equivalent stages of normal thymocyte development. [42] [43] [44] [45] The clinical features most closely associated with T-cell ALL are high blood leukocyte counts, CNS involvement, and radiographic evidence of a thymic mass in about one-half of cases at presentation. Historically, patients with T-cell ALL have had an adverse prognosis in comparison with patients with B-lineage ALL. With the wider use of intensive chemotherapy, however, the outlook for patients with T-cell leukemia appears improved. [46] In addition, expression of specific antigens, including CD2, CD5, and CD10, may identify subgroups of T-cell ALL with better responses to therapy. [46] [47] [48]

The human antigen-specific TCR molecule is a heterodimer composed of disulfide-linked - and -polypeptide subunits, each encoded by gene families containing variable, joining, and constant sequence elements that rearrange at the DNA level to generate diversity, in a manner analogous to the immunoglobulin genes. Hence, rearrangement of the TCR- chain genes can be used to establish clonality and lineage derivation within leukemias of T-cell progenitors. Although TCR- chain genes

are generally in a germline configuration in B-lineage leukemic cells, about 10% of cases possess aberrant rearrangements. [49] [50] Clonal rearrangements of the TCR- and chain genes are even less restricted to the T-cell lineage, being observed in a significant number of B-lineage ALL cases. [51] [52]

Mixed-lineage Leukemia

Acute mixed-lineage leukemias are defined by blast cells that co-express markers of both the lymphoid and myeloid lineages. Two distinct forms of these leukemias are recognized: those with lymphoid morphology, which co-express myeloid-associated antigen, [53] [54] [55] [56] and those with myeloid morphology and reactivity to myeloperoxidase staining, which co-express cell-surface antigens normally restricted to lymphoid cells. [57] [58] The origin of mixed-lineage leukemias has not been established. One possibility is malignant transformation of pluripotent progenitor cells that retain the ability to differentiate into both the myeloid and lymphoid lineages; another is immortalization of rare progenitor cells that normally co-express features of both lineages; and a third is aberrant gene expression due to specific genetic alterations. [59]

Controversy exists over whether cases of lymphoid leukemia with expression of one or more myeloid cell-surface antigens (e.g., CD13, CD33, or CD14) have an adverse prognosis. However, Pui et al. [56] found that in the 16% of childhood ALL cases with expression of myeloid antigens, the prognosis was no worse than in cases without this feature. This finding has recently been supported by Uckun et al., [60] who also found that myeloid expression in ALL was not an adverse prognostic feature. The expression of T-lymphoid properties, such as cytoplasmic T3 and cell-surface CD2 and CD7, by predominantly myeloid leukemias appears to identify patients with a lower likelihood of complete remission with standard AML therapy but who respond well to induction agents commonly used in ALL therapy. [58]

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GENETIC BASIS OF LYMPHOID LEUKEMIA

Multiple somatically acquired genetic abnormalities are responsible for the malignant transformation and disordered cell

growth and differentiation seen in ALL. These abnormalities include microscopically evident chromosomal rearrangements as well as lesions detectable only by molecular analysis of lymphoblast DNA. The ability to identify these changes in a precise, consistent manner and to relate them to the clinical course of the disease has led to risk-specific therapy for ALL. It is worth noting that most children with leukemia have normal constitutional karyotypes, indicating that the genetic abnormalities in their leukemic cells are acquired somatically and thus are restricted to the malignant clone.

Chromosomal translocations are found in 75% of ALL cases (Fig. 56-1). [61] [62] [63] They can be broadly classified as recurring lineage-restricted abnormalities, accounting for approximately two-thirds of the translocations in ALL, and as so-called random translocations, which have been identified in single cases only. Molecular characterization of the breakpoints of recurring translocation led to the cloning of genes intimately involved in leukemogenesis. [1] [2] [3] [4]

Transcriptional Control Genes

Molecular studies of the breakpoints of specific chromosomal translocations of human leukemic cells have focused on transcription factor genes (Table 56-1), whose alteration leads to the differentiation arrest and aberrant growth of leukemic lymphoid and myeloid progenitors. [1] [2] Conserved amino acid sequence motifs within the sequence-specific DNA-binding domains of these nuclear trans-activating proteins allow them to be grouped into families, which in many cases appear to be involved in similar regulatory processes. Thus, the transcription factor genes in Table 56-1 are grouped according to shared structural features of their DNA-binding domains: basic region helix-loop-helix (bHLH), cysteine-rich (LIM), homeodomain (HOX), basic regionleucine zipper (bZIP), zinc finger, AT hook minor groove, ETS-like, or runt homology. Another important association is the lineage restriction of transcription factor genes affected by specific chromosomal translocations, suggesting that the proteins they encode may disrupt the differentiation programs of specific lymphoid progenitors. This interpretation implicates transcription factors as preferred targets in leukemia induction. It also suggests that the normal developmental programs of progenitor cells of different lineages are controlled by different regulatory programs.

Rabbitts [64] has aptly described a key group of regulatory transcription factors as the products of master genes. In his model, the nuclear proteins encoded by these genes act positively to up-regulate critical target genes or negatively to interfere with normal regulatory pathways. The net effect is disruption of gene regulatory cascades that control and coordinate the expression of large numbers of proteins required for completion of lymphoid cell differentiation programs. Disruption of transcription factors in leukemic blasts occurs by at least two distinct mechanisms: the dysregulated expression of intact genes and the creation of chimeric transcription factors.

Dysregulated Expression of Structurally Intact Genes

Activation of *MYC* in B-Cell ALL

In B-cell acute leukemia and Burkitt lymphoma, translocation of one allele of the prototypic bHLH gene, *myc*, on chromosome 8 into the vicinity of an immunoglobulin gene, either the heavy chain gene on chromosome 14q32 or the or light chain genes on chromosomes 2 and 22, leads to dysregulation of that allele. [65] [66] [67] [68] [69] [70] [71] [72] [73] In the predominant t(8;14) translocation, the involved *myc* locus is translocated into the heavy chain gene on chromosome 14, adjacent to the coding sequences of the immunoglobulin constant region. [65] [66] [67] The coding sequences of the immunoglobulin variable region generally are reciprocally translocated to the distal tip of chromosome 8. In variant translocations, the *myc* gene remains on chromosome 8, and portions of the respective light chain genes are translocated to that chromosome downstream of the *myc* locus. [68] [69] [70] [71] [72] [73]

Dysregulation of *myc* appears to transform lymphocytes by altering the interactions of the myc protein with several other transcription factors. Normally, myc dimerizes with the MAX transcription factor, which also can heterodimerize with MAD and Mxil. [74] [75] [76] [77] Myc/MAX dimers activate gene expression, [74] [75] whereas MAD/MAX dimers interact with the SIN3 protein to repress transcription. [78] [79] Overexpression of *myc* as a result of t(8;14) or related translocations leads to increased levels of myc/MAX heterodimers relative to MAD/MAX, ultimately causing transformation by the activation of unknown target genes. [80] This model has been supported by the experimental overexpression of myc in transgenic mice and in B lymphoblasts in culture. [81] [82] [83] Although the oncogenic targets of myc/MAX have not yet been identified, the *cdc25* cell cycle phosphatase gene has been implicated. [84]

Figure 56-1 Distribution of chromosomal translocations in ALL.

TABLE 56-1 -- Transcription Factor Genes Affected by Chromosomal Breakpoints in Human Acute Leukemias

Family[a]	Translocation	Affected Gene	Disease
Basic helix-loop-helix (bHLH) proteins	t(8;14)(q24;q32)	MYC	Burkitt lymphoma and B-cell ALL
	t(2;8)(p12;q24)	MYC	
	t(8;22)(q24;q11)	MYC	T-cell ALL
	t(8;14)(q24;q11)	MYC	T-cell ALL
	t(7;19)(q35;p13)	LYL1	T-cell ALL
	t(1;14)(p32;q11)	TAL1	T-cell ALL
	t(7;9)(q35;q34)	TAL2	

Cysteine-rich (LIM) proteins	t(11;14)(p15;q11)	LMO1	T-cell ALL
	t(11;14)(p13;q11)	LMO2	T-cell ALL
	t(7;11)(q35;p13)	LMO2	T-cell ALL
Homeodomain (Hox) proteins	t(10;14)(q24;q11)	HOX11	T-cell ALL
	t(7;10)(q35;q24)	HOX11	T-cell ALL
	t(1;19)(q23;p13)	E2A-PBX1	Pre-B cell ALL
	t(7;11)(p15;p15)	NUP98-HOXA9	AML
Basic-region/leucine-zipper (bZIP) proteins	t(17;19)(q22;p13)	E2A-HLF	EPB ALL
Zinc-finger proteins	t(15;17)(q21;q11-22)	PML-RAR	APL
	t(11;17)(q23;q21)	PLZF-RAR	APL
	t(5;17)(q32;q12)	NPM-RAR	APL
	t(3;v)(q26;v)	EVI1	AML
AT hook minor groove binding proteins[b]	t(4;11)(q21;q23)	MLL-AF4	EPB ALL
	t(9;11)(p21;q23)	MLL-AF9	AML (monocytic)
	t(11;19)(q23;p13.3)	MLL-ENL	ALL or AML
	t(11;19)(q23;p13.1)	MLL-ELL	AML
ETS-like (TEL, ERG)	t(12;21)(p13;q22)	TEL-AML1	ALL
	t(12;22)(p13;q11)	TEL-MN1	AML
	t(16;21)(p11;q22)	FUS-ERG	AML
Runt homology (AML1)	t(8;21)(q22;q22)	AML1-ETO	AML
	t(12;21)(p13;q22)	TEL-AML1	ALL
	t(3;21)(p26;q22)	AML1-EVI1	AML
	t(3;21)(p26;q22)	AML1-EAP	AML
	inv(16)(p13;q22)	CBF-MYH11[c]	AML

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; APL, acute promyelocytic leukemia; EPB, early pre-B.

Modified from Rabbitts, [2] *with permission from Nature. Copyright 1994 Macmillan Magazines, Ltd.*

[a] Based on DNA-binding domain.
[b] Partial list of MLL fusions.
[c] Lacks a DNA-binding domain, but forms a complex with AML1.

Activation of *bHLH, LIM*, and *HOX11* Genes in T-Cell ALL

In leukemias with a T-cell phenotype, chromosomal breakpoints consistently involve the TCR- chain enhancer (7q34) or the enhancer (14q11). These enhancers, which are highly active in committed T-cell progenitors, cause dysregulated expression of transcription factors located at the breakpoint on the reciprocal chromosome involved in these phenotype-specific rearrangements (Table 56-1). Within the bHLH class of transcriptional regulators, translocations in T-cell ALL affect the *myc*,[85] [86] [87] *TAL1/SCL*, [88] [89] [90] *TAL2/SCL2*, [91] and *LYL1* [92] genes. Presumably, dysregulated expression of these bHLH proteins in T lymphoblasts, like *myc* in B cells, causes transformation via altered expression of unknown target genes.

The best characterized of these genes is *TAL1*, which is altered by t(1;14) or by site-specific deletions in approximately one-fourth of childhood T-ALL cases. [93] [94] [95] [96] [97] [98] *TAL1* is expressed in a lineage-specific fashion and heterodimerizes with E2A. [99] Although not expressed in T cells, TAL1 is required for the generation of all hematopoietic lineages, which suggests that it is an essential regulator of early hematopoietic development. [100] [101] [102]

Another gene family whose expression can be altered by translocation into the proximity of the T-cell receptor loci is the LIM domain genes, *LMO1/RBTN1/TTG1* and *LMO2/RBTN2/TTG2*.[103] [104] [105] Both of these genes encode proteins that possess duplicated cysteine-rich LIM domains but lack homeobox DNA-binding domains as found in other transcription factors in this family, suggesting that the LIM domain may function in protein-protein rather than protein-DNA interactions. In support of this model, LMO2 interacts with TAL1 in erythroid cells and in T-cell leukemias. [106] [107] [108] Moreover, homozygous disruption of *LMO2* in mice causes the same phenotype as described previously for *TAL1* knockouts, indicating that a multiprotein complex involving LMO2, TAL1, and potentially other proteins is required for normal hematopoietic development.[102] [109] In addition, overexpression of *LMO1* or *LMO2* in thymocytes of transgenic mice leads to T-cell lymphomas, recapitulating human T-cell tumors.[110] [111] [112] [113] [114]

The homeodomain gene *HOX11*, located on chromosome 10, band 24, is one of the more interesting proteins activated by translocation into the vicinity of the TCR loci.[115] [116] [117] [118] [119] Within the homeodomain transcription factor superfamily, *HOX11* is most closely related to *HLX*, a recently described murine gene homeobox gene expressed in specific hematopoietic cell lineages and during embryogenesis. [120] *HOX11* is normally expressed during embryogenesis in specific regions of the branchial arches and ectoderm of the pharyngeal pouches of the developing hindbrain in the mouse, but not by developing thymocytes or resting or activated T cells.[121] Homozygous disruption of *HOX11* in the mouse results in asplenia in otherwise normal mice.[121] Although essential for the genesis of the spleen, the role

of HOX11 in the development of the branchial arch and hindbrain structures is apparently compensated for by other proteins, as the nervous system develops normally in these mice. When expressed in transgenic mice, HOX11 interferes with the G_2/M cell cycle checkpoint by interacting with specific phosphatases, demonstrating a mechanism by which HOX11 alters the cell cycle.[122] In contrast to loss of *LMO1* and *TAL1*, the development of the hematopoietic system is not affected by loss of *HOX11*. However, overexpression of Tal1 in T lymphocytes of transgenic mice results in T-cell lymphomas and leukemias similar to those caused by t(10;14) and t(7;10) in humans.

Other Genes Activated by Translocation

Transcription factors are not the only genes activated by translocation to the sites of the immunoglobulin or TCR genes. In cases of B-precursor ALL carrying t(5;14), the *IL-3* gene is activated by juxtaposition with the immunoglobulin heavy chain locus. [123] [124] In T-cell ALL, the *TAN1* gene, which shares homology with the *Drosophila notch* gene, undergoes dysregulated expression following its relocation to the TCR- locus as a result of t(7;9). [125] Similarly, relocation to the TCR- locus activates expression of the LCK tyrosine kinase genes in T-cell ALL cases with t(1;7). [126] [127] [128]

Chimeric Transcription Factor Genes

Formation of chimeric proteins whose functional domains come from two normally separate genes represents a second, more prevalent mechanism of aberrant transcription factor activation. Thus, chromosomal translocations may produce a chimeric protein by fusing the DNA-binding, dimerization, and trans-activation regions

of discrete genes, a process facilitated by the molecular structure of transcription factors.

E2A-PBX1 Fusion Genes in Pre-B ALL

A well-known example of a chimeric transcription factor with oncogenic potential is the *E2A-PBX1* rearrangement, which results from a t(1;19)(q23;p13) chromosomal translocation in human pre-B-cell ALL. This translocation fuses the *E2A* gene on chromosome 19, which encodes a bHLH transcription factor, to a homeobox gene (*PBX1*) on chromosome 1, leading to the expression of several forms of hybrid E2A-PBX1 oncoproteins (Fig. 56-2).[129] [130] [131] *PBX1* is related to the *Drosophila exd*, a homeotic gene that plays a role in segmental development through its ability to heterodimerize with and alter the downstream regulatory programs of the products of the HOM-C major homeobox genes.[4] [132] [133] [134] [135] [136] The hybrid proteins resulting from t(1;19) retain the amino-terminal trans-activation domains of E2A (AD1 and AD2), but not the bHLH DNA-binding/protein interaction domain. [130] [131] [137] The bHLH domain is replaced by the homeobox DNA-binding domain of PBX1, enabling the fusion protein to function as a chimeric transcription factor. [138] [139] [140]

The transforming potential of *E2A-PBX1* was first demonstrated by the rapid induction of AML in lethally irradiated mice repopulated with bone marrow stem cells that had been infected with recombinant retroviruses containing *E2A-PBX1* genes.[141] The fusion has also been shown to transform NIH-3T3 fibroblasts and induce T-cell lymphomas in transgenic mice.[142] [143] The transgenic mouse model also demonstrated lymphopenia in both B cells and T cells, suggesting induction of apoptotic cell death that preceded malignant transformation in T cells expressing E2A-PBX1 proteins. [142] Additional studies of

Figure 56-2 Proposed role of the E2A-PBX1 chimeric protein in ALL. Joining of the chromosome fragments produced by the t(1;19) translocation creates a fusion gene consisting of *E2A* and *PBX1* coding segments. The resulting protein binds to the DNA sequences normally recognized by the homeobox PBX1 protein; however, its effector (or trans-activator) region may interact with the transcription machinery in a manner analogous to that of the normal E2A protein. Consequently, transcription may be dysregulated and contribute to the development of ALL.

the fusion have shown that deletion of one of the E2A activation domains diminishes its transforming activity, but deletion of the PBX1 homeodomain has no effect. [143] [144] However, the homeodomain and flanking sequences are required for interactions with other HOX proteins and for optimal binding of E2A-PBX1 to specific DNA sequences.[145] [146] [147] [148] [149] It thus appears that complex interactions between E2A-PBX1 and other HOX proteins target the fusion protein to specific target genes whose activation is critical to lymphoid cell transformation.

Chimeric *E2A-PBX1* transcripts can be readily detected by using the reverse polymerase chain reaction (PCR) to amplify junctional sequences from leukemic cell RNA.[150] [151] In our analysis of 17 cases with t(1;19),[150] 10 of 11 pre-B cell cases expressing cytoplasmic immunoglobulin (cIg) heavy chains had typical *E2A-PBX1* chimeric transcripts with identical junctions. By contrast, none of the six cases of t(1;19)-positive, cIg ALL had evidence of detectable *E2A-PBX1* chimeric transcripts, suggesting that t(1;19) in these cases affects entirely different loci on chromosomes 1 and 19. Patients whose leukemic blasts express *E2A-PBX1* have a poor prognosis when treated with conventional antimetabolite-based therapy. [32] However, the poor prognosis of these patients can be overcome by the use of more intensive therapy, demonstrating the importance of detecting the *E2A-PBX1* fusion at diagnosis.

E2A-HLF Fusion Genes in Early Pre-B ALL

We and others have identified a second fusion gene, created by t(17;19), that combines *E2A* elements with those of a previously unidentified hepatic leukemia factor gene (*HLF*),[152] [153] which belongs to the PAR subfamily of bZIP transcription factors (Fig. 56-3).[154] [155] [156] [157] Although little is known about the normal function of PAR proteins, their similarity to the *C. elegans* CES-2 protein, which regulates the death of specific nerve cells in the developing worm, suggests a role in cell survival.[158] [159] [160]

The hybrid E2A-HLF protein expressed by leukemic cells contains the two E2A trans-activation domains linked to the DNA-binding/protein-protein interaction domain of HLF.[152] [153] As predicted by the absence of other PAR proteins in hematopoietic cells, the E2A-HLF fusion binds DNA as a homodimer. [161] Like E2A-PBX1, E2A-HLF can transform NIH-3T3 fibroblasts, a process that requires the HLF leucine zipper domain and the E2A trans-activation domains. [162] E2A-HLF can also induce lymphoid tumors in transgenic mice.[163]

Recent work from our laboratory has indicated that E2A-HLF functions in leukemogenesis by inhibiting apoptosis. Expression of a dominant-negative form of E2A-HLF in t(17;19)-carrying cell lines blocked E2A-HLF function and resulted in apoptosis. [158] In normal pro-B lymphocytes, expression of E2A-HLF reversed IL-3-dependent and p53-induced apoptosis. These results suggest that a normal apoptotic pathway in B lymphoblasts, perhaps regulated by a CES-2-like protein, is interrupted by E2A-HLF in t(17;19) leukemias.[158] [160] In these cases, E2A-HLF may activate target genes normally repressed by CES-2-like proteins, causing abnormal cell survival and leukemic transformation.

Because t(17;19) is seen in less than 1% of ALL cases, its clinical significance is unclear. [164] However, of seven patients whose blasts expressed *E2A-HLF*, each died of disease despite aggressive therapy.[161] [165] [166] [167] It seems likely that resistance to chemotherapy in these cases is mediated by the role of E2A-HLF in inhibiting apoptosis.

MLL Fusion Genes

Translocations involving chromosome 11 band q23 occur in approximately 80% of infant ALL cases, 5% of AML cases, and 85% of secondary AML cases that occur in patients treated with topoisomerase II inhibitors. [63] [168] [169] [170] [171] [172] [173] [174] [175] [176] [177] The gene that is bisected by 11q23 translocations is designated *MLL* and encodes a 431-kd protein that shares sequence homology with the *Drosophila trithorax* protein, a regulator of homeotic gene function during embryogenesis. [178] [179] [180] [181] *Trithorax* regulates homeotic genes in the *Antennapedia* and *Bithorax* complexes of the fly and is required for normal head, thorax, and abdomen development.[182] *MLL* shares three regions of homology with *trithorax*, including two central zinc finger domains and a 210-amino acid C-terminal segment. [178] [179] [180] [181]

Figure 56-3 Schematic diagram of the recently discovered E2A-HLF hybrid transcription factor. This protein, a result of the t(17;19) translocation in pro-B lymphoblasts, combines the trans-activation domain of the E2A protein with the bZIP DNA-binding and dimerization domain of HLF, a protein of the bZIP family that appears to regulate gene expression in hepatocytes and renal cells. Because HLF is not usually expressed in hematopoietic cells, its unscheduled presence in lymphoid progenitors may serve as an oncogenic stimulus. *(From Inaba et al.,[161] with permission.)*

This C-terminal region also shares homology with the *Drosophila enhancer of zeste*, which, like *trithorax*, regulates genes in the *Antennapedia* and *Bithorax* complexes.[183] Other structural features of MLL include three AT hook domains near the N-terminus, which are thought to bind the minor groove of DNA in AT-rich regions, and a 47-amino acid region of homology with the noncatalytic domains of human DNA methyltransferase. [184] [185]

Translocation breakpoints cluster in an 8.5-kb region of *MLL* between exons 5 and 11 and fuse the N-terminal region of MLL, containing the AT hook and methyltransferase domains, to a variety of partner proteins. [177] [179] [180] [186] [187] [188] [189] [190] [191] [192] [193] Although the role of the partner protein in leukemogenesis has not been determined, it appears that at least some of the partners may contribute functional domains to the fusion. For example, t(4;11), t(9;11) and t(11;19)(q23;p13.3) fuse MLL to AF-4, AF-9, and ENL, respectively. [179] [180] [186] [187] [191] [194] All three of these partners are small serine- and proline-rich proteins with nuclear localization signals, suggesting that they may function as transcriptional transactivators. An unrelated gene, *ELL*, is fused to *MLL* by t(11;19)(q23;p13.1).[188] [189] ELL was also independently isolated as an RNA polymerase II elongation factor. [195] Still other partners, however, may simply act to truncate MLL and interfere with its normal function.[193]

Homologous recombination techniques have been used to create mice lacking one or both copies of *Mll*, the murine homologue of *MLL*.[196] *Mll* heterozygous mice

demonstrated the effects of haploinsufficiency, including anemia, thrombocytopenia, and reduced numbers of B lymphocytes. In addition, these mice had homeotic transformations of the cervical, thoracic, and lumbar regions that were the result of shifts in the pattern of *Hox* gene expression, establishing a crucial role for *Mll* in *Hox* gene regulation. These results are also consistent with other data suggesting a role for *Hox* genes in normal hematopoiesis.[197] [198] Mice with *Mll* homozygously inactivated died in utero and demonstrated no *Hox* gene expression, further supporting a key role for *Mll* in *Hox* regulation.

Corral et al.[199] used similar recombination techniques to generate an *Mll-AF9* fusion gene in embryonic stem cells and then introduced these cells into blastocysts to create chimeric mice. Although cells of various tissues expressed *Mll-AF9* under the control of the normal *Mll* promoter, transformation occurred only in myeloid cells, consistent with the association of *MLL-AF9* with AML in humans. However, the chimeric mice developed AML after a latency period of 412 months, suggesting that additional mutations may be required for the fully transformed phenotype. Chimeric mice that expressed a truncated *Mll*, rather than *Mll-AF9*, did not develop malignancy, indicating that the fusion is essential for transformation.

Molecular techniques to detect *MLL* fusions in ALL include Southern blot analysis and RT-PCR. Southern blots can detect essentially all *MLL* rearrangements regardless of the partner gene involved, and this technology has proved to be more sensitive and specific than standard cytogenetics.[172] [174] [200] RT-PCR assays are even more sensitive than Southern blotting and have the advantage of detecting specific fusion transcripts.[201] [202] [203] [204] However, RT-PCR is limited to detection of fusions for which the partner gene has been cloned and sequenced. Clinically, it is important to identify all patients with *MLL* gene rearrangements, as these rearrangements are associated with dismal outcomes despite aggressive chemotherapy.[172] [174] [205] [206] [207] [208] [209] Thus, patients with *MLL* rearrangements are candidates for bone marrow transplantation in first remission, or for other highly intensive therapies.

TEL-AML1 Fusion Gene in Early Pre-B ALL

Although t(12;21) is detected by cytogenetics in less than 1 in 1,000 ALL cases, the *TEL-AML1* fusion gene it creates is actually the most common genetic lesion in pediatric ALL, occurring in one-fourth of B-lineage cases.[210] [211] [212] [213] [214] The chimeric protein contains the HLH domain of TEL fused to nearly all of AML1, including both the trans-activation domain and the DNA- and protein-binding runt homology domain. Although the role of *TEL-AML1* in lymphoid transformation has not yet been elucidated, it is interesting that both genes are involved in a variety of hematopoietic malignancies. *TEL* was first identified in a fusion with the platelet-derived growth factor receptor- gene (*PDGFR*) created by t(5;12) in chronic myelomonocytic leukemia[215] and is also fused to *abl* and *MN1* by t(9;12) and t(12;22), respectively.[216] [217] AML1 is the DNA-binding component of the AML1-CBF- transcription factor complex that is targeted by t(8;21), t(3;21), and inv(16) in AML.[218] This complex is thus the most common target of chromosomal translocations in leukemia (Fig. 56-4).

It seems likely that a lack of expression of genes normally turned on by *AML1* plays a role in *TEL-AML1*-induced transformation. Evidence in support of this model includes the interference of AML1-dependent transcriptional trans-activation by TEL-AML1 in vitro.[219] In addition, homozygous disruption of the murine *AML1* gene or *CBF* gene results in a lack of definitive hematopoiesis, indicating that genes regulated by AML1 are essential for normal hematopoietic development.[220] [221] [222] [223] [224]

We and others have recently demonstrated that *TEL-AML1* expression is associated with an excellent prognosis, with event-free survivals of approximately 90% (Fig. 56-5).[225] [226] [227] [228] In addition, the prognostic impact of *TEL-AML1* was independent of age and leukocyte count and remained favorable across several different protocols.[226] [227] Thus, *TEL-AML1* expression identifies a large but previously unrecognized subset of B-precursor ALL patients who may be candidates for less intensive therapy.

Figure 56-4 The transcription factor complex AML1/CBF is the target of a variety of translocations, including t(3;21), t(8;21), t(12;21), and inv(16). TA and DBD represent the transactivation and DNA-binding domains of AML1. In AML, the t(8;21) fuses the DNA-binding domain of AML1 to ETO, whereas the t(3;21) fuses the same portion of AML1 to either EAP or EVI1. In other cases of AML, the inv(16) fuses CBF to MYH11. In ALL, the t(12;21) creates a chimeric protein consisting of the helix-loop-helix portion of TEL fused to nearly all of AML1. *(Adapted from Shurtleff SA, Buijs A, Behm FG et al: TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subset of patients with an excellent prognosis. Leukemia 9:1985, 1995, with permission.)*

Figure 56-5 Kaplan-Meier estimates of event-free survival for patients with rearranged or germline *TEL*. The 5-year event-free survival estimates are shown above each curve. *(Adapted from Rubnitz et al.,[226] with permission.)*

Developmental Biology of Oncogenic Transcription Factors

A surprising connection has emerged from studies of oncogenic transcription factors and the developmental proteins regulating segmentation in *Drosophila*. The DNA-binding domains of these proteins often show striking homology (Table 56-2), an observation that carries important implications for the types of DNA sequence elements recognized by many transcription factors.

The classes of *Drosophila* segmentation genes shown in the first column of Table 56-2 reflect findings made over the last decade on the molecular control of pattern formation during *Drosophila* embryogenesis.[229] [230] According to this model, gap proteins are expressed in broad domains, approximately three segments wide, in response to gradients of maternal cytoplasmic polarity proteins. *Gap* genes are among the first to be transcribed in the embryo and earned their name from the characteristic spaces produced in segmentation patterns by mutations of these genes. Gap proteins act in concert to regulate the expression of pair-rule genes, which are transcribed in the primordia of every other segment and form repetitive segmental divisions in the embryo, and are expressed in a pattern of seven stripes along an anterior-to-posterior axis. Mutations of pair-rule genes generally cause the deletion of portions of every other segment. Pair-rule proteins regulate the expression of segment polarity genes, which are responsible for the formation of certain repeated structures, such as the boundary regions, common to each segment. Mutations of these genes cause the deletion of a portion of each segment, which is repetitively replaced by a mirror-image structure from the same segment. Pair-rule proteins interact with gap proteins and other regulators to control the expression of homeotic genes, which encode proteins that determine the unique structures (e.g., antennae, wings, legs) expressed by each segment. Thus, each of these classes of genes encodes transcriptional regulatory proteins whose expression is determined as a hierarchical cascade in the developing embryo.[231] Ultimately, these transcription factor genes regulate the developmental programs of structural genes that define the three-dimensional form of the unfolding embryo.

The structural similarities shown in Table 56-2 among the proteins regulating segmentation pattern in *Drosophila* and the oncogenic transcription factors activated by chromosomal translocation in acute leukemia suggest that these two classes of proteins may share functional properties as well. An obvious difference, however, is that the *Drosophila* proteins have been identified by the morphologic effects of recessive mutations that cause loss of function, whereas in ALL the proteins arise from dominant lesions that activate expression from one copy of the affected gene or genes, most of which are normally quiescent in lymphoid cells. The suggestion from this body of research is that the morphologic changes associated with lymphoid and

TABLE 56-2 -- Oncogenic Transcription Factors in Human Leukemia: Convergence with Proteins Regulating Segmentation Pattern in *Drosophila*

Drosophila Protein	Morphogenetic Role	Common DNA-Binding Domain	Leukemia Protein	Activating Translocation
Giant	Gap	bZIP	HLF	t(17;19)(q22;p13)
Krüppel	Gap	Zinc finger	PLZF	t(11;17)(q23;q21)
Runt	Pair-rule	Undefined	AML1	t(8;21)(q22;q22)
Antennapedia	Homeotic	Homeobox	HOXA9	t(7;11)(p15;p15)
Extradenticle	Homeotic	Homeobox	PBX1	t(1;19)(q23;p13)
Trithorax	Homeotic gene regulator	AT hook	MLL	t(4;11)(q21;q23) t(9;11)(p21;q23) t(11;19)(q23;p13)

myeloid cell differentiation are regulated by proteins analogous to those regulating the segmentation pattern during *Drosophila* embryogenesis. Hence, the proteins participating in leukemogenesis apparently interfere with a network of hematopoietic transcription factors leading to arrested cell development at stages corresponding to those of early lymphoid or myeloid progenitors. Recent evidence implicating the major *HOX* genes in the control of hematopoiesis as well as embryogenesis, together with the established roles of *Drosophila* segmentation genes in controlling *HOM-C* gene expression, suggests that the *HOX* loci may serve as proximal targets of a wide spectrum of hybrid and dysregulated transcription factors in the acute leukemias. [4] In this model, the oncogenic transcription factors inappropriately activate or suppress the expression of *HOX* genes, which in turn regulate gene programs with pleiotropic effects on normal hematopoietic cell development.

Tyrosine Kinase Genes: *bcr-abl*

The 22q chromosomal marker, which arises from the t(9;22)(q34;q11), was originally identified in patients with chronic myelogenous leukemia (CML), but is also found in about 4% of childhood cases and 25% of adult cases of ALL. [16] [232] [233] [234] The t(9;22) breakpoints on the distal tip of the long arm of chromosome 9 are variable in CML and may occur over a distance of more than 100 kb within the *abl* proto-oncogene, upstream of the tyrosine kinase domain. [235] [236] [237] By contrast, the CML breakpoints on chromosome 22 are confined to a 5.8-kb region of DNA known as the major breakpoint cluster region (M-bcr). [238] This region of chromosome 22 is within a gene called *bcr* [238] [239] that encodes a 160-kd phosphoprotein with undetermined function. As a result of the translocation, a *bcr-abl* fusion gene is produced, consisting of 5 (upstream) sequences from *bcr* and 3 (downstream) sequences of *abl*. The 8.5-kb fusion transcript found in CML encodes a 210-kd hybrid protein (p210) that is activated as a tyrosine-specific protein kinase similar to the v-abl protein. [240] [241] [242] [243]

Although t(9;22) is identical by karyotyping in CML and ALL, molecular studies of the *bcr* and *abl* proto-oncogenes, which are rearranged in both diseases, have revealed potentially important differences. [244] [245] [246] In ALL, the rearrangement produces a 6.5- to 7.0-kb fusion transcript and a 185- to 190-kd hybrid protein (p190), which are distinct from the products of the rearranged *bcr-abl* fusion gene in CML. [244] [245] [246] The breakpoints on chromosome 22 in ALL cases are not within the 5.8-kb region of *bcr* that contains the breakpoints in CML, but lie further upstream within the *bcr* gene, in a minor breakpoint cluster region (m-bcr). [247] [248] [249] The ALL fusion protein includes N-terminal bcr amino acids but lacks the internal residues found in the CML fusion proteins near the *bcr-abl* junction.

N-terminal sequences of *abl* are replaced in activated forms of the gene, with the Moloney virus *gag* gene in the case of v-*abl* [250] [251] [252] and with *bcr* in the 9;22 translocation of CML. [240] [241] [242] [243] Both the v-*abl* and the *bcr-abl* fusion genes can transform pre-B cells, but the *bcr-abl* fusion gene is unable to transform fibroblasts unless it is also fused to the *gag* gene. [253] Thus, N-terminal alterations influence not only the ability of *abl* to function as a lineage-specific transforming gene but also the tyrosine kinase activity of its protein product.

When expressed in murine hematopoietic precursors, both p190 and p210 induce a syndrome similar to CML. [254] [255] [256] [257] Ras signaling is required for transformation, and activated abl is coupled to ras by the GRB2, SHC, and CRKL adapters, resulting in activation of Jun kinase. [258] [259] [260] Transformation by bcr-abl also involves myc, cyclin D1, and the signal transducer STAT5. [261] [262] [263] Hence, multiple signaling pathways and cell-cycle-regulated genes are involved in transformation of hematopoietic cells by bcr-abl.

Expression of *bcr-abl* is associated with an extremely poor prognosis in ALL patients despite treatment on contemporary protocols. [233] [264] [265] Until recently, it was believed that the only curative treatment for Philadelphia chromosome-positive ALL was bone marrow transplantation in first remission. However, results from St. Jude indicate that a subset of these patients, primarily those with low leukocyte counts at diagnosis, are, in fact, cured with intensive chemotherapy. [266] [267]

Tumor Suppressor Genes

Much attention has been focused on recessively acting oncogenes whose products normally suppress tumor formation in differentiating cells of a particular lineage. Loss of function of a tumor suppressor protein, occurring through deletion or mutational inactivation of both chromosomal loci of the gene that encodes it, leads to malignant transformation. Knudson first proposed that inactivation of both alleles of a single locus is needed to initiate the development of retinoblastoma, basing his ideas on the observed frequencies of hereditary and sporadic forms of this disease. [268] Allelic loss of defined regions of many different chromosomes has been linked to specific types of human tumors. [269] [270] By analogy with the findings in retinoblastoma, a reasonable hypothesis is that each of these regions harbors a tumor suppressor gene whose product is uniquely involved in the inhibition of cell cycle progression and promotion of terminal differentiation of the normal cells that give rise to these different types of tumors. Tumor suppressor genes that play an important role in ALL include *p53* [271] [272] [273] [274] [275] [276] and the *p16* locus. [277] [278]

p53, which is located on chromosome 17, band p13, is mutated or lost through chromosomal deletion in a wide variety of human tumors, [274] including colon cancer, [275] [276] lung cancer, [279] breast cancer, [280] and osteosarcoma. [281] Heritable cancer associated changes of the *p53* tumor suppressor gene occur in families with Li-Fraumeni syndromean unusual aggregation of sarcomas, brain tumors, leukemias, adrenocortical carcinomas, and premenopausal breast cancers. [282] [283] [284] [285] *p53* encodes a 53-kd transcription factor that functions as a cell cycle checkpoint. [271] [272] [273] [274] [275] [276] [282] [283] [284] [285] *p53* expression is increased by DNA damage, blocks cell division at G_1 to allow DNA repair, and activates apoptosis in cells that have suffered DNA damage. [271] [286] [287] [288] [289] [290] [291]

p53 is also inactivated in a variety of hematopoietic malignancies, including B-cell ALL and Burkitt lymphoma, but is mutated or deleted in less than 3% of pediatric B-precursor or T-cell ALL cases at diagnosis. [292] [293] [294] It thus appears to play a limited role in the etiology of pediatric leukemia. *p53* mutations, however, are seen in approximately 25% of relapsed T-cell ALL cases, suggesting a role for *p53* inactivation in the development of resistant disease. [292] [293] In addition, *p53* mutations were detected in three of ten ALL patients in whom induction therapy failed or who sustained early relapse, further supporting a role for *p53* inactivation in disease progression. [295] [296]

The cyclin-dependent kinase inhibitors (CDKIs), which include p15 (INK4B/MTS2), p16 (INK4A/MTS1/CDKN2), p18 (INK4C), p19 (INK4D), p21 (WAF1/CIP1/SDI1/CAP20), p27 (KIP1), and p57 (KIP2), compose a family of tumor suppressors that negatively regulate the cell cycle by inhibiting cyclin-dependent kinase (CDK) phosphorylation of pRB. [297] For example, *p15* and *p16* encode highly homologous proteins that function as specific cell cycle inhibitors by inhibiting activated cyclin D-cdk4/6 complexes. These complexes control the cell cycle by phosphorylating and inactivating pRB, leading to release of transcription factors necessary for entry into S phase. Thus, inactivation of *p16* is predicted to increase the proportion of cells entering S phase and hence increase cell proliferation. In fact, while the roles for *p15, p18, p19, p21, p27*, and *p57* in malignancy appear to be limited, *p16* is among the most commonly mutated genes in human cancer. [277] [278]

Deletions of *p16* were initially detected in nearly one-half of

tumor-derived cell lines, suggesting that *p16* is a tumor suppressor gene. [277] [278] Subsequent analysis of primary tumor samples, however, revealed a much lower frequency of *p16* deletions (1020%) and very few point mutations, suggesting that inactivation in some cases occurs during the establishment of permanent cell lines in vitro. Homozygous deletion of *p16* has been detected in approximately one-fourth of pediatric B-precursor ALL cases and three-fourths of T-cell cases. [298] [299] [300] [301] [302] [303] [304] [305] [306] [307] [308] [309] In addition, *p16* deletions are associated with higher leukocyte counts at diagnosis and may represent an independent prognostic factor in pediatric ALL. [301] [303] [309]

Direct evidence for the role of the *INK4a* locus in tumorigenesis was recently provided by the targeted disruption of exon 2 of *p16* in mice. [310] *p16*-deficient mice developed tumors (primarily lymphomas and fibrosarcomas) that were enhanced by the topical application of carcinogens and ultraviolet light. [310] A major breakthrough, however, has shed new light on the role of *INK4a* as a tumor suppressor locus. [311] [312] This locus, which encodes the p16 protein, also encodes a protein from an alternative reading frame, designated $p19^{ARF}$. [311] Interpretation of *p16* knockout experiments was uncertain because targeted disruption of exon 2 of *p16* also disrupted $p19^{ARF}$. Further genetic analysis showed that selective disruption of $p19^{ARF}$ reproduced the phenotype described for *p16*-null mice, indicating that $p19^{ARF}$ is a bona fide tumor suppressor. [311] In addition, embryonic fibroblasts from $p19^{ARF}$-null mice did not senesce and were efficiently transformed by Ha-*ras*, indicating a crucial role for $p19^{ARF}$ in cell cycle control and immortalization. It is likely that both *p16* and $p19^{ARF}$ are tumor suppressors, acting through either the pRB

or p53 pathways in different tumor subsets.[312]

Identification of recurring chromosome deletion syndromes in human ALL indicates that other tumor suppressor loci may be involved in this disease. These syndromes, which affect the long arm of chromosome 6, the short arm of chromosome 9, or the short arm of chromosome 12, can be found in leukemic cells from approximately 10% of patients with ALL, making them among the most frequent cytogenetic abnormalities in this disease. Functional deletion can result either from interstitial deletion of the involved chromosome arm or from derivative chromosomes that result from unbalanced chromosomal translocations. For each chromosome, the deleted regions overlap a single target region, which may contain key genes of the tumor suppressor type, whose loss could be an important step in leukemic transformation.

Deletions of the long arm of chromosome 6 are consistently found in about 10% of cases of ALL. [63] Interstitial deletions affecting bands 6q15q24 have been reported most frequently; translocations with breakpoints within this region are also common. Band q21 of chromosome 6 seems to be involved in each of the abnormalities, suggesting that the target gene(s) resides in this region. Deletions of chromosome 6q occur with equal frequency in pro-B, pre-B, and T-cell cases.

Deletions or translocations involving the short arm of chromosome 12 are also found in about 10% of ALL cases, with most clustered around band 12p13. [63] These cases generally have a B-precursor phenotype, and blast cells usually express CD10 and HLA-DR on the cell surface. Abnormalities of the short arm of chromosome 12 are rarely found in T-cell cases. Translocations involving chromosome 12p13 may be balanced or unbalanced and can involve multiple different donor chromosomes. Molecular studies, however, have revealed that the majority of translocations involving 12p13 are cryptic 12;21 translocations, resulting in the *TEL-AML1* fusion.[210] [211] [212] [213] [214] In the cases with unbalanced translocations, DNA sequences distal to the breakpoint are lost from the affected homolog and subsequently from the leukemia cell genome, so the result is similar to interstitial deletion. The frequency of deletions involving the 12p13 region suggests that these lesions primarily inactivate one allele of a tumor suppressor gene in this chromosomal region. Although the *TEL* gene is a common target of deletion in these cases, there is also evidence for a second tumor suppressor gene in the 12p13 region. [313] [314] [315] [316] [317] [318]

Deletions affecting the short arm of chromosome 9 are associated with T-cell disease, bulky lymphadenopathy, splenomegaly, hyperleukocytosis, and a high risk of treatment failure, but they may also occur in patients with standard-risk early pre-B ALL. [319] [320] The critical region of deletion appears to be the 9p2122 region, suggesting the presence of a potential tumor suppressor gene.

Mutated *ras* Genes

Activation of cellular proto-oncogenes by point mutation is difficult to detect, because such lesions lack the cytogenetic abnormalities that signal other forms of transforming alterations. Genes of this type must be identified in experimental systems, so that investigators know in advance the type of activating point mutations that are likely to occur in human tumors. The prototypic genes of this class are genes of the *ras* family, with mutations affecting defined amino acids of the corresponding proteins. Human tumor DNAs were initially found to contain activated homologs of either the H- *ras* or K-*ras* genes,[321] [322] [323] proto-oncogenes that had already been identified on the basis of their homology with viral oncogenes. Gene transfer methods identified an additional member of the *ras* gene family, called N-*ras*, [324] [325] that had not been previously observed as a component of a transforming retrovirus.

Proto-oncogenes of the *ras* familyH-*ras*, K-*ras*, and N-*ras*encode 21-kd proteins that are associated with the inner surface of the cytoplasmic membrane.[326] These proteins bind guanidine nucleotides and function as intermediates in signal transduction pathways that regulate the growth of cells. The *ras* proto-oncogenes are activated to transforming oncogenes by somatic mutations that alter the amino acids specified by codons 12, 13, or 61. [327] Mutated *ras* genes also bind guanine nucleotides, but have diminished capacity to hydrolyze GTP to GDP. [328] [329] [330] Transforming properties of activated ras proteins may result from their inability to hydrolyze GTP, which could play an important role in modulating signal transduction.

The transforming potential of human *ras* genes activated by point mutation has been documented in experimental systems. The *ras* oncogenes will transform NIH-3T3 murine fibroblasts in vitro, and will collaborate with other oncogenes to transform primary cultures of embryonic fibroblasts. [331] [332] [333] [334] In addition, their role in mammalian tumorigenesis has been documented in carcinogen-induced animal tumor model systems.[335] [336] [337]

Activated N-*ras* genes appear to be preferentially involved in hematopoietic malignancies. They were detected in the myeloid cell lines HL-60, KG1, and Rc2A, [338] [339] in fresh leukemic cell samples from patients with AML and CML,[340] [341] [342] and in lymphoblastic leukemias with a T-cell immunophenotype.[343] In AML, N-*ras* gene mutations involving codon 13 or 61 were found in approximately 20% of cases, regardless of morphologic subtype. [340] [344] Mutation of codon 12 of the K-*ras* gene was also observed in two of 37 cases studied.[344] In a study of lymphoblasts from children with ALL, 2 of 19 patients showed mutated N-*ras* genes, both involving codon 12.[345] Mutated *ras* genes have also been documented in patients with preleukemic syndromes, indicating the potential involvement of activation of these genes in an early stage in the biogenesis of some leukemias.[346]

Gene Amplification

Gene amplification at the DNA level provides the cell a means to increase expression of critical genes whose products are ordinarily tightly controlled. Clinically important examples of

proto-oncogene amplification have been documented in solid tumors of adults and children. For example, the N- *myc* gene is amplified 10- to 300-fold in tumor cells in about one-third of cases of childhood neuroblastoma; such amplification has been linked to an advanced stage of disease and a poor prognosis. [347] However, the cytogenetic hallmarks of gene amplificationdouble-minute chromatin bodies and homogeneously staining regionsare rarely found in karyotypes of human leukemia cells, making it unlikely that consistently amplified cellular proto-oncogenes will be identified in this disease.

Abnormalities of Leukemia Cell Ploidy

Abnormalities of chromosome number, which generally occur in the absence of specific chromosomal translocations, have important prognostic implications in childhood ALL.[348] [349] [350] In an analysis of patients treated in the St. Jude Total Therapy studies IX and X, chromosome number as determined by karyotyping was the strongest single predictor of outcome: patients with hyperdiploid >50 chromosomes had the best response to therapy. [350] Hyperdiploid ALL patients can also be identified rapidly by flow cytometric measurement of the DNA of leukemic blast cells, in which nuclei stained with a DNA-specific dye are analyzed for the amount of DNA per cell.[351] This DNA measurement (DNA index, or DI) is expressed as the ratio of the cellular DNA content of G_1 /G_0 -phase leukemic cells compared with that of normal diploid cells. Using this technique, we showed that among patients treated on our Study X protocol, DI 1.16, corresponding to 53 chromosomes, was the most favorable prognostic factor.[351] More recently, the favorable impact of hyperdiploidy has been confirmed for patients treated on the Pediatric Oncology Group AlinC 14 protocol[352] and St. Jude studies XI[34] and XII.[353] The improved outcome for hyperdiploid patients may be due, at least in part, to increased sensitivity of hyperdiploid blasts to antimetabolites and to increased accumulation of methotrexate polyglutamates. [354] [355]

Specific subsets of the hyperdiploid group of patients may have even better outcomes than the group as a whole. For example, Harris et al. [356] have demonstrated that among patients with DI 1.16, those with trisomies of both chromosomes 4 and 10 had a better outcome than those without these trisomies. Similarly, we have shown that patients with 5667 chromosomes fare better than those with 5155 chromosomes.[357] In contrast to the hyperdiploid group, patients with a near-haploid or hypodiploid <45 line appear to have a poor prognosis and may require alternative therapies. [358]

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IMPLICATIONS OF GENETIC FINDINGS FOR LEUKEMIA THERAPY

Childhood ALL: A Model for Gene-based Risk Assessment

Chromosomal and molecular genetic abnormalities in the leukemic blasts of children with ALL are among the best predictors of response to currently available chemotherapy. Initially performed by time-consuming and labor-intensive cytogenetic methods, the classification of chromosomal abnormalities in leukemic lymphoblasts has been aided by flow cytometric techniques (to detect hyperdiploidy) and by clinically applicable molecular techniques. [355] Thus, at St. Jude Childrens Research Hospital, leukemic blast cells from each new case of childhood ALL are examined for the ploidy of leukemic stem lines, *MLL* and *TEL* gene rearrangements, and expression of the *MLL-AF4, TEL-AML1, E2A-PBX1*, and *bcr-abl* fusion transcripts. With the availability of these tests, uniform treatment of pediatric ALL patients is no longer optimal. Rather, modern trials emphasize risk-based therapy to reduce toxicity in good-risk patients and to ensure appropriate therapy for patients at a high risk of relapse.

Currently, we propose a risk classification scheme for childhood ALL based largely on genetic features of leukemic blasts (Table 56-3).[353] [360] According to this scheme, patients with hyperdiploidy or *TEL-AML1* are in a low-risk group and are candidates for antimetabolite-based therapy. The intermediate-risk group includes patients with standard-risk age and leukocyte count as defined by the NCI criteria, [361] if they have no prognostically important genetic features. The high-risk group includes patients with high-risk age and leukocyte count, [361] T-cell phenotype, *E2A-PBX1*, or a slow, early response to therapy. Patients in the very high-risk groupthose with *MLL* gene rearrangements or expression of *bcr-abl*are eligible for bone marrow transplantation in first remission. This risk classification scheme provides a means to incorporate the molecular genetic findings of the past decade into therapy trials that seek to define optimal treatment intensity.

Therapy Targeted to Oncogenic Transcription Factors

Improved understanding of the leukemia-inducing pathways mediated by aberrantly controlled transcription factors has provided a mechanistic underpinning for the positive responses of acute promyelocytic leukemia (APL) patients to therapy with all-trans-retinoic acid (ATRA) and arsenic trioxide

TABLE 56-3 -- Clinical Risk Assignment in Childhood ALL by Genetic Characterization of Leukemic Lymphoblasts

Risk Group	Features	Patients Affected (%)	Recommended Therapy
Low risk	Hyperdiploid (DI >1.16)	20	Conventional anti-metabolite-based
	TEL-AML1 fusion	20	
Intermediate risk	Standard-risk age/leukocyte count,[a] without clinically significant genetic risk features	15	Intensified anti-metabolite based
High risk	*E2A-PBX1* fusion	6	Intensive multi-agent chemotherapy
	T-cell ALL	15	
	High-risk age/leukocyte count,[a] without clinically significant genetic risk features	15	
Very high risk	*bcr-abl* fusion	3	Allogeneic bone marrow transplantation in first remission
	MLL rearrangement	4	
	Induction failures	2	

[a] Standard- and high-risk refer to NCI criteria.[361]

Figure 56-6 (Figure Not Available) The effects of all- *trans*-retinoic acid (ATRA) and arsenic trioxide ($As_2 O_3$) on the blast cells of acute promyelocytic leukemia. ATRA interaction with PML-RAR causes degradation of the fusion protein and reassembly of PML into nuclear structures called PODs. $As_2 O_3$ induces even more rapid degradation of PML-RAR and eliminates native PML. ATRA leads to promyelocyte differentiation, while $As_2 O_3$ induces programmed cell death. *(Adapted from Look AT: Arsenic and apoptosis in the treatment of acute promyelocytic leukemia. J Natl Cancer Inst 90:86, 1998, with permission.)*

($As_2 O_3$).[362] [363] [364] [365] [366] [367] [368] The vast majority of APL patients have a t(15;17) that produces PML-RAR fusion proteins, whose DNA and ligand-binding domains, encoded by the *RAR* gene on chromosome 17, are linked to amino acids encoded by the *PML* gene on chromosome 15.[369] [370] [371] [372] [373] PML-RAR fusion proteins appear to interfere with normal myeloid cell development, leading to arrested differentiation in the promyelocyte stage and promoting the survival of the leukemic cells.[374] In response to pharmacologic doses of ATRA, the fusion proteins are degraded and the leukemic cells differentiate into mature myeloid cells with a limited life span (Fig. 56-6 (Figure Not Available)). [363] [364] [365] This therapy is specific for APL blasts that express PML-RAR fusion proteins; it is ineffective for other types of myeloid leukemia. Although resistance to ATRA develops quickly, within 34 months on the average, this specific modulator of PML-RAR chimeric receptors has proved a useful adjunct to cytotoxic chemotherapy for inducing remissions and improving disease-free survival in APL patients. [375]

Recently, $As_2 O_3$ was shown to be effective in patients with APL that was resistant to ATRA.[367] [368] In contrast to ATRA, which causes terminal differentiation of leukemic promyelocytes, $As_2 O_3$ rapidly induces apoptosis (Fig. 56-6 (Figure Not Available)). [376] [377] Because the apoptotic effects of $As_2 O_3$ occur in cells sensitive or resistant to ATRA, the incorporation of this agent into front-line protocols may improve the outcome of APL patients. In addition, it may be especially useful in the treatment of relapsed patients.

Whether better understanding of the mechanisms of action of other chimeric or dysregulated leukemogenic transcriptional control proteins will lead to improved therapy is difficult to answer. Unfortunately, most transcription factors do not contain convenient ligand-binding domains, so the model provided by chimeric PML-RAR proteins in APL will be difficult to reproduce in leukemias with the activated transcription factor genes listed in Table 56-1 . Nonetheless, new drugs may be found that

interfere with the modulation of gene expression by specific transcription factors and lack many of the side effects of available cytotoxic agents. Other approaches, such as antisense strategies to block chimeric transcripts and gene therapy to introduce dominant negative inhibitors of protein-protein or protein-DNA interactions essential for transcription factor function, could yield novel therapies. [4]

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AML DEVELOPING AFTER THERAPY FOR ALL

With the wide use of genotoxic drugs, including the epipodophyllotoxins, in recent clinical trials, an apparent increase has been observed in the incidence of secondary AML developing in patients treated for ALL. [378] [379] [380] [381] Epipodophyllotoxin-induced secondary AML is characterized by rearrangements of the *MLL* gene, a short incubation period without a myelodysplastic phase, and a monoblastic morphology. [200] [378] [380] [382] [383] [384] [385] In addition, the risk of secondary AML is influenced by the schedule of epipodophyllotoxin administration and the concomitant use of other agents. [379] [380] [381] Unfortunately, secondary AML induced by epipodophyllotoxins is generally not responsive to chemotherapy, suggesting that allogeneic bone marrow transplantation is warranted for this group of patients.

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FUTURE DIRECTIONS

The immediate applications of the emerging molecular information include a redefinition of risk classification schemes to emphasize the roles of somatically acquired genetic abnormalities that carry a defined prognosis and likelihood of therapeutic failure. Patients are assigned to treatment according to their initial clinical features and, increasingly, the genetic and biologic properties of their leukemic cells. We are now in a position to view ALL as a group of heterogeneous diseases defined by discrete molecular lesions. As these lesions have been systematically analyzed in larger numbers of patients, it has been possible to devise new classification schemes for ALL that reflect prognosis with exquisite precision. The opportunity is now at hand to improve therapy through randomized trials coordinated on a nationwide or even worldwide scale that focus on key subsets of acute leukemia patients whose lymphoblasts harbor specific chimeric fusion genes. The development of new drugs based on the molecular biology of ALL is clearly a priority for the future, and will likely take the form of compounds developed to specifically interfere with oncoproteins expressed by each patients leukemic blasts.

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Chapter 57 - Clinical Manifestations of Acute Lymphoblastic Leukemia

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Before the institution of modern chemotherapy, acute lymphoblastic leukemia (ALL) was a uniformly fatal disease, most patients surviving only 23 months. With current chemotherapy, most children with ALL have prolonged disease-free survival and up to approximately 70% are considered cured. Although most adults also attain complete remission with chemotherapy, 3- to 5-year actuarial survival rates range from only 2035%. [1] [2] [3]

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EPIDEMIOLOGY

Acute lymphoblastic leukemia is the most common malignancy of childhood. In the pediatric population, ALL is five times more frequent than acute nonlymphoblastic leukemia (ANLL). In contrast, ALL accounts for less than 1% of all adult malignancies, and for less than 20% of the acute leukemias in this population. [4] [5] [6] In children, the peak incidence of ALL occurs at approximately 2 to 3 years of age. [7] In adults, the greatest number of cases occur in those older than 65 years.

Some children are at particular risk for development of ALL. Children with certain chromosomal abnormalities, including Downs syndrome, [8] Blooms syndrome,[9] Fanconis anemia, [10] and ataxiatelangiectasia,[11] are all at higher risk than the general population for development of leukemia. In addition, siblings, especially twins, of children with leukemia are at greater risk for development of leukemia, although this risk may be only approximately twice that of the general population. [12] [13] Some cases of childhood ALL may be related to hereditary or acquired mutations in the *p53* gene.[14]

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CLINICAL MANIFESTATIONS

Patients with ALL present most frequently with signs and symptoms of the uncontrolled growth of leukemic cells in bone marrow, lymphoid organs, and other sites of extramedullary spread. Bone marrow involvement results in varying degrees of anemia, thrombocytopenia, and granulocytopenia that may be manifested by pallor and fatigue, petechiae, purpura or bleeding, and fever. Liver, spleen, and nodal enlargement is present in most patients and are the most common sites of extramedullary disease spread. Both hepatosplenomegaly, which occurs in approximately two thirds of patients, and lymphadenopathy, clinically detectable in over half of presenting cases, are usually asymptomatic. Bone pain, however, is a common presenting feature, particularly in the young child with ALL whose first symptom may be the onset of a limp or refusal to walk. [15]

Symptoms may be present from a few days to several weeks before the diagnosis of ALL is made, although in some cases a relevant clinical history may precede diagnosis by several months. The nonspecific nature of the signs and symptoms of ALL occasionally leads to delay in diagnosis. In addition, because ALL may imitate a variety of disorders, there may be diagnostic confusion. For example, arthralgias arising from leukemic infiltration of the joints may be confused with juvenile rheumatoid arthritis or osteomyelitis. In rare cases, ALL has presented with unusual manifestations such as aplastic anemia or hypereosinophilia (Table 57-1 (Table Not Available)). [16] [17]

Extramedullary Spread

Acute lymphoblastic leukemia frequently involves organs other than bone marrow. Most patients have some extramedullary disease at diagnosis and extramedullary relapse is a known complication of the disease. The incidence of occult extramedullary involvement in patients presumed to be in clinical remission is difficult to ascertain, but has been estimated to be as high as 50%. [18] [19] [20] Actual organ dysfunction secondary to leukemic involvement is rare and usually is seen in patients with progressive, end-stage disease. The occurrence of an extramedullary relapse is significant because it frequently heralds the development of bone marrow relapse. The most commonly affected extramedullary sites of disease include the central nervous system, testes, lymph nodes, liver, spleen, and kidney. Of these sites, the central nervous system (CNS) and the testes have the greatest clinical significance.

Central Nervous System

Central nervous system leukemia is presumed to develop either from hematogenous spread, through seeding of the meninges by circulating leukemic cells, or by direct extension from involved cranial bone marrow. [21] [22] [23] The meninges are the primary site of disease, but, particularly in advanced disease, other sites within the brain parenchyma and spinal cord may be involved. [24] In patients with clinically overt CNS leukemia, signs and symptoms are usually caused by increased intracranial pressure and include headache, nausea and vomiting, lethargy or irritability, papilledema, and nuchal rigidity. Cranial nerves, most commonly the seventh, third, fourth, and sixth, may be involved and may on rare occasions be an isolated site of CNS relapse. The hypothalamicobesity syndrome, in which infiltration of the hypothalamus produces hyperphagia and pathologic weight gain, is a rare complication of CNS leukemia. Because of its varied symptomatology, CNS leukemia must be

TABLE 57-1 -- Some Unusual Clinical Presentations of Acute Lymphoblastic Leukemia

(Not Available)

Adapted from Nesbit,[116] with permission.

considered as a possible diagnosis in any patient with ALL in whom neurologic signs and symptoms develop.

The diagnosis of CNS leukemia is made by evaluation of cerebrospinal fluid (CSF) obtained by lumbar puncture. CNS leukemia is often classified into three groups: CNS 0, in which there are <5 WBC/l of CSF and no blasts are seen; CNS 2, in which there are <5 WBC/l of CSF, but blasts are present; and CNS 3, in which there are 5 WBC/l of CSF and blasts are present, or cranial nerve palsies are noted. [21] In symptomatic patients, the opening CSF pressure is usually increased, the CSF cell count is usually elevated, the CSF protein is frequently elevated, and the CSF glucose may or may not be decreased. CSF examination after concentration of cells by cytocentrifugation reveals the presence of leukemic lymphoblasts. The heightened awareness of CNS leukemia has led to routine lumbar puncture surveillance of patients undergoing treatment. As a consequence, in many patients, the diagnosis of CNS leukemia is being made earlier in the course of CNS disease. In such cases, the CSF opening pressure, cell count, protein, and glucose may all be normal and only careful examination of a cytocentrifuged CSF specimen may help identify leukemic lymphoblasts. Other techniques have been explored in an attempt to improve the ability to diagnose CNS leukemia in equivocal cases, including terminal deoxynucleotidyl transferase (TdT) determination and the use of monoclonal antibodies and flow cytometry. Contrast-enhanced magnetic resonance imaging or computed tomography scanning may also be useful.

Central nervous system involvement is relatively uncommon at diagnosis. Less than 5% of children and up to 15% of adults have evidence of CNS disease on initial evaluation. [24] Unless adequate CNS preventive therapy is administered, however, CNS disease eventually develops in most patients. This is presumed to be related, in part, to the bloodbrain barrier, which effectively makes the CNS a pharmacologic sanctuary and prevents many systemically administered antileukemic agents from penetrating adequately into the CNS. For this reason, specific CNS treatment, in the form of intrathecal chemotherapy or cranial radiation, is required to prevent the development of CNS leukemia. Once present, CNS leukemia, although usually controllable in the short term, frequently recurs. The most important consequence of CNS leukemia is that it places patients at a high risk of subsequent bone marrow relapse. Therefore, the development of effective means of preventing CNS disease has become an important focus of clinical investigation, particularly (but not solely) in childhood ALL, where the incidence of CNS disease historically has been higher than in adults.

Testicular Leukemia

As the survival of patients with ALL has increased, so has the incidence of testicular involvement, particularly in children, where testicular leukemia has become a major site of disease recurrence. This complication occurs in approximately 1015% of boys undergoing chemotherapy. The testes are also a site of late relapse in a substantial proportion of boys who had previously successfully completed a full chemotherapy regimen. [25] [26] Although clinically evident testicular involvement is rare at initial diagnosis, occult testicular disease has been reported in up to 25% of newly diagnosed boys. [27] When clinically overt, testicular leukemia presents as a painless testicular enlargement that is usually unilateral. Diagnosis of testicular involvement is made by wedge biopsies, which should be done bilaterally because of the high incidence of contralateral testicular involvement. [28] Testicular leukemia is characterized by infiltration of leukemic cells into the interstitium; involvement of the seminiferous tubules occurs in more advanced disease. [29] Although it had been believed that the testes are a leukemic sanctuary site, protected from systemic

chemotherapy by a bloodtestes barrier, animal studies suggest this is not the case. [30]

Testicular disease frequently occurs as an isolated clinical relapse in patients in bone marrow remission. The actual incidence of true isolated testicular disease, however, may be less than previously believed. In one study, a high percentage of patients with testicular relapse who were presumed to be in bone marrow remission were found to have occult leukemia in other abdominal sites, including lymph nodes, liver, and spleen, when evaluated by exploratory laparotomy. [31] Thus, the frequent diagnosis of testicular involvement may simply reflect the relative ease with which recurrence can be detected clinically at this anatomic site. This point is underscored by the observation that the most successful treatment regimens for testicular recurrence use both bilateral testicular radiation (usually 2,400 cGy) and intensive systemic reinduction and retreatment. [31] [32] [33] [34]

As in the case of CNS leukemia, testicular recurrence frequently is followed by systemic relapse. This fact, and the observation that occult disease can be detected in up to 15% of boys who have successfully completed a full treatment course, led to the practice of performing routine testicular biopsies during maintenance treatment or immediately before its completion. Histopathologically, however, testicular biopsies are notoriously difficult to assess and are associated with a relatively high false-negative rate. This has caused investigators to question the wisdom of performing surveillance testicular biopsies, in view of their relative inability to predict eventual testicular relapse. [34] [35] [36] [37]

Lymph Nodes

Nodal involvement is a characteristic feature of ALL and is often responsible for bringing the patient to medical attention. Leukemic involvement usually results in obliteration of the normal microscopic structure of the node. Typically, the lymphadenopathy is generalized and enlarged nodes are painless and freely moveable. Nodal enlargement is an indirect measure of tumor burden and has been associated with prognosis. The presence of massive lymphadenopathy or a large mediastinal mass, a particular feature of patients with T-cell disease, has been associated with a poor prognosis. [38] [39] [40]

Liver and Spleen

Hepatosplenomegaly is common in newly diagnosed patients with ALL. As in the case of nodal enlargement, there is a correlation between the extent of hepatic and splenic enlargement and prognosis, significant enlargement being linked to a poor outcome. [40] [41] Pathologically, these organs show diffuse enlargement secondary to infiltration by leukemic lymphoblasts. In the spleen, the normal distinction between red and white pulp is lost. In the liver, leukemic infiltration of the portal areas is common. Even in cases associated with marked hepatomegaly, liver function abnormalities, if present, are usually mild.

Kidneys

Renal enlargement at diagnosis is common and represents diffuse infiltration by leukemic blast cells. [42] [43] Preferential involvement of the cortex occurs. Renal dysfunction, in the absence of the development of uric acid nephropathy, is a rare phenomenon.

Differential Diagnosis

In the differential diagnosis of ALL, the clinician must include a variety of malignant and nonmalignant disorders, some of

TABLE 57-2 -- Differential Diagnosis of ALL

Nonmalignant disorders
Aplastic anemia
Myelodysplastic syndrome (a)
Myelofibrosis (a)
Autoimmune diseases (e.g., systemic lupus erythematosus) (a)
Infectious mononucleosis
Juvenile rheumatoid arthritis (c)
Idiopathic thrombocytopenic purpura (c)
Leukemic reactions secondary to infection
Malignant disorders
Other leukemias
Hodgkins and non-Hodgkins lymphoma
Bone marrow metastases from solid tumors (e.g., neuroblastoma) (c)
Multiple myeloma (a)

Where indicated, symbols denote disorders that are to be particularly considered in the differential diagnosis of children (c) or of adults (a).

which are listed in Table 57-2 . ALL must be distinguished from other malignancies, including both Hodgkins and non-Hodgkins lymphomas, and those solid tumors that may exhibit metastatic spread to bone marrow. For example, the morphologic appearance of neuroblastoma in the bone marrow may be difficult to differentiate from that of ALL, especially if pseudorosettes are not present. Infectious mononucleosis, immune thrombocytopenic purpura, aplastic anemia, and infectious causes of lymphocytosis (e.g., pertussis) are other conditions that may mimic ALL. Careful evaluation of a bone marrow aspirate using special stains as well as a panel of monoclonal antibodies usually permits the clinician to make the definitive diagnosis of ALL (see later).

LABORATORY EVALUATION

Hematologic Findings

Over 90% of patients with ALL have clinically evident hematologic abnormalities at diagnosis. These usually reflect the degree to which normal marrow is replaced with leukemic cells. Anemia, usually normochromic and normocytic and characteristically accompanied by a low reticulocyte count, is present in approximately 80% of cases. In approximately 50% of patients, the initial leukocyte count is elevated; in up to 25% it is >50,000/ml at presentation. Patients with a profoundly elevated leukocyte count at diagnosis (>50,000/ml) have a particularly poor prognosis. Despite the elevation in leukocyte count at diagnosis, however, many patients present with severe neutropenia (<500 granulocytes/mm^3) and are at significant risk of serious infection. [44] Thrombocytopenia is extremely common; over three fourths of patients present with platelet counts <100,000/ml. Only approximately one third of patients have a platelet count <50,000/ml at diagnosis. Although petechiae and purpura are present in many patients, severe bleeding is unusual at initial presentation, even when the platelet count is <20,000/ml, unless fever, infection, or an accompanying coagulopathy, such as disseminated intravascular coagulation, are also present.

Examination of the peripheral blood smear reveals the presence of leukemic lymphoblasts in most patients at diagnosis. Definitive diagnosis (see panel) usually requires examination of the bone marrow, which is usually hypercellular and infiltrated with leukemic lymphoblasts. Technically the presence of greater than 5% leukemic blasts cells confirms the diagnosis. Most institutions, however, require at least 25% blast cells before definitive diagnosis is rendered. More than three fourths of patients have greater than 50% lymphoblasts in their bone marrow at initial presentation.

Morphologic Classification

Acute lymphoblastic leukemia cells manifest significant heterogeneity and have been subclassified on the basis of differences in their appearance under the light microscope. The most widely used system, developed by the FrenchAmericanBritish (FAB) Cooperative Working Group (Table 57-3) divides lymphoblasts into three categories. L1 lymphoblasts are small, with scanty cytoplasm and inconspicuous nucleoli (Plate 57-1A). L2 lymphoblasts in general are larger, although they may demonstrate considerable variation in size, and have more prominent nucleoli and abundant cytoplasm (Plate 57-1B). Lymphoblasts of the L3 type are large, manifest deep cytoplasmic basophilia and prominent cytoplasmic vacuolation, and are identical cytomorphologically to Burkitts lymphoma cells (Plate 57-1C). The L1 morphology is predominant in childhood ALL, occurring in approximately 85% of childhood cases. The L2 subtype is more common in adults. [45] Lymphoblasts of the L3 are characteristic of only 12% of ALL cases. Although there is no apparent correlation between the FAB L1 and L2 morphologic types and immunologic cell surface markers, cells of the L3 variety possess cell surface immunoglobulin and other characteristic B-cell markers. [46] [47] [48] The association between FAB classification and prognosis is discussed later.

Immunophenotyping

Immunophenotyping now plays a major role in the diagnosis of the acute leukemias. The use of monoclonal antibodies specific for various stages of B-cell, T-cell, and myeloid differentiation enables the clinician to determine more definitively whether a leukemia is lymphoid or myeloid in origin. In most

TABLE 57-3 -- FAB Classification of Lymphocytic Leukemia

Cytologic Features	L1	L2	L3
Cell size	Small cells predominate	Large, heterogeneous in size	Large and homogeneous
Nuclear chromatin	Homogeneous in any one case	Variable; heterogeneous in any one case	Finely stippled and homogeneous
Nuclear shape	Regular; occasional clefting or indentation	Irregular; clefting and indentation common	Regular, oval to round
Nucleoli	Not visible, or small and inconspicuous	One or more present, often large	Prominent; one or more
Amount of cytoplasm	Scanty	Variable, often moderately abundant	Moderately abundant
Basophilia of cytoplasm	Slight or moderate, rarely intense	Variable, deep in some	Very deep
Cytoplasmic vacuolation	Variable	Variable	Often prominent

From Bennett et al., [9] with permission.

Box 57-1. DIAGNOSTIC EVALUATION OF THE PATIENT WITH ACUTE LEUKEMIA

The diagnostic evaluation of a patient with acute leukemia is a comprehensive process which includes a detailed history and complete physical examination, morphologic and laboratory assessment of peripheral blood and bone marrow, blood chemistries, comprehensive clotting studies (prothrombin time, partial thromboplastin time, thrombin time, and fibrinogen), a lumbar puncture and CSF examination, and other studies necessary to ensure that the newly diagnosed patient will receive optimal supportive care.

HISTORY AND PHYSICAL EXAMINATION

A detailed history is obtained to determine the nature of presenting symptoms and their duration. Specific attention is paid to signs and symptoms of anemia, thrombocytopenia, and neutropenia. Information is garnered regarding possible adverse environmental exposures (e.g., chemicals, radiation) and a detailed family history that includes the occurrence and nature of any malignancies among family members is obtained. On physical examination, signs that reflect leukemic marrow involvement and its sequelae are sought. The degree of pallor, evidence of bleeding (e.g., petechiae or purpura), and the presence of any signs of infection are noted. The degree of lymphadenopathy and hepatosplenomegaly is carefully assessed and documented. The pattern of lymph node enlargement provides helpful information. Generalized lymphadenopathy is more common than regional enlargement. In children, palpable, small axillary, cervical, and inguinal nodes are common, but enlargement of posterior auricular, epitrochlear, or supraclavicular nodes is abnormal. Careful examination of the optic fundi to rule out evidence of retinal hemorrhages secondary to thrombocytopenia or leukemic infiltration is important. The oropharynx is evaluated, including the gingivae, a common site of hypertrophy in patients with acute myelomonocytic or monocytic leukemia.

HEMATOLOGIC EVALUATION

A blood count usually reveals evidence of varying degrees of anemia, thrombocytopenia, and anemia. These changes are also detectable on peripheral blood smear, which, in most patients, reveals the presence of leukemic cells. In most cases, bone marrow aspirate should be obtained before the start of therapy. The posterior iliac crest is the preferred site for marrow aspiration. In cases where aspiration does not yield sufficient material for evaluation, bone marrow biopsy is performed. Bone marrow biopsy also is helpful in assessment of the degree of marrow cellularity. Sternal marrows are rarely required and, because of the risk of retrosternal bleeding, the sternum is not an appropriate site for marrow biopsy. Once the diagnosis of leukemia is confirmed, definitive assignment of the type of leukemia is crucial if appropriate treatment is to be delivered. In the past, the distinction between ALL and ANLL was based solely on the morphologic assessment of a bone marrow specimen. Although this is the appropriate initial step in evaluating a bone marrow specimen, additional diagnostic techniques must always be used. These include histopathologic evaluation with special cytochemical stains, biochemical assessment (i.e., TdT determination), comprehensive immunophenotyping, and cytogenetic analysis.

Special histochemical stains may be particularly helpful in delineating ALL from acute myeloid leukemia (see table) and should be performed routinely in the diagnostic evaluation of bone marrow aspirate specimens.[106] The myeloperoxidase

Morphologic, Cytochemical, and Biochemical Characteristics Helpful in Distinguishing ALL from Acute Myeloid Leukemia (AML)

Characteristic	ALL	AML
Nuclear/cytoplasmic ratio	High	Low
Nuclear chromatin	Clumped	Spongy
Nucleoli	02	25
Granules		+
Auer rods		±
Cytoplasm	Blue	Blue-gray
Cytochemical reaction		
Peroxidase		+
Sudan black B		+
Periodic acidSchiff	±	
Naphthyl ASD chloracetate esterase		±
-Naphthyl acetate esterase		±
-Naphthyl butyrate esterase		
Terminal deoxynucleotidyl transferase	+[a]	

Table provides information on characteristics that may be useful in differentiating ALL from AML (see text for details). Wide variation in morphology is encountered in both disease categories. Diagnostic evaluation should include more refined classification of disease according to FAB subtype.

[a] Tdt is usually negative in typical FAB L3 ALL.

stain detects myeloperoxidase in primary granules and is considered specific for cells of the myeloid lineage. Classically, an acute leukemia in which 3% of cells in the bone marrow are myeloperoxidase positive has been considered to be myeloid, although this definition is arbitrary and may cause confusion in cases of mixed lineage leukemia. The Sudan black stain sometimes has been used as a substitute for myeloperoxidase, but because rare cases of ALL demonstrate Sudan black positivity, the myeloperoxidase stain is preferred. The periodic acid-Schiff (PAS) stain is positive in approximately 50% of cases of ALL and often shows a characteristic pattern of block positivity. The PAS reaction may be negative in ALL, however, and has been positive in rare cases of acute myeloid leukemia, limiting its diagnostic utility. Naphthyl AS-D chloroacetate esterase is an enzyme found in cells of neutrophilic lineage that may help define myeloid disease but is generally considered less sensitive than the myeloperoxidase stain. The -naphthyl acetate esterase and -naphthyl butyrate esterase stains are helpful particularly in defining monocytic lineage.

Analysis of TdT present in lymphoblasts of T- and B-cell precursor lineage, but not usually found in mature B-cell ALL or in ANLL, is also a helpful diagnostic procedure. Detailed immunophenotyping using a panel of monoclonal antibodies is undertaken in all newly diagnosed cases of acute leukemia. Use of a sufficiently comprehensive monoclonal antibody panel capable of detecting the major lymphoid (both B- and T-cell), myeloid, and platelet-related antigens enables a definitive lineage assignment to be made in most cases of acute leukemia. Molecular phenotyping provides useful information regarding the status of the immunoglobulin or T-cell receptor gene rearrangement in lymphoid leukemic cells. However, because immunoglobulin gene rearrangement has been observed in T-cell ALL and T-cell receptor gene rearrangement in B-cell precursor ALL, molecular phenotyping cannot be used to distinguish definitively between immunologic subtypes of ALL. Cytogenetic analysis, *Box continued on following page*

including evaluation of both chromosomal number and structure, is routinely performed and may reveal characteristic chromosomal abnormalities.

When this overall diagnostic strategy is used, it is rare to encounter a leukemia that defies classification.

LUMBAR PUNCTURE

Lumbar puncture should be performed in all newly diagnosed patients to rule out CNS leukemia. In patients with marked thrombocytopenia, platelet transfusions are

When this overall diagnostic strategy is used, it is rare to encounter a leukemia that defies classification.

LUMBAR PUNCTURE

Lumbar puncture should be performed in all newly diagnosed patients to rule out CNS leukemia. In patients with marked thrombocytopenia, platelet transfusions are given to ensure that the platelet count is optimal (>40,000/mm^3) before performing the lumbar puncture. In addition to measurement of CSF opening pressure, the CSF cell count, protein, and glucose are determined. Examination of a cytocentrifuged specimen of CSF is always performed because this technique increases diagnostic sensitivity (see text).

OTHER DIAGNOSTIC AND LABORATORY STUDIES

A chest radiograph is routinely obtained in newly diagnosed patients. When significant mediastinal enlargement is detected, computed tomography can help to define its extent more accurately. Patients presenting with significant bone pain undergo radiologic examination of the involved site. All newly diagnosed patients have a comprehensive battery of blood chemistries performed, including uric acid, electrolytes, calcium, phosphorus, blood urea nitrogen, serum creatinine, and liver function tests (including lactate dehydrogenase). In addition, magnetic resonance imaging or computed tomographic imaging of the brain at diagnosis may be useful, particularly as a baseline for comparison in the event of CNS relapse or toxicity.

SUPPORTIVE CARE

The blood type of all patients is determined and appropriate cross-matching instituted if transfusion is required. Packed red cell transfusions are used to maintain an adequate hemoglobin. Platelets are usually administered prophylactically to maintain the platelet count >20,000/mm^3. If the patient becomes refractory to random donor platelets, single-donor or human leukocyte antigen-matched platelets may be useful.

All newly diagnosed patients receive allopurinol and vigorous hydration to prevent uric acid nephropathy and complications from tumor lysis. In patients with extremely high initial white blood cell counts (e.g., >100,000/ml), leukapheresis or, in very young patients, exchange transfusion is sometimes helpful, although its role is controversial, especially in ALL. [107] [108]

Newly diagnosed patients who are febrile receive an extensive evaluation to rule out infection. The febrile neutropenic patient is placed on broad-spectrum antibiotic coverage.

The diagnosis of leukemia places extraordinary stress on patient and family alike. Starting at the time of initial diagnosis, careful attention is given to evaluating the psychosocial profile of patient and family so that appropriate psychosocial support for both can be instituted. Optimal psychosocial support requires a concerted, coordinated effort of physician, nurses, social workers, clergy, and other skilled health care personnel.

cases, immunophenotyping also permits assignment of the relative stage in the process of B- or T-cell differentiation from which the leukemic clone is believed to have arisen. An example of the type of monoclonal antibody panel used in the immunophenotypic diagnosis of the acute leukemias is shown in Table 57-4 . Approximately 8085% of childhood ALL is believed to develop from the monoclonal proliferation of B-cell precursors. In contrast, only approximately 12% of cases manifest surface immunoglobulin and are classified as mature B-cell ALL. The remainder of cases are of T-cell origin. A variety of classification schemes have been developed that define both B-cell precursor ALL and T-cell ALL according to their degree of differentiation or maturation. One prognostically useful system, devised by the Pediatric Oncology Group, separates childhood ALL into T-cell, B-cell, and B-cell precursor disease. [49] Approximately one third of patients with B-cell precursor ALL have demonstrable cytoplasmic immunoglobulin (preB-cell ALL) and, in the past, were considered to have a worse prognosis. However, current data suggest that the poor outcome noted in these patients is associated with a high incidence of the t(1;19) translocation (see later). [50]

The development of recombinant DNA technology permits identification of immunoglobulin gene and T-cell receptor gene rearrangement in leukemic cells. Although it is possible to relate the patterns of immunoglobulin gene rearrangement and T-cell receptor gene rearrangement to the stages of development of B-cell precursor and T-cell ALL, respectively, the occurrence of immunoglobulin gene rearrangement in some cases of T-cell ALL and the presence of T-cell receptor gene rearrangement in cases of B-cell precursor ALL undermine the value of molecular phenotyping to assign B- or T-cell lineage. [51] [52] [53]

Most cases of ALL express surface antigens and molecular markers that help to identify them as derived from a specific lineage. However, cases of lineage infidelity, or biphenotypic leukemias, exist in which leukemia cells exhibit markers of more than one cell type on the same leukemic cell. Simultaneous expression of lymphoid and myeloid markers also occurs both in childhood and adult ALL and may be associated with a poor prognosis, although this issue remains unsettled (see later). [47] [54] [55] [56] [57]

Other Laboratory Studies

At the time of diagnosis, many patients with ALL have elevated serum uric acid levels, a by-product of the increased purine metabolism of leukemic cells. The degree of uric acid elevation reflects the extent of tumor burden, higher levels occurring in patients with high initial leukocyte counts and pronounced lymphadenopathy and hepatosplenomegaly. Hyperuricemia must be corrected by vigorous use of hydration and administration of the xanthine oxidase inhibitor allopurinol to prevent uric acid nephropathy and renal failure. Administration of urate oxidase, an enzyme that catalyzes the oxidation of uric acid to allantion, has been investigated as another means of rapidly reducing uric acid concentrations. [58]

Abnormalities in a number of lysosomal enzymes are also evident at diagnosis. Serum lactate dehydrogenase (LDH) is frequently elevated. The degree of elevation appears to correlate with tumor burden and also with prognosis. [35] [59] In addition, isoenzyme I, an acid hydrolase hexosaminidase, is frequently elevated in B-cell precursor ALL.[60]

A variety of metabolic abnormalities, including elevated serum levels of calcium, potassium, and phosphorus, may be observed in the newly diagnosed patient with ALL. These are more frequently encountered in those patients with a high leukocyte

TABLE 57-4 -- Monoclonal Antibodies Commonly Used to Immunophenotype Leukemia[a]

CD	Antibody	Predominant Reactivity
T cell		
CD1	T6	Thymocytes
CD2	T11	Pan-T
CD3	T3	Pan-T
CD4	T4/Leu3	T-helper/inducer
CD5	T101/Leu1	Pan-T, B-chronic lymphoblastic leukemia
CD7	Leu9	Pan-T
CD8	T8/Leu2	T-cytotoxic/suppressor
CDw29	4B4	T4+/4B4+=helper inducer T4+/2H4+=suppressor inducer
B cell		
CD19	B4	Pan-B

CD20	B1	Pan-B
CD21	B2	C3dR
CD24	BA1 PCA-1	Pan-B Plasma cells
Myeloid		
CD11c	LeuM5	Monocytes, hairy cell
CD13	My7	Pan-myeloid
CD14	LeuM3/MY4/MO2	Monocytes
CD15	LeuM1	Monocytes, granulocytes
CD33	My9	Pan-myeloid
Miscellaneous		
CD9	BA2	Hematopoietic progenitor/leukemic blasts
CD10	CALLA/J5	Acute lymphoblastic leukemia/Burkitts/follicular lymphoma
CD34	My10/HPCA-1	Hematopoietic progenitor cells/ human T-lymphotropic virus-infected cells
CD41a	Plt-1	Platelets/megakaryocytes
CD45	T-200/LCA	Pan-leukocyte
	T9	Transferrin receptor/proliferating cells

Data courtesy of Jane Trepel, Ph.D., Medicine Branch, National Cancer Institute.

[a] CD classification number, corresponding antibodies, and their predominant reactivity are listed.

count and extensive tumor burden. Hypercalcemia may be due either to extensive infiltration of bone or to the ectopic release of a parathormone-like substance by leukemic lymphoblasts.[42] Hyperphosphatemia may accompany the extensive destruction of tumor cells. It may occur either as a result of ineffective leukopoiesis or as a consequence of chemotherapy induced tumor lysis.[61] Hyperkalemia also can occur as a result of extensive leukemic cell lysis. Spurious hyperkalemia, the result of the release of potassium from leukemic cells during the process of clotting in vitro, must be ruled out as a cause of high potassium levels.[62] Lactic acidosis may also be present at diagnosis or relapse.[63]

Other laboratory abnormalities are also occasionally present at diagnosis. Low serum immunoglobulin levels at diagnosis have been reported in up to 30% of children with ALL and have been associated with a poor prognosis.[64] [65] Significant coagulation abnormalities are not a typical feature of ALL at diagnosis. Although disseminated intravascular coagulation may occur, it is infrequent. Clotting abnormalities are observed, however, in many patients under active treatment with L-asparaginase.[66]

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PROGNOSTIC FACTORS

A number of clinical and laboratory features evident at diagnosis have prognostic value for predicting the remission duration of patients treated for ALL (Table 57-5). The identification of these prognostic factors has provided a means of stratifying patients into different risk groups and tailoring treatment accordingly. This approach has become an important feature of many current treatment protocols, particularly in children. In assessing the relative prognostic value of any feature, it is important to determine whether it functions as an independent prognostic determinant rather than a dependent. Multivariate analysis is helpful in this regard. Advances in technology, especially in molecular genetics, have led to more precise definitions of some risk factors. In addition, changes in treatment strategies may result in previously significant risk factors losing their prognostic value.

The initial leukocyte count at diagnosis has proven to be an important prognostic factor in virtually all ALL studies. In general, the prognosis is inversely related to the leukocyte count. Those patients with initial leukocyte counts greater than 50,000/ml have a particularly poor prognosis. [67] [68] [69] The relationship between white count and prognosis appears to be linear and continuous.

Age at diagnosis also has prognostic importance. Patients who are very young (<2 years) and older patients (>10 years) tend to have a worse prognosis. [70] [71] [72] [73] Among children, infants younger than 12 months of age have the poorest prognosis; their disease has a number of unique biologic features that in part may explain their relative resistance to therapy. [74] In adults, progressively older age is associated with lower rates of remission induction and shorter remission duration. [2] [75]

Cytogenetic analysis also provides important prognostic information. [76] [77] [78] [79] [80] [81] [82] The association between chromosomal number and prognosis is well characterized. Patients with hyperdiploidy (>50 chromosomes [79] or DNA index >1.16 [80]) have a favorable prognosis. Investigators in the Pediatric Oncology Group found that the combined trisomy of chromosomes 4 and 10 independently predicts favorable outcome in B-cell precursor ALL. [81] In contrast, patients with hypoploidy and pseudodiploidy fare less well. [79] Near-tetraploidy also appears to be associated with a poor prognosis, although this may not be an independent factor because many patients with this abnormality also have T-cell disease. [82]

In addition to chromosome number, abnormalities in chromosome structures also convey important prognostic information. A number of chromosomal translocations are associated with both a high rate of induction failure and early relapse, including the t(8;14) translocation associated with B-cell ALL, the t(9;22) found in Philadelphia chromosome-positive ALL, the t(1;19) occurring in B-cell precursor ALL, and the t(4:11) translocation occurring most frequently in infants. Furthermore, technical advances have led to the identification of previously unappreciated, prognostically significant gene rearrangements. For example, the t(12;21) translocation, which results in rearrangement of the *tel* gene, is present in approximately 25% of

TABLE 57-5 -- Factors Associated with Prognosis in ALL

Initial white blood cell count
Age at diagnosis
Gender
Cytogenetics
Immunophenotype
FAB morphology
Mediastinal mass
Organomegaly and lymphadenopathy
Hemoglobin level
Ethnicity
Platelet count
Serum immunoglobulins
Rapidity of leukemic cytoreduction

cases of B-cell precursor ALL and is associated with improved outcome. [84]

Immunophenotype also correlates with prognosis. [85] [86] [87] ALL patients with either mature B- or T-cell immunophenotypes have a worse prognosis than patients with B-cell precursor ALL. The prognostic influence of T-cell phenotype is less striking after adjustment for its association with high initial leukocyte count. The intensity of treatment appears to influence the degree to which T-cell phenotype influences prognosis. In intensive treatment protocols, the prognostic influence of T-cell phenotype has not been evident. [38] [41] [88] [89] [90] Highly aggressive treatment also improves the outcome of patients with B-cell ALL. [91]

The expression of myeloid markers on ALL cells may be associated with a poor prognosis in children with ALL, although this is controversial. [47] [55] [56] There is also a relationship between morphologic subtype and prognosis. The FAB L3 subtype is associated with B-cell ALL and thus conveys a poor prognosis. In many studies of childhood ALL, the L2 subtype has also been associated with a poor prognosis, [38] [48] [92] [93] whereas the L1 subtype is associated with a more favorable outcome. This finding has not been universal, however, and may not be a helpful distinction in adult ALL. [1] [94] [95] [96]

Race and gender may also influence outcome. Female patients have a more favorable prognosis. This appears to be related to the impact of testicular relapse and to the higher incidence of T-cell disease in male patients. [68] [72] [97] Ethnicity appears to be an important prognostic determinant. African American and Hispanic patients have a lower remission induction rate and a higher likelihood of experiencing bone marrow relapse. [94] [98] [99] The reasons for this poor outcome are related in part to the more frequent recurrence of very elevated initial leukocyte counts, mediastinal masses, and L2 morphology in this population. [88] [94] [100] However, even when adjusted for age, initial white blood cell count, and so forth, there still appears to be an increased risk of treatment failure for nonwhite patients. [101]

Tumor burden can be assessed indirectly by evaluating the degree of hepatosplenomegaly and lymphadenopathy. In numerous studies, hepatomegaly,

splenomegaly, and mediastinal mass have been demonstrated to have prognostic importance, although on multivariate analysis they are found to be closely dependent on the initial leukocyte count. Despite this, hepatomegaly and splenomegaly have been useful to some investigators in the prospective definition of risk groups. For example, the German BFM study group uses a risk factor index, computed on the basis of initial leukocyte count and measurement of hepatosplenomegaly, to define different treatment groups for childhood ALL. [102]

In addition to the features already noted, a variety of other characteristics have been found to have prognostic value. In children, low serum immunoglobulins, particularly low IgM levels, are associated with poor event-free survival. [64] [65] [103]

Perhaps one of the most significant prognostic factors is the response to initial treatment, although, strictly speaking, it is not a presenting clinical or laboratory feature of patients with ALL. Nonetheless, patients who fail to achieve complete remission after completing an initial course of induction therapy have markedly reduced remission duration and survival. The rapidity of initial cytoreduction appears to be extremely important. For example, the presence of residual leukemia on day 7 or 14 of induction therapy has been associated with shorter event-free survival compared with patients whose marrow shows no evidence of residual disease at that time. [38] [69] [88] [104]

As noted earlier, current treatment regimens for childhood ALL use prognostic factors to define different risk groups that are subsequently treated according to their relative risk of failure. In turn, improved treatment strategies may reduce the impact on survival of a given prognostic factor. In most centers, patients with poor risk features receive more intensive treatment, whereas those with good risk features receive treatment

TABLE 57-6 -- Uniform Assessment of Risk Factors in Childhood ALL

Risk Factor	Associated with Better Outcome	Associated with Worse Outcome
Age	1.09.99 years	<1 or 10 years
White blood cell count	<50,000/l	50,000/l
DNA index	>1.16	1.16
Trisomy 4 and 10	Present	Absent
M1 marrow day 7 or 14	Yes	No
Peripheral blasts at day 8	Absent	Present[a]
Cytogenetics		t(1;19)
		t(9;22)
		t(4;11)
Immunophenotype	B-cell precursor	T cell[a]
Central nervous system leukemia	CNS 0	CNS 2[a] CNS 3

CNS, central nervous system; CNS 0, no blasts in CSF; CNS 1, <5 WBC/l with blasts; CNS 3, 5 WBC/l with blasts or cranial nerve palsy.

[a]In some studies.

designed to be effective yet minimize treatment-related adverse sequelae. The use of different prognostic factors for stratification of risk groups may lead to difficulty in comparing the results of different approaches to therapy. A workshop at the National Cancer Institute has proposed a uniform approach to risk factor assessment based on a combination of clinical and laboratory criteria (Table 57-6). [105] The success of this risk-based strategy and the general principles of treatment for the patient with ALL are detailed in Chapter 58 .

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Chapter 58 - Treatment of Childhood Acute Lymphoblastic Leukemia

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INTRODUCTION

Advances in the treatment of childhood acute lymphoblastic leukemia (ALL) have been the product of clinical trials and the prospective evaluation thereof (Fig. 58-1). Further advances in the treatment of this disease and attempts to limit therapy-related toxicity require ongoing prospective studies. Thus, in the 1990s, no child with newly diagnosed ALL should receive individualized treatment; rather every child with this diagnosis should be enrolled in a trial to ensure optimal treatment and subsequent advances for future generations of children with leukemia.

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HISTORY AND PHILOSOPHY OF TREATMENT

Prior to 1947, when the first complete remission in childhood ALL was attained by Farber and coworkers, [1] the median duration of survival from the time of diagnosis was 2 months.[2] The development of a model for the scientific methodology of clinical therapeutic research in childhood ALL has been elegantly summarized in detail by Frei.[2] During the 1950s drugs such as 6-mercaptopurine, methotrexate, and corticosteroids were found to be active in leukemia-bearing mice [3] and subsequently in human leukemias.[4] The first controlled clinical trials were conducted by Frei and associates, [5] who ushered in the era of single-agent (and soon thereafter combination-agent) antileukemic chemotherapy trials (Table 58-1).[6]

Active drugs introduced in the 1960s and 1970s, such as the anthracyclines (doxorubicin and daunorubicin), asparaginase, and the epipodophyllotoxins (VP-16 and VM-26), usually underwent initial evaluation in patients whose leukemia had become resistant to the drugs in Table 58-1 . Complete remission rates of 2448% with doxorubicin [7] and 5060% with asparaginase [8] [9]

Figure 58-1 Event-free survival of children with ALL treated between 1973 and 1991 on Dana-Farber Cancer Institute/The Childrens Hospital-based protocols. Abbreviations: CCR, complete continuous remission; F/U, follow-up. *(Adapted from Sallan et al,[15] with permission.)*

TABLE 58-1 -- Chemotherapy of Childhood ALL: Historical Perspective

	Frequency of Complete Remission (%)
Single agents	
Prednisone	57
Vincristine	55
6-Mercaptopurine	27
Methotrexate	21
Cyclophosphamide	18
Combination agents	
Prednisone + vincristine	85
Prednisone + 6-mercaptopurine	81
Methotrexate + 6-mercaptopurine	45
Vincristine + prednisone + methotrexate + 6-mercaptopurine	94

From Freireich and Frei,[6] with permission.

were achieved. The efficacy of these agents, as measured by the frequency of complete remissions, may have been underestimated because they were tested in patients with previously treated and potentially drug-resistant disease. To evaluate antileukemia agents in newly diagnosed rather than in relapsed patients, the concept of an investigational window was developed in the early 1980s. Patients treated with an investigational window receive a single agent 35 days before the onset of multiagent chemotherapy. Using this approach, the Dana-Farber Cancer Institute (DFCI) group evaluated the pharmacokinetics of different preparations of asparaginase in previously untreated patients, [10] and also demonstrated that high-dose methotrexate was more effective than conventional-dose methotrexate.[11]

Critical to the pioneering efforts of early clinical trials was an understanding of the principles of the first-order cytokinetic effect of chemotherapeutic agents and the need for clonal eradication. In the early 1960s, Skipper and associates initiated a series of studies that addressed the quantitative biology of leukemia in mice and its perturbation by chemotherapy.[12] The first-order cytokinetic effect of chemotherapy on tumor (leukemia) cells means that for a given treatment there is a constant fractional reduction of leukemia cells that is independent of the total leukemia burden. The cytokinetic model for antileukemia therapy is depicted in Figure 58-2 .

Figure 58-2 A cytokinetic model for antileukemia therapy relating to hypothetical cell kill with components of treatment.

Using the mouse leukemia models of Skipper and colleagues, it was shown that the time of death following treatment was a precise measure of the number of leukemia cells persisting at the end of treatment.[12] [13] They further demonstrated that having only one L1210 leukemic cell present was enough to cause the death of the animal. This observation led to the understanding of the need for clonal eradication. Moreover, their models provided important observations with respect to drug resistance, combination chemotherapy, and the cycling of chemotherapeutic agents. By the 1960s, effective systemic chemotherapy and the development of improved supportive care with blood products and antibiotics resulted in an increased percentage of complete remissions and an increase in the duration of remissions. The ability to support a patient through prolonged myelosuppression has permitted clinical investigations that have demonstrated the importance of using maximal tolerable doses of drugs.[14] [15]

Box 58-1. THE THERAPEUTIC APPROACH TO CHILDHOOD ALL

The proper treatment of all children with newly diagnosed ALL must begin in a specialized pediatric hematology/oncology center and must subsequently be continued or closely supervised by experienced specialists. As soon as the diagnosis is confirmed and the patient stabilized with appropriate supportive care (hydration, blood products, antibiotics, etc.), chemotherapy should be instituted. Such treatment should be part of a prospective clinical protocol to ensure that each of the children with this disease ultimately contributes to the development of more effective and less morbid treatment regimens.

Conceptually, early treatment should be with high doses of multiple drugs to ensure maximal leukemic cell kill and to address the issues of drug resistance. Although it is impossible to assess the importance of early intensive treatment on the basis of only the percentage of complete remissions or the time to induce a complete remission, long-term results support the use of intensive induction therapy. We use vincristine, prednisone, doxorubicin, methotrexate, asparaginase, and intrathecal cytosine arabinoside. Each agent is individually cytotoxic to leukemic cells and, in combination, the agents presumably are maximally cytotoxic. The dose-limiting aspect of induction therapy is the potentially additive side effects of the drugs; for example, doxorubicin and methotrexate both can result in damage to the gastrointestinal mucosa, and therefore the doses of each must be modified.

After obtaining a complete remission, attention is focused on CNS treatment and further reduction of the total (currently immeasurable) residual leukemic cells (the *minimal residual leukemia*). The treatment of the CNS should be maximally effective and minimally harmful, a balance that is difficult to attain (and controversial among clinical investigators). We treat all children with intensive systemic chemotherapy, including repeated doses of prednisone and high-dose asparaginase, which are also effective for CNS leukemia. Patients at higher risk of CNS relapse (white blood cell count >50,000/mm^3, age <1 or >10 years, and T-cell ALL), who comprise 40% of all patients, are treated with cranial radiation (18 Gy) and concurrently two-drug (methotrexate and cytosine arabinoside) intrathecal therapy. For standard-risk patients, we are currently conducting a randomized trial comparing cranial radiation and concurrent intrathecal drugs with intensive intrathecal therapy (without radiation). For all patients, intrathecal therapy is continued intermittently for 2 years.

Intensive systemic therapy with multiple drugs (including vincristine, prednisone, asparaginase, 6-mercaptopurine, and methotrexate or doxorubicin, depending on the risk group of the patient) is administered after CNS treatment for approximately 6 months. Thereafter continuation therapy (with vincristine, prednisone, 6-mercaptopurine, and methotrexate) is administered to complete a total of 2 years of treatment. Bone marrow transplantation in initial complete remission is recommended for children with ALL who have lymphoblasts that express the Philadelphia chromosome [t(9;22)] (34% of patients) and for those whose leukemia is refractory to initial induction chemotherapy (12% of patients). Infants are treated as high-risk patients, but their therapy includes an extra month of intensification with high-dose cytosine arabinoside and high-dose methotrexate. Children with mature B-cell ALL (SIg+) are treated on a separate protocol that features B-cell-specific drugs.

All current antileukemia therapy is leukemia nonspecific, which means that all the drugs have acute toxicities and many of them also have long-term adverse effects. Thus, treatment programs must be balanced with regard to the risk/benefit ratio. The first goal must be eradication of the leukemic clone. When identical treatment is used, patients characterized as being at standard risk of relapse have a higher likelihood of successful outcome (cure) than those characterized as high-risk patients. Therefore, it is our practice to gradually diminish the potential toxicity in new therapy programs for the standard-risk group, while always being mindful of the risk of more treatment failures. On the other hand, as long as the high-risk group is inadequately treated (too many relapses), we believe that treatment of these patients, albeit associated with known acute and late toxicity, should be intensified. Despite that, efforts need to be made to modify both short- and long-term devastating toxicities (stroke, congestive heart failure, learning disorders, second malignant neoplasms) even in these high-risk patients while at the same time attempting to increase their overall survival. Thus, we are searching for less toxic forms of asparaginase and less cardiotoxic ways of administering anthracyclines, and altering methods for the delivery of cranial radiation. Hopefully, in the near future we will be able to use more specific antileukemia therapy, measure the number of residual leukemic cells, and base the intensity and duration of therapy on individual needs.

In the 1960s, the incidence of central nervous system (CNS) leukemia as an initial site of relapse became progressively more common, [16] [17] and the concept of the CNS as a pharmacologic sanctuary (i.e., an anatomic space that is poorly penetrated by systemically administered chemotherapeutic agents) emerged. Several avenues of approach to the problem of CNS leukemia and its treatment and prevention have been explored. These include intrathecal administration of drugs, [18] craniospinal radiation,[19] cranial radiation plus intrathecal drugs, [20] and high doses of systemically administered drugs that result in therapeutic concentrations in the cerebrospinal fluid (CSF). [21] [22] The optimal delivery of CNS treatment remains controversial.

Current regimens for the treatment of ALL result in high proportions (>95%) of children achieving complete remission and favorable long-term outcome for the majority of patients. This is true despite the fact that, with few exceptions, the drugs used for the treatment of ALL in the 1990s were all available by the late 1960s. Philosophically, treatment should be with curative (not palliative) intentwhich means that all complications of the disease and its therapy need be vigorously treated, but that the major effort should be directed at eradication of disease rather than relief of symptoms (see box on therapeutic approach).

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SUPPORTIVE CARE

Supportive care is an important determinant of outcome in children with ALL. Before instituting chemotherapy, patients must be assessed and treated for any metabolic disturbances, hematologic abnormalities, and presumed or documented infections.

Rapid turnover of leukemia cells before and immediately after the initiation of chemotherapy leads to the release of intracellular contents, which can overwhelm the bodys normal excretory mechanisms. The resultant metabolic disturbances include hyperkalemia, hyperuricemia, hyperphosphatemia, and hypocalcemia. Patients with high levels of uric acid are at risk for the development of acute renal failure secondary to uric acid deposition in the kidney. [23] To ensure that uric acid remains in solution within the renal tubule for optimal excretion, intravenous hydration (usually with twice the maintenance volumes of fluids) and urinary alkalinization (pH 78) (usually with 0.25 N $NaHCO_3$) should be instituted immediately after diagnosis. If urinary alkalinization is difficult, $NaHCO_3$ -containing solutions can be increased to 0.5 N strength. In addition, administration of a xanthine oxidase inhibitor such as allopurinol should be started prior to institution of antileukemia drugs. Allopurinol prevents the formation of uric acid during cell lysis. For patients with white blood cell counts >200,000/mm^3 , leukapheresis has been advocated to prevent hyperviscosity and lysis-related problems. [24]

Thrombocytopenia, in association with neutropenia and anemia, is a common presenting feature of ALL; [25] however, active bleeding is a relatively unusual feature at the time of diagnosis. Despite this fact, most investigators recommend prophylactic platelet transfusions for patients with thrombocytopenia, as opposed to transfusions only for active bleeding. Prophylactic transfusions have frequently been given when platelet counts fall below 15,00020,000/mm^3 . A recent study in adults with acute myeloid leukemia (AML) has demonstrated the safety of decreasing the prophylactic platelet transfusion threshold to a platelet count of 10,000/mm^3 in clinically stable patients. [26] Any active hemorrhage associated with a platelet count of <100,000/mm^3 should be treated with platelet transfusions. Similarly, symptomatic anemia should be treated by transfusion of packed red blood cells. Most investigators recommend prophylactic red cell transfusions for a hematocrit below 2025 volume percent. Stabilization of these two hematologic parameters should take no longer than 1224 hours and therefore should not delay the start of antileukemic therapy.

Patients should also be treated intensively for any documented or presumed infection before beginning chemotherapy. [27] For a temperature above 38.5°C, broad-spectrum intravenous antibiotic coverage should be administered immediately after obtaining cultures, while awaiting laboratory confirmation of an infectious etiology. Chemotherapy should be started as soon as possible after diagnosis despite the need for antibiotics. Although newly diagnosed patients might not have severe neutropenia (granulocyte counts <500/mm^3), the usual state of marrow replacement with lymphoblasts and the anticipated marrow hypocellularity associated with antileukemia treatment make such antibiotic recommendations prudent. One series documented bacteremia in 21% of episodes of fever and neutropenia in children receiving chemotherapy. [28]

Various approaches have been used to prevent or reduce infections in patients once they have started treatment. Prophylaxis with trimethoprim-sulfamethoxazole, usually from the time of complete remission, successfully prevents *Pneumocystis carinii* pneumonia. [29] The optimal use of hematologic growth factors, such as granulocyte colony-stimulating factor (G-CSF), remains a matter of controversy. In prospective randomized studies, the use of G-CSF in children [30] [31] and adults [32] [33] receiving intensive therapy for ALL was associated with a shorter duration of neutropenia and a tighter adherence to the planned treatment schedule. However, although some investigators reported a significantly decreased incidence of severe infections in patients treated with G-CSF during ALL therapy, [30] [33] others have not confirmed this finding. [31] [32] Moreover, a recent study found that G-CSF treatment did not prolong survival or reduce the cost of supportive care for children with ALL. [31]

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CONCEPTS OF THERAPY

Therapy for children with newly diagnosed ALL usually includes four componentsremission induction, intensification, continuation, and CNS treatment. The *remission induction* component of therapy is designed to rapidly destroy measurable leukemia cells and minimize residual leukemia burden (i.e., the total number of leukemia cells in the body). The *intensification* component (also called *consolidation* therapy) is designed to further reduce the total body leukemia cell burden and address issues of antileukemic drug resistance. Such treatment usually consists of higher doses of the same drugs used during remission induction, or of high doses of different drugs. When induction drugs are used again in the same doses, the treatment is known as *re-induction* therapy. The *continuation* component is designed to eradicate the residual leukemia cell burden. In the past, this part of treatment was referred to as maintenance therapy. However, because the current concept is to eradicate all remaining leukemia cells, as opposed to maintaining a low tumor burden, the newer terminology is preferred. The CNS treatment component is used to address the issue of *pharmacologic sanctuary sites* (i.e., areas of the body, such as the brain and spinal cord, that are not well penetrated by conventional doses of most antileukemia drugs).

An important concept in the treatment of ALL in children is risk-directed therapy. Clinical trials conducted during the 1970s and early 1980s established groups of patients whose risk of subsequent relapse varied according to different characteristics. Current clinical trials use these outcome predictors to stratify therapy. For example, children with the least likelihood of relapse can be treated on protocols that modify or eliminate certain intensive components of therapy in order to decrease morbidity. In contrast, individuals at a greater risk of relapse can be more intensively treated, thus limiting the potential higher risks of such intensified therapy to only these patients.

For many years, pediatric cooperative groups and institutions have applied prognostic factors differently when defining risk categories. A more uniform approach to risk classification

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was proposed and agreed on at a National Cancer Institute sponsored workshop held in 1993. [34] For patients with B-precursor ALL, the standard or better risk category was defined as age between 1 and 9 years and initial leukocyte count <50,000/mm ^{3} , as this population has a predicted 4-year event-free survival of approximately 80%. Presenting features used by some investigators to further identify patients with standard-risk" disease includes a hyperdiploidy (DNA index >1.16) and trisomy of chromosomes 4 and 10. The remaining patients, with a predicted 4-year event-free survival rate of approximately 65%, were considered to have high-risk ALL. Other characteristics used by the various cooperative groups to classify patients as high risk include T-cell phenotype, adverse cytogenetic translocations, such as t(9;22),t(4;11) and t(1;19), overt CNS leukemia at diagnosis, and slow early response to induction chemotherapy. [34]

One must be mindful of the potential misuse of pejorative terms such as good-risk, standard-risk, or high-risk patient groups. Philosophically, ALL is a high-risk disease inasmuch as 2030% of affected children are unsuccessfully treated and die as a result of their disease. [35] [36] [37] Ultimately, treatment is the most important prognostic factor, and the importance of a particular presenting feature in predicting outcome depends on the therapy delivered to that patient.

Remission Induction

As soon as the patient has been stabilized by the supportive measures discussed above, antileukemia chemotherapy is begun. The goal of induction therapy is to rapidly induce a complete remission. *Hematologic remission* is defined as attainment of a normocellular bone marrow with 5% or fewer blasts and peripheral blood without lymphoblasts and with a granulocyte count exceeding 500/mm ^{3} and a platelet count exceeding 100,000/mm ^{3} . *Complete remission* is defined as achievement of the above criteria plus the absence of any signs and symptoms of extramedullary leukemia. The duration of most induction regimens is 34 weeks.

A complete remission must be induced before the next component of therapy is begun and is therefore essential in prolonging survival. Using a two-drug regimen of weekly vincristine and daily prednisone, 8090% of children with ALL will achieve remission at the end of 1 month of therapy (see Table 58-1).[38] [39] With the addition of a third agent, such as asparaginase or an anthracycline, the remission rate increases to approximately 95%. [40] [41] In addition to improving remission rates, intensified three-drug induction regimens also prolong remission duration. The importance of induction intensity in determining overall survival was demonstrated in a study in which children were randomly assigned to receive identical therapy except for induction drugs; one group received vincristine and prednisone and the other received those two drugs plus an anthracycline. Although the complete remission rates for both groups exceeded 90%, there was long-term benefit for the more intensively treated group (event-free survival for the two groups at 16 years was 37% and 63%, respectively). [41] [42] In theory, the more intensive induction regimen may prevent the emergence of drug-resistant leukemia clones by an initial leukemia cell lysis of greater rapidity and magnitude. [43] Thus, most investigative trials currently use at least three drugs (e.g., vincristine, prednisone, and asparaginase). [40] The benefit in terms of long-term survival of using four or more drugs during induction is widely accepted in higher risk patients, [44] but less clear in lower risk patients. [45] Even with very intensive induction regimens, very few toxic deaths are reported. [35] [36]

Pharmacologic sanctuary sites, such as the CNS, should be treated during induction. Some commonly used systemic drugs such as glucocorticoids, asparaginase, and high-dose methotrexate affect CNS leukemia cells, but intrathecal agents such as cytosine arabinoside and methotrexate are also recommended. Intrathecal cytosine arabinoside has the advantage of having no additive myelosuppression because it is immediately inactivated by deaminases when it enters the blood.

Failure to achieve remission after 1 month of therapy (induction failure) is uncommon, occurring in fewer than 5% of patients. Successful treatment of refractory ALL has been reported with the use of drugs such as cytosine arabinoside, teniposide, daunomycin, and idarubicin. [46] [47] [48] [49] However, even after achieving remission, overall survival for patients with a history of initial induction failure is very poor, [50] and these patients should be considered candidates for allogeneic bone marrow transplantation (BMT) or other intensive therapies.

Even for patients who achieve remission within 1 month, the rapidity of response to induction chemotherapy, as measured by clearance of peripheral and bone marrow lymphoblasts, is an important predictor of outcome. In an era of relatively less intensive induction therapy it was shown that the time required to enter complete remission had prognostic significance; shorter times of induction were associated with more favorable outcomes. [51] In more recent studies, investigators have reported that response to a short course of corticosteroids, as measured by the absolute peripheral blast count after 1 week of prednisone (plus intrathecal methotrexate), is one of the most sensitive predictors of subsequent event-free survival. [36] [52] Others have demonstrated that the rapidity with which a patient clears peripheral blasts after multiagent induction chemotherapy also has important prognostic significance, with slow responders having a significantly worse outcome. [53] [54] The Childrens Cancer Group (CCG) has reported that the presence of leukemia in bone marrow specimens obtained 7 or 14 days after beginning multiagent chemotherapy strongly correlates with poor outcome,[55] [56] although the adverse prognosis of slow early responders can be improved with intensification of postinduction therapy for these patients. [57]

Although clinical complete remission is the goal of multidrug induction therapy, it must be recognized that clinical remissions are not *biologic* cures. Even after the induction of complete remission, leukemia cells remain in the marrow, undetected by light microscopy. Indirect evidence for this observation was derived from early clinical trials in which chemotherapy was stopped after induction of a clinical complete remission; all patients relapsed within 6 months. [58] According to the principle of first-order cytokinetic killing of leukemia cells, a fixed proportion (99%, 99.9%, 99.99% or more) of cells are destroyed with any given dose of drugs and a fixed proportion (1%, 0.1%, 0.01% or fewer) of leukemia cells remains after each dose of therapy. It is the remaining tumor burden that necessitates postinduction treatment. The use of molecular biology techniques, such as the polymerase chain reaction, may aid in assessing whether the rate or degree of initial cell kill will be a predictive factor for long-term survival. Some recent studies suggest that high levels of molecularly detectable residual disease in patients in clinical remission at the end of induction predicts poor outcome, [59] [60] although this result has not been confirmed by others. [61] [62]

Intensification Chemotherapy

Postinduction intensification is a common component of most pediatric protocols. The goals of intensification therapy are to further reduce the disease burden and to adjust the intensity of treatment based on the risk of subsequent relapse (i.e., to formulate risk group-specific therapy). Further reduction of the disease burden necessitates intensive cytoreductive treatment, which can be given at the time of complete remission because the bone marrow is relatively normocellular at the beginning of this phase of treatment.

A wide variety of agents and schedules have been used during

the intensification phase of treatment in pediatric trials. The Pediatric Oncology Group has reported a significant improvement in the outcome of patients treated with an early intensification phase consisting of intermediate-dose or high-dose antimetabolite therapy, [63] [64] [65] an observation confirmed by others.[37] [66] The DFCI group has shown that early intensification with weekly high-dose asparaginase improves survival for all patients, [67] and that the use of doxorubicin during intensification with asparaginase favorably influences the outcome of high-risk patients, particularly those with T-cell disease. [35] [68] The Berlin-Frankfurt-Munster (BFM) group and CCG have demonstrated that patients benefit from a delayed intensification, consisting of an intensive re-induction cycle after a period of an interim maintenance regimen.[36] [44] [45] [69] The United Kingdom ALL group reported that patients treated with both an early and delayed intensification cycle had a superior outcome when compared with patients who received only one of these cycles.[70] Thus, although most clinical trials demonstrate the importance of intensification therapy, the optimal treatment schedule remains under investigation.

Continuation Therapy

Nearly all current treatment regimens for ALL use a phase of continuation therapy, in which patients are treated with less intensive chemotherapy to complete at least 2 years of therapy.[35] [36] [37] [44] [45] [64] [65] [70] Most continuation regimens consist of weekly low-dose methotrexate and daily oral 6-mercaptopurine. Some groups add regular pulses of vincristine and corticosteroid to this regimen, [35] [44] [45] although the benefit of these pulses remains controversial. [69] [71] Researchers at the St. Judes Childrens Research Hospital have reported that higher risk patients benefit from an intensified continuation therapy consisting of rotating pairs of non-cross-resistant drugs.[37]

The optimal duration of therapy remains unknown. Most investigators continue to treat patients for a total of 23 years, based on results of older studies, in which patients received therapy that was less intensive than current regimens. [72] One recent attempt to shorten therapy duration to 18 months from 24 months resulted in a significant increase in relapses. [69] Ongoing studies of minimal residual disease may help to clarify the optimal therapy duration for patients in the era of intensive, multiagent chemotherapy. Leukemia cells from the majority of patients display clonal abnormalities not found in their normal hematopoietic cells. Current efforts to evaluate minimal residual disease have focused on the identification of these clones at diagnosis by probing for chromosomal translocations or patient-specific immunoglobulin or T-cell antigen receptor gene rearrangements. [73] Once patient-specific clones are identified, molecular techniques, such as polymerase chain reaction, can be used to detect extremely small numbers of residual leukemia cells in subsequent samples.

Initial clinical studies of minimal residual disease in childhood ALL have yielded conflicting results. Many studies suggest that the presence of detectable residual disease late in treatment or at the completion of therapy is predictive of subsequent relapse, [62] [74] [75] although others have reported that molecularly detectable leukemia can persist even after the completion of therapy in patients who remain in clinical remission. [61] [76] Additionally, the absence of detectable residual leukemia at the end of chemotherapy is insufficient to ensure that the patient is cured. [77] Investigation of more sensitive quantification techniques in larger, prospective trials is necessary before the prognostic significance of minimal residual disease testing can be established.

In addition to quantitative levels of residual disease, differences in the proliferative and growth potentials of the remaining leukemia cells are also important considerations in determining optimal duration of therapy. For example, clinical trials of rapidly proliferating, mature B-cell ALL have shown that short, intensive regimens (lasting only 23 months) have been quite effective. [78] [79] On the other hand, disease with cells that have a lower proliferative activity might benefit from a longer course of therapy.

Treatment of the CNS

Treatment of the CNS is usually initiated during induction therapy, and definitive CNS treatment is usually begun immediately after achieving complete remission (to prevent seeding from the CNS to the periphery). This concept is based on data from the 1960s that demonstrated most children who developed CNS leukemia did so during the first year of therapy. However, some clinical trials have delayed definitive CNS treatment for several months to permit more intensive systemic therapy. [37] [80] In these studies, there was no increase in CNS relapse, except in patients who had presented with leukemia blasts in their CSF at diagnosis. [81] There is also variability among investigators regarding the duration of CNS treatment. It remains uncertain whether treatment only during induction and after induction sanctuary therapy is adequate or whether prolonged intermittent CNS treatment is more effective. However, trials with the lowest reported incidence of CNS relapse used prolonged CNS treatment.[82]

Optimal CNS treatment should provide maximum antileukemia efficacy with minimum morbidity. Current CNS treatment strategies include intrathecal chemotherapy, high-dose systemic chemotherapy, or cranial radiation. Much of the controversy surrounding the use of cranial radiation originated from studies of previously irradiated survivors of childhood ALL, who have been noted to have learning disabilities, neuroendocrinologic abnormalities, and an increased incidence of second malignant neoplasms.[83] [84] Based on these concerns, some investigators have replaced cranial radiation with extended intrathecal therapy and have found that it provides adequate CNS prophylaxis when delivered in the context of effective systemic therapy, especially in lower risk patients. [85] [86] [87] However, the neurocognitive sequelae of patients treated in this manner has not been well delineated. Uncertainties about the relative efficacy and morbidity of CNS treatment options have resulted in a wide range of treatment philosophies. For example, by the middle 1980s, the proportion of children receiving cranial irradiation as a component of primary treatment varied from 0%[88] to 100% [70] among different national and international trials. Based on our own experience [82] and that of others,[85] [89] we recommend cranial radiation and intrathecal drugs (methotrexate and cytosine arabinoside) for the 40% of children whom we classify as high-risk patients.

For patients who are treated with cranial radiation, the optimal dosage and schedule of radiation also remain under investigation. Although early studies used a dose of 2,400 cGy, subsequent trials demonstrated that 1,800 cGy is as effective in preventing relapse [90] and may be associated with fewer neurocognitive sequelae.[91] The BFM group has successfully used a radiation dose of 1,200 cGy in the treatment of intermediate-risk patients. [36] [69] The DFCI group is testing whether 1,800 cGy delivered in hyperfractionated (twice daily) rather than conventional (daily) doses maintains efficacy while reducing late toxicity.

Systemic therapy plays an important role in the prevention of CNS leukemia. Penetration of the CSF by drugs has been clearly demonstrated with the use of glucocorticoids[92] and very high doses of methotrexate and cytosine arabinoside. [21] [22] It has also been shown that systemically administered asparaginase, whose efficacy is a function of asparagine depletion, effectively lowers CSF asparagine levels, [93] and lowers the number of lymphoblasts in the CSF of children with CNS disease.

Approximately 1520% of patients will present with detectable CNS leukemia (defined as the presence of any blast on a

cytospin preparation, regardless of CSF white blood cell count). [81] These patients are at increased risk for CNS relapse. [81] For these children, we recommend treatment with twice weekly intrathecal cytosine arabinoside until the CSF no longer has detectable blasts. Thereafter, at the time of complete remission, the CNS is treated with cranial radiation and intrathecal drugs (usually methotrexate and cytosine arabinoside).

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SPECIAL TREATMENT CONSIDERATIONS

Subgroups of patients have been identified who have an extremely poor prognosis, even when they are treated with current, intensive regimens. These patients are often considered candidates for alternative treatments, including allogeneic BMT. For instance, the Philadelphia chromosome, t(9;22)(q34;q11), detectable in approximately 5% of children with ALL,[94] portends a dismal prognosis, with long-term event-free survival rates of 040%. [94] [95] [96] [97] Although some preliminary studies suggest patients with Philadelphia chromosome-positive ALL and low presenting leukocyte counts may be successfully treated with intensive chemotherapy, [98] most investigators recommend allogeneic BMT in first remission for all patients with this chromosomal translocation. [99] [100]

Patients diagnosed with ALL during infancy (<1 year of age) also represent a special patient population because of their poor prognosis, as well as their increased vulnerability to the toxic effects of therapy. Infants appear to have a biologically different form of the disease, characterized by molecularly detectable rearrangements of the *MLL* gene on chromosome 11q23.[101] [102] [103] [104] [105] Their overall outcome is significantly worse than that for older children with ALL, with reported long-term event-free survival rates <20%.[106] [107] [108] Some investigators currently recommend allogeneic BMT in first remission for infants with ALL, especially those with detectable *MLL* gene rearrangements.[105] [108] The DFCI group has observed an improvement in the event-free survival infants treated with a regimen including early intensification with high-dose methotrexate and cytosine arabinoside and delayed cranial radiation (delivered at age 1 year). [109]

One to two percent of children with ALL present with mature B-cell phenotype, characterized by surface immunoglobulin, L3 morphology, and a cytogenetically detectable translocation of the *myc* photo-oncogene on chromosome 8, usually t(8;14)(q24;32), but sometimes t(2;8)(p11-p12;q24) or t(8;22)(q24;q11). Such patients fair poorly and should be treated with regimens used for advanced stage Burkitts lymphoma. [78] [79]

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LATE EFFECTS OF TREATMENT

The treatment of children with ALL has resulted in prolonged, event-free survival for 7080% of patients. [35] [36] [37] However, even for these successfully treated children, long-term effects of the disease and its treatment often result in organ toxicity of varying but clinically significant magnitude. Detailed analyses of the long-term complications of therapy have been thoroughly summarized by Green.[110] The consequences of treatment to the normal tissue are a function of the organ system involved and the type of therapy. The most common, and often most problematic, late effects involve the CNS, [83] but other problems include cataracts,[111] cardiac abnormalities,[112] hepatic toxicity,[113] short stature,[114] obesity,[115] abnormalities of gonadal function and reproduction, [116] and second malignant neoplasms.[117] [118]

Low and low average intelligence quotients (IQs) have been frequent findings in survivors of ALL. [119] [120] [121] [122] More detailed neuropsychological studies have demonstrated that learning disabilities are related to a slow speed of processing information, distractibility, and difficulty in dealing with complex or conceptually demanding material.[123] [124] The severity of neuropsychological sequelae varies, depending on both treatment and patient characteristics. For example, younger children (<5 years old) are more vulnerable than older children, [119] [120] [122] and in some studies, girls are more vulnerable than boys. [123] [125] [126]

Most CNS toxicity has been attributed to cranial radiation. [119] [120] [121] [124] The most severely impaired long-term survivors are those who received cranial radiation doses (2,4002,800 cGy) that are higher than doses used in current treatment regimens (1,2001,800 cGy). [91] [127] Microcephaly has also been reported as a late effect of CNS treatment and was found to depend upon the radiation dose.[128]

The contribution of systemic and intrathecal chemotherapy to the development of neurocognitive toxicities has not been fully assessed. It has been demonstrated that systemic chemotherapy affects the severity of radiation-associated neuropsychological sequelae. In one study, escalating doses of systemic methotrexate in children who had received cranial radiation were associated with development of leukoencephalopathy. [129] In another series, IQ decline was associated with combined therapy of high-dose systemic methotrexate and 1,800 cGy cranial radiation, but not with either therapy alone. [130] Moreover, evidence suggests that cognitive deficits are present in long-term survivors treated without cranial radiation, [131] and in one study, there were no significant differences in the severity and range of deficits between irradiated and nonirradiated patients. [126]

Neuroendocrine abnormalities are also frequently observed in survivors of childhood ALL. In some studies, survivors are shorter than expected for their age. [114] [132] [133] The greatest impact in final height has been noted in patients treated with 2,400 cGy cranial radiation, with less severe growth failure noted in patients whose CNS prophylaxis treatment included 1,800 cGy or no cranial radiation. [132] One study showed normal linear growth in children treated without cranial radiation. [134] Young age and female sex are associated with greater growth failure. [114] [132] In girls, puberty may occur early, and in both sexes, normal pubertal growth spurts may be blunted.[133] [135] In some studies, short stature in survivors of childhood ALL has been associated with growth hormone deficiency, [136] [137] suggesting that growth hormone replacement may have a potential therapeutic role. Others have failed to show impaired growth hormone secretion in similar survivor populations. [138] Moreover, controversy surrounding possible additive cardiac toxicity (especially in patients with anthracycline-induced cardiomyopathy), [139] as well as the possible risk of secondary leukemias in patients previously treated with chemotherapy and radiation, [140] necessitates that growth hormone be prescribed with great care.

Small, nonprogressive posterior subcapsular cataracts, which do not impair vision or require surgical treatment, have been reported in over 50% of children treated for ALL.[111] Although the cataracts were thought to be related to the administration of corticosteroids, the patients described had also received cranial radiation (which included treatment of the posterior half of the globes and the optic nerves).

Echocardiographic abnormalities, particularly increased afterload and decreased contractility, are common late effects of anthracycline therapy. [112] [141] The mechanism of this toxicity is impairment of myocardial growth. The severity of cardiac dysfunction is correlated with higher cumulative doses of anthracycline and higher dose rates.[112] [141] [142] [143] Patients treated at a young age, females, and those with Down syndrome appear to be more vulnerable to anthracycline-associated cardiac toxicity.[142] [143] Despite these echocardiographic abnormalities, most long-term survivors treated with anthracycline do not develop overt congestive heart failure,[141] [143] although longer follow-up is needed to fully assess the risk of late symptomatic cardiac disease.

Late occurring hepatotoxicity is a relatively uncommon effect of ALL therapy. [113] [144] Despite the large number of patients treated with regular doses of methotrexate and 6-mercaptopurine,

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there have been few reports of fibrosis or other abnormalities. [113]

Ovarian and testicular function are relatively unaffected by most antileukemia therapy, [133] [135] [145] with the possible exception of programs that use alkylating agents (such as cyclophosphamide),[146] [147] high doses of cytosine arabinoside,[146] or prophylactic gonadal irradiation. [147] Several normal children have been born of patients successfully treated for childhood ALL. [148]

Second malignant neoplasms, including malignant gliomas,[84] [117] [118] treatment-related AML,[118] [149] [150] and carcinomas of the parotid and thyroid glands, [117] [118] have been reported in survivors of ALL. Patients treated on regimens that include epipodophyllotoxins [149] [150] or alkylating agents (such as cyclophosphamide) [151] have a much higher risk of secondary AML than do patients who were treated without these agents. [152] Although cranial or craniospinal radiation has been associated with the development of solid tumors within the radiation field, the risk of secondary malignancies, including brain tumors, from radiation for ALL appears to be relatively low. For instance, fewer than 1% of patients treated on DFCI protocols with cranial radiation (none of whom received alkylating agents or epipodophyllotoxins) developed a second malignancy. [153]

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TREATMENT OF RELAPSED ALL

The recurrence of ALL is a life-threatening event. Factors that influence subsequent treatment include whether the recurrence is: (1) in the bone marrow or at an extramedullary site, such as the CNS, testis, ovary, eye, or a combination of sites;[154] and (2) after a relatively short initial remission (<1824 months), or after a longer first remission.[154] [155] [156] [157] [158] [159] The latter factor is sometimes thought of as the distinction between recurrence during therapy and recurrence after cessation of therapy;[154] [157] however, variable lengths of treatment programs make this criterion a less sensitive determinant of the outcome of subsequent therapy. Our observation has been that the time to relapse was more important than whether the relapse occurred while the patient was on or off therapy. [160] The presence of circulating blasts at the time of the relapse has been shown to be a significant predictor of adverse outcome. [161]

Depending on the intensity of initial therapy, induction of second complete remissions can be expected in 7090% of children, with conflicting data pertaining to whether some patients who were initially more intensively treated have a lower likelihood of second remission. [155] [156] [162] [163] [164] [165] Second induction treatment usually consists of multiple drugs, most often combinations of vincristine, prednisone, asparaginase, and an anthracycline (with or without methotrexate, an epipodophyllotoxin, and cytosine arabinoside). [155] [162] [164] [165] If the CNS has been involved as a site of relapse, intrathecal therapy with one or more drugs (methotrexate, cytosine arabinoside, hydrocortisone, thiotepa) is usually recommended on a weekly or twice weekly basis until the CSF is clear. Thereafter, when the patient is in complete remission, subsequent CNS treatment ("reprophylaxis") is indicated. [166] Local therapeutic approaches for isolated CNS relapses include intrathecal chemotherapy,[167] [168] craniospinal radiation,[168] [169] cranial radiation with intrathecal drugs,[167] and systemically administered high doses of methotrexate or cytosine arabinoside. [170] [171] Testicular relapses are usually treated with bilateral gonadal irradiation as well as with systemic chemotherapy. [172] [173] [174] [175]

After a second complete remission has been attained, the options for subsequent treatment include BMT and chemotherapy. Many trials of the latter have been unsuccessful at producing long-term survival,[163] [176] [177] especially in patients who relapse within 18 months of diagnosis and those with T-cell disease. [156] [164] The most successful chemotherapy trial results have been achieved with patients whose initial complete remissions exceeded 1830 months. The reported event-free survival of this subset of patients after treatment with intensive chemotherapy in second remission ranges from 36% to 65%. [156] [178] [179]

Sibling-matched allogeneic BMT for relapsed ALL has resulted in long-term survival in 2560% of patients. [157] [158] [180] [181] [182] [183] [184] [185] Transplantation in second remission has generally been more favorable than in third or subsequent remission. [181] In many published reports, the most common cause of failure after allogeneic BMT is recurrence of leukemia.[157] [182] [183] [184] Therapy-related toxicity, including infections and severe graft-versus-host disease, is also a major cause of death, occurring in 1530% of patients.[158] [183] [184] [185] [186] In recent years, the incidence of treatment-related complications appears to be decreasing with improvements in supportive care.[183] In some trials, duration of initial remission is an important predictor of outcome after allogenic BMT, with improved survival observed in patients with longer initial remissions.[157] [185] Other investigators report that patients with short initial remissions fare as well as those with longer initial remissions. [156] [183]

Because there are no randomized trials comparing chemotherapy and allogeneic BMT in relapsed ALL, published comparisons of the two modalities have generally been complicated by variability of patient selection and treatment. A retrospective, matched-pair analysis comparing outcome after sibling-matched allogeneic BMT with that achieved after chemotherapy demonstrated that BMT was associated with improved leukemia-free survival. [185] Others have also observed that sibling-matched allogeneic BMT confers a survival advantage, especially in patients with short initial remissions. [156] [176] [183]

For patients without matched sibling donors, alternative transplant options include autologous and matched-unrelated donor BMT. Autologous BMT is an attractive alternative because of the absence of graft-versus-host disease or need for long-term immunosuppression. Several investigators have studied autologous BMT, often in conjunction with ex vivo purging of residual leukemia in harvested marrow via immunologic or pharmacologic techniques. [159] [160] [187] [188] [189] For patients with long initial remissions, results achieved after autologous transplantation are comparable to those observed after allogeneic BMT, with event-free survival ranging from 40% to 50%.[160] [186] [188] [190] Patients with short initial remissions fare less well. [190] Relapse is the most common cause of treatment failure after autologous BMT, with a lower incidence of toxicity-related deaths than is observed after allogeneic BMT. [186]

Transplants from human leukocyte antigen-matched unrelated donors have been successful for some children with recurrent leukemia. [189] [191] [192] In studies that include patients treated after multiple relapses, event-free survival ranges from 20% to 30%. [189] [191] [192] The risk of treatment-related morbidity and mortality is substantial, often secondary to graft-versus-host disease.

Isolated extramedullary relapses, usually CNS or testicular, are manifestations of systemic disease. Therefore, in addition to therapy directed to the local site of disease (i.e., twice weekly intrathecal drugs for CNS or bilateral testicular irradiation for testicular relapse), intensive systemic therapy is also indicated. Given the high propensity for hematologic relapse after an isolated CNS relapse, [193] [194] we recommend BMT as postremission therapy for these patients. Emerging evidence suggests that intensive chemotherapy regimens may also provide adequate postremission therapy for patients with an isolated CNS relapse, especially those with late-occurring relapses. Recent studies report long-term event-free survival of 4570% for patients treated in this manner. [167] [168] [175] For patients with isolated testicular relapses, systemic chemotherapy and testicular irradiation have resulted in prolonged second remissions in >80% of patients with late-occurring relapses. [172] [173] This approach has been less successful in patients whose testicular relapses occur during or soon after cessation of initial therapy, with long-term

event-free survival ranging from 20% to 43%.[172] [174] Therefore, we recommend BMT for this subset of patients.

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FUTURE DIRECTIONS

Future approaches to the treatment of childhood ALL include redefinition of prognostic factors, so that more intensive regimens are reserved for children at high risk of relapse and attempts to diminish long-term toxicity are addressed in patients who are successfully treated with current therapies. The use of molecular techniques may identify biologically distinct leukemia subtypes that may supplement or replace the epidemiologic factors currently used to determine risk-based therapy. Areas of investigation include the evaluation of more specific treatments (e.g., monoclonal antibodies linked to toxins), [195] the stimulation of leukemia-specific immunity,[196] the use of antiangiogenesis factors,[197] and the documentation of minimal residual disease.[73] The possibility of developing patient-specific treatment regimens based on in vitro cytotoxicity assays also is a promising new approach to the improved treatment of this disease. [198] [199]

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Chapter 59 - Acute Lymphocytic Leukemia in Adults

Dieter Hoelzer

Acute lymphocytic leukemia (ALL) is a malignant disease characterized by the accumulation of lymphoblasts. Two thirds of children and approximately one third of adults with the disorder can be cured with therapy currently available. Prognostic factors for leukemia-free survival (LFS) allow adult ALL patients at presentation to be stratified into low- and high-risk groups. This stratification is necessary for the recognition of patients who require more intensive chemotherapy or bone marrow transplantation (BMT) as curative therapy during first remission.

The difference in outcome of treatment between children and adults is probably not due to the presence of a different disease, but rather is a result of the higher frequency in adults of ALL subtypes associated with adverse prognoses, such as

Philadelphia (Ph) chromosome-positive ALL.[1] [2] Furthermore, a different pharmacology of cytostatic drugs and the higher rates of hematologic and nonhematologic toxicity contributes to their poorer outcome, particularly in ALL patients older than 50 years of age.

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EPIDEMIOLOGY

According to a report from the National Cancer Institute, the age-adjusted overall incidence of ALL in the United States was 1.5/100,000 in whites and 0.8/100,000 in blacks. The male-to-female ratio was 1.4:1.0. After a first peak in children younger than 5 years of age (5.3/100,000), the incidence decreases continuously. There is a slight increase after the age of 35 years and a second minor peak is reached in the age group of 8084 years (2.3/100,000). In the subsequent reporting periods from 1973 to 1987, there was a slight increase of age-adjusted incidence rates from 1.2 to 1.4. [3]

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BIOLOGIC AND MOLECULAR ASPECTS

Preferred Approach to Diagnosis

Classification of the phenotype of the blast cells in acute leukemia requires morphologic and cytochemical evaluation, immunophenotyping, cytogenetic analysis, and molecular genetic analysis. Morphology remains the means by which acute leukemia is initially detected and together with cytochemical reactions is the major aid in distinguishing between ALL and acute myeloid leukemia (AML). For more precise subclassification of ALL into B or T lineages, immunologic techniques must be used to detect lineage-specific antigens as well as surface or intracytoplasmic molecules. The presence of terminal deoxynucleotidyl transferase (TdT) activity in leukemic blasts can facilitate the diagnosis of ALL. Cytogenetic analysis is a prerequisite for diagnosis of ALL because it has strong prognostic value. Molecular genetic techniques for confirmation of diagnosis in particular subsets of ALL (e.g., *bcr-abl*-positive ALL), as well as to evaluate therapeutic efficacy by detection of minimal residual disease (MRD), are of increasing importance.

Morphology

The cytologic features of leukemic blast cells in ALL and their division into L1 to L3 according to the French-American-British (FAB) classification are discussed in Chapter 57 . The distribution of L1 and L2 subtypes is of minor relevance for prediction of outcome. The subtype L3, observed in up to 5% of adult patients, should be distinguished because it is indicative for B-cell ALL, with different treatment option, but should be confirmed by surface marker analysis.

Cell Surface Marker Analysis

Acute lymphoblastic leukemia is divided into subtypes by immunologic criteria based on the presence of specific receptors or antigens on the cell surface of leukemic blast cells. Within the B or T lineage ALLs, the subtypes are defined according to their stage of differentiation. [4] For more details on the immunologic classification of ALL, see Chapters 56 and 57 .

The frequency of and definition of ALL subtypes in adult and childhood ALL are compared in Table 59-1 . In the German multicenter trials for childhood ALL (BFM) and adult ALL (GMALL), the phenotypic characteristics of 1,756 children and 946 adults with ALL were analyzed prospectively. B-precursor ALL is more frequently found in children, whereas T-lineage ALL has a higher incidence in adults. Within the B and T lineages, the more immature subtypes, early pre-B ALL and pre-T ALL, occur more frequently in adults. [4]

B-Lineage ALL

Early pre-B ALL, also termed *pre-pre-B ALL* or pro B, lacks B-, T-, and pre-B-cell markers but expresses human leukocyte antigen (HLA)-DR, TdT, and CD19, and has rearranged immunoglobulin genes. It occurs in approximately 10% of adult and 5% of childhood ALL. *Common ALL* is the major immunologic subtype in childhood as well as in adult ALL. It comprises >50% of cases of adult ALL. Common ALL is characterized by the presence of CD10 (formerly, CALLA) and a glycoprotein (gp100/CD10). Common ALL blast cells do not express markers that characterize relatively mature B cells such as cytoplasmic immunoglobulins or surface membrane immunoglobulins. The blast cells are positive for CD19 and TdT.

Pre-B ALL is characterized by the expression of cytoplasmic immunoglobulin, which is absent in common ALL, but is identical to common ALL with respect to the expression of all other cell markers (Table 59-1). Only very rarely may CD10 be absent in this subtype. Pre-B ALL comprises nearly 10% and 15% of adult and childhood ALL, respectively.

Mature B-cell ALL is found in approximately 4% of adult and 3% of childhood ALL patients. The blast cells express surface antigens of mature B cells, including surface membrane immunoglobulin. CD10 may be present, as well as occasionally cytoplasmic immunoglobulin.

T-Lineage ALL

Approximately 25% of adult ALL cases have blast cells with a T-cell phenotype. All cases express the T-cell antigen gp40 (CD7), and they may, according to their degree of T-cell differentiation, express other T-cell antigens (e.g., the E rosette receptor [CD2] or the cortical thymocyte antigen T6 [CD1]). A minority

TABLE 59-1 -- Immunologic Classification of Childhood and Adult Acute Lymphocytic Leukemia (ALL)

	Children (%; N = 1,756[4])	Adults (%; N = 946[4])	Surface Marker
B-lineage			
Early pre-B ALL	5	11	HLA-DR+, TdT+, CD19+
Common ALL	65	51	HLA-DR+, TdT+, CD10+, CD19+
Pre-B ALL	15	10	HLA-DR+, TdT+, CD10±, cytoplasmic immunoglobulin+
B-cell ALL	3	4	HLA-DR+, CD10±, CD19+, immunoglobulin+
T-lineage			
Pre-T ALL	1	7	TdT+, cytoplasmic CD3+, CD7+
T-cell ALL	11	17	TdT+, cytoplasmic CD3+, CD1a/2/3+

+, positive; ±, positive or negative; HLA, human leukocyte antigen; TdT, terminal deoxynucleotidyl transferase.

of T-cell ALL blast cells may also express CD10 together with T-cell antigens. In most cases of T-cell ALL, one or more of the T-cell receptor genes is rearranged.

These properties make it possible to classify T-cell ALLs according to their stage of differentiation into early T-precursor ALL (or pre-T ALL), 7% and 1% of adult and childhood ALL, respectively, and more mature T-cell ALL, 17% and 11% of adult and childhood ALL, respectively. [5]

Cytogenetic Analysis

Cytogenetic abnormalities are the most important independent prognostic variables for predicting the outcome of adult ALL. In two recent studies, clonal chromosomal aberrations could be detected in approximately 6285% of adult ALL patients; [6] [7] 1538% of the cases had normal metaphases. With careful attention to collection of the bone marrow cells and their rapid transport and preservation, the success rate in analysis of ALL marrow can be increased, and the identification of clonal abnormalities can be achieved in >90% of cases.

The major cytogenetic abnormalities in ALL are clonal translocations (t[9;22], t[4;11], t[8;14], t[1;19] or t[10;14]), and other structural abnormalities (9p, 6q, or 12p abnormalities). If none of the structural aberrations is present, the abnormalities can be classified according to the modal chromosomal number (<46, 46 with other structural abnormalities, 4750, >50; Table 59-2).

The demonstration of chromosomal abnormalities in ALL is relevant for several reasons: the presence of such defects may confirm the diagnosis if a karyotype specific for ALL is found; and chromosomal abnormalities are closely correlated with clinical features and immunologic subtypes and are independent prognostic variables for predicting LFS.

Molecular Genetic Analysis

Molecular analyses, detecting gene rearrangements in ALL by the polymerase chain reaction (PCR), Southern blot analysis, or fluorescent in situ hybridization with chromosome-specific DNA probes, are useful approaches in establishing a more precise diagnosis and defining the quality of remission, and may also give insights into the pathophysiology of the leukemic process (e.g., the mechanisms of leukemic cell stimulation by bcr-abl fusion proteins).

The most frequent molecular markers in ALL are bcr-abl and

TABLE 59-2 -- Chromosomal Abnormalities in Adult Acute Lymphocytic Leukemia

	Frequency (N = 793)[6] [7]
Clonal abnormalities	6885%
Normal diploid	1534%
Numeric aberrations	
Hypodiploid	48%
Hyperdiploid (4750)	715%
Hyperdiploid (>50)	78%
Near tri/tetraploid	35%
Structural aberrations	
t(9;22)	1129%
t(4;11)	34%
t(8;14)	5%
t(1;19)	23%
t(10;14)	3%
9p abnormalities	515%
6q abnormalities	46%
12p abnormalities	45%

ALL1-AF4. The Ph chromosome t(9;22)(q34q11) results from a translocation involving the breakpoint cluster region of the *bcr* gene on chromosome 22 and the *abl* gene on chromosome 9. The *bcr-abl* gene rearrangement can be demonstrated by molecular techniques. PCR analyses revealed an incidence of 2026% *bcr-abl*+ ALL in adult [8] [9] [10] compared with 3% in childhood ALL patients. One third of adult ALL patients with a Ph chromosome show M-*bcr* rearrangements (resulting in a 210-kd protein), similar to patients with chronic myeloid leukemia (CML), whereas two thirds have m-*bcr* rearrangements (resulting in a 190-kd protein). It is noteworthy that *bcr-abl* is more frequently detected than the corresponding chromosome abnormality (t[9;22]) because of occasional difficulties in obtaining sufficient material for cytogenetic analysis.

The most frequent form of 11q23 abnormality in ALL is t(4;11)(q21;q23). The involved gene on chromosome 11 is named *MLL* for mixed lineage leukemia. Synonyms are *ALL-1*, *HRX*, and *HTRX1*. The *MLL* gene is fused to a gene located on chromosome 4 that is named *AF-4* (also referred to as *FEL*). The translocation is frequently detected in infant leukemia and in patients with the early pre-B subtype (CD10 negative). The overall incidence in adults is approximately 6%. [11]

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ETIOLOGY

The cause of ALL remains unknown. However, certain factors are related to its development. [12]

Genetic Predisposition

In epidemiologic studies, patients with some rare congenital chromosomal abnormalities showed an increased risk for development of acute leukemias, including ALL. In children with leukemia (predominantly AML), there is a 20-fold higher incidence of Downs syndrome than would be expected. There is also an increased risk of ALL associated with inherited disorders such as Klinefelters syndrome, Fanconis anemia, Blooms syndrome, ataxia-telangiectasia, and neurofibromatosis. The impact of genetic predisposition on the pathobiology of ALL may also be inferred from reports of the simultaneous development of ALL in identical twins. Such observations may also indicate that the disease is caused by an intrauterine event affecting both twins. [13]

Irradiation

The incidence of acute leukemias, mainly AML, but also ALL, was increased almost 20-fold in survivors of the atomic bomb explosions (>1 Gy exposure) in Japan, [14] with a peak incidence occurring 67 years after the radiation exposure. Induction of leukemia by emissions from nuclear power stations has also been raised as a possible environmental leukemogenic risk. An increased incidence of leukemia in children living in the vicinity has been observed, but it could not be directly related to radioactive emissions. [15]

Whether radiation exposure after the Chernobyl accident led to an increased incidence of leukemia is still controversial. So far, there is no evidence for a major increase in the Chernobyl region or in countries with high levels of contamination. [16] [17]

Chemical

The risk for development of ALL may also be increased after exposure to chemical agents such as benzene or other agents capable of producing bone marrow aplasia, including chemotherapeutic drugs. Secondary, therapy-related AMLs can occur after exposure to alkylating agents such as cyclophosphamide, epipodophyllotoxins, topoisomerase II inhibitors, and, rarely, anthracyclines [18] when used extensively in the treatment of ALL. Rarely, secondary ALL occurs in people with a history of chemotherapy

or irradiation as a second neoplasm. [19] Future treatment strategies might involve reduction of exposure of the patient to these drugs.

Viral

There is no direct evidence that a virus causes human ALL. Indirect findings, however, suggest involvement of a virus in the pathogenesis of two lymphoid neoplasias. [20] In the endemic African type of Burkitts lymphoma, the Epstein-Barr virus, a DNA virus of the herpes family, has been implicated as a potential causative agent. [21] The endemic infection with human T-cell leukemia virus I (HTLV-I) in Japan and the Caribbean has been shown to be an etiologic agent for adult T-cell leukemia/lymphoma. [22]

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CLINICAL MANIFESTATIONS

Most adult patients initially present with clinical symptoms resulting from bone marrow failure. Physical findings such as pallor, tachycardia, weakness, and fatigue are due to anemia; petechiae or other hemorrhagic manifestations are attributable to thrombocytopenia; infectious complications are due to neutropenia. Clinical signs of leukemia related directly to infiltration of organs with leukemic blasts, such as lymphadenopathy, splenomegaly, and hepatomegaly, are present in most patients but are infrequently the problems for which the patient first seeks medical advice.

Symptoms and clinical manifestations of 938 adult ALL patients, 1565 years of age, entering two consecutive German multicenter trials, are given in Table 59-3 . One third had infection or fever at presentation, and one third presented with hemorrhagic episodes. Weight loss was observed only occasionally. Approximately one half of the patients presented at diagnosis with lymphadenopathy, splenomegaly, and hepatomegaly, and hilar lymph node enlargement or a thymic mass (detected on chest radiographs or computed tomography scans in approximately 14% of patients). Most (85%) patients with mediastinal masses had T-cell ALL. Massive thymic enlargement can cause dyspnea, especially when associated with pleural effusions. Although 7% of ALL patients at presentation had central nervous system (CNS) involvement (as demonstrated by leukemic blast cells in the cerebrospinal fluid), only 4% of these initially had CNS symptoms such as headache, vomiting, lethargy, nuchal rigidity, and cranial nerve or peripheral nerve dysfunction.

TABLE 59-3 -- Symptoms and Clinical Signs at Diagnosis of Acute Lymphocytic Leukemia in 938 Adult Patients

Sign or Symptom	Patients (%)
Symptoms	
Infection/fever	36
Hemorrhage	33
Physical findings	
Lymphadenopathy	57
Splenomegaly	56
Hepatomegaly	47
Mediastinal mass	14
Central nervous system involvement	7
Other organ involvement	9
Pleura	2.9
Bone	1.2
Pericardium	1.0
Retina	1.0
Skin	0.6
Tonsils	0.6
Lung	0.5
Kidney	0.4
Testis	0.3

TABLE 59-4 -- Laboratory Findings (Leukocyte Counts) at Time of Diagnosis of Acute Lymphocytic Leukemia in 938 Adult Patients

	Patients (%)
White blood cell count ($\times 10^6$ /L)	
<5,000	27
5,00010,000	14
10,00050,000	31
50,000100,000	12
>100,000	16
Leukemic blast cells in peripheral blood	
Present	92
Not present	8
Leukemic blast cells in bone marrow	
<50%	3
5190%	51

>90%	46
Bone marrow aspirable	84

Virtually any organ can be infiltrated by ALL blast cells, and approximately one tenth of the patients had such organ involvement (Table 59-3). Most often a pleural effusion was observed, and this occurred almost exclusively in those patients with mediastinal enlargement and T-cell ALL. Some of those patients also had a pericardial effusion. Bone or joint pain was rarely observed compared with childhood ALL; bone lesions could be found in only 1.2% of cases. Initial involvement of the testis was very rare (<1%). Lymphoblastic infiltration of retina, skin, tonsils, lung, or kidney was observed only occasionally, particularly in mature B-cell ALL and to a lesser extent in T-cell ALL. Such organ infiltration can result in the typical clinical pattern associated with high-grade non-Hodgkins lymphoma (NHL).

LABORATORY EVALUATION

The peripheral blood cell values at diagnosis of the same cohort of patients are shown in Tables 59-459-6.

The leukocyte count (Table 59-4) was elevated in 59%, 14% had normal counts, and 27% had leukopenia. In 92% of the patients, leukemic blast cells were seen in the blood smear. Thus, aleukemic leukemias account for only a small proportion of

TABLE 59-5 -- Peripheral Blood Counts at Time of Diagnosis of Acute Lymphocytic Leukemia in 938 Adult Patients

	Patients (%)
Neutrophils (× 10^6 /L)	
<500	23
5001,000	14
1,0001,500	9
>1,500	54
Platelets (× 10^6 /L)	
<25,000	30
25,00050,000	22
50,000150,000	33
>150,000	15
Hemoglobin (g/dl)	
<6	8
68	20
810	27
1012	24
>12	21

TABLE 59-6 -- Coagulation Parameters at Time of Diagnosis of Acute Lymphocytic Leukemia in 938 Adult Patients

	Patients (%)
Fibrinogen (mg/dl)	
<100	4
>100	96
Prothrombin time (%)	
<50	7
5075	34
75100	34
>100	25
Partial thromboplastin time (sec)	
<30	33
3040	53
4050	11
>50	3

cases of adult ALL. With automated blood counting, the diagnosis may be missed in patients with normal or decreased white blood cell (WBC) counts and with low blast cell contents. For this reason, the need for microscopic examination of blood smears in people suspected of having acute leukemia should be stressed. An elevated blood count >100,000 × 10^6 /L was observed in 16% of the patients, and occasionally WBC counts >500,000 × 10^6 /L have been observed. In general, a high WBC count is found more frequently in T-cell ALL patients compared with B-lineage ALL.

Neutrophils (Table 59-5) <500 × 10^6 /L were seen in 23% of the patients and thrombocytopenia <25,000 × 10^6 /L in only 30% of patients, corresponding roughly with

the symptoms of infection and bleeding present at diagnosis. Anemia at diagnosis is observed in most adult ALL patients.

Bone marrow aspiration or biopsy is mandatory for diagnosis of ALL. In <15% of patients, the bone marrow cannot be aspirated and a biopsy must be performed. Dry taps are due to densely packed blast cells, fibrosis, or inadequate technique; the first two resolve after therapy. Most patients have >50%, or even >90% of blast cells in the bone marrow (Table 59-4). In <3% of cases, the blast cells constitute <50% of the nucleated marrow cells.

An initial lumbar puncture should be done to determine whether the CNS is involved. However, if there is a risk of bleeding due to a very low platelet count or of blast cell contamination due to a high leukemic blast content in the peripheral blood, lumbar puncture should not be performed. When the leukocyte count in the spinal fluid is low, or the morphologic detection of blasts is inconclusive, demonstration of an immunologically defined blast cell population can confirm a diagnosis of CNS involvement.

The most frequent metabolic abnormality is an increased serum uric acid level in approximately one half the patients; hypercalcemia was rare. Serum lactate dehydrogenase may be elevated as a result of cell destruction in patients with a large tumor mass, particularly in B-cell ALL. In a small proportion of patients (Table 59-6), the initial fibrinogen level was <100 mg/dl. Disseminated intravascular coagulation in ALL was rarely observed at diagnosis.

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DIFFERENTIAL DIAGNOSIS

Difficulty is rarely experienced in establishing the diagnosis of ALL. The differentiation from lymphocytosis, lymphadenopathy, and hepatosplenomegaly in viral infections and other acute or chronic leukemias can usually be done by lymphocyte surface markers.

Aleukemic pancytopenic ALL patients without blast cells in peripheral blood (<10%) have to be distinguished from those with aplastic anemia, which may also be a preleukemic syndrome. In contrast to ALL, in aplastic anemia the bone marrow is hypocellular.

In rare cases with low bone marrow infiltration, an arbitrary distinction between ALL and NHL is usually chosen according to the degree of infiltration, above or below 25%.

Mixed or hybrid leukemias are those in which blast cells express lymphoid as well as myeloid antigens; they may also be termed biphenotypic or bilineage leukemias. Biphenotypic leukemias are defined as those in which markers of lymphoid and myeloid lineages are coexpressed on the same leukemic cells. Bilineage leukemias are those with two populations of blast cells that have either lymphoid or myeloid antigens and might be allocated to a treatment strategy either for ALL or for AML.

Occasionally difficulties can occur in distinguishing Ph/bcr-abl-positive ALL from primary lymphatic blast crisis of CML. Sometimes final diagnosis can be done only after treatment initiation. In ALL patients achieving complete clinical remission (CR), the peripheral blood count shows normal values, whereas CML cases revert to a chronic phase with pathologic left shift.

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THERAPY

Initial Evaluation and Supportive Therapy

At diagnosis, the evaluation of an adult with ALL should include a history and a careful physical examination. Speed in clinical evaluation and diagnosis is important in order to initiate supportive measures and to decide on appropriate therapy. Only in a few cases is the leukemic process so far advanced that immediate treatment of leukemia is necessary (e.g., in patients with symptoms due to a large mediastinal mass and pleural effusions or to a rapidly progressing B-cell ALL).

A few general measures should be initiated at once. Sufficient fluid intake to guarantee urine production of 100 ml/hour throughout induction therapy should be maintained to reduce the danger of uric acid formation. Parenteral fluid administration may be required when the patients oral intake is inadequate because of nausea or difficulty in swallowing. Placement of an implantable port system is advantageous when anticipating a long period of induction therapy or when part of the therapy will be carried out on an outpatient basis.

Patients should receive allopurinol to reduce the formation of uric acid and avoid the danger of urate nephropathy. Allopurinol should be given at a dose of 300 mg/day, which may be increased to 600 mg/day if high leukocyte counts or organomegaly persist. The dose of allopurinol has to be reduced when 6-mercaptopurine is given because it potentiates the action of 6-mercaptopurine.

Approximately one third of adult patients present with infection and bleeding. They are at high risk of infectious and hemorrhagic complications during the induction period because thrombocytopenia and granulocytopenia are aggravated by chemotherapy.

In general, platelet transfusions should be given in response to bleeding episodes and to prevent bleeding when platelet counts fall to <20,000 (10^6/L), especially during febrile periods. Most often 48 U/day of platelets are given until bleeding ceases. HLA-matched platelets are given to patients who become refractory to random donor platelets. The incidence of fatal hemorrhage during induction therapy has been significantly lowered by these measures.

Infection Prophylaxis

The use of more intense cytostatic regimens has resulted not only in improved response rates of malignancies but in higher morbidity and mortality rates due to infections.[23] Long-term

neutropenia is the most important risk factor, but CD4 lymphopenia, antibody deficiency, and multiple immunosuppression in allogeneic transplantation also lead to severe and lethal infections. Whereas formerly gram-negative microorganisms were the leading cause of febrile neutropenia, in the last decade gram-positive bacterial infections have increased, especially those due to more frequent use of indwelling catheters. Fungal infections also occurred more frequently. [24] The successful management of febrile neutropenia is based on hygienic procedures including general body hygiene, low-germ food, reverse isolation or high-efficiency particulate air filtration, antibiotic prophylaxis, sufficient diagnostics, and consequent empirical infection prophylaxis.

For antibacterial prophylaxis, cotrimoxazole or fluorochinolones, both mainly directed against gram-negative organisms, have mostly been used. [25] Cotrimoxazole also reduces the incidence of *Pneumocystis carinii* pneumonia, which occurs in approximately 20% of patients without prophylaxis. [26] Although antifungal prophylaxis with oral amphotericin B solution or triazoles may successfully reduce candidal colonization and prevent local candidal infections, the prophylactic procedures for reducing systemic mycoses are disappointing. Fluconazole has been reported to reduce only systemic *Candida albicans* infections in BMT patients, but increased non-*albicans* infections, especially *Candida krusei*, have been found. Because of its high lethality, *Aspergillus* infection is particularly feared. Attempts to prevent aspergilloses have included prophylaxis with itraconazole, intravenous low-dose amphotericin B, liposomal amphotericin B, and inhalation of aerosolized amphotericin B, but in randomized studies no benefit could be shown.[27]

The diagnostic procedures in febrile neutropenia include physical examination, cultures of blood, urine, sputum, and other sites of suspected infection, and imaging procedures such as chest radiography or abdominal ultrasound. High-resolution computed tomography of the lungs is especially important for the early diagnosis of *Aspergillus* pneumonia. Fungal pneumonias also may be microbiologically proven with bronchoalveolar lavage. More recently, PCR has been studied for use in the early detection of fungal infections.

The standard treatment of febrile neutropenia is the empiric administration of broad-spectrum antibiotics without waiting for microbiologic results. As initial therapy, combinations such as -lactam antibiotics plus aminoglycosides have been the gold standard, but monotherapy (e.g., with carbapenems or cephalosporins of class 3 and 3a) has been suggested to be equally effective. [28] Considering the increasing problems due to systemic fungal infections, empiric antimycotic treatment for persisting fever despite adequate antibacterial therapy in patients with pulmonary infiltration has been introduced. [29] Standard antifungal therapy is conventional amphotericin B, which may be combined with 5-flucytosine. Other formulations of amphotericin B, such as liposomal amphotericin B, the colloidal dispersion or the lipid complex, have been introduced to reduce amphotericin B toxicity. [30]

Hematopoietic Growth Factors

The use of hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) is a valuable component of supportive therapy during the treatment of ALL (Table 59-7). There is no indication that these CSFs stimulate leukemic cell growth in a clinically significant manner.

Most clinical trials demonstrate that the prophylactic administration of G-CSF significantly accelerates neutrophil recovery, [31] [32] [33] [34] [35] and several prospective, randomized studies also show that this is associated with a substantially reduced incidence and duration of febrile neutropenia and of severe infections. [32] [34] [35] The enhanced marrow recovery allows closer adherence to the dose and schedule of chemotherapeutic regimens. However, it still remains open whether the increased dose intensity translates into an improved LFS.

The advantage of G-CSF administration is particularly evident in selected high-risk patients receiving multiple treatment cycles, [32] whereas clinical effects appear to be negligible in patients at low risk of infectious complications. [36] Conversely, the results of the St. Jude trial show that even in cases of acute leukemia with a >40% probability of severe neutropenia, not all patients benefit from G-CSF treatment. [37] A comparison of this study[37] with other trials [34] [35] highlights the great importance

of CSF scheduling. When CSFs are first given at the end of a 4-week chemotherapy regimen, potential benefits are limited. Therefore, it is noteworthy that G-CSF may even be given in parallel with chemotherapy without aggravating the myelotoxicity of these specific regimens, [33] [34] [35] [38] and that this scheduling is an important determinant of the clinical efficacy.

An accelerated neutrophil recovery time, decreased rate of documented infection, and a shortened duration of intravenous antibiotic use and hospital stay could reduce costs of treatment. This has to be weighed against the expense of the hematopoietic growth factors. So far, the limited number of trials that have performed a cost-benefit analysis of G-CSF administration have demonstrated slightly decreased [39] or unchanged[37] overall treatment costs. On the other hand, the authors believed that

TABLE 59-7 -- Application of Granulocyte Colony-Stimulating Factor (G-CSF) in Adult Acute Lymphocytic Leukemia

Author	Year	Patients	Chemotherapy	Growth Factor	N	Days Until Neutrophil Count >500 × 10^6 /L	Incidence of Infections (%)	Early Death (%)	CR Rate (%)
Kantarjian et al.[31]	1993	CR1	Consolidation (VP, HAM)	G-CSF	14	14 <0.001	2	0	NR
				Historical controls	14	18	4	14	NR
Ottmann et al.[38]	1993	PR/ CR1	Induction II (C, AC, MP, radiation, i.th)	G-CSF	15	8	40	0	NR
Scherrer et al.[33]	1993	De novo	Induction (V, P, A, D, C, AC, MP)	G-CSF	16	15[a] <0.01	NR	NR	94
				Historical controls	21	23			Only CR
Ottmann et al.[34]	1995	CR1/ PR	Induction II (C, AC, MP, radiation, i.th)	G-CSF	37	8[a]	43	0	NR
				Control	39	12.5	56	3	NR
Geissler et al.[35]	1997	De novo	Induction I (V, P, D, A)	G-CSF	23	16 <0.005	40	4	96
				Control	22	24	77	9	80

CR, complete remission; CR1, first remission; PR, partial remission; VP, etoposide; C, cyclophosphamide; AC, cytosine arabinoside; MP, 6-mercaptopurine; V, vincristine; P, prednisone; A, L-asparaginase; D, daunorubicin; NR, not reported.

[a] >1,000 × 10^6 /L.

the CSF administration contributed substantially to quality of life. [39]

Chemotherapy

Chemotherapy of ALL is usually divided into several phases, beginning with remission induction. The objective of induction chemotherapy is to achieve CR, that is, eradication of leukemia as determined by morphologic criteria and, more recently, also by molecular markers. Although the induction phase is usually well defined, postremission therapy can be subdivided into intensification and maintenance phases. Usually prophylactic CNS treatment is added (Table 59-8).

Remission Induction Therapy

Exact diagnosis and management of initial complications are the prerequisites for successful induction therapy. A cautious cell reduction phase is recommended for patients with a large leukemic cell burden or a high leukocyte count (>25,000 × 10^6 /L). Patients with extreme leukocytosis (>100,000 × 10^6 /L) have been treated initially with leukapheresis. However, these patients

TABLE 59-8 -- Overall Treatment Results in Adult Acute Lymphocytic Leukemia in Larger Studies[a]

Group	Author	Year	N	Age	Induction	Consolidation	Maintenance	CR	MRD	LFS
MDACC[75] [163]	Cortes et al., Kantarjian et al.	1990,95	112	30	V, AD, DX, C	M, A, AD, HDAC, V, P, D, MP, HdM	AD, V, AC, P/auto BMT, HdM, D, V, MP, P	82%	NR	26% at 3y
GATLA[52]	Lluesma-Gonalons et al.	1991	137	30	V, P, D		MP, M, V, P	80%	10	20% at 5y
GATLA[52]	Lluesma-Gonalons et al.	1991	145	29	V, P, D, A, C, AC, MP	AD, V, DX, A, AC, C, MP	M, MP, V, P	78%	28	34% at 6y
CALGB 8011[53]	Ellison et al.	1991	277	33	V, P, A, D,	MP, M, [AC, D]	MP, M, V, P	64%	21	29% at 9y
CALGB 8513[73]	Cuttner et al.	1991	164	32	V, P, Mi/D, HdM	V, P, Mi/D, HdM, AC, MP, A		64%	11	18% at 3y
UCSF[69] [76]	Linker et al.	1991,97	109	1550	V, P, D, A	V, P, A, D, IdM, VM, AC	M, MP	88%	NR	42% at 5y
EORTC[164]	Stryckmans et al.	1992	106	27	V, P, AD, [HDAC]	A, HdC, [M, TG, AC]	MP, M, P, V, AD, BCNU, C	74%	32	40% at 8y
L+B+V OPAL/HEAVD[165]	Bassan et al.	1992	212	27	V, P, A, AD [C;HDAC]		MP, M, C	71%	23	32% at 10y
GMALL 01[166]	Hoelzer et al.	1993	368	25	V, P, A, D, C, AC, M, MP	V, DX, AD, AC, C, TG	MP, M	74%	24	35% at 10y
GMALL 02[166]	Hoelzer et al.	1993	562	28	V, P, A, D, C, AC, M, MP	V, DX, AD, AC, C, TG, VM, AC	MP, M	75%	27	39% at 7y
UKALL IX[167]	Durrant	1993	266		V, P, A, (MP, M)/D		MP, M, V, P	68%		22% at 8y
FGTALL[115]	Fière et al.	1993	581	33	V, P, D/R, C, [amsa, AC]	D/R, AC, A	MP, M, V, C, P, D/R, DT, BCNU	76%	19	17% at 5y
BGMT[168]	Attal et al.	1995	135	31	V, P, A, D; C, AC, MP	HdM, AC,allo/auto BMT	[Interleukin-2]	93%	24	44% at 3y
CALGB 8811[169]	Larson et al.	1995	197	32	V, P, A, D, C	C, MP, AC, V, A, M, AD, DX, TG	MP, M, V, P	85%	32	30% at 5y

GIMEMA 0183[55]	Mandelli et al.	1996	358	31	V, P, A, D	V, IdM, IdAC, P, VM, AC	MP, M, V, P[A, AC, VM, IdAC]	79%	20	25% at 10y
GIMEMA 0288[54]	Mandelli et al.	1996	767	28	V, P, A, D, C [HDAC,Mi]	V, HdM, IdAC, DX, VM	MP, M, V, P[AC, Mi, VM, HDAC, HdM, DX]	82%	28	34% at 6y
HOVON[60]	Dekker et al.	1997	130	35	V, P, A, D	HDAC, amsa, MP, VP		73%	NR	28% at 5y
SAKK[79] [170]	Wernli et al.	1994,97	140	31	V, P, D, M, A, HDAC, VP	allo/auto BMT; >50y:HDC		69%	NR	21% at 5y
UKALL XA[56]	Durrant et al.	1997	618	>15	V, P, D, A	[AC, VP, D, TG]	MP, MTX, V, P	82%	27	28% at 5 y

[X,Y], with or without; X/Y, either/or; CR, rate of complete clinical remission; MRD, median remission duration; LFS, leukemia-free survival; V, vincristine; P, prednisone; A, L-asparaginase; D, daunorubicin; amsa, amsacrine; M, methotrexate; MP, 6-mercaptopurine; AC, cytosine arabinoside; C, cyclophosphamide; DX, dexamethasone; TG, thioguanine; AD, doxorubicin; DT, dactinomycin; BCNU, carmustine; R, rubidazone; Mi, mitoxantrone; BMT, bone marrow transplant; HdM, high-dose M; IdM, intermediate-dose M; HDAC, high-dose AC; IdAC, intermediate-dose AC; VM, teniposide; VP, etoposide; VD, vindesine; NR, not reported; MDACC, M.D. Anderson Cancer Center; GATLA, Argentine Group for Treatment of Acute Leukemia; CALGB, Cancer and Leukemia Group B; UCSF, University of California, San Francisco; EORTC, European Organisation for Research and Treatment of Cancer; L + B + V, London (St. Bartholomews Hospital) + Bergamo (Ospedale Riuniti) + Vicenza (Ospedale San Bartolo); GMALL, German Multicenter Trials in Adult ALL; UKALL, United Kingdom (Medical Research Council) Trials in ALL; FGTALL, French Group for Treatment of Adult ALL; BGMT, Bordeaux, Grenoble, Marseille, Toulouse; GIMEMA, Gruppo Italiano Malattie Ematologiche Maligne dellAdulto; HOVON, Dutch Hematology-Oncology Working Group for Adults; SAKK, Swiss Group for Epidemiology and Clinical Cancer Research.

[a] Since 1990, >100 patients, follow-up >3 y.

TABLE 59-9 -- Induction Therapy for Acute Lymphocytic Leukemia According to the GMALL Protocol

Drug	Dosage	Day
Pretherapy (for patients with a large leukemic mass or a high leucocyte count)		
Vincristine	2 mg (absolute) IV	1
Prednisone	3 × 20 mg/m^2 PO	17
Phase I		
Vincristine	2 mg (absolute) IV	1, 8, 15, 22
Prednisone	3 × 20 mg/m^2 PO	128
Daunorubicin	45 mg/m^2 IV (0.5 h)	1, 8, 15, 22
L-asparaginase	5,000 U/m^2 IV (0.5 h)	1528
Methotrexate	15 mg (absolute) IT	1
Phase II		
Cyclophosphamide	1,000 mg/m^2 IV	29, 43, 57
Cytosine arabinoside	75 mg/m^2 IV or SC (1 h)	3134, 3841, 4548, 5255
6-Mercaptopurine	60 mg/m^2	2957
Methotrexate	15 mg (absolute) IT	31, 38, 45, 52

GMALL, German Multicenter Trials in Adult Acute Lymphocytic Leukemia; IV, intravenous; PO, oral; IT, intrathecal.

can also be managed with vincristine and prednisone (Table 59-9) in nearly all cases without complications. Thus, leukapheresis in adult ALL is not recommended except in rare instances, such as when leukemia occurs during pregnancy. For mature B-cell ALL, initial treatment with cyclophosphamide and prednisone for 1 week usually results in lysis of large tumor masses.[40]

Standard induction therapy for ALL in most studies consists of vincristine, prednisone, asparaginase, and an anthracycline. The combination of vincristine and prednisone alone produces CR rates of approximately 3667%, but a median remission duration of only 37 months. Prednisone and prednisolone have been most frequently administered, although dexamethasone shows a higher antileukemic activity in vitro and a better penetration to the cerebrospinal fluid. [41] The addition of anthracyclines, daunorubicin, or doxorubicin (Adriamycin) increases the CR rate to 7085%. [42] Asparaginase does not affect the CR rate but probably improves LFS, and if not used during induction therapy, it is often included as part of the consolidation treatment. Evidence comes from pediatric studies where the addition of asparaginase to vincristine and prednisone led to improved CR rates in relapsed ALL patients. [43] LFS also was improved by the administration of postinduction doses of asparaginase.[44] [45] The addition of asparaginase to conventional induction therapy did not improve the CR rate in one trial in adult ALL. There was, however, a trend toward a higher LFS in patients treated with asparaginase. [46]

The next step in improving induction therapy was the addition of cyclophosphamide and cytosine arabinoside (AC; Table 59-8). This does not raise the overall CR rate but possibly improves the quality of remission. Cyclophosphamide and AC therapy is particularly useful as initial treatment for special ALL subgroups such as T-cell ALL.

High-dose Treatment

A more recent strategy is to add high-dose AC (HDAC; 13 g/m^2 , usually for 12 doses) after the standard induction therapy. This approach has resulted in a median CR rate of 79% (Table 59-10), which is not superior to that obtained with conventional treatment, and it remains uncertain whether and for which subgroups it may be beneficial for LFS. The initial, relatively high early mortality rate is now decreasing with better handling and improved supportive care.

A new approach is the up-front administration of HDAC before conventional induction treatment, which in two preliminary reports yielded high remission rates of 8494% (Table 59-10). [47] [48] The aim of high-dose treatment, however, is not only to improve the CR rate but, even more, to increase the quality of remission. This means that a lower tumor load should lead to a better LFS.

Failure During Induction Therapy or Refractory ALL

Fifteen to 20% of adult ALL patients do not achieve CR after induction therapy, in contrast to <5% of children with ALL. Less than 10% of adult ALL patients die during the induction period. Mortality during induction is age dependent, increasing

TABLE 59-10 -- High-dose Cytarabine in Induction Therapy of Adult Acute Lymphocytic Leukemia

Author	Year	N	Induction		CR	ED
Secondary						
Bassan et al.[165]	1992	54	V, P, A, AC	HDAC 2 g/m^2 × 12	67%	20%
Cassileth et al.[56]	1992	89	V, P, D	HDAC 3 g/m^2 × 12	69%	
Willemze et al.[62]	1995	32	NP, amsa, P	HDAC 1 g/m^2 × 12	72%	16%
Kantarjian et al.[171]	1995	128	C, V, AD, DX, HdM	HDAC 3 g/m^2 × 4	91%	
Kaufmann et al.[172]	1996	43	V, P, D	HDAC 24 g/m^2 × 3	69%	
Kaufmann et al.[172]	1996	42	V, P, A, D, VP	HDAC 2 g/m^2 × 3	77%	
Wernli et al.[170]	1997	140	V, P, D, A, M, VP	HDAC 13 g/m^2 × 12	78%	11%
Upfront						
Weiss et al.[48]	1996	37	HDAC 3 g/m^2 × 5	V, P, M, A, HdMi	84%	8%
Smedmyr et al.[47]	1997	65	HDAC 3 g/m^2 × 6	C, D, VP, BM	94%	

ED, early death; BM, betamethasone; see Table 59-8 for other abbreviations.

with age from <3% in adolescents to 2030% in patients >60 years of age. The main cause of death in approximately two thirds of the patients is infection, in part fungal infection. The remaining nonresponders may achieve a partial remission or may be refractory to standard treatment. The number of patients (approximately 10%) who are refractory to chemotherapy is steadily decreasing with the use of more intensive induction regimens. These, however, lead more frequently to aplasia or toxic death, which stresses the need for implementation of optimal supportive treatment to overcome hematologic and nonhematologic toxicity in older patients.

Intensification Therapy

Consolidation therapy refers either to high-dose chemotherapy, to the use of multiple new agents, or to readministration of the induction regimen. These measures are aimed at eliminating clinically undetectable residual leukemia after induction chemotherapy and thereby preventing relapse as well as emergence of drug-resistant cells.

In adult ALL, intensification therapy can prolong LFS (see Table 59-8). Intensification schedules include teniposide, etoposide, m-amsacrine, mitoxantrone, idarubicin, and HDAC, or intermediate- or high-dose methotrexate (HDMTX). Allogeneic or autologous BMT should also be considered as a form of intensive postinduction therapy.

It is generally assumed that early intensification therapy is effective in prolonging remission and preventing relapse in ALL. [49] Evidence comes from nonrandomized trials. In earlier studies, the results of treatment of patients who did not receive consolidation therapy were reported to be poor. [50] [51] Historical comparisons confirmed this observation. Thus, the addition of a six-drug consolidation cycle led to a significantly improved LFS compared with a previous trial without consolidation. [52]

More recent randomized trials failed to demonstrate a clear advantage of different consolidation regimens. The difficulty in assessing the efficacy of certain consolidation schemes is evident from a recent Cancer and Leukemia Group B study. Therapy consisting of two consolidation courses with DA 7+3 (daunorubicin × 7 days + AC × 3 days) and DA 5+2, in addition to 6-mercaptopurine and methotrexate, was not superior to conventional maintenance alone. [53]

In two studies of the Gruppo Italiano Malattie Ematologiche Maligne dellAdulto (GIMEMA) focusing on the impact of intensified versus conventional maintenance treatment, no advantage could be demonstrated for the intensified regimen. [54] [55] In a randomized trial of the Medical Research Council (MRC), however, there was a reduction of relapse risk in patients receiving early or late intensification. [56] Despite these uncertainties, consolidation is now part of almost all adult ALL studies.

High-dose Chemotherapy

High-dose chemotherapy has been used mainly to overcome drug resistance or to achieve therapeutic drug levels in the cerebrospinal fluid.

HDAC.

Although there is considerable experience with HDAC for the treatment of ALL [42] [57] it still remains uncertain what dose is optimal. HDAC has been included in several trials in adult de novo ALL as part of consolidation therapy [58] [59] [60] [61] or during induction and consolidation treatment. [62] With a weighted mean for the LFS of 30% (2650%), the results are not superior to trials without HDAC.

It is unknown whether specific subgroups of ALL profit from a HDAC treatment; thus, excellent results are achieved for pediatric B-cell ALL, with LFS of >80% [63] and apparently for adult pre pre-B ALL, with LFS of 5060%. [64] For other adult poor-risk groups such as late responders, Ph+ ALL, or pre-T ALL, the value of HDAC remains to be determined.

An additional argument for the use of HDAC might be its effectiveness in treating CNS leukemia. There is evidence that in ALL and NHL, higher levels of AC triphosphate can be reached with 3 g/m^2 compared with the lower dose of 1 g/m^2 AC; in addition, with the higher dose, the cerebrospinal fluid can be cleared of blast cells. [65] Thus, for high-risk adult ALL patients, HDAC (3 g/m^2) still seems to be justified despite a higher associated morbidity.

High-dose Methotrexate.

The use of HDMTX has been extensively studied for the treatment of childhood ALL and, to a lesser extent, adult ALL. [66] Intermediate doses (0.5 g/m^2), high doses of 18 g/m^2 , and even doses of 33 g/m^2 have been used. HDMTX appears to be effective in preventing systemic and testicular relapses. [67]

The effect of HDMTX on CNS leukemia may contribute to the favorable results reported with its use. HDMTX at a dose of 6 g/m^2 resulted in an 80% CR rate in children with CNS relapse, [68] indicating that systemic application yields cytotoxic levels in the cerebrospinal fluid.

Several studies have investigated the efficacy of HDMTX as consolidation [69] [70] [71] [72] or during consolidation and induction treatment [73] [74] in combination with other chemotherapeutic agents in adult ALL. The weighted mean for LFS of six studies is 41%, with a wide range (3156%). Most favorable results have been achieved in small trials with HDMTX as part of intensive multidrug consolidation regimens.

The combination of cycles having HDAC with cycles having HDMTX during consolidation was reported from six trials with a weighted mean for LFS of 32% (1748%). [54] [55] [75] [76] [77] Thus, there is some evidence that the inclusion of HDAC and HDMTX as part of multidrug consolidation treatment may improve overall results.

Maintenance Therapy

The optimal duration and form of maintenance therapy in adult ALL is unknown. Because the aim of maintenance or continuation therapy is to eliminate minimal

residual disease (MRD), the optimal form of maintenance therapy will not be identified until reliable methods for detection of MRD are available. Standard maintenance is based on combination treatment with 6-mercaptopurine and methotrexate. The potential effect of further intensification cycles for specific subgroups of ALL remains open. In a large, multicenter Italian study (GIMEMA 0183), after intensive consolidation treatment patients were randomly assigned to postconsolidation therapy with conventional maintenance therapy or to additional alternating treatment courses of different intensity. [54] In this report, there was no difference in the survival rate at 10 years between the treatment groups, which may suggest that after adequate early consolidation therapy the intensity of the maintenance therapy has no influence on survival. However, attempts to omit maintenance altogether after induction and consolidation therapy have resulted in inferior results. [60] [73] [78] [79] Prospective trials with maintenance schedules adapted to immunologic subtypes of ALL (e.g., longer for common ALL, shorter for T-cell ALL, and none for B-cell ALL) or to cytogenetic subgroups (e.g., Ph*/bcr-abl*-positive ALL chemotherapy maintenance vs. interferon-/interleukin-2) are needed.

Prophylaxis of CNS Leukemia

Central nervous system leukemia occurs in 6% (110%) of patients with adult ALL at diagnosis, with a higher incidence in T-cell ALL (8%) and mature B-cell ALL (13%).[80] Treatment and prophylaxis of CNS leukemia may consist of intrathecal methotrexate alone or in combination with AC or prednisone, similar intraventricular therapy administered by an Ommaya reservoir, cranial irradiation, or systemic treatment with HDAC or HDMTX.

Adult ALL patients who do not receive specific prophylactic CNS treatment have a CNS relapse rate of 30% (2932%), [80] similar to that observed in children without CNS prophylaxis.[81]

With intrathecal chemotherapy alone, the rate of isolated and combined CNS relapses could be reduced to 13% (819%). Intermittent application during maintenance therapy improves the efficacy compared with application of only a few doses during induction treatment. [82] In most adult ALL trials, additional prophylactic CNS irradiation (24 Gy) has been included. This combined approach further reduces the CNS relapse rate to 9% (319%). There is some evidence that early irradiation after remission induction is superior to delayed irradiation during consolidation treatment. [80] It is questionable whether systemic high-dose treatment alone provides sufficient CNS prophylaxis because the CNS relapse rate is approximately 14% (1016%).

In many recent trials, combined treatment approaches have shown high efficacy. For high-dose chemotherapy together with intrathecal therapy, the rate of CNS relapses was 7% (216%); with additional CNS irradiation, the relapse rate was 6% (113%). The efficacy of intensified CNS prophylaxis was also demonstrated in a retrospective analysis from the M.D. Anderson Cancer Center, where the lowest CNS relapse rate (2%) was achieved in a trial with early high-dose chemotherapy and intrathecal therapy for all patients. [75]

Because the risk for CNS relapse is associated with other prognostic factors such as T-cell ALL, B-cell ALL, extreme leukocytosis, high leukemia cell proliferation rate, high serum lactate dehydrogenase levels, and extramedullary organ involvement, a risk-adapted CNS prophylaxis has been suggested. [75] This approach, however, in contrast to childhood ALL, is not widely used in adults.

Prophylactic treatment of the CNS may result in acute or chronic neurotoxicity. Adverse effects include febrile reactions, arachnoiditis, leukoencephalopathy, and subclinical dysfunctions, including learning disabilities. These adverse reactions occur primarily in children and are only subclinical in adults. [83]

Therapy for Relapsed and Resistant Leukemia

Patients who fail to achieve CR or those who relapse subsequently have been treated with a variety of protocols (summarized in Welborn [84] and Bassan et al.[85]). The repetition of regimens including vincristine, anthracyclines, and steroids, similar to standard induction treatment in earlier studies, led to CR rates of 61%. [84] [85] Use of single-agent HDMTX, HDAC, and probably the anthracycline derivatives mitoxantrone and rubidazone resulted in a second remission in 30% of patients, whereas other agents such as amsacrine, teniposide, and etoposide are effective in 1015%. [49]

High-dose AC has been extensively studied in relapsed adult ALL. From several small pilot studies comprising 90 patients in total, the weighted mean remission rate was 37%.[86] Higher CR rates (5060%) were achieved with combination regimens that included HDAC and mitoxantrone, amsacrine, or vincristine plus steroids, again with a wide variation that may be attributed to patient selection and nonuniform intensity of pretreatment. Because HDAC is increasingly administered during front-line treatment, its efficacy during relapse treatment may be impaired. Therefore, new combinations with idarubicin (4664% CR rate) [87] [88] [89] [90] or fludarabine (6783% CR rate)[91] [92] have been evaluated. Median remission duration or survival was reported by only a few groups, but did not exceed 5 months and 16%, respectively. The combination of moderate- to high-dose methotrexate with asparaginase and folinic acid rescue produced CR rates of 51%, and was well tolerated. [84]

The most significant predictive factor for treatment response in relapsed patients is the duration of first remission. Patients with longer previous remission (>18 months) have a higher CR rate and longer remission duration compared with those with a short previous remission (<18 months). [93] [94]

For all chemotherapy regimens, the duration of second remission is usually short (<6 months), and the long-term survival rate with chemotherapy alone is <5%. Thus, the only curative chance for adult patients with relapsed or resistant ALL is BMT, and the major aim of relapse treatment is the induction of a second remission with sufficient duration to prepare BMT.

BMT in Adult ALL

Bone marrow transplantation is one of the postremission strategies for eradication of residual disease in ALL. Prognostic factors for remission duration after chemotherapy (i.e., age, WBC count, immunophenotype) are also predictive for the outcome after BMT. [95] Ph-positive ALL seems to be an exception, in which results with chemotherapy alone are very poor, but results with allogeneic BMT are much more favorable, with an LFS rate of 3040%. [96] [97] [98] [99]

Allogeneic BMT from Sibling Donors

The outcome of allogeneic BMT for ALL depends on the age and remission status of the patient. [100] The best results have been obtained with patients transplanted during the first remission. In a total of 875 patients, the LFS rate was 45%, albeit with wide variations (2166%). The relapse probability (RP) was 29% and the treatment-related mortality rate (TRM) 33% (Table 59-11). In more recent studies, results are apparently improving, most probably because of a reduction in TRM. [101] There is evidence that graft-versus-leukemia effects are also present in ALL because several studies have shown that the RP is lower in patients with limited graft-versus-host-disease (reviewed in Appelbaum [102]).

TABLE 59-11 -- Recent Results of Bone Marrow Transplantation in Adult Acute Lymphocytic Leukemia

BMT	Disease Stage	N	Leukemia-Free Survival[a]	Relapse Probability[a]
Allogeneic	CR1	875	45% (2161%)	29% (1050%)
	CR2	104	26% (1541%)	64% (6271%)
	Rel/refr	231	18% (1232%)	71% (5778%)
Autologous	CR1	759	43% (1575%)	57% (2767%)
	CR2	465	28% (1031%)	74% (6988%)
	Rel/refr	27	8%	90%
MUD (mostly children)		65	39% (2353%)	16%
	Rel/refr	25	20%	52%

MUD, matched unrelated donor; Rel, relapsed; refr, refractory; CR1, first remission; CR2 second remission.

[a]Pooled data from literature.

After allogeneic BMT in second remission, the LFS rate is 26% (1541%). The RP of 64% is still high. Improvements may be achieved by increasing remission quality, such as with intensified conditioning regimens or transplantation of peripheral blood stem cells (PBSC) to induce a graft-versus-leukemia reaction. In advanced ALL (refractory or in relapse), allogeneic BMT results in an 18% (1232%) long-term survival rate.

Mismatched Related or Unrelated BMT

Only one third of potential BMT candidates with ALL have an HLA-identical sibling donor. To extend the possibilities of allogeneic BMT by enlarging the number of bone marrow donors available, mismatched BMT from related donors or matched BMT from unrelated donors has been increasingly used. With BMT from partially mismatched family donors, two groups have reported quite favorable results (LFS 3853%) in pediatric and adult patients with relapsed ALL, although the TRM and the rate of graft failure were significantly higher compared with fully matched allogeneic BMT. [103] [104]

Bone marrow transplantation from matched unrelated donors can also result in long-term survival. In a recent report from the Seattle group, including children, the LFS was 39% for ALL in second remission and 10% for ALL transplanted in relapse. The cumulative TRM for all leukemias was 39%, whereas the RP was 41%. [105]

In ALL as a whole, the results of matched unrelated BMT seem encouraging, particularly when it is considered that these are often patients with advanced stages of disease. However, the median age in these series is low, and all studies include children. It remains to be shown in larger studies what the results are for adult patients only.

Autologous BMT

Another attempt to overcome the limited availability of bone marrow donors is autologous BMT. [100] This form of BMT is associated with a risk of reinfusing residual leukemic cells and also of a higher relapse rate owing to the lack of a graft-versus-leukemia effect. Purging of the marrow graft is of interest because it may reduce the leukemia cell burden. Several methods are in use, such as monoclonal antibodies reactive to surface markers on leukemic cells with or without complement, chemotherapeutic drugs such as 4-hydroperoxycyclophosphamide and mafosfamide, or immunomagnetic beads. Purging with immunomagnetic beads can reduce tumor load by 2 log for either bone marrow or PBSC grafts in ALL. [106] [107]

The results for autologous BMT in first remission are surprisingly good, with an LFS of 42% (1575%) and a low TRM (<20%) in a literature survey of 906 patients (Table 59-11). Similar results with an LFS rate of 42% in standard-risk ALL (N = 280) and 40% in high-risk ALL (N = 174) have been reported from the European Bone Marrow Transplant Group (EBMT).[108] There is still a high RP of 50%. In addition to purging, the administration of maintenance treatment after autologous BMT may contribute to a reduction of RP. Favorable results therefore have been achieved in a trial with autologous BMT or PBSC transplantation in adult ALL patients in first CR followed by a 2-year maintenance treatment with 6-mercaptopurine and methotrexate.[109]

For patients with autologous BMT in second CR, the LFS in a large cohort of 513 patients was 22%, which is clearly superior to chemotherapy, but the RP of 71% is still high (Table 59-11).

PBSC Transplantation

2A recent strategy for the treatment of adult ALL is to transplant autologous or allogeneic PBSCs. [110] Several studies have shown that neutrophil and platelet recovery is faster after PBSC transplantation compared with autologous or allogeneic BMT, and that transfusion dependence is reduced. In Ph+ ALL, PBSC transplantation provides the particular advantage that Ph-negative stem cells can be collected in some patients, [111] whereas autologous bone marrow grafts are almost always Ph positive.[107] [112] Whether these advantages will result in a higher LFS remains open. Furthermore, in heavily pretreated patients, the collection of a sufficient number of stem cells from the peripheral blood may be difficult. In a preliminary report from the EBMT in ALL patients in first remission, the LFS rate was 41% after PBSC transplantation, compared with 35% after autologous BMT, with a similar RP of 60%. [113]

Comparison of Different Transplantation Procedures and Chemotherapy

When comparing these results with those achieved by chemotherapy, it is obvious that all patients in second remission should receive transplants, because the outcome after BMT is clearly superior to the 5% cure rate achieved with chemotherapy alone.

The use of BMT during first remission of ALL remains more controversial. The LFS rate of 45% after BMT is superior to that obtained with chemotherapy alone; however, when these results were adjusted for age, risk factors, and time to BMT (thereby excluding early relapses), the differences between BMT and chemotherapy were no longer significant in a comparative analysis of BMT patients from the International Bone Marrow Transplant Registry and chemotherapy patients treated according to the GMALL protocol (34% vs. 32%). This was predominantly due to a higher TRM in the transplant group (53% vs. 5%), whereas the RP was higher in the chemotherapy group (30% vs. 66%).[114]

The French study group conducted a randomized trial with autologous BMT versus chemotherapy in patients without sibling donor and allogeneic BMT for all patients with sibling donor. The LFS rate in patients treated with allogeneic BMT (44%) was not significantly superior to that in those treated with chemotherapy (32%) or autologous BMT (39%). [115] In high-risk patients, however, there was a significantly better outcome for patients treated with allogeneic BMT (39%) compared with those in the control group (14%).[116]

Preferred Approach to BMT

Taking all results together, it appears advisable that low-risk groups having an LFS of approximately 50% after chemotherapy should receive BMT only after relapse and second remission. For high-risk adult ALL patients, allogeneic BMT in first remission results in a survival advantage compared with chemotherapy alone. For veryhigh-risk patients (such as Ph+ ALL) without a compatible donor, BMT from a matched unrelated donor is recommended in younger patients, whereas older patients should receive autologous BMT. With improving strategies for supportive care and prophylaxis of graft-versus-host disease, the TRM after matched unrelated BMT might be reduced without losing the advantage in terms of relapse probability.

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PROGNOSIS

The major risk factors in attaining a CR are advanced age and Ph+ ALL. Prognostic factors are of greater importance for duration of remission and survival (Table 59-12). Appreciation of the impact of such risk factors (Table 59-13) can result in the generation of risk-adapted treatment protocols for adult ALL, a strategy already well established for childhood ALL (Table 59-14).

TABLE 59-12 -- Outcome of Adult Acute Lymphocytic Leukemia According to Subgroups

Subgroup	Incidence	No. of Patients	Clinical Remission Rate[a]	Median Remission Duration[a] (mo)	Leukemia-free Survival[a]
Overall		4,474	75% (6386%)	23 (1141)	31% (1344%)
Age (y)					
1520			8295%		3265%[b]
2050			80%		35%
5060			3663%		20%
>60			3365%		010%
Subtype					
T-cell ALL	24%	621	81%	25	46%
pre-pre-B-ALL	11%	57	75%		50%
Common ALL	57%	881	80%	22	30%
B-cell ALL	3%	89	77%		58%
My+ ALL	29%	258	62%		30%
Cytogenetics					
Ph/*bcr-abl*+ALL	24%	352	66%	9	016%
Risk group					
High	70%			813	025%
Low	30%			>24	50%

[a] *Pooled data from published studies.*
[b] Results from pediatric studies.

Age

There is a continuous decline in the CR rate from 95% in children to 4060% in patients >5060 years of age. [1] [117] Most trials demonstrate that increasing age is also associated with shorter remission duration and survival. [53] [118] [119] [120] [121]

It is difficult to define an age limit where a change in prognosis occurs, but in almost all studies LFS is inferior in patients older than 5060 years of age. This age limit seems to be practical because patients younger than 50 years of age are candidates for intensified treatment approaches such as BMT, whereas for the older group, new strategies need to be explored, carefully weighing the gain in survival against quality of life. Thus, elderly patients >5055 years of age who have achieved a CR and are in good clinical condition are potential candidates for autologous BMT. PBSC transplantation is even applied in patients older than 60 years of age if in a biologic good condition.

Immunophenotype

The immunophenotype is an independent prognostic variable in ALL, despite the fact that with modern treatment strategies the previously poor prognosis of phenotypes such as T-cell ALL and mature B-cell ALL has been changed. In ongoing recent

TABLE 59-13 -- Adverse Prognostic Factors for Remission Duration in Adult Acute Lymphocytic Leukemia[a]

Late achievement of complete remission; >4 wk
High WBC; >10,000>30,000>100,000/× 10^6 /L
Advanced age; >35>50>60 y
Immunologic subtypes

Pre-T-ALL	
Pre-pre-B-ALL (?)	
Chromosomal abnormalities and corresponding molecular markers	
Ph+ ALL (t[9;22]), (*bcr-abl*)	
t(4;11) [*ALL1-AF4*]	

[a] *Analysis and cut-off varies by study.*

trials, there is a tendency to use the immunophenotype to adjust treatment regimens accordingly (e.g., for B-cell ALL).

T-Lineage ALL

Results for T-cell ALL have improved to CR rates of >80% and an LFS of 46%, compared with earlier results with remission duration of <10 months and survival rates <10%. In vivo and in vitro evidence has accumulated to suggest that AC and cyclophosphamide, in addition to the usual cytostatic drugs for ALL, are mainly responsible for this improvement. The inclusion of AC and cyclophosphamide pulses during continuation therapy was beneficial in childhood T-cell ALL. In adult T-cell ALL as well, the combination of AC and cyclophosphamide added to the conventional drugs improved the CR and LFS rates. [122] [123] Increased CR and LFS rates for T-cell ALL were also observed in a Cancer and Leukemia Group B (CALGB) study using the addition of cyclophosphamide to the conventional induction regimen of vincristine, prednisone, daunorubicin, and L-asparaginase (VPDA).[124] HDMTX contributes to the improvement of survival in children, [125] [126] as does HDAC.[127] HDAC improved the prognosis for adult ALL patients with high WBC counts and T-cell ALL. [128] For adults, the benefit of HDAC and HDMTX in T-cell ALL has to be confirmed in larger trials.

B-Lineage ALL

In common ALL, CR rates in adult trials have improved to 80% or more, but patients still relapse in most studies over a period of up to 56 years, and only one third survive. This may in part be explained by the fact that approximately 30% of adult patients with common ALL are Ph/ *bcr-abl* positive and their prognosis remains poor. The results, however, also are not encouraging for adult patients with Ph-negative common ALL. Higher doses of anthracyclines given in induction and reaching a certain cumulative amount may be beneficial.[129] The outcome of patients with pre-B ALL seems to be similar to that of common ALL.

Adults with the subtype pre-pre-B ALL or the t(4;11) translocation have a poor prognosis, similar to childhood ALL patients. [130]

TABLE 59-14 -- Preferred Approach to the Risk-adapted Treatment of Adult Acute Lymphocytic Leukemia

	Low-risk ALL	High-risk ALL	Very-High-risk ALL	Mature B-Cell ALL

WBC, white blood cell count; CNS, central nervous system; BMT, bone marrow transplant. See Table 59-8 for other abbreviations.

[a] For patients with a large leukemic mass or a high leukocyte count.

[b] C 200 mg/m^2 × 5, P 60 mg/m^2 × 5.

With intensive regimens including HDAC and mitoxantrone as consolidation therapy, the results for adults seem to improve. [11]

Complete remission rates for adults with B-cell ALL a decade ago were low, and remission duration and survival was short. In nine studies with a total of 63 patients, the weighted mean CR rate was 44%, and most patients relapsed rapidly, reflected by a median remission duration of 11 months and a very low survival rate. [131] A change was brought about by innovative childhood B-cell ALL studies that significantly improved outcome, with CR rates of 8094% and an LFS rate of 63% (weighted mean).[40] The drugs responsible for the improvement were high doses of fractionated cyclophosphamide, HDMTX (0.58 g/m ^{2}), and HDAC in conjunction with the conventional drugs for remission induction in ALL, given in short cycles at frequent intervals over 6 months.

The application of these childhood B-cell ALL protocols in original or modified form also brought a substantial improvement for adult patients with B-cell ALL. CR rates now range from 60100% and LFS rates from 2065%.[40] Adverse prognostic factors are late CR (more than two cycles of chemotherapy), high WBC (>50 × 10 ^{9} /L), and age >50 years. Patients with these high-risk features are candidates for BMT. B-cell ALL has a higher incidence of CNS involvement at diagnosis and of CNS relapse. Therefore, effective measures against CNS disease, such as HDMTX and HDAC as well as intrathecal therapy, are important components of treatment regimens. Maintenance treatment has been omitted. Because relapses occur almost exclusively within the first year in childhood as well as in adult B-cell ALL studies, patients thereafter can be considered to be cured.

Myeloid Antigen-Positive ALL

Whether myeloid antigen expression in ALL has an adverse impact on outcome is not established. In childhood ALL, myeloid coexpression has apparently no prognostic relevance (reviewed by Drexler and Ludwig [132]). Some adult studies indicated an inferior outcome for myeloid antigen-positive ALL patients both for CR rate and LFS, but more recent studies do not confirm this.

Cytogenetics

Chromosomal abnormalities markedly correlate with outcome in ALL independent of other features. [6] [7] [133] Hypodiploidy and translocations, particularly the t(9;22) Philadelphia chromosome, are unfavorable findings in adults.

Philadelphia chromosome/*bcr-abl*-positive ALL has the worst prognosis in children as well as in adults. In 13 studies with a total of 384 patients, the weighted mean CR rate is 67% (4476%). The median remission duration in all series is short (511 months) and the survival rate, from 016% at 35 years, is extremely poor in all reports.

Treatment options for Ph/*bcr-abl*-positive ALL in adults include HDMTX or HDAC; so far, no convincing progress for this subgroup of patients has been reported. The use of biologic response modifiers found successful in CML, such as

-interferon, was initially encouraging but has not fulfilled its promise. In a study using a combination of -interferon and interleukin-2 after autologous BMT, although there was a two- to threefold increase in T-cell subsets and a five- to sevenfold increase in natural killer cell activity, this did not result in an apparent clinical benefit.[134] The only curative chance for adult Ph/*bcr-abl*-positive ALL patients is therefore BMT.

The adverse impact of the translocation t(4;11) seems to have changed with new treatment modalities (see pre-pre-B ALL in section on B-lineage ALL).

The translocations t(8;14), t(8;2), and t(8;22) are present in most cases of mature B-cell ALL [135] and in Burkitts lymphoma.[136] [137] These translocations have lost their poor prognostic impact because of the improved treatment for patients with B-cell ALL or Burkitts lymphoma. Patient numbers are too small to evaluate whether there is any difference in prognosis for these three translocations.

Other Prognostic Factors

With regard to gender, several studies have observed a higher remission rate in female patients, but these results have not reached statistical significance in most adult ALL reports. When sex-related differences were reported, they were in favor of female patients (e.g., in long-term outcome). Extensive organ involvement at presentation (e.g., hepatomegaly, splenomegaly, CNS leukemia, and mediastinal involvement) has been found in some studies to have an adverse influence on remission duration. CNS leukemia is considered an adverse prognostic factor [138] [139] in some reports. However, in other studies, [140] [141] the LFS for patients with initial CNS involvement was not inferior. Mediastinal mass, although indicative of a large tumor burden, had no adverse influence on either CR rate or remission duration in several adult ALL series. Most ALL patients with mediastinal tumor have the T-cell ALL subtype, so that any adverse influence of mediastinal mass is probably outweighed by the recent improvement in treatment for T-cell ALL.[123] The rare cases with mature B-cell ALL and mediastinal mass, which have to be distinguished from a mediastinal large B-cell lymphoma with sclerosis but no bone marrow involvement, have an apparently poor outcome. [40] Splenomegaly, hepatomegaly, or hepatosplenomegaly at the time of diagnosis had an adverse impact only in single studies. An adverse influence of organ enlargement on CR rate [118] was not correlated with an adverse effect on remission duration, [142] [143] although overall survival might be shortened.

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FUTURE DIRECTIONS

Multidrug Resistance

Circumvention of multidrug resistance (MDR) is an alternative treatment strategy. The *MDR* gene encodes for a membrane glycoprotein, p170, which is thought to function as an efflux pump.[144] The expression of this gene confers resistance to some chemotherapeutic agents, including vinca alkaloids, toxoids, anthracyclines, and epipodophyllotoxins. [145] Ten to 50% of patients with ALL at diagnosis and 1560% at relapse express *MDR*.[146] In adults, the incidence of MDR positivity increases markedly after relapse (10% at diagnosis, 50% after relapse). [147] The expression of MDR is associated with a lower CR rate (56% for MDR-positive vs. 93% for MDR-negative; $P = 0.05$) and a higher relapse rate (100% vs. 46% respectively; $P = 0.05$). This results in a survival advantage for MDR-negative patients. [147] Pharmacologic inactivation of a 190-kd P-glycoprotein, which is coded for on chromosome 16p13, may be more clinically relevant than MDR. [148]

Minimal Residual Disease

Recent advances in molecular and immunologic techniques have allowed the detection of MRD, defined as leukemic cells undetectable by morphologic examination. Methods to detect MRD include cytogenetics, cell culture techniques, flow cytometric sorting and immunophenotyping, and detection of leukemia-specific DNA or RNA frequencies by Southern blot tests or PCR (the reader is referred to summaries [149] [150] [151] [152]). Rearrangements of the immunoglobulin (Ig) heavy chain gene or T-cell receptor genes are one target for MRD detection. The clonal nature of ALL is manifested by a specific and unique rearrangement of these genes in all malignant cells, whereas normal cells have a germ-line configuration. Amplification of such leukemia-specific markers by PCR can identify one leukemic cell among 10^5 normal cells. The major limitation with this technique is that because the rearrangement is unique for each clone, specific probes must be generated for each patient. Recent attempts to detect antigen receptor gene rearrangements by monitoring two or more functional regions of multiple genes found that PCR detection of MRD is possible in approximately 90% of ALL patients and that false-negative results due to clonal evolution may be reduced to 10% or less. [153] [154]

The detection of a leukemia-specific chromosomal translocation by PCR is a tool for MRD with a high sensitivity (10^6). More than 50% of adult ALLs have identifiable chromosomal translocations (e.g., *bcr-abl*, *E2A-PBX1*, *MLL-AF4*, and *TEL-AML1*), which can be detected by PCR analysis.

Detection of MRD by immunologic methods, such as multiparameter flow cytometric analysis measuring characteristic constellations of two or more cell surface antigens, has also been proposed (sensitivity of 10^4). [155]

Clinical studies evaluating MRD by PCR analysis have shown a widespread pattern. After induction chemotherapy with a 3- to 4-log reduction in the number of leukemic cells, even when morphologic (clinical) CR is achieved, some residual disease can be documented. In *bcr-abl*-positive ALL, most patients in clinical CR still have MRD at a molecular level, [112] [156] and most of them relapse.

Persistence of MRD or repeated positive results indicates a high likelihood of subsequent relapse. An increase in leukemic cells can also be detected by PCR before a relapse becomes clinically evident. However, the absence of MRD during or after treatment does not guarantee continuous remission. [157]

Interpretation of MRD is complicated even further by the fact that PCR studies have shown the presence of the *bcr-abl* fusion gene or the *bcl*-2 gene in apparently healthy people at a very low level. [158] [159] This indicates that the expression of a fusion transcript is not necessarily identical with malignant proliferation.

Thus, for practical clinical reasons, it is not only whether MRD is present, but the kinetics of disappearance after treatment, and the subsequent confirmation of negative results, that might be of greater clinical relevance. In most studies of patients with continuous CR, a successive decline of patients with positive MRD has been shown. A substantial number of patients, however, may remain MRD positive even at the end of therapy without subsequent relapse. [160] The kinetics of MRD in the first weeks of induction therapy appear to be very predictive. A level above one target cell in 10^3 normal cells after the end of induction treatment is associated with a high risk of relapse. [161] [162] Apart from possible technical reasons for error in the detection of MRD, there is a need for larger, prospective studies to evaluate the potential of MRD determination.

Detection of MRD could have several consequences for ALL, such as the redefinition of CR in patients who are in morphologic CR but with detectable MRD, or the evaluation of the effectiveness of specific treatment elements, such as consolidation, maintenance, or bone marrow purging. Furthermore, if

there is no MRD, treatment could be stopped, or alternative treatment approaches such as BMT could be considered in patients with positive MRD and therefore a high likelihood of relapse. Evaluation of MRD in ALL therefore may eventually lead to an individualization of therapy.

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Chapter 60 - Myelodysplastic Syndrome

Peter L. Greenberg

INTRODUCTION

Terminology describing the indolent myeloid clonal hemopathies has been refined since it was initially published in 1953. [1] Numerous terms, including preleukemia (or hemopoietic dysplasia), refractory anemia with excess of blasts, subacute or smoldering myeloid leukemia, oligoleukemia, and myelodysplastic and dysmyelopoietic syndromes,[2] [3] [4] [5] [6] [7] [8] [9] have been used to describe patients with refractory cytopenias whose marrows showed morphologic evidence of dysplastic changes in at least two of the three hemopoietic cell lines and in whom the disease showed a propensity to undergo transformation into acute myeloid leukemia (AML). The term myelodysplastic syndrome (MDS) is currently accepted as describing this clinical entity. It should be recognized, however, that although the leukemic transition in these patients morphologically resembles de novo AML, the AML transformation developing from MDS is generally more resistant to standard induction chemotherapy. The poor prognosis of patients with this leukemic transformation is similar to the poor prognosis of patients with chronic myeloproliferative disorders and leukemic transformation (Fig. 60-1).

In these MDS patients, variations in marrow morphology and in the potential for survival and transformation into AML have been recognized. To aid in categorizing patients, the disease was subclassified by the French-American-British (FAB) Cooperative Group, which formulated a set of criteria based on marrow morphology, including the proportion of myeloblasts and the degree of derangement of the hemopoietic cell lines. [10] This morphologic classification scheme subdivides the disease into five subgroups: refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMML) (Table 60-1). The first four entities are characterized by abnormal marrow myeloid cell differentiation patterns (dysplasia) with RA or RARS patients having <5% blasts, associated with dysplasia of the three hemopoietic cell lines, RAEB patients having between 5% and 20% blasts, and RAEB-T patients having 2130% blasts. In contrast, AML is considered to be present if the patients marrow contains >30% blasts. This morphologic characterization has been helpful for assessing the prognosis of patients. However, because these entities evolve into aggressive disease, an additional criterion necessary for categorization is the *pace* of such progression. This characteristic helps distinguish relatively indolent MDS from the disease that evolves rapidly to frank AML. Therefore, stability of the peripheral blood cell counts and marrow morphology for at least 26 weeks aids in categorizing the condition as MDS. The inclusion of CMML in MDS is problematic, as CMML is predominantly a myeloproliferative rather than a myelodysplastic disorder and may (depending on marrow and blood findings) be more like either chronic myeloid leukemia with an excess of monocytes or RAEB with monocytosis (see later).

In most patients with primary MDS the disease develops de novo. However, an increasing number of patients who had previously been treated with chemotherapy or chemotherapy plus radiation therapy for other malignancies, or who had been extensively exposed to a variety of marrow toxins, are developing

Figure 60-1 Evolutionary transitions occurring in clonal myeloid hemopathies. Ess., essential.

a secondary form of MDS.[11] This secondary MDS generally has a more aggressive course than primary MDS.

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PATHOGENESIS

MDS provides a clinical model for evaluating the evolution of a relatively benign clonal myeloid hemopathy into a frankly malignant neoplasm, a form of AML. Biologic data, particularly marrow cytogenetics, in vitro myeloid progenitor cell proliferation characteristics, and clonal analysis utilizing restriction fragment length polymorphisms (RFLPs), indicate that biologically the marrow stem cells of MDS patients are derived from a myeloid malignant clone. [12] [13] [14] These laboratory approaches have been used to attempt to improve our understanding of the mechanisms underlying MDS.

Cells derived from hemopoietic stem cellsneutrophils, monocytes, erythrocytes, and plateletsare clonally derived in MDS. Evidence for this breadth of hemopoietic lineage involvement has been obtained using karyotypic markers [15] [16] [17] [18] [19] [20] [21] or by RFLP analysis using X-linked inactivation assays. [15] [18] [19] [20] [22] [23] [24] [25] In some studies, combined cytogenetic and X-linked inactivation studies have been performed, [19] [20] and results have been concordant in identifying clonal derivation of the hemopoietic cells. With these techniques, data have also been reported concerning the origin of lymphoid cells in MDS patients. Lymphoid cells appear to be part of the abnormal clone in some but not all cases, as clonal T cells [16] [18] [21] [22] [25] and both clonal[21] and polyclonal B cells[23] have been demonstrated in MDS.

Cytogenetic Abnormalities

Cytogenetic abnormalities in the marrow cells of patients with de novo MDS are found in
4060% at diagnosis, whereas >80% of patients with secondary MDS have abnormal karyotypes.[26] Single or complex chromosomal abnormalities may be present initially and evolutionary changes may occur during the course of the disorder. These genetic derangements reflect the multistep process believed to underlie the evolution of MDS. Generally, the more aggressive the stage of the disorder, in terms of rapidity of clinical course and high number of marrow blasts, the more complex is the abnormal karyotype. Major

TABLE 60-1 -- Myelodysplastic Syndrome Subtypes: FAB Cooperative Group Criteria

FAB Subtype	Bone Marrow Blasts (%)	Peripheral Blood Blasts (%)	Auer Rods	Monocytes >1 × 109/L	Ring Sideroblasts > 15% of Nucleated Erythroid Cells
RA	< 5	1			
RARS	< 5	1			+
RAEB	520	< 5			±
CMML	20	< 5		+	±
RAEB-T	21 30 or	5 or	±	±	±

karyotypic aberrations, including many structural changes, rings, and dicentrics, are commonly seen in RAEB and RAEB-T, but only sporadically in RA.

Both structural and numerical chromosomal changes may be found in MDS.[27] [28] [29] Compared with AML, MDS is often associated with chromosome deletions as a primary karyotypic anomaly. Deletions leading to gene loss suggest that a recessive mechanism plays a role in the origin of leukemic transformation. [30] With regard to specific associations between chromosome markers and the FAB morphologic subgroups, apart from the 5q chromosome as sole karyotypic anomaly that is often present in RA, most other aberrations are distributed throughout all MDS subgroups. Chromosome 5q with other abnormalities, however, is unusual in CMML. Monosomy 7 is rarely observed in RA patients. The most frequently occurring clonal chromosomal abnormalities in MDS involve chromosomes 5, 7, 8, 11, 12 and 20.[26] [31] [32] The prevalence of chromosomal anomalies in secondary MDS is higher and the abnormalities are more complex, the most common single abnormality being monosomy 7, followed by 5q, trisomy 8, monosomy 5, and 20q.

Chromosomal abnormalities identify areas of the genome that are susceptible to damage or that may be important in the pathogenesis of disease. The frequent occurrence of the 5q chromosomal abnormality implicates one or more genes residing on chromosome 5q in the development and maintenance of abnormal hemopoiesis. Within the critical regions involved in the deleted region in the 5q abnormality cluster several genes coding for hemopoietic growth factors (HGFs) (Table 60-2). In addition, other genes, such as EGR1, which encodes the GTPase-activating protein for p21 ras, are present in this chromosome region. [33] The interferon regulatory factor-1 (IRF-1) gene was found to be deleted in the 5q syndrome. [34] Since activated forms of this gene decrease cell proliferation, its loss as well as the loss of those for the HGFs may result in the abnormal cell proliferation occurring in this syndrome. Other as yet unidentified tumor suppressor genes may also be deleted in 5q chromosomes.

A detailed analysis of chromosomal aberrations shows similarities between those present in MDS and in other myeloid clonal hemopathies, such as AML and myeloproliferative disorders (MPDs).[26] These observations suggest that a continuum exists between differing FAB subgroups of MDS and also that similar genetic events of the same stem cell may result in a variety of clinical conditions, such as MDS, AML, and MPD.

Secondary MDS is associated with an abnormal karyotype in >80% of cases. As in primary MDS, recurrent aberrations significantly involve chromosomes 5 and 7 with partial or total monosomy, although with a differing prevalence (Table 60-3).[12] [26] [35] Loss of material from the long arm of chromosomes 5 and 7 may also be due to unbalanced translocations typically observed in secondary MDS/AML. [12] [26] [35] [36] [37] [38] [39] MDS or AML may arise as a secondary disorder, suggesting biologic relatedness between secondary MDS and AML. Accordingly, both are characterized by the same chromosomal aberrations and a very short survival. Regions commonly involved in secondary MDS and AML have been delineated, in addition to deletions of chromosomes 5 and 7. Regions on chromosomes 3p1421, 6p23, 12p11p12, 17p11p12, 19q13, and 21q2122 frequently undergo structural rearrangements such as deletions and translocations. [26] [35] The pathogenetic implications of these abnormalities have not been established.

Hematopoietic Regulatory Interactions

MDS marrow hemopoietic precursors demonstrate uncoupling between their proliferative and differentiative abilities, leading

TABLE 60-2 -- Human Hemopoietic Growth Factors and Receptors

Factor	Molecular Weight (kd)	Gene Location	Receptor	
			Molecular Weight (kd)	Gene Location
Interleukin-3	1530	5q2331	140	: X,Y-PAR[b]
				: 22q13.1
GM-CSF	1830	5q2331	45, 84	: X,Y-PAR[b]
				: 22q13.1
G-CSF	20	17q1112	150	1p35
M-CSF	7090	1p1321	160	5q2333
c-kit ligand[a]	40	12q2224	145	4q12
Erythropoietin	39	7q1122	5560	19p
Interleukin-1	17	2q1321	68, 80	2q12
Interleukin-6	21	7p21	80	1q21
Interferon-	1725	9	110, 130	21
Interferon-	1725	9	110, 130	21
Interferon-	1725	9	54	6

Abbreviations: G, granulocyte; M, macrophage; CSF, colony-stimulating factor.

[b]Pseudo-autosomal region of the X,Y chromosome.

[a]Stem cell factor, mast cell growth factor.

TABLE 60-3 -- Karyotypic Abnormalities in Primary and Therapy-Related Myelodysplastic Syndromes

Karyotype	Patients (%)		*P* Value
	Primary MDS (n = 371)	Therapy-Related MDS (n = 65)	
Normal	43	12	<0.01
Chromosome 5 or 7 abnormalities	23	55	<0.01
Trisomy 8	27	11	0.29
t(8;21), inv 16	1	2	NS
Other abnormalities	14	5	0.04
Insufficient metaphases	10	12	NS
Missing	2	3	NS

Abbreviation: NS, not significant.

Modified with permission from Kantarjian et al.[11]

to ineffective hematopoiesis due to defective differentiation. The lesion underlying this defect resides predominantly at the level of the hemopoietic stem cell, and phenotypic expression of such aberrant cells is clonal in nature. In vitro hemopoietic progenitor cell assays have allowed analysis of the hemopoietic stem cell/progenitor cell compartments and the humoral HGFs that regulate hemopoietic cell proliferation and differentiation.

The biochemical and functional nature of HGFs and their receptors have been characterized and assessment of HGF-receptor interactions have allowed the start of analysis of their mechanisms of action (see Chap. 14). Chromosomes coding for HGFs and their receptors (Table 60-2) are often deranged in MDS, suggesting possible pathogenetic relevance of these findings. [40] [41] [42] [43] [44] [45] [46]

Hematopoiesis

Despite the more indolent nature of MDS relative to AML, many in vitro myeloid progenitor cell abnormalities evident in AML are also present in MDS. These biologic parameters have been useful for evaluating pathogenetic mechanisms and prognosis in MDS patients. [40] [47] The colony-forming capacities of all of the marrow hemopoietic precursor cells (colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte [CFU-GEMM], burst forming unit-erythroid [BFU-E], CFU-E, CFU-granulocyte/macrophage [CFU-GM], CFU-megakaryocyte [CFU-Mk]) are quite low or absent in most MDS patients, [40] [47] [48] [49] [50] [51] as well as in most AML patients. Also as found in leukemic patients, an increased proportion of CFU-GMs are of light buoyant density, and abortive myeloid cluster formation and defective cellular maturation occur within the colonies. [40]

In MDS, in vitro hemopoietic regulatory derangements have been shown in progenitor cell assays, which demonstrate decreased responsiveness of hemopoietic precursors to proliferative and differentiative stimuli as well as diminished production of certain HGFs (Table 60-4). [40] These findings suggest that the ineffective hemopoiesis existing in MDS may be related to such biologic abnormalities. In MDS, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) have greater myeloid proliferative effects in vitro than G-CSF, whereas G-CSF has greater myeloid differentiative effects. [52] This is particularly evident in patients with RAEB or RAEB-T and those with normal cytogenetics. These in vitro studies have provided a biologic rationale for the differentiation induction therapy used to potentially counter the relative uncoupling between proliferative and differentiative signals existing in MDS. Such marrow myeloid progenitor cell assays also provide prognostic information and therefore complement evaluation of marrow morphology and cytogenetics in the analysis of disease progression in these patients.

In vitro studies and preclinical data indicate that a critical lesion underlying leukemia and MDS relates to an uncoupling of proliferation and differentiation at the level of the hemopoietic stem cell. [53] Induction of differentiation in culture diminishes the ability of leukemic stem cells to self-generate in vitro and reduces in vivo leukemogenicity in murine models. [54] [55] [56] Studies with these murine models have demonstrated increased survival of animals with leukemia after in vivo treatment with inducers of myeloid differentiation such as G-CSF, MGI-2, or vitamin D. [53] [54] [55] [56] [57] [58] Other studies have also demonstrated differentiation of colonies assayed from human normal and leukemic and MDS marrow after in vitro exposure to retinoids and vitamin D. [51] [56] [59] [60] The in vitro myeloid differentiative and proliferative responses of normal and MDS cells to pharmacologic levels of G-CSF and GM-CSF [52] are consistent with the high proportion of in vivo neutrophil responses following the therapeutic use of these agents in MDS. Increased numbers of marrow CFU-GM post treatment have also been demonstrated in patients who experience such neutrophil responses. [61]

In vitro erythroid progenitor cells in MDS have suboptimal responses to EPO. [51] [62] [63] Analysis of the relationship between EPO levels in MDS and the patients erythroid progenitors indicates that the anemia in MDS is not attributable to an abnormality in the capacity of EPO to induce the generation of CFU-E but is influenced by the size of the BFU-E population: a severe deficiency in the BFU-E population results in insufficient influx of EPO-responsive cells. [62] G-CSF markedly augments the in vitro EPO responsiveness of BFU-E in normal and MDS marrow. [63]

Hemopoietic growth factor production has also been analyzed in MDS. Marrow cells and peripheral blood T cells from MDS patients provide decreased amounts of GM-CSF, IL-3, M-CSF, and IL-6.[64] Blood cell production of G-CSF is predominantly generated by microbe-stimulated monocytes. [65] Decreased levels of monocyte-derived G-CSF have been demonstrated in MDS patients but (as most of these patients are elderly) also from elderly control subjects. [65] Thus, the defective production of a variety of HGFs, in addition to decreased precursor cell responsiveness to some of these factors, may contribute to the ineffective granulopoiesis in MDS patients. The effects of IL-1 and IL-6 in combination with GM-CSF on the in vitro colony formation of myeloid progenitors from patients with

TABLE 60-4 -- Effects of Recombinant Human GM-CSF and G-CSF in Phase I/II Clinical Trials of Patients with Myelodysplastic Syndrome

Parameter Evaluated	GM-CSF [217] [218] [219] [220] [221]	G-CSF [228] [229]
Short-term treatment		
Duration	714 days × 15 courses daily, IV or sc	4256 days daily sc
Daily dose	30750 mg/m^2	0.13 mg/kg
No. of patients	45	18
FAB subtypes		
RA/RAEB/RAEB-T/CMML	14/26/5	2/16/0
Responses		
Neutrophils	38 (84%)	16 (89%)
Reticulocytes	14	5
Platelets	1	
Marrow maturation	9	16
Increased blasts	12	4
Progression to AML	7	5
Long-term treatment		
Duration	29 wk	628 mo
Persistent neutrophil responses/patients	1/5 patients	10/11 patients

From Greenberg,[222] with permission.

MDS indicate that neither IL-1 nor IL-6 alone has colony-stimulating activity, and in normal bone marrow cultures these factors did not alter the stimulatory effects of GM-CSF.[66] However, in the majority of the MDS cases, an enhancing effect of IL-6 or IL-1 in combination with GM-CSF was observed. Other studies indicate that the actions of IL-1 and IL-6 on hematopoiesis are mainly indirect and are mediated by enhanced production of GM-CSF, G-CSF, or IL-3 by accessory cells. [67]

Tissue growth factor- (TGF-) inhibits early normal and leukemic progenitor cell proliferation in vitro. [40] [68] [69] [70] [71] Interferon- (IFN-) IFN-, and IFN- are also inhibitory against both normal and leukemic hemopoietic progenitors. [72] [73] By contrast, prostaglandin E has been shown to have less inhibitory activity for MDS and AML hemopoietic precursors than for normal cells. [74] [75] Similarly, MIP1a and other regulators inhibit normal early stem cells [76] [77] [78] [79] [80] but have a lesser effect on leukemic precursors. Thus, the selective functional nature of these inhibitors may permit a growth advantage of leukemic over normal cells. Further, tumor necrosis factor- (TNF-) generation is increased in MDS and AML and, in addition to its ability to increase CSF production, may inhibit hemopoietic precursor development. [81] [82] These findings suggest that in addition to defective responsiveness of hemopoietic precursors to stimulatory cytokines, enhanced levels of inhibitory cytokines may play a role in the abnormal hemopoiesis in MDS.

Immunologic cell dysfunction has also been demonstrated in MDS patients. In these individuals, T cells, natural killer (NK) cells, and B cells all appear to be defective in proportion and function. Deficiencies have been documented in T-helper cell populations and in the mitogenic response of T cells. [83] [84] [85] These abnormalities also appear to contribute to the decreased HGF production in MDS.

Molecular AbnormalitiesApoptosis

Altered regulation of marrow cell survival has been proposed as a mechanism to explain the ineffective hematopoiesis with associated cytopenia that is characteristic of MDS, as well as the frequent subsequent evolution to AML that occurs in many of these patients. Apoptosis (programmed cell death) is an active cellular process that regulates cell population size by decreasing cell survival and that differs from necrosis both morphologically and biochemically. [86] [87] [88] [89] [90] [91] Apoptosis is characterized by low molecular weight oligomeric DNA fragmentation patterns and specific cellular morphologic changes. Morphologic evidence of increased apoptosis has been demonstrated in MDS, particularly in the early subtypes. [92] Recent studies from a number of laboratories have demonstrated increased levels of apoptosis in MDS marrow cells.[93] [94] [95] [96] [97] Some investigators have found this process most evident within the CD34+ hemopoietic precursor cell population, [94] whereas others have found it predominantly within more differentiated cells and in stromal cells. [93] Flow cytometric data indicate that marrow CD34+ cells (containing the hematopoietic progenitor cells) from early MDS (e.g., refractory anemia) have an increased proportion of fragmented (apoptotic) DNA compared with cells from patients with more advanced stages of MDS or AML.[94] Treatment of MDS patients with the cytokines G-CSF and EPO, alone or in combination, enhanced effective hematopoiesis in vivo and was associated with decreased levels of apoptosis. [94] [97] Increased endogenous marrow levels of the inhibitory cytokine TNF- have been reported in MDS, which appears to enhance the level of apoptosis in these patients. [95]

Several of the genes responsible for the regulation of apoptosis have been identified. They include the proto-oncogenes *bcl*-2 and c-*myc*.[98] [99] [100] [101] [102] [103] *bcl*-2 and certain members of the *bcl*-2 gene family (*bcl*-2, *bcl*-X_L and *Mcl*-1) are anti-apoptotic.[98] [99] [100] [101] In contrast, other members of the *bcl*-2 family (*bax*, *bcl*-X_S , *bad*), c-*myc*, and *p53* act as potent inducers of apoptosis for hemopoietic cells. [102] [103] [104] The intracellular ratio of c-*myc* to *bcl*-2 oncoproteins is increased in MDS CD34+ cells compared to those from normal and AML CD34+ cells.[94] On in situ immunohistochemical staining, *bcl*-2 expression within early myeloid precursors in MDS marrow correlated, in both proportion and absolute number, with initial MDS stage (i.e., higher levels in later-stage disease), progressed over time, and was associated with evolution to AML.[105] These findings are consistent with the hypothesis that an altered balance between cell death and cell survival programs is associated with the increased degrees of apoptosis present in MDS hematopoietic precursors and may contribute to the ineffective hemopoiesis in this disorder. In contrast, decreased apoptosis is associated with enhanced leukemic cell survival as MDS progresses toward AML.

Oncogenes

Conversion of normal cells into those that have preleukemic and ultimately leukemic characteristics is a multistep process requiring the accumulation of genetic lesions. Additional oncogenes to those involved in apoptosis are abnormally expressed in many MDS patients. The *fms* gene encodes the CSF-1 receptor (M-CSF receptor). Mutations of *fms* have been observed in 1218% of MDS patients,[30] with the highest prevalence in the CMML subgroup, consistent with the observation that M-CSF is a monocytic growth factor. The *ras* family of proto-oncogenes encodes a GTP-binding protein. [106] [107] The importance of these genes lies in their role in mitogenic and differentiation-related signal transduction. [136] Mutations of *ras* may give rise to abnormal protein products that have the capacity to transform cells into a malignant phenotype. Mutational activation of *ras* occurs at codons 12, 13, and 61, which correspond to the GTP-binding sites. [108] [109] [110] Mutations of *ras* have been detected in MDS patients, with a wide range of frequencies (333%) reported. [108] [109] [110] [111] These mutations are somatically acquired, and mutant-bearing cells may be present as minor populations that may be lost on progression to AML, indicating that they are unlikely to be initiating events in transformation. The incidence of *ras* mutations varies between FAB types, with the highest occurrence in CMML. In some studies, *ras* mutations may be associated with a poor prognosis in terms of survival or progression to AML.[111] Mutations of the *p53* tumor suppressor gene (anti-oncogene) have been detected at a low frequency in MDS. [29] [30] [112] Thus,

although mutations of these cell growth regulatory genes have been described in MDS, their role in disease progression is currently unclear.

CLINICAL MANIFESTATIONS

MDS generally presents as a refractory cytopenia, predominantly in the elderly, with >80% of the patients being older than 60 years of age. [113] [114] After 70 years of age, the prevalence of MDS is approximately 2245 per 10^5 population,[113] [114] [115] indicating that MDS is as prevalent as the other most common hematologic malignancies of the aged, chronic lymphocytic leukemia (CLL) and multiple myeloma (Fig. 60-2).[113] [114] [115]

Blood and Bone Marrow

Anemia/Fatigue

Nearly all MDS patients are anemic, and many have associated fatigue. The anemia is usually macrocytic with a low reticulocyte response. In this elderly patient population, it is necessary to exclude vitamin B_{12} or folate deficiency as a cause of the macrocytic anemia. The anemia of MDS is usually due to ineffective erythropoiesis. In addition, patients may have abnormal iron metabolism, [116] disordered globin chain synthesis, [117] decreased levels of red cell enzymes, [118] increased levels of hemoglobin

Figure 60-2 Age-specific incidence of myelodysplastic syndrome in comparison with acute and chronic leukemias. (Data extrapolated from references [113] [114] [115] .)

(Hb) F,[119] Hb H inclusions,[120] [121] abnormal red cell antigens,[122] or a positive Ham test.[123] Low levels of pyruvate kinase may occur,[118] with associated hemolysis. Together with the requirement for long-term red cell transfusions, the abnormalities in iron metabolism may lead to hemochromatosis with subsequent organ (hepatic, pancreatic islet cell, testicular, cardiac) dysfunction.

Morphologic evidence of dyserythropoiesis in MDS includes ringed sideroblasts, multinuclear fragments, bizarre nuclear shapes, internuclear bridging, mitosis, abnormal dense chromatin or fine chromatin with asynchronous cytoplasm, and abnormal cytoplasmic features (intense basophilia, Howell-Jolly bodies). The red cells may demonstrate anisocytosis, poikilocytosis, nucleated forms, and acanthocytes (Fig. 60-3). Erythroid precursors show megaloblastoid changes (Figs. 60-4A and 60-5A). Because of the macrocytosis in these patients, vitamin B_{12} and folic acid deficiency need to be excluded. The occurrence of five or more siderotic granules is considered pathologic, and when these granules constitute more than one-third of the nuclear rim, the term ringed sideroblast has been used (Fig. 60-4B).[124] The disruption of mitochondria by these siderotic granules and the observed changes in heme synthesis relate to the ineffective

Figure 60-3 Myelodysplastic syndrome morphology: peripheral blood smears showing abnormal red blood cell morphology. **(A)** Macrocytic and macro-ovalocytic cells in refractory anemia (RA). **(B)** Dimorphic red blood cells (normocytic/microcytic and macrocytic) in RARS. **(C)** Dysplastic neutrophils showing hyposegmentation and hypogranularity. **(D)** Monocytes in CMML. (Wright stain; ×1,000 [orig. mag.]). *(Courtesy of Dr. James W. Vardiman, University of Chicago Hospitals.)*

Figure 60-4 Myelodysplastic syndrome morphology: bone marrow aspirates. **(A)** Megablastoid dyserythropoiesis and **(B)** ringed sideroblasts in RARS (Prussian blue stain). **(C)** Dysgranulopoiesis with increased blasts, hypogranular neutrophils, and hypolobated megakaryocytes in RAEB. **(D)** Abnormal monocytes and neutrophils in CMML. (Wright stain, ×1,000 [orig. mag.]). *(Courtesy of Dr. James W. Vardiman, University of Chicago Hospitals.)*

erythropoiesis, premature erythroid destruction, and RA in this disorder. Very rarely, sideroblastic anemia will respond to pyridoxine. RA is differentiated from RARS by the presence of >15% ringed sideroblasts. If erythroid precursors account for >50% of bone marrow cells, with >30% of the myeloid cells being blasts, the diagnosis of erythroleukemia is made (M6 variant of AML) (see Chap. 53).

Neutropenia/Infections

A substantial proportion (
60%) of MDS patients are neutropenic and are also often unable to mount an appropriate inflammatory response to infection. This likely is due to the poor myeloid marrow responsiveness to and decreased production of HGFs. Qualitative abnormalities occur in MDS neutrophils. [125] [126] [127] [128] [129] [130] In addition, because HGFs play important roles in mature neutrophil and monocyte cell functions, a decreased production of HGFs contributes to the phagocyte abnormalities noted in these patients.[40]

Morphologic abnormalities of granulocytes are common in MDS and are present in both the peripheral blood and bone marrow. The most common abnormalities are hypogranulation (which may be associated with a negative peroxidase reaction), low levels of leukocyte alkaline phosphatase, and hyposegmentation of the polymorphonuclear leukocytes with abnormal chromatin condensation (Pelger-Huët-like anomaly) (Figs. 60-3C and 60-4C). Occasionally the chromatin appears excessively clumped, leading to an appearance of nuclear fragmentation associated with a loss of segmentation. A critical finding of dysplasia within the marrow is nucleocytoplasmic asynchrony in abnormal early myeloid cells (type III blasts or abnormal promyelocytes), with granular cytoplasm, a reticulated nucleus possessing a nucleolus, and a prominent perinuclear Golgi zone. The proportion of type I marrow myeloblasts determines the subtype of MDS (Table 60-1) and is a major reflection of the differentiative abnormality present.

Infections are common in MDS. Approximately 10% of patients present with evidence of infection, and in 21% it is the cause of death. [131] [132] Most infections in MDS are bacterial, usually involving host organisms, and are associated with neutropenia. Defective adhesion, phagocytosis, and bacterial killing are commonly found. [125] [126] [127] [128] The migration of qualitatively abnormal granulocytes into sites of infection often leads to poorly resolving abscesses, which may be occult. [129] In MDS, blood monocytes are derived from the abnormal clone, and in CMML they proliferate in a poorly controlled manner. Certain monocyte functions are impaired, including

phagocytosis, which is decreased.[130] Further altering inflammatory responses, NK cells in MDS are reduced in number and are functionally immature, with impaired ability to produce interferon. [133] [134] [135]

Thrombocytopenia/Bleeding

Thrombocytopenia is common (
60% of cases) in MDS and occasionally becomes severe. In
5% of cases it is the only cytopenia observed. [113] Giant and agranular platelets are often seen, and platelet function may be abnormal, as reflected in prolonged

Figure 60-5 Myelodysplastic syndrome morphology: bone marrow biopsies and aspirates. **(A)** Dyserythropoiesis in RA. **(B)** Dysmegakaryopoiesis in RAEB. **(C)** RARS with erythroid hyperplasia (hematoxylin-eosin, ×200 [orig. mag.]). **(D)** Myeloid hyperplasia with increased immature forms and abnormal megakaryocytes in RAEB (×400 [orig. mag.]). **(E)** Atypical localization of immature myeloid precursors. *(Courtesy of Dr. James W. Vardiman, University of Chicago Hospitals.)*

bleeding times (even without thrombocytopenia) and reduced platelet aggregation. [136] Progressive thrombocytopenia is often a marker of disease evolution. The risk of hemorrhage during surgery or after trauma is increased because of the defective platelet function. Thus, bleeding in these circumstances may occur at relatively normal platelet counts, and platelet transfusions should be administered under these conditions. Splenomegaly occurs in
10% of MDS patients and may contribute, by sequestration of platelets, to the thrombocytopenia.

Common morphologic abnormalities of megakaryocytes in MDS occur and include micromegakaryocytes, mononuclear megakaryocytes, multiple small nuclei separated by strands of nuclear material, dysmorphic nuclear features, and hypogranularity (Figs. 60-4C and 60-5B and D). The 5q syndrome is frequently associated with morphologic abnormalities of megakaryocytes, which are usually small with single eccentric round nuclei.

Clinical Variants

Rheumatic and immunologic processes occur uncommonly in association with MDS. Their features include cutaneous vasculitis, peripheral neuropathy, and lupus-like syndromes.[137] A subgroup of MDS patients have acute seronegative inflammatory arthritis temporally related to the discovery of cytopenia. [138] Episodes of oligoarthritis or polyarthritis may occur, along with such systemic features as fever, pleuritis, pericarditis, and hemolytic anemia. The arthritis and systemic features often respond to corticosteroid therapy.

The association of MDS and lymphoid malignancies, particularly

B-cell neoplasias, has been reported. Chronic lymphocytic leukemia, hairy cell leukemia, lymphocytic lymphoma, multiple myeloma, large granular lymphocytic leukemia, and T-helper cell lymphoma all have been diagnosed simultaneously with MDS. [139] The mechanism whereby such associations occur is unknown but suggests involvement of a common hematolymphoid stem cell in the inciting transformation. After chemotherapy with alkylating agents, secondary MDS may develop in some patients and contribute to problematic cytopenias.

The 5q syndrome is an MDS variant, usually seen in RA, that exhibits not only characteristic morphologic featuresmacrocytic anemia, normal or high platelet counts, nonlobulated micromegakaryocytes, and hypoplastic erythroid cellsbut also results in a clinical course in which, when it is the sole karyotypic abnormality, evolution to acute leukemia is uncommon.[33] [37] [38] [39] Superimposed cytogenetic lesions, however, are associated with a poorer prognosis.

Overlap SyndromesHypocellular MDS

Although most patients with MDS have hypercellular or normocellular bone marrows, a small subgroup of MDS patients (<15%) have marrow hypoplasia at the time of diagnosis.[140] [141] Differentiation of this syndrome from either aplastic anemia or hypoplastic AML may be difficult. When marrow cellularity is low, recognizing hemopoietic dysplasia from either the aspirate or the biopsy specimen may be problematic. Most hypocellular MDS cases fit into the categories of RA and RAEB. [140] [141] [142] CMML, which classically is characterized by a hypercellular bone marrow, has not been reported to have a hypocellular variant. A potentially useful means of identifying hypocellular MDS is the finding of an associated clonal cytogenetic abnormality characteristic of MDS (e.g., 5, 7, 5q, 7q, +8). [143] [144] [145] In a recent large series of patients with aplastic anemia, clonal chromosomal abnormalities were present in only 4% of individuals but were those frequently seen in MDS or AML. [143]

MDS with Fibrosis

Myelofibrosis refers to a generalized increase in the number and thickness of reticulin fibers that is detected with a silver stain of the bone marrow biopsy specimen.[146] Mild to moderate myelofibrosis has been reported in up to 50% of MDS cases, with marked fibrosis occurring in <15% of cases. [146] [147] [148] Myelofibrosis occurs in all FAB subclassifications of MDS. [149] [150] The incidence of myelofibrosis in therapy-related MDS is greater than in primary MDS. [185] [186] Marked fibrosis has been reported to occur in up to 50% of cases of therapy-related MDS, with mild fibrosis occurring in up to 85% of such cases. [149] [150]

The syndrome of MDS with myelofibrosis is characterized by the abrupt onset of pancytopenia without organomegaly but with substantial red cell anisopoikilocytosis, a hypercellular bone marrow with myelofibrosis, trilineage dysplasia, atypical megakaryocyte proliferation with hypolobated forms, and increased numbers of marrow blasts.[148] [149] [150] Occasionally a leukoerythroblastic peripheral blood picture is evident. The patients usually experience a rapidly progressive clinical course. [151]

The distinction between this entity and MPDs may not be clear-cut. Other diagnostic entities that should be excluded before making a diagnosis of MDS with myelofibrosis are primary myelofibrosis, the accelerated phase of CML, post-polycythemia vera myelofibrosis with myeloid metaplasia, AML (especially acute megakaryoblastic leukemia; AML-M7), AML with trilineage dysplasia, and acute myelofibrosis. [152] A period of myelodysplasia may herald a leukemic transformation in up to 50% of patients who develop acute leukemia as a terminal event of polycythemia vera. [153] [154] In most cases this event is related to previous cytotoxic therapy and therefore resembles other therapy-related myelodysplastic disorders, although a myelodysplastic phase has also been reported in polycythemia vera patients treated by phlebotomy alone.[153] [154]

Chronic Myelomonocytic Leukemia

Some patients have disorders with features of both MDS and chronic MPD from the outset. Included in this group are those with leukocytosis, hypercellular bone marrows with variable amounts of dysplasia, and splenomegaly. Some of these patients (i.e., those with monocytosis) may have CMML. Although CMML is defined as an MPD by the FAB group, it also serves as a prototype of those disorders that straddle MDS and chronic MPD. The FAB criteria for CMML include a peripheral monocytosis exceeding 1×10^3 /mm^3 , increased numbers of monocytic cells in the bone marrow (Figs. 60-3D and 60-4D), dysplasia in either the erythroid, megakaryocytic, or granulocytic series, <5% circulating blasts, and <30% marrow blasts (Table 60-1).[10] [155] Pericardial, pleural, synovial, and ascitic effusions have been reported in CMML.[156] These serous effusions appear to be associated with high peripheral monocyte counts.

CMML was categorized as an MDS primarily because of the dysplasia that occurs in some patients and because cytopenias of one or more peripheral blood elements are not uncommon.[155] However, the nosologic position of CMML as a subtype of MDS is not accepted by all investigators, because myeloproliferative features predominate in a substantial portion of the patients. [157] [158] Because of these disparate findings, the disorder has been subdivided into two forms, a proliferative and a nonproliferative form. The proliferative form of CMML (with WBCs >12,000/mm^3 , hepatosplenomegaly, constitutional symptoms) is more akin to an MPD than to MDS.[158] This disorder differs in its major clinical features from the nonproliferative subtype of CMML (i.e., in MDS patients who have monocytosis but relatively low WBC counts and who previously were likely considered to have RAEB or RA with monocytosis). Clinical outcomes in patients with CMML are more closely related to

the proportion of marrow blasts than to peripheral monocyte numbers. These latter features include marked monocytosis or neutrophilic leukocytosis (in nearly 50% of patients), tissue infiltration by monocytes, splenomegaly (50%), and hepatomegaly (up to 20%). [105] [157] [158] [159] In addition, the behavior in vitro of marrow progenitor cells in CMML resembles that of chronic MPD, with increased numbers of colonies and clusters, rather than the pattern of MDS, in which progenitor cells proliferate poorly in short-term cultures.[40] Therefore, CMML may be considered a disorder that encompasses features of both chronic MPD and MDS.

Atypical Chronic Myeloid Leukemia

Another disorder, Ph^1 chromosome-negative CML, in some cases, has features of MDS as well as those of an MPD.[155] [160] [161] These features include dysplastic granulocytes, a low percentage of basophils, a sum of promyelocytes, myelocytes, and metamyelocytes that is >10%, and, often, monocytosis and thrombocytopenia. In a retrospective study of cases previously diagnosed as Ph^1 chromosome-negative CML, 60% of the patients had significant dysplasia, and 3040% of the cases met the criteria for CMML. Subsequent studies, however, demonstrated that, in some cases of Ph^1 chromosome-negative CML, the breakpoint cluster gene (*BCR*) on chromosome 22 was rearranged as in Ph^1 -positive CML, despite the absence of a cytogenetically detectable Ph^1 chromosome.[162] Patients lacking the Ph^1 chromosome t(9;22) but possessing the *BCR* rearrangement have morphologic and clinical features indistinguishable from those of Ph^1 chromosome-positive CML. The remaining patientsthose exhibiting some features of CML but lacking the Ph^1 chromosome and *BCR* rearrangementform a heterogeneous population that includes patients with features of both MDS and chronic MPD. Some, but not all, of these patients have

findings that meet the criteria for CMML. Thus, many of these patients defy placement into current classification schemas and have been designated as having atypical CML.

Secondary Myelodysplastic Syndrome

Secondary (i.e., therapy-related and toxic chemical-related) MDS is emerging as a significant clinical problem and may cause morbidity and mortality with or without progression to AML.[162] [163] [164] [165] [166] [167] [168] [169] The increasing incidence of secondary MDS and AML reflects a number of factors, including a longer period of risk after the successful treatment of solid tumors, more intensive treatment regimens combining high-dose chemotherapy and irradiation, broader utilization of adjuvant chemotherapy and irradiation for the treatment of solid tumors, and environmental pollution and exposure to chemicals and carcinogens (particularly organic solvents)[163] [164] [165] [166] [167] [168] [169] in industrialized nations. Generally these patients have a poorer prognosis than those with primary MDS. The major organic solvent implicated in leukemogenesis is benzene, and its association with disease pathogenesis is related to the intensity and duration of exposure to the carcinogens. Recent studies have indicated that mutations in enzymes that detoxify benzene, with a concomitant increase in toxic metabolites capable of damaging DNA, are associated with enhanced susceptibility to benzene poisoning and the development of leukemia. [170] [171] [172] [173]

In secondary MDS, abnormal karyotypes are evident in almost all patients. Multiple chromosomal aberrations are the rule, and chromosomes 5 and 7 are most frequently (85%) involved (Table 60-3). Accumulating experience in therapy-related leukemia suggests that chemotherapeutic agents may have different leukemogenic potential, associated with differing pathophysiologic processes. The classic therapy-related leukemia involving chromosome 5 and 7 abnormalities, and implicating alkylating agents and irradiation, remains the most common form. The benzene-induced cytogenetic abnormality is frequently trisomy 9. [12] [164] [172] Two additional forms of therapy-related leukemia have recently been described, one attributed to exposure to topoisomerase II-active chemotherapeutic agents and involving the chromosome 11q23 locus and the other involving the chromosome 21q22 locus. [167] [169]

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THERAPY

A variety of treatment approaches have been used in MDS, with supportive care generally the mainstay of therapy in the community. Patients should be treated as needed with antibiotics for infection and with red cell and platelet transfusions for symptomatic anemia and thrombocytopenic bleeding. Long-term red cell transfusion support may lead to iron overload and secondary hemochromatosis. Adverse effects from the needed transfusion support include hepatic pancreatic, gonadal, and cardiac complications. When patients have had, or are anticipated to have, prolonged red cell transfusion requirements, prophylactic or therapeutic desferrioxamine chelation treatment should be considered. Such treatment may delay or occasionally reverse iron-related organ damage. [174]

As MDS is relatively indolent and occurs predominantly in the elderly, the development of treatment modalities for the cytopenia that would not cause excessive toxicity remains a therapeutic challenge. Elderly patients frequently have concomitant medical illnesses that markedly limit therapeutic options. Patients with abnormal cytogenetics, abnormal in vitro marrow myeloid colony growth patterns, and deranged clinical and marrow morphologic features have worse prognoses. These abnormalities generally correlate with the more advanced FAB classifications. Because of this variability in prognosis in subgroups of patients with MDS, stratification by risk factors is necessary in order to determine the efficacy of various treatment options.

Chemotherapy

Chemotherapeutic options for this disease have ranged from intensive cytotoxic therapy to low-dose therapy with DNA synthesis inhibitors such as cytosine arabinoside (Ara-C) or hydroxyurea. Intensive chemotherapy in these generally elderly individuals is often poorly tolerated. In addition, a high proportion of these patients have marrow cells that express the P-glycoprotein, which confers multi-drug resistance (MDR). [175] [176] Thus, in addition to MDS patients having poor marrow recovery after marrow hypoplasia induced by intensive therapy, difficulty often exists in eradicating their abnormal clonal cells. In secondary MDS, the increased frequency of MDR positivity, unfavorable cytogenetics, and treatment-related stromal damage contributes to the relatively poor therapeutic responses, compared with responses in patients with de novo AML. [206] [207] In an attempt to improve therapeutic responses in high-risk MDS patients or those with AML after MDS (i.e., those with an increased degree of MDR expression), several multicenter trials using MDR-modulating agents (e.g., cyclosporine, PSC833, quinine) plus chemotherapy have demonstrated encouraging results. [177] [178] The French Cooperative Leukemia Groups Phase III randomized study with quinine plus chemotherapy indicated improved responses in the MDR-positive patients when compared with responses in patients who received chemotherapy alone. [179]

In studies evaluating the use of standard intensive induction chemotherapy in MDS, variable complete remission rates of 1351% have been reported, but with significant morbidity and a high number of toxic deaths. [180] [181] [182] [183] [184] Better results have been achieved in younger patients and in those with favorable karyotypes. In addition to the severe toxicity of this treatment the remission rates and durations of the responses are generally less than in patients with de novo AML.

Numerous studies have evaluated the use of low-dose Ara-C to treat these patients, generally with 1020% of these individuals achieving a complete remission. [185] [186] However, the durability of these responses is usually short, and in a randomized trial evaluating low-dose Ara-C versus supportive care alone, the complete remission rate was only 8%, and no improvement in survival was noted. [187] In addition, marrow toxicity, particularly thrombocytopenia, has resulted in significant difficulties in the management of these patients. Organomegaly, skin lesions, and serous effusions in CMML may respond to low-dose oral chemotherapy.

Early results of clinical trials using the relatively low-intensity chemotherapeutic agents 5-azacytidine, [188] decitabine,[189] and topotecan[190] have shown responses in 3040% of MDS patients. However, the initial trials with topotecan used relatively high doses of the drug, which were associated with significant toxicity. Recently, improved responses have been reported with lower topotecan doses in combination with cytarabine. [191] A report of a randomized, controlled CALGB Phase III trial of 5-azacytidine versus observation in MDS showed increased responses and decreased AML evolution in the azacytidine arm, whereas survival did not differ in the two groups.[192] Clinical trials are ongoing to assess the relative efficacy of these agents.

Bone Marrow Transplantation

Several investigators have reported the use of allogeneic bone marrow or peripheral blood stem cell (PBSC) transplantation for MDS patients. [193] [194] [195] [196] [197] Because of restrictions based on patient age, performance status, and donor availability, only a small

proportion of this patient population is currently eligible for this procedure. The median age of individuals (generally 2939 years) in these protocols is much lower than that of most MDS patients (>65 years). In a recent large study, the probabilities of disease-free survival, relapse, and non-relapse-related mortality at 4 years were 41%, 28%, and 43%, respectively.[193] Multivariate analysis indicated that younger patients (<40 years old) and patients with low marrow blast counts (<5%) had better prognoses (62% disease-free survival). However, for older patients (>40 years old) and those with >5% blasts (i.e., patients with RAEB or RAEB-T), the disease-free survival rate was only 17% and 32%, respectively; 51% of patients with >5% blasts experienced disease relapse. For patients in this study with secondary MDS, only two of eight individuals remain alive and disease free. In seven MDS patients with associated myelofibrosis, none survived after bone marrow transplantation.

In a study by the European Bone Marrow Transplantation Group, the disease-free survival after bone marrow transplantation in patients with RAEB and RAEB-T was somewhat better, approximately 5074%.[194] The report did not give the ages of individuals who responded to therapy. In another study, a disease-free survival rate of 40% was reported for 15 MDS patients who underwent bone marrow transplantation.[195] Nine (60%) of these patients died of transplant-related complications in the first 100 days. Three of nine RAEB or RAEB-T patients experienced relapse. An increased percentage of marrow blasts had a negative impact on outcome, as only two of ten patients in this group survived, compared to six of ten patients with a lower percentage of blasts before transplantation. In recent reviews of the experience at the Fred Hutchinson Cancer Center and the International Bone Marrow Transplant Registry, patients with complex cytogenetic abnormalities had significantly higher relapse rates and a shorter disease-free survival, whereas those with normal cytogenetics had a better outcome. [196] [197] Taken together, these data suggest that matched sibling allogeneic marrow transplantation may be useful for a portion of relatively young patients with low-risk MDS. However, other forms of therapy should be considered in patients with more advanced forms of MDS (RAEB, RAEB-T) and complex cytogenetics, particularly if they are elderly.

HLA-matched unrelated donor marrow or PBSC transplants have been performed in an increasing number of MDS patients. Recent data from Seattle show 2-year disease-free survival, risk of relapse, and risk of nonrelapse transplant-related mortality rates of 38%, 28%, and 48%, respectively. [198] Relapse rates were significantly higher in patients with RAEB-T or MDS-related AML. Mortality not associated with relapse was higher in older patients. Further studies using newer preparative regimens are ongoing to determine the possible improved tolerance of allogenic stem cell transplantation for high-risk MDS patients.

Hormonal Therapy

Corticosteroids have been used in a small number of MDS patients, with 10% exhibiting transient responses.[199] In individuals who responded there has often been a correlation with in vitro marrow culture studies demonstrating T-cell-mediated inhibition of hemopoiesis. Androgens have also been used to treat MDS patients. However, survival data indicate that patients who received the androgens did no better than those treated by observation and supportive care.[200] A randomized trial of androgens versus low-dose Ara-C or observation indicated similar survival in these groups.[201] Treatment with danazol has been used in some individuals with MDS who were believed to have immune-associated thrombocytopenia.[202] The platelet counts in some patients improved; however, the rate of such responses was generally quite low, and clinically useful responses were rare.

Nonspecific Differentiation-Inducing Agents/Hematopoietic Stimulants

The use of differentiation-inducing agents for treating myeloid malignancies is based on a large body of in vitro marrow culture studies and preclinical data indicating that such drugs may diminish self-replication of abnormal cell clones concomitant with enhancing their differentiation.[53] [54] [55] [56] [57] [58] [59] [60] [203] A variety of differentiation-inducing agents have been used for treating MDS, including retinoic acid [204] [205] [206] and vitamin D,[207] as well as some HGFs (discussed later). Although trials of 13-cis-retinoic acid showed an increase in neutrophils in 3050% of MDS patients, a randomized trial demonstrated that survival was not improved.[205] Similar results, indicating a low response rate, were reported in a trial that used all-trans-retinoic acid.[206] The use of 1,25-dihydroxyvitamin D_3 to treat MDS was found not to be beneficial.[207]

Recent studies with a small number of MDS patients who received phosphothiol amifostine demonstrated hematopoietic responses in 3050% of treated patients.[208] [209] Although the mechanisms whereby this agent improves hematopoiesis are not clear, the drug is well tolerated and may be useful as a low-intensity treatment option. Clinical trials with this agent are ongoing.

Interferons

Because of its effect on enhancing monocytic differentiation of myeloid cells, IFN- has been used to treat MDS patients. In a study using high and low doses of IFN-, 30 MDS patients (18 with RAEB, 10 with RAEB-T) were treated.[210] The median survival of these patients was 12 months. Two patients had partial responses, and in 12 individuals transformation to AML occurred. No clear differences were demonstrated between patients receiving the high- and low-dose therapy. Clinical benefit with regard to number of infectious episodes or improvement in blood cell counts was not demonstrated, and no complete responses were noted. In another study, none of 25 MDS patients had complete responses to IFN-, although three patients had good responses.[211]

IFN- has also been used to treat patients with MDS. In one study of 14 patients (three with RA, nine with RAEB, two with CMML), myelosuppression was noted in 11 individuals, and in five of them the disease evolved to AML.[212] No sustained improvement in blood cell counts occurred in these patients. In other studies, three of ten low-risk MDS patients[213] and two of eight high-risk patients[214] had transient responses. These data do not support using IFN- or IFN- in treating MDS.

Hemopoietic Growth Factors

The HGF have critical physiologic roles in controlling hemopoiesis in vivo.[40] [215] [216] Several of these growth factors, including IL-3 and GM-CSF, have predominantly proliferative effects on early hemopoietic cell compartments, whereas others, such as G-CSF and M-CSF, have major differentiative as well as proliferative effects on later, more lineage-restricted precursor cells (i.e., granulocytes and monocytes, respectively). Each hemopoietic cell lineage appears to be regulated by both proliferative and differentiative stimuli. These factors have been used to attempt to improve the cytopenias and natural history of MDS.

Colony-Stimulating Factor-Granulocyte/Macrophage

The results of five Phase I/II therapeutic trials that used recombinant human GM-CSF short term to treat primary MDS have been reported.[217] [218] [219] [220] [221] [222] Generally treatment was administered for 714 days, with one to five courses of therapy. In combined

data from these studies, neutrophil counts improved in 38 of 45 patients treated with GM-CSF, associated increases in marrow myeloid maturation occurred in nine, 14 had increased reticulocyte counts (with three of these individuals having decreased RBC transfusion requirements), and eight had transient increases in platelets (Table 60-4). In 12 patients an increase in the percentage of marrow blasts or peripheral blood blasts (or both) was noted. In seven patients progression to AML occurred; particularly affected were individuals with >15% marrow blasts (i.e., those with RAEB or RAEB-T).

A trial that used GM-CSF to treat patients with secondary MDS (i.e., MDS developing after prior cytotoxic chemotherapy for other malignancies) demonstrated neutrophil responses in eight of ten evaluable patients.[223] Several patients enjoyed a transient improvement in platelet numbers, and in three active infections resolved. Transformation to AML occurred in four patients after 511 months of therapy, and three other patients died of the underlying malignancy. The median survival of these patients, 11 months, reflected the poor prognosis in this group of patients.

A multicenter study[224] provided information regarding the use of GM-CSF in relatively low-risk MDS patients (RA, RAEB patients with <10% marrow blasts) for longer periods (up to 2 months). Eighty-two patients (50 with RA, 32 with RAEB) received either of two different fixed daily dose levels of GM-CSF. These untreated low-risk MDS patients would have been expected to have relatively good prognoses, with median survivals of several years and a low incidence of evolution to AML. Nearly all of the MDS patients treated with GM-CSF responded with increased neutrophil counts. However, only 35% of the patients completed 8 weeks of treatment, and the drug was discontinued in the others because of progression of disease and the development of local pulmonary infiltrates, flu-like syndromes, hyperleukocytosis, or bone pain. In 25% of patients platelet counts decreased during GM-CSF administration to <50% of baseline values, whereas two patients had increases in platelet counts. Six patients had progressive disease, in two of whom with RAEB acute leukemia developed; erythroid responses did not occur. No differences in responses were noted between the two dose levels of GM-CSF used. The impact of GM-CSF on progression of disease could not be addressed because of the small number of patients and the short duration of treatment. Similar results were noted in a preliminary report of another multi-institutional randomized trial of GM-CSF treatment for periods of up to 6 months versus observation in 21 patients with MDS, with crossover occurring in patients with infections.[225] A decrease in infections in the GM-CSF group was reported. Severe toxic effects requiring a reduction in or discontinuation of the GM-CSF dose occurred in a substantial proportion of the patients. No improvements in platelet counts or hemoglobin levels were noted. Additional patient accrual and longer follow-up will help to determine the impact of this therapy on transformation to AML. These studies demonstrated some of the potential difficulties and benefits of chronic administration of a predominantly proliferative myeloid growth factor, such as GM-CSF, to patients with MDS.

The effects of GM-CSF plus low-dose Ara-C were evaluated in small Phase II studies.[226] The MDS patients were randomly assigned to receive either GM-CSF alone or GM-CSF plus low-dose Ara-C for 2 weeks, repeated monthly for three cycles. Patients receiving GM-CSF alone generally had increases in leukocyte and neutrophil counts, with only one patient having an improvement of platelets. In the group receiving both GM-CSF and low-dose Ara-C, the leukocyte count also increased during administration of the combined treatment, whereas the platelet count declined significantly. In patients who received three courses of marrow combined treatment, partial short-term responses occurred, with a somewhat decreased percentage of blasts. In a Phase III trial, the EORTC leukemia group compared these two drugs given sequentially or simultaneously.[227] Limited responses and no differences in duration of responses were seen in the two arms of the study, and the treatment-related mortality was 16%. These partial transient improvements with combined GM-CSF and low-dose Ara-C suggest that further studies using myeloid growth factors combined with low-dose cytotoxic chemotherapy are needed to validate this approach in this clinical setting. It will be important to analyze long-term as well as short-term outcomes in these individuals, to determine which subtypes of patients may respond and the degree of supportive care needed during such therapy.

Granulocyte-Colony Stimulating Factor

Treatment of 18 MDS patients (two with RA, nine with RAEB, seven with RAEB-T) has been reported. Subcutaneous injections of G-CSF, were administered daily,

with escalating dosage levels every 2 weeks, from 0.1 to 3.0 mg/kg/day, for a 2-month period. [228] [229] Sixteen patients, including those with severe neutropenia, had significant elevations into the normal range of both WBC counts and absolute neutrophil counts. In five patients a greater than twofold increase in reticulocyte counts occurred, and three of 12 red cell transfusion-dependent patients had decreases in transfusion requirements. Improved marrow myeloid maturation was noted in 16 of 18 patients. No significant changes in other blood cell counts were found during treatment in 17 of 18 patients (Table 60-4). After G-CSF treatment was discontinued, peripheral blood WBC counts returned to baseline levels over 24 weeks.

The results of a short-term trial of G-CSF in 40 MDS patients (20 with RA, 20 with RAEB/RAEB-T) have been reported. [230] Twenty of 22 patients (7-day treatment) and 16 of 18 patients (14-day treatment) had substantial improvement in neutrophil levels, which was associated with decrements in percentage of marrow blasts in eight of 13 evaluated patients. In seven of 11 patients with infections prior to G-CSF therapy, the infections resolved after treatment with G-CSF and antibiotics.

As a result of the clinical response and the tolerance of MDS patients for short-term G-CSF treatment, prolonged maintenance therapy was administered, and 10 of the 11 patients studied had persisting improvements in neutrophil counts for 628 months. [229] Marrow granulocytic maturation improved in seven of nine patients. Two of four red cell transfusion-dependent patients had reductions in their transfusion requirements. Platelet counts were generally not altered by this therapy. Neutrophil function (in vitro chemotaxis and phagocytosis), which was maintained or improved after 2 months of treatment, was further augmented in five patients after an additional 6 months of G-CSF therapy. A significant reduction in infection risk was retrospectively demonstrated in responding patients who achieved an absolute neutrophil count of >1,500/mm^3 after G-CSF treatment, compared to the number of pretreatment episodes. Toxic responses to G-CSF were minimal. In five of 18 patients treated long term, four initially with RAEB-T, the disease converted to AML after 616 months of the study. In this study, chronic G-CSF administration was well tolerated and effective in eliciting persistent improvement in neutrophil counts, in vitro function, and marrow myeloid maturation, and possibly in decreasing bacterial infections and red cell transfusion requirements in MDS patients. A Phase III multi-institutional randomized trial evaluating 102 patients with high-risk MDS (RAEB/RAEB-T) compared long-term G-CSF administration with observation in an attempt to determine the impact of G-CSF on the natural history of the disease. [231] Preliminary data from this study showed no difference in the frequency of evolution to AML between the two arms. However, a decreased survival was observed in the RAEB patients receiving G-CSF. No change in the incidence of infections was noted in the two arms.

TABLE 60-5 -- Effects of Recombinant Human IL-3 Treatment in Myelodysplastic Syndrome

Parameter Evaluated	Reference [232]	Reference [233]
Treatment duration	15 days, × 13 courses	28 days
Daily dose	250500 mg/m^2 SC	301,000 mg/m^2 IV
No. of patients	9	13
FAB subtypes		
RA/RAEB-RAEB-T/CMML	6/3/0	5/5/3
Responses		
Leukocytes	9	8
Neutrophils	7;3[a]	6;3[a]
Reticulocytes	3; transfusions 1/9	1
Platelets	3;[b] transfusions 2/4	2
Blasts	2 PB; 1 BM	1 BM
Progression to AML	1	0

Abbreviations: BM, bone marrow; PB, peripheral blood

From Greenberg,[222] with permission.

[a]Increased ANC from <1,000/mm^3 to >1,000/mm^3 .
[b]Unsustained.

These data demonstrate the lack of impact of G-CSF on the natural history of MDS.

Evidence of Clonal Responses

Cytogenetic evaluations and investigations analyzing RFLP were performed in several studies. [24] [229] [231] to determine whether selective responses to HGFs of normal versus abnormal clones occurred in MDS. The persistence of cytogenetic abnormalities and clonal hematopoiesis after treatment with GM-CSF and G-CSF indicated that these cytokines induced differentiation of the abnormal clone. With one exception in which polyclonal hemopoiesis developed after GM-CSF treatment, [231A] most evidence has indicated that GM-CSF or G-CSF does not preferentially stimulate normal marrow stem cells to proliferate and does not have the ability to eradicate the cytogenetically abnormal clone by inducing terminal differentiation.

Interleukin-3

Two short-term studies have reported the effects of IL-3 therapy in 22 MDS patients. [232] [233] These studies indicated modest improvements in neutrophil counts; however, the improvements were not as great as those demonstrated with G-CSF or GM-CSF (Table 60-5). Further, only limited responses occurred in the other cell lines. Similar results were found in a longer-term study that treated low-risk (RA/RARS) MDS patients for 3 months with IL-3. [234] Transient improvement in platelet counts occurred in two of five patients who initially had counts of <50,000 platelets/mm^3 . These data indicate that IL-3 will need to be combined with other HGFs in order to substantially mitigate the cytopenia of MDS.

Interleukin-6

Fifteen low-risk MDS patients and one with CMML were treated with IL-6 in an attempt to improve their platelet counts. [235] Preliminary information indicated that platelet responses occurred in five of 16 patients, including two patients with platelet counts of <50,000/mm^3 . Moderate to severe toxicity with constitutional symptoms occurred, without an improvement in leukocyte number, and anemia worsened in a substantial proportion of these patients. The durability of these platelet responses and the long-term tolerance for this agent remain to be determined.

Erythropoietin

Serum EPO levels may be suboptimally elevated in MDS patients relative to the degree of anemia. [236] Thus, recombinant human EPO therapy has been instituted in an attempt to correct the hypoproductive anemias associated with MDS. A number of published reports have detailed the erythroid responses of MDS patients to this form of treatment. Results of the initial seven studies utilizing EPO in MDS [237] [238] [239] [240] [241] [242] [243] indicated that 14 (19%) of 75 patients responded to EPO (Table 60-6). A relatively higher response rate was found in patients with endogenous serum EPO levels of <100 mU/ml. [241] Extremely poor responses

TABLE 60-6 -- Erythroid Responses to Recombinant Human Erythropoietin in Myelodysplastic Syndrome

MDS Subtype		Epo Dose (U/kg)	Responses/Patients (%)	Responder Serum Epo Levels (mU/ml)	References
RA/RARS	RAEB/RAEB-T				
4	4	200400 IV, 3×/wk	2/8 (25)	694, 919	[237]
2	0	50500 IV, 3×/wk	0/2		[238]
7	5	2001,000 IV, 3×/wk	5/12 (42)	360 (mean)	[239]
17	0	8001,600 IV, 2×/wk	4/17 (24)	16, 515, 589, 1030	[240]
11	1	50250 SC, 3×/wk	0/12		[241]
10	4	80640 SC, 3×/wk	1/14 (7)	1,750	[242]
8	2	6090 SC daily	2/10 (20)	49, 199	[243]
59	16		14/75 (19)		

Modified with permission from Greenberg.[222]

TABLE 60-7 -- Erythroid Responses to G-CSF plus EPO in MDS

FAB Group	No. of Patients	CR[a] (%)	PR[b] (%)	Total Responses (%)
RA	30	17	10	27
RARS	31	26	19	45
RAEB	32	22	16	38
RAEB-T	5	20	0	20
All groups	98	22	14	36

From Hellstrom-Lindberg et al.,[247] *with permission from Blackwell Science, Ltd.*

[a]CR, complete response: hemoglobin increased to 11.5 g/dl.
[b]PR, partial response: 100% reduction in red cell transfusions/stable hemoglobin or hemoglobin increased to 1.5 g/dl.

occurred in patients with the RARS subtype of MDS.[241] Generally, the patients needed relatively high doses of EPO (>200 U/kg/day) to achieve responses. This limited in vivo responsiveness of MDS marrow cells to EPO is not totally unexpected, as the defective erythroid precursors in MDS have demonstrated suboptimal in vitro responses to EPO alone.[40] [62]

HGFs such as G-CSF are synergistic with EPO in vitro, enhancing normal and MDS marrow BFU-E numbers or responsiveness to EPO, [244] suggesting their potential to provide more prominent in vivo erythroid responses in combination than can be achieved with either agent alone. EPO is a relatively late-acting factor that acts predominantly on CFU-E and on a portion of the earlier BFU-E that generate CFU-E. Two clinical studies describing the effects of combination therapy with G-CSF plus EPO to treat the anemia of MDS[245] [246] have shown an enhanced proportion of responses. Therapy was initiated with G-CSF at 1 g/kg (0.33 g/kg) administered by daily subcutaneous injection and adjusted to either normalize or double the neutrophil count. EPO was then administered by daily subcutaneous injection of 100 U/kg and dose-escalated to 150300 U/kg every 4 weeks in one study, or kept at 120 U/kg/day in the other study, while G-CSF was continued. Ten (42%) of 24 patients in one study[245] and 8 (38%) of 21 in the second study[246] demonstrated substantial erythroid responses, including patients with prior RBC transfusion requirements. Responses were more frequent in patients with less advanced pancytopenia, lower endogenous EPO levels, [245] [246] or responsive marrow BFU-E.[244] Patients with ringed sideroblasts, who generally respond poorly to EPO alone, had an increased response rate (60% in this study). These findings suggest synergistic in vivo effects of G-CSF and EPO for the anemia of patients with MDS.

To determine which pretreatment variables predicted erythroid responses to EPO plus G-CSF combination therapy, 98 patients in a multi-institutional trial were evaluated.[247] Overall, 36% of patients showed responses to treatment, particularly those with the RARS subtype of MDS (Table 60-7). In multivariate analysis, the major clinical variables predicting responses to the combination therapy were baseline serum EPO levels and initial red cell transfusion requirement. With pretreatment serum

TABLE 60-8 -- Model for Predicting Erythroid Responses to G-CSF plus EPO in MDS Patients

Variable	Scores[a]					
	3	2	1	0	+1	+2
Serum EPO (U/l)	>500				100500	<100
RBC transfusions (U/mo)		2				<2

Predictive Score	Response Group	
	Type	% Responders (pts.)
>+1	High	74 (22/29)
±1	Intermediate	23 (7/31)
<1	Low	7 (3/34)

From Hellstrom-Lindberg et al., [247] *with permission from Blackwell Science, Ltd.*

[a]Multivariate logistic regression analysis: weighted logistic coefficients.

EPO levels used as a ternary variable (<100, 100500, or >500 U/L) and red cell transfusion requirement as a binary variable (<2 or 2 units per month), a statistically weighted analysis provided a predictive score for erythroid response. Based on this score (Table 60-8), patients were separated into three groups: one group with a high probability of erythroid responses (74% of responders), one group with an intermediate probability (23%), and one group with a low probability (7%). This predictive scoring system appears helpful for decision making regarding the use of these cytokines for treating the anemia of MDS. A recent extension of this trial indicated good durability of the responses (median, 24 months) for patients receiving maintenance therapy with these drugs. [248]

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PROGNOSIS

Marrow Histologic Features

The FAB morphologic classification, developed in 1982, has been useful for helping to determine prognosis in MDS. Although MDS is a heterogeneous disorder with an incidence evolution to AML varying between 10% and 50%, [10] there has been a moderate degree of consistency in prognostic findings in relation to survival and evolution to AML in a number of major large studies when FAB subgroup morphologic criteria were used (Table 60-9 , Fig. 60-6).[249] [250] [251] [252] [253] [254] [255] [256] Patients with RAEB and RAEB-T have had relatively poor prognoses, with median survivals generally ranging from 5 to 12 months, in contrast to RA or RARS patients, who have had median survivals of about 36 years. The proportion of individuals in whom disease transformed to AML varied similarly: in the high-risk RAEB and RAEB-T patients this incidence was 4050%, whereas in the low-risk group it was 515%. In a study evaluating time to disease evolution, 25% and 55% of patients with RAEB and RAEB-T, respectively, experienced transformation to AML at 1 year, and 35% and 65% at 2 years. In contrast, for patients with RA the incidence was 5% and 10% at 1 and 2 years. None of the

TABLE 60-9 -- Myelodysplastic Syndrome: Survival and Leukemic Evolution Related to Morphologic Subgroups[a]

	FAB Subgroups				
	RA	RARS	RAEB	RAEB-T	CMML
Median survival (mo)	43	73	12	5	20
Transformation to AML (%)	15	5	40	50	35
Proportion of patients (%)	25	15	35	15	10

[a]Meta-analysis of patient results from references [249] [250] [251] [252] [253] [254] [255]

Figure 60-6 Survival **(left)** and freedom from AML evolution **(right)** in patients with myelodysplastic syndrome who were evaluated by the International MDS Workshop, in relation to their FAB classification subgroup (Kaplan-Meier curves). RA, refractory anemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; RAEB-T, RAEB in transformation; CMML, chronic myelomonocytic leukemia. *(From Greenberg et al., [256] with permission.)*

patients with RARS experienced leukemic transformation within 2 years.

According to studies using the FAB classification, the major prognostic feature for survival of CMML patients (as for the other MDS subgroups) is the percentage of marrow blasts.[253] [255] [257] The median survival of CMML patients with <5% marrow blasts was 53 months, whereas for those with 520% blasts it was 16 months (similar to the median survival of patients with RAEB). Monocytosis of >2.6 × 10^9 /L and abnormal cytogenetics also correlated with poor survival. Controversy exists regarding whether the hypocellular subtype of MDS has prognostic significance, as these patients have been reported either to experience disease progression to AML less frequently and to have a longer survival, or to have a clinical outcome similar to that of other MDS patients. [140] [141] [142] A tendency toward hypocellularity in therapy-related MDS has been reported, and these patients generally have short survival times. [150] MDS patients with myelofibrosis generally have shorter survivals than MDS patients without fibrosis.[151] According to FAB proposals, the presence of Auer rods in myeloid cells implies the diagnosis of RAEB-T. [10] However, an adverse influence of Auer rods per se has not been demonstrated.

Mortality in MDS is due to a variety of causes, including evolution to AML, infection, or bleeding complications related to the patients dominant cytopenia. Because most patients are elderly, concomitant nonhematologic diseases associated with an elderly patient population also contribute substantially to the mortality. In RAEB and RAEB-T patients, AML is the cause of death in 2055% of patients, whereas infection and hemorrhage due to marrow failure cause 3650% of deaths and nonhematologic causes account for 1020%.[249] [250] [251] [252] [253] [254] [255] In RA and RARS, these figures are somewhat reversed, with AML causing death in 029%, infection and hemorrhage causing death in 1544%, and nonhematologic causes accounting for 2542% of deaths.

As adjuncts to FAB morphologic categorization, other evaluative systems using clinical or cytogenetic features have also been used for prognostic assessment. In all of these systems the proportion of type I marrow blast cells is the most useful clinical prognostic marker in MDS, contributing to the striking differences in survival and progression to AML observed among the FAB subtypes.[249] [250] [251] [252] [253] [254] [255] [256] [257] [258] Survival shortens as the percentage of marrow blast cells increases. [253] [254] [256] These studies have reported clear differences in survival for patients with <5% and 5% marrow blasts, with the survival curves of patients with 1020% and 2030% marrow blasts being almost identical (Fig. 60-7). The percentage of marrow blast cells was a significant factor in the differences in the risk of AML developing (Fig. 60-7). The cumulative probability of evolution to AML for patients with >20% marrow blasts was nearly 100% at 30 months,

Figure 60-7 Survival **(left)** and freedom from AML evolution **(right)** in patients with myelodysplastic syndrome, in relation to percentage of marrow myeloblasts (Kaplan-Meier curves). *(From Greenberg et al., [256] with permission.)*

Figure 60-8 Survival **(left)** and freedom from AML evolution **(right)** in patients with myelodysplastic syndrome, in relation to the number of cytopenias initially present (Kaplan-Meier curves). The two

heavy lines in each graph indicate patients with no or one cytopenia; the two light lines indicate patients with two or three cytopenias, respectively. *(From Greenberg et al.,[256] with permission.)*

a finding emphasizing that the border between RAEB-T and AML is somewhat arbitrary.[267] These data indicate that the use of a cutoff figure of 10% marrow blasts (i.e., including RAEB and RAEB-T patients with >10% blasts) adds substantial prognostic significance to the current FAB criteria.

Despite multiple disparate prognostic risk analysis systems for evaluating clinical outcome for MDS patients, imprecision persists with such analyses. In an attempt to improve upon these systems, an International MDS Risk Analysis Workshop combined cytogenetic, morphologic, and clinical data from 816 patients with untreated primary MDS.[256] The data were collated from seven large previously reported risk-based studies that had generated prognostic systems, and analyzed centrally. [256] A global analysis performed on the patient data and on critical prognostic variables was re-evaluated to generate a consensus prognostic system, one that used a more refined marrow cytogenetic classification. On univariate analysis, the major variables affecting evolution to AML were percentage of marrow myeloblasts, number of cytogenetic abnormalities, and number of cytopenias (Figs. 60-7 and 60-8); for survival, in addition to the above, variables included age and sex. Cytogenetic subgroups associated with relatively good outcomes were: normal cytogenetics, deletion of the Y chromosome alone, del(5q) alone, or del(20q) alone; poor: a complex karyotype (i.e., three or more abnormalities) or chromosome 7 anomalies; intermediate: other abnormalities (Figs. 60-9 and 60-10). Multivariate analysis combined this cytogenetic subclassification with percentage of marrow blasts and number of cytopenias to generate a prognostic model termed the International Prognostic Scoring System (IPSS) (Table 60-10). Weighting these variables by their statistical power allowed separation of patients into distinctive risk subgroups (low, INT-1, INT-2, high) for evolution to AML and survival (Fig. 60-11). Stratification for age further improved analysis of survival (Table 60-11 , Fig. 60-12).

Compared with prior risk-based classifications, the IPSS has provided an improved mechanism for evaluating prognosis in MDS. This effect was the result of several features of the International MDS Workshop model: the more refined cytogenetic categorizations, inclusion of cytopenias, improved subdivision of marrow blast percentages, the four defined outcome subgroups, and the separate stratification for age. The prediction of leukemia-free survival has also been possible with this method (Table 60-12). This classification system should prove

Figure 60-9 Freedom from AML evolution in 759 patients with myelodysplastic syndrome, in relation to their individual cytogenetic categories (Kaplan-Meier curves). *(From Greenberg et al.,[256] with permission.)*

Figure 60-10 Survival **(left)** and freedom from AML evolution **(right)** in patients with myelodysplastic syndrome, in relation to their risk-based categorical cytogenetic subgroupsgood, intermediate, or poor (Kaplan-Meier curves). Good denotes normal, del(5q) only, del(20q) only, or Y only. Poor denotes a complex karyotype (i.e., 3 anomalies) or chromosome 7 abnormalities. Intermediate denotes other abnormalities. *(From Greenberg et al.,[256] with permission.)*

TABLE 60-10 -- International Prognostic Scoring System for MDS

Prognostic Variable	Survival and AML Evolution Score Value				
	0	**0.5**	**1.0**	**1.5**	**2.0**
Marrow blasts (%)	<5	510		1120	2130
Karyotype[a]	Good	Intermediate	Poor		
Cytopenias[b]	01	23			

Risk Category	Combined Score
Low	0
INT-1	0.51.0
INT-2	1.52.0
High	2.5

Modified from Greenberg et al.,[256] with permission.

[a]Good = normal, Y, del(5q), del(20q); poor = complex (3 abnormalities) or chromosome 7 anomalies; intermediate = other abnormalities.
[b]Neutrophils < 1,800/l, platelets < 100,000/l, hemoglobin < 10g/dl.

Figure 60-11 Survival **(left)** and freedom from AML evolution **(right)** in patients with myelodysplastic syndrome, in relation to their classification by the International Prognostic Scoring System (IPSS) for MDS: Low, Int-1, Int-2, or High (Kaplan-Meier curves). *(From Greenberg et al.,[256] with permission.)*

TABLE 60-11 -- Age-Related Survival and AML Evolution in MDS Patients Within the International Prognostic Scoring System Subgroups

	Median Survival (yr)				
	No. of Patients	**Low**	**Int-1**	**Int-2**	**High**
Total pts.: No. (%)	816	267 (33%)	314 (38%)	176 (22%)	59 (7%)
		5.7	3.5	1.2	0.4
Age 60 yr	205 (25%)	11.8	5.2	1.8	0.3
>60 yr	611	4.8	2.7	1.1	0.5
70 yr	445 (54%)	9.0	4.4	1.3	0.4
>70 yr	371	3.9	2.4	1.2	0.4

	25% AML Evolution (yr)				
	No. of Patients	**Low**	**Int-1**	**Int-2**	**High**
Total pts.: No. (%)	759	235 (31%)	295 (39%)	171 (22%)	58 (8%)
		9.4	3.3	1.1	0.2
Age 60 yr	187 (25%)	> 9.4 (NR)	6.9	0.7	0.2
> 60 yr	572	9.4	2.7	1.3	0.2

70 yr	414 (55%)	> 9.4 (NR)	5.5	1.0	0.2
>70 yr	345	> 5.8 (NR)	2.2	1.4	0.4

Abbreviation: NR, not reached.

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useful for more precise design and analysis of therapeutic trials in MDS.

Studies analyzing plastic-embedded marrow biopsy specimens have suggested that the identification of small clusters of blast cells in central marrow regions, referred to as ALIP (abnormal localization of immature myeloid precursors) (Fig. 60-5E), rather than in paratrabecular locations had prognostic relevance. [251] [252] [253] [254] [255] [256] [257] [258] ALIP-positive patients had significantly shorter survival in all subtypes of MDS, including those patients with <5% blasts. ALIP-positive patients were somewhat more common in RAEB, RAEB-T, and CMML.

Marrow Cytogenetics

Approximately 4060% of patients with primary MDS have abnormal marrow karyotypes by conventional karyotyping methods; [256] [258] [259] however with more refined techniques, chromosome abnormalities have been detected in up to 73% of cases. [260] Clinical correlative studies with multivariate analyses have established that marrow cytogenetic features are an independent prognostic factor in MDS, complementing analyses based on clinical patient features. The highest prevalence of aberrations is concentrated in the poor prognostic subgroups of the FAB classification (RAEB and RAEB-T). In early studies, MDS patients with normal karyotypes had better prognoses than patients with abnormal chromosomes. However, investigations using more precisely defined abnormal karyotypes have modified this conclusion. Current data indicate that patients with poor prognoses are those with marrow cell clones that have complex chromosome abnormalities [256] [259] [260] [261] [262] [263] [264] [265] or who have a single chromosome abnormality involving 7 or 7q or +8 (Figs. 60-9 and 60-10). Conversely, patients with the 5q deletion

Figure 60-12 Survival according to age 60 years **(left)** or >60 years **(right)** of myelodysplastic syndrome patients in relation to their classification by the International Prognostic Scoring System (IPSS) for MDS: Low, INT-1, INT-2, or High (Kaplan-Meier curves). *(From Greenberg et al.,[256] with permission.)*

TABLE 60-12 -- Survival of Myelodysplastic Syndrome Patients with or Without AML Evolution: Leukemia-Free Survival

Subgroups	No. of Patients	Patients Died	Patients Died with Leukemia	Patients Died Without Leukemia
		No. (%)	No. (%)	No. (%)
Low	235	113 (48)	22 (19)	91 (81)
Int-1	295	181 (61)	55 (30)	126 (70)
Int-2	171	147 (86)	49 (33)	98 (67)
High	58	51 (88)	23 (45)	28 (55)
Total	759	492 (65)	149 (30)	343 (70)

Modified from Greenberg et al.,[256] with permission.

(i.e., del (5) (q12q33)) as the sole abnormality have relatively longer survivals. [33] [37] [38] However, the 5q abnormality combined with other karyotypic derangements is indicative of a poor prognosis. Survival was >2 years in cases with either normal chromosomes or 5q, 12 years in cases of trisomy 8, and <1 year in cases with either deletions of chromosome 7 or multiple changes.

In the International MDS Workshop patients, individuals with del(20q) only, del(5q) only, Y, or normal karyotypes had improved outcomes. [256] These findings regarding del(20q) as the sole abnormality are similar to those recently reported for a smaller group of MDS patients. [266] In a recent report in which patients with del(20q) had complex karyotypes, an advanced MDS stage or AML and a poor prognosis were noted. [267] Together, these data suggest that del(20q) may be associated with a favorable outcome when it occurs as a sole abnormality, but has a less favorable prognosis in the setting of a complex karyotype. This phenomenon is analogous to that observed with del(5q).

Loss of the Y chromosome in elderly men has been described in the marrows of patients with hematologic malignancies, but this abnormality has also been noted in marrow samples from hematologically normal elderly men.[268] Thus, this finding alone does not indicate the presence of a myeloid clonal hemopathy. However, once the diagnosis is established by other means, this feature conferred an improved clinical outcome. Conversely, chromosome 7 anomalies and complex cytogenetic abnormalities (variously defined) have been associated with poor prognoses. [256] [259] [261] Multiple clones were common in this group. The presence of abnormalities of chromosomes 5 and 7 has been associated with poor outcomes in MDS and AML.[259] [261] Other anomalies were associated with intermediate risk. These cytogenetic correlative findings also suggest that genomic instability and biologically important genes may be present on these chromosomes which alter survival and the potential for leukemic evolution in MDS patients.

The importance of karyotypic analysis for prognosis has been further demonstrated. When cytogenetic features were omitted from the MDS Workshop analysis, relatively poorer discrimination of clinical outcome occurred. [262] In this analysis, a substantial proportion of IPSS INT-1 and INT-2 patients would have been inaccurately categorized as low risk had cytogenetics not been included. In addition, cytogenetic analysis is potentially helpful in distinguishing patients who are likely to have evolving AML, rather than the more indolent MDS. Certain types of cytogenetic abnormalities, particularly recurring translocations, are rarely seen in MDS [259] [263] [264] [265] but are not uncommon in AML,[259] [263] [264] [265] such as trisomy 21, t(8;21), or 11q abnormalities. The International MDS Workshop data also indicated a very low incidence of these abnormalities in MDS.[256] Consistent with this thesis is the recent report indicating that t(8;21) myelodysplasia is an early presentation of M2 AML, with rapid clinical progression.[269]

The IPSS was compared to other MDS prognostic systems to determine its relative discriminatory ability for assessing the natural history of the disease. Thus, compared to prior prognostic systems for categorizing patents with MDSthe FAB system (based on marrow blast percentage), [10] the Spanish system (marrow blasts, age, platelet count),[253] and the Lille system (platelet count, marrow blasts, karyotype) [263] the IPSS effectively discriminated between the defined subgroups of the earlier categorization systems and provided an improved tool for predicting survival and AML evolution. [256]

The greater prevalence of complex chromosomal abnormalities in patients with secondary MDS partly explains their poor prognosis. [259] [261] The acquisition of a chromosomally abnormal clone in MDS patients with a previously normal karyotype or of additional karyotypic changes in a previously abnormal clone is associated with progression to a more aggressive FAB subtype or AML evolution and early death. [270] [271] However, karyotype stability does not preclude transformation to AML, as the majority of MDS patients do not show new chromosomal abnormalities at the time overt AML appears.[270] [271]

In vitro Myeloid Colony Assays

Based on the behavior of myeloid progenitor cells in vitro, MDS may be divided into leukemic and nonleukemic patterns. [40] Leukemic-type growth includes micro- or macrocluster formation with defective maturation or blasts within the aggregates, single persisting blasts, or very low colony formation (<2 colonies per 10^5 marrow cells). Nonleukemic growth is marked by persisting colony formation, even if moderately decreased in frequency. As shown in Table 60-13 , six studies involving 179 MDS patients

TABLE 60-13 -- Prognosis of Myelodysplastic Syndrome: Utility of in vitro Marrow Myeloid Clonogenic Culture Studies

Growth Patterns	Incidence (%)	Transformation to AML (%)	Median Survival (mo)
RAEB-T (n = 80)[272] [273]		51 (4560)	9 (711)
Nonleukemic growth	33 (2738)	31 (2933)	20 (1525)
Leukemic growth	68 (6273)	60 (5070)	7 (58)
RAEB (n = 17)[274] [275]		41	14
Nonleukemic growth	70	29	21
Leukemic growth	30	100	10
RA (n = 82)[273] [276] [277]		39 (3544)	24 (920)
Nonleukemic growth	54 (3074)	20 (2140)	47 (950)
Leukemic growth	46 (2670)	60 (5080)	8 (410)

Values are means and ranges of means for cited studies.

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with differing FAB morphologic subtypes demonstrated correlation between clinical outcome and in vitro marrow growth. [40] [272] [273] [274] [275] [276] [277] When patients were stratified according to their in vitro myeloid growth patterns, subgroups of MDS patients with nonleukemic growth patterns had a 2031% incidence of transformation to AML and 20- to 47-month median survivals. In contrast, MDS patients with leukemic growth patterns had a 60100% incidence of transformation and 7- to 10-month median survivals. MDS patients with single hemopoietic cell line defects, such as idiopathic sideroblastic anemia or idiopathic neutropenia with a low propensity to leukemic evolution, had normal marrow granulopoietic growth parameters. Patients who died without experiencing transformation to AML generally did so as a result of infectious or bleeding complications. The decreased marrow granulopoiesis in vitro (low frequency of normal CFU-GM) may reflect the diminished ability of the marrow to respond to the demand for new cells. Factors other than in vitro growth patterns contribute to transformation, as not all patients with abnormal clonal growth had poor prognoses. A correlation has been demonstrated between in vitro myeloid growth patterns, abnormal marrow cytogenetics, and poor prognoses. [40] The findings of decreasing CFU-GM plating efficiency, a higher proportion of light-density CFU-GM, and increased cluster/colony ratios are functional evidence of clonal evolution and prognostic information as these diseases progress toward acute transformation.

An in vitro colony assay permits growth of blast cell progenitors from marrow and blood of patients with AML. [278] Whereas these colonies are generally not present in cells from normal individuals, the majority of patients in a study of MDS had these circulating blast cell progenitors, some of which were in active cycle. [279] Further investigations correlating this in vitro feature with peripheral blasts, clinical status, and subsequent course will be important to determine the significance of this finding.

Therapeutic Directions

Because of the generally elderly age of patients with MDS, the relatively poor complete responses to most standard therapeutic modalities, and the variability of prognoses of patients with these disorders, the treatment of these individuals remains problematic. Needing evaluation are growth factors required to augment platelet levels in an attempt to modify a major morbidity related to the thrombocytopenia that occurs in this disease. Until results of these trials are available, the extended use of these and other HGFs in MDS should be considered experimental.

THERAPEUTIC OPTIONS FOR MDS

Supportive care: Antibiotics, transfusions, iron chelation as needed

Hemopoietic growth factors (HGFs): Eythropoietin (EPO), G-CSF, GM-CSF

Biologic response modifiers (BMRs): Amifostine, pentoxyfylline, lisofylline, interferon-, ATG, cyclosporin, retinoids, vitamin D analogs

Low-intensity chemotherapy: Low doses of cytarabine, topotecan, decitabine, azacytidine, hydroxyurea

High-intensity therapy: AML induction chemotherapy ± MDR modulator (i.e., rAML therapy); bone marrow or peripheral blood stem cell (PBSC) transplantation; allogeneicmatched sibling or matched unrelated donor transplantation; autologous

Combinations

MDS MANAGEMENT APPROACHES

The International Prognostic Scoring System is described in Greenberg et al. [256] (see Table 60-10 for scoring method). For patients lacking cytogenetic analysis, High risk includes categories RAEB-T, and RAEB and CMML in those with >10% marrow blasts. Therapy generally should be performed in the context of clinical trials. Options are given in order of preference. If patients initially in the Low or Int-1 risk category progress to Int-2 or High risk category, their therapeutic options correspondingly are those of the higher risk categories. The age determination may be based on the patients biologic or chronologic status, according to the particular institutions guidelines for high-intensity therapy, including bone marrow transplantation. Performance status is based on the institutions guidelines, with patients evaluated after remediable conditions have been corrected.

Treatment definitions: rAML induction therapy denotes chemotherapy induction for resistant or refractory AML (i.e., with MDR-1 modulators, or as part of clinical trials). Post-induction therapy includes rAML consolidation performed in the context of clinical trials. Low-intensity therapy includes chemotherapy, cytokines, and biologic response modifiers. Supportive care includes transfusions, antibiotics as needed, and the treatment of symptomatic anemia with erythropoietin with or without G-CSF, as indicated in the previous box. For bone marrow transplantation, allogeneic marrow transplantation or (PBSC transplantation) from an HLA-matched sibling is preferred, but transplants from an HLA-matched unrelated donor may be considered if no HLA-matched sibling donor is available.

IPSS RISK CATEGORY HIGH OR INTERMEDIATE-2

Age <60 years

Performance status good

Bone marrow transplantation

rAML induction with BMT at remission (versus other post-induction therapy at remission)

Low-intensity therapy

Supportive care

Performance status poor

Supportive care

Low-intensity therapy

Age >60 years

Performance status good

Low-intensity therapy

rAML induction with post-induction therapy at remission

BMT

Supportive care

Performance status poor

Supportive care

Low-intensity therapy

IPSS RISK CATEGORY INTERMEDIATE-1 OR LOW

Age 60 years *and* performance status good

Low-intensity therapy

BMT

rAML induction with BMT at remission (versus other post-induction therapy at remission)

Supportive care

Age >60 years *or* performance status poor

Supportive care

Low-intensity therapy

MANAGEMENT OF ANEMIA IN MDS

DIAGNOSTIC EVALUATION

Determine etiology of the anemia (peripheral smear, marrow)

Assess iron status (serum tests, marrow)

Rule out coexisting causes of anemia (e.g., GI bleeding, hemolysis, renal disease, nutritional deficiency, drug toxicity)

Obtain serum erythropoietin (EPO) level

TREATMENT OF SYMPTOMATIC ANEMIA (generally Hb <10 gm/dl)

Treat coexisting causes

Replete iron, folate, etc. as needed

For hypoproductive anemia:

1. RBC transfusions (leuko-reduced)
2. For patients lacking ringed sideroblasts, serum EPO <200500 mU/ml:
   a. rHu erythropoietin (concomitant treatment with oral iron may be beneficial, even without iron deficiency) 150300 U/kg/day SC × 23 months (20,000 U/ml EPO vial may be useful). If response, continue EPO and gradually decrease dose to tolerance.
   b. For nonresponders, consider adding rHuG-CSF, 1 g/kg/day SC (multi-doses may be used from G-CSF vial, stored refrigerated) (0.33 g/kg/day, general range) keeping neutrophil count in normal range (multi-doses may be used from G-CSF vial, stored refrigerated).
   c. Assess response at 23 months. For erythroid responders, continue drugs with gradual decrease of doses to tolerance; for nonresponders, discontinue the drugs.
3. For patients with ringed sideroblasts, serum EPO <500 mU/mL:
   a. Use combination of G-CSF plus EPO, with above doses.
   b. Assess response at 46 weeks. For erythroid responders, continue drugs with gradual decrease of doses to tolerance; for nonresponders, discontinue the drugs.
4. For patients with serum EPO >500 mU/mL or EPO nonresponders:
   a. Use RBC transfusions (leuko-reduced)
   b. Relevant clinical trials

The standard of care for MDS remains supportive therapy and treatment of symptoms. However, subgroups of MDS patients (based on age, clinical condition, and prognostic risk category) are considered for other specific forms of therapy. Using these criteria, some management strategies have been suggested. The National Comprehensive Cancer Network (NCCN), a consortium of medical institutions in the United States, recently attempted to provide evidence-based guidelines for developing management guidelines for a variety of neoplasms. [280] A panel of investigators from medical centers affiliated with the NCCN convened and helped to develop and expand such guidelines for MDS. The panel suggested use of the IPSS classification system, age, and performance status as major determinants for therapeutic strategies. Based on these critical variables and on evidence-based data, therapeutic approaches that attempt either to change the diseases natural history or to treat the anemia of MDS have been designed (see boxes). As there have been few controlled trials indicating improved survival or freedom from AML evolution with treatment, compared to supportive care, the panel strongly suggested that most of the approaches currently in use should be evaluated in clinical trials. The IPSS classification system improves the ability to define clinical outcome in MDS and also provides a framework for future studies to determine the possible role of molecular determinants (e.g., oncogenes, tumor suppressor genes, cytokine expression, and responsiveness) for evaluating prognosis in this disorder. Appropriately designed clinical trials evaluating these treatment approaches are needed to improve the management of patients with this disorder.

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Chapter 61 - Polycythemia Vera

Ronald Hoffman

INTRODUCTION

Polycythemia vera is a hematologic malignancy that leads to excessive proliferation of erythroid, myeloid, and megakaryocytic elements within the bone marrow. Vaquez[1] first described this clinical entity in 1892, noting the characteristic physical findings. At the turn of this century, Cabot [2] and Osler[3] independently associated the name polycythemia vera with this newly described clinical disorder. Polycythemia vera is a clonal, chronic, progressive myeloproliferative disorder, often of insidious onset, characterized by an absolute increase in red cell mass and also usually by leukocytosis, thrombocytosis, and splenomegaly. [4] [5]

New insight into the pathogenesis of this disorder has been gained. Studies have been performed that demonstrate the hypersensitivity of hematopoietic progenitor cells derived from the malignant clone to a number of regulatory factors. [6] [7] [8] [9] In the absence of evidence for changes in the number or structure of receptors for the known hematopoietic growth factors, this heightened responsiveness has been attributed to an undefined event that occurs at the level of a cytokine receptor or an event triggered following activation of these cytokine receptors. [10]

Polycythemia vera differs from many other hematologic malignancies, in that prolonged survival is enjoyed by most patients if the excessive production of red blood cells and platelets can be controlled. [11] This prolonged survival, however, is punctuated by the development of other syndromes, such as myelofibrosis and acute leukemia (Table 61-1).[11] [12] [13] Frequently patients present asymptomatically to a physician only to find that they

TABLE 61-1 -- Evolution of Polycythemia Vera

Stage	Clinical Findings
Asymptomatic	Splenomegaly
	Isolated erythrocytosis
	Isolated thrombocytosis
Erythrocytotic phase	Erythrocytosis
	Thrombocytosis
	Leukocytosis
	Splenomegaly
	Thrombosis
	Hemorrhage
	Pruritus
Inactive phase	No longer requires phlebotomy or chemotherapy
	? Iron deficient
Postpolycythemic myeloid metaplasia	Anemia
	Leukoerythroblastosis
	Thrombocytopenia or thrombocytosis
	Enlarging splenomegaly
	Systemic symptoms (fever, weight loss)
Acute myeloid leukemia	

have either splenomegaly, isolated erythrocytosis, or thrombocytosis; left untreated, these patients will become symptomatic, owing to the consequences of the excessive production of red blood cells or platelets, or both. After a number of years, the erythrocytotic phase of the disease frequently becomes inactive, and the patient may no longer suffer from the sequelae of excessive red cell production. Subsequently, these patients can develop so-called spent-phase or postpolycythemic myeloid metaplasia, which is frequently indistinguishable from another myeloproliferative disorder, agnogenic myeloid metaplasia. [11] [12] [13] Finally, a significant proportion of these patients will eventually go on to develop acute myeloid leukemia.

The transition from one phase of this myeloproliferative disorder to another is not necessarily unidirectional. A number of cases of presumed agnogenic myeloid metaplasia have been described in which chemotherapy treatment has resulted in a striking decrease in marrow fibrosis associated with the development of an elevated red cell mass and a syndrome which was virtually indistinguishable from de novo polycythemia vera. [14]

The constantly changing clinical picture of this malignant hematologic disorder requires careful observation and treatment to deal with the numerous problems that can be encountered.

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EPIDEMIOLOGY

Polycythemia vera is a rare disorder, with an annual incidence in Western Europe and the United States of approximately 517 cases per 1 million population per year.[15] [16] [17] Actual determination of its prevalence is a difficult process because of the need for an extensive diagnostic evaluation to differentiate this disorder from other causes of spurious or absolute erythrocytosis. [18] The prevalence of polycythemia vera has been stated by several investigators to be higher among American Jews and lower among African Americans.[15] [19] [20] The incidence of the disorder is greater among Ashkenazi Jews, who originate from eastern and central Europe, than among Arabs and Sephardic Jews.[21] Interestingly, extremely low occurrence rates have been reported from Japan, where the incidence was found to be two cases per million per year.[22] These findings suggest that important environmental or genetic factors might be involved in the biogenesis of this disorder. One notable exception to the low prevalence of polycythemia vera in Japan has been the higher incidence observed among populations exposed to atomic bomb explosions. [23] The possibility that radiation exposure is an etiologic factor in the generation of polycythemia vera was also raised by the observation in the United States of four cases of polycythemia vera 1020 years after a nuclear explosion to which 3,000 military observers were exposed. [24]

An epidemiologic investigation focused on occupational exposure among petroleum refinery and chemical plant workers has revealed an increased incidence of polycythemia vera relative to the general population. [25] In this study, the increased incidence of polycythemia vera was linked to similar increases in the frequency of multiple myeloma and non-Hodgkins lymphoma, suggesting involvement of a putative environmental toxin that may have broad hematopoietic toxicity. [25]

The importance of genetic factors in the origin of this disease is further emphasized by several case reports of polycythemia vera within families. [26] [27] [28] [29] [30] [31] [32] A greater than expected prevalence has been reported in the parents of patients with this disorder. [30] In addition, one family of three sisters all having the disorder has been reported.[31] An additional report describes a father and son who were both exposed to organic solvents and in whom polycythemia vera subsequently developed.[32] These forms of familial polycythemia vera must be distinguished from primary familial congenital polycythemia (PFCP) which is characterized by isolated erythrocytosis and is inherited as an autosomal dominant. The erythrocytosis in these patients is manifested at birth. [33] [34] [35] [36] To date, there have been seven different reported mutations of the erythropoietin receptor reported in patients with PFCP or probable PFCP. [33] [34] [35] [36] [37] Each of these mutations are located in exon 8 of the gene for the erythropoietin receptor resulting in a truncated protein that lacks a portion of the negative regulatory domain that leads to progenitor cell hypersensitivity to erythropoietin. Although this was originally perceived as a benign disorder, there have been recent reports of premature cardiovascular events, including myocardial infarction, ischemic cerebrovascular events, and intracranial hemorrhage occuring in these patients. [36] [37] The reports of families in which multiple members have polycythemia vera have also raised the possibility that a genetic predisposition occurs in concert with several additional external insults which might lead to the development of polycythemia vera. Slightly more males than females develop this disorder; the male/female ratio is approximately 1.2:1. [15] The average age at diagnosis is 60 years,[15] and the disease is extremely rare in patients <30 years of age. In several large studies, 5% of patients with polycythemia vera were <40 years of age, 1% were <25 years old at diagnosis, and 0.1% were calculated to be <20 years old. [4] [12] [38] To date, only a handful of patients with polycythemia vera have been reported who presented with this disorder during childhood. [39]

BIOLOGIC AND MOLECULAR ASPECTS

Considerable speculation has centered on the pathobiology of the erythrocytosis that characterizes polycythemia vera. [10] [40] London et al.[41] established that the expanded red cell mass of polycythemia vera was due to a two- to threefold increase in the production of red blood cells by a hyperplastic marrow and was not attributable to prolongation of the red cell life span. Granulocyte and platelet production are also increased in this disorder. This overly exuberant production of all cellular elements of the blood suggests that the basic defect resides at the level of the cell from which each of these cells originates, the pluripotent hematopoietic stem cell.

The hematopoietic growth factor, erythropoietin (EPO) is considered to be the physiologic regulator of the later phases of erythropoiesis. [42] Alterations in its production are followed by adjustments in the rate of formation of red blood cells. [43] In humans, EPO production is controlled by the relative supply of oxygen to the kidney, the major site of erythropoietin formation.[42] Studies in animals have indicated that in response to anemia the liver may contribute 1025% of the total EPO mRNA. EPO production appears to be regulated by alterations in the rate of gene transcription. [44] [45] Hypoxic stimulation of the oxygen sensor present in renal interstitial cells results in increased production of hypoxia inducible factor-1 (HIF-1), which is the major enhancer of EPO gene transcription. [46] EPO stimulates red cell production by increasing proliferation, decreasing apoptosis, and promoting differentiation of erythroid progenitor or precursor cells. [47] [48] Committed erythroid progenitor cells ultimately originate from a pluripotential hematopoietic stem cell also capable of producing myeloid and megakaryocytic elements. [42] Steel factor, the ligand for c-*kit*, is also strongly implicated in the earliest steps of erythropoietic development from the pluripotential hematopoietic progenitor cell. [49] [50] Interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF) and insulin-like growth factor-1 (IGF-1) may contribute to the early proliferative expansion of these multipotential progenitors. [49] [50] [51]

Alterations in EPO production can lead to the development of anemia and erythrocytosis. [43] [52] An increased red cell mass, for instance, can be the result of EPO elaboration resulting from chronic hypoxia or can be the consequence of EPO secretion by a tumor or cyst, which is independent of physiologic control mechanisms.[52] [53] By contrast, decreased production of EPO is an important component in the pathobiology of the anemia of chronic renal failure. [54] This deficiency was the initial focus for pharmaceutical development of EPO, an endeavor that has been highly successful. [54]

A large body of information has been accumulated over the years addressing EPO physiology in patients with erythrocytosis. [52] [53] A variety of assays for quantitation of levels of this hormone have been developed. EPO concentrations in the past have been estimated by in vivo bioassays using posthypoxic polycythemic mice. [55] Following phlebotomy of a healthy person, urinary EPO excretion increases, and an inverse logarithmic relationship between hematocrit and EPO excretion rate is observed.[43] Patients with secondary erythrocytosis due to chronic hypoxia have either normal or increased basal values, but all have increased values following reduction of hematocrit to normal levels by phlebotomy. [43] By contrast, urinary EPO excretion is invariably subnormal in patients with polycythemia vera, which demonstrates that this disorder is not a result of excessive EPO production. [43] [56]

Following the purification of EPO and its subsequent cloning, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques for quantitating this hormone have been developed and have now entered widespread clinical use. [56] [57] [58] These assays have proven useful in further defining the role of EPO in the biogenesis of erythrocytosis.

A variety of studies have documented serum EPO levels in patients with primary polycythemia vera, secondary erythrocytosis, and relative polycythemia, and in normal adults. Serum EPO levels have been shown to be subnormal in patients with polycythemia vera, elevated in all cases of secondary erythrocytosis, and normal in patients with relative polycythemia (Fig. 61-1)[39] [56] [57] [58] Numerous studies have indicated that classification

Figure 61-1 Serum EPO levels in polycythemia vera, relative to secondary polycythemia. The middle bar represents median, the boxes, quartiles, and the end bars, 95% range. The open circles represent individual values outside the 95% range. *(From Birgegaard and Wide,[58] with permission from Blackwell Science, Ltd.)*

of 90% of patients with erythrocytosis can be achieved by quantitating serum EPO. [43] [56] [57] [58]

These studies have indicated that patients with polycythemia vera most frequently exhibit serum EPO levels below the 95% confidence intervals for the range observed in normal controls. [56] [57] [58] The subnormal serum EPO levels are maintained even after several phlebotomies have been performed that normalize the serum hemoglobin concentration in polycythemia vera patients. [56] [57] [58]

In 1951 Dameshek [5] postulated that chronic myeloid leukemia (CML), polycythemia vera, primary thrombocythemia, and agnogenic myeloid metaplasia with myelofibrosis were related disorders, which he called myeloproliferative syndromes. He concluded that these disorders resulted from a generalized hyperresponsiveness of marrow cells to myelostimulatory factors and speculated that these disorders were neoplastic in origin. [5]

Since the mid-1970s, a substantial amount of data has accumulated that conclusively demonstrates polycythemia vera to be the result of a neoplastic proliferation of hematopoietic cells.[59] The cellular origin of the disorder was first established by the analysis of glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in African-American women who were heterozygous for this X-linked gene (Fig. 61-2). This approach was based on the random irreversible inactivation of one X chromosome in each female somatic cell during embryogenesis. Inactivation of the same X chromosome occurs in the progeny of these cells. [60] A normal African-American female heterozygous for G6PD will therefore have approximately equal populations of marrow cells with a different G6PD isoenzyme. [60] The G6PD isoenzymes can be readily distinguished by electrophoretic methods.

This approach was exploited in a seminal study by Adamson and coworkers[61] in an effort to determine the cellular origin of polycythemia vera. They presumed that cells composing a tumor that arises from a single cell in a G6PD heterozygote would express a single isoenzyme type, whereas a neoplasm originating from multiple cells would express both isoenzyme types. [60]

Figure 61-2 Schematic presentation of X chromosome inactivation in an embryo heterozygous at the G6PD locus (Gd^{b}/Gd^{a}). In this diagram, the maternal X chromosome bears an A gene for G6PD(X^{A}) and the paternal X chromosome a B gene (X^{B}). During embryogenesis, one X chromosome in each somatic cell is randomly inactivated, so that half the somatic cells will have an active X^{A} and the other half an active X^{B}. Inactivation is fixed for a particular cell and its progeny. All progeny of an active X^{A} gene will express X^{A}. Female G6PD heterozygotes are mosaics, with some

cells active X^A and others X^B . A tumor with clonal origin will consist entirely of either X^A or X^B cells and therefore will contain only G6PD type A or B but not both. Tumors with multicellular origin will contain both X^A and X^B cells and therefore both G6PD types. *(From Fialkow,[290] with permission.)*

TABLE 61-2 -- Relative Amounts of G6PD Isoenzymes in Various Mesenchymal Tissues in Two Patients with Polycythemia Vera

Tissue	%A%B	
	Case 1	Case 2
Skin	55:45	55:45
Lymphocytes	85:15[a]	55:45
Erythrocytes	100/0	100/0
Granulocytes	100/0	100/0
Platelets	100/0	100/0

From Adamson et al.,[61] with permission.

[a]Contaminated with erythrocytes.

These investigators found that circulating red cells, granulocytes, and platelets obtained from African-American females who were G6PD heterozygotes express the same isoenzyme, while skin and cultured marrow fibroblasts obtained from these same patients demonstrate both isoenzymes[61] [62] (Table 61-2). They concluded that polycythemia vera represented a clonal proliferation of neoplastic hematopoietic stem cells and was not multicellular in origin or the consequence of excessive proliferation of normal hematopoietic stem cells. [61]

The clonality of blood cell production in polycythemia vera has subsequently been confirmed using restriction fragment length polymorphisms of the active X chromosome.[63] [64] [65] A monoclonal pattern of X chromosome inactivation has been defined in red cells, granulocytes, monocytes, and platelets in females with polycythemia vera.[63] [64] [65] Such studies confirm the clonal cellular origin of the disorder.

Presently, it remains controversial whether lymphocytes are also the product of the malignant clone in polycythemia vera. This data is of some importance since it would provide insight into whether polycythemia vera is a disorder that originates at the level of a myeloid stem cell that is capable of producing red cells, granulocytes, platelets, and monocytes or rather originates at the level of a totipotent stem cell that is capable of producing not only myeloid cells but also lymphocytes. Alternatively, the presence of polyclonal lymphocytes in patients with polycythemia vera could be attributed to a neoplastic event occuring at the level of a totipotent stem cell resulting in a limited or total inability to give rise to lymphocytes. In a limited number of cases of polycythemia vera lymphocytes have been shown to originate from the malignant clone.[64] [65] The basis and significance of this apparent heterogeneity of cellular origin of polycythemia vera remains unknown.

On the basis of their knowledge that red cell production in polycythemia vera is not associated with excessive EPO production, several investigators have hypothesized that the erythroid progenitor cell in this disorder is no longer subject to physiologic regulators. [10] With the development of clonal assay systems for hematopoietic progenitor cells, it has become possible to analyze the effect of EPO on in vitro colony formation.

Two classes of erythroid progenitor cells, the burst-forming unit-erythroid (BFU-E) and the colony-forming unit-erythroid (CFU-E) present in the marrow and peripheral blood of patients with polycythemia vera were studied. Prchal and Axelrad[66] first reported that polycythemia vera bone marrow can form substantial numbers of erythroid colonies in vitro in the absence of exogenous EPO, whereas normal human bone marrow is incapable of forming such colonies without the addition of EPO. These erythroid colonies have been termed endogenous colonies.[66] [67] When both polycythemia vera and normal bone marrow were subsequently assayed in the presence of EPO, polycythemia vera marrow was characterized by a higher cloning efficiency.[66] [67] [68] Mixed colony formation has also been shown

to be enhanced in polycythemia vera.[68] Mixed colonies originate from multilineage progenitor cells. Their formation by polycythemia vera marrow, but not by normal marrow, also occurs in vitro in the absence of exogenous EPO.[69] These observations suggest that the altered response to EPO in polycythemia vera is characteristic not only of erythroid progenitor cells but of more primitive hematopoietic progenitor cells as well. Cell cycle analysis of polycythemia vera colony-forming unit-multilineage (CFU-GEMM) BFU-E, CFU-E, and colony-forming unit-granulocyte/macrophage (CFU-GM) revealed another cell progenitor abnormality; [69] [70] a higher proportion of polycythemia vera progenitor cells were in the synthetic phase of the cell cycle than was observed in normal subjects. [69] [70]

Further insight into the cellular defects in polycythemia vera was provided by the studies of Prchal et al. [70] (Table 61-3). These workers cloned marrow cells from African-American female G6PD heterozygotes with polycythemia vera both in the presence and in the absence of exogenous EPO and demonstrated that the erythroid colonies that formed in the absence of exogenous EPO contained the same G6PD isoenzyme type as that expressed by peripheral blood elements. [71] Thus, the so-called endogenous colonies arose from the abnormal clone that was responsible for supplying red cells, granulocytes, and platelets to the peripheral blood. When exogenous EPO was added, increasing numbers of colonies were formed containing cellular elements expressing the other G6PD isoenzymes; presumably these colonies originated from cells not involved in the malignant process. Similarly, small numbers of granulocyte/macrophage colonies not originating from the polycythemia vera clone were also observed in these assays. These data collectively indicate the existence of both malignant and nonmalignant populations of hematopoietic progenitor cells in polycythemia vera marrow. The relative frequency of the neoplastic clone in relationship to normal progenitor cells was further examined by Adamson and coworkers,[62] who, by monitoring the proportion of neoplastic erythroid clones and their numerical relationship to normal clones over a period of several years, showed disease progression to be associated with a significant decline in the frequency of normal colony-forming cells and increasing preponderance of the neoplastic clone. [62]

The clonal assay systems first used to obtain endogenous erythroid colonies contained serum contaminated with trace amounts of EPO. [67] This EPO contamination led to confusion about the responsiveness of the polycythemia vera cells to the actions of this hormone. Two conflicting hypotheses were entertained, one suggesting that proliferation of abnormal populations of erythroid progenitor cells is completely independent of EPO and the other consistent with hypersensitivity of polycythemia progenitors to EPO. Using an anti-EPO antiserum to remove trace amounts of EPO present in serum, Zanjani et al. [67]

TABLE 61-3 -- G6PD Isoenzyme Analysis of Erythroid Colonies Cloned from Marrow Cells of G6PD Heterozygotes with Polycythemia Vera

Erythropoietin (U/ml)	Patient 1		Patient 2	
	Colonies[a]	A/B[b]	Colonies[a]	A/B[b]
0	15	19/0	36	27/0
0.25	32	21/1	75	12/0
1.0	47	28/3	115	22/1
5.0	68	26/10	156	44/2
10.0			161	30/8

From Prchal et al.,[70] with permission.

[a]Colonies per 10^5 cells.

[b]Number of individual colonies analyzed of specific G6PD isoenzyme type per 10^5 cells.

concluded that erythroid progenitor cells from polycythemia vera patients do not proliferate in the absence of EPO but are, in fact, abnormally sensitive to the actions of this hormone. This increased responsiveness allowed these cells to form colonies in the presence of serum containing small amounts of EPO. By constructing EPO dose-response curves from polycythemia vera marrows and comparing them with those obtained from normal marrow cells, Eaves and Eaves[72] drew similar conclusions. Their studies showed that most polycythemia patients possess two distinct populations of erythroid progenitor cells: a normally EPO-responsive population and a population of cells similar in proliferative and maturational behavior in vitro but requiring little or no EPO. [72] These investigators suggested that because of the exquisite EPO sensitivity of the malignant clone, the proliferation of the normal progenitor cells in vivo was at a disadvantage. [72] The EPO dependence of polycythemia vera progenitor cells was further demonstrated by Casadevall and coworkers, [73] who used a serum-free culture system, which no longer was contaminated with EPO. They were unable to demonstrate endogenous erythroid colony formation by normal or polycythemia vera marrow and were able to show that polycythemia vera erythroid progenitor cells were exquisitely sensitive to EPO as compared with normal progenitors [74] (Fig. 61-3). These data collectively indicated that the abnormality in the erythroid progenitor cell in polycythemia vera is not only quantitative but also qualitative.

More recently, using semipurified populations of blood and bone marrow BFU-E, Krantz and colleagues [6] [7] demonstrated that the increased responsiveness of these marrow progenitor populations extends to their response to steel factor, IL-3, and GM-CSF. These studies also demonstrated that bone marrow fractions enriched for granulocyte/macrophage progenitors as well as megakaryocyte progenitors from the patients had a heightened responsiveness to IL-3 and GM-CSF.

The dogma that the hyperresponsivity of polycythemia vera progenitor cells to a variety of growth factors (EPO, IL-3, GM-CSF, steel factor) is the underlying defect that leads to polycythemia vera has, until recently, been universally accepted. [59] [163] [166] [167] [168] [169] [170] [171] [172] Several groups have, however, provided data that challenges this hypothesis. Fisher et al. used neutralizing monoclonal antibodies that bind to either EPO or EPO receptors to study the dependence of polycythemia vera progenitor cells on EPO.[74] Their results indicate that both EPO-dependent and EPO-independent erythroid progenitors are present in the blood and marrow of patients with polycythemia vera. The EPO-dependent progenitors had sensitivity to EPO similar to that of normal controls. In contrast, the EPO-independent progenitor cells cloned from the hematopoietic tissues of polycythemia vera patients in the presence of high concentrations of anti-EPO and anti-EPO receptor monoclonal antibodies appeared to be truly independent of free EPO. [74] The EPO-independent progenitors comprised approximately 30% of the total polycythemia vera erythroid progenitors and were not hypersensitive to EPO. [74] Furthermore, Correa et al., using an improved serum-free medium, have shown that peripheral blood BFU-E from polycythemia vera patients are not hypersensitive to EPO but actually exhibit a greater than 100-fold sensitivity to insulin-like growth factor-1 (IGF-1).[9] In addition, this IGF-1 hypersensitivity could be abolished by an anti-IGF-1 receptor antibody indicating that the effect was mediated via the IGF-1 receptor.[9] Recently this same group demonstrated that tyrosine phosphorylation of the subunit of the IGF-1 receptor was increased in polycythemia vera cells in the absence of exogenous IGF-1 and that phosphorylation of polycythemia cells occurred more rapidly at lower concentrations of IGF-1 attaining a higher level of phosphorylation than normal cells.[75] Usually, most circulating IGF-1 is bound to specific high-affinity IGF binding proteins, which can regulate the activity of this hormone.

Figure 61-3 Comparison of the EPO dose-response curves in serum-free cultures of colonies derived from CFU-Es and BFU-Es of marrow cells obtained from normal (dotted line) patients or individuals with polycythemia vera (solid line). *(From Casadevall et al.,[73] with permission.)*

Although plasma IGF-1 levels are not increased in polycythemia vera, the levels of IGF binding protein-1 (IGFBP-1) was reported to be strikingly elevated in the plasma of polycythemia vera patients.[76] The cause of this elevation remains unknown. In addition, IGFBP-1 alone was shown to be capable of stimulating BFU-E formation.[76] The increase in circulating IGFBP-1, a positive regulator of erythropoiesis, could account, in part, for the excessive erythropoiesis that characterizes polycythemia vera. One possible hypothesis that might result in a resolution of this apparently conflicting data is to suggest that the polycythemia vera progenitors respond to IGF-1 and IGFBP-1 by increasing the sensitivity of various classes of hematopoietic progenitor cells and/or stem cells to a variety of other growth factors including EPO, IL-3, and SCF. Alternatively, IGF-1 and/or IGFBP-1 might be capable of promoting the autocrine production of hematopoietic growth factors by polycythemia vera stem/progenitor cells. The importance of this new data remains uncertain and will require further investigation in a number of different laboratories before a unifying hypothesis can be generated.

Many investigators have examined the possibility that the hematopoietic defect in polycythemia vera could be accounted for by genetic alteration of cytokine receptors expressed by affected hematopoietic cells.[75] [76] [77] [78] [79] [80] [81] A growing body of information indicates that the EPO receptor is structurally similar to the normal EPO receptor and that a mutation in the EPO receptor is not directly involved in the pathobiology of polycythemia vera. [75] [76] [77] [78] Furthermore, no significant differences in the number, dissociation constant, or internalization rate of receptors for steel factor has been detected in the erythroid progenitor cells of polycythemia vera patients.[71]

Chiba et al. have, however, recently presented data which supports a potentially novel role of the EPO receptor in the pathobiology of polycythemia vera. [81] The major EPO receptor protein is encoded by an mRNA comprising exons I through VIII with all the introns spliced out and is designated EPOR-F. Another EPO receptor mRNA species has been reported, termed EPOR-T, and has a dominant negative function against EPOR-F, possibly by preventing homo-oligomerization of EPOR-T.[82] [83] Transgenic mice overexpressing EPOR-T are anemic, indicating that the absence of EPOR signaling through EPOR-T results in defective erythropoiesis.[83] This group has shown that EPOR-T mRNA is markedly decreased in the blood cells of patients with polycythemia vera and hypothesized that this might account for the EPO hypersensitivity observed in polycythemia vera. [81] They suggest that an acquired defect in regulation of the EPO receptor transcript system might account for the profound decrease in EPOR-T mRNA in polycythemia and in part lead to a shift in the maturation profile of erythroid progenitor cells. Since both forms of the EPO receptor are known to be expressed by normal CFU-GEMM, which are capable of producing not only erythroid cells but also megakaryocytes, granulocytes, and macrophages, dysregulation of these two forms of the EPO receptor at the level of the CFU-GEMM might account for the leukocytosis and thrombocytosis characteristic of polycythemia vera. This attractive hypothesis will require confirmation and additional study.

The increased sensitivity of polycythemia vera erythroid progenitor cells to a wide variety of growth factors and the structural integrity of the receptors for these growth factors have led a growing number of groups to attempt to define an as yet unknown molecular abnormality within a common intracellular signaling pathway in the progenitor cells of patients with polycythemia vera. [84] [85] [86] [87] [88] Growth factors upon binding to their receptors initiate tyrosine phosphorylation of their receptors, and transduction of their appropriate signals occurs through shared downstream pathways such as RAS/MAP kinase and JAK/STAT to targets in the nucleus. [89] [90] [91] Tyrosine phosphorylation in the signal pathways occurs by a delicate balance between protein tyrosine kinase (PTK) and protein tyrosine phosphatases (PTP). [89] [90] [91] [92] Dai et al. have reported that incubation of normal erythroid progenitor cells in the presence of

orthovanadate, an inhibitor of PTP, results in the formation of increased numbers of erythroid colonies and enhanced protein tyrosine phosphorylation. [86] These studies suggest that a defective PTP, caused by an acquired genetic mutation, might lead to polycythemia vera. The SHP-1 gene encodes an intracellular phosphatase which is expressed primarily in hematopoietic cells. [89] [90] [91]

This phosphatase is an extremely attractive candidate gene, the abnormal function of which could lead to the development of the polycythemia vera phenotype. SHP-1 has been shown to negatively regulate signaling from a number of receptors including the receptors for EPO, IL-3 and steel factor. [89] [90] [91] In addition, several forms of the EPO-receptor defect that accounts for PFCP are associated with defects that delete or mutate the negative regulatory domain containing the SHP-1 binding site.[35] [36] [37] Furthermore, the erythroid progenitor of mice cells with a defective SHP-1 gene (motheaten) are hypersensitive to EPO. [92] [93] Asimakopoulos et al. have reported, however, that the levels of SHP-1 protein are normal in granulocytes isolated from patients with polycythemia vera. [85] By contrast, Wickrema et al. have recently shown that purified CFU-E from polycythemia vera patients have diminished or absent expression of SHP-1 mRNA, suggesting that the hyperproliferation of the malignant clone might be in part due to defective expression of SHP-1. [88] The precise role of SHP-1 in the pathogenesis of polycythemia vera requires further careful study. Interestingly, Sui et al. have recently isolated a potentially novel membrane-associated PTP distinct from SHP-1, the activity of which was reported to be increased threefold in isolated erythroid progenitor cells. [87] These authors suggested that hyperactivation of this novel PTP might play a role in biogenesis of polycythemia vera. Further studies of intracellular signaling in polycythemia vera during the next decade are likely to provide new insight into the genetic basis for polycythemia vera.

Certain clonal chromosomal abnormalities have been found in some polycythemia vera patients at diagnosis (see the section, Laboratory Evaluation). Because these

karyotypic abnormalities are not present in most patients, it remains unclear what role the putative products of these clonal chromosomal aberrations might play in the biogenesis of polycythemia vera. Some have conjectured that multiple etiologies of this disorder might be possible, because of the presence of different nonrandom karyotypic abnormalities in a number of cases. However, in one case of polycythemia vera characterized by the presence of trisomy 8, endogenous erythroid colonies derived from bone marrow cells were analyzed by fluorescence in situ hybridization in order to determine the number of colonies containing cells with this cytogenetic abnormality.[94] The bone marrow progenitors that presumably represent the malignant clone were found to produce not only colonies containing cells with trisomy 8, but also colonies with a normal complement of chromosome 8. This study strongly suggests that the acquisition of trisomy 8 occurs as a secondary event during the biogenesis of the malignant polycythemia vera clone.[94]

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ETIOLOGY AND PATHOGENESIS

The most frequent cause of mortality in polycythemia vera patients is vascular thrombosis. [12] [95] [96] This increased thrombotic tendency is a direct consequence of the expanded red cell mass that characterizes this disorder. [105] Although the contributions of coexisting thrombocytosis and qualitative platelet abnormalities to this thrombotic tendency have remained uncertain, there is increasing evidence that abnormal platelet metabolism occurs in all polycythemia vera patients.

A markedly increased number of thrombotic events in polycythemia vera patients >70 years of age, particularly those with a history of prior thrombosis, has been reported. [103] [104] The relationship between risk of thrombosis and age suggests that coexisting vascular disease is an important contributory factor in the development of thrombosis in polycythemia vera patients. In a study of the natural history of 1213 patients with polycythemia vera for 20 years, the relationship between age and the incidence of thrombotic events was further defined. [104] These results indicate definitively that the risk for thrombosis increases with age, from 1.8 events/100 patients per year in patients younger than 40 years of age to 5.1 events/100 patients per year in those older than 70 years.

This information does not negate the increased incidence of thrombotic incidents also observed in younger patients with this disorder. [106] In a series of 58 polycythemia vera patients diagnosed at <40 years of age, a disturbingly high incidence of life-threatening thrombotic events was observedin fact, 7 of the 10 patients in this series who died during the period of observation died from thrombotic events4 from Budd-Chiari syndrome, 1 from a pulmonary embolism, and 2 from cerebral thrombosis. [81] Therefore, although a significant factor, preexisting atherosclerotic disease is not the sole etiologic factor in the genesis of thrombosis in polycythemia vera. Some have suggested an important role for smoking as a secondary factor leading to the increased incidence of thrombotic events in this patient population. [103]

The principal hemorrheologic abnormality in polycythemia vera is an elevated whole blood viscosity. [99] The blood viscosity in polycythemia vera is higher than that of normal controls at all shear rates. [99] In a retrospective analysis of the records of 69 polycythemia vera patients with a history of vascular thrombosis, Pearson and Wetherley-Mein [97] demonstrated a strong correlation between hematocrit level and development of thrombotic episodes, including many cerebrovascular occlusions (Fig. 61-4). Thomas et al. showed that cerebral blood flow is reduced in patients with polycythemia vera in whom the hematocrit is 5362%. [100] These abnormalities were observed even in patients with hematocrits at the lower levels of normal, 4652%. [100] Reductions in cerebral blood flow were correctable with phlebotomy. Reduction of the hematocrit by relatively small amounts frequently led to substantial improvements in whole blood viscosity and cerebral blood flow. Some polycythemia vera patients apparently still maintain a higher than normal whole blood viscosity despite the normalization of the hematocrit, suggesting that an increase in hematocrit may not be the only factor responsible for increased blood viscosity. [101]

A number of possible explanations have been suggested for the observed relationship between hematocrit and development of thrombotic events in polycythemia vera patients. Turrito and Weiss [102] presented evidence to indicate that platelet adhesion and thrombus formation on the vascular subendothelium are determined in part by the rate at which platelets are transported to the vascular surface. In a polycythemic condition in which increased numbers of red cells are present, a greater number of intercellular collisions between red cells and platelets occur. These collisions could lead to increased platelet movement in a direction perpendicular to blood flow. This facilitation of platelet transport to the vessel wall may be an important factor in the development of thrombosis. [102] An alternative explanation for the association between hematocrit level and risk of thrombosis is based on the knowledge that blood viscosity is particularly sensitive to hematocrit levels. [103] [104] Increased hematocrits lead to increased blood viscosity, in turn leading to increased peripheral vascular resistance and an actual reduction in blood flow to a variety of organs, predisposing them to the development of thrombosis. [103] [104]

Additional factors have been implicated in the development of thrombosis in polycythemia vera patients. Almost all patients with this disorder are iron-deficient. [105] Decreased red cell deformability has been said to accompany iron deficiency, leading

Figure 61-4 Relationship of hematocrit to number of vascular occlusive episodes per 10 years in patients with polycythemia vera. *(From Pearson and Wetherley-Mein, [97] with permission.)*

to increased blood viscosity and a decreased ability of red cells to pass through small-bore polycarbonate filters. [106] [107] Such abnormalities have been shown by Tillman and Schröter [108] and Yip et al. [109] to be due to increased membrane stiffness rather than to reduced surface/volume ratio, as has been suggested by others. This increased membrane stiffness, however, might be counterbalanced by the effect of a reduced red blood cell size on the adherence of blood platelets to arteriolar subendothelium. Aarts et al. [110] have shown that red cell size is a major determinant of platelet adherence, with larger red blood cells leading to increased and smaller red cells to decreased platelet adherence. Whether the increased membrane stiffness associated with iron deficiency is counterbalanced by the decreased platelet adherence associated with smaller red blood cells is yet to be determined.

Study of patients with hemoglobinopathies due to abnormal oxygen binding who have secondary erythrocytosis provides further support to the belief that hematocrit elevations are not the sole cause of the thrombotic tendency in polycythemia vera. A survey of 200 patients with these types of hemoglobinopathies has not demonstrated a higher incidence of myocardial ischemia or any other form of thrombosis, even though the red cell mass is frequently as elevated as that in patients with polycythemia vera. [111]

Thrombocytosis and qualitative platelet abnormalities occur frequently and are likely to be important contributory factors to the development of thrombosis. [13] [112] [113] Dawson and Ogston [114] have implicated uncontrolled thrombocytosis as a cause of thrombosis in these patients, but this relationship has not been confirmed by Kessler et al. [112] or Berk et al. [95] Increased plasma and urinary thromboxane production has been linked to increased platelet activation in these patients. [113] A low-dose aspirin regimen selective for inhibition of platelet cyclo-oxygenase has been found to suppress increased thromboxane production in vivo and to clinically benefit patients with polycythemia vera. [115] [116]

Polycythemia vera patients are also at an increased risk of developing life-threatening hemorrhagic complications. [117] [118] [119] Abnormalities in platelet function and number have been implicated as the cause of this hemorrhagic tendency. [12] [13] Qualitative platelet abnormalities frequently found in these patients include platelet hypofunction as demonstrated by defective in vitro platelet aggregation, acquired storage pool disease, platelet membrane defects, increased platelet reactivity as demonstrated by enhanced platelet aggregation, increased plasma -thromboglobulin levels, and shortened platelet survival. [117] [118] [119] [120] With platelet counts >1000 $\times 10^9$ /l, the development of acquired von Willebrand syndrome has been reported and is associated with life threatening hemorrhagic episodes. [118] [119]

Despite conflicting data, no clear clinical relationship between platelet number or function and the incidence of hemorrhage or thrombosis in polycythemia vera has been found. [95] [97] [99] In primary thrombocythemia, however, reduction of excessive platelet numbers has been shown to reduce the risk of the thrombotic events. [121] In addition, selected patients with polycythemia vera have been afforded prompt resolution of vascular complications such as erythromelalgia or transient ischemic

attacks following institution of platelet antiaggregating agents or cytoreduction. [121] [122] [123] It is important to emphasize that erythromelalgia does not resolve in polycythemia vera patients with phlebotomy alone or with anticoagulation but requires the use of platelet antiaggregating agents or reduction of platelet numbers. [123] What distinguishes the clinical course of these patients from that of others is unknown. These reports, coupled with the knowledge of abnormal thromboxane metabolism of platelets in polycythemia vera, provide substance to the belief that platelets contribute to generation of the thrombotic and hemorrhagic tendencies observed in polycythemia vera (Fig. 61-5).[117] [118] [119] [120]

The high number of white cells which occurs in 5060% of polycythemia vera patients may also have a detrimental effect on the rheology of the microcirculation in polycythemia vera.[99] Although a variety of clinical assessments of platelet function have been used to identify patients who are potentially at a high risk of developing a life-threatening hemorrhagic or thrombotic

Figure 61-5 Pathophysiology of arteriolar and arterial thrombosis and inflammation as well as hemorrhage as a multicellular process in polycythemia vera. *(Modified from Michiels JJ,[117] with permission.)*

event, the results of these studies to date have been very disappointing. [117] [118] [119] [120] One is left with the impression that the etiology of thrombosis and hemorrhage in polycythemia vera is multifactorial and that the available tools are inadequate to identify those patients at highest risk.

A major cause of morbidity and mortality in polycythemia vera results from the transition from the polycythemic phase of the disease to postpolycythemic myeloid metaplasia (PPMM) and to acute leukemia. PPMM is characterized by cytopenias, myelofibrosis, and extramedullary hematopoiesis. [123] [124] [125] [126] In a variety of myeloproliferative disorders, the fibroblastic component of the bone marrow has been shown not to be directly involved in the malignant process but to be a reactive event to the neoplastic clone.[59] Several investigators have suggested that release of growth factors, particularly platelet-derived growth factor from megakaryocytes or platelets, which are present in abundance in patients with myeloproliferative disorders, might be responsible for this fibroblastic proliferation. [127] [128]

Whether the use of any particular therapeutic agents for treatment of polycythemia vera accelerates the development of the spent phase remains hotly debated. Some have suggested that the use of radioactive phosphorus (^{32} P) favors such a transition, although others have not noted any relationship between the treatment modality used and more rapid progression to PPMM.[129] [130] [131] [132] Najean et al. have reported the long-term follow-up of polycythemia vera patients and noted a high incidence of myelofibrosis in patients treated by phlebotomy alone. [133] [134] In this study, myelofibrosis was rarely observed before the 10th year of follow-up, but occured in 20% of patients surviving after 15 years and 50% surviving after 20 years. Messinezy and Pearson have also reported that of 20 patients known to have survived 15 years with polycythemia vera, 25% developed myelofibrosis.[135] These studies suggest that myelofibrosis will be a major problem due to the increased survival of polycythemia vera patients. Surprisingly, in the Italian Natural History Study with a follow-up of 20 years, myelofibrosis was a rare cause of death. [96] After treatment with alkylating agents or irradiation during the proliferative phase, patients with PPMM have been reported to be more likely to transform to acute leukemia.[136]

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CLINICAL MANIFESTATIONS

The principal clinical manifestations of polycythemia vera are a direct consequence of the excessive proliferation of cellular elements of the various hematopoietic cellular lineages involved in the neoplastic process. With the current widespread use of laboratory screening tests during routine patient examinations, increasing numbers of persons are being diagnosed with polycythemia vera before the development of symptoms related to this neoplastic process. Symptomatic patients with polycythemia vera may present to a physician with a myriad of nonspecific complaints, including headache, weakness, pruritus, dizziness, excessive sweating, visual disturbances, paresthesias, joint symptoms, epigastric distress, and weight loss. [4] [11] At diagnosis, in fact, one-third of patients have already lost 10% of their body weight, presumably secondary to the hypermetabolism associated with this disorder. [10] Arthropathies that are frequently observed in these patients are largely due to the clinical manifestations of gout. The hyperproliferative bone marrow state characteristic of polycythemia vera and the increased nucleoprotein degradation are contributing factors in the development of hyperuricemia.

The principal findings on physical examination of a patient with polycythemia vera include ruddy cyanosis, conjunctival plethora, hepatomegaly, splenomegaly, and hypertension. [4] [11]

Untreated patients are at particularly high risk of both thrombotic and hemorrhagic events. [4] [11] [12] [117] [118] [119] [120] In several large series of patients with polycythemia vera, thrombosis was the cause of death in 3040% of patients (Table 61-4). [4] [11] [95] [96] Patients may present with deep venous thrombosis in the lower extremities, pulmonary embolism, or cerebrovascular, coronary, and peripheral vascular occlusions. [122] [123] [124] It is not unusual for patients with polycythemia vera to develop thromboses at unusual anatomic sites; in particular, thromboses are relatively frequent in the splenic, hepatic, portal, and mesenteric vessels. [4] [11] [117] [120]

TABLE 61-4 -- Fatal and Nonfatal Thrombotic Events (*n*= 254) During Follow-up in 1,213 Patients with Polycythemia Vera

Type of Complication	Nonfatal Event	Fatal Event
	n (%)	*n*(%)
Arterial thrombosis	101(50.5)	44(81.5)
Myocardial infarction	28(14.0)	27(50.0)
Ischemic stroke	19(9.5)	17(31.5)
Transient ischemic attack	39(19.5)	
Peripheral arterial thrombosis	15(7.5)	
Venous thrombosis or embolism	77(38.5)	10(18.5)
Deep venous thrombosis	35(17.5)	
Superficial thrombophlebitis	37(18.5)	
Unknown	5(2.5)	
Unknown	22(11.0)	
Total	200(100)	54(100)

From Gruppo Italiana Studio Policitemia,[96] with permission.

Reisner et al. have demonstrated by echocardiography that cardiac valvular abnormalities are common in patients with myeloproliferative disorders. [137] In fact, aortic or mitral valve lesions were found in 77% of patients with polycythemia vera. The most common echocardiography lesion was leaflet thickening in 40% of patients, and vegetation occurred in 16%. The presence of such lesions were closely associated with the occurrence of arterial or venous thrombosis or embolism. Such vascular abnormalities may account for a significant number of thrombotic events in this patient population. [137]

A particularly serious thrombotic event associated with polycythemia vera is Budd-Chiari syndrome, [106] [138] [139] [140] [141] resulting from hepatic venous or inferior vena caval thrombosis and obstruction. This syndrome is characterized by hepatosplenomegaly, ascites, edema of the peripheral extremities, jaundice, abdominal pain, and distension of superficial abdominal veins due to resultant portal hypertension. [140] Routine biochemical determinations of hepatocellular function and injury are frequently of little diagnostic value in patients with suspected Budd-Chiari syndrome. [140] Liver scans using ^{99}Tc-labeled sulfur colloid may provide important diagnostic information, a characteristic pattern being central accumulation of the tracer and an enlarged hypertrophic caudate lobe of the liver, with apparent diminished or even absent tracer accumulation over the right lobe. [140] Percutaneous liver biopsy specimens usually reveal intense congestion and cellular atrophy. Hepatic venous and inferior vena cava catheterization are key diagnostic procedures that indicate the sites of venous obstruction. [140]

Of patients with Budd-Chiari syndrome reported in the literature, 10% have had coexisting polycythemia vera. [138] [140] Therefore, anyone who develops Budd-Chiari syndrome should be suspected of having polycythemia vera, and that diagnosis should be quickly excluded. It has recently been emphasized that patients with polycythemia vera can present with portal or hepatic vein thrombosis with normal hemoglobin or hematocrit valves. Such patients have leukocytosis, thrombocytosis, or splenomegaly. [141] The diagnosis of inapparent polycythemia vera in this patient population can only be made after red cell volume measurements are performed. [141] Gastrointestinal bleeding and/or an increase in plasma volume that is a consequence of splenomegaly often accounts for the normal blood counts in such patients with Budd-Chiari syndrome. The factors operational in the polycythemia vera patient that lead to the development of hepatic vein thrombosis are thought to be multiple. Splenomegaly causes increased portal blood flow while extramedullary hematopoiesis within the hepatic sinusoids frequently obstructs hepatic blood flow. [140] These processes are surely important contributory factors, in addition to the other previously discussed risk factors that lead to the development of thrombosis in this patient population. [120]

Neurologic abnormalities occur in almost 6080% of untreated or poorly controlled polycythemia vera patients and include transient ischemic attacks, cerebral infarction, cerebral hemorrhage, fluctuating dementia, confusional states, and choreic syndromes. [114] [117] [122] [123] [142] [143] In addition, complaints of dizziness, paresthesias, visual disturbances, tinnitus, and headache have been attributed to the increased blood viscosity [100] [143] and reduced cerebral blood flow caused by

erythrocytosis. The transient neurologic symptoms can also be the consequence of small infarcts in the region of the basal ganglia, which can be detected by computed tomography.[143] These small infarcts are known as lacunae and result from the occlusion of small penetrating arteries, which are particularly susceptible to thrombosis.[143] Cerebrovascular thrombosis occurs more often in polycythemia vera patients than in the general population. [120] [122] [143] [144] Symptoms due to intermittent carotid or vertebral basilar artery insufficiency (or both) occur so frequently in polycythemia vera that Millikan et al. [145] suggest that every patient with focal cerebrovascular insufficiency should at least have a complete blood count to rule out an underlying myeloproliferative disorder.

Cavernous sinus thrombosis is usually associated with a primary infectious etiology involving a focus in the face, throat, mouth, ear, or sinuses. [146] Aseptic cavernous sinus thrombosis is an extremely rare phenomenon that has been reported in patients with polycythemia vera. [147] [148] These patients present with monocular blindness and the characteristic features of ipsilateral cavernous sinus thrombosis, and only retrospectively is the diagnosis of polycythemia vera made. [148] Therefore, patients found to have this symptom complex who have no known infectious predisposing causes should be carefully evaluated to rule out this diagnosis.

Thrombosis of large-caliber arteries is a relatively rare event in polycythemia vera patients, but there have been case reports of thromboses within the chambers of the heart leading to refractory congestive heart failure and acute aortic occlusion. [149] [150] Such catastrophic thrombotic events in the heart or large vessels would suggest that cardiac catheterization be performed with some caution. [150]

Polycythemia vera frequently presents with symptoms due to peripheral vascular disease. [117] [151] [152] [153] In these cases, patients may first be seen by surgeons or dermatologists.[151] Intense redness or cyanosis of digits with or without burning, classical erythromelalgia, digital ischemia with palpable pulses, or thrombophlebitis without other known cause may be the presenting symptoms.[123] [152] [153] [154] [155] [156] [157]

Erythromelalgia is characterized by burning pain in the digits, an objective sensation of increased temperature, and relief by cooling. [123] [153] [154] [155] [156] [157] [158] Polycythemia vera is the most common cause of erythromelalgia and is one of the few disorders in which digital ischemia with or without ulceration may exist in the presence of palpable pulses.[156] Other disorders that can lead to this abnormality include embolism, trauma, cutaneous infarction, neuritis, infection, and various types of arteritis.[156] Painful and ulcerating toes and fingers have frequently been observed to be a presenting symptom in patients with polycythemia vera. [123] [153] [154] [155] [156] [157] [158] The likelihood that arterial insufficiency is the cause of such ulceration is quite small in patients who have a palpable dorsalis pedis and posterior tibialis pulses; in this situation, the possibility of an underlying hematologic disorder such as polycythemia vera should be entertained. Foot pain at rest is a distressing but not widely recognized symptom of polycythemia vera. In patients with this complaint, peripheral pulses are of normal character and cutaneous circulation appears to be adequate.[123] [153] [154] [155] [156] [157] [158] The pain is most severe at night, is dull in nature, and occurs primarily in the feet or legs. These symptoms have been shown to be the result of platelet activation and aggregation in vivo, which preferentially occurs in arterioles. [123] If untreated,

erythromelalgia can progress to ischemic acrocyanosis or gangrene. Phlebotemy alone in polycythemia vera does not improve erythromelalgia. [123] [157] [158] These symptoms can be abolished by reducing the platelet counts to normal levels and can be rapidly reversed after the institution of antiplatelet aggregation therapy. Therefore, the cause of erythromelalgia appears to be closely linked to abnormal arachidonic acid metabolism that occurs within platelets in this disorder. [123]

Hemorrhagic complications are the cause of death in 210% of polycythemia vera patients. [12] [15] [117] [118] [119] [120] The gastrointestinal tract is the most frequent site of hemorrhage. These patients have an increased incidence of peptic ulcer disease and, frequently, esophageal varices due to portal hypertension. Cerebral hemorrhage is a common cause of morbidity and mortality.[142] As many as 3040% of patients with polycythemia vera experience some sort of hemorrhagic event, [12] [119] [159] which can be relatively trivial, such as epistaxis or gingival hemorrhage, or can be life-threatening, such as gastrointestinal hemorrhage or hematomas involving vital organs.[12] In large part this hemorraghic tendency has been attributed to qualitative platelet abnormalities. [117] [118] [119] [120]

Bleeding events frequently occur with the use of antiinflammatory agents; [160] [161] an association between the hemorrhage and the use of high doses of these platelet-paralyzing drugs has been made in almost one-third of such instances. [159] [160] [161] Low-dose aspirin therapy has, however, been recently shown not to lead to an increased incidence of life-threatening hemorrhagic events. [115] [116] [120] Spontaneous bleeding in patients with polycythemia vera is relatively rare, [159] [160] [161] although spontaneous retropharyngeal hematomas leading to acute upper airway obstruction or hematomas in the groin have been reported. [162]

The polycythemia vera patient who undergoes a surgical procedure is at a very high risk of developing postoperative complications. [163] [164] [165] In one series of 62 major operations on 54 patients with polycythemia vera, postoperative complications occurred in 47%; [165] 52% of complications were due to hemorrhage, 18% to thrombosis, and 14% to hemorrhage and thrombosis.[165] The postoperative mortality in this patient population was 18%. [165] In another series of 15 patients, 5 suffered serious complications secondary to thrombosis and hemorrhage.[164] Data analysis of polycythemia vera patients undergoing surgery has shown the complication rate to be highest in those who had uncontrolled erythrocytosis before surgery. Patients with inadequately controlled disease had a 79% incidence of complications, while in those who enjoyed adequate hematologic control prior to surgery the rate of perioperative and postoperative complications was reduced to 28%. [163] In addition, duration of disease control was an important factor in decreasing surgical risk; [163] a prolonged period of effective disease control prior to surgery reduced the complication rate to 5%.[163] Complication rates following surgery can therefore be dramatically reduced by appropriate therapeutic interventions with normalization of blood counts. The chief deterrent to such an approach has been the failure by physicians to recognize the risk associated with polycythemia vera in the surgical setting.

Generalized pruritus occurs in approximately 50% of cases of polycythemia vera. [4] [15] [167] Water contact, such as during showers or bathing, induces attacks of intolerable pruritus. [168] There appears to be no clear relationship between the degree of the pruritus and severity of the disease, [169] and 20% of patients continue to experience itching despite reduction of their red blood cell masses to the normal range. [169] The degree of pruritus is so severe in some patients that they are unable to tolerate bathing at all and find it necessary to substitute gentle skin swabbing or to simply not bathe. The etiology of the pruritus in polycythemia vera remains uncertain. Several groups have attempted to implicate elevated blood and urine histamine levels in its pathobiology. [167] [169] Steinman et al.[168] have presented data suggesting that water exposure in patients with polycythemia vera actually leads to elevated histamine levels, which would provide an explanation for the exacerbation of the pruritus frequently observed with water contact. Jackson et al. [169] have been able to establish a strong correlation between skin mast cell numbers and the severity of itching. However, the failure of the pruritus to respond to antihistamine therapy in many patients suggests that abnormally high histamine levels probably do not constitute the sole factor in its development. [171]

Iron deficiency has also been implicated as a factor contributing to pruritus in polycythemia vera patients who are almost invariably iron deficient. [171] Iron substitution therapy has resulted in symptomatic improvement,[171] but this approach is less than optimal because it frequently results in uncontrollable erythrocytosis.

Several investigators have suggested that patients suffer from serious nonhematologic symptoms secondary to iron deficiency. [173] [174] [175] Rector et al.[175] evaluated these sequelae in patients with polycythemia vera who were uniformly iron deficient and followed these patients for >25 years. Their data are unique in that they indicate that quantitative evaluation of symptoms fails to provide convincing evidence that undue fatigue occurs as a result of iron deficiency. [175] In fact, treadmill performance by six patients was equivalent to that observed among normal subjects.[175] None of the patients experienced dysphagia or the esophageal changes associated with chronic iron deficiency.[175] The one symptom that was observed to occur regularly, particularly in women, was picophagia, a pica consisting of compulsive ice eating.[175]

PPMM occurs in 550% of patients with polycythemia vera.[12] [13] [96] [124] [125] [126] [131] [132] The transition to this stage of the disease occurs, on average, 10 years after initial diagnosis, but in individual cases it can occur after either shorter or longer intervals. [123] [124] [125] Najean et al. have reported that 15 years or more after initial diagnosis, this complication is a major clinical problem affecting almost 50% of patients. [133] [134] PPMM is characterized by (1) increasing splenomegaly, (2) teardrop red blood cell morphology, (3) extensive bone marrow fibrosis, (4) a leukoerythroblastic blood picture, and (5) a normal or decreasing red blood cell mass. The patients may be entirely asymptomatic but often complain of fatigue, dizziness, weight loss, and anorexia. [124] [125] [126] Splenomegaly can lead to abdominal pain due to repeated splenic infarcts and to early satiety due to mechanical obstruction of the upper gastrointestinal tract.

The anemia that characterizes the spent phase is primarily a result of splenic pooling, ineffective erythropoiesis, and extramedullary production of red blood cells with a shortened red cell survival. Occasionally, the anemia is exacerbated by folate or iron deficiency. [125] [126] [176] Before assuming that a patient has entered the spent phase, it is prudent to assess bone marrow iron stores. Replacement therapy with iron may lead to the resurgence of erythropoiesis and prevent the faulty categorization of disease progression.

Bleeding abnormalities due to thrombocytopenia or qualitative platelet abnormalities are especially common during this phase of the disease. Frequent instances of

epistaxis or ecchymoses occur,[126] and gastrointestinal hemorrhage due to esophageal varices arising from portal hypertension is a recurrent problem. [124] [125] [126] Frequently, patients suffer from generalized wasting characterized by progressive asthenia and weight loss. Severe hyperuricemia, leading to secondary gout or uric acid nephropathy, may also complicate the clinical course.

Patients with PPMM are at high risk for the development of acute leukemia.[96] [132] [136] [177] Of those patients who enter the spent phase of polycythemia vera, 2050% will eventually undergo leukemic transformation.[124] [125] [126] Approximately 70% of patients who enter the spent phase will be dead 3 years after this transition; however, the other 30% of these patients will have a much longer survival, averaging 6.5 years. [124] [125] [126]

The leukemic transformation of polycythemia vera has been

extensively described.[136] [178] [179] [180] [181] [182] [183] The possibility that a relationship exists between the therapeutic modality used during the erythrocytotic phase and the frequency of development of acute leukemia has been a point of heated discussion. [178] [179] [180] [181] [182] [183] [184] [185] [186] [187] [188] [189] [190] Some of the controversy surrounding this question was formerly due to a lack of understanding of the basic origins of polycythemia vera. Clinical hematologists in the 1950s and 1960s frequently thought of polycythemia vera as a benign hematologic abnormality and believed that therapeutic interventions either with alkylating agents or radiotherapy were solely responsible for the development of acute leukemia. That concept has proved erroneous, and polycythemia vera, like the other myeloproliferative disorders, has been shown to be a clonal malignant hematologic disorder.[59] The evolution to acute leukemia can therefore be thought of as a natural consequence of this malignant disorder, which can be accentuated by the therapeutic interventions discussed above.

Further insight into relationships between acute leukemia and polycythemia vera has been best provided by the published results of the Polycythemia Vera Study Group (PVSG), which described a randomized trial comparing the use of phlebotomy, chlorambucil, and ^{32}P for the treatment of this disorder.[184] The incidence of acute leukemia was approximately 1.5% in patients treated with phlebotomy alone, 17.5% in patients treated with chlorambucil, and 10.9% in patients treated with ^{32}P after over 15 years of follow-up.[185] [186] [187] [188] The incidence of acute leukemia in the patients treated with phlebotomy alone is therefore much higher than that expected in a normal age-matched control group, again indicating that leukemia is a natural evolutionary event in the clinical course of an individual with polycythemia vera. The incidence of acute leukemia can be increased, however, by institution of therapy with either alkylating agents or ^{32}P.[185] [186] [187] [188] The time course for the development of acute leukemia appears to be dependent on the treatment used to control the polycythemia. The development of acute leukemia in patients treated with phlebotomy in the Polycythemia Vera Study Group trial was limited to the first five years of treatment, suggesting that the development of acute leukemia is not due solely to the prolongation of survival. In contrast, analysis of the hazard function (Fig. 61-6) was virtually flat for patients treated with chlorambucil from years two to seven after randomization; however, the risk for acute leukemia became alarmingly high after 10 years of study, suggesting that the risk of acute leukemia increases with time, even after the drug has been stopped.[188] One half the cases of acute leukemia in the chlorambucil arm occured in the first five years with the remainder equally split between the second and third five-year periods. In contrast, 60% of the cases of acute leukemia in the group treated with radioactive phosphorus occurred six to 10 years after randomization. Of particular concern is the high incidence of leukemia in patients who were initially treated with radioactive phosphorous and then switched to maintenance therapy with hydroxyurea, previously thought to be a nonleukemogenic agent. [188] [189] These findings suggest that a combination of radiotherapy (^{32}P) and chemotherapy (hydoxyurea) may particularly augment the risk of leukemia.[188] [189] Approximately 3050% of patients with polycythemia vera who develop acute leukemia have previously entered the spent phase. [128] [188] [189] In contrast, approximately 50% of patients progress directly from the erythrocytotic phase to acute leukemia.[136] [177] [185] [186] [187] [188] The phenotype of the leukemia cells that characterize the leukemic phase is overwhelmingly myeloid.[136] [186] Unusual cases of lymphoblastic transformation of polycythemia vera have, however, been reported, as have rare cases of biphenotypic leukemias. [191] [192] [193] [194] Another adverse effect solely associated with the use of chlorambucil has been the development of large cell lymphoma in 3.5% of patients. [185] [186] [187] [188]

The development of leukemia sometimes can be abrupt, however. In some instances, a preleukemic phase characterized by refractory anemia with excess blasts has been described.[178]

Figure 61-6 Hazard function for development of acute leukemia in PVSG protocol 01. The function shown is the instantaneous rate of acute leukemia at the midpoint of the study year, given survival without acute leukemia at the start of the year. The open circles represent the chlorambucil-treated patients, closed circles represent the ^{32}P-treated patients and the half-closed circles represent the phlebotomy. *(Data from Berk et al.)*[185]

In fact, one-half of such cases of acute leukemia in one series were preceded by a myelodysplastic disorder. [178]

In addition, the incidence of nonhematological malignancies has been shown to be increased in patients treated with either chlorambucil or radioactive phosphorous but not with phlebotomy therapy.[185] [186] [187] [188] The rates of nonhematological cancers were highest in sites in the skin and gastrointestinal tract. These malignancies are presumed to be treatment-related.

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LABORATORY EVALUATION

Laboratory evaluation of the patient with erythrocytosis involves the careful use of a broad range of diagnostic studies. These studies must be employed in a rational manner, or the evaluation can become extremely costly. Since polycythemia vera is a panmyelosis, the overwhelming number of patients have elevated hematocrits, white blood cell counts, and platelet counts.[4] Hematocrit values over 50% in males and over 45% in females are abnormal and require further evaluation. In order to document the absolute increase in red cell mass, the performance of a blood volume study with direct quantitation of both the red cell mass and plasma volume is frequently necessary.[195] A hematocrit greater than 60% in men or greater than 55% in women is almost always associated with an absolute erythrocytosis.[196] [197] In such cases, it is frequently unnecessary to order blood volume studies. In men suspected of polycythemia with a hematocrit below 60% and women with hematocrits below 55%, blood volume measurements are required to determine whether the elevated hematocrit is actually due to an expanded red cell mass.[196] [197] Occasionally, an elevated red cell mass can actually be present in the face of a normal hematocrit. In cases of splenomegaly due to portal hypertension, an expanded plasma volume may mask an elevated red cell mass.[141] [167] In addition, iron deficiency can also lead to a fall in hematocrit in polycythemia vera making the diagnosis difficult.[141] Polycythemia vera is associated with a 2030% frequency of gastric ulcers and gastritis which can be associated with blood loss.[141] In this situation, thrombocytosis may be exacerbated as a consequence of the

iron deficiency. Iron supplementation and correction of the bleeding lesion may permit the diagnosis to be established. Administration of iron to such patients must be performed carefully, thereby avoiding a rapid increase in red cell mass, which can be associated with a high risk of thrombosis. Lamy et al. have recommended that increased hematocrit and hemoglobin levels do not constitute the only criteria for proceeding with a red cell mass determination in order to make a diagnosis of polycythemia vera.[141] They suggest that red cell mass determinations be performed in patients with normal hemoglobin and hematocrit values with portal or hepatic vein thrombosis, splenomegaly, leukocytosis, or thrombocytosis. The use of red cell measurements in these settings is intended to aid in the diagnosis of so-called inapparent polycythemia vera.[141]

Indirect calculations of the red cell mass from plasma volume measurements assume a normal body venous hematocrit ratio. Unfortunately, this ratio may be abnormal in patients with spurious polycythemia vera, and calculations derived from venous packed red cell volumes may overestimate the red cell mass.[195]

The criteria for establishing a red cell mass has previously been based upon values expressed as ml/kg of total body weight. Because adipose tissue is considerably less vascular than lean tissue, it may not contribute equally to the red cell mass.[195] [196] Red cell mass measurements, therefore, based solely on body weight can be misleading.[195] Red cell mass measurements more closely correlate with lean body mass than total body weight. Measured red cell mass values in obese subjects are regularly lower when expressed as ml/kg total body weight than those observed in lean individuals.[195] In order to overcome this difficulty, the International Council for Standardization in Haematology has presented formulas to calculate normal red cell mass values, based upon lean body mass, surface area, height, and weight.[195] Implementation of this approach is suggested due to the high incidence of obesity in our society.[195] Modification of the Polycythemia Vera Study Groups criteria for definition of elevation of the red cell mass has been suggested. An elevated red cell mass is now defined as being 25% greater than the mean predicted value of a red cell mass for that individual rather than basing evaluations on the volume per kg of total body weight.[195]

Leukocytosis is present in approximately two-thirds of cases, and thrombocytosis is observed in 50%.[4] Abnormalities of red blood cell, white blood cell, and platelet morphology are frequently observed. The morphologic red blood cell changes observed during the erythrocytotic phases are characteristic of iron deficiency and include microcytosis, hypochromia, and frequently polychromatophilia.[181] Some anisocytosis and poikilocytosis can be seen. Fetal hemoglobin levels and the number of red cells containing fetal hemoglobin, known as F cells, may be increased.[198] The white blood cells are characterized by normal morphology, although the numbers of basophils, eosinophils, and immature myeloid forms can be increased.[4] Platelet morphology is also quite striking in polycythemia vera. Frequently, megathrombocytes (platelets having the size of red blood cells) are seen on the peripheral blood smear. Patients frequently have platelet counts of $<1 \times 10^{6}$ /mm[3] , but it is not unusual to observe a patient with a platelet count higher than this value. The PPMM phase of the disease is characterized by a leukoerythroblastic blood picture, with the appearance in the peripheral blood of teardrop red blood cells, myelocytes, metamyelocytes, and, rarely, blasts and promyelocytes in addition to nucleated red blood cells in the peripheral blood.[124] [125] [126]

Platelet aggregation studies and bleeding times are frequently abnormal in patients with polycythemia vera, but many patients have normal values.[119] Prothrombin time (PT) and partial thromboplastin times (aPTT), as well as fibrinogen levels, are usually normal.[199] Profound abnormalities of both the PT and aPTT are, however, frequently reported. This is largely a laboratory artifact caused by the extreme erythrocytosis which results in a relative smaller volume of plasma being present in the whole blood sample. Coagulation assays are performed on blood anticoagulated with sodium citrate and the citrate concentration in the anticoagulant is calibrated to chelate the plasma calcium and inhibit coagulation reactions. All coagulation assays include the addition of calcium chloride to neutralize the excess citrate and provide free calcium to mediate coagulation reactions. In patients with extreme erythrocytosis, the ratio of citrate in the collection tube to the volume of plasma is too high; therefore, excess citrate is present and the standard amount of calcium chloride added during the performance of the PT and aPTT is insufficient to neutralize the excessive citrate and the coagulation assays are frequently artifactually prolonged. In order to avoid this problem, one should calculate the relative amount of plasma compared to normal and remove the corresponding volume of sodium citrate from the blood collection tube. Normal values can then be confidently anticipated in patients with erythrocytosis. Tytgat et al.[199] have detected a shortened fibrinogen half-life in some polycythemia vera patients and a significantly increased fractional catabolic rate of the plasma fibrinogen pool per day. Boughton and Dallinger[200] have confirmed these findings. In addition, elevated platelet -thromboglobulin and plasma -thromboglobulin levels are observed. The constellation of findings is indicative of increased platelet turnover rates.

An acquired von Willebrand defect (vWF) occurs frequently in patients with polycythemia vera and primary thrombocythemia who have extreme elevations of platelet numbers. This syndrome is characterized by a normal or prolonged bleeding time, a normal factor VIII level and normal vWF antigen level but abnormal vWF ristocetin cofactor actively associated with a decrease or absence of large vWF multimers.[119] [201] [202] This acquired defect resembles type II vWF disease. Since the molecular size of vWF is a major determinant of its adhesive function and the larger multimers are most active in achieving hemostasis, the deficiency of large vWF multimers is associated with a bleeding tendency. The decrease in the frequency of large vWF multimers occurs in patients with platelet counts over 1000×10^{9} /l.[201] [202] This abnormality has been reported not only with patients with severe thrombocytosis due to myeloproliferative disorders, but also in patients with reactive thrombocytosis.[202] An inverse correlation between the proportion of large vWF multimers and platelet numbers has been observed.[118] [202] In addition, normalization of the platelet count is accompanied by restoration of a normal vWF multimer pattern.[118] These findings suggest that thrombocytosis of any etiology may favor the adsorption of larger forms of vWF multimers onto platelet membranes, resulting in their removal from the circulation and subsequent degradation by platelet-associated proteases.[118] Although patients with myeloproliferative disorders frequently have bleeding tendencies, this is not the case in secondary thrombocytosis, possibly because of the limited periods of extreme thrombocytosis observed in such patients.[118] Patients with polycythemia vera with clinical courses punctuated by hemorrhagic events, extreme thrombocytosis, and acquired von Willebrand syndrome should not receive aspirin therapy if they are suffering from a thrombotic episode but should be phlebotomized and platelet reduction therapy should be initiated.[118] Patients with polycythemia vera and acquired vWF syndrome suffer from recurrent bleeding from mucous membranes and the digestive tract and easy bruisability. These symptoms frequently resolve with normalization of the

platelet count.

Deficiencies of one or more natural anticoagulants have been observed in patients with polycythemia vera and thrombosis. [203] These studies indicate that either familial or acquired antithrombin III deficiency, protein C or S deficiency, or resistence to activated protein C may contribute to the hypercoagulable state observed in polycythemia vera.[203] Such inherited disorders

associated with the erythrocytosis of polycythemia vera provide a scenario that frequently favors thrombosis in affected individuals.

The leukocyte alkaline phosphatase activity level is elevated in 70% of patients. [4] Serum B_{12} concentrations have been found to be elevated in 40% of patients, whereas serum B_{12} -binding proteins are elevated in 70% of cases. [4] Hyperuricemia occurs in an overwhelming number of patients, while elevated histamine levels are also frequently observed.[166] Bone marrow aspirates and biopsies obtained at the diagnosis of patients with polycythemia vera are hypercellular and display characteristic erythroid, granulocytic, and megakaryocytic hyperplasia. [105] [132] [204] [205] [206] This is not a uniform finding, as 13% of the pretreatment biopsies in one series demonstrated an initial marrow cellularity of <60%, which lies within the normal range. [97] The cellular elements are frequently morphologically normal. Iron stores are almost uniformly absent in pretreatment biopsy specimens.[105] [132] [204] [205] Significant increases in bone marrow reticulin may be present in biopsies obtained early in the course; but also may develop during the erythrocytotic phase and may be present for long periods before the onset of the spent phase. [105] [204] [205] The presence of increased reticulin at diagnosis is not predictive of imminent development of PPMM. In those patients who enter the spent phase of the disease, a moderate to marked increase in reticulin fiber is observed, either simultaneously with or within 1 year of this clinical transformation. [105] [132] [204] [205]

Several investigators have attempted to use bone marrow biopsy morphology as a differential diagnostic tool to discriminate between polycythemia vera and secondary forms of erythrocytosis. The marked hypercellularity and megakaryocytic hyperplasia that are the hallmarks of myeloproliferative disorders are useful parameters for distinguishing such individuals (Fig. 61-7).[204] [205] It is imperative to use the bone marrow biopsy rather than to aspirate specimens for this purpose. [105] [132] [204] [205]

The pathologic appearance of the spleen in polycythemia vera depends on the stage of the disease at which that organ is examined. [206] Spleens from patients in the erythrocytotic phase of the disease are characterized by striking congestion with mature erythrocytes. [206] Small numbers of hematopoietic precursor cells are frequently present. By contrast, spleens examined during the PPMM phase are characterized by prominent numbers of foci of extramedullary hematopoiesis, with representation of all marrow precursor elements.[206]

TABLE 61-5 -- Study of Diagnostic Utility of Marrow Erythroid Progenitor Cell Cultures in Patients Suspected of Polycythemia Vera Based on Standard Clinical Criteria

Diagnosis	Standard Criteria[a]	Endogenous Erythroid Colonies[b]
Group A[c]		
Polycythemia vera	46	43 (93%)
Secondary polycythemia	12	0
Unclassifiable polycythemia	29	18
Total	87	61 (70%)
Group B[c]		
Secondary polycythemia	5	0
Unclassifiable polycythemia	16	4 (25%)
Total	21	4 (20%)

From Lemoine et al.,[207] with permission.

[a]Standard criteria were criteria proposed by the PVSG and criteria routinely used for the diagnosis of causes of secondary polycythemia. [4]
[b]Endogenous erythroid colonies derived from bone marrow cells developing without exogenous EPO.
[c]Group A is defined to include male patients having a red cell mass of 36 ml/kg and female patients with a red cell mass of 32 ml/kg. Group B is defined as male patients having a red cell mass of 3036 ml/kg and female patients with a red cell mass of 2532 ml/kg.

The proliferative capacity of polycythemia vera hematopoietic progenitor cells has been shown to be a useful laboratory adjunctive study. [207] [208] [209] Lemoine et al.[207] prospectively used the formation of endogenous erythroid colonies as a diagnostic tool to confirm the diagnosis of polycythemia vera in the evaluation of a group of patients with isolated erythrocytosis (Table 61-5). In this study, the clinical course of those patients with marrow cells that formed endogenous colonies was very similar to that of patients who met standard criteria for the diagnosis of polycythemia vera. [207] Slightly >70% of these patients developed difficulties that eventually required treatment. Conversely, those patients whose marrow cells did not form colonies in the absence of EPO enjoyed a benign clinical course not requiring therapeutic intervention. The utility of such assays has also been emphasized by Shih et al. who studied patients with idiopathic thrombocytosis who did not meet the diagnostic criteria for polycythemia vera. [210] Shih et al. reported that a high proportion

Figure 61-7 Photomicrograph of bone marrow biopsy obtained from polycythemia vera patient in spent phase demonstrating hypercellularity and increased number of megakaryocytes (× 160).

of patients with normal or reduced hematocrits associated with marked isolated thrombocytosis (>1 × 10^6 /mm[3]) who had marrow cells that formed endogenous erythroid colonies eventually developed polycythemia vera within a median of 24 months. [210] These findings require confirmation because other laboratories have reported endogenous erythroid colonies in 50% of patients with primary thrombocythemia. [208] [209] It is important to emphasize that although the presence of endogenous colonies in the blood is indicative of polycythemia vera, the inability to detect these colonies does not entirely eliminate the diagnosis. [211] Assays of bone marrow cells are indicated when endogenous colonies are not detectable in the peripheral blood. [211] Due to the lack of quality assurance in the performance of such clonal erythroid progenitor cell assays in some laboratories, some reservations concerning their widespread use as diagnostic tools have been expressed. [209]

Laboratory evaluation of patients with polycythemia vera has been further facilitated by the more widespread availability of quantitative assays of EPO. [56] [57] [58] At diagnosis, serum EPO levels in polycythemia vera are either reduced or at the lower limits of normal. Even following normalization of the hematocrit, the serum EPO level in polycythemia vera remains low in two-thirds of patients. [56] [57] [58] [212]

The occurrence of nonrandom cytogenetic abnormalities in polycythemia vera is not unexpected, as this is a feature of most hematologic malignancies (Fig. 61-8).[213] [214] [215] [216] [217] [218] [219] [220] Such abnormalities have been observed, with no single characteristic chromosomal abnormality defined; abnormalities of chromosomes 1, 5, 7, 8, 9, 12, 13, and 20 have been detected. [213] [214] [215] [216] [217] [218] [219] [220] These cytogenetic abnormalities can be categorized according to the phase of disease (onset, erythrocytotic, or spent phase) in which they are most frequently observed. [213] [214] [215] [216] [217] [218] [219] [220] Also, certain cytogenetic abnormalities are clearly a result of treatment-induced mutation, associated with the use of ^{32} P or alkylating agents (Fig. 61-8).[213] [214] [215] [216] [217] [218] [219] [220] The frequency of detection of cytogenetic abnormalities in polycythemia vera is a cumulative function, with clonal cytogenetic abnormalities being observed in 820% at diagnosis; in 35 55%

following a number of years of treatment; and in >80% in those patients in whom acute leukemia eventually develops. [218] [219] [220] Therefore, clonal progression from a normal to abnormal karyotype, especially acquisition of abnormalities involving chromosomes 5 and 7, is an important adverse prognostic parameter. [218] [219] [220]

Trisomy of chromosomes 8 and 9 and deletion of the long arm of chromosome 20 (20q) are most frequently observed at diagnosis. [213] [220] In several studies, the single most common abnormality was 20q (q11),[213] [214] [215] [216] [217] [218] [219] [220] which is a recognized genomic constitutive fragile site subject to mutagenic insult in vitro. [221] This cytogenetic defect is common but is not diagnostic of polycythemia vera. The 20q (q11) deletion is due to an interstitial deletion. [222] [223] [224] [225] [226] To date, an extensive search for a putative tumor suppressor gene at the site of the 20q deletion that might play a role in the biogenesis of polycythemia vera has been unrewarding.[226] Using the microsatellite polymerase chain reaction spanning the common deleted region on 20q, this cytogenetic abnormality has been shown to be absent in peripheral blood granulocytes but to be simultaneously present in most marrow metaphases. These cytogenetically normal granulocytes have, however, been shown to be clonal in origin in females with polycythemia vera. [224] Such data suggest that 20q deletions occur as secondary events in polycythemia patients with preexisting clonal hematopoiesis and that the absence of cells with 20q in the peripheral blood results from selective influences such as preferential retention or destruction within the marrow of granulocytes carrying this abnormality. [224]

Serial cytogenetic evaluation of previously karyotypically normal patients indicate that other abnormalities most often acquired with progression of the disease are 13q, 12q, and 1q (Fig. 61-8).[218] [219] [220] These particular abnormalities do not necessarily herald transition to acute leukemia, but leukemia or myelofibrosis was observed in 8 of 12 patients with the 1q abnormality in one study. [201] Deletion of the long arm of chromosome 13 has been observed during the myelofibrotic phase of polycythemia vera, but this abnormality has been detected also during the erythrocytotic phase. It does not appear, therefore, that a chromosome 13q abnormality heralds disease transition.[217] PPMM occurring after the use of chemotherapy is often accompanied by additional karyotypic abnormalities, with chromosomes 5 and 7 being most frequently involved.[214]

One can conclude that chromosomal abnormalities such as

Figure 61-8 Common chromosome abnormalities in untreated and treated patients with polycythemia vera at time of chromosome study, shown by number of years after initial diagnosis. Abn, abnormal. *(From Diez-Martin et al.,[216] with permission.)*

+8,+9, and 20q might be related to the biogenesis of the disease rather than to the treatment employed. [219] [220] In addition, it appears that, at best, in some patients, an abnormal clone (abnormalities of chromosome 5 or 7) and hypodiploidy develop as a consequence of therapy with chemotherapeutic agents. [218] [219] [220] Diez-Martin et al. have also suggested that patients with a cytogenetic abnormality at diagnosis have a statistically significantly poorer survival rate than those in whom a normal karyotype is observed.[218] This influence of cytogenetics on prognosis has not been confirmed by others. [215] [219] [220]

DIFFERENTIAL DIAGNOSIS

In the overwhelming number of patients, establishing the diagnosis of polycythemia vera is not difficult. Characteristically, the patient will present with erythrocytosis, leukocytosis, and thrombocytosis.[4] The bone marrow biopsy shows hypercellularity with trilinear cellular hyperplasia. [105] [204] [205] A direct determination of a red cell mass is not universally necessary.[196] [197] Many patients with a raised hematocrit, however, will require blood volume studies to document an elevated red cell mass.[196] [197] A hematocrit greater than 60% on several occasions in men or greater than 55% in women, however, is associated in the preponderance of cases with an elevated red cell mass.[196] [197] In those situations, it would be reasonable not to proceed with blood volume studies. If the hematocrit is below 60% in men or below 55% in women, blood volume studies are required. It has become increasingly apparent that an elevated hematocrit should not be the only criteria for proceeding with a red cell mass determination.[141] Splenomegaly can lead to an expanded plasma volume, which can mask the diagnosis of polycythemia. It is suggested that red cell mass determinations be performed in patients with normal hemoglobin or hematocrit determinations in the context of portal vein thrombosis, isolated leukocytosis, or splenomegaly.[141] The presence of splenomegaly is an important finding on clinical examination, and adjunctive laboratory findings include normal arterial oxygen saturation, elevated leukocyte alkaline phosphatase activity, elevated serum vitamin B_{12} levels and B_{12}-binding proteins.[4] Splenic sizing by ultrasound can be useful in documenting splenic enlargement when the spleen is not palpable by physical examination. [227]

It is initially important to discriminate polycythemia vera from the large number of other causes of secondary erythrocytosis (see Chap. 25). Two tests that are especially useful for this purpose are a determination of the ability of bone marrow cells to form erythroid colonies in the absence of exogenous EPO, and quantitation of serum EPO levels by either RIA or ELISA assays.[56] [57] [58] [212] The availability of newly defined genetic markers employing DNA restriction fragment length polymorphisms to establish clonality of a bone marrow proliferative disorder may also expedite the diagnosis in affected females. [63] [64] [65] The impact of these new diagnostic tools in establishing the diagnosis of polycythemia vera in an individual patient is now apparent.

Clinical criteria for the diagnosis have been defined by the PVSG and have been used successfully to obtain a uniform patient population with polycythemia vera for evaluation of therapeutic modalities.[13] [184] It is important, however, for the clinician to realize that some patients undoubtedly have a myeloproliferative disorder resembling polycythemia vera but do not fulfill all the diagnostic criteria of the PVSG. [141] The diagnostic criteria of the PVSG do not include determination of elevation of the red cell mass based upon mean predicted values, a measurement of bone marrow proliferative capacity, a measurement of serum EPO, or ultrasound documentation of splenomegaly. More usefuland, it is hoped, more flexiblecriteria for the diagnosis of polycythemia vera are suggested in Table 61-6 . There will always be unusual cases with clinical characteristics that cannot be pigeonholed into a particular diagnostic category. If asymptomatic, these patients should be followed carefully until the

TABLE 61-6 -- Clinical and Laboratory Criteria for Diagnosis of Polycythemia Vera[a]

1. Elevated red blood cell mass of (>25% above mean predicted value)
2. Normal arterial oxygen saturation (92%) in the presence of erythrocytosis as defined in criterion 1
3. Splenomegaly
4. Thrombocytosis (platelet count of 400,000/mm^3) and leukocytosis (white blood cell count of 12,000/mm^3)
5. Bone marrow hypercellularity associated with clustered mature megakaryocytes with hyperlobulated nuclei and absent iron stores
6. Low serum EPO levels (<3.0 U/l) in the presence of an increased red blood cell mass as defined in criterion 1
7. Abnormal marrow proliferative capacity as manifested by formation of erythroid colonies in the absence of exogenous EPO

[a]The presence of criterion 1 and any three additional criteria are diagnostic of polycythemia vera.

disorder evolves into a more recognizable entity. This would appear prudent in order to avoid unnecessary therapeutic interventions. If these patients have serious symptoms, the individual physician must make treatment decisions on the basis of the risk/benefit ratio for that patient.

A particularly difficult dilemma occurs when evaluating patients with isolated pure erythrocytosis. These patients have elevated red blood cell masses, normal white blood cell counts, normal platelet counts, no evidence of splenomegaly, and no evidence of any recognizable cause of secondary erythrocytosis. Russell and Conley[228] and Modan and Modan,[229] as well as Najean et al.,[230] believe that these cases represent a clinical entity distinct from polycythemia vera. In contrast, Pearson and Wetherley-Mein[231] have suggested that at least some of these cases can be reclassified as polycythemia vera at a later time in their clinical course. In the report of Najean et al., 7 of 51 such patients after prolonged follow-up developed a clinical picture similar to that of polycythemia vera or agnogenic myeloid metaplasia with myelofibrosis, or both.[230] The clinical course of patients with primary pure erythrocytosis was also frequently complicated by thrombotic vascular episodes. These patients were treated with myelosuppressive therapy to avoid additional complications. [230] Of these 51 patients, five eventually developed acute myeloid leukemia.[230] On the basis of this information, a significant number of these patients appear to have a disorder that closely resembles polycythemia vera and should be treated accordingly. The familial congenital form of polycythemia, characterized by increased sensitivity of erythroid progenitor cells to EPO, can be easily distinguished from polycythemia vera. These patients frequently present in childhood, and this disorder is characterized by isolated erythrocytosis that is associated with an increased risk of thrombotic events.[33] [34] [35] [36] [37] There is a strong family history of polycythemia.[33] [34] [35] [36] [37] In contrast to polycythemia vera, the marrow progenitor cells are hypersensitive to EPO, yet no colonies form in the absence of EPO. [33] [34] [35] [36] [37]

Polycythemia vera must also be distinguished from an endemic, familial form of congenital polycythemia that occurs commonly in the Chuvash population of the Russian Federation.[232] The extreme elevations of hemoglobin in these patients are accompanied by elevated EPO levels. [232] This disorder is thought to be inherited as an autosomal recessive trait.[232] Patients frequently present with a history of fatigue and headaches and are plethoric on physical examination. The recently reported mortality of more than 10% of a very young cohort of patients over a period of 10 years of observation indicates that this form of polycythemia is not benign.[232] Chuvash polycythemia is thought to be due to an abnormality in the oxygen sensing mechanism resulting in increased EPO production. Chuvash polycythemia is the only endemic form of familial and congenital polycythemia described to date. [232]

The ability of bone marrow cells to form endogenous erythroid colonies has also been used to analyze etiologic factors in the development of Budd-Chiari syndrome.[133] [233] Valla et al.[233] studied the marrow proliferative capacity of 20 patients with this syndrome and observed endogenous erythroid colony formation in 16 cases. In two of these 16 patients, it was quite obvious that a myeloproliferative disorder was the underlying etiologic factor in the development of hepatic vein

thrombosis. This abnormality in the other 14 patients suggests that the development of Budd-Chiari syndrome may represent, in some cases, a forme fruste of a myeloproliferative disorder.[233]

Polycythemia vera must also be differentiated from the other myeloproliferative disorders, such as CML, primary thrombocythemia, and agnogenic myeloid metaplasia with myelofibrosis. Such classification has major prognostic implications and influences important therapeutic decisions. With the distinctive cytogenetic abnormalities and molecular genetic abnormalities that are unique to CML (Philadelphia chromosome, *bcr-abl* gene fusion), these two disorders should not be difficult to differentiate.[59] A less complex test, the leukocyte alkaline phosphatase activity, may also be of assistance. The leukocyte alkaline phosphatase score is elevated in polycythemia vera but is decreased in patients with CML.[4] [59] In addition, the incidence of elevated red cell masses in patients with CML is low.

Patients with agnogenic myeloid metaplasia with myelofibrosis can present with abnormalities that are virtually indistinguishable from those of patients with PPMM. [124] [125] [126] The survival of patients with the latter disorder is much shorter than that of patients with the former condition. [124] [125] [126] Some patients with presumed agnogenic myeloid metaplasia may actually develop, after the institution of chemotherapy, the clinical features of polycythemia vera. [14] A preceding history of polycythemia vera permits differentiation between these two situations. Primary thrombocythemia and polycythemia vera with marked thrombocytosis can easily be confused. When the red blood cell mass is used as a definitive diagnostic test, a distinction between primary thrombocythemia and the erythrocytotic phase of polycythemia vera is usually readily apparent. [234] [243] This measurement, however, can be normal or actually low in the patient with polycythemia vera who is iron-deficient because of bleeding or excessive phlebotomy. [133] To avoid this difficulty, Iland et al. [234] have developed a logistic regression algorithm on the basis of clinical characteristics; this algorithm appears useful in differentiating primary thrombocythemia from polycythemia vera in cases in which either a red blood cell mass quantitation is unavailable or iron deficiency cannot be excluded. Because the marrow cells of over 50% of patients with primary thrombocythemia form endogenous erythroid colonies, hematopoietic progenitor cell assays are not useful in differentiating these two disorders. [209]

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THERAPY

Dramatic prolongation of survival over that expected from the natural history of untreated polycythemia vera has been achieved with several therapeutic strategies. [134] [185] [186] [187] [188] Historical evidence for an untreated median survival of approximately 18 months is derived from descriptive accounts. [12] [192] Median survivals of >10 years are now commonplace with optimal management.[134] [185] [186] [187] [188]

A series of studies performed over a 17-year period (19671984) by the PVSG has answered several very important questions regarding the efficacy and associated complications of particular therapeutic modalities. These investigations have aided in the identification of optimal therapy for individual patients, which must be selected on the basis of age and co-morbid disease status in order to minimize treatment-related complications. [184] [185] [186] [187] [188]

The first PVSG randomized trial (01 trial) examined three

GENERAL PRINCIPLES OF THERAPY

1. Etiology of erythrocytosis must be correctly categorized in order to be certain the patient has polycythemia vera. This will avoid inappropriate exposure of patients with nonmalignant disorders to radiation or chemotherapy.
2. Therapy should be individualized.
3. Initially blood volume should be reduced to normal as rapidly as possible. The speed of phlebotomy will depend on patients general medical condition (250500 ml every other day). Elderly patients with compromised cardiovascular or pulmonary systems should be more carefully phlebotomized (twice a week), or smaller volumes of blood should be removed.
4. Hematocrit should be maintained at 4245%.
5. Excessive doses of chemotherapeutic agents should be avoided. Supplementary phlebotomy rather than potentially toxic doses of chemotherapeutic agents should be used in order to avoid excessive marrow and systemic toxicity.
6. Hyperuricemia is treated with allopurinol (100300 mg/day).
7. Pruritus is treated with cyproheptadine 416 mg/day; if unsuccessful, interferon- therapy 3.0×10^6 units, subcutaneously three times a week should be instituted.
8. Elective surgery or dental procedures should be delayed until red cell mass and platelet counts have been normalized for 2 months. If emergency surgery is contemplated, phlebotomy and cytopheresis should be pursued.
9. Women and men who are contemplating having children should be treated by phlebotomy plus low-dose aspirin therapy (40 mg/day or with interferon-) in order to avoid teratogenic effects of chemotherapy and radiotherapy. Such avoidance will also prevent deleterious effects on fertility. During pregnancy therapy is frequently not necessary; if it is, phlebotomy plus low-dose aspirin should be exclusively used. If phlebotomy control is inadequate, treatment with interferon- should be pursued.

treatment arms: (1) phlebotomy alone, to maintain the hematocrit at <45%; (2) intravenous ^{32}P, 2.3 mCi/m[2] repeated every 12 weeks if needed (maximum 5 mCi per dose), supplemented by phlebotomy to maintain the hematocrit at less than 45%, and (3) myelosuppression with chlorambucil 10 mg/day PO for 6 weeks, then daily on alternate months, with necessary dose reductions and supplemental phlebotomy. [184] [185] More than 400 patients were randomly assigned to this protocol. Median survival from entry into the study until death was 9.1 years for patients treated with chlorambucil, 10.9 years for those treated with ^{32}P, and 12.6 years for the phlebotomy group.[185] [188] Long-term survival was inferior for patients treated with chlorambucil when compared to those treated with ^{32}P or phlebotomy.[185] [188] An early finding was the appearance during the first 5 years of a significant excess of deaths from acute leukemia in the chlorambucil arm which has reached 17% after 15 years of follow-up.[185] [186] [187] [188] As a result, the chlorambucil arm was discontinued, and patients were assigned randomly to one of the other two arms. Even though no statistical difference in overall survival between ^{32}P and phlebotomy alone was apparent through the first 10 years, the morbidity and mortality associated with each type of therapy were attributable to distinctly different causes. [185] [186] [187] [188] Thrombosis as a cause of death was much more frequent in the phlebotomy-only group during the first 57 years of

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follow-up.[186] Analysis of factors associated with thrombosis revealed that the performance of phlebotomy, the rate of phlebotomy, advancing age, and history of previous thrombosis were statistically significant factors predictive of this outcome. [187] In contrast, the use of^{32}P led to a lower rate of thrombosis during the first 5 years, but the incidence of leukemias, lymphoma, and nonhematologic malignancies increased during the next 5 years to nearly 10%. [185] [186] [187] [188] Following a

15-year period of observation, the incidence of leukemia and lymphoma in the chlorambucil group had risen to 17%. [185] [186] [187] [188] A statistically significant increase in skin and gastrointestinal cancers occurred in the ^{32}P and chlorambucil-treated cohorts, as compared with the group treated with phlebotomy alone. [185] [186] [187] [188]

Given the paradox of equal 10-year survivals, but demise

due to distinct causes, in comparable populations of patients treated by phlebotomy alone or by phlebotomy plus ^{32}P, the PVSG pursued strategies to reduce the thrombotic risk in the phlebotomy-only group. One study attempted reduction of the thrombotic risk by combination antiplatelet therapy. It appeared possible that therapy directed toward altering platelet function might reduce the frequency of thrombosis. [160] Therefore, a randomized trial was performed in which phlebotomy supplemented with the platelet antiaggregating agents aspirin and dipyridamole was compared with ^{32}P (PVSG trial 05). The outcome for the phlebotomy/aspirin/dipyridamole-treated group was disappointingly inferior to the ^{32}P results.[160] In fact, more thromboses occurred in the former than in the latter group, but surprisingly, there was also a significantly greater incidence of severe gastrointestinal hemorrhages. [160]

Renewed impetus for the use of nonchemotherapeutic agents for the treatment of polycythemia vera was provided by an extensive natural history study of 1,213 patients reported by the Gruppo Italiano Studio Policitemia. [96] They showed that the ageand sex-standardized mortality rate of patients with polycythemia vera was 1.7 times greater than the mortality of controls in the general Italian population. [96] In addition, four times as many patients who had previously received ^{32}P, alkylating agents, or hydroxyurea died of cancer compared to patients treated with phlebotomy alone. [96] When this group combined the total number of deaths and the number of nonfatal myocardial infarctions and strokes, they found an unsatisfactory risk:benefit profile in patients treated with chemotherapeutic agents and suggested that antithrombotic strategies, such as low-dose aspirin, be carefully evaluated for use in the care of polycythemia vera patients. [96]

In light of more recent knowledge of the dose requirements for selective antiplatelet therapy with aspirin, it appears that the PVSG study of platelet antiaggregating agents might have failed due to the use of excessive aspirin dosages (900 mg/day). Such aspirin doses appear to diminish vascular endothelial production of the platelet antiaggregatory factor, prostacyclin I_2 (PGI_2).[113] [115] [116] [119]

Polycythemia vera platelets are known to have a generalized abnormality of arachidonate metabolism that is characterized by enhanced synthesis of thromboxane A_2 which likely reflects stimuli to platelet activation. [113] [117] [119] The exact mechanism responsible for enhanced platelet synthesis of thromboxane A_2 in polycythemia vera remains unknown and requires further investigation. A low-dose aspirin regimen (50 mg/day for 7 to 14 days) has been shown to suppress greater than 80% of the excretion of the metabolites of thromboxane A_2 .[113] Recently a pilot study was performed in which the toxicity of low-dose aspirin therapy in polycythemia vera patients was evaluated.[115] A very-low-dose aspirin regimen (40 mg/day) was chosen to prevent thrombosis yet minimize the risk of bleeding. After follow-up of the low-dose aspirin treatment group and control group for a mean of 16 ± 6 months, low-dose therapy was shown not to be associated with an increased incidence of bleeding complications. Aspirin therapy was well tolerated and was associated with complete inhibition of platelet cyclooxygenase activity. [115] A large, randomized, placebo-controlled clinical trial testing the risk:benefit ratio of low-dose aspirin therapy in preventing thrombotic episodes in polycythemia vera is currently being pursued.[115] The results of this trial are eagerly awaited. Until the results of this trial become available, the employment of phlebotomy therapy alone is not terribly attractive since the addition of low-dose aspirin therapy appears to be innocuous and possibly effective in diminishing the risk for thrombotic events.

It is interesting to compare the results of the PVSG with those of a randomized trial of ^{32}P versus busulfan for the treatment of polycythemia vera conducted by the European Organization for Research and Treatment of Cancer (EORTC). [234] [235] The two studies were comparable in size, design, and duration of follow-up. In the EORTC trial induction courses of busulfan 46 mg/day for 48 weeks were compared with ^{32}P treatment.[221] Patients treated with busulfan enjoyed a survival advantage over ^{32}P-treated patients.[235] [236] This difference was due primarily to a threefold greater incidence of fatal thrombotic events in the ^{32}P group. Interestingly, the incidence of leukemia in both groups was very low (<2%), with an overall malignancy rate of <10% (involving mostly solid tumors). [235] [236]

It is reasonable to conclude from this trial that busulfan is a myelosuppressive agent of limited leukemogenic potential when used on an intermittent schedule. [235] [236] A retrospective study of patients in England treated with phlebotomy and intermittent busulfan supports these same conclusions. [237]

After the disappointing results experienced with the alkylating agent, chlorambucil, the PVSG began a nonrandomized phase II investigation of hydroxyurea, an S-phase-specific ribonucleotide reductase inhibitor. [238] [239] The hope was that this agent would be nonleukemogenic. Of 53 patients with polycythemia vera treated with hydroxyurea who had never received other forms of myelosuppression, after follow-up for a median period of 8.6 years and a maximum follow-up of 795 weeks, 5.4% developed acute leukemia in comparison with 1.5% of patients treated with phlebotomy alone on the original PVSG randomized study. [188]

In a comparable trial reported by Sharon et al., 71 patients were treated with hydroxyurea for a mean duration of 7.3 years. [240] Remarkably, the incidence of thrombosis was only 6%, indicating the impressive potential of hydroxyurea to lower the incidence of thrombosis in patients with polycythemia vera, confirming an observation previously made by Kaplan et al.[239] The incidence of leukemia in the Israeli trial was 5.6%. [241] Several other reports are also available reporting the incidence of acute leukemia in polycythemia vera patients treated with hydroxyurea. [183] [240] [241] [242] [244] [245] [246] The incidence varies from 10.5% in the 30 patients treated by Weinfeld et al. to 6.2% in the 65 patients treated by Lovfenberg et al. and 10% in the 150 cases treated by Najean et al. [183] [240] [241] [242] [244] [245] [246] Furthermore, in a randomized trial including 296 patients comparing the efficacy of hyroxyurea to piprobroman, an alkylating agent, for the treatment of polycythemia vera, the incidence of leukemia was similar in both treatment arms; the incidence of PPMM was, however, statistically greater in the patients treated with hydroxyurea.[245] One must conclude that the leukemogenic potential of hydroxyurea in this patient population remains inadequately evaluated and that each of the published series are limited in size and duration of follow-up. [183] [188] [240] [241] [242] [244] [245] [246] In lieu of a randomized trial, the author concludes that the incidence of leukemia is somewhat higher in hydroxyurea-treated patients than in phlebotomy-treated patients. However, the documented ability of hydroxyurea to lower the incidence of thrombotic events, even in the face of increasing documentation of its leukemogenic potential, still makes this drug a very useful chemotherapeutic agent in patients with disease that cannot be controlled with phlebotomy alone. Excessive myelosuppression, macrocytosis, hypersegmentation of polys, stomatitis, leg ulcers, creatinine elevations, and jaundice have been attributed to the use of hydroxyurea. [245] [247] In one series, apthous and leg ulcers occured in 20% of patients, usually after 5 years of hydroxyurea maintenance therapy.[245] In addition, the use of hydroxyurea requires good patient compliance and careful monitoring of blood counts to avoid the sequelae of excessive myelosuppression.

Some clinical investigators have suggested that a program of phlebotomy alone would be the most appropriate for younger patients, in whom the risk of cerebrovascular or cardiovascular events might be predicted to be low. [11] [95] [174] In fact, analysis by Najean et al.[98] of a series of patients with polycythemia vera presenting <40 years of age indicated that such suppositions are erroneous. A striking incidence of serious thrombotic events was observed, with Budd-Chiari syndrome (hepatic vein thrombosis) and cerebrovascular accidents being the leading

causes of death.[98] [115] These investigators recommended a program of myelosuppression for the treatment of these young patients. There was a low incidence of acute leukemia in this group of 58 patients, who were followed for >15 years (only one developed acute leukemia). This finding is surprising, because most patients received either ^{32}P, an alkylating agent, or hydroxyurea as supplemental therapy.[98] The use of hydroxyurea in patients who had previously been treated with ^{32}P is to be discouraged because this form of sequential therapy is associated with an especially high risk of leukemia. [189] Strong consideration should be given to the use of phlebotomy therapy in conjunction with low-dose aspirin therapy in addition to the use of such apparently nonleukemogenic drugs as interferon- or anagrelide in this younger patient population.[98]

Silverstein et al.[248] [249] have used anagrelide, a selective inhibitor of platelet production, to treat thrombocytosis in polycythemia vera patients with thrombotic or hemorrhagic complications. This agent appears to be nonleukemogenic and acts by impairing megakaryocyte maturation. [250] [251] Its use leads to a selective reduction in platelet numbers, and it has been effective in patients refractory to hydroxyurea and interferon. [248] [249] [252] [253] [254] This drug does not effectively control the erythrocytosis or systemic symptoms associated with polycythemia vera.

Recently, Pettit et al. have reported that anagrelide usage in polycythemia resulted in a complete resolution of thrombocytosis as defined as a platelet count less than 600,000 or a 50% reduction of pretreatment levels in 66% of 113 patients with polycythemia vera. [252] In nearly all responders, platelet reduction was observed within a

week. The time to complete response generally ranged between 17 and 25 days.[252] The dose of anagrelide required to control thrombocytosis remained constant over time in most patients. When anagrelide is discontinued, platelet counts returned to pretreatment levels within 5 to 7 days. The average daily dose required to control thrombocytosis in polycythemia vera patients was 2.4 mg.[252] Anagrelide should not be used in pregnant patients because it can easily cross the placenta, leading to adverse effects on the platelet count of the fetus.[252] The incidence of life-threatening hemorrhagic and thrombotic events that accompany severe thrombocytosis in patients with myeloproliferative disorders has been shown to be dramatically reduced following the reduction of platelet numbers resulting from the use of anagrelide.[253]

Approximately 1520% of patients treated with anagrelide discontinued the medication due to nonmyelosuppressive side effects.[248] [249] [252] [253] [254] The spectrum of adverse effects involved neurologic (headaches and dizziness), cardiac (vasodilation, fluid retention, congestive heart failure, palpitations, and tachycardia), and gastrointestinal (nausea) toxicities. These toxicities reflect the novel mechanism of action of anagrelide as a cyclic nucleotide phosphodiesterase inhibitor. Anagrelide should be used with caution in patients with known or suspected cardiac disease because of its ability to promote fluid retention.[252] [253] [254] Because a large fraction of the patients with polycythemia vera are elderly, careful attention to fluid status should be maintained in order to avoid patients slipping into congestive heart failure following the initiation of anagrelide.

Another potential approach to controlling the erythrocytosis and thrombocytosis in polycythemia vera involves use of interferon-.[247] [255] [256] [257] [258] [259] Interferon- has global effects on hematopoiesis.[247] [260] It inhibits both in vitro erythropoiesis and thrombopoiesis and antagonizes the action of platelet-derived growth factor, which likely plays a major role in the pathobiology of myelofibrosis.[127] [128] Silver has suggested that treatment with interferon potentially might alter the natural history of polycythemia by interrupting the development of PPMM and myelofibrosis.[247] Reports of small groups of patients treated with interferon indicate that the erythrocytosis can be controlled in 6 to 12 months, virtually eliminating the need for phlebotomy in approximately 70% of cases.[247] [255] [256] [257] [258] [259] In addition, interferon is capable of eliminating or reducing the degree of splenomegaly, leukocytosis, and thrombocytosis in the majority of polycythemia vera patients.[247] [255] [256] [257] [258] [259] Importantly, interferon treatment has been shown to be capable of diminishing the severity of pruritus in 80% of patients.[247] [255] [256] [257] [258] [259] Occasionally the effect on pruritus can occur in days.[242] The mechanism by which interferon eliminates pruritus remains unknown. The usual dose of interferon used to control polycythemia varies between 1.9 and 25 × 10^6 units/weekly with a median maintenance dose to 10.5 × 10^6 units/weekly administered subcutaneously in divided doses three times a week.[258] [259] A loss of biological effect can be attributed to the development of interferon- neutralizing antibodies which may be overcome by retreatment with lymphoblastoid interferon- N3.[247] Disease control persists in most patients only as long as interferon- therapy is continued. Rare patients have, however, enjoyed sustained remissions after discontinuation of interferon.[247] Silver has recently reported remarkable results in 16 patients who had been followed for more than 6 years.[247] In this patient group, albeit limited in size, no patient has developed a malignancy or any thrombohemorrhagic event during the treatment period.[247] Although these results are promising, treatment of larger numbers of patients for longer periods of time is required before the results of long-term interferon therapy in the treatment of polycythemia vera can be appropriately evaluated.[247] Although one would anticipate that this form of therapy would be non-leukemogenic, insufficient data is available to justify this conclusion. In addition, although interferon- has a profound effect on PDGF and megakaryocytopoiesis, no data is available to suggest that its use delays or prevents the development of PPMM. Interferon- has, however, been reported to be capable of suppressing the abnormal clone in polycythemia vera, similar to its ability to induce cytogenetic remissions in chronic myeloid leukemia.[261] Intolerance to therapy with interferon- leads to drug withdrawel in 14% of patients.[243] [258] [259] Initially, all patients suffer flu-like symptoms, which are controllable with acetaminophen or aspirin. Such symptoms usually resolve spontaneously after several months of therapy. More serious side effects, including high fevers, severe asthenia, and reversible lower extemity bilateral neuritis, may require cessation of interferon- therapy. It is anticipated that further evaluation of interferon- therapy will be pursued in the future.

Symptomatic management of the polycythemia vera patient may be complicated by the occurrence of intractable pruritus.[4] [163] [165] [166] [262] Classically the pruritus occurs on exposure to sudden body cooling, especially after a warm bath,[4] [166] [169] and is experienced by as many as 4060% of patients treated with phlebotomy.[262] The frequency of pruritus appears to be somewhat lower in patients treated with myelosuppressive agents.[262] This observation is related to the probable relationship between pruritus and degranulation of tissue mast cells and circulating basophils.[163] [164] [165] Some uncontrolled studies have attributed pruritus to hyperhistaminemia or severe iron deficiency, with relief associated with the use of histamine antagonists or ferrous sulfate.[163] [165] [166] Iron replacement is frequently not possible since it can lead to dangerous elevations of the red cell mass. In a number of instances, however, iron replacement has been possible with disease control with interferon-.[247] [255] [256] [257] [258] [259] In one study, myelosuppressive therapy in combination with aspirin resulted in relief from pruritus.[169] The association between pruritus and tissue infiltration by mast cells would appear to explain the response of occasional patients to photochemotherapy with psoralens and ultraviolet irradiation.[263] [264] At present optimal therapy for pruritus appears to be interferon- therapy.[247] [255] [256] [257] [258] [259] Taylor reported that significant pruritus control occurred in 83% of patients treated with appropriate doses of interferon-.

[259] This use of interferon- represents a significant advance in the treatment of a frequently disabling symptom in this patient population.

Budd-Chiari syndrome is a catastrophic illness, which can lead to significant morbidity and mortality in a patient with polycythemia vera.[134] Patients with myeloproliferative disorders are at a high risk of developing this syndrome.[134] Independently, use of oral contraceptive pills is a risk factor for its development; a number of cases of hepatic vein thrombosis have been reported in nonpolycythemic women taking oral contraceptives.[265] Although no data are available, one must be concerned about use of oral contraceptives in women with polycythemia vera.

The optimal approach to the problem of Budd-Chiari syndrome is obviously preventive and involves maintenance of normal blood values in the patient with polycythemia vera.[138] [139] [140] Once the Budd-Chiari syndrome develops, the prognosis without treatment is dismal. The goals of therapy are to prevent further propagation of thrombus, relieve the intense hepatic congestion, and manage the severe ascites that often plague these patients.[140] If untreated, these patients often have a slowly progressive course, with deterioration and death within 3.5 years.[132] Spontaneous resolution of the hepatic vein occlusion rarely occurs. Diuretics may be of value in the treatment of the ascites but do not affect the long-term outcome.[140] Thrombolytic therapy may be expected to have some role in the treatment of patients with acute thrombosis.[132] Anticoagulant therapy may have a role in the prevention of further clot formation, but there has been no definitive evidence that such therapy promotes resolution of established thromboses.[140]

The clinical deterioration of patients with Budd-Chiari syndrome results from damage to the hepatocytes from necrosis associated with marked elevation in sinusoidal pressure, coupled with ischemia from reduced hepatic arteriole perfusion.[132] The only rational therapeutic intervention therefore involves some sort of portal decompression to achieve effective reduction of sinusoidal pressure.[138] [140] A variety of surgical procedures resulting in portal-splenic decompression have been shown to be of value in patients with Budd-Chiari syndrome.[138] [140] [266] [267] Transjugular intrahepatic portosystemic shunt placement has recently been used as a bridge to liver transplantation.[268] [269] [270] Liver transplantation is a potential option for treatment of those patients with continued hepatic decompensation.[271] The hematologic consequences of polycythemia must be aggressively treated in the post-transplant setting, since the hepatic vein occlusion may recur in the transplanted liver.[140]

The performance of any surgical procedures on patients with polycythemia vera is, as previously discussed, accompanied by excessively high morbidity and mortality.[163] [164] [165] Elective surgery should not be contemplated unless the patients hematologic values have been normalized for several months.[163] [164] [165] The longer the hematologic control has been in effect, the lower the incidence of postoperative complications. If emergency surgery is required, the patient should be phlebotomized rapidly until a normal hematocrit is reached and platelets should be available in case excessive perioperative or postoperative bleeding occurs.[163] [165] Following both emergency and elective surgery, the patient should be mobilized as soon as possible and strong consideration should be given to anticoagulation with low-molecular-weight heparin, unless the patient has some contraindication. Dental extractions can also result in excessive hemorrhage and should not be performed unless the patient is under strict hematologic control.[163] [165]

Perhaps the most difficult and frustrating period encountered during the clinical course of a patient with polycythemia vera is the development of PPMM.[123] [124] [125] [126] With more extensive follow-up of patients treated with a variety of therapeutic strategies (1520 years), PPMM appears to be becoming a greater problem in long-term survivors.[133] [134] [135] These patients are frequently symptomatic, as a result of the sequelae of anemia, infection, bleeding, and splenic enlargement.[124] [125] [126] Because few of these patients have been treated in a uniform, controlled fashion, it is difficult to make strict therapeutic recommendations.

The anemia that characterizes the spent phase is usually multifactorial in origin. An important factor is splenic pooling of red cells and expansion of the plasma volume, which occurs as a consequence of splenomegaly.[272] It is therefore important in these patients to obtain red blood cell mass measurements and to quantitate the degree of anemia directly in order to detect those patients who have a low hematocrit but normal red cell mass. Folate or iron deficiency may be important in anemic patients and should be corrected.[124] [125] [126] Almost 20% of patients with PPMM develop overt hemolytic anemia.[124] [125] [126] Some of these patients respond to prednisone therapy, but most require splenectomy. By far the most common cause of anemia is ineffective erythropoiesis,[273] and these patients often require

transfusion therapy. Androgen therapy may be effective in stimulating effective hematopoiesis and diminishing transfusion requirements. [274] Iron overload syndrome secondary to ineffective erythropoiesis or transfusion therapy is a dangerous possibility. In this situation, some consideration should be given to chronic iron chelation therapy.

The clinical course of these patients is frequently punctuated by pressure symptoms secondary to splenic enlargement and repeated splenic infarcts. [124] [126] [275] [276] [277] Treatment must be directed toward decreasing the expansion of a rapidly enlarging spleen. Small doses of busulfan, hydroxyurea, or interferon- therapy may result in the relief of such symptoms;[124] [125] [126] [278] radiotherapy in small doses is also sometimes helpful. [279] [280] Unfortunately, its effect is frequently transient. [126] [279] The chemotherapy or radiation dose must be carefully determined because overzealous use may lead to granulocytopenia and thrombocytopenia. Often, as a last resort, splenectomy is the only reasonable therapeutic maneuver. This procedure in advanced disease is associated with an operative mortality of 25% and should be performed by only the most experienced of surgeons.[275] [276] [277] Surgical intervention should not be inappropriately delayed. [275] [276] [277] Splenectomy in such patients is frequently complicated by excessive hemorrhage and infected hematomas.[275] [276] [277]

Thrombocytopenia may lead to life-threatening hemorrhage in PPMM patients. [124] [125] [126] [280] Its development is due to ineffective thrombopoiesis and/or platelet sequestration by an enlarged spleen and patients may respond to splenectomy. [275] [276] [277] [280] Bleeding due to qualitative platelet abnormalities has been noted in these patients;[119] when it is severe, platelet transfusions are often required, although because of marked splenomegaly such transfusions frequently do not increase platelet numbers. The clinician therefore must follow the extent of hemorrhage as a means of determining the effectiveness of such transfusions. Disseminated intravascular coagulopathy occasionally complicates PPMM and can lead to life-threatening hemorrhage. [275] After careful laboratory documentation, replacement therapy with fresh frozen plasma should be pursued; if that is unsuccessful, a course of low-dose heparin therapy is warranted. If bleeding increases with institution of heparin therapy, the heparin should be quickly discontinued. [275] Some mention should also be made of the use of biological response modifiers for the treatment of PPMM. A number of investigators have suggested that interferon might be useful in the treatment of agnogenic myeloid metaplasia and might be successful in reducing spleen size.[254] [258] [259] [280] Experience with this drug for the treatment of PPMM is limited but occasional remarkable results have been reported. [254] [258] [259] [280]

Information is available concerning the treatment of those patients who develop acute leukemia following polycythemia

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vera.[186] [262] [281] [282] [283] The overwhelming majority of such cases involve myeloid leukemias, but a small number of patients have a lymphoblastic phenotype. [186] [191] [192] [193] [194] The optimal treatment of such patients is unknown. In the elderly, the choice not to institute chemotherapy is a reasonable option, since results with treatment are so poor. The PVSG has treated 13 patients, irrespective of phenotypic markers, with vincristine and prednisone without a successful response. [186] This group treated the remaining nine patients with a regimen including cytosine arabinoside and adriamycin and were able to achieve a complete remission in one patient and a partial remission in another.[186] The survival for the 13 patients, however, was 32 days from the time of institution of chemotherapy. [186] Hoyle et al., [281] however, have reported more favorable results with an induction regimen of daunomycin, cytosine arabinoside, and 6-thioguanine.

Rare prolonged remissions of acute leukemia following polycythemia vera have been reported in the literature. [282] Some investigators have suggested that the poor prognosis of these patients merits immediate treatment with high-dose cytosine arabinoside in combination with either daunomycin or adriamycin. [281] Because these patients are frequently elderly, poor results with standard regimens have been reported. Alternative approaches to standard therapy are required in these elderly patients with secondary leukemias.[186]

Polycythemia vera occurs infrequently during the childbearing years. [262] When it does, it has been reported to lead to an increased incidence of fetal wastage, with 30% of pregnancies in polycythemia vera patients terminating in spontaneous abortions. [262] [284] [285] In addition, pre-eclampsia occurs more frequently in these women.[262] [284] [285] Pregnancy in polycythemia vera patients is frequently associated with a gradual normalization of blood values, and it is not unusual for a woman who has required extensive therapy for control of her disease to no longer require phlebotomies during pregnancy. [262] [284] [285] Delivery appears not to be complicated by excessive hemorrhage or by an increased risk of venous thrombosis.[262] [284] [285]

The normalization of the hematocrit during pregnancy in polycythemia vera has been associated with lowering of the red cell mass into the normal range in the few patients in whom these measurements have been performed.[262] Although some degree of hematocrit normalization can be explained by expansion of the plasma volume or by nutritional deficiencies that occur during pregnancy, it is unlikely that these factors can be solely responsible. [262] It seems more reasonable to assume that the high estrogen levels characteristic of pregnancy suppress erythropoiesis. In the few male patients with polycythemia vera who have been treated with estrogen, suppression of red cell production has been noted. [262] After termination of the pregnancy, the patients hematologic values slowly drift back to their previously elevated values in parallel with the return to normal estrogen levels. [262] Because pregnancy is usually associated with spontaneous control of the polycythemic state, no specific therapy is required, except for careful observation. If needed, therapy should be limited to phlebotomy and low-dose aspirin therapy because of the mutagenic effects of chemotherapeutic agents.[287] If this management strategy therapy is unsuccessful, interferon therapy is suggested because it is not known to be leukemogenic or teratogenic and does not cross the placenta. [287]

Because polycythemia vera is ultimately a stem cell disorder, it should be potentially curable by bone marrow transplantation. [60] Stobart et al.[288] reported a successful allogeneic transplant of an adolescent with polycythemia vera while De Revel has successfully performed an allogeneic transplant in a 48-year-old female with PPMM.[289] The proper role of allogeneic bone marrow transplantation for polycythemia vera has yet to be defined. Its use should likely be limited to high risk young patients.

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PROGNOSIS

The prognosis of a patient with polycythemia vera depends on the nature and severity of the complications that occur during the clinical course of that particular patients disorder.[12] [13] [96] [117] [120] [131] [133] [177] [187] [188] In addition, an individual patients prognosis depends on the duration of the erythrocytotic phase or the time for transition to PPMM or acute leukemia.[11] [96] [117] [120] [131] [133] [177] [187] [188] Survival is also influenced by whether appropriate treatment is instituted during the erythrocytotic phase of the illness. Patients who have uncontrolled erythrocytosis are at an extremely high risk for the development of thromboses. [97] [117] [120] The median survival from onset of symptoms may be as short as 1.5 years in untreated patients.[11] Determination of the optimal management of patients with polycythemia vera has been a difficult task, as the disease, when treated, is associated with a survival of 1015 years. Studies of new potential therapeutic interventions therefore require prospective study with prolonged follow-up before meaningful results can be generated. [185] [186] [187] [188]

The PVSG has shown quite conclusively that survival is significantly poorer for patients treated with chlorambucil than for those treated with either ^{32}P or phlebotomy.[185] [186] [187] [188] On the basis of this study, chlorambucil is not a desirable choice. [185] [186] [187] [188] In this particular study, at least, median survival from entry until death was 10.9 years for ^{32}P-treated patients, 9.1 years for chlorambucil-treated patients, and 12.6 years for the phlebotomy-treated group. [185] [186] [187] [188]

Thrombosis occurred predominantly among the phlebotomy-treated patients, especially during the first 4 years of this study. [185] [186] [187] [188] However, longer follow-up demonstrates a greater number of deaths due to both hematologic and nonhematologic malignancies in patients treated with either of the myelosuppressive regimens.[185] [186] [187] [188] One is left with a serious dilemma in defining treatment for a particular patient. Those who are treated with phlebotomy alone for the first 57 years of their course appear to be at a very high risk of dying from thrombosis. However, with more prolonged follow-up after this 57-year period, patients treated with ^{32}P or one of the other alkylating agents would appear to be at a much higher risk of dying from a malignant disorder. [185] [186] [187] [188]

Therefore, determining the optimal management for a particular patient remains problematic. In a young patient, who potentially will have a more prolonged course lasting several decades, it appears best to treat with phlebotomy and low-dose aspirin if possible. In patients with serious hemorrhagic or thrombotic complications, however, this may not be possible. This situation warrants supplemental therapy with interferon-, anagrelide, or hydroxyurea. Concern over the potential leukemogenic activity of hydroxyurea is warranted because we are only now becoming aware of the true leukemogenic potential of this drug. [183] [188] [241] [242] [245] In some patients, the effectiveness of hydroxyurea in reducing the incidence of thrombotic complications might, however, outweigh the enhanced risk of leukemic transformation experienced by hydroxyurea-treated patients. In elderly patients, who have a more limited survival because of their age, the use of either ^{32}P or hydroxyurea appears warranted.

FUTURE DIRECTIONS

Future directions in the treatment of patients with polycythemia vera are highly dependent on a more comprehensive understanding of the pathophysiology of this clonal neoplastic disorder. Further definition of oncogenic alterations in intracellular signaling within these malignant cells should permit the development of more specific therapies. Further definition of risk factors for the development of fatal thrombotic and hemorrhagic events will be important for the rational design of therapeutic

interventions to prevent these complications. [96] Completion of the large randomized trial testing the ability of low-dose aspirin to reduce the incidence of thrombotic complications in patients treated with phlebotomy will be of major importance. [115]

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Chapter 62 - Chronic Myelogenous Leukemia

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INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal expansion of hematopoietic progenitor cells characterized clinically by myeloid hyperplasia, leukocytosis with basophilia, and splenomegaly. An initial chronic phase of expanded clonal myelopoiesis is followed by inevitable progression to accelerated phase and, finally, to fatal blast crisis. The characteristic Philadelphia chromosome translocation, t(9;22)(q34;q11), juxtaposes the c- *abl* oncogene from chromosome 9 with the breakpoint cluster region (*bcr*) on chromosome 22 resulting in the generation of aberrant *bcr/abl* transcripts. The abnormal *bcr/abl* tyrosine kinase gene product has enhanced activity compared to the wild type c-*abl* tyrosine kinase and is believed central to the pathogenesis of CML.

In 1960, Nowell and Hungerford described the characteristic Philadelphia chromosome associated with CML, the first recurrent chromosomal abnormality associated with a specific human malignancy.[1] They reported that myeloid cells from patients with CML showed a deletion of the long arm of chromosome 22. Subsequently, the translocation t(9;22)(q34;q11) was defined and the abnormal *bcr/abl* gene product identified.[2] [3] [4] [5] This highly consistent genetic abnormality provides a marker for the study of the disease and may provide a target for specific therapy.

The incidence of CML is 1/100,000 population, accounting for 1520% of all leukemias in adults. [6] [7] [8] The peak incidence occurs between the fifth and sixth decades, with a male:female ratio of 1.4:1.[6] [8] The increased incidence of subsequent development of CML in persons exposed to the atomic bomb at Hiroshima and Nagasaki implicates radiation as a potential contributory cause in the etiology of CML. [9] [10] The later development of CML has also been described in patients undergoing radiation therapy for ankylosing spondylitis and for cervical cancer. [11] [12] However, there are usually no predisposing factors identified in individual patients presenting with CML.

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PRESENTATION AND CLINICAL MANIFESTATIONS OF CHRONIC PHASE CML

Clinical Features

Traditionally, CML is described as a phasic disease with most patients presenting in chronic or stable phase, the median duration of which is 34 years. Chronic phase disease then progresses to accelerated phase and subsequently to blast crisis. In symptomatic patients presenting in chronic phase, the most common presenting features are anemia, splenomegaly, bleeding, and constitutional symptoms such as fatigue, lethargy, weight loss, or low-grade fever [13] [14] (Table 62-1). However,

TABLE 62-1 -- Clinical Features at Presentation of CML

Splenomegaly	Constitutional symptoms
Asymptomatic	Fatigue
Left upper quadrant discomfort/pain	Anorexia/weight loss
	Sweats/low grade fever
Early satiety	Bone pain[a]
Anemia	Hyperleukocytosis
Hepatomegaly	Priapism
Purpura	

[a]Uncommon in chronic phase CML.

2040% of patients with CML present with asymptomatic leukocytosis, thrombocytosis, or splenomegaly discovered on routine investigation. [14] [15]

Splenomegaly is present at diagnosis in 8590% of patients. Patients may also present with symptoms due to splenic enlargement such as satiety, left upper quadrant pain or discomfort, and abdominal swelling or bloating. Mild to moderate hepatomegaly is frequent but lymphadenopathy is uncommon. In recent years, there has been an increase in the proportion of patients presenting with lower white blood cell (WBC) counts and a lesser degree of splenomegaly. However, male patients may present with higher WBC counts and larger spleens, [14] and priapism secondary to hyperleukocytosis may be the presenting feature. Generally, there is no increased risk of infections in chronic phase CML, although abnormal neutrophil and natural killer cell function are described. [16] [17]

Laboratory Features

Peripheral blood leukocytosis is characteristic of chronic phase CML (Table 62-2). Generally the WBC count is >25 × 10^9 /L and is not uncommonly 100300 × 10^9 /L. The differential WBC count shows granulocytes at all stages of maturation and peripheral blood basophilia with or without eosinophilia. Mild anemia is common. Although thrombocytosis is present at presentation in 3050% of patients, thrombotic complications are unusual, [14] in contrast to thrombocytosis associated with polycythemia rubra vera or essential thrombocytosis. [14] [18] Indeed, purpura may be the presenting feature of CML, occurring in 15% of patients despite normal or increased platelet counts, and abnormal platelet function is described. [14] [18] [19] Thrombocytopenia is unusual, occurring in <10% of patients at presentation.

The marrow is hypercellular due to extreme myeloid hyperplasia with a differential WBC count showing a biphasic peak of myelocytes and mature neutrophils. The myeloid:erythroid ratio is increased (1030:1). The blast percentage in the bone marrow is <10% and myeloid maturation appears morphologically normal with little or no dysplasia. There may be absolute monocytosis but the monocyte percentage is usually <3%. Megakaryocytes may be increased with megakaryocyte dysplasia. Pseudo-Gaucher cells or sea-blue histiocytes may be present. [20] The bone marrow biopsy confirms the myeloid hyperplasia and there may be an increase in reticulin fibrosis. Occasionally, moderate or even severe myelofibrosis is present.

The leukocyte alkaline phosphatase (LAP) score is almost uniformly low in chronic phase CML but may increase with therapy or with progression of disease. Serum lactic dehydrogenase and uric acid levels may be elevated. There are increased serum levels of vitamin B_{12} and transcobalamin. Although clinically

TABLE 62-2 -- Laboratory Features at Presentation of CML

Peripheral Blood	**Bone Marrow**
Neutrophil leukocytosis with left shift	Myeloid hyperplasia
	Blasts <10% in chronic phase
Basophilia/eosinophilia	
Thrombocytosis	Minimal/no dysplasia
Anemia	Increased megakaryocytes
Blasts <10% in chronic phase	Myelofibrosis (mild/moderate)
	Monocytes usually <3%
Leukocyte alkaline phosphatase (LAP) score	
Lactic dehydrogenase	
Uric acid	
Vitamin B_{12} /transcobalamin	

	Partial thromboplastin time
Cytogenetic analysis	Philadelphia chromosome positive (9095%)
Molecular analysis	*bcr/abl* positive (>95%)

significant coagulopathy is unusual, the partial thromboplastin time may be prolonged.

Differential Diagnosis

At presentation, the differential diagnosis of chronic phase CML most often involves distinction from reactive leukocytosis or a leukemoid or leukoerythroblastic reaction due to infection, inflammation, or metastatic carcinoma. The presence of more than sparse erythroblasts in the peripheral blood is unusual in CML. Likewise, tear-drop poikilocytes are not characteristic of CML and may indicate myelofibrosis or bone marrow infiltration. An absolute basophilia is characteristic of CML. [21] The LAP score may help distinguish CML from reactive states. This score is almost uniformly low in CML and is usually increased in other myeloproliferative disorders and in reactive leukocytosis. It may, however, also be low in paroxysmal nocturnal hemoglobinuria and in hypophosphatasia, although these conditions are not difficult to differentiate from CML. A definitive diagnosis may be made by the demonstration of the characteristic 9;22 chromosomal translocation by cytogenetic or molecular analysis of the peripheral blood or marrow.

Thrombocythemic presentation of CML (57% of cases) should be considered in the evaluation of patients presenting with unexplained thrombocytosis, especially in the presence of splenomegaly.[22] Atypical cases of CML may occasionally be difficult to distinguish from myelodysplastic syndromes, especially chronic myelomonocytic leukemia (CMML).[23] The French-American British Cooperative Leukaemia Group has published guidelines for distinguishing CML from CMML and atypical CML.[24] CMML is characterized by the presence of an absolute monocytosis with marrow trilineage dysplasia and absence of the Philadelphia chromosome.

Chronic neutrophilic leukemia is a rare myeloproliferative disorder characterized by neutrophil leukocytosis and an indolent course. The LAP score is normal or high and there is no basophilia. Splenomegaly, if present, is mild to moderate. Recently, the *bcr/abl* breakpoint in chronic neutrophilic leukemia has been defined. [25] The clinical course of chronic neutrophilic leukemia is more benign than that of typical CML, and treatment is usually not required.

Juvenile CML and CMML

Juvenile CML should be distinguished from juvenile CMML.[26] Juvenile CML usually presents at the ages of 1014, and the clinical features are similar to the adult form of the disease. In most cases of juvenile CML the Philadelphia chromosome can be detected. Juvenile CMML is characterized by splenomegaly, fever, hepatomegaly, generalized lymphadenopathy with eczematoid rashes, leukocytosis with prominent monocytosis, anemia, and thrombocytopenia. The marrow shows myeloid hyperplasia with monocytosis and dyserythropoiesis. The karyotype is usually normal. Serum fetal hemoglobin levels are markedly increased and serum muramidase levels are raised. Most patients are under 2 years of age at presentation and the response to treatment is poor, with survival usually <2 years.

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ACCELERATED PHASE CML

Survival in patients with CML is usually determined by the time to progression to accelerated phase (usually 34 years from presentation) and subsequent blast crisis. Accelerated or advanced phase CML is characterized by progressive myeloid maturation arrest, increasing resistance to therapy, an increase in bone marrow and peripheral blood blasts and basophils, clonal cytogenetic evolution, extramedullary disease,

TABLE 62-3 -- Features of Advancing CML

WBC count difficult to control with conventional chemotherapy [a]
Anemia or thrombocytopenia unresponsive to chemotherapy
Increasing splenomegaly
>10% blasts in the peripheral blood or bone marrow
>20% blasts plus promyelocytes in the peripheral blood or bone marrow
>20% basophils plus eosinophils in the peripheral blood or bone marrow
Persistent thrombocytosis
Additional clonal chromosomal changes [b]
Extramedullary disease
Myelofibrosis

[a] The relevance of this feature in patients treated with interferon is uncertain.
[b] See text for discussion of the significance of additional clonal cytogenetic abnormalities.

thrombocytosis or thrombocytopenia, myelofibrosis, and increasing splenomegaly despite therapy. Many patients with advancing CML remain asymptomatic until the late stages of disease, but some experience fever, night sweats, weight loss, and symptoms of splenomegaly, bone pain, or anemia.

Criteria for defining acceleration of CML have been proposed by the International Bone Marrow Transplant Registry but a consensus has not been reached [27] [28] (Table 62-3). In addition, the applicability of such criteria in the era of interferon therapy and stem cell transplantation has not been established.

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BLAST CRISIS

Blast crisis, resembling acute leukemia, develops in 7585% of patients with CML and is associated with an extremely poor prognosis. Survival averages only 36 months. The presence of >30% blasts in the bone marrow or peripheral blood is generally accepted as evidence of transition to blast crisis. Others accept a definition of >30% blasts plus promyelocytes in the bone marrow or peripheral blood as blast crisis. [29]

During blast crisis, constitutional symptoms such as bone pain, sweats, fever, weight loss, and anorexia are more prominent than in the chronic or accelerated phases of CML.[14] Complications of blast crisis include infection, anemia, thrombocytopenia, and extramedullary disease. The most common sites of extramedullary disease are lymph nodes, skin, bone, and the central nervous system. Occasionally, extramedullary disease may be the first manifestation of blast crisis, without evidence of progression in the bone marrow. However, it almost invariably heralds the onset of blast crisis in the marrow within a short period of time.

In 50% of cases the leukemic cells in blast crisis show morphologic and immunophenotypic characteristics of myeloblasts. Occasionally, erythroblasts or immature basophils or eosinophils predominate. In 25% of cases, the blasts are lymphoid, with immunophenotypic characteristics of pre-B lymphoblasts, and a further 25% are undifferentiated or biphenotypic. [30] Thus, for patients presenting in blast crisis the differential diagnosis includes Philadelphia chromosome-positive acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL). In addition 20% of adults and 4% of children presenting with ALL and rare patients with AML have the Philadelphia chromosome detected on cytogenetic testing or DNA analysis. Occasionally, patients with presumed Philadelphia chromosome-positive ALL revert to chronic phase CML with persistent Philadelphia chromosome positivity following induction chemotherapy, and the diagnosis of lymphoid blast crisis of CML is made retrospectively.

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PROGNOSTIC EVALUATION

A number of studies have identified prognostic factors in patients with CML. The International Chronic Granulocytic Leukemia Prognosis Study Group examined presenting features in 678 cases of CML and established age, splenic size, platelet count, percentage of blasts, and percentage of basophils in the peripheral blood or bone marrow as significant factors influencing survival. The presence at diagnosis of peripheral blood blasts 15%, peripheral blood blasts plus promyelocytes 30%, peripheral blood basophils 20%, thrombocytopenia $<100 \times 10^9$ /L or of myelofibrosis has been identified as features with independent adverse prognostic significance.[14] [27] Models have been generated defining high-, medium-, and low-risk patients with significant differences in survival based on the various prognostic features.[31] [32] [33] [34] [35] The Sokal score has gained wide acceptance.[34] However, the relevance of such classifications in the era of interferon therapy is as yet undefined, and it may be more useful to define prognosis on the basis of individual features of advancing disease. [36] For patients treated with interferon, a response to therapy is an important prognostic factor.

The site of the breakpoint in the *bcr* region has been suggested to have prognostic significance,[37] [38] but this has not been confirmed.[39] The significance of clonal cytogenetic evolution is discussed below.

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CYTOGENETIC FINDINGS IN CML

Cytogenetic analysis reveals the classical 9;22 translocation in 9095% of patients with CML. A variant form of this translocation is identified in 5%. The most commonly identified variant translocations involve the distal part of chromosome 22 but without involvement of chromosome 9. Complex translocations involving chromosomes 9 and 22 and a third chromosome may also occur.

In many Philadelphia chromosome-negative patients (as assessed by conventional cytogenetic methods), the characteristic *bcr/abl* translocation can be demonstrated by molecular analysis.[40] [41] [42] [43] The presentation, clinical features, prognosis, and response to therapy of these patients do not differ from those with typical Philadelphia chromosome-positive disease. A small number of patients with CML are *bcr/abl* negative on molecular analysis. These patients are typically older, have a higher incidence of thrombocytopenia, lower WBC counts, and less prominent basophilia than patients with typical Philadelphia chromosome or *bcr/abl* positive disease.[41] The prognosis for this group of patients is poorer than that for typical CML, with a reported median survival of 25 months. [40]

Additional chromosomal abnormalities are present in advanced phase CML in 80% of cases. These typically involve duplication of the Philadelphia chromosome translocation, isochromosome 17, trisomy 8, or trisomy 19.[27] [44] The relationship of clonal evolution to disease progression in the absence of other clinical or laboratory features of acceleration remains a subject of debate. Traditionally, clonal cytogenetic abnormalities in addition to the 9;22 translocation have been considered to be independently associated with a worse prognosis. [34] [35] [45] More recently, however, it has been recognized that clonal evolution in chronic phase without other features of acceleration may not necessarily be associated with an adverse prognosis. [46] The significance may depend somewhat on the specific additional chromosomal abnormality, the proportion of metaphases that are abnormal, and the time of appearance of the additional abnormality. Isochromosome 17 is almost always associated with disease evolution to accelerated phase and a poor prognosis. However, loss of the Y chromosome may be associated with improved prognosis.[47] In one study, the appearance of an additional chromosomal abnormality >25 months after diagnosis, its presence in >25% of metaphases, and other concomitant features of advancing disease indicated a poorer prognosis. [46]

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PATHOGENESIS OF CML

Molecular Genetics

The cytogenetic hallmark of CML, a reciprocal translocation between chromosomes 9 and 22 (t(9;22)(q34;q11)), results in the juxtaposition of the c- *abl* oncogene on chromosome 9 with the *bcr* region of chromosome 22 and the resulting formation of a chimeric *bcr/abl* gene. In most cases of CML, this chimeric gene expresses an 8.5-kb hybrid mRNA transcript giving rise to a 210-kd fusion protein (p210 *bcr/abl*) with transforming activity for hematopoietic cells and the ability to cause CML-like myelopoiesis in mice.[5] [48]

The human *abl* proto-oncogene, a large gene (approximately 225 kb in length) located on 9q34 contains sequences homologous to the v- *abl* sequences in the Abelson murine leukemia gene associated with its transforming activity. [49] [50] At transcription, exon 1a is spliced out, resulting in fusion of exon 1b to exon 2 (the first common exon).[51] Two mRNA species are normally generated, resulting in two proteins differing by 19 amino acids at the amino terminal. Both are known as p145 and have intrinsic tyrosine kinase activity. The precise breakpoint on the *abl* gene in CML is variable and is distributed over an area >200 kb between exon 1b and exon 2.[49] The translocation results in most of the *abl* gene (exons 211) being translocated to chromosome 22.[3] [4] [5] [50]

The *bcr* region on chromosome 22 spans approximately 135 kb containing 23 exons. Two *bcr* proteins are encoded and are widely expressed in normal tissues. In CML, the fusion protein product of *bcr/abl* varies in size, depending on the site of the *bcr* breakpoint. Three breakpoints are described, M- *bcr*, m-*bcr*, and -*bcr* (Fig. 62-1). The breakpoint is usually within a 5.8-kb segment, the major breakpoint cluster region (M- *bcr*), either between *bcr* exons b2 and b3 or between exons b3 and b4.[52] [53] As a result, the *bcr/abl* translocation leads to the production of two different fusion gene protein products, that is, two variant 210 kd proteins compared to the normal c-*abl* product (145 kd). In 40% of CML patients, the mRNA transcript has a b2a2 junction, and in 40% it is b3a2. [54] Alternative splicing in 20% of patients results in the presence of both fusion proteins, b2a2 and b3a2. [51] When the breakpoint is within the minor breakpoint cluster region (m- *bcr*), p190*bcr/abl* is produced. Here the breakpoint is in the first intron of the *bcr* gene between exons e1 and e2, resulting in fusion of the first exon of *bcr* (e1) to a2.[55] This 190-kd variant *bcr/abl* protein, usually associated with ALL, is present in low amounts in some CML patients and in rare cases is the predominant mRNA expressed.[54] Recently, co-expression of p210 and low levels of p190 have been shown to be more common than previously recognized in CML.[54] [56]

Figure 62-1 Structure of the normal *bcr* and *abl* genes. The three named breakpoints in *bcr* associated with CML are indicated by arrows. The lower part of the figure shows the structure of the possible *bcr-abl* mRNA fusion transcripts. (*Courtesy of Dr. J. M. Goldman.*)

A third breakpoint cluster region, downstream of M- *bcr*, has also been described, -*bcr*, which results in a protein of 230 kd.[57] Novel variants of *bcr/abl* continue to be described, including one joining exon e6 of *bcr* to exon a2 of *abl*,[58] associated with Philadelphia chromosome-negative CML and a further variant joining exon e2 to a2.[59]

Clinical and Molecular Correlations

Strong evidence suggests that the position of the breakpoint in the *bcr* region influences the disease phenotype in CML. [55] In rare cases of CML, p190 is the predominant gene product. These patients have a prominent monocytic component, are generally older, have no palpable splenomegaly and only moderate leukocytosis.[60] It has also been suggested that co-expression of p210*bcr/abl* and p190*bcr/abl* might correlate with disease progression, and the appearance of m-*bcr/abl* mRNA in patients with M-*bcr* expression may be associated with progression to lymphoid blast crisis. [60] [61] [62] However, it has now been recognized that co-expression of p210*bcr/abl* and p190*bcr/abl* is much more common than previously recognized and is not necessarily associated with disease progression. [54]

The -*bcr* breakpoint is present in rare instances, and some cases resemble chronic neutrophilic leukemia rather than typical CML. [57] The *bcr/abl* breakpoint in chronic neutrophilic leukemia has also been described between exons c3 and c4 of *bcr*, resulting in a c3/a2 junction [25] and is associated with a more benign course than typical CML. Presentation of CML with very high platelet counts (1,0002,000 × 10^9 /L) and only moderate leukocytosis is associated with the b3a2 type of *bcr/abl* mRNA transcript.[22]

bcr/abl Expression and Regulation

The functions of the normal *abl* and *bcr* genes are not well understood, and, consequently, the molecular events causing the disordered proliferation and differentiation and clonal expansion of hematopoietic cells characteristic of CML are poorly delineated. However, evidence strongly suggests that the *bcr/abl* fusion gene product is directly involved in leukemogenesis. The *abl* gene is normally located in the nucleus and has low tyrosine kinase activity. Overexpression of p145 *abl* results in inhibition of cell cycle progression at the G_1/S interphase. The activity and intracellular localization of *abl* is regulated by integrins, and it has been postulated that the normal role of *abl* is in transmission of integrin signals involved in cell cycle control to the nucleus. [63] In CML, the fusion of the first exon of *abl* with the first exon of *bcr* leads to relocation of the fusion protein to the cytoplasm with markedly increased tyrosine kinase activity. Up-regulation of *abl* kinase activity in CML is mediated through binding of the first exon of the *bcr* molecule (essential for transforming activity) to the SH2 regulatory domain of *abl*, the major tyrosine phosphorylation site.

Both the p210 and p190*bcr/abl* gene products have enhanced tyrosine kinase activity compared with the normal c- *abl* product (P145)[64] and there is increasing evidence that the abnormal protein is directly involved in cellular transformation to the CML phenotype. [48] [65] The introduction of a retrovirus vector expressing p210*bcr/abl* into human factor-dependent cell lines can result in the generation of factor-independent cells. [66] In addition, the expression of an activated tyrosine kinase in multipotent hematopoietic progenitors causes a delay in maturation, altered responsiveness to growth factors, and a delay in apoptosis induced

by the absence of growth factor.[67] [68] Thus, *bcr/abl* can induce cell proliferation and transformation of immature hematopoietic cells and suppress apoptosis in vitro. Most importantly, a disease resembling CML has been observed in mice transplanted with hematopoietic stem cells containing a *bcr/abl* construct, and the resulting experimental leukemia can be transplanted to secondary recipients. [48]

bcr/abl Substrates

Current investigation of the mechanisms underlying the development of CML include attempts to identify molecules that interact with *bcr/abl* and the signal-transduction pathways by which *bcr/abl* proteins, which are primarily cytoplasmic, can transduce signals to the nucleus to exert transforming activity. In addition to increased autophosphorylation activity of *abl*, the fusion protein appears to change the normal pattern of phosphorylation of key regulatory proteins. [69] [70] [71] Such *bcr/abl* substrates may act as intermediates in tyrosine kinase signaling cascades regulating expression of genes affecting cell growth and differentiation. Putative targets for *bcr/abl* include important regulatory factors such as Grb-2, Shc, p21*ras*, p120GAP, ph-P53, p160*bcr*, CRKL, c-*myc*, c-*myb*, p120CBL, *bcl*-2, and P1-3 kinase.

Many of these proteins are involved in pathways that lead to the activation of the *ras* oncogene. Activated (GTP-bound) p21*ras* has an established role in signal transduction and growth regulation and is required for the proliferation of CML cells. Expression of p210 *bcr/abl* in fibroblasts simultaneously activates p21*ras* and inhibits the GTPase-promoting activity of p120GAP; down-regulation of p210 *bcr/abl* in CML cells with *bcr/abl* antisense oligonucleotides abrogates these effects. [72] Thus, p210*bcr/abl* activity is necessary for the maintenance of p21*ras* in the active GTP-bound form, regulated by the GTPase-activating protein, p120GAP. [70] The SH2 phosphorylation domain of p210*bcr/abl* may also link p210*bcr/abl* to the adaptor protein Grb-2, also leading to *ras* activation. [73]

Other potentially important substrates for p210 *bcr/abl* include ph-P53, which complexes with *bcr/abl* in K562 cells, the *bcr* protein itself, and the phosphorylated adaptor protein CRKL, which is phosphorylated by and also forms specific complexes with p210 *bcr/abl*, [74] [75] [76] although its role, if any, in transformation is unknown. p120CBL is bound by p210*bcr/abl*-bound CRKL and appears to be an important substrate of *bcr/abl* forming complexes with CRKL and phosphatidylinositol-3 kinase (p-13-kinase). [77] [78] This kinase has been suggested as an important downstream effector of *bcr/abl* tyrosine kinase in CML cells, is regulated by *bcr/abl*, and has been demonstrated to be important for proliferation of Philadelphia chromosome-positive cells in vitro. [79]

Although little is known regarding control of p210 *bcr/abl* expression, its transcriptional regulation may be important in the pathogenesis of CML. [80] This is supported by evidence that Philadelphia chromosome-positive colonies arising from hematopoietic progenitors may transcribe the aberrant *bcr/abl* mRNA to a variable degree or express normal *abl* mRNA. [81] The function of the reciprocal fusion gene *abl/bcr* (expressed in 60% of CML patients) is unknown. [82]

Apoptosis in CML

Growing evidence suggests that regulation of hematopoietic cell survival by programmed cell death or apoptosis may play an important role in normal erythroid and myeloid differentiation. Thus, abnormalities in the regulation of cell death may lead to disordered hematopoiesis, contributing to the development of malignancy. It is postulated that the *abl* kinase is normally involved in regulation of apoptosis. Expression of *bcr/abl* in CML appears to confer resistance in vitro to chemotherapeutic and other DNA-damaging agents and prolongs the growth factor-independent survival of CML progenitors by inhibition of apoptosis. [83] [84] [85] Additionally, *bcr/abl* expression may influence hematopoietic cell cycle distribution following DNA damage with delayed transition through G2/M, perhaps allowing time for DNA repair. [85]

Further information regarding the role of p210 *bcr/abl* in leukemogenesis has been generated using antisense oligonucleotides specific for *bcr/abl* sequences. Antisense oligonucleotides can down-regulate *bcr/abl*, suppress Philadelphia chromosome-positive leukemia cell growth in mice, specifically reduce colony formation of early hematopoietic progenitors from CML patients, and decrease cell proliferation in CML-like lines, perhaps by rendering the cells sensitive to apoptosis. [84] [86] [87] [88] A relationship between *bcr/abl* function and *bcl*-2 has been suggested. Transformed, factor-independent and tumorigenic murine hematopoietic cells expressing *bcr/abl* are rendered resistant to apoptosis by virtue of increased *bcl*-2 expression. [89] Furthermore, when *bcl*-2 expression is suppressed, *bcr/abl*-positive cells revert to factor dependence and nontumorigenicity.

These studies have led to the hypothesis that the *bcr/abl* gene serves an anti-apoptotic function resulting in an expanded myeloid population without stimulation of proliferation. However, the importance of p210-mediated suppression of apoptosis in the pathogenesis of CML is uncertain and inhibition of apoptosis alone is unlikely to account for the characteristic clonal expansion. [90] Furthermore, conflicting results are reported. Despite reports of defects in cell cycle control and apoptosis, other studies have failed to identify differences in survival of normal and CML progenitors following exposure to apoptotic stimuli. [91]

The Bone Marrow Milieu and the Pathogenesis of CML

Current evidence suggests that CML is a disease of the primitive pluripotent stem cell with megakaryocytes, erythroid precursors, monocytes, macrophages, eosinophils, basophils, at least some B lymphocytes, and (in some cases) T lymphocytes involved in the malignant clone. [92] [93] During the chronic phase the leukemic myeloid cells mature normally and have a near-normal life span. However, assays of the peripheral blood reveal a significant increase in erythroid, megakaryocytic, and pluripotent progenitors. These are actively cycling, in contrast to the quiescent population of progenitors present in normal blood. [94] These abnormalities may reflect either an expansion of the progenitor cell population or abnormal trafficking and release of progenitors from the marrow.

Long-term marrow cultures have been used to study the relationship between hematopoietic precursors and the marrow microenvironment and to characterize very primitive benign and malignant cells in normal and CML hematopoiesis. In patients with CML, the marrow mononuclear cell population contains both Philadelphia chromosome-negative and -positive primitive progenitors, both of which can give rise to granulocyte/macrophage progenitors in long-term culture. [94] [95] [96] In vitro culture studies have identified abnormalities in the proliferation and differentiation of CML progenitors, their interactions with marrow stroma, and their requirements for and responsiveness to growth factors and negative growth regulators, although the significance of many of these observations remains uncertain. [97] [98] [99]

Progenitor Cell/Stroma Interactions

Abnormalities of stromal/progenitor cell interaction may be central to the pathogenesis of the leukemic hematopoiesis in CML. [97] [98] Specifically, abnormal cytoadhesion or anchorage properties of malignant progenitors is hypothesized to contribute to the disorderly maturation and proliferation of CML progenitors.

[100] [101] [102] CML progenitor cells display defective adherence to normal marrow stroma and to purified fibronectin, perhaps due to adhesion receptor abnormalities, although benign Philadelphia-negative progenitors in CML retain their ability to adhere to stroma. [103] -1-Integrin receptor-mediated adhesion mechanisms especially are defective in CML. [104] [105] In addition, CML progenitors have decreased expression of the adhesion molecule lymphocyte activation antigen-3. [106] The cytoplasmic distribution of the p210 *bcr/abl* fusion protein is hypothesized to be directly involved in these adhesion abnormalities, perhaps by inducing phosphorylation of the intracytoplasmic domains of integrins or other adhesion molecules. [99] [104] [105] These abnormalities in hematopoietic progenitor cell adhesion may, at least in part, contribute to the abnormal proliferation, egress from the marrow, and trafficking of cells in CML.

The CML progenitors may show different growth patterns and responsiveness to humoral factors. Although in vitro observations have uncertain relevance to in vivo growth patterns, differential responses are seen to a number of cytokines including stem cell factor and macrophage inflammatory protein 1-. [95] [105] [107] Further characterization of the complex interactions between marrow stroma and both leukemic and normal progenitor cells and the function and substrates of the *bcr/abl* gene products will be required to enhance our understanding of the pathogenesis of leukemic hematopoiesis in CML.

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MOLECULAR ASPECTS OF DISEASE EVOLUTION

Although additional cytogenetic changes are commonly identified as CML progresses, the molecular events responsible for the transition to the accelerated and blastic phases are unknown. It is believed that the inevitable evolution of CML into accelerated phase and blast crisis is a multistep process involving sequential genetic events. Abnormalities of the tumor suppressor gene, p53, are detectable in 1015% of patients with myeloid blast crisis, [108] [109] and introduction of mutant p53 into hematopoietic progenitor cells from patients with CML can promote their proliferation in vitro and can even lead to the growth of factor-independent colonies, suggesting a potential role for p53 in the evolution to accelerated phase of CML in at least some cases. [110] Changes in the retinoblastoma and *ras* genes have also been described in a small number of cases. [111] [112] However, alterations in tumor suppressor genes or in specific oncogenes appear to be uncommon. New evidence suggests that genomic instability or spontaneous errors in DNA replication, as demonstrated by the detection of microsatellite instability, may play a role in disease evolution. [113]

Most patients with phenotypical lymphoid blast crisis have clonal rearrangement of the immunoglobulin genes and some also have rearrangement of the T-cell receptor genes. [114] [115] More recently, homozygous deletion of the p16 tumor suppressor gene has been identified in 4050% of lymphoid blast crises of CML, but not in myeloid blast crisis. [116] [117] p16 (also known as the CDKN-2 gene) inhibits a critical cycle-dependent kinase (CDK-4), which in turn regulates a primary cell cycle checkpoint before cells are committed to DNA synthesis. Sequential studies have shown homozygous deletion of p16 is acquired in association with progression to blast crisis in at least some cases. [117] A defect in cell cycle regulation (involving G_2/M arrest or the inhibition of apoptosis following DNA damage) may allow the accumulation of additional cytogenetic events and survival of damaged cells, thus contributing to disease evolution. [85]

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THERAPY OF CML

The first chemotherapeutic agent widely used for the therapy of CML was busulfan. [118] The effectiveness of this agent was confirmed in 1968 in a randomized trial. [119] The use of hydroxyurea was first reported in 1966 [120] and both agents are still widely used for control of chronic phase CML. Interferon, introduced in the mid-1980s, [121] [122] was the first agent shown to prolong survival, delay the transition to accelerated phase, and achieve cytogenetic remission in a proportion of patients. During the same decade, allogeneic stem cell transplantation (SCT) became established as the treatment of choice for younger patients with a matched sibling donor and remains the only proven curative treatment. Recently, autologous transplant using chemotherapy-primed peripheral blood stem cells has been performed. Despite these advances, treatment of the accelerated and blast crisis phases of CML remains unsatisfactory, with most patients dying from CML. A treatment pathway is outlined in the box and accompanying algorithm.

Conventional Cytotoxic Chemotherapy

Single-agent therapy with hydroxyurea or busulfan can control chronic phase CML and maintain normal WBC counts but is unlikely to significantly prolong survival. In many centers, hydroxyurea has replaced busulfan for initial control of CML, due to the potential for prolonged bone marrow hypoplasia associated with busulfan, and the superiority of hydroxyurea over busulfan has been demonstrated. [123] Hydroxyurea inhibits the enzyme ribonucleotide reductase, interfering with DNA synthesis. The usual starting dose is 2 g/day, although higher doses (34 g/day) can be used for short periods in patients presenting with very high WBC counts. The dose is titrated in the individual patient to the WBC count. Aplasia, if it occurs, is transient and usually reversible within days of discontinuation of the drug, unlike that associated with busulfan. Allergic reactions may rarely occur. Other complications include oral aphthous ulceration and nail dystrophy. At higher doses, nausea, vomiting, and diarrhea may occur. Teratogenicity has been reported in patients taking hydroxyurea during the first trimester of pregnancy.

Therapy with busulfan is usually commenced at 46 mg/day. Close monitoring of the WBC count is essential and it is advisable to discontinue therapy when the WBC count reaches 2030 × 10^9 /L, because the count will continue to fall for 23 weeks following discontinuation. When the WBC count stabilizes, busulfan therapy may be reinstituted at lower doses (13 mg/day) or intermittent courses may be used. Prolonged and occasionally irreversible aplasia may complicate busulfan therapy. Other toxicities include aspermia, amenorrhea, and a late pulmonary syndrome characterized by cough, fever, pulmonary infiltrates, and respiratory failure. A wasting syndrome similar to Addisons disease may also occur.

Other chemotherapeutic agents have also been investigated for the therapy of CML. Homoharringtonine has shown activity in some patients, including some hematologic and cytogenetic responses in accelerated phase disease. [124] However, complete elimination of Philadelphia chromosome-positive cells from the marrow using conventional chemotherapy is unusual, occurring in only 25% of patients. [125] [126] [127] Intensive chemotherapy similar to induction therapy for AML has been used in CML. Although complete hematologic responses and major cytogenetic responses can be achieved in some patients, these have generally been transient. [125]

Interferon Alpha

Interferon- is now considered standard therapy for most newly diagnosed patients with chronic phase CML. Initial reports indicated that interferon- therapy results in hematologic control in approximately 7080% of patients in chronic phase with a complete cytogenetic response in 1015% of patients and suggested that time to development of blast crisis and overall median survival could be prolonged. Achievement of a major

A TREATMENT PATHWAY FOR PATIENTS WITH CML

Numerous approaches to the therapy of newly diagnosed chronic phase CML are available, ranging from use of hydroxyurea as a single agent until disease progresses to aggressive use of allogeneic transplantation shortly after presentation. The use of interferon- is associated with low early mortality and prolonged survival in a subset of recipients, but arguably may jeopardize efficacy of subsequent allogeneic stem cell transplantation. On the other hand, early use of hematopoietic stem cell transplantation may be curative but is associated with hospitalization and a high incidence of early morbidity and mortality. Of course, the therapeutic decision-making process should include the wishes of a well-informed patient. A description follows of one approach to the treatment of CML drawing on our current understanding of the therapeutic options.

Patients presenting with CML receive leukapheresis or hydroxyurea or both to lower the WBC count, if necessary. Selected patients seeking the potential advantages of early transplantation may wish to proceed directly to allogeneic or autologous transplantation without a trial of interferon- (*dashed line*). Other patients wishing to pursue an initial nontransplant approach (*solid line*) receive a trial of high-dose interferon- (5 million units/m^2 /day) with appropriate dose modification. Cytosine arabinoside may be used as an adjunct to interferon-. Patients who achieve a major or complete cytogenetic remission (66% Philadelphia chromosome-negative metaphases in the bone marrow) by 1 year and who tolerate interferon- without major side effects remain on this therapy. Patients who fail to achieve major or complete cytogenetic remissions as well as patients with clinical or hematologic evidence of advancing disease and patients who cannot tolerate interferon- become eligible for alternative therapeutic approaches. Transplant approaches include allogeneic stem cell transplantation for patients with a suitably HLA-matched related or unrelated donor and autologous transplantation. Nontransplant approaches include hydroxyurea, interferon-, or novel chemotherapy.

cytogenetic response, defined as <35% Philadelphia chromosome-positive cells in the marrow, was associated with a statistically significant improvement in survival. [15] [128]

Randomized trials have compared interferon- with conventional chemotherapy for patients with chronic phase CML [126] [127] [129] [130] (Table 62-4). Most of these have shown a survival advantage for patients treated with interferon-. [126] [127] [129] The Italian Cooperative Study Group on CML reported results of therapy in 322 chronic phase patients randomly assigned to receive conventional chemotherapy (hydroxyurea or busulfan) or interferon- $_{2a}$. Therapy with interferon- induced more karyotypic responses than conventional chemotherapy (30% compared with 5%). A complete cytogenetic response was achieved in 8% and a partial cytogenetic response in

11% of interferon--treated patients. Interferon- therapy was associated with a longer time to progression to accelerated phase or blast crisis (median >72 months compared with 45 months) and with longer survival (median 72 compared with 56 months)[126] (Fig. 62-2 (Figure Not Available)).

Similarly, in the multicenter Medical Research Councils study of 527 chronic phase patients randomized to receive either interferon- or chemotherapy (busulfan or hydroxyurea) survival was significantly improved for patients treated with interferon-. Patients with a cytogenetic response to interferon- survived significantly longer than those not responding. In addition, a survival benefit was seen in the interferon- group even in the absence of a cytogenetic response. [129] [131]

TABLE 62-4 -- Randomized Studies of Interferon in Chronic Phase CML Patients

Reference	Randomization	n	Complete Hematologic Response	Major Cytogenetic Response	Median Survival	Cytogenetic Response Associated with Increased Survival
[130]	Interferon vs hydroxurea or busulfan	513	31%	10%	66 mo	No
[126]	Interferon vs hydroxurea or busulfan	322	62%	19%	72 mo	Yes
[129]	Interferon vs hydroxurea or busulfan	527	68%	11%	61 mo	Yes
[127]	Interferon vs busulfan	117	39%	7.5%	65 mo	Yes
[134]	Interferon/cytarabine vs interferon	721	66%	41% (12 mo) 54% (24 mo)	86% 3-y survival	Yes

However, the German CML Study Group, in a randomized trial of 513 patients receiving interferon-, hydroxyurea, or busulfan, failed to demonstrate an overall survival advantage for patients treated with interferon- compared with those receiving hydroxyurea (median survival 5.5 years compared with 4.7 years). Survival was improved compared to patients treated with busulfan (median survival 3.8 years). Patients with a complete hematologic response to interferon- had a survival advantage compared to partial responders or nonresponders. In contrast to other reports, achievement of a complete cytogenetic response to interferon- was not associated with improved survival.[130] It has been speculated that differences in dosing regimens may partly explain these discrepancies. [132] [133]

More recently, the use of interferon- in combination with other agents has been explored. [134] [135] Previous data suggested that cytarabine (cytosine arabinoside) given subcutaneously could reduce the number of Philadelphia chromosome-positive cells in the bone marrow. [136] [137] In the largest study reported to date, a multicenter trial conducted by the French Chronic Myeloid Leukemia Study Group, 721 chronic phase patients were randomized to receive either interferon- (5 million units/m [2] /day) and hydroxyurea or interferon-, hydroxyurea, and cytarabine (20 mg/m[2] /day subcutaneously for 10 days each month). The rate of hematologic response was greater in the interferon-cytarabine group (66% compared to 55%). Major cytogenetic responses were observed 12 months after randomization in 41% of patients in the interferon-cytarabine group compared with 24% in the interferon alone group. In patients receiving interferon- with cytarabine, cytogenetic response was complete in 15% compared with 9% for those receiving interferon- alone. At 24 months, a major cytogenetic response had been achieved in 54% compared with 41%. In both groups, landmark analysis showed a significant survival advantage for patients with a cytogenetic response (complete or partial) compared to those with no response or a minor response.[134] Low-risk patients by the Sokal score had a greater probability of cytogenetic response. Overall survival after 3 years was significantly greater (86%) for patients treated with interferon- and cytarabine compared with 79% for those treated with interferon- alone.

These studies have had a major impact on the approach to therapy of patients with CML. A recent meta-analysis confirms the survival advantage for patients with chronic phase CML treated with interferon-.[138] In summary, treatment with interferon- in chronic phase CML can result in hematologic responses in 7080% of patients. Cytogenetic responses, either partial or complete, occur in up to 50% of patients and these may be major and durable in 1530% with prolonged high-dose therapy. In most studies to date, achievement of a major cytogenetic

Figure 62-2 (Figure Not Available) Survival of CML patients randomly assigned to receive conventional therapy (hydroxyurea or busulfan) or interferon- $_{2a}$. (*From Italian Cooperative Study Group on Chronic Myeloid Leukemia,[126] with permission. Copyright 1994, Massachusetts Medical Society. All rights reserved.*)

response is associated with improved outcome, with survival of up to 90% in patients with a complete cytogenetic response.

Despite its demonstrated value for patients with chronic phase CML, interferon- therapy may be up to 200 times more costly than conventional chemotherapy [126] and up to 25% of patients discontinue therapy as a result of toxicity. [130] Side effects of interferon- include myalgia, arthralgia, impotence, weight loss, headache, and fever. Most patients experience an influenza-like syndrome at the onset of therapy. Gradually escalating doses over a number of weeks, premedication with acetaminophen, and administration of interferon- in the evening may alleviate these side effects significantly. Neurologic toxicity may occur, usually manifested by memory loss and depression, and is more prominent in older patients. Autoimmune phenomena including immune thrombocytopenia, autoimmune hemolysis, Raynauds phenomenon, lupus erythematosus, and hypothyroidism are also described. Up to 22% of patients develop anti-interferon antibodies, and neutralizing antibodies may be associated with a poor response to therapy.[139] Although interferon- therapy results in equivalent response rates in older patients, toxicity may be increased in this group. [140] Toxicity may also be increased if interferon- is combined with other agents. [130] [134] Thus, patient tolerance and compliance remain significant problems.

The optimal dose for interferon- therapy in CML has not yet been established but is currently being addressed in randomized trials. [131] [141] The most commonly used dose in clinical trials is 5 million units/m [2] /day. It has been argued that administration of lower daily doses of interferon- or use of thrice weekly interferon- dosing can be effective. Such regimens would probably reduce the toxicity of interferon-; however, their efficacy has been called into question. The median time to hematologic response is 68 months (many centers combine interferon- with hydroxyurea for initial control of disease) and to cytogenetic response is 2224 months. [126] [142]

Debate also continues regarding the precise role of interferon and its interaction with other therapies including SCT, where interval between diagnosis and transplant may be a critical prognostic factor for both survival and relapse. Some studies have suggested that prior interferon- therapy may adversely affect post-transplant outcome, although this remains controversial.[143] [144] [145] [146] In addition, even chronic phase patients with a complete cytogenetic response to interferon- usually have residual *bcr/abl*-positive cells detectable by reverse transcriptase-polymerase chain reaction (RT-PCR). [147] Thus, interferon- is unlikely to lead to continuing complete molecular remission, and its curative potential is not yet established.

Mechanism of Action of Interferon Alpha

The mechanism of action of interferon- remains unclear. There is evidence in vitro for antiproliferative effects, normalization of adhesion, progenitor/stroma interactions, and promotion of natural killer cell cytotoxic activity. [104] [105] [106] Interferon- causes a dose-dependent increase in adhesion of primitive long-term culture-initiating cells and committed colony-forming cells from CML marrow to normal stroma, possibly mediated by restoration of normal -integrin function. [104] [105] Interferon- may also modulate local paracrine release of growth factors in the stromal environment, with an increase in inhibitory cytokines such as the interleukin-1 receptor antagonist, transforming growth factor, and MIP-1. There may also be increased T-cell reactivity, although specific anti- *bcr/abl* T-cell reactivity induced by interferon remains unproven. Prevention of transcription of p210 *bcr/abl* mRNA and hence expression of the p210*bcr/abl* tyrosine kinase has also been suggested as a possible mechanism of action.[148] Others have implicated induction of apoptosis of Fas-positive CML progenitors via up-regulation of the Fas-receptor/Fas-ligand system by interferon.[149] [150] However, the relevance of these findings to the restoration of normal hematopoiesis in CML by interferon remains undefined.

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TREATMENT OF THE ACCELERATED PHASE OF CML

With the onset of disease acceleration, increasing doses of hydroxyurea or interferon- are typically required for control, and median survival is 818 months. Interferon- may be combined with hydroxyurea, although the impact of this approach to treatment has not been addressed by randomized trials. Almost all of the large studies to date have examined the role of interferon- in patients early in the course of disease and in chronic phase. Factors influencing the response to therapy with interferon- are poor performance status, splenomegaly, anemia, and the presence of symptoms at diagnosis as well as elevated WBC count, bone marrow basophil percentage, or blast percentage in the peripheral blood. [128] A complete hematologic response is seen in <40% of patients in accelerated phase and major cytogenetic responses are rarely achieved.[15] [151] Thus, interferon- may not play a major role in the treatment of patients with accelerated phase disease, and the predominant cause of death remains blast transformation. Aggressive combination chemotherapy similar to that used in acute leukemia may result in complete hematologic remission in 2530% of patients, but is not associated with significant prolongation of survival. [152]

The complications of accelerated phase disease may require attention. Splenic pain may respond to splenic irradiation, although neither irradiation nor splenectomy prolong survival in most cases. In patients with thrombocytosis, Anagrelide may be useful to control the platelet count without affecting the WBC count. [153] Hyperviscosity symptoms due to hyperleukocytosis may be treated in urgent cases with leukapheresis. Therapy of priapism is difficult and surgical intervention is often complicated by impotence.

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TREATMENT OF BLAST CRISIS

With the development of blast crisis, survival is usually limited to weeks or months. Most patients develop myeloid blast crisis (with occasional basophil, eosinophil, or erythroid predominance) that responds poorly to a combination chemotherapy regimen similar to that used for AML. Occasionally a lasting second chronic phase can be established using aggressive combination chemotherapy.[154] However, complete remission is achieved in <20% of patients. The results of SCT in blast crisis are similarly poor.[155] Tumor lysis syndromes may occur during aggressive treatment of blast crisis with associated hypocalcemia, hyperphosphatemia, hyperkalemia, hyperuricemia, renal failure, and acidosis.

In 2535% of cases of blast crisis the blasts show markers of lymphoid or biphenotypic differentiation. Patients with lymphoid blast crisis may respond to chemotherapy regimens containing vincristine and prednisone, similar to those used in ALL, with approximately 60% of patients responding. However, the overall survival of patients with lymphoid blast crisis remains only 46 months.[29]

ALLOGENEIC STEM CELL TRANSPLANTATION

Related Donor Transplantation

Long-term disease-free survival can be anticipated in 4570% of CML patients undergoing related donor SCT in chronic phase. [155] [156] [157] The beneficial effects are most evident in younger patients transplanted early in the course of disease (optimally within 1 year of diagnosis). More than 70% of this good-risk group experiences long-term disease-free survival. [155] [158] Indeed,

Figure 62-3 Probability of hematologic relapse for patients undergoing related donor marrow and unrelated donor marrow transplant for CML. (*Data from the University of Minnesota Bone Marrow Transplant Program,[161] with permission.*)

in some long-term studies, median survival for younger patients following allogeneic transplant for CML now approaches 10 years. [155] [156] [158]

Allogeneic SCT is generally performed following preparation with cyclophosphamide (60 mg/kg/day for 2 days) combined with total-body irradiation (single dose, fractionated or hyperfractionated). [156] Cyclophosphamide (50 mg/kg/day for 2 days) may be combined with busulfan (4 mg/kg/day for 4 days).[159] Alternative regimens that may be equally effective include high-dose etoposide in combination with fractionated total-body irradiation. [160] However, longer-term follow-up of patients transplanted with alternative preparative regimens may be necessary to gauge their effectiveness as it is increasingly recognized that late relapses following related donor SCT are not uncommon.[161] Pretransplant splenectomy does not improve the post-transplant outcome and may be associated with an increased risk of relapse.[162] [163] [164] Splenic irradiation has also been studied in a randomized multicenter study by the European Bone Marrow Transplant Group. No benefit was demonstrated in standard-risk patients. [165]

Pretransplant patient and disease characteristics have been analyzed to determine their association with outcome. Factors independently associated with an increased risk of relapse or death following related donor SCT include increased patient age and a longer interval between diagnosis and transplant. [155] [166] In a study of 137 consecutive related donor SCT recipients with CML at the University of Minnesota, the 5-year disease-free survival for patients undergoing transplant within 1 year of diagnosis was significantly better than that for patients transplanted beyond 1 year from diagnosis. [155] Other factors associated with an adverse outcome include SCT performed in accelerated phase of CML, with long-term survival of only 2040% of patients. [155] [158] [159] [167] [168] The International Bone Marrow Transplant Registry reported 35% survival in accelerated phase patients and 12% for patients undergoing transplant in blast crisis. [168] The recognition of a poor prognosis for patients undergoing transplant late in the course of disease or with advanced disease is of some importance because as many as one-third of patients in economically advanced countries undergo transplant in the less favorable accelerated or blastic phases of CML, and the interval between diagnosis and transplant varies widely.[169] T-cell depletion is associated with a decreased incidence of graft-versus-host disease (GVHD), but an increased risk of graft failure and relapse and is generally not undertaken for patients with a matched sibling donor. [170]

The probability of relapse at 5 years approaches 20% for recipients of unmanipulated, matched sibling donor stem cells, and approximately 10% of survivors free of disease at 5 years may subsequently undergo late hematologic relapse [155] [161] [171] (Fig. 62-3). Pretransplant factors independently predicting increased risk of post-transplant relapse include increased patient age, a prolonged interval between diagnosis and transplant, and prior splenectomy [155] [161] [164] [171] (Table 62-5). The International Bone Marrow Transplant Registry has reported a 40% relapse rate at 3 years in patients with CML receiving syngeneic stem cells compared with 7% in sibling donor transplant recipients. [172] For patients receiving busulfan-containing regimens, low plasma busulfan levels may be associated with an increased risk of relapse.[173] The rate of relapse is higher for recipients of T-cell-depleted marrow and for those receiving non-T-cell-depleted marrow with mixed T-cell chimerism post-transplant.[174] [175] Patients developing GVHD (acute or chronic GVHD) have a decreased risk of relapse. [155] [161] [176]

Unrelated Donor Transplantation

A human leukocyte antigen (HLA)-matched related donor is available for <35% of potential SCT recipients, and unrelated donor SCT should be considered for young patients without a matched sibling donor. This approach can result in successful outcome for patients with CML. Engraftment is achieved in 90% of patients, and overall survival following transplant of unmanipulated, unrelated donor stem cells is approximately 3545% at 2 years. [177] [178] [179] Results are highly correlated with risk factors. Selected patient groups with good risk factors, such as younger age, HLA match, and short interval for diagnosis to transplant in chronic phase, may have a <70% survival rate at 3 years. Recipients of unrelated donor marrow, although having increased transplant-related mortality and poorer overall long-term survival, rarely have hematologic or cytogenetic relapse of CML. This supports the concept that an ongoing graft-versus-leukemia effect related to alloreactivity between donor and host is important in preventing relapse. [161] [180] [181] The University of Minnesota reported a hematologic relapse rate at 5 years of only 3% for recipients of non-T-cell-depleted unrelated donor stem cells compared with a 20% risk of relapse following related donor

TABLE 62-5 -- Risk Factors for Relapse of CML Following Allogeneic Transplantation

Pretransplant Factors	Transplant Characteristics and Peritransplant Factors	Post-transplant Factors
Age	Related donor[a]	Absence of chronic GVHD
Interval from diagnosis to transplant	T-cell depletion[b]	*bcr/abl* positivity >6 mo post-transplant
Splenectomy	Plasma busulfan levels	Persistent *bcr/abl* positivity post-transplant
	Radiation dose	

[a]Compared to unrelated donor recipients.
[b]T-cell depletion is associated with a higher risk of relapse in both related donor and unrelated donor marrow recipients.

transplant[161] [171] (Fig. 62-3). An HLA-A or -B serologic mismatch between donor and recipient has an independent adverse effect on survival. [177] [178] [182] [183] The results of unrelated donor transplantation in children with CML have also been reported. Engraftment is usually successful in this group of patients (>95%). However, transplant-related mortality may be as high as 40%, and the reported long-term event-free survival is only 50%. [184]

In view of the associated toxicity, alternative approaches to unrelated donor transplant are being explored. T-cell depletion of unrelated donor stem cells can result in successful engraftment and perhaps a decreased incidence of GVHD and improved long-term survival. However, this may be achieved at the cost of an increased rate of CML relapse approaching that seen following related donor SCT, especially for patients undergoing transplant with advanced CML. [180] [185]

New Approaches to Allogeneic Transplantation

Recent studies suggest that the use of allogeneic peripheral blood progenitor cells (including cryopreserved peripheral blood cells) may increase the speed of myeloid and immunologic reconstitution following transfusion. [186] [187] Successful and sustained engraftment and similar short-term outcome to patients undergoing transplants with stem cells obtained from marrow have been reported, although the long-term influence of this approach on survival, the incidence of GVHD, and the risk of relapse have yet to be determined. Successful transplants have also been described using umbilical cord blood or placental blood stem cells in both related donor and unrelated donor recipients. Most reported studies describe the use of this approach in children, but successful engraftment in adults also appears possible. [188] [189] Use of nonmyeloblastic-preparative regimen with the potential to spare regimen-related toxicity is being tested and may be particularly applicable to older recipients.

Post-transplant Relapse and the Graft-versus-Leukemia Effect

Studies of minimal residual disease post-transplant have demonstrated that the leukemic clone is not always eradicated by the preparative regimen. It is believed that a continuing graft-versus-leukemia effect related to alloreactivity between donor and host is important. [161] [180] Cells positive for *bcr/abl* are detectable by PCR in many post-transplant patients, with a significantly higher incidence in patients receiving T-cell-depleted stem cell grafts compared with patients receiving unmanipulated stem cell grafts.[190] [191] It has also been demonstrated that *bcr/ abl*-positive cells persisting following transplant have both proliferative and myeloid differentiation capacity in vitro and therefore the capacity to contribute to relapse. [192] There is a lesser incidence and persistence of *bcr/abl*-positive cells in patients who develop either acute or chronic GVHD.[190] [192] Close monitoring of *bcr/abl* post-transplant can provide useful information regarding the risks of subsequent relapse, which may lead to therapeutic intervention. Patients PCR positive for *bcr/abl* 612 months following transplant and those with persistent positivity or rising numbers of *bcr/abl* transcripts have a significantly increased risk of subsequent cytogenetic and hematologic relapse. Earlier or transient positivity may not be associated with an increased risk of relapse compared to PCR-negative patients. [190] [193]

Therapeutic options for patients who relapse following allogeneic transplant have included second transplant, interferon-, and donor leukocyte infusions (DLIs). The graft-versus-leukemia effect, pioneered by Kolb, has been exploited to induce complete molecular remission using DLI and this has become the treatment of choice for most patients with CML who relapse post-transplant.[194] [195] [196] [197] Complete remission can be re-established in up to 75% of patients. In addition, these remissions are durable and the probability of continuing complete remission at 23 years is almost 90%. [194] Collins et al. surveyed results from 25 North American transplant programs regarding the use of and outcome following DLIs in 80 patients. [197] A complete response was observed in 60% of patients with CML. Factors associated with disease response included cytogenetic or chronic phase relapse. Seventy-six percent of patients receiving DLI therapy after molecular only or chronic phase hematologic relapse experienced complete response compared to 33% for patients with accelerated phase relapse and 17% for patients with blast phase relapse. The actuarial probability of remaining in complete remission at 2 years was 90%. Response also correlated with the occurrence of GVHD following the infusion of donor leukocytes. Most studies have shown that patients with cytogenetic or molecular relapse but without hematologic evidence of disease have a higher rate of complete remission compared with those with frank hematologic relapse.[195] [197] Sixty percent of patients develop GVHD and up to 20% of infusions are complicated by marrow aplasia.[197] This often fatal complication is associated with lack of demonstrable residual donor hematopoiesis in the transplant recipient prior to DLI.[198] These risks must be balanced against the potential benefits of re-establishing remission.

The appropriate dose and schedule for DLI infusion, whether a dose response exists, and the specific effector cell subpopulations responsible for the observed graft-versus-leukemia effect have yet to be determined.[199] [200] Future efforts will focus on identification of the effector cell populations that mediate the graft-versus-leukemia effect, particularly those specific subsets that might contribute to graft-versus-leukemia without causing GVHD.

Minimal Residual Disease in CML

The availability of successful therapy for post-transplant relapse of CML and the need to identify complete responders in patients treated with other modalities including interferon have heightened the importance of the wider clinical application of sensitive molecular biologic techniques for detection of minimal residual disease.[201] [202] [203] [204] Fluorescence in situ hybridization (FISH) techniques are especially useful for characterizing Philadelphia chromosome-negative but *bcr/abl*-positive patients.[201] [202] Hypermetaphase FISH allows analysis of greater numbers of metaphases per sample than conventional FISH and may be especially useful for monitoring patients treated with interferon, where large numbers of metaphases may be difficult to obtain. [202] RT-PCR may have increased sensitivity compared to FISH; however, reliable quantitative techniques suitable for general application have not yet been developed. An important limitation to both FISH and PCR techniques is their inability to detect clonal cytogenetic evolution, although increased *bcr/abl* chimeric mRNA expression may precede disease evolution.[193] [203] [205] In addition, the recent demonstration of low levels of *bcr/abl* expression in peripheral blood WBCs from healthy individuals raises concerns regarding the clinical implications of very low levels of *bcr/abl* transcripts post-transplant.[206]

Autologous Stem Cell Transplantation

The marrow in patients with CML contains a primitive population of progenitors that includes both Philadelphia chromosome-negative and -positive cells. [207] Given the unavailability of HLA-matched donors for many patients with CML and the toxicity of unrelated donor transplant in older patients, autologous SCT has been developed as an alternative approach to therapy. [208] [209] [210] [211] [212] [213] Several nonrandomized studies have suggested a survival advantage for patients treated with autologous transplant.[209] [210] [211] Indeed, in a number of studies survival compares favorably with that following allogeneic transplant. [210] [212]

McGlave et al. reported the combined results of autologous SCT for CML from eight transplant centers. Among 200 patients,

median survival was 42 months and the importance of age, interval between diagnosis and transplant, and phase of disease in determining transplant outcome was confirmed.[210] Although autologous SCT can result in (occasionally durable) Philadelphia chromosome-negative hematopoiesis, even patients with cytogenetic complete remission subsequently relapse. [210] [212] [214] [215] [216]

Current efforts are directed toward attempting to enrich the autologous stem cell graft for benign hematopoietic progenitors. In vivo enrichment for benign hematopoietic progenitors can be achieved by treatment with high-dose chemotherapy and granulocyte colony-stimulating factor (G-CSF) followed by collection of peripheral blood stem cells for autologous reinfusion. [211] [217] [218] Major cytogenetic responses (i.e., collections of Philadelphia chromosome-negative peripheral blood stem cells) can be achieved in up to 30% of cases by these means, and Philadelphia chromosome-negative long-term culture-initiating cells are present in such collections.[218] These techniques have resulted in the temporary establishment of benign hematopoiesis following reinfusion. The level of Philadelphia chromosome-positive cells present on hematopoietic recovery depends on the numbers of Philadelphia chromosome positive cells in the infused graft. [211] [217] [219]

There appear to be high numbers of normal, nonleukemic, long-term culture-initiating cells in the peripheral blood early in the course of CML, suggesting that early collection of peripheral blood stem cells for reinfusion may be advantageous. [220] [221] Indeed, recent reports indicate that results are better when peripheral blood stem cell mobilization and infusion are performed early in the course of CML. [222] [223] [224] In view of the potential for associated toxicity and mortality associated with the mobilization of peripheral blood stem cells with high-dose chemotherapy, [217] [224] it is of note that Philadelphia chromosome-negative progenitor cells can be mobilized using recombinant human G-CSF alone in patients treated with interferon. G-CSF mobilized blood cells enriched for the Philadelphia-negative progenitor population have been successfully used in autografting. [225] [226]

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NEW STRATEGIES FOR THERAPY OF CML

Selection and Expansion of Benign Progenitors

Future therapy for CML may involve improved selection and expansion ex vivo of benign progenitors for reinfusion. [211] [227] It is known that the CD34+, HLA-DR fraction of resting marrow is enriched for Philadelphia-negative progenitors. It may therefore be possible to identify and isolate sufficient nonleukemic progenitors from the chromosome marrow to use for transplantation on the basis of their lack of HLA-DR expression, especially early in the disease. [94] [220] [227] [228] Alternative approaches include the functional isolation of cells with characteristics of hematopoietic stem cells using differential responsiveness to cytokines, [97] [229] and long-term culture of CML cells on stem cell factor-deficient stroma, which may favor the growth of benign progenitors. [107] The CD34+ populations contain only 15% of long-term culture-initiating cells. This proportion is increased to 40% in stroma non-contact culture conditions, and further enrichment may be achieved by supplementation with macrophage inflammatory protein 1 and interleukin-3. The development of this methodology has implications not only for the in vitro characterization of primitive progenitor cells, but may provide the ability to expand and enrich for Philadelphia chromosome-negative cells in vitro intended for subsequent in vivo infusion. [96] [227]

Antisense Oligonucleotides

Complementary antisense deoxyoligonucleotide sequences can hybridize to target mRNA and inhibit translation either by physical blockade of ribosome function or, following transport to the nucleus, by specific RNA degradation. Antisense oligodeoxynucleotides targeted to *bcr/abl*, which can cause decreased *bcr/abl* transcript levels and decreased CML cell growth in culture (perhaps by inducing apoptosis), are currently undergoing investigation for their potential use as ex vivo purging agents for autologous SCT. [230] [231] It has been suggested, however, that antisense-mediated inhibition of p210 *bcr/abl* mRNA and induction of apoptosis may not occur via sequence-specific mechanisms, [90] [232] [233] and there are a number of technical barriers to their successful therapeutic application. [234] Experimental autografts using in vitro purging with both *bcr/abl* and c-*myb* antisense oligonucleotides (which have been shown in marrow culture to result in the growth of *bcr/abl*-negative colonies) have resulted in successful engraftment and partial cytogenetic remission following reinfusion in patients with CML. [235] Others have investigated the use of antisense oligodeoxynucleotides in combination with chemotherapeutic agents in animal models. In a SCID mouse model of CML, the use of mafosphamide in combination with antisense phosphorothioate oligodeoxynucleotide significantly retarded the development of leukemia. [86] [236]

Tyrosine Kinase Inhibitors

The abnormal *bcr/abl* gene product has enhanced tyrosine kinase activity compared to the wild type c-*abl*. Recently, *abl*-specific tyrosine kinase inhibitors have been identified with the ability to selectively inhibit in vitro proliferation of leukemic colony-forming units-granulocyte/macrophage from CML progenitors (but not their normal counterparts). [237] [238] The tyrosine kinase inhibitor, genistein, can suppress both normal and CML cell proliferation and colony formation in marrow culture, with relative sparing of primitive long-term culture-initiating cells, and a decrease in the percentage of *bcr/abl*-positive progenitors, perhaps by induction of apoptosis. [237] Further preclinical testing is needed before the potential of this and other tyrosine kinase inhibitors for the treatment of CML is defined.

Gene Therapy for CML

Technical advances in gene therapy are likely to lead to novel approaches to stem cell transplant in patients with CML. One approach currently under investigation is the retroviral transduction of normal, Philadelphia chromosome-negative cells in patients with CML with a drug resistance gene linked to a *bcr/abl* antisense gene. [208] [239] [240] Selective therapy could then be applied following autologous transplant to eliminate residual Philadelphia chromosome-positive cells while sparing normal progenitors.

Immunomodulation

The characterization in vitro of apparently leukemia-specific immune responses to CML cells has led to speculation that in vivo immunomodulatory treatment may be possible. [241] Immunogenicity of amino acid peptides representing the p210 *bcr/abl* fusion region and peptide binding to major histocompatibility complex class I alleles have been demonstrated and peptide-specific CD4+ T-cell lines that recognize *bcr/abl*-expressing cells have been generated. [242] [243] [244] The p210 b3a2 gene product can be degraded and expressed for presentation by major histocompatibility complex class II molecules at the surface of leukemic cells, thus acting as a tumor-specific antigen. These observations may lead to therapy of CML using human T-cell-mediated recognition of tumor-associated antigens. [244] [245]

Others have explored the antileukemic potential of interleukin-2-activated natural killer (NK) cells and cytotoxic T cells. [17] [246] [247] Autologous NK cells can suppress progenitor growth from CML but not normal marrows, and large-scale expansion ex vivo of autologous activated NK cells now appears feasible, allowing future use for in vivo immunotherapy or in vitro marrow purging. [248] Recently, generation of dendritic cells in culture from patients with CML with the capacity to stimulate autologous T cells with cytotoxic and antiproliferative activity against CML cells but low activity against normal marrow cells has also been possible. [249] These approaches may lead to the development of adoptive immunotherapy of CML using expanded autologous antileukemic NK or cytotoxic T cells with enhanced activity against CML cells. These in vitro observations may precede the development of new strategies using immune recognition and tumor-specific cytotoxicity for immunotherapy of CML. [241]

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THERAPEUTIC OPTIONS FOR PATIENTS WITH CML: SUMMARY

Initial control of the WBC count is most easily achieved using hydroxyurea. For most patients, early institution of therapy with interferon- is appropriate. Recent reports indicate that the use of interferon- in combination with other agents such as cytarabine may result in improved hematologic and cytogenetic responses, and survival may be prolonged. Patients responsive to interferon- may choose to continue interferon- therapy rather than risk the morbidity and mortality associated with allogeneic transplant. However, debate continues regarding the precise role of interferon- and its interaction with other therapies including SCT, where interval between diagnosis and transplant influences both survival and relapse rates. Even chronic phase patients with a complete cytogenetic response to interferon almost all have residual *bcr/abl*-positive cells detectable by RT-PCR, and its curative potential is not established. For younger patients with an available HLA-matched related donor, SCT may be curative and, therefore, early consideration should be given to the patients suitability for allogeneic transplantation. For patients lacking a matched related donor, unrelated SCT or autografting, with or without purging, may also be considered. The use of autologous stem cells may be appropriate for patients without a histocompatible donor who do not achieve a cytogenetic response to interferon, although, as with other approaches to therapy, results are likely to be improved by transplant early in the course of disease. Randomized studies comparing interferon therapy with autografting or allogeneic transplantation may be appropriate for selected groups of patients. Unfortunately, treatment in accelerated phase and blast crisis of CML remains unsatisfactory; for these patients, the predominant cause of death remains blast transformation.

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Chapter 63 - Agnogenic Myeloid Metaplasia

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INTRODUCTION

Agnogenic myeloid metaplasia (AMM) is a chronic, malignant hematologic disorder characterized by splenomegaly, a leukoerythroblastic blood picture, teardrop poikilocytosis (dacryocytes), varying degrees of marrow fibrosis, and extramedullary hematopoiesis. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] This disorder was first described in 1879 by Heuck,[11] who reported the presence of marrow fibrosis and extramedullary hematopoiesis in the liver and spleen of two patients. AMM has also been referred to by a variety of other terms, including myelofibrosis, myelosclerosis, idiopathic myeloid metaplasia, and osteosclerosis. [6] Fibrosis of the bone marrow is not unique to AMM and may accompany many other disorders[9] [10] (Table 63-1). In AMM, the marrow fibrosis is thought to be a response to a clonal proliferation of hematopoietic stem cells. This syndrome frequently leads to progressive marrow failure, [12] but whether the marrow failure is a consequence of the excessive marrow fibrosis or of the underlying hematologic malignancy remains unknown. Dameshek[13] in 1951 included AMM among the myeloproliferative disorders. This hypothesis was largely based on clinical observations of patients with polycythemia vera, chronic myeloid leukemia (CML), and primary thrombocythemia who developed marrow fibrosis and a clinical picture resembling AMM. In addition,

TABLE 63-1 -- Conditions Associated with Myelofibrosis Nonmalignant Conditions

Nonmalignant conditions
Infections: tuberculosis, histoplasmosis
Renal osteodystrophy
Vitamin D deficiency
Hypoparathyroidism
Hyperparathyroidism
Gray platelet syndrome
Systemic lupus erythematosus
Scleroderma
Radiation exposure
Osteopetrosis
Paget disease
Benzene exposure
Thorotrast exposure
Gaucher disease
Malignant Disorders
Agnogenic myeloid metaplasia
Other chronic myeloproliferative disorders: polycythemia vera, chronic myeloid leukemia, primary thrombocythemia
Acute myelofibrosis
Acute myeloid leukemia
Acute lymphocytic leukemia
Hairy cell leukemia
Hodgkin disease
Acute myelodysplasia with myelofibrosis
Multiple myeloma
Systemic mastocytosis
Non-Hodgkin lymphoma
Carcinoma: breast, lung, prostate, stomach

Dameshek[13] noted that each of these myeloproliferative disorders frequently terminates in a leukemic phase.

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EPIDEMIOLOGY

Few epidemiologic studies are available to estimate the actual incidence of AMM. From an examination of the number of cases encountered at a variety of medical centers, one can arrive at some estimate of the frequency of the disorder. The Mayo Clinic cared for 137 patients with AMM from 19601965; 100 cases were observed at the Ohio State University over a 16-year period; and 65 patients were seen from 19681980 at Barnes Hospital in St. Louis. [1] [5] [6] [8] Recently, the incidence rate of AMM in Olmstead County, Minnesota has been determined to be 1.33 patients/100,000 population/year. [14] The median age at diagnosis was 67.0 years and the Kaplan-Meier survival was 52.4% at three years. [14]

In Japan AMM is considered a rare disorder, with only 84 cases per 100,000 at autopsy. [15] [16] This figure was obtained by monitoring autopsy protocols from all Japanese medical schools, universities, general hospitals, and research institutes over a 10-year period. [15] The autopsy incidence of myelofibrosis among survivors who were 10,000 m from the hypocenter of the atomic bomb explosion at Hiroshima was 18 times the incidence reported from the remainder of Japan. [17] These patients became symptomatic an average of 6 years after the bomb blast. [17] Such data indicate a strong link between excessive radiation exposure and development of AMM, which is further substantiated by the high incidence of myelofibrosis in patients who have received the contrast material Thorotrast (which contains ^{232}Th, a radioactive element with a half-life of 1.41×10^{10} years). [18] Thorotrast is taken up and retained indefinitely by cells of the reticuloendothelial system, which results in continuous irradiation of the liver, spleen, lymph nodes, and bone marrow. Chronic exposure to several industrial solvents, including benzene and toluene, has also been associated with development of AMM. [19] [20] [21] AMM has been reported as a complication of chronic benzene poisoning since the chemical was first used in the leather and shoe industries during the 1930s and 1940s. [19]

The average age at diagnosis of AMM is approximately 60 years, most patients being diagnosed at 5069 years of age. [1] [2] [3] [4] [5] [6] [7] [8] [14] In several series, males have been reported to be affected more frequently than females, but others have failed to confirm this male predominance. [1] [2] [3] [4] [5] [6] [7] [8] [14] Rarely, AMM has been reported in the pediatric age group. [22] In several children, idiopathic myelofibrosis presented in early childhood and in one family, twins were affected. [23] No evidence of genetic transmission exists.

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BIOLOGIC AND MOLECULAR ASPECTS

Ward and Block[7] originally proposed that AMM represents a response of an intrinsically normal stem cell to an unidentified stimulus, while Dameshek [13] conjectured that the abnormal fibroblastic proliferation in AMM is not an integral part of the primary disorder.

These conflicting hypotheses were tested in studies using a

variety of genetic markers to define the cellular origin of AMM. Jacobson et al. [12] demonstrated in a black female AMM patient who was heterozygous for glucose-6-phosphate dehydrogenase (G6PD) that circulating hematopoietic cells were derived from a common hematopoietic stem cell and that the bone marrow fibroblasts were nonclonal in origin. Using x-chromosome gene probes, Lucas et al. [24] have confirmed the clonal origin of hematopoiesis in AMM. Furthermore, using a similar molecular biologic approach Anger et al. [25] reported that in each of three cases of AMM suitable for clonal analysis a clear-cut monoclonal X-inactivation pattern was observed. Furthermore, Greenberg et al.[26] studied the cytogenetic composition of bone marrow fibroblasts of an AMM patient who had a clonal cytogenetic abnormality in unstimulated peripheral blood cells, which, however, was absent in the marrow fibroblasts.

The fully developed fibrosis of the bone arrow in AMM is frequently preceded by a hypercellular phase of variable duration. Because characteristic blood cell findings of the cellular phase of AMM do not exist, the diagnosis depends on the demonstration of atypical megakaryocytes and an increase of fibers within marrow biopsies and the exclusion of other myeloproliferative disorders. Kriepe et al. [27] have used an analysis of x-linked restriction fragment length polymorphisms of blood cells of patients at various stages of AMM, to demonstrate clonality of hematopoiesis not only in advanced stages of AMM but also in the cellular phase. In addition, an N- *ras* mutation present in hematopoietic cells in an AMM patient indicated that T and B cells were also involved in the malignant process in some patients, perhaps providing some explanation for the immunologic abnormalities in AMM. [28] These studies indicate that AMM is a clonal hematologic malignancy and that the marrow fibrosis represents a secondary nonneoplastic reaction of marrow stromal cells. [24] [25] [26] [27] [28] [29] If myelofibrosis is truly an epiphenomenon of the neoplastic hematopoietic cell proliferation it may be expected to disappear if this cell population is eradicated. Reversal of myelofibrosis has, in fact, been observed following allogeneic bone marrow transplantation and following long-term administration of chemotherapy or interferon. [30] [31] [32] Such findings indicate that the bone marrow fibrosis in AMM is not irreversible and is clearly a consequence of the neoplastic cellular proliferation.

The concept that the primary defect in AMM resides in hematopoietic stem cells or progenitor cells is further supported by the observations of a number of investigators concerning the number of unilineage and multilineage hematopoietic progenitor cells present in the blood of AMM patients. [33] [34] [35] [36] [37] [38] Circulating assayable progenitor cells are increased from 9- to 20-fold above numbers present in normal peripheral blood. [33] [34] [35] [36] [37] [38] Patients with secondary myelofibrosis have a much smaller (threefold) increase in circulating assayable progenitor cells. [34] Secondary myelofibrosis is thought to result from insults to the marrow microvascular system rather than from an intrinsic hematopoietic cell defect. [9] [10] Erythroid and megakaryocyte colony formation has been observed to occur in the absence of added exogenous cytokines in AMM, a finding common to other myeloproliferative disorders. [33] [34] [35] [36] [37] [38] [39] The hypothesis that marrow fibrosis in AMM is a secondary process is further supported by the work of Castro-Malaspina et al., [40] Wang,[41] and Hirata et al.[42] These groups have each found that fibroblasts derived from marrow explants obtained from AMM patients displayed the same physical and proliferative characteristics as normal marrow fibroblasts. Both AMM and normal marrow fibroblasts exhibited anchorage and serum dependence, contact inhibition of growth, and similar production of hematopoietic colony-stimulating activities. These data suggest that marrow fibroblasts and their precursor cells in AMM patients do not differ from those of normal subjects.

Many of the peripheral blood abnormalities associated with AMM may be attributed to the extramedullary hematopoiesis which is characteristic of this disorder. Extramedullary hematopoiesis had previously been attributed to the reactivation of quiescent hematopoietic stem cells, which are retained at sites of prior embryonic hematopoiesis, especially the spleen.[7] [13] This hypothesis has been recently questioned by the observation that the spleen is not a prominent site of fetal hematopoiesis in humans and by the observation that extramedullary hematopoiesis occurs in a wide variety of sites in AMM that cannot be accounted for by the fetal reversion hypothesis.[43] [44] [45] Intravascular hematopoiesis within the sinusoids of the bone marrow is a conspicuous finding in AMM. [46] It is likely that this finding is the result of marrow fibrosis distorting the marrow sinuses and allowing entrance of hematopoietic precursors or progenitor/stem cells into the sinusoids and access to the circulation.[46] [47] Such cells are normally filtered from the peripheral blood by the spleen and destroyed. If the number of such primitive cells exceeds the capacity of the spleen, these cells are thought to be worked into the peripheral blood, leading to a leukoerythroblastic blood picture, or to lodge in potential sites of extramedullary hematopoiesis.[44] [45] The characteristic changes of the marrow vascular architecture consist of increased quantities of collagen type IV deposits associated with endothelial cell proliferation. [46] [47] Moreover, sinusoidal hyperplasia and hypervascularity, resulting in increased blood flow, occurs. [47] The excessively dilated marrow sinusoids in AMM contain prominent intraluminal foci of hematopoiesis. [47] [48] Thiele et al.[46] pursued a morphometric analysis of marrow vascular structures and collagen type IV deposits in AMM. In comparison to normal controls and patients with polycythemia vera, a significant increase in the number of marrow sinusoids as well as subendothelial collagen type IV in AMM was observed. [46] Furthermore, evolution of the fibro-osteosclerotic changes in AMM was accompanied by a striking accumulation of collagen IV and a marked luminal expansion and irregularity. Thiele et al. [46] have hypothesized that neovascularization of the bone marrow stroma in AMM is likely mediated by megakaryocyte -granule constituents. Transforming growth factor- (TGF-), for instance, has a profound effect on angiogenesis.[49] [50] The evolution of the fibro-osteosclerotic process in AMM appears to be a coordinated process closely related to the vascular proliferation and also modulated by growth factors present within abnormal megakaryocytes.

Groopman [51] first hypothesized that growth factors released from neoplastic hematopoietic cells in AMM were capable of stimulating marrow fibroblast proliferation and suggested that the megakaryocyte was the primary source of such proliferation factors. The role of megakaryocytes in the development of fibrosis in AMM is further supported by (1) the megakaryocytic hyperplasia with dysplastic or necrotic megakaryocytes which characterizes this disorder, (2) increased circulating megakaryocytes and megakaryocyte progenitors that are present in AMM, (3) the association of marrow fibrosis and acute megakaryocytic leukemia, and (4) the presence of myelofibrosis in gray platelet syndrome, an inherited disorder of platelet -granules. [52] [53] [54] Castro-Malaspina et al.[55] subsequently showed that megakaryocyte-enriched marrow cell homogenates and platelet homogenates induced DNA synthesis by human marrow fibroblasts. This group hypothesized that ineffective megakaryocytopoiesis in AMM results in liberation of excessive amounts of such growth factors, leading to marrow fibroblast expansion and collagen synthesis.[55] Platelet-derived growth factor (PDGF), TGF-, and epidermal growth factor (EGF), each of which is contained within platelet and megakaryocyte -granules, are known to stimulate marrow fibroblast proliferation. [54] [55] [56] [57] [58] [59] [60] [61] [62] [63] [64] In fact, TGF- enhances type I and type III procollagen and fibronectin synthesis by marrow fibroblasts.[57] Kimura et al.[59] have presented data to suggest that myeloproliferative disease fibroblasts are more sensitive to human serum mitogens than normal

marrow fibroblasts. The PDGF content of platelets from AMM patients is known to be decreased, indicating that a release or leakage of such growth factors by marrow

megakaryocytes may occur.[58] Burstein et al.[60] were, however, unable to detect any connection between marrow fibrosis and plasma levels or platelet content of platelet factor 4. Their studies indicated that if -granule constituents were important in the development of marrow fibrosis, their release or leakage would likely occur within the marrow cavity. Such local effects could result in fibrosis without leading to increased concentrations of -granule constituents in the general circulation. [60]

Martyré et al.[61] have further examined the possibility that platelet -granule constituents may account for marrow fibrosis in AMM. In these studies, AMM platelet PDGF and TGF- levels were found to be 2.03.0 and 1.53.0-fold higher respectively in AMM than in normal controls while EGF levels in AMM were similar to that of control platelets.[61] The role of PDGF and TGF- in the biogenesis of AMM is likely not restricted merely to promoting fibroblastic proliferation but is also related to the effect of these two growth factors on synthesis, secretion, and degradation of extracellular matrix components. [56] [57] Martyré et al. have presented additional data which strengthens the hypothesis that TGF- plays a role in the development of progressive fibrosis in AMM. [52] [53] [61] This group documented the increased expression of TGF- mRNA in peripheral blood mononuclear cells isolated from AMM patients and localized this to peripheral blood megakaryocytes of AMM patients. [61] These data strongly implicate TGF- as a major player in the biogenesis of fibrosis in AMM. [52] [53] [61]

TGF- enhances fibronectin and collagens type I, III, IV as well as chondroitin/dermatan sulphate and proteoglycan gene expression. [63] [65] TGF- decreases the synthesis of various collagenase-like enzymes that degrade extracellular matrices, while at the same time stimulating the synthesis of protease inhibitors such as plasminogen activator inhibitor I. [66] The net effect of these complex interactions is the accumulation of extracellular matrix, which likely contributes to further progression of fibrosis.[63]

Additional growth factors are likely involved in the development of progressive fibrosis in AMM. Circulating megakaryocytic cells and platelets from AMM patients have been shown to possess high levels of basic fibroblast growth factor (bFGF). [67] [68] Since bFGF is devoid of a secretion peptide signal, bFGF is not present in media conditioned by megakaryocytic cells from AMM patients. Basic fibroblast growth factor is a potent angiogenic factor and is a mitogen for human bone marrow stromal cells.[68] Elevated platelet, megakaryocyte, and serum bFGF levels have been reported in AMM patients with progressive fibrosis. [67] [69] Basic fibroblast growth factor could be released or leaked from dysplastic and necrotic AMM megakaryocytes or platelets. These findings suggest that bFGF might also contribute to the progressive fibrosis and pronounced angiogenesis frequently observed in AMM. [67] [69]

Recently, platelets have been shown to store and release the calcium-binding protein, calmodulin. [67] [69] Extracellular calmodulin is a mitogen for a variety of cells including fibroblasts.[67] [69] Urinary calmodulin excretion has been shown to be significantly elevated in patients with AMM in comparison to other myeloproliferative disorders without fibrosis.[69] These data suggest that abnormal secretion or leakage of calmodulin in addition to bFGF, PDGF, TGF- and EGF from defective megakaryocytes and/or platelets may also be involved in the pathogenesis of bone marrow fibrosis. [67] [69]

The role of the megakaryocyte and platelet in the development of marrow fibrosis was further clarified by the transplantation of marrow cells genetically modified to overexpress thrombopoietin (TPO) into normal mice.[70] [71] These animals possessed increased numbers of platelets and megakaryocytes and developed a syndrome characterized by marrow myelofibrosis, osteosclerosis, extramedullary hematopoiesis, and elevated numbers of circulating hematopoietic precursors. [70] [71] In these mice, TGF- and PDGF levels in platelet-poor plasma were elevated 23-fold higher than that in normal control mice. [70] [71] Retransplantation of these mice with normal marrow cells resulted after 1215 weeks in reduction of platelet and megakaryocyte numbers and reversal of the marrow myelofibrosis and osteosclerosis. [71] These data dramatically demonstrate the role of megakaryocytes and platelets and their intracellular growth factors in the generation of marrow fibrosis. Remarkably, transgenic mice overexpressing TPO in the liver but not the marrow have similar degrees of megakaryocytic hyperplasia and thrombocytosis but do not develop marrow fibrosis.[72] The discrepancy between these two animal models emphasizes the unique consequences that marrow TPO generation might play in the generation of marrow fibrosis. Furthermore, Ozaki et al. have reported recently that TPO was capable of enhancing the production of megakaryocyte PDGF, PF-4 and TGF-.[73] Whether TPO actually serves as an autocrine or paracrine growth factor for megakaryocytes in AMM will likely be the subject of considerable future investigation.

Megakaryocytes are not the only cells capable of releasing cytokines that promote marrow fibrosis. Monocytes/macrophages from patients with AMM have been shown to produce greater quantities of TGF- and interleukin-1 (IL-1) than normal controls. [74] IL-1 and TGF- are both fibroblast mitogens that induce extracellular matrix protein production. Rameshwar et al. have shown that monocyte adhesion to extracellular matrix proteins leads to the overproduction of IL-1 and TGF- by AMM monocytes.[74] The monocyte adhesion molecule CD44 appears to be involved in the induction of fibrogenic cytokines by mediating the interaction between monocytes and accumulated extracellular matrix protein deposits.[74] The relative role of megakaryocyte/platelet or macrophage/monocyte-derived fibrogenic cytokines in the pathogenesis of AMM requires further study.

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ETIOLOGY AND PATHOGENESIS

The etiology of AMM remains unknown. In experimental systems the exposure of a variety of animal models to chemical agents, industrial solvents, hormones, viruses, immunologic stimuli, and ionizing radiation have led to the development of AMM. [6] A model of AMM has been established in the rabbit, [75] [76] [77] [78] in which saponin administered intravenously induces extramedullary hematopoiesis and myelofibrosis over a period of weeks. [76] Interestingly, mice and rats fail to respond to the same doses of saponin. [75] Hoshi and Weiss [75] have provided ultrastructural evidence to suggest that saponin causes damage to the endothelium of marrow vascular cells, rendering them incompetent. A series of hemorrhagic events appears to occur, leading to release of normoblasts into the peripheral blood, marrow hypoplasia, fibrosis, and regeneration. [75] This effect is accompanied by the appearance of increased numbers of hematopoietic progenitor cells in the blood and spleen and a simultaneous depletion of such cells in the marrow. [75] Such marrow vascular injury may be a common link leading to the development of myelofibrosis.

Progress has recently been made in defining the nature of the connective tissue matrix present in normal marrow and in patients with AMM. [10] Reticulin fibrosis of the marrow represents an exaggeration of the fibrous pattern of normal marrow. In contrast, collagen fibrosis occurs in primary and secondary AMM and results in the disruption and obliteration of the sinusoidal architecture of the bone marrow. Bone marrow reticulin has been shown to be composed of types I and III collagen and fibronectin. [78] [79] [80] Charron et al. [81] documented a constant increase in marrow collagen content during the course of AMM. The increment in collagen was highest in those patients in whom the

disease was of longest duration. [81] As compared with collagen extracts from normal individuals, AMM extracts showed a moderate increase in neutral soluble collagen and a larger increase in polymeric collagen. [81] Charron et al. [81] also noted changes in the pattern of marrow collagen deposition during the course of AMM. Early in the course there is a higher percentage of newly synthesized fibers, and later more polymeric collagen is present, presumably as a result of progressive cross-linking and insolubilization. Both Charron et al. [81] and Gay et al. [79] claim that type III collagen preferentially increases in the early stages of the disease but that it is subsequently replaced by type I collagen. Serum procollagen NH_2 -terminal peptide III (PC III), which is cleared extracellularly during collagen biosynthesis, is increased in most AMM patients. [82] This finding supports the concept that type III collagen synthesis is increased in AMM. [83] [84] [85] Some investigators have suggested that PC III levels do not reflect the extent of marrow fibrosis in AMM, [83] [84] yet PC III elevation in a longitudinal study of patients with AMM was found to be a sensitive marker of disease activity. [85] [86] PC III levels were observed to fall in patients responding to chemotherapy and to rise 12 weeks prior to elevations in white cell and blast cell counts in patients unresponsive to chemotherapy. [86] Bone marrow fibrosis appears to depend not only on the accumulation of collagen but also on the establishment of an equilibrium between collagen production and destruction. PC III levels would therefore be expected to reflect collagen synthesis more closely than total marrow collagen content.

Extensive deposition of collagen type VI in AMM has been documented. [54] This structural component forms a linkage between individual collagen type 1 and/or collagen type III fibers. In addition, advanced marrow fibrosis is associated with increased deposition of fibronectin, tenascin and citronectin. In normal bone marrow, collagen type IV and laminin are limited to discontinuous sinusoidal membranes while myelofibrotic stroma possesses continuous sheets of both proteins resulting from neovascularization and endothelial cell proliferation. Reilly has suggested that this altered basement membrane structure might facilitate the excessive migration of marrow progenitor/stem cells from the marrow in AMM. [54]

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CLINICAL MANIFESTATIONS

Table 63-2 lists the symptoms of patients with AMM at presentation.[6] [8] Approximately 20% of patients are entirely asymptomatic

TABLE 63-2 -- Summary of Symptoms and Physical Findings of Patients with AMM Detected at Diagnosis

Symptom or Finding	Incidence (%)		
	Varki et al. [8]	Silverstein [6]	Visani et al.[214]
Asymptomatic	21	30	16
Fatigue	71	58	47
Fever	5	10	5
Symptoms due to enlarged spleen	11	23	48
Bleeding	20	17	5
Gout/renal stones	13	6	NR
Weight loss	39	15	7
Night sweats	21	6	NR
Pallor	NR	60	NR
Petechiae/ecchymoses	20	15	NR
Splenomegaly	89	90	99
Hepatomegaly	64	70	39
Peripheral edema	13	NR	NR
Evidence of portal hypertension	2	6	2
Lymphadenopathy	2	10	1
Jaundice	0	4	NR

NR, not reported.

and come to medical attention because of an enlarged spleen detected during routine physical examination or because of an abnormal peripheral blood smear. The most common symptoms in AMM are a result of anemia, which leads to complaints of weakness, fatigue, dyspnea on exertion, and palpitations. With enlargement of the spleen, various syndromes characterized by abdominal discomfort may emerge.[87] Pressure of the spleen on the stomach may lead to delayed gastric emptying and early satiety.[87] Patients may merely complain of a dull, heavy sensation in the left upper quadrant. Splenic infarction may produce pain of extreme severity, simulating an acute abdominal emergency. Severe diarrhea in patients with AMM may be particularly disabling; because studies of gut flora and motility have been unrevealing, it appears that the pressure of the spleen on the colon or small bowel may be responsible for this symptom. Rarely, the development of AMM can be preceded by the appearance of multiple cutaneous edematous plaques and nodules characteristic of Sweet syndrome, a cutaneous process occuring in response to a number of hematologic malignancies.[88] In addition, extramedullary hematopoiesis can rarely occur in the skin, manifesting as nontender, occasionally pruritic red, pink or violaceous plaques, papules, or hemangioma-like nodules. These dermal infiltrates, when biopsied, are composed of combinations of myeloid, erythroid, and megakaryocytic cells.[85] [90]

Bleeding problems may complicate the clinical course of AMM patients. Bleeding may be trivial, as manifested by petechiae and ecchymoses, or may be life-threatening as a result of uncontrollable esophageal bleeding. [1] [2] [3] [4] [5] [6] [7] [8] It may be secondary to thrombocytopenia or to poor platelet function. [1] [2] [3] [4] [5] [6] [7] [8] Bleeding may be only initially encountered during a surgical procedure such as splenectomy; in this case, the bleeding diathesis may be secondary to nonapparent disseminated intravascular coagulopathy (DIC) and has the potential for catastrophic consequences. [6]

In addition to the preceding three major modes of clinical presentation of AMM, occurrence of isolated sites of ectopic myeloid metaplasia has been reported, particularly in the pulmonary, gastrointestinal, central nervous, and genitourinary systems. [1] [2] [3] [4] [5] [6] [7] [8] Such patients present with cough and large lung tumors, headache, or paralysis secondary to brain tumors or spinal cord tumors, urinary tumors present in either the bladder or kidney, small bowel obstruction, or intractable ascites secondary to ectopic implants of hematopoietic tissue in the gut or peritoneum. [1] [2] [3] [4] [5] [6] [7] [8] [91] [92] [93] [94] [95] [96] [97] [98]

Myeloid metaplasia of the renal pelvis, ureters, and bladder, as well as renal parenchymal infiltration, have been observed. In addition, expansion of hematopoietic tissue at the urethral meatus may be confused with a urethral carbuncle. [95] Such strategically localized sites of extramural hematopoiesis may lead to renal failure or obstruction of both kidneys and bladder dysfunction. [95] Ascites occurring in a patient with AMM may result from peritoneal or mesenteric implants of extramedullary hematopoietic tissue and/or from portal hypertension. [96] [97] If the ascites is secondary to peritoneal implants the fluid is always exudative and sterile and frequently contains myeloid, erythroid, and megakaryocytic elements. [97] Such cytologic studies should routinely be performed on ascitic or pleural fluid obtained from patients with AMM.

Nonspecific systems are not infrequent in AMM and include fever, night sweats, anorexia, and weight loss. [5] Table 63-2 lists the prominent physical findings in patients with AMM. [5] [8] Splenomegaly serves as the hallmark of the disease. Its extent may vary, but massive splenomegaly, with the organ occupying the entire left side of the abdomen and extending into the pelvis, may occur in 35% of patients. Hepatomegaly occurs in almost 70% of cases, [1] [2] [3] [4] [5] [6] [7] [8] and lymphadenopathy is observed in 1020%, but the degree of nodal enlargement is frequently only moderate. [5] [8] Other important physical findings include pallor, peripheral edema, jaundice, and bony tenderness. Acute

Figure 63-1 Peripheral blood smear showing normoblasts and teardrop poikilocytes in AMM. (Wright stain, ×700.)

monoarticular inflammation due to secondary gout is seen in 6% of patients. [5] Peritoneal implants of extramedullary tissue may lead to development of ascites. [95] [96] Portal hypertension may occur and is a result of massive increases in hepatic blood flow and intrahepatic obstruction. [3] [5] [6] [7] [87] [98] [99]

Clinical features of portal hypertension, such as ascites or esophageal varices, are known to occur in 918% of patients with AMM. [96] Occasionally, cirrhosis or evidence of thrombosis of the portal or hepatic veins has been reported. [98] [99] The liver histologic findings in AMM have previously been considered normal or to be characterized by minimal portal fibrosis, which has led to the view that the splenomegaly in AMM may be a result of increased hepatic blood flow with resultant portal hypertension. Wanless et al., however, analyzed a large autopsy series of patients with AMM and found frequent thromboses in small or medium-sized portal veins as well as in extrahepatic portal veins in patients with portal hypertension. [98]

LABORATORY EVALUATION

Careful examination of the peripheral blood smear (Fig. 63-1) and bone marrow (Fig. 63-2) permits ready diagnosis of AMM. The presence of a leukoerythroblastic blood picture with teardrop poikilocytosis (dacryocytes) strongly suggests this diagnosis. A leukoerythroblastic blood picture characterized by the presence of nucleated red blood cells and immature myeloid elements is seen in 96% of cases. [1] [2] [3] [4] [5] [6] [7] [8] Megathrombocytes and megakaryocytic fragments are a constant finding. The teardrop erythrocytes (dacryocytes) have been noted to decrease in number following splenectomy or institution of chemotherapy, [100] [101] which has led some to suggest that splenic fibrosis may lead to development of these red cell changes. [100] [101] In approximately 60% of patients, hemoglobin levels drop to <10 g/dl.[1] [2] [3] [4] [5] [6] [7] [8] The degree of anemia is difficult to estimate by hemoglobin or hematocrit determinations, since individuals with large spleens often have expanded plasma volumes. Such alterations in hemodynamics may lead to apparent anemia, which is largely dilutional in nature. Of patients with decreased red cell masses, 95% have normochromic normocytic red cell indices.[102] The anemia is due to both ineffective red cell production and shortened red cell survival. [103] [104] [105] [106] Ineffective red cell production in AMM can be demonstrated by ferrokinetic studies, which are characterized by increased iron turnover but decreased incorporation of radioactive iron into circulating red cells. [103] [104] [105] [106] Silverstein[6] found ineffective iron incorporation into red cells to occur in 90% of patients with AMM. Morphometric analysis of bone marrow

Figure 63-2 Marrow section from a patient with diffuse myelofibrosis and osteosclerosis. (H&E, ×135.) (*From Silverstein,[6] with permission.*)

biopsies have demonstrated a significant reduction of erythroid precursors. Erythroid hypoplasia is more common than previously appreciated. [107]

Fifteen percent of patients have major hemolytic episodes during their clinical course. [6] The cause of the hemolytic anemia is usually multifactorial, with contributions from hypersplenism, a defect in red cells resembling that in paroxysmal nocturnal hemoglobinuria, and antierythrocyte autoantibodies. [103] [104] [105] [106] [107] [108] [109] [110] In one series, 55% of patients with AMM had a positive acid hemolysis or sucrose hemolysis test, or both, and 10% had decreased haptoglobin levels and hemosiderinuria, suggestive of intravascular hemolysis. [109] [110] Hypochromic microcytic anemia resulting from iron deficiency secondary to blood loss may develop in 5% of AMM patients. The etiology of the blood loss may be leaking esophageal varices, duodenal ulceration, gastritis, or intravascular hemolysis. Occasionally, a patient with AMM may develop an occult malignancy or a site of extramedullary hematopoiesis within the gastrointestinal tract, which may serve as a bleeding source.[111] Macrocytic anemia may complicate AMM.[112] Folic acid absorption is normal in these patients, but the folic acid deficiency is probably due to increased utilization.[112]

Leukopenia can occur in 1325% of patients, while leukocytosis is seen in one-third. [1] [2] [3] [4] [5] [6] [7] [8] In one series,[8] the mean white count was 16,600/mm^3 . Occasional blast cells and granulocytes with the pseudo-Pelger-Huët anomaly are frequent findings. [6] The leukocyte alkaline phosphatase score was studied in 78 patients by Silverstein and Elveback [113] and was high in 41 patients (>100), normal in 17 patients (30100), and low in 20 patients (<30). Such a distribution of leukocyte alkaline phosphatase scores has been reported by others. [8]

In the Mayo Clinic series of 169 patients, platelet counts of <100,000/mm^3 were observed in 31% of patients, while platelet counts of >800,000 were observed in 12%.[6] Defective platelet aggregation is common, and platelets frequently do not respond to either collagen or epinephrine. [6] [114] Didsheim and Bunting[114] reported that 5 of 10 patients with AMM had prolonged bleeding times. A variety of qualitative platelet anomalies documented by abnormal in vitro aggregation patterns has been documented in AMM.[115] In 15% of patients, abnormalities suggestive of ongoing DIC are found, including decreased platelet numbers, decreased levels of coagulation factors V and VIII, and increased fibrin-split products. [6] Usually when DIC occurs in AMM, it produces no symptoms and unfortunately may only become clinically apparent following surgical intervention. Associated liver dysfunction may also be a contributory factor to prolongation of the prothrombin time. Additional laboratory abnormalities are quite frequent. In one series lactic acid levels were elevated in 95% of patients, bilirubin levels in 40%, uric acid in 60%, and alkaline phosphate and serum glutamic oxaloacetic transaminase levels in 50%. [8] Patients with AMM have been noted to have decreased levels of total cholesterol. [116] [117] The ratio of high-density lipoprotein cholesterol to low-density lipoprotein cholesterol has been shown to be diminished. [116] [117]

Ferrokinetic and red cell survival studies may be useful in defining sites of extramedullary hematopoiesis and determining the cause of anemia in AMM. [6] [103] [104] [105] [106] Furthermore, the presence of erythroid hyperplasia, intense hemolysis, increased plasma volume, and significant ineffective erythropoiesis appears to define a subpopulation of patients with an especially poor prognosis. [6]

A variety of immunologic abnormalities have been reported in AMM, including the presence of antinuclear antibodies, elevated rheumatoid factor titers, direct Coombs test positivity, lupus-type circulating anticoagulants, hypocomplementemia, marrow lymphoid nodules, and increased circulating immune complexes. [118] [119] [120] [121] [122] In one series of 50 patients with AMM, increased quantities of circulating immune complexes were detected [118] and found to be associated with increased disease activity as manifested by increased transfusion requirements, bone pain, and fever. [118] Some investigators have suggested that abnormalities of the complement system may be important in disease progression of AMM,[121] while others have hypothesized that low levels of C3 may predispose these patients to develop serious bacterial infections.[118] A remarkably high incidence of monoclonal gammopathies has been reported in AMM, with such benign gammopathies occurring in 810% of patients in some series.[123] [124] Ten cases of the simultaneous occurrence of a plasma cell dyscrasia and AMM have been reported. [6] [123] [124]

Successful bone marrow aspiration is unusual, being accomplished in only 6 of 48 cases in one series, with the tap completely dry in 50% of cases. [8] A bone marrow biopsy is necessary in all cases to assess the amount of residual hematopoietic cellular tissue and the degree of marrow fibrosis. Table 63-3 (Table Not Available) summarizes the appearance of bone marrow biopsies in the series of Varki et al. [8] Some caution should be taken in using repeated bone marrow biopsy specimens to diagnose AMM. Cases in which a marrow biopsy was performed at the site of a previous bone injury during the healing process frequently results in confusion because of primary callus formation at that site. [125] Intense myelofibrosis with new bone formation has been noted at such sites. Marrow biopsies performed at the site of previous biopsies or aspirates may potentially lead to an erroneous diagnosis of AMM. It is recommended that marrow biopsies intended for diagnosis of AMM be performed at a site previously spared. [125] Most marrow biopsies in AMM are hypercellular and are remarkable for increased numbers of megakaryocytes (Table 63-3 (Table Not Available)). Bone marrow fibrosis and osteosclerosis were seen in 67% and 54% of cases, respectively. [8] The three characteristic morphologic features include: (1) patchiness of the hematopoietic cellularity and the reticulin fibrosis, some microscopic fields being cellular and others depleted of hematopoietic cells and

TABLE 63-3 -- Bone Marrow Biopsy Findings at Diagnosis of Patients with AMM

(Not Available)

From Varki et al.,[8] with permission.

the amount of reticulin varying from field to field; (2) increased numbers of megakaryocytes, which often are arranged around and within the sinuses and not always clustered in groups. The megakaryocytes are large with irregular, roundish, cloud-like nuclei; [126] [127] and (3) distended marrow sinusoids frequently containing intravascular hematopoiesis.[48] [126] [127] In Wolf and Neimans series, morphologic evidence of progression of fibrosis was present in only 1 of 21 cases in which sequential biopsies were obtained.[48] In addition, no connection was observed between bone marrow cellularity and fibrosis and splenic size. [48] By contrast, Lohmann and Beckman[128] observed progressive fibrosis in 18 of 20 patients who did not have maximal myelofibrosis at the time of the initial biopsy. Thiele et al. [126] presented data to indicate an early hyperplastic subtype of AMM with no or minimal medullary reticulin and another phase with conspicuous fibrosis and osteosclerotic changes of the marrow. They concluded based on a careful histomorphometric evaluation of the bone marrow that in a subset of patients there was a progressive fibro-osteosclerotic process during the evolution of the disease that was paralleled by an increase in small megakaryocytes with irregular perimeters and megakaryocytes with naked nuclei.[126] However, this same group emphasizes that in 25% of the patients, there was no progression in the degree of marrow fibrosis over time.[126]

Morphologic examination of the spleen reveals foci of extramedullary hematopoiesis in the sinusoids of the red pulp, where megakaryocytes, myeloid elements, and nucleated erythroid elements are seen.[48] Follicular atrophy in the white pulp frequently occurs. [48] Pathologic examination of the liver reveals hematopoietic cellular elements within the sinusoids. Sinusoidal dilation is a frequent finding, as well as prominent intrahepatocyte and Kupffer cell hemosiderin deposition. A marked increase in the hepatic reticulin network has also been observed. [48] [98]

Clinical features of portal hypertension such as ascites or esophageal varices are known to occur in 918% of patients with agnogenic myeloid metaplasia. [96] [97] [98] [99] The liver histologic findings have usually been considered normal or to be characterized by minimal portal fibrosis, which has led to the view that the splenomegaly in AMM may be due to increased hepatic blood flow with resultant portal hypertension. Wanless et al., [98] however, analyzed a large series of patients with polycythemia vera and AMM at autopsy. In those patients with portal hypertension, thrombotic lesions in small or medium-size portal veins as well as extrahepatic portal veins were observed. In addition, nodular regenerative liver hyperplasia occurred in 14.6% of cases and correlated closely with the presence of portal vein lesions. They have concluded that thrombosis is the most likely cause of portal venous obliteration and portal hypertension in AMM and that clinically significant thrombosis confined to small intrahepatic veins or large hepatic veins should be considered in any patient with AMM. In this autopsy series portal and hepatic venous disease occurred even in the absence of signs of portal hypertension. Such a finding is consistent with subclinical thrombosis with recanalization occurring fairly commonly in this patient group.[98]

Approximately 3075% of patients with AMM have karyotypic abnormalities at diagnosis. [129] [130] [131] [132] [133] [134] It is important to perform cytogenetic analysis on this patient population in order to discriminate AMM from CML. [135] [136] Detection of a Philadelphia chromosome and/or a *bcr/abl* fusion gene indicates that the myelofibrosis is secondary to CML. [135] [136] In those cases that are Philadelphia chromosome negative, it is prudent to perform a PCR for BCR/ABL to exclude the diagnosis of chronic myeloid leukemia. Chromosomal abnormalities in AMM have been reported to involve chromosomes 1, 2, 513, 15, 17, 18, 20, and 21 and the Y chromosome.[129] [130] [131] [132] [133] [134] In a recent report by Reilly et al., three cytogenetic patterns (del [13q], del [20q] and partial trisomy 1q) were seen in 65% of all cases with an abnormal karyotype.[134] Partial or complete losses of chromosomes, particularly chromosomes 5 and 7, appear to be associated with the use of chemotherapeutic agents for treatment of AMM.[129] [130] [131] [132] [133] [134] An association between erythroid hypoplasia in AMM and a defect on chromosome 11 has been reported.[137] It is not unusual for a leukemic transformation of AMM to be preceded by additional cytogenetic abnormalities; a finding consistent with a multistep process leading to a leukemic transformation.[138]

On radiographic examination the characteristic features of AMM are a diffuse increase in bone density and increased prominence of the bony trabeculae. This increased bone density may be patchy and can produce a mottled appearance. In 2566% of patients with AMM, such abnormalities have been reported. [6]

Magnetic resonance imaging (MRI) is a promising noninvasive means of evaluating the bone marrow status of patients with AMM. MRI can portray the conversion or reconversion of fatty to cellular marrow.[139] [140] Fibrotic marrow is easily distinguished from cellular marrow by its strikingly low signal intensity with all pulse signals. [139] Kaplan et al.[139] reported that marrow patterns in the proximal femurs of AMM patients correlate with the clinical severity of the disease and that MRI of the proximal femurs may be useful in both staging and evaluating the progression of the disease process. Marrow MRI has been successfully used to follow the progression of this disorder.[140]

DIFFERENTIAL DIAGNOSIS

A patient with hepatosplenomegaly, peripheral cytopenias, teardrop poikilocytosis, and leukoerythroblastosis and marrow fibrosis likely suffers from AMM. It must be appreciated, however, that a number of other disorders besides AMM may lead to this clinical picture [9] [10] (Table 63-1). Secondary myelofibrosis frequently occurs in patients with lymphoma or metastatic carcinoma of the stomach, prostate, lung, and breast. [9] [10] [141] [142] [143] [144] Successful treatment of Hodgkin disease or breast cancer has resulted in reversal of such marrow fibrosis. [141] [142] [143] [144] One should be extremely careful of making the diagnosis of AMM in a patient who has a previous history of a primary neoplasm. The demonstration of carcinoma cells in the marrow establishes that metastatic carcinoma is the cause of the marrow fibrosis. Careful breast examination and mammography are indicated in all women suspected of having AMM, in order to rule out the possibility of metastatic breast cancer. The finding of blastic or lytic bone lesions in patients with myelofibrosis suggests the presence of an underlying carcinoma.

Disseminated tuberculosis and histoplasmosis have also been associated with the development of secondary myelofibrosis. [6] [145] Caseating or noncaseating granulomas observed on bone marrow biopsy suggest the presence of these infectious disorders. Identification of the causative organisms by culture techniques should be pursued.

A number of other primary hematologic disorders can also be accompanied by marrow fibrosis. The peripheral blood and marrow findings that allow one to distinguish these disorders have recently been reviewed by Dickstein and Vardiman (Table 63-4).[45] A variant myelodysplastic syndrome with myelofibrosis has been described by Pagliuca et al.[146] These patients frequently present with cytopenias and have cellular dysplastic abnormalities indistinguishable from those of other patients with myelodysplasia. Their marrows, however, are characterized by the presence of marrow fibrosis and a striking megakaryocytic hyperplasia, with a predominance of small hypolobulated forms, in some cases surrounding fibrosis. Reticulocytopenia is characteristic of these patients as well as teardrop red blood cells and a leukoerythroblastic blood picture. [146] Unlike patients with AMM, patients with myelodysplasia and marrow fibrosis do not have hepatic or splenic enlargement extending

TABLE 63-4 -- Distinguishing Features of Myeloid Malignancies Presenting with Marrow Fibrosis

Disorder	Splenomegaly	WBC	Teardrop Poikilocytosis	Marrow Fibrosis	%Marrow Blasts	Bilineage or Trilineage Dysplasia
MDS with fibrosis	0-±	, Normal or	0-±	+	<30	+
Acute megakaryocytic leukemia	0-±	, Normal or	0-±	+	30	+
Transitional AMM/MDS or AMM in accelerated phase	+	, Normal or	+	+	<30	+
AMM	+	, Normal or	+	+	<30	0
CML, chronic phase	+		0-±	0-+	<30	0
CML, accelerated phase	+		0-±	0-+	<30	0-+

Modified from Dickstein and Vardiman,[45] with permission.

beyond 3 cm below the costal margin. [146] The overall survival of patients with this variant of myelodysplasia has been reported to be 30 months, with death resulting from the effects of cytopenias or transformation to acute leukemia. [146] Two additional studies have indicated that the presence of myelofibrosis in patients with myelodysplasia is associated with a particularly short survival (9.6 months) as compared with patients with myelodysplasia without fibrosis (17.4 months). [147] [148]

Hairy cell leukemia can also be confused with AMM. [8] In one study, 5 of 61 patients who had originally been diagnosed as having AMM were shown retrospectively to have had hairy cell leukemia. [8] Hairy cell leukemia can present as pancytopenia with splenomegaly and is associated with a dry marrow tap. In one series, marrow reticulin content was increased in 26 of 29 patients with hairy cell leukemia. [149] The presence of hairy mononuclear cells possessing tartrate-resistant acid phosphatase or the appropriate phenotype in the peripheral blood or marrow should facilitate differentiation of AMM from hairy cell leukemia. This exercise is important because of the different modalities of treatment that can be successfully employed for hairy cell leukemia.

Myelofibrosis can occur in patients with other myeloproliferative disorders, especially polycythemia vera and CML, and less frequently primary thrombocytopenia. [150] [151] [152] [153] In CML, progressive marrow fibrosis may herald the onset of accelerated disease or blast crisis. [150] [151] [152] [153] Myelofibrosis in CML occurs in two distinct patterns, one in which patients present with CML and significant associated marrow fibrosis, and a second in which the myelofibrosis develops late in the course of the CML.[136] The myelofibrosis in this latter group appears at a mean of 36 months after the diagnosis of CML, is associated with a mean survival of 4.9 months from the detection of myelofibrosis, and therefore represents an ominous prognostic sign. [136]

Postpolycythemic myeloid metaplasia occurs in 515% of patients with polycythemia vera. [150] [151] [152] This transition occurs, on average, 10 years after the initial diagnosis of polycythemia vera is made, but in individual cases it may appear after either shorter or longer intervals. [150] [151] [152] AMM is clinically indistinguishable from postpolycythemic myeloid metaplasia except for the previous history of erythrocytosis in the latter group. Of patients with postpolycythemic myeloid metaplasia, 2550% will develop leukemia, and 70% will be dead within 3 years of this transition. [150] [151] [152] Postpolycythemic myeloid metaplasia represents a transitional myeloproliferative syndrome with relatively grave prognostic implications. Myelofibrosis has also been reported following primary thrombocythemia. [153]

Acute myelofibrosis represents a clinical entity distinct from AMM. [154] [155] [156] Patients characteristically present with pancytopenia, fever, absence of clinically significant splenomegaly, minimal or absent teardrop poikilocytosis, and fibrotic bone marrow. [154] [155] [156] The bone marrow is characterized by the appearance of immature myeloid elements, and the blast cells frequently express megakaryocytic phenotypic properties. [154] [155] [156] Although the number of circulating blast cells is frequently low, these cells have been reported to range from 1355% of the total number of leukocytes. [154] [155] [156] Survival ranges from 1 to 9 months following diagnosis. Acute myelofibrosis is a form of acute megakaryoblastic leukemia. Its distinction from AMM is vital, since aggressive chemotherapy and possibly bone

marrow transplantation are the treatments of choice for acute myelofibrosis.

Pacquette et al. have reported that up to 12% of patients who present with myelofibrosis may suffer from an underlying autoimmune disorder such as systemic lupus erythematosus.[157] The appearance of the marrow in autoimmune myelofibrosis is indistinguishable from AMM. Autoimmune myelofibrosis occurs predominantly in females. The clinical spectrum of autoimmune myelofibrosis is broad. Patients may present with myelofibrosis in the setting of established systemic lupus or in patients with minimal manifestations of an autoimmune disorder.[157] [158] The presence of teardrop erythrocytes or a leukoerythroblastic blood picture in a patient with lupus may suggest autoimmune myelofibrosis. Such patients universally have a positive antinuclear antibody (ANA) test or an elevated anti-DNA titer. [157] [158] Since the physical manifestations of autoimmune diseases may not be evident, all patients with myelofibrosis should have an ANA test. This diagnosis is important because the cytopenia and marrow fibrosis in autoimmune myelofibrosis may partially resolve with steroid therapy. [157] [158]

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THERAPY

The optimal forms of treatment have not yet been defined. A conservative approach to management is generally accepted, asymptomatic patients being observed and therapeutic intervention being reserved for those patients with symptoms. [159] [160] An alternative approach proposed by Pegrum et al. [161] provides for the institution of single-agent chemotherapy early in the course of the disease. Chemotherapeutic regimens used have included busulfan (24 mg/day), 6-thioguanine (2040 mg/day), and a combination of chlorambucil (15 mg/day) and prednisone (30 mg/day), administered intermittently for a course of 34 weeks with a 2-week rest interval between courses.[1] [2] [3] [4] [5] [6] [7] [8] [159] [160] [161] [162] [163] [164] [165] [166] [167] [168] Such an approach is associated with reduction in spleen size, and some authors have claimed reversal of marrow fibrosis with reduction in the amount of teardrop red cells, poikilocytosis, and leukoerythroblastosis. [98] [159] [160] [161] [162] [163] [164] [165] [166] [167] [168]

Hydroxyurea appears to be a particularly useful agent for the treatment of AMM. Its use has been reported to be associated with significantly reduced platelet production and a

THERAPY FOR AGNOGENIC MYELOID METAPLASIA

When initially encountering a patient with presumed AMM, one must be certain that this process is not secondary to carcinoma invading the marrow or involvement of the marrow with tuberculosis. After a careful history and physical examination, appropriate cultures, chemistries, and radiographic studies should be performed to exclude these secondary forms of myelofibrosis. An antinuclear antibody screen should be performed on all patients with myelofibrosis to avoid missing an immune-mediated myelofibrosis that is treatable with steroids.

The treatment of AMM is dependent on the major manifestations of the disease in the individual patient. One must consider treatment of anemic patients, patients with symptoms resulting from splenomegaly, those with bleeding abnormalities, those with portal hypertension, and those with ascites, bone pain, or symptoms due to hypermetabolism.

Blood volume studies should be performed in mild to moderately anemic patients with symptoms. Those who have normal red cell masses and marked increases in plasma volume have a dilutional form of anemia requiring no treatment. Nutritional deficiencies of iron or folate are easily diagnosed and treated. Treatment for ineffective erythropoiesis includes use of androgens, the androgens of choice being oxymethalone in doses of 50 mg tid or danazol (400600 mg/day). Occasional hypoproliferative anemias may respond to low-dose dexamethasone (2 mg/day). Those patients who demonstrate a shortened red cell survival benefit from addition of prednisone at a dose of 60 mg/day. In patients with iron deficiency anemia, every attempt should be made to determine the underlying etiology of blood loss. If a patient develops esophageal varices or peptic ulcer disease, these lesions must be specifically treated. Occasional patients may develop an underlying occult gastrointestinal carcinoma.

In those patients with symptoms resulting from splenomegaly, treatment will depend on the severity and nature of these difficulties. Various measures to reduce the size of the spleen have been used to alleviate uncontrollable splenic pain; these measures may include the use of radiotherapy, chemotherapy in the form of busulfan or hydroxyurea, or the use of interferon-. Approximately two-thirds of patients treated with these chemotherapy agents or biological response modifiers will have some reduction of spleen size, but on discontinuation of chemotherapy, rapid enlargement of the spleen usually occurs.

Individuals with acute splenic infarction who do not respond to administration of analgesics may gain pain relief from local irradiation over the spleen at doses of 0.250.50 Gy/day for 45 days. Those with huge, painful spleens in whom splenectomy is too risky a procedure may benefit from a cautious trial of radiotherapy. Patients with symptoms resulting from a profoundly enlarged spleen are best treated by splenectomy.

Whenever a patient is considered a candidate for splenectomy, an extensive preoperative evaluation must be pursued. A patient must be considered an acceptable surgical risk in terms of cardiac, hepatic, renal, and metabolic function. All patients must have an extensive preoperative evaluation of the coagulation system, which should include assays for coagulation factors V and VIII, fibrin-split products, platelet count, and bleeding time. Patients with qualitative platelet abnormalities remain candidates for splenectomy, but successful surgical intervention may require use of adrenal steroids before surgery and platelet transfusion at the time of surgery. In patients with nonapparent DIC, surgery is definitely contraindicated. The operation itself must be considered a major procedure. Only a senior staff surgeon who has performed this type of surgery many times should attempt such an operation.

In treating patients with myelofibrosis, one must understand that bleeding may be multifactorial in origin. Invariably, patients with myelofibrosis will have qualitative platelet ab- normalities. In addition, thrombocytopenia and nonapparent DIC may lead to a hemorrhagic diathesis. In those patients who have qualitatively abnormal platelet function, platelet transfusions are suggested for serious bleeding or when preparing a patient for surgery. Patients with thrombocytopenia secondary to marrow failure and hypersplenism can often be managed by administering danazol (600 mg/day). In those patients with life-threatening thrombocytopenia who do not respond to danazol, splenectomy should be seriously considered. Patients with nonapparent DIC require no treatment. In those patients who develop DIC secondary to infection or other identifiable etiologies, treatment is best directed toward controlling the underlying etiology of the coagulopathy and initiating replacement therapy with platelets and fresh frozen plasma.

Portal hypertension may complicate myelofibrosis in 68% of patients. Careful assessment of patients with this potentially catastrophic problem is mandatory. Portal hypertension in AMM may be secondary to increased blood flow from the spleen to the liver (i.e., forward-flow portal hypertension) or secondary to an intrahepatic block due to postnecrotic cirrhosis or intrahepatic extramedullary hematopoiesis. Forward-flow portal hypertensions cause is correctable by splenectomy whereas portal hypertension due to an intrahepatic defect requires some form of portal-systemic shunt procedure. The indications for portosystemic decompression include upper gastrointestinal bleeding and refractory ascites. Some patients can be decompressed with a transjugular intrahepatic portosystemic stent. [230] If portal hypertension secondary to intrahepatic obstruction persists, a splenorenal shunt is the decompressive procedure of choice. [231] Limited survival in patients treated medically with variceal bleeding has been observed but prolonged survival, >3 years, in patients treated surgically has been reported.

Patients with ascites, bone pain, or hypermetabolism present unique problems. If the ascites is due to portal hypertension, management should be directed toward relieving the hypertension. Development of ascites in AMM may also, however, be a result of seeding of the peritoneal cavity with extramedullary hematopoiesis. In all patients with myelofibrosis who develop ascites, paracentesis should be performed and careful study of the ascitic fluid pursued. If megakaryocytes are found in the samples of ascitic fluid, the ascites is likely due to peritoneal implants of myeloid tissue. These implants are treated by intraperitoneal instillation of cytosine arabinoside initially at a dose of 0.77 mg/kg/day which can be advanced to 13.5 mg/kg/day or by abdominal radiation. Administration of fractional doses of radiation

Patients with ascites, bone pain, or hypermetabolism present unique problems. If the ascites is due to portal hypertension, management should be directed toward relieving the hypertension. Development of ascites in AMM may also, however, be a result of seeding of the peritoneal cavity with extramedullary hematopoiesis. In all patients with myelofibrosis who develop ascites, paracentesis should be performed and careful study of the ascitic fluid pursued. If megakaryocytes are found in the samples of ascitic fluid, the ascites is likely due to peritoneal implants of myeloid tissue. These implants are treated by intraperitoneal instillation of cytosine arabinoside initially at a dose of 0.77 mg/kg/day which can be advanced to 13.5 mg/kg/day or by abdominal radiation. Administration of fractional doses of radiation at 0.25 Gy/day with rotation into the four quadrants of the abdomen is also extremely effective. Radiation therapy to a total dose of 510 Gy may be very rewarding.

Patients with myelofibrosis who develop severe bone pain have an extremely guarded prognosis. Treatment with etidronate at a dose of 6 mg/kg per day on alternative months has resulted in complete recovery from bone symptoms in a few patients.[232] This complication frequently represents leukemic transformation in evolution. In these patients, biopsies at sites of pain may reveal pure populations of leukemic blasts. The pain appears to be the result of invasion of the periosteum by blast cells. Bone pain may also be treated with local radiation for several days.

Symptoms resulting from hypermetabolism such as weight loss, sweating, and asthenia are not unusual. Such patients may benefit from low-dose hydroxyurea or interferon- therapy. All patients less than 50 years of age with symptomatic AMM should be evaluated for allogeneic marrow transplantation. Identification of an appropriate donor and rapid referral to an expert stem cell transplant unit should be expedited.

reduction of megakaryocytic abnormalities, as well as a significant reduction of myelofibrosis. [167] [168] Some investigators have hypothesized that hydroxyurea-induced suppression of megakaryocytopoiesis causes a reduction in platelet-derived fibrogenic cytokines resulting in reduced fibroblast proliferation and deposition of reticulin.[166] Manoharan et al.[162] [163] have shown that moderate doses (2030 mg/kg) of hydroxyurea given twice or three times weekly are effective and safe in AMM patients requiring treatment. Furthermore, similar reports have appeared following the use of busulfan; Chang and Gross [168] have, in fact, reported three patients with AMM who responded to daily busulfan therapy with the achievement of hematologic remission and reversal of myelofibrosis and myeloid metaplasia. Not only did hematologic parameters, including the hemoglobin and hematocrit levels, improve, but a reduction of teardrop erythrocytes and lessening of leukoerythroblastosis was noted as well as reversal of myeloid metaplasia and marrow fibrosis. Most importantly the quality of life of these individuals improved. [168] Judicious use of busulfan is recommended in this setting, however, because of its potential for producing delayed marrow suppression. The long-term effects of such early chemotherapeutic intervention on prognosis and frequency of leukemic transformation of AMM remains unknown. Selection of such an aggressive therapeutic approach at present appears unwarranted, but this strategy clearly merits further investigation. A randomized clinical trial comparing early use of chemotherapy with a supportive approach is needed to clarify the merit of these two different strategies.

Interferon- has also been suggested as a drug that might alter the course of AMM. [169] [170] [171] [172] It is useful in suppressing thrombocytosis and inhibiting the activity of PDGF which stimulates the proliferation of fibroblasts. Dalla et al. reported a case of AMM with anemia, thrombocytopenia and hepatosplenomegaly that was treated for over two years with interferon- with remarkable results.[171] The patient became asymptomatic and transfusion-independent, and repeat marrow biopsy revealed no fibrosis. Bourantos et al. have reported the use of the combination of interferon, GM-CSF, and erythropoietin in seven patients with AMM. [172] Each of these patients had an aspirable marrow with megakaryocytic hyperplasia but only mild to moderate deposition of reticulin fibers. [172] Six of seven patients enjoyed marked reductions in spleen size.[172] Each of these patients had a 1g/dl increase in hemoglobin concentration. In one patient the degree of marrow fibrosis was diminished. These authors suggested that interferon might decrease the rate of fibrosis if administered chronically during the early course of this disease. [172] This hypothesis will require careful testing.

Symptomatic therapy is indicated for patients with the following conditions: (1) symptoms attributable to anemia, (2) pressure symptoms related to splenomegaly, (3) bleeding problems or life-threatening thrombocytopenia, (4) significant hyperuricemia, and (5) portal hypertension and life-threatening gastrointestinal bleeding. [6] Hyperuricemia should be aggressively treated in all patients with AMM. Hydration and chronic administration of allopurinol (300 mg/day) are suggested. [6]

Anemia is a common problem in patients with AMM. It is usually multifactorial in origin, contributing factors being folate deficiency, iron deficiency, ineffective erythropoiesis, erythroid hypoplasia, and hemolysis. [103] [104] [105] [106] Kinetic studies and red cell survival studies using radioisotopes can be helpful in defining the etiology of the anemia. It is also important in some instances to directly measure the patients red cell mass to be assured that the low hemoglobin level is not merely dilutional in origin. [103] [104] [105] [106] Transfusion therapy with packed red cells is clearly indicated in those patients who are symptomatic from their anemia. Patients with documented nutritional deficiencies should receive either folate or iron supplementation, or both. Chronic transfusion therapy will frequently be required; one should try to attain a hemoglobin level at which symptoms resolve. Since AMM is a chronic disease, long-term transfusion therapy potentially may lead to development of iron overload syndrome. Serious consideration should be given to early institution of iron chelation therapy. In a handful of patients, a reduction of transfusion requirements was noted following desferrioxamine therapy. This iron chelator can promote transferrin receptor expression on erythroblasts. [173]

The remainder of approaches to the anemic patient deal with therapeutic interventions designed to avoid or diminish the number of transfusions administered. Ineffective erythropoiesis in AMM may be treated with anabolic steroids. [1] [2] [3] [4] [5] [6] [7] [8] [174] [175] [176] [177] [178] Gardner and Nathan[174] have recommended that all patients with AMM and anemia should receive a trial of androgens. A number of preparations have been suggested, including testosterone enanthate (600 mg/wk IM), stanozolol (12 mg/day PO), nandrolone (3 mg/kg/wk IM), fluoxymesterone (10 mg tid PO), and oxymethalone (50 mg qid PO). It is unknown whether any of these preparations is superior to the others, but a good response, as defined by a decrease or total avoidance of transfusion therapy, occurs in about 50% of patients. A course of 36 months of androgen therapy is indicated in order to identify responsive patients, [174] but the development of hepatic dysfunction or virilizing side effects may limit long-term androgen administration. Besa et al.[177] have indicated that patients with associated chromosomal abnormalities are less likely to respond to androgen therapy. Danazol, a synthetic attenuated androgen, has recently been shown to be useful in reducing the requirement for red cell transfusion support and correction of thrombocytopenia. [178] Danazol appears to be potentially effective therapy in AMM which is mainly characterized by marrow failure. [168] In addition, low dose dexamethasone has also been reported to be useful in the treatment of transfusion-dependent AMM. [179] Whether these cases represent steroid responses of autoimmune-induced myelofibrosis and not classical AMM remains unknown. Erythropoietin therapy alone does not appear to improve the anemia associated with AMM.[180] [181]

Corticosteroids (e.g., prednisone 1 mg/kg/day PO) have also been successfully employed for treatment of the hemolytic anemia associated with AMM. [1] [2] [3] [4] [5] [6] [7] [8] [182] Bouroncle and Doan[1] reported favorable results in approximately 25% of patients, while Silverstein [6] reported a 2 g increase in hemoglobin in 29% of men and 52% of women. Folate should be simultaneously administered to all such patients. Bouroncle and Doan [1] reported even more encouraging results with simultaneous administration of busulfan and corticosteroids, with clinical responses lasting 612 months. Once patients have reached a peak response, tapering of prednisone should be initiated to determine an acceptable maintenance dose. Frequently the hemolytic process recurs following such tapering. Appropriate patients should be referred for splenectomy at that time.

Pressure symptoms secondary to splenic enlargement can be treated initially with cytotoxic chemotherapy. Busulfan, chlorambucil and prednisone, 6-thioguanine, radioactive phosphorus, or hydroxyurea (1520 mg/kg three times a week) have been used for this purpose. [1] [2] [3] [4] [5] [6] [7] [8] [161] [162] [163] [164] [165] [166] [167] [168] In the Mayo Clinic series a significant reduction in spleen size with relief of pressure symptoms occurred in 70% of patients receiving chemotherapy. [6] Responses are unfortunately short-lived, lasting a median of only 4.5 months. [6] Only 16% of those patients with long-term maintenance therapy enjoyed sustained relief of symptoms.[6] Hematologic toxicity was not infrequent and often necessitated cessation of therapy.

Splenic irradiation has also been frequently used for treatment of the painful big spleen syndrome. [6] [7] [182] [183] [184] [185] [186] Irradiation in fractions of 0.151 Gy administered either daily or by an intermittent fractionation schedule (two or three times per week) to a total dose per treatment course of 2.56.5 Gy may be effective. [6] [7] [182] [183] [184] [185] [186] Responses are transient, lasting an average of 3.5 months, and hematopoietic toxicity is frequently significant.

Wagner et al.[184] have recommended for this purpose simultaneous treatment with splenic irradiation and oral hydroxyurea and have obtained some promising preliminary results. Silverstein[6] has reported that splenic irradiation is especially useful for treatment of splenic pain of sudden onset and also for treatment of ascites due to implants of hematopoietic tissue. Radiation therapy should be considered as a temporary measure to be employed in patients who are too ill to tolerate splenectomy or chemotherapy. In one series, the median duration of response to radiation was 6 months. [182] The toxicity of splenic irradiation is limited to myelosuppression with significant prolonged cytopenias occurring in 43% of cases. [182]

Radiotherapy offers a viable treatment option and at times may be the therapy of choice for the treatment of peritoneal or pleural implants leading to ascites or pleural effusions and extramedullary hematopoiesis in vital organs leading to organ dysfunction. [186] Because of the inherent sensitivity of myeloid tissue to irradiation and

profound marrow suppression that may occur following irradiation, therapy is usually initiated at low doses (2025 cGy/day) with modification of the dose as the clinical situation dictates.[186] Recently, an alternative approach using intraperitoneal administration of cytosine arabinoside has been used to treat ascites in AMM. [187] Therapy is initiated at 0.77 mg/kg/day to achieve 30 mg/2 liters of peritoneal fluid. The dosage is advanced so that the patient eventually receives 1 gram or 13.5 mg/kg/day. Abdominal pain is the most common adverse effect associated with this approach.[187]

Parmeggiani et al.[188] reported the use of interferon- (IFN-) for the treatment of painful splenomegaly in AMM. [188] Splenic pain and pressure symptoms disappeared with a decrease in splenic size, but peripheral blood counts deteriorated. Gilbert has, however, reported a similar reduction in spleen size with maintenance IFN- therapy but without hematologic compromise.[189] Barosi et al.[190] have suggested that IFN- might be useful in treating thrombocytosis that follows splenectomy in AMM patients. Several investigators have noted, however, that although IFN- has cytoreductive activity in AMM, its use is often limited by debilitating toxic effects such as severe flu-like symptoms. Martyré et al.[191] also reported that IFN- administration results in the reduction of PDGF and TGF- levels present within AMM platelets. These investigators have suggested that such an approach might lead to a cessation or slowing of the marrow fibrosis. [191] The appropriate role of the IFNs in the treatment of AMM as previously mentioned requires further investigation.

The role of splenectomy in the management of AMM remains a controversial issue.[192] [193] [194] [195] [196] Splenectomy is indicated in patients with hemolysis, thrombocytopenia, painful splenomegaly, recurrent splenic infarctions, and traumatic splenic rupture refractory to other therapeutic modalities. [6] Crosby[192] and Mulder et al.[193] have suggested that splenectomy be performed in every patient with AMM immediately after the diagnosis is made. However, Benbassat et al. [194] reviewed the role of splenectomy in AMM by evaluating 321 published AMM splenectomy cases and concluded that this procedure should be considered only in selected individuals for specific indications. Table 63-5 shows the combined results from two representative published series of splenectomized patients. These series indicate that for specific indications splenectomy is an excellent therapeutic choice. [195] [196] It is important to note, however, that 10 of the 72 patients in these series died postoperatively and that significant postoperative morbidity resulting from septicemia and hemorrhage was present. [194] [195] [196] [197] Benbassat et al.[196] further explored the risks and benefits of splenectomy by reviewing and performing a formal decision analysis of 15 reports in the literature dealing with splenectomy in AMM that were published after 1970. They determined that the operative mortality was 13.4% and early morbidity caused by atelectasis, hemorrhage, thromboembolism,

TABLE 63-5 -- Indications for and Responses to Splenectomy in Patients with AMM

Indication	Combined Series	
	No. of Patients	No. of Responders
Painful splenomegaly	40	38
Refractory thrombocytopenia	13	6
Refractory hemolytic anemia	19	11
Portal hypertension	11	9

Data from Silverstein and Remine[195] and Brenner et al. [200]

and subphrenic infections was 45.5%. [196] However, anemia was improved in 70% of patients, painful splenomegaly in 97% of patients, thrombocytopenia in 56% of patients and portal hypertension in 83% of individuals. [196] Splenectomy was not demonstrated to alter survival but was recognized to improve the quality of life. In several series, an extraordinarily high rate of leukemic transformation was noted following splenectomy. [197] [198] [195] Whether this was a function of the absence of the spleen or more likely the consequence of the natural history of the disease is unknown. Splenectomy should be considered for symptomatic patients after they have been informed of the operative mortality and chances of palliation. [196] Ferrokinetic measures of total erythropoiesis and of plasma volume previously claimed to be useful guides for the choice of splenectomy have little value for predicting outcome. [197] [200] [201] The clinician should not agonize over the decision of whether or not to perform splenectomy on a patient with AMM. Splenectomy often results in improved quality of life due to resolution of cytopenias or relief from painful splenomegaly.[194] [195] [196] [197] [198] [199] [200] It is important to be aware that compensatory hepatic myeloid metaplasia may accelerate following splenectomy, leading to rapid enlargement of the liver.[8] [199] [202] [203] Occasionally this massive myeloid metaplasia and sinusoidal dilation can result in liver failure and death. [202] [203] This complication can, however, frequently be treated with chemotherapy.[202] 2-chlorodeoxyadenosine (2-CdA) has been shown to be particularly effective in controlling progressive hepatomegaly or symptomatic thrombocytosis after splenectomy in AMM.[204] This drug has been administered at 0.050.1 mg/kg/day as a 7-day continuous infusion for four to five courses at monthly intervals. This therapy resulted in control of thrombocytosis in 78% of patients and significant reduction in liver size in over 50% of cases for a prolonged period (428 months).[204] Surgical morbidity following splenectomy can be minimized by careful selection of patients with respect to cardiac and renal status, improved surgical technique, aggressive blood bank support with platelet transfusions and early postoperative mobilization of the patient. Platelet transfusions should be administered to the bleeding patient following splenectomy even if the platelet count is normal because AMM is frequently associated with qualitative platelet abnormalities.

Excessive bleeding in patients with AMM can be a result of either thrombocytopenia, qualitative platelet defects, or DIC. [6] Based on his clinical observations, Silverstein[6] [195] has suggested that splenectomy is contraindicated in patients with DIC. Actively bleeding patients with consumption coagulopathy should receive platelet and plasma replacement therapy. Low-dose heparinization has resulted in improvement in occasional patients. [6] Platelet transfusions are suggested in bleeding patients who are thrombocytopenic or have a normal platelet count but who are known to have qualitative platelet abnormalities.

Bone marrow transplantation is a potentially useful therapeutic tool in young patients with AMM who have an appropriate

donor available. [31] [205] [206] [207] [208] Successful transplantation is associated with gradual resolution of marrow fibrosis and normalization of hematopoiesis. [31] [209] [210] Allogeneic bone marrow transplantation offers the best chance for cure and should be seriously considered in all patients <40 years of age. [209] [211] The feasibility of successful bone marrow transplantation was questioned by Rajantie et al. [209] who reported that severe marrow fibrosis can adversely affect post-transplantation hematopoietic reconstitution. Soll et al., however, challenged this conclusion and, after an analysis of 203 patients with marrow fibrosis caused by a variety of hematological malignancies who underwent allogeneic transplantation, concluded that myelofibrosis did not enhance the risk of failure to engraft but merely delayed the time to reach platelet transfusion independence by three days. [210]

Splenectomy before transplantation may hasten hematological reconstitution, but removal of the spleen may not be essential. Although graft failure is not usually a problem following allogeneic transplantation in AMM, splenectomy may be indicated in those patients receiving T-cell depleted grafts. [207] [208] [211] Recently Guardiola et al. has reported on the results of 12 patients with the average age of 40 who received allogeneic transplantation for AMM. [208] Eleven patients engrafted with median times to achieve neutrophil and platelet engraftment of 17 and 29 days, respectively. [208] The four-year overall and event-free rates were 71% and 59%, respectively. [208] Two patients were in complete remission 10 and 5 years following transplantation. [207] Allogeneic marrow transplantation appears to be the only means of curing AMM. This conclusion was confirmed in a larger series of 40 patients with AMM who underwent allogeneic bone marrow transplantation, in whom a three-year survival rate of 60% was observed and four patients were alive more than 10 years following transplantation. [211] This study also emphasized that this patient population is at a high risk for the development of graft-versus-host disease. [211]

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PROGNOSIS

The median overall survival from the time of diagnosis of AMM varies from series to series but is approximately 5 years [1] [2] [3] [4] [5] [6] [7] [8] [212] (Fig. 63-3). Individual survival has been reported to range from 1 year to >30 years. [1] [2] [3] [4] [5] [6] [7] [8] Several clinical and biological parameters that are characteristic of patients at diagnosis have been used to identify subgroups of patients with different outcomes. [213] [214] [215] [216] [217] Some authors have suggested that there are two subpopulations of patients in AMM: short-lived and long-lived. Such efforts at developing prognostic parameters have met with conflicting results with the exception that anemia at presentation is consistently associated with short survival. [217] Recently Dupriez et al. have developed an extremely simple scoring system based on two adverse prognostic factors, Hgb <10g/dl and WBC <4,000 or >30,000/mm ^{3} and were able to separate patients into three groups (Table 63-6).[216] The low-risk groups (0 factor), intermediate risk (1 factor), and high-risk (2 factors) were associated with a median survival of 93, 26, and 13 months, respectively. Reilly has demonstrated, in 106 patients with AMM with successful marrow karyotypic analysis, the prognostic significance of cytogenetic abnormalities[134] (Fig. 63-4). Thirty-five percent of cases exhibited clonal abnormalities, and 65% had a normal karyotype in this study. Kaplan-Meiers long-range plot analysis defined age ($P < 0.01$), hemoglobin ($P < 0.001$), white blood cell count ($P < 0.06$), platelet count ($P < 0.0001$), and abnormal karyotype ($P < 0.001$) as adverse prognostic parameters. [134] Reilly was able to define a prognostic schema by combining age, karyotype, and hemoglobin level (Table 63-7).[134] Median survival times varied from 180 months for the good risk group (age < 68 Hgb 10, abnormal karyotype). [134]

Figure 63-3 Overall survival from time of diagnosis of 141 patients with AMM. (*From Silverstein, [6] with permission.*)

TABLE 63-6 -- LILLE Scoring System for Predicting Survival in Agnogenic Myeloid Metaplasia

Adverse Prognostic Factors			
Hb <10 g/dl			
WBC <4 or >30 × 10^9 /L			
The scoring system (number of adverse prognostic factors)			
Factor No.	**Risk Group**	**Cases (%)**	**Median Survival (mo)**
0	Low	47	93
1	Intermediate	45	26
2	High	8	13
Reprinted with permission from Dupriez et al.[216]			

Knowledge of the prognosis of an individual patient may be useful in defining high-risk patients in whom experimental forms of therapy might be indicated. In one series of 106 cases of AMM, 62 patients had died after prolonged follow-up. [217] The primary causes of death were determined in 53 cases and included infection (16 cases), leukemic transformation (9 cases), heart failure (9 cases), bleeding (7 cases), hepatic failure due to massive myeloid metaplasia of the liver (5 cases), portal hypertension (4 cases), and renal failure, pulmonary embolism, and post-transplant graft-versus-host disease (1 case each). [217] The incidence of acute leukemia as a terminal event ranges from 522% of patients depending on the series cited. [1] [2] [3] [4] [5] [6] [7] [8] [218] [219] [220] [221] [222] Approximately one-half of the patients who develop acute leukemia have received previous treatment with alkylating agents or radiotherapy, suggesting that the evolution into acute leukemia might be part of the natural history of AMM.[196] [223] [224] [225] The actuarial cumulative risk of death from leukemic transformation at 1 and 5 years following diagnosis has been reported to be 2% and 16% respectively. [216] Immunologic and morphologic characterization of the blast phenotype comprising these leukemias reveals that a typical myeloid phenotype is most commonly detected; other cell lineages, such as megakaryocytic, erythroid, lymphoid, and even stem cell phenotype may also be involved, leading to the existence of both mixed myeloid and hybrid transformations. [222] [223] [224] [225] [226] [227] Megakaryoblastic transformations have been detected in one-third of cases in one series, an incidence clearly higher than that found in de novo acute myeloid leukemia. [225] Survival following these blast transformations is limited, a phenomenon that is probably a result of patient age and the aggressive biology of these leukemias. Successful leukemia induction of these patients is a rare event. [228]

Figure 63-4 Kaplan-Meier plots (survival in months, X axis; percent survival, Y axis) demonstrating age, hemoglobin g/dl, and total leukocyte count (WCC, platelet count (10 ^{9} /1) and karyotype) to be individual predictors of survival. N, normal; A, abnormal. (*From Reilly et al.,[134] with permission from Blackwell Science, Ltd.*)

TABLE 63-7 -- Prognosis of Patients of AMM Based on Presence of Anemia and Cytogenetic Abnormalities

Age (yr)	**Hb (g/dl)**	**Karyotype**	**Survival (mo) (95%CI)**[a]	**(n)**[b]
<68	10	Normal	54 (46,62)	(7)
		Abnormal	22 (14,30)	(7)
	>10	Normal	180 (6,354)	(25)
		Abnormal	72 (32,112)	(11)
>68	10	Normal	44 (31,57)	(13)
		Abnormal	16 (5,27)	(14)
	>10	Normal	70 (61,79)	(22)
		Abnormal	78 (26,130)	(7)

From Reilly et al.,[134] with permission from Blackwell Science, Ltd.

[a]95% confidence intervals.
[b]Number of patients.

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FUTURE DIRECTIONS

Rapid strides have been made in understanding the biosynthesis of collagen in myelofibrosis. If it is indeed true that PDGF and TGF- play a major role in promoting fibroblastic proliferation in patients with AMM, agents capable of neutralizing the biologic functions of such growth factors may be of potential clinical use. This hypothesis was tested in a clinical trial of suramin, an antiparasitic agent that antagonizes the activity of TGF-. [227] In a phase II trial suramin was administered in a 24-hour continuous infusion at dose levels of 280350 mg/m ^{2} for 510 days every 5 weeks. No biologic evidence or clinical evidence of suramin activity in AMM was observed.[229] Instead progressive splenomegaly and severe pancytopenia were observed in all patients. [229] Further exploration of the use of biologic response modifiers or drugs that might alter the fibrotic process in AMM will likely be pursued in the future. In addition, because fibrogenic cytokines that are released from megakaryocytes and platelets play such a critical role in AMM biogenics, it remains possible that suppression of megakaryocytopoiesis in this disorder might alter its natural history.

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Chapter 64 - Primary Thrombocythemia

Ronald Hoffman

INTRODUCTION

Primary thrombocythemia is a chronic myeloproliferative disorder characterized by a sustained proliferation of megakaryocytes, which leads to increased numbers of circulating platelets. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] In addition to platelet counts in excess of 600,000/mm ^{3} , this disorder is characterized by profound marrow megakaryocyte hyperplasia, splenomegaly, and a clinical course punctuated by hemorrhagic or thrombotic episodes or both. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] Primary thrombocythemia is a clinically heterogeneous disorder with up to two-thirds of patients who meet the criteria for diagnosis being asymptomatic at presentation. [17]

Primary thrombocythemia was first reported in 1934 by Epstein and Goedel, [18] who described a patient with an elevated platelet count who suffered from repeated hemorrhagic episodes. [18] This disease entity has been referred to by a variety of names, including essential thrombocythemia, idiopathic thrombocythemia, essential thrombophilia, and essential thrombocytosis. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17]

Originally, many clinical investigators questioned whether primary thrombocythemia represents a distinct clinical entity. [19] However, extensive descriptions of larger series of patients have provided information to overcome this initial skepticism. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] Dameshek [20] in 1951 speculated that primary thrombocythemia may represent one of the myeloproliferative disorders. Subsequent laboratory investigations have confirmed this concept and clearly demonstrated that the disorder is a clonal hematologic malignancy. [21] [22] [23] [24] [25]

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EPIDEMIOLOGY

The true incidence of primary thrombocythemia is unknown, because extensive epidemiologic studies are not available. Although many investigators have indicated that this disorder is very rare, information from several institutions suggests that it occurs considerably more frequently than originally believed. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] Following the incorporation of a platelet channel in automated complete blood cell counts, extreme thrombocytosis has been shown to be a relatively common occurrence in a general hospital setting. [26] Thus, 94 cases of newly diagnosed primary thrombocythemia were identified from 19611982 at the Saint Louis Hospital in France, while 61 patients with this disorder were discovered from 1974 through 1987 at the Medizinische Poliklinik in Munich. [13] [14] The incidence of primary thrombocythemia in Olmstead County in southeastern Minnesota is reported to be 2.38 patients/100,000 population/year. [27] Primary thrombocythemia and polycythemia vera appear to have approximately a 1:4 relative incidence. [27]

The disorder appears to affect primarily middle-aged people, with an average age at diagnosis of 5060 years. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] In most series, there appears to be no sexual predilection; although in several reports, a higher prevalence in females has been noted. [7] [12] [13] [14] [15] [16] [28] [29] This discrepancy might be due to an equal frequency in older patients of both sexes but to a second peak frequency at around 30 years of age for women. [13] This predisposition of young women to develop primary thrombocythemia has been reported previously. [27] [28] [29] [30] [31]

Genetic transmission of the disorder is unusual, although several families with multiple members having primary thrombocythemia have been described. [32] [33] [34] [35] In two of these families, thrombocythemia occurred in three successive generations, with patients ranging in age from 2 to 62 years. [32] [35] An abnormality of thrombopoietin production or in the thrombopoietin receptor has been suggested as the cause of these inherited disorders. [34] A Dutch family with 11 family members and a Japanese family with 8 family members were reported with a hereditary form of primary thrombocythemia which was inherited in an autosomal dominant fashion. The thrombopoietin receptor in the Dutch family was normal, yet there was a G to C transversion in the splice donor site of intron 3 of the thrombopoietin gene. [36] All of the affected members of both the Japanese and Dutch families were shown to have elevated thrombopoietin levels. In each family, a point mutation in the thrombopoietin gene was thought to lead to systemic overproduction of thrombopoietin, leading to a hereditary form of primary thrombocythemia. [36] Several cases of primary thrombocythemia have also been reported in patients in the pediatric age group, but this is an extremely unusual finding. [37] [38] [39] [40]

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ETIOLOGY

The mechanisms leading to thrombocytosis in primary thrombocythemia are poorly understood. Patients with primary thrombocythemia have normal or near-normal platelet survival.[31] [32] The thrombocytosis is due to increased platelet production by megakaryocytes. Effective platelet production is increased as much as 10-fold and is associated with an increase in megakaryocyte clustering, volume, nuclear lobe number, and nuclear ploidy. [41] [42] [43] [44]

Analysis of circulating blood cells of females with primary thrombocythemia who were heterozygotes for isoenzymes of glucose-6-phosphate dehydrogenase has revealed that platelets, erythrocytes, and neutrophils express a single isoenzyme type. [21] [22] [23] [24] In addition, Raskind et al.[24] have presented data to indicate that B cells can also be involved in this neoplastic process. Such findings indicate that primary thrombocythemia is a clonal hematopoietic disorder originating at the level of the pluripotential hematopoietic stem cell. The clonal origin of primary thrombocythemia has subsequently been confirmed using restriction fragment length polymorphisms of X chromosome genes.[25] [45]

Such clonal analyses have revealed that a significant proportion of nonclonally derived leukocytes exist in addition to the clonally derived population of leukocytes in patients with primary thrombocythemia.[25] [45] [46] In one study of 42 patients with primary thrombocythemia, 31 patients exhibited clonality of at least one hematopoietic lineage whereas the remaining 11 patients had polyclonal origins of all lineages studied. [25] The biogenesis of polyclonal primary thrombocythemia remains ill-defined. It is possible that small numbers of normal hematopoietic stem cells persist to account for this admixture of nonclonal populations. Primary thrombocythemia patients with polyclonal hematopoiesis appear to have a clinical picture identical to that of patients with monoclonal hematopoiesis. [25] [46]

In one patient, Anger et al.[45] determined that although the total leukocyte fraction was clonally derived, the T-lymphocyte population was nonclonal in origin. In fact, most studies indicate that T lymphocytes are of polyclonal origin in primary thrombocythemia patients. [25] Interestingly, in some patients, monoclonality of hematopoiesis is restricted to platelets despite the polyclonal origin of the other lineages. Other studies, however, have indicated a common origin of granulocytes, platelets, and B lymphocytes in this disorder. [23] Such studies raise the possibility that the malignant transformation leading to primary thrombocythemia occurs at a number of stages along the hematopoietic cellular hierarchy. [47] Such a model would predict that in some cases a primitive stem cell would be the site of the initial oncogenic event, while in others this event might occur in a more differentiated progenitor cell. Data supporting such a hypothesis were supplied in an analysis of two sisters with primary thrombocythemia.[48] In one sister, granulocytes, monocytes, and T lymphocytes were clonally derived; in the other sister, the granulocytes fraction was monoclonal, but the T lymphocytes were polyclonal in origin. This study confirms the heterogeneity of the affected hematopoietic cell at which primary thrombocythemia might originate.

The biologic behavior of megakaryocyte progenitor cells present either in the marrow or in the peripheral blood of patients with primary thrombocythemia has been extensively studied.[49] [50] [51] [52] [53] [54] [55] [56] [57] [58] In each of these studies, the use of serum-containing culture systems has led to the detection of increased numbers of assayable progenitors. These data support the concept that the principal abnormality is an expansion of the progenitor pool. In addition, colonies were noted to appear in the absence of exogenous cytokines.[48] [49] [50] [51] [52] [53] [54] [55] [56] [57] [58] A subpopulation of colony-forming unit-megakaryocyte (CFU-MK) assayed from patients with primary thrombocythemia was also shown to remain responsive to addition of cytokines. [49] [50] [51] [52] [53] [54] [55] [56] [57] [58] [59] [60] [61] [62] [63] [64] [65] [66] [67] [68] [69]

Several laboratories have demonstrated that megakaryocyte progenitor cells isolated from primary thrombocythemia patients are hypersensitive to the action of several cytokines including IL-3, IL-6, but not GM-CSF. [58] [59] Furthermore, investigators have explored the possibility that the defect in primary thrombocythemia might actually be due to resistance to inhibitors of megakaryocytopoiesis. [57] Zauli et al. have shown that the primary thrombocythemia CFU-MK have a significantly lower sensitivity to the inhibitory effects of low doses of TGF- and a limited response to the inhibitory effects of high concentrations of autologous platelet lysates. [60] TGF- was shown to be primarily responsible for the inhibitory activity present in such platelet lysates. [69] Some combination of increased sensitivity to growth factors that promote platelet production and disrupted sensitivity to negative regulators of thrombopoiesis at the level of the megakaryocyte progenitor cell might account for the thrombocytosis that is characteristic of primary thrombocythemia.

Using serum-free cultures, a number of laboratories have reported conflicting data about spontaneous megakaryocyte colony formation by CFU-MK from patients with primary thrombocythemia in the absence of the addition of exogenous cytokines.[54] [55] [56] [57] [58] Several groups have attempted to utilize this in vitro characteristic as a diagnostic tool to discriminate between primary thrombocythemia and reactive thrombocytosis. [54] [55] [56] [57] [58] [59] Because there is some degree of spontaneous megakaryocyte colony formation by normal marrow in such serum-free culture systems, these findings remain difficult to interpret. [61] No evidence of an autocrine regulatory defect involving IL-3, IL-6, GM-CSF, or thrombopoietin has been detected to account for the expansion of the megakaryocyte progenitor cell pool in primary thrombocythemia.[58] [62]

Erythroid progenitor cells in primary thrombocythemia can also proliferate in response to the small amount of cytokines present in serum alone. Erythroid colony formation in the absence of exogenous erythropoietin is a hallmark of the proliferative defect that characterizes polycythemia vera. [63] Burst-forming unit-erythroids can be assayed from both marrow and peripheral blood of patients with primary thrombocythemia and form erythroid colonies in the absence of exogenous erythropoietin.[64] [65] [66] [67] Such an abnormality in these nonpolycythemic patients probably indicates an underlying defect shared by progenitor cells in many myeloproliferative disorders.[64] [65] [66] [67] These observations allow one to question the claim that endogenous erythroid colony formation can be useful in discriminating between primary thrombocythemia and masked polycythemia vera.[68] This does not diminish the use of such erythroid colony assays to discriminate between myeloproliferative disorders characterized by extreme thrombocytosis and reactive thrombocytosis.

Careful study of one woman with primary thrombocythemia, who entered a spontaneous clinical remission while pregnant, has indicated the existence of normal hematopoietic progenitor cells in the marrow of patients with primary thrombocythemia. [69] This information suggests the coexistence of normal and neoplastic hematopoietic progenitor cells in primary thrombocythemia.[68] This hypothesis is further corroborated by the finding, in some patients, of polyclonal granulocytes, indicating a normal stem cell population alongside the thrombocythemic stem cell clone. [25] [46] [47] Thus, during active phases of the disease the neoplastic cells apparently predominate over normal progenitor cell populations.

Thrombopoietin is the primary physiological regulator of megakaryocytopoiesis and platelet production. This growth factor acts by binding to its cell surface receptor, c-mpl.[70] The thrombopoietin receptor is expressed by CD34+ hematopoietic progenitor cells, megakaryocytes and platelets. [70] Recently, data have been provided that thrombopoietin and its receptor pathways are not directly linked to the defect that underlies primary thrombocythemia. [71] [72] [73] Normal or slightly elevated thrombopoietin levels have been observed in patients with primary thrombocythemia. [71] [72] [73] Furthermore, expression of the thrombopoietin receptor and its mRNA were shown to be dramatically reduced in the platelets of patients with primary thrombocythemia. [72] In addition, the thrombopoietin receptor-mediated signaling cascade was shown not to be constitutively activated in platelets isolated from patients with primary thrombocythemia. [72] Further studies of the molecular pathology

underlying primary thrombocythemia are needed to gain a better understanding of the origins of this disorder.

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PATHOBIOLOGY

Most clinical sequelae of primary thrombocythemia are related to hemorrhagic and thrombotic episodes, which frequently punctuate the clinical course of individual patients.[74] [75] [76] [77] Thrombotic complications occur most frequently in older patients and patients with a previous history of a thrombotic event, whereas hemorrhagic events occur almost exclusively in individuals with high platelet counts (>1000 × 10^9 /l).[1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [77] A subset of young patients who are asymptomatic and remarkably free of such complications has also been described.[29] [38] [78] Such age-related differences in the frequency of these events have been attributed to the coexistence of vascular disease in older patients.[79] [80] This hypothesis remains controversial since other studies have described younger patients with a significant incidence of vascular thrombosis.[13] [81] Microvascular thrombosis causing digital or central nervous system ischemia leads to a variety of clinical syndromes closely associated with primary thrombocythemia.[9] [74] [75] [76] [82] [83] [84]

Past studies have concluded that the degree of elevation of the platelet count in primary thrombocythemia is an important determinant of the frequency of thrombotic and hemorrhagic events.[1] [4] [5] These conclusions were, however, based on observations of a limited number of patients. Four different studies have failed to define a relationship between the frequency of thrombotic complications and platelet numbers.[13] [14] [85] [86] The relationship between frequency of bleeding episodes and platelet counts is also cloudy. In two studies, patients with extreme thrombocytosis (>1000 × 10^9 /l) were reported to have a much higher incidence of hemorrhagic events, although such a relationship was not confirmed in a third study.[13] [77] [79] [86]

Recently, some progress has been made in further defining the relationship between platelet numbers and the risk for thrombotic and hemorrhagic events in primary thrombocythemia.[74] [77] [87] [88] [89] [90] Cortelazzo has reported in a randomized trial of patients at high risk of developing a thrombotic event (>60 years of age and/or a previous history of thrombotic episode) that reduction of platelet numbers was highly effective in preventing additional thrombotic events.[87] Furthermore, Cortelazzo et al. have shown that the incidence of thrombotic events is closely correlated with the duration of thrombocytosis.[76]

Several groups have now confirmed that the degree and duration of bleeding in this patient population correlates with the platelet count.[72] [82] [83] [84] Bleeding events appear to occur exclusively when the platelet counts are excessively high and stop when the platelet count falls to normal.[77] [88] [89] [90] The clinical spectrum of bleeding in primary thrombocythemia patients closely resembles that observed in von Willebrand disease.[82] [83] [84] Several groups have now shown that high platelet counts (>1000 × 10^9 /l) are associated with an acquired von Willebrand syndrome and that reduction of platelet numbers is associated with correction of the von Willebrand defect and cessation of bleeding episodes.[88] [89] [90] An increase in the number of circulating platelets appears to favor the adsorption of larger von Willebrand multimers onto platelet membranes, resulting in their removal from the circulation and their subsequent degradation.[89] The laboratory features of acquired von Willebrand syndrome in primary thrombocythemia is characteristic of a Type II deficiency, a prolonged bleeding time, a normal Factor VIII C:VWF Ag ratio, decreased ristocetin cofactor activity, and a decrease or absence of large von Willebrand factor multimers.[90]

Platelets in patients with primary thrombocythemia have been known for a considerable time to be qualitatively abnormal.[4] [7] [74] [75] [76] [77] [88] [90] While both increased and decreased platelet reactivity has been described,these findings have not been definitively associated with thrombohemorrhagic complications with two noteworthy exceptions; erythromelalgia, where the prompt relief of symptoms by cyclo-oxygenase inhibitors provides direct evidence that prostaglandins play a role in the development of vascular occlusion, and acquired von Willebrand syndrome, which is a major cause of bleeding in patients with primary thrombocythemia.[4] [7] [75] [82] [83] [84] [85] [86] [87] [88] [89] [90] Prolongation of the bleeding time has been reported in 719% of newly diagnosed patients with primary thrombocythemia.[77] A close correlation between prolongation of the bleeding time and the occurrence of hemorrhage in primary thrombocythemia patients does not always exist. This is in contrast to correction of the bleeding time in those patients with primary thrombocythemia, extreme thrombocytosis, and acquired von Willebrand syndrome following reduction of platelet numbers to the normal range.[75] [90] It is important to emphasize that aspirin prolongs the bleeding time of patients with primary thrombocythemia to a greater degree than that of normal controls.[77] [79] Abnormal platelet aggregation has been reported in 35100% of patients with primary thrombocythemia.[77] Such abnormal aggregation studies are not related to prolongation of the bleeding time or to the incidence of episodes of hemorrhage or thrombosis. In primary thrombocythemia, platelet aggregation is classically defective in response to epinephrine, ADP and collagen but usually normal with arachidonic acid and ristocetin.[77] Characteristically, in primary thrombocythemia, the first wave of aggregation is diminished and the second wave of aggregation is absent in response to epinephrine.[77] Interestingly, preincubation of primary thrombocythemic platelets with thrombopoietin corrects in part the impaired aggregation in response to epinephrine, ADP, and collagen.[91] Usuki et al. have proposed that circulating thrombopoietin levels in this disorder might regulate platelet function and responses to agonists in vivo.[91] An acquired form of platelet storage pool disease occurs frequently in primary thrombocythemia. Platelet -granule content and release are abnormal resulting in elevated plasma levels of platelet factor-4 and -thromboglobulin.[77] Because the content of -granule constituents has been reported to be normal in megakaryocytes isolated from primary thrombocythemia, the synthesis of these molecules is not thought to be abnormal, but rather, the release of granule constituents is thought to be a consequence of platelet activation.[77] The finding of an acquired storage pool defect again does not correlate with platelet numbers or with the occurrence of clinical symptoms. A variety of individual functional

Figure 64-1 Proposed pathophysiologic mechanisms of platelet-mediated inflammatory ischemic arthrothrombotic processes in primary thrombocythemia. (*From Michiels et al.,*[75] *with permission.*)

platelet abnormalities have been demonstrated.[92] [93] [94] [95] [96] [97] [98] [99] A defect in the metabolism of arachidonic acid by lipoxygenase has been documented, as have decreased numbers of platelet receptors for prostaglandin D_2 and adrenergic receptors for epinephrine.[77] [97] [98] Platelets from patients with thrombotic episodes have been found to be capable of increased generation of thromboxane B_2 and to have an increased affinity for fibrinogen.[99] [100] Elevations in -thromboglobulin and serum thromboxane B_2 levels in primary thrombocythemia patients are suggestive of the presence of enhanced in vivo platelet activation and possibly thrombin generation. These same abnormalities are not detected in patients with secondary thrombocytosis and may provide some explanation for the high incidence of thrombosis associated with primary thrombocythemia.

The survival of platelets in patients with erythromelalgia and thrombosis has been shown to be reduced to 4.2 ± 0.2 days in comparison to normal platelet survivals in asymptomatic thrombocythemia patients (6.6 ± 0.3 days) and patients with reactive thrombocytosis (8.0 ± 0.4 days).[101] Treatment of erythromelalgia with aspirin increased mean platelet survival from 4.0 ± 0.3 days to 6.9 ± 0.4 days and was associated with a significant elevation of platelet numbers.[101] These findings suggest that erythromelalgia results from platelet-mediated thrombosis of the arterial microvasculature of the extremities (Fig. 64-1).[75] [101] Complete correction of this ischemic circulatory defect is associated with the use of platelet cyclo-oxygenase inhibitors such as aspirin and indomethacin.[85] Agents that do not inhibit platelet cyclo-oxygenase, such as coumadin, sodium salicylate, dipyridamole, sulfinpyrazone and ticlopidine are not active in the treatment of this disorder. Dazoxiben is capable of inhibiting platelet malondialdehyde and thromboxane B_2 synthesis but does not correct the symptoms of erythromelalgia.[76] [77] [85] These findings suggest

that prostaglandin endoperoxides play a role in the generation of platelet-associated thrombosis in primary thrombocythemia (Fig. 64-1).[75]

Abnormalities of platelet membrane constituents have also been demonstrated in primary thrombocythemia. Most commonly, decreased concentrations of glycoprotein (GP) Ib and also reduced concentrations of GPIIb and GPIIIa have been reported. [77] A remarkable patient with primary thrombocythemia with a prolonged bleeding time was shown to have an acquired deficiency of glycoprotein Ia-IIa, the putative collagen receptor. Collagen-induced platelet aggregation was totally absent.[93] The mechanisms underlying these acquired membrane defects as well as their clinical significance remain unclear.

One intriguing explanation for the increased risk of thrombosis in patients with primary thrombocythemia has been proposed by Lee and Baglin. [94] These investigators demonstrated that the total amount of thrombin generated on the platelet surfaces of patients with primary thrombocythemia was markedly greater than that generated on the platelet surfaces of normal controls or patients with reactive thrombocytosis. [94] The molecular basis of this abnormality has not been defined, but it remains possible that an abnormal membrane structure of primary thrombocythemia platelets may account for the enhanced thrombin potential that may lead to a relatively high thrombotic risk.

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CLINICAL MANIFESTATIONS

The presenting symptoms of patients with primary thrombocythemia are quite variable. Many patients (1267%) reach medical attention fortuitously, as a result of the extreme degree of thrombocytosis detected when obtaining a routine blood cell count. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] In two series, in fact, 7684% of cases were asymptomatic and the diagnosis was made incidentally (Table 64-1 (Table Not Available)). [76] [102] Most patients present with symptoms related to small- or

TABLE 64-1 -- Thrombosis and Hemorrhage in 100 Patients with Primary Thrombocythemia

(Not Available)

From Cortelazzo et al.,[76] with permission.

large-vessel thrombosis or minor bleeding. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] Presentation with a major bleeding episode is unusual. [103] After thrombocytosis has been detected, 1337% of patients relate symptoms resulting from hemorrhagic events, while 2284% of patients report thromboembolic complications. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [76] [104] Table 64-1 (Table Not Available) lists the symptoms at diagnosis from one large series of patients. [76] The thrombotic events primarily involved the microvasculature, with thrombosis of large vessels also occurring. Neurologic complications are common: [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [81] [104] [105] [106] Table 64-2 lists representative neurologic complaints, [82] of which headache was the most common, with paresthesias of the extremities a close second. There was an extremely high incidence of transient ischemic attacks involving both the anterior and posterior cerebral circulation. [105] [106] [107] These attacks have a sudden onset, last for a few moments, and are frequently associated with a pulsatile headache. [105] [106] [107] The various symptoms occur sequentially rather than simultaneously and can be preceded or followed by erythromelalgia. Transient neurological symptoms include unsteadiness, dysarthria, dysphoria, motor hemiparesis, scintillating scotomas, amaurosis fugax, vertigo, dizziness, migraine-like symptoms, syncope, and even seizures. [105] The syndrome is caused by platelet-mediated ischemia and thrombosis in end-arterial microvasculature. It is not unusual for these symptoms to eventually progress to definitive cerebral infarcts. [106]

Microvascular occlusions involving the toes and fingers are frequent. [74] [75] [76] Such events can lead to digital pain, enhanced by warmth; distal extremity gangrene, (Fig. 64-2) and classic erythromelalgia. [9] [74] [75] [83] [84] [108] [109] The term *erythromelalgia* refers to a syndrome of redness and burning pain in the extremities. [109] Venous thrombosis is not observed in these patients. Erythromelalgia, which is characterized by a burning pain and a dusky congestion of swollen extremities, is usually preceded by paresthesias. [75] Cold provides relief to these symptoms and heat intensifies the symptoms. Patients prefer to wear shoes or slippers without socks and elevate their feet. [75] These symptoms may progress in intensity and lead to peeling of the skin in affected appendages or affected toes or fingers, which then may become cold and ischemic with a dark purplish tinge. Erythromelalgia symptoms are asymmetric in the majority of cases. [70] Symptoms related to coronary artery disease or transient ischemic attacks may precede or accompany the onset of erythromelalgia. [75] [108] Occasionally, hemorrhagic episodes may occur in patients experiencing erythromelalgia. [75] Platelet counts in patients with erythromelalgia are frequently below 1000×10^9 /l except in those patients with concomitant occurrence of erythromelalgia and hemorrhage. [75] [83] [84] [108] The relief of such pain for several days after a single dose of aspirin is diagnostic of erythromelalgia. [75] [83] [84] The specific microvascular syndrome of erythromelalgia is readily explained by platelet-mediated arteriolar inflammation and occlusive thrombosis leading to acrocyanosis and even gangrene. [75] [83] [84] Skin biopsies from affected sites reveal arteriolar lesions without involvement of venules, capillaries, or nerves. [83] The arteriolar endothelial cells are swollen and the vessel walls thickened by cellular swelling and deposition of

TABLE 64-2 -- Frequency of Neurologic Complaints Associated with Primary Thrombocythemia

Manifestations	Patients (N)
Headache	13
Paresthesias	10
Posterior cerebral circulatory ischemia	9
Anterior cerebral circulatory ischemia	6
Visual disturbances	6
Epileptic seizures	2
Total number of patients:	33

From Jabaily et al.,[82] with permission.

Figure 64-2 Gangrene of the toe in a patient with primary thrombocythemia.

intracellular material. [83] In contrast to atherosclerotic circulatory obstruction, arterial pulses in patients with erythromelalgia remain normal. [83] [84]

Although thrombosis of the microvasculature is generally more frequent, thrombosis of large veins and arteries in patients with primary thrombocythemia still occurs commonly. [13] [14] [110] In one series, 51% of patients had symptoms related to large-vessel thrombosis, mostly in the arteries of the legs (30%), the coronary arteries (18%), and the renal arteries (10%). [14] Involvement of the carotid, mesenteric, and subclavian arteries is not unusual. In the same series, 7% of patients suffered from venous thrombosis involving either the splenic vein, hepatic veins, or veins of the legs and pelvis. [14] Unexplained thrombosis of the hepatic veins leads to Budd-Chiari syndrome, while thrombosis of the renal vein can result in the development of the nephrotic syndrome. [104] [111] Priapism is a rare complication of primary thrombocythemia, presumably caused by platelet sludging in the corpus cavernosum. [105] [112] In addition, myocardial ischemia or infarction, or both, associated with normal coronary angiograms has been reported in patients with primary thrombocythemia, as has a high incidence of anginal symptoms. [113] [114] [115] A high incidence of aortic and mitral valvular lesions has been reported in patients with myeloproliferative disorders, including primary thrombocythemia. [115] These valvular lesions resemble previous descriptions of nonbacterial thrombotic endocarditis and may be the origin of the peripheral arterial emboli observed in these patients. [115] In addition, acute renal failure has been observed after thrombosis of renal arteries and veins in one patient with primary thrombocythemia. [116] Pulmonary hypertension secondary to alveolar capillary plugging by platelets and megakaryocytes has also been reported in two patients with primary thrombocythemia. [117] [118]

Hemorrhagic problems plague many patients with primary thrombocythemia;[1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] the primary site of bleeding is the gastrointestinal tract.[5] [74] [75] [79] [90] [119] At least 40% of patients with primary thrombocythemia may have duodenal arcade thrombosis with sloughing of duodenal mucosa, simulating a duodenal ulcer.[5] Other sites of bleeding may be the skin, eyes, urinary tract, gums, tooth sockets (following extraction), joints, or brain. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] Bleeding most often is not severe but occasionally may require red cell transfusion support. [1] [8] [13] The postoperative period appears to be an extremely precarious time, with a high incidence of bleeding episodes following surgical insult. [113] The syndrome of hemorrhagic thrombocythemia is closely correlated with a significant increase of platelet counts in excess of 1000 × 10^{9} /l and is associated with pseudohyperkalemia.[75]

In one large study in which 97 primary thrombocythemia

patients were followed for an average of 7 years, the incidence of thrombohemorrhagic complications was significant, although these complications were rarely life-threatening.[120] Of the 97 patients, 26 had some type of hemorrhage, mostly of gastrointestinal or mucosal origin; 33 of the 97 patients had some form of thrombosis, mainly in the peripheral arteries. Risk factors for atherosclerotic disease, especially cigarette smoking, increased the risk of thrombosis. [80] [120] It is important to emphasize that individual patients can suffer from both thrombotic and hemorrhagic episodes and that patients are not necessarily consistent bleeders or clotters.[74] [75] [79] Constitutional symptoms, such as weight loss, sweating, low-grade fever, and pruritus, can occur in 2030% of patients. [10]

A clinically relevant controversy revolves around the understanding of the risk of thrombohemorrhagic events in asymptomatic patients with primary thrombocythemia who are less than 40 years of age.[29] [31] [38] [78] [82] This group represents 25% of the patients studied in a 13-year period at one institution and 34% of those studied at another institution. [78] In one series of 57 such adults with a mean follow-up period of 4.7 years (range from 5 months to 20 years) 43% remained asymptomatic, whereas thrombotic complications developed in another 43%.[78] Hemorrhagic complications occurred in 11% of the patients and 4% suffered a spontaneous abortion (2 out of the 16 pregnancies).[38] The thrombotic complications were life-threatening in only 5% of the patients, all of whom recovered. The two deaths that occurred were not attributable to primary thrombocythemia.[38] The most common thrombotic complications were migraine headache in 20% of the patients and erythromelalgia in 5% of the patients. None of the hemorrhagic episodes were life-threatening. [38] In this series, young patients enjoyed a low incidence of life-threatening complications and a favorable long-term prognosis, findings confirmed by another large series. [29] [38] [73] However, in another study of 44 patients under the age of 45, serious thrombotic or hemorrhagic complications occurred in 23% of patients and 4% died as a consequence of thrombotic events. [79] In addition, one patient experienced a serious hemorrhagic event associated with the development of acquired von Willebrand syndrome. This group did not consider young age as a favorable prognostic factor and favored treatment of asymptomatic young individuals. [79] It is generally agreed that age >60 years is clearly associated with a greater risk of thrombosis.[76] Management of asymptomatic patients between the ages of 40 and 60 years therefore remains problematic, since there is little question that the symptomatic patient requires some sort of therapeutic intervention.

The outcome of pregnancy in patients with primary thrombocythemia has recently been the subject of intense investigation. [121] [122] [123] [124] In an excellent review of the literature, Greisshammer et al. reported that of 106 pregnancies in 57 women, the rate of spontaneous abortion was 43% in comparison to the 15% rate expected in the general population.[121] Placental thrombosis leading to placental infarction was the most common pathological event leading to placental insufficiency, intrauterine growth retardation, and even fetal death.[121] The platelet count usually remains the same during the pregnancy, although a progressive decline in platelet count, which returns to its previous elevated level postpartum has been reported in 20% of cases. [121] [123] A significant fall in platelet count has been reported to be associated with a successful pregnancy.[124] The basis of the decrease of platelets during pregnancy is unknown, but the decrease is generally greater than the 20% reduction in platelet counts associated with normal pregnancies. [124] In the large majority of cases, the fetal losses occur during the first trimester. [121] [122] [123] [124] In one series, 65% of the miscarriages occurred in 17% of women.[122] Thus, a previous history of spontaneous abortion may be the greatest risk factor for the development of subsequent spontaneous abortions.[122] Excessive bleeding during delivery appeared to be an extremely rare event. [121] [122] [123] [124]

Physical examination is relatively unremarkable in most cases. Most patients are not severely ill at diagnosis, a median Karnofsky score of 90% being reported in one series.[10] [12] Splenomegaly is detectable in 4050% of patients, and approximately 20% have hepatomegaly. [10] [12] During the course of the disorder, a further increase in the degree of hepatosplenomegaly does not appear to occur. [10] [12]

Although splenomegaly is the more common finding in primary thrombocythemia, splenic atrophy due to silent autoinfarction of the spleen may lead to an acquired form of hyposplenism.[6] [7] The appearance of Howell-Jolly bodies and target erythrocytes in the peripheral blood smear suggests the occurrence of splenic autoinfarction.

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LABORATORY EVALUATION

The hallmark of primary thrombocythemia is a sustained and unexplained elevation of the platelet count. The level of thrombocytosis required for the diagnosis is arbitrarily determined; the range is 450,0001,000,000/mm^3 , depending on the series cited.[1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] Table 64-3 lists the laboratory findings reported in one large series of patients with this disorder. [13] These findings may be considered representative, as only patients with platelet counts of >600,000/mm^3 were evaluated, and relatively rigorous diagnostic criteria were used. Accompanying leukocytosis was a common finding in this series, as in others reported in the literature.[1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] Occasional immature myeloid precursor cells (myelocytes or metamyelocytes) and nucleated red blood cells can be seen in the peripheral blood of 25% of patients. [10] [12] In one series, mild eosinophilia (>400/mm^3) and basophilia (>100/mm^3) were present in more than one-third of patients.[10] [12]

The most common morphologic abnormalities are variations in red cell size and shape and the presence of megathrombocytes. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] The mean leukocyte alkaline phosphatase activity in one series was 79, with levels below 20 being observed in only three patients. [10] [12] Table 64-4 summarizes the bone marrow biopsy findings.[10] [12] In this careful study performed by the Polycythemia Vera Study Group, marrow cellularity was increased

TABLE 64-3 -- Laboratory Findings Associated with Primary Thrombocythemia

Findings	Patients	
	N	%
Hemoglobin level		
>16 g/100 ml	6	6
1216 g/dl	64	68
<12 g/dl	23	24
Leukocytosis		
<8 × 10^9 /l	23	24
812 × 10^9 /l	41	44
1220 × 10^9 /l	22	22
2029 × 10^9 /l	8	8
Platelet count		
<1 × 10^9 /l	37	38
11.5 × 10^{12} /l	37	39
>1.5 × 10^{12} /l	20	22
Reticulin fibrosis	38/70	54
Normal cytogenetics	49/51	99
Platelet aggregation		
Normal	4/64	6
Decreased		
After ADP	19/64	30
After collagen	6/64	9
After ADP and collagen	35/64	55

From Bellucci et al.,[13] with permission.

TABLE 64-4 -- Bone Marrow Biopsy Findings in Primary Thrombocythemia

Parameter	Normal	Slightly Increased	Moderately Increased	Markedly Increased
Cellularity	4	7	22	4
Megakaryocyte number	0	3	10	24
Erythroid elements	6	12	15	4
Myeloid elements	7	11	17	2
Reticulin content	29	7	1	0

From Iland et al.,[10] with permission.

in almost 90% of patients. In two-thirds of patients, marked megakaryocytic hyperplasia, morphologically bizarre megakaryocytes with nuclear pleomorphism, and clustering of megakaryocytes were frequently present.[10] [12] [28] [32] Enlargement of megakaryocytes with multilobulated nuclei and their tendency to cluster in small groups along sinuses is the hallmark of primary thrombocythemia.[125] Granulopoiesis and erythropoiesis are not remarkable except for increased cell numbers. Reticulin content was increased in 25% of patients, but collagen fibrosis was not evident. [10] [12] In 7080% of patients, iron stores were present in the marrow, albeit at reduced levels.[14] [125] Almost all patients have normal serum ferritin levels.[125] Cervantes et al.[126] have suggested that the absence of iron stores in 30% of patients

may merely be an epiphenomenon of a chronic myeloproliferative syndrome, and not truly reflective of an iron deficiency state. Bleeding times are prolonged in 1020% of patients.[13] [77] Platelet aggregation studies are frequently abnormal, most often demonstrating impaired aggregation in response to epinephrine, ADP and collagen but not to arachidonic acid and ristocetin. [77] Spontaneous platelet aggregation has been reported to occur frequently in such patients, but this has not been a universal finding.[13] [77]

About 25% of patients with primary thrombocythemia have been reported to have elevated uric acid levels at diagnosis. [10] [12] [14] The average value of the serum potassium at diagnosis is usually within the normal range, although 23% of patients have been reported to have pseudohyperkalemia and falsely elevated phosphorus concentrations.[127] [128] The laboratory features of acquired von Willebrand syndrome associated with excessively high platelet count and a bleeding tendency have been previously described in detail. This syndrome in patients with primary thrombocythemia is associated almost uniformly with a platelet count >1000 × 10^{9} /l, a prolonged bleeding time, a normal factor VIII coagulant activity and von Willebrand antigen level but a decreased von Willebrand factor-ristocetin cofactor activity and collagen binding activity as well as a decrease or absence of large von Willebrand factor multimers simulating a type II von Willebrand factor deficiency. [88] [89] [90] An enhanced thrombotic risk in primary thrombocythemia patients has been associated with a reduction in the concentration of one of the natural anticoagulants including protein S, antithrombin III protein C, and resistance to activated protein C resulting from an associated genetic defect in factor V. [128] These studies indicate that such a genetic deficiency of one of the natural anticoagulants may further contribute to the thrombotic tendency of patients with primary thrombocythemia. Pseudohypoxemia has also been observed in primary thrombocythemia patients with extreme degrees of thrombocytosis. [129] The serum B_{12} level can be increased in 25% of cases. [10] [12] [13]

In primary thrombocythemia, marrow karyotypes are characteristically normal.[10] [11] [12] [13] [14] [130] The absence of the Philadelphia chromosome rules out the diagnosis of chronic myeloid leukemia (CML).[131] [132] Aneuploidy is seen in the minority of cases. In fact, analysis of 170 cases of primary thrombocytosis revealed a definite chromosomal abnormality in only 5.3% of cases. Marker chromosomal abnormalities, such as 1q , 20q , 21q , or $1q^{+}$, have been reported, but no consistent chromosomal abnormality has been identified. [10] [11] [12] [13] [14] [130] [131] [132] [133] [134] [135] [136] [137]

DIFFERENTIAL DIAGNOSIS

The diagnosis of primary thrombocythemia is one of exclusion (Table 64-5). Primary thrombocythemia must be distinguished from reactive or secondary forms of thrombocytosis and from other myeloproliferative disorders, such as polycythemia vera, agnogenic myeloid metaplasia, and CML, which are also frequently characterized by thrombocytosis.[1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] Patients with myelodysplastic syndromes can also present with a moderate or marked degree of thrombocytosis; this is especially true of patients with the 5q syndrome and of patients with idiopathic acquired sideroblastic anemia. [138] [139] The causes of secondary or reactive forms of thrombocytosis are numerous. In a hospital population, patients with extreme thrombocytosis (>1000 × 10^9 /l) are not particularly rare in adult or pediatric patient populations. [26] [140] [141] [142] [143] Examination of the blood smear is important to avoid confusion with so-called pseudothrombocytosis. This occurs in a number of conditions in which platelet-sized particles that are red or white cell fragments (CLL, TTP, Hgb-H disease, microspherocytosis) are erroneously enumerated as platelets by automatic particle counting. [144] Confirmation of increased numbers of platelets by examination of the peripheral smear will

TABLE 64-5 -- Criteria for Diagnosis of Primary Thrombocythemia

1. Platelet count >600,000/mm^3 on two different occasions, separated by a 1-month interval
2. Absence of identifiable cause of reactive thrombocytosis
3. Normal red cell mass, measured red cell mass <25% above the normal predicted value
4. Absence of significant fibrosis of the marrow (>1/3 cross-sectional area of the bone marrow biopsy)
5. Absence of the Philadelphia chromosome and the fusion *bcr/abl* gene by PCR; absence of clonal cytogenetic abnormalities associated with myelodysplastic disorders
6. Presence of splenomegaly by physical examination, ultrasonography, or scan
7. Bone marrow hypercellularity, as shown by a bone marrow biopsy and the presence of megakaryocytic hyperplasia with clusters of multilobulated large megakaryocytes
8. Absence of iron deficiency, as documented by the presence of stainable marrow iron and/or normal serum ferritin
9. In females, demonstration of clonal hematopoiesis by means of restriction fragment length polymorphism analysis of genes present on the X chromosome
10. Presence of abnormal marrow hematopoietic progenitor cells, as determined by the formation of endogenous erythroid and/or megakaryocytic colonies with increased sensitivity to IL-3
11. No elevation of plasma C-reactive protein and IL-6 levels
Patients who meet criteria 15 and 3 of criteria 611 should be considered to have primary thrombocythemia.

avoid misdiagnosis and unnecessary clinical evaluation. In one series of 280 patients with extreme thrombocytosis encountered over a 5-year period at a university hospital, reactive thrombocytosis accounted for 82% of cases of extreme thrombocytosis, whereas thrombocytosis due to myeloproliferative disorders accounted for only 14% of cases.[142] In fact, of the patients with myeloproliferative disorders, primary thrombocythemia accounted for only 29% of the cases. Reactive thrombocytosis was more common in all age groups except those in the eighth decade and higher. The causes of extreme thrombocytosis outlined in this series are listed in Table 64-6 .[142] Importantly, in this series, the mean peak platelet count for patients with reactive thrombocytosis (1,195 × 10^9 /l) was significantly lower than that of patients with thrombocytosis secondary to a myeloproliferative disorder (1,808 × 10^9 /l).[138] This shows that a significant number of patients with reactive thrombocytosis have platelet counts greater than 1,000 × 10^9 /l and that it is impossible to distinguish between reactive thrombocytosis and thrombocythemia caused by a myeloproliferative disorder, based solely on the degree of thrombocytosis. [140] [141] [142] Thrombotic and hemorrhagic events infrequently occur in patients with reactive thrombocytosis.[140] [141] [142] [143] These findings are in contrast to the enhanced risk of these two complications in patients with primary thrombocythemia. This relatively high frequency of extreme thrombocytosis in an acute care hospital emphasizes the need for caution in making a diagnosis of primary thrombocythemia. A number of groups have shown that reactive thrombocytosis may be a consequence of the elaboration of known cytokines in response to the underlying inflammation or neoplastic disorder.[145] [146] [147] [148] Elevated levels of IL-1, IL-6, GM-CSF and G-CSF have been detected in such patient populations but not in individuals with thrombocytosis caused by an underlying myeloproliferative disorder. [72] [73] [145] [146] [147] [148] [149] Elevation of thrombopoietin has not only been found to be elevated in patients with reactive thrombocytosis but also in patients with primary thrombocythemia. [73] C-reactive protein is an acute phase reactant, the hepatic synthesis of which is mediated by IL-6.[141] C-reactive protein levels are high in patients with high levels of IL-6. [145] In one series, 81% of patients with reactive thrombocytosis had elevated IL-6 or C-reactive protein levels, whereas patients with uncomplicated thrombocytosis secondary to a myeloproliferative disorder had undetectable IL-6 levels.[145] Low levels of both IL-6 and C-reactive protein are strongly indicative of the thrombocytosis

TABLE 64-6 -- Etiologic Conditions Associated with Extreme Thrombocytosis

Total Cases		**280**
Reactive thrombocytosis		231
Infection (%)	72 (31)	
Post-splenectomy (or hyposplenism) (%)	43 (19)	
Malignancy (%)	33 (14)	
Trauma (%)	32 (14)	
Inflammation (noninfectious) (%)	21 (9)	
Blood loss (%)	13 (6)	
Uncertain etiology (%)	9 (4)	
Rebound (%)	8 (3)	
Myeloproliferative disorders		38
CML (%)	16 (42)	

PT (%)	11 (29)	
PV (%)	5 (13)	
IMF (%)	2 (5)	
Unclassified (%)	4 (11)	
Uncertain etiology		11

CML, chronic myeloid leukemia; PT, primary thrombocythemia; PV, polycythemia vera; IMF, idiopathic myelofibrosis.

From Buss et al.,[142] with permission of Excerpta Medica, Inc.

TABLE 64-7 -- Clinical and Laboratory Features Helpful in Distinguishing Primary Thrombocythemia from Reactive Thrombocytosis[a]

Feature	ET	RT
Chronic platelet increase	+	
Known causes of RT		+
Thrombosis or hemorrhage	+	
Splenomegaly	+	
BM reticulin fibrosis	+	
BM megakaryocyte clusters	+	
Abnormal cytogenetics	+	
Increased acute phase reactants		+
Spontaneous colony formation[b]	+	

From Tefferi and Hoagland,[150] with permission.

[a]Acute phase reactants include C-reactive protein and fibrinogen: BM, bone marrow; ET, essential thrombocythemia; RT, reactive thrombocytosis.
[b]Erythroid colonies.

being the consequence of an underlying myeloproliferative disorder rather than being reactive thrombocytosis. [145] The clinical and laboratory features useful in distinguishing primary thrombocythemia from reactive thrombocytosis are outlined in Table 64-7 .[150]

A number of investigators have constructed lists of diagnostic criteria useful in identifying individuals with primary thrombocythemia. [10] [13] [29] [30] [150] [151] [152] [153] [154] No such list is infallible. Table 64-5 lists useful diagnostic criteria, but these criteria are arbitrary. Red cell mass and plasma volume studies are frequently necessary to differentiate primary thrombocythemia from polycythemia vera. In males with thrombocytosis and a hematocrit <60% or in a female with a hematocrit <55%, such studies should be performed. Values greater than these values are associated with an elevated red cell mass, and radioisotope studies are unnecessary to document the presence of an elevated red cell mass.[155] Bone marrow karyotypic analysis is imperative in every patient to exclude the diagnosis of CML. [131] [132] This step is necessary because the natural history of these disorders is very different, and early therapeutic intervention with bone marrow transplantation for appropriate patients with CML is potentially curative.[131] [132] In one series, six women presented with the clinical picture of primary thrombocythemia without anemia, marked splenomegaly, or extreme leukocytosis characteristic of CML.[132] Each of these patients was shown to have a Philadelphia chromosome on karyotypic analysis, and five of the six entered the accelerated phase or blast crisis within 57 years of diagnosis. [132]

A highly instructive case was reported by Morris et al. [131] The patient was a 23-year-old woman who presented with a syndrome indistinguishable from primary thrombocythemia.[131] Although no Philadelphia chromosome was detected, the patient did develop blast crisis after four years of follow-up. By Southern blot analysis, she was shown to have the BCR-ABL fusion gene, suggesting that she actually suffered from CML and not from primary thrombocythemia.[132] This case emphasizes the importance of molecular studies when evaluating patients suspected of having primary thrombocythemia. [131]

Blickstein et al., in a large series of patients with Philadelphia chromosome negative primary thrombocythemia, confirmed these findings. [156] They examined the BCR-ABL status using a two-step nested PCR assay of the bone marrow cells in 25 patients with Philadelphia negative primary thrombocythemia and found that 12 were positive for BCR-ABL.[156] The clinical characteristics of these two groups of patients were identical and after a median follow-up of just less than 2 years there had been no cases of leukemic transformation.[156] This high rate of BCR-ABL positivity in this group is surprising, and further

follow-up of this patient population will be required to determine the eventual rate of leukemic transformation. This study does, however, again emphasize the need for molecular diagnostics to exclude the diagnosis of CML in patients with isolated thrombocytosis.

The presence of clonal hematopoiesis at least in one lineage quickly establishes the diagnosis of primary thrombocythemia. [21] [22] [23] Such studies may be particularly useful in young females with thrombocytosis.[25] Probes for a variety of genes on the X chromosome can be informative for clonal analysis of blood cell production in >72% of American females.[25] In such patients, analysis of restriction fragment length polymorphisms can be used to establish a pattern of clonal hematopoiesis, which is indicative of a hematologic malignancy and establishes the diagnosis of primary thrombocythemia in a young female with thrombocytosis. [24] Polyclonal hematopoiesis is found in all cases of reactive thrombocytosis. Polyclonal hematopoiesis, however, does not exclude the diagnosis of primary thrombocytosis because in several series, almost one-third of patients who met the clinical criteria for primary thrombocythemia had polyclonal hematopoiesis in all studied lineages.[25] [45] The biogenesis of this polyclonal form of primary thrombocythemia is poorly understood.

A number of diagnostic tests, including splenic volume estimates using ultrasound evaluations and assays of bone marrow progenitor cells (erythroid or megakaryocyte), have been suggested as useful means of differentiating reactive thrombocytosis from primary thrombocythemia. [54] [55] [56] [57] [58] [157] Insufficient numbers of patients as well as a lack of long-term follow-up make it impossible to assess the clinical value of such tests.

As has been previously discussed, the lack of quality control in many laboratories in the performance of such clonal assays for hematopoietic progenitor cells is of concern and limits the utility of such information gained by their performance. [54] [55] [56] [57] [58] The presence of endogenous erythroid colonies or megakaryocyte progenitors with increased sensitivity to hematopoietic growth factor is, however, helpful in distinguishing reactive thrombocytosis from thrombocytosis due to a myeloproliferative disorder.[54] [55] [56] [57] [58] Such assays are of limited use in distinguishing primary thrombocythemia from the other myeloproliferative disorders. [58]

At times it is impossible to define the cause of an individual patients thrombocytosis. In an asymptomatic patient, the resolution of this problem is easy: one should simply provide follow-up and determine whether the degree of thrombocytosis increases. If additional clues to the cause of the thrombocytosis are subsequently revealed, a diagnosis will become apparent. In a patient with thrombohemorrhagic difficulties, one must make a presumptive diagnosis of the cause of the thrombocytosis and then, after weighing the benefits versus the risks of various treatment plans, determine whether reduction of platelet numbers or simple observation is indicated. Some reassurance is provided by the report of Schilling, who followed a large cohort of patients, each with platelet counts of $>1 \times 10^6/mm^3$, for 18 months. None of the patients with reactive thrombocytosis developed a cerebrovascular accident, thrombophlebitis, or peripheral arterial thrombosis. [140] These findings are consistent with the conclusions of Buss et al. that thrombohemorrhagic complications are rare in patients with secondary thrombocytosis. [86] [142]

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THERAPY

The treatment of patients with primary thrombocythemia is controversial and remains largely problematic, yet new insight into the management of such patients has recently been gained. [15] [30] [150] Handling of primary thrombocythemia patients with life-threatening hemorrhagic or thrombotic episodes is more straightforward and is best approached by plateletpheresis in combination with institution of myelosuppressive therapy. [158] [159] [160] [161] Rapid plateletpheresis using continuous or discontinuous flow centrifugation devices has proved effective in preventing additional morbidity in patients with primary thrombocythemia. [158] [159] [160] [161] In this situation, immediate physical removal of large numbers of platelets is preferred. Since chemotherapeutic agents generally require 1820 days before platelet counts can be reduced to normal levels, Taft et al. [158] have recommended reducing the platelet count to 500,000/mm^3 by each plateletpheresis and suggest that achievement of such a goal requires the passage of two blood volumes over a 34-hour period.

Such a therapeutic approach has been employed to treat acutely ill patients with problems such as cerebrovascular accidents, myocardial infarction, transient ischemic attacks, or life-threatening gastrointestinal hemorrhage. [158] [159] [160] [161] Long-term plateletpheresis has proved an ineffective means of controlling thrombocythemia, presumably because of the rapid rate of production of platelets. [159] Therefore, most clinicians begin by administering a chemotherapeutic agent that has a rapid onset of action, such as hydroxyurea, simultaneously with the institution of plateletpheresis. [161] If plateletpheresis is not an available therapeutic option, administration of nitrogen mustard (0.4 mg/kg) is usually effective in lowering the platelet count within several days. [160]

In those patients found to have primary thrombocythemia and who are clearly symptomatic, little controversy exists as to the need for lowering the platelet count. The large number of thrombotic complications that occur in patients with primary thrombocythemia who smoke, point to the urgent need for these patients to stop smoking immediately. [80] Most investigators try to normalize the platelet count or to reach a platelet count at which the patients symptoms resolve. Although major bleeding episodes requiring hospitalization are rare, those patients with extreme thrombocytosis (>1,500 × 10^9 /l), acquired von Willebrand syndrome, and history of hemorrhagic episode are clearly at risk for developing additional bleeding complications. [88] [89] [90] These patients require reduction of the increased platelet counts to the normal range with use of a variety of chemotherapeutic agents, anagrelide or interferon-. Such patients should avoid exposure to aspirin even if they are suffering hemorrhagic complications and thrombotic episodes simultaneously. [88] [89] [90] Thrombotic complications are more frequent, especially when thrombocytosis is less marked. Symptoms of functional ischemia are the most common clinical presentation, although the majority of patients are asymptomatic at diagnosis (Table 64-1 (Table Not Available)). [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [30] [76] Many authors have attempted to identify factors associated with an increased risk of thrombotic complications. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [30] [76] Patients older than 60 years of age or with a previous history of vascular occlusive episode have significantly greater risk of developing additional thrombotic episodes. [76] Such patients define a high-risk population who merit therapeutic intervention. A clear relationship between platelet numbers and the frequency of thrombotic events has never been established. [76] Recently, Cortellazzo et al., in a randomized trial of high-risk patients, reported that cytoreductive therapy (platelet count to 600 × 10^9 /l) with hydroxyurea was effective in preventing additional thrombotic episodes. [87] Another situation that requires treatment is discomfort due to erythromelalgia or progression of erythromelalgia to frank gangrene. [75] Such patients respond within days to low-dose aspirin therapy and/or platelet reduction therapy. [75] The therapeutic strategy to be employed with asymptomatic patients less than 60 years of age remains controversial. The risk of the development of thrombotic events, the leukemogenic potential of chemotherapeutic agents used to treat thrombocytosis, and the ability of cytoreductive therapy or a platelet antiaggregating therapy to reduce the incidence of thrombotic events must be considered before embarking on treatment of the asymptomatic younger (<60) patient with primary thrombocythemia. Groups of patients have

PERSONAL APPROACH TO THERAPY FOR PRIMARY THROMBOCYTHEMIA

The optimal therapy for patients with primary thrombocythemia remains uncertain. Certain concepts, however, apply to all patients. One is that all patients with primary thrombocythemia should stop smoking to minimize the risk factors associated with atherosclerotic disease. Indiscriminant use of high doses of nonsteroidal anti-inflammatory drugs should be avoided because this practice can lead to an increased risk of hemorrhage. Use of such agents is particularly common in the elderly age group in which primary thrombocythemia is common.

In a randomized trial of high-risk patients, cytoreductive therapy has been shown to lessen the chance of developing additional thrombotic events. High-risk patients include patients older than 60 years of age and patients with a history of a previous thrombotic episode including erythromelalgia, transient ischemic attacks, or large vessel thrombosis. At present, no therapy is indicated in asymptomatic patients less than 60 years of age. If a patient has a platelet count 1,500 × 10^9 /l and the acquired von Willebrand syndrome, platelet reduction therapy is also indicated to avoid the high risk of hemorrhage. Patients with this acquired von Willebrand syndrome should clearly avoid the use of aspirin.

In those patients requiring platelet reduction therapy, the choice between the use of anagrelide, interferon- and hydroxyurea therapy is based upon patient age, ease of administration, and drug-related toxicity. In patients over 60 years of age, hydroxyurea therapy is the treatment of choice, while in younger patients, I prefer to initiate therapy with anagrelide. This choice is based upon the ability to administer anagrelide orally rather than resorting to parenteral administration for interferon-. If a patient cannot tolerate anagrelide, I then start therapy with interferon-. If the patient cannot tolerate interferon- or fails to respond, I feel comfortable treating the symptomatic patient less than 60 years of age with hydroxyurea. Although I remain concerned about the leukemogenic potential of hydroxyurea, this potential danger is far less than that of the alkylating agents or ^{32}P. Those patients who initially receive hydroxyurea and no longer respond to this agent or suffer toxicity and require another agent should not receive long term ^{32}P or melphalan therapy. This sequence of administration is associated with an extremely high risk of leukemic transformation. Those patients who have had a trial of hydroxyurea and require further treatment should receive either anagrelide or interferon-. Doses of each of these agents required for disease control will, of course, be dependent on the target platelet level that I hope to achieve. I believe that strict control to a platelet count 350 × 10^9 /l may lead to greater protection from thrombosis than merely reduction to a level 600 × 10^9 /l. I realize that there are no randomized trials to support this approach but the available anecdotal information is compelling. My success in achieving this goal is obviously dependent on the ability of the patient to tolerate the agents used. In the younger patient if such strict control is not achievable due to poor compliance or toxicity associated with anagrelide or interferon- therapy, I am satisfied with continuing therapy and maintaining platelet counts 600 × 10^9 /l. In patients who suffer from thrombotic episodes, especially episodes involving the microcirculation or large vessels, I administer low-dose aspirin (100 mg/day). This dose of aspirin does increase the number of bleeding episodes to a modest degree but is effective in treatment of thrombotic events. This low-dose aspirin therapy is given in addition to an agent which results in platelet reduction.

Hydroxyurea can be started at a dose of 1 g/day and then adjusted to achieve the target platelet count (<350 × 10^9 /l) without developing leukopenia. Anagrelide is initiated at 0.5 mg qid and increased by 0.5 mg/day every 57 days, if platelet counts do not begin to drop. The usual dose, however, is 2.02.5 mg/day. There are patients who do not tolerate either hydroxyurea or anagrelide. In this patient group, interferon- therapy is initiated at 3 million units three times per week subcutaneously. Another choice is busulfan at 4 mg/day for two-week courses every time the platelet count rises above the normal range. Busulfan therapy is reserved for patients older than 60 years of age.

In general, I do not routinely treat patients younger than 40 years of age, unless they have already have had thrombohemorrhagic symptoms or have significant risk factors for atherosclerotic disease. Complications even in young, otherwise healthy, patients with prolonged platelet counts of >2.0 × 10^6 /mm^3 are unusual.

In certain situations, in young, low-risk patients, treatment should be instituted. Surgery can increase the risk of thrombosis, and use of anti-inflammatory agents can increase the risk of bleeding postoperatively. Under these circumstances, the platelet count should be lowered to the normal range. In pregnant patients with primary thrombocythemia, low-dose aspirin therapy is the first treatment option. If the patient develops symptoms as a result of thrombosis within the vasculature, platelet reduction therapy is necessary and interferon- therapy is the treatment of choice. Since interferon does not cross the placenta, it likely will not be teratogenic. Both hydroxyurea and busulfan have been successfully used to treat myeloproliferative disorders during pregnancy, but they are probably teratogenic if used during the first trimester. If such agents are needed, they should be instituted after the first trimester.

In a patient with primary thrombocythemia and a serious acute hemorrhagic event, the site of bleeding should be immediately determined, and any antiplatelet aggregating agents stopped. Although the platelet count may be high, these platelets should be considered to be qualitatively abnormal, leading to defective hemostasis. The patient may be suffering from acquired von Willebrands syndrome. In patients with acquired von Willebrand syndrome, DDAVP or Factor VIII concentrates containing von Willebrand factor can be used immediately as chemotherapy is being administered. If acquired von Willebrands syndrome is not present, the transfusion of normal platelets is suggested. In those patients with persistent hemorrhage, immediate reduction of the platelet count can be achieved by plateletpheresis. Hydroxyurea at 24 g/day for 35 days should be administered immediately, then reduced to 1 g/day. Any patient receiving hydroxyurea should be monitored for the onset of granulocytopenia and/or thrombocytopenia. Reduction of platelet counts is usually observed within 35 days of hydroxyurea treatment.

In contrast, patients with acute arterial thrombosis require immediate institution of platelet antiaggregating agents. Aspirin at a dose of 100 mg/day is suggested. Patients with erythromelalgia or transient ischemic attacks will have a rapid cessation of symptoms following the use of low-dose aspirin. In a patient with a life-threatening arterial thrombosis, the platelet count should be lowered with either a combination of apheresis and hydroxyurea or with hydroxyurea alone, depending on the severity of the event. If the arterial thrombosis involves the microcirculation and is not life-threatening (transient ischemic attacks or erythromelalgia), immediate low-dose aspirin therapy is indicated and platelet reduction therapy (hydroxyurea, anagrelide or interferon-) can be initiated using standard dose and schedule.

TABLE 64-8 -- Effect of Primary Thrombocythemia Treatment Options on Development of Acute Leukemia

Category	Myelosuppressive Therapy	Number of Patients	Number Developing Acute Leukemia
A	None	7	1
B	HU only	22	1[a]
C	AA or ^{32}P	34	4[a]
D	HU AA &/or ^{32}P	7	5[a]
E	AA or ^{32}P HU	21	1[a]
	Totals	91	12

HU, hydroxyurea; AA, alkylating agent.

From Murphy et al.,[30] with permission.

[a]P <.006 (Fishers exact test) for B, C, E versus D.

been successfully treated with a variety of chemotherapeutic agents, including busulfan, chlorambucil, pipobroman, thiotepa, radioactive phosphorus, hydroxyurea, nitrogen mustard, uracil mustard, and CCNU (lomustine).[6] [7] [8] [9] [10] [11] [12] [25] [30] [162] [163] [164] [165] [166] [167] [168] Many of these agents have been used to treat a variety of myeloproliferative disorders and solid tumors, and their use has been associated with an increased risk of leukemia.[30] [169] Only a few Phase III studies comparing the efficacy of such agents for the treatment of primary thrombocythemia have been completed.

In a study of a small group of patients, the response to either melphalan or radioactive phosphorus therapy was studied during the first year of therapy.[165] The only

conclusion that could be drawn from this study was that time to response as defined by platelet count reduction was considerably shorter for patients receiving melphalan than for those receiving radioactive phosphorus. [165] Intermittent use of busulfan (4 mg/day, until the platelet count fell to 400,000/mm ^{3} , followed by a series of 2-week courses when the platelet count rose to >400,000/mm ^{3}) has proven to be a relatively nontoxic and effective regimen. [157] These conclusions were based on a lengthy examination of the course of 37 patients. [162]

During the 1980s and 1990s, hydroxyurea became the drug of choice for the treatment of primary thrombocythemia. [29] [30] [87] [168] [170] The impetus for this practice was based upon the capacity of ^{32} P and alkylating agents such as melphalan and busulfan to induce acute leukemia. [30] [169] The popularity of the antimetabolite, hydroxyurea, for the management of primary thrombocythemia was due to belief in the early 1970s that it was nonleukemogenic. [29] [30] Hydroxyurea is administered at a dose of 15mg/kg initially with adjustment of the dose in order to achieve a maintenance dose to maintain a platelet count at a level at least below 600 × 10 ^{9} /l. [81] The use of this drug in a high-risk group of patients with reduction of platelet numbers to less than 600 × 10 ^{9} l has resulted in reduction of thrombotic events when compared to a control population. [87] The reduction of platelet numbers to this level did not entirely eliminate the occurrence of additional thrombotic episodes. One center has reported that more aggressive platelet reduction to less than 350 × 10 ^{9} /l was associated with no recurrence of minor or major thrombotic events involving the peripheral, coronary or cerebral circulation. [170] These findings require confirmation in a large well-controlled randomized clinical trial. Hydroxyurea usage is associated with some toxicity including dose-related neutropenia, nausea, stomatitis, and hair loss. [170] Each of these problems resolve with withdrawal of the drug and dose reduction. Hydroxyurea is also not universally successful in controlling the thrombocytosis. Resistance to hydroxyurea has been reported in 1117% of cases. [170] The risk of evolving to acute leukemia is extremely low in untreated patients. [13] [14] [28] [30] [171] Unfortunately, hydroxyurea has not proven to be nonleukemogenic, with over 20 cases of acute leukemia reported in patients with primary thrombocythemia treated with hydroxyurea alone. [30] [166] [171] [172] [173] [174] In fact, some of the strongest evidence for the leukemogenic potential for hydroxyurea has been provided by the Polycythemia Vera Study Group, which had previously been the most vocal advocate of hydroxyurea therapy for the treatment of primary thrombocythemia. [28] [30] A cohort of 29 patients with primary thrombocythemia were treated with hydroxyurea between 1977 and 1982. [30] These patients met the diagnostic criteria for this disorder as established by this cooperative group. [28] Although 5 patients were lost to follow-up, the median follow-up time for the remaining patients was 7.3 years (Table 64-8). Disturbingly, 6 of the 24 patients developed acute leukemia. One patient developed acute leukemia 2.4 years after treatment, whereas other cases occurred after 6.7 to 9.7 years of follow-up. One of these patients was treated with hydroxyurea alone while 5 others were switched from hydroxyurea to either ^{32} P or an alkylating agent. [32] Another group of 62 patients were treated by this group initially with ^{32} P or melphalan. Of these patients, 21 were later switched to hydroxyurea therapy. After the analysis of patients in all of these groups, the predicted probability of developing acute leukemia was determined to be 21.6% at 10 years with the greatest likelihood being between 5 and 10 years after diagnosis. [20] With careful analysis of this data, the subgroup of patients who were initially treated with hydroxyurea but subsequently switched to an alkylating agent or ^{32} P had an incredibly high incidence of acute leukemia (Table 64-9). [30] By contrast, those patients initially treated with ^{32} P or melphalan, whether or not they were eventually treated with hydroxyurea, had a

TABLE 64-9 -- Incidence of Thrombotic and Bleeding Complications in 68 Patients with Primary Thrombocythaemia Who Had Long-term Follow-up According to Treatment Strategy

Treatment	Duration of Follow-up (person-years)	Thrombotic Complications		Bleeding Complications	
		Events (n)	Events/100 Person-Years	Events (n)	Events/100 Person-Years
Careful observation	127	27	32.3	2	1.6
Low-dose aspirin	139	5	3.6[a]	10	7.2[d]
Cytoreduction	113	10	8.9[b]	2	1.8[e]
Low-dose aspirin and cytoreduction	40	0	0[c]	4	10.0[f]
Total	419	42		18	

From Van Genderen et al., [189] with permission from Blackwell Science, Ltd.

[a] P <0.001 (^{2} = 17.3, 1df), for comparison with careful observation (thrombosis).
[d] P = 0.032 (^{2} = 4.6, 1df), for comparison with careful observation (bleeding).
[b] P = 0.014 (^{2} = 6.0, 1df), for comparison with careful observation (thrombosis).
[e] P = 0.92 (^{2} = 0.01, 1df), for comparison with careful observation (bleeding).
[c] P = 0.003 (^{2} = 8.6, 1df), for comparison with careful observation (thrombosis).
[f] P = 0.014 (^{2} = 6.0, 1df), for comparison with careful observation (bleeding).

lower incidence of leukemia (Table 64-9). [30] One can conclude from these studies and those of others that hydroxyurea therapy alone is likely less leukemogenic than alkylating agents or ^{32} P alone, but that its use does present some enhanced risk for the development of leukemia. [28] [30] [166] [175] Also, if several classes of agents are to be used, the sequence of their use appears to be of great significance. Hydroxyurea therapy followed by ^{32} P or an alkylating agent appears to be a highly risky sequence. [30] By contrast, ^{32} P or melphalan followed by hydroxyurea therapy appears to offer far less risk for the development of leukemia. [30] There are no known explanations for these observations. In those patients who cannot tolerate hydroxyurea or cannot be controlled with its continued use, analgrelide or interferon- therapy appears to be a wise choice. [170] When considering the risk-benefit ratio, one can conclude that hydroxyurea therapy is indicated in patients >60 years of age with a previous history of a thrombotic episode with significant cardiovascular risk factors. Such nonleukemogenic drugs as interferon- or anagrelide appear to be good choices in symptomatic patients less than 60 years of age. Whether platelet-reducing agents, such as analgrelide or interferon, will be equally effective in eliminating symptoms in patients less than 60 years of age remains unknown and will require the performance of large randomized trials. These patients are reported to have a typical form of dysgranulopoiesis characterized by hypolobulated polys, small vacuoles in polys, and p53 mutations. [172] A high proportion of the acute myeloid leukemias and myelodysplastic disorders occurring in primary thrombocythemia patients treated with hydroxyurea alone have morphologic, cytogenetic, and molecular characteristics of the 17p syndrome. [172]

Interferon- has been employed to treat the thrombocytosis associated with myeloproliferative disorders with increasing frequency during the last decade. [176] [177] [178] [179] [180] [181] [182] [183] [184] [185] Interferon- acts by directly inhibiting megakaryocyte colony formation and secondarily by inhibiting the expression of thrombopoietic stimulating cytokines such as GM-CSF, G-CSF, IL-3, and IL-11 and by stimulating the production of negative regulators of megakaryocytopoiesis such as IL-1ra (receptor agonist) and MIP-1a. [158] In a total of 212 patients treated in a total of 11 different clinical trials, a response rate of approximately 90% has been reported. [183] Therapy was administered to outpatients, most frequently at an initial dose of 3 million units daily, and usually produced a rapid decrease in platelets within a period of two months. [183] The mean time to complete response with a daily dose of 3 million units daily was about 3 months. [183] Interferon- was effective in patients who had received other chemotherapeutic agents and in patients resistant to conventional cytotoxic drugs. [176] [177] [178] [179] [180] [181] [182] [183] [184] [185] In the majority of patients, the interferon dose required to maintain a normal platelet count during maintenance therapy was lower than the induction dose. [171] [172] [173] [174] [175] [176] [177] [178] [179] [186] [187] In one study, 61% of patients required 3 million units three times a week, 15% once a week and 24% daily. [187] In addition, sustained remissions that persisted for 336 months were achieved with interferon- therapy in 916% of patients. Interferon- is reported to be nonmutagenic and to not cross the placenta, making it a useful drug for the treatment of the symptomatic pregnant patient with primary thrombocythemia. [182] [183] Reduction in platelet numbers with interferon results in a marked improvement in clinical symptoms. Toxicity, especially in older patients, and the need for parenteral administration, limit the utility of interferon-. [184] The side effects are well known. They include flu-like symptoms during induction therapy, including fever, bone and muscle pain, fatigue, lethargy, and depression. Symptoms are frequently controlled with acetaminophen. [176] [177] [178] [179] [180] [181] [182] [183] [184] [185] [187] [188] Long-term administration of interferon- can result in mild weight loss, alopecia, and late development of autoimmune thyroiditis leading to hypothyroidism and autoimmune hemolytic anemia. [176] [177] [178] [179] [180] [181] [182] [183] [184] [185] [187] [188] Patients may develop neutralizing antibodies to recombinant interferon, leading to a concomitant rise in platelet numbers. [185] In such a situation, use of leukocyte interferon- results in an excellent response. [185] In one review, it was reported that 25% of 273 patients failed to continue to receive interferon therapy either because of poor compliance or side effects. [183] In order to gain a more comprehensive evaluation of the clinical utility of interferon- a prospective clinical trial comparing interferon- to hydroxyurea is presently being pursued in patients with primary thrombocythemia. [183] Until the results of this trial are available, interferon- should be considered a reasonable alternative to hydroxyurea in a patient less than 60 years of age who has suffered a previous thrombotic episode. [179] In addition, interferon- is of use in patients who are in the childbearing years. [183]

The use of platelet antiaggregating agents remains an exciting area of investigation. Patients with primary thrombocythemia have an increased predisposition to

hemorrhage, which is likely potentiated by the use of drugs that affect platelet function. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [74] [77] [79] [89] Transient ischemic attacks and erythromelalgia associated with primary thrombocythemia have been reported to respond rapidly to aspirin alone, to aspirin and dipyridamole in combination, or to indomethacin alone. [75] [83] [84] [108] [109] In erythromelalgia, symptoms disappear for 24 days after administration of a single dose of aspirin. [83] Although these agents surely have a role in the treatment of these specific complications, their use should be pursued with extreme caution because of the increased risk of hemorrhage. Kessler et al.[85] have, in fact, determined that 32% of bleeding episodes in patients with extreme thrombocytosis and myeloproliferative disorders occurred concurrently with the use of anti-inflammatory agents. [85] By contrast, Hehlmann et al.[14] have reported the treatment of 46 patients with primary thrombocythemia with 250 mg/day of aspirin without any bleeding complications.

Aspirin, particularly in lower doses, 100 mg/day has been shown to be useful in preventing thrombotic episodes in patients with primary thrombocythemia. [189] Risk for bleeding associated with aspirin can be minimized if it is administered solely to patients with platelet counts <1,000 × 10^9 /l and/or the absence of a bleeding history.[183] Van Genderen et al. have retrospectively analyzed a group of 57 patients receiving aspirin alone or in combination with a chemotherapeutic agent (Table 64-9).[189] Their data suggests that low-dose aspirin 100500 mg/day is not only effective in the treatment of erythromelalgia and transient ischemia attacks but also reduces the recurrence of other thrombotic events in symptomatic patients.[189] Impressively, in this admittedly small population of high-risk patients with a mean follow-up of only 6.2 years, myelosuppressive therapy, plus low-dose aspirin therapy totally eliminated the incidence of thrombotic complications. [189] These findings are encouraging and are supportive of the judicious use of aspirin in patients with a past history of a thrombotic episode. Low-dose aspirin therapy must be restricted to patients with a platelet count <1,000 × 10^9 /l. The diagnosis of an acquired von Willebrand syndrome should be excluded prior to aspirin use and considered a contraindication to the use of aspirin. [77] [189] These recommendations merit testing in a large randomized trial in the future.

Of great interest has been the increased use of a novel drug, anagrelide, for the treatment of primary thrombocythemia. [170] [182] [190] [191] [192] [193] [194] Anagrelide is a member of the imidazo(2,1-b)quinazolin-2-1 series of compounds. During studies in humans, it was noted that anagrelide in small doses produced profound thrombocytopenia. The drug acts primarily by reducing megakaryocyte size and ploidy and at therapeutic doses, it does not have a major antiproliferative effect. [192] Anagrelide, therefore, appears to lower platelet counts primarily by interfering with the maturation of megakaryocytes. [195] A major study of 577 patients treated with anagrelide has confirmed its usefulness.[191] Anagrelide in low doses was effective in lowering the platelet count in 93% of patients. Most importantly, it was effective

despite resistance to previous therapy. Resistance to anagrelide therapy has not been documented. [170] [182] [190] [191] [192] [193] [194] The recommended initial dose is 0.5 mg orally two to four times a day. The dose should be increased by 0.5 mg/week in order to control thrombocythemia. [182] The dose of anagrelide should not exceed 10 mg/day or 2 mg/dose.[182] Excessive use will result in predictable thrombocytopenia. The median maintenance dose in patients with primary thrombocythemia is 2.0 mg/day administered in divided doses.[194] By 1997, data on over 3,000 patients with a variety of myeloproliferative disorders complicated by extreme thrombocytosis were available.[182] In addition, follow-up of over 500 patients for over 5 years had been reported. [194] Anagrelide has been shown to be an effective drug in the treatment of primary thrombocythemia resulting in a median time to response of 2.5 to 4 weeks. An effect on platelet numbers is usually noted in 6 to 10 days. [182] [190] [191] [192] [193] [194] Anagrelide leads to a reduction in hematocrit in 36% of patients but it has no effect on white cell numbers. [176] [182] [183] [184] [185] Most important, the reduction in platelet numbers initiated by anagrelide use has been reported to be associated with a decrease in symptoms attributable to the thrombocythemia. [194] Silverstein reported that anagrelide use in 1,700 evaluable patients reduced the incidence of thrombohemorrhagic episodes due to thrombocytosis associated with myeloproliferative disorders from 0.66 symptoms per patient before therapy to 0.07 symptoms per patient after 2830 months of therapy. [194] The most common side effects of anagrelide resulted from its vasodilatory and positive inotropic actions. These effects resulted in complaints of headache, dizziness, fluid retention, and palpitations.[182] [189] [190] [191] [192] [193] [194] The vasodilatory effect leads to reduced renal blood flow resulting in fluid retention. In addition, gastrointestinal complications such as nausea, abdominal pain, and diarrhea are preeminent. These side effects usually develop within 2 weeks of initiation of therapy and frequently diminish in severity or resolve within 2 weeks of continued therapy. Because of its ability to promote fluid retention and the development of tachyarrhythmias, anagrelide therapy should be avoided in patients with cardiac disease and should be administered carefully to elderly patients. [194] If congestive heart failure or arrhythmias other than tachycardia develop, anagrelide therapy should be discontinued. [194] Dose reduction can be used to lessen the degree of tachycardia or fluid retention. Acetaminophen may be useful for treatment of the headaches. Silverstein claims that patients with nausea, diarrhea, and abdominal pain are usually lactose-deficient and that the use of LactAid results in resolution of such symptoms. [194] Although most adverse effects are mild or moderate, in one series therapy was discontinued in 16% of patients because of intolerable side effects especially headache, nausea, fluid retention, and rarely, frank congestive heart failure. [188] Anagrelide has no mutagenic activity but its use is not currently advised during pregnancy. Because of its small molecular weight, it is believed to be capable of crossing the placenta and eventually to lead to fetal hemorrhage.[195] Anagrelide does not appear to be leukemogenic because no cases of leukemic transformation have been attributed to its use during 54.7 months of observation.[194] In the review of Silverstein, four cases of acute leukemia developed while patients were receiving anagrelide for 73, 189, 276, and 300 days.[194] It is unknown what myeloproliferative disorders these patients suffered from, but the short duration of therapy and the mechanism by which anagrelide acts make it unlikely that these cases of leukemic transformation were anagrelide-related. [194] Anagrelide, therefore, appears to be a highly effective drug for the treatment of young, symptomatic patients with primary thrombocythemia. Anagrelide has also been successfully used to treat children with primary thrombocythemia.[189]

A continuing clinical controversy revolves around the question of whether any treatment is indicated in patients with primary thrombocythemia in whom the platelet count elevation is initially detected fortuitously and who remain largely asymptomatic. Such a decision is particularly important, as the use of most chemotherapeutic agents is associated with an increased risk of the development of leukemia, and the use of platelet antiaggregating agents is not without risk. [169] [170] The need for such treatment can be questioned, since in several studies a relationship between frequency of thrombotic episodes and degree of platelet elevation has not been established.[13] [77] [86] One should not lapse into a false sense of security in deferring therapy, however, since the course of primary thrombocythemia can include infrequent but dangerous thromboembolic complications, and patients may function normally for long periods of time without experiencing a life-threatening event. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] If the clinician feels compelled to use some therapeutic intervention in the young asymptomatic patient, low-dose aspirin (100 mg/day) appears to be effective in the treatment of microvascular complications and its use is associated with limited toxicity. [189] Still, it would seem reasonable to withhold therapy in younger, asymptomatic patients until the development of a clinically significant thrombotic or hemorrhagic event. The elderly patient (>60 years) with other significant risk factors for cardiovascular complications is probably best served by immediate institution of therapy. In addition, platelet reduction therapy is indicated in a patient with excessive thrombocytosis (platelet count >1,500 × 10^9 /l) and a documented acquired von Willebrand syndrome. [90] Such an individual is at a very high risk of suffering a serious hemorrhagic episode.

The management of a pregnant patient with primary thrombocythemia remains problematic.[121] [122] [123] [124] A review of the literature disclosed that of 106 pregnancies in 57 women with primary thrombocythemia, the rate of miscarriage was 43% as compared to an anticipated rate of 15% in a normal control population. [121] The most frequent complication was spontaneous abortion resulting from placental infarction secondary to thrombosis. [121] [122] [123] [124] In one series, 65% of the miscarriages occurred in only 17% of the women at risk.[122] A previous history of spontaneous abortion appears to be the greatest risk factor for the development of subsequent miscarriages. Remarkably, the incidence of maternal hemorrhagic or thrombotic episodes was extremely low. [121] [122] The maternal platelet count frequently is stable throughout the pregnancy or, in a subpopulation, actually falls. This drop in platelet numbers has been associated with a good outcome. [124] The major goal of any therapeutic intervention in a pregnant patient with primary thrombocythemia should be the prevention of the vaso-occlusive events that lead to placental infarction, intrauterine fetal growth retardation and, in some cases, fetal death. In one large series, there was no significant relationship between the fetal outcome and the degree of maternal thrombocytosis or presence of disease complications. [122] In this series, there were no instances of excessive bleeding or other related complications during delivery.[122] This group did not recommend the use of therapeutic plateletpheresis and, in fact, claimed that specific therapy (aspirin, heparin, or plateletpheresis) did not alter the clinical course. [122] However, of the 21 patients reported in the literature who did not receive any therapy, 12 experienced either spontaneous abortion or intrauterine deaths. [121] Low-dose aspirin (100 mg/day), because of its profound effect on events involving the microcirculation, such as erythromelalgia and transient ischemic events, has been used with increasing frequency in pregnant patients during the first and second trimester. [121] [196] It is recommended that aspirin be discontinued at least 1 week before delivery in order to avoid bleeding complications during delivery or during the postpartum period. [121] Because of the high risk of bleeding in patients with platelet counts greater than 1000 × 10^9 /l with acquired von Willebrand syndrome aspirin therapy is contraindicated.[121] There is limited experience reported in the literature with aspirin therapy alone and although the results are promising, the sample size is too small to confirm a beneficial effect.[121] [197] The observed true birth rate, however, was 75% in those receiving aspirin as compared to 43% in the group in the literature who received no therapy.[121] Treatment with low-dose aspirin during

the first trimester combined with subcutaneous heparin during the second and third trimesters has been reported in a small series to be a promising strategy. [194] This regimen requires further evaluation. Chemotherapeutic drugs are to be avoided during the period of conception and especially during the first trimester. [121] Both

hydroxyurea and busulfan are known teratogens in animals.[121] In addition, busulfan and hydroxyurea reduce fertility in males.[121] With the availability of newer agents to treat primary thrombocythemia, it is difficult to justify the use of chemotherapeutic agents during the time of conception or during the first two trimesters of gestation. Interferon- therapy is not known to be leukemogenic or teratogenic, and because it does not cross the placenta, its use may be considered during pregnancy.[178] The manufacturers of interferon- still advise that interferon- not be used during pregnancy because adverse effects on the fetus cannot be ruled out.[184] The effect of interferon- on male infertility remains uncertain. Anagrelide therapy should be avoided in the pregnant patient because of its potential to lead to fetal hemorrhage.[198] Since none of the strategies described above have been tested in large clinical trials, one must develop a therapeutic strategy for an individual patient. In the patient who has no history of spontaneous abortions and is totally asymptomatic but is found to be pregnant, no therapy is presently indicated although low-dose aspirin therapy (100 mg/day) can be initiated without appreciable risk and can be continued throughout the pregnancy. Its use should be discontinued one week before delivery. Aspirin therapy is contraindicated in patients with platelet counts >1,500 × 10^{9} /l and/or patients with acquired von Willebrand syndrome. If a patient is symptomatic from a thrombohemorrhagic episode or if the platelet count is rising to levels above 1,500 × 10^{9} /l, therapy with a platelet-reducing agent is indicated. Plateletpheresis therapy can be attempted but if incomplete control is obtained, interferon- therapy is at this point the best option. Several successful pregnancies have been reported with interferon- therapy.[121] The patient with a history of a previous spontaneous abortion appears to be at a particularly high risk of developing subsequent spontaneous abortions if left untreated.[122] At the minimum, low-dose aspirin therapy in this patient population is a reasonable therapeutic approach.

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PROGNOSIS

The probability that a patient with primary thrombocythemia will survive 10 years is within the range of 6480%. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] In a large study from Spain with extensive follow-up, there was no substantial difference between the survival probability of patients with primary thrombocythemia and that of the control population.[199]

Table 64-10 (Table Not Available) presents the causes of death in one series of 61 patients with primary thrombocythemia. [14] Death predominantly ensued from thrombotic complications.[14] Transformation to acute myeloid leukemia has been reported with increasing frequency in patients with primary thrombocythemia and is

TABLE 64-10 -- Causes of Death in Primary Thrombocythemia

(Not Available)

From Hehlmann R, Jahn M, Baumann B, Kopcke W: Essential thrombocythemia: clinical characteristics and course of 61 cases. Cancer 61:2487, 1988. Copyright 1988 American Cancer Society. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

an important cause of mortality.[30] [171] [172] [173] [174] [175] [196] [200] The rate of leukemic transformation ranges from 310%.[28] [32] The blast cell phenotype that characterizes this transformation can be either myeloid, myelomonocytic, megakaryocytic, mixed lineage, or even lymphoblastic. [26] [32] [171] [172] [173] [174] [175] [186] [187] [196] [200] [201] [202] A limited number of patients have been reported who developed acute leukemia and who had not been previously treated with chemotherapeutic agents. [30] [171] [172] [201] [202] Many investigators have concluded that the transformation of primary thrombocythemia into acute leukemia is a rare event, which can be accelerated by the administration of chemotherapeutic agents.[32] [171] [172]

Transformation of primary thrombocythemia to a clinical stage that resembles agnogenic myeloid metaplasia with or without myelofibrosis has been well-documented.[9] [30] In fact, 6% of the patients documented unequivocally as having primary thrombocythemia by the criteria of the Polycythemia Vera Study Group went on to develop myelofribrosis.[30] Three additional patients who met these diagnostic criteria went on to develop polycythemia vera. [28] [30] These studies emphasize the potential of primary thrombocythemia to eventually evolve into clinical picture that resembles one of the other myeloproliferative disorders. This does not represent an instance of misdiagnosis but rather the natural evolution of the underlying hematological malignancy. According to the data mentioned above, the risk of transformation into acute leukemia in this patient population is greater than that in normal individuals. This is a phenomenon shared with the other myeloproliferative disorders.

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FUTURE DIRECTIONS

Primary thrombocythemia is now firmly established as a hematologic malignancy with its own distinct clinical manifestations and associated complications. Better means of identifying patients at risk for developing fatal thrombotic or hemorrhagic complications are necessary to provide the basis on which to develop the optimal care of such patients. The ability to reduce the incidence of thrombohemorrhagic episodes with cytoreductive therapy in high-risk patients is now well-established. [87]

Multi-institutional comparisons of the efficacy of such promising agents as interferon- and anagrelide for the treatment of young patients with a prior thrombotic episode are needed. The use of low-dose aspirin therapy to reduce the number of episodes of erythromelalgia and transient ischemic attacks is now well-established.[74] Whether such therapy should be used in combination with anagrelide, interferon-, or hydroxyurea remains uncertain. Another pressing question that requires resolution is the degree of platelet reduction that is required for optimal management to be achieved. Whether reduction of platelet numbers below 350 × 10^9 /l rather than using 600 × 10^9 /l as a therapeutic target will further reduce the rate of thrombotic events will require further study. [170]

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Chapter 65 - Myelodysplastic Syndromes and Myeloproliferative Syndromes in Children

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Myelodysplastic syndromes (MDS) and myeloproliferative syndromes (MPS) are clonal disorders observed only rarely during the childhood years. Unlike acute leukemia, in which abnormalities of both proliferation and differentiation are apparent at diagnosis, MDS and MPS are characterized by abnormalities of differentiation and proliferation, respectively. The propensity of patients with these disorders eventually to acquire acute leukemia, with abnormalities of both proliferation and differentiation, suggests that MPS and MDS represent an initial step in the process of leukemogenesis.

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MYELODYSPLASTIC SYNDROMES

Myelodysplastic syndromes are characterized by ineffective hematopoiesis (peripheral cytopenia with a hypercellular bone marrow) and morphologic evidence of abnormal differentiation in at least one, and often multiple, cell lines. The likelihood that a patient with an MDS will acquire acute nonlymphocytic leukemia (ANLL) accounts for the commonly used terms preleukemia and smoldering leukemia. Although MDS occur most often in adults, 6 of 37 children with ANLL had an MDS before diagnosis. Thus, MDS may precede ANLL in children as often as in adults. [1]

Five types of MDS are described in the 1982 French-American-British classification. [2] Children, however, usually present with refractory anemia with an excess of blasts (RAEB) or with RAEB in transformation (RAEBt). Although the other forms of MDS seen in adults may progress at a variable rate to ANLL, the two forms seen in childhood, RAEB and RAEBt, progress rapidly to ANLL. The main features of RAEB and RAEBt are anemia and an excessive number of blasts in the marrow. MDS and ANLL have similar abnormalities of differentiation, but the proliferative abnormality is less apparent in MDS. Recent evidence, however, suggests a rapid proliferative rate, counterbalanced by high levels of apoptosis. [3] Rapid turnover thus may increase the likelihood of a second mutagenic event resulting in an overt leukemia.

Biologic and Molecular Aspects

Cells of multiple hematopoietic lineage are involved in MDS, and chromosomal abnormalities have been noted in 5060% of such patients. [4] Complex chromosomal aberrations in MDS have been associated with rapid progression to ANLL and poor prognosis. In one review, 7 of 16 children in whom chromosome studies were performed had detectable chromosomal abnormalities.[5] Children with an MDS and monosomy 7 often appear to have features of an MPS initially (see later). However, at the time of their leukemic conversion, they may be clinically indistinguishable from patients with MDS only.

Monosomy 7 is the most common chromosomal aberration in children with MDS, followed by trisomy 8, deletion 5q, and aberration of chromosome 3.[6] Monosomy 7 may occur in constitutive preleukemic syndromes such as Fanconis anemia and Kostmanns agranulocytosis as they evolve to ANLL. [7] Families with ataxia and monosomy 7 have been described in which the two phenomena do not always coassociate, suggesting a role for this lesion as a second step causing progression rather than initiation of disease. Secondary point mutations in the granulocyte colony-stimulating factor (G-CSF) receptor have been noted in leukemic cells (but not normal tissue) of children in whom ANLL developed while receiving G-CSF for Kostmanns syndrome. [8]

In one study, mutations of the *ras* oncogene were identified in 1530% of childhood MDS.[9] In one patient, such a mutation was detectable during the MDS phase but not the leukemic phase of disease, suggesting a role in the initiation but not the progression of disease.

Assays of hematopoietic progenitor cells, performed in vitro with marrow cells from patients with RAEB and RAEBt, are characterized by increased numbers of abortive clusters of myeloid cells exhibiting defective maturation, similar to that found in ANLL. [10] In acute leukemia, expansion of an abnormal clone proceeds at the expense of normal cells. In the MDS, normal and abnormal cells may coexist for a prolonged period. [11] The abnormal hematopoiesis of MDS may reflect the growth pattern of the abnormal clone itself or its effect on normal progenitors, perhaps mediated by inhibitors of hematopoiesis. Leukemic proliferation in a patient with an MDS may result from a karyotypic change in the original clone or from a subclone of malignant cells, which slowly gains predominance.

Clinical Manifestations

In contrast to adults, who are often asymptomatic initially, most children with an MDS are symptomatic. Fever, pallor, hemorrhage, and infection are most frequently seen.[1] [12] Except in children with monosomy 7, hepatosplenomegaly is not common. Macrocytic anemia and pancytopenia are classically found in adults with an MDS. Children, however, may present with a normocytic, normochromic anemia,[1] with macrocytosis and ovalocytosis appearing later, followed by poikilocytosis and anisocytosis. Nucleated red blood cells (RBCs) and a low reticulocyte count may be seen. White blood cell (WBC) changes, including Pelger-Huet abnormalities, hypersegmentation, and hypogranularity, may be subtle. Peripheral blasts may be seen with a normal marrow examination, and hypogranular platelets are noted.

The marrow of patients with an MDS is often hypercellular, which is indicative of ineffective hematopoiesis. Megaloblastoid changes of erythroid and myeloid precursors are common. Decreased numbers of megakaryocytes have been reported in children, [1] [13] although megakaryocytes are often increased in adults. Auer rods were present in 12 of 21 children in one study. [1]

Vitamin B_{12} levels were increased in six children tested in one study. [1] In another study, three of nine children had decreased leukocyte alkaline phosphatase activity.[5] Other neutrophil abnormalities noted in adults include decreased myeloperoxidase, chemotaxis, phagocytosis, and bactericidal activity. [14] RBC abnormalities include decreased RBC enzymatic activity, abnormal

iron metabolism, increased fetal hemoglobin, and abnormal expression of RBC antigens. [15] [16]

Differential Diagnosis

The myelodysplastic syndromes are characterized by the presence of anemia with or without other cytopenias and a hypercellular, dyserythropoietic marrow. The high likelihood of leukemic transformation in children with classic presentations of MDS justifies the use of the term preleukemia. Two forms of preleukemia occur commonly in children: pre-ANLL and preacute lymphoblastic leukemia (pre-ALL; Table 65-1). As in acute leukemias, the age frequency of pre-ANLL is approximately constant throughout the childhood period, whereas pre-ALL appears most often in children aged 16 years. Symptoms and peripheral blood findings may be similar, but the marrow findings usually differ, marrow hypoplasia being more common in pre-ALL. Although ineffective erythropoiesis and myelopoiesis may occur in either, ineffective megakaryopoiesis is unique to pre-ANLL. The morphology of marrow precursor cells is abnormal in pre-ANLL and normal in pre-ALL, whereas karyotypic abnormalities are common in pre-ANLL and rare in pre-ALL.[12]

A review of 760 pediatric marrow samples identified 7 children with hematopoietic dysplasia, [17] of whom 1 died of hemorrhage and 4, including 2 with family histories of childhood leukemia, acquired ANLL. One child had Shwachmans syndrome, which has a known association with hematopoietic dysplasia and progression to ANLL.[18] Six of the seven children had constitutional abnormalities, including skin abnormalities (five), short stature (four), unusual facies (four), mental retardation (three), and endocrinopathy (two). The otherwise normal child had a hydrocele. The incidence of leukemia is also increased in other constitutional disorders with

hematologic manifestations, including Kostmanns agranulocytosis, Downs syndrome, Blooms syndrome, Diamond-Blackfan syndrome, and Fanconis anemia. [13] [19]

TABLE 65-1 -- Preleukemia in Childhood

	Pre-ANLL	Pre-ALL
Patient population		
Sex	M > F	F > M
Age	All	16
Signs and symptoms		
Pallor	+	+
Fever/infection	+	+
Bleeding/bruising	+	+
Hepatosplenomegaly	+	+
Peripheral blood		
Anemia	++	++
Granulocytopenia	+	++
Thrombocytopenia	+	+
Morphology	Macrocytosis	Normal
	Ovalocytosis	
	Pelger-Huët WBCs	
	Hypogranular WBCs	
	Hypogranular platelets	
Marrow		
Hypoplasia (all cell lines)		+
Erythroid hyperplasia	++	
Myeloid hyperplasia	+	
Megakaryocyte hyperplasia	+	
Abnormal maturation	+	
Chromosomes		
Detectable abnormality	+	

ANLL, acute nonlymphocytic leukemia; ALL, acute lymphocytic leukemia; ++, >75%; +, 3075%; +/, 1530%; , <15%.

Therapy and Prognosis

Myelodysplastic syndromes of childhood are rapidly progressive; children rarely die of unrelated causes. Of 26 children with MDS, overt leukemia developed in 23, and 3 died in a preleukemic state. [5] The median preleukemic phase in children is short, lasting 12 months. The rapidity of disease progression necessitates consideration of treatment options beyond supportive care.

Maturational therapies have been used primarily in adults with an intent of inducing differentiation of abnormal cells into normally functional cells. Agents used include low-dose cytosine arabinoside (which may actually exert its effect through cytotoxic clonal suppression), retinoic acid, and vitamin D_3. In general, these therapies have been disappointing. Hematopoiesis improved temporarily (327 months) in one half of patients treated with low-dose cytosine arabinoside, but hospitalizations increased and survival was unchanged. [20] [21] Retinoic acid enhanced in vitro granulocyte-macrophage colony-forming unit (GM-CFU) and erythroid burst-forming unit (BFU-E) cloning efficiency and increased the granulocyte count of some patients, but transfusion requirements were unchanged. [22] Our ability to modify the preleukemic disorder by induction of normal cellular maturation appears limited, most likely because the cellular defect is more complex than a simple block of differentiation.

Success has been greater with regimens used for ANLL. Aggressive chemotherapy in adult MDS patients results in prolonged bone marrow aplasia with few responses. [23] Better remission rates have been noted in younger patients. [24] [25] Of 11 children with MDS treated with intensive chemotherapy, 6 achieved complete remission, and 2 of these remained in complete remission at 48 and 69 months (i.e., 18% survived for longer than 2 years). Allogeneic bone marrow transplantation (BMT) from a human leukocyte antigen (HLA)-matched sibling has resulted in long-term survival of 4080% of patients [26] [27] and is the preferred therapy, when feasible. A recent study in children showed a disease-free survival rate of 69% at 4 years. [28] Hasle et al compared outcome after conventional ANLL therapy to outcome after BMT in children with MDS, noting a 3-year survival rate of 35% versus 74%, respectively. [29] This improved outcome suggests that a marrow donor should be sought for all children with MDS. Six of 10 children undergoing unrelated donor transplantation have survived a median of 1.2 years post BMT. [30]

The use of biologic response modifiers such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and the interferons has been studied in patients with MDS in the hope of affecting the control mechanisms that regulate hematopoiesis. [31] [32] [33] [34] [35] [36] Studies of these agents have shown definitive improvement in neutrophil counts in most patients, with occasional improvement in platelet counts and hemoglobin. Concerns regarding the risk of stimulating a leukemic clone limit use of these agents in pediatric MDS to situations of life-threatening risk, such as serious infection.

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MYELOPROLIFERATIVE SYNDROMES

Classic MPS, including adult-type chronic myeloid leukemia (ACML), polycythemia vera (PV), essential thrombocythemia (ET), and agnogenic myeloid metaplasia with myelofibrosis (AMMM), are disorders that were initially thought to be pure proliferations of granulocytes, RBCs, platelets, and fibroblasts, respectively. In 1951, they were grouped together as myeloproliferative syndromes by Dameshek, who noted that, to variable degrees, stimulation of all hematopoietic cells occurs. [37] These syndromes are now known to be clonal disorders, with aberrant regulatory control of hematopoietic precursors causing excessive proliferation. [38] [39] [40]

Classic MPS most frequently occur in adults, but MPS

unique to childhood also exist. Such syndromes include juvenile myelomonocytic leukemia (JMML; previously known as juvenile CML), the MPS of monosomy 7 in childhood, familial CML, the transient MPS of infants with trisomy 21, and childhood forms of myelofibrosis. Study of the biology of these syndromes gives clues to the pathogenesis of leukemia. Biologic and clinical features of MPS in children are discussed.

Disorders Characterized by Leukocytosis

Table 65-2 lists the salient characteristics of ACML, JMML, familial CML, and the MPS of monosomy 7.

Adult Chronic Myeloid Leukemias

Biologic and Molecular Aspects

Adult chronic myeloid leukemia is characterized primarily by granulocytosis, often in association with thrombocytosis, marrow hyperplasia, and fibrosis. A reciprocal translocation, with breakpoints involving the *bcr* oncogene of chromosome 22 and the *abl* oncogene of chromosome 9, results in the abnormal Philadelphia chromosome, which encodes for chimeric *bcr*/*abl* mRNA.[41] [42] Two common molecular rearrangements between the *bcr* and *abl* genes occur in adults (*mbcr* exons 2 or 3 are joined to *abl* exon II). Most children have the *bcr* exon 2 joined to *abl* exon II.[40] The translocated chromosome 9 is usually of paternal origin, whereas the translocated chromosome 22 is of maternal origin.[43] The bcr/abl protein product is an abnormal tyrosine kinase [44] that produces clonal expansion by blocking apoptosis (genetically programmed cell death). [45] Occasional patients have variant translocations that may result in the formation of a similar abnormal protein.

Clinical Features

Adult chronic myeloid leukemia accounts for 13% of childhood leukemia and is the most common form of CML in children. [46] [47] It occurs primarily in the older child. Generalized malaise, weakness, weight loss, fever, pallor, and hepatosplenomegaly are frequently the presenting findings. Hyperleukocytosis has been found to be a more prominent feature in patients younger than 20 years of age (median presenting WBC count = 360,000/mm ^{3}) compared with older patients (137,000/mm ^{3}), [48] resulting in an increased incidence of central nervous system, retinal, and pulmonary dysfunction. Arthritis and priapism may occur.

In addition to leukocytosis with a WBC count >100,000/mm ^{3} and the appearance of some immature myeloid elements, such abnormalities as thrombocytosis, erythrocytosis, eosinophilia, and basophilia may be seen. The bone marrow shows myeloid hyperplasia but normal maturation. Other abnormalities include increased vitamin B_{12} levels and decreased leukocyte alkaline phosphatase levels.

The course of ACML is variable, but ultimately blast crisis intervenes within 10 years (median, 23 years), [47] [49] [50] at which time a florid leukemic picture develops. Such a transformation may be heralded by increased fatigue, pallor, and splenomegaly. Although the blast crisis is usually of myeloid origin, lymphoid blast crisis does occur. Responses to standard therapeutic agents are usually brief.

Therapy

The traditional therapy of ACML has been palliative, using agents such as busulfan and hydroxyurea to lower the granulocyte count and prevent complications. More intensive chemotherapy rarely eradicates the abnormal clone, never for more than a short time. Recombinant -interferon induces hematologic and cytogenetic remissions in CML in adults [51] [52] and in children. [53] However, the *bcr*/*abl* rearrangement can usually be detected in such patients if a sensitive assay is used. [54] [55] Combination regimens (e.g., -interferon and low-dose cytarabine) may show improved clinical response rates as well as higher rates of cytogenetic response. [56] Nonetheless, allogeneic BMT remains

TABLE 65-2 -- Chronic Myeloid Leukemia in Childhood

	ACML	JCML	MPS of Monosomy 7	Familial CML
Patient population				
Sex	M = F	M > F	M > F	M = F
Age	>5	<2	<2	<2
Neurofibromatous		+		
Signs and symptoms				
Fever/infection	+	+	++	+
Bleeding/bruising		+	+	+
Splenomegaly	++	++	++	++
Lymphadenopathy		+	+	+
Rash		+	+	

Central nervous system symptoms	+			
Philadelphia chromosome	++			
Hematology				
Anemia	++	++	++	++
Leukocytes	++	++	++	++
Leukocytosis	+			+/
Monocytosis		++	++	+
Thrombocytopenia	+			
Thrombocytosis	+			
Fetal hemoglobin		++	+/	+/
Leukocyte alkaline phosphatase	++	++	+/	++
Course of disease				
Blast crisis	++	+/	++	+
Type	ANLL (ALL)	Peripheral erythroblasts	ANLL	ANLL
Survival	3 yr	<9 mo	16 yr	<2 yr or recovery

ACML, adult chronic myeloid leukemia; JCML, juvenile chronic myeloid leukemia; MPS, myeloproliferative syndrome; CML, chronic myeloid leukemia; ANLL, acute nonlymphocytic leukemia; ALL, acute lymphocytic leukemia; ++, >75%; +, 3075%; +/, 1530%; , <15%.

the only known curative approach.[57] The outcome of 68 children with CML diagnosed from 1977 to 1994 showed 42% survival after BMT (62% if transplantation occurred within 3 years of diagnosis) versus 10% without BMT (median follow-up, 5.5 years). [58] Although patients may remain clinically well during the chronic phase, it is not possible to predict when blast crisis will intervene. Thus, early BMT is recommended for children with HLA-matched siblings. For those without such donors, transplantation from matched unrelated donors should be sought. A recent cohort of 366 patients with CML (median age, 34 years) showed a 2-year disease-free survival rate of 51% in those transplanted in the first chronic phase. [59] Because results in children are usually better than in adults, the benefit of initiating an early donor search is apparent.

Juvenile Myelomonocytic Leukemia/Juvenile Chronic Myeloid Leukemia

Biologic and Molecular Aspects

In spite of the similarity in nomenclature, ACML and JMML/JCML are very different disorders, both biologically and clinically. Peripheral blood and marrow monocytosis is more characteristic of JMML/JCML, resulting in the now preferred name of juvenile myelomonocytic leukemia (JMML). [60] Colony-forming assays produce large numbers of macrophage colony-forming units (M-CFU) from blood or marrow of patients with JMML without a requirement for exogenous growth factors.[61] Recent evidence indicates that this is attributable to marked sensitivity of JMML cells to GM-CSF as well as to tumor necrosis factor- (TNF-) and interleukin-1B.[62] [63] The primary abnormality may be alteration in *ras* pathways, either mutations in the gene itself or defective regulation of the gene, resulting in proliferation induced by GM-CSF-initiated nuclear signaling. [64] [65] Monocytes in JMML appear to release interleukin-1, [66] which then augments the effects of GM-CSF and TNF-. TNF- may play a role in disease progression by inhibiting normal hematopoietic colony growth. [63] JMML occurs with increased frequency in neurofibromatosis. Neurofibromin, the protein altered in JMML is normally a negative regulator of *ras*; thus, loss of the effect of neurofibromin allows for increased *ras* activity with consequent enhancement of GM-CSF-induced proliferation. [66] [67]

The Philadelphia chromosome is not present in JMML. Other chromosomal abnormalities have been noted in 18% of patients. [68] Monosomy 7 has been reported in a clinical syndrome much like JMML.[69] [70] The course of that disorder appears different, and these patients are probably best categorized as having a distinct MPS. However, the similarity of the disorders has resulted in a tendency to group these patients together, which perhaps accounts in part for the conflicting clinical and biologic information reported.

Clinical Features

Children with JMML are younger than those with ACML. Most are younger than 2 years of age, and 95% are younger than 4 years of age. [47] [49] [71] At the time of presentation, malaise, bleeding, and fever are common. Occasional patients present with cough, tachypnea, and wheezing, with an interstitial pulmonary pattern observed on chest radiography. Leukemic pulmonary infiltrates have been reported. [72] Physical examination shows splenomegaly, pallor, hepatomegaly, and lymphadenopathy. A facial eczematoid rash may occur, particularly in patients with neurofibromatosis (which has been associated with JMML). [47] [72] [73] [74] [75] [76] Biopsy shows a leukemic infiltrate in the dermal layer. The rash may precede the diagnosis of JMML by 1 year.

Laboratory findings include thrombocytopenia, anemia, and an elevated leukocyte count (usually <100,000/mm ^{3}) with a prominent monocytosis. Occasional blasts may be noted in the peripheral blood, but myeloid cells in all stages of development are noted. The bone marrow shows myeloid and erythroid hyperplasia with myeloid/erythroid ratios of 2:1 to 5:1 (lower than in ACML). [49] Dysplastic features are not present. Other laboratory features include increased fetal hemoglobin levels and glucose-6-phosphate dehydrogenase activity levels, and decreased I antigen, carbonic anhydrous activity, and hemoglobin A $_2$, all of which are consistent with an increased number of fetal RBCs. Leukocyte alkaline phosphatase activity is decreased.

Juvenile myelomonocytic leukemia is associated with an acute deteriorating course, with death from bone marrow failure occurring within 9 months. Increasing myeloid blasts appear, and thrombocytopenia with progressive splenomegaly occurs terminally. An increase in pronormoblasts without evidence of leukemic marrow infiltration has been noted in some patients.[47] [49] In patients with JMML in association with neurofibromatosis, deletion of the whole or part of chromosome 7 may be an important step in the evolution of JMML into the accelerated or blast phase. Erythroid progenitor cells remain responsive to erythropoietin, even at this terminal erythroblastic period.[76]

Two patients have been reported with classic features of JMML who remitted spontaneously. They were found to have serologic evidence of persistent Epstein-Barr virus (EBV) infection.[75] Serologic tests should be performed to rule out an EBV infection before chemotherapy is administered. Monosomy 7 has also been noted in patients with persistent EBV infection.[77]

Further biologic studies will be necessary to understand the relationship of monosomy 7 and EBV infection to JMML-like disorders. The association of neurofibromatosis, monosomy 7, and JMML suggests a multistep mechanism of oncogenesis.[78] [79]

Therapy

Early reports suggested that agents active in ACML (e.g., busulfan) were ineffective in JMML, whereas 6-mercaptopurine was efficacious, although not curative. [47] In 1987, an intensive chemotherapeutic regimen used for the treatment of acute myelomonocytic leukemia was reported to induce complete remissions in all four children to whom it was administered.[80] However, all except one child had continued inhibition of normal hematopoietic colony formation and autonomous M-CFU-derived colony formation when marrow cells were assayed in vitro. More recently, 13- *cis*-retinoic acid has been shown be effective with only minimal toxicity in the treatment of JMML by virtue of its ability to reduce both the spontaneous proliferation and the G-CSF sensitivity of JMML cells. [81]

As in myelomonocytic leukemia, more intensive regimens may be necessary to improve survival rates. Allogeneic BMT has resulted in survival of 6 of 14 children with

JMML for more than 0.5 to more than 11.5 years.[82] This is currently the treatment of choice if an acceptable bone marrow donor can be found.

Myeloproliferative Disease of Monosomy 7

Biologic and Molecular Aspects

In patients with ANLL, monosomy 7 suggests involvement by the leukemic process of a pluripotent hematopoietic stem cell. [83] Monosomy 7 has also occurred in some patients who have been reported as having JMML involving a multilineage progenitor. [84] Because the natural history of those with monosomy 7 differs from that of other patients with JMML, the MPS of monosomy 7 is considered here as a distinct disorder.

Clinical Features

Patients have been reported with monosomy 7 with a clinical presentation similar to JMML. [70] [78] [85] Both syndromes present in children younger than 2 years of age. Pallor, lymphadenopathy, hepatosplenomegaly, infection, facial rash, and petechial bleeding are frequently present. The hematologic picture is characterized

by leukocytosis with monocytosis, anemia, and thrombocytopenia. However, leukocyte alkaline phosphatase activity is reduced in JMML, and hemoglobin F levels, which are increased in JMML, are variable in patients with monosomy 7. It is important to distinguish these two disorders because of their different natural histories. The course of JMML is brief, with most patients dying within 9 months. Patients with monosomy 7 often present initially with repeated bacterial infections and acquire ANLL after a latent period of 36 years. Those patients with monosomy 7 who are diagnosed while their disorder is evolving to ANLL may appear clinically identical to patients with RAEBt or chronic myelomonocytic leukemia, and may be classified as such.

Therapy

Patients with the MPS of monosomy 7 eventually acquire ANLL. Chemotherapy has not proved effective. Allogeneic BMT remains the only means of curing such patients.

Familial Chronic Myelogenous Leukemia

In 1965 Randall et al reported a large kindred of nine cousins afflicted with an MPS characterized by early onset (age 5 months to 4 years), hepatosplenomegaly, anemia, thrombocytopenia, and leukocytosis (with a monocytosis documented in some). [86] The marrow, liver, and spleen of these patients showed marked granulocytic and slight erythroid hyperplasia. Leukocyte alkaline phosphatase levels were low in all patients tested as well as in parents and grandparents. Three of these patients died of complications, whereas six eventually improved (up to 14 years from diagnosis). Other sibling groups with JMML-like illnesses have been reported,[87] [88] particularly in association with monosomy 7. [89] Cerebellar ataxia was also noted in two families described with monosomy 7-associated JMML. [90] [91] The genetic predisposition is not limited to chromosome 7. [92] Thus, a multistep mechanism involving another constitutive gene as well as loss of chromosome 7 may result in leukemia. [93] Potential marrow donors for transplantation should be examined for chromosomal abnormalities in addition to other hematologic abnormalities similar to that of their sibling.

Myeloproliferative Syndrome Associated with Trisomy 21

Newborns with trisomy 21 may present with an MPS that appears morphologically identical to ANLL. The WBC count may be as high as 400,000/mm ^{3} , with peripheral myeloblasts accounting for up to 95% of the WBCs. Anemia, thrombocytopenia, hepatosplenomegaly, and skin infiltrates may be seen. [94] Spontaneous remission occurs within a few months in some of these patients, but others have a persistent leukemia. Three female neonates with Downs syndrome and transient MDS have been shown to have clonal disorders by analysis of methylation patterns of the X chromosome by restriction fragment length polymorphism. [95]

Of 15 neonates with transient MPS, all with trisomy 21 as the only abnormality, leukemia later developed in 1 at 18 months of age. [96] At that time, trisomy 8 and another marker chromosome were present. Some patients have been reported to have chromosomal abnormalities other than trisomy 21 that were no longer detectable as the MPS resolved. [97] [98] These findings are suggestive of a clonal leukemic or preleukemic disorder that remits, possibly as regulatory influences allow the normal trisomic hematopoietic cells to gain dominance over the abnormal clone. In one infant with a transient MPS, an extra chromosome C was noted in 6% of his cells. [99] This abnormal clone persisted as a minor cell line until 26 months of age, at which time leukemia appeared, and the extra chromosome was present in 93% of the cells. The extra C chromosome may have caused genetic instability, predisposing the cells to leukemic transformation. Alternatively, the clone may have been a truly leukemic line that was initially suppressed but then expanded. Transient MPS with trisomy 21 in blast cells has occasionally been noted in phenotypically normal infants,[100] suggesting an increased proliferative potential in cells with trisomy 21.

Granulocyte-macrophage colony-forming unit assays have been performed in an attempt to determine whether the transient disorder can be differentiated from persistent leukemia.[101] [102] [103] [104] In several children with the transient form, the GM-CFU cloning efficiency was normal. In one patient, abortive myeloid clusters were observed, a finding common in ANLL. This patient had a persistent leukemia. The numbers of patients who have been studied with such in vitro techniques are too few to permit routine use of hematopoietic colony assays to predict outcome. Children with this disorder should receive supportive care as long as possible to determine whether the abnormality is transient or whether the patient has a true leukemic process.

Disorders Associated with Myelofibrosis

Some characteristics of the three myelofibrosis-associated myeloproliferative disorders are listed in Table 65-3 .

Agnogenic Myeloid Metaplasia with Myelofibrosis

Agnogenic myeloid metaplasia with myelofibrosis is characterized by myelofibrosis, myeloid metaplasia (splenomegaly), and a leukoerythroblastic blood picture. AMMM is virtually unheard-of in children, although there is one published case in which the child clearly fits the criteria for AMMM. [105]

Acute Myelofibrosis

Acute myelofibrosis (AMF) in adults presents as a rapidly fatal disorder with nonspecific symptoms of fatigue and weight loss. Splenomegaly is absent. The peripheral smear shows pancytopenia with morphologically normal cells, but the marrow shows bizarre megakaryocytes with fibrosis. [106] Unclassifiable blast cells may be present. Electron microscopic examination and platelet peroxidase assays reveal features of megakaryoblasts, suggesting that this may be a variant of acute megakaryocytic leukemia. Classic AMF is rare in childhood. [106] However, a number of children have been reported with a similar syndrome characterized by myelofibrosis, unclassifiable blast cells, and bizarre megakaryocytes. [107] [108] [109] [110] [111] [112] [113] Unlike adults with this disorder, children commonly have splenomegaly, and a leukoerythroblastic blood smear may be seen. These children are acutely ill and survival is brief. This childhood form of AMF (C-AMF) occurs most commonly in toddlers (<3 years of age) with trisomy 21 (Table 65-3). Trisomy 21 is also known to be associated with acute megakaryocytic leukemia. Although marrow infiltration with leukemic blasts is less prominent, C-AMF

TABLE 65-3 -- Myelofibrosis

	AMMM	AMF	C-AMF
Patient population			
Age	Adult	Adult	<4
Trisomy 21			+

Hepatosplenomegaly	+		+
Leukoerythroblastosis	+		+
Marrow fibrosis	++	++	++
Survival	10 yr (median)	<1 y	<1 y

AMMM, agnogenic myeloid metaplasia with myelofibrosis; AMF, acute myelobrosis in adults; C-AMF, child acute myelofibrosis; ++, >75%; +, 3075%; +/, 1530%; , <15%.

overlaps with childhood acute megakaryocytic leukemia in clinical symptomology, marrow findings, and the population at risk. [114]

Children with C-AMF treated with chemotherapeutic regimens used for ANLL have had prolonged remissions, [114] as did a child who received an allogeneic BMT. [107] This information also suggests that C-AMF is a type of acute megakaryocytic leukemia.

Congenital Myelofibrosis

Two siblings were found to have myelofibrosis and myeloid metaplasia at the ages of 7 and 8 weeks. [115] No evidence of a clonal disorder was present. A constitutional abnormality may have caused abnormal regulation of marrow fibroblast function or megakaryocytic proliferation.

Disorders Characterized by Erythrocytosis or Thrombocytosis

Polycythemia Vera

Biologic and Molecular Aspects

Polycythemia vera is a clonal disorder resulting in an increase in RBC mass. An increased proliferative response to erythropoietin has been noted for the erythroid colony-forming units (CFU-E) and BFU-E of patients with PV, [116] [117] [118] allowing them to establish dominance over normal hematopoietic precursors.

Clinical Features

Polycythemia vera is rarely seen in children, with only 0.1% of patients younger than 20 years of age. [119] Fewer than 20 children have been reported, and not all have clearly been documented to have PV. [120] [121] [122] [123] [124] [125] [126]

During the proliferative phase, hyperplasia of all marrow elements is present, leading to varying degrees of thrombocytosis and leukocytosis. Splenomegaly is common. When making the diagnosis of PV, it is necessary to exclude causes of relative, or spurious, polycythemia and secondary polycythemia. Erythrocytosis may cause plethora, cardiac symptoms (dyspnea and hypertension), and symptoms of disturbed cerebral circulation (dizziness and paresthesias). Thrombosis and hemorrhage are due to the combination of abnormal platelet function and thrombocytosis. Granulocytic proliferation is associated with increased histamine turnover, causing gastrointestinal symptoms and pruritus. Hyperuricemia and hypermetabolic symptoms of weakness and weight loss are common. During this phase, thrombohemorrhagic events are of greatest concern. In children, serious complications have been noted, including hypersplenism, splenic infarction, hypertension, strokes, and hemorrhage.[121]

A minority of patients progress to the stable phase, during which blood counts normalize without therapy. Eventually they enter the spent phase of postpolycythemic myeloid metaplasia, characterized by extensive marrow fibrosis, hepatosplenomegaly, and peripheral cytopenias. [127] Leukemia most often arises in patients with postpolycythemic myeloid metaplasia.

Therapy

Therapeutic modalities used during the proliferative phase in an attempt to decrease the incidence of thrombohemorrhagic phenomena have included phlebotomy, radioactive phosphorus (^{32}P), chlorambucil, and hydroxyurea. [128] Current recommendations by the Polycythemia Vera Study Group are to use hydroxyurea, a nonalkylating myelosuppressive agent, for older patients at risk for thrombosis. Such agents are to be avoided in younger patients and those at low risk for thrombosis.[129] More recently, -interferon has been shown to decrease the need for

TABLE 65-4 -- Causes of Thrombocytosis in Children

I:	Infection/immune disorder
S:	Surgery/splenic dysfunction
T:	Trauma/thrombosis
O:	Oncologic (lymphoma, neuroblastoma, acute megakaryocytic leukemia)
P:	Pharmacologic (epinephrine, exogenous or endogenous steroids, Vinca alkaloids, leucovorin)
U:	Unclassifiable diseases (histiocytosis, sarcoid, Caffey disease)
P:	Proliferative disorders essential thrombocytopenia, polycythemia vera, adult chronic myeloid leukemia
A:	Anemia (iron/vitamin E deficiency, hemolytic, megaloblastic)
BLEED:	Hemorrhage

Data from Addiego et al.[141] and Schwartz and Cohen.[145]

phlebotomy,[130] perhaps by inhibiting the production of hematopoietic cytokines and stimulation of negative regulators. [131] Angrelide, an inhibitor of platelet release, has also resulted in disappearance of thrombocythemia-related symptoms in 80% of patients with PV and ET. [132]

Essential Thrombocythemia

Clinical Features

Essential thrombocythemia is a clonal disorder that causes thrombocytosis (Table 65-4 lists causes of thrombocytosis in children). ET is rarely reported in children,[133] [134] although one study claimed that 13 of 94 patients (14%) with ET were younger than 20 years of age. [135] Approximately one third of young patients present with thrombohemorrhagic events, including transient cerebral ischemia, peripheral vascular ischemia, deep vein thrombosis, and priapism. Pruritus, splenomegaly, and hepatomegaly may occur but are less severe and less frequent in ET than in PV. Laboratory abnormalities related to the hyperproliferative hematopoietic state include elevations in granulocyte count, leukocyte alkaline phosphatase activity, and vitamin B_{12}, uric acid, and cholesterol levels. [135] [136] Platelet aggregation is abnormal,[133] [135] [137] and platelet clumps may be seen on the peripheral blood smear, with megakaryocytic hyperplasia in the marrow.

The course of ET in children is relatively benign. Of 10 children described in the literature, 1 died of leukemia after radioactive ^{32}P treatment, [134] [138] and idiopathic myelofibrosis developed in 1.[139] Among adults, 80% survive more than 100 months, with 5 of 95 treated patients experiencing a leukemic conversion. [135] Children

appear to have a more benign course than that of adults, [140] [141] perhaps because they are more tolerant of thrombocytosis regardless of etiology.

Therapy

Asymptomatic children need not be treated. Treatment with hydroxyurea should be considered for those who have had thrombohemorrhagic episodes. -Interferon has been used successfully to control thrombocytosis in ET. [142] Another potentially useful agent for the treatment of thrombocytosis in ET is anagrelide. [132] [143] [144]

CONCLUSION

Myeloproliferative disorders of childhood include those classic for adults (e.g., CML, PV, AMMM, and ET), as well as some unique to childhood. The increased risk of leukemic conversion in these MPS and in the MDS suggests that an abnormality of proliferation or differentiation may be the first in a two-step process of leukemogenesis.[145] Understanding the biologic processes

involved may help improve our ability to treat these patients.

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Chapter 66 - Pathobiology of Non-Hodgkin Lymphomas

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The Revised European-American classification of lymphoid neoplasia (REAL classification), introduced in clinical practice in 1994, has provided a new classification of lymphoid malignancies based on the stage of differentiation of the tumor (see Chap. 69). [1] According to this classification, non-Hodgkin lymphomas (NHL) include lymphoid neoplasms derived from mature lymphoid cells. Thus, NHL are distinct from tumors of precursor lymphoid cells, as represented by most acute lymphoblastic leukemias, although NHL do include leukemic disorders, such as chronic lymphocytic leukemia or L3-type lymphoblastic leukemia, which display biologic features consistent with mature lymphoid cells. Some lymphomas, however, such as the lymphoblastic lymphomas, which present predominantly as solid masses but are derived from precursor lymphoid cells, are biologically related to the acute lymphoblastic leukemias.

Despite their common origin from mature lymphoid cells, NHL constitute an extremely heterogeneous group of diseases. [1] First, NHL may be derived from either the B-cell or the T-cell lineage. Second, within each lymphoid lineage, NHL may be derived from cells at different stages of maturation. Finally, NHL differ in terms of pathogenetic pathways and patterns of molecular alterations. This chapter provides an overview of the main biologic mechanisms contributing to lymphomagenesis and summarizes the molecular basis of the clinico-pathologic heterogeneity of NHL. Overall, many advances have been made in the understanding of the pathogenesis of B-cell NHL, whereas, with few exceptions, the molecular basis of T-cell NHL remains relatively undefined.

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HISTOGENESIS OF NON-HODGKIN LYMPHOMAS

The histogenesis of NHL can be assessed by identifying the precise cellular subset from which a given NHL category is derived. This is achieved by defining the lineage and the precise differentiation stage of the various types of NHL and by comparing it with the features characteristic of the different maturation stages of normal lymphocytes. In most parts of the world, with the exception of some regions of the Far East and of the Caribbean, B-cell NHL represents 85% of all NHL and thus far exceeds the number of cases of T-cell NHL.[2] This section will focus on current knowledge on the histogenesis of B-cell NHL; relatively little is known concerning most T-cell NHL.

B lymphocytes are generated in the bone marrow as a result of a multistep differentiation process (see Chap. 8).[3] [4] [5] Precursor B cells usually undergo immunoglobulin (Ig) gene rearrangements of the H chain locus followed by rearrangements of the L chain locus. [3] [4] [5] If precursor B cells express a functional surface antibody acting as antigen receptor, they are positively selected into the peripheral B-cell pool comprising naive B cells. [3] [4] [5] Cells failing to express a functional antigen receptor are eliminated within the bone marrow. [3] [4] [5] For many B cells, the subsequent maturation steps are linked to the histological structure of the germinal center (GC) (Fig. 66-1). [3] [4] [5] [6] [7] The GC is characterized by a dark zone, composed of a dominant growth of rapidly proliferating B cells, and a light zone, in which nonproliferating B cells are selected and induced to differentiate through interactions with follicular dendritic cells and T helper cells. Within the GC, antigen-activated B cells accumulate somatic point mutations within their rearranged H and L chain genes (a phenomenon known as somatic hypermutation) which modify the affinity of their surface antibody to the antigen. [3] [4] [5] [6] [7] [8] Only B cells that have acquired mutations leading to high-affinity binding are positively selected and differentiate into memory B cells or plasmablasts; the majority of B cells are eliminated by apoptosis within the GC. [3] [4] [5] [6] [7] [8]

Until recently, only a few markers were available for histogenetic studies of B-cell NHL, and these markers did not allow one to define with certainty the relationship between the GC and a given NHL category. More recently, the use of somatic hypermutation as a specific marker of B-cell transition through the GC has allowed the definition of two broad histogenetic categories of B-cell NHL: (1) NHL devoid of somatic Ig hypermutation, which may be derived either from pre-GC B cells or from B cells that have achieved maturation without transiting through the GC; (2) NHL associated with somatic Ig hypermutation and thus putatively derived from GC or post-GC B cells. NHL generally devoid of somatic Ig hypermutation include mantle cell lymphoma (MCL) and a substantial fraction of B-cell chronic lymphocytic leukemia (B-CLL) (Fig. 66-1).[9] [10] [11] NHL generally associated with somatic Ig hypermutation include follicular lymphoma (FL), B-lineage diffuse large cell lymphoma (B-DLCL), Burkitt lymphoma (BL), lymphoplasmacytoid lymphoma (LPL), mucosa-associated lymphoid tissue NHL (MALT-NHL), splenic lymphoma with villous lymphocytes, and primary effusion lymphoma (Fig. 66-1).[12] [13] [14] [15] [16] [17] [18] Extranodal lymphomas associated with somatic Ig hypermutation are postulated to originate from B cells that have transited through the GC and subsequently migrated to the involved extranodal site.

Figure 66-1 Model of B-cell NHL histogenesis based on the maturation steps of normal B cells. Upon release from the bone marrow, naive B cells migrate to peripheral lymphoid organs, namely the lymph nodes. A lymphoid follicle, composed of the germinal center (GC) and the follicular mantle, is represented together with the surrounding marginal zone. Upon entering the GC, B cells transform into centroblasts, proliferate, and mature into centrocytes. These events are coupled to somatic hypermutation of Ig genes and the isotype switch of the Ig is produced. GC B cells that have accumulated Ig mutations increasing their affinity for antigens, are positively selected by antigen and therefore survive and exit the GC successfully. Conversely, cells that are not positively selected by antigens undergo apoptosis within the GC and are eliminated. Cells that have exited the GC (post-GC B cells) have two fates, differentiation into plasma cells or into memory B cells. Based on the absence or presence of somatic Ig hypermutation, B-cell NHL may be divided into two broad histogenetic categories: (1) B-cell NHL derived from pre-GC B cells and devoid of Ig mutations, exemplified by mantle cell lymphoma (MCL) in the figure; and (2) B-cell NHL derived from B cells that have transited through the GC and harbor Ig mutations, exemplified in the figure by follicular lymphoma (FL), diffuse large cell lymphoma (DLCL), and Burkitt lymphoma (BL).

Little is known concerning the histogenesis of T-cell NHL. Normal T-cell ontogeny, however, has been investigated in detail. [19] T-cell precursors reside in the thymus, where they undergo maturation and selection. They usually are divided into three stages according to the sequential expression of differentiation antigens: stage I and II thymocytes reside in the cortex of the thymus, whereas stage III thymocytes are located in the medulla. [19] Stage III thymocytes are characterized by the mutually exclusive expression of CD4 and CD8, giving rise to $CD4^+$ /CD8 and $CD8^+$ /CD4 mature T cells (post-thymic T cells). [19] At the genetic level, T-cell maturation is accompanied by rearrangement of the and chain genes of the T-cell receptor (TCR), followed by rearrangements of the TCR chain gene, and at a later step, of the TCR chain gene.[20] The different stages of thymocytes give rise to the T-cell lymphoblastic lymphomas, which are biologically related to T-cell acute lymphoblastic leukemias.[1] Mature, i.e., post-thymic, T cells give rise to a variety of peripheral T-cell tumors that are recognized to be histogenetically heterogeneous, although the biologic basis for this heterogeneity remains poorly understood. [1]

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GENERAL MECHANISMS OF LYMPHOMAGENESIS

The pathogenesis of NHL is a highly complex process involving genetic alterations in the tumor clone itself as well as biological alterations in the host. Four main mechanisms of lymphomagenesis are recognized. These include (1) accumulation of gene alterations in the tumor genome; (2) infection of the tumor clone by an oncogenic virus; (3) stimulation and selection of tumor cells by an antigen; and (4) immunodeficiency of the host. The precise role of these mechanisms varies substantially in the pathogenesis of different NHL types, and not all mechanisms are operational during the development of a given category of lymphoma. Yet, since NHL are monoclonal processes, it is generally understood that clonal accumulation of genetic lesions is a prerequisite for lymphomagenesis that is involved in the pathogenesis of all categories of NHL. The one exception to this rule may be represented by lymphoproliferative disorders occurring in transplant recipients, which, at least in a proportion of cases, may be a consequence of virus-driven expansions of genetically normal lymphoid cells.

GENETIC LESIONS

As in most human cancers, the genetic lesions involved in NHL include the activation of proto-oncogenes and disruption of tumor suppressor genes. In contrast to many types of epithelial cancers, the genome of lymphoma cells tends to be relatively stable and is not subject to the generalized random instability that characterizes many types of epithelial cancers.[21] In addition, lymphomas generally lack microsatellite instability, which is caused by defects in DNA mismatch repair genes observed in some hereditary cancer predisposition syndromes as well as in a fraction of sporadic solid cancers. [22] [23] Historically, detection of recurrent, non-random chromosomal abnormalities by karyotypic analysis of NHL metaphases has been a major advance in the identification and cloning of most genetic alterations of NHL.[21] [24]

Activation of Proto-oncogenes by Chromosomal Translocation

Chromosomal translocation represents the main mechanism of proto-oncogene activation in NHL. As in most types of hematopoietic neoplasms, chromosomal translocations in NHL are the consequence of reciprocal and balanced recombination events between two specific chromosomal sites. These translocations are frequently characteristic of a specific clinico-pathologic category of NHL and are clonally represented in each tumor case. All NHL chromosomal translocations that have been cloned to date share a common feature, i.e., the presence of a proto-oncogene mapping to the vicinity of one of the two chromosomal recombination sites. In contrast with neoplasms of precursor lymphoid cells, chromosomal translocations associated with NHL do not generally lead to coding fusions between two genes (Fig. 66-2). Rather, chromosomal translocations of NHL juxtapose the proto-oncogene to heterologous regulatory sequences derived from the partner chromosome (Fig. 66-2). The heterologous regulatory regions implicated in NHL translocations may be derived from antigen receptor loci as well as from other loci, which are expressed at sustained levels in normal cells corresponding to the differentiation stage of the lymphoma (Table 66-1). The common consequence of the translocation is the deregulated expression of the proto-oncogene, which may be categorized as homotopic deregulation or heterotopic deregulation. Homotopic deregulation occurs when the proto-oncogene is also expressed by the normal cells giving rise to the lymphoma, although in normal cells the proto-oncogene is expressed in a tightly regulated manner, which contrasts with the deregulated expression found in tumors. Heterotopic deregulation occurs when the proto-oncogene is not normally expressed in the normal cellular counterparts from which the lymphoma is derived. The one exception to the deregulation model of NHL translocations is represented by the t(2;5) of T-cell anaplastic large cell

Figure 66-2 Models of chromosomal translocations in non-Hodgkin lymphoma. The two genes involved in the translocation event are represented by the relative coding sequences, identified by the rectangle, and by regulatory sequences (RS). The two genes are identified by different colors (red boxes and white boxes). Upper panel: germline configuration of the two genes involved in the translocation. The coding sequence of each of the two genes is proximal to its physiologic regulatory sequences, which coordinate the normal expression of the gene. Lower panel: Chromosomal translocations may lead to two different consequences. In the case of transcriptional deregulation, the normal regulatory sequences of the proto-oncogene are removed and substituted with regulatory sequences derived from the partner chromosome. The proto-oncogene coding sequence (red rectangle) is thus juxtaposed to heterologous regulatory sequences (white circle). In NHL, the novel regulatory regions most commonly are derived from the immunoglobulin gene loci which are consistently expressed at high levels in mature B cells. In the case of fusion transcript formation, part of the coding sequence of the two genes involved is fused together, generating a novel fusion protein with biochemical properties distinct from the native proteins.

lymphoma, which causes the fusion of two genes and gives rise to a chimeric transcript (Table 66-1).

The precise genetic mechanisms driving the chromosomal recombination events are poorly understood. It has been proposed that translocations involving antigen receptor loci may constitute errors of the machinery involved in antigen receptor gene rearrangement in normal lymphoid cells. [25] This hypothesis is corroborated by the evidence that, among B-cell NHL, chromosomal breakpoints within the Ig loci are frequently located within joining (J) or switch (S) sequences, which represent the DNA sequences implicated in physiologic Ig gene rearrangements during B-cell development. [26] Based on this hypothesis, translocations would be the consequence of errors in the enzymatic process physiologically mediating the recombination of antigen receptor genes. In pathologic conditions, this same process would join sequences from different chromosomes instead of sequences within the same antigenic receptor locus. This model, though probably valid for a number of NHL translocations, is challenged by the relatively frequent occurrence of chromosomal translocations that do not involve antigen receptor loci and by the absence of Ig signal recombination sequences at the chromosomal breakpoints in a number of cases. [21] [24] [26] At present, it cannot be determined whether the sites involved in such translocations contain sequences that are aberrantly recognized by recombination enzymes or whether they follow a completely independent mechanism.

The pathogenetic role of chromosomal translocations is demonstrated by in vitro transformation studies as well as by tumorigenicity assays in vivo and reconstruction experiments in transgenic animal models. These experimental models have sometimes led to the conclusion that, both in vitro and in animal models, the sole translocation is sufficient to cause neoplasia. [27] [28] [29] [30] In humans, however, the situation is more complex. When investigated with high-sensitivity techniques, chromosomal translocations characteristic of NHL also commonly occur at a certain rate in normal lymphoid cells of individuals without lymphoma and/or with no increased relative risk of developing lymphoma. [31] [32] [33] This observation suggests that, in naturally occurring tumors, chromosomal translocations contribute to but do not necessarily lead to neoplasia, and that a permissive biological environment may also be required.

The detailed molecular characterization of the most frequent chromosomal translocations of NHL is discussed in the section of this chapter dedicated to the pathogenetic overview of each NHL type.

Other Mechanisms of Proto-oncogene Alteration

In addition to chromosomal translocations, other mechanisms of proto-oncogene activation can also occur in NHL. Proto-oncogene amplification is substantially less common than in epithelial cancers, yet it occurs in some cases of high-grade NHL, as exemplified by the instance of REL amplifications in B-DLCL. [34] Amplification may involve many other unknown chromosomal sites, which are likely to be revealed by the extensive use of advanced cytogenetic techniques such as comparative genomic hybridization. Point mutations can alter the coding

TABLE 66-1 -- Chromosomal Translocations of Non-Hodgkin Lymphomas

NHL Histologic Type	Translocation	Proto-oncogene Involved	Mechanism of Proto-oncogene Activation	Proto-oncogene Function
Lymphoplasmacytoid lymphoma	t(9;14)(p13;q32)	*PAX*-5	Transcriptional deregulation	Transcription factor regulating B-cell proliferation and differentiation

Follicular lymphoma	t(14;18)(q32;q21)	*BCL*-2	Transcriptional deregulation	Negative regulator of apoptosis
	t(2;18)(p11;q21)			
	t(18;22)(q21;q11)			
Mantle cell lymphoma	t(11;14)(q13;q32)	*BCL*-1/cyclin D1	Transcriptional deregulation	Regulator of the early phases of cell cycle
B-lineage diffuse large cell lymphoma	t(3;various)(q27;various)	*BCL*-6	Transcriptional deregulation	Transcriptional repressor implicated in formation and function of germinal centers
Burkitt lymphoma	t(8;14)(q24;q32)	c-*MYC*	Transcriptional deregulation	Transcription factor regulating cell proliferation, differentiation, and apoptosis
	t(2;8)(p11;q24)			
	t(8;22)(q24;q11)			
T-cell anaplastic large cell lymphoma	t(2;5)(p23;q35)	*NPM/ALK*	Fusion protein	*ALK* is a tyrosine kinase

sequence of the proto-oncogene, as in the case of c-*MYC* and *BCL*-2, and thus alter the biological properties of the proto-oncogene product. [35] [36] Alternatively, mutations may affect the proto-oncogene regulatory sequences, as in the case of c-*MYC* and *BCL*-6, thus altering their sensitivity to factors normally regulating the expression of the proto-oncogene through binding to its regulatory sequences. [37] [38] Mutations of the *RAS* genes, which represent the most frequent proto-oncogene alteration in human neoplasia, are virtually absent among NHL. [39] Mutations activating unknown oncogenes may be a frequent event in NHL and their identification is the focus of current investigations.

Inactivation of Tumor Suppressor Loci

Disruption of tumor suppressor loci in NHL occurs through mechanisms similar to those associated with other human cancers and generally leads to biallelic inactivation. Biallelic inactivation is most frequently achieved through deletion of one allele and mutation of the other, although variable combinations of allelic deletion, mutation, and methylation may occur in some cases. The tumor suppressor gene most frequently involved in the pathogenesis of NHL is *p53.* [40] Other tumor suppressor genes involved in lymphoproliferative disorders of mature lymphoid cells include *p16,* a regulator of cell cycle, and *ATM* (for *ataxia teleangectasia mutated*), a gene with multiple functional domains which is also responsible for the hereditary immunodeficiency syndrome ataxia-teleangectasia. [41] [42]

In addition to the aforementioned tumor suppressor genes, NHL frequently carry specific chromosomal deletions, which conceivably are the site of tumor suppressor loci that have not yet been identified. [21] [24] The most frequent of these deletions involve the long arm of chromosomes 6 (6q) and 13 (13q). [43] [44] [45] [46] [47] The putative pathogenetic role of 6q deletions in B-cell NHL is suggested by the observation that 6q deletions may occur as the sole cytogenetic abnormality in some cases. [48] In addition, 6q deletions may have prognostic value as markers of poor outcome in these disorders. [24] With respect to del(13q), it represents the most frequent genetic change detected in B-CLL and is likely to be a very early alteration in the pathogenesis of this lymphoproliferative disorder. [45] [46] [47]

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INFECTION BY ONCOGENIC VIRUSES

An additional molecular mechanism implicated in NHL development is infection of the tumor clone by oncogenic viruses. Three oncogenic viruses have been associated with NHL development, including two herpesviruses, Epstein-Barr virus (EBV) and human herpesvirus type-8 (HHV-8), and a retrovirus, human T-cell lymphotropic virus-I (HTLV-I).

Historically, EBV was initially identified in cases of endemic BL of African children. [49] The currently known spectrum of EBV-infected NHL, however, also includes sporadic BL (sBL) and AIDS-related NHL, in addition to endemic BL (eBL). [50] [51] [52] [53] Upon infection of a B cell, the EBV genome is transported into the nucleus, where it is predominantly present as an extrachromosomal circular molecule (episome). [50] The cohesive terminal repeats of EBV, constituted of a variable number of tandem repeats (VNTR) sequence, mediate the formation of the circular episomes. [50] Because of the heterogeneity of the EBV termini, the precise number of VNTR sequences enclosed in the newly formed episomes displays a marked degree of variation, thus providing a constant clonal marker of a single infected cell. [54] EBV infection of NHL is generally characterized by a latency pattern, in which the virus is not replicated and expresses a limited subset of viral genes. [50] The pathogenetic relevance of EBV in lymphomagenesis is substantiated by at least two sets of data. First, EBV is able to significantly alter the growth of B cells in vitro and in vivo. [50] Second, EBV-infected NHL generally harbor one single form of fused EBV termini, consistent with the hypothesis that infection has preceded and may thus have contributed to clonal expansion. [51] [53] [54] Finally, a fraction of EBV-infected NHL express the EBV encoded proteins LMP-1 and EBNA-2, which are well-known transforming agents for B cells. [52] [55] [56] Despite this body of evidence, two observations complicate our understanding of the pathogenetic role of EBV in lymphomagenesis. First, most healthy individuals are infected by EBV, although the risk of NHL in the general population is low. [57] Second, some types of EBV-infected NHL, namely BL, fail to express the EBV-encoded transforming proteins LMP-1 and EBNA-2. [52] [55]

HHV-8 is a gamma herpesvirus initially identified in tissues of AIDS-related Kaposi sarcoma and subsequently found in multicentric Castleman disease and a peculiar type of lymphoma known as primary effusion lymphoma (PEL). [58] [59] [60] [61] [62] [63] Phylogenetic analysis has shown that the closest relative of HHV-8 is herpesvirus saimiri (HVS), a gamma-2 herpesvirus of primates associated with T-cell lymphoproliferative disorders. [64] [65] Like the other gamma herpesviruses, HHV-8 is also lymphotropic, since it can be found in lymphocytes both in vitro and in vivo. [59] [60] [61] [62] [66] [67] Lymphoma cells naturally infected by HHV-8 harbor the HHV-8 genome in its episomal configuration and display a marked restriction of viral gene expression, suggesting a pattern of latent infection. [65] HHV-8 carries several genes that may behave as oncogenes, including a gene homologous to the cellular D-type cyclins, a G-protein coupled receptor (GPCR) displaying constitutive activation, and several genes encoding for molecules displaying high homology with cellular cytokines (IL-6) and chemokines (MIP-1, MIP-II). [68] [69] [70] Although several of these genes are expressed by HHV-8 positive lymphoma cells, including viral IL-6 and viral cyclin D, their precise contribution to lymphomagenesis is still under investigation. [68] [69] [71]

HTLV-I is a member of the lentivirus group which can immortalize normal T cells in vitro and can cause adult T-cell leukemia/lymphoma (ATLL). [72] [73] [74] Unlike acutely transforming retroviruses, the HTLV-I genome does not encode a viral oncogene. [72] [73] [74] Furthermore, this retrovirus does not transform T cells by cis-activation of an adjacent cellular proto-oncogene, because the provirus appears to integrate randomly within the host genome. [72] [73] [74] Rather, the pathogenetic effect of HTLV-I seems to result from viral production of a *trans*-regulatory protein (HTLV-I *tax*) that markedly increases expression of all viral gene products and transcriptionally activates the expression of certain host genes, including IL-2, the alpha chain of the IL-2 receptor (CD25), c- *SIS,* c-*FOS,* and GM-CSF. [75] [76] [77] [78] [79] The central role of these genes in normal T-cell activation and growth, coupled with direct experimental evidence, support the notion that *tax*-mediated activation of these host genes represents an important mechanism by which HTLV-I initiates T-cell transformation. [80] In addition, there are suggestions that *tax* may mediate DNA damage as a consequence of either inactivation of the *p53* checkpoint or a repression of DNA repair functions. [81] [82] Recently, it has been shown that *tax* may abrogate a mitotic checkpoint by targeting the TXBP181 cellular gene, a homolog of the yeast mitotic checkpoint MAD1 protein. [83] These features of *tax* are consistent with the fact that ATLL cells are karyotypically abnormal and frequently present as pleomorphic multinucleated giant cells. [84] [85]

Apart from EBV, HHV-8, and HTLV-I, other viruses have been postulated to contribute to lymphomagenesis. These include human immunodeficiency virus (HIV) and hepatitis virus C (HCV). [86] [87] In contrast to EBV, HHV-8, and HTLV-I, however, other viruses do not infect the lymphoma clone, and thus do not contribute directly to lymphomagenesis. Rather, they may be responsible for the hosts conditions, such as immunodeficiency

in the case of HIV, which predisposes the patient to develop lymphoma through indirect mechanisms.

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ANTIGENIC STIMULATION AND SELECTION

The role of antigenic stimulation and selection in the pathogenesis of lymphoid malignancies has been investigated in particular depth in the case of B-cell NHL. [88] The survival of normal B cells depends upon the expression and proper function of surface Ig molecules serving as antigen receptors. [3] [4] [5] [6] [7] [8] When a virgin B cell has left the bone marrow and entered the GC, it will die within a few days unless its receptor is engaged by antigen. [3] [4] [5] [6] [7] [8] If antigen receptors are succesfully engaged, B cells residing in the GC will be induced to activate and proliferate. [3] [4] [5] [6] [7] [8] Given the fundamental role played by antigen stimulation in B-cell development and mitogenesis, it is generally agreed that challenging surface Ig by antigenic stimuli may play a role in the development of malignancies of mature B cells. This notion is supported by the following experimental evidence: (1) presence of somatic mutations of Ig genes, typical of the antigen-driven process; (2) biased usage of specific families of Ig genes, consistent with stimulation and selection by specific antigens; (3) identification of antigenic specificity of the antigen receptors expressed by NHL cells.

The mechanism of Ig gene hypermutation leads to introduction of somatic mutations in Ig genes, which can occur either in the complementarity determining region (CDR) or in the framework region (FWR). [8] [88] Because contact with an antigen is mediated primarily by aminoacid residues in the CDR, mutations replacing the aminoacids in the CDR (defined as R mutations) may potentially alter antigen binding and be positively selected in this fashion. As a result of selection, Ig genes for B cells that have been positively selected by antigen often harbor a disproportionate number of R mutations in their CDR. [8] [88] Studies of the distribution of somatic hypermutation of Ig genes throughout the clinico-pathologic spectrum of B-cell NHL have shown that somatic Ig gene hypermutation characterizes several types of B-cell NHL, including FL, B-DLCL, BL, MALT-NHL, and LPL. [12] [13] [14] [15] [16] [17] [88] [89] [90] [91] [92] [93]

Biased usage of Ig genes in B-cell NHL has also been taken to reinforce the concept of antigen stimulation and selection in B-cell NHL pathogenesis. [94] Given the high number of Ig variable gene segments, a randomized rearrangement process would potentially give rise to a diverse heavy chain repertoire with an equal representation of all Ig gene segments. The expressed human Ig repertoire, however, is not an equal representation of all Ig V segments present in genomic DNA. [94] Rather, a biased subset of V_H segments is overrepresented in antibody repertoires of adult B cells, possibly as a result of antigen selection favoring certain classes of V genes. [95] [96] Similarly, a restricted set of V_H genes is frequently expressed by neoplastic and autoimmune B cells. [94] For example, B-cell NHL frequently express Ig genes containing V segments that are frequently implicated in the generation of autoantibodies, indirectly suggesting that selection by autoantigens may have been involved in lymphoma development. [94] [97] [98]

The characterization of the precise nature of the selective force and the antibody reactivity of NHL with given antigens has helped clarify antigen stimulation and selection in lymphomagenesis. These studies have been performed only in a few cases. In the case of FL and BL, the demonstration of expression of Ig with autoantibody reactivity has suggested the possibility that self-antigens may play a role in B-cell NHL growth. [90] [91] [99] [100] [101]

The precise timing and duration of antigen selection during the clonal expansion of NHL has not been clarified. Also, it is not known whether continued antigen binding may be required for tumor cell growth. Although it appears that in some NHL Ig gene hypermutation is an ongoing phenomenon persisting after full neoplastic transformation, other NHL associated with Ig hypermutation no longer express surface antigen receptor molecules. [1] [16] [93] Thus, for some NHL, antigen selection may be required for the maintenance of the tumor clone, whereas in other cases antigen selection may have played a role in the early phases of tumor development and/or in cells representing the precursor population of the tumor.

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IMMUNODEFICIENCY

The close relationship between decreased immunosurveillance and NHL is documented by the observation that many immunodeficiency syndromes are associated with a high relative risk for lymphoma, including most congenital immunodeficiencies, acquired imunodeficiency syndrome (AIDS), and iatrogenically induced immunodeficiencies in transplant recipients. [102] [103] [104] [105] [106] [107]

Immunodeficiency-related NHL are represented by high grade lymphomas, which frequently affect unusual extranodal sites, including the central nervous system. [102] [103] [104] [105] [106] [107] The overwhelming majority of lymphomas associated with the hosts immune disruption are of B-cell origin, with the exception of some congenital immunodeficiency conditions, namely ataxia-teleangectasia, which carries a significant risk for T-cell malignancies. Studies in HIV-infected individuals and transplant recipients have determined that at least three factors related to immunodeficiency influence lymphomagenesis: (1) the degree of immunodeficiency; (2) the duration of immunodeficiency; and (3) the specific type of immunosuppression. [102] [103] [104] [105] [106] [107] These observations may explain why immunodeficiency-related lymphomas are highly heterogeneous and why different immunodeficiency syndromes lead to different types of lymphoma. This notion is best exemplified by the differences in the histologic and molecular spectrum of lymphomas arising in the context of AIDS as opposed to lymphoproliferative disorders developing in transplant recipients. [102] [103] [104] [105] [106] [107]

The precise mechanisms by which immunodeficiency causes lymphoma are complex and not well understood. In the context of AIDS, for example, the development of EBV-infected NHL has been partly explained by the fact that one of the AIDS-associated immunologic defects selectively impairs the immunosurveillance against EBV-infected B cells, which are present in increased numbers in the patients blood and lymphoid organs and may be responsible for minor clonal B-cell expansions that precede the neoplastic transformation. [108] [109] Another mechanism that has been deemed relevant in immunodeficiency-related NHL is the focal in situ failure of T-cell response. In fact, immunodeficiency-related B-cell NHL usually display a markedly lower representation of tumor infiltrating T-lymphocytes (T-TIL) when compared to NHL occurring in immunocompetent hosts. [110] The role of T-TIL in tumor containment is well-established and the magnitude of T-TIL response in B-cell NHL biopsies has been suggested to be an independent predictor of clinical outcome. [111]

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PATHOGENETIC HETEROGENEITY OF NON-HODGKIN LYMPHOMAS

The clinico-pathologic heterogeneity of NHL is reflected by a high degree of heterogeneity in the molecular pathogenesis of these disorders. This section provides an overview of the molecular pathways associated with B-cell and T-cell NHL classified according to the Revised European-American Lymphoma (REAL) classification of lymphoid neoplasia.[1] Whereas several B-cell NHL and some T-cell NHL types are known to be associated with well-defined genetic alterations in a consistent and selective fashion, the molecular pathogenesis of other lymphomas is less well defined. In some cases, the only available knowledge is based on karyotypic studies revealing recurrent

cytogenetic abnormalities not yet clarified at the molecular level. The following descriptions focus on those NHL categories for which molecular studies have provided a certain amount of knowledge (Table 66-1).

Small Lymphocytic Lymphoma/B-Cell Chronic Lymphocytic Leukemia

The molecular pathogenesis of small lymphocytic lymphoma/B-cell chronic lymphocytic leukemia (SLL/B-CLL) has been elucidated only in part. In particular, none of the cancer-related genes known to date has been shown to be associated consistently and selectively with SLL/B-CLL. [112] Based on karyotypic and deletion mapping studies, it is likely that the 13q14 chromosomal region may harbor a novel tumor suppressor gene that is involved in the pathogenesis of a high frequency of SLL/B-CLL cases.[45] [46] [47] In fact, analysis with sensitive molecular tools has revealed that deletions of 13q14 occur in approximately 60% of cases, but the relevant tumor-suppressor gene has not been identified.[45] [46] [47] Among known cancer-related genes, mutations of *p53* occur in 10% of the cases of SLL/B-CLL.[112] [113] The frequency of *p53* inactivation increases substantially in cases that have evolved to Richter syndrome, suggesting that *p53* may be at least partially responsible for this aggressive clinical transformation.[113] Despite initial suggestions, it is now well established that true cases of SLL/B-CLL (i.e., $CD5^+$, $CD23^+$ according to the REAL classification) are consistently devoid of *BCL*-1 and *BCL*-2 rearrangements.[1] [112] From a diagnostic standpoint, this notion has practical implications. For example, leukemic processes apparently resembling B-CLL, but harboring *BCL*-1 rearrangements, should be classified as MCL (see section later in this chapter).

In addition to del(13)(q14), chromosomal abnormalities associated with SLL/B-CLL include trisomy 12 and deletion of 6q. [114] [115] Trisomy 12 occurs in 3040% of cases and correlates with poor survival, whereas deletions of 6q define a subset of SLL/B-CLL cases displaying prolymphocytic features. [114] [115] The genes involved in trisomy 12 and del(6q) that lead to SLL/ B-CLL are not known.

Lymphoplasmacytoid Lymphoma

Approximately 50% of LPL are associated with a t(9;14)(p13;q32) translocation (Table 66-1).[116] The translocation appears to display a preferential clustering with LPL cases associated with Waldenstrom macroglobulinemia. The chromosomal breakpoints of t(9;14)(p13;q32) involve the Ig_H locus on chromosome 14q32, and, on chromosome 9p13, a genomic region containing the *PAX*-5 (Paired Homeobox-5) gene.[117] *PAX*-5 encodes a B-cell-specific transcription factor involved in the control of B-cell proliferation and differentiation. [118] Presumably, the juxtaposition of *PAX*-5 to the Ig_H locus in NHL carrying t(9;14)(p13;q32) leads to deregulated expression of the gene, thus contributing to tumor development. Apart from t(9;14)(p13;q32), no other genetic lesion has been detected at significant frequencies in LPL.

Mantle Cell Lymphoma

MCL is typically associated with t(11;14)(q13;q32) (Table 66-1).[111] The translocation juxtaposes the *BCL*-1 locus at 11q13 with the Ig_H locus at 14q32.[119] [120] [121] The t(11;14)(q13;q32) consistently leads to homotopic deregulation of *BCL*-1 (also known as *CCND*1 or *PRAD*1), a gene located in proximity to the breakpoint and encoding for cyclin D_1 , a member of the D-type G_1 cyclins, which regulate the early phases of cell cycle. [122] [123] [124] [125] [126] The consistent and selective clustering of *BCL*-1 overexpression with NHL carrying t(11;14)(q13;q32) strongly suggests that this gene is indeed the critical component of the translocation.

The precise contribution of cyclin D_1 to cell cycle regulation is still under investigation. Like other D-type cyclins, cyclin D_1 is thought to act primarily as a growth factor sensor integrating extracellular signals with the cell cycle machinery. [127] The pathogenetic role of *BCL*1 activation in human neoplasia is suggested by the ability of cyclin D_1 overexpression to transform cells in vitro and contribute to B-cell lymphomagenesis in transgenic mice. [128] [129] [130]

The application of strict phenotypic criteria to the classification of B-cell lymphoproliferations has revealed that the distribution of *BCL*-1 rearrangements, and consequent cyclin D_1 overexpression, are selectively restricted to MCL throughout the clinico-pathologic spectrum recognized by the REAL classification (70% of the cases).[1] [11] [131] [132] Because the diagnosis of MCL may be difficult on pure histologic grounds, the frequency and specificity of this genetic alteration provide excellent markers for diagnosis of MCL. In particular, because cyclin D_1 is not generally expressed by normal B cells, positive expression of cyclin D_1 in the context of a lymphoproliferative disorder has come to represent a highly specific marker for MCL in the clinical practice. [132] The precise identification of MCL among non-follicular small B-cell lymphomas is clinically relevant, since MCL is a far more aggressive disease and displays a significantly shorter survival than other histologically related forms.[133] [134]

Other genetic alterations may be also involved in MCL. Inactivation of *p53* occurs in a subset of cases and is a marker of poor prognosis.[135] Inactivation of *p16,* by deletion, mutation, or hypermethylation, is detectable in approximately half of the cases belonging to an aggressive MCL variant characterized by a blastoid cell morphology.[136]

FOLLICULAR LYMPHOMA

The genetic hallmark of FL is represented by chromosomal translocations of *BCL*-2 which are detected in 8090% of the cases independent of cytologic subtype. [1] Other genetic lesions may also occur, especially in FL cases that have undergone histologic progression to a high grade NHL.

Chromosomal Translocations Involving the *BCL*-2 Gene

Translocations involving 18q21 are the most common translocation in human lymphoid malignancies. Typically, these translocations juxtapose 18q21 to Ig_H [t(14;18)(q32;q21)], although occasionally 18q21 is translocated to Ig [t(2;18)(p11;q21)] or Ig [t(18;22)(q21;q11)] (Table 66-1). Breaks at 18q21 are present in virtually all FL and approximately 30% of B-DLCL. In t(14;18), the rearrangement joins the *BCL*-2 gene at its 3 untranslated region to an Ig_H J segment (Fig. 66-3).[137] [138] [139] [140] [141] Each of these translocations leads to deregulated expression of *BCL*-2, and, consequently, constitutively high levels of *BCL*-2 protein within the cells.[142] [143] Two main breakpoint cluster sites are known in t(14;18)(q32;q21). [137] [138] [139] [140] [141] Approximately 70% of the chromosome 18 breakpoints cluster within the major breakpoint region, conventionally known as MBR. The remaining cases usually break in the more 3 distant minor cluster region of *BCL*-2, known as mcr. Rare cases break in an alternative cluster region known as the 5 breakpoint cluster. [144]

The *BCL*-2 gene encodes a 26-kDa integral membrane protein that has been localized to mitochondria, smooth endoplasmic reticulum, and perinuclear membrane. [145] Whereas most proto-oncogenes of lymphoid noplasia directly enhance cell growth, *BCL*-2 has no ability to promote cell cycle progression or cell proliferation but rather controls the cellular apoptotic threshold by preventing programmed cell death. [145] [146] [147] [148] In normal cells, the topographic restriction of *BCL*-2 expression to GC zones of surviving B cells suggests that *BCL*-2 drives the emergence of long-lived memory B cells.[149] Indeed, *BCL*-2 transgenic animals show

Figure 66-3 Schematic representation of *BCL*-2 translocations. The germline configuration of the *BCL*-2 gene, mapping to 18q21, is shown in the upper panel of the figure. In its germline configuration, *BCL*-2 is composed of three exons with a large intron between exon 2 and exon 3. The coding region of the *BCL*-2 gene is indicated by red boxes, whereas non-coding exons (or portions of exons) are indicated by white boxes. The location of the major breakpoint region (MBR), where most *BCL*-2 breakpoints fall, is indicated by an arrow. The bottom panel of the figure depicts the molecular consequences of t(14;18)(q32;q21), which causes the juxtaposition of *BCL*-2 to the Ig_H locus. Both the genomic configuration of a representative translocated *BCL*-2 allele and the resulting cDNA are shown. The translocation breakpoint within *BCL*-2 most frequently involves the MBR (as in the case of the figure), although approximately 20% of cases break within a region located 3 of the MBR, and designated as minor cluster region (MCR; not shown in the figure). Within the Ig_H locus, the breakpoint involves J_H sequences. Notably, the *BCL*-2 coding sequence of the translocated *BCL*-2 allele is intact. The resulting cDNA is represented by the fusion of *BCL*-2 exons 13 (at the 5 side of the transcript) with J_H and C exons derived from Ig_H (at the 3 side of the transcript). Since the *BCL*-2 coding region is preserved, the hybrid *BCL*-2/Ig_H transcript gives rise to a wild type and normally sized *BCL*-2 protein.

markedly protracted secondary immune responses and an extended lifetime for memory B cells in the absence of antigen. [150]

BCL-2 is only one member of a family of apoptotic regulators, which also includes *BAX* and *BCL*-X.[145] It is now clear that *BCL*-2 exists as part of a high molecular weight complex generated through heterodimerization with *BAX.*[151] [152] The inherent ratio of *BCL*-2 to *BAX* determines the functional activity of *BCL*-2. When *BAX* is in excess, *BAX* homodimers dominate and cell death is accelerated; conversely, when *BCL*-2 is in excess, as in NHL carrying *BCL*-2 rearrangements, *BCL*-2/*BAX* heterodimers are the prevalent species and cell death is prevented. The fine biochemical pathways through which *BCL*-2 exerts its anti-apoptotic function involve activation of an antioxidant pathway at sites of free radical generation and/or regulation of endoplasmic reticulum-associated Ca^{++} fluxes.[153] [154]

The precise contribution of *BCL*-2 deregulation to FL development is complex. The translocation is thought to occur early in ontogeny in a pre-B cell representing a precursor of FL.[89] In contrast to other genetic lesions occurring in pre-B cells and leading to B-cell lineage acute lymphoblastic leukemia by providing a differentiative block of the target cells, *BCL*-2 rearrangements are permissive of B-cell maturation at the stage of $sIgM^+/sIgD^+$ B cell. The pathogenicity of *BCL*-2 lesions in the context of FL is substantiated by the ability of *BCL*-2-specific antisense oligonucleotides to inhibit the growth of human B-cell NHL bearing *BCL*-2 translocations.[155] In vivo, however, the *BCL*-2 transgene leads to a pattern of polyclonal hyperplasia of mature, long-lived B cells resting in G_0, which, despite morphologic similarities, contrasts with the consistent monoclonality of human FL. [156] Hence the view that *BCL*-2 activation is not sufficient for FL development, and that other genetic lesions or host factors are required. A strong candidate for a second event is chronic antigen stimulation and selection that would synergize with *BCL*-2 in driving FL expansion.[88] [89] With time, and similar to what is observed in the human disease, a fraction of *BCL*-2 transgenic mice progress to develop aggressive, clonal large cell lymphomas that have acquired additional genetic lesions. [157]

Other Genetic Lesions

Other proto-oncogenes involved in lymphomagenesis, such as c-*MYC, BCL*-6, or *BCL*-1, are not involved in FL. Deletions of chromosome 6 at 6q27 occur in approximately 20% of cases.[43] Over time, a significant fraction of FL evolves into an aggressive lymphoma with a diffuse large cell architecture. This histologic shift is almost consistently accompanied by the accumulation of *p53* mutation and deletion in addition to the pre-existent *BCL*-2 lesion.[158] In some cases, transformation is accompanied by inactivation of *p16* by deletion, mutation, or hypermethylation. [136] In very rare cases, the histologic progression of FL involves c-*MYC* rearrangements or 6q deletions.[158] [159]

Mucosa-Associated Lymphoid Tissue (MALT) NHL

Understanding of the molecular pathogenesis of MALT-NHL is still in its early stages. In the case of gastric MALT-NHL, the majority of tumors are associated with *Helicobacter pylori* (*H. pylori*) infection.[160] It has been suggested that gastric MALT-NHL may be dependent upon antigen stimulation by *H. pylori,* because malignant lymphoid cells respond to *H. pylori* antigens and the lymphoma may regress upon eradication of infection. [160] The potential role of antigen in MALT-NHL pathogenesis is further supported by the observation that MALT-NHL cells harbor the genotypic clue of antigen-experienced B cells, i.e., somatic hypermutation of Ig genes. [15] [161] [162] Whether the development of MALT-NHL arising in body sites other than the stomach is also dependent upon antigen stimulation and selection remains an open question. In this respect, it is remarkable that thyroid MALT-NHL is generally a sequela of Hashimoto thyroiditis, an autoimmune process causing the exposure of B cells to thyroid-derived autoantigens. [163]

Among genetic alterations commonly involved in other NHL types, only *BCL*-6 rearrangements and *p53* mutations have been detected in MALT-NHL, though at low frequency.[164] [165] Cytogenetic studies, however, have pointed to several abnormalities recurrently involved in these tumors. The most frequent of these abnormalities

are trisomy 3 and t(11;18).[166] [167] The genes implicated in these aberrations have not yet been identified.

B-Lineage Diffuse Large Cell Lymphoma

According to the REAL classification, the term B-lineage diffuse large cell lymphoma (B-DLCL) includes more than one disease entity, consistent with the striking heterogeneity of morphology, clinical presentation, and response to treatment. [1] Part of this heterogeneity may reflect the heterogeneous natural history of the disease, which may arise de novo or, alternatively, may develop from the histologic transformation of an FL. [1] Molecular investigations have confirmed the heterogeneity of B-DLCL at the genotypic level.[168] All B-DLCL represent clonal expansions of mature B cells reflecting features of GC centroblasts. [1] Because of the extreme complexity of the biological interactions regulating the physiologic proliferation and differentiation of mature B cells residing in the GC, it is likely that B-DLCL development is influenced by a multitude of events regulating GC function

and development. [4] [6] [7] As in most other types of NHL, however, it is now clearly established that development of B-DLCL is associated with specific molecular lesions involving proto-oncogenes and tumor suppressor genes.

Chromosomal Translocations Involving *BCL*-6

Cytogenetic studies of NHL have demonstrated that chromosomal alterations affecting band 3q27 are a frequent recurrent abnormality in B-DLCL (Table 66-1).[169] [170] These alterations are predominantly represented by reciprocal translocations between the 3q27 region and several alternative partner chromosomes, including, though not restricted to, the sites of the Ig genes at 14q32 (Ig_H), 2p11 (Ig), and 22q11 (Ig) (Fig. 66-4). [169] [170] The variability of the partner chromosomes juxtaposed to 3q27 in B-DLCL translocations suggests that these abnormalities belong to the group of promiscuous translocations, which involve a fixed chromosomal breakpoint, on one side, and, on the other side, different chromosomal partners in different cases.

Cloning of the 3q27 chromosomal breakpoints revealed that the *BCL*-6 gene is involved in the overwhelming majority of B-DLCL cases harboring 3q27 breaks irrespective of the partner chromosome participating in the translocation. [171] [172] [173] [174] [175] The *BCL*-6 gene is a transcriptional repressor belonging to a family of transcription factors containing zinc fingers, a protein sequence motif that is able to mediate the protein binding to specific DNA sites. [176] [177] The aminoterminal region of the *BCL*-6 protein contains a domain, termed *POZ,* which is homologous to domains found also in several other zinc-finger transcription factors. [178] Apparently, the *POZ* domain acts as a protein-protein interface implicated in homo/hetero-dimerization processes. [178] These structural features of the *BCL*-6 protein are consistent with functional studies indicating that *BCL*-6 can indeed function as a transcriptional repressor that inhibits the expression of genes carrying its specific DNA-binding motif.[179]

The pattern of *BCL*-6 protein expression in human tissues is highly specific and high levels are specifically found in B cells. [180] In particular, *BCL*-6 expression is topographically restricted to

Figure 66-4 Chromosomal sites implicated in der (3)(q27) and leading to rearrangement of *BCL*-6. The variability of the partner chromosome juxtaposed to 3q27 in B-DLCL suggests that these chromosomal abnormalities belong to the group of promiscuous translocations. The thick arrows point to some sites that are involved more frequently than others. (In many cases, indicated by ?der(3)(q27) in the figure, the partner chromosomal site cannot be identified with precision by conventional cytogenetics.)

Figure 66-5 Schematic representation of genetic lesions affecting the *BCL*-6 gene in B-DLCL. In its germline configuration, the *BCL*-6 gene is composed of 10 exons. The coding region of *BCL*-6 is indicated by color boxes, and the noncoding exons (or portions of exons) are indicated by white boxes. The physiologic *BCL*-6 promoter is indicated by an arrow. Rearrangements of *BCL*-6 by chromosomal translocation occur in approximately 35% of B-DLCL cases and cause substitution of the BCL-6 promoter with heterologous promoters derived from the chromosomal partner. The breakpoint sites (indicated by arrows) span the *BCL*-6 first exon and its adjacent sequences on both sides. The majority of breakpoints map to 3 sequences in the immediate vicinity of the *BCL*-6 first exon (indicated by thick arrow). In addition to rearrangements, *BCL*-6 may be also affected by small mutations clustering in the proximity of the *BCL*-6 first exon/first intron border.

the GC, where *BCL*-6 is expressed by both centroblasts and centrocytes, whereas expression of *BCL*-6 is absent in pre-GC B cells (virgin B cells) and post-GC B cells (memory B cells and plasma cells).[180] The observation that *BCL*-6 is expressed within the GC, but not before entry into or following exit from the GC, led investigators to postulate that *BCL*-6 may be needed for GC development and survival, whereas its downregulation may be necessary for further differentiation of B cells. [180] [181]

Clarification of the precise role of *BCL*-6 in physiologic immune processes has been further clarified by knock-out animal models carrying biallelically disrupted *BCL*-6 genes.[182] [183] Mice carrying the *BCL*-$6^{/}$ phenotype consistently fail to form GC. Consistent with the lack of GC formation, *BCL*-$6^{/}$ mice also display impaired T-cell dependent antigen-specific IgG responses. Overall, these animal models unequivocally demonstrate that *BCL*-6 is a key regulator of GC formation and B-cell immune response.

Chromosomal translocations involving band 3q27 truncate the *BCL*-6 gene within its 5 flanking region, within the first exon or within the first intron (Fig. 66-5), making these alterations readily detectable as rearrangements by Southern blot hybridization analysis of tumor DNA. [171] [172] [173] [174] [175] In conventional molecular assays, *BCL*-6 rearrangements are detectable in 35% of cases of B-DLCL and in a small fraction of FL. [165] [184] [185] Conversely, with the exception of a tiny fraction of FL and MALT-NHL, *BCL*-6 rearrangements are consistently absent in all other types of lymphoid neoplasms. [184] [185] The coding domain of the *BCL*-6 gene is left intact in all cases displaying *BCL*-6 rearrangements, whereas the 5 regulatory sequences, which contain the *BCL*-6 promoter, are either truncated or, alternatively, completely removed.[171] [172] [173] [174] [175] In all *BCL*-6 rearrangements, the entire coding sequence of *BCL*-6 is juxtaposed downstream to heterologous sequences which, based on cytogenetic data, may originate from different chromosomal sites in different patients (Fig. 66-4). The common functional consequence of *BCL*-6 translocations is the juxtaposition of heterologous promoters to the *BCL*-6 coding domain, a mechanism called promoter substitution (Fig. 66-5). [186] [187] The substitution of the *BCL*-6 promoter by heterologous regulatory sequences causes deregulated *BCL*-6 expression in B-DLCL carrying *BCL*-6 rearrangements. One feature shared by

the heterologous promoters linked to rearranged *BCL*-6 alleles is that they are physiologically active in normal B cells and are not downregulated during the late stages of B-cell differentiation. [181] [186] [187] Thus, *BCL*-6 rearrangements may prevent downregulation of *BCL*-6 and, in turn, block the differentiation of GC B cells toward plasma cells. According to this model, B-DLCL cells carrying *BCL*-6 rearrangements would thus be frozen at the stage of GC cells.

In addition to rearrangements, the *BCL*-6 gene may be altered by other mechanisms in B-DLCL as well as in other types of B-cell NHL. In approximately 70% of B-DLCL and 50% of FL, the *BCL*-6 gene is affected by multiple, often biallelic, mutations that selectively cluster within the 5 non-coding regions of the gene (Fig. 66-5).[38] [188] The DNA sequences most frequently affected by mutations lie in close proximity to the *BCL*-6 promoter region and overlap with the major cluster of chromosomal breaks at 3q27, suggesting that mutations and rearrangements may be selected by their ability to alter the same region which conceivably regulates the normal expression of *BCL*-6. The cumulative frequency of mutations and rearrangements approaches 100% of B-DLCL cases, indicating that structural alterations of the 5 non-coding regions of *BCL*-6 might be a sine qua non for the development of these lymphomas.[38] [188]

Other Genetic Lesions in B-DLCL

Several other genetic lesions have been detected in B-DLCL. In contrast to *BCL*-6 rearrangements, however, these additional lesions are either not specific for B-DLCL, being detected also in other NHL types, or occur at low frequencies in B-DLCL cases. Approximately one third of B-DLCL cases display chromosomal rearrangements of *BCL*-2.[24] [165] [189] These translocations are entirely similar to the ones associated with FL and lead to the deregulated expression of *BCL*-2. [137] [138] [139] [140] [141] These alterations appear to be mutually exclusive with *BCL*-6 rearrangements, and tend to associate with B-DLCL cases that are derived from the histologic transformation of FL.[158] [165] *REL,* a member of the NF-kB/REL family of transcription factors, also appears to be involved in the pathogenesis of B-DLCL. [34] Activation of *REL* occurs through gene amplification and appears to be a relatively frequent event, occurring in 20% of B-DLCL cases, preferentially in those with

extranodal involvement.[34]

Among tumor suppressor genes, inactivation of *p53* frequently associates with cases resulting from the histologic transformation of FL. [158] In these cases, mutations of *p53* are acquired during the transformation from FL to B-DLCL. [168] Given the role of *p53* in controlling cell proliferation and genomic stability, it is likely that disruption of normal *p53* functions may contribute to tumor progression directly, by providing FL cells with a high proliferative rate, or indirectly, by allowing the accumulation of additional genetic lesions. Deletions of the long arm of chromosome 6 are also frequently detected in B-DLCL, although the gene(s) involved is not known. [43]

Based on the knowledge outlined here, a pathogenetic model for explaining B-DLCL heterogeneity is proposed in Figure 66-6 .

Burkitt Lymphoma

Burkitt lymphoma (BL) is classified into two main epidemiologic variants, including sporadic BL (sBL) and endemic BL (eBL). [1] Cases of sBL occur in North America and Europe, whereas cases of eBL occur primarily in Equatorial Africa and Papua New Guinea. Some BL cases present with massive involvement of the bone marrow and peripheral blood. Such cases, though traditionally termed acute lymphoblastic leukemia L3-type (L3-ALL), should be classified as BL because of their biologic features and their response to therapy. In 1994, the REAL classification of B-cell neoplasia formally acknowledged

Figure 66-6 Model of molecular pathways in B-DLCL development. Three main pathogenetic pathways may be recognized in B-DLCL. The first two molecular pathways are designated de novo pathways since, in these instances, B-DLCL develops without a pre-existent follicular lymphoma. One de novo pathway implicates the *BCL*-6 gene and occurs in approximately 35% of B-DLCL. The second de novo pathway involves presently unknown genetic lesions (indicated by the question mark), although some cases may harbor alterations of *REL.* The third pathway, designated as a transformation pathway, implicates the transformation of a pre-existing follicular NHL to a B-DLCL histology. Cases of B-DLCL belonging to this pathway harbor rearrangements of *BCL*-2 and mutations of *p53.* Whereas the *BCL*-2 rearrangement is already present in the follicular NHL phase, *p53* mutations are gained during histologic transformation.

that L3-ALL and classical BL represent different manifestations of the same disease. [1]

Despite the epidemiologic and clinical heterogeneity of BL, all BL variants, including sBL, eBL, and L3-ALL, consistently share a common genetic background represented by chromosomal translocations between c-*MYC* and one of the Ig loci. Other genetic lesions associate at variable frequencies with cases of BL and include infection of the tumor clone by EBV, inactivation of *p53* and *p16,* and mutations of *BCL*-6 5 noncoding regions.

Breaks at 8q24 and c-*MYC*

Chromosomal breaks at 8q24 are found in 100% of sBL, eBL, and L3-ALL (Table 66-1).[1] Historically, translocations of 8q24 have provided the first example of the involvement of proto-oncogenes in tumor-associated chromosomal abnormalities and have thus constituted the paradigm for the study of other chromosomal translocations involving antigen receptor loci. [190] In BL, all 8q24 breaks lead to a common final consequence, deregulated expression of the c-*MYC* proto-oncogene.[190] [191] [192] [193] [194] [195] Depending on the Ig locus involved, the c-*MYC* gene may be involved in three distinct translocations. In 80% of cases the translocation involves Ig_H , leading to t(8;14)(q24;q32) (Fig. 66-7).[190] [191] [192] [193] [194] [195] In the remaining 20% of cases, c-*MYC* juxtaposes either to Ig , leading to t(2;8)(p11;q24) (15% of cases), or to Ig , leading to t(8;22) (q24;q11) (5% of cases). Breaks at 8q24 selectively cluster with all BL variants throughout the spectrum of B-cell neoplasms recognized by the REAL classification. [1]

The product of the c-*MYC* proto-oncogene is a ubiquitously expressed nuclear bHLH-LZIP phosphoprotein that functions as a transcriptional regulator controlling cell proliferation, differentiation, and apoptosis. [190] [196] Expression of c-*MYC* is rapidly induced in quiescent cells upon mitogenic induction, suggesting that c-*MYC* plays a role in mediating the transition from quiescence to proliferation. [196] In addition to mediating cell

Figure 66-7 Schematic representation of chromosomal translocations involving the c-*MYC* gene. In its germline configuration (upper panel), c-*MYC* is composed of three exons. The coding region of c-*MYC* is indicated by red boxes, and noncoding exons (or portions of exons) are indicated by white boxes. The c-*MYC* promoters are indicated by arrows. The molecular consequences of the most frequent c-*MYC* translocation, i.e., t(8;14), are depicted in the middle and lower panels. Two molecular subtypes of t(8;14) are recognized, which tend to associate with sBL (middle panel) and eBL (lower panel), respectively. For each molecular subtype of t(8;14), both the genomic configuration and the resulting cDNA of a representative translocated c-*MYC* allele are shown. In the case of t(8;14) of sBL (middle panel), the chromosome 8 breakpoint involves sequences within c-*MYC,* which is deprived of its exon 1. Since the physiologic promoters are removed by the translocation, transcription is driven by an otherwise silent promoter located within c-*MYC* intron 1 (indicated by an arrow in the figure). On chromosome 14, the breakpoint maps in the proximity of the switch (S) region. Notably, the translocation leaves intact the gross configuration of the coding sequence of c-*MYC.* However, at the nucleotide level, c-*MYC* translocated alleles frequently harbor point mutations within the exon 2 coding sequence, leading to amino acid substitutions in the c-*MYC* protein. The cDNA encoded by translocated c-*MYC* alleles includes the exons 2 and 3 of the gene, as well as an abnormally transcribed sequence of intron 1 starting from the novel transcriptional initiation site within c-*MYC* intron 1. Because the c-*MYC* coding region is intact, a normally sized c-*MYC* protein is translated by t(8;14) of sBL. In the case of t(8;14) of eBL (lower panel), the c-*MYC* breakpoint involves sequences on chromosome 8 at an undefined distance (>100 kb) 5 to c-*MYC* and sequences on chromosome 14 within or in proximity to the Ig J_H region. The internal genomic configuration of the translocated c-*MYC* allele is thus apparently preserved. However, c-*MYC* alleles involved by t(8;14) of eBL consistently harbor small mutations mapping to the exon 1/intron 1 border, where c-*MYC* regulatory regions are located. In addition, point mutations within exon 2 coding sequence are also present. The cDNA transcribed by translocated c-*MYC* alleles of eBL includes c-*MYC* exons 13 and gives rise to a normally sized c-*MYC* protein.

proliferation, c-*MYC* is also implicated in blocking differentiation. [196] In the absence of a supportive microenvironment providing adequate concentrations of growth stimuli, however, proliferation and/or block of differentiation are replaced by cellular apoptosis. [197] [198] [199] [200] The biochemical mechanisms by which c-*MYC* achieves its various functions have been clarified to a certain extent. In physiologic in vivo settings, c-*MYC* is mainly engaged in heterodimeric complexes with the related protein *MAX* which stimulates transcription and cell proliferation. [201] [202] [203] [204] [205] [206] In addition to c-*MYC, MAX* can also form homodimers as well as heterodimers with *MAD* and *MXI*1, two bHLH-LZIP proteins that act as negative regulators of transcription. [207] [208] Because the levels of *MAX* tend to be stable throughout the cell cycle, the ratio of c-*MYC*/*MAX* heterodimers is controlled by the relative abundance of c-*MYC, MAD,* and *MXI*1.[201] [202] [203] [204] [205] [206] [207] [208] As the ratio of c-*MYC* to *MAD* or *MXI*1 varies, the promoter activity of target genes is expected to be modulated in either a positive (when c-*MYC* levels are high) or negative (when *MAD* or *MXI*1 levels are high) fashion. Therefore, in lymphoid tumors associated with c-*MYC* deregulation, it is conceivable that constitutive expression of c-*MYC* leads to the predominance of *MYC*/*MAX* heterodimers, thus inducing positive growth regulation.

Although fairly homogeneous at the microscopic level, c-*MYC* translocations display a high degree of heterogeneity when dissected at the molecular level (Fig. 66-7). The t(8;14) breakpoints are located 5 and centromeric to c-*MYC,* whereas they map 3 to c-*MYC* in t(2;8) and t(8;22).[190] [191] [192] [193] [194] [195] In the instance of t(8;14), the exact location of the breakpoint sites of chromosomes 8 and 14 contributes further heterogeneity. [209] [210] In eBL, the breakpoint sites on chromosome 8 preferentially involve sequences at an undefined distance (>200 kb) 5 to c-*MYC,* whereas on chromosome 14, the breakpoints fall within or in proximity to the Ig JH region. In sBL, the t(8;14) breakpoints fall within or immediately 5 (<3 kb) to c-*MYC* on chromosome 8 and within the Ig S regions on chromosome 14.

The common functional effect of t(8;14), t(2;8), and t(8;22) is that c-*MYC* translocated alleles undergo constitutive expression in tumor cells, whereas under physiologic conditions c-*MYC* levels are tightly regulated during B-cell proliferation and differentiation. [211] [212] [213] Chromosomal translocations cause c-*MYC* deregulation by at least two distinct mechanisms. First, translocated c-*MYC* alleles are juxtaposed to heterologous regulatory elements derived from Ig loci. [190] [191] [192] [193] [194] [195] Second, the 5 regulatory regions of c-*MYC* are consistently affected by structural alterations

that are supposed to modify their responsiveness to cellular factors regulating c-*MYC* expression (Fig. 66-7). In the case of sBL and L3-ALL, the t(8;14) chromosomal translocation depletes the c-*MYC* gene of its first exon.[190] Conversely, in cases carrying breakpoints located either 5 or 3 to the c-*MYC* gene, such as eBL harboring t(8;14) and all cases carrying t(2;8) or t(8;22), a 400-bp region spanning the first exon/first intron junction, and containing several potentially relevant regulatory domains, is consistently mutated in translocated c-*MYC* alleles.[37]

In addition to transcriptional deregulation, oncogenic conversion of c-*MYC* is also thought to stem from amino acid substitutions in c-*MYC* exon 2 affecting the N-terminal transcriptional activation domain of the gene (Fig. 66-7).[36] [214] The high prevalence of these mutations suggests a biologic role for these alterations in lymphomagenesis. Although under normal conditions the activity of the c-*MYC* transactivation domain is suppressed by protein-protein interactions with the pRB-related protein p107, c-*MYC* proteins carrying exon 2 mutations are able to escape the p107-mediated modulation. [215] [216] The mechanism of resistance of mutant c-*MYC* proteins to p107-mediated suppression is not known, although it is not caused by the disruption of the physical interactions between c-*MYC* and p107.[216] Rather, c-*MYC* exon 2 mutations may confer resistance to p107-mediated phosphorylation, which is essential for the p107 suppression effect. [216] [217]

Several lines of experimental evidence document that the deregulated expression of c-*MYC* can influence the growth of B cells in vitro and in vivo. In vitro, the expression of c-*MYC* oncogenes transfected into EBV immortalized human B cells, a potential natural target for c-*MYC* activation in EBV positive BL, leads to their malignant transformation.[28] In addition, antisense oligonucleotides directed against translocated c-*MYC* alleles are able to revert the tumorigenicity of BL. [218] [219] In vivo, the targeted expression of c-*MYC* oncogenes in the B-cell lineage of transgenic mice leads to the development of B-cell malignancies at a high rate. [27]

Other Genetic Lesions in Burkitt Lymphoma

In addition to c-*MYC* translocations, the molecular pathogenesis of BL also involves infection of the tumor clone by EBV, inactivation of the *p53* and *p16* tumor suppressor genes, mutations of the 5 noncoding regions of *BCL*-6, and deletions of 6q. Infection by EBV occurs in virtually all cases of eBL and in approximately 30% of cases of sBL.[49] [50] [51] [52] The consistent monoclonality of EBV infection in BL, as assessed by molecular analysis of EBV terminal repeats, suggests that infection precedes clonal expansion of the tumor, consistent with a pathogenetic role of the virus. [49] [50] [51] [52] [54] Notably, however, BL cells fail to express the EBV transforming antigens LMP-1 and EBNA-2, rendering the role of EBV infection unclear. [50] [52] Inactivation of *p53* is detected in approximately 3040% of BL cases, independent of their geographic origin or the presence of EBV infection. [113] Inactivation of *p16* occurs in 3040% of BL through mutation, deletion or hypermethylation. [220] Mutations of the *BCL*-6 5 noncoding regions occur in approximately 40% of BL, confirming the histogenetic derivation of the disease from GC B cells. [221] As in many other NHL types, BL is also associated with deletions of 6q.[43] In vitro models indicate that accumulation of genetic lesions follows a temporal order in BL pathogenesis. [222] Thus, translocations of c-*MYC* and mutations of *BCL*-6 represent early events, whereas *p53* mutations occur late in BL pathogenesis. [222]

Immunodeficiency-Related NHL

AIDS-Related Lymphomas

AIDS-related non-Hodgkin lymphomas (AIDS-NHL) are invariably derived from B cells and are primarily classified into three main clinicopathologic categories, including AIDS-related Burkitt lymphoma (AIDS-BL), AIDS-related diffuse large cell lymphoma (AIDS-DLCL), and AIDS-related primary effusion lymphoma (Table 66-2).[86] [103] [104] [107] Based on the presence or absence of immunoblastic features, AIDS-DLCL may be further categorized as large noncleaved cell lymphoma (LNCLL) or immunoblastic lymphoma plasmacytoid (IBLP).[86] [103] [104] [107] Based on the site of origin, AIDS-NHL are generally grouped into systemic AIDS-NHL and AIDS-related primary central nervous system lymphoma (AIDS-PCNSL).[86] [103] [104] [107] Systemic AIDS-NHL may be either AIDS-DLCL or AIDS-BL. Conversely, AIDS-PCNSL display a uniform morphology consistent with an architecture of diffuse large cells.

The different categories of AIDS-NHL are associated with distinctive molecular pathways (Table 66-2).[86] [104] [107] [223] Cases of AIDS-BL consistently display activation of c-*MYC* by chromosomal translocations that show structural similarities to those found in sBL patients. [53] Rearrangements of *BCL*-6 are consistently absent in this AIDS-NHL type. [224] AIDS-BL also frequently harbor mutations of *p53* (60%), mutations of *BCL*-6 5 noncoding regions (60%), and, in 30% of cases, infection of the tumor clone by EBV.[53] [188] The EBV-encoded antigens LMP-1 and EBNA-2 are not expressed by AIDS-BL.[55] [225] In addition to genetic lesions and EBV infection, stimulation and selection by antigens, frequently represented by autoantigens, appear to be a prominent feature of AIDS-BL. [90] [91] [107] [223] One puzzling feature of AIDS-BL is that AIDS is the only immunodeficiency syndrome associated with this type of lymphoma. In fact, patients affected by other types of immunodeficiency syndromes, such as congenital or iatrogenically induced immunodeficiencies, do have a high relative risk of NHL development, but these NHL

TABLE 66-2 -- Clinical and Biological Heterogeneity of AIDS-NHL

Histology[a]	Occurrence[b]	Immunodeficit	Predominant Genetic Lesion	EBV		HHV-8 Infection
				Infection	LMP-1 Status	
AIDS-BL	Early	Mild	c-*MYC*	30%		
AIDS-DLCL						
AIDS-LNCCL	Late	Marked	*BCL*-6	30%		
AIDS-IBLP	Late	Marked	?	90%	+	
AIDS-PCNSL	Late	Very marked	?	100%	+	
AIDS-PEL	?	Marked	?	90%		100%

[a]AIDS-BL, AIDS-related Burkitt lymphoma; AIDS-DLCL, AIDS-related diffuse large cell lymphoma; AIDS-LNCCL, AIDS-related large noncleaved cell lymphoma; AIDS-IBLP, AIDS-related immunoblastic lymphoma plasmacytoid; AIDS-PCNSL, AIDS-related primary central nervous system lymphoma; AIDS-PEL, AIDS-related primary effusion lymphoma.
[b]Time of lymphoma occurrence since HIV infection.

are consistently B-DLCL or PCNSL, and not BL. In this respect, it is intriguing that AIDS-BL may also develop in the presence of relatively normal numbers of CD4 cells.

AIDS-DLCL display several genotypic differences compared to AIDS-BL.[86] [104] [107] [223] First, the most frequent genetic alteration detected in AIDS-DLCL is infection by EBV, which occurs in approximately 6070% of the cases and associates frequently, though not always, with expression of LMP-1. [53] [55] [225] Second, AIDS-DLCL displays rearrangements of *BCL*-6 in 20% of cases.[224] Mutations of *BCL*-6 5 noncoding regions occur in 70% of AIDS-DLCL.[188] AIDS-DLCL can be segregated into two distinct histogenetic categories based on the expression pattern of the *BCL*-6 protein: the CD138/syndecan-1 antigen and the EBV-encoded LMP-1. [55] [226] AIDS-DLCL associated with the *BCL*-6^{+} /syndecan-1 /LMP-1 phenotype tend to display a LNCCL morphology and closely reflect the phenotype of GC B cells. [55] [226] Conversely, *BCL*-$6^{/}$ syndecan-1^{+} /LMP-1^{+} AIDS-DLCL are morphologically IBLP and reflect a post-GC stage of B-cell differentiation. [55] [226]

All AIDS-PCNSL harbor EBV infection.[56] [227] However, only a fraction of AIDS-PCNSL, namely those with IBLP morphology, express the LMP-1 transforming protein of EBV.[56] Both systemic AIDS-DLCL and AIDS-PCNSL may be divided into two phenotypic categories, based on the expression pattern of *BCL*-6, syndecan-1, and LMP-1.[56] Apart from EBV infection, AIDS-PCNSL are also characterized by mutations of *BCL*-6 in 60% of cases.[56] Although some reports have suggested that HHV-8 may be related to PCNSL pathogenesis in immunocompromised patients, extensive analysis of AIDS-PCNSL has unequivocally ruled out this hypothesis. [56] [228]

The last type of AIDS-NHL that has been characterized at the molecular level is primary effusion lymphoma (PEL), also termed body cavity-based lymphoma. [59] [60] [61] [62] [229] This is a novel lymphoma characterized by HHV-8 infection and clinically presenting as effusions in the serosal cavities of the body (pleura, pericardium, and peritoneum) in the absence of solid tumor masses.[59] [60] [61] [62] [229] PEL is consistently derived from B cells that reflect a pre-terminal stage of B-cell differentiation. [55] [60] [61] [62] [229] [230] Infection of the tumor clone by HHV-8 occurs in 100% of cases and is a sine qua non for diagnosis of the disease. [59] [60] [61] [104] [107] [223] [229] In addition to HHV-8, cases of PEL frequently carry co-infection of the tumor clone by EBV. [55] [60] [61] [104] [107] [223] [229]

Post-transplant Lymphoproliferative Disorders

Post-transplant lymphoproliferative disorders (PTLPD) are a major cause of morbidity and mortality among transplant recipients. [102] [103] [106] [231] [232] The occurrence of

PTLPD depends substantially upon the type, degree, and duration of immunosuppressive therapy administered. PTLPD frequently involve multiple sites in the same patient, are characterized by frequent extranodal location, and have a rapid course and a high mortality rate. [231] [232] [233] [234] [235] The precise histologic classification of PTLPD is a matter of current debate.[235] It is generally agreed that PTLPD are highly pleomorphic proliferations of large cells frequently resembling immunoblasts. [105] [106] [233] [235] Upon reduction of immunosuppressive therapy, a fraction of PTLPD will regress, questioning the truly malignant nature of the disease. [105] [106] [233] [235]

Because PTLPD are virtually always derived from B cells, immunogenotypic studies of Ig genes have allowed the definition of their clonality status. [106] [233] [234] [236] Many PTLPD are monoclonal B-cell proliferations. Multiple, separate PTLPD from different sites in the same patient, even when arising in the same organ, may display different Ig gene rearrangement patterns. A fraction of PTLPD is constituted of oligo- or polyclonal proliferations throughout the entire course of the disease.[233] [234] [235] [236] Molecular genetic analysis has established that PTLPD of organ transplant recipients are generally of host origin. [237] Conversely, PTLPD arising after allogenic bone marrow transplantation originate from donor cells. [106] Defining the donor-versus-recipient origin of PTLPD is a fundamental requirement in view of the progressively larger clinical use of adoptive immunotherapy strategies based on infusion of cytotoxic T-lymphocytes directed against EBV antigens expressed by PTLPD.[238]

The molecular pathogenesis of PTLPD is poorly understood. In virtually all PTLPD, there is evidence of EBV infection as demonstrated by expression of LMP-1 and EBNA-2.[106] [233] [234] [236] Monoclonal PTLPD generally display a monoclonal pattern of EBV infection, whereas oligo- and polyclonal PTLPD are associated with polyclonal infection by the virus. Mutations of *p53* and N-*RAS,* as well as rearrangements of *BCL*-6 and c-*MYC,* have been reported in scattered cases of PTLPD characterized by a monoclonal proliferation pattern, but are absent in oligo- and polyclonal PTLPD. [106] [233] [234] [236] Overall, oligo- and polyclonal PTLPD are generally regarded as lymphoproliferative disorders solely driven by EBV in the context of immunodeficiency. The genotypic nature of PTLPD has been proposed to influence the disease prognosis.[106] [233] [234] [236] Monoclonal PTLPD cases containing genetic lesions other than EBV infection usually progress in spite of reduced immunosuppression. Conversely, oligo- or polyclonal PTLPD devoid of genetic lesions may regress by simply decreasing the level of immunosuppression.

T-Cell Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) is a specific category of T-cell NHL composed of large pleomorphic cells that usually express the CD30 antigen. [1] ALCL is characterized by frequent cutaneous and extranodal involvement. [1] Conventional karyotypic analyses of ALCL cases have shown a unique translocation involving bands 2p23 and 5q35 in a substantial fraction of ALCL cases. [21] The cloning of the t(2;5)(p23;q35) translocation has demonstrated that it involves the fusion of the nucleophosmin/B23 (*NPM*) gene on 5q35 to a novel anaplastic lymphoma kinase (*ALK*) on 2p23.[239] As a consequence of this translocation, the *NPM* and *ALK* genes are fused to form a chimeric transcript that encodes a hybrid protein (p80) in which the aminoterminus of *NPM* is linked to the catalytic domain of *ALK*.[239] Two distinct oncogenic effects are thought to be caused by the t(2;5) translocation. First, the *ALK* gene, which is not physiologically expressed in normal T lymphocytes, undergoes heterologous expression in lymphoma cells, conceivably because of its juxtaposition to the promoter sequences of *NPM,* which are physiologically expressed in T cells. Second, based on the activation model of other tyrosine kinase oncogenes, one would predict that the truncated *ALK* constitutively phosphorylates intracellular targets to trigger malignant transformation.

The pathogenic role of *NPM/ALK* rearrangements is supported by studies in vitro and in vivo. First, overexpression of p80 in in vitro models induces neoplastic transformation of target cells, substantiating the notion that the p80 kinase is in fact aberrantly activated. [29] Second, retroviral-mediated gene transfer of *NPM/ALK* in vivo causes T-cell lymphoid malignancies in mice. [30] In such animal models, *NPM/ALK* selectively transforms lymphoid cells of T-cell origin, whereas the growth properties of other hematopoietic cells remain unaffected. [30]

The distribution of *NPM/ALK* rearrangements throughout the spectrum of NHL is highly selective, being virtually restricted to T-cell lineage ALCL. [240] [241] [242] Within this category, *NPM/ALK* rearrangements seem to be preferentially associated with cases occurring during childhood (88% positivity), although they are also detected in a large fraction of adult cases (60%). [240] [241] [242] Despite initial suggestions, *NPM/ALK* rearrangements are consistently negative in HD. [240] [242]

Adult T-Cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma (ATLL) is associated with HTLV-I infection of the tumor clone in 100% of cases. [72] [73] The

period between infection and onset of clinical disease is generally quite long, varying between 10 to 40 years. [72] [73] The pathogenetic effect of HTLV-I in ATLL appears to be due to the viral production of a *trans*-regulatory protein (*tax*) that markedly increases expression of all viral gene products and transcriptionally activates the expression of certain host genes, including IL-2 and the chain of the IL-2 receptor. [72] [73] [74] [75] [76] [77] [78] [79] [80] Indeed a property of ATLL cells is the constitutive high levels of expression of IL-2 receptors, which are known to mediate growth signals to T cells. [80]

The consistent monoclonality of ATLL, the long latency period between HTLV-I infection and the development of disease, as well as the small proportion of HTLV-I-infected individuals developing ATLL (<5% lifetime risk), have led to the notion that HTLV-I alone is not sufficient for ATLL development and that other genetic alterations may be required for the biogenesis of this disease. [72] [73] This concept has been validated by the observation that ATLL cells frequently carry *p53* mutations as well as a number of other cytogenetic abnormalities.[85] [243] On these grounds, it has been proposed that tumor development begins with an initial phase of polyclonal T-cell proliferation, conceivably mediated by the *tax*-induced overexpression of IL-2 and IL-2R. This proliferative process then predisposes the infected T-cell population to the accumulation of additional genetic events leading to the monoclonal outgrowth typical of ATLL patients.

T-Cell Prolymphocytic Leukemia

T-cell prolymphocytic leukemia (T-PLL) frequently carries cytogenetic abnormalities of chromosome 11, the most common abnormalities being monosomy 11, partial or terminal deletions of 11q, and unbalanced translocations involving the 11q arm. [21] The gene relevant to these abnormalities has been recently identified and has been shown to correspond to *ATM* (for *ataxia teleangectasia mutated*), a gene that is also responsible for the hereditary disorder ataxia-teleangectasia. [41] [244] [245] Whereas *ATM* is mutated in the germline of ataxia-teleangectasia patients, it is altered somatically in cases of T-PLL. [244] [245] Mutations of *ATM* in T-PLL associate with deletion of the other allele and lead to the absence, premature truncation, or alteration of the *ATM* gene product, consistent with the inactivation model of tumor suppressor genes.[244] [245] Substantial evidence suggests that *ATM* might be involved in cell cycle regulation and DNA repair, which in fact have been shown to be defective in cells with biallelic *ATM* inactivation.[41]

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CLINICAL IMPLICATIONS OF GENETIC LESIONS OF NON-HODGKIN LYMPHOMAS

The use of genetic lesions as clinical tools has been well-established in at least three settings. First, genetic lesions integrate morphologic and immunohistochemical diagnosis of lymphoma. Second, genetic lesions may allow follow-up of minimal residual disease by highly specific and highly sensitive technologies. Third, genetic lesions may provide the rationale for designing novel therapeutic strategies directly aimed at correcting the molecular defect of the lymphoma.

Genetic lesions may serve as markers for NHL diagnosis because of their selective association with specific clinicopathologic categories of lymphoma. Examples include the selective association between rearrangements of *BCL*-1/cyclin D_1 and MCL, *BCL*-6 and B-DLCL, c-*MYC* and BL, and *NPM/ALK* and $CD30^+$ T-cell ALCL, as well as HHV-8 infection and PEL. [1] [229] The use of genetic markers may be particularly valuable in classifying traditionally difficult cases displaying doubtful morphologic features. Thus, for example, the detection of *BCL*-1/cyclin D_1 activation in non-follicular small-cell NHL is considered the most specific indicator in the diagnosis of MCL. [132] Similarly, infection by HHV-8 is the most reliable marker of PEL in the differential diagnosis of lymphomatous effusions. [229] The widespread use of molecular diagnostics for NHL is likely to substantially refine classification of these disorders. Ideally, NHL should be grouped into homogeneous categories displaying the same pattern of molecular lesions. At the same time, some genetically distinct NHL categories may represent distinct diseases that may require distinct therapeutic options. The REAL classification represents a major advance toward this aim, and all future proposals for NHL classification should consider tumor genotype among the leading criteria for diagnosis. [1] Furthermore, it is conceivable that advanced cytogenetic techniques, such as fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH), may reveal novel genetic alterations of lymphoma, which will help further refine future lymphoma classifications.

The second well-established application of molecular diagnosis to NHL management relies on the fact that genetic lesions constitute a specific and sensitive marker for evaluation of minimal residual disease. At present, the availability of PCR strategies for large-scale analysis of minimal residual disease is restricted to rearrangements of *BCL*-1, *BCL*-2, and *NPM/ALK.* [246] The development of routine PCR assays for rearrangements of *BCL*-6 and c-*MYC* is in progress. Several factors account for the difficulties in developing PCR assays for minimal residual disease evaluation in NHL as compared to acute leukemias, such as the lack of chromosomal clustering in many NHL translocations (e.g., c-*MYC* translocations) and/or the heterogeneity of chromosomal partners juxtaposing the involved oncogene (e.g., *BCL*-6 translocations).

In addition to the contribution to the diagnosis and molecular follow-up of lymphomas, the study of the molecular pathogenesis of these disorders may provide other potential applications in the future. One such application lies on the fact that subtle differences in the tumor genotype may influence the prognosis of NHL belonging to the same histologic category. [114] [247] [248] A further implication is the possibility of developing therapeutic strategies targeted at the very genetic lesions that are responsible for tumor development. Such therapy should, by definition, be largely specific for the lymphoma cells, and hence devoid of the major side effects presently encountered with antineoplastic therapy. Preliminary reports from in vitro studies do substantiate the validity of gene targeting in controlling the tumorigenic behavior of NHL. [155] [218] [249]

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Chapter 67 - Pathology and Histogenesis of Hodgkin Disease

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The nature of the Reed-Sternberg (RS) cell and the cellular origin of Hodgkin disease (HD) have been an enigma for more than 150 years. Some authorities have doubted that HD represents a malignant process, in part because of its variable clinical behavior and multiple histologic types. Recent progress in immunology, cell culture, and the molecular biology of isolated RS cells has helped identify the RS cell as a part of the malignant cell population. The remaining cell types, which comprise the bulk of the HD lesion, appear to represent a combination of RS cell precursors, host inflammatory cells, and cells recruited to the site in response to cytokines liberated by RS cells. Somehow the mixture of these cell types and stromal elements determines the natural history of HD, thereby influencing the treatment and prognosis of the disease. This chapter re-evaluates the

morphologic hallmarks of HD in light of new knowledge about the RS cell and its cellular immunology. Immunopathologic methods for the more accurate diagnosis of HD are emphasized. Recent information regarding the role of Epstein-Barr virus (EBV) in HD is presented. Finally, cytogenetic abnormalities and non-Hodgkin lymphomas (NHL) associated with HD are discussed in the context of the histogenesis of HD.

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HISTOPATHOLOGY

The histopathology of HD is more complex and variable than that of the NHL. To simplify the histopathologic interpretation of HD and make it clinically relevant, Lukes et al[1] formulated a histopathologic classification of HD based on six different types: (1) lymphocytic or histiocytic (L&H), nodular; (2) L&H, diffuse; (3) nodular sclerosis; (4) mixed cellularity; (5) diffuse fibrosis; and (6) reticular (cellular) types. At the Rye conference on HD, [2] the nodular and diffuse subtypes of L&H were included under a single heading of lymphocyte predominance type; the diffuse fibrosis and reticular types were combined under the heading of lymphocyte depletion. In 1989, Wright[3] argued that clinicopathologic correlations since the late 1960s justify a return to the subtypes of HD proposed originally by Lukes and co-workers.

Recently, hematopathologists have noted that lymphocyte predominance HD includes two major types, one corresponding to the original lymphocyte predominance nodular type that contains L&H variants of RS cells, and a second type corresponding to lymphocyte-rich classic HD; in this latter, lymphocyte-rich type, the RS cells and natural history more closely resemble those of mixed cellularity and nodular sclerosing types. [4]

A definite relationship among histologic types, clinical stages, and survival has been demonstrated. [1] Nodular sclerosis is associated with a good prognosis and a marked propensity to involve the mediastinum, and is often limited to stage I or II disease. Lymphocyte predominance, nodular, has an excellent prognosis and is commonly restricted to localized stage I disease in the neck or inguinal areas. Lymphocyte predominance, diffuse, also has a strong tendency for presentation with limited-stage disease and a recurrence rate that may be significantly lower than that for patients with a nodular histologic type, when strict morphologic and immunophenotypic criteria are used for diagnosis. [5] Lymphocyte depletion has a poor prognosis and usually presents with advanced stage III or IV disease and a high frequency of bone marrow and abdominal lymph node disease. Some cases originally classified as lymphocyte depletion type, on review with immunohistochemical studies, have been reclassified as large cell lymphoma, which may account for their poor outcome. Nevertheless, there remain cases with numerous RS cells or diffuse fibrosis, particularly in older male patients or immunosuppressed patients, including those with human immunodeficiency virus (HIV) infection, which are bona fide HD. Mixed cellularity has an intermediate prognosis, commonly presenting with abdominal disease and relatively infrequently being associated with mediastinal disease.

These relationships were confirmed and extended in a study of 719 patients with HD who underwent staging laparotomy with splenectomy between April, 1969 and December, 1986 at the Harvard Joint Center for Radiation. Mauch et al[6] showed that the mediastinum and left and right sides of the neck are the most common sites of involvement in patients with nodular sclerosis or mixed cellularity types, each site being involved in 60% of cases. By contrast, the mediastinum was involved in only 8% of patients with lymphocyte predominance. Nodular sclerosis and mixed cellularity appeared to spread by contiguity, whereas lymphocyte predominance was limited to one site of involvement in approximately 50% of patients and was least likely to spread in a contiguous fashion.

A review of 9,418 microscopically confirmed HD cases accessioned from 1973 to 1987 by the Surveillance, Epidemiology and End Results (SEER) program of the National Cancer Institute revealed a dramatic increase in the incidence of nodular sclerosis, particularly in adolescents and young adults, whereas mixed cellularity remained stable. The incidence of HD in the elderly decreased, most likely because of improved ability to recognize many cases of NHL that would have been misclassified as HD.[7]

Histologic Types

Nodular Sclerosis

In most series, nodular sclerosis is the most frequent type of HD [1] [6] [7] [8] [9] (Plate 67-1). It is characterized by interconnecting, dense bands of collagen that circumscribe abnormal lymphoid nodules. As a result, the cut surface of lymph nodes show tan nodules of lymphoid tissue bulging out from gray-white connective tissue. This gross appearance should lead to a high suspicion of nodular sclerosing HD, which can sometimes be confirmed in the surgical suite by demonstrating RS variants in touch imprints of the lymph node. In permanent tissue sections stained with hematoxylin and eosin, eosinophilic collagen bands of varying width surround blue lymphoid nodules, a pattern that can be recognized with the naked eye by holding the slide up to the light. The collagen is birefringent in polarized light, which may aid in its recognition and quantitation. It appears to emanate from a thickened capsule or from the adventitia of blood vessels, or both. [1] [8] Microscopically, there are large spaces around the RS variants, called lacunar cells. The lacunar artifact, best seen in formalin-fixed tissue, results from retraction of cytoplasm of the RS cells from surrounding tissues (Plate 67-1). The lacunar cells appear distinctive by virtue of pale, sometimes transparent cytoplasm and relatively small nuclei and nucleoli compared with diagnostic RS cells. The nucleoli may be basophilic, in contrast to the eosinophilic nucleoli of RS cells. Lacunar cells may have one, two, or many nuclear lobes. Usually only an occasional lacunar cell closely resembles an RS cell.

The amount of collagen sclerosis may vary considerably from marked (visible to the naked eye) to minimal, in which case the diagnosis is suspected mainly from the presence of distinctive lacunar cells. This latter type, with minimal or absent sclerosis, has been referred to as the cellular phase of nodular sclerosis. [8] [10] The frequent finding of typical sclerotic lesions elsewhere in the same patient seemed to justify recognition of the cellular phase as part of the spectrum of nodular sclerosis.[8] This concept was supported by the study conducted by Strum and Rappaport,[11] who observed progression from the cellular phase to advanced sclerosis in serial biopsies from five of seven patients. Colby et al [9] found that patients with the cellular phase of nodular sclerosis had clinical features and overall survival rates similar to those of patients with mixed cellularity HD, but a relapse-free survival rate similar to that of nodular sclerosis patients. Recognizing that sampling sometimes demonstrates lymph node involvement in the cellular phase without collagen bands, Lukes [10] recommended that for reproducibility, the diagnosis of nodular sclerosis should be made only when the two criteria of collagen band formation and lacunar cells are met.

Colby et al[9] described a fibroblastic variant of nodular sclerosis. They found the number of fibroblasts to be prognostically more significant than the number of lymphocytes; numerous fibroblasts correlated with a shorter relapse-free survival. This grading system is not in widespread use. Dorfman [12] has indicated that mediastinal biopsies from patients with the fibroblastic variant of nodular sclerosis may be misinterpreted as malignant fibrous histiocytoma.

In 1986, Strickler et al[13] described a syncytial variant of nodular sclerosis in which cohesive clusters or sheets of RS cells are

found (Plate 67-2). Syncytial foci may be found anywhere in the node, but often occur in interfollicular areas and in the trabecular sinuses of the lymph node. The atypical HD giant cells often surround zones of necrosis and may be associated with numerous granulocytes. This histologic picture may be mistaken for metastatic carcinoma, malignant melanoma, thymoma, and large cell NHL.[10] The distinction of nodular sclerosing HD can be confirmed by immunophenotypic studies, as described later in this chapter.

The British National Lymphoma Investigation (BNLI) recognized that within the spectrum of nodular sclerosis there is wide variation in the relative numbers of

pleomorphic HD giant cells and small lymphocytes.[14] The BNLI subclassified nodular sclerosis into grades I and II; grade II shows areas of lymphocyte depletion or numerous pleomorphic HD giant cells. Grade II, which accounts for 2030% of all cases of nodular sclerosis, has a significantly worse prognosis than that of grade I. The prognostic significance of this grading of nodular sclerosis has been confirmed in a multivariate analysis. [15] Patients with nodular sclerosis grade I who relapse have a more successful salvage and longer period of survival than do patients with grade II nodular sclerosis. [16] Histologic subclassification of nodular sclerosis therefore appears to be clinically relevant and important in planning therapy. Subclassification of nodular sclerosis into grades I and II was found to be reproducible at a rate of 81.6% by a panel of 4 experienced pathologists reviewing biopsy results of more than 1,000 patients in the German National Trial on Hodgkin disease. [17]

Nodular sclerosis occurs most often in women, a predilection that is present across all age groups. The prognostic advantage of nodular sclerosing HD is largely restricted to patients in clinical stages I and II. There is no survival advantage of patients with nodular sclerosis in stages III and IV, with the possible exception of patients with stage IV disease on the basis of contiguous spread to the lung or anterior chest wall. [18]

Lymphocyte Predominance

Lymphocyte predominance occurs in both diffuse and nodular forms[1] (Plates 67-3 and 67-4). The cut surface of the lymph node has a diffuse or faintly nodular fish-flesh appearance. Both nodular and diffuse types consist primarily of small lymphocytes and of benign epithelioid histiocytes. Other cellular elements, such as eosinophils and plasma cells, are rare or absent. Necrosis and fibrosis are also negligible, and diagnostic RS cells are rare (Plate 67-3). There are, however, distinctive variants of RS cells, which have large folded, twisted, or multilobated nuclei and relatively small nucleoli (Plate 67-4). These variants of RS cells have become known as L&H cells or, because they resemble popcorn, as popcorn cells. L&H/popcorn variants are sufficiently distinctive that some authorities rely on their appearance to make a diagnosis of HD with lymphocyte predominance (LPHD) in the absence of diagnostic RS cells.

Because of the rarity of diagnostic RS cells, the diffuse form of LPHD can be mistaken for well differentiated lymphocytic lymphoma or chronic lymphocytic leukemia.[10] When histiocytes predominate, the differential diagnosis includes lymphoepithelioid lymphoma (Lennerts lymphoma) and granulomatous inflammation. Lennerts lymphoma can be distinguished by a spectrum of atypical lymphoid cells and clustering of histiocytes. Diffuse LPHD differs from nodular LPHD mainly in having a much smaller amount of follicular dendritic cells. [19] Granulomatous inflammation is often accompanied by necrosis not found in LPHD. In nodular LPHD, epithelioid histiocytes may be numerous within the nodules or surrounding them in a wreathlike arrangement. The diagnosis of LPHD is made when there are L&H/popcorn variants of RS cells. LPHD can occasionally present at extranodal sites, particularly Waldeyers ring, spleen, liver, or bone marrow; in such instances, confusion with low-grade NHL or T-cell-rich B-cell lymphoma may occur. [20]

Nodular LPHD is often found in association with progressively transformed germinal centers (PTGC) [21] [22] [23] [24] (Plate 67-5), which occur in follicular hyperplasia when secondary follicles become large and the border between the germinal center and lymphocytes of the mantle zone becomes indistinct. In PTGC, the small lymphocytes of the mantle zone appear to infiltrate and gradually overrun the germinal centers. PTGC may precede, follow, or coexist with nodular LPHD, but the condition is not sufficient for a diagnosis of LPHD. [23] [24] It is essential that the diagnosis of nodular LPHD not be made unless diagnostic RS cells or their L&H/popcorn variants, or both, are found in some of the lymphoid nodules. Indeed, the only difference between PTGC and the nodules of nodular LPHD is the absence of L&H/popcorn cells in PTGC.[25]

Regula et al[26] emphasized clinical differences between the nodular and diffuse subtypes of LPHD. Patients with the diffuse form tend to have a course similar to that of the mixed cellularity and nodular sclerosing types of HD. By contrast, patients with nodular LPHD have significantly more relapses, independent of stage or treatment and occurring continuously for many years after initial therapy. Trudel et al [27] noted differences in the clinical presentations of diffuse and nodular LPHD: nodular LPHD usually involves a single anatomic site, whereas the diffuse form often presents with more extensive disease. These differences were not confirmed when immunophenotypic criteria were used to confirm the diagnosis of the diffuse type.[5]

Nodular LPHD sometimes progresses to large cell NHL.[21] , [28] [29] [30] Miettenen et al[28] reported that 5 of 51 patients with nodular LPHD acquired large cell NHL 411 years after the diagnosis of nodular LPHD, and that only 1 of these patients had received radiation therapy. Hansmann et al [29] observed the simultaneous presence of (n = 11) or subsequent transition into (n = 3) a large B-cell lymphoma in 14 nodular LPHD cases. Retrospective follow-up of these secondary large cell lymphomas in patients with nodular LPHD revealed a longer survival time than that of primary B-type large cell lymphomas and other secondary large cell lymphomas. Sundeen et al[30] described seven cases of nodular LPHD in which mononuclear (L&H) RS cell variants occurred in large confluent sheets resembling large cell lymphoma. These findings were interpreted as histologic progression of nodular LPHD, with uncertain biologic significance. Six of these patients were in complete remission after radiation or chemotherapy, suggesting a good prognosis for large cell lymphomas occurring in patients with LPHD.

In a study of 2,836 cases of LPHD, the German Hodgkin Study Group found that immunohistochemistry disproved the expert panels morphologic diagnosis of LPHD in 25 of 104 cases. Moreover, 13 cases originally not classified as LPHD had an LPHD-like immunophenotype. Immunohistochemically confirmed LPHD cases showed a significantly better freedom from treatment failure and survival than classic HD ($P = 0.033$). Significance of survival for LPHD cases improved from $P = 0.047$ (morphologic classification) to $P = 0.0071$, when classified by immunohistochemistry. The authors concluded that immunohistochemical analysis should be mandatory for HD biopsies suspected of being LPHD when a modified therapy protocol, different from that used for classic HD, is used to treat LPHD. [31]

Lymphocyte Depletion

Lymphocyte depletion HD includes two major types: diffuse fibrosis and reticular[1] (Plate 67-6). Both types are characterized by a depletion of lymphocytes, and focal necrosis is common. The diffuse fibrosis type is characterized by amorphous proteinaceous material or disorderly fibrils without mature birefringent collagen, or both, whereas the reticular type shows numerous diagnostic RS cells (Plate 67-6) or RS cell variants of the

pleomorphic and sarcomatous types. When the pleomorphic variants predominate, the process may be mistaken for a poorly differentiated nonlymphoid neoplasm. A morphologic distinction between the reticular type of lymphocyte depletion HD and Ki-1 (CD30)+ anaplastic large cell lymphoma (ALCL) can be difficult and is aided by application of immunohistochemical techniques. [32] [33]

Neiman et al[34] noted that 13 patients with lymphocyte depletion had a distinctive clinicopathologic syndrome of rapidly fatal disease with fever, pancytopenia, lymphocytopenia, and abnormal hepatic function, often without peripheral lymphadenopathy. In patients with the diffuse fibrosis type, the diagnosis was commonly made by bone marrow examination, at laparotomy, or at autopsy. Patients with the reticular type more often had peripheral lymphadenopathy. Lymphocyte depletion HD is predominantly subdiaphragmatic, with extensive involvement of liver, spleen, retroperitoneal lymph nodes, and multiple bone marrow sites, often accompanied by bone marrow hypoplasia and pancytopenia. Thus, multiple bone marrow biopsies and liver biopsies can frequently establish the diagnosis of lymphocyte depletion HD.

Bearman et al[35] found no clinical or survival differences between reticular and diffuse types of lymphocyte depletion HD. However, Greer et al [36] reported that patients with diffuse fibrosis more often had bone marrow involvement and less frequently had peripheral lymphadenopathy than patients with the reticular type. Among patients who received chemotherapy, median survival was longer for patients with diffuse fibrosis.

Kant et al[37] found that of 39 patients treated for lymphocyte depletion HD at the National Cancer Institute between 1964 and 1976, 10 actually had large cell NHL and 13 had nodular sclerosing HD. Only 3 of 10 patients with NHL experienced complete remissions, and their median survival was only 7 months. Complete remissions were attained by 67% and 85% of patients with lymphocyte depletion and nodular sclerosing HD, respectively, and the median survival had not been reached in either group with a median follow-up of 14 years. These results suggest that more accurate diagnosis and classification of HD is needed not only for proper care of individual patients but for accurate analysis of new treatment protocols.

Mixed Cellularity

Mixed cellularity is the second most common histologic type of HD and shows a relatively high frequency of abdominal involvement. [6] [38] Reactive histiocytes, eosinophils, neutrophils, plasma cells, and small lymphocytes are numerous, and small foci of necrosis are common. Among the benign cellular elements are numerous diagnostic RS cells (Plate 67-7). In diffusely involved lymph nodes, the architecture is usually obliterated, and the capsular and subcapsular sinuses are compressed. In focally involved lymph nodes the sinuses may remain patent. Mixed cellularity HD comprises a heterogeneous histologic group representing the

center of a spectrum of lesions whose appearance ranges from that of lymphocyte predominance on one extreme to that of lymphocyte depletion on the other. Lukes[10] intended mixed cellularity also to serve as a catch-all classification for those lesions that lack typical features of the remaining types. For example, HD in which lymph nodes are focally or partially involved and lack characteristics of nodular sclerosis are included in the mixed cellularity type. A morphologic variant of mixed cellularity with numerous epithelioid histiocytes has been described. [39] Awareness of this morphologic variant helps to distinguish it from lymphoepithelioid (Lennerts) lymphoma, angioimmunoblastic T-cell lymphoma, and granulomatous inflammation.

Reed-Sternberg Cells

Diagnostic RS cells have two or more nuclear lobes and huge, inclusion-like nucleoli (Plate 67-8). The classic RS cell has a symmetric mirror-image nucleus. Other RS cells have multiple separate nuclei or an elongated and often twisted nucleus, which, when cut in thin tissue sections, simulates a multinucleated cell. Some RS cells are truly multinucleated. RS cell nucleoli are of relatively uniform density and eosinophilic to amphophilic in staining quality. Nuclear chromatin is condensed to the nuclear membrane, resulting in a clear halo around the nucleolus. The cytoplasm is variable in appearance but is usually abundant and eosinophilic to amphophilic in staining quality.

Reed-Sternberg cell variants are associated with the different histologic types of HD (see Table 67-1 and Plates 67-1 , 67-4 , and 67-6). The L&H/popcorn variant is characteristic of the lymphocyte predominance type, the lacunar variant is typical of nodular sclerosis, and the pleomorphic or sarcomatous variant is found in lymphocyte depletion.

In general, the number of diagnostic RS cells is inversely proportional to the number of small lymphocytes; hence, diagnostic RS cells are rare in lymphocyte predominance, frequent in lymphocyte depletion, and easily found in mixed cellularity types. In nodular sclerosis, the number of diagnostic RS cells is quite variable and may be low. This is rarely a problem for diagnosis, because lacunar variants in a background of collagen sclerosis are virtually diagnostic of nodular sclerosis HD.

Diagnostic Problems

Because of wide variation in the character and frequency of RS cells, HD may sometimes be confused with benign lymphadenopathies,

TABLE 67-1 -- Summary of Morphology and Phenotype of Reed-Sternberg Cells in Different Histologic Types of Hodgkin Disease

Histologic Type	Reed-Sternberg Cell Variant	Appearance	Pattern	Phenotype
Mixed cellularity	Classic	Bilobed or multilobed nucleus, huge, inclusion-like, eosinophilic nucleoli	Diffuse or interfollicular	CD30+, CD15+, CD45
Nodular sclerosis	Lacunar cells	Pale, retracted cytoplasm	Nodular with collagen bands	CD30+, CD15+, CD45
Lymphocyte predominance	Popcorn cells	Wrinkled, twisted nuclei, small nucleoli	Nodular, rarely diffuse	CD30+/, CD15, CD45+, CD20+, EMA+
Lymphocyte depletion	Sarcomatous	Pleomorphic hyperchromatic nuclei, nucleoli often indistinct	Diffuse with fibrosis	CD30+, CD15+, CD45

with NHL, and even with epithelial and soft tissue malignancies. [9] , [40] ,[41] Lukes et al[40] reported cells indistinguishable from RS cells in infectious mononucleosis. Strum and Rappaport[41] expanded the list of conditions containing RS-like cells to include rubeola, myositis, thymoma, anticonvulsant-induced lymphadenopathy, carcinomas of the lung and breast, malignant melanoma, malignant fibroxanthoma, and various hematopoietic neoplasms. Colby et al [9] added angioimmunoblastic lymphadenopathy, toxoplasmosis, and malignant histiocytosis to the list of disorders mimicking HD.

Coppleson et al[42] found a high degree of interobserver and intraobserver disagreement in the histologic classification of HD. These investigators recommended the use of a panel of pathologists for the reproducible classification of HD.

In an expert hematopathology panel review of initial diagnostic material from a Southwest Oncology Group trial of advanced HD, Miller et al [43] found that 13% of 287 cases had been misdiagnosed as HD. The most common error was to confuse other malignant lymphomas with HD (14 of 21 patients), particularly in the case of large cell lymphomas with pleomorphic features and RS-like cells. Lennerts lymphoma and angioimmunoblastic lymphadenopathy were also confused with HD. Mixed cellularity and lymphocyte depletion HD were the types most frequently diagnosed incorrectly (14 of 21), and nodular sclerosis was least frequently mistaken for other disorders (2 of 21). One of the two patients mistakenly diagnosed as having nodular sclerosing HD actually had a sclerosing carcinoma; the other had a dermatopathic lymphadenopathy with multinucleate cells, which on review were found not to be RS cells. Atypical clinical presentations, particularly unusual extranodal sites of disease, characterized the group most often incorrectly diagnosed as HD. Overdiagnosis of HD is particularly common in elderly patients. [44]

Should We Continue to Subclassify HD?

Advances in treatment have diminished the earlier prognostic significance of the histologic subtypes of HD, and the question has been raised: Should we bother subclassifying HD?[45] For now, the answer seems to be yes. Histologic classification continues to identify subcategories at greater or lesser risk of relapse. Classification also helps to predict the most likely sites and extent of disease, and in so doing influences decisions about staging and therapy. Correlations of HD histology with new biologic markers will likely provide new insights into the etiology and pathophysiology of HD.

Pathologic Staging

Improved therapy protocols have placed greater emphasis on more accurate staging of HD. Staging considerations include recognition of early or focal involvement of abdominal lymph nodes, distinction of HD from nondiagnostic granulomas, and recognition of HD in the spleen and at extranodal sites, usually the liver and bone marrow.[46] In addition, HD may present at unusual extranodal sites such as the skin in patients with acquired immunodeficiency syndrome (AIDS), and at various sites, including body fluids, in patients who relapse from HD.

Clinically occult lymph node involvement is most often found in splenic hilar and celiac lymph nodes. [8] [38] Early microscopic involvement of abdominal lymph nodes typically occurs in the interfollicular areas (Fig. 67-1), where it resembles the pattern described as interfollicular HD, sometimes encountered in initial biopsy specimens.[47] At least one diagnostic RS cell or variant thereof should be found before a staging lymph node is considered to be involved by HD. Occasionally, prominent immunoblasts in a perifollicular location may cause confusion with HD.

Splenic involvement almost always is apparent on gross inspection of the spleen sliced at thin (34-mm) intervals. The number of splenic nodules should be noted because patients with five or more nodules may have a more unfavorable prognosis. [48] Splenic lesions begin in the white pulp and appear as more prominent than usual malpighian corpuscles. Microscopically, the lesions of HD are first evident near the central artery in the periarterial lymphatic sheath [49] (Plate 67-9). Usually inflammatory cells are increased; patients with nodular sclerosis are commonly found to have bands of collagen.

Liver involvement histologically begins in the portal areas, where increased numbers of small lymphocytes, inflammatory cells, and occasional, often infrequent, HD giant cells are present. It is generally accepted that once the diagnosis of HD has been established, a mononuclear variant of the RS cell with a huge nucleolus is sufficient to confirm HD in the liver and other extranodal sites. [10]

Liver involvement almost never occurs in the absence of splenic HD, except in patients with AIDS in whom HD develops. [50]

Figure 67-1 Splenic hilar lymph node: interfollicular Hodgkin disease.

Liver function tests are poor predictors of hepatic involvement. The frequency of positive liver biopsies is doubled by performing laparotomy or peritoneoscopy after negative percutaneous biopsy.[51]

Bone marrow involvement occurs in no more than 10% of untreated patients with HD.[52] It is almost always detected in the biopsy and only rarely in the aspirate. This result is most likely due to the focal nature of bone marrow involvement and the increase in reticulum fibers in areas of HD. Bone marrow involvement is probably never recognized as isolated RS cells. Consequently, it can usually be detected at low and medium microscopic magnifications as nodular aggregates of inflammatory cells or fibrosis (Plate 67-10). RS cells or their variants may be confirmed at medium and high power (Plate 67-11). They must be distinguished from megakaryocytes, which lack large, inclusion-like nucleoli. Confirmation of the presence of RS cells may be possible by use of immunohistochemical stains that detect Ki-1 (CD30) antigen (Plate 67-12).

In a patient with HD, bone marrow fibrosis is presumptive evidence of marrow involvement by HD. Further sections of the biopsy should be made until RS cells or their variants can be found. Bone marrow fibrosis is usually not related to previous treatment, which more often causes bone marrow hypoplasia.

Isolated sarcoid-like granulomas may occur in any of the staging sites and should not be considered evidence of HD unless accompanied by RS cells. [53] These granulomas most often occur in a perivascular location (Plate 67-13). In one study, they were found to be associated with a more favorable prognosis and are thought to represent a host immune response to HD.[54] Isolated granulomas in HD seldom contain areas of necrosis, but probably should be stained for acid-fast bacilli and fungi to exclude opportunistic infection, which can occur with higher incidence in immunocompromised patients with HD than in the normal population.

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HISTOGENESIS

Nature of the Malignant Cell

Origin of RS Cells from Germinal Center B Lymphocytes

Molecular genetic studies support an origin of the RS cell from crippled germinal center B lymphocytes, particularly

centroblasts. RS cells from HD tissues of 10 untreated patients were isolated and studied by polymerase chain reaction (PCR) amplification of immunoglobulin (Ig) genes by Rajewskys group.[103] Clonally related rearrangements of Ig V_H genes were found in 9 of the 10 cases of mixed cellularity and nodular sclerosis. Rearranged V_H genes from RS cells carry a high load of somatic mutations. Stop codons in some in-frame V_H gene rearrangements suggest that RS cell precursors are derived from germinal centers where they have acquired crippling mutations that prevent antigen selection, but escape apoptosis, possibly through a transforming event such as that mediated by EBV LMP1 (see following). B cells normally are selected for expression of high-affinity antibodies. Only cells that fulfill this criterion are allowed to persist and leave the germinal center to become memory B cells.[104] Because B cells that bear V_H gene rearrangements that render them nonfunctional by somatic hypermutation can occur only within the germinal center, it is likely that RS cells that carry such rearrangements originate from precursor cells that had undergone a transforming event before entry into, or within, the germinal center, enabling them to escape apoptotic death in the absence of antigenic selection. Two other groups found that RS cells could be either monoclonal or polyclonal with respect to Ig genes or X-chromosome inactivation. [105] [106]

Studies of L&H variants of RS cells in nodular LPHD are consistent with a germinal center origin for these cells as well. Sixteen cases of nodular LPHD were found to contain L&H cells with molecular features of clonal B lymphocytes. L&H cells with identically rearranged V_H genes were isolated from the same or different nodules in nodular LPHD, [107] [108] and from different lymph nodes from the same patient,[108] supporting the hypothesis that nodular LPHD is a clonal B-cell lymphoproliferative disorder.

These recent studies using single-cell PCR amplification of Ig genes are consistent with the original observation of Weiss et al, who reported that HD tissues frequently contain clonal immunoglobulin gene rearrangements.[109] The recent PCR results are also in agreement with the earlier finding that the frequency of Ig gene rearrangements detected in HD is higher in cell suspensions enriched for RS cells. [110] Thus, both earlier studies on whole HD tissues and single-cell PCR amplification of Ig genes indicate that RS cells are derived from germinal center B lymphocytes in most cases of HD.

Possible Origin of RS Cells from Other than B Lymphocytes

Several lines of evidence suggest that RS cells are not derived from germinal center B cells in all cases of classic HD. Clonal Ig rearrangements have not been found in all HD cases.[103] In 1015% of classic HD cases, RS cells express T-cell differentiation antigens (CD3, T-cell receptor-f1, CD4), [56] [57] [69] [70] [71] or protein products (granzyme B or perforin) of cytolytic T lymphocytes or natural killer cells. [72] [73] [74] RS cells also can express antigens associated with granulocytes or monocyte/macrophages.[111] [112] [113] It is possible that these antigens or cytolytic proteins are acquired from surrounding inflammatory cells in the microenvironment of RS cells. However, this hypothesis would not explain the expression of such antigens on tumor cell lines derived from HD. [114] [115] [116] [117] [118]

Some studies [58] [119] [120] [121] [122] have shown similarities between RS cells and interdigitating reticulum cells, which are antigen-presenting cells in interfollicular regions of lymph nodes. The L428 cell line has been shown to be capable of antigen presentation. [114] RS cells express antigens shared with activated interdigitating reticulum cells,[120] and both cell types have similar reactivities with CD15 antibody and peanut agglutinin. [111] Pinkus et al showed similar expression of fascin by RS cells and interdigitating reticulum cells.[58] Moreover, RS cells closely resemble interdigitating reticulum cells in their cytomorphology and location in affected lymph nodes. [58] [121] [122] Fascin expression can be induced in B cells by EBV, [123] but this does not explain the high levels of fascin expression by RS cells in cases of HD where EBV is not detected.

Delsol et al [124] hypothesized derivation of RS cells from follicular dendritic reticulum cells (FDRC). Delsol and co-workers supported this hypothesis by observations of the intimate association of RS cells with the lymph node FDRC network, expression of CD21 antigen by both FDRC and RS cells, expression of IgG Fc receptors, and the presence of polyclonal IgG and IgE in the cytoplasm of both FDRC and RS cells.

Epstein-Barr Virus

An association between EBV and HD has been suggested by epidemiologic and serologic studies that reveal an increased risk of HD in patients with a history of infectious mononucleosis, elevated EBV antibody titers, and an altered serologic pattern of antibody response to EBV, before and after diagnosis of HD. [125] EBV was first demonstrated directly in HD tissues by immunoperoxidase staining of EBV nuclear antigen in RS cells in a single case of mixed cellularity HD after a chronic EBV infection.[126] Subsequently, EBV genomes have been demonstrated in RS cells in approximately one half of HD cases.[127] [128] [129] There is evidence that EBV is clonally integrated into RS cells, which indicates that infection occurred before or at the time of malignant transformation, and thereby implicates EBV directly in the pathogenesis of these cases of HD. Using a combined approach of slot blot, Southern blot, and in situ hybridization, Weiss et al [126] and Anagnostopoulos et al[129] demonstrated monoclonality of EBV-infected cells and localization of EBV nucleic acid to RS cells and their variants. EBV is associated at highest frequency with the mixed cellularity histologic type, less often with nodular sclerosis, and only infrequently with LPHD. [130] EBV is most commonly found in HD of children and older patients, only rarely in young adults, [131] [132] and occurs at highest frequency in HD of underdeveloped countries. [133]

Reed-Sternberg cells commonly express the EBV gene product LMP1,[134] which can confer a growth advantage on these cells. [135] LMP1 up-regulates expression of both CD40 and *bcl*-2, which prevent apoptosis of germinal center cells, and this could be the transforming event that confers immortality on precursors of Hodgkin/RS (H/RS) cells in germinal centers.[136] In HD, EBV infection is more highly associated with expression of *bcl*-x, a *bcl*-2-related protein.[137] *Bax*, a cell death-inducing gene, is frequently expressed in H/RS cells, but usually is expressed together with *bcl*-2, *bcl*-x, or *mcl*-1, another antiapoptotic protein.[138] Frequent expression of *Bax* may explain the relatively good therapeutic responses usually obtained in HD.

Latent membrane protein 1 engages signaling proteins of the TNF receptor superfamily. [139] Transfection of LMP1 into cell lines promotes multinuclearity of the EBV-negative HD cell line L428, and this effect appears to be mediated by NF- B activation. [140] LMP1 is recognized by cytotoxic T cells, which normally limit the growth of EBV-infected cells.[141] This could explain the better overall survival of HD patients whose tumor cells express LMP1. [142] Somewhat unexpectedly, increased numbers of cytotoxic T cells in HD tissues, identified by expression of granzyme B and CD8, has been associated with an unfavorable clinical outcome of HD

patients.[143] This may reflect impaired ability of cytotoxic T cells in HD patients to kill H/RS cells. Frisan et al found an inverse correlation between the presence of EBV in H/RS cells and EBV-specific cytotoxic lymphocytes in HD nodes.[144] Although EBV-specific cytotoxic T cells were detectable in three EBV-negative HD cases, none of six EBV-positive cases had detectable EBV-specific cytotoxic activity. One patient with EBV-positive HD from whom peripheral blood was available had circulating EBV-specific cytotoxic T cells. These observations suggest that local factors in the lymph node environment

of H/RS cells can suppress virus-specific immunity. Indeed, T cells in patients with HD have a defect in cytolytic function associated with down-regulation of the T-cell receptor chain, possibly in response to transforming growth factor-.[145] Failure of H/RS cells to express major histocompatibility class I antigens may also contribute to defective immune surveillance by cytotoxic T cells in HD.[146] There is also evidence for a genetic defect in immunity,[147] so that HD could result from the inability of certain affected people to control an EBV infection that in others of normal immunocompetence would go unnoticed or produce only a benign syndrome of infectious mononucleosis. An exaggerated defect in T-cell immune surveillance contributes to the high frequency of advanced HD in patients with AIDS.[148] Immunotherapies with bispecific monoclonal antibodies that bridge human effector cells to H/RS cells deserve evaluation as a means to overcome the impaired immune surveillance in HD.[149]

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CYTOGENETICS

Chromosome analyses of fresh HD tissue have usually revealed two populations of cells, one having a diploid modal chromosome number that is thought to represent the nonmalignant small lymphocyte population, and the other having a pseudodiploid or often hypotetraploid number that appears to represent the malignant cells. Seif and Spriggs[150] were the first to demonstrate marker chromosomes as evidence of a clone of malignant aneuploid cells in HD. Subsequent studies confirm that HD cells are usually aneuploid and frequently clonally related. [151] By use of simultaneous fluorescence immunophenotyping and interphase cytogenetic analysis, Weber-Matthiesen et al have shown that numeric chromosome abnormalities are present in CD30+ H/RS cells in all cases of HD. [152] In each case, there is considerable variability in chromosome numbers, demonstrating that karyotype instability is a characteristic of malignant cells in HD. Cytogenetic markers are consistent with a lymphocytic origin of HD. [153] The most common breakpoints found by Cabanillas et al[153] were at 11q23, 14q32, 6q11-q21, and 8q22-q24, which are also common breakpoints in B- and T-cell lymphomas. Moreover, the 11q23 breakpoint is also seen in a type of childhood B-cell acute lymphocytic leukemia characterized by the presence of aberrant myeloid and monocytic antigens, [154] a possible similarity to HD. Tilly et al [155] found that some of the chromosome regions involved in HD are shared with NHL of either B-cell (e.g., 14q32, 8q24, 6q, and 11q21-q23) or T-cell origin (e.g., 4q28, 7q31-q35, and 3q27), and demonstrated that significant differences occurred more often between HD and diffuse B-cell NHL than between HD and T-cell NHL. Schouten et al [156] found recurrent breakpoints at 4q32-q34, 6q24, 12q13, 12q23-q24, and 13p11-q13; three patients had two or more clones, and one had subclones. In HD cell lines, Fonatsch et al [157] found nonrandom marker chromosome abnormalities that are frequently associated with proto-oncogenes and other genes. Among the frequently involved sites are 1p21-p22, associated with N-*ras*, B-*lym*, and L-*myc*; 7q11.2-q36, associated with *met* and the T-cell receptor -chain gene; 11q21-q23, associated with c-*ets*1, CD3, , and epsilon chains; 14q32, associated with immunoglobulin heavy-chain gene; 15p12, associated with the nucleolus organizing region; and 21q21-q22, associated with c-*ets*2.

Rearrangement of *bcl*-2 can occur as a result of translocations involving chromosomes 14 and 18. [158] Rearrangements of the *bcl*-2 gene have been detected in approximately 40% of HD cases, and it has been suggested that these rearrangements may be involved in the pathogenesis of HD. [159] To determine whether the presence of translocations between chromosomes 14 and 18 explain the observed *bcl*-2 rearrangements in HD, Poppema et al[160] performed cytogenetics and *bcl*-2 gene rearrangement analyses on biopsy specimens from 28 consecutive untreated patients with HD. Although 11 patients had chromosome abnormalities in the chromosome 14q region, and 6 of these had involvement of the 14q32 region that comprises the gene encoding for immunoglobulin heavy chain, only 1 patient had a t(14;18) translocation, whereas almost 40% of these 28 patients showed *bcl*-2 rearrangements by a PCR method. Thus, although most cases of HD contain a clonal population with an abnormal karyotype, comprising RS cells, and the 14q32 region is frequently involved, a t(14;18) is extremely infrequent in HD.

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ASSOCIATION OF HODGKIN DISEASE WITH NON-HODGKIN LYMPHOMAS

Hodgkin disease can follow, coexist with, or precede a variety of lymphocytic malignancies. [30] [161] [162] [163] This interrelationship has stimulated the hypothesis that both HD and NHL can be derived from a common stem cell. Validation of this hypothesis would support a lymphoid origin for RS cells. Among the lymphocytic malignancies repeatedly associated with HD are chronic lymphocytic leukemia (CLL), follicular lymphoma, diffuse large cell or immunoblastic lymphoma, and mycosis fungoides. The most common association between HD and NHL is that of nodular LPHD and B-cell large cell lymphoma. This association is consistent with a B-cell origin for L&H cells in nodular LPHD, and suggests that the diffuse large cell component represents a form of histologic progression of nodular LPHD. [30] Meittenen et al[28] described five cases of diffuse large cell lymphoma in 51 patients, occurring 411 years after the diagnosis of non-LPHD. Sundeen et al [30] described seven cases of localized large cell lymphoma that were coexistent with nodular LPHD.

Momose et al[162] described 13 cases of CLL/small lymphocytic lymphoma in which RS cells were present. The RS cells in 12 of 13 cases contained EBV RNA, but the surrounding neoplastic lymphocytes were negative for EBV RNA. In each of these cases, the NHL was of B-cell phenotype. Weisenberg et al described eight additional cases of HD associated with CLL, including two of the lymphocyte predominant type. [164] Ohno et al showed identical sequences of the Ig heavy chain complementarity region III in H/RS cells and leukemic cells in two cases of HD arising in patients with CLL, supporting the concept that H/RS cells arising in classic HD often are derived from transformed B cells.[165]

Some cases of nodular LPHD are associated with the development of a T-cell lymphoma. [166] A report from the British National Lymphoma Investigation indicates that 2.2% of nodular LPHD cases are associated with B-cell lymphomas and 1.6% are associated with T-cell lymphomas. [167]

More than 20 cases of HD associated with mycosis fungoides, a T-cell malignancy, have been reported. [161] [168] In one such case, molecular genetic and immunophenotypic studies indicated that the RS cells and mycosis fungoides cells were derived from a common T-cell clone. [163] Together, these studies of HD and NHL or CLL in the same individual support the hypothesis that the RS cell of HD is most often derived from a B lymphocyte and less frequently from a T lymphocyte.

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CYTOKINES

It has been hypothesized that the different histologic types of HD, and systemic symptoms, are a consequence of cytokines secreted by the malignant RS cells. [55] [169] [170] IL-1 and granulocyte colony-stimulating factor have been demonstrated in culture supernates of HD cell lines. [171] [172] Eosinophilia may occur in response to IL-5 secreted by RS cells.[173] Nodular sclerosis may be explained by the stimulation of collagen synthesis by transforming growth factor- secreted by RS cells [174] [175] and eosinophils.[176] IL-6, which stimulates growth of B cells and plasma cells, was found in cell cultures and tissue sections of HD but did not correlate with the intensity or number of RS cells stained

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or degree of plasma cell infiltration.[177] IL-9, which stimulates T-cell proliferation, appears to be uniquely associated with RS cells and tumor cells in CD30+ ALCL. [178] Kretschmer et al[179] demonstrated synthesis of both TNF- and TNF- (lymphotoxin) by HD cell lines, and TNF- protein and mRNA directly in HD tissue specimens. Sappino et al[180] demonstrated high levels of lymphotoxin mRNA in Northern blots of tissue extracts from LPHD. TNF- and lymphotoxin have a broad range of biologic activities, which include neutrophil and eosinophil recruitment, [181] macrophage activation,[182] stimulation of fibroblast growth,[183] and endothelial cell/leukocyte interactions, [184] each of which can contribute to the pathologic features and symptoms of HD. Interferon (IFN)- mRNA and protein are produced by the HD cell line SUP-HD1 derived from a patient with nodular sclerosing HD.[185] IFN- causes fever and chills, which are symptoms of HD. IFN- also induces lymphocyte proliferation, activation of phagocytes, recruitment of monocytes and T lymphocytes, and formation of multinucleated giant cells, which may account for some histologic features of HD.[186]

Hodgkin/RS cells express several members of the TNF receptor superfamily, including CD30, CD40, CD95, CD120a, CD120b, and 4-1BB, [187] and the corresponding ligands are produced by surrounding inflammatory cells. [188] CD30 antigen is expressed preferentially, if not exclusively, on activated lymphocytes, supporting the hypothesis that RS cells are derived from activated lymphocytes.[55] The normal counterparts of CD30+ cells are found around, between, and at the inner margin of germinal centers.[55] [189] Stein et al[55] and Froese et al[190] have shown that CD30 can be induced, together with the IL-2 receptor (CD25), on normal B and T cells by exposure to phytohemagglutinin, human T-leukemia viruses, EBV, or *Staphylococcus aureus*. [55] [190] The 120-kd CD30 antigen develops from an intracellular precursor of 90 kd by N- and O-glycosidic glycosylation, and can be shed from the cell surface as a soluble CD30 antigen of approximately 90 kd. [191] CD30 antigen expression appears to represent a late event in lymphocyte activation. [45] [192]

CD30L and CD40L are expressed as membrane-bound proteins by activated T cells. CD30L has been localized to T cells and granulocytes that surround H/RS cells.[193] Recombinant CD30L and CD40L enhance release of IL-6, TNF-, and lymphotoxin from cultured H/RS cells. [194] CD40L, but not CD30L, induces IL-8 secretion and enhances expression of costimulatory molecules, intercellular adhesion molecule (ICAM)-1 (CD54) and B7-1 (CD80), by cultured H/RS cells. [60] CD40L is also a critical signal for T-cell-dependent activation of B cells. CD30L was found to enhance proliferation of T-cell-like HD cell lines HDLM-1, -2, and -3, and L540) but not B-cell-like HD cell lines KMH2 and L428.[195] CD30L expressed by eosinophils stimulates the proliferation of the HD cell line HDLM-2. [196]

The cytokine hypothesis does not appear to provide an adequate explanation of the influence of environmental factors and patient age on the relative frequency of the different types of HD. For example, lymphocyte predominance and nodular sclerosis are more common in children from developed countries, whereas the less favorable mixed cellularity and lymphocyte depletion types are relatively more common among older patients and among children from underdeveloped countries. [197] [198] [199] These observations suggest that nutrition and host immune factors may also contribute significantly to the histopathology of HD.

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SUMMARY

Hodgkin disease is a malignant disorder in which the proportion of RS cells and their variants in affected tissues correspond to the clinical grade of malignancy. RS cells have a distinctive immunologic profile that is characteristic of activated lymphocytes and allows distinction of HD from NHLs with overlapping morphologic features but different natural histories and therapeutic responses. Recurrent chromosomal abnormalities and clonal relationships between HD and NHL are both consistent with a lymphocytic origin for the malignant cell in HD. In most cases, H/RS cells are derived from crippled germinal center B cells (centroblasts) that are rescued from apoptosis by a transforming event, possibly induced by EBV-LMP1, which up-regulates antiapoptotic proteins. Although LMP1 is a target for cytotoxic T cells, the latter are defective in their ability to respond to H/RS cells because of genetic and local microenvironmental factors in HD tissues. Cytokines released by H/RS cells and surrounding inflammatory cells are responsible for the different histologic types of HD and symptoms experienced by patients with HD.

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Chapter 68 - Hodgkin Disease: Clinical Manifestations, Staging, and Therapy

Carol S. Portlock
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INTRODUCTION

Hodgkin disease (HD) is a unique malignancy of uncertain etiology and cell type that has become a prototype for curable neoplasms. Although the disease may involve the entire lymphoid system, the pattern of presentation and spread is usually predictable and initially localized, lending itself to the successful use of extended field radiotherapy. Since the first description of this disorder by Thomas Hodgkin in 1832, there has been much conjecture as to its nature and, in fact, whether it represents a true malignancy at all.

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INCIDENCE AND EPIDEMIOLOGY

In the United States, the number of new HD cases in 1997 was estimated to be 7,500.[1] The median age of diagnosis is 2631

years. A bimodal pattern of age distribution frequency for HD has been characteristically seen in the United States, with one peak at 2029 years, followed by a trough at 4059 years and a second peak at 60 years. It is now recognized that the second bimodal frequency peak was the result of pathologic misclassification. [2] The most recent data from the National Cancer Institutes Surveillance, Epidemiology, and End Results Program (SEER) indicate that these cases were largely aggressive non-Hodgkin lymphomas (NHL), rather than HD, mixed cellularity or lymphocyte depletion subtypes, as often reported. The nodular sclerosis (NS) subtype is the most common diagnosis overall, with >75% of cases occurring at <40 years of age and without a second peak thereafter. The other histologic subtypes, lymphocyte predominance (LP), mixed cellularity (MC), and lymphocyte depletion (LD), occur with low but gradually increasing frequency in all age groups. [3]

The incidence of HD is approximately 1.4-fold-greater in males. A male predominance has been noted in children <10 years old and again >50 years of age. Between the ages of 10 and 40 years, the ratio is reversed, with females predominating. This corresponds to the peak incidence of NSHD, which is more frequent in females. HD is primarily a malignancy of the white population in the United States, with whites accounting for more than 90% of all cases. In addition, the incidence is associated with small family size and a high standard of living in childhood, as well as a high level of maternal school education. [4] [5] Other possible risk factors include human immunodeficiency virus (HIV)[6] infection, Epstein-Barr virus (EBV) [7] infection, genetic predisposition, [8] [9] environmental exposure to herbicides, and certain occupations (woodworking, livestock, and meat processing). [10]

The etiology of HD remains uncertain (see Chap. 67). Both a possible genetic susceptibility and an infectious agent, particularly EBV, have been considered. Recent epidemiologic data support both. However, each may play a role in different subsets of disease.

Twin studies have demonstrated that the risk of HD is significantly increased among identical twins as compared to fraternal twins. [8] However, overall, familial HD occurs in less than 5% of all HD patients. In reviewing 328 patients with familial HD, only one major incidence peak was identified between ages 1534 years as compared to the broader incidence of sporadic HD.[9] A study of 60 patients from such HD families revealed no excess positive concordance for the presence of EBV in tumor tissue or in EBV serologies of patients from the same family.[11] Moreover, only 1 of 10 monozygotic twin pairs was concordant for EBV. These data suggest that EBV does not play an important role in familial HD.

On the other hand, EBV is consistently identified in the majority of HD cases occurring in developing countries. In developed countries, EBV is seen in pediatric and older adult cases and less frequently in young adults with nodular sclerosing histology. For example, in Kenya, 100% of pediatric cases were EBV Latent Membrane Protein-1 (LMP-1) positive, and 25 of these cases had type 1 and/or type 2 EBV strains documented. [12] In this same study, only 63% of Kenyan adults were LMP-1 positive. A recent epidemiologic analysis of 1546 HD cases with known EBV tumor status revealed an increased odds ratio for EBV in mixed cellularity versus nodular sclerosis; children versus young adult males versus females; and Hispanics versus whites. [7]

When EBV is present in HD, the monoclonal EBV genome is detectable in all tumor cells. Why it is not consistently identified in all HD and what role the virus plays in lymphomagenesis remain to be elucidated.

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CLINICAL MANIFESTATIONS

HD usually presents with supradiaphragmatic lymph node involvement and only later with generalized lymphatic and extralymphatic disease. Regional disease presenting below the diaphragm is distinctly unusual (<10% of all cases). [13] Lymph node involvement above the diaphragm frequently includes the anterior mediastinum and may become very large (>10 cm) without causing major symptoms. Complaints of dry nonproductive cough, substernal discomfort, and decreased exercise tolerance are common in this situation. Enlarged lymph nodes in the supraclavicular, cervical, and/or axillary regions are typically noted. Occasionally infraclavicular, submandibular, or even preauricular disease may be present. Waldeyers ring adenopathy is distinctly unusual and suggests a NHL. Lymph nodes are characteristically firm and rubbery and are often bulky. Local inflammation or erythema is uncommon, although lymphadenopathy will occasionally wax and wane spontaneously or appear to respond partially to antibiotic therapy.

Mauch et al[14] performed a detailed analysis of the initial sites of anatomic involvement, histopathologic findings, and clinical features in 719 patients with HD who underwent staging laparotomy and splenectomy. Table 68-1 (Table Not Available) presents the initial sites of involvement above the diaphragm, with the mediastinum, left side of neck, and right side of neck each present at diagnosis in >50% of patients with NSHD and MCHD. One of these sites of involvement was present in 92% of their patient population. The spleen was involved in 27% of patients (documented by splenectomy), while upper and lower abdominal nodes were found in 14% and 11% of patients, respectively. A strong association between splenic involvement and upper and lower abdominal disease was found. Only 5% of patients without splenic involvement had abdominal lymph node involvement. By contrast, 61% of patients with splenic involvement had abdominal lymph node disease. [14]

Bulky lymph node disease may result in certain regional complications. For example, tracheal or bronchial compression by massive mediastinal disease can result in complaints of cough or shortness of breath, often made worse in a supine rather than an upright position; obstructive pneumonia may also develop with hilar nodal compression. Extension of mediastinal and hilar adenopathy into adjacent lung, pericardium, chest wall, and pleura may be identified on chest radiograph and by computed tomography (CT) scanning. Pericardial and pleural fluid collections are usually asymptomatic, and cytologic examination rarely reveals Reed-Sternberg cells. Chest wall extension may be so great that adjacent sternal or rib destruction occurs, and palpable parasternal disease may be appreciated on physical examination.

In spite of bulky intrathoracic disease presentations, it is rare

TABLE 68-1 -- Presenting Sites of Involvement Above the Diaphragm in Descending Order of Prevalence

(Not Available)

From Mauch P, Kalish L, Kadin M et al: Patterns of presentation of Hodgkins disease: implications for etiology and pathogenesis. Cancer 71:2062, 1993. Copyright 1993 by the American Cancer Society. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

for patients with HD to have superior vena cava syndrome, upper-extremity venous thrombosis, or phrenic nerve or recurrent laryngeal nerve entrapment. These clinical manifestations are more likely found in patients with NHLs.

Bulky intra-abdominal lymph node involvement is less common in HD and, when identified, is often associated with regional presentations (inguinal and iliac nodes) below the diaphragm. Ureteral obstruction, lower-extremity lymphedema, and venous thrombosis are rare complications and again suggest a NHL. Moreover, lymph node involvement rarely occurs in the mesentery or as isolated retroperitoneal adenopathy.

The spleen is often an occult site of HD spread (see the Staging section of this chapter). Massive splenomegaly is rare, as are such complications as splenic infarct or hypersplenism. In addition, clinical and radiologic evidence of splenomegaly does not always indicate disease involvement.

Extranodal spread of HD may occur by two separate means: (1) direct extension from a nodal mass (e.g., hilar adenopathy extending into lung parenchyma) and (2) hematogenous dissemination (e.g., bone marrow disease). Regional extranodal extension usually occurs in the presence of local bulky adenopathy. For example, in the lung this may lead to a confusing picture of obstructive pneumonia and infiltrative pulmonary HD. Along the lumbosacral spine, the epidural cord or nerve root may be compressed in addition to direct invasion of one or more vertebral bodies.

Hematogenous spread may be manifested by such findings as multiple pulmonary nodules or diffuse infiltrative disease of the liver and/or bone marrow. Extranodal sites that are rarely involved in HD include skin, the gastrointestinal tract, and the central nervous system. Although recognized more often in patients with the acquired immunodeficiency syndrome (AIDS) and HD, [6] [15] these extranodal disease sites suggest a NHL. The presence of disseminated extranodal disease is generally accompanied by generalized lymphadenopathy and splenic involvement. Because all such presentations are uncommon, it is important to document these extranodal sites pathologically, which usually requires a cutting-needle or open biopsy, since aspiration cytology is rarely if ever positive. Clinical judgment must be used in assessing the risk versus benefit of performing an extranodal site biopsy. For example, in a patient with multiple pulmonary nodules and mediastinal and hilar adenopathy, lung biopsy is rarely indicated.

HD may have associated systemic symptoms such as unexplained fever of greater than 101°F, drenching night sweats, and/or weight loss of >10% body weight during the previous six months. Such B symptoms correlate with disease stage, increasing in frequency with extent and bulk of tumor, and also correlate with overall prognosis. Other systemic manifestations of HD without known prognostic significance include generalized pruritus (often severe and difficult to treat symptomatically without control of the HD) and lymph node pain after ingestion of alcoholic beverages. These systemic complaints may be the first indication of active HD, but more often they are elicited after the diagnosis has been established.

Patients with HD will often be anergic to skin test antigens at first diagnosis. Hyporesponsiveness increases with increasing disease stage. In addition, T-cell number, T-cell helper (CD4)/suppressor (CD8) ratio, and T-cell in-vitro response to antigen may be altered. Although cell-mediated immunity may be mildly abnormal, patients with HD rarely develop opportunistic infections prior to treatment. An exception, however, is the increased frequency of herpes zoster, seen in previously untreated as well as in treated patients. [16]

B-cell function appears to be normal in HD at diagnosis. Pneumococcal vaccination, for example, results in normal antibody response as long as subsequent treatment is delayed 1014 days. Splenectomy does not interfere with an adequate antibody rise. In contrast, overwhelming bacterial sepsis with encapsulated

organisms is still a potential risk among patients with HD after splenectomy or irradiation to the spleen. [17] [18]

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STAGING

Clinical and Pathologic Staging

Once a diagnosis has been established with an adequate biopsy and hematopathologic interpretation, systematic clinical staging should be initiated (Table 68-2). During a complete history and physical examination, the presence of systemic symptoms and the extent of lymph node involvement can be assessed. A chest radiograph will define the bulk of intrathoracic adenopathy and the possible presence of pulmonary extension, postobstructive pneumonia, or rarely, disseminated lung disease. CT scanning of the chest, abdomen, and pelvis will identify enlarged lymph nodes and their relationship to normal anatomic structures (e.g., pericardium, chest wall, kidney). Possible extranodal involvement (e.g., lung, liver, bone) may also be imaged. Although hepatosplenomegaly may be identified, its presence does not necessarily confirm disease involvement since nonspecific enlargement of these organs may occur.

The lymphogram complements the negative or equivocal

TABLE 68-2 -- Clinical Staging Evaluation

1. Detailed history for unexplained fever, weight loss, night sweats, and pruritus
2. Physical examination to document all areas of lymphadenopathy; size of liver and spleen; bony tenderness; neurologic evaluation
3. Laboratory studies
a. CBC, differential and platelet count, erythrocyte sedimentation rate
b. Serum alkaline phosphatase, lactate dehydrogenase
c. Renal function, including uric acid
d. Liver function tests
4. Radiologic studies
a. Chest radiograph
b. Bipedal lymphogram
c. Computed tomography of the chest and whole abdomen, including the pelvis
d. Gallium scan
e. Bone scan/bone radiographs obtained when areas of bone pain or tenderness are present
5. Biopsy studies
a. Diagnostic biopsy of lymph node: review with experienced hematopathologist
b. Bone marrow biopsy
c. Biopsy of suspicious disseminated extranodal sites (i.e., pulmonary or liver lesions), if clinically indicated
6. Under special circumstances
a. Magnetic resonance imaging
b. Ultrasound

abdominal and pelvic CT scan, since it may identify abnormal lymph nodes of borderline size, as well as identifying intranodal architecture. The lymphogram is more sensitive in detecting para-aortic disease (8598% rate of true-positive results as compared with 4065% for CT). [19] [20] However, the lymphogram is not accurate above L2L3 and is therefore unable to assess celiac axis, porta hepatis, or mesenteric (rare in HD) adenopathy; CT scanning is more accurate in assessing these nodal areas. Thus, both abdominal CT scanning and a bipedal lymphogram are recommended as part of the initial staging procedures.

Although uncommon at diagnosis, bone marrow involvement should be excluded before regional therapy is initiated. Percutaneous bone marrow biopsies are indicated as part of routine clinical staging. They are more likely to be positive in patients with B symptoms, clinical stage III or IV disease, or mixed cellularity Hodgkin disease (MCHD) or lymphocyte depletion Hodgkin disease (LDHD) and in patients with leukopenia or thrombocytopenia. Routine laboratory blood work may reveal leukocytosis, often with eosinophilia, a mild thrombocytosis, and/or anemia with a reactive bone marrow picture. The alkaline phosphatase may be elevated nonspecifically or in association with bone and/or bone marrow or liver involvement. Elevation of lactate dehydrogenase is an indication of bulky disease and appears to have prognostic significance at high levels.

Other staging studies that are often used include gallium scanning and magnetic resonance imaging (MRI). Gallium scanning is most useful in the anterior mediastinum and chest when evaluating treatment outcome of bulky disease.[21] If initial and post-treatment studies indicate gallium avidity with a persistent mediastinal mass, the possibility of residual HD mandates biopsy confirmation. The gallium scan is less useful in the abdomen because of bowel uptake, although SPECT (Single Photon Emission Computed Tomography) imaging will often clarify the uptake. MRI of the bone marrow in relapsed HD may be a particularly useful method of detecting occult disease.[22] Although MRI may provide excellent imaging of nodal and extranodal sites, its superiority to CT scanning has not been proven nor shown to be cost-effective. A new imaging technique that appears to hold promise is PET (positron emission tomography) scanning. [23]

The Ann Arbor staging classification[24] for HD represented an important step in therapeutic decision making in the treatment of HD. The Cotswolds modification [25] of the Ann Arbor staging method, which recognizes both a clinical and a pathological stage, is presented in Table 68-3 . Staging designations are determined according to the number of lymph node regions (not sites) involved and whether there is disease on one or both sides of the diaphragm. Systemic or B symptoms include fever, night sweats, and weight loss. The E lesion is defined as a direct extranodal extension of lymph node disease that can potentially be encompassed in radiation portal, such as hilar node extension in lung parenchyma.

Clinical staging includes the initial biopsy site and all other abnormalities detected by noninvasive methods, including physical examination and radiologic studies. Pathologic staging requires biopsy confirmation of potentially abnormal sites (e.g., liver or bone). In the past, pathologic staging also referred to findings at staging laparotomy, performed in those patients with clinically limited supradiaphragmatic disease. This latter procedure required biopsy of para-aortic, celiac, porta hepatis, and other suspicious lymph nodes, and splenectomy (with placement of clips on the splenic pedicle), liver wedge and needle biopsies, and bone marrow biopsy (if not done prior to laparotomy). Pneumococcal vaccine is routinely administered prior to surgery.

Although staging laparotomy has been widely applied in HD, its indications have decreased significantly in recent years, and it is now rarely indicated. Successful treatment of many clinically staged patients has become feasible with the use of combination chemotherapy to treat uncertain intra-abdominal sites, plus limited radiotherapy fields to known disease above the diaphragm. Moreover, very favorable subsets of patients with early-stage HD may be identified in whom the risk of intra-abdominal disease is <510% and in whom primary irradiation may be considered without laparotomy confirmation and without the need for combination chemotherapy.

Historically, following complete clinical and pathologic staging, the distribution of patients with HD is as shown in Table 68-4 .[26] Prognostic factors not reflected in the Ann Arbor staging system that may influence treatment decisions and outcome include bulk of disease, site and number of nodal regions involved, distribution of intra-abdominal stage III adenopathy, extent of splenic involvement, and blood tests such as erythrocyte sedimentation rate (ESR) or lactic dehydrogenase (LDH).

In 1989, an international multidisciplinary committee[27] recommended

TABLE 68-3 -- Cotswolds Staging Classification of Hodgkin Disease

Classification	Description
Stage I	Involvement of a single lymph node region or lymphoid structure
Stage II	Involvement of two or more lymph node regions on the same side of the diaphragm (the mediastinum is considered as a single site, whereas hilar lymph nodes are considered bilaterally); number of anatomic sites should be indicated by a subscript (e.g., stage II_3)
Stage III	Involvement of lymph node regions or structures on both sides of the diaphragm
III_1	With or without involvement of the spleen, splenic, hilar, celiac, or portal nodes
III_2	With involvement of para-aortic, iliac, and mesenteric nodes
Stage IV	Involvement of one or more extranodal sites in addition to a site for which the designation E has been used (see the following designations)
	Designations applicable to any disease stage
A	No symptoms
B	Fever (temperature, >38°C), drenching night sweats, unexplained loss of >10% body weight within the preceding 6 months
X	Bulky disease (a widening of the mediastinum by more than one-third or the presence of a nodal mass with a maximal dimension of >10 cm)
E	Involvement of a single extranodal site that is contiguous or proximal to the known nodal site
CS	Clinical stage
PS	Pathologic stage (as determined by laparotomy)

Adapted from Lister and Crowther,[25] with permission.

TABLE 68-4 -- Clinical Characteristics of Patients Treated for Hodgkin Disease at Stanford (19681988), According to Histologic Subtype

	Lymphocyte Predominance	Nodular Sclerosis	Mixed Cellularity	Lymphocyte Depletion
No.	78	1,301	282	13
Percentage	5%	78%	17%	1%
Age range	465	282	481	1165
Median age	31	26	30	42.5
Stage				
I	42%	6%	13%	0
II	38%	51%	26%	8%
III	19%	31%	46%	38%
IV	0	12%	15%	54%
B symptoms	3%	33%	30%	62%

Data from Hoppe.[26]

further modifications in the Ann Arbor staging classification to reflect changes in clinical staging criteria, the increased use of new diagnostic tests such as CT scanning, newly recognized prognostic factors, and their impact on therapeutic decisions. Table 68-5 summarizes the Cotswolds Staging Classification recommendations for clinical imaging criteria.[25] [27] This staging system acknowledges the value of CT and other imaging modalities in defining disease extent. However, it was again emphasized that biopsy confirmation is required if treatment recommendations might be altered by an equivocal study. This new staging system also clarified issues of disease distribution and bulk. In regional disease, the number of involved sites is denoted by a subscript (e.g., stage II_3). For stage III presentations, anatomic extent of intra-abdominal adenopathy is defined and denoted by subscript, with stage III_1 assigned to patients with spleen or splenic hilar, celiac, or portal node involvement; and stage III_2 to those with para-aortic, iliac, inguinal, or mesenteric involvement. Bulky disease is defined by maximum dimension (>10 cm) or by at least one-third mass/thorax ratio, designated by the subscript X. In the setting of bulky intrathoracic disease, contiguous spread to adjacent extranodal tissues is clearly distinguished from disseminated extranodal involvement (e.g., multiple pulmonary nodules). This common clinical stage II presentation would be designated with the subscript X for bulky or E for extranodal extension of lymph node disease into adjacent tissues (e.g., chest wall, pericardium, and sternum) and with a numerical subscript for the number of nodal sites involved above the diaphragm.

TABLE 68-5 -- Cotswolds Meeting Recommendations for Clinical Imaging Criteria

1. Lymph node involvement
CT size criteria of >1.5 cm for a positive study
2. Spleen involvement
Requires unequivocally palpable spleen or equivocally palpable spleen plus radiologic enlargement or splenic defects
3. Liver involvement
Multiple focal defects confirmed by two imaging modalities
4. Definition of bulky dimension

	10 cm in largest dimension
	Mediastinal mass greater than one-third of the internal transverse diameter of the thorax at T5T6 [a]

From Lister et al,[27] with permission.

[a]Although the Cotswolds definition of a bulky mediastinal mass uses the internal transverse diameter of the thorax at T5T6, most investigators define a large mediastinal mass as greater than one-third of the maximal intrathoracic diameter.

Staging Laparotomy: Is This Procedure Still Indicated?

The Ann Arbor and Cotswolds staging systems are based on both clinical and pathologic staging. The extent to which disease is pathologically confirmed depends on the suspected sites involved and whether that information would influence treatment decisions. Most HD patients have involvement limited to lymph node areas. Therefore, suspected stage IV sites should be pathologically confirmed (e.g., liver lesions) if these sites are not unequivocally involved by disease (e.g., multiple obvious lung nodules or a positive bone radiograph with blastic involvement).

Moreover, the anatomic distribution of lymph node involvement in HD is not random. Supradiaphragmatic disease with or without intra-abdominal involvement is the rule, and regional disease limited to subdiaphragmatic sites is uncommon. During the 1960s, the extent of intra-abdominal involvement was recognized to be underestimated in many cases, since the disease status of the spleen could not be reliably assessed by clinical means alone. Staging laparotomy was introduced to more adequately evaluate the suspected clinical findings and to determine whether radiation alone could be used for treatment.

Surprisingly, the accuracy of clinical abdominal disease assessment was often poor in spite of CT and lymphogram imaging. [13] [28] More than 40% of clinical stage IIIA patients were downstaged (negative laparotomy findings), and the extent of inaccuracy rose to 68% for patients <40 years of age with LPHD or NSHD. Likewise, for patients whose disease was classified as clinical stage I and II, staging laparotomy yielded evidence of intra-abdominal disease in approximately one-third of patients. Those factors most likely to be associated with upstaging include male sex, presence of B symptoms, and two or more sites of disease involvement above the diaphragm.

Of equal importance, however, was the observation that certain subgroups had a very low likelihood of change in stage with laparotomy. Those with clinical stage IA disease presenting in the mediastinum had virtually no risk of intra-abdominal involvement. Females with clinical stage IA at other supradiaphragmatic sites had a 6% risk, whereas males with similar clinical characteristics were at an equally low risk only if they had LPHD or interfollicular HD. Other histologic subtypes were associated with positive laparotomy findings in up to one-third of clinical stage IA males. [28]

For clinical stage II patients, female sex conferred a significantly lower risk of intra-abdominal disease (22% vs. 36%) than for males. Moreover, a favorable subset (females 26 years of

TABLE 68-6 -- Correspondence of Clinical and Pathologic Staging

	Correspondence
90%	1. CS IA, females
	2. CS IA, males with LPHD or interfollicular HD
	3. CS I, mediastinum only
	4. CS II, females with 3 disease sites, and age 26 years
	5. CS IIIB, with MCHD or LDHD, and age >40 years
80%	1. CS IIIB
	2. CS IVB

Abbreviations: CS, clinical stage; LP, lymphocyte predominance; HD, Hodgkin disease; MC, mixed cellularity; LD, lymphocyte depletion.

Data from Leibenhaut et al[13] and Mauch et al.[26]

age with fewer than three disease sites) was identified by the Stanford group; [13] laparotomy is positive in <10% of this subset. Such laparotomy data make it possible to identify subsets of patients in whom there is good correspondence between clinical and pathologic staging; these subsets are summarized in Table 68-6 .

For patients with favorable clinical stage I and II presentations, a correspondence of 90 with pathologic staging suggests that primary irradiation without staging laparotomy may be administered. This approach is valid if other treatment parameters are satisfactory for primary irradiation (i.e., no bulk disease at any site). [29] Similarly, for patients with clinical stages IIIB and IVB, correspondence is 8090%, suggesting that few if any of these patients would be downstaged with complete staging laparotomy evaluation. In this situation, combination chemotherapy with or without irradiation would be the primary treatment approach chosen.

For all other presentations of clinical stages I and II as well as equivocal clinical stage IIIA, it has been previously recommended that staging laparotomy be performed, if primary irradiation alone was to be used. [30] [31] [32] [33] In these situations, the correspondence was low enough (80%) that many patients would be inaccurately staged and treated with primary irradiation if the clinical stage was not pathologically confirmed.

As discussed below, however, the recommendation of staging laparotomy is no longer made in those instances of low correspondence. Instead, such patients are managed with combination chemotherapy and limited irradiation, obviating the need to accurately stage the abdomen.

In summary, the concept of clinical and pathologic staging in HD remains a valid tool in selecting curative treatment strategies. By keeping in mind the likelihood of clinical and pathologic stage correlations, an accurate definition of subsets of patients is possible.

For limited disease with high correspondence, primary irradiation may be considered based on clinical stage. For advanced disease and high correspondence, combination chemotherapy is recommended. For patients diagnosed with early stage disease by clinical staging, but whose disease has uncertain correspondence with historical laparotomy data, combined modality therapy is indicated.

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THERAPY

The prognosis for patients with all stages of HD has improved dramatically since 1960 as a result of advances in precise staging, knowledge of important prognostic factors, the development of supervoltage radiotherapy, and the use of effective combination chemotherapy. The improved results achieved with modern therapy after accurate staging are possible only with experienced teams of pathologists, surgeons, radiotherapists, hematologists, and oncologists working closely together. This approach can produce the excellent cure rates now possible, while minimizing acute and long-term morbidity from treatment. It should be emphasized that the goal of treatment is to obtain the highest overall possible cure rate for all patients, minimizing side effects whenever feasible. Thus, freedom from first or even second relapse must be considered in the evaluation of both disease-free and overall survival when the results of past and current trials are analyzed. For example, an early-stage patient who has relapsed after radiation therapy alone may be cured with chemotherapy administered at relapse. The challenge for physicians caring for patients with all stages of HD is to weigh carefully the toxicity/benefit ratio for each treatment regimen and to design a treatment strategy that maximizes the overall survival rate.

Radiotherapy for Early-Stage Disease

The long-term survival of patients with early-stage HD is >85% in multiple studies using various staging techniques and treatment modalities. [34] [35] [36] [37] [38] [39] [40] [41] [42] Definitive radiotherapy has long been considered standard treatment for the vast majority of patients with stage III HD because of the excellent long-term survival with minimal complications. The results of radiotherapy for laparotomy-stage I and II patients are dependent on close attention to important technical considerations for radiotherapy, including total radiation dose per field; size, shape, and number of treatment fields and careful definition of involved sites; use of appropriate voltage devices (e.g., linear accelerators); treatment simulation with individually shaped blocks for careful protection of normal tissues, such as the lungs and heart; equal doses from anterior and posterior fields; and use of cone-down fields and careful matching techniques with appropriate cord blocks. [35] [43] [44] [45] [46] [47]

Kaplan[47] provided the critical data to indicate that radiotherapy of HD follows a dose-response curve. Permanent eradication of any given known site of involvement is achieved in the vast majority of cases with doses of 3,6004,400 cGy delivered at a dose rate of 1,000 cGy/wk. Prophylactic radiation to a total dose of 3,0003,600 cGy is delivered to apparently uninvolved areas treated for subclinical disease. Use of a linear accelerator in the 48 MeV range provides the advantage of precise beam margins with reduced lateral scatter, increased depth-dose, and improved skin tolerance. [46] It is important to define carefully the extent of disease and fields to be treated, using appropriate treatment simulators. Proper technique also includes the use of carefully shaped lead blocks to protect such vital structures as the lungs, heart, and spinal cord; frequent port films; and dose verification using precise dosimetry.

Current radiotherapy practice continues to emphasize the treatment of multiple lymph node chains within a few very large, carefully shaped fields. Figure 68-1 illustrates the radiation fields most frequently delivered in clinical practice. The mantle field treats the cervical, supraclavicular, infraclavicular, axillary, hilar, and mediastinal lymph nodes to the level of the diaphragm in one contiguous treatment volume. Prophylactic whole lung irradiation to a dose of 1,5001,650 cGy in 150-cGy fractions is administered by some radiotherapists when the ipsilateral hilum is involved with HD. [44] [46] Although this technique decreases relapse in the treated fields for patients with hilar disease, the routine use of whole-lung irradiation remains controversial. The para-aortic field covers the splenic pedicle, the spleen (if not removed at laparotomy), and the celiac and para-aortic lymph nodes from the diaphragm to the level of the aortic bifurcation. The mantle (M) and para-aortic fields are frequently referred to as extended-field (EF) or subtotal nodal (or lymphoid) irradiation (STNI). The radiotherapy approach known as total nodal irradiation (TNI) or total lymphoid irradiation, involves treating the mantle, para-aortic, and pelvic fields.

Figure 68-1 Radiotherapy fields commonly used for HD. Total nodal irradiation consists of **(A)** a mantle and an inverted Y or **(B)** three fields. **(C)** Subtotal nodal irradiation after splenectomy consists of a mantle and para-aortic fields. (*From Rosenberg,[43] with permission.*)

As discussed in the following sections, inclusion of pelvic irradiation as part of TNI has disappeared. In addition, pelvic irradiation affects large amounts of bone marrow and therefore may subsequently preclude the use of full-dose chemotherapy. Pelvic radiotherapy may also increase the risk of infertility.

Delivering radiotherapy to apparently uninvolved lymph node regions has long been advocated because of the orderliness and contiguity of spread for patients who present with early stage disease. [48] [49] [50] [51] [52] This approach is based on the knowledge of sites of relapse of HD when treated only with involved field radiotherapy, the limitations of our diagnostic techniques in discovering minute or microscopic foci of subclinical disease, and the advantage of avoiding overlapping fields. In the initial Stanford trials, [37] patients with stage I and IIA disease were randomly assigned to involved field (IF) irradiation, EF irradiation, or TNI. A highly significant difference in disease-free survival was observed in favor of either EF irradiation or TNI, although overall survival was similar (80% at 15 years). The studies from the Joint Center for Radiation Therapy (JCRT) in laparotomy stage IIIA HD have been reviewed by Mauch et al [34] At a median follow-up of 9 years, review of the 315 patients treated with mantle and para-aortic irradiation (M + PA) revealed a freedom from relapse of 82% and an overall survival of 90%. These results are illustrated in Figure 68-2 . More than 80% of patients treated with mantle and para-aortic irradiation have been continuously relapse-free and therefore have not required the use of salvage chemotherapy. Pelvic recurrences were noted in only 3% of cases, indicating that it is reasonable to omit the pelvic field from the radiotherapy port. Other investigators using comparable staging and radiotherapy techniques have reported similar results, which are summarized in Table 68-7 .

In the JCRT series, mediastinal size was the only factor that significantly predicted freedom from relapse. [34] Patients with large mediastinal masses were significantly less likely to have freedom from relapse than were those with less or no mediastinal involvement (53% vs. 83%, respectively). However, the survival of these two groups of patients was not significantly different. The approach to patients with large mediastinal masses is discussed in a subsequent section.

Gospodarowicz et al[40] reported the long-term results of treating 521 clinical stage I and II patients with radiation alone at the Princess Margaret Hospital (Table 68-7). The overall actuarial survival for all irradiated patients was 78% at 10 years and 63% at 20 years, while the cause-specific survival was 87% and 83%, respectively. The use of mantle radiotherapy rather than IF radiotherapy was associated with a significant improvement in the 10-year relapse-free survival (RFS) (M = 66%, IF = 51%) and in the 10-year actuarial survival (M = 80%, IF = 59%). There was further improvement in the 10-year RFS with the use of EF

Figure 68-2 Actuarial freedom from relapse (FFR) and survival for stage IA and IIA patients treated initially with mantle and para-aortic irradiation. Actuarial survival curves are shown censored for unrelated deaths (marked HD) and for survival, including deaths from all causes. (*From Mauch et al, [34] with permission.*)

radiotherapy (76%), but this effect was not statistically significant when EF was compared with mantle radiotherapy alone in a multivariate analysis adjusting for other prognostic features. In a low-risk or very favorable cohort of patients (defined as clinical stage I and IIA, <50 years of age, with LP or NS histology, erythrocyte sedimentation rate (ESR) <40, no large mediastinal mass, and no E lesion), the 10-year survival rate was 91%, with an RFS of 77%. The 10-year RFS for patients in this favorable subgroup was 80% for mantle radiotherapy, compared with 87% for EF radiotherapy (not statistically significant). The overall conclusions reached by Gospodarowicz et al[40] were that the role of laparotomy and splenectomy in defining a favorable group of patients for treatment with radiotherapy alone may be offset by patient selection, using multiple clinical prognostic factors and by employing EF radiotherapy.

An alternative approach to the staging and treatment of early stage HD was proposed by Cosset et al, [39] reporting for the European Organization for the Research and Treatment of Cancer (EORTC). In the EORTC H2 trial, NSHD patients were randomized to either staging laparotomy and splenectomy followed by mantle and para-aortic radiotherapy, or to clinical staging and STNI plus splenic irradiation (Table 68-7). At 12 years, RFS was 68% for STNI alone and 76% for laparotomy-STNI. Overall survival was virtually identical. In the subsequent H5 trial, a favorable prognostic group was defined with all the following characteristics: no B symptoms, age <40 years, ESR <70, LP or NS histology, and either clinical stage I or II without mediastinal involvement. All patients in this favorable group first underwent laparotomy and splenectomy. If negative, patients were randomly assigned to mantle irradiation alone or to mantle plus para-aortic radiotherapy. There was no difference in either RFS or overall survival between the two arms (Table 68-7). Therefore, in patients with all these initial favorable prognostic factors and with no infradiaphragmatic extension (i.e., negative laparotomy), the addition of para-aortic irradiation did not yield any benefit. The EORTC H6 trial then addressed the need for laparotomy in the favorable subgroup: patients in the first arm of the study underwent laparotomy with treatment adapted to the pathology findings; if the laparotomy was negative, mantle radiotherapy was given (plus para-aortic field for MC or LD histology); if the laparotomy was positive, combined-modality treatment was administered. Patients in the second arm of the study without laparotomy directly received mantle, para-aortic, and spleen radiotherapy (STNI + spleen). In this trial, the end point was survival and not RFS. At 5 years, RFS was significantly lower in the STNI group than in the laparotomy (plus adapted treatment) patients, as expected. However, because of the efficacy of salvage chemotherapy, 5-year overall survival was similar (Table 68-7). [39] Based on these studies, the EORTC decided to give up staging laparotomy for supradiaphragmatic disease and subsequently used clinical staging. On the basis of their data, they also defined a small cohort of patients who could be safely treated with a mantle field alone without laparotomy. This subgroup is very restrictively defined and only includes female NSHD or LPHD patients with clinical stage I, <40 years of age, without B symptoms, with an ESR of <50, and without bulky mediastinal involvement. [39]

Similar results were reported by Fuller et al [50] for the M.D. Anderson group in a small number of prognostically favorable patients, who had supradiaphragmatic disease with either no mediastinal disease or limited mediastinal disease without hilar involvement and no B symptoms. These patients were treated with mantle radiotherapy alone. The British National Lymphoma Investigational Trials [49] as well as Mauch et al[52] have confirmed the equal effectiveness of more limited radiotherapy fields (i.e., mantle) for favorable subsets of stage IIIA patients.

Thus, laparotomy-staged patients with pathologic stage IIIA supradiaphragmatic disease of the NS or LP histology who do not have mediastinal involvement can be treated with mantle radiotherapy alone. Stage I patients with these histologies and a small mediastinal mass may also be treated with a mantle field. However, for pathologic stage IIIA patients not meeting these criteria, mantle and para-aortic fields should be used. In clinically staged patients (i.e., no laparotomy), the risk of undetected intra-abdominal disease may be up to 3050%. Except in very favorable subgroups, as defined by Gospodarowicz et al, [40]

TABLE 68-7 -- Stage I and II Hodgkin Disease: Comparison of Different Radiation Therapy Strategies

Investigators	Stage	Follow-up	Comment	Treatment	FFP/RFS (%)	Survival (%)
Rosenberg and Kaplan[37]	I,IIA	15 yr	All patients (lap)	IF	32	79
				sTNI/TNI	80	80
Mauch et al[34]	I, IIA	14 yr	All patients (lap)	M + PA	82	90
			SMM	M + PA	86	93
			LMM	M + PA	53	88
			NS/LP	M + PA1	88	97
			MC/LD	M + PA	80	84
Gospodarowicz et al[40]	I, II	10 yr	All patients (no lap)	M	66	80
				M + PA	76	NA
	I, II	10 yr	Very favorable[a] (No lap)	M	80	88
				M + PA	87	97
Cosset et al[39]	I, II	12 yr	All patients	M + PA (lap)	76	79
				M + PA + spleen (no lap)	68	77
	I, II	9	Favorable[b] (no lap)	M	69	94
				M + PA	70	91
	I, II	5	Favorable[b]	M (lap negative)	89	90
				M + PA + spleen (no lap)	79	94

Abbreviations: FFP, freedom from progression; RFS, relapse-free survival; M, mantle radiotherapy; PA, para-aortic radiotherapy; sTNI, subtotal nodal irradiation; TNI, total nodal irradiation; lap, staging laparotomy and splenectomy; ESR, erythrocyte sedimentation rate; SMM, small mediastinal mass; LMM, large mediastinal mass; NS, nodular sclerosis; LP, lymphocyte predominance; MC, mixed cellularity; LD, lymphocyte depletion; NA, results not available.

[a]Very favorable: clinical Stage IA or IIA with age <50, or lymphocyte predominance, ESR <40, no LMM, and no E lesion.
[b]Favorable: age <40, ESR <70, NS or LP histology, clinical stage IA or clinical stage IIA without mediastinal involvement. Unfavorable: one or more of the following: age 40, ESR 70, MC or LD histology, clinical stage II with mediastinal involvement.

SUMMARY OF INITIAL THERAPY RECOMMENDATIONS BY STAGE OF HODGKIN DISEASE

The recommended therapy for a patient with HD must be individualized. Advances in histopathologic classification, precise staging procedures, and selection of appropriate aggressive therapy have led to continuous improvement in both failure-free survival and overall survival. Any treatment recommendations must be viewed with the understanding that the management of HD is dynamic, constantly undergoing change and refinement. The goal is to provide each patient with the best probability of cure and the least possibility of long-term toxicity.

With an early stage presentation one must first consider whether the disease presentation is associated with a low likelihood of occult intra-abdominal involvement. Staging laparotomy is no longer indicated because historical data is adequate to define clinically favorable and unfavorable subsets. For very favorable subsets of clinical stage IA and selected IIA patients, primary irradiation, either a mantle field, or mantle, para-aortic, and splenic fields, depending on their clinical prognostic factors may be administered. For other favorable and unfavorable subsets with clinical stage I and IIA disease, ABVD and limited irradiation is indicated. For early-stage asymptomatic patients with bulky mediastinal masses, radiotherapy alone is not an acceptable option. A combined modality approach is appropriate, and treatment is initiated with one of the currently accepted regimens for advanced-stage disease. In this situation chemotherapy is given to maximal tumor response, which is then followed by limited-field radiotherapy.

Stage IIIB disease, with or without bulky mediastinal mass, should be treated with combined modality therapy.

Patients with subdiaphragmatic early stage HD are generally treated with combined modality therapy since the risk of intra-abdominal disease is high and a favorable subset is not defined for primary irradiation of clinically staged patients.

Combined modality therapy is an attractive alternative for the management of early- and intermediate-stage disease, particularly with the use of short-course ABVD or other chemotherapy and limited field irradiation. Future investigation will clarify the role of chemotherapy alone in these settings.

For patients with clinical stage IIIA disease, combined modality therapy or chemotherapy alone should be used. From the limited data available, it would appear that chemotherapy alone using any one of the accepted combinations for advanced disease is as effective as combined modality therapy for most patients with extensive stage IIIA disease. Although the results with combined modality therapy for IIIB and IV disease are excellent in terms of freedom from progression and overall survival, they do not appear significantly different from the results obtained with chemotherapy alone. Thus, for the vast majority of patients with stage IIIB or IV disease, chemotherapy alone is recommended as initial treatment.

For many years, MOPP remained the standard of care for the treatment of patients with stage IIIB or IV disease. However, data from recent randomized trials indicate that ABVD should now be considered the standard drug combination for treatment of Hodgkins disease. In advanced disease, other alternating regimens such as MOPP/ ABVD and MOPP/ABV hybrid have not been shown to be superior and may have significantly greater acute and late toxicity. Administration of radiotherapy to all sites of pretreatment involvement, once a complete remission has been obtained with chemotherapy for advanced disease, does not result in improved failure-free or overall survival and cannot be recommended. Limited-field irradiation to initial sites of bulky disease may be used in selected clinical situations.

The treatment of HD remains one of the great triumphs of cancer therapy since the middle 1960s. The overall 5-year survival rate for all patients has increased to 75% today. Thus, a treatment strategy for each patient should be designed to provide the highest probability of overall cure, while limiting late toxicities when possible. Moreover, it is important to counsel patients regarding secondary cancer and cardiac prevention (e.g., to stop smoking, to modify diet, and to exercise) and detection (mammography).

the EORTC,[39] Liebenhaut et al,[13] Mauch et al[28] and others, it is now recommended that most clinical Stage I and IIA patients receive combination chemotherapy and limited field irradiation. Similarly, for clinically staged IIIA patients with large mediastinal masses, combined modality therapy should be used as initial treatment.

The presence of constitutional or B symptoms has long been recognized as an important prognostic factor in HD. This observation is based primarily on data from patients with advanced disease, since patients with early-stage disease rarely present with B symptoms. In the past, this has led to different treatment recommendations for surgically staged patients with supradiaphragmatic stage IIIB disease. Investigators now recommend the routine use of combined modality therapy without laparotomy for these patients,[34] [51] [53] [54] [55] and this approach is no longer considered controversial.

In one of the few randomized trials for this subset of patients, the Stanford investigators treated laparotomy staged IBIIB patients with either TNI alone or TNI followed by six cycles of adjuvant MOPP (mechlorethamine, vincristine [Oncovin], procarbazine, prednisone) chemotherapy.[35] This small controlled trial showed no difference in either freedom from progression or overall survival, with 78% of all patients surviving >10 years from initial treatment. Crnkovich et al [53] reviewed the treatment records of 180 patients with pathologic stage IBIIB HD treated at Stanford and the JCRT. In this retrospective review, the two most important disease characteristics predictive of relapse were the number and type of B symptoms and the presence of a large mediastinal mass. Patients with both fever and weight loss had a 7-year freedom from relapse of 48% and an overall survival of only 57%. The poor prognosis in this subset was apparent for treatment either with radiation alone or with combined modality therapy. The presence of night sweats alone had no adverse effect on outcome. Among patients treated with radiation alone, there was similar freedom from relapse and overall survival irrespective of whether pelvic irradiation was included in the initial treatment fields. Combined modality therapy improved the 7-year freedom from relapse when compared with radiotherapy alone (86% vs. 74%, $P = 0.02$), whereas overall survival in the two treatment groups was virtually identical (88% and 89%, respectively). It was concluded that the presence of B symptoms was not necessarily a poor prognostic factor in patients with pathologically staged IAIIB HD, since their overall survival approached that of surgically staged IAIIA patients.[53] Thus, patients with stage IB or IIB disease can be treated effectively with either combined modality therapy or, after staging laparotomy, with mantle and para-aortic radiotherapy alone. The presence of both fever and weight loss and/or

a large mediastinal mass requires initial combined modality therapy. As previously emphasized, staging laparotomy and primary irradiation is no longer recommended as the best alternative since combination chemotherapy has proven to be well tolerated and spares the patient a major surgical procedure. Moreover, as the Stanford study demonstrated, combined modality therapy resulted in superior disease-free survival for stages IBIIB.

Radiation and Combined Modality Therapy for Large Mediastinal Masses

It is now well-recognized that a subgroup of stage I and II patients with large mediastinal masses have a significantly greater risk of relapse than patients with little or no mediastinal disease when treated with radiotherapy alone.[56] The most commonly accepted definition of a large mediastinal mass is a mass greater than one-third of the maximal intrathoracic diameter. However, this measurement alone is not a sufficient criterion for selecting which patients should be treated by definitive irradiation alone rather than by combined modality therapy. Other important selection criteria that should be considered in the evaluation of patients with a large mediastinal mass include (1) location of the mass: wide versus long, anterior and superior mediastinum versus mid-mediastinum; (2) involvement of multiple E sites; and (3) estimated volume of normal tissue that would need treatment with shrinking radiation fields. Treatment of all patients with a large mediastinal mass with initial radiotherapy alone results in a RFS of approximately 50%. However, in 1990 Hoppe and Behar reported a Stanford study [57] of a small series of selected patients with bulky mediastinal disease, who were carefully staged with CT scans to determine tumor volume, and then were laparotomy-staged (some patients were irradiated before surgery). These patients were treated with aggressive mantle radiation, which often included prophylactic treatment to the lungs. This treatment resulted in a 7-year freedom from relapse in 80% of patients and an overall survival of 100%. Careful monitoring of the tumor response during radiotherapy and the use of a shrinking field technique helped the Stanford group keep complications to a minimum. However, radiotherapy alone is inappropriate for most patients with early-stage HD who present with large mediastinal masses. Treatment with initial radiotherapy in these patients often requires extensive irradiation of the heart and lungs in order to include the large mediastinal mass in the radiotherapy port, which causes significant subsequent morbidity.

Although it must be recognized that patients with early-stage HD and large mediastinal masses treated with initial combined modality therapy achieve significantly better freedom from relapse at 10 years (83%) than do those treated by radiotherapy alone (49%), overall survival is virtually identical (8283%). [34] Despite the similarity in overall survival, most investigators concur that a 50% risk of relapse is too high, and therefore recommend combined modality therapy as the initial approach. Patients treated with radiotherapy alone tend to relapse in the initially irradiated field, as well as in adjacent untreated lymph nodes, while extranodal

relapses are seen primarily in the lung.

Therefore, for patients with large mediastinal masses, a combined modality approach is recommended. In the subgroup of patients who will receive initial combined modality therapy, staging laparotomy is not indicated, and treatment is initiated with ABVD (Adriamycin, bleomycin, vinblastine, dacarbazine) or one of the other regimens commonly used for advanced disease. Chemotherapy is given to maximal tumor response, as judged by chest radiographs and CT scans, after which two additional cycles of consolidation chemotherapy are given. Once maximal benefit from chemotherapy has been obtained, limited radiation therapy (generally to the mediastinum alone or to a mantle field) is used. With this combined modality approach, approximately 80% of patients will remain disease-free beyond 5 years. The medical and psychological advantage to this approach, with its high rate of RFS, cannot be underestimated. One unresolved question is whether patients with stage III and IV disease who have large mediastinal masses at presentation and in whom initial chemotherapy is the treatment of choice should receive consolidation radiotherapy to the mediastinum to decrease the risk of relapse in this one particular site. [58]

It is also important to recognize that most patients with large mediastinal masses treated either with radiation therapy or with chemotherapy plus radiation will have a residual mediastinal abnormality after treatment. Jochelson et al [59] noted that mediastinal abnormalities were common at the end of radiation or combined modality therapy for HD, and that they did not necessarily indicate persistent active disease or an increased risk of relapse. Persistent radiologic abnormalities may continue to improve in up to one-half of patients for >1 year after therapy. The use of high-dose gallium scanning may be particularly helpful when a residual mediastinal mass is documented, especially if the gallium scan is positive before treatment and reverts to normal following the completion of radiotherapy or combined modality treatment.[60] On the other hand, a persistently positive gallium scan after treatment is associated with a high likelihood of relapse and indicates the need for biopsy. [61] In young patients (<25 years old), a regenerating thymus during the 6 months after treatment may present as an enlarging anterior mediastinal mass. [33] This can be a diagnostic problem, as this mass may be gallium-positive.

Radiation and Combined Modality Therapy for Subdiaphragmatic Early-Stage Disease

HD below the diaphragm is a relatively rare clinical presentation, occurring in <5% of patients. Patients with early-stage subdiaphragmatic presentation tend to be older men with MC pathology.[13] [62] [63] Overall freedom from relapse and survival are similar to those of patients with supradiaphragmatic presentations, if compared stage for stage.[62] [63] [64] However, relapses tend to occur later in subdiaphragmatic presentations.

Historically, clinically staged IIIA patients with negative para-aortic nodes on lymphangiogram had a 10% incidence of splenic involvement at laparotomy, as compared with a 52% incidence of splenic involvement when the para-aortic nodes were positive. [13] [62] In subdiaphragmatic clinical stage IIB disease, risk of splenic disease was even higher (89%).

The M.D. Anderson group [65] treated 60 patients with infradiaphragmatic presentations. In 22 patients with inguinal/femoral or pelvic disease who were treated with irradiation alone, the 10-year freedom from progression and overall survival were 86% and 90%, respectively. In this retrospective analysis, the small numbers of patients with abdominal disease who were treated with radiotherapy plus MOPP fared better than did similar patients treated with radiotherapy alone. Patients with subdiaphragmatic pathologic stage IA were generally treated with inverted Y radiotherapy that included the splenic pedicle. In patients with pathologic stage IIA and II_S A with limited splenic involvement, TNI (including the mediastinum) and combined modality therapy appeared to be of comparable efficacy on the basis of limited data.[13] [62] [65]

For all subdiaphragmatic clinical stage I and II presentations, the incidence of possible intra-abdominal involvement is high enough to recommend combined chemotherapy and limited irradiation. Although results with primary irradiation appear similar, this approach requires staging laparotomy, which is no longer warranted.

Complications of Radiotherapy

Complications of radiotherapy are related to the technique employed, dosage administered, and irradiated volume. [43] [46] Mantle

and para-aortic radiotherapy or TNI is technically difficult and potentially hazardous. The complications of radiation are also directly related to the skill and experience of the radiotherapist. The acute side effects of radiotherapy include transient nausea and vomiting, dysphagia, and marrow suppression. These side effects subside shortly after radiotherapy is completed. Temporary alopecia may be expected in the occipital regions on either side of the mantle field. Late potential side effects of radiation include elevation of thyroid-stimulating hormone in 2550% and clinical hypothyroidism in 1020% of patients; [66] [67] symptomatic radiation pneumonitis in <5%; transient myelitis (generally manifested as electric-like shocks in limbs on neck flexion, known as Lhermitte sign); and rarely, radiation pericarditis or chronic restrictive fibrosis. Persistent myelosuppression is also a rare late complication. Radiation-induced decrease in bone growth has been noted in children. Radiotherapy appears to accelerate coronary artery disease.[68] [69] [70] The Stanford group has reported a 3.1 increased relative risk of death, primarily from late fatal myocardial infarction.[70] Variables affecting risk included: radiation dose (3000 cGy, no increased risk; >3000 cGy, relative risk was 3.5), lack of cardiac shielding, young age at radiotherapy (particularly younger than 20), and increasing duration of follow-up. Of 49 patients dying of myocardial infarction, 53% had a documented smoking history. The incidence of second neoplasms following radiotherapy alone or combined modality therapy is discussed in a separate section.

Combined Modality Therapy or Chemotherapy Alone for Early- and Intermediate-Stage Disease

Combinations of radiotherapy and chemotherapy in the treatment of early-stage HD have been used since about 1970, with the goal of increasing the cure rate. It is reasonable to assume that chemotherapy, effective in curing most patients with advanced disease, should be even more effective for subclinical disease that might be present after radiotherapy. Relapses after radiotherapy frequently occur in areas of extranodal disease, whereas relapses after chemotherapy most frequently occur in sites of pretreatment involvement, including bulky lymph node areas.

Combined Modality Therapy

Combined modality therapy for clinical stage I and II HD is shown in Table 68-8 . Randomized trials conducted at Stanford[37] [41] in pathologic stage I-IIA HD demonstrated that adjuvant chemotherapy could replace prophylactic irradiation to areas of occult subclinical disease. IF radiotherapy followed by MOPP or phenylalanine mustard, Alkeran, vinblastine (PAVe) chemotherapy achieved freedom from relapse and overall survival results comparable to those of TNI or STNI. [37] [41] [71] In a small study from Stanford,[41] [72] asymptomatic patients with pathologic stage I, II, and IIIA HD were randomly assigned to involved field radiotherapy plus vinblastine, bleomycin, methotrexate (VBM) chemotherapy versus STNI. Initially, there was no difference in either freedom from relapse or overall survival. However, follow-up results of that study[73] reveal freedom from progression at 10 years of 98% for VBM + IF vs. 78% for STNI. Overall survival of all patients, regardless of treatment arm, is 100% at 10 years. The VBM combination was empirically developed because of its presumed lower long-term morbidity (i.e., less effect on fertility and lower risk of leukemia). However, pulmonary toxicity is a potential risk in this bleomycin- and methotrexate-containing regimen. Although less toxic than either adjuvant MOPP or PAVe, the VBM combination is untested in large numbers of patients with advanced HD. A randomized study by the Stanford group [73] comparing sTNI vs. VBM plus IF in 78 clinically staged patients has again demonstrated comparable outcomes at 4 years median follow-up, freedom from progression was 92% for radiation alone and 87% for the combined modality regimen. These excellent results will need confirmation in a larger prospective study before VBM + IF can be considered an effective alternative in favorable presentations of early stage HD.

More recent trials confirm the data that combined modality therapy may substitute for primary irradiation. The EORTC [74] has compared a novel chemotherapy regimen of epirubicin, bleomycin, vinblastine, prednisone (EBVP) for six cycles plus IF versus STNI in 333 clinically staged favorable HD patients. This H7 study revealed a 5-year event-free survival of 90% versus 81% in favor of combined modality therapy.

The Milan group[75] has confirmed that IF irradiation is adequate as consolidative irradiation following ABVD. Santoro et al treated 103 clinical stage I and II patients with four cycles of ABVD followed by either IF or sTNI irradiation. With median follow-up of 38 months, freedom from progression was 95% versus 94%, with 100% of patients surviving.[75]

Cosset et al[39] reported the updated EORTC results in clinical stage III patients classified as having any one or more of the following unfavorable prognostic factors: B

symptoms, age 40 years, ESR 70, MC or LD histology, and/or clinical stage II with mediastinal involvement. In these patients, laparotomy was not performed. Patients were randomly assigned to either TNI or three cycles of MOPP, followed by mantle radiotherapy

TABLE 68-8 -- Clinical Stage I and II Hodgkin Disease: Combined Modality Therapy

	#	FFP	DFS	OS	Actuarial Analysis	Median Follow-up
Stanford[73]						
PS I-IIIA	72					9 yrs
sTNI vs.		78%		100%	10 yrs	
VBM + IF		98%		100%	10 yrs	
CS I-IIA	48					4 yrs
sTNI vs.		92%		90%	5 yrs	
VBM + IF		87%			5 yrs	
Milan[75]						
CS I-IIA	103					38 mos
ABVD + sTNI vs.		94%		100%	4 yrs	
ABVD + IF		95%		100%	4 yrs	
EORTC[74]						
CS I-II	333					6 yrs
favorable sTNI vs.			81%	95%	5 yrs	
EBVP + IF			90%	98%	5 yrs	

and a further three cycles of MOPP (Table 68-7). There was a significant difference in freedom from relapse at 9 years in favor of combined modality treatment (83% versus 66%), but overall survival showed only a borderline advantage for the combined modality patients (Table 68-7). In patients <40 years of age, no difference in long-term survival could be detected between the two forms of treatment.

A second EORTC trial in this unfavorable cohort randomized patients to MOPP for three cycles, mantle radiotherapy, MOPP for three cycles; or to ABVD for three cycles, mantle radiotherapy, ABVD for three cycles. At 5 years, RFS was significantly lower in the MOPP group (79%) than in the ABVD group (89%), but no difference in overall survival was noted (groups were virtually identical at 89%). [39] The M. D. Anderson group[50] [76] reported similar results with combined modality therapy using two cycles of MOPP and IF radiotherapy.

The obvious conclusion can be drawn from these studies that staging laparotomy is unnecessary if combined modality treatment is to be used as initial therapy. Moreover, when treatment is selected on the basis of prognostic factors in stage I and II disease, virtually all patients can be managed without staging laparotomy, using primary irradiation for very favorable disease settings and combined modality therapy for all other presentations. Questions remaining in this patient group include: which combination chemotherapy regimen should be utilized (ABVD as standard vs. others, such as VBM, [72] [73] EBVP,[74] EVA,[77] and NOVP[78]); what should be the duration of chemotherapy (short course, 34 cycles, vs. full course, 6 cycles); and finally, is the adjuvant regional irradiation necessary?

Less conclusive prospective data is available regarding combination chemotherapy alone in early stage Hodgkins disease. The National Cancer Institute (NCI) has conducted a pilot study in patients with pathologic stages IBII and IIIA, comparing IF radiotherapy followed by six cycles of MOPP, as compared to MOPP alone. [79] The small number of patients in each stage precludes definitive conclusions from this study. However, to date, there are no significant differences in either disease-free survival, freedom from relapse, or overall survival among these two groups.

Pavlovsky et al[80] reported the results of a randomized trial of cyclophosphamide, vinblastine, procarbazine, prednisone (CVPP) chemotherapy for six monthly cycles versus the same chemotherapy plus IF radiotherapy (3,000 cGy) in 277 patients with clinical stage III HD (Table 68-7). One or more of the following factors were considered prognostically unfavorable: age >45 years, more than two lymph node areas involved, or bulky disease. In the favorable group without any of these adverse prognostic factors, disease-free survival (77% vs. 70%) and overall survival (92% vs. 91%) were similar at 7 years for CVPP versus radiotherapy plus CVPP. These results are similar to comparable series of patients treated with mantle and para-aortic radiotherapy alone. Among patients in the unfavorable prognostic subgroup reported on by Pavlovsky et al[80] those treated with radiotherapy plus CVPP had a longer disease-free survival (75% vs. 34%) and overall survival (84% vs. 66%) than those patients treated with CVPP alone. However, the results with CVPP chemotherapy alone in this subgroup appear distinctly inferior to those reported in a small series of early stage patients treated with MOPP at the NCI. [81]

Longo et al[81] recently reported the updated results of a randomized trial of MOPP chemotherapy alone versus mantle and para-aortic radiotherapy in 106 patients with surgically staged IA, IB, IIA, IIB, and III_1 A disease (Table 68-7). With a median follow-up of 7.5 years, 96% of MOPP-treated patients achieved complete remission, with 7 (13%) relapses. Of the radiotherapy-treated patients, 96% achieved a complete response, but 17 (35%) relapsed. However, the radiotherapy arm of the study contained more patients with large mediastinal masses than did the chemotherapy arm. Four MOPP-treated patients (7%) and 10 radiotherapy-treated patients (20%) have died. Although the curves of complete response duration significantly favor MOPP, overall survival is not significantly different. [81] When patients with either large mediastinal masses or stage III_1 A are excluded from the analysis, there are no significant differences in either disease-free survival (67% for radiation vs. 82% for MOPP) or overall survival (85% for radiation vs. 90% for MOPP). Patients treated with MOPP had significantly more hospital admissions, episodes of febrile neutropenia, and documented infections and received more blood transfusions. The results from this study do not permit one to conclude that MOPP alone can be safely substituted for mantle and para-aortic radiotherapy for early-stage HD.

Biti et al[82] updated the results of a small randomized trial comparing MOPP alone with mantle and para-aortic radiotherapy for 89 patients with pathologic stage IIIA disease (Table 68-7). Complete remission was obtained in all radiotherapy patients compared with 40 of 44 (91%) of the MOPP patients. With a median follow-up of 8 years, overall survival was significantly higher in the radiation therapy group (93%) as compared to 56 percent in the MOPP group ($P < 0.001$), whereas freedom from progression and disease-free survival were similar. Relapsing patients had a much higher salvage rate in the radiotherapy group, reflected in the significantly improved overall survival. True recurrences (in sites of initial presentation) were significantly more frequent in the chemotherapy patients than in those treated with radiotherapy, who relapsed mainly in previously uninvolved sites. Early relapses (within 1 year from the end of treatment) were also more frequent in the chemotherapy group. Moreover, treatment-related complications were more severe in the chemotherapy group. [83] These data suggest that curative radiotherapy may achieve better local control of HD than does MOPP chemotherapy. Whether combined modality therapy is better than chemotherapy alone when utilizing regimens not containing MOPP, is the important question today in early stage Hodgkin disease. This answer must await future prospective study.

The role of primary irradiation in stage IIIA HD has previously been controversial. Table 68-9 summarizes the treatment results with either radiotherapy alone (generally mantle plus para-aortic irradiation or TNI), combined modality therapy, or chemotherapy alone. The use of combined radiation therapy and chemotherapy is associated with both a lower risk of relapse and improved survival for most patients with stage IIIA disease, as compared with radiation therapy alone. [84] [85] Previously, it has been reported that pathologic stage III_1 A (pathologic involvement of the spleen and/or upper abdominal nodes) and minimal splenic involvement could be successfully treated with either mantle and para-aortic radiotherapy or with TNI alone. [86] However, even in such patients, Marcus et al have recently reported a significant survival advantage for combined modality therapy. [31] Overall, among 93 patients with PS III_1 A retrospectively analyzed, freedom from relapse at 10 years was 39% for STLI, 55% for TNI, and 94% for MOPP plus STLI. Ten-year actuarial survival was 89% for combined modality patients versus 78% for STLI and 70% for TNI. Therefore, primary irradiation alone is no longer indicated in any subset of stage IIIA HD.

The most important question pertaining to the treatment of stage IIIA patients today is whether combined modality therapy is superior to chemotherapy alone.

Investigators from the NCI reported a 94% 10-year disease-free survival in a small number of stage IIIAIVA patients treated with MOPP alone. [87] Lister et al[88] reported a 10-year freedom from relapse rate of 85% in patients treated with mechlorethamine, vinblastine, procarbazine, and prednisone (MVPP) chemotherapy alone, as compared to 60% with TNI. However, there were no significant differences in overall survival between these two modalities. Crowther et al [89] compared MVPP chemotherapy alone with the same

TABLE 68-9 -- Stage IIIA Hodgkin Disease: Treatment Results

Investigators	Stage	Treatment	FFR (%)	Survival (%)
Stein et al[84]	III_1 [a]	XRT	60	76
		CMT	92	88
	III_2 [b]	XRT	20	41
		CMT	84	84
Mauch et al[92]	$IIII_1$	XRT	53	73
		CMT	92	97
	III_2	XRT	15	44
		CMT	73	66
Hoppe et al[85]	$IIII_1$	XRT	59	91
		CMT	93	89
	III_2	XRT	63	83
		CMT	77	90
Mauch et al[92]	Minimal splenic involvement[c]	XRT	62	100
		CMT	92	92
	Extensive splenic involvement[d]	XRT	35	58
		CMT	81	77
Hoppe et al[85]	Minimalsplenic in-volvement[c]	XRT	80	80
		CMT	80	85
	Extensive splenic involvement[d]	XRT	30	70
		CMT	85	85
Marcus et al[31]	IIIA, minimal splenic involvement[c]	sTNI	39	78
		TNI	55	70
		CMT	94	89
Henkelmann et al[93]	III_1	MOPP × 2 + STLI/TNI	84	86
Lister et al[88]	AII IIIA	TNI	60	83
		MVPP	85	90
Crowther et al[89]	AII IIIA	MVPP	82	87
		MVPP + XRT	72	84
Santoro et al[90]	AII IIIA	MOPP × 3, XRT,	65	NS
		MOPP × 3	92	NS
		ABVD × 3, XRT, ABVD × 3		

Abbreviations: XRT, radiotherapy (either mantle and para-aortic or TNI); TNI, total nodal irradiation; STLI, subtotal lymphoid irradiation; CMT, combined modality therapy (generally extended field radiotherapy + MOPP); MVPP, nitrogen mustard, vinblastine procarbazine, prednisone; MOPP, nitrogen mustard, vincristine, procarbazine, prednisone; ABVD, Adriamycin, bleomycin, vinblastine, decarbazine; NS, not stated; FFR, freedom from relapse.

[a] III_1 , pathologic involvement of any of the following sites: spleen; splenic hilar, celiac, porta hepatis nodes.

[b] III_2 , pathologic involvement of para-aortic nodes, iliac, or inguinal nodes with or without involvement of the spleen and upper abdominal nodes.

[c] Minimal splenic involvement: <5 splenic nodules.

[d] Extensive splenic involvement: 5 splenic nodules.

chemotherapy regimen plus radiotherapy. There were no significant differences in either disease-free survival or overall survival between these two arms. Thus, it appears that MOPP- based chemotherapy alone is as effective as combined modality therapy for patients with stage IIIA disease.

In a small randomized study from Milan of 63 stage IIIA patients treated with three cycles of either MOPP or ABVD followed by extensive irradiation and then in some cases by three additional cycles of the same chemotherapy, the 7-year results indicated both improved complete response rates and freedom from progression with ABVD plus RT.[90] Moreover, it was reported that irreversible gonadal dysfunction as well as acute leukemia occurred only in patients subjected to MOPP, while cardiopulmonary studies failed to document a significant laboratory difference between the two treatment arms. These results suggest that ABVD as part of a combined modality therapy program is at least as effective as MOPP, with decreased morbidity.

The radiation dose and field size employed in combined modality therapy of bulky and intermediate stage Hodgkins disease continues to be defined. Low-dose RT (20002400 cGy) combined modality regimens have been utilized in pediatric patients and in adults with advanced stage disease. A recent prospective comparison of 2000 cGy versus 4000 cGy RT as extended field adjunctive therapy following combination chemotherapy of Stages IIIIA (bulky mass, E lesion, or massive splenic involvement) has revealed no significant differences in failure-free or overall survival at 5 years. [91] The bulky site was always treated with 4000 cGy. Among 26 relapsing patients, 18 failed in the bulky site or in extranodal sites. Acute toxicities were greater in the 4000 cGy group. Loeffler et al have concluded that 2000 cGy is sufficient as adjuvant RT in nonbulky sites, as part of a combined modality regimen. [91] This important report will require confirmation in larger studies before this dose schedule can be considered standard.

Patients with clinical or pathologic stage IIIB disease are generally treated with combination chemotherapy alone, as described in subsequent sections. However, selected small series suggest a possible role for combined modality in these patients. Mauch et al [92] treated 43 stage IIIB patients with combined modality therapy (IF radiotherapy plus MOPP). At 12 years they observed excellent freedom from relapse (79%) and overall survival (76%). Rosenberg and Kaplan, [37] reporting for the Stanford group, performed a series of prospective trials in pathologic stage IIIB disease, in which, again, small numbers of patients were treated with each combined modality therapy regimen. TNI plus MOPP resulted in only a 51% freedom from progression rate and 52% overall survival at 10 years. The Stanford investigators then proceeded to use two courses of initial chemotherapy, followed by TNI, followed by four cycles of the same chemotherapy (split-course technique). In this series of 42 patients, freedom from relapse of 82% and overall survival of approximately 72% at 8 years were observed. [37] Henkelmann et al[93] treated 26 patients with pathologic

stage III_1 B disease with two cycles of MOPP and TNI. They reported 78% freedom from progression and 91% overall survival at 10 years. In the randomized study conducted by Santoro et al,[90] a total of only 28 patients were treated with three cycles of either MOPP or ABVD, followed by IF radiotherapy, and then with three cycles of the same drug regimen. In this series, 78% of IIIB patients achieved complete remission with MOPP, as compared to 94% with ABVD. Freedom from progression also favored the ABVD patients (92% at 7 years), as compared with MOPP (65%).

Chemotherapy of Advanced Disease

MOPP-Containing Regimens

Since the late 1960s, a dramatic improvement has been observed in the prognosis of patients with advanced HD resulting from the development of curative combination chemotherapy. Until recently, MOPP represented the standard against which all alternative chemotherapy or combined modality regimens were judged. The landmark studies of DeVita et al[87] [94] at the NCI demonstrated that >50% of patients with advanced HD are cured with chemotherapy alone. An update of the NCI data has been reported by Longo et al, [95] who reanalyzed 188 of the original NCI patients after a median of 14 years of follow-up. A complete remission rate of 84% was reported; also, 66% of the complete responders had remained disease-free for>10 years. Thus, 54% of patients were continuously free of disease, and 48% were alive between 9 and 21 years from the end of treatment. It is important to note that 19% of the complete responders died of intercurrent illnesses while free of HD.

The major factors adversely affecting complete response rate in the NCI-MOPP trials were B symptoms, male sex, advanced-stage disease, and lower than projected rate of vincristine administration. [95] The most important factors predicting duration of complete response were B symptoms, age, rapidity of complete response (patients requiring five cycles or less had significantly longer remissions), number of extranodal sites of disease, and liver or pleural involvement. Maintenance chemotherapy after achieving complete remission had no influence on either disease-free or overall survival.

In the NCI-MOPP trials, most patients required six cycles of chemotherapy, but approximately 2025% of patients required two or more additional cycles. Restaging studies were generally performed after the fourth cycle, and if they were negative, two more cycles were administered. However, if continued tumor response was observed between cycles 4 and 6, two additional cycles of chemotherapy were administered followed by repeat staging studies. [33] Thus, it is current practice to administer six to eight cycles of chemotherapy, using the general guideline of treating to clinical or pathologic complete response (usually reassessed first after four cycles) plus two additional cycles. In the case of a stable residual mass that is unchanged by radiography or CT scan after two or more additional cycles of chemotherapy, the patient is classified as a clinical complete responder and chemotherapy is stopped. Further evidence for complete response in this situation is gained if the tumor mass went from gallium scan-positive before chemotherapy to gallium scan-negative after chemotherapy. These same principles apply to the duration of therapy for the newer drug combinations described later in this section.

The NCI-MOPP trials and subsequent studies over the next decade continued to use the end points of complete response, disease-free survival, and overall survival as measures of efficacy. However, determination of complete response may be difficult and ambiguous in the setting of advanced HD. This is particularly true in bulky disease, where a residual mass on radiography is frequently observed after six to eight cycles of combination chemotherapy. Disease-free survival refers only to patients who achieve a complete remission and does not reflect the outcome of the whole population of patients treated. Moreover, some investigators censor out patients who die from causes other than HD from their disease-free survival curves, artificially improving their results. Therefore, the most accurate and definitive end point for determining the effectiveness of any chemotherapy regimen is failure-free survival, also referred to as freedom from progression. Failure-free survival is defined for the entire group of patients treated and therefore counts as an event the following parameters: progressive disease, relapse from complete or partial remission, and death from any cause. Overall survival remains the most important end point and reflects not only the effectiveness of the primary chemotherapy regimen but also the efficacy of any secondary or salvage treatments.

Although MOPP revolutionized the treatment of advanced HD, there is ample opportunity for improvement in these results, since 1530% of patients do not achieve initial complete remission, and 3040% of complete responders eventually relapse. Although multiple studies with MOPP confirmed its efficacy in the treatment of advanced HD, numerous investigators attempted to design alternative combinations aimed at improving the therapeutic index of MOPP by reducing toxicity and attempting to improve the cure rate. These trials are described in detail in several reviews, [33] [46] [96] and selected alternative chemotherapy regimens are listed in Table 68-10 . For example, the BCVPP (BCNU [carmustine], cyclophosphamide, vinblastine, procarbazine, prednisone) regimen adds BCNU (carmustine or 1,3 bis[2-chloroethyl 1]-1-nitrosourea), while substituting cyclophosphamide for nitrogen mustard and vinblastine for vincristine. In a prospective controlled trial conducted by the Eastern

TABLE 68-10 -- Selected Chemotherapy Regimens for Advanced Hodgkin Disease

ABVD
Doxorubicin (Adriamycin) 25 mg/m^2 IV, days 1, 15
Bleomycin 10 U/m^2 IV, days 1, 15
Vinblastine 6 mg/m^2 IV, days 1, 15
Dacarbazine 375 mg/m^2 IV, days 1, 15
MOPP (NCI)
Nitrogen mustard 6 mg/m^2 IV, days 1, 8
Vincristine (Oncovine) 1.4 mg/m^2 IV, days 1, 8
Procarbazine 100 mg/m^2 PO, days 114
Prednisone 40 mg/m^2 PO, days 114
MOPP/ABVD
Month 1
Nitrogen mustard 6 mg/m^2 IV, days 1, 8
Vincristine (Oncovine) 1.4 mg/m^2 IV, days 1, 8 (max = 2 mg/dose)
Procarbazine 100 mg/m^2 PO, days 114
Prednisone 40 mg/m^2 PO, days 114
Month 2
Doxorubicin (Adriamycin) 25 mg/m^2 IV, days 1, 15
Bleomycin 10 U/m^2 IV, days 1, 15
Vinblastine 6 mg/m^2 IV, days 1, 15
Dacarbazine 375 mg/m^2 IV, days 1, 15; alternate one cycle of monthly MOPP with one cycle of monthly ABVD
MOPP/ABV hybrid
Nitrogen mustard 6 mg/m^2 IV, day 1
Vincristine (Oncovine) 1.4 mg/m^2 IV (max = 2 mg/dose)
Procarbazine 100 mg/m^2 PO, days 17

Prednisone 40 mg/m^2 PO, days 1–14
Doxorubicin (Adriamycin) 35 mg/m^2 IV, day 8
Vinblastine 6 mg/m^2 IV, day 8
Bleomycin 10 U/m^2 IV, day 8, repeat every 28 days

Cooperative Oncology Group (ECOG), Bakemeier et al [97] compared BCVPP directly with MOPP. Although the complete response rates were virtually identical, the duration of complete response and the overall survival of the complete responders were significantly longer with BCVPP. Moreover, despite inclusion of the additional myelosuppressive agent BCNU, there were no significant differences in hematologic toxicity. Both severe and life-threatening neurotoxicity and gastrointestinal toxicity were significantly reduced on the BCVPP arm. Reporting for the Southeast Oncology Group, Gams et al [98] achieved similar results with BCVPP.

Several other alternatives to MOPP that are considered useful under specific circumstances emerged during the mid- to late 1970s. The MVPP combination substitutes vinblastine for vincristine (Oncovin) and has been used for patients who cannot tolerate the neurotoxicity of vincristine. [99] The LOPP regimen substitutes chlorambucil (Leukeran) for nitrogen mustard. A controlled trial compared LOPP with MOPP and achieved equivalent results with less toxicity for LOPP. [100] However, the results with both arms are inferior to other reports. The ChlVPP protocol [101] [102] substitutes chlorambucil for mustard and vinblastine for vincristine. Nausea, vomiting, and neurotoxicity are substantially reduced with ChlVPP, which makes it a valuable regimen, particularly for elderly patients. Selby et al [101] updated the British experience with ChlVPP in 229 previously untreated patients, 85% of whom entered complete remission. The disease-free survival of complete responders was 71% at 10 years,

and the 10-year overall survival for all patients was 65%. Acute toxicity was mild, and the 10-year actuarial risk of leukemia was 2.7%. Reporting for four research groups that pooled their data on the use of ChlVPP, Anderson et al [102] observed an 89% and 72% complete response rate in stage IIIA and IIIB/IV patients, respectively. Failure-free survival and overall survival at 5 years were 67% and 78%, respectively, for the stage IIIA patients. For the IIIB/IV group, 5-year failure-free survival was 51%, and 5-year overall survival was 63%. Patients aged 50 years with all stages of disease did especially poorly on ChlVPP therapy, making this less-toxic regimen much less useful in the older age population. [102] Although the BCVPP, MVPP, and ChlVPP regimens are attractive alternatives to MOPP, these combinations are generally no longer recommended as initial therapy.

The identification of the ABVD regimen as an active non-cross-resistant combination in relapsed HD led to the investigation of this new and important regimen in the previously untreated patient. In the original comparison of MOPP and ABVD as initial treatment, [103] the complete response rate with MOPP was 63%, as compared to 72% with ABVD. In addition, disease-free and overall survival were similar. Bonadonna et al [104] then proceeded with a controlled randomized trial in which 88 patients with stage IV HD previously untreated by chemotherapy were treated in a 12-month cycle with either MOPP alone or MOPP alternating every month with ABVD (Table 68-11). Although the complete remission rate with MOPP/ABVD was 89% compared to 74% with MOPP, this difference was not statistically significant, undoubtedly because of the small number of patients entered in this trial. The 8-year actuarial results also indicated that MOPP/ABVD was superior to MOPP in terms of freedom from progression (64% vs. 36%, respectively) as well as disease-free survival of the complete responders (73% vs. 45%). Tumor mortality (only deaths from HD included) was also significantly reduced on the MOPP/ABVD arm. However, the overall survival (including deaths from all causes) at 8 years was 76% on MOPP/ABVD versus 62% on MOPP. This difference is not statistically significant, but again this may be due to the small sample size in this study. Favorable prognostic factors in this trial include the fact that 28% of patients relapsed after radiotherapy alone and 30% were asymptomatic. Although this study has been criticized for the lower-than-expected freedom from progression and disease-free survival in the MOPP-alone arm, it is interesting to note that there were no differences in the overall survival rate between MOPP results seen in this study and the MOPP results from the NCI. However, a larger patient population and confirmatory clinical trials were clearly required before the apparent superiority of MOPP/ABVD could be accepted.

Two confirmatory trials have been completed and the results reported (see Table 68-11). Canellos et al [105] described the 5-year results of the Cancer and Acute Leukemia Group B (CALGB) controlled trial comparing MOPP, ABVD, and MOPP/ABVD in 361 eligible patients with HD stage III $_2$ AIVB. Patients received a minimum of six cycles of treatment with either MOPP or ABVD, which included two additional cycles after complete remission was obtained; MOPP/ABVD patients received six cycles of each combination for a total of 12 cycles. While this study has been criticized for the reduced doses of MOPP administered, doses were adjusted on the basis of observed toxicity and were not arbitrarily reduced. The complete response rate for MOPP/ABVD was 83%, compared with 67% for MOPP. However, the complete response rate for ABVD of 82% was similar to that with MOPP/ABVD. With a median follow-up of >4 years, these data have been reported in terms of 5-year actuarial results. Failure-free survival reported with MOPP/ABVD (65%) was significantly improved as compared to MOPP (50%) but was similar to that obtained with ABVD alone (61%). The overall 5-year survival for the treatment groups was similar and was not statistically significant: MOPP 66%, ABVD 73%, and MOPP/ABVD 75%. To date there is no advantage of MOPP/ABVD over ABVD alone. The ABVD regimen is attractive because of its relative lack of gonadal toxicity and absence of acute leukemia. However, on the question of ABVD or MOPP/ABVD versus MOPP, the CALGB trial clearly showed the superiority of MOPP/ABVD or ABVD alone.

The ECOG compared the MOPP/ABVD regimen with BCVPP alone and with BCVPP followed by low-dose consolidation radiotherapy administered to sites of pretreatment involvement in patients achieving a complete response. [106] In this protocol, each of the three chemotherapy regimens was administered for a minimum of eight cycles (or to complete response plus two cycles). Since there were no differences in complete response rate, failure-free survival, or overall survival at 6 years between the two BCVPP arms, they have been combined and compared directly with MOPP/ABVD. The complete response rate on MOPP/ABVD of 80% was significantly better than the 69% complete response rate on the combined BCVPP arms. Although there are no significant differences in complete response duration, both failure-free survival and overall survival were

TABLE 68-11 -- Chemotherapy for Advanced Hodgkin Disease

Group	Regimen	N	CR (%)	FFS (%)	OS (%)	Median Follow-up (years)
NCI-US[95]	MOPP	188	84	(55)	48	14
Milan[104]	MOPP	43	74	36	64	8
	vs MOPP/ABVD	45	89	65[a]	84[a]	
ECOG[106]	BCVPP ± RT	130	69	47	63	6
	vs MOPP/ABVD	98	80[a]	59[a]	73[a]	
CALGB[105]	MOPP	123	67	50	66	5
	vs ABVD	115	82[a]	61[a]	73	
	vs MOPP/ABVD	123	83[a]	65[a]	75	
Intergroup[111]	MOPP ABVD	344	79	54	71	7.3
	vs MOPP/ABV hybrid	347	83[a]	64[a]	79[a]	
NCI Canada[112]	MOPP/ABVD ± RT	148	76	67	83	5.75
	vs MOPP/ABV hybrid ± RT	153	83	71	81	
Milan[113]	MOPP/ABVD	211	91	67	74	9
	vs MOPP/ABVD hybrid	204	89	69	72	
CALGB[114]	MOPP/ABV		71	65	87	3
	ABVD	856	73	67	85	3

Abbreviations: N, number of eligible patients; CR, complete response rate; FFS, failure-free survival for all eligible patients entered in study; OS, overall survey for all patients; RT, radiotherapy. See Tables 68-9 and 68-10 for definitions and doses of the chemotherapy regimens.

[a]P 0.05 for the comparison of the experimental arm compared with the MOPP-type regimen.

significantly improved on MOPP/ABVD. At 6 years, the failure-free survival on MOPP/ABVD was 59% versus 47% on the combined BCVPP arms. At 6 years, 73% of the MOPP/ABVD patients were alive, compared with 63% of those receiving either of the BCVPP regimens. Importantly, the MOPP/ABVD data from ECOG [106] are virtually identical to those reported by CALGB [105] and by Bonadonna et al[104] All three of these prospectively randomized trials provide strong evidence of the superiority of MOPP/ABVD over MOPP.

The MOPP/ABVD regimen was based on clinical empiricism (i.e., the therapeutic limitations of MOPP and the apparent effectiveness of ABVD as salvage therapy in MOPP-refractory patients) and preceded the mathematical model proposed by Goldie et al [107] The Goldie-Coldman hypothesis predicts that the efficacy of treatment will be enhanced by the earliest introduction of the most rapid alternation of all active single agents in a chemotherapy regimen to decrease drug resistance. Klimo and Connors[108] [109] [110] reported the updated results of a MOPP/ABV hybrid regimen (Tables 68-8 and 68-11) designed to test the Goldie-Coldman hypothesis prospectively. In this regimen, decarbazine is omitted, the adriamycin dose is increased, prednisone is administered for 14 days, and MOPP is given on day 1, while ABV is given on day 8. Treatment is administered for a minimum of six courses of therapy, with patients in complete remission at that time receiving two additional courses. In patients who have a partial response limited to a single nodal area, local radiotherapy is administered, whereas for more extensive residual disease further MOPP/ABV is given. In the original report, of the 76 evaluable patients, 97.5% achieved complete remission, 84% on chemotherapy alone and 13% with IF radiotherapy. In Klimo and Connorss 1988 report,[109] only seven patients (9.5%) relapsed, most of these patients being males with large mediastinal masses and B symptoms. Actuarial RFS of the 74 complete responders was 94% with a median follow-up of almost 4 years; overall survival for all patients was 79%. The toxicity of this regimen was acceptable, with only one death from toxic effects and <10% of patients requiring hospitalization for a suspected or proven systemic infection. In this study, 40% of patients were asymptomatic, 17% had stage II disease, and the very high complete response rate included patients who received radiotherapy for residual disease. Nonetheless, the results with the MOPP/ABV hybrid regimen represented a potential increase in the cure rate for advanced HD. In addition, it remained to be determined whether the MOPP/ABV hybrid was equivalent or superior to alternating monthly MOPP/ABVD.

Three confirmatory trials have now been reported (Table 68-11). The Intergroup trial led by ECOG compared the MOPP/ABV hybrid regimen to sequential MOPP followed by ABVD, in which MOPP alone is administered to the point of complete remission or stable partial response followed by three cycles of ABVD in consolidation.[111] No radiotherapy was administered in either arm of the Intergroup trial. The complete response rate on the MOPP/ABV hybrid was 83%, compared with 79% on the sequential regimen ($P < 0.02$). The 8-year failure-free survival (FFS) of 64% on the hybrid program was also superior to the 54% 8-year FFS on the sequential arm ($P = .01$). Moreover, the 8-year overall survival of 79% for MOPP/ABV hybrid was significantly better than the 71% overall survival on the sequential MOPP followed by ABVD ($P = 0.02$). There was no difference in the incidence of severe or very severe leukopenia between the two arms, while significantly more patients on the sequential MOPP/ABVD arm had thrombocytopenia. Infectious and pulmonary complications were greater on the hybrid arm, whereas there was no difference in neurotoxicity. Myelodysplasia or acute myelogenous leukemia was documented in nine patients receiving sequential MOPP/ABVD and in one patient receiving MOPP/ABV hybrid. ($P = 0.01$) Thus, the Intergroup trial provides definitive evidence for the superiority of MOPP/ABV hybrid over MOPP, even when MOPP is augmented by ABVD given as late consolidation to complete responders.

The NCI of Canada has compared the MOPP/ABV hybrid regimen with Bonadonnas alternating monthly MOPP/ABVD, with radiotherapy administered in both arms to selected patients with residual nodal disease. [110] [112] The most recent analysis at 5 years reveals that the complete response rates were similar: 83% on the hybrid and 76% on MOPP/ABVD.[112] At 5 years, the FFS for both regimens is not significantly different (Table 68-11). This important trial demonstrates the equivalence of the MOPP/ABV hybrid regimen and alternating monthly MOPP/ABVD. In an earlier report from the NCI of Milan, Viviani et al [113] also compared alternating half-cycles of MOPP and ABVD with their original MOPP/ABVD program and found no significant differences between these two eight-drug regimens (see Table 68-11).

ABVD as Standard Chemotherapy

Thus, the cumulative results of prospective trials comparing MOPP or MOPP-containing regimens with ABVD alone (as described previously and as outlined in Table 68-11) have demonstrated that ABVD must now be considered the standard drug combination for advanced HD. The first report suggesting ABVD superiority was by the Milan group comparing MOPP with MOPP/ABVD. The CALGB subsequently compared MOPP versus MOPP/ABVD versus ABVD, confirming the superiority of the ABVD-containing regimens and demonstrating the equivalence of MOPP/ABVD and ABVD. MOPP/ABVD was then compared to MOPP/ABV hybrid by the NCI Canada and found equivalent.

Finally, the most recent pivotal intergroup study is that led by CALGB comparing ABVD versus MOPP/ABV hybrid in which the outcomes of 856 prospectively randomized patients were reported with three-year median results. [114] Although follow-up is still short, outcomes were equivalent between the two regimens (complete response 73% vs. 71%; FFS 67% vs. 65%; and overall survival, 85% vs. 87%). However, toxicity was significantly greater with the MOPP-containing hybrid regimen for pulmonary, hematologic, and infectious complications. Moreover, secondary malignancies were significantly more frequent with the hybrid regimen.

A summary algorithm of Table 68-11 may be outlined as
ABVD MOPP/ABV hybrid = MOPP/ABVD > MOPP
The selection of ABVD as the standard regimen is based on equivalent, or better, complete response rates, failure-free survival and survival as compared with MOPP-containing regimens. Moreover, toxicities of infertility, secondary leukemia, and complications of myelosuppression are significantly reduced. ABVD is not without serious potential toxicity, however, and must be used with caution.

Unfortunately, ABVD does not cure all patients with advanced HD, and new strategies are clearly needed. One approach is a short-course, dose-intense regimen such as Stanford V[115] or BEACOPP (see Table 68-12).[116] Both combination programs utilize involved field adjuvant irradiation. Preliminary results have been encouraging, and BEACOPP has been prospectively compared with COPP/ABVD in 321 patients revealing significant improvement in CR (89% vs. 76%), with decreased progression (7% vs. 16%), and death rate (5% vs. 13%). Neither BEACOPP nor Stanford V has been compared to date with ABVD.

Autologous stem cell transplantation has an important place in the standard management of relapsed and refractory HD. Its role in upfront management of poor risk subsets is yet to be defined. The first question is whether a poor risk group can be identified that justifies no initial trial of standard therapy. As

reported by Hasenclever and Diehl, [117] prognostic factors that have been reported as adversely affecting outcome in advanced HD include age 45 years, male gender, Stage IV presentation, albumin <4.0 mg/dl, hemoglobin <10.5 gm/dl, white blood cell count 15,000/l and lymphocyte count <600/l or <8%. In this prognostic model, the cumulative number of factors correlates well with 5-year failure-free survival. For example, FFS at five years is 84% in patients with no poor risk factors, 60% for those with 3 factors, and 42% for those with five or more factors. Such predictive modeling may assist in the design of future clinical trials comparing ABVD with dose-intense regimens.

A pilot study by Carella et al has reported 22 patients experience with MOPP/ABVD induction followed by ASCT consolidation in high-risk HD. [118] A historical comparison with MOPP/ABVD has revealed an improved outcome in the high risk ASCT-treated patients (77% disease free at 86 months as compared to 33% at 89 months for MOPP/ABVD alone). This important question will require a prospective randomized trial with long-term follow-up, as it can be anticipated that ASCT may produce a greater frequency of immediate and late effects that could negatively influence survival outcome in otherwise cured patients.

Since it is well known that most relapses following chemotherapy for advanced HD occur in nodal sites, other investigators have evaluated the additive role of low-dose radiotherapy administered following chemotherapy to sites of pretreatment involvement. Prosnitz et al [119] updated their combined modality results for 102 previously untreated patients with stage IIIBIV disease and for 82 patients who relapsed after initial treatment with radiotherapy. During the initial years of their studies, induction chemotherapy with mechlorethamine, vincristine, vinblastine, procarbazine, prednisone (MVPP) for 6 months was followed by low-dose radiotherapy (1,5002,500 cGy) to all disease sites present prior to chemotherapy. Subsequently, induction chemotherapy for poor-risk advanced-stage patients was changed to MOPP/ABVD and ultimately to a randomization between MVVPP and MOPP. The results of the three induction chemotherapy regimens were similar. The overall

complete response rate was 82%, with a 5-year relapse-free survival rate of 70%. However, the 15-year actuarial overall survival of all treated patients was 54%, which is not significantly different from that reported by the NCI for MOPP alone. Of the 184 patients, 17 died of causes other than HD, 11 with secondary malignancies.[119]

ECOG attempted to reproduce the results reported by Prosnitz et al in two separate controlled studies, reported by Glick et al [106] [120] [121] In the first of these trials, sequential bleomycin-MOPP (Bleo-MOPP) followed by ABVD was compared with the same induction chemotherapy with Bleo-MOPP followed by low-dose radiotherapy administered according to the technique used by Prosnitz et al [119] The overall complete response rate in both arms was identical (75%), but the 8-year freedom from progression on the sequential Bleo-MOPP/ABVD arm was 62%, compared with 44% on the Bleo-MOPP/radiotherapy arm.[121] This difference is statistically significant. In addition, the 8-year overall survival rate of 78% for all patients on the sequential Bleo-MOPP/ABVD arm was significantly better than that achieved on the radiotherapy arm (65%). These results were not known at the time a second ECOG study was initiated in 1981. The Prosnitz radiotherapy technique was again used in an ECOG protocol in which alternating monthly MOPP/ABVD was compared with BCVPP alone or followed by low-dose radiotherapy. Again, no differences in complete response rates, disease-free survival, or overall survival were noted in the two BCVPP arms. [106] The German Hodgkins Study Group has also reported a prospective comparison of consolidative combination chemotherapy (COPP/ABVD) versus low-dose involved field RT (2000 cGy) in 100 complete responders to COPP/ABVD.[122] Involved field RT resulted in no significant improvement of failure-free survival or survival in patients with advanced Hodgkins disease. Thus, three large-scale randomized trials have failed to confirm any significant benefit for administration of low-dose radiotherapy to sites of pretreatment involvement once a complete remission has been obtained with chemotherapy.

Salvage Therapy for Advanced Disease

The choice of salvage therapy for HD relapsing after initial treatment is one of the most difficult challenges and must be individualized to the clinical circumstances of the relapse. Four broad categories of treatment failures after initial therapy have been identified: (1) patients with early-stage disease relapsing from primary radiotherapy; (2) patients whose initial complete response to front-line chemotherapy lasted >1 year; (3) patients whose initial complete response to chemotherapy lasted <1 year; and (4) patients who fail to achieve a complete remission with initial chemotherapy. The treatment of choice for the first category is one of the accepted chemotherapy regimens used as initial treatment for stage III or IV disease. Thus, ABVD, MOPP/ABVD, or the MOPP/ABV hybrid regimen should be used in full doses with the realistic expectation of achieving complete response rates, failure-free survival, and overall survival as good as, or better than, that achieved with initial chemotherapy for stage IIIB and IV disease. Although the risk of significant myelosuppression is recognized under these circumstances, salvage chemotherapy results in the cure of 50% of patients who relapse after radiotherapy alone. [46] [123] As the risk of secondary leukemia will approach 10% at 10 years in patients receiving MOPP salvage regimens, ABVD is the first choice, if possible. However, patients relapsing after receiving mantle radiotherapy may experience an increased risk of cardiac or pulmonary toxicity when ABVD is used alone as a salvage treatment. Therefore, close cardiac and pulmonary monitoring is warranted.

Even for patients whose initial complete response to chemotherapy lasted >1 year, retreatment with the same or a cross-over regimen (e.g., MOPP-treated patients receiving MOPP a second time or crossing over to ABVD at relapse) will result in a durable remission in only one-third or less of cases. [124] [125] [126] [127] [128] [129] [130] The NCI recently updated their experience using retreatment with MOPP in patients with recurrent HD who had previously achieved complete remission with the same initial chemotherapy.[124] Among patients with long initial remissions (>1 year), RFS at 10 years was 45%, but the development of second neoplasms and other treatment-related mortality reduced the overall survival to 24%. Among patients with a short (<1-year) initial remission, only 11% survived >10 years. The follow-up results from the Milan group on long-term salvage therapy with ABVD are similar to the NCI data. [125] Those patients whose initial response to chemotherapy lasted <1 year or who fail to achieve a complete response with initial chemotherapy represent a very poor risk group. Although complete response rates ranging from 1372% have been reported with a variety of standard dose salvage regimens, prolonged disease-free survival is achieved in significantly <20% of patients. [124] [125] [126] [127] [128] [129] [130]

High-dose combination chemotherapy with autologous bone marrow or peripheral stem cell transplantation (ASCT), or both, has become the standard salvage approach for most patients relapsing after initial chemotherapy. This recommendation is based on (1) high rates of durable complete remission with ASCT regimens; (2) low rates of morbidity and mortality in selected patients with the availability of growth factors to hasten nadir myelosuppression recovery; and (3) poor outcomes in most patients treated with standard-dose salvage chemotherapy regimens.

ASCT in relapsed or refractory HD has several technical

components that can influence outcome. These include timing of the intervention (first or later relapse); type of stem cell rescue product (bone marrow or peripheral blood stem cells, or rarely allogeneic bone marrow); type of cytoreductive induction chemotherapy prior to ASCT to demonstrate chemoresponsive disease (e.g., ifosfamide/cisplatin-based regimens); type of preparative regimen (e.g., CBV [cyclophosphamide, BCNU, VP-16]; or BEAM [BCNU, VP-16, cytosine arabinoside, melphalan]); the use of consolidative irradiation; and the use of growth factors and other supportive care measures.

The Stanford group has recently summarized their experience with ASCT in recurrent/refractory HD. A case-matched comparison with conventional salvage therapy revealed significant benefit of ASCT at 4 years, in terms of failure-free progression, 62% versus 32%, although overall survival was not yet significantly improved (54% vs. 47%). [131] Among 119 patients transplanted,[132] adverse prognostic factors prior to ASCT included systemic symptoms, disseminated lung or bone marrow disease, and incomplete response to re-induction chemotherapy prior to ASCT. At four years, patients without adverse factors had an 85% freedom from progression as compared to 41% for those with one or more poor prognostic factors. Survival outcomes were not significantly affected by the kind of conditioning regimen employed, whereas involved field irradiation appeared to improve freedom from relapse in those with nodal involvement prior to ASCT. [133]

The University of Nebraska and M.D. Anderson groups have identified the number of prior chemotherapy regimens (two or fewer vs. three or more) and the absence of bone marrow disease as important prognostic variables in predicting significantly improved survival. Among 128 patients, failure-free survival at 4 years (37%, 31%, 10%) significantly favored patients who had failed one (35 patients), two (45 patients), or three or more (48 patients) chemotherapy regimens, respectively. [134] The Nebraska group has also reported their results of ASCT in 85 patients in first relapse of HD. [135] An overall failure-free survival of 40% at 5 years was reported. The 5-year failure-free survival was 32% for patients relapsing at <18 months after initial diagnosis compared to 47% for those with a relapse of >18 months after diagnosis. An early death was reported in 4% of patients, and overall survival at 5 years was 44%. It was concluded that ASCT should be considered in any patient relapsing after initial chemotherapy, regardless of the remission duration.

Most investigators agree that autologous transplantation is the standard salvage therapy, when possible, for refractory Hodgkin disease and for those patients relapsing within one year of conventional chemotherapy. The small randomized trial of the British National Lymphoma Investigation group [136] has conclusively demonstrated significantly improved event-free survival (at 3 years, 53% vs. 10%) among 40 patients randomized to ASCT with BEAM vs. Mini-BEAM without ASCT, respectively. All patients had refractory or early relapsing disease. Nevertheless, even in this study, overall survival was not yet significantly better for the ASCT group.

For relapses beyond one year, the question is somewhat more difficult as elucidated by Lohri and Connors. [137] However, as the acute toxicities of ASCT are better managed and mortality rates decline, ASCT has emerged as the standard, potentially curative, treatment option for the vast majority of refractory and relapsed patients.

For patients ineligible for ASCT, or in selected patients with very long first remissions, retreatment with the initial drug regimen or a second non-cross resistant conventional drug combination may be utilized. In addition to those listed in Table 68-12 , regimens developed by the Milan group such as lomustine or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), VP-16, and prednimustine (CEP) or ifosfamide/etoposide regimens may be considered. Following CEP, an ifosfamide/etoposide-containing regimen, or retreatment with MOPP/ABVD in

TABLE 68-12 -- New Short-Course Regimens in Hodgkin Disease

BEACOPP[116]		
Bleomycin	10 mg/m^2	day 8
Etoposide	100 mg/m^2	day 13

Doxorubicin	35 mg/m^2	day 1
Cyclophosphamide	650 mg/m^2	day 1
Vincristine	1.4 mg/m^2	day 8
Procarbazine	100 mg/m^2 PO	day 17
Prednisone	40 mg/m^2 PO	day 114
Repeat cycles every 3 weeks for 8 cycles		
Stanford V[115]		
Doxorubicin	25 mg/m^2 IV	day 1, 15
Vinblastine	6 mg/m^2 IV	day 1, 15
Mechlorethamine	6 mg/m^2 IV	day 1
Vincristine	1.4 mg/m^2 IV	day 8, 22
Bleomycin	5 U/m^2 IV	day 8, 22
Etoposide	60 mg/m^2 IV	day 15, 16
Prednisone	40 mg/m^2 PO	QOD
Repeat cycles every 28 days for 3 cycles		

those with initial CR greater than 12 months, Bonfante et al have reported a second complete remission was achieved in 89% and, at 8 years, the freedom from progression was 44%, with overall survival of 54%.[138] These results appear to compare favorably with that of ASCT in second remission and further challenge the question of ASCT timing. The salvage chemotherapy regimen of etoposide, vinblastine, and doxorubicin (EVA) has also shown significant activity in recurrent HD. [139] Forty-five patients who were refractory or in relapse following MOPP as the only prior therapy were treated. The complete response rate was 40%. The median time to treatment failure was 10 months, with 31% progression-free at 42 months.

Thus, the treatment of choice for patients failing to achieve complete remission with initial chemotherapy alone or relapsing after an initial complete remission to chemotherapy for advanced disease is high-dose chemotherapy (e.g., CBV) plus ASCT. Since ASCT appears to be more effective in a setting of low-volume chemotherapy-responsive disease, it would appear that the optimal approach to these subgroups of patients would be to use a conventional-dose chemotherapy regimen to maximum clinical response (with or without involved field radiotherapy to the sites of residual disease) before employing ASCT. With this approach, a significant survival advantage and a realistic increase in the long-term cure rate may be expected.

Although salvage chemotherapy with non-cross-resistant regimens, combined modality therapy, or high-dose chemotherapy with ASCT is generally recommended for relapse from a chemotherapy-induced complete remission, extended-field radiotherapy alone may be curative in a small subset of this population. Several retrospective and selected series have been reported, [140] [141] from which it has been concluded that comprehensive salvage radiotherapy is of benefit for patients who have long disease-free intervals after initial chemotherapy and who have relapsed only in nodal sites without systemic dissemination of their disease. High-dose extended-field radiotherapy in this situation generally included the areas of nodal recurrence as well as covering adjacent nodal sites in the same manner as would be used in treating patients with early-stage HD. Therefore, most patients received either mantle and para-aortic radiotherapy or TNI. All patients in these trials had relapsed or had failed to obtain a complete remission after combination chemotherapy. The 5-year disease-free survival in these three series ranged from 25% to 48%, and the 5-year overall survival ranged from 30% to 70%. [140] [141] These results are not significantly different from those obtained with salvage chemotherapy alone in

patients who had had a complete remission of >1 year with initial chemotherapy. However, for selected patients with nodal disease at recurrence, who have had prolonged initial remission with chemotherapy and for whom either salvage chemotherapy with a non-cross-resistant regimen or high-dose cytoreductive chemotherapy plus ASCT is not an option, extended-field radiotherapy alone may be curative.

Complications of Chemotherapy

A major complication of combination chemotherapy is bone marrow suppression, with increased risk of infection and, rarely, hemorrhage. Peripheral blood counts are monitored carefully during chemotherapy, and drug doses are adjusted according to the degree of myelosuppression. However, with the availability of growth factor support, dose modification may not be necessary. As a cautionary note, drug dose reductions made simply for the purpose of decreasing subjective toxicity are inappropriate. Significant myelosuppression is seen with all the chemotherapy regimens commonly used for advanced disease or as part of combined modality programs.

Treatment-induced sterility, more commonly seen in males, is a frequent and often permanent side effect of chemotherapy. This is of particular importance because long-term survival is observed in most patients treated for advanced disease with combination chemotherapy. Irreversible sterility in males after treatment with MOPP and MOPP-containing regimens has been reported in a high percentage of patients, regardless of age, whereas drug-induced ovarian failure among females, resulting in premature menopause, is much more common in older patients. [142] [143] [144] The ABVD combination produces significantly less infertility than does MOPP. Children conceived after treatment for Hodgkins disease appear normal without increased rates of stillbirth, congenital malformation, or cancer. [144]

The incidence of congestive heart failure does not appear to be significantly increased with ABVD, but the dose of adriamycin is frequently limited to 300 mg/m ^{2} . Patients treated with ABVD and mantle radiotherapy have a significantly higher incidence of post-irradiation paramediastinal fibrosis; persistent dyspnea on exertion has been observed in a small number of these patients at 3 years after completion of therapy. [90] [145] Significant nausea and vomiting may occur with MOPP and with the MOPP-containing and ABVD regimens. The use of selective 5-HT3 (hydroxy tryptamine) receptor antagonists such as odansetron or granisetron has diminished, but not fully eliminated, these toxicities. These drug programs may produce serious psychological problems that require effective counseling as well as antiemetic agents. Mild to moderate peripheral neuropathy is commonly seen with vincristine, and muscular weakness is an indication to discontinue this drug. Aseptic necrosis of the femoral heads and rarely, the humeral heads, is an unusual late complication related to corticosteroid therapy. [43]

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SECOND PRIMARY NEOPLASMS

Patients cured of HD are at an increased risk of the development of second primary cancers. The most widely reported neoplasm is acute myeloid leukemia, generally thought to result from combination chemotherapy or from treatment with both chemotherapy and radiation. An increased risk of solid tumors, usually attributed to irradiation, is emerging as a significant problem, as patients cured of HD are being followed for longer times. [41]

Tester et al[146] reviewed the NCI experience in 473 previously untreated patients, among whom 34 subsequent second malignant neoplasms were observed. At 10 years of follow-up, these investigators reported no increased risk of the development of leukemia in patients treated with radiotherapy alone. The estimated risk of leukemia following MOPP chemotherapy alone was 2%, and that following initial planned combined radiotherapy and MOPP chemotherapy was 6%, increasing to 9% in patients treated initially with radiotherapy who received MOPP chemotherapy at relapse. The 10-year estimated risk of solid tumors was 7% overall, with all treatment groups having similar risks. In contrast to other reports,[147] a greater risk of leukemia in patients who began treatment for HD at 40 years of age was not found. No case of acute leukemia occurred at >11 years after treatment. Actuarial analysis showed a peak onset of leukemia-related complications between 3 and 9 years after first treatment.[146]

Valagussa et al,[148] reporting for the Milan group, retrospectively reviewed the records of 1,329 patients, in whom a total of 68 new cancers were documented. None of the 19 cases of acute myeloid leukemia reported was observed in patients treated with radiotherapy alone. The 12-year estimate of leukemia development by treatment was 1.4% for chemotherapy alone, 10.2% for radiotherapy plus MOPP, 0% for radiation plus ABVD, and 4.8% for radiation plus other drug regimens. The incidence of leukemia was particularly high in patients who received salvage MOPP after relapse from radiotherapy. Valagussa and co-workers [148] also noted a positive association between increasing age and the risk of second malignancies, especially leukemia, which had been reported initially by the Southwest Oncology Group.[147] The overall risk of NHL in the Milan series was only 1.3%, and that of solid tumors, excluding basal cell carcinomas, was 6.7%. The failure to document an increased risk of leukemia in patients treated with ABVD chemotherapy alone or with radiotherapy plus ABVD is an important observation, since it appears that ABVD is more effective than MOPP in its ability to achieve long-term disease-free survival as initial chemotherapy for advanced disease. [105]

In a case-controlled study of 163 cases of leukemia following treatment for HD, Kaldor et al [149] observed that the use of chemotherapy alone to treat HD was associated with a relative risk of leukemia of 9.0, as compared with the use of radiation alone; patients treated with both modalities had a relative risk of 7.7. After treatment with more than six cycles of combination chemotherapy including nitrogen mustard and procarbazine, the risk of leukemia was 14 times as high as after radiotherapy alone. The use of radiotherapy in combination with chemotherapy did not increase the risk of leukemia above that produced by the use of chemotherapy alone. The peak in leukemia risk came approximately 5 years after the start of chemotherapy, and a large excess risk as compared with other treatment modalities persisted for 8 years after its discontinuance. Patients who had undergone splenectomy had at least twice the leukemia risk of patients who had not undergone this surgical procedure. Interestingly, Tura et al [150] have confirmed the observation that splenectomy, in addition to exposure to MOPP chemotherapy, increases the risk of secondary leukemia. In their study of 503 patients treated with MOPP plus irradiation, only 1 of 145 without splenectomy developed leukemia as compared to 21 of 358 splenectomized patients (5.9%). Moreover, an increasing number of MOPP cycles correlated with increasing risk of secondary leukemia, as reported by other groups.

A review by Tucker et al[151] of the Stanford series of 1,507 patients treated since 1968 indicated that 83 second cancers occurred >1 year after diagnosis. The mean 15-year actuarial risk was 18% for all second cancers, of which 13% were solid tumors. The risk of leukemia appeared to reach a plateau level of 3.3% at 10 years, whereas NHL continued to increase to 16% by the end of the follow-up period. Although these investigators noted that there was no increased risk of leukemia after radiotherapy alone, the risk of leukemia was significantly higher after either adjuvant chemotherapy or chemotherapy alone. The risk of a second solid tumor did not vary significantly according to treatment category, but the data did suggest that the risk of solid tumors after therapy for HD continues to increase with time.

The Stanford group has recently updated this data base, examining the risk of breast cancer in a cohort of 885 women with a mean follow-up of 10 years. [152] The overall relative risk (RR) of breast cancer was 4.1, and age at irradiation was a significant prognostic factor. For girls treated before age 15, RR was 136; at 1524 years, RR was 19; at 2429 years, RR was 7; and interestingly, at >30 years, RR was not elevated (0.7). Length of follow-up was also correlated with RR: <15 years, RR was 2.0; >15 years, RR was 13.6. This finding explains the lack of significant breast cancer risk observed in the earlier Stanford analysis by Tucker et al [151] Most breast cancers occurred within, or adjacent to, prior irradiation portals. Yahalom et al [153] from Memorial Sloan-Kettering also confirmed the increased risk of breast cancer in patients irradiated for HD. In this series, patients in whom breast cancer developed were more likely to be younger (median age 43 years) and to have bilateral disease, and involvement of the medial half of the breast was more frequent. The median interval from the treatment of HD to the diagnosis of breast cancer was 15 years.

Van Leeuwen et al[154] assessed the risk of second cancers in 744 patients admitted to the Netherlands Cancer Institute from 1966 to 1983. Among the 69 second cancers observed were lung cancer (14 cases), leukemia (16 cases), myelodysplastic syndrome (6 cases), and NHL (9 cases). The overall relative risk of the development of lung cancer was 4.9%. Excess lung cancer risk was noted only in treatment regimens including radiotherapy, and all lung cancers arose in irradiation fields. Kaldor et al[155] reported the results of a collaborative group of population-based cancer registries and major treatment centers using a case-control study. Patients treated with chemotherapy had about twice the risk of the development of lung cancer than that of patients treated with radiotherapy alone or both modalities. Among patients treated with radiation alone, the increased risk was related to estimated radiation dose to the lung. There was also a strong association between cigarette smoking in this population and the risk of lung cancer.

Mauch et al have reported the relative risks of second solid tumors in 794 patients with Pathologic Stage IAIIIB disease treated with radiotherapy alone or RT plus chemotherapy.[156] Risk increased with number of years post-treatment (3.7 at <5 years; 7.4 at 15 years) and was greater in patients treated at a young age (absolute excess risk at age of therapy: 69.6 if <17 years; 49.9 if 1739 years; and 5.7 if >40 years at age of therapy). Solid tumor sites included breast, gastrointestinal tract, lung, head and neck, and melanoma.

It is evident from these studies and others in the literature that treated HD patients have an increased risk of second neoplasms. The incidence of leukemia appears lowest in patients treated with radiotherapy and/or ABVD, whereas the development of solid tumors increases significantly over time in those receiving RT. The risk of leukemia is seen in patients treated with chemotherapy alone using a MOPP-containing regimen, with initial combined modality therapy using adjuvant MOPP, and with salvage alkylating agent-based chemotherapy following radiotherapy relapse. The peak risk of leukemia occurs at 58 years after initiation of chemotherapy, and no cases of leukemia have been observed at >10 years after the end of treatment. The risks of the development of solid tumors or acute leukemia must be taken into consideration in planning the initial treatment for an individual patient. Any predicted improvement in disease-free survival from initial therapy must be balanced against the possible risk of a second neoplasm at a later date.

It is reasonable to attempt to diminish the late treatment-associated risks of HD therapy with the following preventive strategies: counsel patients to stop smoking, encourage exercise and a cardiac prevention diet, monitor females for breast cancer with annual mammography beginning approximately seven years after RT (particularly important in women under age 30 at the time of irradiation) decrease sun exposure, monitor thyroid status yearly if patients have been exposed to prior neck irradiation, and suppress chemical hypothyroidism.

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Chapter 69 - The Pathologic Basis for the Classification of Non-Hodgkin Lymphomas

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INTRODUCTION

The histogenetic principles upon which the classification of human neoplasia has traditionally been predicated dictate that the malignant lymphomas, as neoplastic derivatives of the immune system, be classified in conformity with our understanding of the functional heterogeneity of lymphoid cells that make up the normal immune system. In view of the rapidity with which our understanding of immune system biology has grown in recent decades, it is not surprising that the evolution of lymphoma classifications has been swift and somewhat turbulent. In the span of some 40 years, these classifications have progressed from schemes recognizing only two essential cellular components (lymphocytes and histiocytes) to ones that now distinguish immature (precursor) from mature (peripheral) cells of B-, T-, and natural killer (NK) cell lineage, with additional phenotypic heterogeneity defined within each of these categories. The conjoint influences of monoclonal antibody technology and molecular biology have been largely responsible for these refinements, and it has been gratifying to note that neoplastic cells do indeed recapitulate the functional attributes of their presumptive normal cellular counterparts, thereby validating such an approach to tumor classification. However, our knowledge of the origin and function of certain specific constituents of the immune system, and of their neoplastic derivatives, remains rudimentary at best. In particular, the / T-cell and cytotoxic T-cell and NK cell malignancies, as well as lymphomas deriving from the whole of the extranodal immune system, have only recently become the objects of clinical investigation, in part because of their relative rarity and in part because of the lack, until recently, of the tools for their recognition. Our ability to classify these neoplasms has not progressed apace with the much more common node-based peripheral B-cell neoplasms.

In this chapter a very brief appraisal of the evolutionary history of lymphoma classifications, followed by a discussion of the normal developmental and functional anatomy of the immune system, will serve as a prelude to a review of the cardinal morphologic, immunophenotypic, and molecular genetic characteristics of the non-Hodgkin lymphomas.

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HISTORICAL PERSPECTIVE ON LYMPHOMA CLASSIFICATIONS

The optimal classification of lymphoid neoplasia has been the focus of one of the most contentious debates in the history of nosology since the mid-1960s. While all could agree that a valid classification should be easily taught, readily learned, scientifically valid, and clinically relevant, a consensus as to how this might best be achieved proved elusive. The Rappaport scheme, initially introduced by Henry Rappaport in 1956, subdivided tumors with respect to growth pattern (nodular vs. diffuse), cell type (lymphocytic vs. histiocytic), and degree of differentiation (well differentiated vs. poorly differentiated vs. undifferentiated) and gained wide acceptance in the United States by both clinicians and pathologists. [1] The principal competing classifications were those of Karl Lennert, which became known as the Kiel classification and gained equally widespread endorsement in Europe, [2] and the Lukes and Collins classification, proposed by Robert Lukes and Robert Collins in the United States. [3] These classifications differed from the Rappaport classification in advocating a more rigorous reliance on the principles of cellular immunology as the conceptual basis for lymphoma classification. In the Kiel schema of 1974, which antedated the advent of monoclonal antibody technology, lymphoid tumors were related to discrete stages of lymphoid differentiation on the basis of their characteristic morphologic features. [4] [5] [6] [7] Each of these classifications spawned various congeners, rapidly leading to a surfeit of nomenclatures without any readily apparent intercorrelation. This awkward situation eventually led to an unprececented retrospective collaborative project between hematopathologists and oncologists treating lymphomas. This initiative culminated in the publication of the Working Formulation for Clinical Usage in 1982 (Table 69-1). [8] In essence the Working Formulation represented a modification of the Rappaport scheme, adapting some terminology from the immunologically based scheme of Lukes and Collins. Although it was intended only as a compendium of equivalencies among the various prevailing classification schemes and had been designed with the primary purpose of facilitating interinstitutional clinical dialogue, the Working Formulation quickly gained de facto acceptance as a classification and was favored by oncologists, who appreciated its grouping of lymphoid neoplasia into three clinically prognostic grades. The Working Formulation, like the Rappaport scheme, eschewed any formalized reliance on immunology or the then developing monoclonal antibody technologies, maintaining that tumor classification should be accomplished on a purely morphologic basis. The Kiel classification, in contrast, persisted in its pursuit of a precise histogenetic classification in accordance with the emerging principles of immune biology and was quick to embrace immunohistochemistry as an adjunctive tool in that quest. [9] These refinements were incorporated in the updated Kiel classifications of 1988 and 1992 (Table 69-2), as well as in later updates. [10] [11] [12] [13] Thus, the polyglot prehistory of lymphoma classifications became distilled over the past decade and a half into a largely bilingual era, which nonetheless preserved the essential dichotomies and controversies of the earlier period. More recently, as monoclonal antibody technology and molecular biology have contributed steadily to our understanding of lymphoid biology, the argument for an immunologically and genetically based classification of lymphoid neoplasia has become more and more compelling. Indeed, the nascent but certain prospect of specific, genetically based therapies virtually mandates such a classification. In accordance with these goals and the desire for a single consensus classification of lymphoid neoplasms, a proposal was recently offered by an international group of 19 hematopathologists under the rubric of the Revised European-American Lymphoma (REAL) classification (Table 69-3). [14] Unlike the Working Formulation, this project was undertaken without any systematic review of clinical data, and the resulting proposal was stated merely to reflect a listing of those entities which the participating pathologists considered to be well-defined pathologic or clinical entities that

TABLE 69-1 -- Working Formulation of Non-Hodgkin Lymphomas for Clinical Use (1982)

Low Grade
A. Malignant lymphoma, small lymphocytic
Consistent with CLL
Plasmacytoid
B. Malignant lymphoma, follicular, predominantly small cleaved cell
Diffuse areas
Sclerosis
C. Malignant lymphoma, follicular, mixed, small cleaved, and large cell
Diffuse areas
Sclerosis
Intermediate Grade
D. Malignant lymphoma, follicular, predominantly large cell
Diffuse areas
Sclerosis
E. Malignant lymphoma, diffuse, small cleaved cell
Sclerosis
F. Malignant lymphoma, diffuse, mixed, small, and large cell
Sclerosis
Epithelioid cell component
G. Malignant lymphoma, diffuse, large cell
Cleaved cell
Non-cleaved cell
Sclerosis
High Grade
H. Malignant lymphoma, large cell, immunoblastic
Plasmacytoid

Clear cell
Polymorphous
Epithelioid cell component
I. Malignant lymphoma, lymphoblastic
Convoluted cell
Nonconvoluted cell
J. Malignant lymphoma, small non-cleaved cell
Burkitt
Follicular areas
Miscellaneous
Composite
Mycosis fungoides
Histiocytic
Extramedullary plasmacytoma
Unclassifiable
Other

TABLE 69-2 -- Updated Kiel Classification of Non-Hodgkin Lymphomas (1992)

B Cell	T Cell
Low-grade Malignant Lymphomas	
Lymphocytic	Lymphocytic
Chronic lymphocytic leukemia	Chronic lymphocytic leukemia
Prolymphocytic leukemia	Prolymphocytic leukemia
Hairy cell leukemia	Small cell, cerebriform
Lymphoplasmacytic/cytoid (immunocytoma)	Mycosis fungoides/Sézary syndrome
Plasmacytic	Lymphoepithelioid (Lennert lymphoma)
Centroblastic-centrocytic (follicular ± diffuse; diffuse)	Angioimmunoblastic
Centrocytic (mantle cell)	T-zone lymphoma
Monocytoid, including marginal zone cell	Pleomorphic, small cell (HTLV-I±)
High-grade Malignant Lymphomas	
Centroblastic	Pleomorphic, medium-sized, and large cell (HTLV-I±)
Immunoblastic	Immunoblastic (HTLV-I±)
Burkitt lymphoma	Large cell anaplastic (Ki1+)
Large cell anaplastic (Ki1+)	
Lymphoblastic	Lymphoblastic
Miscellaneous	
Other rare types of lymphoma may be separately identified for T-cell and B-cell lymphomas, respectively.	

TABLE 69-3 -- Revised European-American Lymphoma Classification (1994)

B-Cell Neoplasms
Precursor B-cell neoplasm
Precursor B-lymphoblastic leukemia/lymphoma
Peripheral B-cell neoplasms
B-cell chronic lymphocytic leukemia/prolymphocytic leukemia/small lymphocytic lymphoma
Lymphoplasmacytoid lymphoma/immunocytoma
Mantle cell lymphoma
Follicle center lymphoma, follicular
Provisional cytologic grades: I (small), II (mixed), III (large)
Provisional subtype: diffuse, predominantly small cell
Marginal zone B-cell lymphoma
Extranodal (MALT type ± monocytoid B cells)
Provisional subtype: nodal (± monocytoid B cells)
Provisional entity: splenic marginal zone lymphoma (± villous lymphocytes)
Hairy cell leukemia
Plasmacytoma/myeloma
Diffuse large B-cell lymphoma
Subtype: primary mediastinal (thymic) B-cell lymphoma
Burkitt lymphoma
Provisional entity: high-grade B-cell lymphoma, Burkitt-like
T-Cell and Putative NK Cell Neoplasms
Precursor T-cell neoplasm
Precursor T-lymphoblastic lymphoma/leukemia

Peripheral T-cell and NK cell neoplasms
T-cell chronic lymphocytic leukemia/prolymphocytic leukemia
Large granular lymphocyte leukemia
T-cell type
NK-cell type
Mycosis fungoides/Sézary syndrome
Peripheral T-cell lymphomas, unspecified
Provisional categories: medium, mixed, large, lymphoepithelioid
Provisional subtypes: hepatosplenic T-cell lymphoma subcutaneous panniculitic T-cell lymphoma
Angioimmunoblastic T-cell lymphoma
Angiocentric lymphoma (nasal T/NK cell lymphoma)
Intestinal T-cell lymphoma (± enteropathy)
Adult T-cell lymphoma/leukemia
Anaplastic large cell lymphoma (T/null)
Provisional: anaplastic large cell lymphoma, Hodgkin-like
Hodgkin Disease
Lymphocyte predominance (nodular ± diffuse)
Nodular sclerosis
Mixed cellularity
Lymphocyte depletion
Lymphocyte-rich classic Hodgkin disease (provisional subtype)

could be recognized with reasonable reproducibility by experienced hematopathologists. [15] This proposal is, in essence, the Kiel classification, modified to incorporate extranodal lymphomas and Hodgkin disease, as well as a different conceptual approach to the still challenging classification of postthymic and extrathymic T-cell and NK cell lymphomas.[16] The REAL classification of 1994 is used as a structural framework for this chapter. The reader is advised, however, that classifications are constantly evolving, and the REAL classification is already undergoing further modifications, to be published as the WHO classification of lymphoid neoplasia.

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LYMPHOPOIESIS AND THE FUNCTIONAL ANATOMY OF THE IMMUNE RESPONSE

Lymphoid cells of all lineages derive from a common progenitor cell that differentiates into precursor cells of B-, T-, and NK cell lineage. In fetal life, hematopoiesis is initiated in blood islands, followed shortly by the liver and spleen. This function is gradually transferred to the primary, or generative, lymphoid organs of the adult, where precursor lymphoid development proceeds. These generative organs are the bone marrow and, in the instance of T cells, the thymus as well. The precursor stage of lymphoid ontogeny is antigen independent, that is, it takes place in the absence of antigenic stimulation. During this early phase of differentiation B and T cells rearrange antigen receptor genes and ultimately express on their surface a functional and clonally specific immunoglobulin or T-cell receptor. [16] [17] [18] [19] [20] [21] [22] [23] [24] [25] [26] [27] [28] [29] [30] [31] [32] [33] [34] [35] [36] [37] [38] [39] [40] [41] [42] [43] [44] This process is mediated by the concerted activities of several lymphocyte-specific recombinases. Recombinatorial diversity is further enhanced by the enzyme terminal deoxynucleotidyl transferase (TdT), which has the capability of randomly adding nucleotide bases to the newly formed genetic recombination sites in the absence of substrate direction. [45] Expression of TdT thus constitutes a highly useful marker for precursor lymphocytes without respect to lineage and is a definitive marker of precursor cell neoplasiathat is, lymphoblastic lymphoma/leukemia. The antigen receptor proteins, though membrane spanning, consist of only rather short intracytoplasmic tails, and must become associated with an appropriate membrane signal transducer, CD79a in the case of B cells and CD3 (aided by the proteins) in the case of T cells. [45] [46] [47] [48] [49] [50] [51] [52] [53] It is likely that these transducer proteins are expressed on the cell surface prior to the expression of the respective antigen receptors and are, in fact, requisite for the transport of the antigen receptor proteins to the cell surface. The subset antigens, CD4 and CD8, additionally form part of the T-cell receptor complex and define MHC class II and MHC class I restriction, respectively. [54] [55] [56] [57] [58] [59] [60] Although most CD4+ T cells function as helper cells and the majority of CD8+ T cells are functionally cytolytic or suppressor, there are exceptions in both directions.[61] [62] [63] Once a viable antigen receptor complex has been expressed by the developing cell, thereby rendering it capable of antigenic interaction, the cell acquires resistance to programmed cell death through the cellular expression of bcl-2 and related proteins and migrates to the secondary, or peripheral, lymphoid organs as a TdT naive (i.e., non-antigen-primed) B- or T-lymphoid cell. [64] [65] Those cells that express either nonfunctional or self-reactive antigen receptors are eliminated in the primary lymphoid organs through apoptosis. [66] [67] [68] [69] [70] [71]

Peripheral B cells comprise two principal populations of cells, distinguished by their expression of the CD5 antigen. [72] CD5+ B cells are rare in the adult, accounting for less than 510% of B cells, but they are the predominant B-cell population in the fetus, in which they appear to constitute a rather primitive but effective first line of defense against foreign antigens.[73] The immunoglobulin receptors of these naive cells are of IgM or IgD type (or both) and are characterized by highly selective utilization of a few variable region genes that have not yet undergone the mutational process of affinity maturation, with the result that these cells express only a limited antibody repertoire of broad specificity but low affinity. [74] In the adult these cells recirculate and, in tissues, home to the primary follicle and follicular mantle zone.[75] CD5+ B cells are implicated in autoimmune disease, in which they are frequently numerically expanded, [76] and are the presumptive precursors of the two common CD5+ B-cell malignancies, B-cell chronic lymphocytic leukemia (B-CLL, also known as small lymphocytic lymphoma/leukemia) and mantle cell lymphoma. [77]

The second, numerically preponderant, population of peripheral B cells is characterized by lack of CD5 antigen expression. This group includes a subpopulation of naive B cells as well as follicle center B cells and post-germinal center effector and memory B cells. With the exception of the naive B-cell subgroup, and in contrast to the CD5+ B-cell population, these cells generally show evidence of positive germinal center selection in the form of affinity maturation of immunoglobulin receptors with or without heavy chain class switch. This large family of B cells constitutes the precursors of virtually all B-cell lymphomas other than B-CLL and mantle cell lymphoma.

Peripheral T and NK cells, like their B-cell counterparts, can be segregated into families based on functional differences as well as differences in immunophenotypic profiles and T-cell receptor expression. [78] T cells are distinguished from NK cells by the presence of a functional surface T-cell receptor complex, including CD3, and absence of (or infrequent) expression of NK antigens. Mature T cells are further subdivided into two populations by virtue of their expression of either the / or the / heterodimeric T-cell receptor. Commercially available monoclonal antibodies to the and chains now readily permit this distinction to be made without resort to more complicated molecular analyses.[79] In lymphoid development, the process of T-cell receptor rearrangement is initiated by recombination of the / receptor genes. [80] The signals through which certain T cells remain permanently committed to / lineage, while the vast majority of precursor T cells undergo subsequent rearrangement of the / T-cell receptor genes to become / T cells, are presently unknown. [81] Indeed, it remains a matter of conjecture whether rearrangement of the / and / receptor genes occurs serially or in parallel in T-cell populations. It is evident, however, that, like CD5+ B cells, / T cells manifest very selective variable region gene usage and that the pattern of gene usage is correlated with specific patterns of tissue tropism. [82] Moreover, / T cells exhibit a remarkable propensity for extranodal localization, a property that appears to be recapitulated by their malignant counterparts. In the human adult, / T cells constitute fewer than 5% of peripheral lymphoid cells. Whereas in mice, / T cells compose the majority of intraepithelial T cells in the gut and a significant proportion of intraepidermal T cells, this does not appear to be the case in humans, in whom CD8+ / T cells predominate in these sites. [83] / T cells appear to mediate non-MHC-restricted immune interactions, largely of a cytotoxic nature, mediated by perforin and serine esterases. [84]

The much larger population of MHC-restricted / T cells can be further segregated based on patterns of expression of the subset antigens, CD4 and CD8. CD4+/CD8+ and CD4/CD8 phenotypes are largely thymic, or precursor, phenotypes and are rarely encountered in normal peripheral T cells, except for / T cells, which are typically double negative but may occasionally express CD8 or, very exceptionally, CD4. Cells expressing a CD4+/CD8 phenotype predominate over those with the inverse phenotype and are the precursors of the majority of node-based peripheral T-cell lymphomas. Normal CD4+ helper T cells function largely through lymphokine secretion, a property reflected in the polymorphous morphology of their derivative neoplasms (e.g., histiocyte aggregation and activation, vascular proliferation, eosinophil attraction). In contrast,

normal CD4/CD8+ T cells generally subserve a suppressor or cytotoxic function. Some, known as NK-like T cells, express NK cell antigens in addition to a surface T-cell receptor complex and possess perforin and granzyme-containing cytoplasmic granules that mediate cytolysis, analogous to the situation in NK cells. True NK cells lack both a T-cell receptor and surface CD3 but may express the CD3epsilon chain within the cytoplasm and usually express the CD56 NK-associated antigen. NK and NK-like T cells, both benign and malignant, can be recognized morphologically by their distinctive cytoplasmic azurophilic granules, which house the cytolytic proteins perforin and granzymes. NK cell lymphomas, like / T-cell lymphomas and / T-cell lymphomas that manifest a cytolytic phenotype, tend to localize to extranodal sites.

The secondary lymphoid organs constitute the lymph nodal network, the spleen, and various extranodal immune systems, including the mucosal and cutaneous lymphoid tissues. These organs collectively represent the principal sites of antigen concentration and are the preferential homing targets for naive lymphoid cells. Each of these organs is characterized by a functional anatomy that (1) allows for the constant sampling of lymph or blood (or both) for the presence of foreign antigens and (2) optimizes B-, T-, and accessory cell interactions in response to these challenges. Whereas T lymphocytes for the most part constitute a recirculating pool of cells that continually traffic between extranodal sites and lymph nodes and exert their influences locally via cytokines and cytolytic granule-associated proteins, B cells are largely confined to the secondary lymphoid organs, whence they exert their effector function remotely via secreted antibody molecules. T cells respond only to protein antigens, with a further requirement that these proteins be processed into short peptides and be presented on the surface of an appropriate

antigen-presenting cell (including macrophages, B cells, and dendritic cells) in conjunction with MHC molecules. [85] [86] [87] In contrast, B cells have the capacity to engage both protein and nonprotein antigens, which may be soluble, that is, non-cell-associated.

In the lymph node, the cortex, with its component primary and secondary lymphoid follicles, represents the principal B-cell region. The primary follicle is composed of small lymphocytes, predominantly naive B cells, with lesser numbers of memory B cells and small numbers of T lymphocytes and follicular dendritic cells. This structure thus represents both the precursor and the residuum of the germinal center reaction. The T-cell- and antigen-dependent phase of the immune response is initiated when a naive B cell, with its primitive (i.e., nonmutated) clonal antigen receptor, encounters antigen and, prompted by T-cell help, transforms into a primary B-cell blast (possibly the normal cellular counterpart of Burkitt lymphoma), which migrates into the B-cell follicle and there undergoes massive clonal expansion, giving rise to the secondary follicle center, or so-called germinal center. [88] [89] [90] In so doing, this cell down-regulates its expression of the bcl-2 protein and becomes susceptible to programmed cell death. In the basal, darkly staining zone of the germinal center, this primary B-cell blast differentiates into the cell morphologists recognize as the centroblast, characterized by a vesicular chromatin pattern, membrane-associated nucleoli, and basophilic cytoplasm. It is at this stage of transformation that the process of somatic mutation of immunoglobulin variable region genes occurs, resulting in affinity maturation of the primitive immunoglobulin receptor.[91] Heavy chain class switch is also initiated at this time, producing B cells of differing isotype but identical antigen specificity. The centroblast eventually transforms into the cell known as the centrocyte, recognized by its angulated nuclear contour, inconspicuous nucleoli, and scant cytoplasm, and migrates into the apical, lightly staining zone of the germinal center. This region is rich in follicular dendritic cells, which bind, immobilize, and retain immune complexes via complement and Fc receptors and in this form present antigen to follicle center B cells. It is at this stage that the process of antigen selection occurs, ensuring (1) the survival of those centrocytes in which somatic mutation has yielded an antibody of appropriate high affinity and (2) the elimination of other B cells. [92] If a centrocyte can sustain a stable interaction with antigen as a consequence of a high-affinity receptor, the centrocyte then ingests and processes that antigen and, in turn, presents the modified antigen on its surface in relation to MHC molecules. This composite antigen is in turn recognized by helper T cells, which respond by expressing a variety of surface determinants and secreted factors, including the CD40 ligand, that collectively induce the centrocyte to increase the density of its surface immunoglobulin expression and to up-regulate bcl-2 protein expression, which is under post-transcriptional control in the cell. This latter event effectively rescues the cell from apoptotic degradation and allows it to further differentiate into an effector cell of either plasmacytic or memory type. Plasma cells are terminally differentiated B cells in which alternative splicing of primary RNA transcripts of immunoglobulin receptor genes has resulted in loss of hydrophobic transmembrane domains, causing a switch from membrane-bound immunoglobulin to cytoplasmic and secreted immunoglobulin. Those cells that are not positively selected in the germinal center succumb to programmed cell death and phagocytotic elimination by the so-called tingible body macrophages of the germinal center.

The T-cell-rich zone of the lymph node is termed the paracortex. This region is composed predominantly of small T lymphocytes, most of which are CD4+ helper cells and are associated with a specific form of antigen-presenting cell referred to as the interdigitating reticulum cell. The latter cell is closely related to the Langerhans cell of the epidermis and performs two functions critical to T-cell activation. First, it is responsible for the proteolytic conversion of native protein antigens into MHC-associated peptide fragments, which are then presented to T cells for cognate interaction (essentially the same function performed by centrocytes in the germinal center reaction, as discussed above). Second, the antigen-presenting cells express and/or secrete a number of co-stimulatory molecules that maximize the T-cell response. Naive T cells enter lymph nodes either from afferent lymphatics or from the bloodstream. The latter route of access is mediated by specific homing receptors (selectins) for cuboidal endothelial cells that line specialized paracortical venules known as high endothelial venules. Similar venules are present in the mucosal immune system. Thus, particular patterns of adhesion molecule expression determine the preferential homing of naive T cells to lymph nodes, where the recognition and activation phases of the primary immune response take place with the aid of antigen-presenting cells. [93] [94] Following activation, altered profiles of adhesion molecule expression diminish the affinity of T cells for nodal endothelium, resulting in the displacement of activated T cells to sites of inflammation, where endothelial cell expression of ligands for activated T-cell integrins has been amplified by inflammatory cues. If activated T cells encounter antigen in these sites, adhesion molecule interaction is further strengthened and T cells are retained locally, where they accomplish the effector phase of the T-cell immune response. [95] [96] [97]

Whereas the lymph node system is the primary venue for immune responses to lymph-borne antigens, the spleen functions as the major site for immunologic responses to blood-borne antigens, and this function is reflected in its structure. The splenic arterioles terminate in a vast meshwork of red pulp sinusoids and cords, through which the blood is filtered prior to gaining re-entry into the venous circulation. This sojourn provides ample opportunity for the detection of foreign antigens by splenic lymphoid tissue, known as the splenic white pulp. The white pulp comprises T- and B-cell-rich sectors, just as in the lymph node, but with some differences. The periarteriolar

lymphoid sheath constitutes the principal T zone of the spleen and is composed of CD4+ and CD8+ / T cells in a ratio of approximately 2:1, in addition to antigen-presenting cells, including dendritic cells and macrophages. B-cell follicles, which may demonstrate germinal center formation, are situated adjacent to the periarteriolar lymphoid sheath and are in most respects analogous to their nodal counterparts. Surrounding both sectors is a specialized zone of B cells, macrophages, and helper T cells termed the marginal zone.[98] This zone, which is normally present only in the spleen, mesenteric lymph nodes, and other components of the mucosal immune system, appears to be particularly important in the immunologic response to nonprotein antigens, notably bacterial cell wall polysaccharides.[99] [100] Humoral immunity affords the only substantive host defense against such antigens, as these antigens cannot be recognized by T cells. Marginal zone B cells are distinguished by their surface expression of IgM in the absence of IgD. The splenic red pulp, in contrast to the white pulp, is selectively populated by cytotoxic T cells and contains a resident population of / T cells.

The mucosal immune system is most fully developed in the gastrointestinal tract, where it comprises the Peyers patches of the distal ileum, intraepithelial lymphocytes throughout the length of the intestine, and lymphoid cells distributed in the lamina propria of the entire gastrointestinal tract. Remote components of this system include the mesenteric lymph nodes and the spleen. The lymphoid follicles of the Peyers patches exhibit a functional organization similar to that of the spleen, with central germinal centers surmounted by a marginal zone, which intervenes between the germinal center and the overlying dome epithelium. Intraepithelial lymphocytes in the dome epithelium constitute an admixture of B- and T-lymphoid cells, the former tending to occur in small aggregates and the latter as singly distributed cells. T cells surrounding the lymphoid follicle are largely CD4+ helper cells. When protein antigen reaches the Peyers patches, B and T lymphocytes are stimulated to respond as in other lymphoid tissues. Two cytokines, TGF- and IL-5, both secreted by T cells, are implicated in mediating IgA isotype switch in activated B cells. The greater numbers of IL-5-producing T cells in Peyers patches as compared with other lymphoid organs and selective homing of IgA-producing B cells to Peyers patches account at least in part for the concentration of IgA-expressing B cells in this site. Intraepithelial lymphocytes elsewhere in the gastrointestinal tract are predominantly of T-cell lineage, with CD8+ cells predominating. Approximately 10% of intraepithelial lymphocytes in humans are of / lineage. Regardless of receptor class, intraepithelial T cells display restricted usage of variable region gene families with resultant limited diversity of antigen receptors, presumably reflecting an evolutionary origin to deal with commonly encountered luminal antigens. [101] [102] Cytolytic granule-associated proteins have been demonstrated in intraepithelial lymphocytes.[103] Finally, lymphoid cells of the lamina propria consist largely of CD4+ T cells that most probably home to this site following antigen recognition and activation in mesenteric lymph nodes. Lesser numbers of activated B-lymphoid cells and polytypic plasma cells, as well as eosinophils, mast cells, and macrophages, are also present in the lamina propria.

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NON-HODGKIN LYMPHOMAS

Neoplasms of Precursor B and T/NK Cells

Precursor B-Lymphoblastic Leukemia/Lymphoma

The vast majority of cases of precursor B-cell neoplasia manifest an acute leukemic presentation with preferential involvement of peripheral blood and bone marrow. However, a small proportion of cases, perhaps 510%, present as solid tumors with a predilection for skin, bone marrow (osteolytic lesions), and lymph nodes. [104] This disease is most common in children and young adults, with a mean age at onset significantly less than that of precursor T-lymphoblastic lymphoma/leukemia, but may be observed in older adults as well. Although aggressive, this disease is frequently curable by existing therapeutic modalities. Approximately 80% of cases of acute lymphoblastic leukemia (ALL) and 1520% of cases of lymphoblastic lymphoma are of precursor B lineage.

The neoplastic cells of precursor B-lymphoblastic leukemia/lymphoma infiltrate in a diffuse pattern and exhibit an immature cytology, characterized by intermediate size, finely stippled chromatin, inconspicuous nucleoli, and scant cytoplasm. Nuclear contours may be either round or convoluted (Fig. 69-1A). The mitotic rate is high, consonant with developmental stage. The distinction between precursor B- and precursor T-cell neoplasms cannot be established on histologic grounds and must be supported by immunophenotypic studies. Differential diagnostic considerations based purely on morphologic observations include acute myelogenous leukemia (AML)/granulocytic sarcoma (extramedullary myeloid cell tumor) and blastic variants of mantle cell lymphoma. Immunophenotypic analysis may be required for distinction.

Precursor B-lymphoblastic leukemia/lymphoma, like its normal cellular counterpart, demonstrates consistent immunoreactivity for early B-cell antigens such as CD19, CD79a, and CD10, as well as nuclear positivity for the enzyme TdT. [105] Surface immunoglobulin expression is uniformly lacking, whereas heavy chains may be demonstrable in the cytoplasm. The stem cell antigen CD34 is often detectable, and the myeloid markers CD13 and CD33 may be infrequently encountered. Immunoglobulin heavy chain gene rearrangements are detectable in a majority of cases; light chain rearrangement is less frequent. [106] Incomplete rearrangements of the T-cell receptor genes may also be observed. [107]

Cytogenetic analysis may provide prognostic information. Karyotypic alterations associated with an unfavorable outcome include t(1;19), t(9;22), and abnormalities of 11q13. Cases with more than 50 chromosomes, in contrast, appear to have a favorable prognosis. Immunophenotypic profiles have also been correlated with outcome. In particular, myeloid antigen expression is said to confer an unfavorable prognosis, [108] as is lack of expression of the CD10 or CD34 antigens.

Precursor T-Lymphoblastic Lymphoma/Leukemia

Neoplasms of precursor T-cell lineage constitute roughly 15% of cases of ALL and 8085% of cases of lymphoblastic lymphoma, in contrast to precursor B-cell neoplasia, as discussed above. [109] These are tumors of adolescents and young adults, with a male preponderance. The disease typically presents as a bulky mediastinal mass, often attended by supradiaphragmatic lymphadenopathy, and is usually disseminated at diagnosis, with frequent involvement of the bone marrow, central nervous system, and gonads. The distinction of leukemic from lymphomatous disease is arbitrary, generally based on a threshold of 25% marrow blasts, but is now of lesser therapeutic importance, as most patients are currently treated on leukemia protocols.

The histologic and cytologic features of precursor T-lymphoblastic lymphoma/leukemia are identical to those described for neoplasms of precursor B lineage (Fig. 69-1B). Involvement of the nodal paracortex may be exceedingly subtle. Evidence of early nodal infiltration is often best sought in the trabecular structures and capsule of the node, where it is more readily appreciated by virtue of its striking single file pattern. A subset of cases of T-lymphoblastic lymphoma/leukemia distinguished by marked eosinophil infiltration has been associated with the subsequent development of AML or myeloproliferative

Figure 69-1 Lymphoblastic lymphoma/leukemia. **(A)** An example of cutaneous precursor B-cell lymphoma is composed of primitive intermediate-sized cells with convoluted nuclear contours. **(B)** Cytologic features in **(A)** are indistinguishable from those of the nodal precursor T-cell lymphoma shown here.

disease. The t(8;13)(p11;q12) and t(8;13)(p2123;q14) translocations appear to represent recurring abnormalities in such cases. [110] [111] [112]

Immunophenotypic studies are requisite for lineage assignment in lymphoblastic lymphoma/leukemia. In concert with its precursor B-cell counterpart, precursor T-lymphoblastic lymphoma/leukemia displays consistent immunoreactivity for the intranuclear enzyme TdT, which is now readily demonstrable using monoclonal antibodies in paraffin sections. Apart from this, the neoplastic cells may manifest an immunophenotypic profile corresponding to any discrete stage of thymic maturation, with leukemic cases tending to be less phenotypically mature than cases with a lymphomatous presentation. [113] [114] [115] [116] [117] The early T-cell differentiation antigen CD7 is almost uniformly present. Cytoplasmic CD3, with or without surface CD3, is often detectable, while the CD2 and CD5 antigens are less commonly observed. [118] CD1a is variably expressed, and the subset antigens CD4 and CD8 are expressed either in concert (double positive) or not at all (double negative), in contrast to tumors of peripheral T-cell lineage, in which the subset antigens have segregated. The CD10 antigen is present in about one-third of cases. NK-associated antigens (CD16 and CD57) are rarely encountered but have been associated with clinically aggressive disease in the small number of cases reported. [119] [120] [121] [122] [123] [124] [125] [126] [127]

T-cell receptor genes show an inconstant pattern of rearrangement. Neoplastic cells may express either the / or the / T-cell receptor or may fail to express T-cell receptors altogether. [128] Immunoglobulin gene rearrangements are variable and often incomplete. [129]

Cytogenetic abnormalities are common and typically involve T-cell receptor gene loci, in particular 14q11 (TCR-), 7q34 (TCR-), and 7p15 (TCR-). With the possible exception of t(9;17)(q34;q23), which was associated in one study with mediastinal disease and a rapidly progressive clinical course without leukemic conversion, and of the translocations associated with eosinophilia and subsequent transversion to myeloid disease, none of the karyotypic abnormalities appears to have prognostic relevance.

Neoplasms of Peripheral B and T/NK Cells

Peripheral B-Cell Lymphomas

CD5+ B-Cell Lymphomas

B-Cell Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma/Prolymphocytic Leukemia

This category comprises a group of related disorders distinguished histologically by a preponderant proliferation of small lymphocytes and prolymphocytes, admixed in varying proportion.[130] [131] [132] The disease affects older adults and is usually leukemic at presentation, often with concomitant involvement of lymph nodes, spleen, and liver; in this circumstance the term B-cell chronic lymphocytic

leukemia (B-CLL) is preferred. When the clinical presentation is nonleukemic, a diagnosis of small lymphocytic lymphoma is made and is understood to connote a B-cell neoplasm having all of the morphologic and immunophenotypic attributes of B-CLL. Approximately 10% of cases of B-CLL/small lymphocytic lymphoma manifest a terminal high-grade histologic transformation, referred to as Richter syndrome. [133] [134] This most often takes the form of a large B-cell or immunoblastic lymphoma, but may also evince paraimmunoblastic[135] or lymphoblastic morphology. A Hodgkin-like transformation, associated with Epstein-Barr virus (EBV), has also been described and appears to be less clinically aggressive. [136] [137] Alternatively, the disease course may be attended by a progressive increase in prolymphocytes in the peripheral blood, a phenomenon termed prolymphocytoid transformation. Prolymphocytic leukemia, by contrast, is a disease that typically presents de novo and manifests with splenomegaly, minimal lymphadenopathy, and very high white cell counts, with prolymphocytes constituting at least 55% (and usually a very much higher proportion) of lymphoid cells. [138] [139]

A definitive morphologic diagnosis of B-CLL leukemia and small lymphocytic lymphoma is most readily established in tissue biopsies, preferably lymph nodes. Despite the nomenclatural emphasis on the small lymphocyte, which generally exhibits a round nuclear contour and mature condensed chromatin pattern, the most characteristic and diagnostically reliable feature of these tumors is the invariant presence of prolymphocytes and paraimmunoblasts, frequently aggregated in proliferation centers. [140] The paraimmunoblasts are activated and proliferating cells, distinguished from small lymphocytes by their larger size, vesicular nuclear chromatin, and single, usually central nucleoli (Fig. 69-2). So characteristic are these cells that alternative diagnoses should be entertained if they cannot be identified in a reasonably representative biopsy specimen. Plasmacytoid differentiation is evident in a minority of cases and may be accompanied by a paraprotein. If proliferation centers are identified in such cases, they should nonetheless be classified as B-CLL (i.e., not as lymphoplasmacytoid lymphoma/immunocytoma) and will most often exhibit an immunophenotypic profile and clinical evolution typical of this disease.

The diagnosis of prolymphocytic leukemia, on the other hand, generally requires the availability of cytologic preparations such as peripheral blood and bone marrow aspirate smears or imprint preparations of lymph nodes or spleen. As previously stated, the diagnosis is based on the identification of a preponderant population of prolymphocytes, which display a less condensed chromatin pattern than small lymphocytes and small but definite single nucleoli (Fig. 69-3). This disease pursues a much more accelerated clinical course than that of the other indolent diseases included in this category. Indeed, a strong argument can be advanced for its separation from this grouping, as has been the case historically and as has been proposed in the WHO classification of lymphoid neoplasia.

The immunophenotype of this family of diseases is highly constant. B-CLL/small lymphocytic lymphoma expresses low-density monotypic surface immunoglobulin of IgM type, with or without IgD. Cytoplasmic immunoglobulin detection is variable and correlates with plasmacytoid differentiation. B-cell antigens such as CD19 and CD20 are expressed in association with both CD5 and CD23, in contradistinction to mantle cell lymphoma, which manifests a CD5+, CD23 phenotype. Neoplastic cells are nonimmunoreactive for the CD10 antigen and for FMC7, the latter commonly expressed in a host of other low-grade B-cell lymphomas. Expression of the CD11c antigen is variable and may identify a clinically distinctive subset. Prolymphocytic leukemia differs from this classic phenotype by a higher density surface immunoglobulin, variable CD5 expression, and more consistent expression of the CD22 antigen.

Cytogenetic studies have revealed the presence of trisomy

Figure 69-2 Small lymphocytic lymphoma. Note the distinctive admixture of small lymphocytes, prolymphocytes (medium-sized cells with small nucleoli), and paraimmunoblasts (large cells with prominent central nucleoli) that is the diagnostic sine qua non of this entity.

Figure 69-3 Prolymphocytic leukemia. A peripheral blood smear demonstrates the dominant population of prolymphocytes that characterizes this clinically aggressive B-cell leukemia. Neoplastic cells have a relatively mature chromatin pattern, but nucleoli are uniformly discernible.

12 in about one-third of cases, and abnormalities of 13q in roughly one-quarter. [141] [142] The t(11;14) is occasionally but infrequently encountered in the morphologic setting of B-CLL/small lymphocytic lymphoma.

Mantle Cell Lymphoma

The entity now termed mantle cell lymphoma [143] was initially described as centrocytic lymphoma in the Kiel classification and as intermediate lymphocytic lymphoma or mantle zone lymphoma in the United States. The characterization of this disease as a clinicopathologic entity proceeded more rapidly in Europe than in the United States.[144] However, the entity has now gained widespread acceptance through substantiating immunophenotypic and molecular genetic data and mounting evidence of a distinctly poor clinical outcome not predicted by its low-grade histology. Mantle cell lymphoma is largely a disease of adults, like other malignant lymphomas of small B lymphocytes, from which it differs by showing a distinct predilection for males and an aggressive clinical course with a median survival of approximately 3 years.[145] [146] [147] The disease is typically disseminated at presentation and has a propensity to involve Waldeyers ring and the gastrointestinal tract, where it accounts for most cases of lymphomatous polyposis.[148] Roughly 25% of cases are overtly leukemic at diagnosis. The disease is currently believed to arise from a small population of CD5+ peripheral B cells that selectively home to the follicular mantle. Transformation to large cell lymphoma is exceedingly rare, if it occurs at all.

Universal recognition of mantle cell lymphoma as a morphologic entity was delayed in part because of its rather subtle and occasionally deceptive cytological and architectural attributes. The disease most commonly demonstrates a diffuse growth pattern, but may also grow in a mantle zone configuration surrounding reactive germinal centers,[149] [150] and may even manifest a purely nodular or follicular pattern devoid of benign germinal centers, mimicking that of follicular lymphoma. [151] The neoplastic cells in the usual case are slightly larger than small lymphocytes and exhibit irregular nuclear contours, a less condensed chromatin pattern, indistinct nucleoli, scant cytoplasm, and a variable mitotic rate. At one extreme of the cytologic spectrum, so-called lymphoblastoid variants are composed of somewhat larger cells with stippled chromatin and a high mitotic rate, whereas at the opposite extreme, rare examples may closely simulate small mature lymphocytes. [152] [153] [154] A cardinal feature of mantle cell lymphoma regardless of cytologic or architectural pattern is the virtual absence of large transformed cells having features of centroblasts, immunoblasts, or paraimmunoblasts. Epithelioid histiocytes are often singly distributed among the neoplastic cells and may further aid in the recognition of this disease (Fig. 69-4).

Neoplastic mantle cells are characterized immunophenotypically by the presence of moderate-density monotypic surface immunoglobulin of IgM type, usually with coexisting IgD. In contrast to most other B-cell lymphomas, light chains are expressed in the majority (at least 65%) of cases. The tumor cells co-express B-cell antigens and CD5 but do not express the CD10 antigen. CD23 is not expressed, a feature of utility in the distinction from B-CLL/small lymphocytic lymphoma. Staining for the cyclin D1 protein, deregulated as a consequence of the t(11;14) observed in the majority of cases of mantle cell lymphoma, provides a highly specific marker for this disease, as this protein is not known to be expressed by any normal lymphoid population and is only very exceptionally detected in lymphomas of other histologic types.[155]

The translocation t(11;14)(q13;q32) characterizes the vast majority of cases of mantle cell lymphoma and results in overexpression of the cyclin D1 protein, which is involved in the complex regulatory mechanism of cell cycle progression. [62] [156] [157] [158] [159] [160] [161] [162] [163] [164] [165] [166] The breakpoints in this translocation are widely dispersed over a considerable nucleotide span, rendering this rearrangement less susceptible to polymerase chain reaction (PCR) detection than other recurring chromosomal translocations in hematolymphoid malignancies. [167]

CD5 B-Cell Lymphomas

Lymphoplasmacytoid Lymphoma/Immunocytoma

Lymphoplasmacytoid lymphoma, or immunocytoma, is largely a disease of older adults, usually associated with a serum paraprotein of IgM type and variably accompanied by the clinical syndrome of Waldenströms macroglobulinemia. The spleen, lymph nodes, and bone marrow are frequent sites of disease; extranodal involvement may also be seen. An absolute lymphocytosis is less a feature of this disease than of B-CLL. [168] [169]

The criteria currently proposed for a diagnosis of lymphoplasmacytoid lymphoma or immunocytoma are more restrictive than hitherto and rely to a certain extent on the rigorous exclusion of other entities (small lymphocytic lymphoma, follicle center lymphoma, marginal zone B-cell lymphoma, mantle cell lymphoma) that may on occasion be associated with plasmacytoid differentiation. [170] Thus defined, this entity most closely approximates the lymphoplasmacytic type of immunocytoma as elaborated by Lennert. These tumors are composed of a spectrum of small lymphocytes, plasmacytoid cells, and plasma cells and do not manifest any of the histologic hallmarks of other specific types of B-cell lymphoma (Fig. 69-5). The growth pattern is diffuse and often interfollicular. Lymph node sinuses often remain patent or even ectatic.

Lymphoplasmacytoid lymphoma is characterized by the simultaneous presence of surface and cytoplasmic immunoglobulin of the same isotype, most commonly IgM without IgD. The neoplastic cells express B-cell antigens but generally fail to co-express CD5. CD10 is not expressed, and expression of CD23 is variable. [7]

The t(9;14)(p13;q32), with breakpoints involving the *pax-5* gene on the short arm of chromosome 9, has recently been reported in cases diagnosed as plasmacytoid variants of small lymphocytic lymphoma. [171] [172] The *pax-5* gene encodes a transcription factor known as B-cell-specific activator protein (BSAP), which is normally expressed throughout B-cell development, but not in terminally differentiated plasma cells. [173] [174] [175] [176] [177] It is unclear whether such cases correspond to what would be classified as lymphoplasmacytoid lymphoma in the REAL classification.

Follicle Center Lymphoma, Follicular

Follicle center lymphoma and large B-cell lymphoma are the most frequently diagnosed lymphoma subtypes in the United States, each accounting for approximately one-third of all cases. Follicle center lymphomas are largely diseases of adults, having a peak incidence in the fifth and sixth decades. Exceedingly rare cases are observed in teens and young adults. The clinical presentation is usually nodal, and bone marrow involvement is documented at diagnosis in the majority of patients. The natural history of

Figure 69-4 Mantle cell lymphoma. **(A)** A typical example is composed of a diffuse proliferation of highly monotonous angulated cells with scant cytoplasm and a virtual absence of blasts. **(B)** A lymphoblastoid variant exhibits a more primitive nuclear chromatin. **(C)** Cyclin D1 staining highlights a mantle zone configuration in this mantle zone variant. Non-neoplastic germinal centers are negatively stained and are surrounded by a collar of dark-staining tumoral cells. **(D)**. In contrast is the nodular variant shown here, in which the neoplastic population has effaced the central germinal centers, leaving only vestiges focally discernible (unstained) in this cyclin D1 stain. This pattern is easily mistaken for follicle center lymphoma.

Figure 69-5 Lymphoplasmacytoid lymphoma/immunocytoma. A bone marrow aspirate smear contains small lymphocytes, plasmacytoid cells, and plasma cells. Mast cells (arrows) are often conspicuously increased in this lymphoma.

this disease is characterized by a continuous risk of histologic progression or transformation, usually to diffuse large B-cell lymphoma. Extranodal presentations, though infrequent, appear to be most common in the skin, with a particular predilection for the scalp. Molecular genetic evidence suggests, however, that fundamental biologic differences exist between primary cutaneous follicle center lymphoma and nodal follicle center lymphoma, despite their apparent morphologic similarities.

Histologically this disease is easily recognized as a consequence of its characteristic follicular growth pattern in lymph nodes. It must be emphasized, however, that tumors deriving from any of the zones of the B-cell follicle (e.g., mantle cell lymphoma, marginal zone B-cell lymphoma, nodular lymphocyte-predominant Hodgkin disease) may exhibit a nodular pattern, and thus it is imperative that the diagnosis be based on an assessment of the cytologic composition of the neoplastic follicles. Follicle center lymphomas invariably display the full spectrum of follicle center differentiation and are composed of a polymorphous admixture of centrocytes and centroblasts in varying proportion (Fig. 69-6). [178] [179] Several schemes for grading follicle center lymphomas, based on estimates of the relative or absolute numbers of centrocytes and/or centroblasts, have been proposed but have generally proved to be poorly reproducible and of questionable clinical utility. [180] [181] [182] [183] The follicular growth pattern may be accompanied by a component of diffuse growth. [184] [185] Rarely, the pattern may be entirely diffuse; follicle center origin is more difficult to establish in such cases. Sclerosis may be a prominent feature of this disease and is especially common in retroperitoneal presentations.

Follicle center lymphomas usually express surface immunoglobulin, which may be of any isotype, reflecting the phenomenon of heavy chain class switch that normally occurs during germinal center differentiation. Approximately 10% of cases are surface immunoglobulin negative but express other B-cell-associated antigens, confirming their B-cell lineage. These tumors typically express the CD10 antigen but are CD5 and CD23 variable. Staining for the *bcl*-2 protein, which is constitutively expressed in most follicle center lymphomas as a consequence of the t(14;18), is useful in distinguishing malignant from benign follicle centers but is of no utility in differentiating follicle center lymphoma from other types of low-grade B-cell lymphoma, many of which also express the protein. [186] [187] [188] [189] It should also be borne in mind that a fraction of follicle center lymphomas, particularly those of large cell type, cannot be demonstrated to express the *bcl*-2 protein, and nonimmunoreactivity for *bcl*-2 should not therefore exclude a diagnosis of follicle center lymphoma if all other features are typical. This pattern of more frequent *bcl*-2 expression by centrocytes than centroblasts recapitulates the normal process of germinal center differentiation and may perhaps indicate intrinsic biologic differences between low- and high-grade follicle center lymphomas. [190]

The cytogenetic and molecular genetic hallmark of follicle center lymphoma is the t(14;18)(q32;q21), which is observed in at least 85% of cases and leads to overexpression of the anti-apoptosis protein, *bcl*-2. [31] [191] [192] The practical consequence of this event is to confer a survival advantage on the cell, resulting in the steady accumulation of long-lived cells that are at risk for secondary genetic events. [193] [194] These secondary events, particularly those inducing cellular proliferation (e.g., c-*myc* activation), may correlate with histologic progression and disease acceleration. [195] [196] [197] [198] [199] [200] Bcl-2 breakpoints are highly clustered into two breakpoint regions, termed the major breakpoint region and the minor cluster region, facilitating detection by simple PCR strategies. [201] Accumulating evidence suggests that clinical outcome may correlate with the specific breakpoint site.

Marginal Zone B-Cell Lymphoma

As presently conceived, this category encompasses two closely related disorders that are distinguished on the basis of their patterns of clinical disease. By far the more frequent is the extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) type (Fig. 69-7). This disease, initially characterized by Isaacson, represents the neoplastic derivative of the mucosal immune system, whose cytologic and homing properties it recapitulates. [202] [203] The stomach and salivary glands are the most frequent sites of involvement, although disease may be encountered in any extranodal site distinguished by the presence of a columnar or cuboidal epithelium. [204] [205] [206] [207] The disease is strongly associated with *Helicobacter pylori* infection in the stomach and with autoimmune manifestations in other

sites (Sjögren syndrome, Hashimoto thyroiditis).[208] [209] MALT lymphoma is a disease of adult life and is characterized by an exceptionally indolent clinical course, which, in conjunction with certain histologic features, delayed its recognition as a clonal lymphoid neoplasm. The disease is most often localized at presentation, although simultaneous or sequential involvement of multiple mucosal sites is often observed. [210] [211] The frequency of nodal dissemination is difficult to evaluate, as nodal disease may remain clinically silent for protracted periods and may be difficult to distinguish from a reactive process on a purely histologic basis. Marrow infiltration is uncommon. The demonstration that some gastric MALT lymphomas can be induced to regress in response to antibiotic therapy directed at *H. pylori*, coupled with the association with autoimmune disease, has led to the hypothesis that continued antigenic stimulation is critical to both the development and the maintenance of these distinctive tumors.[212] [213]

Figure 69-6 Follicle center lymphoma. **(A)** The follicular growth pattern is easily recognized, but can be closely simulated by lymphomas deriving from any of the zones of the B-cell follicle. **(B)** Neoplastic follicles invariably comprise an admixture of centrocytes and centroblasts.

The second disease included in this category is nodal marginal zone B-cell lymphoma, also referred to as monocytoid B-cell lymphoma. [214] [215] [216] [217] [218] [219] It now appears that the majority of these tumors represent nodal dissemination of underlying extranodal MALT lymphomas, which may not be clinically evident at the time of lymph node biopsy.[220] [221] [222] Nonetheless, occasional cases of apparently primary nodal marginal zone B-cell lymphomas are encountered and are histologically indistinguishable from low-grade B-cell lymphomas of MALT type with secondary nodal involvement (Fig. 69-8). Nodal involvement may be either

Figure 69-7 Gastric marginal zone B-cell lymphoma of MALT type. **(A)** The neoplastic proliferation consists of pale-staining cells of small to intermediate size that infiltrate in a marginal zone pattern around reactive-appearing germinal centers. Note the greatly attenuated rim of darkly stained apparent mantle zone lymphocytes that separates the neoplastic cells from the germinal centers. **(B)** A hallmark lymphoepithelial lesion shows partial destruction of a gland by clusters of neoplastic B cells, which in this instance evince plasmacytic differentiation.

Figure 69-8 Nodal marginal zone B-cell lymphoma. The cytologic composition and pattern of infiltration are indistinguishable from secondary nodal involvement by an extranodal marginal zone B-cell lymphoma of MALT type. Compare with Figures 69-7 and 69-9 .

localized or generalized, and marrow involvement may be seen.[223] Finally, it should be noted that focal monocytoid B-cell differentiation may be observed in other forms of low-grade B-cell lymphoma, most commonly follicle center lymphoma.

The unifying cytologic feature of both diseases is the marginal zone B cell, a small to medium-sized cell with abundant pale cytoplasm, which is commonly admixed in varying proportion with monocytoid B cells (similar-appearing cells with distinctly reniform nuclear contours), small lymphocytes, and plasma cells. [224] [225] [226] [227] Large transformed cells are commonly present, and overt large cell transformation may occur. The neoplastic proliferation is almost invariably attended by reactive follicle centers, about which tumor cells infiltrate in a marginal zone pattern, often with secondary involvement of the follicle centers themselves (so-called follicular colonization).[228] In lymph nodes, the pattern of infiltration may as well be sinusoidal or parafollicular. Lymphoepithelial lesions, consisting of epithelial units partially or entirely disrupted by discrete islands of small lymphoid cells, are a consistent finding in mucosal sites. Isotypic plasmacytic differentation may be extensive, causing diagnostic confusion with plasmacytoma.[229] The latter, however, consistently lacks a significant B-lymphocytic component and is not characterized by the presence of reactive germinal centers.

Marginal zone B-cell lymphomas express monotypic surface immunoglobulin, which may be of IgM, IgG, or IgA type. Cytoplasmic immunoglobulin is detectable in close to one-half of cases, reflecting plasmacytoid differentiation. There is no expression of CD5, CD10, or CD23, and expression of CD11c and CD43 is variable.

Karyotypic data are available in only a small number of cases because of the difficulty in obtaining analyzable metaphases. The translocations t(11;18)(q21;q21) and t(1;14)(p22;q32) have both been reported in extranodal cases, [230] [231] [232] [233] [234] as have a variety of numerical abnormalities, with trisomy 3 being most frequent. [235]

Splenic Marginal Zone Lymphoma (+/ Villous Lymphocytes)

In common with the marginal zone B-cell lymphomas of nodal and extranodal sites, these tumors present in adult life, with a slight female predominance. Patients exhibit little lymphadenopathy, but in contrast to patients with nodal and extranodal marginal zone tumors, frequently manifest marrow involvement and low-degree peripheral blood dissemination. Peripheral blood manifestations closely simulate those of so-called splenic lymphoma with villous lymphocytes; the precise histogenetic relation between these two entities remains uncertain. [236] [237] [238] As in other marginal zone B-cell lymphomas, plasmacytoid differentiation may occur and may be accompanied by a serum paraprotein. This disease pursues an indolent clinical course, with sustained remissions reported after splenectomy.

Involved spleens demonstrate white pulp expansion with selective involvement of the marginal zone. [239] Neoplastic cells resemble normal marginal zone B cells in possessing round to mildly irregular nuclear contours and relatively abundant pale cytoplasm (Fig. 69-9). Not infrequently, the neoplastic marginal zone is separated from a central reactive germinal center by what appears to be a preserved rim of small mantle zone B cells with scant cytoplasm. Immunophenotypic characterization, however, reveals that these apparent mantle zone cells lack IgD expression and may, therefore, represent a differentiated marginal zone population. [240] Just as in nodal and extranodal marginal zone B-cell lymphomas, the neoplastic proliferation may eventually infiltrate and obscure the follicle center, making recognition of the tumors marginal zone origin less apparent histologically. Concomitant red pulp involvement is usual, though of a variable degree.

The immunophenotype is similar to that described for nodal and extranodal marginal zone B-cell lymphomas. Cytogenetic reports are generally lacking.

Plasmacytoma/Plasma Cell Myeloma

These tumors are defined as being of purely plasmacytic origin, i.e., lacking a discernible lymphoid component as detected either morphologically or immunohistochemically. The majority of such tumors correspond to the clinical definition of plasma cell myeloma, or so-called multiple myeloma. A minority, however, arise as solitary bone or extranodal tumors, or less commonly as nodal neoplasms (Fig. 69-10). This disease may progress to overt myeloma, the incidence appearing to be greatest in patients with osseous involvement.

The majority of plasmacytomas are composed of well-differentiated plasma cells and are clinically low-grade tumors. [241] A small number of cases exhibit immature or plasmablastic cytomorphology, characterized by prominent nucleoli, variable pleomorphism, and a high mitotic rate. Such cases may be impossible

Figure 69-9 Splenic marginal zone lymphoma. The spleen shows selective involvement of the white pulp, with secondary infiltration of the red pulp. The white pulp infiltrate surrounds atrophic germinal centers in a marginal zone pattern, as in Figures 69-7 and 69-8 . A narrow rim of small, darkly staining cells is interposed between the germinal center and the neoplastic population. Although these cells appear morphologically to represent mantle zone B cells, their immunophenotype is not typical and suggests that they may rather represent a differentiated population of marginal zone B cells.

Figure 69-10 Plasmacytoma. The lymph node is diffusely infiltrated by mature-appearing plasma cells. An isotypic B-lymphocytic component is not observed.

to differentiate from immunoblastic lymphoma,[242] and may pursue a more aggressive clinical course.

Like their benign counterparts, the neoplastic cells express monotypic cytoplasmic immunoglobulin without surface immunoglobulin and do not express B-cell antigens.[243] [244]

Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphomas as presently defined collectively account for approximately one-third of adult non-Hodgkin lymphomas. This likely reflects our current ignorance of the biologic diversity of these lymphomas, as many as 40% of which arise in extranodal sites. There is mounting evidence, best exemplified at present by primary mediastinal large B-cell lymphoma, that extranodal lymphomas are not in any simple way analogous to their morphologic counterparts in lymph nodes. Additionally, diffuse large B-cell lymphoma represents the common morphologic expression of a variety of transformed low-grade B-cell lymphomas, including small lymphocytic lymphoma, follicle center lymphoma, marginal zone B-cell lymphoma, and nodular lymphocyte-predominant Hodgkin disease (nodular paragranuloma). [245] [246] It is likely that the next decade will see considerable progress in the recognition of clinically relevant subsets of diffuse large B-cell lymphoma. [247] [248] Although generally a disease of adult life, large B-cell lymphomas may occur in childhood. Untreated, the disease is aggressive, but contemporary chemotherapeutic regimens now yield complete remissions in 7580% of cases, with durable relapse-free survival rates approximating 50%.

As defined by the REAL classification, this category encompasses both the diffuse large cell and large cell immunoblastic categories of the Working Formulation, which had been separately distinguished as intermediate- and high-grade tumors, respectively, in the Working Formulation (Fig. 69-11). There remains considerable controversy regarding the appropriateness of this decision, as some data from European groups suggest that immunoblastic lymphoma, when defined by the Kiel criterion of greater than 90% immunoblastic composition, is indeed a more clinically aggressive disease than centroblastic lymphoma. Also included in the single category of diffuse large B-cell lymphoma for the time being are tumors composed of large cleaved cells (large centrocytes), multilobated cells (many of such tumors arise in extranodal sites),[249] [250] and anaplastic B cells. Large B-cell lymphomas may be infiltrated by substantial numbers of non-neoplastic T lymphocytes or histiocytes (T-cell or histiocyte-rich large B-cell lymphoma, respectively), giving rise to diagnostic confusion with both peripheral T-cell lymphoma and Hodgkin disease.[251] [252] [253] [254] [255] [256] [257]

The majority of diffuse large B-cell lymphomas express monotypic surface immunoglobulin, an important exception being primary mediastinal large B-cell lymphoma (discussed later). Cytoplasmic immunoglobulin is detectable in a minority of cases. Expression of either the CD5 or the CD10 antigen is exceptional. [258]

Chromosomal alterations involving the 3q27 locus of the *bcl*-6 gene are strongly associated with diffuse large B-cell lymphoma, occurring in approximately 40% of cases.[198] [259] [260] [261] [262] [263] [264] [265] [266] [267] The

Figure 69-11 Diffuse large B-cell lymphoma. **(A)** B-immunoblastic lymphoma is composed of a uniform population of immunoblasts having prominent central nucleoli and plasmacytoid cytoplasm. **(B)** T-cell-rich large B-cell lymphoma is readily identified in sections stained for CD20 (L26), which strongly and uniformly marks the minor population of neoplastic large B cells. **(C)** Intravascular large B-cell lymphoma is shown here involving a lesion of Kaposi sarcoma in a patient with AIDS.

-6 protein functions as a transcription factor[268] [269] [270] [271] and is normally expressed exclusively on follicle center B cells. [272] [273] Constitutive expression of the protein is induced during lymphomagenesis by heterologous promoters juxtaposed to the *bcl*-6 coding sequence as a consequence of chromosomal translocation.[274] Neoplastic cells are thereby arrested at a germinal center stage of development. Other molecular abnormalities in diffuse large B-cell lymphoma include rearrangement of the *bcl*-2 gene in roughly 2530% of cases.[275] [276] [277] [278] [279] [280] These alterations are entirely analogous to those seen in follicle center lymphomas and presumably identify cases that have transformed from follicular histology. Interestingly, rearrangements of *bcl*-2 and *bcl*-6 appear to be mutually exclusive, suggesting that these represent distinct transformation pathways. Available clinical data tend to substantiate this interpretation, indicating a favorable prognosis for *bcl*-6-associated disease and an unfavorable prognosis for *bcl*-2-associated disease.

Primary Mediastinal Large B-Cell Lymphoma

This variant of large B-cell lymphoma is thought to derive from a minor population of B cells that normally reside in the thymus. [281] [282] [283] [284] Although certain morphologic attributes have been proposed as characteristic of this variant, the histologic features are inconstant and the disease is therefore largely defined by clinical parameters. Like other diffuse large B-cell lymphomas, it is composed of large transformed B cells, which may resemble centroblasts, immunoblasts, large centrocytes, multilobate large cells, or even Reed-Sternberg cells. Typically the cytoplasm is pale-staining, and the tumor is often accompanied by fine comparmentalizing sclerosis (Fig. 69-12). [285] [286] [287] [288] This disease exhibits a striking predilection for women in the fourth decade of life, who commonly present with superior vena cava syndrome. Curiously, this syndrome is exceedingly rare in Hodgkin disease, even in the setting of bulky mediastinal disease. Chemotherapy now produces cure rates similar to those for other forms of large B-cell lymphoma.[289] Relapse patterns, however, are distinctive, with a high incidence of visceral dissemination involving the kidneys, adrenals, liver, ovaries, and central nervous system.

In contrast to other diffuse large B-cell lymphomas, this variant commonly fails to express surface immunoglobulin while retaining expression of other pan-B-cell antigens, such as CD19, CD20, and CD22.[290] Recurring chromosomal translocations have not been described, although abnormalities of the c- *myc* locus have been reported.

Burkitt Lymphoma

Burkitt lymphoma is the most common pediatric non-Hodgkin lymphoma in the United States, accounting for roughly 35% of cases. [291] The disease is less frequent in adults, in whom it is often encountered in the setting of immunodeficiency. The clinical presentation is generally extranodal, with a predilection for the jaw in African subjects and for the ileocecal region and mesentery in non-African subjects. Leukemic presentations with involvement of peripheral blood

and bone marrow (ALL, French-American-British [FAB] L3 type) are relatively infrequent. Burkitt lymphoma is the most highly proliferative of all non-Hodgkin lymphomas and is clinically aggressive, but curable with appropriate multiagent chemotherapy. [292]

The morphologic features of Burkitt lymphoma are highly distinctive. The tumor is composed of a monomorphous population of intermediate-size cells with round nuclear contours, multiple nucleoli, and moderately abundant basophilic cytoplasm. Imprint preparations reveal prominent cytoplasmic lipid vacuoles. The classical starry sky pattern is conferred by the presence of numerous macrophages ingesting apoptotic debris resulting from the high rate of programmed cell death (Fig. 69-13).

Burkitt lymphoma cells express surface immunoglobulin of the IgM type, in conjunction with pan-B-cell antigens, and additionally express the CD10 antigen. [293] CD5, CD23, and the *bcl*-2 protein are not expressed.

Translocations involving the c-*myc* gene on chromosome 8q24 are observed in virtually all cases of Burkitt lymphoma, although both the precise breakpoint and the reciprocal chromosome may vary.[294] The most frequent translocation, t(8;14) (q24;q32), occurs in approximately 80% of cases and juxtaposes the c- *myc* gene with

immunoglobulin heavy chain regulatory loci on chromosome 14. Variant translocations involve the light chain genes on chromosomes 2 and 22 in the remainder of cases.

Figure 69-12 Primary mediastinal large B-cell lymphoma. These tumors cannot be reliably distinguished from other forms of large B-cell lymphoma on purely morphologic grounds, although the pattern of compartmentalizing sclerosis seen here is typical.

Figure 69-13 Burkitt lymphoma. The distinctive starry sky pattern reflects the presence of numerous phagocytic histiocytes ingesting apoptotic debris in this most rapidly proliferating of all lymphomas.

In cases of endemic (African) Burkitt lymphoma, the heavy chain breakpoint lies within the joining region gene cluster, suggesting an early differentiational event, whereas the breakpoint in nonendemic disease occurs in the switch regions, implicating a later transformation event. [295] [296] A further difference in the biology of endemic versus nonendemic disease relates to the frequency of detection of EBV genomes in neoplastic cells. EBV genomes are present in nearly all cases of endemic Burkitt lymphoma but in only 1520% of cases of sporadic disease, with an intermediate incidence in AIDS-related Burkitt lymphoma. [297] Finally, mutations of *p53* are frequently encountered in Burkitt lymphoma.[133]

High-grade B-Cell Lymphoma, Burkitt-like

This category is afforded provisional recognition in the REAL classification pending further study of criteria for its recognition and the systematic analysis of clinical outcome. It corresponds in large part to those lymphomas classified as small non-cleaved cell lymphoma, non-Burkitt type, in the Working Formulation and is defined loosely as a lymphoma whose morphologic attributes are intermediate between those of Burkitt lymphoma and diffuse large B-cell lymphoma. [298] [299] The immunophenotype differs from that of classic Burkitt lymphoma in that cytoplasmic immunoglobulin may be expressed, while the CD10 antigen is typically not expressed. The molecular profile of these tumors in one study suggested a closer relation to large B-cell lymphoma than to Burkitt lymphoma, in that c- *myc* rearrangements were

not identified, whereas *bcl*-2 rearrangements were found in about 30% of cases.[300] [301] [302]

A special form of high-grade B-cell lymphoma, strongly AIDS associated, has been recently described under the terms body cavity-based lymphoma and primary effusion lymphoma. As the names imply, these lymphomas are characterized clinically by presentation as effusions involving serosal cavities, without concomitant solid tumors.[303] These lymphomas are invariably of B-cell lineage, as confirmed by antigen receptor gene rearrangement analysis, but typically fail to express common pan-B-cell antigens. Virtually all cases show evidence of HHV-8 infection, [303] [304] [305] [306] often with EBV co-infection. Abnormalities of known B-cell lymphoma-associated oncogene loci are not detected.

Peripheral T- and NK Cell Lymphomas

/ T-Cell Lymphomas

T-Cell Chronic Lymphocytic Leukemia/Prolymphocytic Leukemia

This rare variant of peripheral T-cell neoplasia is characterized clinically by a leukemic presentation, often with a very high white cell count, splenomegaly, and hepatomegaly. Lymphadenopathy is variable; primary nodal presentations are unusual. Cutaneous and mucosal manifestations are not infrequent. The disease pursues a moderately aggressive course and is not generally curable with existing regimens. [307] [308]

Neoplastic cells are of small to medium size and, in most instances, demonstrate at least some nuclear irregularity, prominent nucleoli, and relatively abundant cytoplasm. In lymph nodes the pattern of infiltration is paracortical or diffuse. Proliferation centers, typical of B-CLL, are not observed. High endothelial venules may be prominent and are commonly infiltrated by neoplastic cells (Fig. 69-14). Splenic red pulp and hepatic sinusoids are selectively involved, and the marrow tends to be diffusely involved.

The immunophenotype is that of a mature T cell; loss of pan-T-cell antigens is unusual. Thus, most cases express CD2, CD3, CD5, and CD7. The majority of cases express the CD4 subset antigen. Occasional cases are double positive (i.e., CD4+ and CD8+). CD25 is not expressed, in contrast to adult T-cell lymphoma/leukemia.

Karyotypic analysis has shown a striking correlation between this histology and inv14(q11;q32). This abnormality has been reported in as many as 75% of cases. An association with trisomy 8q has also been noted.

Mycosis Fungoides/Sézary Syndrome

Mycosis fungoides and Sézary syndrome are primary cutaneous T-cell lymphomas that differ in their propensity for peripheral blood dissemination. These entities tend to be disorders of adults and are typified by the presence of multiple cutaneous plaques or by diffuse exfoliative erythroderma. Nodal involvement is rare in the early stages of disease and, when it develops, usually heralds a terminal phase. [309] [310] [311] Histologic progression to large cell lymphoma, frequently with anaplastic morphology, may occur late in the disease course.

In the skin, mycosis fungoides is recognized by its characteristic bandlike distribution in the superficial dermis. Epidermotropism, with formation of so-called Pautrier abscesses, is the histologic hallmark (Fig. 69-15). Spongiosis is absent. Neoplastic cells are predominantly of small size and exhibit cerebriform nuclear contours. A minor population of larger, hyperchromatic cells is often admixed; transformed cells with vesicular nuclei and prominent nucleoli are less commonly observed. Nodal involvement, when seen, is paracortical and the neoplastic infiltrate is invariably accompanied by a proliferation of CD1a+ interdigitating reticulum cells. [312]

Neoplastic cells typically express the CD2, CD3, and CD5 antigens but fail to express CD7 in at least two-thirds of cases.

Figure 69-14 T-cell chronic lymphocytic leukemia/prolymphocytic leukemia. The neoplastic population consists of small lymphoid cells with small but conspicuous nucleoli. High endothelial venules are prominent and are permeated by neoplastic cells.

The vast majority of cases demonstrate CD4 subset restriction, though rare CD8+ cases are encountered. [313] CD25 is not expressed.

Peripheral T-Cell Lymphoma, Unspecified

As currently formulated, this represents the largest single category of peripheral T-cell lymphomas in the REAL classification, reflecting not

Figure 69-15 Mycosis fungoides. Cerebriform cells exhibit pronounced epidermotropism, with formation of Pautrier microabscesses.

a homogeneous disease entity but rather the difficulty in defining morphologic, immunophenotypic, or genetic parameters that would allow for precise and reproducible subclassification of these tumors. This category encompasses all of the pleomorphic subtypes of the updated Kiel classification, as well as T-zone lymphoma, lymphoepithelioid cell lymphoma (Lennert lymphoma), and T-immunoblastic lymphoma of that scheme. A number of clinical studies have now documented the generally more aggressive nature of peripheral T-cell lymphomas as compared to lymphomas of B-cell derivation and have shown stage to be a more accurate predictor of outcome than morphologic subtype.[314] [315] [316] [317] [318] [319] [320] As a whole, patients with these diseases are adults who present with generalized lymphadenopathy. Cutaneous involvement is not uncommon, and visceral involvement is found with some frequency. Peripheral blood manifestations include the presence of circulating lymphoma cells and eosinophilia. Collectively, patients with peripheral T-cell lymphomas appear more prone to disease relapse than do patients with B-cell tumors of comparable grade. Histologic progression may occasionally be observed in sequential biopsies, though this appears less frequent than in B-cell lymphomas. Hemophagocytic syndromes producing profound cytopenias may supervene as terminal events.[321] EBV has been etiologically associated with a variable proportion of peripheral T-cell lymphomas but is most strongly correlated with angiocentric T/NK cell lymphomas of nasal type.

As might be expected of such a heterogeneous group of disorders, the morphologic manifestations are highly varied, although with some points of commonality.[322] Peripheral T-cell lymphomas as a group tend to be characterized by both pleomorphism (cytologic variability among cells of the same lineage or clone) and polymorphism (intermingling of cells of different lineage), the latter apparently the consequence of lymphokine secretion by neoplastic T cells. Most of the unspecified peripheral T-cell lymphomas are composed of pleomorphic lymphoid cells of intermediate to large size, admixed with varying numbers of eosinophils, plasma cells, histiocytes, and epithelioid cells (Fig. 69-16A). Cells closely resembling the Reed-Sternberg cell of Hodgkin disease may be seen. High endothelial venules may be a prominent feature of these proliferations, and interdigitating reticulum cells are often increased in number. Lymphoepithelioid cell lymphoma (Lennert lymphoma) is composed of small lymphoid cells with minimal pleomorphism and is distinguished by the presence of small multifocal aggregates of epithelioid cells (Fig. 69-16B).[323] [324] Thus, neoplastic expansions of post-thymic T cells simulate in all respects the biologic and morphologic aspects of the normal T-cell immune response. Nodal involvement is either diffuse or interfollicular. Marrow involvement is relatively frequent.

The immunophenotype is that of a post-thymic T cell, with variable loss of T-cell antigens, most commonly CD7. The majority of cases exhibit CD4 subset restriction. Occasional cases are CD8 subset restricted; rare cases may be either double negative or double positive.

T-cell receptor genes are rearranged in the vast majority of cases. Incomplete rearrangements of immunoglobulin heavy

Figure 69-16 Peripheral T-cell lymphoma, unspecified. **(A)** This typical example demonstrates a pleomorphic spectrum of lymphoid cells in a polymorphous milieu of histiocytes, plasma cells, and eosinophils. **(B)** Lymphoepithelioid cell lymphoma (Lennert lymphoma), in contrast, is characterized by a minimally pleomorphic proliferation of small T lymphocytes associated with multifocal aggregates of epithelioid histiocytes.

and occasionally light chain genes may additionally be seen. Most cases express the / T-cell receptor, as detected by the antibody F1; a minority express the / receptor, identified by the antibody TCR-. Cytogenetic abnormalities involving the T-cell receptor gene loci are frequent.[325] [326] [327] [328]

Subcutaneous Panniculitis-like T-Cell Lymphoma

This variant of peripheral T-cell lymphoma describes a distinctive clinical syndrome and has been provisionally recognized in the REAL classification. Patients with this disease present with numerous subcutaneous nodules, generally distributed over the extremities and unassociated with lymphadenopathy.[329] The disease does not appear to be EBV related and manifests a particularly high incidence of terminal hemophagocytic syndromes, which are the usual cause of death in fatal cases.

Biopsy specimens demonstrate a panniculitic pattern of disease in which atypical lymphoid cells infiltrate between adipocytes of the subcutis in a manner reminiscent of that seen in panniculitis (Fig. 69-17). The dermis is usually uninvolved. Destruction of adipocytes may produce fat necrosis and a foreign body giant cell reaction. In contradistinction to simple panniculitis, however, the lymphoid population is cytologically malignant, mitotically active, and shows prominent karyorrhexis. Most cells are of medium size and exhibit irregular nuclear contours and hyperchromasia. Large cells may be admixed. Angioinvasion is usually not a salient feature, although necrosis is frequent.

The neoplastic cells have a mature T-cell phenotype and may express either CD4 or CD8 subset antigens. EBV sequences are not identified in neoplastic cells. T-cell receptor genes are consistently rearranged. Rare cases of / T-cell lymphoma may present with a similar clinical and morphologic picture.

Angioimmunoblastic T-Cell Lymphoma

Initially described as an abnormal immune response under a variety of names (angioimmunoblastic lymphadenopathy with dysproteinemia, immunoblastic lymphadenopathy, lymphogranulomatosis X), this condition appears in most cases to represent a bona fide T-cell lymphoma with distinctive clinical and morphologic features.[330] Patients typically present with generalized lymphadenopathy, constitutional symptoms, a skin rash, and polyclonal hypergammaglobulinemia. Although prolonged remissions may be obtained in some cases with steroids, intensive chemotherapy is typically required for disease control. Histologic progression to large cell (immunoblastic) lymphoma, which may be of either T or B lineage, may occur.[331] Molecular or karyotypic analyses of sequential biopsies or of simultaneous biopsies from different sites have revealed unstable and fluctuating clonal expansions, lending some credence to the notion that these proliferations may indeed arise out of the background of an abnormal immune response.

The morphologic features are highly characteristic. Involved lymph nodes appear lymphoid depleted at low magnification and consistently demonstrate a marked proliferation of high endothelial venules.[332] The venules are often surrounded by a cuff of hyaline material, which can be highlighted by PAS staining. The neoplastic proliferation comprises small lymphocytes, immunoblasts, and a distinctive population of pale-staining or clear cells, which tend to aggregate along vessel walls (Fig. 69-18). Plasma cells, eosinophils, and histiocytes may be interspersed in varying number. Germinal centers are typically absent or inconspicuous. Irregularly expanded aggregates of follicular dendritic cells, recognized by immunohistochemical staining with appropriate antibodies (e.g., CD21 or CD35), constitute a consistent and relatively specific immunologic lesion in angioimmunoblastic T-cell lymphoma.

The immunophenotype is that of a post-thymic T-cell, usually CD4+, without other distinguishing characteristics except for the expanded follicular dendritic cell aggregates previously mentioned. EBV sequences are detectable in a high percentage of cases.[333] [334]

Molecular analysis of antigen receptor gene configuration reveals the presence of clonally rearranged T-cell receptor genes in the majority of cases.[335] Immunoglobulin gene rearrangements are encountered in a smaller percentage of cases, with or without corresponding T-cell receptor rearrangement.[336] Numerical abnormalities, particularly trisomy 3 and trisomy 5, have been noted in cytogenetic studies.[337] [338]

Intestinal T-Cell Lymphoma

This highly aggressive, primary intestinal form of T-cell lymphoma afflicts adults, many of whom have a history of gluten-sensitive enteropathy. In some cases, however, it constitutes the initial manifestation of enteropathy, whereas in others an association with enteropathy is never established.[339] [340] [341] Presenting complaints include abdominal pain and weight loss. The jejunum is the most frequent site of disease, with multifocal ulceration, often with perforation, being typical. Mass lesions are not always discernible. Regional lymph node involvement is common. The disease typically pursues an accelerated and intractable course.

Biopsies and resected specimens demonstrate constant infiltration

Figure 69-17 Subcutaneous panniculitis-like T-cell lymphoma. **(A)** The interstitial pattern of neoplastic infiltration between subcutaneous adipocytes simulates panniculitis. Tumoral cells, however, are cytologically atypical and often show karyorrhexis. **(B)** Terminal hemophagocytic syndromes are particularly common in this form of T-cell lymphoma. A bone marrow biopsy from such a patient shows hypocellularity with prominent phagocytosis of erythrocytes and hematopoietic precursors by benign-appearing histiocytes.

Figure 69-18 Angioimmunoblastic T-cell lymphoma. **(A)** Relative lymphoid depletion and a conspicuous haphazard vascular proliferation are the cardinal low-power attributes of this lymphoma. **(B)** Aggregates of clear cells, often perivascular, are frequently observed.

of the mucosa and variable degrees of infiltration of the bowel wall by an atypical lymphoid population that may range from small to large in size. Anaplasia may be observed. Small intraepithelial T cells are usually prominently distributed in the adjacent mucosa, which may or may not show changes of villous atrophy (Fig. 69-19). [342]

Figure 69-19 Intestinal T-cell lymphoma. An ulcerated, transmural tumor in the jejunum is associated with prominent atrophy and blunting of adjacent villi.

Neoplastic cells express T-cell antigens, including CD3. The mucosal lymphocyte antigen CD103 is consistently expressed. [343] [344] [345] T-cell subset antigens are variably expressed, with most cases exhibiting a CD4/CD8 or a CD4/CD8+ phenotype. TCR- genes are clonally rearranged.

Adult T-Cell Lymphoma/Leukemia

The clinical and morphologic profiles of this disease are protean, and thus the disease is defined serologically by the presence of antibodies to HTLV-I. The disease is endemic in southwestern Japan and the Caribbean basin and occurs most commonly in an acute form characterized by lymphadenopathy, hepatosplenomegaly, cutaneous disease, and peripheral blood involvement. [346] [347] [348] [349] [350] [351] [352] In this form the disease is frequently accompanied by hypercalcemia and lytic bone lesions. Median survival does not exceed 1 year. A lymphomatous variant is characterized by lymphadenopathy or by tumor formation in visceral sites, whereas chronic and smoldering forms are manifest by lesser degrees of lymphocytosis, often accompanied by a skin rash. [353] These variant forms of disease generally follow a more protracted disease course.

The most characteristic and easily recognizable manifestation of adult T-cell lymphoma/leukemia occurs in the peripheral blood, where neoplastic cells stand out by virtue of their multilobate nuclei (so-called cloverleaf or flower cells) and basophilic cytoplasm. In lymph nodes the infiltration is diffuse and may be composed of cells of small, intermediate, or large size, often in combination (Fig. 69-20). Reed-Sternberg-like cells may be present. [354] Cutaneous infiltrates frequently show epidermotropism, making distinction from mycosis fungoides difficult or impossible on morphologic grounds. Bone marrow

Figure 69-20 Adult T-cell lymphoma/leukemia. A pleomorphic infiltrate of medium-sized to large cells infiltrates the paracortex and surrounds a benign germinal center. Neoplastic cells expressed a CD4+, CD7, CD25+ immunophenotype.

involvement is diffuse and may be discordantly sparse in relation to the level of lymphocytosis observed in the peripheral blood. Biopsy of lytic lesions typically reveals only increased bone resorption in the absence of significant tumoral infiltration; it appears to represent a remote effect.

Tumor cells exhibit a T-cell phenotype but generally lack the CD7 antigen and, in contrast to other peripheral T-cell lymphomas, express high levels of the CD25 antigen (IL-2 receptor). Most cases are CD4+, though rare CD8+ cases have been reported. [355]

T-cell receptor genes are clonally rearranged. All cases show clonal integration of HTLV-I genomes. [356] [357]

Anaplastic Large Cell Lymphoma

Two distinct clinical syndromes are included in this category and likely represent different disease entities, as evidenced by their differing clinical behavior, immunophenotypes, and genetic features. [358] [359] [360] The classic form is a systemic disease manifested by lymphadenopathy, with or without cutaneous involvement. The disease may affect any age group, but a strong predilection for onset in childhood and adolescence is evident. In this form, anaplastic large cell lymphoma corresponds to most cases previously described as malignant histiocytosis. In addition, many cases formerly classified as lymphocyte-depletion Hodgkin disease would now be categorized as anaplastic large cell lymphoma. The clinical outcome following intensive therapy appears to be more favorable than that for other forms of peripheral T-cell lymphoma, with survival rates not differing substantially from those of other types of large cell lymphoma. [361] [362] The second form of anaplastic large cell lymphoma is a primary cutaneous lymphoproliferation that is cytologically malignant but pursues an indolent clinical course, frequently punctuated by spontaneous remissions. This disease overlaps considerably with, and is often indistinguishable from, the syndrome described as lymphomatoid papulosis. [363] [364] [365] It is primarily a disease of adults and carries an attendant risk of transformation to a disseminated lymphomatous phase of disease, currently placed at about 20%.

The morphologic picture of anaplastic large cell lymphoma does not differ appreciably in the two variants. The neoplastic population comprises a pleomorphic spectrum of large cells, many with horseshoe- or wreath-shaped nuclei, prominent nucleoli, and abundant, rather basophilic cytoplasm. Cells closely resembling Reed-Sternberg cells may be found. [366] In lymph nodes, the infiltrate assumes a cohesive pattern with a predilection for sinusoidal involvement (Fig. 69-21 A, B). [367] These features not infrequently lead to a diagnosis of metastatic disease. Variant morphologies have been described and include a monomorphous type with minimal anaplasia, a small cell variant, and the so-called lymphohistiocytic variant in which a characteristic plasmacytoid-appearing histiocyte overwhelmingly predominates and obscures the minor population of anaplastic large cells (Fig. 69-21 C, D). [368] [369]

The immunophenotype of these tumors is distinctive. [370] The CD30 antigen, which initially defined these lymphomas, is almost always expressed, but it may be expressed by other lymphomas of both B- and T-cell type. [371] [372] [373] [374] [375] Leukocyte common antigen (CD45) and epithelial membrane antigen are variably expressed, [376] [377] whereas the CD15 (LeuM1) antigen is infrequently expressed. [378] The vast majority of cases exhibit either a T-cell or null phenotype and genotype and express cytolytic granule-associated proteins, indicative of cytolytic T-cell function. Histiocyte-specific markers are not expressed. [379] It remains controversial whether rare cases with a B-cell phenotype exist; at present the REAL classification recommends that only cases with a T- or null cell phenotype be classified as anaplastic large cell lymphoma. [380] Cases with anaplastic morphology and a B-cell phenotype are classified simply as diffuse large B-cell lymphomas.

Cytogenetic studies of anaplastic large cell lymphoma and of cases earlier classified as malignant histiocytosis [381] [382] have shown a strong association with the t(2;5)(p23;q35), particularly in pediatric populations. [383] [384] [385] [386] [387] [388] [389] Molecular studies in several laboratories have since confirmed the highly selective nature of this association. The t(2;5) translocation yields a chimeric p80 protein in which the amino-terminus of nucleophosmin (NPM) on 5q35 is fused to the catalytic domain of anaplastic lymphoma kinase (ALK) on 2p23. [390] This translocation is readily detected by DNA-based PCR strategies, [391] [392] and antibodies are now available that permit detection of the p80 protein. [393] [394]

A primary cutaneous form of CD30+ lymphoproliferative disorder with anaplastic large cell morphology is recognized under the clinical term lymphomatoid papulosis. Although morphologically indistinguishable from anaplastic large cell lymphoma, this disease is characterized by the presence of one or several cutaneous nodules, rarely exceeding 2 cm in size, that exhibit the remarkable property of spontaneous regression, often followed by similar episodes of recurrence and spontaneous remission. Up to 20% of patients may eventually develop disseminated lymphoma. These lesions differ immunophenotypically from classic anaplastic large cell lymphoma by their expression of the cutaneous lymphocyte antigen and lack of expression of epithelial membrane antigen. Cytolytic granule-associated proteins, as

observed in most cases of anaplastic large cell lymphoma, have recently been demonstrated. Cytogenetic and molecular studies to date have shown these cases to lack the t(2;5)(p23;q35) typical of anaplastic large cell

Figure 69-21 Anaplastic large cell lymphoma. **(A)** Neoplastic cells, here stained for CD30 (BerH2), exhibit a striking propensity for sinusoidal infiltration. **(B)** Parenchymal involvement is often syncytial, composed of anaplastic cells with horseshoe-like and otherwise bizarre nuclear contours. **(C)** Malignant cells are inconspicuous in the so-called lymphohistiocytic variant of anaplastic large cell lymphoma. In such cases a cytologically distinctive histiocyte with plasmacytoid morphology predominates, conferring a deceptively benign appearance. The *NPM/ALK* rearrangement was detected in this case by PCR. **(D)** CD30 staining in the same case revealed the presence of rare malignant cells with anaplastic morphology.

lymphoma. Thus, despite considerable morphologic similarity, these appear to represent distinctive clinical entities that mandate different therapeutic approaches.

/ T-Cell Lymphomas

Hepatosplenic / T-Cell Lymphoma

The function of / T cells remains poorly understood, but there is good experimental evidence in mice suggesting that / and / T cells represent distinct cell lineages. / T cells are the first to develop ontogenetically and exhibit highly selective utilization of variable region genes, limited diversity, and a remarkable predilection for localization to particular anatomic sites (often epithelial) that appears to correlate with variable region gene usage. [395] TCR- knockout mice develop normal numbers of / T cells; and, conversely, normal numbers of / T cells are found in TCR- knockout mice. Moreover, molecular analysis of /-expressing T cells has shown a high frequency of out-of-frame rearrangements of region genes and germline region sequences in deleted DNA loops, indicating that such cells could not have expressed a functional / receptor. [396] The / receptors associate with the CD3 transmembrane signaling complex but, unlike their / counterparts, are only infrequently coupled with either the CD4 or the CD8 antigen. Although the antigenic specificity of / T cells is unknown, it appears that these cells have a non-MHC-restricted cytolytic capability that may have evolved to recognize a limited number of high-frequency antigens encountered at epithelial surfaces and to provide a first line of defense against microbial organisms at these sites prior to the recruitment of a more specific immune response. [397] Interestingly, while / T cells constitute the majority of intraepithelial and intraepidermal lymphocytes in mice, they appear to represent less than 10% of such cells in humans. [398] [399]

The classification of lymphomas derived from cytolytic or NK-like T cells, true NK cells, and / T cells will surely undergo significant revision in the coming years. As a group these

Figure 69-22 Hepatosplenic / T-cell lymphoma. Sinusoidal infiltration by rather featureless small to medium-sized cells is the hallmark of this disease in the spleen, as well as in the liver and bone marrow.

lymphomas are distinguished by their propensity to involve extranodal sites and, with a few exceptions, to pursue an aggressive clinical course. As is evident from the schema proposed by the International Lymphoma Study Group, the morphologic, immunophenotypic, and other criteria that have historically been well suited to the classification of B-cell lymphomas have proved disappointing in the study of T-cell lymphomas, with the result that new entities have been accorded provisional recognition on the basis of what appear to be reproducible clinical patterns of disease. As additional immunophenotypic and molecular data accrue, it is likely that these disease boundaries will shift or be largely redrafted.

The so-called hepatosplenic / T-cell lymphoma has become the prototype of the / T-cell lymphomas. [400] This is a disease predominantly affecting young men, who present with massive hepatosplenomegaly and insignificant lymphadenopathy. [400] [401] [402] [403] The clinical course is abbreviated, despite initial response to chemotherapy, with median survival not exceeding a few years.

Morphologically, the disease is recognized by its distinctive pattern of infiltration in splenic and hepatic sinusoids (Fig. 69-22). [404] [405] Bone marrow involvement is common but may be subtle, requiring immunohistochemical staining to highlight its sinusoidal predilection. The neoplastic cells are most commonly of small to intermediate size, although some variation from this spectrum may be seen in individual cases. The chromatin is less condensed than that of a mature lymphocyte, and nucleoli are inconspicuous. The tumoral cells are otherwise generally featureless, with round or minimally irregular nuclear contours and a small amount of cytoplasm. [406]

The immunophenotypic profile of the neoplastic population is that expected of / T cells, with positivity for the TCR- protein. Tumoral cells most commonly express a CD4/CD8 phenotype, as is typical of the vast majority of normal / T cells, but may occasionally exhibit a CD4/CD8+ phenotype. Cytolytic proteins such as those identified by TIA-1 are often demonstrable, and NK-associated antigens may also be detected.

Molecular studies confirm clonal rearrangement of the TCR- and TCR- genes in most cases. Karyotypic analysis has identified the isochromosome 7q as a recurring chromosomal aberration in a high percentage of cases. [407] [408]

/ T-cell lymphomas with a predilection for cutaneous rather than hepatosplenic presentation have now been well characterized. [409] [410] [411] [412] [413] [414] These lymphomas closely mimic in morphologic terms the disease described above as subcutaneous panniculitis-like T-cell lymphoma and differ from the hepatosplenic variant of / T-cell lymphoma in utilizing V2 rather than V1 genes. The latter feature recapitulates the pattern observed in normal / T cells, in which the V1 subset shows selective homing to spleen and thymus, while the V2 subset homes to lymph nodes, peripheral blood, tonsils, and skin. [415] In addition, a clinically and histologically identical form of hepatosplenic T-cell lymphoma has been shown recently to express the / T-cell receptor, to harbor the i7q cytogenetic abnormality, and to be characterized by an equally aggressive clinical course. [416] Thus, it must be conceded that, for the moment, a rational classification of many of the peripheral T-cell lymphomas, whether on clinical, morphologic, or immunologic grounds, remains elusive.

NK Cell Lymphomas

Large Granular Lymphocyte Leukemia

The indolent clinical syndrome connoted by the term large granular lymphocyte leukemia is shared by two morphologically indistinguishable variants of this disease, one derived from CD8+ T cells and the other from cells of NK type. [417] [418] Patients affected by these diseases are generally adults, with a roughly equal sex distribution. Peripheral blood smears typically demonstrate a mild to moderate lymphocytosis with a discernible increase in large granular lymphocytes, characterized by their small to intermediate size, oval nuclei, and abundant pale blue cytoplasm containing coarse or fine azurophilic granules (Fig. 69-23). Neutropenia is a consistent hallmark of the T-cell variant and may be seen as well in the NK form, in which it tends to be less pronounced. [419] Bacterial infections, consequently, may pose a recurring clinical problem in these patients. Anemia may additionally be seen in the T-cell form. Hepatosplenomegaly is almost always present, whereas significant lymphadenopathy is infrequent. Various autoimmune manifestations may accompany the disease, with rheumatoid arthritis being especially common. Bone marrow disease is interstitial in distribution and may be subtle. Splenic involvement is localized to the red pulp and may be difficult to identify in routine preparations. Imprints are helpful in recognizing the cytoplasmic azurophilic granules, which are not identifiable in tissue sections. Liver biopsies reveal

Figure 69-23 Large granular lymphocyte leukemia. A peripheral blood smear shows a mild lymphocytosis consisting of small cells with relatively abundant pale cytoplasm containing prominent azurophilic granules. Neutropenia is additionally evident.

a sinusoidal pattern of infiltration. [420] The disease course is stable and prolonged in most instances, although aggressive evolution has been reported on occasion and appears particularly common in EBV-associated Asian cases. [421]

Immunophenotypic and molecular studies are useful adjuncts in the diagnosis of these lymphoproliferative disorders. The T-cell variant exhibits a CD3+, CD8+, CD16/57 variable +, CD56 immunophenotype and has productively rearranged T-cell receptor genes, usually /. In contrast, the NK form of disease fails to express surface CD3 (but may express cytoplasmic CD3), uniformly expresses CD56, and has not undergone productive T-cell receptor gene rearrangement. T subset antigens are usually not expressed in this variant, although CD8 may be detectable in a fraction of cases. [422] [423]

Angiocentric (Nasal-Type) T/NK Cell Lymphoma

This disease was known historically by the names lethal midline granuloma and polymorphic reticulosis, and was initially included in the spectrum of diseases conceptualized under the rubric of angiocentric immunoproliferative lesion, which included so-called lymphomatoid granulomatosis. [424] [425] [426] As now more rigorously defined, this disease is considered to represent a neoplastic proliferation of NK or related T cells. [427] [428] [429] [430] It is the most strongly EBV associated of all peripheral T-cell lymphomas[431] [432] [433] and is endemic to Asia and certain parts of Latin America. [434] The disease may affect any age group, and demonstrates a particular predilection for the nose and palate. Other extranodal sites, including the skin and testis, may be primarily involved. [435] [436] [437] [438] The clinical course is variable and difficult to predict. [439] [440] At least some cases previously diagnosed as angiocentric immunoproliferative lesion in the lung (lymphomatoid granulomatosis) have since been shown to represent EBV-associated large B-cell lymphomas with a marked T-cell response, and are excluded from the category of angiocentric T/NK cell lymphoma.[441]

The cytologic spectrum observed in this lymphoma is broad, ranging from cases composed predominantly of intermediate-sized cells to cases with large numbers of immunoblasts. Plasma cells are often interspersed; other inflammatory components, including eosinophils, may be seen as well. Regardless of cytologic composition, these cases exhibit in common a distinctive angiocentric and angiodestructive pattern of growth, resulting in large geographic zones of necrosis, which may prove diagnostically frustrating in small biopsy specimens (Fig. 69-24).

The typical immunophenotypic profile includes immunoreactivity for CD2 and CD56, with variable positivity for CD5 and nonimmunoreactivity for surface CD3 as its most salient features.[442] Cytoplasmic positivity for the CD3epsilon chain may, however, be detected. The molecular configuration of the T-cell receptor genes is almost invariably germline.[443]

Aggressive NK Cell Lymphoma/Leukemia

Rare cases of NK cell neoplasia phenotypically indistinguishable from nasal-type angiocentric lymphoma have been reported in young adults presenting with fever, cytopenias, mild lymphocytosis, and hepatosplenomegaly. These cases demonstrate significant

Figure 69-24 Nasal angiocentric T/NK cell lymphoma. **(A)** Extensive necrosis, displayed here, is a common finding in these tumors. **(B)** Angiodestructive growth is characteristic, but may be focal and difficult to demonstrate in small biopsies.

morphologic overlap with indolent large granular lymphoproliferative disorders, including the presence of cytoplasmic azurophilic granules, but are characterized by a highly aggressive clinical course and poor therapeutic response. [444] [445] [446] [447] [448] [449] [450] They are variably EBV associated.

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Chapter 70 - Clinical Manifestations, Staging, and Treatment of Non-Hodgkin Lymphoma

Koen van Besien
Fernando Cabanillas

INTRODUCTION

The term non-Hodgkin lymphoma (NHL) encompasses all malignancies originating from the lymphoid system with the exception of classic Hodgkin disease. The lymphoid system is extremely complex. Cells belonging to it are located in highly specialized organs such as the lymph nodes, but also are distributed throughout practically every organ in the body, with some exceptions such as the central nervous system (CNS) and stomach. The three major cell typesnatural killer (NK) cells, B cells, and T cellscan undergo expansion, maturation, and selection on exposure to an appropriate antigen. Expansion of abnormal clonogenic cells can occur in any of these cell types, in different subtypes of cells, and at different stages of maturation. It is therefore not surprising that NHL has many different subtypes with different behavior, and can be localized in or spread to any organ. In many respects, NHL is not one but many different disorders, each as dissimilar from the others as Hodgkin disease is from other NHLs. Also, some forms of NHL defy classification schemes and straddle the border between different disease categories. Burkitt lymphoma and T-cell lymphoblastic disease may be appropriately called mature B-cell acute lymphocytic leukemia (ALL) and T-cell ALL, respectively. There is very little difference between small lymphocytic lymphoma and chronic lymphocytic leukemia (CLL); and the entity termed Waldenström disease may be interchangeable in many cases with immunocytoma. Some lymphomas are difficult to categorize as either Hodgkin disease or NHL. Finally, some disorders may be at the border between lymphoid malignancy and inflammatory disorders. This is true for some cases of indolent NK cell proliferation and for some cases of severe gluten enteropathy, in which a clonal T-cell proliferation can be noted, or for Sjögren syndrome, in which a clonal B-cell proliferation is commonly found.

Despite the extreme variability in clinical presentation, some clinical and pathologic features are common to practically all lymphoid malignancies. These features include the frequent localization to the principal lymphoid organsthe lymph nodes, bone marrow, and blood. Although NHL does not always originate in these organs, it almost always involves them at some time during the course of the disorder. Other common features include a particular morphology, the expression of a lymphoid immunophenotype (either NK, B, or T cell), and T-cell receptor or immunoglobulin gene rearrangement. Importantly, almost all NHLs are sensitive to commonly used chemotherapeutic agents or radiation therapy (or both).

Because of the diverse subentities, many of which are clinically relevant, the classification of NHL is a challenge for the practicing physician. A number of classifications have been proposed over the years, leading to considerable confusion. The Working Formulation was an attempt by the National Cancer Institute to provide common ground in classification and reporting. [1] It continues to represent a useful scheme for the majority of cases. The Working Formulation was based on purely morphologic criteria and clinical outcomes. The immunophenotype was not used at all because the technique was not widely available in the 1970s. The Working Formulation formed the basis for a number of large cooperative studies and was widely accepted. Our knowledge of the pathogenesis of NHL, however, has progressed rapidly over the past decade. Advances in immunology, molecular biology, cytogenetics, and immunophenotyping have been applied to the characterization of lymphomas and have profoundly influenced our thinking. New entities are recognized that do not have a place in the Working Formulation. Furthermore, some recognized morphologic entities were found to include heterogeneous disease subgroups, such as diffuse small cleaved cell lymphoma, which includes what is now recognized as mantle cell lymphoma as well as some cases of peripheral T-cell lymphoma. A new classification of NHL, the Revised American-European Lymphoma (REAL) classification, was proposed in 1994. [1] It provides a framework for understanding currently recognized lymphoma subtypes and allows the design of specific treatment strategies for a number of newly recognized disorders. [2] The REAL classification will be used throughout this chapter. It represents, as its authors acknowledge, a work in progress. [3] Since its application, additional proposals have been published for the classification of skin lymphomas,[4] and important new insights have been gained regarding angiocentric lymphomas. [5] [6] A classification of NHL for clinical use is provided in Table 70-1 . It is derived from the REAL classification but attempts to arrange the disorders

TABLE 70-1 -- Clinical Classification of Lymphoid Malignancies

Indolent B-cell lymphomas
Chronic lymphocytic leukemia
Lymphoplasmacytoid lymphoma/immunocytoma/Waldenström macroglobulinemia
Hairy cell leukemia
Marginal zone B-cell lymphoma
Nodal: Monocytoid B-cell lymphoma
Extranodal: MALT lymphoma
Spleen/peripheral blood: Splenic lymphoma with villous lymphocytes or splenic marginal zone lymphoma
Follicle center lymphoma, grade I and II
Mantle cell lymphoma of mantle zone type
Primary cutaneous follicle center lymphoma
Aggressive B-cell lymphomas (intermediate risk)
Prolymphocytic leukemia
Mantle cell lymphoma (diffuse, nodular, and blastic variants)
Follicle center lymphoma grade III
Diffuse large B-cell lymphoma

Primary mediastinal large B-cell lymphoma
Primary cutaneous large cell lymphoma
Lymphomatoid granulomatosis
Immunoproliferative small intestinal disease
Plasmacytoma/plasma cell leukemia
Very aggressive B-cell lymphoma
Precursor B-lymphoblastic lymphoma/leukemia
Burkitt lymphoma/B-cell ALL
Plasma cell leukemia
Indolent T-cell and NK cell lymphomas
T-cell large granular lymphocyte leukemia
Chronic NK cell lymphocytosis
Mycosis fungoides/Sézary syndrome
Smoldering and chronic adult T-cell leukemia/lymphoma, HTLV-1 related
Aggressive T-cell and NK cell lymphomas (intermediate risk)
T-cell prolymphocytic leukemia
Peripheral T-cell lymphoma, unspecified
Angiocentric sinonasal lymphoma
Intestinal T-cell lymphoma
Anaplastic large cell lymphoma (T- and null cell type)
Hepatosplenic T-cell lymphoma.
CD56+ T-cell large granular lymphocyte leukemia
Very aggressive T-cell and NK cell lymphomas
Adult T-cell lymphoma/leukemia (HTLV-1 related)
Precursor T-lymphoblastic lymphoma/leukemia
Aggressive NK cell lymphoma
CD3+, CD33, DR+, promyelocyte-like NK cell leukemia
ALL-like NK cell leukemia
Undifferentiated myeloid/NK cell leukemia
Note: Disorders that are not discussed in this chapter are in *italic*.
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according to their expected clinical behavior. More recently, essentially the same group of pathologists who devised the REAL classification has proposed several modifications in a new scheme that is known as the World Health Organization (WHO) classification.

In many cases, especially for extranodal lymphomas, patient management is influenced not only by stage and histology, but also by the site of disease. The second part of this chapter provides an overview of the differential diagnosis and management of specific types of extranodal lymphoma.

EPIDEMIOLOGY AND ETIOLOGY

Approximately 50,000 cases of NHL are diagnosed yearly in the United States, representing 4% of all cancers, and NHL causes 4% of all cancer deaths annually. The incidence of NHL has been increasing steadily over the past decades, in part in relation to the AIDS epidemic but also for other unknown reasons. [7] [8] In the United States, NHL is more common in men than in women and among whites than in other racial groups, for reasons that are unknown but may in part be genetic. The racial differences are more pronounced for some subsets of the disease, such as follicular lymphoma, which constitutes a large percentage of cases in Western countries but is relatively rare in developing countries. Genetic features may also be important in other lymphoid disorders, such as CLL, which is virtually absent in Asian populations, and multiple myeloma, which is increased in African Americans. On the other hand, some entities that are particularly common in specific countries appear to be related to viral infections such as Epstein-Barr virus (EBV; e.g., NK-cell lymphomas in South America and Asia, or Burkitt lymphoma in Africa), HTLV-1 (e.g., adult T-cell leukemia/lymphoma in some Caribbean countries and Asia) or hepatitis C (e.g., B-cell lymphoma, especially immunocytoma in northern Italy and Japan).

In most cases NHL is a sporadic disease without specific etiologic factors. Epidemiologic studies have, however, revealed important environmental and dietary risk factors.

The incidence of NHL is higher among agricultural workers than among the general population. [9] [10] A number of studies have implicated the use of pesticides and especially the herbicide 2,4-D in the increased risk for NHL. [9] The use of pesticides has increased steadily in the United States over the past 40 years, with extensive exposure through use in homes, gardens, and lawns, as well as indirectly through food and water. [11] It is conceivable that the increasing incidence of NHL may be related to such increased exposure to pesticides. In addition to exposure to pesticides, nitrate contamination of the groundwater is a significant problem in some parts of the country that is related to an increased incidence of NHL and that affects both farmers and the general population. [12]

In addition to farming, employment in other occupations has been linked to an increased risk for NHL. [13] These occupations include professions with exposure to chemicals such as chemists, dry cleaners, printing workers, wood workers, beauticians, and cosmetologists. Exposure to phenoxyacetic acid, chlorophenols, and solvents, particularly benzene, has been associated with an increased

risk. The use of hair dyes, especially permanent hair color products, has been associated with an increased risk of NHL among users of dark colors. [14] [15] [16] [17] In one study, the use of hair dyes was estimated to account for 20% of the cases of NHL. [15] Dietary factors such as an increased intake of proteins or a decreased intake of vitamins and vegetables have sometimes been associated with the development of NHL. The strongest dietary association was described in two European studies that found a correlation between milk intake and an increased incidence of NHL. A high intake of milk (>2 glasses per day) was associated with a twofold greater risk of NHL. [18] [19] In one study from Nebraska this risk was restricted to men. [20]

Blood transfusion has been associated with a 1.5- to 2.5-fold increased risk for NHL. [21] [22] [23] This increased risk may be related to the transmission of infectious agents or to an immunosuppressive effect of transfusion. Exposure to radiation has not generally been thought to be a major risk factor for NHL, [24] but exposure to some chemotherapeutic agents is. [13] Familial cases of NHL have been observed and are sometimes associated with inherited immunodeficiency. [13] The risk for indolent lymphoma is 3.3 times increased in persons with a family history of leukemia or lymphoma. [25]

Immunosuppression is associated with an increased incidence of NHL. This is best exemplified by the increased incidence of NHL in AIDS (see Chap. 75). Other situations of relative immune deficiency such as rheumatoid arthritis, Sjögren syndrome, and organ transplantation are associated with an increased risk of NHL. In many cases the increased risk is related to reactivation of EBV.

Some infectious agents are associated with a great increase in risk for NHL. Some agents are associated with various types of NHL, while others are associated with one specific subtype. The most widely implicated organism in lymphomagenesis is EBV, which is associated with many different types of NHL in immunocompetent as well as immunocompromised subjects, and with Hodgkin disease. Infection with HTLV-1 can cause a specific form of lymphoma/leukemia; HTLV-1 may also be implicated in other forms of NHL. *Helicobacter pylori* is probably the etiologic agent leading to gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and infection with *Borrelia burgdorfferi*, the etiologic agent of Lyme disease, is associated with some forms of skin lymphoma. Recently, infection with hepatitis C virus (HCV) has been associated with an increased risk for B-cell NHL. These and other infectious associations with and causes of NHL are discussed later in the chapter.

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CLINICAL PRESENTATION

Many subtypes of NHL are recognized (Table 70-1), often associated with specific clinical features. In general, the signs and symptoms of NHL are dependent on the rate of tumor cell proliferation and on the initial localization. For clinical purposes, therefore, the division into indolent, aggressive, and very aggressive lymphoma continues to apply.[2]

Patients with nodal indolent lymphoma often present with widespread central and peripheral adenopathy, including involvement of unusual sites such as epitrochlear nodes. Some subtypes, the MALT lymphomas, have typically extranodal localizations. A third group of indolent lymphomas is characterized by peripheral blood involvement and splenomegaly. Patients with indolent lymphoma may have few or no symptoms and may seek medical attention because they have noticed a lump or because of mild discomfort. In cases of indolent nodal lymphoma, a prolonged history of waxing and waning adenopathy can often be elicited. A staging workup will frequently reveal bone marrow involvement and sometimes involvement of the peripheral blood. Constitutional symptoms (night sweats, fever, or >10% weight loss), elevations in lactate dehydrogenase (LDH) levels, or anemia are distinctly uncommon in indolent NHL. When these symptoms occur, the possibility of disease transformation should be entertained.

Extranodal indolent lymphomas of the MALT type are commonly located in the stomach, parotid gland, thyroid, or lung. In contrast to nodal disease, they often represent localized (i.e., stage I-II) disease. Lymphoproliferative disorders with a leukemic picture often have limited lymph node involvement but pronounced splenomegaly. If symptoms occur they are due to splenomegaly (e.g., abdominal discomfort) or to hypersplenism. Sometimes there is neutropenia or thrombocytopenia without splenomegaly. Such patients usually come to medical attention because of infection related to neutropenia, or because abnormal cells are detected on routine examination of the peripheral blood smear.

In contrast to indolent lymphomas, aggressive lymphomas are commonly symptomatic at diagnosis and affected patients have a relatively short disease history. They commonly present with pain, obstructive symptoms (swelling of legs or the superior vena cava syndrome) or constitutional symptoms. Patients with T-cell lymphoma more commonly present with extranodal involvement and severe constitutional symptoms. Sometimes T-cell NHL is associated with hemophagocytosis. Peripheral adenopathy can occur but is not usually widespread, and involvement of epitrochlear or occipital nodes is uncommon. Many patients do not have peripheral adenopathy, but radiologic examinations may reveal extensive retroperitoneal adenopathy or sometimes anterior mediastinal adenopathy. Disease localization is frequently suggestive of specific histologies, although the diagnosis ultimately depends on biopsy. Lymphoma of the nasopharyngeal area with destruction of midline facial structures (so-called lethal midline granuloma) is often due to an EBV-associated T-cell or NK cell lymphoma. Pulmonary or skin involvement may be due to lymphomatoid granulomatosis. Small intestinal involvement in a patient with a history of gluten enteropathy is almost certainly due to T-cell lymphoma. The laboratory and radiologic workup of patients with aggressive NHL can reveal various stages of disease. More extensive disease is associated with a worse prognosis.

Mantle cell lymphoma is somewhat different from other aggressive lymphomas in that it is often widespread at the time of diagnosis and fairly reminiscent of follicle center lymphomas at presentation. In contrast to what is seen in follicle center lymphomas, involvement of the intestine (lymphomatous polyposis) is common.

The very aggressive (high-grade) lymphomas are typically disorders of young adults and children, although older patients are occasionally seen. Burkitt lymphoma is one of the most rapidly proliferating malignancies known. Patients typically present with rapidly enlarging abdominal masses. In African Burkitt lymphoma, jaw masses are common. Symptoms such as pain, fever, or sweats are common. In American Burkitt lymphoma abdominal masses are common and such a mass can often be palpated in the abdomen. Bone marrow and peripheral blood involvement is extremely common, and examination of the bone marrow and blood may permit the diagnosis to be made. Serum LDH levels can be extremely elevated. Levels 510 times normal are not unusual. These extreme levels are rarely seen in other lymphomas. Uric acid may be elevated at diagnosis, and renal function may be impaired owing to ongoing spontaneous tumor lysis. CNS involvement at diagnosis is not uncommon but is not per se an adverse prognostic feature.

Lymphoblastic lymphoma of T-cell origin also occurs in young adults who present with rapidly enlarging mediastinal masses. As in Burkitt disease, the diagnosis can often be made by examination of the bone marrow and blood, thus allowing expedited treatment.

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STAGING AND RESTAGING OF NHL

History and Physical Examination

The staging of NHL begins with a thorough history in which such symptoms as fever, weight loss, night sweats, and painful areas are reviewed. Any remote history of adenopathy should also be recorded, because indolent lymphomas frequently are associated with a several-year history of waxing and waning nodes. The physical examination should emphasize the cervical, supraclavicular, axillary, inguinal, and epitrochlear nodes, as well as the abdominal cavity, liver, and spleen. The epitrochlear nodes are occasionally involved by indolent NHLs, whereas such involvement is uncommon in aggressive disease. The size of these nodes must be measured and recorded to aid in post-therapy follow-up. Other areas of emphasis in the history and physical examination depend on the particular presentation of the disease.

Biopsy and Invasive Procedures

Adequate tissue sampling is required for the correct diagnosis of NHL and should include enough material for immunophenotyping, cytogenetic studies, and molecular studies. Immunophenotyping is essential for correct assignment of diagnosis according to the REAL classification. In some cases, however, such as T-cell-rich B-cell lymphoma or lymphomatoid granulomatosis, an excess of reactive T cells may obscure a minority of malignant B cells. In other situations an inflammatory process

may be confused with NHL. Therefore, in difficult cases the diagnosis may depend on demonstration of clonality in the B or T cells. This may require studies of immunoglobulin gene or T-cell receptor gene rearrangement. In addition, in a number of diseases the demonstration of oncogene rearrangement such as that of *bcl-2* (follicular lymphoma) or of *bcl-1* (mantle cell lymphoma) helps in establishing a diagnosis. Cytogenetic analysis of the lymph nodes or bone marrow can also be helpful. Some NHLs are associated with typical cytogenetic abnormalities (e.g., t(8;14) in Burkitt lymphoma, t(14;18) in follicular lymphoma, t(2;5) in anaplastic large cell lymphoma, t(11;14) in mantle cell lymphoma, and trisomy 3 or trisomy 18 in marginal zone lymphoma).

In some cases, especially when patients have abdominal tumors, an incisional or excisional biopsy, which provides the ideal material for diagnostic purposes, may require laparotomy. Core needle biopsy has been used in lieu of a lymph node biopsy in such cases. These biopsies provide sufficient information in certain cases, but adequate assessment of lymph node architecture is not always possible and may occasionally lead to an erroneous diagnosis. [26] [27] [28] Fine needle aspiration (FNA) is also an alternative, but only an expert cytologist with experience in the cytologic diagnosis of NHL should attempt this for the establishment of a primary diagnosis. Total reliance on FNA for the diagnosis of NHL is risky and frequently leads to erroneous diagnosis. Some tumor types, such as Burkitt lymphoma and lymphoblastic lymphomas, with very well defined cytologic, cytogenetic, molecular, and immunophenotypic features lend themselves more to this approach than do others. FNA is more commonly used for the diagnosis of recurrent or persistent disease after therapy and to rule out transformation of indolent lymphoma.

In general, the analysis of peripheral blood or bone marrow that is involved does not allow complete assessment of lymphoma, and biopsy of lymph nodes or tumor masses, if present, should be pursued as well. There are, however, some exceptions: (1) In CLL and in other lymphomas characterized by a leukemic picture, the diagnosis can be made by immunophenotyping of cells in the peripheral blood. Lymph node biopsy is not warranted unless mantle cell lymphoma or disease transformation is suspected. (2) Burkitt disease and lymphoblastic lymphoma often manifest as abdominal or mediastinal masses, respectively. These diseases require immediate treatment, and an accurate diagnosis can often be established from examining the bone marrow or peripheral blood, or both. No further staging beyond a chest radiograph, computed tomography (CT) of abdomen and pelvis, bone marrow aspirate, lumbar puncture, and routine blood tests is required in these disorders. In fact, unnecessary attempts at invasive diagnostic procedures may lead to significant delays and compromise the patients ability to tolerate chemotherapy. In the presence of a rapidly growing tumor mass in the characteristic clinical context of Burkitt or lymphoblastic lymphoma, whenever possible minimally invasive procedures, including FNA with morphologic, flow cytometric, and cytogenetic analyses of tissue, should be used to allow prompt initiation of therapy.

Bone marrow biopsy provides valuable staging information and should be performed routinely in NHL. The yield of bilateral bone marrow biopsies is 15% higher than that of a unilateral biopsy in indolent NHL, and such biopsies are routine practice in several institutions. In many cases of large cell lymphoma, an indolent histology is encountered in the bone marrow, leading to the diagnosis of discordant (divergent) lymphoma, which has important therapeutic implications. [29] [30] The distribution of marrow involvement can be very irregular or patchy. In some cases, magnetic resonance imaging (MRI) can be useful in detecting occult marrow involvement.

Diagnostic lumbar puncture is part of the diagnostic workup of patients with highly aggressive lymphomas or blastic variants of mantle cell lymphoma. It should also be considered for patients with aggressive histologies and high-risk factors for CNS relapse (such as high LDH levels or involvement of multiple extranodal sites) or specific localizations, such as testicular lymphoma, and for those with large cell lymphoma involving the bone marrow.

Gastrointestinal (GI) workup and evaluation of Waldeyer ring may be useful in patients with lymphoma that has a high propensity to disseminate to these areas, specifically MALT and mantle cell lymphomas. Liver biopsy is needed only when liver involvement is suspected on the basis of laboratory or radiologic test results.

Imaging Studies

Chest radiography and CT of the abdomen, and pelvis are a routine part of the workup of a patient with NHL. Chest CT should be performed in cases in which the chest x-ray findings are questionable or whenever a mediastinal mass is detected. In the latter instance, occult pulmonary or pleural disease is common and can be detected with CT. Ultrasound (US) of the liver should be considered for patients with abnormal results on liver function tests and a normal CT scan, because US occasionally has greater sensitivity than CT. Lymphangiography is more sensitive in demonstrating lower abdominal and pelvic adenopathy than CT and should be considered in the treatment planning for patients with indolent NHL if total lymphoid irradiation is considered for the treatment.

The gallium scan is increasingly used in the staging of patients with aggressive histologies, who will commonly have residual changes on CT after completion of treatment. It is abnormal at the time of diagnosis in the large majority of patients. Persistent gallium positivity in a residual mass after completion of treatment indicates a high risk of persistent disease. [31] [32] [33] False positive results occur commonly in the pulmonary hilar areas after therapy and in areas where previous involvement of bone existed. The latter are due to inflammatory changes. Hence, interpretation of gallium scans in bone is not reliable. The pulmonary hilar changes must be interpreted in conjunction with the chest radiograph, which will not show hilar enlargement in cases of false positivity on gallium studies. On the other hand, the absence of gallium uptake in residual masses is not a sensitive predictor of prolonged remission. Gallium scanning has also been used in indolent lymphoma and

appears to be useful in indicating persistent disease, although its role in these histologic types is less established. [34]

Because of its ability to discriminate to some extent between benign and malignant tissue, MRI has been used in a fashion similar to gallium scanning to detect residual lymphoma in persistent masses after completion of chemotherapy.[35] It appears to have a sensitivity similar to that of gallium scanning. MRI is also used to detect marrow involvement that cannot be demonstrated by biopsy because of inhomogeneous distribution of the marrow involvement. [36]

Blood Tests

In addition to routine blood tests, it is essential to measure serum LDH and $_2$ -microglobulin levels. These values are indirect indicators of tumor burden and proliferative activity and important indicators of disease prognosis. HIV or HTLV-1 serology, hepatitis B and C, and EBV serology should be considered in the workup for many patients because of their association with NHL and because of implications for treatment. Serum levels of -fetoprotein or -human chorionic gonadotropin are necessary in the evaluation of young males with an isolated mediastinal mass. Markedly elevated levels of either of these markers indicate the presence of a mediastinal germ cell tumor and may obviate thoracotomy.

Evaluation of Response to Therapy

In aggressive lymphomas the rate of response to treatment is an important prognostic feature.[33] [37] Disease progression or lack of response are extremely poor prognostic features, as is the commonly observed kinetic failure, in which progression occurs after an initial response but before administration of the next cycle of chemotherapy. It is therefore customary to repeat abnormal staging studies after the first two cycles of chemotherapy. Restaging involves the repetition of any tests that yielded abnormal results at baseline, including physical examination, radiologic examination, bone marrow biopsies, and blood tests. If there is a change in clinical status, earlier restaging may be indicated. After treatment is completed, complete restaging is usually repeated. If the gallium scan became normal earlier during treatment, there is no need to repeat it. MRI could constitute an alternative to gallium scanning, especially if the initial gallium scan was normal. FNA can also be used to evaluate residual masses after therapy. It is customary to repeat staging procedures at regular intervals after treatment is completed. Although restaging after treatment is useful for reassurance of the patient and for evaluation of clinical studies, it has not been shown to be helpful in detecting most instances of recurrence of large cell lymphoma before the development of other signs such as an elevated LDH level or symptoms. [38] The gallium scan may be a useful adjunct in routine follow-up to detect recurrence of large cell lymphoma.

In indolent lymphomas, the rate or rapidity of the response to chemotherapy is usually slower, and therefore restaging is needed at less frequent intervals. We usually perform restaging after three to four cycles of treatment in patients with indolent follicle center lymphomas.

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PROGNOSTIC FACTORS

The histologic type is a major determinant of prognosis in patients with NHL. The implications of histology for the treatment and prognosis of NHL are discussed in conjunction with each of the different subtypes. But factors other than disease histology and treatment play a major role in determining the prognosis for an individual patient. It is extremely important to recognize these prognostic factors in order to determine the optimal management, predict outcome for an individual patient, and interpret and design clinical studies. Diffuse large B-cell lymphoma is the subtype of lymphoma in which the impact of prognostic factors has been studied most thoroughly, and to a certain degree it has served as a paradigm for the study of prognostic factors in other types of NHL.

Prognostic Factors in Diffuse Large Cell Lymphoma

Prognostic factors in diffuse large cell lymphoma can be divided into those that are related to the disease and those that are related to the patient or the patients interaction with the disease.[39] [40] Factors related to the patient include advanced age and poor performance status, which are adverse prognostic factors. The poor prognosis related to age may be due to a lesser tolerance for chemotherapy or perhaps to increasing immunocompromise with advancing age. Even when adequate chemotherapy is administered, the complete remission rate and survival rate in older patients are less than in younger cohorts. [41] Performance status is related in part to the patients disease and may be an indicator of the degree of tumor bulk and proliferation. In part it is related to the patients general health and appears to correlate with the patients ability to tolerate treatment.

A number of parameters related to the bulk and proliferative capacity of the lymphoma are also predictive of outcome. They include Ann Arbor stage, size of tumor masses, number of extranodal sites, and LDH and $_2$ -microglobulin levels. Such parameters are only surrogates for important biologic differences between different NHL patients. Recently there has been interest in defining more exactly the biologic determinants of disease that may relate to prognosis. The presence of certain molecular or cytogenetic abnormalities in the genome of the lymphoma cells or the expression of certain adhesion molecules may be related to prognosis. Such abnormalities, while not routinely determined in the assessment of NHL patients, provide insight into the pathogenesis of the disorder and may ultimately provide valuable leads for treatment. Some of the newly defined prognostic parameters are shown in Table 70-2 . The recognition of certain histologic subtypes that are commonly included in studies of aggressive lymphoma may also add further prognostic information. Ki-1-positive anaplastic large cell lymphoma is associated with an improved prognosis in recent studies, while other subtypes of T-cell lymphoma are associated with a worse prognosis. Similarly, immunoblastic lymphoma, although often grouped with diffuse large cell lymphoma, may have a worse prognosis.

In addition to prognostic factors that can be recognized prior to initiation of treatment, some prognostic information becomes available only after treatment has started. The response to chemotherapy is a very important feature. A slow response to chemotherapy predicts a poor outcome. [33] [37] If a patient with an aggressive histologic type fails to achieve a complete remission after two or three cycles of chemotherapy, the chance for long-term disease-free survival is decreased. A source of confusion in evaluating response is the presence of a residual mass after therapy. Therefore it is helpful to evaluate those cases with gallium scan, MRI, or biopsy, either FNA or incisional. [31] [35]

Prognostic Models

Prognostic models are developed with the intent of predicting the prognosis of a patient prior to initiating treatment. A good prognostic model should satisfy a number of criteria. The most important ones are (1) by dividing instances of disease into favorable and unfavorable categories, it should facilitate therapeutic decisions, and (2) it should be simple and reproducible.

The International Prognostic Index (IPI) and the Age-Adjusted International Index result from an international cooperative effort and have become increasingly accepted as the tool to determine prognosis in patients with large cell lymphoma. [42] The International Prognostic Index was devised using a very large database of patients treated with standard doxorubicin-containing regimens. It is based on five parameters: age, performance status, serum LDH, involvement of more than one extranodal site, and stage of disease (the mnemonic is APLES). A point is assigned for each of the following characteristics: (1) age >60, (2) performance score of 2, (3) increased serum LDH level, (4) involvement of more than one extranodal site, and (5) Ann Arbor stage III or stage IV disease. In principle, the International Prognostic Index applies only to patients with aggressive NHL. It has also been applied to patients with other types of NHL and appears to provide information in those subtypes as well. [43] [44] [45] [46] The major limitation of the IPI is that approximately one-half of patients fall into an intermediate-prognosis category. In these patients only very limited prognostic information is gained, and therapeutic decisions are not facilitated (Table 70-3).

Another index, called the tumor score, was developed at M.D. Anderson Cancer Center. [47] It is a simple system based on five reproducible parameters. One point is assigned for each of the following: (1) the presence of constitutional symptoms, (2) stage III or IV disease, (3) any tumor mass >7 cm, (4) LDH >1.10 over the normal level, and (5) $_2$ -microglobulin increased >1.5 above the normal level. This system divides the patients in two prognostic groups. All patients with a tumor score of

TABLE 70-2 -- New Prognostic Parameters in Aggressive NHL

Prognostic Feature	Author, Year	Method	Comments
Abnormalities in chromosomes 7 and 17	Cabanillas et al., 1989[530]	Cytogenetic analysis	Poor prognosis associated with abnormalities of chromosome 7 and chromosome 17. No multivariate analysis
Ki-67	Miller et al., 1994[531]	Monoclonal antibody, detects nuclear antigen associated with cell proliferation	High Ki-67 is associated with 18% 1-year survival. Independent factor in multivariate analysis of small patient sample[a]
bcl-2 protein expression	Hermine et al., 1996; [532] Hill et al., 1996[533]	Immunohistochemical staining for expression of *bcl-2* protein	High expression of *bcl-2* is associated with reduced disease-free survival and overall survival in diffuse large B-cell lymphoma; also associated with an increased risk for relapse. Independent factor in multivariate analysis [a]
Ploidy and proliferative activity	Winter et al., 1996[534]	Flow cytometric analysis	No association between aneuploidy and outcome in aggressive NHL. Increased mitotic index is associated with decreased survival but is not an independent predictor[a]

CD44v	Stauder et al., 1995[535]	Variant isoform of adhesion protein, immunohistochemical analysis	Expression of variant isoform of CD44 is associated with decreased survival in aggressive lymphoma. Independent predictor in multivariate analysis[a]
Plasma level of TNF and TNF receptor p55 and p75	Warzocha et al., 1997[536]	ELISA	High levels of TNF, p55, and p75 are associated with decreased survival and freedom from progression in aggressive lymphomas. Independent predictor in multivariate analysis[a]
Rearrangement of *bcl-6*	Offit et al., 1994[537]	Southern blot	Improvement in survival and disease-free survival in aggressive lymphomas with rearranged *bcl-6*. Independent predictor in multivariate analysis[a]
Mutation of *p53*	Ichikawa et al., 1997[559]	Polymerase chain reaction	Mutations of *p53* are associated with decreased rate of complete responses and decreased overall survival. Independent predictor in multivariate analysis[a]
Serum level of $_2$ -microglobulin	Preti et al., 1993[514]	Radioimmunoassay	Increased level of $_2$ -microglobulin is associated with a worse prognosis. Independent predictor in multivariate analysis[a]
Serum level of interleukin-6	Preti et al., 1997[538]	ELISA	Increased level of interleukin-6 is associated with decreased complete response rate and survival. Independent predictor in multivariate analysis[a]
ICAM-1 expression	Terol et al., 1998[539]	Immunostaining	Increased expression of ICAM-1 is associated with lymphoma dissemination and decreased survival. Independent predictor in multivariate analysis[a]

[a]Multivariate analysis incorporates the parameters evaluated in the International Prognostic Index, in addition to the one studied.

>3 have a very poor prognosis when treated with CHOP-bleomycin and are currently treated on investigational protocols. Those with tumor scores of <3 have a disease-free survival rate of 80% when treated with conventional regimens. Therefore, the prognostic information gained from this model is in general greater than that obtained with the IPI.

The same prognostic parameters that apply in aggressive lymphoma are usually found to be valid in indolent lymphomas, [48] and therefore the IPI has been applied to indolent malignancies. [43] [45] [46] In indolent lymphomas, however, the IPI lacks sufficient sensitivity to predict the majority of failures. When the index is used, only 812% of cases will be assigned to a high-risk subgroup. [43] [45] [46] [48] The vast majority of patients will fall into favorable and intermediate-favorable subgroups. Several groups have proposed other prognostic models. [49] [50] [51] A simple model based on serum LDH and $_2$ -microglobulin levels has been found to be useful. It divides patients into three prognostic groups according to whether serum levels of neither, one, or both factors are elevated. [49] It has excellent discriminatory power and was confirmed in an independent population to be easily applicable and predictive of outcome. [48] As in other types of lymphoma, further research is needed to identify parameters that can accurately predict the prognosis in indolent lymphomas.

TABLE 70-3 -- Prognostic Indices in Large Cell Lymphoma[a]

Risk Category	Score	Patients in Risk Group (%)	Complete Responses (%)	5-Year Disease-Free Survival for Patients with Complete Responses (%)	5-Year Survival (%)
International Prognostic Index[42]					
Low	0 or 1	35	87	70	73
Low-intermediate	2	27	67	50	51
High-intermediate	3	22	55	49	43
High	4 or 5	16	44	40	26
Age-Adjusted International Index[42]					
Low	0	22	92	86	83
Low-intermediate	1	32	78	66	69
High-intermediate	2	32	57	53	46
High	3	14	46	58	32
Tumor score[47]					
Low	02	61	91	92[b]	83[b]
High	3	39	46	46[b]	24[b]

Age-Adjusted International Index: one point each is assigned for performance status <1, elevated lactate dehydrogenase level, stage III or stage IV disease.

Tumor score: one point each is assigned for elevated lactate dehydrogenase level >110%, elevated $_2$ -microglobulin >3, presence of constitutional symptoms, stage III or stage IV disease, each mass >7cm.

[a]International Prognostic Index: one point each is assigned for age >60, performance status <1, elevated lactate dehydrogenase level, involvement of more than one extranodal site, stage III or stage IV disease.
[b]3-year survival.

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PRESENTATION AND MANAGEMENT OF B-CELL LYMPHOMAS

Management and Diagnosis of Burkitt Lymphoma and High-grade B-Cell Burkitt-like Lymphoma

In the Working Formulation three disease entities were included among the high-grade lymphomas: lymphoblastic lymphoma, immunoblastic lymphoma, and diffuse small noncleaved cell lymphoma. Lymphoblastic lymphoma is usually of T-cell type and will be discussed later. The term immunoblastic lymphoma is not used in the REAL classification. Immunoblastic lymphoma of B-cell origin is currently thought to be closely related to diffuse large cell lymphoma and is treated similarly. A recent report from the Kiel group suggests that immunoblastic B-cell lymphomas have a worse prognosis than other diffuse B-cell large cell lymphomas. [52] This may prompt re-evaluation of this issue. A number of subtypes of T-cell immunoblastic lymphoma are currently classified as anaplastic T-cell lymphoma or as angioimmunoblastic T-cell lymphomas.

Three variants of Burkitt disease are recognized. The endemic (African form) is closely associated with EBV infection, in which patients often present with jaw or abdominal masses.[53] [54] The sporadic (American) form is infrequently associated with EBV infection. Patients with sporadic Burkitt disease often present with abdominal disease and bone marrow involvement.[55] A third variant, AIDS-related Burkitt lymphoma, is discussed in Chapter 75 , which deals with AIDS-associated lymphomas.

Diffuse small noncleaved cell lymphoma has been subdivided in the REAL classification into Burkitt lymphoma and high-grade B-cell lymphoma, Burkitt-like. [55] The latter includes some cases that might have been classified as large cell lymphoma in the past. The clinical significance of this distinction is unclear. The morphology of Burkitt lymphoma cells is very typical and is similar to the L3 subtype of ALL. The cells are mature B cells with expression of surface immunoglobulin. The disease is associated with chromosomal translocations involving the c-*myc* oncogene and immunoglobulin genes.[56] Some cases of Burkitt-like NHL lack these rearrangements and may have rearrangements of *bcl-2.*[60]

Burkitt lymphoma is more common among children and young adults. Patients present with rapidly proliferating tumors, and frequently with bone marrow and peripheral blood involvement. CNS involvement at diagnosis is not uncommon. The serum LDH level is often extremely elevated, and hyperuricemia and renal impairment may be found at diagnosis. Staging should be limited to allow expedited treatment. [55] [56] In addition to a diagnostic biopsy with cytogenetic studies and immunophenotyping, staging studies should always include a bone marrow biopsy and lumbar puncture, as these two tests have both prognostic and therapeutic implications. Cytogenetic and molecular analysis as well as immunophenotyping allow one to establish the diagnosis with certainty and to rule out alternative diagnoses such as aggressive NK cell leukemia. If the bone marrow or peripheral blood sample is diagnostic, a biopsy of tumor masses should generally be avoided. It is unnecessary and may lead to considerable delay in initiating treatment. If the bone marrow examination is negative, diagnostic FNA should be considered in cases with a typical presentation. Surgical resection used to be recommended as part of the treatment of patients with Burkitt disease. [57] [58] Currently, given the high efficacy of intensive chemotherapeutic regimens, surgical debulking is in general not indicated. [59]

The curative treatment of endemic Burkitt disease with cyclophosphamide represented one of the first successes of chemotherapy. [61] This single-agent treatment resulted in cure for a minority of patients and is no longer used. For patients without bone marrow or CNS involvement, cure can often be achieved with regimens that include both alkylating agents and antimetabolites and that were designed for large cell lymphoma, [62] [63] [64] but patients with extensive disease usually do not benefit from such approaches. Burkitt disease is very closely related to B-cell ALL. A number of intensive treatment regimens designed for pediatric B-cell ALL have now been used in adult Burkitt lymphoma. Successful regimens include high doses of cyclophosphamide (13 g/m ^{2} per course) or ifosfamide combined

MANAGEMENT OF VERY AGGRESSIVE LYMPHOMA IN THE ADULT

The large majority of very aggressive lymphomas are either T-cell lymphoblastic or B-cell (Burkitt) lymphoma. The differential diagnosis between the two entities is usually obvious from the clinical picture. T-cell lymphoblastic lymphoma presents with a mediastinal mass, whereas Burkitt lymphoma often presents with large abdominal masses or other more peripheral masses; mediastinal masses are rare in Burkitt lymphoma. The staging workup should be expedited. Often examination of the peripheral blood or bone marrow, or both, can provide the diagnosis. Immunophenotyping and molecular and cytogenetic analyses of the bone marrow or peripheral blood, or both, are essential. Burkitt lymphoma is characterized by an L3 morphology, a mature B-cell phenotype (with expression of surface immunoglobulin), and t(8;14), t(2;8), or t(8;22). Lymphoblastic lymphoma has an L1 morphology, a T-cell phenotype, is TdT positive, and usually has diploid cytogenetics. The differential diagnosis includes other forms of very aggressive lymphoma, such as NK cell lymphoma or adult T-cell lymphoma, or different types of ALL. In cases without bone marrow involvement, the diagnosis may require a surgical procedure. In some cases with a suggestive clinical picture, FNA is preferred, as it is less invasive and provides adequate tissue for diagnosis in this disorder. The staging workup is completed with a laboratory workup that includes determinations of serum LDH (often very elevated) and $_2$ -microglobulin levels, liver and kidney tests and coagulation tests. Chest radiography and CT of the abdomen are usually performed. A lumbar puncture is also essential.

Other tests can be considered if clinically indicated, but their performance should not delay the initiation of treatment.

A central line is placed and D5W + 100 mEq sodium acetate at 100150 ml/hour is administered to alkalinize the urine. Allopurinol, 600 mg, is given PO daily for 2 days; thereafter it is continued at 300 mg/day PO until the tumor lysis syndrome has resolved. The chemotherapy regimen used at M.D. Anderson consists of the hyper-CVAD regimen.

The first cycle consists of cyclophosphamide, 300 mg/m^2 given IV over 3 hours every 12 hours for six consecutive doses (total dose 1,800 mg/m^2); Mesna, 600 mg/m^2 given by continuous IV infusion over 24 hours per day for 3 consecutive days (Mesna is started 1 hour before cyclophosphamide and continued for 12 hours after the last dose); adriamycin, 25 mg/m^2 given by continuous IV infusion over 24 hours for 2 days (total dose, 50 mg/m^2); and vincristine, 1 mg given by continuous IV infusion over 24 hours for 2 days (total dose, 2 mg). Vincristine and adriamycin are started after the last dose of cyclophosphamide. Vincristine, 2 mg by IV push, is repeated on day 11. Decadron, 40 mg/day PO, is given on days 14 and 1114. G-CSF, 5 g/kg, is given daily starting 24 hours after the last dose of adriamycin. It is continued until granulocytes are >30,000/l or until day 21 (whichever comes first).

This regimen is very effective in inducing remissions in high-grade NHL. Tumor lysis, especially in patients with Burkitt lymphoma, is very common, and in the first days of treatment the patient should be monitored extremely closely. Electrolyte abnormalities should be corrected, and if renal failure ensues, dialysis should be initiated. In our experience the acute renal failure associated with tumor lysis is usually profound but rapidly and completely reversible. To avoid opportunistic infection, ciprofloxacin, acyclovir, and fluconazole can be given preventatively.

One day after completion of G-CSF therapy, and provided a complete remission is achieved, we proceed with the next cycle of treatment. This consists of methotrexate, 200 mg/m^2 given IV over 2 hours, then 800 mg/m^2 given IV over 22 hours on day 1. Leucovorin, 50 mg IV or PO, is given 12 hours after the completion of methotrexate, followed by 15 mg IV or PO every 6 hours for 8 doses. Ara-C, 3 g/m^2 IV over 2 hours, is given every 12 hours for four doses on days 2 and 3. At the end of the methotrexate infusion, 24 hours and 48 hours after completion, the methotrexate level is checked. If the level is >1 M at 24 hours or >0.1 M at 48 hours, the leucovorin dose is increased to 50 mg IV every 6 hours until the level is <0.1 M. G-CSF, 5 g/kg, is given starting on day 1 until the ANC is >30,000/l or until day 21 (whichever comes first). Then it is held for 1 day and the next cycle is started. Alkalinization with D5W and sodium acetate, steroid eye drops, and prophylactic antibiotics are used in this regimen.

These cycles are alternated every 3 weeks (or faster if possible) for eight cycles. Intrathecal prophylaxis consists of methotrexate, 12 mg on day 2, and Ara-C, 100 mg on day 7. This is continued for four cycles (total of eight injections). Patients with CNS involvement at diagnosis receive intrathecal treatment with alternating methotrexate and Ara-C twice a week until the CSF clears. Then they receive prophylaxis during all cycles of their treatment (total of 16 injections). Cranial radiation therapy is not routinely used in this protocol.

Maintenance treatment is offered to patients with lymphoblastic lymphoma, but it is omitted in Burkitt disease. It consists of 6-mercaptopurine, 50 mg PO t.i.d. one-half hour before meals daily; methotrexate, 20 mg/m^2 IV weekly; vincristine, 2 mg IV monthly; and prednisone, 100 mg daily for 5 days every month. This treatment is continued for 24 months. Doses are adjusted for myelosuppression, mucositis, liver dysfunction, neuropathy, or steroid side effects.

with vinca alkaloids, steroids, and anthracyclines. They also include drugs with good CNS penetration such as Ara-C and high-dose methotrexate, as well as intrathecal chemotherapeutic prophylaxis. Radiation treatment to the brain is not used in most protocols, except on occasion in patients with CNS involvement at diagnosis. Treatment is extremely intensive, with a rapid succession of myelosuppressive regimens. Maintenance treatment is considered unnecessary, and treatment is usually completed within 3 months. The prognosis has steadily improved even for patients who present with CNS involvement. [65] [66] [67] [68] [69] [70] [71] [72] [549] A number of recently reported trials in adults and treatment outcomes are summarized in Table 70-4 . The trials that include higher doses of alkylating agents appear to be associated with a somewhat superior outcome. The outcomes are also better in those trials that include a large proportion of patients without bone marrow involvement (i.e., the outcomes for patients with mature B-cell leukemia are inferior to the outcomes for patients with classic Burkitt lymphoma) and for younger patients.

High-dose chemotherapy followed by autologous transplantation has been successful in children and young adults with persistent or recurrent Burkitt lymphoma. [73] [74] [75] This has prompted the use of autologous bone marrow transplantation during the first remission. [75] Given the excellent results of modern

TABLE 70-4 -- Recent Treatment Results in Adults with Diffuse Small Noncleaved Cell Lymphomas

Author, Year	Regimen	N	Median Age, Year (range)	Bone Marrow Involvement	CNS Involvement	Complete Responses	Treatment-Related Deaths	Relapse from Complete Response	Percentage Disease-Free Survival
				No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	
Soussain et al., 1995[69]	LMB84-86-89	65	30 (1765)	25 (38)	12 (18)[b]	58 (89)	9 (14)[c]	8 (12)	74[a]
Todeschini et al., 1997[67]	Hyper-CVAD	8	35	7 (87)	1 (15)	8 (100)	1 (15)	1 (15)	75
Hoelzer et al., 1996[72]	BNHL83-86[d]	59	34 (1565)	59 (100)	8 (14)	41 (69)	7 (12)	13 (22)	50[e] [f]
Magrath et al., 1996[66]	89-C41	20	25 (1859)	3 (15)	?	20 (100)	0	0	100
Kantarjian et al., 1995[540] [549]	Hyper-CVAD	26	58 (1779)	26 (100)	11 (42)	18 (70)	5 (19)	6 (23)	12 (58)

[b] Seven of 12 are disease free.
[c] Four treatment-related deaths were related to autologous bone marrow transplantation.
[a] Thirteen patients received autologous bone marrow transplants during the first complete response.
[d] Treatment regimen included ifosfamide and prophylactic cranial irradiation.
[e] Six patients received bone marrow transplants during the first complete response, with two treatment-related deaths.
[f] CNS involvement is not an adverse prognostic factor.

chemotherapy in this disorder, consolidation with high-dose chemotherapy may be unnecessary in the large majority of patients during first complete remission and is generally not recommended any longer except as salvage treatment.

The induction treatment of Burkitt lymphoma is frequently complicated by massive tumor lysis syndrome and requires special precautions. Patients should receive allopurinol and should undergo hydration and alkalinization. Electrolyte abnormalities such as hyperkalemia, hyperphosphatemia, and hypocalcemia should be monitored and aggressively corrected. Some groups prefer to give an initial low-dose cytoreductive treatment, to prevent tumor lysis. [69] [72] This is followed 1 week later by more intensive chemotherapy.

Presentation and Management of Aggressive B-Cell Lymphomas

The most common variant of aggressive NHL is diffuse large B-cell lymphoma. It accounts for 40% of all B-cell lymphomas. [76] Diffuse large cell lymphoma and a small subset of follicle center lymphoma, the so-called follicle center grade III histology, are aggressive disorders and will be discussed together.

Diffuse Large B-Cell Lymphoma and Follicle Center Lymphoma Grade III

These entities roughly correspond to the intermediate grade histologies in the Working Formulation and also to some cases of immunoblastic lymphoma. They have a fairly similar natural history, have often been grouped together for the purpose of prospective studies, and are usually treated in a fairly uniform fashion. Within the category of diffuse large cell lymphoma, several disease entities have been recognized with particular clinical, histologic, immunophenotypic, or cytogenetic characteristics. Some histologic variants such as T-cell-rich B-cell lymphoma have an outcome similar to that of diffuse large cell lymphoma and are similarly treated.[77] [78] Another entity, primary mediastinal B-cell large cell lymphoma, has a somewhat different natural history and will be briefly discussed. It is likely that further progress will lead to the recognition of other subtypes with clinical relevance.

Clinical Presentation and Natural History

The median age of patients presenting with aggressive B-cell lymphoma is 60 years, but it is considerably lower for some subgroups, such as those with primary mediastinal B-cell lymphoma. Large cell NHL can arise de novo or as a transformation of (sometimes undiagnosed) low-grade lymphoma. Nearly half of patients present with localized nodal disease (stage I or II disease) and half with widespread disease. Many patients are symptomatic at the time of diagnosis, and with increasing tumor bulk constitutional symptoms develop. B-cell large cell lymphoma is usually a nodal disease, but it can also occur in extranodal organs such as the skin, stomach, or CNS. Such extranodal presentations may have a somewhat different biology and require specific consideration for their treatment. They are discussed in another section. The lymphadenopathy in cases with nodal disease is often localized to the retroperitoneum or mediastinum, rendering a diagnostic biopsy difficult. Untreated, the disease has a poor prognosis and leads to death within 1224 months. The diagnosis of this disorder depends on an adequate biopsy. Treatment planning requires rigorous staging and assessment of prognostic factors. With appropriate treatment, 40% of patients can be cured. [79] The mainstay of treatment is combination chemotherapy, but radiation therapy has a role in selected patients. The treatment of elderly patients, of patients with recurrent disease, and of those with adverse prognostic features presents specific problems and is discussed separately.

Chemotherapy in Diffuse Large B-cell Lymphoma

The alkylating agents, corticosteroids, and vincristine were the first effective agents for the treatment of aggressive NHL. [80] The CHOP regimen, in which doxorubicin is added to the combination, was then shown to result in improved survival.[81] [82] The 1970s and early 1980s saw the testing of numerous third-generation regimens built on the CHOP backbone but with the addition of other drugs. A number of these regimens are shown in Table 70-5 . Encouraging results were reported in pilot studies that included predominantly young patients. Unfortunately, four large randomized multicenter studies failed to show in a more representative population a significant advantage for any of these regimens (Table 70-6). [83] [84] [85] [86] Similarly, increments in the dose of doxorubicin, [87] or the infusional administration of etoposide and doxorubicin (the EPOCH regimen), could not be shown to result in improved disease-free or overall survival. [88] On the other hand, two recent reports indicated a statistically significant benefit for the second-generation regimens. [89] [90] In one study, MACOP-B was compared with CHOP and no difference was detected at the time of initial publication. [86] A later analysis of the same patient population did detect a benefit in both survival and disease-free survival for patients receiving MACOP-B.[89] A second study with a median follow-up of 10 years indicated benefit of a six-drug regimen over a four-drug regimen (Table 70-6).[90] Although the latter two studies indicate that there may be some benefit to second-generation regimens, any such benefit is likely to be influenced by the selection of patients participating in the study and is unlikely to be very large.

MANAGEMENT OF AGGRESSIVE NHL IN THE ADULT

The modern management of aggressive NHL begins with an accurate diagnosis, which should include a review by an expert hematopathologist as well as immunophenotyping and, if possible, cytogenetic assay. Aggressive NHL includes the cell types indicated in Table 70-1 . The treatment strategy for these disorders is similar, with the exception of mantle cell lymphomas and immunoproliferative small intestinal disease, which are unique entities and require different management.

Once a diagnosis is firmly established and other staging studies have been completed, it should be determined whether there is a reasonable chance for cure with the CHOP regimen and whether the patients cardiac function is adequate for the administration of anthracyclines. Determination of the chance for cure with CHOP can be made using the prognostic models already discussed. Most clinicians use the IPI to determine this, but the inclusion of $_s$ -microglobulin as used in the tumor score model can add discrimination to this model.[514] For these purposes we use the M.D. Anderson tumor score system as previously described.

If the chance for cure with CHOP is >75%, this regimen should be used if at all possible because it is well tolerated and simple to administer. For patients whose cardiac function is compromised, the decision is more difficult. Possible alternatives in these cases include the use of non-anthracycline-containing regimens such as ESHAP or IMVP-16, or both, given in alternating fashion.

MANAGEMENT OF PATIENTS WITH A FAVORABLE RISK PROFILE

Within this category are included only those cases with favorable prognostic features such as a tumor score of 02 (or IPI of 01). In general, these patients are treated with the CHOP regimen, but they can be divided into two subsets: those who had localized disease on presentation (i.e., Ann Arbor stages III) and those with more advances stages (IIIIV). The length of treatment may vary accordingly. Localized disease can be considered an ultrafavorable presentation, with a cure rate of 85%. Treatment may be very brief and consist of only three courses of CHOP followed by involved-field irradiation. [91] [93] [550] Patients with advanced Ann Arbor stage disease (the second subset) can be treated with six courses of CHOP without radiation therapy. Their prognosis is also excellent with this approach.

MANAGEMENT OF PATIENTS WITH AN UNFAVORABLE RISK PROFILE

These patients include anyone with a tumor score >2 (or anyone with an IPI score >3). The outcome for patients with disease with adverse prognostic features when managed with the classic CHOP regimen is poor, and their cure rate is <30%. [42] [47] There is no standard regimen that has been tested in a randomized setting and shown to be more effective than CHOP for these patients. We have used the alternating triple-therapy or ATT regimen, consisting of alternating courses of ASHAP, M-BACOS, and MINE for a total of nine cycles. Patients with bulky disease received consolidative radiation to areas of bulky involvement. This regimen, when compared with the historical experience with CHOP, showed a statistically significant benefit for patients <61 years old. [515] Although this study was not randomized, we used prognostic factor analysis to compare the two protocols, thus adjusting for potential bias in patient selection. Others have used intensive chemotherapy and autologous stem cell transplantation for patients with unfavorable risk factors. Currently we are comparing, in a randomized study, the ATT regimen with an intensive chemotherapy regimen followed by autologous stem cell transplantation.

Those patients with an adverse prognostic profile (i.e., tumor score >2) and >60 years old represent the most challenging group. When these patients were treated with ATT they did not show any benefit as compared with CHOP. Their cure rate as well as their 3-year survival rate has been only 28%. [513] [515] Recently the substitution of idarubicin for doxorubicin in a randomized study using the ATT regimen has been associated with a 50% 3-year survival in older patients with a tumor score >2, compared with 35% for doxorubicin.[115] This is currently our standard treatment in older patients with poor prognostic features.

All randomized studies antedated the routine use of prognostic models, and therefore their conclusions may not apply to the current treatment of patients. It is now well established that CHOP is highly effective in subsets of patients with so-called good prognostic features, which includes those aged <60 years with an Age-Adjusted International Prognostic Index of 0 or 1. For such patients the expected complete response rate with a conventional anthracycline-containing regimen is at least 80%, and two-thirds of the remissions are durable.[42] Their expected survival at 5 years is 69%. An alternative treatment strategy for patients with good-prognosis stage I or II disease consists of the administration of three to four cycles of CHOP or other anthracycline-based chemotherapy, followed by radiation treatment of the involved field. This strategy, when tested in a randomized study, has yielded superior long-term disease control with minimal toxicity. [91] [92] [93] [550] It is unlikely that second- or third-generation regimens will further improve the prognosis in those groups, and CHOP chemotherapy can continue to be used. By contrast, among patients with several adverse prognostic features, neither CHOP nor any of its derivatives is likely to result in high durable remission rates. Whenever possible, such patients should be enrolled in investigational protocols.

Treatment of Aggressive, Poor-Prognosis NHL with High-Dose Chemotherapy

CHOP chemotherapy or any of its derivatives is unlikely to be beneficial in patients with an IPI of >2. One of the most interesting avenues of research, at least for younger patients with poor-prognosis NHL, is that of high-dose chemotherapy and autologous bone marrow

TABLE 70-5 -- Commonly Used Anthracycline-Containing Chemotherapy Regimens for Aggressive NHL[a]

Name	Frequency of Administration	No. of Cycles	Cyclophosphamide	Adriamycin	Vincristine[b]	Prednisone	Methotrexate[c]	Bleomycin	Etoposide	Cytarabine	Procarbazine
CHOP[82]	21	68	750 mg/m^2	50 mg/m^2	1.4 mg/m^2	100 mg for 5 days					
CAP-BOP[555]	21	68	650 mg/m^2	50 mg/m^2	1.4 mg/m^2 on day 15	100 mg for 7 days (days 1521)		10 U/m^2 on day 15			100 mg/m^2 for 7 days
m-BACOD[556]	21	68	600 mg/m^2	45 mg/m^2	1 mg/m^2 on day 8	Decadron, 6 mg/m^2 for 5 days	200 mg/m^2 on days 8 and 15	4 mg/m^2			
MACOP-B[557] [d]	14	6	350 mg/m^2	50 mg/m^2	1.4 mg/m^2	75 mg for 14 days	400 mg/m^2 on day 8 (every other cycle)	10 U/m^2 on day 8 (every other cycle alternates with methotrexate)			
ProMACE CytaBOM[541]	28	68	650 mg/m^2	25 mg/m^2	1.4 mg/m^2 on day 8	60 mg/m^2 for 14 days	120 mg/m^2 on day 8	5 U/m^2 on day 8	120 mg/m^2	300 mg/m^2 on day 8	
LNH-84 induction[100] [e]	14	4	1,200 mg/m^2	75 mg/m^2		60 mg/m^2 for 5 days		5 U/m^2 on days 1 and 5			
EPOCH[135] [f]	21	68	750 mg/m^2 on day 6	10 mg/m^2 on days 15	0.4 mg/m^2 on days 14	60 mg/m^2 on days 14			50 mg/m^2 on days 14		

Hyper-CVAD, first cycle[540] [549]	21	6	300 mg/m^2 q 12 hr for 6 doses with mesna	25 mg/m^2 IV on days 4 and 5	0.1 mg on day 4, 1 mg on day 5, and 2 mg on day 11	Decadron, 40 mg on days 14, 1114						

Note: This table does not provide all details regarding supportive care for the regimens described. See specific references.

[a] All drugs are administered on day 1, unless otherwise specified.
[b] The dose of vincristine in most regimens is limited to 2 mg total dose.
[c] Regimens containing methotrexate commonly include leucovorin rescue.
[d] MACOP-B requires antifungal and *Pneumocystis carinii* prophylaxis.
[e] Consolidation LNH-84: methotrexate, 3 g/m^2 during weeks 1 and 3; ifosfamide, 1,500 mg/m^2, and etoposide, 300 mg/m^2, during weeks 5 and 7; asparaginase, 5,000 IU/m^2 during weeks 9 and 10; and cytarabine, 100 mg/m^2 for 4 days during weeks 11 and 13.
[f] EPOCH is given as infusional therapy over 96 hours.

TABLE 70-6 -- Randomized Studies of Combination Chemotherapy Regimens in Patients with Newly Diagnosed Aggressive Lymphomas

Study, Year[a]	Inclusion Criteria[b]	Median Follow-up	Chemotherapy[c]	N	Median Age, Year (range)	Complete Response (%)(*P*)	Disease-Free Survival (%) (*P*)	Survival (%) (*P*)
Longo et al., 1991 [541]	FLCL, DMCL, DLCL, IBL, DSNCC (non-Burkitt)	5 yr	ProMACE-MOPP	99	46 (1583)	74	54	53
			ProMACE-CytaBOM	94	47 (1880)	86 (0.048)	69 (0.082)	69 (0.046)
Fisher et al., 1993[83]	FLCL, DSCCL, DMCL, DLCL, IBL, DSNCC; stage II bulky disease, stage III, stage IV; no prior LGL	35 mo	CHOP	225	56 (1579)	44	41	54 (0.90)
			m-BACOD	223	57 (1881)	48 (NS)	46	52
			ProMACE-CytaBOM	233	54 (1781)	56 (NS)	46 (0.35)	50
			MACOP-B	218	57 (1979)	51 (NS)	41	50
Gordon et al., 1992[84]	DMCL, DLCL, IBL; stage III, stage IV	4 yr	CHOP	174	±60	51		48
			m-BACOD	151	±60	56 (NS)		49 (NS)
Sertoli et al., 1994[85]	Age 1565 yr; DMCL, DLCL, IBL; stage II bulky disease, stage III, stage IV	41 mo	ProMACE-MOPP	114	50 (1668)	49.1	36.4	45.2
			MACOP-B	107	47 (1767)	52.3 (NS)	36.1 (.5)	52.3
Cooper et al., 1994[86] [b]	FLCL, DSCCL, DMCL, DLCL, IBL; stage I bulky disease, stage II, stage III, stage IV	3.3 yr	CHOP	111	53 (1672)	59	32	51
			MACOP-B	125	54 (2272)	51 (0.3)	44 (0.47)	56 (0.69)
Wolf et al., 1997[89] [d]	FLCL, DSCCL, DMCL, DLCL, IBL; stage I bulky disease, stage II, stage III, stage IV	6.5 yr	CHOP	111	53 (1672)	51 (0.3)	**30**	**41**
			MACOP-B	125	54 (2272)	59	**42 (0.045)**	**54 (0.035)**
Meerwaldt et al., 1977[90]	DSCCL, DMCL, DLCL, IBL; stage III, stage IV	10 yr	CHVmP	140	55 (2072)	49	**23**	**22**
			CHVmP + VCR + Bleo		57 (1672)	72	**34 (0.024)**	**34 (0.024)**
Meyer et al., 1993[87]	FLCL, DSCCL, DMCL, DLCL, IBL, DSNCL (non-Burkitt); any constitutional symptoms; stage II bulky disease, stage III, stage IV	65 mo	S-BACOP[e]	119	58	61	53	60
			esc-BACOP[f]	119	58	59 (0.78)	51 (0.97)	48 (0.17)
Khaled et al.,1997[88]	IGL, HGL	NS	CHOP	40	45	67	**71**	**54**
			EPOCH	38	(1975)	50	**42 (0.04)**	**32 (0.09)**

[g] All significant values are given in **bold**.

[a] All studies excluded patients with severe prior medical conditions, prior chemotherapy, or life-threatening illness.
[b] FLCL, follicular large cell lymphoma; DMCL, diffuse mixed cell lymphoma; DLCL, diffuse large cell lymphoma; IBL, immunoblastic lymphoma; DSNCC, diffuse small noncleaved cell lymphoma; DSCCL, diffuse small cleaved cell lymphoma; LBL, lymphoblastic lymphoma; LGL, low grade lymphoma; IGL, intermediate-grade lymphoma; HGL, high-grade lymphoma.
[c] CHVmP: cyclophosphamide, adriamycin, VM26, prednisone; VCR: vincristine; Bleo: bleomycin. For other chemotherapy regimen abbreviations, see Table 70-5 footnote.
[d] Same study, with updated follow-up.
[e] S-BACOP: adriamycin, 50 mg/m^2 per cycle.
[f] esc-BACOP: adriamycin, 80 mg/m^2 per cycle.

TABLE 70-7 -- Studies of Autologous Transplantation versus Conventional Chemotherapy as Initial Treatment for Aggressive NHL

Study, Year	Inclusion Criteria[a]	Randomization[b]	No. Enrolled	Chemotherapy[c]	No. Randomized[d]	Disease-Free Survival (%) (95 % CI)	Survival (%) (95 % CI)	P Value;[e] Comments
Haioun et al., 1994 and 1997[100] [101]	Age 1655 yr; WF: IGL or HGL; one of the following: PS2-4, >1 ENS, tumor >10 cm, BM involvement, CNS involvement, DSNCCL, LBL	Patients in complete remission after induction	916	LNH-84 induction and consolidation	273	54 (4761)	67 (6173)	DFS: 0.20; surv.: 0.80
					(HI, H: 111)	HI, H: 39 (2850)	HI, H: 52 (4262)	**HI, H: DFS: 0.01; surv.: 0.06**
				LNH-84 induction; CBV + ABMT	268	62 (5668)	69 (6375)	
					(HI, H: 125)	HI, H: 59 (4969)	HI, H: 65 (5674)	
Verdonck et al., 1995[104]	Age 1560 yr; WF: IGL and IBL; stages IIIV	Partial response after CHOP × 3 but no BM involvement	286	CHOP × 8	34	72 (6282)	85 (7991)	DFS: 0.5; surv.: 0.12
				CHOP × 3, CYTBI + ABMT	35	60 (4872)	56 (4666)	

Gianni et al., 1997,[102]	Age 1760 yr; B-cell DLC and IBL; bulky (>10 cm) stage I or II disease, any stage III, stage IV without BM involvement	Upon initiation of chemotherapy	98	MACOP-B	50	49 (3265)	55 (3673)	**DFS: 0.004; surv.: 0.09**
				Intensive sequential + SCT	48	84 (7197)	81 (6891)	
Martelli et al., 1996[103]	Age 1560 yr; WF: IGL and HGL	Partial response	286	DHAP	27	52 (4361)	59 (5068)	NS. Mediastinal disease is favorable factor
		F-MACOP		BEAC + ABMT	22	73 (6482)	73 (6482)	
Pettengell et al., 1997[105]	Age 1560 yr; WF: IGL and IBL; more than one of the following: stage III or IV disease, increased LDH, KPS <80	Sequential studies		VAPEC-B × 11 wk	34	35	35	**DFS: 0.01; surv.: 0.01**
				VAPEC-B × 7 wks, ifosfamide Ara-C × 3, BuCy + ABMT	33	61	64	

[a] WF, working formulation; IGL, intermediate-grade lymphoma; HGL, high-grade lymphoma; ENS, extranodal site; BM, bone marrow; CNS, central nervous system; DSNCCL, diffuse small noncleaved cell lymphoma; LBL, lymphoblastic lymphoma; IBL, immunoblastic lymphoma; LDH, lactate dehydrogenase.
[b] The subgroup of patients who were randomized between high-dose chemotherapy and conventional chemotherapy (for details, see text); F-MACOP, variant of MACOP-B without bleomycin but with 5-fluorouracil.
[c] MACOP-B, see Table 70-2; VAPEC-B, doxorubicin, cyclophosphamide, vincristine, bleomycin, etoposide, prednisone; DHAP, see Table 70-8; BEAC, BCNU, etoposide, cytarabine, melphalan; CYTBI, cyclosphosphamide and total body irradiation; BuCy, busulfan-cyclophosphamide; SCT, salvage chemotherapy; ABMT, autologous bone marrow transplantation.
[d] HI and H, high-intermediate-risk category and high-risk category by the International Prognostic Index (see Table 70-14).
[e] Significant values are in **bold**.

transplantation. A number of prospective studies have established the feasibility of this approach and reported encouraging response rates. [94] [95] [96] [97] [98] [99] Several randomized prospective studies of high-dose chemotherapy have been recently completed. They are summarized in Table 70-7 .

In a large French study,[100] patients with poor prognostic features who achieved complete remission after four induction cycles of conventional chemotherapy were randomized to intensification or sequential chemotherapy. The 3-year disease-free survival rate was 52% (4559%) in the sequential chemotherapy arm and 59% (5266%) in the autologous bone marrow transplantation arm ($P = 0.46$). When the International Index was applied, approximately 50% of patients were found to be in a high-intermediate or high-risk category. For these patients, the 5-year disease-free survival rate was 39% after conventional chemotherapy, versus 59% for those in the autologous bone marrow transplantation arm ($P = 0.01$).[101]

Gianni et al. reported a comparison between MACOP-B and an innovative high-dose sequential chemotherapy program, supported by growth factors and autografting for patients with bulky or advanced stage diffuse large B-cell lymphoma but without bone marrow involvement. [102] They found an improved survival rate and disease-free survival rate in patients who received high-dose chemotherapy.

Martelli et al. randomized 49 patients whose response to front-line treatment was only a partial remission to receive either DHAP chemotherapy or high-dose chemotherapy and autologous stem cell transplantation. [103] The complete response rate was significantly higher for patients who received high-dose chemotherapy (96% vs. 59%, $P < 0.001$). The disease-free survival rate at 3 years was 73% in the autologous bone marrow transplantation group versus 52% in the DHAP group (P = NS). The difference was not statistically significant because of the small numbers of patients randomized.

In a Dutch study, [104] patients with stages IIIV lymphoma who had a partial remission after three induction cycles of CHOP chemotherapy, but no bone marrow involvement, were randomized to either three additional cycles of CHOP or to autologous bone marrow transplantation after conditioning with cyclophosphamide and total body irradiation. Sixty-nine patients were randomized, 34 to autologous bone marrow transplantation, 35 to CHOP chemotherapy. In this study no benefit in either survival rate or disease-free survival rate could be shown for autologous bone marrow transplantation. Because neither a biopsy nor a gallium scan was required at the time of randomization, it is likely that many of the so-labeled partial responses were actually complete responses with residual fibrotic masses.

These data and others[97] [98] [105] suggest that high-dose chemotherapy improves the outcome of younger patients with NHL and some adverse prognostic features, such as bulky disease, by approximately 1020%. In the absence of a study protocol, consolidation with high-dose chemotherapy appears justified in younger patients with adverse prognostic features. It should be acknowledged, however, that the studies on which such recommendations are based used rather stringent selection criteria. These criteria often included good performance status, young age, and absence of bone marrow involvement. The subset of patients with the most adverse prognostic features was therefore almost certainly excluded from such studies. Improved supportive care and blood stem cell support have dramatically decreased the morbidity and mortality of high-dose chemotherapy, and it is now possible to evaluate this technology in less selected and older patients. Whether any benefit will be achieved in such patients remains to be demonstrated.

Radiation Therapy in the Treatment of Large Cell Lymphoma

Radiation treatment was the first effective treatment approach for NHL and was also the first approach to be studied systematically. [106] Response rates correlate with the dose of radiation and are optimal with doses of at least 40 Gy. At these doses, response rates of up to 80% are observed, and some of the responses, especially among patients with localized disease, are durable. [107] Currently, the use of radiation therapy as a sole treatment modality in aggressive lymphoma has been abandoned because of the high recurrence rates both in and outside the radiation field; however, in nonbulky limited-stage disease, the most effective approach is that of combining three or four cycles of chemotherapy with localized radiation. [91] [92] [93] [550]

In some studies, radiation to bulky tumor masses has also been used as consolidation treatment after chemotherapy in patients with widespread disease. This practice is based on the observation of frequent recurrence in sites of bulky disease but has never been evaluated in a randomized study. Only one randomized study, reported in abstract form, has evaluated the use of adjuvant radiation therapy in patients with bulky stage I and stage II disease. [108] All patients were treated with eight cycles of CHOP. Those who achieved a complete response were then randomized to receive or not to receive 3,000 cGy of radiation to areas of previously involved disease. With a median follow-up of 6 years, the disease-free survival was 58% for those receiving CHOP, versus 73% for those receiving CHOP plus radiation ($P < 0.04$). The overall survival rate was 70% versus 84% ($P = 0.06$).

Treatment of Older Patients with Aggressive Non-Hodgkin Lymphoma

Half of the patients with aggressive lymphoma are >60 years old. There are specific challenges in treating these older patients. The mainstay of treatment has been the CHOP regimen. The outcome of CHOP chemotherapy is worse with increasing age, in part because of decreased tolerance, a high frequency of intercurrent infections, and perhaps differences in disease biology and a higher incidence of tumor resistance. Because of concerns over toxicity, a number of alternative regimens have been designed with an intent to reduce toxicity. In general they incorporate either no anthracyclines, or different or reduced doses of these agents. [109] [110] [111] However, four prospective randomized studies specifically designed for older patients clearly indicate that anthracyclines are an essential component for the treatment of NHL in the elderly, and that not all anthracyclines are equivalent in their activity. Bastion et al. reported the results of a French randomized study of chemotherapy for patients over the age of 68 with aggressive NHL.[41] The median age was 75. Patients were randomly assigned to cyclophosphamide, 750 mg/m^2 , teniposide, 75 mg/m^2 , and prednisone, 40 mg/m^2 , for 5 days (CVP), or to the same regimen with added pirarubicin, an anthracycline derivative (CTVP). Two hundred twenty patients received CVP and 233 received CTVP. The complete remission rate and progression-free survival rate were significantly better with the CTVP regimen. The 5-year survival rate was 26% with CTVP versus 19% with CVP ($P < 0.05$). The treatment-related mortality was 13% overall. This treatment-related mortality was higher than would be expected in a younger age group, but there were no significant differences in treatment-related mortality between the treatment groups.

Sonneveld et al. compared, in patients older than 59, CHOP chemotherapy with CNOP, in which doxorubicin, 50 mg/m^2, was replaced with mitoxantrone (Novantrone), 10 mg/m^2.[109] The complete remission rate, survival rate, and disease-free survival rate were significantly better with CHOP chemotherapy, indicating that mitoxantrone in the doses used in this study is less effective than doxorubicin for the treatment of NHL. The severity of toxicity was similar in both arms of the study.

In a cooperative study from the European Organization for the Research and Treatment of Cancer (EORTC), patients aged 70 years and older were randomized to receive six cycles of CHOP or six cycles of a regimen consisting of etoposide, prednimustine, and mitoxantrone (VMP).[112] Sixty patients in each arm were assessable for response. Response rates and complete remission rates were higher for patients receiving CHOP. The number of treatment-related deaths in both treatment groups was comparable. At 2 years the progression-free survival rate was 25% with VMP versus 55% with CHOP ($P = 0.002$). The overall survival rate was 30% with VMP versus 65% with CHOP ($P = 0.004$). Finally, in a small prospective study from Canada, weekly one-third-dose chop was not

found to be less toxic than full-dose CHOP and was possibly less effective.[113]

These studies and a number of smaller studies indicate that despite lower response rates than in younger patient populations, a significant proportion of older patients derive prolonged benefit from anthracycline-based chemotherapy. In a report from the Cancer Center of British Columbia, Vancouver, 80% of older patients were in good enough condition to receive chemotherapy and derive benefit from it.[114] By far the major reason for mortality in older patients with aggressive NHL is disease progression. Reducing the intensity of chemotherapy regimens does not have a major effect on toxicity but does reduce efficacy. Hence, more effective regimens, rather than attenuated regimens, need to be explored. In this regard, the use of growth factor support may allow further dose intensification, which may conceivably result in higher remission rates and longer durations of remission. Such a strategy will need to be explored in prospective trials. Other new approaches include the use of shortened, 8-week treatment regimens that have been well tolerated and result in response rates that are comparable to those obtained with CHOP,[109] [110] [114] or the use of new, potentially more effective drug combinations. In one study, the use of idarubicin-containing regimens in older patients was shown to result in higher response rates and reduced toxicity compared with doxorubicin-based regimens.[115]

For patients with limited disease, short courses of chemotherapy followed by radiation therapy to the involved field represent an attractive, nontoxic, and highly effective option.[91] [92] [93] [550] For patients with recurrent disease after conventional chemotherapy, options such as high-dose chemotherapy with stem cell transplantation have become possible with the availability of growth factors and the reduced risk associated with these treatments.[116]

Primary Mediastinal Large B-Cell Lymphoma

Primary mediastinal large B-cell lymphoma is a subset of diffuse large cell lymphoma with a specific biology and age distribution. The disease usually affects patients in their 20s and 30s, and women are more frequently affected than men. Patients with primary mediastinal large B-cell lymphoma represent a fairly large percentage of the younger patients with diffuse large B-cell lymphoma. Patients present with large anterior mediastinal masses, often extending into the pulmonary parenchyma or associated with pleural effusions, or both.[117] [118] [119] [120] Initially the disease is often limited to the thorax or supraclavicular lymph nodes (Fig. 70-1). The differential diagnosis includes Hodgkin disease, lymphoblastic lymphoma, thymoma, and, in males, germ cell tumor. Disseminated disease is characterized by infiltration of parenchymal organs such as the liver and kidney and frequent CNS involvement. Spread to other lymph node areas, on the other hand, is uncommon. Histologically, a diffuse infiltrate of large lymphoid cells is present, often in a background of fibrosis. Immunophenotyping reveals a B-cell phenotype. Expression of CD21 and of surface immunoglobulins is absent. In contrast to other types of aggressive NHL, serum levels of $_2$-microglobulin are rarely elevated.[121] The International Prognostic Index is not very useful for predicting outcome in this disorder.[122] [123] Recognized adverse prognostic features are (1) extrathoracic disease, (2) a mediastinal mass >10 cm, (3) in some series, the presence of a pleural effusion, and (4) a partial response to initial treatment, as evidenced by residual gallium positivity or histologic evidence of residual disease.

The disease is usually treated with anthracycline-containing regimens followed by radiation therapy to the involved field.[122] [124] In most large series, the disease-free survival rate is approximately 50%. In some series an improved survival rate has been attributed to the use of third-generation regimens such as MACOP-B,[125] [126] or other more intensive treatment regimens.[123]

Figure 70-1 Primary mediastinal large B-cell lymphoma. **(A)** On CT, a large anterior mediastinal mass with necrosis is noted. This radiologic picture can be confused with Hodgkin disease, mediastinal germ cell tumor, or rarely thymoma. Lymphoblastic lymphoma is usually associated with a more symmetric mass. **(B)** Chest radiograph shows widening of the mediastinum with a large mass extending more to the left lung.

High-dose chemotherapy and autologous stem cell transplantation have also been used with good success as consolidation of initial response in this disorder.[99] Patients with recurrent or refractory primary mediastinal lymphoma have a relatively favorable outcome with autologous stem cell transplantation.[127] [128] Radiation treatment is ineffective if used as the only salvage treatment, even in cases with recurrences localized to the mediastinum.[122]

Treatment of Patients with Recurrent or Refractory Large Cell Lymphoma

Forty percent of patients with aggressive NHL fail to achieve remission with conventional chemotherapy, and half of those who achieve remission will relapse. Salvage chemotherapy regimens

TABLE 70-8 -- Non-Anthracycline-Containing Regimens Used in the Treatment of Large Cell Lymphoma

DHAP:[129]	Cisplatin, 100 mg/m^2 by IV continuous infusion over 24 hours on day 1
	Ara-C, 2g/m^2 IV q 12 hr × 2 doses on day 2
	Dexamethasone, 40 mg IV × 4 doses on days 14
	Repeat q 21 days
MIME:[132]	Methyl-gag, 500 mg/m^2 IV on days 1 and 14
	Ifosfamide, 1 g/m^2 /day IV on days 15
	Methotrexate, 30 mg/m^2 IV on day 3
	Etoposide, 100 mg/m^2 /day × 3 days
	Mesna
	Repeat q 21 days

MINE:[134]	Ifosfamide, 1.3 g/m^2 /day IV on days 13
	Mitoxantrone, 8 mg/m^2 IV on day 1
	Etoposide, 65 mg/m^2 IV on days 13
	Mesna
	Repeat q 21 days
ESHAP:[130]	Etoposide, 60 mg/m^2 /day IV on days 14
	Methylprednisolone, 500 mg/day IV on days 14
	Cisplatin, 25 mg/m^2 /day IV on days 14
	Ara-C, 2 g/m^2 IV on day 5
	Repeat q 21 days
CEPP(B):[558]	Cyclophosphamide, 600 mg/m^2 IV on days 1 and 8
	Etoposide, 70 mg/m^2 /day IV on days 13
	Procarbazine, 60 mg/m^2 PO on days 110
	Prednisone, 60 mg/m^2 PO on days 110
	(Bleomycin, 15 U/m^2 on days 1 and 15)
	Repeat q 21 days
LNH-84 Induction (see Table 70-5), followed by: LNH-84 consolidation:[100]	
	Methotrexate, 3 g/m^2 during weeks 1 and 3 (with leucovorin rescue)
	Ifosfamide, 1,500 mg/m^2 (with Mesna), and etoposide, 300 mg/m^2 IV during weeks 5 and 7
	Asparaginase, 5,000 IU/m^2 IV weeks 9 and 10
	Cytarabine, 100 mg/m^2 /day × 4 days during weeks 11 and 13
Hyper-CVAD, second[540] [549]	Methotrexate, 1 g/m^2 IV on day 1
	Cytarabine, 3 g/m^2 IV q 12 hr on days 2 and 3

are therefore of major importance. A number of consecutive trials of salvage chemotherapy, which were mainly based on the use of non-cross-resistant chemotherapeutic agents, have been reported (Table 70-8). The DHAP regimen resulted in response rates in 60% of patients. [129] Subsequently ESHAP was developed, which has a superior outcome but less toxicity. [130] [131] Regimens combining ifosfamide and etoposide with methyl-GAG (MIME) or with methotrexate (IMVP-16) are also active salvage regimens. [132] [133] Alternating an ifosfamide/etoposide regimen with a platinum/cytarabine regimen, as in the combination of MINE (Mesna-ifosfamide-etoposide-Novantrone) and ESHAP, appears superior to either regimen alone. [134] The M.D. Anderson regimens are designed for patients who have been previously exposed to anthracyclines. Others have utilized anthracycline-containing regimens with different infusion schedules, such as EPOCH. [135]

A number of newer salvage regimens utilize higher-dose chemotherapy with growth factor support. They include the high-dose ifosfamide/VP-16 regimen or regimens utilizing cyclophosphamide and carboplatin. [136] [137] These regimens result in somewhat higher response and complete remission rates and are often used at the same time for stem cell mobilization. At present it is unclear which, if any, of the commonly used salvage regimens is superior. In any case, patient characteristics such as age and performance status and disease characteristics such as stage, response to initial treatment, and duration of initial remission are more important determinants of response than the salvage chemotherapy regimen used. For patients with disease that is truly refractory to anthracycline-containing regimens (i.e., patients who fail to achieve a partial remission in response to initial treatment or those whose disease progresses during treatment), the rates of response to salvage regimens are low. [132] [138]

Patients with chemotherapy-sensitive recurrences, i.e., those whose disease is responsive to salvage chemotherapy, are usually offered consolidation with high-dose chemotherapy and autologous stem cell transplantation. [79] [139] [140] A randomized study (Parma study) has indicated that durable remissions can be obtained in 50% of such patients, which is significantly better than the rate of durable remissions obtained with conventional doses of DHAP chemotherapy. [141] The treatment options for patients with truly refractory disease are limited. With the possible exception of primary mediastinal B-cell lymphoma, autologous stem cell transplantation is often followed by rapid recurrence. The use of allogeneic transplantation in a subset of younger patients with HLA-compatible donors has been explored. [142] [143] [144] [145] It appears that durable remissions can be obtained in a substantial fraction of such patients and that treatment-related morbidity and mortality can be decreased by improvements in supportive care. The majority of patients, however, do not qualify for allogeneic transplantation because of age, performance status, or lack of a donor. They should be considered for investigational treatments. Paclitaxel is a drug with limited toxicity that can induce transient responses. [146] Nucleoside analogs such as fludarabine or 2-CDA have little or no effect on aggressive lymphomas. Interferon, commonly used for patients with indolent lymphoma, is used only rarely for those with aggressive lymphoma, mainly because of low response rates when it is used as a single agent.

Mantle Cell Lymphoma

Mantle cell lymphoma was first recognized as a separate entity in the Kiel classification. In Europe it was known as centrocytic lymphoma, and in the United States as lymphocytic lymphoma of intermediate differentiation or as mantle zone lymphoma. [147] The description of an association with t(11;14)(q13;q32) in a large percentage of these cases led to the recognition of mantle cell lymphoma as a unique biologic entity. [148] [149] [150] In the REAL classification the term mantle cell lymphoma was proposed, indicating the close relationship with the normal counterpart, the B cell of the mantle zone. [1]

The clinical features of mantle cell lymphoma have been described only recently. [147] [151] [152] [153] [154] [155] [156] The usual age of patients with mantle cell lymphoma is 5070 years. Patients often present with widespread disease; bone marrow involvement and peripheral blood involvement are common, but the lymphocyte count usually does not exceed 20,000/l. The spleen is often involved. Liver involvement occurs, and diffuse GI involvement is fairly typical for mantle cell lymphoma. Sometimes this form of lymphoma presents as multiple lymphomatous polyposis. Waldeyers ring involvement also occurs frequently.

MANAGEMENT OF RECURRENT OR REFRACTORY NHL

GENERAL PRINCIPLES

Management of the patient with relapsed NHL should begin with a repeat biopsy, which is meant not only to establish objectively the existence of a relapse but also to determine the disease histology. It is not uncommon for indolent NHL to relapse with a large cell lymphoma. On the other hand, the large cell lymphomas can also relapse with an indolent cell type, which most likely occurs because at diagnosis there might have been divergent histologic types present that could have gone undetected until a relapse occurred. [518]

Once the cell type is determined, a thorough staging evaluation is performed. This is necessary in order to determine the response to salvage therapy later on. The same tests are performed as in the initial staging of the disease. Cardiac function tests are important in determining whether anthracyclines can be used again and whether high-dose cyclophosphamide can be utilized. Laboratory tests such as serum creatinine determinations will also help to decide whether platinum can be used as part of the salvage regimen. Serum LDH and $_2$ -microglobulin levels can also be used as predictors of prognosis in the salvage setting. [134] The bone marrow biopsy, platelet count, and WBC count predict the patients tolerance of intensive therapy.

Management depends on various factors. In addition to the histologic type and extent of disease, these factors include the history of prior therapy, the quality of the response to front-line therapy, and the patients age, performance status, cardiac function, renal function, and bone marrow function. In general, salvage chemotherapy regimens for patients with disease relapse should include active agents that were not used in the front-line combination. However, patients with NHL that relapses more than a year after completion of frontline treatment can still respond to the same chemotherapy regimen. [518] Cytarabine/platinum-based combinations (e.g., ESHAP) are effective in patients with both indolent and aggressive NHL who have received initial CHOP therapy.

MANAGEMENT OF RECURRENT AGGRESSIVE AND HIGHLY AGGRESSIVE NHL

The easiest to manage category comprises younger patients who have received only limited treatment and have chemotherapy-sensitive disease. For cases in this category, an attempt at salvage with a conventional dose regimen such as ESHAP (etoposide, Solumedrol [methylprednisolone], high-dose Ana-C, and platinum), MINE (Mesna, ifosfamide, Novantrone [mitoxantrone], and etoposide), ICE, EPOCH,[130] [134] [135] or a variant of these has a reasonable chance of producing a second remission, especially if favorable prognostic factors are present, such as low LDH and $_2$ -microglobulin levels. Once a response has been achieved, the current consensus is to proceed with harvesting of either stem cells or bone marrow, followed quickly by high-dose chemotherapy with a regimen such as BEAM that includes BCNU, etoposide, Ara-C, and melphalan.[137] [141] The latter is preferred over BEAC (same regimen but with cyclophosphamide instead of melphalan) because it avoids exposure to high-dose cyclophosphamide, which is potentially cardiotoxic. [280] Stem cells can be harvested at the time of recovery from myelosuppression from the conventional dose regimen or after chemotherapy mobilization. We have used intermediate-dose ifosfamide and intermediate-dose etoposide plus G-CSF with great success to generate stem cells.[137] High-dose chemotherapy with autologous peripheral blood stem cell rescue can yield cure rates in the range of 3050% for those with disease sensitive to the conventional dose salvage regimen that pre- cedes the high-dose chemotherapy. [128] [137] [141] Some disease subsets, in particular primary mediastinal lymphoma, may be more sensitive to chemotherapy dose escalation. [127] [128] Those with disease that fails to respond to a conventional dose salvage regimen have experienced a poor outcome with high-dose chemotherapy regimens. [128] [139] Their management remains in the realm of clinical research. Allogeneic bone marrow transplantation might have a role in these cases because of the decreased recurrence rate associated with this procedure and a potential graft-versus-lymphoma effect. [142] [143] [144] [145] [519] [520]

Older patients who have received only one prior regimen and who do not have chemotherapy-refractory disease can usually be treated for salvage with a conventional dose regimen. DHAP is the most commonly used combination.[129] [131] ESHAP has been found to be less toxic and more effective in a series of sequential salvage regimens tested at the M.D. Anderson Cancer Center.[131] A newer regimen, MINE-ESHAP, has been the most active of all the salvage combinations tested at the M.D. Anderson Cancer Center.[134] It starts with MINE, which is consolidated with ESHAP. Mitoxantrone has moderate cardiotoxicity, especially in elderly patients who have received a significant amount of anthracyclines in the past. In addition, the ESHAP regimen is nephrotoxic. Candidates for this treatment should therefore undergo a cardiac scan and their kidney function should be reasonable (serum creatinine <2 mg/dl is a minimum requirement). Consolidation with high-dose chemotherapy should be considered in selected patients who are in good general condition. Given the improvements in supportive care, it can be used even in some patients in their early 70s.

The most difficult to treat are patients who have received extensive prior therapy and who have disease refractory to front-line therapy. Usually their response to salvage regimens is also poor, irrespective of the dose intensity. [133] [134] Such patients are seldom able to mount any type of significant response to salvage treatment, and consequently other novel treatment strategies are necessary.

Newer agents with moderate activity in recurrent aggressive NHL include paclitaxel (Taxol) [146] [521] and topotecan.[522] More recently, the monoclonal antibody rituximab, directed against the CD20 antigen, which is expressed in the vast majority of B-cell NHLs, has produced a 37% response rate as a single agent in relapsed large cell lymphoma.[523] In selected patients allogeneic transplantation may have a role, [142] [145] [519] [520] and autologous transplantation may have a role in some patients with primary mediastinal B-cell lymphoma.[127] [127]

MANAGEMENT OF RECURRENT INDOLENT LYMPHOMAS

Salvage therapies for relapsed indolent lymphoma span many options, including high-dose chemotherapy with autologous peripheral blood stem cell rescue, allogeneic bone marrow transplantation in young patients with a matched sibling donor, monoclonal antibody treatments, purine analog-based therapy, or other active chemotherapy regimens, including Ara-C and cisplatin combinations, as well as the watch-and-wait strategy.

Because of the indolent nature of these disorders and the wide disparity of available alternatives, selection of the best option becomes difficult. The patient, aided by the physician, must assume an active role in the decision-making process. In reaching this decision, two chief factors must be considered: the patients age and the expected survival if the

disease is managed with conventional dose therapy. The choice of a treatment alternative associated with a high mortality and morbidity, such as allogeneic bone marrow transplantation in a patient whose expected survival is long even when the disease is managed conservatively, has to be weighed against the potential for cure. Despite evidence suggesting a graft-versus-lymphoma effect, the potential for cure at this point remains to be proved. [143] [265] [266] [267] [268] [269] [270] The expected survival after relapse is a function of various prognostic factors. In one study, the median survival of patients with indolent lymphoma after a first or second relapse was 36 months, and after a third relapse it was 14 months.[524] Features associated with a short median survival after relapse were the presence of constitutional symptoms, a bulky tumor mass, more than two relapses, LDH >400 mg/ml (normal, 225 mg/ml), and hemoglobin <10 g/dl. Whenever one of these variables was present, the median survival was 28 months, and when two or more were present, it was 8.5 months. On the other hand, when none of these adverse features was present at relapse, the median survival still had not been reached at 6 years.

Management of Patients Less Than 65 Years Old

The most difficult decision in treating patients <65 years old is whether to proceed with an experimental and risky approach such as allogeneic bone marrow transplantation, should an HLA identical donor be available. Other alternatives include an aggressive approach such as high-dose chemotherapy with autologous peripheral blood stem cell rescue, conventional dose regimens (with higher-risk strategies for the future), or the watch-and-wait posture. The use of allogeneic bone marrow transplantation in recurrent indolent NHL is usually reserved for patients <55 years old because they better tolerate this treatment.

Whenever a donor is not available, the possibility of high-dose chemotherapy with autologous peripheral blood stem cell rescue has to be considered. This approach is experimental, because there are no solid data to prove that it prolongs survival or results in cures. Available data show that at 4 years, the failure-free survival of relapsed patients treated with high-dose chemotherapy is 45%. [257] [525] [526] Although this rate appears at first glance to be superior to the rate achieved with standard management, these studies did not control for patient selection. Further, there is no clear plateau in the failure-free survival curve to indicate a likelihood of cure in these cases. One of the major limitations of this strategy is the potential for transplanting tumor cells. Purging with antibodies directed against B-cell antigens can reduce this risk but is not always totally effective in removing malignant cells. [261] [264]

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The use of conventional dose regimens includes anything from single-agent chemotherapy with drugs such as fludarabine to combinations such as the FMD (fludarabine, mitoxantrone, Decadron) regimen (Table 70-11), Ara-C/platinum combinations, or ifosfamide-based regimens, [130] [134] [235] [242] [527] [528] [529] all of which have significant activity but none of which has been demonstrated to be curative in recurrent indolent lymphomas. The new monoclonal anti-B-cell antibody rituximab has added another treatment option for these patients. [248] [249] [250] [251]

Patients More Than 65 Years Old

The choice of treatment for patients >65 years old is simpler because it only involves selecting among the different standard dose regimens available. The introduction of the therapeutic anti-CD20 monoclonal antibody rituximab is an important advance for this age group, whose tolerance of chemotherapy is frequently compromised by decreased bone marrow function. [251] The FMD combination has also been used sucessfully in all age groups and has shown a 94% response rate (Table 70-11). Future trials will likely involve the use of rituximab in combination with chemotherapy regimens.

Abnormalities in serum immunoglobulin levels (either hypo- or hypergammaglobulinemia) can be detected in a large percentage of the patients. Monoclonal gammopathy, mainly of the IgM type, was reported to occur in 25% of patients. [157]

Morphologically, the lymphoma cells are usually small cleaved cells with a pattern of differentiation that is intermediate between that of CLL and follicle center lymphomas. [1] [147] Blastoid and pleomorphic variants are recognized as well. [158] The growth pattern is either nodular, diffuse, or mantle zone. [151] [152] The experienced hematopathologist can often make the correct diagnosis by recognition of the typical cleaved cells in the blood smear or from the lymph node histology. [147] [159] The immunohistology reveals a typical CD5+, CD19+ phenotype like B-cell CLL, which is crucial for the diagnosis. The cells express sIgM and often sIgD. In contrast to CLL, they lack expression of CD23 and have more intense sIG and CD20 staining. Immunoglobulin gene rearrangement is consistently present. [1] [147] Although *bcl-1* rearrangements are present in at most two-thirds of cases, [160] [161] [162] [163] [164] overexpression of cyclin D1 (*PRAD* oncogene) and the presence of t(11;14) by fluorescence in situ hybridization (FISH) can often be demonstrated. [162] [165] [166] [167] Mutations in the *p53* oncogene have also been described in a subset of patients and are associated with a worse prognosis. [168] [169] [170]

The prognosis of mantle cell lymphoma is worse than that of CLL or follicular lymphoma, and in general the responses to chemotherapy are less durable than those achieved in other types of diffuse lymphoma. [151] [152] [153] [154] [155] The estimated 10-year survival in a recent series was only 8%. [153] One subset, the mantle zone variant (i.e., proliferation of disease limited to the mantle zone of the follicle) is characterized by an indolent behavior, a good response to conventional chemotherapy, and a prolonged survival. [147] [151] Bone marrow involvement is less common in this subtype than in the diffuse and nodular variants of mantle cell lymphoma. The diffuse and nodular variants respond poorly to conventional anthracycline-containing regimens and are associated with a median survival of 3 years. [151] Blastoid and pleomorphic variants as well as those with mutations of the *p53* gene have an even worse prognosis, [153] [168] [169] [170] and patients may be at high risk for CNS recurrence. [171] Treatment with platinum-containing regimens or fludarabine has also been disappointing. [147] [172] Recently the hyper-CVAD regimen (see box on management of very aggressive lymphoma) as originally proposed for the treatment of childhood ALL has been applied to the treatment of mantle cell lymphoma. This regimen has resulted in encouraging response rates of good duration. [173] Similar results have been reported with VAD plus chlorambucil by Gressin et al. [174] Preliminary results indicate that maintenance therapy with interferon may have a role in the treatment of this disorder. [172]

Many centers have recommended autologous or allogeneic transplantation as consolidation of remission. [147] [175] [176] Future follow-up is needed to clarify whether this tactic will result in improvement in long-term outcome. [172] A high recurrence rate was reported in one recent series. [177] [178]

Follicle Center Lymphoma, Grade I or II, and Small Lymphocytic Lymphoma

Several subtypes of indolent B-cell non-Hodgkin lymphomas are recognized. Their immunophenotypic, cytogenetic, and clinical characteristics are summarized in Table 70-9 . The majority of cases belong to the group of follicle center lymphomas and to the small lymphocytic lymphomas. [3] These entities were grouped in the Working Formulation as low-grade NHL. They are usually considered to have a similar prognosis and may benefit from the same treatments. Follicle center lymphoma grade I, as defined by the REAL classification, corresponds to follicular small cleaved cell lymphoma of the Working Formulation. Follicle center lymphoma grade II corresponds to follicular mixed cell lymphoma and to some cases of follicular large cleaved cell lymphoma. Follicle center lymphoma grade III corresponds to the majority of cases of follicular large cell lymphomas and has a more aggressive behavior. [179] [180] Usually there is at least partial follicular architecture in follicle center lymphomas, but diffuse areas may be present, and occasionally the entire biopsy specimen shows a diffuse pattern. This finding may connote a somewhat worse prognosis. The follicle center lymphomas are characterized by the presence of t(14;18), leading to rearrangement of the *bcl-2* oncogene in the majority of cases. Small lymphocytic lymphoma is a variant of chronic lymphocytic leukemia and has similar morphologic, immunophenotypic, and cytogenetic characteristics. It presents almost always with bone marrow involvement but has by definition no involvement of the peripheral blood. The treatment of indolent non-Hodgkin lymphomas is one of the more controversial

MANAGEMENT OF B-CELL INDOLENT NHL

As with any other NHL, management of the indolent forms should begin with a careful review of the biopsy slides by an experienced hematopathologist. The indolent NHLs include the cell types listed in Table 70-1 . Among these, the follicular types and the small lymphocytic lymphomas are the most common, but recently marginal zone lymphomas have been added to this group of disorders. They share the indolent behavior of the former, but their clinical presentation and management may be very different. This discussion addresses only the follicle center grade I and II lymphomas and small lymphocytic lymphomas.

The single most important factor in determining the management of an indolent NHL is the Ann Arbor disease stage. For this reason, thorough staging is critical. Of all the staging tests, the bone marrow biopsy is the most important because the skeleton is the most commonly involved extranodal organ in these disorders. For this reason, many investigators prefer to perform bilateral bone marrow biopsies. Once the Ann Arbor stage is determined, the patients can be divided into those with stage IV disease, which is the most common presentation, versus all others.

MANAGEMENT OF EARLY STAGES

Early-stage indolent NHL refers to Ann Arbor stages IIII presentations. Some investigators have traditionally managed the Ann Arbor stage III presentations the same way as the stage IV cases. There is enough information, however, to suggest that stage III presentations should be treated more like the early stage III cases because their prognosis is superior to that of the stage IV presentations; furthermore, in contrast to stage IV, there seems to be a potential for cure with standard therapy.

For patients with stage III disease we use COP-Bleo chemotherapy (cyclophosphamide, 100 mg/m^2 IV on day 1; vincristine, 2 mg IV on day 1; and prednisone, 100 mg PO daily × 5; plus bleomycin, 15 units IV). This regimen is administered for ten cycles. After the third cycle, radiation is administered to involved fields. In general, we use a dose of 45 Gy for the upper torso, 30 Gy for the lower torso and the abdominal sites. For patients with high-risk disease (high LDH levels, bulky disease, extranodal involvement) and for patients with small lymphocytic lymphomas, adriamycin, 50 mg/m^2 IV, is given with every cycle to a total dose of 450 mg/m^2 (CHOP-bleomycin). In patients >60 years old, no bleomycin is administered. [201]

For patients with stage III disease we use CHOP-bleomy- cin for eight cycles (adriamycin is capped at 400 mg/m^2), plus two courses of COP. Bleomycin is not given to patients >60 years old. After cycle 2, cycle 4, and, if needed, after cycle 6, radiation therapy is administered to all involved lymph node areas. [203] After radiation to the abdomen, doses of CHOP-bleo are reduced by 20%. This regimen has resulted in prolonged disease-free survival in 50% of patients.

MANAGEMENT OF ADVANCED DISEASE

The optimal management of patients with advanced disease (i.e., stage IV indolent lymphoma) is controversial. Treatment options range from watchful waiting to intensive chemotherapy and autologous bone marrow transplantation. No treatment option has been convincingly shown to improve survival, and the treatment options for specific patients are dictated by patient and physician preference or by investigational protocols. All treatment options are discussed in the text of this chapter. Here we detail the investigational approach that is pursued at the M.D. Anderson Cancer Center.

In an attempt to obtain durable remissions, an intensive chemotherapy regimen was designed that incorporates three different alternating non-cross-resistant regimens. The first combination, CHOP-bleomycin, is based on doxorubicin and cyclophosphamide. This is followed by an Ara-C-platinum-based regimen (ESHAP). The third combination, NOPP, consists of mitoxantrone, 10 mg/m^2 IV on day 1; oncovin, 2 mg IV on day 1; procarbazine, 100 mg/m^2 PO daily for 14 days; and prednisone, 100 mg PO daily for 5 days. This regimen is administered at approximately monthly intervals for a total of 12 cycles and is followed by maintenance treatment with interferon for 1 year. In a series of 138 patients treated with this regimen, approximately two-thirds achieved complete remission, and by PCR analysis, 13 of 19 patients achieved PCR-negative status in the blood. [516] PCR negativity had seldom been observed after CHOP-bleomycin therapy in stage IV follicular lymphoma, and achievement of PCR negativity may correlate with long-term failure-free survival. [517] Prolonged follow-up will be necessary to demonstrate any impact of this regimen on overall survival. More recently, we have compared this regimen in a randomized study with the fludarabine-mitoxantrone-dexamethasone regimen (FMD).[242] In a preliminary analysis, clinical and molecular response rates were equivalent with both regimens, but the toxicity of FMD was much less than that of ATT.[560] The FMD regimen currently constitutes our standard of care.

TABLE 70-9 -- Differential Diagnosis and Characteristics of Indolent B-Cell Lymphoma

Lymphoma	Peripheral Blood Involvement	Paraprotein	Bone Marrow Involvement	Lymph Node Involvement; Histology	Phenotype[c]	Typical Cytogenetic and Molecular Abnormalities
B-CLL/SLL	Common; WBC: 10,000200,000/mm^3	Rare	Common	Common; diffuse	SIG+, CIG±, **CD5**+, CD10, **CD23**+, CD43+	Trisomy 12 (30%)
Lymphoplasmacytoid lymphoma	Rare	IgM	Common	Common; diffuse	SIG+, **CIG**+, CD5, CD10, CD23, CD43±	NA
Mantle cell lymphoma	Common; WBC 10,00030,000/mm^3	Rare[b]	Common	Common; mantle zone, nodular, or diffuse	SIG+, CIG, **CD5**+, CD10±, **CD23**, CD43+	t(11;14), *bcl-1* rearrangement
Follicle center lymphoma	Rare	Rare	Common	Common; follicular, diffuse areas	SIG+, CIG, CD5, CD10±, CD23, CD43	t(14;18), *bcl-2* rearrangement
Monocytoid B-cell[a]	Rare	IgM or IGD similar to monocytoid B cell	Common	Common; diffuse, interfollicular, marginal zone, occasionally follicular colonization	SIG+, CIG±, CD5, CD10, CD23±, CD43±	Trisomy 3, trisomy 18, rearrangement 1q21 or 1p34
Splenic lymphoma with villous lymphocytes[a]	Common; WBC: 10,00025,000/mm^3 , villous lymphocytes	IgM or IGD similar to monocytoid B cell	Common	Unusual; for pattern, see monocytoid B-cell lymphoma		
MALT[a]	No	Rare	Rare	Unusual; for pattern, see monocytoid B-cell lymphoma		

Adapted from Harris et al.,[1] with permission.

[c]The most specific immunophenotypic characteristics are indicated in **bold**. SIG, surface immunoglobulin; CIG, cytoplasmic immunoglobulin.
[b]Paraprotein: presence of a monoclonal immunoglobulin in serum and subtype of paraprotein, if detected.
[a]Subtypes of marginal zone lymphoma.

issues in hematology. The controversy can be attributed to the rather prolonged natural history of this disease entity and the difficulties associated with demonstrating the impact on survival of most of the available treatment strategies.

Natural History

Patients with low-grade follicular lymphomas or small lymphocytic lymphomas typically present with widespread lymphadenopathy and bone marrow involvement in 75% of cases. The median age is 60, but there is a wide age range. [181] [182] [183] [184] Patients are often relatively asymptomatic at presentation and may come to medical attention because of a lump or because of vague complaints. Despite its rather benign clinical presentation, most patients affected by indolent lymphomas will die from the disease and not from other causes. There is a mortality rate of 8% per year associated with this disorder and the median survival is 9 years. [185] Disease transformation to a more aggressive histologic type is the most common terminal event. [186]

Differential Diagnosis

Follicular lymphomas should be differentiated from mantle cell lymphomas, especially from the nodular and mantle zone variants. Lymphocytic lymphoma may pose differential diagnostic confusion with other diffuse lymphomas of small cells, such as diffuse mantle cell lymphoma, marginal zone lymphoma, and immunocytoma. (Marginal zone lymphomas are discussed in detail later.) Immunocytoma in many cases represents Waldenström macroglobulinemia or is closely related to it. It is considered in more detail in Chapter 76 . Whenever a biopsy specimen reveals a diagnosis of immunocytoma, serum and urine electrophoresis are indicated. Even in the absence of a monoclonal spike, treatment similar to that for Waldenström disease should be considered. Low-grade lymphoma should also be differentiated from polyclonal lymphoproliferative disorders and from inflammatory and infectious conditions. Expert histopathologic analysis that includes immunophenotyping, cytogenetic analysis, and occasionally molecular analysis can be extremely helpful in the differential diagnosis of these disorders.

Treatment of Stages I and II Follicle Center Lymphomas

Approximately 20% of patients with follicle center lymphoma present with stage I or II disease. Such patients tend to survive longer than those with more advanced disease. They are frequently treated with radiation therapy to the involved field, and a considerable fraction obtain durable remissions. [184] [187] [188] [189] [190] [191] [192] [193] [194] In contrast to more advanced disease, the curability of these disorders is accepted and treatment is generally recommended.

The treatment alternatives for stage III presentations include radiation therapy alone and combined modality using chemotherapy and radiation. [195] Radiation, when used alone, has ranged from involved-field irradiation to treatment of more extensive fields, such as by total nodal or central lymphatic radiation techniques. [184] [187] [188] [189] [190] [191] [192] [193] [194] [196] [197] [198] [199] [200] The latter encompasses all lymph node-bearing areas, including the mesenteric nodes. No comparative trials have been conducted to determine which of these radiation therapy techniques is superior, but the published literature suggests that techniques that involve more extensive radiation fields are associated with a better failure-free survival rate. [196] The use of combined modality therapy with COP-bleomycin and radiation to involved fields (or CHOP-bleomycin and radiation to involved extranodal sites) has been associated with an outcome similar to that with total nodal radiation. [201] At this time it is not known whether radiation alone or combined modality management is superior because no prospective studies have compared these two modalities directly. Although long-term disease-free survival is attainable and there is a hint of a plateau in the failure-free survival curve with all of these modalities, not all investigators accept that such patients are truly cured because occasional relapses have been described even after 15 years.

Treatment of Stage III Disease

The published literature on stage III presentations is scarce. Most investigators have treated these patients in the same way as patients with stage IV disease and most of the available literature has combined the results of treating stage III disease with the results of treating stage IV disease, thus making it difficult to analyze the outcome. Three studies, however, have reported on them separately. [200] [202] [203] At Stanford University, 66 patients with stage III disease were treated with either total nodal irradiation or total body irradiation. [202] In addition, 13 patients were given CVP chemotherapy. At 10 years, 50% were alive and 40% were disease free. A study conducted at the University of Wisconsin included 29 patients with stage III disease who were treated by central lymphatic irradiation alone. [200] At 5 years, 61% of patients were free of disease. At the M.D. Anderson Cancer Center, with a combination of CHOP-bleomycin and radiation therapy to involved regions, approximately 55% of patients achieved a disease-free state beyond 5 years, with a plateau in the curve that suggests potential curability. [203] However, follow-up beyond 10 years will be necessary to prove that the patients treated in these studies were cured.

Treatment of Stage IV Disease

The treatment of stage IV indolent NHL is controversial. [204] Patients with stage IV disease represent the majority of patients with follicle center cell lymphomas and almost all patients with small lymphocytic lymphomas. Both disorders have a similar behavior and prognosis, and many studies have included patients with both types of histologies. Among the many treatment options available are watchful waiting, conventional chemotherapy, interferon treatment, intensive chemotherapy, high-dose chemotherapy with autologous or allogeneic transplantation, and a number of newer drugs.

Watchful Waiting

Watchful waiting as a treatment option is based on the disappointing results of several chemotherapy studies that used alkylating agents or combination chemotherapy with anthracyclines. Although respectable response rates and even complete remissions were observed, few if any responses were durable, and in comparison with historical control patients who were untreated, no improvement in survival was thought to have resulted. [204] [205] Many experienced physicians believe, therefore, that asymptomatic patients with low-grade lymphomas should not be treated at diagnosis, but rather that treatment should be withheld until symptoms develop. [204] [206] [207] Immediate treatment according to this paradigm may even be contraindicated as it may result in the selection of more resistant clones and disease transformation.

The data implying a lack of survival benefit from chemotherapy are based on retrospective analyses and comparison with historical control patients and should therefore be interpreted with caution. Two studies have prospectively compared watchful waiting and immediate intervention. Young et al. compared watchful waiting followed by ProMACE-MOPP chemotherapy when necessary at the time of progression with immediate treatment with ProMACE-MOPP at diagnosis. [208] In a preliminary analysis, median survival was similar in both treatment groups, but more patients remained free of disease in the arm treated aggressively (51% vs. 12%). These results have not yet been updated. Brice et al. [209] reported a French multicenter study in patients with low-tumor-burden follicular lymphoma. Patients were randomized between no initial treatment, prednimustine, or interferon-. With a median follow-up of 45 months, no difference in survival could be demonstrated between the three study groups. No combination chemotherapy arm was included in this series.

Other investigators believe that watchful waiting is appropriate

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only for a selected subgroup of older patients with low tumor burden, and that most younger patients should be offered participation in research protocols to develop more effective treatments and identify prognostic features so that treatment can be directed more effectively toward those with the worst prognosis.

Conventional Chemotherapy

No convincing survival benefit has been shown for patients treated with conventional chemotherapy, and in general, only temporary remissions are anticipated. The most commonly used drugs include either chlorambucil given intermittently by mouth or the combination of cyclophosphamide, vincristine, and prednisone (CVP). In randomized studies, response rates were higher with CVP, but no improvement in survival could be demonstrated, perhaps because of the high salvage rate that can be achieved after relapse from single-agent therapy. [210] [211] [212] [213] The median duration of remission with CVP ranges from 1.53 years, but few remissions are durable. Anthracyclines have also been used but have not been shown to significantly increase complete remission rates, remission duration, or survival. [214] [215] On

the other hand, patients who will receive maintenance treatment with interferon may benefit more if they have received induction treatment with an anthracycline-containing regimen (discussed in the following sections).

Several groups have attempted to devise potentially curative approaches to the treatment of indolent NHL. One approach consists of the rapid cycling of non-cross-resistant chemotherapeutic agents, based on the Goldie-Coldman model. Others have utilized induction chemotherapy with CHOP followed by consolidation with high-dose cyclophosphamide and total body irradiation for patients with poor prognostic features. [216] These approaches do not have sufficient follow-up to allow an assessment of their validity and probably should not be recommended outside an investigational protocol.

Interferon

Interferon is a biologic agent that influences the proliferation of a number of hematologic malignancies and induces remissions in patients with low-grade NHL, especially in those with follicular lymphoma. [217] [218] [219] [220] [221] [222] [223] [224] [225] Interferon has been mainly used in combination with chemotherapy, either during the induction of remission or as maintenance treatment after remission has been achieved with alkylating agents (Table 70-10).

The use of interferon concurrently with chemotherapy in remission induction has been evaluated in four randomized studies. The two studies that used both anthracyclines and alkylating agents as induction treatment indicated an improvement in disease-free survival for the patients receiving interferon. In two studies an advantage in overall survival was also found for patients treated with interferon. [226] [227] [228] [551] In the two studies that used a chemotherapy regimen without anthracyclines, the effects of both induction and maintenance interferon were studied through double randomization. In a preliminary analysis of one study, the combination of induction and maintenance interferon with an alkylating agent for induction showed an advantage in progression-free survival over treatment with an alkylating agent alone. [229] In the second study, which is reported as not showing a benefit, the results have been analyzed only for the two induction arms but not for the maintenance interferon arms. [230] Furthermore, the interferon dose used in that study was lower than the standard doses. [230]

Three studies evaluated the use of interferon only as maintenance treatment for patients who had achieved a remission with chemotherapy. They all indicated an improvement in disease-free survival. [231] [232] [233] Interestingly, in one of these studies, the advantage for interferon maintenance was more pronounced if the remission had been induced with an anthracycline-containing regimen rather than with the CVP regimen. [231] No improvement in survival has been demonstrated in these three studies, in part because of limited follow-up. A recent update of the study by McLaughlin et al. [203] revealed that with longer follow-up, a survival advantage of borderline statistical significance has been achieved. [234]

The majority of the data indicate a significant role for interferon in the treatment of indolent lymphomas. When used concurrently with anthracycline-based chemotherapy, it has a role in remission induction and in maintenance treatment. When used in combination with anthracycline-containing chemotherapy regimens, it has a significant impact on survival. [542] [551] [552]

Nucleoside Analogs

Fludarabine and 2-CDA are nucleoside analogs with considerable activity in indolent lymphoid malignancies. Fludarabine was initially tested in CLL and 2-CDA was tested in hairy cell leukemia. Both agents have also been used in grade I and II follicle center lymphoma and in small lymphocytic lymphoma. [235] [242] A number of studies are summarized in Table 70-11 . Response rates of 4080% have been demonstrated with single-agent 2-CDA or fludarabine. Response rates may be somewhat higher in patients who have not received any prior treatment. Both agents have cumulative hematopoietic toxicity, which may result in mild but prolonged thrombocytopenia. This usually is of limited immediate clinical significance but may be important if consolidation with autologous transplantation is planned, as it may impair the ability to collect stem cells. Both agents are profoundly immunosuppressive and their use can be complicated by opportunistic infections, such as *Pneumocystis carinii* pneumonia, recurrent herpes, or occasionally fungal pneumonias.

Recently the combination of nucleoside analogs with other agents has been evaluated. The combination of fludarabine, mitoxantrone, and decadron was tested in a Phase II trial of 51 patients with recurrent indolent NHL. Twenty-four patients (47%) achieved a complete remission and 24 (47%) achieved a partial remission. [242] The median failure-free survival time for patients with complete remissions was 21 months. Opportunistic infections occurred in some of the initial patients but could be prevented with the prophylactic use of trimethoprim-sulfamethoxazole. Similarly, the combination of 2-CDA and mitoxantrone was tested in 23 patients with recurrent indolent NHL and resulted in complete responses in 22% and partial responses in 48%. [236] The median duration of a complete response was 15 months. 2-CDA has an advantage over fludarabine in that it can potentially be administered as an oral agent. On the other hand, combination chemotherapy with 2-CDA, mitoxantrone, and prednisone resulted in an unacceptable incidence of opportunistic infections. [236]

Monoclonal Antibody Treatments

A number of monoclonal antibodies have been raised against surface epitopes of NHL and have been used for therapy for NHL. [243] Different approaches have been followed, including (1) labeling antibodies with radioactive isotopes, (2) attachment of cell toxins (immunotoxins) to antibodies, and (3) the infusion of unlabeled (naked) antibodies, sometimes raised as anti-idiotypic antibodies against the patients NHL. [244] The dose-limiting toxicity of radiolabeled antibodies is myelosuppression,[245] whereas immunotoxins can cause hepatic toxicity and a vascular leak syndrome.[246] Some of the unconjugated antibodies (Campath-H1) are also myelosuppressive and can cause profound immunosuppression. [247] All monoclonal antibodies derived from mouse cell lines can give rise to the development of human anti-mouse antibodies (HAMA), which may prevent their repeated administration. [243] Many of these agents are still investigational, but promising results have been recently reported with four types of antibodies.

Idec-C2B8 (Rituximab) is a chimeric anti-CD20 therapeutic antibody. The CD20 antigen is expressed in 95% of B-cell lymphomas and thus has been chosen as the target for this molecule, which has an acceptable toxicity profile and induced responses in 50% of 166 patients with recurrent follicle center lymphoma with a median duration of 13 months. [248] [249] [250] [251] In one

TABLE 70-10 -- Randomized Studies of Interferon Treatment in Indolent Lymphomas

Study, Year	Inclusion Criteria[a]	Median Follow-up	Chemotherapy[b]	N	Response (%) (*P*)	Failure-Free Survival (%) (*P*)	Survival (%) (*P*)
Smalley et al., 1992, [226] Andersen and Smalley, 1993,[227] Smalley et al., 1998[551]	DWDLL, FSCCL, DSCCL, FMCL, FLCL, aggressive disease	10 yr	COPA × 810 COPA × 810 + induction IFN, 6 MU/m^2 on days 2226 each cycle	147 141	86 86 (NS)	39 at 2 yr 19 at 5 yr 62 at 2 yr (0.001) 34 at 5 yr (0.0013)	median 5.7 yr median 7.8 yr (0.04)
Solal-Celigny et al., 1993,[228] 1996[542]	Aggressive follicular lymphoma	5 yr	CHVP, 12 cycles over 18 mo CHVP + IFN, 5 MU SQ 3×/wk × 18 mo	119 123	69 86 (0.006)	Median, 1.5 yr Median, 2.9 yr (0.0002)	56 at 5 yr 70 at 5 yr (0.016)
Peterson et al., 1994[230] [c]	FSCCL, FMCL, stage III or IV	2.7 yr	CTX, 100 mg/m^2 /day PO 3 days/wk until 3 mo past complete or partial response CTX, 100 mg/m^2 /day PO + IFN, 2 MU/m^2 SQ 3 days/wk	531	89 84 (NS)	40 at 3 yr 37 at 3 yr (NS)	80 at 3 yr 78 at 3 yr (NS)

Price et al., 1991[229]	Follicular lymphoma, stage III or IV	2.5 yr	Chlorambucil Chlorambucil + maintenance IFN Chlorambucil + induction IFN Chlorambucil + maintenance and induction IFN	127	71 55 (NS)	[f]	
Unterhalt et al., 1996[231]	FSCCL and FMCL, stages III and IV		Prednimustine + Mitoxantrone Prednimustine + Mitoxantrone + maintenance IFN, 5 MU 3×/wk until progression CVP CVP + maintenance IFN, 5 MU 3×/wk until progression	498	85 86 (NS)	Median, 21 mo Median, 45 mo (0.0033)[f] Median, 16 mo Median, 31 mo	NS NS NS NS
Hagenbeek et al., 1998[232]	FSCCL, FMCL	4.2 yr	CVP × 8 CVP × 8 + maintenance IFN, 3 MU 3×/wk × 12 mo	120 122	79 80 (NS)	87 wk 132 wk (0.12)[e]	70 at 5 yr 70 at 5 yr
McLaughlin et al., 1993,[233] 1996[234] [d]	DWDLL, FSCCL, FMCL, stage IV	9.1 yr	CHOP-Bleo IFN, 3 MU/m^2 3×/wk × 8 wk + CHOP-Bleo + IFN, 3 MU/m^2 3×/wk × 2 yr	96 127	NS 96	28 at 5 yr 47 at 5 yr (0.01)	64 at 5 yr 74 at 5 yr (0.07)

[a]DWDLL, diffuse well-differentiated lymphocytic lymphoma; FSCCL, follicular small cleaved cell lymphoma; DSCCL, diffuse small cleaved cell lymphoma; FMCL, follicular mixed cell lymphoma; FLCL, follicular large cell lymphoma.
[b]COPA: cyclophosphamide, 600 mg/m^2 on day 1; vincristine, 1.2 mg/m^2 on day 1; prednisone, 100 mg/m^2 on days 15; adriamycin, 50 mg/m^2 on day 1, × 810 cycles. CHVP: cyclophosphamide, 600 mg/m^2 on day 1; teniposide (VM-26), 60 mg/m^2 on day 1; prednisone, 40 mg/m^2 on days 15; adriamycin, 25 mg/m^2 on day 1, × 810 cycles; MU, million units.
[c]Second randomization to maintenance or no maintenance interferon. Results of this randomization are unpublished.
[f]In this study no information on survival is provided. But there is an advantage in duration of remission for those receiving interferon throughout the treatment ($P = 0.02$).
[e]$P = 0.054$ when adjusted for other prognostic features.
[d]Not a randomized study, used historical controls. CHOP-Bleo is given for 1 year. Doxorubicin is discontinued at a total dose of 450 mg/m^2.

TABLE 70-11 -- Selected Phase I and Phase II Studies of Nucleoside Analogs for Indolent NHL

Study, Year	Patient Population	Treatment	N	Response	Toxicity	Comments
Kay et al., 1992[238]	Indolent NHL: failed alkylators (34), transformed (6)	2-CDA, 0.1 mg/kg/day × 7 days	40	CR 8/40 (20%) PR 9/40 (23%)	Myelosuppression	
Saven et al., 1995[239]	Indolent NHL, untreated	2-CDA, 0.1 mg/kg/day × 7 days	28	CR 9/28 (35%) PR 14/28 (50%)	Myelosuppression	Actuarial DFS at 36 months: 17%; actuarial survival at 36 months: 70%
Betticher et al., 1996[240]	Follicular NHL, stage IIIIV, untreated	2-CDA, 0.1 mg/kg/day × 7 days	37	CR 5/37 (14%) PR 26/37 (70%)	Myelosuppression	Median TTF: 17 months
Tefferi et al., 1994[220]	Indolent NHL, CLL, Refractory[a]	2-CDA, 14 mg/m^2 /day × 7 days Chlorambucil, 30 mg/m^2 q 2 wks	15	CR 1/15 PR 6/15	Myelosuppression, opportunistic infections	MTD: CDA, 2 mg/m^2 × 7 days; chlorambucil, 30 mg/m^2 q 2 wk
Saven et al., 1996[237]	Indolent NHL, failed alkylators	2-CDA, 0.1 mg/kg/day × 7 days Mitoxantrone, 57.5 mg/m^2 on day 1 Prednisone, 100 mg/day × 5 days	23	CR 5/23 (22%) PR 11/23 (48%)	Myelosuppression, opportunistic infections	Recommended dose: 2-CDA, 0.075 mg/kg × 7 days; mitoxantrone, 5 mg/m^2 on day 1, *no prednisone*
Redman et al., 1992[236]	Indolent and aggressive NHL, failed alkylators	Fludarabine, 25 mg/m^2 /day × 5 days	67	CR 5/67 (9%) PR 23/67 (28%)	Myelosuppression, opportunistic infections	No responses in aggressive NHL; >60% response in indolent NHL
Solal-Céligny et al., 1996[235]	Follicular NHL, stages IIIIV, high tumor burden, untreated	Fludarabine, 25 mg/m^2 /day × 5 days	54	CR 37% PR 28%	Myelosuppression	Median TTF: 9 months
McLaughlin et al., 1996[242]	Indolent NHL, failed alkylators	Fludarabine, 25 mg/m^2 /day × 3 days Mitoxantrone, 10 mg/m^2 IV on day 1 Dexamethasone, 20 mg/day on days 15 PCP prophylaxis	51	CR 24/51 (47%) PR 24/51 (47%)	Myelosuppression, opportunistic infections	Median TTF: 14 months; median survival: 34 months

Abbreviations: 2-CDA, 2-chlorodeoxyadenosine; CR, complete response; PR, partial response; DFS, disease-free survival rate; TTF, time to treatment failure; MTD, maximum tolerated dose; PCP, *Pneumocystis carinii* pneumonia.
[a]One patient with T-cell PLL without prior treatment was included.

study, molecular remissions, i.e., the disappearance of polymerase chain reaction (PCR)-detectable *bcl-2* rearrangement in the bone marrow and blood, were noted in patients treated with Idec-C2B8.[248] This has not been observed after treatment with CHOP. The lack of significant myelosuppression and the short treatment schedule are other attractive features of this new agent. Its major side effects are fever, chills, and occasionally hypotension. These effects are mostly seen during the first dose. Eight cases of anaphylactoid reactions have been reported among more than 12,000 patients treated worldwide. Because the antibody is partially humanized, it does not significantly induce neutralizing antibodies. [252]

Iodine-131 anti-B1 is a nonhumanized mouse antibody that is labeled with radioactive iodine. Its main toxic effect is myelosuppression. It has been used in high doses with autologous stem cell support and also in lower doses that do not induce permanent myelosuppression. [253] In Phase I studies, durable remissions have been obtained in patients with indolent and more aggressive NHL. [253] [254] This drug is currently under investigation in a Phase II study.

Anti-CD52 (Campath-H1) has been mainly tested in Europe for the treatment of CLL and has resulted in a high incidence of responses, some of which are durable. [247] It is a humanized antibody and therefore does not cause the production of neutralizing antibodies. Its main side effect is profound immunosuppression. Reactivation of HSV and several cases of *P. carinii* pneumonia have occurred during treatment with this drug. Anti-idiotypic antibodies are antibodies directed against the idiotypic determinants of the surface immunoglobulin of the patients lymphoma. In most patients such antibodies can be induced by injection of a modified lymphoma immunoglobulin.[244] The generation of such antibodies leads to durable molecular remissions in patients with minimal disease. [553] While currently not applicable in large numbers of patients, these data are of considerable interest.

High-Dose Chemotherapy and Autologous or Allogeneic Transplantation

Patients with recurrent follicular lymphoma have a poor prognosis. The median survival after first recurrence is estimated to be 4.5 years for patients with follicle center cell lymphomas and decreases with successive recurrences. [255] Autologous transplantation has been used as salvage treatment for patients with chemosensitive recurrences of indolent NHL. [256] [257] [258] [259] The initial reports on autologous transplantation indicated a high incidence of durable responses. With increasing follow-up, higher recurrence rates have been documented, [260] as has a considerable risk for secondary myelodysplastic syndromes. [257] [261] [262] [263] Recurrences may in part be due to the reinfusion of occult lymphoma cells, and improved marrow purging techniques may lead to improved results. [264] Recently, allogeneic transplantation has been proposed for the treatment of patients with recurrent low-grade lymphoma. A number of reports indicate the potential for long-term disease-free survival. [145] [265] [266] [267] [268] [269] [270]

Disease Transformation

Disease transformation to a higher-grade malignancy is a common event in follicle center lymphoma. Clinically, transformation occurs in at least 2030% of patients with follicle center lymphoma. It is probably a much more frequent event that is underdiagnosed because biopsies are not always performed at the time of recurrence.[186] [271] [272] [273] [274] [275] [276] [277] [278] Disease transformation should always be suspected at the time of disease progression or recurrence, especially if the serum LDH level is elevated or in the face of rapidly enlarging lymph nodes, constitutional symptoms, or invasion of extranodal sites such as lung, pleura, CNS, or bone. Repeat biopsies should be done to establish the diagnosis and guide further treatment. The use of FNA has facilitated the diagnosis of transformation.

The median survival after disease transformation ranges from 7 to 22 months, but 20% of patients with disease transformation, mainly those with low tumor bulk, low LDH levels, or those who have not been exposed to combination regimens containing alkylating agents and anthracyclines, can achieve prolonged survival. [186] [279] Some patients with transformed disease have successfully undergone high-dose chemotherapy and autologous or allogeneic transplantation. [140] [280]

Marginal Zone Lymphoma

B-cell lymphomas of the mucosa-associated lymphoid tissue (MALT lymphomas), monocytoid B-cell lymphomas, splenic marginal zone lymphomas, and splenic lymphoma with villous lymphocytes are four independently recognized and described entities. However, their morphologic, immunophenotypic, cytogenetic, and molecular characteristics suggest that they are very closely related disorders occurring in different organs, [1] [281] and they have therefore been grouped under the name marginal zone lymphomas. Their normal counterpart is thought to be the marginal zone B cells that are located outside the mantle zone of the lymphoid follicle. This marginal zone is clearly seen in the spleen and in Peyers patches, but not in peripheral lymph nodes. Cells from the marginal zone have the capacity to differentiate into plasma cells and home to specific tissues.

Morphologically marginal zone lymphomas are characterized by a rather heterogeneous infiltrate of cells ranging from centrocytes to monocytoid B cells, small lymphocytes, and plasma cells. When localized in lymphoid tissue, they usually infiltrate the marginal zones. Occasionally there is colonization of the follicles. Extranodal marginal zone lymphomas are characterized by this same pleomorphic infiltrate as well as by the presence of lymphoepithelial lesions, the hallmark of MALT lymphomas. When peripheral blood involvement occurs, monocytoid cells predominate, but villous lymphocytes or cells indistinguishable from CLL can be observed.[282] [283]

Immunophenotypically these disorders are B-cell malignancies. CD5, CD22, CD23, and CD10 expression is absent (in contrast to what is seen in CLL), and 40% of cases express cytoplasmic immunoglobulin. A monoclonal serum IgM or IgD can occur in some cases with nodal and bone marrow involvement but is usually absent in cases of MALT lymphoma. The immunoglobulin genes are consistently rearranged, confirming the clonal nature of the disorder, even when the infiltrate is pleomorphic. No rearrangements of *bcl-1, bcl-2, bcl-6,* or c-*myc* have been consistently demonstrated.[1] Common cytogenetic abnormalities include trisomy 3 in two-thirds of cases, trisomy 18 in 40% of the cases, and structural changes involving 1q21 (in 40% of cases) or 1p34 (in 15% of cases). [281] [284] [285] [286] [287] Although these abnormalities are not pathognomonic for marginal zone lymphomas, they occur at a much higher frequency than in other lymphomas. There are differences in presentation and treatment among the different types of marginal zone lymphomas, as discussed in the following sections.

MALT Lymphomas

The extranodal presentation of marginal zone lymphoma is called MALT lymphoma. [288] [289] [290] [291] MALT lymphomas occur in many organs, including the stomach, small intestine, parotid, bronchial tissue, thyroid, and other nonmucosal organs such as skin, breast, liver, pancreas, and kidney. They are commonly associated with chronic inflammatory conditions such as Sjögren syndrome in the parotid, [292] Hashimoto thyroiditis, or *H. pylori* infection in the stomach.

Patients with MALT lymphomas typically present with indolent and localized, stage I or II disease and without systemic symptoms. In a recent study, the predicted 5- and 10-year survival rates were 87% and 75%, respectively. [289] The few patients with stage III or IV disease appear to have a more aggressive course, with a median survival of 3 years.[153] Also, MALT lymphoma of the thyroid has an unusually aggressive behavior.

Gastric MALT lymphoma is the most common presentation of MALT lymphoma. This entity is similar to what used to be called pseudolymphoma of the stomach. Typically patients are

diagnosed during endoscopy performed for vague abdominal discomfort.[293] The endoscopic findings are those of gastritis or ulcer; a mass is less common. Endoscopic US is very useful in better defining the extent of disease in the stomach and the response to treatment. [294] Although the disease rarely spreads to other organs, dissemination to other parts of the GI system can occur and has been shown to be due to the same tumor clone. [295] Gastric MALT lymphoma usually arises as a consequence of chronic stimulation of MALT by *H. pylori*.[296] [297] The treatment of *H. pylori* with antibiotics results in eradication of the bacteria and in regression of the lymphoma in the majority of patients with indolent MALT lymphoma.[298] [299] [300] The most popular antibiotic regimen is a metronidazole-amoxicillin combination plus omeprazole. A 2-week regimen is considered sufficient treatment to eradicate *H. pylori* and produce an antitumor response in most cases. Clarithromycin has been used mostly as a second-line treatment. Histologic regression of the lesions can take from 3 to 18 months to occur. Those with localized disease in whom antibiotic therapy fails may benefit from radiation therapy, which induces high response rates. [301] Antibiotic therapy has mainly been used in cases with flat mucosal lesions. Large tumor masses also respond well to antibiotics, but less frequently. It is unknown whether patients whose disease has spread to regional lymph nodes respond to antibiotic treatment. Those with more extensive disease can be treated with single-agent chlorambucil or, if this treatment fails, with CHOP or similar regimens. For nongastric MALT lymphomas single-agent chemotherapy or simple combination chemotherapy regimens such as the COP regimen have been used with good success.[291] Radiation therapy has also been used in selected cases. [289] [290] A response to antibiotic therapy has been reported in a case of parotid MALT lymphoma and leads to speculation that such treatment might be effective in some cases of non-GI MALT lymphoma.[302]

Monocytoid B-Cell Lymphoma

Monocytoid B-cell lymphoma is the lymph node form of marginal zone lymphoma. Considerable overlap exists between this disorder and MALT lymphoma, and part of

the difference between the two disorders is semantic. The median age for patients with monocytoid B-cell lymphoma is 60. There is a preponderance of females and an association with autoimmune disorders such as Sjögren syndrome. Patients usually present with stage II disease and involvement of peripheral lymph nodes. In many reported cases there has also been involvement of extranodal organs such as the parotid or stomach; therefore those cases could also be considered stage II MALT lymphoma. Bone marrow or peripheral blood involvement is rare.[283] Monoclonal gammopathies can occur.[303] [304] Patients with monocytoid B-cell lymphoma usually have indolent disease and no systemic symptoms, although disease transformation can occur. No prospective treatment studies have been reported in this disorder. Patients have responded to a variety of chemotherapy regimens. Based on the available information, treatment should be tailored to the individual patient, the disease characteristics, age, and performance status. As in follicular lymphomas, minimal therapy may be indicated for older patients and more aggressive approaches for younger patients

Splenic Marginal Zone Lymphoma and Splenic Lymphoma with Villous Lymphocytes

The third component of marginal zone lymphomas is splenic marginal zone lymphoma, which in most cases represents the same disease as splenic lymphoma with villous lymphocytes.[282] [305] [306] [307] This disorder occurs mainly in the elderly (median age, 70 years) and has a male preponderance. Patients typically present with massive splenomegaly. Bone marrow involvement and involvement of the peripheral blood by abnormal lymphocytes with irregular cytoplasm are almost always present. The white blood cell (WBC) count, however, rarely exceeds 30,000/l. Lymphadenopathy, if present, is not very striking. The majority of symptoms are related to splenomegaly and include abdominal discomfort and cytopenias. A small monoclonal spike is common. It is important to distinguish this disorder from hairy cell leukemia, CLL, prolymphocytic leukemia, Waldenström disease, and mantle cell lymphoma with peripheral blood involvement. The differential diagnosis is based on the clinical picture and on histopathologic studies, including immunophenotyping and cytogenetic assays.

Splenic marginal zone lymphoma is an indolent disorder with, in one series, 78% survival at 5 years, and only one-third of deaths due to lymphoma.[305] For symptomatic patients, splenectomy usually results in rapid improvement. For those unable to tolerate splenectomy, splenic irradiation has been used. Only one-third of the patients responded to single-agent chemotherapy such as chlorambucil. Other drugs such as interferon or nucleoside analogs have not been extensively tested.

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PRESENTATION AND MANAGEMENT OF T-CELL AND NK CELL LYMPHOMAS

Lymphoblastic Lymphoma

T-cell NHLs are clonal proliferations corresponding to different stages of maturation of T cells. By definition, peripheral T-cell lymphoma corresponds to post-thymic stages of development. They lack terminal deoxynucleotidyl transferase (TdT), an enzyme important in T-cell receptor gene rearrangement. By contrast, T-lymphoblastic lymphoma corresponds to an intrathymic or prethymic stage of T-cell development and expresses TdT. [308] Young adults and children are the most commonly affected by T-lymphoblastic lymphoma and present with large mediastinal masses, commonly associated with bone marrow and peripheral blood involvement. As in Burkitt disease a rapid diagnosis is important and should, if possible, be based on the examination of bone marrow or peripheral blood, the latter examination to include immunophenotyping. As in other high-grade lymphoid malignancies, a lumbar puncture is essential. The disease is closely related to T-cell ALL and should be treated as such, even in the occasional case with isolated mediastinal involvement. [309] [310] [311] [312] An alternative treatment is the so-called Stanford regimen. This consists of a CHOP-like induction regimen in which the doses of anthracyclines are increased. [313] This induction treatment is followed by cranial irradiation, consolidation with further CHOP chemotherapy, and ALL-type maintenance treatment. A high incidence of CNS recurrence has been reported after treatment with this regimen.[314] (These issues are discussed in Chaps. 58 and 59 .) A recent randomized trial from Europe indicates an improvement in relapse-free survival for patients undergoing autologous transformation in first remission. [554] Follow-up in this trial, however, is very short. B-cell lymphoblastic lymphoma is an extremely rare entity that in many instances may represent either a blastic variant of mantle cell lymphoma or an extramedullary presentation of common ALL. [308] Its treatment is not well defined, but it has a poor prognosis.

Adult T-Cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma (ATL) is a T-cell malignancy caused by infection with the HTLV-1 virus. HTLV-1 infection is widespread in Japan and in some of the Caribbean islands such as Jamaica, and most affected patients live in these areas or originate from them. The estimated cumulative risk of development of ATL in an individual who has been exposed to HTLV-1 ranges between 0.5% and 7%. Another disorder associated with infection by HTLV-1 is tropical spastic paraparesis, a progressive encephalomyelitis involving the pyramidal tracts.

The usual clinical presentation of ATL is that of an acute disorder, with a leukemic picture characterized by peripheral blood involvement with lymphocytes with convoluted nuclei similar to those of Sézary cells. These cells are usually CD4+ and CD8. Skin involvement and lymph node involvement are common, as is hypercalcemia. The marrow is involved in 60% of cases. In addition to the acute form, three other subtypes are recognized. The smoldering form presents with predominantly skin and lymph node involvement. The peripheral blood usually contains small numbers of abnormal T cells. The chronic leukemia form is associated with increased abnormal lymphocytosis but minimal organ involvement. In the lymph node form, no or minimal blood or marrow involvement is present but the patients present with a lymphoma that is difficult to differentiate from other forms of T-cell lymphoma. [315] Patients with the smoldering type of ATL and some with the chronic leukemia type have a relatively good prognosis even without treatment, although transformation to the acute type can occur. The lymphoma and acute types, on the other hand, have a very poor long term outcome, with a median survival of only 9 months. The disease is very refractory to treatment. In 1995 Gill et al. reported responses in 10 of 19 patients with ATL with a combination of azidothymidine and IFN-. [316] Similar results have been reported from France. [317]

Peripheral T-Cell Lymphomas

Peripheral T-cell lymphomas originate from T cells that have completed thymic maturation and are TdT negative. Many different subtypes of T-cell lymphoma have been recognized, some of which are well characterized and require special treatment. Mycosis fungoides is discussed in Chapter 74 . T-cell CLL or prolymphocytic leukemia is discussed in Chapter 72 . Other well-defined categories include Ki-1-positive anaplastic large cell lymphoma, AILD-like T-cell lymphoma, intestinal T-cell lymphoma, and T-cell lymphoma. Each of these subtypes is briefly discussed below. The majority of patients with T-cell lymphoma cannot currently be categorized in any of these types and are designated in the REAL classification as having peripheral T-cell lymphoma, unspecified. [1] The classification of peripheral T-cell lymphomas is evolving rapidly. Angiocentric lymphomas, which used to be considered T-cell lymphomas, are now known to be of NK cell or of B-cell origin and are discussed in the next section. Lymphomatoid papulomatosis is a benign skin disorder with a propensity to evolve into T-cell lymphoma and is discussed in this section. The diagnosis of T-cell lymphoma depends on immunophenotyping, and the disease may be indistinguishable clinically from B-cell NHL. Nevertheless, a higher incidence of extranodal disease and constitutional symptoms and the fairly frequent occurrence of hemophagocytic syndrome are considered typical of T-cell NHL. Differentiation from HTLV-1related lymphoma can be difficult as well, and HTLV-1 serology should be considered in patients with peripheral T-cell lymphoma.

Peripheral T-Cell Lymphoma of Angioimmunoblastic Lymphoma Type

Angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) is a T-cell malignancy with unique clinical and morphologic features. Patients present with lymphadenopathy, hepatosplenomegaly, skin rashes, profound systemic symptoms, hypergammaglobulinemia, and hemolytic anemia. [318] Many patients have a history of allergic reactions to drugs or prior viral infections. The constitutional symptoms often predominate, and other signs and symptoms such as lymphadenopathy or hemolytic anemia can have an intermittent course. The patients are often initially evaluated because of fever of unknown origin. The diagnosis depends on the demonstration of the typical histologic picture in the lymph nodes. Affected lymph nodes show an effaced architecture caused by a proliferation of small lymphoid cells and endothelial venules intermingled with variable proportions of B immunoblasts, plasma cells, and eosinophils embedded in a network of follicular dendritic cells. [318] Originally AILD was thought to be an autoimmune disorder, but cytogenetic and molecular studies have demonstrated its clonal nature, and currently the designation AILD-related T-cell lymphoma is commonly used.[319] Gene rearrangement studies have established the T-cell origin of the disorder in the majority of cases; occasionally immunoglobulin gene rearrangement, mixed T-cell and B-cell gene rearrangement, or even biclonality as indicated by T-cell receptor gene rearrangement studies has been observed. [320] [321] Some patients with AILD have been reported to ultimately develop B-cell immunoblastic lymphoma. [322] The development of Kaposi sarcoma has also been reported.

Untreated, the disease usually has a rapidly progressive course, but occasionally spontaneous remissions occur. Only one prospective study of the treatment of AILD has been reported.[323] Patients initially received prednisone. Those with life-threatening complications, recurrent disease, or refractory disease received the COPBLAM/IMVP-16 regimen. This is a second-generation anthracycline-containing chemotherapy regimen. Only eight of 28 patients receiving prednisone achieved complete remissions, and only three of these were durable. The complete remission rate in response to COPBLAM/IMVP-16 was 59% (17 of 29 patients treated). The probability of event-free survival at 36 months was 32%. There were no recurrences beyond 1 year after the initiation of treatment, indicating that the disease is curable in a percentage of patients. Because of the low rates of response to steroid therapy and the fact that a substantial number of patients deteriorated while receiving steroids, anthracycline-containing combination chemotherapy is recommended for all patients.

Enteropathy-Associated Intestinal T-Cell Lymphoma

Lymphomas of the small intestine, especially of the jejunum, are commonly of T-cell origin. Such cases represent 7% of all GI lymphomas. T-cell lymphomas often occur in patients with a history of gluten enteropathy or dermatitis herpetiformis, or both, and are therefore called enteropathy-associated T-cell lymphoma (EATCL).[324] Occasionally, patients without a history of prior GI disorders are diagnosed with T-cell lymphoma involving the small intestine, and some patients develop mild or atypical gluten enteropathy after the diagnosis of lymphoma has been made. The term EATCL-like NHL is used to classify such cases. [325] Patients with EATCL present usually in their fifth or sixth decade with worsening symptoms of diarrhea and malabsorption. Many also present with an acute abdominal event such as perforation or obstruction. The entire length of the jejunum may be affected and a mass may be present. A pleomorphic infiltrate of abnormal lymphocytes that have a clonally rearranged T-cell receptor and a variable degree of villous atrophy are present. [1] [325] The lymphoma often involves the abdominal lymph nodes but rarely spreads beyond the abdominal cavity. Systemic symptoms and elevations in LDH levels or other signs of systemic disease are uncommon. The much increased risk of NHL in patients with gluten enteropathy and the occasional observation of monoclonal T cells in the biopsy specimens of patients with otherwise straightforward gluten enteropathy have led to speculation that gluten enteropathy itself represents an indolent form of NHL. [326] [327]

No satisfactory treatment for EATCL exists. There is no response to the institution of a gluten-free diet, and curative surgery is often precluded by the extent of involvement. No prospective studies of chemotherapy are available, but in several retrospective series of intensive combination chemotherapy, the

prognosis was poor. Remissions are usually short, with the median survival <1 year from diagnosis, although occasional long-term survivors have been reported. [325] [328]

Ki-1-Positive Anaplastic Large Cell Lymphoma

In 1982, the presence of a new antigen expressed on Reed Sternberg cells of patients with Hodgkin disease was reported. The antigen was named Ki-1 (CD30 or Ber H2) and was later found to represent a membrane protein that belongs to the family of tumor necrosis factor (TNF) receptor molecules. [329] A number of cases of NHL with anaplastic features were found to display CD30 as well and have been identified as a specific disease entity, termed Ki-1-positive anaplastic large cell lymphoma.[330]

Clinically, three types are recognized, cutaneous Ki-1-positive anaplastic large cell lymphoma, nodal Ki-1-positive anaplastic large cell lymphoma, and HIV-associated Ki-1-positive anaplastic NHL. [331] [332] [333] [334] [335] [336] Some cases of cutaneous involvement are secondary lymphomas occurring after a transformation from mycosis fungoides, lymphomatoid papulomatosis, or Hodgkin disease. The entity of anaplastic Ki-1-positive NHL in association with HIV infection [337] is discussed in the section on HIV-related NHL.

Patients with primary cutaneous disease usually present with localized or solitary nodules or tumors. Lymph node involvement can occur. The disease is fairly indolent, and spontaneous remissions occur in 25% of cases. [4] [331] Cutaneous Ki-1-positive anaplastic large cell lymphoma is only rarely associated with t(2;5) and probably represents a different biologic entity than the nodal form. The management of cases with isolated skin disease should include careful staging and observation to ascertain the behavior of the disease so that lymphomatoid papulosis can be ruled out. In selected cases, local surgery or radiation therapy, or both, may suffice.

The age distribution of nodal anaplastic Ki-1-positive NHL is bimodal, with a first peak in the second and third decades and a second peak after age 60. The majority of patients present with extensive and rapidly progressive disease. Extranodal involvement is fairly common and includes skin involvement (Plate 70-1).[338] Mediastinal masses can occur, but the incidence varies considerably among different studies. Most likely the majority of cases with mediastinal masses represent Hodgkin-related anaplastic large cell lymphoma and not classic anaplastic large cell lymphoma.

Morphologically, anaplastic large cell lymphoma is characterized by a pleomorphic infiltrate with a majority of anaplastic large cells. [1] [330] [332] [339] [340] [341] There is a predilection for involvement of the T-cell areas of the lymph node and of the lymph node sinuses. B-cell areas are commonly spared. Skin involvement is characterized by a nonepidermotropic infiltrate. In most series, the majority of cases have a cytotoxic (CD8) T-cell phenotype, but cases with a null cell phenotype are also common. In such cases distinguishing the entity from carcinoma, melanoma, sarcoma, or Hodgkin disease can be extremely difficult, as the tumor cells may lack expression of all lineage-specific markers. B-cell anaplastic large cell lymphomas with Ki-1 positivity also occur but are not currently recognized by the REAL classification as part of the Ki-1 anaplastic large cell entity. Translocation t(2;5) (p23;q35) is commonly detected in cases of nodal Ki-1 anaplastic large cell lymphoma.[341] This translocation leads to the production of an aberrant tyrosine-kinase resulting from the fusion of the ALK-kinase (anaplastic lymphoma kinase) on chromosome 2 and the *NMP* (nucleophosmin) gene on chromosome 5. The t(2;5) translocation is not found in cases of primary cutaneous anaplastic Ki-1 lymphoma or in Hodgkin disease. [342] [343] [344] [345] [346] [347] [348] [349] Interestingly, expression of the complete, nonrearranged ALK-kinase was reported in a newly recognized subtype of large B-cell lymphoma that does not carry t(2;5) and does not express CD30. [350]

Initial reports indicated a poor prognosis for Ki-1-positive anaplastic large cell lymphoma. Subsequent studies of treatment with intensive anthracycline-based combination chemotherapy have demonstrated that a high proportion of complete remissions can be obtained and that a large percentage of these remissions are durable. [332] [334] [335] [336] [340] In a recent French cooperative group study on the treatment of aggressive lymphoma, the subset of patients with anaplastic large cell lymphoma had a more favorable outcome than other patients.[338]

Lymphomatoid Papulomatosis

Lymphomatoid papulomatosis is a clinically benign, histologically malignant cutaneous lymphoproliferative disorder. [4] [351] In this entity the clinical picture overrides the microscopic appearance of the disorder. Patients typically present with multiple papular or nodular lesions of the skin in different stages of development. The disease has a chronic, recurrent course. Individual skin lesions appear and disappear within 36 weeks. Histologically there is a dermal infiltrate of malignant-appearing anaplastic cells expressing CD30, rendering the diagnostic distinction from anaplastic large cell lymphoma difficult. In other instances there is epidermotropism and infiltration by lymphoid cells with cerebriform nuclei akin to mycosis fungoides. Rearrangement of T-cell receptor genes can be demonstrated in most cases. The clinical criteria for the diagnosis of lymphomatoid papulomatosis are (1) the appearance of multiple small (0.52.0 cm) nodular or papular skin lesions, (2) spontaneous regression followed by recurrence, i.e., waxing and waning, and (3) no systemic or lymph node involvement. Management may consist of observation. If the cosmetic appearance is compromised, either PUVA therapy, topical chemotherapy, or low doses of methotrexate can be used. The initial response rate is high, but response is usually followed by recurrence. There is a very high cumulative risk for development of a secondary large cell lymphoma or Hodgkin disease (up to 80% in one series).[351]

Hepatosplenic T-Cell Lymphoma

Hepatosplenic T-cell lymphoma is a newly recognized disorder. [352] [353] [354] It has been reported mainly in young males who present with aggressive lymphomas involving the liver and spleen. Rearrangement of the T-cell receptor has been demonstrated in these cases. Isochromosome 7q has been described as a specific cytogenetic finding. The cells in this disorder can be CD56 positive, and therefore hepatosplenic T-cell lymphoma should be considered in the differential diagnosis of NK cell leukemia/lymphoma. The disease has a rapidly progressive course, and no effective treatment is known. Several cases of nonhepatosplenic T-cell lymphoma have also been reported. [355] Affected patients present with involvement of skin or mucosal surfaces.

T-Cell Lymphoma, Unspecified

The majority of patients with T-cell lymphoma cannot be classified in any of the above described categories and are classified as having T-cell lymphoma, unspecified. This is a very heterogeneous group of malignancies, some indolent and others more aggressive. [1] [356] Patients with aggressive histologic types are usually treated in a fashion similar to patients with B-cell lymphoma; they more commonly present with extranodal disease and poor prognostic features, and their prognosis is generally somewhat worse than that of patients with aggressive B-cell lymphomas. [44] [76] [357] [358] [359] [360] [361] [362]

Because of the major differences in disease biology between B- and T-cell lymphomas, it is expected that new drugs will be

identified with specific activity in T-cell lymphomas. A number of drugs specific for mycosis fungoides have already been developed (see Chap. 74). Other drugs under evaluation include 2-CDA, retinoic acids, monoclonal antibodies, and Ara-G. 2-CDA has activity against mycosis fungoides, and in a pilot study was also effective in a small number of patients with other T-cell lymphomas.[363] Retinoic acids are differentiating agents. In a Phase I trial from Taiwan, responses were observed in six of 12 patients with T-cell NHL, versus none of six with B-cell NHL.[364] Campath-H1 (anti-CD52) is a humanized monoclonal antibody. It has activity in B-cell malignancies, but has also been tested in T-cell prolymphocytic leukemia. Major responses were observed in 11 of 15 patients.[365] Finally, compound 506U (Ara-G) is a 6-methoxy derivative of arabinosyl guanine that preferentially accumulates in immature T cells and is converted there to Ara-GTP. In a Phase I trial, responses were observed in 19 of 25 patients with T-cell ALL, in four of six patients with T-cell CLL and in eight of 12 patients with peripheral T-cell lymphomas. The response rate in patients with B-cell disorders was only 25%.[366]

Angiocentric Lymphomas

The term angiocentric immunoproliferative lesions was proposed in 1984 to encompass a spectrum of disorders characterized by angiocentric and angiodestructive lymphoid proliferation.[367] These disorders include lymphomatoid granulomatosis in the lung and sinonasal lymphoma in the facial area. Because of similar morphologic and immunophenotypic characteristics, these disorders were thought to represent closely related entities of T-cell origin. This appears not to be the case. Immunoglobulin gene rearrangements have been demonstrated in lymphomatoid granulomatosis, indicating its B-cell origin.[5] Sinonasal lymphoma, on the other hand, is currently thought to be an NK cell disorder.[368] Both lymphomatoid granulomatosis and sinonasal lymphoma are related to infection with EBV.[6] [369]

Patients with lymphomatoid granulomatosis typically present with diffuse pulmonary lesions.[370] [371] Secondary involvement of the skin or CNS is common. Lymphomatoid granulomatosis was originally described as an atypical reactive lymphoid infiltrate in the lung, associated with extensive necrosis and to be distinguished from Wegener granulomatosis. It rapidly became clear that lymphomatoid granulomatosis represented a form of lymphoma with a spectrum of histologic features. Various histologic grades are currently recognized.[371] Grade I is characterized by a polymorphous cellular infiltrate with an angiocentric and often angiodestructive character. Grade II lesions have the same polymorphic infiltrate but also have considerable cellular atypia, and grade III lesions are characterized by monomorphic vascular infiltrates of both large and small lymphoid cells. The lymphocytic infiltrate consists predominantly of mature T cells with a predominance of CD4+ cells, but the malignant cells are of B-cell origin, and the T cells represent merely an inflammatory infiltrate. In situ hybridization studies show EBV infection in the B cells.[5]

Historically, the disease had a poor prognosis, with a median survival of only 14 months.[370] Treatment with cyclophosphamide and steroids results in a high rate of remission, but also a high recurrence rate in patients with grade I histology.[371] Treatment with CHOP or similar regimens results in durable remissions in at least 50% of patients and is preferred by many investigators.[371] [372] [373] Recently, interferon treatment has been reported to be highly effective in a small number of patients, including some in whom chemotherapy had failed.[5]

Sinonasal lymphoma, previously known as lethal midline granuloma and polymorphic reticulosis, is an uncommon disorder in the West but is common in Asia and in the indigenous populations of Central and South America.[302] [369] [374] [375] [376] Patients present with destructive infiltrates of the nose and facial structures. The disease can spread in later stages to other nodal and extranodal sites. Owing to their proximity, the cranial nerves are commonly involved. Prospective studies of treatment are not available in this disorder. The prognosis for sinonasal lymphoma depends on the stage and extent of disease.[377] The treatment is discussed in the section on head and neck lymphomas.

Lymphomas of Large Granular Lymphocytes and Related Disorders

The syndrome of lymphocytosis with large granular lymphocytes (LGLs) was recognized more than a decade ago. Several closely related disorders of large granular lymphocytes, differing in phenotype and in biologic behavior, have since been recognized. New disease entities continue to be reported frequently, resulting in a confusing and still evolving classification.[6] [378] A list of currently recognized entities is given in Table 70-12 .

Immunophenotyping has played a major role in the recognition of these entities, which are either of T-cell or NK cell origin. Analysis of T-cell receptor gene rearrangement may be necessary to establish the diagnosis and to rule out inflammatory disorders. LGL morphology, although typical in some cases, is not always present and cannot constitute the sole basis for diagnosis in these disorders.[379] T-cell disorders usually express most of the pan-T-cell markers, namely, CD2, CD3, CD5, and CD7. They also often express either CD4 or CD8, or both. The NK cell marker CD56 is usually but not always absent. CD16, on the other hand, is commonly present. In cases with CD56 positivity, T-cell receptor gene rearrangement can establish the T-cell origin of the disorder.

NK cell malignancies by definition lack T-cell receptor gene rearrangement. They usually express the NK cell markers CD16 and CD56. They usually lack CD4 and CD8. They also lack the pan-T-cell markers CD3 and CD5 but express CD2 and CD7. Demonstration of clonality in these disorders can be difficult.

Chronic T-cell LGL leukemia (synonyms: chronic T-cell lymphocytosis with neutropenia, T8 chronic lymphocytic leukemia, granulated T-cell lymphocytosis, T-suppressor cell CLL, T-lymphocytosis, and lymphoproliferative disorder of T lymphocytes) is a disorder of T cells characterized by a moderate lymphocytosis (lymphocyte count is usually between 5×10^9 /L and 10×10^9 /L), often accompanied by profound neutropenia.[378] The median age at presentation is 60, and there is a slight male predominance. An indolent, asymptomatic form of this disorder may be fairly common.[379] Symptomatic patients often present initially with recurrent infections due to neutropenia and are often found to have a moderate lymphocytosis. But some patients with very low lymphocyte counts ($<2 \times 10^9$ L) may have demonstrable clonal T-cell populations and may be symptomatic as a result of neutropenia.[380] Moderate thrombocytopenia and anemia are common. Mild splenomegaly and hepatomegaly may be present. Lymphadenopathy is virtually always absent. There is a strong association with rheumatoid arthritis, and large granular lymphocytic leukemia may explain at least some of the cases that were formerly classified as Felty syndrome as well as some cases of cyclic neutropenia. Serologic abnormalities such as a positive rheumatoid factor assay, positive antineutrophil antibody (ANA) assay, and circulating immune complexes are demonstrated in at least half of patients. Hypergammaglobulinemia is frequent, and hypogammaglobulinemia has been noted in occasional patients. T-cell LGL leukemia should be differentiated from a number of viral infections, including acute infectious mononucleosis, acute CMV infection, and early HIV infection, all of which can be associated with lymphocytosis, and from disorders of NK cells.

The circulating lymphocytes often have abundant cytoplasm that contains azurophilic granules. The bone marrow is focally involved by an interstitial lymphoid infiltrate. Sinusoidal liver

TABLE 70-12 -- Classification and Treatment of NK Cell and NK-Cell-like T-Cell Disorders

Disease	Clinical Manifestations	Course	Cell Type	Phenotype[a]	EBV
Undifferentiated NK cell/myeloid leukemia[544]	Acute leukemia (MO or LBL), and extranodal disease	Aggressive	NK, myeloid precursor	**DR, CD33+, CD56+**, CD16, TCRr, TdT	
ALL of NK cell type[545]	Acute leukemia	Aggressive	NK cell precursor	**CD7+**, CD5, **CD2+, CD56±, TCRr**	?
Precursor NK cell leukemia[546]	Acute leukemia (M3 microgranular)	Aggressive	NK cell precursor	**DR, CD33+, CD56+**, CD16, TCRr+	
Aggressive NK cell leukemia[387] [388]	BM, PB, liver, spleen, skin	Aggressive	NK cell	CD13, CD33, CD7+, CD5, CD2+,**CD56+**, CD16±, **TCRr**	±

Aggressive TLGL leukemia/lymphoma[384]	BM,PB, liver, spleen, GI tract	Aggressive	Cytotoxic T cell	**CD3**+, CD5+, CD8+, **CD56**+, **TCRr**+	
Chronic TLGL leukemia/lymphoma[378]	Leukemia, neutropenia, rheumatoid arthritis	Indolent	Cytotoxic T cell	**CD3**+, CD5+, CD8+, **CD56, TCRr**+	
Nasal and nasal-type NK cell lymphoma[302] [369]	Extranodal tumors, sinonasal, skin and subcutis, GI tract, testis	Aggressive	NK cell	CD13, CD33, **CD7**+**, CD5**, CD2+, CD16±, TCRr	+
Lymphomatoid granulomatosis[5]	Lung, skin, brain, GI tract, kidney	Variable	B cell		+
Hepatosplenic T-cell lymphoma[352] [353] [354]	Liver, spleen, focal bone marrow	Aggressive	T cell	TCR+, CD56+	
Intestinal T-cell lymphoma[325]	GI tract	Aggressive	T cell	CD3+, CD56+	

Abbreviations: NK cell, natural killer cell; TLGL, T-cell large granular lymphocyte; BM, bone marrow; PB, peripheral blood; GI, gastrointestinal; TCR, T-cell receptor; TCRr, T-cell receptor gene rearrangement; TdT, terminal deoxynucleotidyl transferase; EBV, Epstein-Barr virus.

Modified from Jaffe,[6] with permission.

[a]The most relevant phenotypic characteristics are indicated in **bold**.

involvement and involvement of the splenic red pulp with sparing of the follicles and even follicular hyperplasia are typical. Immunophenotyping of the peripheral blood reveals usually a CD3+, CD8+, CD16+, CD56 phenotype, suggesting a cytotoxic/suppressor cell origin. T-cell receptor gene rearrangement is present. Rare cases have rearrangement of the T-cell receptor and are CD4, CD8+/. Occasional cases of TCR+, CD4+, CD8 LGL leukemia have been reported as well. Cytogenetic abnormalities have been demonstrated in some patients, but no specific karyotype has emerged. There may be an association with HTLV-1 or HTLV-2 in some cases.

The mechanism for the neutropenia associated with T-cell LGL leukemia is unclear. The marrow typically shows a maturation arrest of the myeloid series. Specific suppression of myelopoiesis by the LGLs, however, has not been demonstrated. Although ANAs are often present, their specificity is somewhat unclear. [378] The thrombocytopenia can be caused by the formation of autoantibodies. Pure red cell aplasia and Coombs-positive hemolytic anemia have also been reported.

T-cell LGL leukemia usually has an indolent but not altogether benign course. In one series, 20% of patients had died after 4 years of follow-up. [381] In other series, survival was better.[379] [382] Differences in prognosis may in part be attributed to inclusion of earlier and asymptomatic cases. The major causes of death are infections related to neutropenia and in some cases progressive lymphoproliferation. Hence, recurrent infections and progressive lymphoproliferation are the two major indications for treatment.

A variety of treatment approaches have been attempted for neutropenia.[378] Splenectomy has been used with variable success. Some patients have responded to granulocyte colony-stimulating factor (G-CSF). In patients with cyclic neutropenia, pulsed steroids have been useful. Low-dose oral methotrexate (520 mg/m ^{2} PO every week) was studied in ten patients. A complete response was achieved in five patients and a partial response in one. [383]

A rare, more aggressive variant of T-cell LGL leukemia has also been described. Patients presented with fever, marked hepatosplenomegaly, and mild lymphadenopathy. Lymphocytosis or bone marrow involvement was present in the majority of patients. The course was rapidly progressive, and five of six patients died within 26 months despite aggressive chemotherapy. Immunophenotyping in these cases revealed a T-cell phenotype with T-cell receptor gene rearrangement and expression of the T-cell receptor and of CD3. There was also aberrant expression of CD56, an NK cell marker, which according to the authors of this report indicated a more aggressive disorder.[384] Subsequently investigators from Japan have reported cases with a similar phenotype but an indolent clinical course. [385]

A disorder called chronic NK cell lymphocytosis shares many clinical features with T-cell LGL leukemia. [378] [386] It is less commonly associated with autoimmune disorders and has an indolent course. Patients typically present with lymphocytosis, usually with morphologic features of large granular lymphocytes. Immunophenotypically the cells are CD3, CD16+ and do not show T-cell receptor gene rearrangement. Clonality is often difficult to demonstrate in NK cell disorders as there is no NK cell receptor and no demonstrable gene rearrangement. Cytogenetic studies however, have demonstrated clonality in a minority of these patients. In others, the NK cell proliferation may be more inflammatory in nature.[378]

CD3, CD56+ LGL leukemias are clinically aggressive disorders of NK cell origin that occur in younger patients. [6] [387] [388] The median age in one series was 39 years. Patients present with fever, massive hepatosplenomegaly, and commonly jaundice. GI tract involvement and CNS involvement are also common. Blood, bone marrow, and other organs are involved by lymphomatous cells. The usual phenotype of these cells is CD3,

CD5, CD7+, CD16+, CD56+, CD8, CD4, suggesting an NK cell progenitor origin. The T-cell receptor is not rearranged. Clonality has been shown in several cases by the demonstration of karyotypic abnormalities. EBV infection of the malignant cells has been demonstrated in several Asian patients. This disorder has a rapidly progressive course, and most patients have died within a short period despite intensive treatment.

Composite, Discordant, and Hodgkin-Related Anaplastic Large Cell Lymphoma

The term *composite lymphoma* is used when two different but related lymphoma entities involve the same lymph node or organ. In many cases, one of the lymphomas represents a transformation of an indolent lymphoma to an aggressive type (such as follicular grade 1 small cleaved to B-cell large cell). However, a small number of cases of true composite lymphoma have been reported. In such cases, two presumably unrelated histologic types are detected in one single node (such as Hodgkin disease and large cell lymphoma).[389] [390] The treatment of such cases is usually dictated by the treatment of the most aggressive component.

More commonly one encounters cases of *discordant* (*divergent*) *lymphoma*, in which different subtypes of lymphoma involve different lymph nodes or extranodal sites. It is common to find involvement of the bone marrow with low-grade malignancy simultaneously with large cell lymphoma in the lymph nodes.[29] There is also an association between the occurrence of mycosis fungoides in the skin and Hodgkin disease in the lymph nodes in which both are derived from the same clone. [391] Other associations are those of B-cell NHL or, rarely, T-cell NHL after Hodgkin disease, [392] [393] of paraproteinemia in Sézary syndrome,[394] and of B-cell immunoblastic lymphoma after AILD. Possible pathogenetic mechanisms for such associations include (1) chronic immunostimulation by cytokines secreted by the original malignancy; this is thought to play a role in secondary lymphomas in AILD; (2) a common clonal origin for the different lymphoid disorders, as has been demonstrated in cases of Hodgkin disease associated with mycosis fungoides;[391] (3) chronic viral infection, which induces sequential neoplasms; [395] or (4) chemoradiotherapy-induced secondary malignancy.

Finally, a newly recognized entity, *Hodgkin-related anaplastic large cell lymphoma*, is an aggressive disease seen in young adults, who often present with large mediastinal masses. The tumor is composed of confluent sheets of lymphoma cells similar to anaplastic large cell lymphoma; but the architectural features, such as the presence of collagen bonds, resemble nodular sclerosing Hodgkin disease. In the newly proposed WHO classification, this entity would be called anaplastic large cell lymphoma-like Hodgkin disease in recognition of the fact that it most likely represents a histologic variant of Hodgkin disease. This disorder appears to have a poor prognosis when treated with regimens designed for Hodgkin disease. [396] [397]

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CASTLEMAN DISEASE AND OTHER LYMPHOMA-LIKE DISORDERS

Occasionally, patients present with lymphadenopathy, skin lesions, or systemic symptoms suggestive of lymphoma but the biopsy is not conclusive. Such cases pose major diagnostic and therapeutic dilemmas. In a retrospective review of 172 such cases with prolonged follow-up, some form of malignancy, including lymphoma, myeloma, thymoma, and Castleman disease, developed in 43%. Other diagnoses included infections (EBV, toxoplasmosis, HIV) in 10% of cases, rheumatologic disorders (Sjögren syndrome, systemic lupus erythematosus) in 4%, and sarcoidosis in 6%. [398]

A number of disorders that are closely related to lymphoma may also pose differential diagnostic problems. They include Castleman disease, [399] [400] [401] true histiocytic lymphoma,[402] [403] and rare polyclonal or reactive disorders associated with lymphadenopathy. [404] [405] [406] [407] An awareness of these disorders is necessary in order to diagnose unusual cases and because some of these entities require specific management. In addition, the study of some cases that are related to mutations in the *fas* gene has provided important information on lymphocyte biology. [405]

Castleman disease consists of a massive growth of lymphoid tissue presenting either as localized or as multicentric disease. Histologic findings include follicular hyperplasia and marked capillary proliferation; in the plasma cell variant, there is a massive accumulation of polyclonal plasma cells in the interfollicular space. The localized form of the disease is usually asymptomatic and is found incidentally on chest radiographs as a mediastinal mass. Cases of abdominal localization have also been reported. Localized Castleman disease is a self-limited process; surgical resection is usually curative. [399]

The multicentric form of Castleman disease is much more aggressive. Patients present with widespread adenopathy or constitutional symptoms, or both. [408] Hepatic or splenic enlargement is present in the majority of cases. Infections, transformation to non-Hodgkin lymphoma, and the development of Kaposi sarcoma are common causes of death. Only limited information is available on the treatment of this disorder. Treatment with prolonged courses of high-dose steroids (e.g., prednisone, 12 mg/kg/day for several months) has been reported to result in durable remission. Other treatment strategies have consisted of the administration of combination chemotherapy regimens, which has resulted in favorable responses in many cases, [409] or of radiation to areas of bulky disease. Responses to interferon treatment have also been reported. Recently, Castleman disease has been shown to be related to infection with human herpesvirus 8 (HHV-8), the virus responsible for Kaposi sarcoma.[410] [411]

DIAGNOSIS AND MANAGEMENT OF LYMPHOMA INVOLVING EXTRANODAL SITES

Approximately 40% of all NHLs present in extranodal sites. The most common sites of extranodal involvement are the gut and the skin, but almost every organ can be affected by NHL. Some sites are associated with unique subtypes of NHL and require specific management. In other sites, various disease histologies can be found, and appropriate management requires an awareness and diagnosis of the exact disease subtype.

Differential Diagnosis and Management of GI Lymphomas

The GI tract is the most frequently involved extranodal localization in NHL. In order of frequency the following sites may be involved: stomach, ileum, jejunum, and, rarely, the colon. Factors predisposing to GI lymphoma are infection with *H. pylori* in MALT lymphoma of the stomach,[293] [296] and celiac disease in EATCL.[324] [325] [326] Both indolent lymphomas and aggressive lymphomas are encountered. A classification and a staging scheme have recently been proposed and are summarized in Tables 70-13 and 70-14 , which list most types of NHL that can occur in the GI tract.[412]

Lymphoma Involving the Small Intestine

In developed countries the differential diagnosis for small intestinal NHL includes MALT NHL, extranodal presentation of mantle cell lymphoma, EATCL, Burkitt disease, or extranodal manifestations of large cell lymphoma. Small intestinal MALT may require surgery for diagnosis and is usually treated by resection. Adjuvant chemotherapy is often administered.[289] The

TABLE 70-13 -- Histologic Classification of Gastrointestinal Non-Hodgkin Lymphomas

B-cell lymphomas
Lymphomas of mucosa-associated lymphoid tissue (MALT)
Low-grade B-cell lymphoma of MALT
High-grade B-cell lymphoma of MALT, with or without evidence of a low-grade component
Mediterranean lymphoma (immunoproliferative small intestinal disease), low grade, mixed, or high grade
Malignant lymphoma centrocytic (mantle cell or lymphomatous polyposis)
Burkitt-like lymphoma
Other types of low- or high-grade lymphoma corresponding to peripheral lymph node equivalents
T-cell lymphomas
Enteropathy-associated T-cell lymphoma
Other types not associated with enteropathy

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management of mantle cell lymphoma is by aggressive combination chemotherapy and was discussed earlier. EATCL NHL is usually treated with aggressive chemotherapy but has a poor prognosis. Burkitt disease can currently be successfully treated with chemotherapy in many cases.

In developing countries, and especially in the Middle East, involvement of the small intestine by a special form of B-cell immunoproliferative disease, immunoproliferative small intestinal disease (IPSID), is common and may be related to chronic GI infection.[413] [414] [415] This disorder is virtually nonexistent in the West and is not included in the REAL classification. Usually persons in the second and third decades of life are affected. There is an association with low socioeconomic status. The disease is preceded by a reversible premalignant phase that is characterized by malabsorption and weight loss. In the blood or GI fluids, or both, a monoclonal protein can usually be detected that represents an aberrant heavy chain. The small intestine shows a lymphoplasmocytic infiltrate, usually with preserved villous architecture (in contrast to EATCL, where the villous architecture has often disappeared). Lymph nodes as well may have a plasmocytic infiltrate, but the lymph node architecture is usually preserved. At this stage the disease often responds to treatment with antibiotics (usually tetracycline). Commonly there is chronic GI infection, suggesting a possible infectious etiology for this disorder. In contrast to MALT lymphoma, a specific infectious etiology has not been demonstrated.

As the disease progresses, it invades the submucosa and may invade the entire intestinal wall and the mesenteric lymph nodes. Although the advanced stages might not respond as well, there have been occasional reports of response to antibiotic therapy. No prospective studies of chemotherapy have been performed in this disease, but patients are usually treated with

TABLE 70-14 -- Staging System for Gastrointestinal Non-Hodgkin Lymphomas

Stage	Description
I	Tumor confined to gastrointestinal tract without serosal penetration (one or multiple primary sites)
II1	Local (gastric, mesenteric) lymph node involvement
II2	Distant (para-aortic, caval) lymph node involvement
IIE	Penetration of serosa to involve adjacent organs or perforation/peritonitis
IV	Disseminated extranodal involvement or a gastrointestinal tract lesion with supradiaphragmatic nodal involvement

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combination chemotherapy (CHOP or similar regimens) and tetracyclines. Prolonged antibiotic therapy is continued after a remission has been achieved. Durable remissions can be obtained in a substantial fraction.

Gastric Lymphoma

In the stomach, the most common form of lymphoma is indolent MALT lymphoma, but aggressive lymphoma either due to transformation of MALT or as an extranodal manifestation of Burkitt disease or diffuse large cell lymphoma can also be encountered. The treatment of gastric lymphoma has evolved considerably recently. Low-grade MALT is now usually treated with antibiotic therapy, especially if the disease is localized. [293] The most popular regimens are a combination of clarithromycin, amoxicillin, and omeprazole or amoxicillin, metronidazole, and omeprazole. Currently, a 2-week regimen is considered sufficient treatment to eradicate *H. pylori* and produce an antitumor response in most cases. Improvement can be expected to occur soon after initiation of antibiotics but can continue for 612 months after the antibiotics are discontinued.

The treatment of localized gastric large cell lymphoma is more controversial. The treatment of choice used to be surgery, which served both diagnostic and therapeutic purposes. It was thought that surgery could prevent chemotherapy-induced complications such as fatal GI tract bleeding. Recent studies indicate that the combination of chemotherapy (CHOP or similar regimens) with or without radiation therapy, or in some instances radiation therapy alone, is equally efficient and less debilitating, and that the risk of perforation with chemotherapy is minimal. [416] [417] [418] [419] [420] Surgery is currently considered unnecessary in the majority of cases.

B-Cell Lymphomas of the Skin

The majority of primary skin lymphomas are of T-cell origin and include mycosis fungoides, Sézary syndrome, and large cell lymphoma (Ki-1 positive or negative). [4] These disorders are discussed in Chapter 74 . Several types of primary skin lymphoma of B-cell phenotype have also been recognized. In their presentation, biology, treatment, and outcome they are somewhat different from other B-cell lymphomas. The classification of primary skin lymphomas of B-cell type as proposed by the EORTC study group is shown in Table 70-15 .[4] The EORTC defined primary cutaneous lymphoma as any lymphoma that is limited to the skin and remains limited to the skin for at least 6 months after initial diagnosis. NHLs with any degree of extracutaneous involvement were excluded from consideration in this classification with the exception of mycosis fungoides and Sézary syndrome. This restrictive definition of primary skin lymphoma may have led to exclusion of a number of cases with a more aggressive clinical behavior.

Three major types of primary cutaneous B-cell lymphoma are recognized by the EORTC: (1) primary cutaneous follicle center cell lymphoma (PCFCCL), (2) marginal zone lymphoma of the skin, and (3) large B-cell lymphoma of the leg. Rarer subtypes include intravascular large B-cell lymphoma (also called malignant angioendotheliomatosis) and plasmocytoma of the leg.

TABLE 70-15 -- EORTC Classification of Primary Cutaneous B-Cell Lymphomas

Follicle center cell lymphoma
Immunocytoma (marginal zone B-cell lymphoma)
Large B-cell lymphoma of the leg
Intravascular large B-cell lymphoma
Plasmocytoma
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Patients with PCFCCL present with localized plaques, papules, and tumors, often limited to the head and neck area or to the trunk. [4] [421] Untreated, the lesions enlarge, but dissemination to extracutaneous sites is uncommon. Histologically, a nodular or diffuse dermal infiltrate consisting of centrocytes and centroblasts is present. In contrast to mycosis fungoides, the epidermis is usually spared. Unlike nodal follicular center cell lymphomas, these NHLs do not have t(14;18) or rearrangements of *bcl-2.* Radiation therapy is the preferred mode of treatment. Only in rare cases with generalized skin lesions should chemotherapy be considered, but in those cases, CHOP chemotherapy is highly efficient. [421]

Marginal zone B-cell lymphomas (primary cutaneous immunocytomas) are considered extranodal marginal zone lymphomas or MALT lymphomas. Patients present with solitary or multiple, localized subcutaneous tumors that preferentially involve the extremities. Histologically a somewhat pleomorphic infiltrate is found that contains small lymphocytes, lymphoplasmocytoid cells, and plasma cells. In the central areas of the infiltrates variable numbers of T cells are intermixed. The lesions show clonal rearrangements of immunoglobulin genes, confirming their B-cell nature. Treatment usually consists of radiation therapy. The prognosis is excellent. [422] In one series the 5-year survival of 12 patients was 100%. [4] *Borrelia burgdorfferi*, the causative agent of Lyme disease, may play a role in the pathogenesis of some cases of PCFCCL and of primary cutaneous marginal zone lymphoma.[4] [422] In contrast to primary cutaneous immunocytomas, secondary cutaneous immunocytomas present with more widespread skin disease and paraproteins, and autoimmune disorders commonly coexist. Their prognosis is not as favorable as for primary cutaneous immunocytoma.

Large B-cell lymphoma of the leg typically affects older patients. Patients present with nodules or tumors on one or both legs. [423] Histologically there is a dermal infiltrate predominantly composed of large B-cells. Localized cases should be treated with CHOP chemotherapy followed by involved-field radiation therapy. [424] This is a more aggressive disorder than PCFCCL and marginal zone lymphoma. The estimated 5-year survival rate is 58%.

Although not included in the EORTC proposal, aggressive B-cell lymphoma can also be encountered in other skin areas (Plate 70-2). In a recent review of 54 patients with primary cutaneous lymphoma (excluding mycosis fungoides and Sézary syndrome), an aggressive histology was found in 50 patients. [424] Thirteen of 27 patients tested had a B-cell phenotype. The outcome of patients treated with anthracycline-based combination chemotherapy was significantly better than that of patients treated with radiation alone (10-year failure-free survival rate of 70% vs. 11%, $P < 0.0001$). On the other hand, the addition of radiation therapy to chemotherapy had no discernible impact on failure-free survival rates.

CNS Lymphoma

Three different entities of CNS lymphoma are recognized. The most common presentation is that of CNS lymphoma associated with AIDS (see Chap. 75). In the non-HIV setting, CNS disease can arise as a complication of systemic lymphoma (secondary CNS lymphoma) or as primary CNS lymphoma. CNS lymphoma should not be confused with extradural lymphoma, which, by compression, can cause CNS symptoms. The latter disorder has a good prognosis when treated with combined radiation therapy and chemotherapy. [425]

Secondary CNS lymphoma is commonly associated with high-grade NHL of Burkitt and lymphoblastic type, and intrathecal and systemic CNS prophylaxis is now usually part of the treatment for these disorders. In contrast, CNS involvement is extremely uncommon in follicular lymphomas or other indolent lymphomas. In aggressive lymphomas, CNS involvement occurs in approximately 5% of patients (Plate 70-3). [426] [427] Factors predictive of this complication include adverse prognostic features such as involvement of multiple extranodal sites and increased LDH levels. [426] Certain subtypes of lymphoma such as testicular lymphoma,[428] sinonasal lymphoma, [429] or aggressive variants of mantle cell lymphoma [171] may have a higher incidence of CNS recurrence than others. Routine CNS prophylaxis has been recommended in high-risk patients, but data on the efficacy of CNS prophylaxis are lacking. The few prospective studies on CNS prophylaxis have failed to demonstrate any survival benefit for patients so treated. [430] [431] The optimal approach to the prevention of CNS relapse of aggressive lymphoma remains difficult and may require a combination of intensive CNS-directed systemic chemotherapy and intrathecal prophylaxis. Secondary CNS lymphoma often occurs in association with, or is rapidly followed by, systemic recurrence and is usually rapidly fatal. In one study, only two of 23 patients with CNS recurrence survived beyond 1 year. [426] A small fraction of patients who respond to either radiation therapy or intrathecal treatment can successfully undergo consolidation treatment with high-dose chemotherapy. [432] [433] [434]

Primary CNS lymphoma is a diffuse infiltrative tumor that spreads along white matter neuronal tracks and through the CSF. [435] It typically affects patients between the ages of 40 and 60, who commonly present with symptoms related to intracranial hypertension, seizures, or generalized or focal neurologic deficits. A significant percentage of patients with primary CNS lymphoma have episodes of uveitis preceding the diagnosis of lymphoma for months to years. The diagnosis of primary CNS lymphoma should therefore be considered in any patient with steroid-resistant uveitis. Another commonly encountered presentation is that of a multiple sclerosis-like

illness, with exacerbations and (corticosteroid-induced) remissions. The radiologic appearance is that of a solitary tumor or multifocal lesions (Fig. 70-2). MRI indicates that many tumors are multifocal at diagnosis. There is a rapid and often complete disappearance of the tumor on CT after treatment with corticosteroids (ghost tumor). CSF abnormalities are common, although lymphoma cells can be demonstrated in the CNS in only one-fifth of cases. Like other brain tumors, the disease rarely spreads beyond the CNS. The classic management scheme consists of cranial radiation therapy. Typically 40 Gy is administered to the brain and the meninges with an additional 20 Gy boost to the tumor bed. Such treatment results in a median survival of at best a year, and a 2-year survival rate of at best 28%, with continued relapses beyond 2 years. [435] [436] [437] Most of the recurrences occur in the

Figure 70-2 Primary CNS lymphoma in a non-HIV-infected patient. Multifocal involvement is typical and occurs in at least one-third of cases.

tumor bed, indicating the limited efficacy of radiation therapy in this disorder. Based on the rates of response to systemic chemotherapy, [438] recent research has focused on the use of systemic chemotherapy prior to radiation therapy or sandwiched around the radiation treatment. Results from some studies have indicated improvement in disease-free survival, [440] while others have been more disappointing. [441] Prolonged remissions after combined chemoradiotherapy have been associated with a high incidence of debilitating encephalopathy, especially in older patients. [440] [548] Some investigators have, therefore, tested combinations of intravenous and intrathecal chemotherapy using agents with good penetration across the blood-brain barrier. [443] [561] Preliminary results of these treatment approaches without radiation are encouraging. Another interesting approach is that of blood-brain barrier disruption with the use of hyperosmolar solutions prior to chemotherapy. This has resulted in durable remissions and preservation of cognitive functioning, sometimes without radiation therapy. [442]

It is now generally accepted that chemotherapy has a role in the treatment of primary CNS lymphoma. The optimal chemotherapy regimen and schedule however, are far from agreed upon. The regimen should probably include drugs with activity across the blood-brain barrier, such as high-dose methotrexate and Ara-C. Other drugs such as cyclophosphamide, cisplatin, and vincristine have only limited penetration across the blood-brain barrier and may therefore have less of a role (although they have been used in some regimens).[435] [438] Primary CNS lymphoma is a relatively rare disorder that is difficult to treat. Ideally all patients should be enrolled in investigational treatment protocols.

Testicular Lymphoma

Testicular lymphoma is a disease of men in their 60s and 70s. It is the most common testicular tumor in that age group. [444] [445] [446] The majority of patients present with disseminated disease (i.e., stage III or IV disease). Most testicular lymphomas have a diffuse histology and an aggressive behavior. [447] The large majority of cases are of B-cell phenotype. This disorder has a high propensity to disseminate to other extramedullary sites, especially to the CNS.

A few patients are diagnosed with isolated testicular involvement, i.e., stage I disease. Simple orchiectomy is insufficient treatment for these patients. Current treatment recommendations include either contralateral orchiectomy or radiation treatment of the contralateral testis and a short course of doxorubicin-based chemotherapy (i.e., four cycles of CHOP). This results in durable remissions in >70% of such patients. [444] [448] [449] The role of pelvic and para-aortic radiation is unclear in this situation, as is that of CNS prophylaxis.

For stage III and IV disease, intensive chemotherapy and intrathecal CNS prophylaxis is recommended. Despite such treatment, only a small fraction of patients achieve durable long-term survival. [428] [444]

Orbital and Ocular Lymphoma

Orbital lymphoma affects the anterior compartment of the orbital cavitythe eyelids, lacrimal gland, and conjunctiva, or the retrobulbar space. [425] Most orbital lymphomas are of MALT origin and have an indolent behavior. A few are of follicular type. [450] [451] [452] The contralateral eye should always be examined carefully because bilateral diseases are common. The usual treatment consists of moderate doses of radiation (2535 Gy), which carries few risks of ocular complications. [425] [453] [454] The few patients with aggressive histologic types may need systemic chemotherapy. The prognosis is good, with an estimated 10-year survival rate of 6075%. Recurrences, if they occur, are systemic or in the contralateral eye.

Ocular lymphoma is a rare disorder that affects the orbital globe, usually the vitreous, retina, and choroid, and is frequently an expression of primary CNS lymphoma.[425] [455] [456] Spread to other parts of the CNS is common. The diagnosis of ocular lymphoma should be considered in patients with steroid-resistant chronic uveitis. Treatment involves bilateral ocular and whole brain radiation therapy, combined with intensive chemotherapy, usually including high-dose Ara-C. The prognosis is poor. As in primary CNS lymphoma, intensive chemotherapy regimens without radiation are being investigated. [561]

Lymphoma of the Thyroid

Lymphoma of the thyroid is usually of MALT origin.[452] [457] It manifests as a rapidly enlarging mass in older women with a history of Hashimoto thyroiditis. [425] [457] [458] In contrast to other types of MALT lymphoma, the majority of cases are aggressive lymphomas. Radiation therapy alone is insufficient treatment and results in a high rate of systemic recurrence, except in patients with small localized masses and provided that the mediastinum is included in the radiation field. [459] Chemotherapy alone has been complicated by a fairly high incidence of local recurrence. [425] [455] Recently excellent results have been reported with combined modality therapy consisting of CHOP-like chemotherapy and involved-field radiation therapy. [459] [460] Surgery is no longer recommended in the treatment of this disorder. [461]

Primary Lymphoma of the Bone

Primary lymphoma of the bone accounts for 5% of all extranodal NHLs.[425] Most patients have aggressive B-cell lymphomas. Historically, radiation treatment has resulted in durable remissions in 4045%. Currently, combined chemotherapy with CHOP-like regimens and radiation therapy is usually recommended and results in excellent long-term outcomes.[462] [463] Children with primary lymphoma of the bone are often treated with chemotherapy alone because of the adverse impact of radiation on growth and the risk of secondary tumors in the radiation field. [464] [465]

Primary Lymphoma of the Lung

Three types of lymphoma can affect the lung: diffuse large B-cell lymphoma, lymphomatoid granulomatosis, and indolent lymphoma of the BALT type (bronchus-associated lymphoid tissue), which is part of the spectrum of MALT lymphomas.[425] Diffuse large B-cell lymphoma should be treated as any other form of aggressive B-cell NHL with combination chemotherapy. Lymphomatoid granulomatosis should be treated with chemotherapy or with interferon and is discussed elsewhere. Like low-grade MALT lymphomas, BALT-type lymphomas are indolent disorders and are usually treated with single-agent or moderately aggressive combination chemotherapy regimens, or even with local radiation therapy. [289]

Primary Lymphoma of the Liver

Large cell lymphoma of the liver is commonly associated with chronic hepatitis C infection but can also occur outside this setting. [425] [466] It should be treated with combination chemotherapy similarly to other types of aggressive NHL. Its prognosis does not appear to be any worse than that of other large cell lymphomas. If liver function is severely affected, the anthracycline dose may have to be reduced. Patients with underlying hepatitis C do not appear to have a worse prognosis. [466] Primary hepatosplenic lymphoma is a recently described entity with a poor prognosis. [352] [353] [354] It was discussed earlier in the section on T-cell NHL.

Lymphoma of the Head and Neck Area

Lymphomas of the head and neck area present special diagnostic and therapeutic difficulties. [425] One should differentiate between NHL of the tonsils or Waldeyers ring, NHL arising in the parotid, NHL of the oral cavity, NHL of the paranasal sinuses, and NHL of the nose. NHLs of tonsil or Waldeyers ring should be considered nodal lymphomas and their treatment should follow the guidelines discussed for each histologic subtype. One peculiarity of these disorders is their frequent spread to the GI tract. A GI workup should therefore be part of the staging workup of these disorders.

Parotid lymphomas are commonly of the MALT type. These are indolent disorders that are found in 14% of cases of parotidectomy. [467] [468] They often arise from the intraparotid lymph nodes rather than from the parotid itself. Many cases are associated with Sjögren syndrome or hepatitis C. [292] [469] Surprisingly, a high incidence of t(14;18), as in follicle center lymphomas, has been documented in these disorders. [470] Excellent survival has been reported after treatment with chemotherapy or radiation therapy, or both. [292] [471] Some cases of parotid lymphoma are of an aggressive histologic type. They are usually treated with doxorubicin-containing combination chemotherapy, often followed by involved-field radiation therapy. [471] Their outcome is predicted by the same prognostic features that apply in other patients with aggressive lymphomas. Patients with a low IPI score have a favorable outcome. Those with more advanced disease have a worse prognosis.

Lymphomas of the oral cavity are rare. They have a diffuse histology and behave aggressively. Their treatment should include combination chemotherapy and local radiation therapy. [425]

Lymphomas of the paranasal sinuses are usually aggressive. No cases of MALT lymphoma have been reported in this disease site. In the past the prognosis was poor, owing to CNS or systemic recurrence. [425] [472] Two recent series indicate that with combined modality treatmenti.e., combination chemotherapy (CHOP) followed by radiation therapyprolonged disease-free survival can be obtained in >50% of patients, and the risk of CNS recurrence can be minimized. [473] [474] As in other aggressive lymphomas, the IPI score is the most significant prognostic factor for patients treated in this fashion. [474]

Lymphoma of the nose or nasopharynx is much more common in Asian countries (Fig. 70-3). Many cases are so-called angiocentric lymphomas of NK cell origin and are associated with EBV infection. [369] [374] [375] Other cases are T-cell or B-cell lymphomas. In a retrospective study of primary nasal lymphoma in Hong Kong, patients with clinically limited disease were treated with radiation therapy or combined radiation therapy and chemotherapy and had an 50% disease-free survival at 5 years. [377] Those with stage III and IV disease were treated with conventional chemotherapy. Their 5-year disease-free survival rate was only 10%. In another series, reported from Mexico, chemotherapy alone for early-stage disease was associated with a poor outcome; combined modality therapy appeared indicated for all patients. [376]

In a series of 113 patients reported from Hong Kong, 45% had an NK-cell phenotype, 21% had a T-cell phenotype, and 34% had a B-cell immunophenotype. Different clinical behaviors and outcomes were associated with the different phenotypes. [302] Patients with NK cell and T-cell lymphoma were significantly younger (median age 53 and 54 years, vs. 64 years for B-cell lymphoma). Patients with NK cell and T-cell lymphoma more commonly had involvement of the nasal cavity and midfacial destructive disease at presentation, whereas B-cell lymphoma was often limited to the nasopharynx alone. Neck node involvement at presentation was more common in B-cell lymphoma. Patients were fairly uniformly treated with anthracycline-based combination chemotherapy followed by radiation therapy, except for a small percentage of patients with

Figure 70-3 Primary nasopharyngeal lymphoma with obstruction of the nasopharynx by tumor.

stage III disease who received radiation therapy alone. Regardless of stage, patients with NK cell neoplasms had the worst prognosis. In these patients there was a high frequency of local recurrence and of systemic dissemination to extranodal organs such as the skin and liver. Hemophagocytic syndrome occurred in four of 51 patients with NK cell lymphoma (vs. one of 62 with other histologic types). The 2-year failure-free survival rate of patients with NK cell lymphoma was 30%, whereas it was 52% for those with T-cell NHL and 41% for those with B-cell NHL ($P = 0.007$ in multivariate analysis).

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LYMPHOMA RELATED TO INFECTION AND IMMUNOSUPPRESSION

AIDS-related lymphomas and lymphoproliferative disorders related to congenital immunodeficiency represent well-documented instances of malignancy related to a deficient immune system. They are discussed in Chapter 75 . A number of other lymphoproliferative disorders related to infection or immunodeficiency are discussed here, because their management or recognition may be of interest to the practicing oncologist.

Post-transplantation Lymphoproliferative Disorders

Post-transplantation lymphoproliferative disorders are well-known complications of solid organ or allogeneic bone marrow transplantation and can occur any time after transplantation. The large majority of cases are B-cell malignancies and are EBV

related.[475] [476] [479] Rare cases of B-cell lymphoma without detectable EBV have also been reported, as have a small number of cases of T-cell post-transplantation lymphoproliferative disorders.[476] [477] [478] The risk for post-transplantation lymphoproliferative disorders is related to the degree of immunosuppression; very high rates have been reported after the use of OKT-3 (an anti-T-cell antibody) in high doses for the prevention or treatment of rejection in solid organ recipients, [480] or after T-cell-depleted allogeneic bone marrow transplantation, especially when ATG is used for further immunosuppression. [481] [482] The incidence of post-transplantation lymphoproliferative disorder is also higher in pediatric transplant recipients than in adults, probably because many children have not been exposed to EBV and therefore have no immunologic memory against the virus.[483] Rarely, EBV-induced lymphoproliferation has been reported in non-transplant patients receiving maintenance chemotherapy for malignant hematologic disorders.[484]

In solid organ recipients, different types of post-transplantation lymphoproliferative disorder are recognized, corresponding to various degrees of EBV-driven lymphoproliferation.[475] The earliest lesions, called plasmocytic hyperplasia, commonly arise in the oropharynx and lymph nodes, are nearly always polyclonal, and usually contain evidence of multiple EBV infections or a minor population of cells infected by a single form of EBV. They lack oncogene alterations. The second type of lesions are called polymorphic B-cell hyperplasia. They are associated with post-transplantation lymphoproliferative disorders occurring within the first 6 months after transplantation and manifest as a polymorphic lymphoproliferation. They usually contain a single form of EBV, and also lack oncogene alterations. This disorder is associated with a poor prognosis. The third type of post-transplantation lymphoproliferative disorder usually occurs >6 months after transplantation, typically in the form of a monomorphic proliferation of diffuse large lymphoma cells or immunoblasts or occasionally as multiple myelomas. This type of lymphoma has alterations of one or more oncogenes in addition to EBV infection and has a better outcome. In most cases the lymphoma originates from recipient cells, but lymphomas of donor cell origin have also been demonstrated.

Clinically, post-transplantation lymphoproliferative disorder manifests in extranodal sites, including sometimes the transplanted organ. The disease presentations range from a slow-growing localized mass that can be surgically resected to rapidly proliferating and widespread disease, sometimes involving the CNS. [476] [477] [480] [483] [485] The treatment options for post-transplantation lymphoproliferative disorder include (1) a reduction in immunosuppression, (2) treatment with antivirals such as ganciclovir and acyclovir, (3) surgery, (4) treatment with monoclonal anti-B-cell antibodies, and (5) chemotherapy.

Reduction of immunosuppression can occasionally result in durable remission. [477] [485] [486] [487] It is most effective in the earliest phase of post-transplantation lymphoproliferative disorder (i.e., plasmocytic hyperplasia) but can also be effective in the other phases. The withdrawal of immunosuppression is associated with a considerable risk of graft rejection. Treatment with antivirals such as high-dose acyclovir or ganciclovir [487] [488] or with IFN- and intravenous immunoglobulins [489] occasionally results in a response in patients with post-transplantation lymphoproliferative disorder. Given the relative safety of antivirals, they are routinely combined with a reduction in immunosuppression as the first step in the treatment of post-transplantation lymphoproliferative disorder.

For patients in whom withdrawal of immunosuppression and provision of antiviral treatments are unsuccessful, surgery, chemotherapy, and monoclonal antibody treatments have been tried. A new treatment approach under investigation is the infusion of genetically modified virus-specific T lymphocytes. [490]

Surgery may result in durable remissions for occasional patients with localized disease. [491] The use of combination chemotherapy and especially the ProMACE-CBOM regimen has been prospectively tested in heart transplant patients with posttransplantation lymphoproliferative disorder. [485] ProMACECBOM was chosen because it contains a lower dose of anthracyclines and a higher and more prolonged dose of steroids than other treatments. It was therefore thought to be minimally cardiotoxic and sufficiently immunosuppressive, thus potentially reducing the risk of congestive heart failure and graft rejection. In three patients with early post-transplantation lymphoproliferative disorder (<6 months after transplantation) no durable responses were observed. Patients died from multi-organ failure or sepsis within days of institution of therapy. By contrast, among eight patients with post-transplantation lymphoproliferative disorder occurring more than 6 months after transplantation, complete remission was obtained in six and no recurrences were observed. The main toxicity of this regimen was moderate neutropenia. Two patients died in remission from infections. Cardiac toxicity was observed in four of seven evaluable patients.

The use of anti-B-cell monoclonal antibodies has been evaluated mainly in Europe. [477] [492] Durable responses were observed in six of eight patients with early post transplantation lymphoproliferative disorder and in two of two with late posttransplantation lymphoproliferative disorder. Toxic effects were minimal. Responses were not observed in two patients who received intrathecal monoclonal antibodies for post-transplantation lymphoproliferative disorder with CNS involvement.

Post-transplantation lymphoproliferative disorder occurring after bone marrow transplantation used to be a uniformly and rapidly fatal complication. [476] [482] Currently, the infusion of small numbers of immunocompetent donor lymphocytes can reverse the lymphoproliferation and rapidly and reliably results in durable remissions. [481]

Lymphoproliferative Disorders Associated with Rheumatoid Arthritis and Sjögren Syndrome

Autoimmune disorders and their treatment are associated with a certain degree of immunosuppression. It is therefore not surprising that some autoimmune disorders have been associated with an increased incidence of NHL. T-cell large granular lymphoma, closely associated with rheumatoid arthritis, was discussed in a previous section. Recently another form of NHL that is possibly EBV related was described in patients with rheumatoid arthritis who were treated with methotrexate. In a literature review, 37 patients were identified who had been treated for rheumatoid disorders with methotrexate and in whom a lymphoproliferative disorder developed.[493] Disease histology was available for 32 patients and was consistent with Hodgkin disease in five patients, diffuse large cell lymphoma in 17, high-grade lymphoma in three, and low-grade lymphoma in seven. Methotrexate withdrawal was evaluated in 16 patients, and responses occurred in nine. Fifteen of the lymphomas were tested for the presence of EBV genome and nine were positive. Responses to methotrexate withdrawal occurred in seven of nine EBV-positive patients but in only one of six EBV-negative patients. It therefore appears that at least in some cases, lymphoproliferative disorders associated with methotrexate-treated rheumatoid arthritis are caused by EBV. The immune systems surveillance for EBV is reduced in rheumatoid arthritis, resulting in a higher than

normal frequency of circulating EBV-positive B cells. [494] Treatment with methotrexate may allow the expansion of EBV-driven clones. Treatment approaches for patients with rheumatoid disorders and lymphoproliferative disorders should include consideration of methotrexate withdrawal in addition to more conventional methods of treatment.

In Sjögren syndrome, the prevalence of NHL may be as high

as 6.4 per 1,000 cases per year (up to 44 times the risk in the general population). [495] The large majority of cases are MALT lymphomas, sometimes presenting with disease transformation.[292] The histologic lesion of Sjögren syndrome is a benign lymphoepithelial lesion composed of a majority of CD4 T cells. A similar CD4 T-cell population may be present in the lacrimal glands, lungs, and kidneys of patients with Sjögren syndrome. It is hypothesized that chronic B-cell stimulation by T cells secreting a variety of cytokines leads to B-cell proliferation and ultimately transformation. Lymphoma related to Sjögren syndrome may be difficult to differentiate from the pathognomonic lymphoid infiltrate of Sjögren syndrome, and the diagnosis may be aided by demonstrating a B-cell immunophenotype. The use of PCR techniques to detect immunoglobulin gene rearrangement has also been tried but remains controversial, because immunoglobulin gene rearrangement can be detected in a large proportion of cases of Sjögren syndrome without evidence of lymphoma and may therefore represent a premalignant event. [496]

In a review of 16 NHLs occurring in patients with Sjögren syndrome, 13 were extranodal lymphomas.[292] Affected sites included the parotid (three cases), stomach (four), skin (three), buccal mucosa (one), and thymus (one). Twelve cases, including the three nodal cases, were classified as low-grade marginal zone lymphomas. Diffuse large cell lymphomas were seen in four cases but represented transformation from MALT in at least two cases. A viral etiology could not be demonstrated. Most patients were treated with chemotherapy. Those with marginal zone histologies responded well, and most had durable remissions. Disease transformation occurred in one patient and an unrelated aggressive T-cell lymphoma occurred in another.

Lymphoma Associated with Hepatitis C Virus Infection

Hepatitis C virus (HCV) is a single-stranded RNA virus that is responsible for most cases of transfusion-associated hepatitis. Shortly after its discovery it was found to be the causative agent of type II essential mixed cryoglobulinemia, a disorder considered to be lymphoproliferative in nature. [497] A high risk for NHL in HCV-positive patients has been demonstrated in a series of studies from Italy. [498] [499] [500] [501] More recently, similar associations have been reported in the United States. [466] [502] The large majority of HCV-associated cases are an indolent form of lymphoma, lymphoplasmacytoid lymphoma or immunocytoma. Some are aggressive lymphomas that transformed from immunocytoma. In all cases a cryoprecipitable IgM is present, and a majority of the patients also have essential mixed cryoglobulinemia. Many have evidence of liver disease or membranous glomerulonephritis (or both). In a series of 31 patients with essential mixed cryoglobulinemia and HCV infection, 38% sooner or later developed NHL, usually of an indolent type and commonly with bone marrow involvement. [499] An increased incidence of B-cell NHL has also been found in patients with HCV infection without evidence of essential mixed cryoglobulinemia. [466] [501] [502] Both large cell and indolent (usually marginal zone) lymphomas are found in such patients. There is a high incidence of primary extranodal lymphomas, and specifically a high incidence of lymphoma of the liver and salivary glands, two target organs for HCV infection.[466] [501]

It is unclear whether the pathogenetic role of HCV is limited to certain subtypes of HCV or whether all subtypes can be associated with NHL. The mechanism by which HCV may cause NHL is not completely defined either. The bulk of the evidence points, however, toward an indirect mechanism of chronic stimulation of B cells by nonlymphoid target cells infected by HCV. The presence of HCV proteins has been demonstrated in the residual parotid epithelium of a patient with parotid lymphoma and in a number of patients with hyperplastic reactive lymphadenopathy or low-grade lymphoma. [469] [503] In no case could the virus be demonstrated in the lymphoma cells.

Lymphoproliferative Disorders Associated with Other Infections

The virus most commonly associated with lymphoma is EBV. This virus was originally discovered because of its association with Burkitt lymphoma. [54] It is also associated with primary immunodeficiency-related lymphomas, Hodgkin disease, post-transplantation lymphoproliferative disorders, rheumatoid arthritis-related lymphomas, some cases of AIDS-related lymphoma, angiocentric lymphomas, lymphomatoid granulomatosis, aggressive NK cell lymphomas, AILD, and many other cases of T-cell lymphoma.[504] In all these cases it is thought that expression of the EBV genome leads to lymphoproliferation. EBV is much more commonly associated with endemic Burkitt lymphoma or intestinal NHL in developing countries than in the West, [505] perhaps as a result of exposure to EBV at a younger age in developing countries or of the immunosuppressive effects of chronic infections or malnourishment. In sporadic nonlymphoblastic T-cell lymphoma, the demonstration of an EBV genome in the malignant cells has been associated with an extremely poor prognosis. [506]

Other viruses are also implicated in the pathogenesis of lymphoproliferative disorders, among them HIV, HTLV-1, HTLV-2, the Kaposi sarcoma virus or HHV-8, and HHV-6. HIV is not directly implicated in lymphomagenesis but, by causing immunosuppression, it allows the proliferation of lymphomas, some of which are driven by EBV or HHV-8. HTLV-1 is the causative agent of adult T-cell leukemia/lymphoma, an aggressive disorder that is discussed elsewhere. In a study from China, HTLV-1 antibodies were found much more frequently in patients with T-cell lymphomas than in the control population. [507] Some cases of T-cell LGL lymphoma have also been associated with HTLV-1 or the related HTLV-2. [378] This indicates that the pathogenetic role of HTLV-1 in NHL may well extend beyond adult T-cell leukemia/lymphoma.

HHV-6 is a ubiquitous herpesvirus that is the cause of exanthema subitum in childhood. [508] Its reactivation causes pancytopenia and CNS infections in bone marrow transplant patients.[509] It has also been associated with some cases of Burkitt-like lymphoma.[510] Kaposi sarcoma virus, or HHV-8, is the causative agent of Kaposi sarcoma. It has recently been associated with a distinct type of lymphoma, primary effusion lymphoma.[511] These lymphomas present as effusions in the peritoneum or pleura without a distinguishable mass. The cells have a distinctive morphology bridging large cell and anaplastic large cell lymphoma. They express CD45, lack B-cell-associated antigen, and lack alterations of *bcl-2, bcl-6, ras*, or *p53*, but show immunoglobulin gene rearrangements. Usually this disorder is associated with HIV infection, but it has also been reported in HIV-negative patients. [512] In the majority of cases both EBV and the HHV-8 genome can be detected in the lymphoma cells. The prognosis of HHV-8-associated primary effusion lymphoma is poor. Most patients are in advanced stages of HIV infection and die within a year. HHV-8 is also the causative agent of some cases of Castleman disease,[410] [411] and it has been implicated in the pathogenesis of multiple myeloma.[513]

Bacteria have also been associated with the pathogenesis of NHL. *Helicobacter pylori* is thought to lead to MALT lymphoma of the stomach, and treatment with antibiotics can reverse the disease.[293] [297] *Borrelia burgdorfferi*, a spirochetal agent and the causative agent of Lyme disease, has been associated with some cases of primary cutaneous B-cell NHL.[423] An as yet unidentified pathogen may be causing immunoproliferative disease of the small intestine, a common disorder in developing countries that often responds to treatment with antibiotics.

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Chapter 71 - Malignant Lymphomas in Childhood

John T. Sandlund Jr.

INTRODUCTION

Malignant lymphomas are the third most common malignancy among children and adolescents.[1] [2] [3] [4] [5] Among children <15 years of age, non-Hodgkin Lymphoma (NHL) is more frequent; however, in patients up to 18 years of age, Hodgkin disease is predominant. [6] NHLs in children are usually extranodal diffuse high-grade tumors, whereas low- and intermediate-grade nodal lymphomas predominate in adults. [7] These differences are speculated to reflect maturational changes in the function and composition of the immune system.[2] The different histologies explain in part the differing clinical features, disease course, and treatment strategies used in adults and children.

The differences in treatment approach and disease subtypes are less striking in adults and children with Hodgkin disease. [8] However, there are significant challenges in the management of children with Hodgkin disease. These primarily comprise the sequelae of therapy, such as radiation-induced bone growth abnormalities, endocrine dysfunction, and chemotherapy-related sterility. Of greater concern are the radiation- and chemotherapy-related second malignancies and late cardiac deaths. Current trials are examining ways to reduce the toxicity of therapy without compromising the excellent outcome generally achieved. These issues, as well as differences between children and adults, in the proportion of histologic subtypes are covered in Chapter 68 , which deals with the management of Hodgkin disease in adults.

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EPIDEMIOLOGY

The incidence of NHL increases steadily throughout life, in contrast to Hodgkin disease, which has a bimodal age distribution with peaks in early and late adulthood. [2] From 1973 to 1991, the average annual incidence of NHL in the United States increased by almost 30%, [6] for reasons that remain unclear. NHL occurs two to three times more often in boys than in girls and is almost twice as common in whites as in African Americans. [2] [9] Specific populations at risk include those with acquired immunodeficiency syndrome (AIDS), those with congenital immunodeficiency syndromes (ataxia-telangiectasia, Wiskott-Aldrich syndrome, or X-linked lymphoproliferative syndrome), and those who have received immunosuppressive therapy (e.g., recipients of bone marrow or organ transplants). [2] [10] [11] [12] Deficient T-cell function probably contributes in part to this increased risk.

There are differences in both the incidence and proportion of histologic subtypes in different parts of the world. [1] [2] For example, the NHLs are very rare in Japan, whereas they account for approximately half of all childhood malignancies in equatorial Africa. Burkitt lymphoma is the predominant histologic subtype in equatorial Africa and northeast Brazil, [13] but comprises approximately one-third of cases in the United States and western Europe. In equatorial Africa, the region of high incidence of Burkitt lymphoma overlaps with the malaria belt. This observation prompted investigators to search for an infectious cause for this disease, leading to discovery of the association

Figure 71-1 Distribution of histologic subtypes of NHL in children and adults. *(From Sandlund et al., [6] with permission.)*

between Epstein-Barr virus (EBV) and this tumor. [2] Although no direct pathogenic role has been demonstrated for this virus, it has been speculated that as a B-cell mitogen, EBV increases the target pool of cells for transformation. An EBV nuclear antigen 1 (EBNA-1) variant has been found associated with the majority of EBV positive Burkitt lymphoma cases, suggesting that this mutation may provide a growth advantage to lymphoma cells by modifying EBNA-1 function in some way. [14] EBV is associated with 90% of African (endemic) Burkitt lymphoma cases, but with only 15% of cases in the United States (sporadic). [2] Disrupted and aberrant expression of the EBV genome has recently been identified in the host genome of sporadic Burkitt lymphoma cells that appeared to be EBV negative by conventional EBNA testing. [15] This finding, together with the 50% EBV-positive rate among Burkitt lymphoma cases in other parts of the world (including South America and Moscow, Russia) suggests that EBV has a widespread role in the pathogenesis of this malignancy. [13] [16] [17]

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CLASSIFICATION

After Thomas Hodgkin described the disease bearing his name in 1832, various schemes emerged to classify the tumors now collectively referred to as the NHLs. In an attempt to reduce the confusion of multiple classification schemes, the National Cancer Institute (NCI) sponsored a workshop to design a single classification scheme for clinical usage. This scheme, now referred to as the NCI Working Formulation, divides the tumors into three grades (low, intermediate, and high) based on their clinical aggressiveness. [7] In contrast to the NHLs in adults, which are primarily low and intermediate grade, the NHLs in children are predominantly diffuse high-grade tumors (Fig. 71-1). The high-grade lymphomas comprise the small noncleaved cell (SNCC), lymphoblastic, and large cell subtypes (Plate 71-1 , panels AC). The clinical and biologic characteristics of NHL in children are summarized in Table 71-1 .

TABLE 71-1 -- Clinical and Biologic Characteristics of NHL in Children

Subtype[a]	Proportion of Cases (%)[b]	Phenotype	Primary Site	Translocation	Affected Genes[c]
Small noncleaved cell (Burkitt)	39	B cell	Abdomen or head and neck	t(8;14)(q24;q32) t(2;8)(p12;q24) t(8;22)(q24;q11)	IgHc-*MYC* Ig c-*MYC* Ig c-*MYC*
Lymphoblastic	28	T cell[d]	Mediastinum or head and neck	t(1;14)(p32;q11) t(11;14)(p13;q11) t(11;14)(p15;q11) t(10;14)(q24;q11) t(7;19)(q35;p13) t(8;14)(q24;q11) t(1;7)(p34;q34)	TCR *TAL1* TCR *RHOMB2* TCR *RHOMB1* TCR *HOX11* TCR *LYL1* TCR *MYC* TCR *LCK*
Large cell	26	B cell, T cell, indeterminate	Mediastinum, abdomen, head and neck, or skin	t(2;5)(p23;q35)	*NPM-ALK*

From Sandlund et al.,[66] with permission.

[a]The subtypes are classified according to the National Cancer Institutes working formulation.
[b]Proportion at St. Jude Childrens Research Hospital; other histotypes account for approximately 7%.
[c]Ig denotes immunoglobulin and TCR (T-cell receptor).
[d]B-cell progenitor variants have also been described.

Small Noncleaved Cell

The SNCC lymphomas have been subdivided into the Burkitt and non-Burkitt subtypes based on the respective absence or presence of heterogeneity in nuclear size. The clinical and biologic irrelevance of this morphologic distinction has led to widespread intechangeable use of the terms Burkitt lymphoma and SNCC lymphoma. [18] Heterogeneity in nuclear size may also blur the morphologic distinctions between the non-Burkitt and large cell subtypes. Burkitt lymphoma is a diffuse B-cell lymphoma expressing surface immunoglobulin (usually IgM, although IgA and IgG have been described), as well as other B-cell-associated antigens, including CD19 and CD20.[2] These lymphomas contain sheets of monomorphic lymphoid cells with basophilic cytoplasm and one or more prominent nucleoi. The starry sky appearance frequently associated with this tumor is caused by tingible body macrophages in the field.

Cytogenetically, Burkitt lymphomas are characterized by the presence of one of three reciprocal chromosomal translocations. [2] [19] [20] [21] The unifying feature in these translocations is the juxtaposition of the c-*myc* proto-oncogene on chromosome 8 with one of the three immunoglobulin genes, resulting in deregulation of the c-*myc* gene. The classical translocation, t(8;14)(q24;q32), which involves the heavy chain immunoglobulin locus, is identified in approximately 85% of cases. Each of two variant translocations, t(2;8)(p12;q24) and t(8;22)(q24;q11), involves one of the two light chain immunoglobulin loci; these account for the remaining 15% of cases.

Expression of the c-*myc* gene is associated with cell proliferation. For example, in normal B cells, mitogenic stimulation results in increased rates of c-*myc* transcription. The c-myc protein induces cell cycle progression from G_1 to S phase through the activation of various target genes. [22] This DNA-binding protein forms heterodimers with related proteins (e.g., MAX, MAD) that subsequently influence cell cycling. [23] [24] [25] [26] In Burkitt lymphoma, it has been speculated that the deregulated expression of c-*myc* results in an increased proportion of MYC-MAX complexes, leading to tumor cell proliferation. [27]

There are various theories about the pathogenic mechanism of c-*myc* deregulation in Burkitt lymphoma.[28] [29] [30] The invariable presence of mutations (truncations or point mutations) in the translocated c-*myc* gene has led to speculation that these mutations result in deregulated expression. The mutations may occur in either the regulatory or coding regions of the gene. For example, mutations in the myc-inhibitory factor binding sites in the first intron regulatory region negate the repressive effect of myc-inhibitory factor binding on transcription. [31] Gu et al.[29] have demonstrated that in cases that contain a coding region mutation in the amino terminal transactivation domain where p107 would normally bind and block transactivation, tumor cells are no longer responsive to p107-mediated functional suppression. Other hypotheses focus on the juxtaposition of c-*myc* to the immunoglobulin loci. It has been suggested that the immunoglobulin gene usurps control over the translocated c-*myc* gene, perhaps through long-range enhancer sequences. [28] [30] It is likely that multiple mechanisms deregulate c-*myc* in Burkitt lymphoma.

The apparent abrogation of the c-*myc* induction of apoptosis suggests that other factors besides deregulation of c-*myc* are involved in the pathogenesis of Burkitt lymphoma.[32] Data supporting this position have emerged from a transgenic mouse model in which insertion of a deregulated c-*myc* gene mimicking the abnormality in SNCC NHL results in the development of B-cell malignancies. [33] [34] The fact that these tumors are monoclonal and take 69 months to develop suggests that additional factors or molecular events are necessary for malignant transformation. The identity and potential role in pathogenesis of other oncogenes or tumor suppressor genes is currently under investigation. [32] [35]

Abnormalities in the p53 gene have been identified in cases of SNCC NHL and B-cell acute lymphoblastic leukemia (ALL). [36] [37] [38] The mutations in these tumors differ from those seen in solid tumors such as lung, breast, and colorectal carcinomas. Beyond the mere association of p53 with these tumors, observed regulatory interactions between c-*myc* and p53 have supported hypotheses regarding the role of p53 in pathogenesis. The frequency of mutations in primary tumor biopsies is

much lower than that reported in cell lines, which are usually established from cells obtained at relapse (33% vs. 70%, respectively). This suggests that p53 mutations in Burkitt lymphomas may be involved in disease progression, reminiscent of its hypothesized role in the progression of adenomas to adenocarcinomas. [37]

Lymphoblastic Lymphoma

The morphology of these tumor cells is similar to that of ALL. The lymphoblasts are small with round or convoluted nuclei, distinct nuclear membranes, inconspicuous nuclei, and a scant rim of basophilic cytoplasm. The vast majority (>95%) are of T-cell immunophenotype. A small percentage have a B-cell progenitor immunophenotype and are associated with cutaneous involvement. [39] [40] [41] [42]

Because of the similarities in cellular origin, immunophenotype, morphology, and clinical features, it is generally assumed that lymphoblastic lymphoma and T-cell leukemia are different presentations of the same disease process; however, this has yet to be proved. [43] Most theories regarding the pathogenesis of lymphoblastic lymphoma are based on studies of T-cell ALL. The reciprocal chromosomal translocations identified in T-cell leukemia and lymphoblastic lymphoma typically involve one of the T-cell receptor genes and result in deregulation of the reciprocal partner gene. [44] [45] [46] [47] [48] [49] [50] [51] [52] The reciprocal partner gene is often a transcription factor gene such as *TAL1,* which is not usually operative in T cells. [45] [46] [51] [52] In T-cell leukemia, a submicroscopic deletion of *TAL1* can be identified in up to 25% of cases, suggesting that this deletion may also be the most common molecular abnormality in lymphoblastic lymphoma. [51] Examples of other genes involved in translocations that have been described in these T-cell malignancies include the *HOX11* transcription factor gene and the *RHOMB* genes whose products are members of a family of proteins that contain a cysteine-rich (LIM) protein-protein interaction domain. [47] [49] [50]

Large Cell Lymphoma

The large cell lymphomas are a heterogeneous group of malignancies that vary in immunophenotype and histology. [53] [54] [55] Immunophenotypically, these lymphomas may be T cell, B cell, or non-B, non-T cell. When classified according to the NCI Working Formulation, the majority are high-grade large cell, immunoblastic, with a smaller percentage being intermediate-grade diffuse large cell. These two groups receive the same therapy with no significant difference in outcome. In the Kiel system, however, up to 50% of cases can be classified as having anaplastic features (abundant cytoplasm, atypical lobulated nuclei, and prominent nucleoli in sheets of adherent cells with sinusoidal involvment). [54] [55] [56] [57] These anaplastic large cell lymphomas appear to represent a unique clinicopathologic entity and are associated with CD30 expression (an activation antigen first identified on Reed-Sternberg cells), [58] the presence of the t(2;5)(p23;q35) translocation, [55] [57] [59] [60] a T-cell or non-T, non-B-cell immunophenotype, and extranodal disease sites (e.g., skin, bone, and soft tissue). Some studies have suggested better overall survival in this group of patients; however, it remains controversial. [61]

The t(2;5) chromosomal abnormality is present in approximately 50% of cases of pediatric large cell NHL. [60] In contrast to Burkitt and lymphoblastic lymphoma, in which the translocation results in deregulated expression of a transcription factor gene by its reciprocal partner gene, the t(2;5) results in fusion of the involved genes (the amino-terminal portion of the nucleophosmin gene, *NPM,* on chromosome 5 with the catalytic domain of the anaplastic lymphoma kinase gene, *ALK,* on chromosome 2) on the der(5) chromosome. [62] This results in a novel chimeric NPM-ALK protein product with properties unlike those of either component. [63] The cytoplasmic localization of this product may inappropriately phosphorylate subtrates involved in normal cell growth and differentiation. The molecular characterization of the t(2;5) has led to the development of a reverse transcriptase-polymerase chain reaction assay that enables the detection of NPM-ALK transcripts, even in patients with no detectable t(2;5) by standard cytogenetics. [64] This technology not only facilitates diagnosis and classification, but theoretically provides a tool for following minimal residual disease.

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CLINICAL FEATURES

The clinical features at presentation vary with both primary site (Table 71-2) and extent of disease spread.[2] [9] [39] [65] [66] There is a striking difference in primary site between the SNCC and lymphoblastic histologic subtypes.[9] The primary site of SNCC NHL may be either abdomen or head and neck, but rarely the mediastinum. In contrast, lymphoblastic NHL may primarily involve the mediastinum or head and neck, but rarely the abdomen. Children with large cell NHL, on the other hand, may present with primary involvement of head and neck, abdomen, or mediastinum.[2] [9] The bone marrow may be involved at diagnosis in any of the histologic subtypes.[67] The central nervous system (CNS) may be involved at diagnosis in both the SNCC and lymphoblastic histotypes, but almost never in large cell NHL.[9] [53] [68]

Children who have a mediastinal mass may present with a spectrum of symptoms ranging from cough to severe respiratory distress caused by direct airway compressionthis condition may be worsened by an associated pleural effusion (Plate 71-1E). Mediastinal disease may also compress the superior vena cava, resulting in swelling of the neck and shoulder (superior vena cava syndrome).[2]

Primary involvement of the abdomen may be associated with nausea, vomiting, and abdominal pain. Abdominal lymphoma often arises from the distal ileum and may result in intestinal obstruction secondary to either intussusception or compression by an expanding mass encasing the bowel lumen (Plate 71-1D). These tumors may invade adjacent structures and be associated with ascites and other intra-abdominal sites of disease, including kidney, liver, and lymph nodes.[2]

TABLE 71-2 -- Distribution of Primary Sites of Tumor in Children and Adolescents with NHL

	St. Jude Childrens Research Hospital	The Hospital for Sick Children
No. of cases	338	102
Intra-abdominal	31%	40%
Mediastinal	26%	23%
Head and neck	29%[a]	11%[b]
Nodal	7%[c]	21%
Other[d]	7%	6%

From Murphy, [8] *with permission.*

[a] Includes Waldeyers ring and/or cervical lymph nodes.
[b] Includes only Waldeyers ring.
[c] Includes cases with primary nodal disease arising outside the head/neck region.
[d] Includes a variety of less common locations, including bone, skin, epidural space, thyroid.

Involvement of the bone marrow may result in pancytopenia with associated pallor and bruising. Involvement of the CNS may be associated with cranial nerve palsies or symptoms of increased intracranial pressure such as headache and vision changes. Cutaneous involvement may also occur and is usually associated with CD30+ anaplastic large cell lymphoma;[55] [61] however, lymphoblastic lymphoma (often non-T-cell immunophenotype) may also involve skin.[40] [41] [42] Bone involvement may be associated with pain or limping (Plate 71-1F).

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LABORATORY EVALUATION

Initial laboratory evaluation should include a complete blood count with differentialthese may identify abnormalities in children with bone marrow involvement. A chemistry profile, which should include electrolytes, blood urea nitrogen, creatinine, lactic dehydrogenase (LDH), calcium, phosphorus, and uric acid is also important, particularly for children with bulky Burkitt lymphoma, who often present with metabolic abnormalities such as hyperuricemia. A screen for human immunodeficiency virus should be performed on all patients with newly diagnosed lymphoma. These individuals may be at increased risk for therapy-related toxicity, specifically, life-threatening infections. Serologic studies for EBV infection may be helpful in children in whom lymphoproliferative disease is highly suspected in the differential diagnosis.

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DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

The differential diagnosis of NHL includes both benign and malignant conditions. If the blood counts are normal and there is no mediastinal mass, a single 1014-day trial of antibiotics for painless enlarged peripheral adenopathy is permissible to treat presumed bacterial adenitis. Other infectious causes for adenopathy simulating lymphoma include histoplasmosis, tuberculosis, and EBV infection, for which serologic and skin testing may be helpful. If the diagnosis of NHL is suspected, consultation with a pediatric oncologist is indicated.

The diagnosis of NHL is usually established by examination of tissue obtained by open biopsy of the involved site. A comprehensive characterization of the biologic features of the tissue will help to distinguish NHL from the small round blue cell tumors, including Ewing sarcoma, neuroblastoma, and rhabdomyosarcoma. Sufficient tissue should be obtained not only for histology, but also for immunophenotypic, cytogenetic, and molecular studies. In some cases, a patient is too unstable to undergo anesthesia for open biopsy, as in children with large anterior mediastinal masses and associated airway compression. In such cases, the diagnosis may be established by parasternal fine-needle aspiration or biopsy with local anesthesia. If there is an associated pleural effusion, thoracentesis with cytologic examination of pleural fluid is usually diagnostic. In cases of large abdominal Burkitt tumors, direct percutaneous aspiration of the mass for cytology and cytogenetics is often diagnostic, as is cytologic examination of associated ascitic fluid obtained by paracentesis. In children with suspected NHL, a bone marrow and cerebrospinal fluid examination may be diagnostic, averting the need for more invasive procedures and possible increased morbidity.

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STAGING

It is imperative that a meticulous staging work-up be performed because therapy is determined in part by location and degree of disease spread. Additionally, because NHL in children grows very rapidly, there should be no unnecessary delay in the staging work-up or in starting appropriate therapy. The staging work-up should include a complete history and physical, including the documentation of the presence or absence of B

TABLE 71-3 -- Stages of NHL[a]

Stage I
A single tumor (extranodal) or involvement of a single anatomic area (nodal), with the exclusion of the mediastinum and abdomen
Stage II
A single tumor (extranodal) with regional node involvement
Two or more nodal areas on the same side of the diaphragm
Two single (extranodal) tumors, with or without regional node involvement on the same side of the diaphragm
A primary gastrointestinal tract tumor (usually in the ileocecal area), with or without involvement of associated mesenteric nodes, that is completely resectable
Stage III
Two single tumors (extranodal) on opposite sides of the diaphragm
Two or more nodal areas above and below the diaphragm
Any primary intrathoracic tumor (mediastinal, pleural, or thymic)
Extensive primary intra-abdominal disease
Any paraspinal or epidural tumor, whether or not other sites are involved
Stage IV
Any of the above findings with initial involvement of the CNS, bone marrow, or both

From Murphy,[70] with permission.

[a]Based on the classification proposed by Murphy.

symptoms (e.g., weight loss and fever) by history. Diagnostic imaging studies should include computed tomography scanning of the chest, abdomen, and pelvis, and bone scanning. Gallium scanning may also be helpful in selected cases, particularly in following residual masses that were gallium positive at diagnosis. The cerebrospinal fluid and bone marrow must be examined in all patients. Bilateral posterior iliac crest aspiration and biopsy increases the chance of identifying marrow involvement, thus reducing the possibility of underestimating the disease stage. [69]

On completion of the above work-up, the stage of disease is usually determined according to the St. Jude Staging System described by Murphy (Table 71-3),[70] which was developed to accommodate the noncontiguous nature of disease spread, predominant extranodal involvement, and involvement of the bone marrow and CNS. Stages I and II are considered limited stage disease, whereas stages III and IV represent advanced stage disease.

THERAPY

General Principles

The dramatic improvement in treatment outcome achieved over the past 25 years is in large part due to the refinement in multiagent chemotherapeutic strategies through sequential clinical trials. [2] [9] [53] [71] [72] [73] [74] [75] [76] [77] [78] [79] [80] [81] [82] [83] [84] [85] [86] [87] [88] [89] [90] [91] [92] [93] [94] [95] [96] [97] [98] [99] [100] [101] [102] [103] [104] [105] [106] [107] [108] The experience at St. Jude Childrens Research Hospital (SJCRH) is shown in Figure 71-2 . The most recent era features both a stage- and histology-directed treatment strategy (see box). A randomized trial performed by the Childrens Cancer Group (CCG) that compared two of the earliest effective treatment regimens for childhood NHL (COMP [cyclophosphamide, Oncovin, methotrexate, prednisone] and LSA_2L_2) provided data supporting this approach. [80] In this trial, children with limited stage disease had an excellent outcome regardless of histology or treatment regimen used. In contrast, among children with advanced stage disease, outcome varied with histology and therapy. Children with SNCC NHL had a better outcome with the cyclophosphamide-based COMP regimen, whereas children with lymphoblastic NHL had a better outcome with LSA2L2, a regimen designed for children with ALL. Among children with large cell NHL, outcome did not vary with treatment protocol. In the United States, subsequent trials built on this histology- and stage-directed approach. In Europe, and more recently in some US trials, an immunophenotype-directed approach is being used. Data suggest that immunophenotype-directed therapy is particularly important for children with advanced large cell NHL, who have varied treatment outcomes with respect to immunophenotype. [54]

There is a minimal role for radiation therapy and surgery in the management of childhood NHL. [2] [9] Prospective randomized trials in children with either limited or advanced stage disease have shown there is no improvement in treatment result when involved-field radiation is included; in fact, this modality may only add to treatment-related morbidity. The use of radiation therapy in certain emergency situations, at relapse, and in the management of CNS disease at diagnosis for certain histotypes will be discussed later. Apart from the initial diagnostic biopsy or the complete resection of an isolated peripheral node, tonsil, or ileocecal primary tumor associated with mesenteric nodes only (see discussion on staging), there is no other clear indication for the surgical management of this disease. Of note, aggressive debulking procedures are not indicated.

To prevent the spread of disease to the CNS, prophylactic intrathecal and high-dose systemic chemotherapy are used in most children. Prophylactic intrathecal therapy may not be necessary in children with limited stage disease, sparing the head and neck region, and in children with advanced stage large cell disease not involving the head and neck bone marrow. Some groups have included cranial radiation for CNS prophylaxis in pediatric lymphoblastic lymphoma; however, this approach is controversial. [101] Among children who present with overt CNS disease, intensification of both intrathecal and systemic chemotherapy is often needed, and, with the exception of Burkitt lymphoma, the addition of cranial irradiation.

Limited Stage Disease

The excellent prognosis for children with limited stage disease has prompted investigators to develop treatment strategies that reduce treatment-related morbidity while maintaining an excellent treatment result (Table 71-4). [71] [73] [74] [96] These studies have examined various ways to reduce treatment intensity. For

Figure 71-2 Event-free survival of patients with NHL treated from 1962 to 1993 at St. Jude Childrens Research Hospital. From 19621974, various chemotherapeutic approaches were used, including radiation therapy in some cases. From 19751978, patients were treated with a single combined-modality protocol. From 19791993, treatment was based on both the stage and histologic subtype of lymphoma. The numbers in parentheses are the total numbers of patients. Because some patients were not in complete remission at year 0 the curves do not begin at 100%. *(From Sandlund et al.,* [66] *with permission.)*

TABLE 71-4 -- Treatment Outcome for Limited Stage NHL

Regimen	Strategy	No. of Patients	Outcome
SJCRH[73]	Decrease intensity	28	86% 2 yr DFS
CCG[96] [a]	Shorten duration	54	98% 2 yr EFS
POG[74]	Delete radiation	131	86% 5 yr CCR
	Delete continuation	113	89% 5 yr CCR
SFOP[71] [a]	Surgical resection	44	96% 3 yr EFS

DFS, disease-free survival; EFS, event-free survival; CCR, continuous complete remission.

[a] Excludes lymphoblastic NHL.

example, in the first of two sequential trials performed by the Pediatric Oncology Group (POG), [74] it was demonstrated that involved-field radiation therapy could be safely deleted from a 33-week chemotherapy regimen that comprised three courses of CHOP (cyclophosphamide, Adriamycin, vincristine, and prednisone) given over a 9-week period, followed by a 24-week maintenance phase consisting of weekly 6-mercaptopurine and methotrexate. In the subsequent trial, they demonstrated that the 24-week maintenance phase could be deleted without compromising the treatment result for those with either large cell or SNCC NHL. The optimal approach for children with limited stage lymphoblastic NHL remains controversial. In the POG trial, one-third of these children treated with the 33-week regimen experienced treatment failure but were successfully salvaged

ALGORITHM FOR MANAGEMENT OF PEDIATRIC NHL

in most cases. French investigators have taken a different approach, treating children with limited stage lymphoblastic NHL with the same regimen used for those with advanced stage disease.[95] Although this approach may reduce the need for retreatment, it also increases the risk of treatment-related morbidity.

Advanced Stage Disease

In the United States, efforts to improve the treatment outcome for children with advanced stage disease have primarily examined strategies to increase treatment intensity in the framework of a histology-directed approach,[53] [54] [72] [75] [76] [80] [81] [82] [83] [84] [85] [87] [88] [89] [90] [91] [92] [93] [98] whereas in Europe, an immunophenotype-directed strategy is generally used.[71] [77] [78] [79] [86] [94] [95] [97] [101] [102] [103] [106] [107] [108]

Advances in the treatment outcome of patients with SNCC NHL represent one of the true success stories in pediatric oncology (Table 71-5). Following the CCG trial that demonstrated the efficacy of the cyclophosphamide-based COMP regimen for advanced stage SNCC NHL, improved treatment results were achieved by including high-dose methotrexate and cytarabine[72] [75] [76] [77] [79] [83] even in regimens given over as short a period as 24 months.[77] [84] In the past decade, further improvement has been achieved by dose intensification of therapy and by the inclusion of additional active agents such as etoposide or ifosfamide.[71] [78] [79] [82] [101] [104]

Most of the regimens used successfully to treat lymphoblastic NHL are similar to or derived from those designed for children

TABLE 71-5 -- Treatment Outcome for Advanced Stage SNCC NHL

Protocol	Stage	No. of Patients	Event-Free Survival	Reference
Total B	III	17	2 yr EFS = 81%	[72]
	IV/B-ALL	4/8	2 yr EFS = 17%	
POG 8617	IV	34	4 yr EFS = 79 ± 9%	[76]
	B-ALL	47	4 yr EFS = 65 ± 8%	
LMB 84[a]	III	167	2 yr EFS = 80% (SE 3)	[77]
	IV/B-ALL (CNS)	34	2 yr EFS = 68% (SE 8)	
LMB 86[a]	B-ALL (CNS)	11	>1 yr EFS = 82% (SD 12)	[78]
	B-ALL (CNS+)	24	>1 yr EFS = 75% (SD 9)	
LMB 89[a]	III	279	3 yr EFS = 93% ± 3%	[71]
	IV/ALL	165	3 yr EFS = 88% ± 4%	
BFM				
81	B-ALL	22	5 yr EFS = 40% (SD 6%)	[79]
83	B-ALL	24	5 yr EFS = 50% (SD 10%)	[79]
86	B-ALL	41	5 yr EFS = 78% (SD 6%)	[79]
90	III	171	6 yr EFS = 86% (SD 3%)	[102]
	IV	23	6 yr EFS = 83% (SD 8%)	
	B-ALL	56	6 yr EFS = 76% (SD 8%)	
CCG				
$LSA_2 L_2$ vs. COMP	III/IV/B-ALL	44	5 yr EFS = 29% (95% CI 1643%)	[80]
	III/IV/B-ALL	93	5 yr EFS = 50% (95% CI 3960%)	
CCG				
COMP vs. D-comp (randomized)	III/IV/B-ALL	175	2 yr EFS = 65%	[81]
CCG[a]				
Orange vs. LMB 86	III/IV/B-ALL	43	12 mo EFS = 83%	[82]
	III/IV/B-ALL	42	12 mo EFS = 84%	[82]
NCI[a]				
77-04	III	30	3 yr EFS = 57% ± 9%	[83]
	IV	9	3 yr EFS = 13% ± 12%	
CODOX/VIPA	III/IV/B-ALL	75	1 yr EFS = 89%	[105]
Boston				
HiC-COM	III	12	2 yr EFS = 95% (CI 5499%)	[84]
	IV/B-ALL	8	2 yr EFS = 50% (CI 1578%)	

[a]Includes patients with B-cell large cell NHL.

TABLE 71-6 -- Treatment Outcome for Advanced Stage Lymphoblastic NHL

Protocol	Stage	No. of Patients	Event-Free Survival	Reference
$LSA_2 L_2$ (modified)	III	24	3 yr EFS = 57%	[85]
POG 7615				
$LSA_2 L_2$ (modified)	III/IV	124	5 yr EFS = 64%	[80]
CCG-551				
BFM 75/81	III/IV	42	4 yr EFS = 78%	[86]
BFM 86/90	III	119	pEFS[a] = 87% (SE 3%)	[106]
	IV	30	pEFS[a] = 90% (SE 6%)	
X-H SJCRH	III/IV	22	4 yr DFS = 73%	[87]

APO (Dana Farber)	III/IV	21	3 yr DFS = 58% ± 23%	[88]
77-04 (NCI)	III	10	4 yr EFS = 70%	[83]
A-COP + (POG)	III	33	3 yr DFS = 54% ± 9%	[89]

DFS, disease-free survival; A-COP, Adriamycin, cyclophosphamide, Oncovin, prednisone; APO, Adriamycin, prednisone, Oncovin.

[a]Median observation time = 4.3 yrs.

with high-risk T-cell ALL (Table 71-6).[80] [85] [87] [88] [89] [95] [99] [100] A St. Jude study demonstrated the benefit of adding teniposide and cytarabine to an otherwise conventional antimetabolite-based regimen.[87] The French cooperative group (SFOP) demonstrated that an excellent response could be achieved by incorporating courses of high-dose methotrexate into an $LSA_2 L_2$ backbone.[95]

TABLE 71-7 -- Treatment Outcome for Advanced Stage Large Cell NHL

Protocol	Stage	No. of Patients	Event-Free Survival	Reference
COMP	III & IV	42	5 yr EFS = 52%	[80]
$LSA_2 L_2$	III & IV	18	5 yr EFS = 43%	[80]
APO	III & IV		3 yr EFS = 65%	[90]
ACOP+	III & IV	22	4 yr EFS = 67%	[91]
COMP vs. D-COMP	III	86	2 yr EFS = 66%	[81]
CHOP	III & IV	21	3 yr EFS 62% ± 11%	[53]
MACOP-B	III & IV	11	3 yr EFS 55% ± 16%	[92]

MACOP-B, methotrexate, Adriamycin, cyclophosphamide, Oncovin, prednisone, bleomycin.

The German Berlin-Frankfurt-Munster (BFM) group has incorporated a reinduction phase into their treatment strategy and in their most recent trial have featured high-dose methotrexate pulses (5 g/m^2) as an early consolidation with very encouraging preliminary results90% event-free survival at 3 years. [101] [106] Although the French and German studies suggest an important role for high-dose methotrexate in the management of advanced stage lymphoblastic lymphoma, the multiagent nature of the regimens studied to date makes the relative value of individual components uncertain.

The optimal approach to the treatment of advanced stage large cell NHL has been a challenge to identify, both because of the biologic heterogeneity of these tumors and the markedly varied treatment strategies reported. In the United States, children with large cell NHL are treated on histology-directed protocols (Table 71-7). [53] [54] [80] [81] [90] [91] [92] Most of these histology-directed strategies are CHOP based, with current trials examining the benefit of adding agents such as carboplatin, high-dose cytarabine, etoposide, ifosfamide, and high-dose methotrexate. Some trials have examined the feasibility of eliminating individual agents with significant late effects, such as Adriamycin or cyclophosphamide.[54] [81] In a POG trial, children with large cell lymphomas of B-cell immunophentotype had a superior treatment result compared with those with a non-B-cell immunophenotype, suggesting that an immunophentotype-directed approach should be explored in future trials. [54] In this regard, European trials[71] [101] for children with large cell NHL have generally been designed according to immunophenotype; children with B-cell tumors are treated like those with SNCC NHL,[107] [108] those with T-cell tumors are treated like those with lymphoblastic lymphoma or T-ALL; and those with CD30+ anaplastic lymphomas are treated with various approaches, including therapy designed for Burkitt lymphoma. [94]

Emergency Situations

Various emergency situations may arise in the management of the child with newly diagnosed malignant lymphoma.[2] Those with a large anterior mediastinal mass may present with severe respiratory distress secondary to direct tracheal compression, a situation that may be worsened by an associated pleural effusion. If the diagnosis has been confirmed, appropriate chemotherapy should be started as soon as possible. If the diagnosis has not been established, efforts should be made to establish it expeditiously (see section on diagnosis). If the degree of respiratory compromise requires emergency management, involved-field irradiation of the tumor mass should be considered, with sparing of peripheral portions of the tumor so that a biopsy can be obtained when the patient is stable. Steroids may also be considered; however, this approach risks altering the tumor histology making it difficult to establish the correct diagnosis.

Children with a large tumor burden, particularly those with Burkitt lymphoma are at increased risk of significant metabolic abnormalities. Specifically, they may present with hyperuricemia, hyperkalemia, hyperphosphatemia, and associated renal dysfunction. This situation may only worsen with the massive tumor cell lysis that follows administration of chemotherapy ("tumor lysis syndrome").[2] Some of these children develop renal failure requiring dialysis. To help prevent this complication, children at risk should be vigorously hydrated (34 L/m^2 /day with D5 1/4 NaCl and 40 mEq/L $NaHCO_3$; there should be no added potassium) and started on allopurinol, a xanthine oxidase inhibitor. The urine pH should be maintained at about 7.0; at a more alkaline pH, phosphorus is less soluble, and at a more acidic pH, uric acid is less soluble. In some cases, mannitol followed by Lasix (furosemide) is required to maintain urine output. Uricolytic agents, such as uricozyme, which has been used for many years in Europe, directly cleaves the uric acid molecule, resulting in a precipitous drop in serum uric acid levels within a few hours. [109] A recombinant form (SR29142) is currently being studied. These agents have significantly reduced the frequency of renal failure and subsequent need for dialysis. They have recently become available in the United States as investigational agents.

Children with NHL may present with epidural masses and associated neurologic deficits caused by spinal cord compression. If the diagnosis is known, chemotherapy should be started as soon as possible. If the diagnosis is not known, or if there is a sluggish response to chemotherapy, low-dose radiation therapy may be considered.[2]

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SALVAGE

Children who fail initial therapy are generally considered to have a poor prognosis, particularly if they have received intensive therapy up front. Current approaches to the management of these patients incorporate intensive multiagent therapy, which may be followed by hematopoietic stem cell or bone marrow transplantation (BMT). Various multiagent regimens have been studied. For example, VIPA[110] (etoposide, ifosfamide, and cytarabine) has been shown to be active in relapsed Burkitt lymphoma, and DHAP (dexamethasone, cytarabine, and cisplatin) has been shown to be active in relapsed large cell NHL. [92] Other regimens, including ICE[111] (ifosfamide, carboplatin, and etoposide) and MIED (methotrexate, ifosfamide, etoposide, and dexamethasone) are under investigation. Children who have chemosensitive recurrent disease are considered candidates for an intensification phase with autologous hematopoietic stem cell support (stem cells obtained by peripheral blood pheresis or bone marrow harvesting) or allogeneic BMT.

Autologous hematopoietic stem cell transplantation has been used successfully in children with relapsed large cell lymphoma [112] and in certain cases of relapsed Burkitt lymphoma.[113] [114] It has recently been reported by the European Lymphoma Bone Marrow Transplantation Registry that among children with poor-risk Burkitt lymphoma, in their experience, two groups of patients responded well to high-dose chemotherapy followed by autologous BMT: those with a partial response to induction therapy and those with chemosensitive relapse. They point out, however, that with intensive modern frontline protocols, postrelapse salvage with high-dose chemotherapy and autologous BMT may be less successful and they suggest that novel approaches including antibody-targeted therapy or the graft-versus-lymphoma effect following an allogeneic BMT approach be explored. [115] There are limited published data on the comparative effectiveness of autologous and allogeneic BMT for children with relapsed NHL; however, most investigators would consider allogeneic BMT strategies in cases of relapsed lymphoblastic or SNCC lymphoma if there is a matched related donor, particularly in cases in which the bone marrow is involved at the time of relapse.

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SUPPORTIVE CARE

Optimal supportive care is critical for the successful management of children with NHL. The increased intensity of many current regimens requires the availability of the appropriate expertise and resources to manage the complications encountered during periods of profound pancytopenia. The management of life-threatening infections requires the availability of appropriate broad-spectrum antibiotics and antifungals and an intensive care facility. The use of prophylactic trimethoprim-sulfamethoxazole is recommended in all patients to reduce the chance of *Pneumocystis carinii* pneumonitis; for patients on aggressive B-cell protocols, treatment should be continued for approximately 6 months after chemotherapy is completed. Access to a blood bank that can quickly provide irradiated leukocyte-depleted packed red blood cells and platelets is also important. The role of colony-stimulating factors (CSF) such as granulocyte-CSF or granulocyte/macrophage-CSF has yet to be fully elucidated; however, they provide the potential benefit of reducing the duration of neutropenia and thus reducing infectious morbidity. The placement of a permanent venous access device, such as a Hickman catheter or subcutaneous port, greatly facilitates the delivery of hydration fluids, chemotherapy, antibiotics, and blood products. Consultation with a nutritionist or nutritional support team regarding need for enteral or total parenteral nutritional support is often necessary, particularly for children on intensive advanced stage B-cell NHL protocols, which are associated with significant gastrointestinal tract toxicity.

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PROGNOSTIC FACTORS

Factors that are associated with treatment outcome include treatment era and tumor burden as reflected by both stage and serum LDH. [2] [9] [116] Those with limited stage (I and II) disease generally have a better prognosis than those with advanced stage (III and IV) disease; however, more intensive therapies for children with advanced stage disease have resulted in approximately equal outcomes in the two groups. Involvement of the CNS and bone marrow, both features of stage IV disease, has historically been associated with a poorer prognosis. However, for certain histologic subtypes, such as Burkitt lymphoma, modern intensive therapy has resulted in a good prognosis for those with marrow involvement, and in some studies, CNS disease has been eliminated as an adverse prognostic factor. [117] Elevated serum LDH (>500 IU/L) and interleukin-2 receptor are associated with outcome. [118] [119] Within specific histologic subtypes, certain biologic features have prognostic value. For example, among those with large cell NHL, those with a B-cell immunophenotype have better treatment results than do those with a non-B-cell immunophenotype (i.e., T cell or non-B, non-T cell). [54] Because prognostic factors are reflective of the specific treatment used, they will continue to change as therapy improves.

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FUTURE DIRECTIONS

Although there have been dramatic improvements in treatment outcome for children with NHL over the past 25 years, approximately 30% of children with these tumors either relapse or do not respond to initial therapy. [6] Additionally, late effects such as anthracycline-related cardiomyopathy, secondary malignancies such as epipodophylotoxin-related acute myeloid leukemia, and endocrine abnormalities such as cyclophosphamide-related azospermia remain a concern. [120] [121] Thus, a major goal for the future is to develop treatment strategies that will provide a cure for the remaining 30% while reducing treatment-related morbidity. There are several areas to target if this goal is to be achieved.

The identification at diagnosis of both clinical and biologic features that predict treatment failure will enable investigators to determine which patients need aggressive or novel therapy. More intensive therapy may require autologous hematopoietic stem cell support provided by either peripheral blood or bone marrow harvesting. The administration of CSFs may be necessary in some cases, although their role in therapy is currently under investigation. Novel approaches may include immunotherapeutic strategies such as tumor vaccines, phenotype-specific immunotoxins, [122] or the use of surface protein-specific cytotoxic T lymphocytesan approach that has been successful in both the prevention and treatment of EBV-related post-transplant lymphoproliferative disease. [123]

The continued investigation of lymphoma-related cytogenetic and molecular abnormalities as well as the pathogenetic mechanisms of malignant transformation are critical. These studies may help to further refine disease classification and provide a means of detecting minimal residual disease during clinical remission. Additionally, these studies may provide clues to novel and more successful treatment strategies, including those directed toward tumor-specific molecular lesions.

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Chapter 72 - Chronic Lymphocytic Leukemia

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Chronic lymphocytic leukemia (CLL) as a distinct clinical entity was first identified in 1903 by Turk, [1] who gave us not only the criteria for its diagnosis but the features that distinguish it from the lymphomas. In 1924, Minot and Isaacs [2] presented a detailed clinical description of CLL. Although several physicians studied this disease in the ensuing decades, it was not until 19661967 that the pathophysiology of CLL was fully explained. Galton [3] and Dameshek [4] independently but virtually simultaneously suggested that the main characteristic of CLL is a progressive accumulation of functionally incompetent, long-lived lymphocytes.

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EPIDEMIOLOGY

Chronic lymphocytic leukemia is the most common form of leukemia in the Western Hemisphere, accounting for about 2530% of all leukemias. Approximately 10,000 new cases are diagnosed every year in the United States. [5] Characteristically, CLL is a disease of advancing age, the incidence being >20 per 100,000 people >70 years of age. [6] However, the disease is being diagnosed in increasing frequency among younger age groups and is no longer considered unusual even in patients 35 years of age. The median age at diagnosis is 55 years. The incidence of CLL is higher among men, with the male/female ratio being nearly 1.7:1. CLL is rarely seen in Japan, China, and other Asian countries. The reason for this wide disparity in incidence of CLL in different parts of the world remains unknown. More than 95% of patients have a B-cell phenotype; T-cell CLL is a rare disease, accounting for only 25% of all cases. Unless otherwise specified, most descriptions of CLL pertain to B-cell disease.

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GENETIC ASPECTS

Chronic lymphocytic leukemia is an acquired disorder. There is one report in the literature of CLL occurring in twin sisters who were monozygous, but not identical. Immunoglobulin gene rearrangements in the CLL cells of these twins were found to differ from each other. [7] Relatives of patients with CLL have an increased frequency of CLL, other B-cell malignancies, and autoimmune disorders. [8] [9] [10] [11] The risk for development of CLL among first-degree relatives of patients with CLL is higher than expected. Consanguinity and chromosomal abnormalities have been proposed as possible explanations. No human leukocyte antigen (HLA) haplotype has been found to be consistently associated with CLL. [12]

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ETIOLOGY AND ONCOGENESIS

The etiology of B-CLL remains unknown. No causal relationship has been found with exposure to radiation, chemicals, and alkylating agents. Human T-cell leukemia/lymphoma virus 1 (HTLV-1) is known to cause adult T-cell leukemia. Although retroviruses and DNA viruses such as HTLV-1 and Epstein-Barr virus are not considered to be etiologic agents for CLL, Mann et al. [13] observed two patients with B-CLL who were HTLV-1 seropositive, but the virus was not present in the cellular genome.

In CLL, there is a progressive accumulation of the leukemic lymphocytes, with no increase in their rate of proliferation. Lymphocytes in CLL are known to be long-lived. The early observations of Galton [3] and Dameshek[4] suggested that CLL lymphocytes are long-lived because they are functionally incompetent. Recent work has demonstrated that CLL represents a classic example of a human cancer resulting from defects in the pathways for programmed cell death or apoptosis. The Bcl-2 family of proteins is known to serve as a key regulator of apoptosis. Bcl-2, the prototype of this family, is a blocker of apoptosis. [14] [15] Leukemia cells from approximately 90% of patients with CLL have high levels of Bcl-2. [15] [16] Thus, nearly 30 years after Galtons and Damesheks observations, we now have an explanation for the longevity of CLL cells and, therefore, the first clues in understanding the pathogenesis of this disease.

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GROWTH AND DIFFERENTIATION

Leukemic cells in CLL are known to be arrested in the G_0 phase of the cell cycle with a relatively rare cell in the peripheral blood showing evidence of being in the active proliferative cycle.[17] [18] CLL cells, arrested in the late stages of B-cell differentiation, reveal certain characteristics in vitro that may explain at least some aspects of the pathophysiology of this disease. When cultured [19] without cytokines and mitogens, in vitro, CLL cells die rapidly by apoptosis. It has been demonstrated that CLL cells lose bcl-2 protein during culture. [20] Addition of interleukin (IL)-4 [21] or interferon (IFN)- [22] to the cultures inhibits the cell death by apoptosis, an observation correlated with a simultaneous increased expression of bcl-2 protein in IL-4treated B-CLL cells. [21] When CLL cells are cultured in the presence of B-cell mitogens or the phorbol ester TPA (12-0-tetradecanoylphorbol-13-acetate), they are capable of plasmacytoid differentiation, suggesting that these cells are not frozen at an intermediate stage in the B-cell differentiation pathway. The process of stimulation of differentiation of CLL cells, however, is complex and requires all the co-stimulatory factors necessary for normal B cells undergoing differentiation in vitro, including various cytokine-producing, non-neoplastic T cells. [23]

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CHROMOSOMAL ABNORMALITIES

Chronic lymphocytic leukemia lymphocytes are resting cells in G_0 that have no spontaneous mitoses in vitro. Until the recent introduction of B-cell mitogens, cytogenetic studies were not possible in CLL because metaphases were only rarely inducible. In the past decade, however, several laboratories have been successful in performing cytogenetic studies in CLL following the availability of a battery of B-cell mitogens, including lipopolysaccharide, TPA, cytochalasin B, pokeweed mitogen, and Epstein-Barr virus supernate, which induce readable metaphases in a large proportion of cases. Juliusson and Gahrton, [24] in an update on the cytogenetic data pooled by the International Working Party on Chromosomes in CLL and drawing on additional data from the published literature, have provided a detailed status report on this subject. In this collected series [24] of

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G-banded metaphase analysis of 1,244 cases of CLL, clonal chromosomal abnormalities were seen in 43% (533 cases), and trisomy 12 was the most frequently observed abnormality, occurring in approximately 15% of all patients studied and approximately one third of all cases with clonal abnormalities. Structural abnormalities involving the long arm of chromosome 13 accounted for 20% of cases with clonal abnormalities, and other, less frequently occurring structural abnormalities involved the long arms of chromosomes 6 and 14.[24]

The study of chromosomal abnormalities in CLL has benefited from recent molecular biology techniques such as fluorescence in situ hybridization (FISH), constructing a high-resolution physical map of yeast artificial chromosome, and comparative genomic hybridization. These studies have identified abnormalities of chromosome 12, chromosome 13q14 and 13q12, and chromosome 11. Fluorescence in situ hybridization has proven to be the most reliable method to detect trisomy 12 abnormality. A study by Garcia Marco et al.[25] revealed 20% of 600 CLL patients with trisomy 12. These investigators found that patients with 12+ had an increased proportion of prolymphocytes and an overall poorer prognosis.

Abnormalities of the long arm of chromosome 13 have been the subject of intense investigations by several workers. These studies reveal a loss of chromosome material on 13q, initially suggesting the involvement of retinoblastoma susceptibility gene (*RB1*) because of frequency of allelic loss in its locus, 13q14. However, it has since been demonstrated that the actual region of deletion is telomeric to *RB1,* close to the marker D13S25.[26] A genomic region of consensus of approximately 300 kilobases has been identified, and it is expected that a candidate tumor suppressor gene for CLL may soon be discovered in this area of minimal deletion at 13q14.[27]

Döhner et al.[28] reported that in a study of 338 patients with B-CLL by FISH, the frequency of chromosomal abnormalities (in decreasing order) was as follows: 13q (54% single and complex and 36% single), 11q (17%), +12q (15%), 17p (8%), and 6q (7%). In an attempt to correlate prognosis with chromosomal abnormalities, Döhner et al.[28] observed that 17p and 11q deleting were associated with rapid disease progression and inferior survival, while in the better prognosis group there was no significant survival difference among patients with normal karyotype, 13q (single abnormality) and trisomy 12.

In a study of more than 200 CLL cases, 20% were found to have deletions of 11q23, a structural abnormality second only in frequency after deletions of 13q1214. [29] Patients with 11q deletions are reported to have progressive disease, tend to be young, may be associated with extensive adenopathy, and have short treatment-free survival.[30]

IMMUNOPHENOTYPIC PROFILE OF LYMPHOCYTES

B Cells

The normal T-cell/B-cell ratio is reversed in CLL. In B-CLL, the B cells usually account for nearly 90% of all lymphocytes. There is a characteristic immunophenotypic profile of B cells, as shown in Table 72-1 .[31] [32] [33] CLL B cells express low surface density of immunoglobulins, usually IgM or IgM with IgD, which are monoclonal, as revealed by expression of only one light chain, either or . These cells form rosettes with mouse erythrocytes. Intracytoplasmic immunoglobulins may be detectable in a few cases. Some B cells have receptors for Fc fragments, for IgG, and for complement (C3d). By using a wide range of monoclonal antibodies, it has been established that B cells of B-CLL stain positively with one pan-T-cell antibody Leu-1 (CD5), [34] [35] [36] while simultaneously also staining with at least one of the B-cell monoclonal antibodies B1 (CD20), B4 (CD19), CD23 (activation marker, low-affinity FcE receptor), and BA1 (CD24). These cells

TABLE 72-1 -- Immunophenotypic Pattern of B-Cell Chronic Lymphocytic Leukemia Cells

Surface immunoglobulin (slg):
Usually IgM or IgM and IgD of low intensity (infrequently, the amount of slg may be so low that the cell is read as slg negative)
Mouse-erythrocytes rosetting
One or more of the following B-cell markers
CD19 (B4), CD20 (B1), CD24 (BA1), CD21 (C3dR), CD23 (activation marker)
Ia
CD5 (pan-T, Leu-1)
Heterogeneous with respect to
CD11c ($_2$ integrin)
Surface adhesion molecules CD54, CD58, L-selectin, and CD25 (interleukin-2 receptor)

express HLA-DR, the major histocompatibility class II antigen. There is a considerable degree of heterogeneity in the expression of CD25 (IL-2 receptor) and CD11c ($_2$ integrin) and surface adhesion molecules CD54, CD58, and L-selectin. [31] [32] [37] The overall interpretation of this phenotypic expression is that CLL cells are relatively mature cells that are arrested at an intermediate stage in the pathway of B-cell differentiation.

The CD5 positivity of B cells in CLL has become a subject of active investigation. A very small subset of normal B lymphocytes is known to be CD5+. [34] It is not clear whether this normal CD5+ B-cell subpopulation is the one that proliferates and accumulates in CLL. [38] Normal CD5+ B cells express activation markers, and >10% of these cells may be in active proliferative cycle, whereas only <1% of CD5+ B cells in CLL are in active cycle [38] (Table 72-1). Investigations in this area, however, do not provide clear evidence that CD5+ B cells constitute a separate B-cell lineage. [39]

T Cells and Natural Killer Cells

The absolute number of T cells in B-CLL may be normal, decreased, or increased according to the total lymphocyte count and percentage of T cells. The interplay of cytokines produced by cell-cell interaction among malignant B cells, macrophages, and T cells may provide an explanation for the heterogeneity of B-CLL. [40] T cells in B-CLL may influence the expansion of the malignant clone by the increased numbers of CD4 and helper T cells in the bone marrow and lymph nodes. In addition, although T cells may otherwise be normal, they have alterations in the T-cell receptor repertoire, as revealed by the observations that within both CD4+ and CD8+ populations in B-CLL there is an increase in oligoclonality. These oligoclonal T cells coexpress CD57 marker, and their expansion is a function of disease duration. [41] T cells also produce several cytokines under the control of accessory cells, which happen to be the malignant B-CLL cells. [42]

The population of large granular lymphocytes (natural killer [NK] cells) is usually decreased. [43] [44] The functional status of T cells and NK cells in CLL remains unresolved and unclear.[45]

Autoimmune Complications

Autoimmune complications are known to occur frequently in CLL and are due to antibodies restricted to blood cell self-antigens. [42] [46] [47] CLL patients also have a significant degree of hypogammaglobulinemia. Autoimmunity and immunodeficiency have been related to B-CLL biologic properties and cellular origin. [42] It has been suggested that CD5+ B cells may play an important role in the production of IgM autoantibodies. An

TABLE 72-2 -- Factors Contributing to Immunodeficiency in Chronic Lymphocytic Leukemia

Reduced serum immunoglobulin
Reduced percentage of CD4+ cells, increased percentage of CD8+ cells (worsening with disease prognosis)
Reduced response to antigens and mitogens

Modified from Foa R: Pathogenesis of the immunodeficiency in B-cell chronic lymphocytic leukemia. In Cheson BD (ed): Chronic Lymphocytic Leukemia: Scientific Advances and Clinical Developments. Marcel Dekker, New York, 1993, p. 147, with permission.

increase in the number of CD5+ B cells has been reported in rheumatoid arthritis and other autoimmune diseases. [48] Autoimmune phenomena in CLL are often directed against hematopoietic cells. A positive direct antiglobulin test has been noted in 35% of CLL cases. Autoimmune hemolytic anemia occurs in 1025% of cases at some time during the course of the disease.[39] In most cases, the autoantibodies against erythrocytes are warm reactive and polyclonal, [48] with or without red-cell-associated C_3 b or C_3 d. Immune thrombocytopenia occurs in a few CLL cases. Pure red cell aplasia and autoantibodies against neutrophils are observed less frequently.

Hypogammaglobulinemia is a not unusual feature of CLL. The levels of serum IgG, IgA, and IgM may all be markedly decreased, or just one or two of the immunoglobulin classes may be involved. The pathogenesis of this complication is poorly understood, but regulatory abnormalities of helper T, suppressor T, NK, and antibody-dependent cellular cytotoxicity cells may play a role. [49] NK cells from patients with CLL with hypogammaglobulinemia were found to cause a decrease in immunoglobulin secretion by normal B cells.[50] It is also possible that a decrease in or inhibition of normal B cells (CD5) results in hypogammaglobulinemia. [39] A monoclonal serum immunoglobulin spike (usually IgM) has been observed in 5% of cases with CLL. Patients with CLL tend to have defective specific antibody responses to infection and to immunization.[51] Infections with encapsulated organisms as well as with gram-negative bacteria are recognized as the most frequent cause of morbidity and mortality in CLL. [51] [52]

A summary of the currently recognized factors that may play a role in the heterogeneous immunologic abnormalities in CLL is shown in Table 72-2 . There are additional factors (such as reduced NK, antibody-dependent cellular cytotoxicity, and lymphokine-activated killer cell activity, increased levels of soluble IL-2 receptors, and reduced IL-2 availability), but their role is far from clearly proven. The available data strongly suggest that the abnormalities of the T- and cytotoxic cell compartments represent a secondary event occurring during the course of CLL. [45]

CLINICAL MANIFESTATIONS

Criteria for Diagnosis of CLL

The National Cancer Institute-sponsored Working Group (NCI-WG) on CLL was charged with the task of developing guidelines for protocol studies in this disease, and in that context it recommended the following three diagnostic requirements: [53] [54]

1. An absolute lymphocytosis in the blood, with a count of 5×10^9 /L, and cells morphologically mature in appearance, sustained over at least a 4-week period.
2. At least 30% lymphocytes in a normocellular or hypercellular bone marrow.
3. A monoclonal B-cell phenotype expressed by the preponderant population of blood lymphocytes with low levels of surface immunoglobulins and simultaneously showing CD5 positivity (a pan-T-cell marker).

The requirement that the lymphocytosis should be sustained over a period of time is included in these diagnostic criteria to exclude those conditions (e.g., infectious mononucleosis, pertussis, toxoplasmosis, and cytomegalovirus infection) in which lymphocytosis is transient. In our view, if a bone marrow lymphocytosis also is required for the diagnosis of CLL, it is not necessary to prove that blood lymphocytosis is of a sustained nature because none of the conditions with transient lymphocytosis is associated with bone marrow involvement.

The International Workshop on CLL (IWCLL) [55] proposed somewhat similar diagnostic criteria, but it requires 10×10^9 /L lymphocytes in the blood for the diagnosis to be made if facilities to obtain phenotyping are not available. IWCLL recommended that a diagnosis of CLL can be made in a patient with $<10 \times 10^9$ /L lymphocytes in the peripheral blood, provided phenotyping is performed and reveals the pattern characteristic of CLL, as described previously.

The FAB Cooperative Group [56] states that when >10% of cells in the blood are large (or prolymphocytic) in appearance, the diagnosis of mixed-cell-type CLL should be considered. In our experience, clearly identifiable forms of prolymphocytic leukemia are a consistent clinical identity. The importance of occasional prolymphocytic-appearing cells in the peripheral blood in CLL is controversial.

Symptoms

The usual complaints of patients with CLL are weakness, easy fatigue, night sweats, fever without infections, weight loss, frequent bacterial and viral infections, increased bleeding tendencies, and exaggerated responses to mosquito or other insect bites. These are symptoms often associated with malignancy and immunodeficiency. Symptoms may be entirely absent, or only some or all may be present in varying severity.

Findings on Physical Examination

The most frequently noted abnormal finding on physical examination is lymphadenopathy. Only a single node-bearing area may be involved, or lymph nodes may be palpably enlarged in the cervical, axillary, and inguinofemoral areas. These nodes may be small (e.g., about 1 cm in diameter) or massively enlarged. The enlarged lymph nodes in CLL are almost always nontender, nonpainful, discrete, firm, and easily movable on palpation. Enlargement of spleen and liver when present may range from barely palpable to 15 or 20 cm below the respective costal margin. In addition, infiltration by CLL cells may be manifested in virtually all other parts of the body, including the meninges and skin.

Laboratory Evaluation

Absolute lymphocytosis in blood, with mature-appearing cells, is one of the two major presenting features of CLL (Plate 72-1). The blood lymphocyte count may range from 5 to 500 $\times 10^9$ L, but in most cases it is $>20 \times 10^9$ /L. Similarly, the differential count of bone marrow aspirate smear may reveal lymphocytes accounting for as little as 30% of all nucleated cells or as much as 99%; in the latter, the marrow is totally replaced by monotonously similar-appearing lymphocytes. The overall cellularity of bone marrow is normal or increased; a hypocellular marrow is not a typical finding in CLL unless it is the result of cytotoxic therapy. Depending on the extent of lymphocytic infiltration, myeloid and erythroid precursors and megakaryocytes may be decreased or normal. Pure red cell aplasia, [57] [58] however, may also occur in CLL. Bone marrow biopsy examination has become virtually routine whenever an aspiration procedure is performed in CLL. Biopsy specimens are characteristically infiltrated with lymphocytes, but the patterns of such infiltration

may be diffuse, nodular, or interstitial [59] [60] [61] [62] [63] [64] (Plates 72-2 , 72-3 , 72-4).

Lymphocytes in the blood and the marrow appear morphologically mature. However, it is now well recognized that functionally they are not mature inasmuch as they are arrested at an intermediate level of differentiation. [65] Lymphocytes in CLL are usually small, with the nucleus filling almost the entire cell, and the nuclear chromatin is dense and clumped and without any discernible nucleolus (Plate 72-1). Occasionally the CLL lymphocyte may be a large cell with a round or somewhat notched nucleus, there may be an indistinct nucleolus, and the cytoplasm may be abundant and slightly basophilic or orthochromatic. Several morphologic variants of CLL have been described in the literature (e.g., prolymphocytic leukemia and Sézary cell leukemia). [56] Preparation of blood films may cause severe morphologic deformities of CLL lymphocytes, recognized as smudge cells (Plate 72-5).

Although CLL is characterized by leukocytosis, the proportion of neutrophils is always reduced, but to a varying degreefrom as low as 1% to as high as 40%. Thus, the absolute neutrophil count may be normal, extremely low, or extremely high, depending on total leukocyte count and percentage of neutrophils.

Approximately 30% of all patients with CLL may have somewhat decreased hemoglobin values or platelet counts, but these values are significantly decreased in only 15% of cases (hemoglobin <110 g/L, platelets $<100 \times 10^9$ /L) at the time of initial diagnosis. Autoimmune complications, including Coombs-positive hemolytic anemia, immune thrombocytopenia, and hypogammaglobulinemia, were discussed previously.

There is no characteristic abnormality of the blood chemistry profile in CLL, but hypercalcemia and abnormal liver and kidney function test results may be encountered.

Differential Diagnosis

The differential diagnosis of CLL includes the entire spectrum of chronic lymphoproliferative disorders. Malignant lymphoma in the leukemic phase may sometimes be indistinguishable from CLL, and the most helpful findings in such situations are phenotypic profiles of lymphocytes. Whereas the fluorescence intensity of surface immunoglobulins on B lymphocytes is bright in lymphomas, it is very faint in CLL; only CLL B lymphocytes form rosettes with mouse erythrocytes. On flow cytometry, B lymphocytes show dual positivity with CD19 and CD5 or CD20 and CD5 both in CLL and in the leukemic phase of mantle cell lymphoma, but CLL cells are CD23+, whereas mantle cell lymphoma cells are CD23.

T-cell CLL and morphologic variants of CLL (e.g., prolymphocytic leukemia, Sézary syndrome, hairy cell leukemia) are distinguished by their respective characteristic phenotypic and microscopic appearances.[56] The phenotypic characteristics useful in differentiating these various disorders are summarized in Table 72-3 .

Prognosis

Just as the initial extent of disease is variable in CLL, the prognosis and clinical course also are extremely variable. Some patients have a rapid downhill course and die within 23 years after diagnosis, whereas others have a very benign, indolent course and live for 10 or 20 years without major problems from CLL. Approximately one half of patients with CLL have a disease course somewhere in between the two extremes. Boggs et al.[66] studied several prognostic factors and concluded that the extent of disease at initial diagnosis correlates inversely with survival.

Clinical Staging System of Rai

Building on the work of Dameshek,[4] Boggs et al.,[66] and of Hansen,[67] my colleagues and I (KRR)[68] were able to devise a clinical staging system in which patients with minimum evidence of disease (those merely satisfying the minimum diagnostic criteria) were considered to be in the earliest stage of disease, whereas those demonstrating significant compromise of bone marrow function (as an index of high leukemic cell burden) were considered to be in advanced stages. This staging system is detailed in Table 72-4 .

The definition of lymphocytosis in our original description consisted of a 15 × 10^9 /L or higher absolute lymphocyte count in blood and 40% of lymphocytes in the marrow differential count of all nucleated cells. However, following the recommendations of the NCI-WG [53] [54] and the IWCLL[55] detailed previously, we suggest that these thresholds be modified to 5 × 10^9 /L and >30% in blood and marrow, respectively. The presence of palpably enlarged lymph nodes was found to be of prognostic value (stage I), but it did not seem to make much difference

TABLE 72-3 -- Markers Useful in Distinguishing Chronic Lymphocytic Leukemias (CLL)

Marker	CLL	Prolymphocytic Leukemia	Hairy Cell Leukemia	Leukemic Phase of Follicular Non-Hodgkin Lymphoma	Mantle Cell Lymphoma	T-CLL	Sézary Syndrome
Surface immunoglobulin	Weak	Strong	Strong	Strong	+	Negative	Negative
Mouse red cell rosetting	++		±	±			
Sheep red cell rosetting						++	++
CD2						++	++
CD3						++	++
CD4						+	++
CD5	++	±			++	++	++
CD7						++	
CD8						+	
CD19/20/24	++	++	++	++			
CD23	++	±					
CD22	±	++	++	+			
CD10		±		+			
CD25			++				
CD38			±	±			

+, incidence at which a marker is positive in >40% of cells in a particular leukemia; ++, 80100% ; ±, 1040% ; , 09% of cases.

Modified from Bennett JM, Catovsky D, Daniel MT et al: The French American British (FAB) Cooperative Group proposals for the classification of chronic (mature) B and T lymphoid leukemia. J Clin Pathol 42:567, 1989, with permission.

TABLE 72-4 -- Rai Clinical Staging Systems

Level of Risk	Stage	Description
Low[a]	0	Lymphocytosis only (in blood and marrow)
Intermediate[a]	I	Lymphocytosis plus enlarged nodes
	II	Lymphocytosis plus enlarged spleen or liver with or without enlargement of nodes
High[a]	III	Lymphocytosis plus anemia (hemoglobin <110 g/L) with or without enlarged nodes, spleen, liver
	IV	Lymphocytosis plus thrombocytopenia (platelets <100 × 10^9 /L) with or without anemia or enlarged nodes, spleen, liver

[a]Modified Rai system.

whether adenopathy was bulky or minimal, or whether it involved a single node-bearing region or was generalized. Median survival correlated inversely with the clinical stage: stage 0, 12+ years; stage I, 8.5 years; stage II, 6 years; stage III, 1.5 years; and stage IV, 1.5 years. [68]

Modified Rai System

Although several investigators confirmed the validity of the staging system of Rai et al. as a reliable predictor of survival time in CLL, many workers found that having as many as five stages in the system made it difficult to plan prospective therapeutic trials. We had, however, acknowledged in our original proposal [68] in 1975 that in the actuarial survival curves, there were indeed only three, not five, distinct patterns: (1) stage 0, (2) stages I and II combined, and (3) stages III and IV combined. [68] Therefore, a formal modification[69] of the staging system was published in 1987, which assigns stage 0 to the low-risk group, stages I and II combined to the intermediate-risk group, and stages III and IV combined to the high-risk group (Table 72-4). The survival curves according to the modified Rai system in a large series of patients with CLL followed in the Hematology Clinic of the University of Barcelona in Spain [70] are shown in Figure 72-1 . These curves are statistically

significantly different from each other and demonstrate that the patients in the low-risk group have the best outlook for survival. The NCI-WG recommends

Figure 72-1 Survival according to the modified Rai staging criteria in 443 patients with chronic lymphocytic leukemia at the Postgraduate School of Hematology, Barcelona, Spain. *(From Montserrat E, Rozman C: Chronic lymphocytic leukaemia: prognostic factors and natural history. Baillieres Clin Haematol 6:849, 1993, with permission.)*

[53] [54] using the modified Rai staging system for prospective therapeutic trials.

Staging System of Binet and Colleagues

The only other staging system that has found wide acceptance in clinical practice is the one devised by Binet et al. [71] This system is similar to the Rai system in concept. Stage C consists of all patients who have anemia (hemoglobin <100 g/L) or thrombocytopenia (platelets <100 × 10^9 /L). All other (non-C) patients are divided into A or B stages depending on the number of lymphoid-bearing areas palpably enlargedtwo or less for A and more than three for B (there are five of these areas: the cervical, axillary, and inguinofemoral nodes; spleen; and liver). This three-stage system is an excellent predictor of survival and is useful in planning therapeutic trials. Binet et al. have observed that the survival times of stage A patients do not differ from those of age- and sex-matched normal members of the French population. Inasmuch as stage A patients include those who have splenomegaly with one area of lymphadenopathy (or two areas of adenopathy without splenomegaly), it is somewhat surprising that the life expectancy of such patients is equal to that of the nonleukemic normal French population. Conceptually, in our opinion, the stage 0 subgroup of Binets A should have a better prognosis than all other (non-stage-0) patients in stage A. In actual practice, both the Rai and the Binet systems are used in clinical management and in therapeutic protocols.

Other Staging Systems

The IWCLL recommends the use of an integrated Binet and Rai system in which each Binet stage (A, B, or C) is subclassified according to the corresponding Rai stage.[55] However, clinicians use either of the two systems without the recommended integration.

Jaksic and Vitales[72] total tumor mass estimation yields a score indicating the size of the spleen and the largest palpable lymph node, while also including a factor related to the blood lymphocyte count. Other systems proposed include those of Mandelli et al., [73] Lee et al.,[74] Baccarini et al.,[75] Skinnider et al.,[76] and Paolino et al.[77] Each of these systems has certain advantages and helps a physician in individual cases, but none has found wide usage, perhaps because the Rai and Binet methods are simple to apply and succeed in segregating large populations of patients with CLL into distinct groups of survival outlook.

Additional Prognostic Features

Numerous clinical, hematologic, and laboratory abnormalities, as well as immunophenotypic and cytogenetic characteristics have been reported to be indicative of adverse prognosis in CLL. Some of these are listed in Table 72-5 . Perhaps only a few of these features have a consistent impact on prognosis, and are described in the following sections.

Lymphocyte Doubling Time

Although, as an indicator of prognosis, the lymphocyte count acts as a continuous variable, we have not found a threshold (such as a cut-off level of 4050 × 10^9 /L) count consistently reliable. However, the rate of increase of the absolute lymphocyte count in the blood of patients with CLL not receiving cytotoxic therapy has proven to be a reliable indicator of disease activity. A serial plotting of blood lymphocyte counts provides a measure of this activity and, either by extrapolation or by actual observation, it can be determined whether the blood lymphocyte count doubles slowly (12 months) or rapidly (<12 months); the latter is associated with a worse prognosis.[78] [79] [80]

TABLE 72-5 -- Factors Associated with a Poor Prognosis

Clinical
Lymphadenopathy
Splenomegaly
Hepatomegaly
"Bulky" disease
Poor performance status
Hematologic
Anemia
Thrombocytopenia
Large and atypical lymphocytes in blood
Diffuse bone marrow lymphocytic infiltration
Laboratory abnormalities
Increased serum lactate dehydrogenase level
Hypoalbuminemia
Increased serum calcium level
Cytogenetic abnormalities
Complex and multiple cytogenetic abnormalities and 17p, 11q
Immunologic
Hypogammaglobulinemia
Immunophenotype (different abnormalities related to poor prognosis (e.g., Smlg+++, CD5, CD23)
Increased serum-soluble CD25 receptors
Increased serum-soluble CD23 receptors
Kinetic parameters
Rapid doubling time
Others
Poor response to therapy

Adapted from Montserrat E, Rozman C: Chronic lymphocytic leukaemia: prognostic factors and natural history. Baillieres Clin Haematol 6:349, 1993, with permission.

Bone Marrow Histopathology

The pattern of lymphocytic infiltration in the bone marrow biopsy specimens (Plates 72-2 , 72-3 , 72-4) can be classified as either diffuse or nondiffuse. The nondiffuse pattern may be nodular, interstitial, or mixed nodular and interstitial. Patients with diffuse infiltration have a worse prognosis than those with nondiffuse. The diffuse pattern is seen most frequently in CLL of advanced clinical stage, whereas a nondiffuse infiltration is the more likely pattern in early stages of CLL. [59] [62] [63] [81]

Immunophenotypic Features

A large, prospective study of flow-cytometric immunophenotyping in Denmark provides the most systematic analysis of the prognostic value of these findings. [82] There were 503 CD5+ and 37 CD5 cases. The survival of CD5 patients was on the borderline of being significantly shorter than that of CD5+ patients. Most CD5 patients had malignant lymphocytes with strong sIgM fluorescence, and were FMC7+ and CD23, indicating that CD5 cases represent an atypical variant of CLL. [82] Among the CD5+ cases, by Cox multiple regression analysis, a few features emerged with independent prognostic importance: higher age, low CD23 expression and high sIgM fluorescence intensity, and advanced clinical stage were all associated with worse prognosis. CD20, CD21, and CD22 expression did not have prognostic importance.[82]

Cytogenetics

A clonal chromosomal abnormality indicates a poorer prognosis compared with a normal karyotype. [24] As noted in an earlier section, recent studies suggest that patients with normal karyotype, single abnormality of 13q and trisomy 12 have better prognoses than those with 17p and 11q. [28]

TABLE 72-6 -- Definition of Smoldering Chronic Lymphocytic Leukemia

Patients in Binets stage A
Nondiffuse lymphocytic infiltration in bone marrow biopsy
Lymphocyte doubling time >12 mo
Blood lymphocyte count 30 × 10^9 /L
Hemoglobin 13 g/dl

From Montserrat E, Rozman C: Chronic lymphocytic leukaemia: prognostic factors and natural history. Baillieres Clin Haematol 6:849, 1993, with permission.

Prediction of Clinical Course in Patients with Early Stages

Neither the Rai nor the Binet system of clinical staging can reliably predict the clinical course of patients in the nonadvanced stages (0, I, and II for the Rai and A and B for the Binet) of CLL. It is widely recognized that there is a group of patients in these stages whose disease course remains indolent for prolonged periods (several years) and another group with a relatively progressive and active course. Several prognostic factors have been tested by numerous investigators since the 1970s. These include a blood lymphocyte count above or below a certain threshold; [75] [83] [84] lymphocyte morphology and size;[79] [85] serum levels of several enzymes (e.g., lactate dehydrogenase[86] and deoxythymidine kinase[87]); serum -microglobulin levels;[88] phenotype of blood lymphocytes;[82] [89] and chromosomal abnormalities.[24] [28] The value of these criteria for predicting the clinical course of CLL has not been universally accepted.

Patients with early stages of CLL whose blood lymphocyte doubling time is long (>12 months) and whose bone marrow biopsy shows a nondiffuse pattern of lymphocytic infiltration tend to have an indolent course of the disease. The Spanish group [90] retrospectively tested certain criteria associated with nonprogressive (or stable) CLL among patients in Binets A stage, which they call smoldering CLL. They noted that (in addition to the two criteria mentioned previously) a relatively low (30 × 10^9 /L) absolute lymphocyte count and a relatively high (13 g/dL) hemoglobin (Table 72-6) characterize the category of smoldering CLL. The patients with smoldering CLL were found to have life expectancies no different than those of an age- and sex-matched control population and a significantly lower risk of disease progression compared with the Binets stage A patients not meeting the criteria of smoldering CLL (and were deemed to have active CLL; [70] Table 72-7).

CLL in the Younger Age Group

Chronic lymphocytic leukemia is a disease of the elderly, with a median age at diagnosis of 55 years. However, with the easy

TABLE 72-7 -- Disease Activity in Smoldering versus Active Chronic Lymphocytic Leukemia (CLL)

	Smoldering CLL[a] (%)	"Active" CLL (Nonsmoldering)[b] (%)	
Risk of progression			
at 3 y	8	57	
95% CI	616	4272	
at 5 y	13	57	
95% CI	324	4272	*P*<0.001
Surviving at 10 y	78	43	
95% CI	5699	2264	*P*<0.05

CI, confidence interval.

Data from Montserrat E, Rozman C: Chronic lymphocytic leukaemia: prognostic factors and natural history. Baillieres Clin Haematol 6:849, 1993, with permission.

[a] 38 patients.

[b] 161 patients.

availability of routine blood counts in todays society, CLL is being diagnosed in increasing frequency both at earlier stages of the disease as well as in younger age groups. Approximately 12% of patients are <50 years of age at diagnosis. Although nearly two thirds of younger patients with CLL are >40 years of age, a Mayo Clinic report shows 18 years as the lower end of the age range.[80] With the advent of newer and more aggressive therapies in CLL, several studies have been conducted to determine whether younger patients with CLL have different outcomes from those in the more typical, older age groups. These studies show that there are no differences in the presenting features, treatment response rates, and median durations of response between the younger and the older age groups. [91] [92] [93] [94] [95] [96] The Mayo Clinic study was confined to the prognostic features of nonadvanced stages of CLL in the younger patients, and it showed that the survival curves for stages 0 and I were virtually superimposable, with a median duration of 140 months, which was significantly longer than the median duration of 60 months for stage II patients. On multivariate analysis of several factors, only clinical stage (0 and I vs. II) and lymphocyte doubling time (>12 vs. 12 months) emerged as prognostically useful among younger CLL patients in the nonadvanced stages.[80] These observations are helpful in planning long-term therapeutic options for these patients, who do not find it particularly reassuring to be informed when they are 40 years of age that their median life expectancy is 10 or 12 years. The same prognosis may not have such a grim impact on a 70-year-old person. Therefore, although it is helpful to know that there are no unique prognostic factors for the younger patients with CLL, the ability of the physician as well as the patient to weigh the risks and benefits of various treatment options is greatly influenced by the patients age.

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THERAPY

Period of Observation Without Cytotoxic Therapy

It is prudent to withhold cytotoxic therapy after the initial diagnosis of CLL has been established. [97] Even after a period of observation, it may be the best operational decision in most patients to continue the policy of no treatment with reassessment of this decision at regular intervals of 36 months. Because the clinical course is highly variable in this disease, the physician can use the therapy-free period to observe whether the disease is stable or progressive in an individual patient. The following criteria are most helpful in these periodic reassessments:

1. Chart the blood lymphocyte counts and determine the rate of increase in their numbers, to estimate whether the doubling time (actual or projected) is long (>12 months) or short (12 months).
2. Continue to reassess clinical stage.
3. Note the pattern of lymphocytic infiltration in bone marrow biopsy specimen.
4. Note the presence or absence of constitutional symptoms. The therapy-free period may be extended indefinitely for those patients whose disease appears to be indolent. A minority of patients have an aggressive course of CLL and require institution of some therapy within 24 weeks from the time of initial diagnosis.

Indications for Therapeutic Intervention

A decision to start antileukemic therapy is made in the presence of any of the following indications: [97]

1. Disease-related progressive symptoms (e.g., weight loss without trying, fever without overt infection, night sweats, weakness, or easy fatigability).
2. Progressively worsening anemia or thrombocytopenia.
3. Autoimmune (Coombs-positive) hemolytic anemia or autoimmune thrombocytopenia.
4. "Bulky" lymphadenopathy that is getting progressively worse, and poses risk to the patient from pressure on underlying tissues, or causes significant cosmetic problems.
5. Massive splenomegaly that is worsening progressively or results in hypersplenism.
6. Progressive hyperlymphocytosis. It is not possible to set a rigid upper threshold for the blood lymphocyte count that must be met before starting therapy, but it is our current practice not to allow this count to be >150 × 10^9 /L. Hyperviscosity syndrome associated with hyperlymphocytosis in CLL can be catastrophic. [98] [99] As noted earlier, the rate of increase of blood lymphocyte count is of importance; thus, a short doubling time (12 months, actual or by extrapolation) is an indication for therapeutic intervention.
7. Increased susceptibility to bacterial infections. [50] [51] This may result from marked hypogammaglobulinemia, in which case intravenous high-dose -globulin therapy has a proven protective effect.[100] Severe neutropenia or agranulocytosis may occur in CLL, and may play a major role in the development of bacterial sepsis.[51]

Choices of Therapeutic Modalities

Alkylating Agents

Chlorambucil and cyclophosphamide are the most frequently used initial drugs of choice. Chlorambucil is administered orally and is readily absorbed from the gastrointestinal tract. There are two methods of treatment with this drug: small-dose continuous therapy (0.07 mg/kg/day with adjustments as needed by monitoring blood counts at weekly or biweekly intervals) or large-dose bolus intermittent therapy (0.7 mg/kg at intervals of 3 or 4 weeks). [101] Both methods are approximately equally effective, markedly reducing the size of previously enlarged lymph nodes or spleen, but patient compliance is perhaps better with intermittent therapy. The premise[102] [103] that the intermittent method may enable recovery of normal hematopoietic elements in the marrow before regrowth of leukemic cells between two successive doses and thus be superior to daily continuous administration of chlorambucil was not conclusively proven to be the case in a randomized trial sponsored by the Cancer and Leukemia Group B (CALGB).[101] Our preference is for intermittent chlorambucil, but both methods are commonly used by physicians caring for patients with CLL. The dose-limiting toxicity is bone marrow suppression, which is reversible if it is recognized promptly by frequent blood counts. Nausea, vomiting, mucositis, and so forth, are not major problems with chlorambucil.

The second most frequently used alkylating agent in CLL is cyclophosphamide, which may be given orally or intravenously. This drug, like chlorambucil, is also given either on a daily basis (50150 mg/day PO) or intermittently (1,0001,200 mg every 24 weeks PO). The intravenous dose is the same as the intermittent oral dose. Cyclophosphamide is equal to chlorambucil in its effectiveness in control of CLL, but some patients who are starting to show refractoriness to the latter respond to the former. Besides bone marrow suppression, chemical cystitis is a major side effect of cyclophosphamide, but is avoidable in most cases by ensuring adequate hydration and advising the patients to urinate frequently after each intermittently administered dose. The incidence of nausea is somewhat greater than with chlorambucil. Both drugs are effective as first-line single-agent therapy in CLL. Other alkylating agents (e.g., melphalan or nitrogen mustard) may also be effective, but they are associated with considerable toxicities and are rarely used in CLL.

Fludarabine monophosphate is a fluorinated analogue of adenine that is resistant to deamination by the enzyme adenosine

deaminase. This drug has proved an effective therapy for patients with CLL who are resistant to alkylating agents. [104] Fludarabine is given intravenously at a dose of 25 mg/m^2 /day for 5 consecutive days every month. Usually four to six treatments at monthly intervals are required to achieve the maximally achievable benefits from this drug. Although fludarabine does not cause nausea, vomiting, and hair loss, prolonged use may result in cumulative myelotoxicity and infectious complications. Precautions against tumor lysis syndrome during the initial phase of therapy with fludarabine are advised. Addition of prednisone does not increase response rates of fludarabine, but may increase the risk of opportunistic infections. [105] [106]

Other Chemotherapeutic Drugs

Alkylating agents and fludarabine are the only cytotoxic drugs used as single agents in CLL, but vincristine, doxorubicin, nitrosoureas, mitoxantrone, and others are administered as part of several combination chemotherapy protocols. Even though vincristine has been administered frequently in CLL (being a part of several treatment schedules tested in the lymphomas and used when a patient with CLL is refractory to single-agent therapy), there is no clear evidence that this drug is

indeed useful in the treatment of CLL. Considering the potential for significant peripheral neuropathy associated with vincristine therapy in patients with CLL, who usually are elderly, we recommend either reduced dosage or avoidance of this drug when using a combination chemotherapy schedule in patients with CLL, who usually are elderly.

Glucocorticosteroids

Glucocorticosteroids (e.g., prednisone) are frequently used in CLL either as single agents in the management of autoimmune hemolytic anemia and thrombocytopenia or as part of combination chemotherapy protocols in other cases. Prednisone has significant lymphocytolytic effect; it causes marked reduction in previously enlarged nodes and spleen, and after an initial phase of causing a further increase in blood lymphocytosis, eventually results in a significant decrease when therapy is continued for several days or a few weeks. Side effects of prednisone use (especially in elderly patients with CLL) that should be kept in mind are increased blood sugar levels, worsening of pre-existing osteoporosis, psychiatric reactions ranging from euphoria to severe depression, and increased susceptibility to infections, particularly reactivation of old healed tuberculous lesions. Prednisone is given orally on an intermittent schedule of 4080 mg/day for 57 days every month. In some patients with persistent chronic hemolysis, a lower-dosage maintenance schedule (e.g., 515 mg/day or twice a week) may be necessary.

Androgens or Anabolic Steroids

Androgens or anabolic steroids[107] have been used in patients with CLL with marked anemia (considered to be due to leukemic infiltration of bone marrow or erythroid hypoplasia) to stimulate erythropoiesis. These agents are not uniformly effective, but in some patients have been very beneficial; therefore, in selected situations, their use is justifiable. Side effects include hepatic toxicity, hirsutism, and prostatism. There have been reports of diethylstilbestrol causing significant reduction in blood lymphocyte counts when used in those patients with prostate cancer who happened also to have CLL,[108] but these results have not been confirmed in any controlled trials in CLL.

Radiation Therapy

The most frequently used form of radiation therapy is splenic irradiation,[109] [110] which in selected patients results in prompt reduction in the size of an enlarged spleen, accompanied by evidence of partial control of overall disease. This treatment is particularly beneficial in patients with marked splenomegaly who are not responsive to chemotherapy. Irradiation of large, bulky lymphoid masses localized in one region, nonresponsive to chemotherapy, is also an effective method of treatment. Experimental therapies, including extracorporeal irradiation of blood,[111] mediastinal irradiation,[112] [113] [114] total-body irradiation,[109] [115] [116] and administration of radioactive isotopes,[117] have proved to be either too toxic or ineffective. A consultation with a radiation oncologist experienced in treating CLL and other hematologic malignancies is recommended in deciding whether radiation therapy is advisable.

Splenectomy

Splenectomy [118] [119] [120] [121] [122] [123] is an effective treatment for CLL in specific clinical situations. Patients with extensive splenomegaly unresponsive to chemotherapy and with significant anemia or thrombocytopenia attributed to hypersplenism (some of these patients are not considered for splenic irradiation because of fear of worsening anemia or thrombocytopenia, and some of them may have failed radiation therapy), and patients whose response to steroid therapy is inadequate in the presence of autoimmune anemia are among those considered to be candidates for splenectomy. Beneficial response to splenectomy may last from a few months to several years. Pneumococcal vaccine should be administered before surgery.

Leukapheresis

Leukapheresis [124] [125] has a very limited role in the long-term management of CLL. When the blood leukocyte count is >500 × 10^9 /L either at diagnosis or during the course of the disease, catastrophic complications of hyperviscosity and thromboembolic phenomena may be avoided by resorting to intensive leukapheresis (using one of the automatic cell-separating machines) while simultaneously providing adequate hydration and initiating cytotoxic chemotherapy. Leukapheresis as the sole therapeutic measure is of no benefit because the reduction in blood lymphocyte count so achieved is very transient.

Criteria for Evaluating Response

The NCI-WG[53] [54] and the IWCLL[55] have separately proposed a series of criteria to assess objectively complete or partial remission in CLL. To qualify for complete remission, a patient must have no adenopathy, no hepatosplenomegaly, no constitutional symptoms, normal hemogram (hemoglobin >110 g/L, platelets >100 × 10^9 /L, absolute lymphocytes <4 × 10^9 /L, and absolute neutrophils >1.5 × 10^9 /L), and the bone marrow must contain <30% lymphocytes. To qualify for partial remission, IWCLL criteria require an improvement in clinical stage (e.g., from Binets C to B or A or from Binets B to A). The NCI-WG recommends a similar approach (i.e., improvement in clinical stage), but in addition defines partial remission by a >50% reduction in the previously enlarged nodes, spleen, or liver, together with a >50% improvement of peripheral blood values over baseline (when they do not approach the levels described for complete remission).

Therapy Based on Clinical Stage

Low-risk (stage 0) patients should not be started on cytotoxic therapy unless significant constitutional symptoms develop or there is evidence of an active clinical course, as described previously.

Intermediate-risk (stage I and II) patients should also be observed without antileukemic therapy until there is evidence of

an active clinical course, as described previously, or of any of the indications for therapeutic intervention, also as enumerated previously. In randomized studies, CALGB[126] and the French Cooperative Group[127] assigned early-stage patients to chlorambucil therapy or observation alone. Both these studies showed that early treatment with chlorambucil did not improve survival, although it did correlate with a somewhat slower progression to the more advanced stages. If treatment is indicated, chlorambucil as a single agent is the appropriate first-line therapy. The therapeutic end point is unclear; we do not know whether pushing treatment to try to achieve a complete remission is necessarily beneficial for the patient. The current practice, however, is to at least try to eliminate whatever indication required initiation of therapy in the first instance, and, if possible, to maximize the best achievable level of response without subjecting the patient to undue toxicity.

High-risk (stages III and IV) patients have a uniformly poor prognosis and should be started on cytotoxic therapy. A large trial by CALGB [101] revealed that if at least a partial (complete if possible) remission is achieved after chlorambucil and prednisone therapy, there is a significant improvement in survival compared with survival in those patients who failed to achieve such a response. This was the first study that defined the therapeutic end point in advanced CLL (i.e., a partial or complete remission). The French Cooperative Group[128] demonstrated a significantly better survival of advanced-stage patients after treatment with COP (cyclophosphamide, vincristine [Oncovin], prednisone) together with low-dose doxorubicin (CHOP), compared with patients who received COP without doxorubicin. [128] The results of this study, however, have not been confirmed by subsequent trials, nor has there been any evidence that a somewhat higher response rate with CHOP provides a survival advantage over COP, or chlorambucil and prednisone therapy. [129] [130] [131]

Fludarabine as Initial Therapy

Among the three nucleoside analogues fludarbine, pentostatin, and 2-chlorodeoxyadenosine that have been used in CLL, only fludarabine has been tested in prospectively controlled clinical trials in previously untreated patients with active disease. These results demonstrate clear superiority of fludarabine in inducing a higher incidence of remissions (both complete and overall) compared with patients treated with chlorambucil or combination chemotherapy. [132] [133] Although use of fludarabine as a front-line agent in CLL has so far not been shown to increase the overall duration of survival, encouraged by the higher response rates, some clinicians are using this drug as the initial therapy. We recommend including fludarabine alongside chlorambucil and cyclophosphamide among the list of drugs worthy of consideration of use in front-line treatment of CLL and making the choice between it and an alkylator on the individual patients clinical status, such as age, co-morbid conditions, and overall goals of therapy (whether it is palliative or likely to be followed by high-dose chemotherapy and stem cell transplantation). Fludarabine, like chlorambucil and cyclophosphamide, is useful in all stages of CLL when therapeutic intervention with drugs is indicated. It is advisable to avoid the

use of glucocorticosteroids with fludarabine because of an increased risk of opportunistic infections.

Second-Line Therapy

Fludarabine

Fludarabine has proven to be an extremely effective drug for patients with CLL who have failed prior therapy with an alkylating agent. [104] [105] [106] A large proportion of such patients obtain objective responses, and responding patients have an improved survival rate.

Combination Chemotherapy

Other multiagent combination chemotherapies (e.g., POACH [COP plus cytosine arabinoside and doxorubicin], [134] devised at the M.D. Anderson Hospital in Houston, TX) or the M-2 protocol[135] (COP plus melphalan and carmustine, developed for treatment of multiple myeloma at the Memorial Sloan-Kettering Cancer Center in New York) also have some promise in the management of advanced stages of CLL, but the results are not uniformly consistent.

Special Therapeutic Issues

High-Dose Intravenous Immunoglobulin

As mentioned earlier, significant hypogammaglobulinemia is frequently observed in CLL, rendering these patients highly vulnerable to bacterial infections. With the availability of purified immunoglobulins for intravenous administration, it is now possible to treat those patients with CLL who are at increased risk of infections. In a multi-institutional, placebo-controlled, randomized trial, [100] it was observed that replacement therapy with intravenous -globulin provides a significant protection from major bacterial infections in patients with CLL who would otherwise be at risk. The -globulin dosage in this study was 400 mg/kg body weight every 3 weeks for 1 year. Although such therapy appears rather expensive, when the potential costs of treating bacterial pneumonia in a hospital inpatient setting are considered, the cost of such preventive measures becomes justifiable in certain selected patients who are at high risk for infections. We have been using a lower dosage (200 mg/kg body weight) of intravenous immunoglobulin, which appears to provide protection from infections at significantly reduced costs. A multicenter trial comparing a high dosage (500 mg/kg/mo) with a lower dosage (250 mg/kg/mo) has been conducted, and the preliminary results show that both dosages are equally effective in preventing bacterial infections in the at-risk patients with CLL. [136] Thus, if the patients are selected for their previous history of major bacterial infections or severe hypogammaglobulinemia and the dosage of intravenous immunoglobulin is lowered, the overall cost of such a preventive measure would not remain as big an issue as was previously considered.[137] This therapy is well tolerated and can be given at the patients home or in outpatient clinics.

Interferon-

Although initial results[138] with IFN- in advanced stages of CLL were very disappointing, subsequently performed studies suggest that this agent may benefit patients in early stages of the disease, or when the tumor burden is relatively low. [139] [140] [141] [142] The clinical benefits from a combination of IFN- and chlorambucil and prednisone have been reported, but need confirmation by additional studies. [143] The mechanism of action of IFN- in CLL is not understood. In vitro, it causes differentiation of CLL cells,[144] and there are also data to suggest that it inhibits apoptosis of CLL cells. However, there is no evidence of direct cytotoxicity from IFN-; it may interfere with cellular interactions necessary for the survival and growth of CLL cells or, alternatively, it may inhibit the proliferation of the small fraction of clonogenic CLL progenitors.[145] Until more data from some of the recently completed trials become available, IFN- should be considered an experimental agent in the treatment of CLL.

Pure Red Cell Aplasia

Pure red cell aplasia is a relatively rare cause of anemia in CLL. Some studies suggest that suppressor cytotoxic T cells exert inhibitory effects on erythroid progenitor cells in the bone marrow.[57] [58] Therefore, an immunosuppressive agent such as

cyclosporine has been proposed for therapy of pure red cell aplasia with the objective of attacking the erythropoiesis-inhibiting effect of suppressor T cells. Since the first report of Chikkappa et al.[146] of successful treatment of pure red cell aplasia in CLL with cyclosporine, others [147] [148] have confirmed these results in a small number of patients. This therapy is well tolerated, the only reported side effect being mild and reversible renal toxicity. A reticulocyte response is noted within 2 weeks of therapy and is soon followed by an increase in hemoglobin levels. We have observed [147] that not only hemoglobin but platelet counts increased in a patient with CLL who had significant, refractory anemia and thrombocytopenia before cyclosporine therapy.

Late Complications and Terminal Events

The quality of life and performance status of patients with CLL gradually deteriorate with progression of the disease, and a refractoriness to all cytotoxic therapies becomes increasingly evident. A marked degree of persistent anemia is not unusual in the last phases of this disease, and the patients require frequent and regular transfusions of packed red cells. Recombinant human erythropoietin may be effective in some patients with anemia. Although platelet transfusions are not routinely recommended in patients with profound degrees of thrombocytopenia who have no evidence of any bleeding, such transfusions are necessary in the presence of bleeding.

Infections

Infections from bacterial, viral, and fungal agents are the most important cause of morbidity and mortality in CLL. [50] [51] There are several factors that contribute to the increased incidence of infections in CLL, but advanced stages or long duration of the disease, hypogammaglobulinemia, and neutropenia (from CLL or from myelosuppressive effects of chemotherapy) are the most significant. The new nucleoside analogues, which have found increasing use in the therapy of CLL, are known to cause marked lymphopenia, especially of the T-helper cell populations. [104] [105] [106] This in turn renders the patients vulnerable to infections with opportunistic organisms. Therapy is directed at identification of the causative organisms and use of the appropriate antibiotics after consultation with an infectious diseases specialist. Judicious use of granulocyte-stimulating factor is helpful in septic patients with chemotherapy-induced neutropenia. Prophylactic use of high-dose intravenous immunoglobulin has been discussed previously. Prophylactic use of antibiotics in neutropenic or hypogammaglobulinemic patients is not recommended.

Richter Syndrome

A large cell lymphoma develops in approximately 110% of patients with CLL. It is known as Richter syndrome because it was Richter who first described this association.[149] In a retrospective review of 1,374 patients with CLL seen at the M.D. Anderson Cancer Center during a 20-year period between 1972 and 1992, Richter transformation was reported to have developed in 2.8%. [150] Richter syndrome is often characterized by sudden clinical deterioration, development of systemic symptoms, and usually a rapid increase in the size of a lymphoid mass at one site. Less frequently, a monoclonal gammopathy or lytic bone lesions are observed. The histologic type of lymphoma is either diffuse large cell or its immunoblastic variant. [150] One of our patients had a lymphoma of Burkitt-type pathology. Immunoglobulin gene rearrangements and light chain isotype analyses in the study at the M.D. Anderson Cancer Center suggest that CLL and Richter syndrome had a common origin, indicating that a bona fide transformation occurred in the CLL cells. [150] The published literature, however, supports both theories: [151] [152] some studies show that lymphoma cells and CLL cells have identical features, whereas others show that lymphoma cells arise de novo with characteristics distinct from those of CLL cells. There have been separate reports of two cases extensively studied with molecular markers, one conclusively proving a common origin and another equally conclusively revealing that CLL cells and lymphoma cells were clonally distinct. [151] [152] These reports prove that diffuse large cell lymphoma may occur both ways: by a transformation of the original clone of CLL cells as well as through development of a new or second malignancy. It is customary to treat patients with Richter transformation with chemotherapeutic agents known to be effective in treating de novo diffuse large cell lymphoma. Therapy has so far proven to be uniformly unsuccessful, and the overall duration of survival of patients is approximately 6 months after Richter transformation.

Prolymphocytoid Transformation

In addition to Richter transformation, prolymphocytoid transformation may occur terminally in approximately 10% of patients with CLL. The morphology of blood lymphocytes changes into that of a large cell with convoluted nucleus, immature-appearing nuclear chromatin, and one or two large nucleoli. Therapy of this complication also is unsatisfactory. Besides the drugs commonly used in therapy of CLL, antilymphoma agents have also been tried, but the success rate has been low. Acute leukemia and multiple myeloma are extremely rare late events in CLL.

Patients with CLL are known to have a higher incidence of development of a second malignancy (e.g., cancer of the gastrointestinal tract, lung, or any other organ) than the general population. Patients with CLL also have a high risk of development of skin cancers.

Paraneoplastic Pemphigus: An Autoimmune Complication

Anhalt et al.[153] have suggested that the term paraneoplastic pemphigus, which is clinically distinct from pemphigus vulgaris and pemphigus foliaceus, be applied to the painful, persistent, and treatment-resistant erosions of the oral mucosa, vermilion borders of the lips, and conjunctiva that appear in patients with various types of cancers, including CLL. These acantholytic mucocutaneous lesions are characterized by autoantibodies that are pathogenic after passive transfer.

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FUTURE DIRECTIONS

Although chlorambucil and cyclophosphamide have been the mainstays of chemotherapy in CLL over the last 2030 years and both drugs induce a high rate of partial remissions, it is also recognized that no treatment available to date has resulted in improvement of the natural history of this disease. The overall median survival time has remained at approximately 6 years. Besides the two nucleoside analogues discussed in the following sections, the future directions in the treatment of CLL are likely to lead us into the areas of bone marrow and stem cell transplantation and monoclonal antibodies.

2-Chlorodeoxyadenosine

2-Chlorodeoxyadenosine (2-CdA) is also a purine analogue like fludarabine, and is resistant to the action of adenosine deaminase. This drug has proven to be extremely effective in inducing lasting remissions in hairy cell leukemia. 2-CdA has been used in a relatively small number of patients with CLL, mostly previously treated and refractory to alkylating agents.[154] The preliminary results are very encouraging, and controlled clinical trials in previously untreated patients with CLL are being planned; the results of such studies will have an impact on the future

therapies of this disease. 2-CdA is given by a 2-hour intravenous infusion at a dose of 0.120.14 mg/kg/day for 5 days every month. Most patients obtain maximally attainable response after 46 months of therapy. Myelosuppression and immunosuppression are the major toxicities of 2-CdA.

Pentostatin

Pentostatin (deoxycoformycin) has a chemical structure somewhat similar to that of 2-Cda and is a potent inhibitor of adenosine deaminase. This drug also seems to offer benefit[155] [156] to previously treated patients with CLL, but it is used less often in CLL than fludarabine or 2-CdA.

Bone Marrow Transplantation as Therapy

With the recognition that CLL is being diagnosed today in increasing numbers in patients in the 35- to 50-year age group, even a relatively long survival outlook of 610 years is not satisfactory for people this young. Simultaneously, we are developing an increasing level of expertise in performing bone marrow transplantation and in managing the various complications of this therapy in several other types of human malignancies. In the United States [157] [158] and in Europe,[159] results of small trials have been reported that show that bone marrow transplantation is a feasible approach in properly selected groups of patients with CLL. An allogeneic bone marrow transplantation is preferred if a sibling is available as an HLA-compatible donor. In the absence of a compatible donor, autologously harvested marrow (after intensive chemotherapy-induced maximal reduction of leukemic cell mass in the patients bone marrow) is reinfused after myeloablation with massive doses of chemotherapy. The preliminary results are encouraging, and in the next few years we expect to learn more about this interesting and promising therapy. [158] [159]

Monoclonal Antibodies

IDEC-C2B8, a chimeric antibody[160] directed against CD20 that is expressed to some extent in most leukemic B cells in patients with CLL, has received approval by the U.S. Food and Drug Administration for treatment of low-grade lymphoma. CALGB has initiated a trial to study the efficacy of this new monoclonal antibody used either simultaneously or sequentially with fludarabine in previously untreated patients with CLL who have active disease.

CAMPATH-1H monoclonal antibody was developed by humanization of rodent variable immunoglobulin regions with human immunoglobulin gene sequences that present fewer xenogenic peptide sequences and are thus less immunogenic.[161] Our own limited experience with CAMPATH-1H has been extremely promising,[162] and we are continuing these studies on a larger number of patients. Osterberg et al. have reported excellent responses with CAMPATH-1H in both previously treated as well as untreated patients with CLL.[163] [164]

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T-CELL CLL

The T-cell variant of CLL, a rare form accounting for only 25% of all CLL cases, is briefly reviewed here. The diagnosis is suspected when confirmation of a lymphocytosis in blood and bone marrow is accompanied by phenotype analysis of blood lymphocytes revealing a preponderance of T cells. [165] Within this rare group of diseases there is considerable heterogeneity with respect to clinical features, clinical course, and phenotypic markers. [165] The most benign end of the spectrum consists of large granular lymphocytic leukemia associated with neutropenia, a T8+, T4, T3+ phenotype (suppressor T lymphocytes), multiple autoantibodies (rheumatoid factor, antinuclear antibodies), splenomegaly, and absence of lymphadenopathy. Cytogenetic, immunologic, and functional studies indicate that this disease results from a clonal proliferation of immature NK cells. [166] The clinical course is variable, but in most cases is rather indolent. Treatment is generally based on corticosteroids, with the addition of alkylating agents if there is evidence of disease progression. The T4 CLL variant [167] [168] usually affects patients <40 years of age and is associated with hyperlymphocytosis and marked generalized lymphadenopathy, frequently involving the skin and central nervous system. The lymphocyte morphology reveals small, mature-appearing cells with a notched nucleus that lacks a nucleolus and without cytoplasmic granules. The phenotype is T3+, T4+l, and T8. The clinical course is aggressive, the response to the usual cytotoxic therapy is inadequate, and overall survival is <2 years. Sézary syndrome is the leukemic manifestation of cutaneous T-cell lymphoma. The lymphocyte phenotype is CD4+, CD2+, CD3+, and CD5+. Treatment is directed to the underlying lymphoma. Adult T-cell leukemia, which is seen in certain areas of Japan, the Caribbean, and in the southeastern United States, is an HTLV-1-associated disease and bears little relation to CLL.

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Chapter 73 - Hairy Cell Leukemia

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Hairy cell leukemia (HCL) is an uncommon chronic lymphoproliferative disorder initially described by Bouroncle and colleagues in 1958. [1] Although the etiology remains unknown, the morphologic features and clinical manifestations have been well defined over the past 40 years. HCL is characterized by splenomegaly, pancytopenia, and infiltration of the bone marrow with lymphocytes that have irregular cytoplasmic projections when identified in the peripheral blood [2] [3] (Fig. 73-1). The disease is now recognized as a clonal B-cell malignancy, as identified by immunoglobulin gene rearrangements. [4] [5] [6] The expression of B-cellassociated surface antigens reflects differentiation between the immature B cell of chronic lymphocytic leukemia and the plasma cell of multiple myeloma. [7] [8] Although many patients have a paucity of symptoms, others have life-threatening pancytopenia, painful splenomegaly, or constitutional symptoms requiring treatment. [9] [10]

Splenectomy, the treatment of choice for many years, led to normalization of the peripheral blood counts in approximately one half of all cases. [11] [12] [13] [14] Interferon- was first introduced for patients with HCL in 1984, [15] and although a high overall response rate was achieved, most responses were partial. [14] [15] [16] [17] [18] [19] [20] [21] [22] Furthermore, patients invariably relapsed when the drug was discontinued. The most remarkable progress has occurred with the introduction of the two purine analogs, 2-deoxycoformycin (2-DCF) [23] [24] [25] [26] [27] [28] [29] [30] [31] [32] [33] [34] and 2-chlorodeoxyadenosine (2-CdA). [35] [36] [37] [38] [39] [40] Most patients with both previously treated and untreated HCL achieve

Figure 73-1 A hairy cell in the peripheral blood smear from a patient who presented with anemia, thrombocytopenia, and splenomegaly. The nucleus of the hairy cell is eccentrically located and exhibits a reticular or netlike chromatin. The cytoplasmic borders are irregular, with fine, hairlike projections. (Wright-Giemsa stain; orig. mag. × 1,000.)

durable complete remission (CR) with either of these agents. The purine analogs appear to have changed the natural history of this disease.

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EPIDEMIOLOGY

In the United States, HCL represents 2% of adult leukemias; approximately 600800 new patients are diagnosed each year. [41] [42] Although there have been several reports of familial HCL, there is no known genetic predisposition. [43] [44] [45] [46] [47] [48] [49] The median age of diagnosis is 52 years, and the disease occurs in men more often than in women by a ratio of approximately 4 to 1. [50] Although the incidence is similar in the United States and Great Britain, [41] [42] classic HCL is rare in Japan, where a distinct variant form occurs. [51] [52] [53]

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ETIOLOGY AND PATHOGENESIS

The etiology of HCL has not been determined. Although some investigators have suggested that exposure to benzene, [54] [55] organophosphorus insecticides, [56] or other solvents[57] may be associated with disease development, this has not been confirmed by other reports. [58] Exposure to radiation, [59] agricultural chemicals,[55] wood dust,[42] and a previous history of infectious mononucleosis[57] have also been suggested as etiologic associations; however, a causal relationship has not been firmly established.

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CLINICAL PRESENTATION

When a middle-aged man presents with splenomegaly, pancytopenia, and circulating hairy cells, the diagnosis is usually clear (Table 73-1). The initial evaluation of a patient with HCL should include a history and physical examination, a complete blood count with differential count, review of the peripheral blood smear, routine serum electrolytes, blood urea nitrogen and creatinine, hepatic transaminases, a bone marrow aspirate and trephine biopsy, tartrate-resistant acid phosphatase (TRAP) stain, and immunophenotyping of peripheral blood or bone marrow aspirate by flow cytometry (Table 73-2).

At the time of diagnosis, most patients present with symptoms related to anemia, neutropenia, thrombocytopenia, or splenomegaly. Approximately 25% of patients present with fatigue or weakness, 25% with infection, and 25% because of the incidental discovery of splenomegaly or an abnormal peripheral blood count. [50]

Patients usually appear well at the time of diagnosis. Invariably, the most common and often sole physical finding is splenomegaly, occurring in approximately 80% of patients.[3] [50] The spleen is palpable 5 cm below the left costal margin in approximately 60% of patients. Hepatomegaly occurs in approximately 20% of patients. Unlike many other lymphoproliferative disorders, peripheral adenopathy is uncommon, with less than 10% of patients presenting with peripheral nodes larger than 2 cm. Although adenopathy is not common at diagnosis, internal adenopathy may develop after a prolonged disease course, [60] [61] and is present in 75% of patients at autopsy.[62]

Patients with HCL are susceptible to both gram-positive and

TABLE 73-1 -- Classic Presentation of a Patient with Hairy Cell Leukemia

Middle-aged man
Splenomegaly (often greater than 5 cm below left costal margin)
Pancytopenia
Inaspirable bone marrow ("dry tap")
Tartrate-resistant phosphatase (TRAP)-positive circulating hairy cells
Bone marrow trephine biopsy showing mononuclear cells separated by clear cytoplasm

TABLE 73-2 -- Initial Evaluation of a Patient with Hairy Cell Leukemia

History and physical examination
Complete blood cell count, differential count
Review of peripheral blood smear
Serum chemistries
Bone marrow aspirate and biopsy
Tartrate-resistant acid phosphatase (TRAP) stain
Immunophenotyping of peripheral blood or bone marrow aspirate

gram-negative bacterial infections,[63] but are also susceptible to atypical mycobacterial infections, [64] particularly *Mycobacterium kansasii*, as well as invasive fungal infections.[65] Acute bacterial infections result from neutropenia, whereas opportunistic infections occur because of cellular immune defects, including monocytopenia. Other opportunistic infections that have been reported include Legionnaires disease, [65] toxoplasmosis, [66] and *Listeria monocytogenes* infection.[67]

Patients with HCL may have associated systemic immunologic disorders[68] including scleroderma and polymyositis,[69] and polyarteritis nodosa.[70] HCL has been associated with other cutaneous lesions such as erythematous maculopapules [71] and pyoderma gangrenosun.[72] [73] An associated coagulopathy manifested by factor VIII antibodies has been reported.[74] Osseous involvement has also been described, primarily lytic lesions in the axial skeleton, usually the proximal femur. [75] [76] Rarely, osteolytic lesions may be associated with paraproteinemia. [77] A rare case of HCL occurring with systemic mast cell disease has been reported.[78]

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LABORATORY EVALUATION

Pancytopenia is present in approximately 50% of patients with HCL at diagnosis, whereas most other patients present with suppression of only one or two cell lines. [3] [50] Most patients with HCL present with leukopenia, although 1020% of patients exhibit a leukemic phase with a white blood cell count >1020 × 10^9 per liter. Monocytopenia is a frequent, but often overlooked finding. [1] [3] [50] [79]

Other laboratory findings include abnormal hepatic transaminases (19%), azotemia (27%), and hypergammaglobulinemia (18%), which is rarely monoclonal. [3] [77] [79] Hypogammaglobulinemia is uncommon.

Hairy cells can be identified in Wrights-stained peripheral blood smears from most patients with HCL. The number of circulating hairy cells is variable, but is usually low. In some patients, hairy cells can be found only after a prolonged search. Bone marrows are often inaspirable, resulting in a dry tap. However, when bone marrow aspiration is successful, hairy cells similar to those in the blood are found.

The morphologic features of hairy cells are characteristic (Fig. 73-1). The neoplastic cells are approximately one to two times the size of a small lymphocyte. The nuclei are usually round, oval, indented, or monocytoid; rarely, they appear convoluted. [80] The nuclei are located in a central or eccentric position. The chromatin pattern is reticular or netlike in appearance; nucleoli are indistinct or absent. The cytoplasm is variable in amount and pale blue-gray in color. The cytoplasmic borders are irregular with fine, hairlike projections or ruffled borders. Occasionally, cytoplasmic granules are present. Rarely, the cytoplasm exhibits rod-shaped inclusions that correspond to ribosomal lamellar complexes, observed ultrastructurally in approximately 40% of cases. [81]

Examination of the bone marrow trephine biopsy plays a critical role in the diagnosis of HCL because of the characteristic histopathologic appearance [82] [83] [84] [85] (Fig. 73-2). The bone marrow is hypercellular in most patients. Hairy cell infiltration may be diffuse

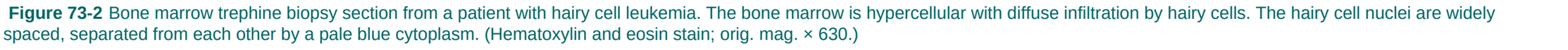

Figure 73-2 Bone marrow trephine biopsy section from a patient with hairy cell leukemia. The bone marrow is hypercellular with diffuse infiltration by hairy cells. The hairy cell nuclei are widely spaced, separated from each other by a pale blue cytoplasm. (Hematoxylin and eosin stain; orig. mag. × 630.)

or focal. In patients with diffuse involvement, large areas of the bone marrow are replaced by hairy cells, with complete effacement of the bone marrow in some patients. When the marrow is focally involved, the infiltrates are randomly situated and may include paratrabecular locations. Residual hematopoietic cells are usually decreased in HCL. The granulocytes are typically more severely reduced than erythroid precursors and megakaryocytes. The hairy cell nuclei are widely separated from each other by abundant, clear or lightly eosinophilic cytoplasm. Mast cells are often numerous. Extravasated red cells are frequently seen and blood lakes, similar to those observed in the spleen, may also be present. The hairy cell nuclei typically merge with the surrounding normal hematopoietic tissue. The complete extent of involvement of the bone marrow by HCL may be difficult to appreciate in routinely stained sections, and is usually accentuated when examined with antibodies to B lymphocytes. Approximately 1020% of patients exhibit a hypocellular bone marrow (Fig. 73-3). The hypocellularity may be severe, with small numbers of hairy cells infiltrating around

Figure 73-3 A markedly hypocellular bone marrow from a patient with hairy cell leukemia who presented with pancytopenia and splenomegaly. A markedly hypocellular bone marrow in hairy cell leukemia may closely resemble aplastic anemia. (Hematoxylin and eosin stain; orig. mag. × 312.)

Figure 73-4 The hairy cells in the hypocellular bone marrow biopsy shown in Figure 73-3 are accentuated by immunostaining for the B-cell antigen CD20. (Orig. mag. × 400.)

fat cells [81] [82] [83] [84] [85] [86] (Fig. 73-4). The morphology of these latter cases strongly resembles aplastic anemia.

Reticulin stains of the bone marrow trephine biopsy almost always show a moderate to marked increase in reticulin fibers. In some cases, the reticulin fibers appear to surround individual hairy cells, with the fibrosis extending into the adjacent, more normal-appearing bone marrow tissue.

Cytochemical demonstration of TRAP activity has been traditionally used to confirm the diagnosis of HCL. [87] In HCL, the enzyme activity resides in isoenzyme 5, one of seven acid phosphatase isoenzymes in human leukocytes. TRAP-positive cells are found in almost all cases of HCL at diagnosis, although the percentage of positive cells varies greatly among patients. A positive TRAP stain in conjunction with a characteristic bone marrow biopsy is essentially diagnostic of HCL. However, a wide variety of other hematopoietic neoplasms, including prolymphocytic leukemia, Sézary syndrome, and adult T-cell leukemia/lymphoma, may rarely show positive reactions. The TRAP reaction is usually performed on peripheral blood cells, marrow aspirates, or touch preparations of bone marrow; a monoclonal antibody against TRAP that is reactive in paraffin-imbedded material has also been described. [88] The introduction of immunophenotyping for the diagnosis of chronic lymphoproliferative disorders has made reliance on the TRAP stain less important.

Hairy cell leukemia is a B-cell neoplasm, as demonstrated by both immunologic and molecular studies. [6] [89] Hairy cells exhibit a mature B-cell phenotype and typically express one or more heavy chains and monotypic light chains. Hairy cells strongly express panB-cell antigens, including CD19, CD20, and CD22, and hairy cells usually lack CD5 expression.[90] The mucosal lymphocyte antigen, CD103, has been shown to be a highly sensitive marker for HCL. [91] CD103 is a member of the integrin family and is also present on intraepithelial T cells and some activated lymphocytes. The presence of CD103, when coexpressed with other panB-cell markers, is strongly suggestive of HCL. Hairy cells also strongly express CD11c, a marker associated with myelomonocytic cells and CD25, the interleukin-2 receptor.[90] [91]

CD20 (L26) and DBA.44, B-cellassociated antibodies, react with hairy cells in fixed, routinely processed tissue sections (Fig. 73-4). Neither is specific for HCL, but these antibodies are useful in highlighting the extent of bone marrow infiltration at the time of diagnosis and following therapy. [92] [93] [94] [95]

Clonal cytogenetic abnormalities are present in approximately two thirds of patients with HCL. The most frequently

involved chromosomes include chromosomes 1, 2, 5, 6, 11, 19, and 20. In particular, chromosome 5 is altered in 40% of patients, most commonly as trisomy 5, pericentric inversions, and interstitial deletions involving band 5q13. [96] [97]

Splenic involvement in HCL is characterized by diffuse infiltration of the red pulp cords and sinuses, with atrophy or replacement of the white pulp. Blood-filled sinuses, lined by hairy cells, are often a prominent but not pathognomonic finding in the spleen; they have been referred to as pseudosinuses. [98]

The liver shows both sinusoidal and portal infiltration by hairy cells. Involved lymph nodes commonly exhibit partial effacement, with hairy cells infiltrating the paracortex and medulla in a leukemic pattern. The leukemic cells often surround residual lymphoid follicles and extend through the capsule.

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DIFFERENTIAL DIAGNOSIS

The differential diagnosis of HCL includes other small B-cell lymphoproliferative disorders associated with splenomegaly, [99] such as prolymphocytic leukemia,[100] [101] [102] splenic marginal zone lymphoma (splenic lymphoma with villous lymphocytes),[103] [104] [105] [106] and HCL variant[107] [108] [109] (Table 73-3). Patients with prolymphocytic leukemia typically present with splenomegaly, but this disorder can usually be readily distinguished from HCL by the marked elevation of the white blood cell count and the morphology of the prolymphocytes. Splenic marginal zone lymphoma exhibits some clinical and morphologic features similar to HCL but, in contrast, the cells usually do not exhibit TRAP positivity, the bone marrow infiltrates are sharply demarcated, and the immunophenotypic profile differs from HCL, including negativity for CD103.[106] HCL variant exhibits morphologic features intermediate between hairy cells and prolymphocytes, and is associated with leukocytosis, lack of monocytopenia, and expression of the interleukin-2 receptor chain, but not the chain (CD25). [110] Finally, systemic mastocytosis may resemble the variant of HCL in which the hairy cells are spindle shaped in the biopsy sections. Immunohistochemical studies show the mast cells, unlike hairy cells, to be negative for B-cell markers and positive for lysozyme and tryptase.[81]

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TREATMENT

Indications

Because HCL usually has an indolent course, treatment may not be required for many months or years after the diagnosis is established. Therapy is indicated when the patient has significant cytopenias; symptomatic organomegaly or adenopathy; infections; or constitutional symptoms such as fever, night sweats, or fatigue. Typical peripheral blood counts that warrant treatment include an absolute neutrophil count of <1,000/l, a hemoglobin <11.0 g/dl, or a platelet count <100,000/l.

Role of Splenectomy

Splenectomy was the first effective therapy for HCL and remained the initial treatment of choice until recently. [11] [12] [13] [14] Although

TABLE 73-3 -- Differential Diagnosis of Hairy Cell Leukemia

Prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia variant
Chronic lymphocytic leukemia
Low-grade lymphoma
Agnogenic myeloid metaplasia
Systemic mastocytosis

MANAGEMENT

For patients with newly diagnosed HCL who require treatment (absolute neutrophil count <1,000/l, hemoglobin <11.0 g/dl, and platelet count <100,000/l or symptomatic organomegaly), we administer 2-chlorodeoxyadenosine (2-CdA) 0.1 mg/kg/day by continuous IV infusion for 7 days as an outpatient procedure by portable pump using a midline percutaneous intravenous central catheter (PICC). If fever of 100.5°F or greater develops while the patient is neutropenic, blood cultures are drawn, urine culture and chest radiography are done, and oral ciprofloxacin 750 mg po bid is administered. If cultures are sterile at 2448 hours, naproxen 250 mg po bid is added for 24 days. 2-CdA is not discontinued. Hematopoietic growth factors are not routinely given. The platelet count is usually the first cell line to recover (within 24 weeks), followed by the white blood cell count, and, finally, the hemoglobin. We repeat the bone marrow at 3 months to assess remission status. We currently do not administer a second cycle of 2-CdA for patients with MRD.

For patients with relapsed HCL who have been previously treated with either splenectomy, interferon, or 2-deoxycoformycin (2-DCF), we administer a repeat cycle of 2-CdA as described previously. For patients who relapse after a single cycle of 2-CdA, we give a second cycle of 2-CdA. For patients with a relapsed HCL previously treated with at least two prior cycles of 2-CdA, we consider either 2-DCF 4 mg/m^2 IV every 2 weeks for 36 months or interferon 2 × 10^6 units/m^2 three times each week for 1218 months.

splenectomy does not produce pathologic remissions in the bone marrow, all three cell lines return to normal in approximately 4070% of patients. [13] [111] This response is maintained for a median of 20 months in approximately two thirds of patients, and the overall 5-year survival rate is approximately 70%. [111] There appears to be no correlation between spleen size and response to splenectomy.

Chemotherapeutic Approaches

A variety of chemotherapy regimens have been used and reported in several small series of patients who have failed splenectomy. [112] Chlorambucil at a dose of 4 mg/day for 6 months produced an improvement in blood counts in some patients; however, neutropenia did not resolve and patients remained at significant risk of infection. Combination chemotherapy with rubidazone, cytosine arabinoside, and cyclophosphamide produced complete pathologic remissions lasting more than 2 years in three of seven patients, but three other patients died of infection during the period of marrow aplasia. [113] More recently, CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy was reported to produce long-lasting normalization of the peripheral blood counts with significantly less morbidity.[114] High-dose chemotherapy with syngeneic (identical twin) bone marrow transplantation has been successfully carried out in one patient who enjoyed a prolonged disease-free survival of more than 5 years. [115] HCL is clearly sensitive to chemotherapy, but toxicity has been significant. Although hematopoietic growth factors may diminish this toxicity, newer therapies such as the purine analogs have made conventional chemotherapy treatment unnecessary.

Interferon

Interferon was first reported to be an effective therapy for patients with HCL in 1984, [15] and since then numerous large studies have confirmed its activity. [15] [16] [17] [18] [19] [20] [21] [22] The precise mechanism of action of interferon is not known, but may be due to a reduction in the production of cytokines such as granulocyte colony-stimulating factor, granulocytemacrophage colony-stimulating factor, interleukin-3, interleukin-6, and tumor necrosis factor, perhaps related to the characteristic monocytopenia associated with interferon treatment. [116] Despite a high overall response rate or 7590%, most patients achieve only partial remission (PR; defined as normalization of all peripheral blood counts). [19] [20] Interferon is commonly administered subcutaneously at a dose of 2 million international units/m ^{2} subcutaneously three times a week for 1218 months. During the first 2 months of treatment, the white blood cell count and hemoglobin are often decreased, occasionally necessitating transfusion. The platelets normalize first in responding patients, followed by the hemoglobin and the white blood cell count. An absolute neutrophil count >1,500/l is achieved after a median of 5 months of therapy. Common toxicities include flulike symptoms, anorexia and fatigue, nausea and vomiting, diarrhea, dry skin, peripheral neuropathies, and central nervous system dysfunction. The latter is usually manifested as depression or memory loss. Elevated hepatic transaminases are the most common laboratory abnormality, other than myelosuppression. The median failure-free survival after discontinuing interferon ranges from 6 to 25 months in different series. [19] [20] [21] [117] Patients with >30% hairy cells in the marrow or a platelet count of <160,000/l at the end treatment have a higher risk of early relapse. [19] [20] [21] In addition, patients who express the CD5 antigen appear to respond poorly to interferon. [118] A recent report suggests that patients can be maintained on long-term interferon at a dose of 3 million units subcutaneously given three times a week with minimal toxicity. Sixty percent of patients have sustained their initial response for a median of 5 years, 9% discontinued therapy early because of unexpected neurologic toxicity, and only 13% stopped therapy because of progressive disease. [119] In summary, treatment of HCL with interferon is effective, resulting in normalization of the peripheral blood counts in most patients. However, CRs are uncommon, and failure-free survival is usually short after discontinuation of treatment.

Purine Analog Therapy

Twenty-five years ago, Giblett and colleagues observed that 30% of children with severe combined immunodeficiency syndrome lacked the enzyme adenosine deaminase (ADA). [120] It appeared that the accumulation of the triphosphorylated form of deoxyadenosine was responsible for lymphocyte destruction. [121] Therefore, the deliberate inhibition of ADA emerged as a potentially useful antileukemic strategy. The methods to accomplish this included the development of agents to bind irreversibly to ADA or to resist the action of the enzyme.

2-Deoxycoformycin

2-Deoxycoformycin was the first agent found to induce a significant number of CRs in HCL. [23] [24] This drug is an irreversible inhibitor of ADA, [122] an enzyme found in all lymphoid cells that is important in purine metabolism. Deoxyadenosine triphosphate metabolites accumulate and are thought to be responsible for the cytotoxicity in HCL. [123] [124]

Several studies using various dosing schedules of this drug have been published (Table 73-4). [23] [24] [25] [26] [27] [28] [29] [30] [31] [32] [33] [34] In an Eastern Cooperative Oncology Group study, 50 patients with HCL were treated with 2-DCF at a dose of 5 mg/m ^{2} /day intravenously (IV) for 2 consecutive days every 2 weeks until maximum response. [27] This therapy resulted in a CR rate of 64% and an overall response of 84%, with most patients achieving maximal response within 6 months. Toxicities were moderate and included nausea, vomiting, skin rash, and conjunctivitis, as well as significant neurologic toxicities in four patients. Neutropenia was life threatening in 70% of patients; however, this was limited to the first two to three cycles of therapy. Mortality as a result of therapy was attributable to infection in 6% of patients. Other investigators have administered a lower dose of 2-DCF and have produced similar results. [25] [26] In one study, patients were treated with 2-DCF at dose of 4 mg/m^2 IV every other week, and CR was attained in 20 of 23 patients (87%). [25] In this study, infections were infrequent and there were no deaths. CD4+ T cells were found to be significantly decreased after treatment. However, this laboratory finding was reversible, and no opportunistic infections or secondary malignancies were noted after 2 years of follow-up. In an additional study, patients were treated with 2-DCF at a dose of 4 mg/m ^{2} IV weekly for 3 consecutive weeks with therapy repeated every 8 weeks. [26] CR was achieved in 25 of 28 patients (89%) after completing two cycles of therapy. Transient neutropenia occurred during the first cycle only, and 12 patients had fever or infection, but no deaths occurred.

A large, prospective, randomized study showed that the CR rate and relapse-free survival rate are significantly better with 2-DCF than interferon. [34] However, the overall survival rate was not different between these two treatments.

2-Chlorodeoxyadenosine

2-Chlorodeoxyadenosine is a purine analog that does not inhibit ADA, but is resistant to the enzyme. This agent accumulates in the lymphoid cells presumably because they are rich in the enzyme deoxycytidine kinase. [121] This enzyme phosphorylates 2-CdA, creating a deoxynucleotide that cannot readily exit the cell. These deoxynucleotides are thought to be responsible for the lack of lymphocyte development in ADA-deficient

TABLE 73-4 -- Activity of 2-Deoxycoformycin in Hairy Cell Leukemia

Reference	No. of Patients	Prior Therapy	Response[a]		
			Complete	Partial	None
Cassileth et al[27]	50	31	32 (64)	10 (20)	8 (16)
Blick et al[25]	10	10	10 (91)	1 (09)	0
Kraut et al[33]	23	13	20 (87)	1 (04)	2 (09)
Johnston et al[25]	28	18	25 (89)	3 (11)	0
Ho et al[30]	33	30	11 (33)	15 (45)	4 (13)
Catovsky et al[31]	148	23	110 (74)	33 (22)	5 (03)
Golomb et al[32]	85	85	36 (42)	35 (42)	14 (15)

[a]Number of patients with particular response to therapy; numbers in parentheses indicate percentages of patients enjoying a particular response to therapy.

TABLE 73-5 -- Activity of 2-Chlorodeoxyadenosine in Hairy Cell Leukemia

Reference	No. of Patients	Prior Therapy	Response[a]		
			Complete	Partial	None
Piro et al[35]	144	75	123 (85)	17 (12)	3 (02)
Estey et al[36]	46	27	36 (78)	5 (11)	5 (11)
Juliusson and Liliemark[37]	16	3	12 (75)	0	4 (25)
Tallman et al[38]	50	15	40 (80)	19 (20)	0
Lauria et al[40]	37	12	29 (78)	8 (22)	0
Hoffman et al[39]	49	28	37 (76)	12 (24)	0

[a]Number of patients with particular response to therapy; numbers in parentheses indicate percentages of patients enjoying a particular response to therapy.

children.[120] 2-CdA was first reported to be effective for HCL by Piro and colleagues in 1990. [35] Twelve patients were treated with a single cycle of 2-CdA at a dose of 0.1 mg/kg/day by continuous infusion for 7 days, and a complete pathologic remission was obtained in 11 of the 12 patients within 8 weeks of treatment. None of the patients had relapsed at a median of 15.5 months of follow-up. Toxicity was minimal. In fact, no patients experienced the usual toxicities of cytotoxic chemotherapy, including nausea, emesis, alopecia, or other constitutional symptoms. Fever was common toward the end of therapy and coincided with a rapid decline in the number of circulating hairy cells. Remarkably, no infections were documented, and it was believed that the fever may be the result of cytokine release from hairy cells. In a recently updated report by the same investigators, 144 patients were treated and 85% achieved a CR [125] (Table 73-5). Even those patients with residual disease evident by routine light microscopic evaluation had complete normalization of their peripheral blood counts. At Northwestern University, 40 (80%) of 50 assessable patients achieved CR and 9 (18%) achieved PR, with minimal toxicity.[126] Hoffman and colleagues reported that 37 (76%) of 49 patients achieved CR. [39] No viral, fungal, or other opportunistic infections were observed other than a single case of dermatomal herpes zoster 16 months after treatment. It is now clear that most patients remain disease free for a prolonged period of time. [126] [127] [128] [129] Alternative routes and schedules of administration to the standard 7-day continuous intravenous infusion have included a 5-day, 2-hour bolus infusion, [129] subcutaneous administration,[130] and oral administration.[129] [131]

Fludarabine monophosphate is a third purine analog that is resistant to ADA. Two brief case reports suggest that fludarabine can produce normalization of the peripheral blood counts in previously treated patients with HCL. [132] [133]

The purine analogs are uniquely effective in patients with HCL. Extremely high CR rates are achievable and the incidence of toxicity is low. These agents should be considered the treatment of choice for HCL.

Immunosuppression with Purine Analogs

Both 2-DCF and 2-CdA are associated with prolonged immunosuppression. [37] [134] [135] [136] With 2-DCF, a decrease in the total lymphocyte count occurs, with a greater reduction in T cells than B cells or natural killer cells. [135] The levels of CD4+ and CD8+ cells decrease to <200 cells/l for at least 6 months after 2-DCF treatment is discontinued. In a series of 15 patients treated with 2-DCF with long follow-up, the median time to recovery of CD4+ lymphocyte counts to normal was 54 months. [136] Treatment with 2-CdA induces similar suppression of CD4+ lymphocyte counts.[127] The median time to recovery of CD4+ lymphocyte counts to normal after 2-CdA was 40 months. Despite such immunosuppression, opportunistic infections, other than an occasional case of herpes zoster, are distinctly uncommon, unless purine analog treatment is associated with corticosteroid exposure. [37]

Prognosis

Several studies have addressed the relapse rate, progression-free survival, and overall survival for patients with HCL treated with 2-DCF and 2-CdA. [126] [127] [128] [137] [138] These studies have indicated that most patients with either previously treated or untreated HCL achieve durable remissions with either multiple cycles of 2-DCF or a single cycle of 2-CdA, and the relapse rates appear low. In the report from Northwestern University, 7 of 52 patients (14%) had relapsed at a median duration of 24 months (range, 1244 months).[126] The progression-free survival rate is 72% at 4 years and 83% for those patients achieving CRs (Fig. 73-5). The overall survival rate is 86% at 4 years. Seymour and

Figure 73-5 Kaplan-Meier curves showing the progression-free survival **(A)** and overall survival **(B)** for 50 patients with hairy cell leukemia treated with a single cycle of 2-CdA.

colleagues from the M. D. Anderson Cancer Center reported that 8 of 40 patients (20%) relapsed at a median of 30 months. [127] The largest number of patients treated with 2-CdA has been reported by Saven and colleagues.[138] Of 349 patients with either previously treated or untreated disease, 91% achieved a CR and 7% achieved a PR. The relapse rate for 341 patients responding to 2-CdA was 19% at 48 months. The overall survival rate was 96% at 48 months.

Several investigators have reported similarly favorable long-term outcomes after treatment with 2-DCF. Kraut and colleagues reported that 11 of 24 patients (45%) had relapsed from CR after treatment with 2-DCF at a median of 30 months.[33] Grever and colleagues reported a relapse-free survival rate of 91% at 7 years among 117 patients achieving CR on the 2-DCF arm of a prospective, randomized study comparing 2-DCF with interferon [34] (Fig. 73-6). A recent update of the long-term outcome of these patients indicated that 76 ± 4% of patients remain alive and disease-free at 8 years, and the overall survival rate was 87 ± 2%. [137] A similar excellent outcome was reported by Catovsky and associates.[31] These investigators observed a 5-year survival rate of 88% among 110 patients treated with 2-DCF. Because of the indolent natural history of this disease, very long follow-up of patients treated with either purine analog will be required to determine if one of the agents offers a

Figure 73-6 Kaplan-Meier curves showing relapse-free survival **(A)** and overall survival **(B)** for 117 and 154 patients, respectively, with hairy cell leukemia treated with multiple cycles of 2-deoxycoformycin.

FOOD AND DRUG ADMINISTRATION-APPROVED TREATMENTS FOR HAIRY CELL LEUKEMIA

Interferon- 2 × 10^6 international units/m^2 subcutaneously three times a week (for 1218 months)
2-Deoxycoformycin (2-DCF; Pentostatin) 45 mg/m^2 IV q 2 weeks until remission (usually 36 months)
2-Chlorodeoxyadenosine (2-CdA; cladribine, Leustatin), a single cycle, 0.1 mg/kg/day continuous infusion for 7 days

substantially longer remission duration or overall survival. However, it seems unlikely that such a prospective, randomized comparison between 2-CdA and 2-DCF will ever be conducted because the CR rates with each agent are so high, and many years and resources would be required to detect even large differences.

Evaluation of Minimal Residual Disease

The remarkable activity of the purine analogs has led to the examination of post-treatment bone marrow biopsies to detect minimal residual disease (MRD) in patients otherwise in CR. Immunohistochemistry using anti-CD20 antibodies in paraffin-embedded biopsy specimens is a useful technique. [92] [93] [94] [95] [139] [140] [141] Depending on the criteria used, 1351% of patients in apparent CR have evidence of MRD. [92] [93] [94] [95] The presence of MRD may predict relapse.[94] However, molecular studies have suggested that the malignant clone of cells may remain even in patients in apparent remission. [142]

Risk of Second Malignancies

Kampmeier and colleagues reported a significantly increased incidence of second malignancies in HCL patients treated with interferon. [143] Among 69 patients treated with interferon-2b followed for a median of 91 months, a second malignancy developed in 13 (19%), half of which were of hematologic origin. However, this association has not been uniformly observed.[144] Given the fact that the purine analogs are immunosuppressive, it will be important to determine if patients treated with either 2-DCF or 2-CdA are at risk for second malignancies. Kurzrock and colleagues reported no excess of second malignancies among 350 patients treated with either interferon, 2-CdA, or 2-DCF. [145]

FUTURE DIRECTIONS

Remarkable progress has been made in the treatment of HCL. Several very effective approaches are now available. The purine analogs have supplanted splenectomy and interferon as the treatment of choice. The long-term outcomes appear equivalent with either 2-DCF or 2-CdA, but very long-term follow-up will be important to determine if one or the other agent offers a significant survival advantage. Future directions will include clinical testing of an oral formulation of 2-CdA [146] and exploring approaches for patients with MRD. Patients who relapse after 2-DCF usually respond well to 2-CdA, [147] but whether the reverse is true will require further study. Finally, although patients who fail purine analogs may respond to interferon, [148] it is not likely to be curative. Novel strategies are needed for this subset of patients, and might include the anti-CD20 monoclonal antibody recently shown to be very effective in patients with other indolent B-cell lymphoproliferative disorders.[149]

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Chapter 74 - Cutaneous T-Cell Lymphomas

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INTRODUCTION

The T-cell non-Hodgkin lymphomas have been recognized to include a wide variety of clinical disorders with differing prognoses. The revised European-American classification of lymphomas (REAL)[1] suggests that more than 10 discrete clinicopathologic entities can be considered part of this family. This chapter focuses on the disorders that would be encompassed by the diagnosis cutaneous T-cell lymphoma (CTCL) (Table 74-1). The most common subtypes of cutaneous T-cell lymphoma are the epidermotropic variants mycosis fungoides (MF)

TABLE 74-1 -- Cutaneous T-Cell Lymphomas (CTCL)

Primary
Indolent
Mycosis fungoides
Mycosis fungoides + follicular mucinosis
Pagetoid reticulosis (Woringer-Kolopp disease)
Large Cell CTCL, $CD30^+$
Anaplastic
Immunoblastic
Pleomorphic
Lymphomatoid papulosis
Aggressive
Sézary syndrome
Large cell CTCL, CD30
Immunoblastic
Pleomorphic
Provisional
Granulomatous slack skin
CTCL, pleomorphic small/medium size
Subcutaneous panniculitis-like T-cell lymphoma
Systemic T-Cell Disorders with Skin Manifestations
Angioimmunoblastic lymphodenopathy
Lymphomatoid granulomatosis
Adult T-cell leukemia and lymphoma
Modified from the EORTC Classification. Blood, July 1997, p. 355.

and the Sezary syndrome (SS). The bulk of this chapter focuses on the approach to these more common disorders, although features that allow the clinician to exclude the distinct variants will also be described.

Epidemiology

MF and SS are the most frequent primary lymphomas involving the skin.[2] [3] Data collected from the Surveillance, Epidemiology, and End Results (SEER) program showed a rapidly increasing incidence from 0.2 cases per 100,000 population in 1973 to 0.4 cases per 100,000 population in 1984. This corresponds to approximately 1,000 new cases each year in the United States. Whether this represents a true increase in incidence or is attributable to a better awareness and therefore more frequent recognition of this disease is not resolved.

The incidence of MF/SS increases with advancing age, as does the incidence of non-Hodgkin lymphomas in general. The average age at presentation is approximately 50 years. Although very young patients have been reported, most patients are at least 30 years of age. [4] [5] MF/SS is seen in all racial groups. There is a 2:1 ratio of black people to white people and a 2.2:1 ratio of men to women with this disorder.

Clusters of cases of MF/SS within families have been reported.[6] Furthermore, an association with histocompatibility antigens AW31, AW32, B8, BW35, and DR5 has been described.[7] However, a solid genetic predisposition or inherited genetic defect has not been demonstrated.

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BIOLOGY AND MOLECULAR ASPECTS

The T lymphocyte is central to the body's ability to mount an immune response and is the precursor of neoplastic cells in MF/SS. These cells spontaneously form rosettes with sheep red blood cells. Sezary cells will respond to phytohemagglutinin and perform T-cell immunoregulatory functions similar to normal lymphocytes. [8] [9] Clonal rearrangements of the T-cell receptor

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(TCR) can be demonstrated by Southern blotting or polymerase chain reaction (PCR) techniques.

The development of monoclonal antibodies directed against a specific cluster of differentiation (CD) antigens has allowed for more precise identification of surface markers on the malignant cell.[10] In most instances, the cells express the pan T-cell antigens CD3 and CD5. The sheep erythrocyte receptor, another pan T-cell marker, has been designated CD2. Early T cells may also express CD7 on their cell surface. This marker is frequently deleted in MF/SS, but may also be deleted in benign dermatoses and inflammatory skin lesions. The majority of cases of MF and SS are of the T-helper memory/effector subset with a $CD4^+$, $CD45RO^+$ phenotype. Occasional cases expressing the suppressor $CD8^+$ phenotype have been described. Low expression of the interleukin-2 receptor (CD25) is detected in less than half the patients with heterogenous expression on the malignant cells.

Cytogenetic analyses have demonstrated both numerical and structural chromosomal abnormalities in MF/SS. [11] Hyperdiploidy and complex karyotypes are common. Nonrandom deletions of chromosomes 1, 6, 8, 10, and 17 have been reported. Regions of the genome that include genes encoding the T-cell receptor do not appear to be involved, suggesting that the genetic basis for malignant transformation in MF/SS appears to be different from that involved in other T-cell malignant disorders.

Modest data exists on the aberrant expression of oncogenes and/or suppressor genes in MF/SS. Mutant forms of the P53 tumor suppressor gene, noted in high grade lymphomas, are rarely seen in MF/SS.[12] LYT-10, a member of the NF-KB family of transcription factors associated with translocations in lymphoid malignancies, is rearranged in a small proportion of cases.[13] However, BCL-2, a gene whose rearrangement is characteristic of follicular B-cell lymphomas and which slows programmed cell death, is overexpressed in MF.[14] In addition, constitutive expression of STAT 3 (a member of the transcription factor family that contributes to the diversity of cytokine responses) has been reported. [15] Altered expression or release of select cytokines or their receptors including IL-1, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12, and TGF-B receptor II has been noted. [16]

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ETIOLOGY AND PATHOGENESIS

The etiology of MF/SS remains unknown. It is considered to be a sporadic disease without compelling evidence of transmissibility. Several viruses have been implicated in the pathogenesis of MF/SS including Human T-cell lymphotropic virus-I/II, Herpes simplex virus, Herpes virus-6, and the Espstein-Barr virus. [13] However, a viral cause of MF/SS has not been proven.

Investigators have suggested that prolonged exposure to contact allergens may lead to enhanced immune responses leading directly or indirectly to the development of MF/SS. Sezary cells respond in vitro to superantigenic exotoxins, and colonization by *Staphylococcus aureus* may influence disease activity.[17] In addition, several reports suggested that exposure to metals or their salts, pesticides or herbicides, and organic solvents (halogenated or aromatic hydrocarbons) could be related to the development of MF/SS.[18] However, two well-designed case-control studies have failed to support these observations. [19] [20]

Various theories have been advanced to explain the epidermotropism of malignant T cells in MF/SS. Organ-specific affinity to skin and other organs has been recognized in subsets of normal T cells. Homing of CTCL cells to the skin is probably mediated by more than one adhesion receptor mechanism. CTCL cells express cutaneous lymphocyte antigen (CLA), a skin homing receptor that interacts with e-selectin on cutaneous endothelium. [21] It has been noted that peripheral blood mononuclear cells bind to cultured keratinocytes exposed to interferon gamma. The MHC II proteins, along with intercellular adhesion molecule 1 (ICAM-1), present on keratinocytes, will attract lymphocytes. Class II MHC proteins recognize the CD4 antigen and ICAM-1 binds to the lymphocyte function-associated molecule 1 (LFA-1). [22]

An additional feature of MF/SS cells is the production of a cytokine profile consistent with T-helper-2 type cells (T_{H2}).[23] The T_{H2} cells produce IL-4, IL-5, and IL-6 and they are inhibited by interferon gamma. The T_{H2} cells are critical for stimulating antibody and eosinophil-mediated responses. Hypergammaglobulinemia and eosinophilia are often seen in advanced cases of MF/SS and are consistent with a T_{H2} profile. Stimulation of T_{H2} cells inhibits the T_{H1} subpopulation of lymphocytes involved in cell-mediated immunity. A cause as well as a consequence of the progression of MF/SS is immune suppression as a result of depletion of this T cell subset. The T_{H2} cytokine profile also explains the decrease in tumor infiltrating lymphocytes during tumor progression.

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CLINICAL PRESENTATION

Alibert reported the first case of MF in 1806.[24] His patient developed a skin eruption that progressed into mushroom-like tumors, prompting the term mycosis fungoides. Later in the nineteenth century, Bazin defined the three classic cutaneous phases (patch, plaque, and tumor stage) of the disease.[25] The recognition of the clinical triad of intensely pruritic erythroderma, lymphadenopathy, and abnormal hyperconvoluted monstrous cells in the peripheral blood led to the description of SS.[26]

More than 50% of CTCL patients have MF. The initial course of patients with MF is usually indolent. Most patients will give a history of antecedent skin lesions, usually nonspecific erythematous patches that can mimic eczema or psoriasis. In many cases, there is an orderly progression from limited patches to more generalized patches, plaques, tumors, and finally nodal or visceral involvement. The characteristic patch lesion is typically poorly demarcated, lightly erythematous, and usually on the trunk or the extremities (Fig. 74-1). Plaque lesions are more erythematous, have well-demarcated margins, and some scaling (Fig. 74-2). They frequently resemble psoriasis. Plaques can arise from patch lesions or previously uninvolved areas of skin. Tumor lesions tend to be later lesions, frequently associated with previous patches or plaques. They can be located on any part of the body. Ulceration of these lesions is common and secondary infection is a major cause of morbidity (Fig. 74-3). Tumors may be the initial presentation in a small percentage of patients (D'emblée presentation).

SS patients present with generalized erythroderma, pruritus, and circulating malignant cells. Peripheral blood usually shows more than 15% hyperconvoluted atypical lymphocytes (Fig. 74-4). Approximately 5% of all newly reported cases of CTCL are SS. In its most advanced form, patients with SS suffer from leonine facies, hyperkeratosis and fissuring of the palms and soles, and severe pruritus and cutaneous pain. Many other entities can clinically mimic this disease including drug eruptions, atopic dermatitis, contact dermatitis, and erythrodermic psoriasis.

A number of variant presentations of CTCL have been described. The following sections discuss the premalignant variants of CTCL.

Poilikoderma Vasculare Atrophicans

Poilikoderma vasculare atrophicans is characterized by reticulate hyperpigmentation and hypopigmentation, telangiectasia, and skin atrophy.[27] The classic presentation includes multiple skin lesions of variable size, affecting covered areas such as the breasts, buttocks, and flexures in a symmetric distribution.

Figure 74-1 Erythematous and scaly patch lesion of MF.

Lesions typically improve with sun exposure. Peak incidence occurs between 40 and 60 years of age. A small percentage of patients will evolve into MF.

Large Plaque Parapsoriasis

Large plaque parapsoriasis is the classic premalignant lesion of MF. It most commonly consists of a few scattered, erythematous to brown, plaques that are usually greater than 6 cm is size.[28] There is a predilection for the buttocks and intertriginous areas. Histologic examination shows a superficial lymphocytic infiltrate with minimal nuclear atypia. Epidermotropism is scant or absent and dermal fibrosis correlates with the chronicity of the process. Plaques can persist for decades before a frank evolution to MF occurs. Approximately 1030% of patients ultimately develop an overt malignant transformation.

Alopecia Mucinosa

Alopecia mucinosa presents with grouped erythematous follicular papules or boggy or indurated nodular plaques, notably devoid of hair (Fig. 74-5).[29] There is a predilection for the head and neck area, especially the forehead, which has the highest density pilosebaceous units. Histopathologic evaluation reveals follicular mucinosis and mucinous degeneration of epithelial cells in sebaceous glands and hair follicles, associated with lymphocytic infiltration. Most patients have a benign course, but lymphoma associated with follicular mucinosis has been

Figure 74-2 Plaque lesion of CTCL.

Figure 74-3 Ulcerated tumors arising from MF plaques.

Figure 74-4 Sezary cells: the nuclei are cerebriform and hyperconvoluted with fine chromatin and scant cytoplasm.

reported in the literature to range from 967%.[30] In general, patients over the age of 40 years with a more generalized cutaneous involvement and a chronic course are more likely to develop associated MF. No cases of MF have been reported in children with alopecia mucinosa, although few reports of Hodgkin disease have been reported in children with follicular mucinosis.[31]

Lymphomatoid Papulosis

Lymphomatoid papulosis is characterized by recurrent crops of self-healing, red-brown, centrally necrotic, asymptomatic papules

Figure 74-5 Follicular mucinosis showing a patch of alopecia with follicular prominence.

and nodules (Fig. 74-6).[32] This entity represents 510% of all CTCL cases. Patients may have a few lesions or more than 100 at a time. Histologic evaluation reveals an atypical $CD4^+$ lymphocytic infiltrate with a variable mixed inflammatory infiltrate (Fig. 74-7). These may be primarily small cerebriform cells similar to those seen in MF (type B) or primarily larger $CD30^+$ cells of the Reed-Sternberg type (type A). A third variety of lymphomatoid papulosis (type C) with sheets of anaplastic large cells has also been reported.[33] T-cell receptor gene rearrangement studies demonstrate a clonal origin. Though the typical course is usually indolent, spanning decades, approximately 30% of patients develop MF, Hodgkin, or non-Hodgkin lymphoma during their lifetime. However, the cumulative risk of developing a lymphoma approaches 80% at 15 years, with negligible risk of transformation during the first five years. [34] A direct link between lymphomatoid papulosis, CTCL, and Hodgkin disease was demonstrated in a patient with the three lymphoproliferative disorders arising from a common T-cell clone as shown by TCR gene studies. [35]

The malignant variant presentations of CTCL are discussed in the following sections.

Pagetoid Reticulosis (Woringer-Kolopp Disease)

This rare condition affecting young adults presents with hyperkeratotic, often verrucous plaques on the lower limb. [36] Biopsies show atypical cerebriform lymphocytes with a perinuclear halo almost exclusively localized within the intraepidermal compartment. [37] This is a localized form of CTCL. Pagetoid reticulosis typically affects a distal limb and extracutaneous dissemination is exceedingly rare. A predominantly CD8 immunophenotype has been reported in pagetoid reticulosis. Although most cases have an indolent protracted course, generalized and sometimes aggressive variants have been reported. [38]

Granulomatous Slack Skin

In granulomatous slack-skin syndrome, clonal $CD4^+$ T cells elicit a reactive granulomatous response that destroys the elastic fibers, rendering skin slack, fibrotic, and inelastic (Fig. 74-8).[39]

Figure 74-6 Lesions of lymphomatoid papulosis appear in crops and consist of ulcerated papules and scars.

Changes characteristic of MF are often found within the epidermis and papillary dermis, while the reticular dermis contains numerous tuberculoid granulomas with multinucleated giant cells. Some patients with granulomatous MF do not have destruction of the elastic fibers with slack-skin changes. The differential diagnosis for such cases includes sarcoidosis and tuberculoid leprosy.

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LABORATORY EVALUATION

The diagnosis of MF/SS is based on light microscopic evidence of a band-like infiltrate involving the papillary dermis containing small, medium-sized, and occasionally large mononuclear cells with hyperchromatic, hyperconvoluted (cerebriform) nuclei and variable numbers of admixed inflammatory cells. [13] [40] Epidermal exocytosis of single or small clusters of neoplastic cells is a characteristic finding (Fig. 74-9). The presence of Pautrier microabscesses is classic, but seen only in a minority of cases. Tumor stage lesions demonstrate a more diffuse superficial and deep, dermal infiltrate with fewer reactive cells, and an absence of epidermotropism (Fig 74-10). The malignant T-cell clone often evolves into large cell morphology during

Figure 74-7 Skin biopsy of lymphomatoid papulosis with a mixture of large atypical lymphocytes with inflammatory cells.

tumor progression, although rare cases show large cell morphology from the early patch lesions. [41] The histologic features in SS may be similar to those of MF. However, the cellular infiltrates in SS are more often monotonous, and epidermotropism may be absent.

Lymph node involvement initially involves the paracortical regions. Progression is associated with small to large clusters of atypical cells with preserved nodal architecture, followed by partial or total effacement of the node by neoplastic cells. Visceral involvement is a late clinical feature. Peripheral blood involvement can be demonstrated in all stages of skin disease, though most prevalent in patients with tumor or erythrodermic presentations. Patients with SS may have circulating neoplastic cells, but be lymphopenic. Bone marrow involvement can often be demonstrated in patients with SS or advanced tumor- or plaque-stage MF, but rarely influences management outside an investigational setting.

The malignant cells are typically $CD3^+$, $CD4^+$, $CD45RO^+$, CD8 , and CD30 by immunohistochemistry. CD7 is often deleted from the early stages. More aggressive variants and advanced CTCL may have multiple pan-T-cell antigen deletions, especially CD2, CD5, and even CD4. T-cell receptor genes are clonally rearranged, and can be demonstrated in most cases by Southern blotting or polymerase chain reaction (PCR) assays when a sufficient malignant infiltrate exists.

Analysis of peripheral blood may reveal an elevated LDH (lactate dehydrogenase) in a small percentage of patients with advanced disease or an aggressive tempo. Eosinophilia and hypergammaglobulinemia is not uncommon is SS patients. A limited number of patients will have an associated monoclonal gammopathy. Elevated serum B_2 microglobulin and IL-2 receptor levels have also been noted in advanced cases.

Imaging studies for classic MF/SS are generally of modest utility. CT scans of the chest, abdomen, or pelvis should be reserved for patients with SS, nodal involvement, or CTCL variants. In an investigational setting electron microscopy, cytogenetics, and molecular analyses have shown that a higher percentage of patients will have occult involvement of internal organs.

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DIFFERENTIAL DIAGNOSIS

Primary CTCL represents a heterogenous group of disorders with considerable variability in histology, phenotype, and prognosis.

Figure 74-8 Lesions of granulomatous slack skin with destruction of the dermal elasticity.

The Kiel Classification, Working Formulation, and the Revised European-American Lymphoma (REAL) classification system were developed for non-Hodgkin lymphomas and are not designed to provide an adequate characterization of the spectrum of CTCL. To address the deficiencies of the previously proposed systems a more clinically useful classification has been developed by the European Organization for Research and Treatment of Cancer (EORTC). [2] A number of other disorders in which malignant T cells infiltrate the skin should be distinguished from MF/SS. These disorders are discussed in the following sections.

CD30-Positive Cutaneous Large T-Cell Lymphoma

Primary cutaneous $CD30^+$ large cell lymphoma ($CD30^+$ LCL) typically occurs in adults presenting with solitary or localized

Figure 74-9 Skin biopsy of mycosis fungoides showing a dermal infiltrate of cerebriform lymphocytes with epidermotropism.

(ulcerating) nodules or tumors (Fig. 74-11). [42] Regional lymph node involvement is seen in 25% of patients at presentation. These primary cutaneous $CD30^+$ LCL are probably closely related to lymphomatoid papulosis, regressing atypical histocytosis, and primary cutaneous Hodgkin disease. The tumor

Figure 74-10 Tumor stage mycosis fungoides with a deep and dense mononuclear cell infiltrate without significant epidermotropism.

Figure 74-11 Lesions of $CD30^+$ large cell lymphoma with ulceration.

has a favorable prognosis and often complete or partial spontaneous regression occurs. This is in contrast to primary noncutaneous $CD30^+$ LCL, which can be seen in children or adults and which carries a poor prognosis. [43] Histopathology consists of diffuse nonepidermotropic infiltrates with cohesive sheets of large CD30-positive tumor cells (Fig 74-12). In most instances the tumor cells have an anaplastic morphology, showing round, oval, or irregularly shaped nuclei, prominent (eosinophilic) nucleoli, and abundant cytoplasm. Less commonly, the neoplastic cells have a pleomorphic or immunoblastic appearance. Reactive lymphocytes are often present. The immunophenotype of this disorder is characteristically $CD4^+$ with the majority (>75%) of neoplastic cells expressing CD30. In contrast to the poor outcome of MF that has transformed to a $CD30^+$ large cell variant, primary CD30-positive cutaneous large T-cell lymphomas are associated with an excellent prognosis. Radiotherapy is the preferred treatment for solitary or localized disease, with combination chemotherapy reserved for patients with generalized skin lesions or extracutaneous dissemination.

CD30-Negative Cutaneous Large T-Cell Lymphoma

These lymphomas tend to have an aggressive clinical course. Patients present with localized or generalized plaques, nodules, or tumors. [44] Histopathology demonstrates that infiltrates are nonepidermotropic with variable numbers of medium-sized to large pleomorphic T cells with or without cerebriform nuclei, and immunoblasts. The tumor cells are $CD4^+$ with CD30 staining negative or restricted to few scattered tumor cells. Multiagent chemotherapy is used in most instances, with radiation therapy reserved for patients with localized disease. The five-year survival rate is less than 20%.

Pleomorphic Small/Medium-Sized CTCL

This is an uncommon entity. Patients typically present with red-purplish nodules or tumors. Histopathology shows a dense, diffuse, or nodular infiltrate with small/medium pleomorphic neoplastic cells within the dermis, often with extension into the subcutis. [34] [45] The neoplastic cells are of the helper phenotype and do not express CD30. Localized disease is typically treated with radiation therapy. Patients with more generalized disease have been treated with regimens used for indolent non-Hodgkin lymphomas.

Figure 74-12 $CD30^+$ large cell lymphoma with sheets of large atypical lymphocytes.

Subcutaneous Panniculitis-like T-Cell Lymphoma

This is an extremely rare entity. Patients present with subcutaneous nodules and plaques. [46] [47] Systemic symptoms are common including fevers, fatigue, and

anorexia. Histopathology reveals a subcutaneous infiltrate with pleomorphic T cells of variable size, mixed with benign macrophages. Tumor cell necrosis, karyorrhexis, and erythrophagocytosis are common findings. Differential diagnosis includes the frequently fatal but non-neoplastic cytophagic histocytic panniculitis. Neoplastic infiltration of deep blood vessels can be noted in some cases. [48] Immunophenotyping reveals post-thymic T-cell markers with both $CD4^+$ and $CD8^+$ subtypes. The prognosis is in general poor despite aggressive chemotherapy.

Angioimmunoblastic Lymphadenopathy

Angioimmunoblastic lymphadenopathy is a rare lymphoproliferative disorder frequently accompanied by hepatosplenomegaly, fever, skin rash, and generalized malaise.[49] Approximately 50% of patients have skin involvement at presentation. Common laboratory features include anemia (often Coombs-positive hemolytic), thrombocytopenia, leukocytosis with lymphopenia, and polyclonal hypergammaglobulinemia. Histologic features of lymph node biopsies include complete architectural effacement with replacement by a diffuse polymorphous cellular infiltrate composed of lymphocytes, immunoblasts, and plasma cells, with or without histiocytes and eosinophils. Prominent arborization of postcapillary venules and atrophic germinal centers are seen.

Skin biopsy findings are nonspecific, revealing a perivascular lymphocytic infiltrate in the dermis with a cytologic composition similar to that of the lymph node. Histologic progression to an appearance of a high grade lymphoma is common. Molecular and immunologic studies usually demonstrate an activated mature clonal T-cell phenotype. The median survival is approximately 1.5 years, and only 30% of patients survive 2 years. Treatment with corticosteroids and/or chemotherapy rarely provides durable control. An association with Kaposi sarcoma has been noted.

Lymphomatoid Granulomatosis

Lymphomatoid granulomatosis is a rare multiorgan disease of the lungs, nasopharynx, joints, peripheral and central nervous system. [50] Cutaneous involvement occurs in 2550% of patients. Though nodules are most common, some patients have nonspecific macules, papules, or ulceration. Histologic evaluation reveals an angiocentric, polymorphous infiltrate of both atypical lymphocytes and histiocytes surrounding and invading blood vessels within the dermis. Molecular and immunologic studies suggest a mature clonal helper T-cell process. However recent reports have suggested a massive reactive T-cell infiltrate driven by a small number of clonal B cells.[51]

Epstein-Barr virus DNA sequences are frequently present, and their role in the pathogenesis of this disorder is being explored. Though the clinical course is variable, the prognosis for patients with diffuse pulmonary involvement or the appearance of high-grade lymphoma is poor, with a median survival of less than 2 years. Treatment that depends on histologic findings and extent and location of disease, may include corticosteroids, radiotherapy, and/or chemotherapy. Recently interferon has been demonstrated to have significant activity against this disease. [52]

Adult T-Cell Leukemia and Lymphoma (ATLL)

ATLL is in most instances a rapidly progressive T-cell neoplasm expressing a helper phenotype. [53] [54] It is endemic in southern Japan

Figure 74-13 Skin lesions in adult T-cell lymphoma/leukemia syndrome.

and the Caribbean islands and is associated with the retrovirus HTLV-1. However, most HTLV-l infected patients remain asymptomatic and only 24% develop ATLL. The clinical presentation is polymorphous and can resemble MF or SS. Cutaneous lesions are variable, ranging from a rash simulating a viral exanthem to large tumors and plaques similar to MF (Fig. 74-13). Advanced stages of the disease, which affects a younger population than seen with MF, are characterized by visceral involvement, immunodeficiency, elevated LDH, and hypercalcemia. Malignant lymphocytes often have convoluted or multilobed nuclei and can be detected in the peripheral blood in 75% of patients. The neoplastic T cells express high levels of the IL-2 receptor (CD25). For purposes of treatment and prognosis it is wise to view ATLL as a spectrum with two subgroups, acute and all others, with treatment, though inadequate, reserved for those with acute ATLL. Therapeutic options include multiagent chemotherapy and antibody or recombinant toxins directed against the IL-2 receptor. Patients with acute ATLL have poor survival rates, with median duration of 46 months.

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PROGNOSIS

The goals of treatment in MF are the relief of symptoms and improvement in cosmetics. Despite some uncontrolled clinical trial results that have been reported to suggest cures in this

disease, the general perception remains that this disease is not curable with standard therapies available today. The disease behaves similarly to other low-grade lymphomas, with periods of remission gradually becoming shorter with subsequent therapeutic interventions. Unlike B-cell low grade lymphomas, however, advanced stage MF is associated with a relatively short median life expectancy. Patients with either significant nodal involvement (LN3 or LN4) or extensive skin involvement (T4) have median life expectancies of 3055 months.[55] [56] Thus, a driving force in the development of treatments for this disease is the goal of altering the natural history for this group of poor prognosis patients. No clinical trial has yet determined that aggressive early therapy is better than sequential palliative approaches or investigational approaches,[57] and thus new treatments continue to be developed and tested for these patients.

Techniques such as flow cytometry, cytogenetic analysis, and determination of nuclear contour indices are specialized methods, which may also improve diagnostic and prognostic specificity. Flow cytometry and cytogenetic analysis are complementary techniques. Flow cytometry allows the detection of cell populations with the normal (diploid) number of chromosomes versus abnormal (aneuploid) numbers, while cytogenetic analysis precisely identifies the individual chromosomal structure and number. Bunn et al.[58] demonstrated that this can be important prognostically in MF/SS, as the presence of aneuploidy during the clinical course was associated with more aggressive behavior of the disease. Hyperdiploid cell clones were demonstrated in patients with large-cell histology, aggressive disease, and shortened survival time.

The nuclear contour index has been used by several groups in an effort to separate benign cutaneous lymphocytic disorders, such as lymphomatoid papulosis and pityriasis lichenoides, from MF/SS.[59] [60] Electron microscopy allows the calculation of a value based on the degree of nuclear folding: this nuclear contour index is significantly greater in patients with MF than in other benign conditions.

Most recently, the density of epidermal Langerhans' cells in biopsy samples, as determined by immunoperoxidase stains, has been identified as a prognostic feature.[61] Epidermal Langerhans' cells are necessary for antigen recognition and processing in the normal immune response. Patients with Langerhans' cell densities greater than 90 cells per square millimeter had a significantly reduced risk of death from MF/SS compared with those with lower densities. Furthermore, there was no prognostic significance identified for the presence or absence of CD30 positive (Ki-1) cells.

Occasionally patients may develop a more clinically aggressive lymphoma concurrent with a change in the histologic appearance of the neoplastic cells. This progression from typical small, convoluted lymphocytes to larger lymphocytes, such as those associated with large-cell lymphoma, has been documented.[62] [63] Whether this conversion is secondary to prior therapeutic modalities used remains uncertain.

The gold standard for the diagnosis of mycosis fungoides is still routine histopathology with adequate clinical correlation. Early lesions of MF are frequently accompanied by heavy infiltrates of benign reactive T cells, hampering the detection of abnormal T-cell clones by any laboratory method. Hence most adjuvant laboratory methods are not helpful at the precise time when they are most needed.

In 1979, the staging committee at an international workshop on MF proposed a staging system based on the international tumor-node-metastasis (TNM) system (Table 74-2).[64] This classification was based on the evaluation of 347 patients and a multivariate analysis of potential prognostic factors. This group identified several independent prognostic factors: extent of skin disease at diagnosis (T), type of lymph node (N) involvement, presence or absence of peripheral blood (PB) involvement, and presence or absence of visceral (M) involvement. The group also

TABLE 74-2 -- TNM Classification of Cutaneous T-Cell Lymphomas

Classification	Description
T skin	
T_0	Clinically or histopathologically suspicious lesions
T_1	Limited plaques, papules, or eczematous patches covering 10% of the skin surface
T_2	Generalized plaques, papules, or erythematous patches covering 10% of the skin surface
T_3	Tumors (one or more)
T_4	Generalized erythroderma
	Pathology of T_{14} is diagnostic of a cutaneous T-cell lymphoma. When more than one T stage exists, both are recorded and highest is used for staging. Record other features if appropriate (ulcers, poikiloderma, scale, etc.)
N lymph nodes	
N_0	No clinically abnormal peripheral lymph nodes
N_1	Clinically abnormal peripheral lymph nodes (record number of sites)
NP_0	Biopsy performed, not CTCL
NP_1	Biopsy performed, CTCL
PB peripheral blood	
PB_0	Atypical circulating cells not present (5%)
PB_1	Atypical circulating cells not present (>5%), record total WBC, total lymphocyte count, and % of abnormal cells
M visceral organs	

M_0	No visceral organ involvement
M_1	Visceral involvement (must have pathologic confirmation), record organ involved

Note: Staging classification: Stage Ia: T_1 , N_0 NP_0 , M_0 ; Stage Ib: T_2 , N_0 NP_0 , M_0 ; Stage IIa: T_{12} , N_1 NP_0 , M_0 ; Stage IIb: T_3 , N_0 NP_0 , M_0 ; Stage III: T_4 , N_0 NP_0 , M_0 ; Stage IVa: T_{1-4} , $N_{0,1}$ NP_1 , M_0 ; Stage IVb: T_{1-4} , $N_{0,1}$ $NP_{0,1}$, M_1 .

translated this staging into a recommended clinical staging system (see note in Table 74-2).

More recently, investigators at the National Cancer Institute retrospectively analyzed 152 patients who underwent uniform pathologic staging. [65] They were able to identify three distinct prognostic groups. Good-risk patients had plaque-only skin disease without lymph node, blood, or visceral involvement, and a median survival of more than 12 years. Intermediate-risk patients had skin tumors, erythroderma, or plaque disease with lymph node or blood involvement (but no visceral disease), and a median survival of 5 years. Poor-risk patients had visceral disease or complete effacement of lymph nodes by lymphoma, and a median survival of 2.5 years.

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THERAPY

Therapy can be conveniently divided into two approaches: (1) topical (skin-directed), such as psoralens with ultraviolet light A (PUVA), topical chemotherapy application (nitrogen mustard or carmustine), and total skin electron beam radiotherapy, and (2) systemic (skin- and viscera-directed), such as interferons, oral or parenteral chemotherapy, photopheresis, retinoids, and investigational new compounds (Table 74-3). No studies have demonstrated that one topical therapy is more effective than another, and patient and investigator preference remain the most important discriminating factor governing choice. However,

TABLE 74-3 -- Therapeutic Options for Mycosis Fungoides

Topical
Ultraviolet Light A with Psoralen (PUVA)
Ultraviolet Light B (UVB)
Total skin electron beam radiation
Topical chemotherapy
Topical retinoids
Systemic
Photophoresis
Interferon alpha
Single agent chemotherapy
Combination chemotherapy
Oral retinoids
Investigational agents

as the biology of the neoplastic cell has become better understood, [66] it has become clear that some therapies may actually have topical and systemic effects through alterations in the body's cytokine milieu and ability to mount a host response against the neoplastic cell. [67] Finally, investigational approaches combining therapies remain active research strategies. The following sections will detail the individual treatment options, discussing mechanisms of action, rationale for use, and outcomes.

PUVA

8-Methoxypsoralen (8-MOP) is a member of a family of photoactivated compounds (furocoumarin derivatives), which may inhibit both DNA and RNA synthesis through formation of mono- or bifunctional thymine adducts, gene mutations, or sister chromatid exchanges. [68] [69] These drugs are only active if the tissue containing the psoralen compound is exposed to ultraviolet A rays (UVA). It has become apparent recently that the mechanism of cell cytotoxicity for many therapies of cancer involves the induction of apoptosis. Yoo et al. have demonstrated that peripheral blood mononuclear cells from Sezary syndrome patients and controls exposed in vitro to PUVA undergo apoptosis.[70] This finding was confirmed with light microscopy, flow cytometry, and electron microscopy, and by gel electrophoresis. Unfortunately, normal and neoplastic lymphocytes were equally sensitive to the apoptosis induction with PUVA (as opposed to with psoralen alone). However, macrophages appeared to be resistant to apoptosis induction, and phagocytized apoptotic lymphocytes (but not non-apoptotic lymphocytes). Thus, apoptosis induction may be the ultimate endpoint yielding benefit, but an immunologic effect due to monocyte phagocytosis and antigen presentation to secondary effector cells may also be present.

Photochemotherapy units with UVA lamps emit a continuous spectrum of long UVA in the range of 320400 nm with peak emission between 350 and 380 nm. Initial exposure times of patients to high output UVA are based on the degree of pigmentation before therapy, history of ability to tan, and the output of the photochemotherapy units. Exposure times are increased with each treatment depending upon the patient's response, and evidence of erythema. The initial UVA dose is between 1.0 to 2.0 J/cm^2 , and can be increased by approximately 0.5 J/cm^2 per treatment as tolerated. UV light-blocking glasses should be worn for 24 hours after administration of 8-MOP. Therapy is typically given three times weekly until complete clearing occurs. The frequency of treatments can then be reduced, but some maintenance therapy (once every two to four weeks) may prolong the duration of remission. As new data emerges regarding the long-term risks of second skin malignancies after PUVA, this maintenance therapy recommendation may change.

Initial trials using PUVA noted benefit in psoriasis patients. [71] Several clinical trials with PUVA for patients with MF soon followed. [72] These studies all demonstrated high rates of remission induction in early stage (patch/plaque stage of disease). The Scandinavian study group reported a 58% complete remission rate for these patients within 412 months of initiation of therapy. [73] Maintenance therapy allowed remission duration of up to 53 months. In these early studies, the same group also reported a surprisingly high rate of objective remission induction in tumor stage patients of 83%. [74] We have recently reported our large modern era results of PUVA therapy for patients with MF.[75] Eighty-two patients were followed for a median of 43 months. An objective response rate was observed in 95% of patients, with a 65% complete clearance rate. The majority (90%) of these patients had early stage disease (stage IAIIA). A single patient with tumor stage disease attained a short remission with PUVA alone. Two of six patients with generalized erythroderma cleared completely (no evidence of circulating neoplastic lymphocytes). Given the difficulty in treating advanced stage patients with PUVA alone, we usually restrict PUVA therapy to patients with stage IAIIB disease (note Table 74-2), monitoring patients with tumor disease closely for progression.

Side effects with PUVA are quite tolerable. Occasional nausea or vomiting due to the psoralen ingestion is observed, and erythema, pruritus, and chronic dry skin is an effect of the UVA light damage to the skin. Long-term PUVA exposure has been associated with a number of late effects. These include dry skin, lichenification, keratosis, and rarely amyloid deposition in the skin. [76] Most important is the late development of iatrogenic (both basal and squamous) carcinomas, [55] [77] secondary malignant melanomas of the skin, [78] and rarely cataract formation. Because the cumulative dose of PUVA is correlated with the risks of second skin neoplasms, routine use of maintenance therapy may be less desirable, especially for patients with an excellent prognosis (stage IA). Despite these problems, the long remissions

induced, the ease of administration, and the lack of interactions with other therapeutic modalities make PUVA an attractive early intervention.

Radiation Therapy

The non-Hodgkin lymphomas in general, and the CTCL in particular, have been shown to be radiosensitive. [79] External-beam radiation has been shown to adequately control local areas of otherwise resistant MF, or provide palliation in cases of bulky tumor lesions. [80] [81] Unfortunately, the cumulative dosage that can be given to patients over time is limited due to normal organ toxicity. In addition, side effects consisting of leukopenia, thrombocytopenia, and radiation dermatitis may prevent long-term therapy with other agents. Newer techniques involving total nodal irradiation, fractionated total body irradiation, or hemibody irradiation may have a role to play in the development of multimodality approaches to this disease.

The limitations of external-beam radiation led to increasing use of electron-beam radiotherapy for cases of MF confined to the skin. Linear accelerator-generated electron beams are scattered by a penetrable plate placed at the collimator site. The energy of the electrons is reduced to 47 MeV and allows adequate field distribution. Because of this low energy level, the beam only penetrates the surface several millimeters to 1 cm (into the dermis). Patients may be treated using six-field or rotational treatments. [82] [83] Thus, the total skin surface can be treated without significant internal organ toxicity. [61] Most patients are able to tolerate total doses of approximately 3,0003,600 cGy over an 810 week period. [62]

An excellent review has recently compared results of external beam therapy at Stanford University with those achieved in Hamilton, Ontario. [84] The results cited in this paper reflect the extensive expertise of both centers in the delivery of this therapy, and may not be applicable to centers with much less

ALGORITHM FOR CARE OF PATIENTS WITH MF/SS

frequent use of the technique. For patients with stage IAIIA (see Table 74-2 , note), nearly 6595% of patients achieved a complete remission. Treatment delivered without adjuvant therapies is associated with a relatively high rate of relapse in patients of all stages except stage IA. Ten-year relapse-free survival rates at the two centers range from 3352% for this good-prognosis group. However, for stage IB and worse disease, 10-year unmaintained remission rates are only 16% or less. Still the benefits of therapy may extend beyond crude estimates of relapse rates or survival. Patients with tumor lesions, generalized erythroderma, peripheral blood or nodal involvement, and even visceral spread can be successfully palliated with electron beam radiation therapy, as well. Side effects, however, can be occasionally extreme, including scaling, dryness of skin, erythema, extremity edema, telangiectasia formation, skin ulceration, and hair or sweat gland loss (frequently permanent). Careful radiation dosimetric techniques are required to ensure adequate skin treatment without excessive normal organ toxicity. [64] We do not use electron beam radiotherapy early in the disease now that other topical therapies have been developed that yield similar response rates with less potential toxicity. Furthermore, the long duration of remissions observed in patients with stage IA disease does not equate with cure, as their life expectancy is essentially the same as age-matched controls, and these patients may simply have indolent biology. One group has reported their experience with multiple courses of therapy. [85]

They re-treated 15 patients with relapsed MF a mean of 41 months after the initial course of electron-beam therapy. All patients had received intervening therapies for disease control. Eleven of the 15 had a complete remission with their first cycle of radiation. The second course achieved 6 complete remissions and 9 partial remissions. The median duration of initial complete remission was 11 months. The median duration of complete remission to the second course of therapy was only 3 months, suggesting radioresistance had developed between the first and second cycles in many patients. In general, toxicity was thought to be tolerable.

Topical Chemotherapy

The earliest therapies for MF focused on treatment of the skin disease. Novel approaches by dermatologists to apply chemotherapy topically to avoid systemic therapy complications were therefore devised. Mechlorethamine hydrochloride was the first topical agent evaluated to demonstrate efficacy in MF. [86] The solution used for topical application contains 1020 mg of mechlorethamine dissolved in 50100 ml of tap water (no vesicant activity at this low concentration). Although several methods may be used for administration, self-administration at home to the entire skin surface is preferred. The concentration may need to be varied depending on patient tolerance and sensitivity. [87] The time to initial response is usually short, approximately 12 weeks, but long-term application is usually required to obtain the maximum response.

Several large studies have been completed demonstrating the benefit of mechlorethamine, especially in early stage disease. [88] [89] One by Vonderheid et al. [68] reported their experience with topical mechlorethamine and found that it compared favorably with electron beam treatment results. Complete remissions were seen in 80%, 68%, and 61% of patients with limited plaque, extensive plaque, and tumor lesions, respectively. The corresponding median duration of remissions were in excess of 15, 5, and 12 months, respectively. It is difficult to draw definite conclusions from these data, as many patients included in the analysis also received intravenous mechlorethamine or methotrexate, and some may have received radiation therapy. In addition, several patients have relapsed as long as 8 years after the completion of therapy, suggesting that follow-up times must be long to compare various topical therapies for early stage disease. Hoppe et al. [69] confirmed these findings using an ointment-based (aquaphor or polyethylene glycol) topical mechlorethamine, which may be associated with a lower incidence of cutaneous sensitivity.

For early-stage disease, therefore, topical mechlorethamine offers an efficient, convenient (outpatient treatment), and relatively inexpensive treatment option. Side effects consist of delayed hypersensitivity in approximately 35% of patients, although ointment-based solutions appeared to offer a reduced risk of allergic contact dermatitis. Once hypersensitivity develops, patients can be desensitized by injecting minute daily doses of mechlorethamine over a period of several weeks. [90] This should only be done in a medical setting with appropriate anaphylaxis precautions observed. Other investigators had difficulty replicating the results using this risky procedure, and now topical desensitization has become much more common. Constantine et al. propose that therapy should be transiently discontinued until clearance of the allergic dermatitis is achieved (using topical steroids if necessary). [91] Then 0.01 to 0.1 mg per 100 ml of the drug should be applied daily for 1 week. If this dilution was tolerated, the dosage was doubled weekly until the dose achieved was often identical to the initial concentration that induced the hypersensitivity.

Some clinicians, however, believe that a mild hypersensitivity reaction may have beneficial antitumor effects. Ratner et al. previously demonstrated that plaque lesions of MF cleared when exposed to topical doses of 2,4 dinitrochlorobenzene, a known universal inducer of delayed hypersensitivity responses. [92] Anergic individuals failed to improve. Other known sensitizing agents yielded similar but less dramatic responses. Thus, some hypersensitivity may be beneficial; the generalized erythroderma and pruritus are usually poorly tolerated when severe, however, and some alteration in therapy is required.

There has been an increased risk of secondary skin cancers in patients receiving long-term mechlorethamine. Of recent concern is the safety of family members or health care workers secondarily exposed to the topical solutions. However, we are unaware of any documented adverse outcomes, and believe this to be a theoretical concern more than a practical one.

Several other topical agents have been tested and shown to be of benefit in the treatment of MF, including corticosteroids, cytarabine, dianhydrogalactitol, dacarbazine, quanazole, teniposide, hydroxyurea, thiotepa, and methotrexate. [93] However, topical carmustine is the only agent that has demonstrated clinical use. [94] A stock solution is created with 300 mg carmustine in 150 ml of 95% ethanol (sufficient for 30 days of treatment). The patient then adds 5 ml of the 0.2% stock solution to 60 ml of room temperature tap water. This solution can then be applied to the general body surface, with the exception of the head, genitals, palms, soles, and intertriginous zones, unless involved by disease. Applications are daily for 26 months if necessary. Brief exposures to double-dose solution can be tried for resistant

disease.

Results in 188 patients with patch/plaque disease demonstrate efficacy similar to that of topical mechlorethamine. [74] For limited patch disease, there was a failure-free rate of 90% at three years. For more extensive patch stage disease the freedom from treatment failure rate at three years was 62%. Some patients have been managed as long as 10 years with the topical carmustine. Side effects of contact dermatitis are less frequent with this agent, but systemic side effects, mainly leukopenia, are more common. This drug may be helpful for the treatment of patients who do not tolerate topical mechlorethamine as there is no cross-sensitivity.

Hamminga et al. have performed one of the few prospective trials comparing total-skin electron beam radiation to topical mechlorethamine. [95] A total of 42 patients with MF localized to the skin (no documented nodal or visceral involvement) were treated. Patients were not randomized to their treatment; rather, the physician based the decision on patient health, availability of the linear accelerator, and the distance the patient lived from the clinic. In patients with minimal skin disease, no difference was noted. In more advanced skin disease, a trend toward superior initial response was seen with electron beam, but there was a high relapse rate in those patients, necessitating subsequent therapy.

Systemic Chemotherapy

Systemic chemotherapy should be reserved for those patients with relapsed or refractory disease after topical interventions, or for those patients with advanced nodal or visceral disease at presentation. Single-agent chemotherapy with alkylating agents, cisplatin, etoposide, bleomycin, doxorubicin, vincristine, and vinblastine have been used for the treatment of MF or the Sezary syndrome.[96] [97] [98] [99]

McDonald and Bertino have reported particularly good results with methotrexate administered intravenously followed by oral leucovorin factor. [100] Patients received 15 mg/kg of intravenous methotrexate every 5 days. If a patient tolerated the lowest dose, each subsequent dose was escalated. After 5 intravenous doses, patients were switched to oral methotrexate (2550 mg) with oral citrovorum as weekly maintenance. All eleven patients achieved good or better clearing (>60%) for a median duration of 24 months. Mucositis and skin ulceration were

the most significant toxicity witnessed. Myelosuppression was mild in general.

Subsequently this group has explored the benefits of adding 5-fluorouracil to the regimen exploiting the synergy between these agents. [101] The methotrexate was administered as a 24-hour continuous infusion at 60 mg/m^2 . Immediately following this infusion, 5-fluorouracil (20mg/kg each 24 hours) was continuously infused for 3648 hours. Oral leucovorin factor (10mg/m^2) was administered intravenously 6 hours after cessation of the methotrexate infusion, and then orally for five additional doses. The methotrexate dose was escalated to a maximum of 120 mg/m^2 , as allowed by toxicity, and response. Ten patients were treated for an average duration of 33 months (range 378 months). The number of cycles administered ranged from 545. All patients achieved a partial remission. Initial cycles were given every 58 days. Once a good response was achieved, cycles were administered every 3 months as maintenance.

Other groups have anecdotally reported success with low doses of oral methotrexate. In general, these regimens appear to be fairly well tolerated.

A new family of compounds, the purine anti-metabolites, has been developed and shown to be active in the treatment of MF. These compounds do not have a single mechanism of action, but all ultimately interfere with intracellular regulation of deoxyribonucleotide pools and this imbalance partially explains their cytotoxicity. This family of drugs includes 2-deoxycoformycin (DCF), fludarabine phosphate, and 2-chlorodeoxyadenosine (2-CdA). [102]

DCF is a transition state inhibitor of adenosine deaminase. Inhibition of this enzyme, necessary for the conversion of adenosine to inosine, results in accumulation of 2-deoxy-ATP and subsequent inhibition of the enzyme ribonucleotide diphosphate reductase necessary for DNA synthesis in dividing cells. DCF is also effective against cells in the resting state, where ribonucleotide diphosphate reductase levels are barely detectable. It has now been shown that deoxy-ATP accumulation in resting lymphocytes results in increased DNA strand breaks over time; this results in the activation of Ca^{++} /Mg^{++} dependent endonuclease that produces double-stranded DNA strand breaks at internucleosomal regions, and also activation of a poly-ADP-ribose polymerase that consumes both NAD and adenosine triphosphate. These perturbations lead to apoptotic cell death.

Fludarabine phosphate represents the fluorinated derivative of ara-A. This compound was known to retain cytotoxic action against leukemias, and was resistant to degradation by adenosine deaminase. Solubility was poor, however, unless the 5'-monophosphate derivative was used; hence fludarabine monophosphate is the 5'-monophosphate form of F-ara-A. Similar to the mechanism of action of cytarabine or ara-A, fludarabine phosphate requires phosphorylation by deoxycytidine kinase to the active triphosphate metabolite F-ara-ATP. Again, this triphosphate derivative inhibits ribonucleotide reductase, resulting in nucleotide pool imbalances, which prevent DNA repair and ultimately cause apoptosis.

2-CdA represents another chemical modification of deoxyadenosine rendering the drug resistant to adenosine deaminase. After activation via deoxycytidine kinase, the triphosphate derivative similarly inhibits ribonucleotide reductase, and accumulates intracellularly perturbing the deoxyribonucleotide pool balance, resulting in DNA damage and cell death.

Enzymes such as cytoplasmic 5'-nucelotidase catalyze the degradation of the active triphosphate derivatives noted previously. Cells with relatively greater levels of the activation enzymes versus degradation enzymes were identified as likely clinical targets. Lymphocytic disorders make good targets for these agents due to their high levels of deoxycytidine kinase, low levels of 5'-nucleotidase, and their dependence upon polymerase alpha for DNA repair. It was known that T-lymphoblastoid cell lines were most sensitive to these drugs, and thus it was thought that T-lymphocyte disorders would be sensitive in vivo to these agents.

A number of studies of small numbers of patients with MF have been performed to date with these drugs. Table 74-4 shows the results of these studies. Three studies used DCF as a single-agent at doses ranging from 410 mg/m^2 daily for three doses every 28 days.[103] [104] [105] Twenty-five patients with MF or Sezary syndrome were included in these studies; overall 3 complete responses (12%) and 12 partial responses (48%) were documented.

The use of fludarabine for the treatment of MF has been more thoroughly tested by von Hoff et al. [106] They treated 33 patients good risk (no prior systemic therapy) or poor risk (prior systemic therapy) with fludarabine alone at doses of 25 or 18 mg/m^2 for the two groups respectively. One complete response and five partial responses were observed for an overall response rate of 18%.

Finally, 2-CdA has been evaluated as a single agent for the treatment of MF or the Sezary syndrome. At Northwestern University we have treated 21 patients who had failed at least one prior therapy. [107] There were three complete responses and three partial responses (overall response rate = 28%). The median duration of response in this heavily pretreated group, however, was only 4 months. Three other groups have also reported results in small numbers of patients for the treatment of MF.[108] [109] [110] A total of 21 patients were reported when the results from these studies were combined. Seven patients achieved responses, giving an overall objective response rate of 33%, remarkably similar to our large single institution result.

The similarity in mechanism of action of these compounds would suggest that toxicity associated with the various compounds would be similar. This has definitely not been the case, however. There are distinct differences in the spectrum of both acute and chronic toxicities with these agents. DCF and fludarabine are associated with higher rates of nausea/vomiting and alopecia then commonly associated with 2-CdA. The most significant toxicity with DCF and fludarabine, however, are neurotoxicity and immunosuppression. Approximately 15% of patients developed sepsis, and 10% developed an opportunistic infection, such as disseminated toxoplasmosis or cytomegaloviral infection, *Pneumocystis carinii* pneumonia infection, atypical mycobacterial infection, and fungemia also have occurred in patients in studies with these drugs. Another 15% developed severe neurotoxicity in the form of confusion, motor weakness, parasthesias, and central nervous system demyelination. 2-CdA therapy is extremely well-tolerated acutely, but may result in somewhat greater myelosuppression than the other agents. This myelosuppression may even be more significant when the agent is used to treat T-lymphocyte disorders compared to B-cell diseases. In the study by Betticher et al., significant decrements in neutrophils and lymphocyte populations occurred in 46% and 41%, respectively. In our study of 2-CdA, we reduced the days

TABLE 74-4 -- Response Rates of Treatment with Purine Anti-metabolites for MF

Author/Drug	Response Rate
ECOG/DCF	66%
Grever/DCF	100%
Dearden/DCF	54%
von Hoff/Fludara	18%
Kuzel/2-CdA	28%
Saven/2-CdA	38%
O'Brien/2-CdA	18%
Betticher/2-CdA	100%

Abbreviations: DCF, 2-deoxycoformycin; Fludara, fludarabine; 2-CdA, 2-chlorodeoxyadenosine; ECOG, Eastern Cooperative Oncology Group.

of therapy delivered by continuous infusion to 5 days, from the usual 7, because of a perception that the toxicity, primarily prolonged thrombocytopenia, was unacceptable. These results suggest that patients treated with these agents should be carefully evaluated for infectious complications, especially opportunistic infections, and that prophylactic antibiotic, antifungal, and/or antiviral therapy should be considered during and following therapy if significant immunosuppression is documented. Additionally, one should carefully consider the value of continuing to administer cycles of therapy if there is not evidence of further improvement in clinical response, because of the risk of patients suddenly developing prolonged cytopenias that may limit further therapeutic approaches.

Combination chemotherapy has often been employed for patients with advanced disease, either at presentation or with progression. Usually alkylating agents are used, in combination with doxorubicin, or vinca alkaloids. [111] [112] Response rates of 80100% have been achieved, with longer duration of remission than those seen with single agent therapy. However, no trial has demonstrated survival benefits in patients treated with more aggressive regimens versus milder palliative therapies alone. Thus, single agent and combination chemotherapy regimens should generally be reserved for use after less toxic interventions have been attempted.

The natural evolution of combination chemotherapy has been to use dose-intensified approaches with hematopoietic reconstitution with autologous [113] or allogeneic bone marrow.[114] There are few reports in the literature of such treatment programs. Of a total of 9 patients (of whom we are aware) who received high-dose chemotherapy with or without total body irradiation and autologous bone marrow transplants, only two had reasonable disease-free intervals (greater than 12 months). Given the propensity for Sezary cells to be detectable despite a lack of clinical symptoms even in early stage disease if sophisticate molecular techniques are used, it is likely that reinfusion of neoplastic cells may occur with autologous bone marrow. The questionable benefit in low-grade B-cell lymphomas of autologous bone marrow transplant similarly suggests that this approach will not benefit patients. Allogeneic bone marrow transplantation, on the other hand, has been presumably curative in small percentages of patients with low-grade B-cell lymphomas. Therefore, this approach should be investigated in the small subset of young patients with HLA identical siblings, who have poor-prognosis disease and demonstrated relapse or resistance to interferon and topical therapies. A single patient treated with cyclophosphamide and total body irradiation was reported to achieve a complete remission to therapy. This patient, however, relapsed by day 70, necessitating additional therapy. The patient remained clear of lesions and alive at least 6 years after the transplant. These reports provide little reliable information regarding the efficacy of such approaches, but do suggest that the procedure can be performed safely without excessive toxicity. Further information is needed.

Photopheresis

An adaptation of the use of psoralens with UVA light called photopheresis has been described by Edelson et al. [115] Patients ingest 0.6 mg/kg of oral 8-methoxypsoralen before a treatment. The treatment consists of routine leukapheresis with isolation of the mononuclear cell fraction. The cells are then exposed to UVA light ex vivo within a special chamber within the pheresis device. In the initial report, Edelson et al. documented an 88.5% loss of lymphocyte viability compared to control patients treated with drug alone. Overall 64% of patients responded to therapy, with the best results in those with generalized erythroderma, and, presumably, higher circulating Sezary cell levels. The mechanism is not thought to be directly cytotoxic, but rather to induce a host immune response to the reinfused altered Sezary cells. This theory would explain the findings of some investigators that patients without leukemic involvement do poorly with this therapy. This treatment modality has given best results in SS patients with erythroderma of short duration and with adequate CD8+ blood counts.

Several other groups have reported their experiences with photopheresis. [116] [117] When the data are analyzed on an intent-to-treat basis, overall response rates of 3652% are observed. Only 1218% achieve complete remissions using this therapy. These investigators attempted to wean patients from therapy as clearing of lesions was documented. Ultimately, most responders developed recurrent disease.

Toxicity is mild and includes occasional nausea, erythematous flares, and temperature elevations. Patients may develop hypotension during leukapheresis, which usually responds to saline infusions.

Interferons

It is now recognized that the most active agent for the treatment of MF is interferon alpha. [118] [119] [120] [121] [122] Dosages and routes of administration have differed among studies. Initially, high dose interferon was used with maximum doses of 3650 million IU. Bunn et al. and Olsen et al. independently demonstrated complete response rates of 1027% in heavily pretreated patients. The duration of response was only 5.5 months. More recent trials of untreated patients with doses of 318 million IU subcutaneously daily have demonstrated response rates of 8092%. From all these studies it appears that a reasonable and tolerable single agent dose is 12 million IU per square meter administered subcutaneously daily. We recommend starting at 3 million IU and gradually increasing as treatment is tolerated by the patient.

Side effects are dose-dependent. Most common adverse effects are constitutional symptoms consisting of fever, chills, myalgias, malaise, and anorexia. Rarely, cytopenias, elevations of liver function tests, renal dysfunction, cardiac dysfunction, or changes in mental status can be seen. Patients need to be monitored closely while on interferon.

Investigational Approaches

Because of the chronic relapsing nature of MF, new therapies with different mechanisms of action are needed to circumvent tumor resistance. A variety of such approaches are under investigation. These include the use of existing or newly developed retinoid compounds [123] or combinations of retinoids with interferons. [124] Other combination modalities under study include retinoids, interferons, and chemotherapy or radiation therapy, [125] and total skin electron beam radiotherapy followed by photophereis or chemotherapy.[126] Given the lack of benefits of combination chemotherapy and radiotherapy previously, the role of such approaches should remain investigational. Another less toxic combination approach has been the simultaneous administration of interferon and phototherapy. [127] Kuzel et al. demonstrated an overall response rate of 92% with this combination in all stages of patients, many of whom had been previously treated with other therapies. The combination of interferon and topical therapy is now being tested against topical therapy alone in a national cooperative group trial.

Another approach to the therapy of this disease has involved new drugs to exploit the biology of these neoplastic cells. Targeted therapies against unique tumor antigens continue to be tested,[128] such as monoclonal antibodies and ligand-toxin fusion proteins. Knowledge of the unique cytokine milieu associated with these neoplastic T-cells has led to trials testing cytokines that may inhibit the growth of these cells, such as IL-12 [129] or IL-2.[103] Finally, vaccine approaches may be practical.[130]

It is clear that a significant amount of basic and practical research has been performed in an attempt to control this disease.

It is certainly hoped that one of the strategies under development or study will lead to treatments that will control the disease and symptomatic effects, or even cure this neoplasm.

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Chapter 75 - AIDS-Related Lymphomas

David T. Scadden

INTRODUCTION

Non-Hodgkin lymphoma is the most lethal complication of HIV disease. This disorder was noted to be associated with HIV infection early in the epidemic and was added to the list of complications defining the acquired immunodeficiency syndrome (AIDS) in 1987. [2] [3] [4] [5] [6] [7] [8] [9] [10] The clinical landscape of HIV infection has changed over the last several years and with these changes have come modifications in both the understanding and treatment of AIDS-related lymphomas. This chapter covers current background information, evolving concepts in pathophysiology, treatment guidelines, and prospects for future development in AIDS-related lymphoma (ARL).

Lymphoid proliferation is common among many different types of immunodeficiencies. Congenital abnormalities of lymphocyte function such as Wiskott-Aldrich, or immunosuppressive therapy employed in the setting of organ transplantation have long been identified as predisposing patients to developing often-fatal lymphoproliferative disease. The incidence of non-Hodgkin lymphoma following organ transplant is related to the severity of immunosuppressive regimen used and is more common in multi-organ transplants for that reason. While the depth of immunosuppression enhances the likelihood of developing lymphoma, immune stimulation may also participate in the lymphomagenic process. This is supported by the association of lymphoma with autoimmune diseases considered reflective of excessive immune activation such as rheumatoid arthritis or sicca syndrome (Table 75-1).[11] [12] [13] [14] [15] [16] [17] [18] [19] [20]

Despite a common association of immunodeficiency with lymphoma, HIV-1 induced immunosuppression and resultant lymphoma do have distinctive features. For example, the relatively high incidence of a polyclonal, prodromal phase in post-transplant patients appears to be relatively infrequent in ARL, although the issue remains controversial.[21] Epstein-Barr virus is virtually uniformly present in the post-transplant lymphomas, whereas it is present only in approximately half of AIDS-related lymphomas. Translocations of *c-myc* are generally not seen in the post-transplant setting, but are common in ARL. The small

TABLE 75-1 -- Magnitude of Relative Risk of Lymphoma[a]

High (RR > 15)	Intermediate (RR > 2)	Low (RR 2)
Multiple transplants	Sibling transplant	Splenecotomy
Cadaver transplant	Mild sicca syndrome	Sarcoidosis
Severe sicca syndrome	Nontropical spruce Crohn disease	Hyperimmunization Asthma
Wiskott-Aldrich syndrome	Short-term HIV infection	Hansen disease
Ataxia-telangiectasia	Rheumatoid arthritis	
Long-term HIV infection		Systemic lupus erythematosus

Abbreviation: RR, relative risk.

From Hoover, [11] with permission.

[a] Risk of lymphoma in patients with the condition relative to a risk of 1.0 to comparable individuals without the condition.

non-cleaved cell- or Burkitt-like lymphoma seen in ARL is not an entity among the other immunodeficiency lymphomas. It is extremely rare in the post-transplant patient, strongly suggestive of unique pathophysiologic characteristics in the setting of AIDS.

The heterogeneity of lymphomas within the HIV-infected population emphasizes the complexity of tumor development. A range of different mechanisms have been evoked, including abnormal B-cell activation, excessive cytokine stimulation, altered microenvironment preferentially supporting lymphomatous cells, the predisposition to the persistence of specific chromosomal abnormalities, and perhaps most important, the inadequate immunologic control of persistent viral infections with transforming potential. Understanding how these factors may result in the manifestation of malignancy offers the potential for unraveling how immunologic function interfaces with immunologic malignancy with implications extending beyond that of the AIDS epidemic.

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EPIDEMIOLOGY

An increased incidence of non-Hodgkin lymphoma (NHL) was first noted among middle-aged unmarried men in the San Francisco area in 1984 and became a criterion for defining an AIDS illness with the first redefinition of the syndrome by the Centers for Disease Control. This association has been reaffirmed in other epidemiological series and includes individuals from virtually every risk group for HIV infection. Unlike the other major neoplastic complication of AIDS, Kaposi sarcoma (KS), NHL is fairly uniform across the spectrum of HIV-infected individuals. [22] [23] There is a slight increase in incidence noted among hemophiliacs [22] [23] [24] that has provided an intriguing link and potential window on other contributing cofactors, but this remains simply a statistical observation at this time. Lower incidences of lymphoma among IV drug abusers in some series probably reflects the early death of such patients from other opportunistic diseases as well as issues related to their overall care, rather than a true biologic difference.

The incidence of NHL in HIV-infected individuals has been variably estimated between 1.6 and 6% per year. [25] [26] [27] The discrepancy in estimates may be related to inclusion of postmortem diagnoses in the series with high estimates. The incidence of lymphoma does increase with worsening immunosuppression and most often is seen in patients with longstanding HIV infection. However, lymphoma may be the presenting symptom of HIV and consideration should be given to this possibility in patients with aggressive lymphomas. Concerns have been voiced that antiretroviral therapy may predispose to the development of lymphoma because of the potential effect of nucleoside analogs on cellular DNA synthesis. However, subsequent studies have failed to note any association and discount the notion that retroviral therapy should be withheld out of concern about induction of lymphoma. [28]

Projected increases in the incidence of NHL in AIDS have not materialized, as other opportunistic disease has been better controlled. [29] How the recent advent of highly active antiretroviral

therapy will affect the incidence of lymphomas remains unclear. Since the introduction of protease inhibitor therapy and combined antiretroviral therapy in 1996, there has been a dramatic change in the overall prognosis for patients with AIDS. The reduction in death rates in the United States has been profound and has been paralleled by markedly reduced frequencies of many opportunistic complications. For example, the rate of cytomegalovirus disease, which had been increasing before the advent of protease inhibitor therapy, has now fallen precipitously and admissions for this disorder are a relative rarity in most US centers. Within the context of AIDS-related malignancies, spontaneous regression of Kaposi sarcoma has been documented and decreased incidence and marked reductions in the number of patients requiring systemic therapy for Kaposi sarcoma have been observed. Firm epidemiologic data regarding the impact of these therapies on lymphoma are yet to evolve. However, clinical impressions suggest that there is a marked reduction in those patients who present with primary CNS lymphoma, generally a manifestation of end-stage immunosuppression. Systemic lymphomas continue to occur and alterations in their frequency appear to be far more subtle than that of primary CNS lymphoma.

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PATHOPHYSIOLOGY

The epidemiology of non-Hodgkin lymphoma is generally changing within the United States, with an increased incidence noted particularly among men. The bases for this change are poorly understood, but a contributing factor is the HIV epidemic and AIDS-related lymphoma. The epidemiology of ARL is influenced by HIV treatment and coincident associated viruses such as EBV and the recently described Kaposi sarcoma herpesvirus (KSHV) also known as human herpesvirus-8 (HHV-8). [30] There are likely other, yet undetected cofactors that account for some of the other subsets of this disease. In particular, the small non-cleaved or Burkitt-like lymphomas represent a subset for study for identification of new, potentially infectious, cofactors contributing to ARL.

HIV-1 itself rarely participates directly in the malignant process, but rather provides the background of immunodeficiency within which lymphomas may arise. A small minority of lymphomas seen in AIDS are of T-cell origin. Molecular analysis of some of these cases has indicated that HIV was either integrated in the T cells or highly expressed in tumor-associated macrophages. The site of HIV integration into the host genome was evaluated and found to be in a common site upstream of the proto-oncogene, *c-fes.* [31] Apart from these relatively uncommon tumors, the transformed cells do not contain HIV-1 DNA. Rather, other mechanisms of lymphomagenesis likely predominate.

Potential contributing underlying abnormalities include most prominently:

1. Inadequate immunologic control of persistent viruses with transforming capability
2. Alteration in regulatory environment for lymphoid growth control
3. Accumulation of genetic abnormalities in target cells

The issue of participating secondary infectious processes in ARL is supported by the spectrum of AIDS malignancies generally (Table 75-2). Tumors with definite or probable increased frequency in HIV disease are notable for the relatively small number of tumor types seen. Unlike tumors outside the setting of HIV disease, where a broad spectrum of epithelial neoplasms occur, the spectrum of malignancy is narrow. Unifying each of these diseases is an association with underlying infectious agents. The herpes virus, KSHV, is associated with both KS and a subset of NHL. It may also participate in the development of

TABLE 75-2 -- AIDS-Associated Tumors

Definite
Kaposi sarcoma
Non-Hodgkin lymphoma
Squamous cell neoplasia of uterine cervix, anus, conjunctiva
Probable
Hodgkin disease
Leiomyosarcoma
Plasmacytoma

plasma cell dyscrasias [32] and it has been implicated in the development of Castleman disease. [33] The squamous cell neoplasia of the uterine cervix in HIV-infected women, of the anus of homosexual men, and of the conjunctiva in individuals in Africa is associated with the human papillomavirus. [34] [35] [36] [37] [38] [39] [40] Epstein-Barr virus is a well known participant in a subset of lymphomas as well as the leiomyosarcoma seen in children [41] and in the Hodgkin disease seen in the setting of HIV. [42] Thus viral pathogens are strongly related to the development of neoplastic complications. The specific mechanisms participating in transforming events are in the process of further evaluation. These offer the potential for novel types of therapeutic intervention and the viral antigens associated with them offer possible immunotherapeutic targets.

The Epstein-Barr virus genome has been reported to be present in 3367% of AIDS-related lymphomas as opposed to 5% of high grade lymphomas outside the setting of HIV. [43] The frequency varies depending on the cell type involved. Immunoblastic tumors of the CNS virtually uniformly have the EBV genome present, whereas only 2034% of the Burkitt-like tumors contain EBV. [44] [45] Recently described oral cavity ARLs with T-cell markers [46] and plasmablastic lymphomas in the oral cavity [47] all appear to have EBV present. Leiomyosarcomas occurring in children with HIV disease, but not in adults, also uniformly contain EBV within the tumor cells. [41]

EBV is thought to infect cells and induce their altered proliferation by expression of virus genes that are part of the virus latent (as opposed to lytic) phase. The pattern of EBV gene expression varies with the clinical and pathologic setting. In patients with primary CNS lymphoma, the EBV latent gene expression pattern is identical to that of post-transplant lymphoproliferative disease, including Epstein-Barr nuclear antigens (EBNA)-2 through EBNA-5 and latent membrane protein (LMP)-1 and -2. [48] [49] In Burkitt lymphoma, only EBNA-1 is usually detectable. [48] [49] [50] [51] For some systemic ARL, a unique combination of EBNA-1 and LMP-1 expression has been reported. [52] Molecular analysis of EBV latent genes has indicated that EBNA-1 is important for transformation due to its effects on transcription and maintenance of the EBV episome. LMP-1 is essential for primary B cell transformation and for transforming effects on non-lymphoid cells. [53] [54] [55] LMP-1 interacts with the signaling pathway used by the tumor necrosis factor receptor family of genes through very specific molecular interactions at its c-terminus. [53] [54] [55] [56] A number of mutations have been noted within the LMP-1 portion of the EBV genome and some data have supported the possibility that these variants are associated with ARL or HIV-related Hodgkin disease. [57] [58] [59] [60] However, controversy on this continues as some studies have indicated that LMP-1 mutations occur with similar frequency in patients with lymphoproliferative disease and in unaffected individuals. [58] The relationship of EBV to transformation is clear, but why certain individuals are more predisposed to develop lymphoma is still unknown. Molecular evolution of the virus within the host remains a tenable, but unproven hypothesis.

An alternative possibility is that some individuals will selectively lose their immunologic reactivity to EBV or other transforming

viruses. A number of immunologically recognized antigens within the Epstein-Barr genome have been characterized as being targets for cytotoxic T lymphocytes. [61] These may be masked by effects of EBV EBNA-1 on antigen presentation through altered processing pathways [41] [62] [63] or through deletion of reactive cytotoxic T lymphocyte (CTL) clones during the course of progressive immunodeficiency. Alteration of the T-cell receptor repertoire is a well documented phenomenon in AIDS and may result in non-homogeneous losses of reactivity to specific foreign or tumor antigens. [64] This phenomenon suggests the potential for clonal repletion as a strategy for intervention and indeed such an approach has been successfully demonstrated in post-transplant patients with EBV lymphoproliferation. [65] [66] [67] Adoptive

transfer of T cells has induced tumor regression in other immunodeficiency-related lymphomas.

The vigor of immunologic response may also be affected by virus gene products. In particular an EBV gene (BCRF-1) has homology with human interleukin 10 (IL-10) and mimics IL-10 function.[68] [69] [70] IL-10 has been noted to inhibit interferon gamma and IL-2 elaboration by helper T-cells (TH-1), cells important in immunologic function.[69] Cell lines and primary tumors isolated from patients with AIDS lymphoma produce a high level of interleukin-10 and elevated circulating levels of interleukin-10 in mouse models of AIDS-lymphoma have been well documented.[68] [70] [71] [72] [73] Other cytokine-related effects include the production of CD40 (a B cell proliferation signal) by HIV-infected bone marrow endothelial cells [74] and IL-6 production by HIV-infected macrophages or by KSHV-infected cells.[75] [76] Indeed increased IL-6 levels have been noted to correlate with malignancy and increased serum CD23 (a marker of activation) to precede it. [77] [78] Dysregulation of cytokines further induces alterations in B-cell biology contributing to a hyperproliferative state.

Another viral pathogen that contributes to ARL is the recently described member of the gamma-herpesvirus family, KSHV. This virus was originally identified by comparative analysis of the DNA content from Kaposi sarcoma (KS) tissue versus uninvolved tissue.[79] This genetic approach to pathogen identification revealed a virus that has now been shown convincingly to be associated with KS, but also with a subset of ARL, Castleman disease, and perhaps plasma cell dyscrasias. [30] [32] [33] [80] The lymphomas are generally restricted to body cavities without tumor masses and are termed primary effusion lymphomas (PEL). Features of this clinicopathologic entity will be further discussed in a separate section, but features of this virus are extremely interesting from the pathophysiologic perspective.

KSHV is known to be capable of infecting primary B cells[81] and is uniformly found in the transformed B cells of PEL. Mechanisms by which this virus participates in the malignant process remain speculative since in vitro infection with KSHV does not result in B cell transformation. However, a number of components of the KSHV genome suggest the potential for this virus to alter B cell physiology. These include a constitutively active chemokine receptor which causes cell lines to have enhanced malignant potential when transfected, a physiologically active cyclin D homologue, a viral interleukin-6 with potent function, a BCL-2 homologue, and CC chemokine homologues.[76] [80] [82] [83] [84] [85] [86] [87] This constellation of different immunoregulatory proteins is highly suggestive of direct mechanisms by which the virus may perturb cell functions, but the details have not yet been clarified. The immunologic response to KSHV at this time is also poorly understood. However, the association of KSHV-related tumors with immunodeficiency of multiple types strongly supports the role of immune function in tumor control.

A large fraction of ARLs do not have evidence for either EBV or KSHV infection. While it has been hypothesized that these may represent cells transformed by still-unknown infectious agents, a number of potential etiologies remain. These include the proliferative drive provided by EBV or HIV-induced cytokine alterations as noted previously. In addition, a gross dysregulation of B-cell biology is evident early on in HIV disease regardless of the subsequent development of lymphoma. [88] This includes the presence of hypergammaglobulinemia, which is perhaps the most common serologic abnormality among HIV-infected individuals.[89] [90] As patients progress in HIV disease, lymphadenopathy with follicular hyperplasia is common and is similarly reflective of disordered regulation of the B-cell lineage. [91] [92] It has been postulated that a portion of the HIV envelope may be capable of inducing T-cell-mediated B-cell activation. [93] [94] This may result in particular outgrowth of HIV-specific B cells. With chronic stimulation transformation may occur and sporadic multiple myeloma with HIV specificity has been reported. [95] [96] [97] Characterization of the variable chain repertoire of immunoglobulins produced by B cells in patients with HIV disease has indicated that preferential use of the VH subsets occurs early in HIV infection[98] and in ARL tumors.[99] These findings suggest that there may be an antigen-driven expansion of selected B-cell clones that may provide the mitogenetic background for mutogenesis to result in malignancy.

A number of specific genetic mutations have been observed in ARL and are associated with specific tumor-types. [100] [101] [102] That is, tumors of small-cell or Burkitt-like histology uniformly exhibit *c-myc* gene rearrangements, may have p53 gene mutations, and do not exhibit BCL-6 rearrangements.[103] [104] [105] [106] This is in contrast to large cell lymphomas, among which approximately one-third will demonstrate BCL-6 rearrangement, 40% *c-myc,* and 25% p53 mutations.[107] The *c-myc* rearrangement that is found most often juxtaposes *c-myc* with the immunoglobulin heavy chain switch region.[105] [108] [109] [110] [111] The involvement of this region suggests that the transforming event has occurred in a B cell that is undergoing transition between heavy chain isotopes: phenomena occurring at a relatively late stage in B-cell ontogeny.[23] [103] [104] [106] [112] [113] [114] The malignant cell therefore appears to be a relatively mature B lymphocyte. In ARL, *c-myc* rearrangement and EBV infection are not necessarily present in association with one another. [103] [104] [105] [106] Similarly, BCL-6 rearrangements are variably associated with the presence of EBV. It is of interest that no coincident *c-myc* and BCL-6 rearrangements have been detected.[107] Molecular abnormalities associated with low-grade lymphomas (such as the 11:14 rearrangement) are generally not seen in ARL.

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CLINICAL MANIFESTATIONS

AIDS-related lymphomas are generally extremely aggressive, high-grade lymphomas of B-cell origin. However, the spectrum of lymphoproliferative disease is broad and is continuing to expand. T-cell malignancies have been reported, including large granular lymphocyte disease, large cell or anaplastic T-cell lymphoma, Sezary syndrome, and angiocentric T-cell proliferation. [97] [115] [116] [117] [118] [119] [120] [121] [122] Virus infection with human T-cell leukemia/lymphoma virus (HTLV-1) has been noted in some cases of T-cell malignancy with coincident HIV infection. [123] In addition, HIV itself has been noted to be integrated in some of these lymphomas, [31] [122] as previously discussed. Oropharyngeal T-cell lymphoma has been described in AIDS patients and is associated with the presence of EBV in the tumor cells. [46]

Castleman disease or angiofollicular lymph node hyperplasia has been reported in patients with advanced HIV disease. [33] This is typically a multicentric variant of Castleman disease that is associated with fever, peripheral adenopathy, weight loss, hepatosplenomegaly, pulmonary symptoms, and anemia or pancytopenia. Both the hyalin-vascular and plasma cell subtypes of Castleman disease have been reported in AIDS patients. There is a very high association of this process with the presence of KSHV in the involved nodes and a high prevalence of coincident or subsequent Kaposi sarcoma. [33] [80] [124] [125] This lymphoproliferative process is often aggressive with poor outcome, although responses to chemotherapy have been reported. [33]

TABLE 75-3 -- Primary Effusion Lymphoma (Body Cavity Lymphoma)

Positive fluid cytology without mass
Distinct phenotype: null cell immunophenotype with rearranged immunoglobulin genes)
HHV8-positive

Plasma cell disorders appear to be increased in the setting of HIV infection. Plasmablastic lymphomas of the oral cavity have recently been described [47] as a relatively uncommon neoplasm, notable for the frequent absence of typical lymphoid markers such as CD45 (leukocyte common antigen) or CD20. Extramedullary plasmacytomas have been reported, as well as overt multiple myeloma. [126] [127] The clinical course of these disorders is highly variable and the clinical approach should be based on standard guidelines for these diseases outside the context of HIV infection.

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HODGKIN DISEASE

Controversy remains regarding the incidence of Hodgkin disease in patients with HIV infection, but data are accumulating to support the notion that the frequency of the disease is increased in infected individuals. [128] [129] The clinical presentation in the setting of HIV infection is often distinct in that the clinical stage is more advanced and the mixed cellularity subtype more common. [6] [117] [129] [130] [131] [132] [133] [134] [135] [136] Stage 34 disease at presentation has been noted in over 80% of HIV-seropositive patients. [131] Extranodal disease is also more common in HIV-seropositive patients with one series reporting a frequency of 63% compared to 29% in seronegative patients. [135] In addition, histopathologic features of Hodgkin disease in AIDS are different both in the frequency of mixed cellularity and the presence of EBV. Virtually all tumors have detectable EBV and express LMP-1. [137] A high frequency of LMP-1 molecular abnormalities has also been noted in patients with AIDS compared to patients with Hodgkin disease who do not have AIDS. [137]

The clinical approach to patients with Hodgkin disease in the setting of HIV infection is similar to that in other contexts with standard staging methods. Treatment strategies should be applied as appropriate for stage, similar to guidelines outside the setting of HIV infection. However, the underlying level of immunosuppression and overall performance status of the patient must be considered. A higher incidence of treatment complications may be expected if the patient has very advanced AIDS, and appropriate prophylaxis for *Pneumocystis carinii* should be undertaken. However, cure of Hodgkin disease has been well demonstrated in the setting of HIV infection and dose attenuation should be contemplated only in those patients with advanced AIDS or those who have demonstrated intolerance to the standard dose regimens.

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NON-HODGKIN LYMPHOMA

The vast majority of lymphomas that occur in the context of HIV are of B-cell origin and are typically aggressive, high-grade malignancies. The lymphomas can basically be broken down into two major subcategories: primary CNS lymphoma and systemic lymphoma that may or may not include CNS involvement. In the systemic disease category, a subset of patients have the primary effusion lymphoma (PEL) mentioned previously (Table 75-3).

These PEL or body cavity lymphomas are generally present in body fluids without associated tumor masses. [30] [138] They have a common immunophenotype in that markers of B or T cells are generally absent from the cell surface, but evidence for immunoglobulin gene rearrangement by analysis of tumor DNA indicates that they are of B-cell origin. These tumors are uniformly associated with the presence of the KSHV genome in the tumor cells. Although many also contain Epstein-Barr virus (EBV), this finding is less uniform. [100] [101] Sero-epidemiologic data regarding this virus remain quite controversial. However, emerging information suggest that this is a virus that can be transmitted sexually and is present in subsets of the North American population with estimates ranging from 520%. [139] [140] Other areas associated with the higher frequency of Kaposi sarcoma, such as the Mediterranean basin and Central Africa, have been noted to have far higher frequencies of seropositivity for KSHV. [139] Patients with PEL often have advanced immunosuppression and present with rapidly evolving pleural or pericardial effusions or ascites. The bowel may be involved [141] and some patients will show dissemination to bone marrow and blood. This tumor generally behaves very aggressively and the outlook with current approaches is unfavorable.

The most common histologic types of NHL in AIDS are small non-cleaved cell types (Burkitt or Burkitt-like) representing approximately one-third of cases, with the rest approximately equally divided among the diffuse large cell and immunoblastic subtypes (Table 75-4). The latter two subcategories would be lumped together under the Revised European-American

TABLE 75-4 -- Histology, Extranodal Sites, and Stage of Lymphoma in AIDS

	Histology				Extranodal Sites				Stage	
	# Pts.	SNCC	IBS/ALC	LC	Gastro	Liver	CNS	Marrow	I, II	III, IV
Levine et al.	27	10	13		6	1	8	5	7	20
Katler et al.	14	7	1	5	2	2	8	4	1	13
Gill et al.	22	16	6		5	5	3	7	1	21
Knowles et al.	89	36	25	25	14	14	19	19	27	57
Ziegler et al.	90	32	24	17	8	8	38	30	38	52
Bermudez et al.	31	8	6	10	6	6	8	10	1	30
Lowenthal et al.	43	16	8	16	11	6	11	13	9	26
Kaplan et al.	84	29	36	17	7	22	10	26	14	69
Kaplan et al.	30	7	16	3	?	?	?	7	12	14
Levine et al.	42	17	14	4	5	11	6	6	11	24
Remick et al.	18	0	7	10	5	?	?	?	5	13
Raphael et al.	113	41	33	35	18	3	22	17		
Kaplan et al.	198	35	17	85	48	51	3	30	61	129
Sparano et al.	50	22	16	12	10	20	4	16		
TOTAL	851	276	222	239	145	149	140	190	187	468

Abbreviations: SNCC, small non-cleaved cell; IBS, immunoblastic; ALC, anaplastic large cell; LC, large cell.

PREFERRED APPROACH

Patients who present with non-Hodgkin lymphoma in the setting of AIDS should be assessed with a standard complete blood cell count and chemistry panel to which should be added a lymphocyte subset analysis and plasma HIV RNA. Patients who have B symptoms and a CD4 count <200 cells/cm^3 should also have blood cultures and blood isolator tubes sent. A CMV serum titer, toxoplasmosis titer, and cryptococcal antigen are also indicated along with sputum analysis for *Pneumocystis carinii* pneumonia. Imaging studies for evaluation of the extent of lymphoma should be completed in the manner employed outside the setting of HIV disease. However, we also recommend imaging of the central nervous system and sampling of the cerebrospinal fluid. Bone marrow aspiration biopsy should be obtained and special stains and cultures performed to exclude opportunistic infections.

For those patients who have a small, non-cleaved or Burkitt-like histology, paranasal involvement, bone marrow involvement, or testicular involvement, we prefer the administration of intrathecal ara-C at 50 mg weekly times 4. This should be started at the initiation of chemotherapy. Chemotherapy, in patients for whom a protocol is not available or practical, is generally standard CHOP unless patients have advanced AIDS or poor bone marrow reserve. In such cases, a modified or half-dose regimen can be justified and is often quite effective.

If patients have active involvement of the meninges, twice weekly ara-C is used until clearing of the CSF of lymphoma cells is achieved and then ara-C is administered monthly for three months. Whole brain radiation is also added.

With relapse there are no standard regimens available. We commonly use a combination of ifosfamide 2 grams/m^2 by continuous infusion and etoposide 150 mg/m^2 /d IV over 1 hour for 2 days each with MESNA support, but encourage consideration of investigational protocols for such patients.

Patients who present with primary CNS lymphoma should receive Decadron 4 mg, 4 times daily until improvement of symptoms and then the dose should be gradually reduced during a course of CNS radiation therapy. Steroids should be tapered as rapidly as tolerated to avoid the complications of prolonged immunosuppression.

In general, all patients undergoing treatment for non-Hodgkin lymphoma should have prophylaxis for PCP. We prefer trimethoprim sulfamethoxazole when tolerated. Our policy regarding antiretroviral therapy awaits the results of ongoing clinical trials; however, we generally prefer the nucleoside analogs, D4T and 3tC with indinavir or nelfinavir protease inhibitors. Alterations in drug metabolism are more likely to be encountered with the use of ritonavir or the non-nucleoside reverse transcriptase inhibitors delaverdine or nevirapine and these should therefore be used with caution. Growth factor support is often required due to poor marrow reserve in patients with AIDS.

Lymphoma (REAL) classification. Low-grade B-cell malignancies are a distinct minority among HIV-infected patients. Such tumors may occur, but with a frequency approximately comparable to the background for the population.

Like high-grade malignancies outside the setting of HIV disease, ARL often involves sites outside the confines of the lymphatic system (Table 75-4). Extranodal disease has been estimated to occur in up to 95% of patients with ARL, with extranodal tissue frequently the source of diagnosis. [4] [6] [8] [104] [134] [142] [143] [144] [145] [146] [147] [148] [149] [150] [151] [152] [153] [154] Disease that is exclusively outside the lymphatic system has been reported in up to 56% of patients. [154]

The frequency of extranodal disease emphasizes the necessity of exercising particular vigilance in evaluating these patients. Unusual symptoms must be assessed with attention to possible direct tumor involvement as the cause. AIDS-lymphoma can also mask or be confused with other opportunistic disease. In particular, the constitutional or B symptoms which are generally seen in lymphoma will suggest possible opportunistic infection. In addition, lymphadenopathy occurs frequently in the course of HIV infection and is often simply evidence of hyperproliferation without transformation. A high index of suspicion must be maintained, particularly for those patients who have coincident B symptoms or who have rapidly progressive asymmetric lymphadenopathy.

Sites of extratumor involvement are favored by particular histologic subtypes. For example, the small non-cleaved cell histology often favors bone marrow or meninges. In contrast, immunoblastic histology often favors the GI tract and brain parenchyma. The most common extranodal sites of involvement are the bone marrow in approximately 25% of patients, central nervous system (CNS) in 23%, gastrointestinal tract in 15%, and liver in 13%. [5] [6] [8] [9] [104] [105] [106] [134] [142] [143] [144] [145] [146] [147] [148] [149] [150] [151] [152] [153] [154]

Although approximately 1525% of patients will present with stage 1 or 2 disease (Table 75-4), the sites of involvement are often extranodal. If local therapies are undertaken, it is highly likely that patients will relapse at remote sites and therefore systemic therapy is generally favored regardless of the stage at presentation.

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DIAGNOSIS AND PROGNOSIS

Staging of patients with systemic AIDS-related lymphoma includes tests performed in other lymphoma populations, such as CT scans of the chest, abdomen and pelvis, bone marrow biopsy and, if bulky mediastinal or abdominal disease is present, gallium-67 scanning. In addition, the frequency of involvement of the CNS supports the use of both radiographic imaging and CSF sampling at the time of initial staging. [143]

Patients who present with constitutional or B symptoms should have a thorough microbiologic workup to address the possibility of coincident opportunistic infections. This should include isolator cultures of the blood for mycobacteria, serologic assays for cryptococcal antigen, cytomegalovirus (CMV) antigen and toxoplasmosis antibody, induced sputum for *Pneumocystis carinii,* and routine blood culture.

The prognostic indicator that has been most predictive in patients with ARL is the stage of immunodeficiency as reflected by the CD4 count at the time of diagnosis. CD4 cells of <100/cm^3 are associated with a shorter survival in several series. In addition, prior AIDS-defining diagnoses, the Karnofsky performance score, and the presence of extranodal disease have been regarded as indicators of outcome. [135] [148] [155] [156] [157] A study involving 192 patients recently completed by the AIDS Clinical Trials Group (ACTG) demonstrated that poor overall survival on multivariant analysis was associated with age >35 years, IV drug use, stages III/IV, and CD4 cells <100. The median overall survival for patients with no or one adverse factor was 46 weeks and 30% were alive at 144 weeks. Patients with three or four factors had a median overall survival of 18 weeks with no survivors at 144 weeks. [158] Although the International Prognostic Index established for other patient populations with lymphomas has not been specifically evaluated within ARL, the results of the ACTG study and other studies suggest that these parameters will likely be useful in assessing AIDS patients as well. [159]

Other features that have been associated with outcome include polyclonality, which in one study was associated with a

more positive outcome. [155] This feature was particularly favorable if tumors were EBV negative and the CD4 counts >200 ccm^3 .

The presence of PEL has generally been associated with a very poor outcome; however, the compiled number of cases with this syndrome remains relatively limited. [138]

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TREATMENT

Treatment of patients with ARL should include several components that distinguish this patient population from others. These include the use of prophylaxis for *Pneumocystis carinii* pneumonia regardless of the CD4 count at initiation of chemotherapy. This practice is generally applied because of the high potential risk during treatment-induced exacerbation of immunosuppression. The high incidence of CNS involvement in a series reported early in the epidemic [145] has led to a controversy as to whether or not CNS prophylaxis should be provided for all patients. There are no definitive data on this point, but with use of CNS prophylaxis the previously observed frequency of CSF relapse has been diminished.[156] No clinical standard can be said to have been defined, although many centers including our own administer either ara-C or methotrexate weekly for 4 weeks by intrathecal injection to at least those patients with Burkitt-like histology, bone marrow involvement, testis involvement, or perinasal sinus disease.

A concern regarding myelotoxicity has led many investigators to use growth factors prophylatically. Bone marrow dysfunction is a common feature of advanced HIV disease and those patients who present with cytopenias at diagnosis are reasonable candidates for prophylactic growth factor use. A small randomized, prospective trial in patients receiving a cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) regimen indicated that patients who received GM-CSF had statistically significantly reduced instances of fever and neutropenia and fewer days of hospitalization than patients who received chemotherapy without growth factor support.[150]

The use of antiretroviral therapy in conjunction with chemotherapy remains controversial. Earlier in the epidemic when zidovudine (AZT) was essentially the only retroviral agent available, the myelotoxic effects of this medication generally precluded its use. Most reports of treatment in patients with ARL are patients who have not received concurrent antiretroviral therapy. A study by Levine and colleagues assessed whether or not antiretroviral DDC could be given concurrently with modified m-BACOD chemotherapy[157] [160] and did not note any enhanced toxicity. In particular, neurotoxicity was not worsened and a favorable effect on HIV p24 antigen levels was noted. Ongoing studies are intended to assess whether or not highly active antiretroviral regimens including protease inhibitors can safely be given in conjunction with chemotherapy. Pharmacokinetic interactions of these medications have not yet been defined. A variable effect on the p450 pathway of drug metabolism has been documented with the use of protease inhibitors or non-nucleoside reverse transcriptase inhibitors and must be considered when they are used with chemotherapy. Although the aggressive treatment of the underlying HIV infection during treatment for lymphoma has theoretic appeal, it should be noted that HIV levels have generally remained stable during the administration of chemotherapy regimens even in the absence of specific antiretroviral drugs. In addition, the threat of HIV viremia is generally one of chronicity as opposed to the acute need for lymphoma treatment. Therefore, antitumor chemotherapy should not be compromised to accommodate antiretroviral therapy. If antiretrovirals are to be given, they should be used at doses that maximally suppress the virus since submaximal suppression may permit the emergence of resistant strains of the virus.

Selecting treatment for lymphoma is largely dictated by the patients overall clinical status. Until recently, a fair amount of skepticism has existed as to whether a curative approach should be undertaken in ARL patients since they all had what was considered a uniformly fatal underlying disease, AIDS. However, with the optimism surrounding the recent developments in anti-HIV therapy, it is now clear that a curative approach should be strongly considered. For those patients, however, who have far-advanced AIDS, are refractory to antiretroviral therapy and have poor performance status, a palliative approach may be appropriate. No specific guidelines in this situation can be provided except that careful consideration should be given to patients history of antiretroviral therapy, their history of HIV-related complications, their current CD4 count, their HIV RNA levels, and most important, their wishes regarding the level of aggressiveness of care.

For those patients in whom a curative strategy is planned, there remains ongoing controversy on how to proceed. Early in the epidemic, the high level of toxicity reported following the use of chemotherapy regimens led to exploration of the minimum necessary dose of chemotherapy needed to achieve a good clinical outcome. A regimen was originally tested that used essentially half-dose mBACOD. Encouraging results of this study [143] led to a randomized Phase III trial by the ACTG. Results of this study indicated comparable tumor outcomes for patients who were treated with a low-dose (modified m-BACOD) compared to those treated with full-dose mBACOD.[156] The rate of complete remission (50% vs. 46%), relapse after complete remission (19% vs. 23%), time to progression (22 weeks vs. 28 weeks), overall median survival (31 weeks vs. 34 weeks), death from AIDS (20 patients vs. 12 patients) and death from lymphoma (24 patients vs. 36 patients) indicated that the standard-dose and low-dose mBACOD groups were equivalent. The major difference between the two arms of the trial was that grade 4 neutropenia occurred with greater frequency in patients receiving the standard dose regimen despite the fact that all such patients received GM-CSF as a part of their treatment program. While the size of this study was relatively large (198 patients), it should be noted that it was conducted before the protease inhibitor era. The overall entry CD4 count was approximately 100 and the overall prognosis for patients was quite poor. The number of patients enrolled was not sufficient to allow subset analysis in those with more preserved immune function, but there is concern that reduced dosing may result in compromised clinical outcomes that should not be risked in such patients, since they do tolerate full-dose regimens quite well. A reasonable approach would be to use standard dose regimens such as CHOP, except for those patients in whom advanced immunosuppression raises concerns about tolerability, and with whom a modified dose intensity may be employed.

In contrast with the effort in the United States, European investigators have evaluated more chemotherapy-intensive regimens for patients with AIDS lymphoma. In a 140-patient study conducted by a French-Italian Cooperative Group using the LNH84 regimen, 65% of patients achieved a complete remission with a relatively low relapse rate of 24%.[161] In a follow-up study patients who had CD4 counts of >100 cells/mm^3 , no prior AIDS-defining illnesses and good performance status were enrolled. The patients were randomized to receive either CHOP chemotherapy or ACVB (doxorubicin 75 mg/m^2 day 1, cyclophosphamide 1.2 grams/m^2 day 1, vindesine 2 mg/m^2 day 1, bleomycin 10 mg days 15, prednisolone 60 mg days 15) with granulocyte-colony stimulating factor (G-CSF) support. More severe toxicity in the ACVB arm, but a higher response rate has been reported.[162] Further analysis of this data is required before firm conclusions can be drawn. However, the potential for dose-intensive regimens for patients in this population with good prognostic factors is suggested and needs to be further explored.

Other trials have used continuous infusion regimens for patients

as first line therapy. The CDE regimen (cyclophosphamide 800 mg/m^2 , doxorubicin 50 mg/m^2 , etoposide 240 mg/m^2 , all over four days) to which ddI was added, resulted in a complete response rate of 58% with a median response duration exceeding 18 months. [163] A follow-up study using this approach is ongoing at present.

Although controversy remains regarding the optimal strategy for treating patients who present with AIDS-related lymphoma, each of the trials indicates the potential for durable response. These data emphasize the need to approach patients with otherwise good prognostic features in a manner focused to maximize cure. However, the fraction of patients failing to respond continues to remain high and the frequency of relapse significant. Development of salvage regimens in AIDS-related lymphoma has been impeded by the generally poor ability of patients to tolerate subsequent rounds of chemotherapy. A small number of studies has indicated that regimens used in other contexts for relapsed high-grade lymphoma may have some impact on relapsed ARL. However, durability of response has been very poor.

Efforts are currently underway to explore other approaches to relapsed lymphoma that again span the spectrum in terms of treatment intensity. A Phase II trial using

the polyamine synthesis inhibitor, mitoguazone, demonstrated a response rate of 23%. [164] This medication has advantages in that it is minimally myelosuppressive and has excellent CNS penetration. However, the agent remains investigational at this time. Other efforts using dose-intensive regimens and studies with antiviral regimens are ongoing.[165] The European experience with more dose-intensive regimens when used with good supportive care has provided rationale for investigating the potential for autologous transplantation and such protocols are underway in ARL patients. This type of approach should be reserved only for clinical trial.

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PRIMARY CNS LYMPHOMA

Patients with HIV presenting with a mass lesion of the brain raise a number of diagnostic possibilities. Abscesses (toxoplasma, mycobacterial, or bacterial) or progressive multifocal leukoencephalopathy (PML) must all be considered in addition to primary CNS lymphoma. [166] [167] [168] Criteria for distinguishing between these entities remains imperfect without tissue sampling. However, for those patients in whom biopsy is either impossible or refused, certain parameters can raise or lower the likelihood of a lymphoma diagnosis. Perhaps the most important among these is the patients toxoplasmosis serologic status and whether the patient has been using trimethoprim-sulfamethoxazole as PCP prophylaxis. The latter is highly effective for toxoplasma and this coupled with a negative toxoplasma titer are strong evidence against toxoplasmosis. More commonly, patients have had prior toxoplasma exposure and have an IgG titer to this agent; differentiating an IgG versus IgM response can weigh the relative likelihood of an acute process. In addition, cultures for mycobacterium, fungus, and serum assay for cryptococcal antigen can be helpful in defining the nature of the CNS process. Some radiographic criteria have been described as being more suggestive of AIDS-lymphoma than toxoplasma brain abscess. These include large size (>2 cm), central location, and lack of multifocality. [144] [169] Lymphoma may cross the midline, but this is extremely rare in the case of infectious causes. Some recent studies have addressed whether high sensitivity assays for Epstein-Barr virus in cells within the CSF may be useful. Data in toxoplasma seronegative patients suggest that the presence of EBV detected by polymerase chain reaction (PCR) strongly correlates with the presence of EBV-related disease (lymphoma) in the CNS.[170] [171] In some centers it has now become acceptable to use positive DNA PCR with radiographic imaging (often including thallium or positron emission tomography (PET) scanning) to begin empiric therapy for lymphoma. In those patients in whom ambiguity remains, but biopsy is not possible, it is reasonable to begin an empiric trial of antibiotic therapy (sulfadiazine or clindamycin and pyrimethamine) directed against toxoplasmosis. Those patients who worsen after day five or fail to improve after 14 days have a higher likelihood of the disease not being due to toxoplasmosis. [172]

The clinical presentation of patients with primary CNS lymphoma can be extremely variable and quite subtle. This is a disease most common in late stage AIDS and in that patient population a high level of vigilance must be maintained for patients who present with neurologic or psychologic symptoms. Treatment of primary CNS ARL has generally been restricted to radiation therapy and steroids. The response rate to radiotherapy is high (6079%), but durable remissions are relatively uncommon.[144] [173] [174] [175] Patients prognoses have often been limited by other opportunistic disease. It may be possible now with improved supportive care to better define the utility of other regimens in this patient population. A recent clinical trial evaluating the combination of systemic chemotherapy with radiation therapy failed to demonstrate any benefit to offset the toxicity of the chemotherapy. However, small studies with high-dose methotrexate have been encouraging. [176] Studies of newer approaches that attempt to exploit the high frequency of Epstein-Barr virus infection with primary CNS lymphoma are ongoing. These trials represent pathophysiologically based methods that include rendering EBV-positive cells sensitive to antiherpes virus agents through chemical or genetic manipulation [177] [178] and represent an evolving trend in the care of patients with ARL. It is important that these approaches be critically evaluated and enrollment of patients in clinical trials is strongly encouraged. If no trial is available the approach must be clearly tailored to the overall status of the patient. Frank discussion of the generally poor prognosis (historically 25 months) in patients with end-stage AIDS should be a part of establishing a treatment plan. In patients with advanced AIDS, palliative measures such as steroids or pain medications alone may be appropriate.

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CONTROVERSY AND FUTURE DIRECTIONS

A number of areas within the context of ARL remain to be fully defined. The need for better understanding of the basis for lymphomagenesis is particularly acute in AIDS because of the potential for novel underlying infectious cofactors that may serve as therapeutic targets. Recent identification of KSHV and its clear relationship with a small subset of these tumors has provided a compelling example of this and represents considerable opportunity for new therapeutic interventions. The presence of multiple viruses now identified in ARL raises the question of what role there might be for specific antiviral medications or immunization. Why some patients develop malignancy while others with EBV or KSHV remain tumor-free is a puzzle rich with possibility. Unraveling such issues will potentially point to mechanisms of screening and subsequent intervention. The molecular biologic basis of cell transformation by infectious cofactors is also a highly appealing area for investigation with potential targets for the small molecule or structurally designed pharmacologic agents currently under vigorous study by the pharmaceutical industry.

More extensive clinical investigation into the immunologic, cytokine, and viral profiles of select patient populations or in animal models with EBV-like disease [179] will likely provide significant insights into the pathology of ARL. Looking for potential interactions of virus and host by intensive laboratory study may identify patients with high risk for ARL development and provide surrogate markers for early intervention.

Issues specifically related to therapy of ARL include: which patients need CNS prophylaxis and what the role is for intensive retroviral therapy during ARL chemotherapy. Therapeutic

trials are currently testing whether non-cytotoxic agents such as monoclonal antibodies directed against lymphoma cells may be combined with standard chemotherapy to enhance the overall outcome for patients.[180] Such strategies will likely enter more widespread clinical practice as monoclonal therapeutics become available for the treatment of lymphomas. Augmentation of immunologic reactivity is compelling in a disease arising from an immunologic incompetent background; however, the specific nature of the intervention is yet to be defined. Some investigators are adding interferon alpha to chemotherapy to augment the immunologic reactivity[181] and interleukin-2 at low doses is also currently being evaluated in clinical trials. [182] The use of specific cell-based approaches to therapy in this setting are based on the striking success of adoptively transferred lymphocytes in patients with EBV lymphoproliferative disease in the transplant setting. [65] [66] [67] Attempts employing similar approaches in patients with ARL are ongoing and may offer an alternative approach to those patients refractory to standard cytotoxic agents. The potential of either supplementing or replacing chemotherapy with biologically directed therapies is a focus of clinical investigation in ARL and, if fruitful, may provide benefit not just for patients with ARL, but for non-HIV-infected lymphoma patients as well. The interplay between immune function and lymphomagenesis is an issue best demonstrated by ARL. Unraveling the details of this relationship will provide principles applicable well outside the scope of the AIDS epidemic.

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Chapter 76 - Multiple Myeloma and Other Plasma Cell Disorders

Guido Tricot

MULTIPLE MYELOMA

Introduction

Multiple myeloma (MM) is the prototype of a clonal B-cell tumor of differentiated and usually slowly proliferating plasma cells, mainly contained within the bone marrow.[1] This disorder is frequently accompanied by monoclonal (M) protein production [2] [3] and either diffuse osteoporosis or lytic bone lesions. [4]

Myeloma has always been associated with destructive osteolytic bone lesions and bone pain. The bone lesions occur in several patterns. Occasionally, patients develop a single osteolytic lesion; some patients have diffuse osteopenia, which mimics the appearance of osteoporosis and is due to myeloma cells being spread diffusely throughout the axial skeleton. In most patients, there are multiple discrete lytic lesions occurring at the site of deposits of nests of myeloma cells [4] (Fig. 76-1).

Multiple myeloma accounts for approximately 1% of all malignant diseases and 10% of hematologic malignancies. In 1996, an estimated 13,000 new cases of myeloma were diagnosed. The annual incidence of myeloma is 34/100,000. The median age of patients with MM is 65 years and only 3% of patients are <40 years of age. Although myeloma can occur at a young age,[5] the diagnosis of myeloma in patients <30 years old should only be made after careful evaluation of all data. The incidence in African Americans is twice that in whites and myeloma affects males slightly more frequently than females. The incidence of myeloma is lowest in Asian populations. MM mortality rates are increasing in the elderly; for example, in the United States, mortality rates among men and women age 85 and older with MM have increased to 143% and 144%, respectively, from 1968 to 1989. These increases may be related to differences in the gene pool of successive elderly cohorts, as adversity to human survival (mainly mortality due to infections) has declined drastically. [6]

Etiology

The cause of MM is unknown. Both genetic and environmental factors have been implicated. A genetic predisposition is suggested by a significantly higher incidence of MM among African Americans. There are also well-documented reports of familial clusters of two or more first-degree relatives with MM. [2] Additional

Figure 76-1 Radiograph of the skull shows multiple osteolytic lesions.

indirect support is provided by murine myeloma models in which only specific mouse strains display markedly increased susceptibility to MM. Repeated antigenic stimulation has also been implicated in the development of MM. However, in a case control study, myeloma patients had fewer chronic inflammatory conditions than did normal controls.[7] Exposure to radiation may increase the risk of MM as evidenced by a higher than expected rate in atomic bomb survivors, radiation workers in England, and patients who received a single course of radiotherapy for ankylosing spondylitis. Although multiple chemicals and occupations have been linked to myeloma,[8] in a case control study, sheet metal work for 10 years or more was the only occupation associated with a significantly increased risk for MM. [9] These sheet metal workers were exposed to numerous potential toxins, including iron compounds, metallic dust, metal oxide fumes, chlorine dioxides, welding fumes, abrasive dust, aluminum compounds, steel dust, and aromatic hydrocarbons. Of all the common medications, only mineral oils used as laxatives or for dermatitis have been associated with an increased risk of MM, but only in women. [10]

Pathogenesis

Normal B-Cell Development

B-cell development occurs initially in the bone marrow, and subsequently in lymphoid tissues, while terminal B-cell differentiation takes place in the bone marrow. In the bone marrow, hematopoietic progenitor cells differentiate into the earliest identifiable cell type committed to the B lineage, the *pro-B cell.* This cell undergoes rearrangement of its immunoglobulin heavy chain genes and is called a *pre-B cell*; it is characterized by the presence of cytoplasmic chains. Subsequent rearrangement of the light chain enables the cell to express surface IgM and the cell becomes an *immature B lymphocyte*. These cells leave the bone marrow and, on entering the peripheral blood, start to express surface IgD and are called *virgin B cells*. They are arrested in G_0 phase of the cell cycle. These virgin B cells enter the lymphoid tissue, where they are exposed to antigen-presenting cells, become activated, and differentiate into *short-lived plasma* cells or *memory B cells*. These memory cells travel from the extrafollicular area of the lymph node to the primary follicles, where they are confronted with antigens, presented by follicular dendritic cells, resulting in the development of a secondary response. At this stage, primary follicles change into secondary follicles containing germinal centers. Through activation by an antigen, the memory B cells differentiate into *centroblasts*, resulting in Ig isotype switching and somatic mutations in the variable region of the immunoglobulin with the generation of higher affinity antibodies. Centroblasts then progress to the *centrocyte* stage and re-express surface Ig. The centrocytes with high affinity antibodies differentiate into either memory B cells or *plasmablasts,* which subsequently move to the bone marrow and terminally differentiate to *plasma cells*. These bone marrow plasma cells produce the vast majority of serum immunoglobulins and have a life span of about 1 month. [11]

Three distinct gene segments, the variable (V_H), diversity (D), and joining region (J_H) genes, encode the variable region of the heavy chain, whereas two segments, variable (V or V) and joining (J or J) region genes, encode the variable fraction of the light chain. The Ig heavy chain (IgH) locus on chromosome 14q32 contains an estimated 100 to 150 V_H genes, 30 D, and 6 J_H gene segments. Because some of the V_H genes are nearly identical, it is likely that 6070 V_H genes are available for rearrangement. These 6070 genes belong to seven families (V_H 17) whose members have >80% sequence homology. Of the 75 known V sequences, only 36 are potentially functional and of the 36 known V sequences, only 24 are functional. Rearrangement of V gene segments is dependent on the protein products of the recombinase-activating genes RAG-1 and RAG-2. Recombination of V genes starts in lymphoid progenitors within the IgH locus of either the maternal or paternal chromosome 14. If the initial V_H -D- J_H rearrangement yields a sequence that cannot be translated, then rearrangement of the IgH locus proceeds on the other allele.

The presence on the B-cell surface of a fully assembled heavy chain rearrangement begins when one of the V genes rearranges to one of the J genes. If light chain rearrangement is unsuccessful on both alleles, light chains will subsequently rearrange. [12]

The Ig heavy and light chains each contain three hypervariable (complementarity determining regions or CDR) segments, which are in direct contact with the antigen. The CDR 3 is the most variable portion of the Ig molecule, because it not only contains the somatic mutations induced by contact with the antigen as for CDR 1 and CDR 2, but it also encompasses the 3 end of V_H , all of D, and the 5 end of J_H . It is therefore an ideal marker to detect a very small population of the malignant clone within a larger population of normal cells.

The Myeloma Stem Cell

The origin of malignant plasma cells remains a mystery. Bone marrow myeloma cells have only limited self-renewal and proliferative capacity in vivo. Although the predominant cell in MM is the plasma cell, data have accumulated showing that other lymphoid cell types are involved in the malignant process. A significant number of peripheral blood lymphocytes bear the unique paraprotein idiotype found in plasma cells [13] [14] [15] [16] and have clonal immunoglobulin gene rearrangements identical to those seen in the bone marrow plasma cells.[17] [18] These data strongly suggest the existence of a precursor compartment. Cloning and sequencing of V_H genes in myeloma shows somatic mutations characteristic of an antigen-driven process with no intraclonal diversity, [19] [20] strongly implying that the malignant clone in MM evolves from a late cell in B-cell development, probably at the level of the memory B cell or plasmablast in the germinal centers of the lymph nodes. [11] [20] Additionally, bone marrow and peripheral blood of myeloma patients contain B-cell populations at different stages of differentiation that are clonally related to the malignant plasma cells, but have a preswitch isotype, consistent with the existence of a myeloma precursor compartment.[21] [22] However, other investigators have found mutations scattered throughout the variable region with no indication of antigen selection, suggesting that MM may derive from B cells that have rearranged during fetal development rather than during adult life.[23] [24]

Role of Adhesion Molecules in Myeloma

Intercellular adhesion molecules (ICAM) mediate cell-cell interactions that result in homing and activation of cells. Many of the differences in localization and growth of cancer cells versus their normal counterparts have been associated with differences in expression of specific adhesion molecules. [25] Neoplastic cells lacking adhesion molecules may proliferate autonomously, without the requirement for cellular interaction for their growth and survival. MM cells adhere to bone marrow stromal cells, although this binding cannot be totally accounted for by the known adhesion molecules. Adhesion molecules are also important for homotypic aggregation, that is, neoplastic cells adhering to each other and creating tumor masses. In myeloma, such homotypic aggregation occurs through CD56: CD56, syndecan-1:collagen and ICAM-1: leukocyte function-associated antigen 1 (LFA-1) interactions. Adhesion molecules can also regulate cytokine secretion; binding of MM cells to bone marrow stromal cells via very late antigen (VLA)-4 and vascular cellular adhesion molecule (VCAM)-1 induces interleukin-6 (IL-6) transcription and secretion. CD40 ligand regulates autocrine

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IL-6 secretion in myeloma cells. Myeloma cells localize to the bone marrow via binding of VLA-4 (CD49d) to VCAM-1 or fibronectin and LFA-1 to ICAM-1 (CD54). VLA-5 (CD49e) has also been associated with adherence of MM cells to the bone marrow. Acquisition of CD11b and LFA-1 combined with loss of CD56, VLA-5, and syndecan-1 expression is associated with egress of tumor cells from the bone marrow and the development of plasma cell leukemia. Myeloma cells, seeding extramedullary, re-express syndecan-1 and may acquire at the metastatic sites previously expressed adhesion molecules such as CD56 and VLA-5.

Cytokines and Myeloma

Interleukin-6 is involved in the proliferation of normal plasmablastic cells and terminal differentiation of these plasmablasts to nondividing plasma cells. Terminally differentiated plasma cells produce large amounts of immunoglobulins (>600 pg/cell/24 hours). In MM, IL-6 is a proliferation factor for immature myeloma cells, but in contrast to normal plasma cells, IL-6 is not a differentiation factor for immature myeloma cells. Myeloma cells lack terminal differentiation. They produce much lower quantities of immunoglobulins (<10 pg/cell/24 hours) and are still able to divide, but at a low rate. [26] Although IL-6 is produced by myeloma cells and an autocrine IL-6 loop is functioning in myeloma,[27] almost all of the IL-6 is produced by the bone marrow environment[26] after adhesion of myeloma cells to stroma and bone cells. Moreover, bone marrow stromal cells from MM patients produce higher amounts of IL-6 than do stromal cells from healthy individuals after triggering with myeloma cells.[28] IL-6 promotes MM cell growth via phosphorylation of the retinoblastoma protein (pRB). [29] Dephosphorylated (activated) pRB blocks transition from G1 to S phase of the cell cycle, whereas phosphorylated (inactivated) pRB releases this growth arrest. Dexamethasone treatment induces apoptosis of myeloma cells, resulting in a decrease of the myeloma protein of >50% in half the patients. This dexamethasone-induced apoptosis is prevented by IL-6. [30] Finally, high levels of IL-6 may contribute to the development of renal insufficiency in MM.[31] In addition to IL-6, oncostatin M, leukemia inhibitory factor, but not IL-11, all using the common signal transducer gp130,[32] as well as IL-10[33] and insulin-like growth factor 1[34] stimulate growth of myeloma cells.

Recently, the excessive production of IL-6 by marrow stromal cells has been attributed to an infection by the Kaposis sarcoma-associated virus (KSHV) of a subset of macrophages, namely dendritic cells in the marrow of patients with multiple myeloma.[35] A homolog to human IL-6 has been identified in the KSHV genome. The viral IL-6 is able to support the growth of plasma cells.[35] Apparently the malignant plasma cells in MM are not infected with KSHV. Rettig et al. have concluded that the viral IL-6 may contribute to the mechanism by which the stromal cells infected with KSHV promote myeloma cell growth.[35] This study provides a unique model of malignancy in which a virus can potentially support the growth of a neoplasm by infecting a nonmalignant cell without directly infecting the malignant clone.

Oncogenes/Suppressor Genes in Myeloma

Dysregulation of oncogenes and suppressor genes, controlling cellular proliferation, growth arrest, and apoptosis contribute to the pathogenesis of most malignancies, including MM. The evolution of MM is a multistep process with alterations in the IL-6/Ras pathway, dysregulation of bcl-2 and myc proteins, and loss of tumor suppressor gene product function such as pRB and p53.[36]

RB Protein

In myeloma cells, pRB seems to be constitutively phosphorylated and IL-6 further shifts pRB from its dephosphorylated to its plosphorylated form. [29] Growth stimulatory signals, such as growth factors, induce the accumulation of cyclins, active in the G1 phase, which in turn regulate the catalytic activity of the cyclin-dependent kinases (CDK). Two classes of CDK inhibitors, the inks (p15,16) and cips/kips (p21, p27, p57) block CDK function. Both p15 and p16 are encoded by genes on chromosome 9p. Deletions of p15/p16 are highly specific for lymphoid malignancies, more specifically of B-cell origin, except for MM. However, hypermethylation causing inactivation of genes is observed for p16 in 75% and for p15 in 67% of MM patients. [37] This high frequency makes it one of the most common genetic abnormalities in MM. Concomitant hypermethylation of both genes occurred in two-thirds of the patients and may be associated with plasmablastic transformation or extramedullary plasmacytomas.[37] Although frequent deletions of the RB gene (50%) have been identified in MM, the majority of these deletions are hemizygous and are not associated with lack of pRB protein expression. [38] [39]

p53

Although the p53 tumor suppressor gene is the most commonly mutated gene in human malignancies, its mutations are uncommon in MM (10%) and are mainly associated with the terminal stage of the disease. In contrast, p53 mutations are very common in myeloma cell lines (80%). The p53 gene participates actively in DNA replication and repair and is considered the guardian of the genome. Because cytogenetics in MM are almost always very complex, it is perplexing that the incidence of p53 mutations is so low in MM. However, p53 function may be altered by other mechanisms, such as high expression of mdm-2 as seen in myeloma cell lines and cells of patients with plasma cell leukemia (K. Anderson, personal communication), or through PAX-mediated transcriptional repression of p53. [40] The PAX-5 gene, encoding for the B-cell specific activating protein (BSAP), necessary for CD19 expression, which is lost on MM cells, is altered in myeloma and results in loss of CD19 expression on MM cells.[41]

P21/Ras

The p21/Ras signaling transduction pathway integrates a variety of growth signals that are critical for cellular proliferation and differentiation. The p21 is a

GTP-binding protein that is activated when GTP is bound and inactivated when GDP is bound. IL-6 is a potent growth and survival factor for myeloma cells that uses gp130 as a signaling transducer molecule. Activation of gp130 not only leads to activation of the Janus kinases, but also to activation of the Ras pathway. Point mutations in Ras, most commonly at codons 12, 13, and 61, result in constitutive activation of Ras, thereby making myeloma cells independent of exogenous IL-6. It has been shown that transduction of an IL-6-dependent cell line (ANBL-6) with mutant Ras makes this cell line IL-6 independent. [42] Ras mutations are very common even at diagnosis of the disease, occurring in 40% of patients, and N-Ras is the most frequently mutated. [43]

Bcl-2 Family

The bcl-2 proto-oncogene was initially identified through the t(14;18) present in 85% of low-grade follicular non-Hodgkins lymphomas. Bcl-2 prevents apoptosis induced by a variety of conditions, such as treatment with glucocorticosteroids, alkylating agents, and radiotherapy as well as by cytokine deprivation, without obviously affecting the rate of cellular proliferation. The bcl-2 family contains not only anti-apoptotic members such as bcl-2 and bcl-x_L but also pro-apoptotic members such as bax,

bcl-x_S and bad. The ratio between anti-apoptotic and pro-apoptotic members determines the ultimate fate of a cell. In myeloma the frequency of t(14;18) involving the bcl-2 gene is quite low (2%), yet almost all myeloma cell lines and fresh myeloma cells have elevated levels of bcl-2, possibly due to hypomethylation of the bcl-2 gene. Bcl-2 is probably a critical survival factor for myeloma cells in the early stage of the disease when the myeloma cells are hypoproliferative, whereas it probably loses its importance in the aggressive proliferative phase of the disease. Bcl-2 levels have been correlated with response to interferon-, but in general, no correlation has yet been established between bcl-2 levels and survival or response to chemotherapy. [44]

C-myc

C-myc is expressed in proliferating cells and is tightly associated with cell cycle progression. However, in the absence of growth stimuli, c-myc expression may lead to cell death. Although rare, c-myc rearrangements have been found in some myeloma patients. However, c-myc deregulation is nevertheless observed in the majority of myeloma patients. Both transcriptional and translational regulatory mechanisms account for this deregulated c-myc expression in myeloma. Approximately 70% of myeloma patients transcribe c-myc mRNA from an unusual promotor, P_0 , instead of the common promoters P_1 and P_2 . Translational up-regulation of c-myc expression is probably the most common mechanism responsible for elevated levels of c-myc in myeloma. This translational up-regulation might be due to a specific mutation in the first exon. [45]

Bone Disease in Myeloma

Skeletal-related abnormalities are the most common cause of morbidity in patients with MM. Approximately 80% develop substantial skeletal dysfunction during the course of their disease. [2] Problems related to skeletal dysfunction include diffuse osteopenia, focal lytic bone lesions, pathologic fractures, incapacitating bone pain, hypercalcemia, loss of height, and restrictive pulmonary disease. Quantitative studies of bone histology have shown that MM is characterized by excessive resorption of bone that occurs only in the vicinity of myeloma cells. At the same time, bone formation is inhibited. [46] Myeloma cells stimulate all phases of bone resorption, specifically, the recruitment, differentiation, and bone resorbing activity of osteoclasts. This excessive resorption of bone can be detected histologically months or even years before bone lesions appear radiographically. [46] IL-1, tumor necrosis factor (TNF-) and IL-6 are produced in large quantities by the microenvironment as a direct consequence of myeloma cells adhering to stromal cells. IL-6, an essential growth factor for myeloma cells, is produced by bone cells. Thus, once myeloma cells invade the bone marrow space a vicious circle starts, whereby myeloma cells trigger the release of osteoclast activating factors, mainly IL-1, that stimulate the osteoclasts. These activated osteoclasts resorb bone and this results in the release of IL-6, which in turn stimulates immature myeloma cells and serves as a survival factor for the more mature myeloma cells. This results in more myeloma cells and more osteoclastic activity (Fig. 76-2).

Clinical Manifestations

The clinical manifestations of MM (Table 76-1) are the direct consequence of marrow infiltration by plasma cells, the production of monoclonal protein in blood or urine, and immune deficiency (Fig. 76-3).

Bone Pain

Bone pain, typically in the back or chest and less often in the extremities, is present at diagnosis in more than two-thirds of patients. The pain is usually aggravated by movement. The patients height may be reduced by several inches because of collapse of one or more vertebral bodies. [2] Sudden onset of back pain in an individual over the age of 40, especially if the complaint is new for the patient, is sufficient to suspect the diagnosis of myeloma and should lead to proper work-up for this disease.

Renal Insufficiency

The two major causes of renal insufficiency in MM are myeloma kidney and hypercalcemia. Myeloma kidney is characterized by

Figure 76-2 Myeloma cells invading the bone marrow adhere to stromal cells and induce the release of osteoclast activity factors. This will result in bone reabsorption and release from the bone matrix of multiple cytokines that will directly or indirectly stimulate the growth of myeloma cells. The net result is more myeloma cells and more bone destruction. FGF, fibroblast growth factor; IGF, insulin-like growth factor; TGF-, transforming growth factor-beta.

TABLE 76-1 -- Clinical Features and Therapy of Multiple Myeloma

Anemia	Due to high levels of IL-6, bone marrow replacement and partially dilutional, if M protein is high Therapy: can often be corrected with erythropoietin
Bone lesions	Mainly due to IL-1, also IL-6 and TNF- Therapy: preventable with bisphosphonates
Hypercalcemia	Due to bone destruction, often causes renal insufficiency Therapy: urgenthydration, bisphosphonates, calcitonin
Infection	T-cell deficiency: Fas ligand on plasma cells (?) B-cell deficiency: TGF- Therapy: prophylactic antibiotics with initial cytotoxic treatment
Renal failure	Dehydration due to hypercalcemia Myeloma kidney: precipitation of light chains in tubules (light chains) Light chain deposition disease (light chain) IL-6 Amyloidosis
Hyperviscosity	Mainly in IgA myeloma and IgG_3 Therapy: plasmapheresis

Amyloidosis	Insoluble M protein: cardiac, renal, and neurologic problems Therapy: eradication of malignant clone
Hypoalbuminemia	IL-6 secretion, dilutional
Neurologic symptoms	Central Compression of tumor on cord Compression of bone fragment on cord Peripheral Amyloidosis

the presence of large, waxy, laminated casts in the distal and collecting tubules. These casts are composed mainly of precipitated monoclonal light chains and are surrounded by multinucleated syncytial epithelial cells (giant cells). The casts result in dilation and atrophy of the renal tubules and eventually the entire nephron becomes nonfunctional and interstitial fibrosis may occur. [47] The extent of cast formation correlates directly with the amount of free urinary light chains, but the actual mechanism of nephrotoxicity from Bence Jones proteinuria is unknown, although dehydration, associated with hypercalcemia, and radiographic contrast medium studies may precipitate myeloma kidney. Myeloma kidney is more often encountered with light chains.

The second most common course of renal insufficiency is hypercalcemia, resulting in hypercalciuria and osmotic diuresis, leading to volume depletion and prerenal kidney failure. In addition, hypercalcemia may cause calcium deposits, leading to interstitial nephritis. Hyperuricemia, as well as nonsteroidal anti-inflammatory agents, reducing the blood flow to the glomeruli may also promote the development of renal failure. Patients with myeloma are very sensitive to nephrotoxic medications such as aminoglycosides, cisplatin, amphotericin B, and cyclosporine A. Immunocyte-derived (AL) amyloidosis in myeloma patients is associated with light chain proteinuria and usually presents as nephrotic syndrome, but can lead over time to renal failure (Chap. 77). Probably underestimated as a cause of renal failure is the immunoglobulin light chain deposition disease, commonly associated with light chain myeloma proteins, in which monoclonal light chains are deposited in renal glomeruli. The histopathologic picture resembles nodular glomerulosclerosis, but electron microscopy reveals granular, electron-dense deposits representing light chains. Myeloma involvement of the kidneys is uncommon, but should be suspected in patients who have renal enlargement not due to amyloidosis. Finally, IL-6 may also contribute to renal failure. [31]

Hypercalcemia

Some myeloma patients have paraproteins that avidly bind calcium, resulting in spurious hypercalcemia. The best solution to deal with these problems is to measure an ionized serum calcium

Figure 76-3 Major clinical and laboratory features of multiple myeloma.

level. Hypercalcemia occurs in 3040% of patients with MM and is usually associated with a large disease burden. Hypercalcemia is the presenting finding in 1530% of patients, who may develop lethargy, polyuria, polydipsia, constipation, nausea, and vomiting. [48] Levels of total serum calcium should be adjusted for serum protein concentrations, especially albumin. Pronounced hypoalbuminemia, caused by an increased level of Il-6, poor food intake, or urinary loss in case of concomitant amyloidosis, can mask frank hypercalcemia.

Neurologic Symptoms

Neurologic symptoms are usually the result of a compression by masses of myeloma cells or bone fragments on the spinal cord or on a nerve. The pain is usually in the thoracic or lumbosacral area. Compression of the spinal cord needs to be considered an oncologic emergency, requiring prompt intervention. It is best diagnosed by magnetic resonance imaging (MRI). In addition to back pain with radicular features, weakness or paralysis of the lower extremities and bowel or bladder incontinence may occur. Peripheral neuropathy is uncommon in MM and is almost always associated with amyloidosis. It is also seen in osteosclerotic myeloma and is sometimes part of the POEMS syndromes (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, and skin changes). Intracranial plasmacytomas usually represent extensions of myelomatous lesions of the skull and may lead to cranial nerve deficits or more infrequently leptomeningeal infiltration (usually only seen in the terminal phases of the disease).

Hyperviscosity

In contrast to Waldenström macroglobulinemia, hyperviscosity is rare in MM, occurring in <10% of patients. Because of a greater tendency for IgA to form polymers, patients with IgA myeloma more often have features of hyperviscosity, resulting in circulatory problems and leading to cerebral, pulmonary, and renal manifestations. Hyperviscosity is often associated with bleeding. Among patients with IgG myeloma, those with IgG 3 subclass are most likely to develop hyperviscosity. The diagnosis can be made by measuring serum viscosity and by fundoscopic examination showing a slow blood flow in often distorted blood vessels. Hyperviscosity can give the clinical and radiologic picture of pulmonary edema, but will only worsen if treated with diuretics. Plasmapheresis is the appropriate treatment for this condition.

Amyloidosis

Amyloidosis is the clinical syndrome that results from the extramedullary deposition of insoluble fibrillar protein. A diagnosis of MM can be made in 20% of cases with AL amyloidosis[49] and about 3% of patients with newly diagnosed MM have overt amyloidosis, [50] but the frequency of asymptomatic amyloidosis in myeloma, as diagnosed with subcutaneous fat aspiration and rectal and bone marrow biopsies is much higher (35%). The most common clinical manifestations are carpal tunnel syndrome or edema due to nephrotic syndrome; less common manifestations are cardiomyopathy, macroglossia, or extensive bruising around the eyelids. The presence of amyloidosis in patients with MM adds to their morbidity and has an adverse effect on survival. [50] When treated with conventional chemotherapy the median survival of patients with both myeloma and amyloid is 12 months.[50]

Infections

Patients with MM have an increased susceptibility to develop infections, because of the associated hypogammaglobulinemia. Myeloma patients are not able to mount a vigorous primary immune response and also have an impaired secondary antibody response to antigens. T-cell, NK-cell, and monocyte defects may accelerate and contribute to the humoral deficiency. The additional immunosuppressive effect of chemotherapy, especially with corticosteroids, further increases infection risk. The average MM patient will suffer from 0.5 to 1.5 infections per year, with the highest frequency during the first 2 months of treatment. Polysaccharide-encapsulated organisms, especially *Streptococcus pneumoniae* and *Hemophilus influenzae*, were initially identified as the major causes of infection. However, it is now clear that enteric gram-negative bacilli are the most common isolates, accounting for about 60%, whereas encapsulated organisms represent <25% of isolates. Oral thrush is the only fungal infection of significance. Other organisms, such as anaerobes, *Mycobacterium tuberculosis*, herpes simplex, and varicella zoster, are uncommon. Early diagnosis and prompt initiation of broad-spectrum antibiotic therapy are critical in patients with MM. Third-generation cephalosporins or extended-spectrum penicillins are used most frequently. Infection prophylaxis with trimethoprim-sulfamethoxazole is effective in decreasing the incidence of infectious complications during the first few months of initial standard-dose chemotherapy. [51] Vaccination against influenza in patients with MM is safe and may have significant benefits. [52]

Extramedullary Disease

Extramedullary plasmacytomas have been found in lymph nodes, skin, liver, spleen, and occasionally kidneys and meninges. This is usually associated with high serum lactic dehydrogenase (LDH) levels and plasmablastic morphology. These patients usually have a poor outcome even with more aggressive treatment approaches.

Bleeding and Anemia

Bleeding problems occur in 15% of IgG and 30% of IgA myelomas and may be due to platelet dysfunction or acquired coagulopathy. Thrombocytopenia, even with extensive bone marrow involvement, is rare in the early phases of the disease. The preserved platelet count might be related to increased IL-6 levels, which are capable of promoting megakaryocyte maturation. Anemia is the consequence of increased IL-6 production by the microenvironment and may be spuriously aggravated by expansion of plasma volume due to large amounts of circulating immunoglobulins. Serum erythroprotein levels are low relative to the degree of anemia, a finding that is probably due to the abundant production of cytokines such as IL-1 and TNF-.

Laboratory Evaluation

The *bone marrow* is involved with an excess of plasma cells (>5%) in approximately 90% of cases (Fig. 76-4); the mean percentage of plasma cells at diagnosis is 30&%.[1] A nodular infiltration is found in <5% of cases.

A *monoclonal protein* (M protein) is evidenced on serum protein electrophoresis as a spike or localized band in the or globulin region in 80% of patients; approximately 10% have hypogammaglobulinemia and 10% have a normal-appearing pattern. The more sensitive immunoelectrophoresis and immunofixation will reveal an M protein in >90% of patients in the serum and 80% in the urine. With these sensitive techniques, an M protein in serum or urine is found in 99% of cases. [2] About 55% of patients have an IgG isotype, 25% have IgA, 1% have IgD, 1% have IgM, and about 20% have only light chain secretion with a / ratio of 2:1. [3] In heavy chain disease, a localized band is often not seen and either immunoelectrophoresis or immunofixation is required for identification of an M protein.

Figure 76-4 The bone marrow is invaded by plasma cells, which are more immature than normal plasma cells as evidenced by a more delicate chromatin structure and the presence of a nucleolus in some of the cells.

Other laboratory manifestations of MM include anemia, present in about two-thirds of patients, increased erythrocyte sedimentation rate in patients with a significant M protein in the serum, elevated creatinine in approximately one-half of cases, and decreased albumin levels, which are associated with increased IL-6 activity.

Diagnosis

Minimal criteria for the diagnosis of overt myeloma include the presence of at least 10% abnormal plasma cells in the bone marrow or histologic proof of a plasmacytoma, the usual clinical features of MM, as outlined above, and at least one of the following abnormalities: serum M protein (usually >3 g/dl), M protein in the urine (usually >1 g/dl), or osteolytic lesions [53] (Table 76-2).

If the diagnosis of MM is suspected, the physician should obtain, in addition to a complete history and physical examination, the following tests: complete blood count with differential, electrolytes, blood urea nitrogen, creatine, calcium, phosphorus, uric acid, LDH, alkaline phosphatase, serum protein electrophoresis, quantitative immunoglobulins, serum immunoelectrophoresis, $_2$ -microglobulin ($_2$ M), C-reactive protein

TABLE 76-2 -- Criteria for the Diagnosis of Multiple Myeloma[a]

Major Criteria
Plasmacytoma on tissue biopsy
Marrow plasmacytosis 30%
Monoclonal protein
IgG > 3.5 g/dl
IgA > 2 g/dl
Bence Jones 1 g/24 hr
Minor Criteria
Marrow plasmacytosis 1029%
Monoclonal protein present but less than the levels defined above
Lytic bone lesions
Decrease in uninvolved immunoglobulins
IgM < 50 mg/dl
IgA < 100 mg/dl
IgG < 600 mg/dl

[a]The diagnosis is confirmed with at least one major and one minor criterium or at least three minor criteria.

(CRP), radiographic survey of the bones, and bone marrow aspirate and biopsy, preferentially with plasma cell labeling index and cytogenetics. In case of a solitary plasmacytoma or severe back pain, an MRI of the head, spine, and pelvis is indicated to evaluate the extent of the disease and the presence of spinal cord compression.

Differential Diagnosis

In patients with monoclonal gammopathies associated with borderline bone marrow plasmacytosis, connective tissue diseases, chronic infections, carcinoma, and lymphoma should be excluded. In most patients, however, the diagnosis of MM is readily established. It is important to distinguish myeloma from monoclonal gammopathy of undermined significance (MGUS), solitary plasmacytoma of the bone, and amyloidosis.

MGUS

Monoclonal gammopathy of undetermined significance is characterized by the presence of a serum M protein of <3 g/dl, fewer than 10% plasma cells in the bone marrow, no or only small amounts of Bence Jones protein in the urine, absence of lytic lesions, anemia, hypercalcemia and renal insufficiency, and most importantly stability of the M protein.[54] MGUS is found in approximately 3% of individuals over the age of 70. Of all patients with monoclonal gammopathies, 62% have MGUS. [54] The majority of patients with MGUS die of unrelated causes (52%), whereas 22% have either no substantial increase in M protein or an increase in M protein without any symptoms; 11% progress to MM.[54] The median interval from recognition of the M protein to the diagnosis of MM is 10 years (range, 229). Currently, no single test can distinguish MGUS from MM. In MGUS the plasma cell labeling index (PCLI) is low, $_2$ M and CRP levels are normal, and CD56 may not be expressed on the plasma cells.[55] It is important to know that 50% of patients with MGUS have an abnormal DNA content either by flow cytometric analysis [55] or fluorescence in situ hybridization.[56] The clinical and laboratory features of patients with MGUS with chromosomal abnormalities are indistinguishable from those without.

Solitary Plasmacytoma of the Bone

Solitary plasmacytoma of bone is uncommon and represents 35% of patients with plasma cell dyscrasias. The most common symptom at diagnosis is pain and the median age of these patients is a decade younger than that in MM. [54]

The diagnosis is based on histologic evidence of a tumor consisting of monoclonal plasma cells with no other lesions on skeletal survey or MRI, a normal bone marrow and absence of an M protein in blood and urine; or, if present, therapy for the solitary lesion should result in disappearance of the M protein. The uninvolved immunoglobulins are usually normal and there is no evidence of anemia or hypercalcemia. Overt MM develops in approximately 50% of these patients with evidence of progressive disease within 3 years in two-thirds of the patients. The 5- and 10-year survival rates are 74% and 45%, respectively. [54] Tumoricidal radiotherapy with 4,0005,000 cGy has been considered the treatment of choice. However, if such a dose is given to the spine, total body irradiation as part of a more intensive treatment approach in case of relapse will not be possible anymore.

Amyloidosis

Immunocyte-derived amyloidosis can occur in the context of MM, but also without myeloma. Primary amyloidosis and amyloidosis associated with myeloma have the same underlying pathologic process, whereby excessive amounts of the Ig produced

by a single clone of B cells are deposited in tissues in a manner and quality sufficient to compromise organ function. In primary amyloidosis, there is a minimal or no clinically detectable increase in plasma cells and no other manifestations of myeloma such as anemia, hypercalcemia, and bone lesions. The diagnosis of amyloidosis is made by staining tissue with Congo red with its classical apple-green birefringence when viewed under polarized light. [47]

Prognostic Factors

Survival of patients with MM is highly variable and ranges from a few months to many years. Without therapeutic intervention the median survival is approximately 7 months.[57] The median survival is 2.53 years with conventional chemotherapy. Only 3.5% of patients survive more than 10 years. [58]

Durie-Salmon Clinical Staging

This staging system[59] (Table 76-3) is based on a combination of factors that correlate with myeloma mass and renal function, whereby patients with a low tumor load and creatinine <2 mg/dl have a median survival of approximately 5 years, whereas patients with a high tumor load and renal sufficiency have a median survival of 15 months. This system has permitted better interpretation of therapeutic trials, based on the composition of the patient population, but it has significant shortcomings, especially in categorizing bone lesions. Moreover, it does not consider important variables such as the proliferative rate of the disease and the quality of response to therapy.

$_2$ M

The $_2$ M level is one of the most significant prognostic factors in myeloma. It reflects both tumor load and renal function and is therefore highly correlated with the Durie-Salmon staging system.[60] However $_2$ M alone predicts survival, regardless of renal function and stage, [61] suggesting that $_2$ M is a reflection of more than these two parameters. It also reflects to some degree disease activity and drug sensitivity. The best cutoff level before any treatment for $_2$ M is 6 mg/L.[60] [61] It is important to remember that $_2$ M levels increase on interferon- therapy.

CRP

Serum CRP[62] concentrations reflect IL-6 activity, a major growth and survival factor for myeloma cells. CRP and IL-6 levels show a linear association and their predictive value largely overlaps. [63]

TABLE 76-3 -- Durie-Salmon Staging System

Stage I	Low tumor mass (<0.6 × 10^{12} /m^2) All of the following Hemoglobin > 10 g/dl IgG < 5 g/dl; IgA < 3 g/dl; Bence Jones < 4 g/24 hr Normal calcium level No or only one lytic bone lesion
Stage II	Intermediate tumor mass (0.61.2 × 10^{12} /m^2) Not fitting stage I or III
Stage III	High tumor mass (>1.2 × 10^{12} /m^2)
	Any of the following Hemoglobin < 8.5 g/dl IgG > 7 g/dl; IgA > 5 g/dl; Bence Jones > 12 g/24 hr Calcium level > 12 mg/dl (adjusted for albumin) Multiple lytic lesions
	A. BUN 3 mg/dl, creatinine 2 mg/dl
	B. BUN 3 mg/dl, creatinine 2 mg/dl

The predictive value of CRP is independent of $_2$ M. Therefore, a combination of these two parameters allows stratification into three groups. A low-risk group with both CRP and $_2$ M < 6 mg/L, an intermediate-risk group with CRP or $_2$ M < 6 mg/L, and a high-risk group with CRP and $_2$ M 6 mg/L. With conventional chemotherapy the median survival in these three groups is 54, 27, and 6 months, respectively. [62] Other acute phase proteins, such as $_1$ -antitrypsin, carry a similar prognostic value as CRP.[64] CRP levels can obviously be a reflection of other ongoing problems than myeloma activity. Almost any infection or inflammation will significantly elevate CRP levels. The prognostic value of CRP is only relevant when no other causes of CRP increase are present. Increased soluble IL-6 receptor levels have been correlated with decreased survival.[65]

PCLI

The PCLI reflects the proliferative activity of plasma cells. Early in the disease most myeloma patients have a hypoproliferative disease, but during disease progression the labeling index may increase substantially. [66] For patients treated with conventional chemotherapy, the PCLI is probably the most important prognostic factor.[67] [68] When PCLI is taken into consideration, CRP values do not provide additional prognostic information. [65] [67] The median survival of patients with low PCLI and $_2$ M was 71 months, compared with only 17 months when both of these parameters were elevated.[65] The median survival of MM patients with a PCLI < 3% was 56 months, compared with 19 months for those with a PCLI 3&%.[68]

Cytogenetics

Cytogenetics have major prognostic importance in acute leukemias. Unfortunately, cytogenetic information is limited in MM because it is a malignancy composed

mainly of well-differentiated B cells with low proliferative activity. Abnormal karyotypes are observed in only 3050% of cases. Typically, previously treated and relapsing patients have a higher frequency of chromosomal abnormalities (3563%) than newly diagnosed patients (2030%) because of the higher growth fraction in advanced disease.[66] It is therefore not surprising that in cytogenetic studies combining newly diagnosed and previously treated patients, abnormal karyotypes have been associated with an inferior outcome.

In contrast to cytogenetics, flow cytometry-derived aneuploidy data and analysis using fluorescence in situ hybridization have found cytogenetic abnormalities in 8090% of patients, irrespective of their disease status. Therefore, the majority of the normal karyotypes in MM are derived from the normal hematopoietic cells and not from the myeloma clone. Karyotypic abnormalities are very complex involving more than three chromosomes in 80% of patients.[49] The most common abnormalities are translocation (51%), followed by multiple trisomies (45%). In patients receiving autotransplants, a particularly poor outcome is observed in those with translocations or abnormalities involving 11q or partial or complete deletion of chromosome 13 (unfavorable karyotype) (Fig. 76-5). On multivariate analysis, the absence of an unfavorable karyotype was the single most important favorable variable for both event-free and overall survival. [49] In contrast, patients with abnormal cytogenetics, excluding the unfavorable karyotypes, had event-free and overall survivals comparable to those with normal chromosomes. It is unclear whether the above-mentioned unfavorable karyotypes would also confer a significantly inferior outcome for patients treated with conventional chemotherapy. Hypodiploidy has been associated with drug resistance[69] and newly diagnosed patients with abnormal cytogenetics have an inferior outcome with standard therapy. [70]

Figure 76-5 Impact of unfavorable karyotypes on outcome. Patients with unfavorable karyotypes have a poor outcome after transplantation when compared with other patients. CR, complete response.

LDH

About 10% of untreated myeloma patients have elevated LDH levels. It is more frequent in patients with a high tumor mass, extramedullary disease, and hypodiploidy. A high LDH level is associated with a short median survival of only 9 months and a low response rate (20%) to standard chemotherapy. [71]

Plasmablastic Myeloma

This MM subtype has 2% plasmablastic myeloma cells in the bone marrow and occurs in approximately 8% of patients with newly diagnosed disease. It is associated with a significantly shorter event-free (1.1 vs. 2.7 years) and overall survival (23.3 vs. 44.6 months). [72] Multivariate analysis has shown that the plasmablastic subtype is a prognostic factor for survival independent of PCLI, CRP, $_2$ M, and creatinine.

Peripheral Blood Monoclonal Plasma Cells

Eighty percent of newly diagnosed MM patients have monoclonal plasma cells in the peripheral blood; 57% of those patients had 4% plasma cells in the peripheral blood. Patients with 4% plasma cells in the peripheral blood have a median survival of 2.4 years versus 4.4 years for those with < 4&%. [73] Patients with a high percentage of plasma cells in the peripheral blood are less likely to have lytic bone disease.

Variant Forms of MM

Smoldering Myeloma

Patients with smoldering myeloma[2] have a serum myeloma protein level >3 g/dl but <4.5 g/dl and >10% atypical plasma cells in the bone marrow, but do not have anemia, renal insufficiency, hypercalcemia, or multiple skeletal lesions. They may have small amounts of M protein in the urine, and the levels of the uninvolved immunoglobulins in the serum are decreased. The PCLI is low.[74] Because these patients are asymptomatic, they should not be treated until there is clear evidence of disease progression or symptomatic disease. The median time to progression in patients with smoldering myeloma is 26 months. Patients with either a lytic lesion of the bone or both serum monoclonal protein >3g/dl and Bence Jones proteinuria have the shortest time to progression (median 10 months) compared to 61 months for those without any of these poor prognostic factors.[73] Despite the markedly different times to disease progression, the response rates and survival after standard chemotherapy are similar.[75]

Plasma Cell Leukemia

Patients with plasma cell leukemia have an absolute plasma cell count in the peripheral blood of 2.0 × 10 ^{9} /L. A distinction needs to be made between *primary,* when diagnosed in the leukemic phase, and *secondary* plasma cell leukemia, when there is leukemic transformation of previously recognized multiple myeloma. Approximately 5% of newly diagnosed MM patients present with plasma cell leukemia.[76] These patients almost always have a high tumor mass and more frequently have thrombocytopenia, hypodiploid plasma cells, high LDH levels, and extramedullary involvement. [76] Primary plasma cell leukemia requires prompt and intensive chemotherapy. Patients with secondary plasma cell leukemia more frequently have IgA myeloma and more lytic bone lesions. These patients are usually refractory to chemotherapy and have a median survival of <2 months.[77]

Osteosclerotic Myeloma

The major clinical feature in osteosclerotic myeloma (POEMS syndrome) is a chronic inflammatory demyelinating polyneuropathy causing predominantly motor disability. It is assumed that the plasma cells secrete a monoclonal immunoglobulin that is toxic to the peripheral nerves and is responsible for the endocrine abnormalities. The bone marrow of these patients usually contains <5% plasma, and hypercalcemia and renal insufficiency are rare. The diagnosis is confirmed by identification of monoclonal plasma cells in the biopsy of an osteosclerotic lesion. When compared to myeloma patients, patients with POEMS have higher serum levels of IL-1, TNF-, and IL-6, but lower levels of TGF-, insufficient to counteract the noxious effects of the proinflammatory cytokines. [78] Other clinical manifestations of this disease are enlargement of lymph nodes, liver, and spleen; low concentration of the monoclonal component; hypertrichosis; and hyperpigmentation. [79] Most M components have light chains. Pathologic findings in the enlarged lymph

nodes and spleen are compatible with Castlemans disease. Solitary bone lesions must be aggressively treated with surgery or radiotherapy. [79] Polychemotherapy should not include neurotoxic agents.

Extramedullary Plasmacytoma

Extramedullary plasmacytomas[2] occur most frequently in the upper respiratory tract, including the nasal cavity and sinuses, nasopharynx, and larynx. They also occur in the gastrointestinal tract and various other organs. The diagnosis is based on the finding of a plasma cell tumor at an extramedullary site and the absence of MM at bone marrow examination, radiography, and appropriate studies of blood and urine. Treatment consists of tumoricidal radiation. Prognosis is favorable and evolution to MM is rare. In stage I disease, therapy may be curative.

Therapy

The approach to therapy for patients with MM is outlined in the accompanying box.

Cytotoxic Therapy

Patients with asymptomatic myeloma usually have low tumor burden and slow disease progression. Their marrows usually contain <30% myeloma cells, serum M protein <5 g/dl, or Bence-Jones proteinuria <1 g/24 hours. They have no or very few bone lesions and no anemia, hypercalcemia, renal insufficiency, or recurrent infections. The PCLI and $_2$M and CRP levels are usually low. These patients should be monitored closely and be treated only in case of clear disease progression. Symptomatic patients or those with anemia, recurrent infections, hypercalcemia, or renal insufficiency require treatment.

Standard-Dose Therapy

Oral melphalan and prednisone introduced approximately 30 years ago have remained the standard therapy for symptomatic myeloma (Table 76-4). They provide control of symptoms and tumor reduction by 50% in approximately half the patients. The usual dose is 9 mg/m^2 of melphalan and 100 mg prednisone given for 4 days every 46 weeks for at least 12 months. Failure to respond to this therapy was thought to be due to poor melphalan absorption, and dose escalation of melphalan with one third has been suggested in patients whose absolute neutrophil count does not fall to <1.5 and 100 × 10^9/L, respectively, 2 weeks after the start of chemotherapy. However, a recent study has reported similar hematologic toxicities in both responders and nonresponders, making it more likely that differences in response are related to drug sensitivity rather than to differences in absorption. [80] Moreover, the quality of response to low-dose melphalan is similar whether melphalan is given orally or intravenously. [81] It is unclear whether the addition of 100 mg prednisone to melphalan is beneficial. The Medical Research Council trials did not find a survival benefit, once the uneven distribution of patients was corrected for $_2$M levels.[82] Patients with IgA myeloma have a significantly inferior outcome with melphalan plus prednisone. [83] The observation of Alberts et al. [84] that the combination of Adriamycin and bis-chloroethyl-nitrosurea was active in patients who had become refractory to melphalan has led to combination chemotherapy regimens. In these combination regimens, vincristine is frequently added to alkylating agents, Adriamycin and prednisone, although the addition of vincristine to melphalan and prednisone has not shown any survival advantage. [82] Most trials have been unable to confirm a clear advantage of combination chemotherapy over melphalan plus prednisone. [83] [85] [86] [87] This

APPROACH TO THERAPY FOR MULTIPLE MYELOMA

Patients who are asymptomatic should not be treated until there is clear evidence of disease progression (hypercalcemia, increase in M protein or anemia, new lytic lesions) or the patient becomes symptomatic. If the physician is not sure whether a patient should be treated, it is rausually better to delay therapy and re-evaluate after 68 weeks. For patients requiring treatment, sufficient evidence is available from randomized studies and pair-mate analysis that high-dose chemotherapy is superior to standard-dose treatment in terms of complete response rate and event-free and overall survival, provided that the patient has adequate cardiopulmonary function, does not have major liver function abnormalities, and has a good performance status, unless the decline in performance status is due to bone pain. Peripheral blood stem cell transplants can be performed safely up to the age of 70 years and also in patients with renal failure, if high-dose melphalan is used as preparative regimen. Usually patients will receive one to three cycles of chemotherapy prior to stem cell mobilization to improve the general condition and to attain a significant tumor reduction. This is best achieved with the VAD regimen or with high-dose dexamethasone pulsing because of the rapidity of response and its stem cell sparing capacity. Before any treatment is given, patients should have a bone marrow aspirate and biopsy performed as well as a skeletal survey. In addition, the following laboratory tests should be obtained: complete blood count, blood urea nitrogen, creatinine, LDH, uric acid, calcium (ionized), CRP, and $_2$M. If possible, cytogenetics and PCLI should be performed on the bone marrow.

Studies are underway to determine whether double transplants are better than a single transplant and whether patients should undergo transplantation initially or after failing first-line treatment. However, peripheral stem cells should always be collected early in the disease before too much alkylating therapy has been given. Patients who are not candidates for high-dose chemotherapy should be treated for at least 12 months or until a plateau phase has been obtained. The duration of the plateau phase can be prolonged by the administration of interferon- maintenance therapy, although it does not result in a longer overall survival. Anemia can be corrected in many patients by the administration of erythropoietin. Hypercalcemia treatment is an oncologic emergency and should consist of fluids and bisphosphonate or gallium nitrate administration. In severe cases, calcitonin may be required for 23 days. To prevent further bone damage, monthly administration of an intravenous bisphosphonate is recommended. During the early stages of chemotherapy, prophylactic antibiotics will decrease the incidence of infections. Because of the high treatment-related mortality, allogeneic bone marrow transplantation should probably only be performed in patients with one or more poor prognostic markers, such as unfavorable cytogenetics elevated $_2$M or CRP levels after two to three cycles of high-dose dexamethasone-containing regimens; >12 months of conventional chemotherapy; or disease refractory to standard chemotherapy, i.e., <50% reduction in M protein.

TABLE 76-4 -- Results of Treatment in Myeloma

	N	Complete Response (%)	>50% Reduction in M Protein (%)	Median Survival (months)	5-Year Survival (%)
Standard Therapy					
Melphalan/Prednisone (SWOG/ECOG)	335	N/A	40	26	18
VMCP/VBAP (SWOG)	115	N/A	53	36	30
VBMCP (ECOG)	220	N/A	72	30	26
VAD (Alexanian)	175	N/A	55[a]	36	30
Dexamethasone (Alexanian)	112	N/A	43[a]	36	N/A
Autotransplants					
Melphalan/TBI (Attal)	100	22	81	60+	52

Melphalan (Powles)	195	53	79	54	40
Double transplants (Barlogie)	123	40	85[a]	62+	60

Abbreviations: ECOG, Eastern Cooperative Onclogy Group; SWOG, Southwest Oncology Group; TBI, total body irradiation; VAD, vincristine, Adriamycin, dexamethasone.

[a] 75% reduction in M protein.

indicates that minor increases in dose intensity are insufficient to change the outcome in myeloma.

Patients responding to standard chemotherapy have a much better survival than nonresponders (43 months vs. 19 months) with no survival advantage for complete responders over partial responders. [88] [89] Most responders attain a plateau phase, that is, a state of tumor quiescence in which the malignant plasma cells appear to be dormant. Therefore, a sine qua non of a plateau phase is a low labeling index. [90] The duration of this plateau phase is variable: a plateau phase of at least 3 months is achieved in 80%, at least 6 months in 75%, at least 12 months in 60%, and at least 24 months in 33% of the responders. [89] The median survival of patients with a plateau phase of 311 months, 1223 months, and 24 months or longer is 21, 46, and 81 months, respectively. [89] Escape from the plateau phase can be slow with no clinical symptoms but a progressive rise in serum or urine M protein or it can be clinically fulminant, sometimes associated with the presence of light chains electrophoretically unassociated with the original heavy chain (light chains escape). [90]

In patients with MM refractory to standard alkylating agents, the VAD regimen, combining continuous infusions of vincristine, Adriamycin, and 4-day pulses of high-dose dexamethasone at 40 mg daily, produces marked cytoreduction of 75% in >50% of patients, with an especially high response rate in relapsing patients (i.e., patients with prior response). [91] High-dose dexamethasone alone in refractory myeloma is equally effective as VAD in terms of survival. [92] Responders to VAD and dexamethasone not only have significant reduction to myeloma protein, but also have a drastic reduction in bone marrow plasmacytosis. VAD-based regimens in previously untreated myeloma patients result in a 55% response rate. [93] Response is usually rapid with near-maximum response occurring after two cycles of treatment. [93] [94] Despite these rapid responses, median survival times were similar for VAD and the previously described combination chemotherapy regimes. [93] [95] When compared to VAD the response rate with pulses of dexamethasone alone was 15% less, but the survival times were similar for the two regimens, indicating that dexamethasone was clearly the most active agent in the VAD regimen. [96] Myelosuppression is uncommon with VAD and the toxicity encountered with this regimen is almost exclusively due to the dexamethasone. The failure of the VAD regimen to have a significant impact on survival is probably due to the lack of effect on the myeloma precursors compartment that has receptors for IL-6 and therefore can be rescued from dexamethasone-induced apoptosis by this cytokine; actually an increase in clonogenic myeloma cells has been observed after treatment with VAD. [97] By adding cyclophosphamide to VAD (CVAD), 40% of VAD-resistant myeloma patients show an objective response with the best results obtained in patients with low LDH, low $_2$M, and primary resistant disease. [98]

Autologous Stem Cell Transplantation

Because minor dose increases in alkylating agents, as used in the combination chemotherapies, had not resulted in improvement of overall survival, it appeared logical to try a more pronounced escalation. This was first piloted by McElwain et al. using a dose of 100140 mg/m^2 of melphalan in nine patients, five previously untreated and four refractory to standard therapy. [99] Three of the five previously untreated patients achieved a biochemical and bone marrow complete remission, compared to a 3% complete response rate with standard therapy. High-dose melphalan therapy induced complete remission in one-third of patients previously untreated [100] and partial responses in approximately 40% of refractory myeloma cases. [101] However, the procedure-related mortality was high at 1520%, mainly due to long duration of aplasia. [100] [102] To correct this problem, high-dose melphalan therapy was supported initially with a bone marrow transplant that could contain up to 30% myeloma cells, [103] based on the concept that myeloma is a hypoproliferative disease and that these myeloma cells could not hamper hematologic reconstitution, while significantly shortening the duration of aplasia. However, the median time to granulocyte and platelet recovery with bone marrow transplantation was still approximately 3 weeks, [104] but bone marrow support allowed further intensification of therapy; the dose of melphalan could be escalated to 200 mg/m^2 or total body irradiation could be added to melphalan 140 mg/m^2.

Further progress was made by supporting high-dose chemotherapy with mobilized peripheral blood stem cells, reducing the median time to both granulocytes >0.5 × 10^9 /L and platelets >50 × 10^9 /L to 14 days. [105] With peripheral blood stem cell support, the transplant-related mortality is <5% and tandem transplants are feasible, with >75% of intended patients completing their double transplants. [105] When considering all patients, 30% achieve a stringently defined complete response (immunofixation of serum and urine are negative) with median duration of event-free and overall survival of 26 and 41 months, respectively. [105] Better results are obtained with tandem transplants in previously untreated myeloma patients with a 40% complete response rate and median event-free and overall survivals of 49 and 62+ months [106] (Table 76-4). Double transplants

appear to be superior to single transplants; with single transplants the complete response rate in unselected patients is only 25% [107] with median event-free survival of only 27 months. [107] However, both the French randomized [107] and a pair-mate comparison [106] have demonstrated that high-dose chemotherapy is superior to standard-dose therapy and therefore should be considered the standard of therapy for patients, at least up to the age of 65, who are in generally good condition, and in patients in whom their poor performance status is due to myeloma bone pain. High-dose chemotherapy with melphalan and stem cell rescue can be given safely to patients with renal failure. Pharmacokinetic studies have shown that the melphalan clearance is not delayed in renal failure. [108]

The most important prognostic variables for event-free and overall survival after autotransplant are cytogenetics, duration of standard-dose therapy, and the $_2$M level prior to the first transplant. [49] A model using these three variables accurately separates patients who will do well and who will do poorly with autotransplants. [49] The median time to clear the paraprotein after autotransplantation is 1.5 months with, very rarely, further reduction in paraprotein seen after 6 months. [109] Although IgA myelomas have a significantly more rapid clearing of their paraprotein and are associated with a higher complete response rate, the event-free and overall survival in these patients is significantly inferior. [105] Although peripheral stem cells should always be collected early in the disease, a current Intergroup trial in the United States is addressing the issue of optimal timing of autotransplantation, that is, upfront versus at the time of failure of standard therapy.

Other issues addressed in ongoing multicenter randomized trials are the benefit of CD34 selection as a means to deplete tumor cells and the benefit of one versus two autotransplants. Although it is still unclear whether MM patients can be cured with autotransplants, based on the lack of a plateau in the survival curves, it seems likely that in the low-risk group of patients (i.e., those with favorable cytogenetics, <12 months of preceding standard therapy, and low $_2$M levels prior to transplantation) a plateau is starting to form. Approximately 2030% of these patients (810% of all patients) may be cured. [49] Selected patients may benefit from further autotransplants after relapse post-transplantation, specifically, patients with low $_2$M at relapse and those with late relapse. [110]

Allogeneic Transplantation

The advantages of allotransplantation in myeloma are the lack of tumor contamination of the graft and the potential of a graft-versus-myeloma effect that has clearly been demonstrated in a few cases recently. [111] [112] However, patients with MM are in general older than those with leukemia, are more immunodeficient by nature of their disease and due to the administration of multiple courses of chemotherapy containing corticosteriods, and are in a more compromised condition because of their bone lesions. This results is a high first-year mortality rate after allotransplantation of approximately 4050&%. [113] [114] The complete remission rate in mainly refractory myeloma patients is 3545% and a progression-free survival of 2035% is seen at 5 years. [114] [115] Favorable prognostic factors for outcome are short duration of standard therapy, low $_2$M level before transplantation, and low tumor load at time of transplant. [113] [115] When a case-matched analysis was performed comparing patients treated with allogeneic versus autologous transplantation, the overall survival was significantly better in the autotransplant group. Again, the main reason for the poorer survival in the allotransplant group was the higher transplant-related mortality that was not compensated for by a higher response rate; however, in patients alive at 1 year post-transplantation, the progression-free survival was significantly better in the autotransplant group. [114] Because of the high treatment-related mortality, allogenic transplantation should probably only be considered in patients with more than one poor prognostic marker, that is, unfavorable cytogenetics, high $_2$M, high CRP, high LDH, high PCLI before transplantation, and disease refractory to standard-dose chemotherapy.

Interferon-

Encouraging results have been reported with interferon- therapy as maintenance therapy after 12 cycles of standard treatment with prolongation of response and survival in MM patients responding to conventional chemotherapy. [116] Although subsequent studies have shown a longer plateau phase with maintenance interferon therapy, in patients responding to standard therapy, they failed to show a survival advantage. [117] Similarly, there are no convincing studies showing any benefit from interferon- therapy post-transplantation. Besides a flulike syndrome, interferon can cause general weakness, autoimmune disorders, impotence, and depression. Prolonged interferon- also makes collection of adequate amounts of peripheral stem almost impossible.

Clarithromycin (Biaxin)

An unexpected and surprising activity of this agent in newly diagnosed and previously treated myeloma patients was reported recently by Durie et al. [118] The trial with clarithromycin was based on good responses seen in mucosa-associated lymphoid tissue lymphomas with t(11;14) associated *Helicobacter pylori* infection, as a result of the detection of a circulating nucleotide sequence, which contains the same Bcl-1/J $_H$ break point as observed in some myeloma patients. Patients received a dose of 500 mg clarithromycin twice daily. Thirty patients were enrolled with the longest follow-up being 1 year. Six patients had a 75% and seven had a 50% reduction in M protein. Responses were associated with improvement in clinical status, hemoglobin, $_2$ M, bone marrow plasmacytosis, and lesions detected by MRI. Responses were also observed in patients failing standard or high-dose chemotherapy. The best responders have been patients with IgA or IgG myeloma. A rebound in disease activity was seen if clarithromycin was discontinued before a maximal response was achieved. Although these results appear promising and this therapy is well tolerated, these data need to be confirmed in a larger study and if patients are started on this treatment, they should be monitored very closely for disease progression and switched to proven treatment modalities if progression occurs.

Management of Complications

Anemia

In patients with hemoglobin levels <10 g/dl with stable disease on standard therapy, erythropoietin therapy induces a response, defined as an increase in hemoglobin levels of 2 g/dl, in approximately 70% of patients with the best results in those with limited prior alkylating agent therapy and low endogenous erythropoietin levels. [119] [120]

Pain

Myeloma bone pain is usually severe and requires morphine derivatives. The pain usually improves remarkably with chemotherapy, especially regimens containing high-dose dexamethasone. Use of local radiotherapy for pain should be discouraged because it may interfere with future treatment plans and the collection of adequate numbers of peripheral blood stem cells. Patients should be encouraged to be as active as possible as soon as the pain is controlled to avoid further decalcification of the bone.

Hypercalcemia

Hospitalization is recommended for patients with calcium levels >12 mg/dl and for those with any other symptoms than mild fatigue and constipation. Hospitalization is certainly required in patients with moderate to severe anorexia, nausea, vomiting, and confusion. Treatment of hypercalcemia is urgent because renal insufficiency develops quickly. Intravenous administration of isotonic saline is the first step in the management of severe hypercalcemia; expansion of intravascular volume will also increase renal calcium clearance. The rate of administration of isotonic saline should be based on the severity of the hypercalcemia, the extent of dehydration, and the tolerance of the cardiovascular system. Usually approximately 3 liters of fluid is given daily and diuretics are used if symptoms of fluid overload occur. In addition to hydration, specific agents to lower calcium levels are required. The most commonly used agent is pamidronate, 90 mg as a 2- to 4-hour infusion. An alternative is gallium nitrate at a dose of 200 mg/m^2 /day by continuous intravenous infusion for 25 days; the infusion can be terminated if normocalcemia has been achieved. In patients with mild and moderate hypercalcemia, normal saline infusions and pamidronate should be sufficient therapy. However, in patients with severe and life-threatening hypercalcemia, calcitonin therapy will also be required to lower the calcium levels rapidly. Calcitonin has the most rapid onset of action (24 hours). It should only be given for 23 days until the pamidronate has had time to start working. The dose of calcitonin is 68 IU/kg every 812 hours. However, calcitonin rarely reduces serum calcium levels to normal and should therefore not be relied on as a single agent. Once hypercalcemia is controlled, standard chemotherapy, preferably with a high-dose dexamethasone regimen, should be started.

Prevention of Further Bone Disease

Bisphosphonates do not only have a role in the treatment of hypercalcemia, but they also contribute to the long-term control of bone disease. The administration of monthly pamidronate at 90 mg as an intravenous infusion over 24 hours in 500 ml normal saline significantly reduces the incidence of skeletal events and alleviates bone pain and improves the quality of life in patients undergoing standard-dose chemotherapy. [121] The most significant differences with a placebo control group were seen in patients who had already received more than one treatment regimen, who had experienced prior skeletal events, and who did not respond to standard therapy. Whether bisphosphonates are useful after a marked tumor reduction, as attained with transplantation, remains to be determined. However, until the studies have been completed, it is probably wise to give monthly pamidronate to all patients with myeloma.

Future Directions

Major progress has been made in the biology and treatment of MM. The role of cytokines and of oncogenes/suppressor genes has become more evident. Aggressive treatment approaches are resulting in higher complete remission rates and extended survival. However, relapses still occur. Further progress in the treatment of myeloma will come from one of the following areas:

1. Application of post-transplant chemotherapy in patients who have received minimal standard therapy and immediately proceed to high-dose therapy while still having chemosensitive disease. The post-transplant chemotherapy needs to consist of agents with a different mechanism of action than the alkylators used in the preparative regimens for the autotransplant. Tubulin-active agents, known to be active in cancers with altered p53 function and known to phosphorylate bcl-2, as well as agents interfering with DNA repair should be used in this setting.
2. Immunotherapeutic interventions to eradicate minimal residual disease post-transplantation. Dendritic cell-based protocols are being designed using either the myeloma protein or MUC-1 present on plasma cells, as tumor-specific or tumor-associated antigens.
3. Intervention into the cell cycle. Myeloma cells differ from normal plasma cells by their lack of terminal differentiation and by their capacity to proliferate. Agents that can either induce terminal differentiation or can prevent cell cycle progression, such as flavoperidol, may be important in the treatment of this disease.

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WALDENSTRÖMS MACROGLOBULINEMIA

The original description of this disease was provided by Waldenström in 1944 when he reported on two male patients with fatigue, a tendency to bleed from the gums and the nasal mucosa, lymphadenopathy, normochromic anemia, and an increased serum viscosity due to high levels of circulating macroglobulin (IgM). These patients lacked the typical signs of myeloma, including lytic bone lesions even on autopsy.

The main characteristics of the disease are the bone marrow infiltration with lymphoplasmacytoid cells and high levels of IgM in the blood. Patients with IgM paraprotein with lytic bone lesions or hypercalcemia and infiltration of the bone marrow with more typical plasma cells should be considered as having IgM myeloma. The median age of patients with Waldenströms disease is 63 with 60% of patients being male. Lymphadenopathy or splenomegaly is present in 40% of patients if computed tomography or MRI of the abdomen is performed. Tumor cells may infiltrate the lungs, the gastrointestinal tract, and the skin. MRI shows bone marrow involvement in >90% of patients. Anemia, monoclonal lymphocytosis in the peripheral blood, and Bence Jones proteinuria occur in the majority of patients. However, Bence Jones proteinuria very seldom exceeds 1 g/24 hours. One-third of the patients have elevated $_2$ M levels and CRP values are increased in the majority (±66%).[122] Abnormal and complex karyotypes are common. The malignant lymphoplasmacytoid cells express surface and cytoplasmic IgM. By flow cytometric analysis these cells express panlymphocyte surface antigens CD19, CD20, and CD22, but are often also positive for CD5 and CD10. CD38 expression is less intense than that on myeloma cells. Normal immunoglobulins are less frequently and less markedly depressed than in myeloma. Cellular immunity including CD4+ counts are usually preserved unless immunosuppressive therapy is given.

Disease in most patients is diagnosed during assessment for complications caused by the macroglobulin such as hyperviscosity syndrome, cryoglobulinemia, cold hemolytic anemia, peripheral neuropathy, amyloidosis, renal disease, and bleeding problems (Fig. 76-6). The serum viscosity is elevated in most patients, but only 1520% have symptoms related to hyperviscosity. Patients with a blood viscosity greater than four times that of water are at risk of developing clinical problems. Because of the expanded plasma volume, intracranial pressure increases, which causes headaches. Other symptoms related to hyperviscosity are dizziness, blurred vision, easy bleeding, and mental status changes. Because of the anemia, the expanded plasma volume, and the increased viscosity, these patients may develop symptoms of congestive heart failure. Fundoscopic evaluation may show distended, sausage-shaped veins, hemorrhage, and papilledema. In contrast to myeloma, where hyperviscosity is almost never seen until the monoclonal paraprotein exceeds 5 g/dl, patients with Waldenströms disease develop hyperviscosity syndrome with an IgM level >3 g/dl. Cryoglobulinemia may cause Raynauds syndrome, arthralgias, purpura, peripheral neuropathy, liver function abnormalities, and renal

Figure 76-6 Major clinical and laboratory features of Waldenströms macroglobulinemia.

failure. For proper diagnosis, blood should be collected, transported, and separated at 37°C before analysis. In approximately 10% of patients, the IgM paraprotein causes cold agglutinin hemolytic anemia, usually associated with a markedly elevated cold agglutinin titer (>1:1,000). The most common target is the I antigen on red blood cells. Bleeding problems may be caused by platelet dysfunction or clotting abnormalities. Hyperviscosity may contribute to the bleeding severity.

About 10% of patients with macroglobulinemia develop chronic, predominately demyelinating sensorimotor peripheral neuropathy. In approximately half of these patients, the IgM antibody is directed against carbohydrate epitopes of myelin-associated glycoprotein (MAG). The presence of anti-MAG antibodies can be detected by enzyme-linked immunosorbent assay. Patients with anti-MAG antibodies usually have a sensory or ataxic polyneuropathy, whereas the other patients with peripheral neuropathy often have a mixed sensorimotor neuropathy. Renal insufficiency is less frequent in Waldenströms disease than in myeloma. IgM may precipitate on the glomerular basement membrane forming deposits that occlude the capillaries. Some patients develop an immunologically mediated glomerulonephritis associated with a nephrotic syndrome. Amyloidosis is rare (<5%), but may result in cardiac, renal, hepatic, and pulmonary complications.

One-third of patients with macroglobulinemia die of causes unrelated to their disease and one-third succumb to infectious complications; other causes of death are bleeding, transformation to high-grade lymphoma, renal failure, or the development of acute myeloid leukemia or a solid tumor. [123] The best prognostic factors are age, hemoglobin, weight loss, and cryoglobulinemia. [123] [124] Asymptomatic patients should be monitored without treatment until complications occur due to either circulating IgM or tumor bulk. Complications such as hyperviscosity, cryoglobulinemia, cold agglutinin hemolytic anemia, or peripheral neuropathy should be treated with plasmapheresis. Because 80% of IgM is intravascular, the level of IgM will be reduced rapidly. However, most patients will require effective chemotherapy to decrease IgM production by the tumor cells. This treatment has most commonly consisted of an alkylating agent and a glucocorticoid. Almost 75% of newly diagnosed patients will show a 50% reduction in M protein. [120] Treatment is usually continued until maximum reduction of IgM is obtained. Approximately 10% of patients attain a complete remission and have a median survival of 11 years. The median survival for all patients is 5 years. [123] [124] Nucleoside analogs such as fludarabine and 2-chloro-deoxyadenosine (2-CDA) have been studied in previously untreated patients. Fludarabine at a dose of 25 mg/m ^{2} intravenously for 5 days every 4 weeks and 2-CDA at a dose of 0.1 mg/kg for 7 days by continuous infusion through a central venous catheter have revealed an 80% response rate with 10% achieving a complete remission.[122] [125] This treatment is well tolerated but results in a marked and sustained reduction of CD4+ lymphocytes, but without a marked increase in life-threatening opportunistic infections. [125] Based on the successes with myeloablative therapy and autologous stem cell support in myeloma, a formal Intergroup trial evaluating the role of autotransplantation in Waldenströms macroglobulinemia will be started.

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HEAVY CHAIN DISEASES

The heavy chain diseases are rare lymphoproliferative disorders of B-cell origin that secrete abnormal immunoglobulins lacking light chains. [2] [126] The major types described are , , and heavy chain disease. The first reported was the heavy chain disease, also called Franklins disease; the most common type is the heavy chain disease, sometimes also referred to as Seligmann disease, of which there are several hundred cases reported in contrast to the types, of which there are approximately 100 cases, and the type, of which there are fewer than 30 documented cases. Infection may play a role in the etiology of some types of heavy chain disease, especially in heavy chain disease. The disease occurs in two forms: the enteric and respiratory forms. The enteric form is prevalent in areas where intestinal infections with parasites, bacteria, and viruses are common, and an occasional patient attains a complete remission after antibiotic therapy. About one-fourth of patients with heavy chain disease have an associated autoimmune disease, such as rheumatoid arthritis, autoimmune hemolytic anemia, Sjögrens syndrome, lupus erythematosus, vasculitis, idiopathic thrombocytopenia purpura, and myasthenia gravis. They usually precede the development of heavy chain disease by many years. Most heavy chain disease proteins have an internal deletion of much of the variable region domain and of the first constant region domain (C_H1 domain). In the absence of light chain expression, the heavy chain is retained within the endoplasmic reticulum or Golgi apparatus. Toxicity can ensue from accumulation of heavy chains within these intracellular compartments ultimately resulting in cell death. Neoplastic B cells producing such abnormal heavy chains may continue to secrete light chains as free monomers or lose expression of light chains altogether.

Heavy Chain Disorder

The median age at onset is 60 years, but occurs before the age of 20 years in about 10% of patients. They usually present with a lymphoma-like illness, but the findings are diverse, ranging from asymptomatic disease to an aggressive lymphoproliferative process. Weakness, fatigue, and fever are common; hepatosplenomegaly and lymphadenopathy occur in about 60% of patients. Anemia is present in 80&%. Other manifestations may be parotid gland swelling, severe soreness of the tongue, skin infiltration, autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, enlargement of the thyroid gland, and neutropenia due to hypersplenism. Serum protein electrophoresis may show a broad band, suggestive of polyclonal gammopathy. The M protein is usually in the -area. Most of the proteins are IgG, but the monoclonal band may vary from a trace to 9 g/dl. Immunoelectrophoresis is necessary for detection of the monoclonal heavy chain. The bone marrow and lymph nodes contain an increased number of plasma cells, lymphocytes, or lymphoplasmacytoid cells. Osteolytic lesions are rare. The clinical course of heavy chain disease varies from a rapidly progressive downhill course to a prolonged stable disease. The median survival is approximately 12 months. Therapy is initiated only for symptomatic patients; a CHOP-like (cyclophosphamide, hydroxydaunomycin, Oncovin, prednisone) regimen is preferred. Treatment results are usually disappointing.

Heavy Chain Disease

Most patients are from the Mediterranean region and develop the disorder in the second or third decade of life. The gastrointestinal tract is most commonly involved, resulting in malabsorption, weight loss, and diarrhea. Plasma cell infiltration is seen in jejunal mucosa and mesenteric and para-aortic lymph nodes. The stomach may also be involved. The bone marrow is usually normal as is serum protein electrophoresis in 50% of cases; in the other patients a small broad band may be present in the $_2$ or regions. Usually this disease is progressive and fatal. Patients with diarrhea secondary to the enteric form of heavy chain disease may require intravenous infusions of fluids, electrolytes, calcium and magnesium, albumin, and antibiotic therapy (e.g., tetracyclines) or metronidazole. The preferred therapy for the more advanced stages of the disease is CHOP.

Heavy Chain Disease

The age of these patients varies between 15 and 80 years. Hepatosplenomegaly is found in most patients. The bone marrow shows increased numbers of lymphocytes and plasma cells. Lytic bone lesions are present in 20% of patients. Serum protein electrophoresis may be normal, except for hypogammaglobulinemia, or show an abnormal band; Bence Jones proteinuria is found in two-thirds of patients. The course is variable and survival may vary from a few months to many years. Treatment with alkylating agents and corticosteroids may be of benefit.

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CRYOGLOBULINEMIA

Cryoglobulins are immunoglobulins that precipitate at temperatures below 37°C (Fig. 76-7), producing high-molecular-weight aggregates. These aggregates dissolve again when heated to temperatures above 37°C.[127] Leukocytoclastic necrotizing vasculitis involving small and medium-sized arteries occurs in patients with cryoglobulinemia leading to a disorder involving multiple body systems. [128]

Cryoglobulins are best detected by drawing venous blood into a preheated syringe (37°C) and separating the serum in a centrifuge that maintains a temperature between 37°C and 39°C. The serum is removed and placed in capillary tubes and incubated at 4°C for 814 days. Samples with cryoprecipitates are examined daily after being centrifuged for 15 minutes. A cryocrit of 0.4% is considered indicative of a positive test because symptoms attributable to cryoglobulinemia are rare at lower values. Normal serum has been reported to contain up

Figure 76-7 Cryoglobulinemia. **(Left)** Precipitate formed at 1°C. **(Right)** Disappearance of precipitate on heating to 37°C. *(From Kyle and Greipp, [153] with permission.)*

to 80 g/ml of cryoglobulins, whereas levels of 500 to 5,000 g/ml of cryoglobulins are frequently associated with symptoms. [129]

Brouet et al. first classified cryoglobulins into three types. [130] Type I cryoglobulins are composed of one class of monoclonal immunoglobulins, most frequently IgM. Type II cryoglobulins are mixed cryoglobulins, which are composed of a mixture of monoclonal and polyclonal immunoglobulins (monoclonal anti-IgG being the most common). These complexes may also include other proteins such as hepatitis C-related proteins, the third component of complement or lipoproteins. [129] Type III cryoglobulins are composed of polyclonal immunoglobins, most commonly polyclonal IgM anti-polyclonal IgG. In type II and type III, the IgMs are antiglobulins acting as anti-IgG rheumatoid factors.[127] [130] To determine the type of cryoglobulin, the serum cold precipitate can be redissolved by reheating to 37°C. The presence of monoclonal or polyclonal immunoglobulins and complexes is then determined by standard electrophoretic analyses described in Chapter 160 .

Type I cryoglobulins are usually associated with underlying lymphoproliferative disorders including low-grade lymphomas, Hodgkins disease, Waldenströms macroglobulinemia, MGUS, angioimmunoblastic lymphadenopathy, and chronic lymphocytic leukemia.[128] Both type II and type III cryoglobulins are associated with lymphoproliferative disorders (indolent lymphoma, Waldenströms macroglobulinemia), autoimmune disorders (systemic lupus erythematosus, Sjögrens syndrome, scleroderma, rheumatoid arthritis), or a variety of infectious disorders (Table 76-5).[127] [128] [129] Patients in whom an underlying etiology cannot be identified are classified as having essential mixed cryoglobulinemia (Table 76-6 (Table Not Available)). Of the 103 cases of cryoglobulinemia reported by Wooten and Jasin, 28% had type I cryoglobulins, 58% had type II cryoglobulins, and 14% had type III cryoglobulins. [128] In addition, 14% of patients with cryoglobulinemia have been reported to have Waldenströms macroglobulemia. Although 15% of patients with Waldenstroms macroglobulinemia have type I cryoglobulins, only 5% are symptomatic. [128] The patients with type II mixed cryoglobulinemia characteristically have serum hypergammaglobulinemia and decreased complement levels due to activation by immune complexes.[127] [129] Wooten and Jasin analyzed seven large series of patients with symptomatic cryoglobulinemia reported in the literature and determined that 6% (138%) eventually developed a lymphocytic lymphoma.[128] Such data have led a number of investigators to suggest that type II cryoglobulinemia is a forme fruste of low-grade lymphoma.

A growing number of studies have consistently implicated hepatitis C virus (HCV) as the major etiologic agent that leads to type II mixed cryoglobulinemia. [131] [132] [133] Approximately 80% of patients with essential mixed cryoglobulinemia have mixed cryoglobulinemia secondary to an HCV infection. [131] [132] Cryoprecipitates in these patients can be dissociated to hepatitic C virons, IgG, and monoclonal IgM rheumatoid factor. In addition, HCV virons or related proteins have been detected in peripheral blood mononuclear cells, cutaneous tissues, and the residual ductal or acinar parotid structures of patients with non-Hodgkins lymphoma of the parotid gland and in glomerular and tubulointerstitial vascular structures of patients with cryoglobulinemia and membranoproliferative glomerulonephritis. [129] [134] [135] [136] [137] Lymphoid aggregates have been demonstrated in the liver and marrow of such patients and are composed of monoclonal B cells, which likely play a role in the immune-mediated liver damage and the development of lymphoma.[134] [135] [136] [137] The phenotype of the B cells within these lymphoid aggregates is consistent with an indolent lymphoma.[134] In patients with hepatitis, cryoglobulinemia is not a rare event, having been documented in 54% of patients with hepatitis C, 15% of patients with hepatitis B, and 4% of normal controls.[133] Recognition that cryoglobulinemia is an HCV-related disorder lends support to the use of antiviral agents such as interferon- in its treatment.

TABLE 76-5 -- Diseases Frequently Associated with Mixed Cryoglobulins

Lymphoproliferative conditions
Chronic lymphocytic leukemia
Non-Hodgkins lymphoma
Angioimmunoblastic lymphadenopathy
Infectious diseases
Viral etiology
Acute and chronic hepatitis
Infectious mononucleosis
Cytomegalovirus infection
Bacterial etiology
Lepromatous leprosy
Subacute bacterial endocarditis
Syphilis
Lymphogranuloma venereum

Parasitic etiology
Echinococcosis
Malaria
Toxoplasmosis
Leishmaniasis
Schistosomiasis
Fungal etiology
Coccidioidomycosis
Autoimmune and immune complex-mediated diseases
Systemic lupus erythematosus
Rheumatoid arthritis
Sjögrens syndrome
Feltys syndrome
Progressive systemic sclerosis
Polyarteritis nodosa
Autoimmune vasculitis
Glomerulonephritis
Autoimmune thyroiditis
Polymyositis
Sarcoidosis
Celiac disease
Pemphigus vulgaris
Disorders of the liver
Primary biliary cirrhosis
Alcoholic cirrhosis

From Dommacco and Sansonno,[129] with permission.

It is recommended that all patients with mixed cryoglobulinemia be tested for HCV and that all patients with HCV be questioned for symptoms associated with essential mixed cryoglobulinemia. Many of the extrahepatic symptoms of HCV are thought to be due to chronic immune complex disease and can frequently be attributed to a mixed cryoglobulinemia syndrome[138] (Fig. 76-8).

Cryoglobulinemia is a rare disorder characterized by symptoms due to systemic vasculitis. Clinical manifestations range from the triad of palpable purpura, arthralgias, and weakness to life-threatening disease involving vital organs such as the kidneys. [127] [129] [131] Symptoms in many patients are first related to

TABLE 76-6 -- Summary of Clinical Manifestations in 1,033 Patients with Essential Mixed Cryoglobulinemia

(Not Available)

From Agnello, [131] with permission.

Figure 76-8 Relationship of symptoms of hepatitis C infection and mixed cryoglobulinemia. *(From Wener et al.,[138] with permission.)*

skin manifestations, which are a consequence of cutaneous vasculitis. These patients present with purpura, leg ulcers, Raynauds phenomenon, edema, and urticaria. The purpura is nonpruritic and appears intermittently on exposed parts of the body. [129] The purpura occurs most frequently following cold exposure, lasts for 12 weeks and leaves a hardened, diffusely hyperpigmented area. [129] Chronic leg ulcers in the supramalleolar regions can be associated with purpura.

Intermittent, symmetrical, nonmigratory arthralgias accompanied by profound weakness are common.[129] Liver involvement occurs in almost 50% of patients and can range in severity from minimal changes to sporadic hepatic necrosis within lobules, to a picture consistent with chronic active hepatitis. Sensory and motor peripheral neuropathy mostly involving the lower extremities may occur. Severe abdominal pain occurs in 20% of patients and is a consequence of intestinal vasculitis. [129] Central nervous system dysfunction including transient hemiplegia, dysarthria, mental confusion, or even coma has been reported. [127] [129] Membranoproliferative glomerulonephritis with subendothelial deposits occurs frequently in patients with mixed cryoglobulinemia. These patients present initially with isolated proteinuria, microscopic hematura, and signs of moderate renal insufficiency or nephrotic syndrome. [137] In 2025% of cases, patients present with acute oliguric renal failure due to massive precipitation of cryoglobulins in glomerular capillary loops with subsequent infiltration with monocytes. [137] Progression to end-stage renal failure occurs in 10% of cases usually several years after the onset of symptoms.[129] [137] The most common causes of death in patients with cryoglobulemenia are systemic vasculitis, infections, and severe hypertension leading to cardiovascular and cerebrovascular accidents. [129]

Frequently mixed cryoglobulinemia will evolve into a lymphoid malignancy. In one series of 31 patients with mixed cryoglobulinemia and HCV infection, 39% of patients had marrow biopsy findings consistent with the presence of a low-grade lymphoma. [139] Early in the course of the disease, this lymphoproliferative disorder is limited but often can evolve to a full-blown non-Hodgkins lymphoma. The overt B-cell lymphomas frequently present as diffuse large cell lymphomas originating in extranodal sites such as the liver, spleen, and salivary glands. [140] [141]

Treatment of the systemic vasculitis that accompanies cryoglobulinemia remains problematic. Avoidance of the cold is a mainstay of therapy. Following the identification of HCV in 80% of the cases of mixed cryoglobulinemia interferon- has

been used extensively alone or in combination with other drugs. [129] [142] [143] [144] Cytotoxic drugs such as cyclophosphamide should be avoided in these virally induced vasculitides; their use should be limited to short pulses in patients with severe forms of the disease. Interferon- is usually administered at a dose of 3 million units daily for 36 months, resulting in a 77% response rate. [142] [144] An alternative regimen of interferon- in which a dose of 3 million units is administered three times a week has resulted in sustained response in 35% of patients. [143] In a randomized study the addition of prednisone to interferon- resulted in little therapeutic benefit. [142] Prednisone therapy alone results in 40% of patients in elevations of serum HCV RNA levels, therefore leading to little justification for long-term prednisone therapy. [142] Significant improvement with interferon- therapy can be achieved in patients with cutaneous vasculitis. The therapeutic efficacy of interferon has been demonstrated to be clearly related to inhibition of HCV replication, and clinical responses have been associated with reduction of levels of HCV RNA to a point where the virus can no longer be detected.[144] The absence of HCV RNA in cryoprecipitates of responsive patients suggests that alterations in the composition of cryoprecipitates occur following treatment.[129]

Although responses to interferon are frequently sustained, 80% of patients relapse within 6 months of cessation of treatment. [142] [143] [144] These data suggest that continuous maintenance therapy is indicated in patients with persistently active disease. In some patients with chronic renal disease reduction of the degree of proteinuria and serum creatinine levels has been achieved with interferon therapy. Most clinicians, however, prefer to initially treat patients with acute renal failure with a combination of steroids, cyclophosphamide, and plasmapheresis therapy. [145] [146] Once these patients have been stabilized, interferon therapy should be instituted.

Plasmapheresis in combination with interferon- therapy has been shown to be particularly effective for treatment of arterial leg ulcers, peripheral neuropathy, or situations where digital ulcers or strokes can be attributed to hyperviscosity or cerebral vasculitis. [129] Case reports of successful treatment of cryoglobulinemia and low-grade lymphoma with fludarabine and 2-chlorodeoxyadenosine have appeared in the literature, yet optimal treatment of overt diffuse large cell lymphoma following HCV and mixed cryoglobulinemia requires further investigation. [147] [148]

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PYROGLOBULINEMIA

Pyroglobulins are immunoglobulins that precipitate when the serum is heated to 5660°C. The precipitation is irreversible. [149] This thermolabile property distinguishes these proteins from Bence Jones proteins, which will redissolve if heating is continued above 56°C. The presence of pyroglobulins is not associated with any symptoms.[149] [150] [151] [152] The presence of pyroglobulinemia is associated most commonly with MM and Waldenströms macroglobulinemia. [149] [150] [151] [152]

Pyroglobulins are found in 23% of patients with monoclonal paraproteins and are not found in normal individuals. [149] These proteins can be detected serendipitously in individuals in whom fibrinogen levels are detected by heat precipitation. This irreversible denaturation of immunoglobulins with heating is likely the result of conformational changes triggered by light chains with increased molecular hydrophobicity. [151] [152]

Combined cryopyroglobulins are unusual immunoglobulins that form a reversible cryoprecipitate at low temperatures and an irreversible gel when heated to 5660°C.[151] This abnormality has been observed in patients with IgG, IgM, IgA, and K or light chain producing myelomas. [151] Symptoms in these patients have been attributed to the cryoglobulin, not the pyroglobulin, although the occasional combination of cyroprecipitation and pyrogel formation suggests the presence of both defects in the same molecule.[151]

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Chapter 77 - Amyloidosis

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The term *amyloidosis* is used to describe a heterogeneous group of extracellular protein deposition diseases in which homogeneous protein molecules aggregate in an ordered structure to make linear, nonbranching fibrils 7.510 nm wide and of indeterminate length. The fibrils are arranged in a characteristic -sheet structure, which gives apple-green birefringence under polarized light when stained with Congo red. [1] Amyloid deposits were first recognized by Virchow in 1854, and were so named because of the apparent affinity of the material for iodine, which suggested a resemblance to starch. For historic reasons, the name has been retained, although it is now clear that all types of amyloid deposits are proteinaceous and not carbohydrate

TABLE 77-1 -- Nomenclature and Classification of Amyloid and Amyloidoses

Amyloid Type	Protein Precursor	Protein Type or Variant	Associated Clinical Syndromes
AL	,	A, A	Primary amyloidosis
			Myeloma and Waldenström macroglobulinemia-associated amyloidosis
AH	IgG 1 (1)	A1	Heavy chain disease associated
AA	ApoSAA	AA	Reactive/secondary amyloidosis
			Familial Mediterranean fever
			Muckle-Wells syndrome
ATTR	Transthyretin		Hereditary amyloidoses:
		e.g., Met30[a]	Familial amyloidotic polyneuropathies
		e.g., Met111[a]	Familial amyloidotic cardiomyopathy
		TTR or Ile122[a]	Senile systemic amyloidosis
AApoAI	Apolipoprotein AI	e.g., Arg26[a]	Familial amyloidotic polyneuropathy
		e.g., Arg60[a]	Familial non-neuropathic (Ostertag type)
AGel	Gelsolin	Asn187[a]	Familial amyloidosis (Finnish)
A_2 -m	$_2$ -microglobulin		Dialysis associated
A	-protein precursor A4	A4 protein	Alzheimer disease
			Down syndrome
		e.g., Gln 695[a]	Hereditary cerebral hemorrhage with amyloid (Dutch type)
ACys	Cystatin-C	e.g., Leu 68[a]	Hereditary cerebral hemorrhage with amyloid (Icelandic type)
AScr	PrP^C (cellular protein precursor)	Prp^{SC} , PrP^{CJD}	Spongiform encephalopathies
		e.g., Leu 102[a]	Gerstmann-Sträussler-Scheinker syndrome
ACal	(Pro)calcitonin	(Pro)calcitonin	Medullary carcinoma of thyroid
AANF	Atrial natriuretic factor	Atrial natriuretic factor	Isolated atrial amyloidosis
AIAPP	Islet-associated peptide	Islet-associated peptide	Amyloid of the islets
			Diabetes mellitus type II
A Lys	Lysozmye	e.g., Thr56	Hereditary non-neuropathic systemic amyloidosis
A Fib	Fibrinogen chain	e.g., Leu554[a]	Hereditary renal amyloidosis

Abbreviations not explained in table: AA, amyloid A protein; L, immunoglobulin light chain; H, immunoglobulin heavy chain; SAA, serum amyloid A precursor; apo, apolipoprotein; TTR, transthyretin.

Data from WHO-IUIS Nomenclature Subcommittee: Nomenclature of amyloid and amyloidoses. Bull World Health Organ 71:105, 1993.

[a]Variant amino acid with corresponding position in the mature protein.

in nature. [2] The fibrillary nature of the amyloid protein was first recognized by electron microscopy in 1959 by Cohen and Calkins. [3] The definition of the cross--pleated sheet supersecondary structure was obtained by x-ray diffraction analysis of isolated amyloid fibrils in 1968. [4] Historically, the amyloidoses were classified as either primary or secondary. The primary amyloidoses were thought to occur in people with no underlying illness, whereas amyloid deposition in the setting of chronic infectious or inflammatory conditions was termed secondary. In 1971, Glenner et al demonstrated the sequence homology of a major protein subunit from the primary amyloid fibril to the variable segment of the immunoglobulin (Ig) light chain. [5] Since then, several other proteins have been identified as major

constituents of amyloid fibrils, and the chemical nature of the amyloidogenic protein is the key by which the current classification of amyloidosis is organized [6] (Table 771). The most life-threatening forms are systemic, with multiorgan involvement, but in a number of subtypes the deposition may be localized to a single organ. These disorders are of special interest to hematologists because one of the most common systemic forms (primary or AL amyloidosis) is a plasma cell dyscrasia causing the tissue deposition of monoclonal immunoglobulin fragments.

PATHOGENESIS

The diverse human proteins that can form amyloid fibrils in vivo each have a normal, unrelated protein precursor (Table 771), but they can all polymerize into insoluble fibrils with similar ultrastructural appearances and identical tinctorial properties. The structure of the fibrils, which comprise the protein core of amyloid deposits, consists of a -sheet structure with the strands perpendicular to the long axis of the fiber. The elucidation of the conformational modifications that drive the conversion of different proteins into an identical pathologic fold is important for understanding the molecular basis of the disease and may enable development of rational approaches to the treatment of amyloidosis.

Amyloid Proteins

Immunoglobulin Light Chains

Primary Structure

Determination of the primary structure of amyloidogenic Bence Jones proteins has been one of the most important accomplishments in the elucidation of the structural basis of AL amyloidosis in the last 20 years. An extensive survey and analysis of the published sequences of amyloidogenic proteins in comparison with a few nonamyloidogenic ones has emphasized the role of critical amino acid positions in conferring this pathologic property. [7] All light chain classes can cause AL amyloidosis, but light chains are involved in amyloid deposition two to three times as often as chains. One of the most intriguing findings is that $_{VI}$ light chains are strictly associated with AL amyloidosis. Comparison of the normal and pathologic light chain counterparts suggests that the amino acid replacements affect the light chain scaffold,[8] because they are located in the well preserved positions in the framework regions of the light chain variable regions. The amino acid replacements identified in amyloidogenic light chains and most strictly related to the disease are also frequently located in the framework regions. [9]

Post-translational Modifications

The two post-translational modifications most frequently correlated with the amyloidogenicity of light chains are proteolytic rearrangement and glycosylation.

Proteolysis.

Extraction of fibrillar light chains from amyloid deposits, even when conducted with mixtures of protease inhibitors, demonstrates the presence of light chain fragments associated with different percentages of complete light chains. It has been demonstrated that light chain fragments circulate in small amounts in the serum of these patients,[10] and that the bone marrow plasma cells from patients with AL amyloidosis secrete light chain fragments of heterogeneous molecular weight. [11] The pathogenic role of proteolytic rearrangement of amyloidogenic light chains is still under discussion, [12] and it cannot be excluded that in some cases this represents a postfibrillogenic event. However, two aspects of the problem must be emphasized: the first is that in vitro, fibrillogenesis of these immunoglobulins is accelerated by proteolytic digestion of the constant domain, [13] and the second is that even though postfibrillogenetic, proteolysis of the constant domain stabilizes the fibrillar structure.

Glycosylation.

A pathogenic effect for glycosylation has been hypothesized on the basis of the high frequency of glycosylated amyloidogenic light chains: >50% of them seem to be sugar linked, compared with 15% of nonamyloidogenic Bence Jones proteins. [14] The possibility that these carbohydrates may have a stabilizing effect on the growing fibril in vivo has been postulated. [15]

Three-Dimensional Structure and Folding Abnormalities

Determination of the tertiary structure of amyloidogenic light chains has been achieved at high resolution in a few cases only. One of the most recently resolved three-dimensional structures is that of light chain Bre. [16] The native structure of BRE is not markedly different from that of the nonpathologic light chain $_{I}$ REI. The only significant modifications are located in the regions that participate in the monomer-monomer contacts (loops 4044 in framework 2) and control dimer formation and stability. New light has been shed on the relationship between structure and the tendency to create amyloid fibrils from analysis of the thermodynamic parameters of the unfolding pathway of amyloidogenic and non-amyloidogenic light chains. Several recombinant V_L domains of amyloidogenic light chains were evaluated, and it was determined that these variants are less stable than the wild-type Rei V_L light chain. [17] This finding was confirmed by a natural amyloidogenic light chain isolated as a dimer and presenting the arginine 61-threonine replacement. [18] Conversion of circulating immunoglobulin light chains to the fibrillar state proceeds through at least one conformational intermediate that is accessible to some, but not all light chains. Structural characterization of this unstable intermediate is required to explain the unresolved mystery of this pathologic phenomenon: why do these light chains self-aggregate into a well ordered fibrillar structure instead of remaining an amorphous aggregate?[19]

Heavy Chain Amyloidosis

After the first demonstration by Eulitz et al [20] that an internally deleted IgG1 heavy chain can create amyloid in vivo, a few other cases of heavy chain disease and amyloidosis have been described.

Light/Heavy Chain Deposition Disease

As in AL amyloidosis, there is a relationship in light/heavy chain deposition disease (L/HCDD) between the structure of these immunoglobulins and the molecular basis of disease in which the light chains accumulate, with very selective tissue tropism, in the basement membrane and produce an amorphous, nonfibrillar protein deposit. Unlike in amyloidosis, light chains are more often responsible for the disease than light chains. Structural data obtained from the few pathogenic light chains in which the V_L region has been completely sequenced indicate frequent amino acid replacements that result in the creation of hydrophobic patches in regions normally exposed to the solvent.[21] It is worth pointing out that the deposits in L/HCDD are positive for carbohydrate stains like periodic acid-Schiff (PAS), and that the light chains responsible for L/HCDD are frequently glycosylated. However, the PAS-positive material found in the basement membrane is not associated with light chain glycosylation, but rather is related to glycoproteins and proteoglycans synthesized locally in the basement membrane and in the mesangial cells. Recently, two monoclonal light chains responsible for L/HCDD were studied for folding stability and self-aggregation tendency. [18] [22] Although the two light chains show significantly different stability under denaturing conditions, both have a high tendency to self-aggregate and create an amorphous precipitate. In one case, in a mouse model, a remarkable tropism for the kidney basement membrane was demonstrated.[18] A multistep process can be hypothesized in which the light chain first binds some as yet unknown components of the basement membrane, where it reaches a high concentration; later, protein aging and local conditions could cause unfolding and self-aggregation, which enhance the deposition of insoluble light chain precipitate.

Protein

The fibrils extracted from the intracerebral and cerebrovascular amyloid deposits of patients affected by Alzheimer disease, Down syndrome, and amyloid angiopathy of the Dutch type are constituted by a 39- to 42-residue peptide known as protein. The amyloid protein precursor (APP) is the source of the peptide found in the Alzheimer brain. It constitutes a family of transmembrane glycoproteins encoded on the long arm of chromosome 21. The secretory form of APP is produced along two pathways: a nonamyloidogenic pathway involving proteolytic cleavage within the A region and an amyloidogenic one, leading to the release of A subunits. The first

step in the genesis of A amyloid is the release of the A fragment from the parent APP molecule. This implies the existence of two distinct proteolytic events to generate the N- and C-terminals of A. Any protease that releases APP and the protein from the membrane is known as a *secretase*. Most secreted APP is released by a putative secretase, which cleaves within the A domain and precludes the release of the intact amyloidogenic peptide. A portion of secreted APP is released by a secretase, which cleaves near the N-terminus of A and produces C-terminal fragments containing the whole A domain. The release of the 39- to 42-residue amyloidogenic polypeptide requires the action of a third enzyme, secretase, which can generate the C-terminus of A after release of the transmembrane domain from the lipid bilayer. The identity of the three secretases is unknown, but may provide a novel therapeutic target. To date, six different mutations in exons 16 and 17 of the APP gene have been correlated with early onset of Alzheimer disease. The mechanism by which mutations leading to amino acid substitution cause -protein amyloidosis is unknown. It has been reported that cultured cells expressing APP cDNA encoding the substitutions Asn595-Lys and Leu596-Met, responsible for hereditary Alzheimer disease in a Swedish family, produce six to eight times more protein than cells expressing wild-type APP. [23] The identification of the effect of these mutations on the rate of -protein production has allowed the preparation of a transgenic mouse that reproduces the histopathologic pattern of the disease and provides an irreplaceable model for biomedical and pharmaceutical investigations. [24]

Prion Protein

In prion-related neurodegenerative diseases like scrapie, Creutzfeldt-Jakob disease, and bovine spongiform encephalopathy, the amyloid protein consists of a prion-related protein of 2730 kd (PrP_{2730}). PrP^{SC} or PrP^{CJD} represents the pathogenic protein,

whereas PrP^{C} is the normal cellular isoform. Mutations in the PrP gene have been linked to familial forms of the disease like the Gerstmann-Sträussler-Scheinker syndrome, the familial Creutzfeldt-Jakob disease, and the fatal familial insomnia. The nuclear magnetic resonance structure of mouse prion reveals an -helical structure and a -sheet strand. [25] The conversion of the helical structure into a -sheet secondary structure represents one of the key events of prion disease, but in this case, at variance with other forms of amyloidosis, the interaction of PrP^{SC} with PrP^{C} induces the transition of nonpathologic PrP^{C} into PrP^{SC}, thereby providing a mechanism of transmission of the disease. [26]

Transthyretin

In certain hereditary systemic amyloidoses (previously termed *familial amyloid polyneuropathy* [FAP]) and senile systemic amyloidosis, the amyloid deposits contain transthyretin (TTR). [27] TTR is a plasma protein that carries thyroxine (T_4) directly and retinol indirectly by binding to the retinol-binding protein. The normal wild type forms fibrils in senile systemic amyloidosis, but most of the variants are associated with an earlier onset and aggressive form of the disease. TTR forms amyloid fibrils in vitro on partial acid denaturation and during pH-mediated reconstitution from a partially unfolded state. [28] Extensive biophysical studies on both the wild type and a few FAP variants delineate a fibrillogenic pathway characterized by a rearrangement of the tetrameric structure and dissociation into a monomeric amyloidogenic intermediate with a native secondary but an altered tertiary structure. [29] A model of the TTR fibril structure has been proposed by Blakes group in Oxford on the basis of x-ray diffraction of the natural homozygous Met30 TTR fibrils. [30] This study defined a novel structure for the amyloid fibril composed of helical sheets that can be continuously hydrogen bonded over long distances along the fibril axis. When the functional activity of amyloidogenic TTR variants was investigated, it was demonstrated that some of them show reduced T_4 affinity, [31] and one TTR variant was not able to bind the retinol-binding protein. [32] Recent data seem to demonstrate that the binding of TTR to T_4 can inhibit in vitro fibrillogenesis of both wild-type and amyloidogenic variants through stabilization of the quaternary structure. [33]

Amyloid A Protein

Amyloid fibrils in secondary amyloidosis and familial Mediterranean fever consist of a protein known as amyloid A (AA) protein. AA protein is a single polypeptide chain of approximately 8 kd that contains 76 residues corresponding to the N-terminal portion of serum AA protein (SAA). SAA in plasma is produced by hepatocytes under the transcriptional regulation of interleukin (IL)-1, IL-6, tumor necrosis factor, and other signal transducers. Serum concentrations can rise from a normal level of 1 gml up to 1,000 times higher within 2448 hours after an inflammatory stimulus. Circulating SAA is the precursor of amyloid fibril AA protein, from which it is derived through proteolytic digestion. It is not known whether the process of fibrillogenesis necessarily requires protein cleavage or if this proteolytic rearrangement occurs after fibrillar aggregation. A persistently high level of circulating SAA is a necessary condition for the deposition of AA amyloid, but it is not known why at similar levels of SAA amyloidosis develops in some people and not in others. It is possible to induce an experimental form of amyloidosis in mice by inflammatory stimulation with silver nitrate associated with a complex of molecules called amyloid enhancing factors, whose composition is not completely clarified, but which certainly contain ex vivo AA amyloid fibrils. This has been the most extensively used in vivo amyloid model for testing drugs and verifying various pathogenic hypotheses. [34]

Lysozyme

Lysozyme is a bacteriolytic glycosidase discovered by Fleming in 1922. In 1993, the Pepys group described in two English families a form of hereditary, systemic, non-neuropathic amyloidosis in which the fibrillar protein was constituted by lysozyme variants presenting the Asp67-His mutation in one family and Ile56-Thr in the second. [35] The structure, function, and folding properties of the two amyloidogenic lysozyme variants have been described. Both have a less stable native fold than the wild type. They aggregate to form amyloid fibrils with transformation of the mainly helical native fold, observed in the crystalline structure, to the amyloid fibril cross- fold. Biophysical studies suggest that a partly folded intermediate is involved in fibrillogenesis; this form presents a different structure from the native and unfolded states and has the characteristics of the molten globule state. [36]

Cystatin C

In patients with Icelandic-type hereditary cerebral amyloid angiopathy, the fibrillar protein is constituted by a variant of cystatin C with Leu68-Gln mutation. This protein, known as trace, is an inhibitor of the cysteine proteinases and consists of a single nonglycosylated polypeptide chain of 120 residues. Dimerization of the variant is temperature dependent, and increasing the incubation temperature from 37°C to 40°C results in a 150% increase in the dimerization rate. These biochemical data demonstrate reduced folding stability in this amyloidogenic protein as well, and have direct clinical relevance because therapy to abort febrile periods in carriers of the disease trait may reduce the in vivo formation of cystatin C aggregates. [37]

Gelsolin

Gelsolin is an ubiquitous cytoplasmic protein that binds actin monomers, nucleates actin filament growth, and severs actin filaments. The variants of gelsolin carrying the Asp187-Asn or the Asp187-Tyr mutation are responsible for hereditary systemic amyloidosis, in which the amyloid deposits are mainly located in blood vessels and basement membranes. The major protein component of the fibrils is a 71-amino-acid fragment containing the actin-binding domain (173243) of secreted plasma gelsolin.

$_2$ -Microglobulin

$_2$ -Microglobulin ($_2$ -m) is a nonpolymorphic, single-chain protein of 99 residues with a single intrachain disulfide bridge. Its level increases dramatically in hemodialyzed patients, and after a few years at a persistent serum level of 4070 mg/l, it precipitates as a fibrillar aggregate. Self-aggregation of unmodified $_2$ -m into a fibrillar structure can occur at physiologic pH and low ionic strength, but the fibril yield is scanty in the method of Connors et al, [38] and most of the protein precipitates in an unstructured aggregate. Other, as yet undetermined factors are involved in making the in vivo transition from the native protein to amyloid fibrils more productive and more efficient. A contributing effect of peripheral blood mononuclear cells has been suggested in experiments on $_2$ -m fibrillogenesis in cell culture. [39] Structural modifications like limited proteolysis [40] and glycation [41] are associated with the pathologic turnover of $_2$ -m in hemodialyzed patients, [42] but their relevance to an increased propensity of this protein to make fibrils is uncertain.

Apolipoprotein AI

Apolipoprotein AI (ApoAI) is the most abundant of the high-density lipoprotein particles. Variants of ApoAI are extremely rare, and some of them have been associated

with amyloidosis.

Interestingly, all mutations introduce an extra positive charge in the amino acid sequence. [43] Recently, two separate families affected with ApoAI amyloidosis have been described in which a deletion mutation of the ApoAI gene was responsible for the expression of amyloidogenic ApoAI. [43] The main protein component in the fibrils is constituted by a (182/92) N-terminal fragment associated with a minor component of the complete molecule that is made up of 243 residues. It is unknown whether the mutations principally cause a perturbation of folding stability or an increased susceptibility to proteolytic cleavage.

Fibrinogen

Non-neuropathic renal amyloidosis can be caused by variants of the fibrinogen chain. The mutations reported thus far affect residue 526, where Glu is replaced by Val, and residue 534, where Arg is replaced by Leu, whereas the deletion of a single residue at the third base of codon 524 results in a frame shift and premature termination of the protein at codon 548. [44] Three families have been described with A chain mutations (Val 526 and Leu 554), without any evidence of a clotting disorder, whereas in a subject with a frame-shift mutation, the level of fibrinogen was lower than in noncarrier mutations, and thrombin time and reptilase clotting time were also prolonged.

Endocrine Amyloid

Polypeptide hormones such as atrial natriuretic factor, insulin, and amylin have fibril-permissive sequences and can easily assume the -pleated sheet conformation. [34] Polypeptide hormones are highly concentrated in secretory granules at the site of their release, and high concentrations may favor protein fibrillogenesis.

Common Constituents

Human Serum Amyloid P Component

Human serum amyloid P component (SAP) binds to all forms of amyloid fibrils and constitutes the most common nonfibrillar protein of amyloid deposits. SAP is a member of the pentraxin family, which includes C-reactive protein, and belongs more generally to the category of animal lectins. [45] The quaternary structure of SAP is formed by 10 identical, noncovalently associated subunits of æ25 kd each, arranged in two pentameric, disklike rings interacting face to face. SAP is a calcium-dependent, ligand-binding protein that binds specific carbohydrates like the 4,6-cyclic pyruvate acetal of -galactose as well as DNA, glycosaminoglycans, and all known types of amyloid fibrils. The physiologic function of SAP is not completely known; however, no deficiency has been described, and it is stably preserved in evolution. SAP is synthesized by hepatocytes and its plasma concentration varies in normal subjects between 8 and 55 mg/l. SAP level remains unchanged in patients affected with amyloidosis of any type. The protein is extremely resistant to proteolytic attack, and even though it probably is not a proteinase inhibitor, it can protect amyloid fibrils from degradation. A functional role in amyloidogenesis has been reported by the Pepys group in experimental murine amyloidosis. [46]

Glycosaminoglycans

That carbohydrates are a part of amyloid deposits has been known for more than a century, and this characteristic is still responsible for the term *amyloid*, which means starchlike. Fibrils isolated by the water extraction procedure and separated from other unrelated tissue components contain 12% by weight glycosaminoglycans. Immunohistochemical studies demonstrate the presence of proteoglycans in the core of amyloid deposits, but in isolated ex vivo fibrils, the glycosaminoglycans are constituted by free polysaccharide chains only. The role played by proteoglycans or glycosaminoglycans in amyloidogenesis is uncertain, but levels of perlecan (a heparan sulfate proteoglycan) increase in the spleen during the induction of AA amyloid in mice, [47] and studies with gold-labeled antibodies to protein core have shown that intact proteoglycan is part of the amyloid fibril itself and that glycosaminoglycans are deposited in a periodic fashion along the fibril. [48] The composition of glycosaminoglycans in amyloid deposits is restricted to heparan sulfate and dermatan sulfate, and their tissue content is strictly correlated with the amount of the amyloid deposits. A direct effect of glycosaminoglycans in the conversion of proteins with different structures to a common anti-parallel -sheet secondary superstructure has been hypothesized and demonstrated, at least in in vitro experiments. [49]

Other Common Constituents

Apolipoprotein E4, $_2$ -macroglobulin, $_1$ -antichymotrypsin, extracellular matrix proteins, and protease inhibitors like inter--trypsin inhibitor have been found to be associated with amyloid deposits. Their role in amyloidogenesis is under investigation. Particular interest has been raised by ApoE. In fact, it has been established that subjects carrying the allele E4 have an increased risk for development of Alzheimer disease in an allele dose-dependent manner. The ApoE4 genotype is correlated with younger age at onset of the disease, and there is a correlation between the ApoE4 genotype and increased deposition of A in blood vessels and plaques. The biochemical mechanisms by which ApoE modulates the onset and progression of the disease are unknown. [50]

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CLINICAL CHARACTERISTICS

The clinical manifestations in this group of diseases are extremely polymorphic, and depend on the specific organ involved and the extent of resultant organ dysfunction. Amyloidosis (both AL and AA) and L/HCDD should be suspected in patients with unexplained renal disease, especially those with nephrotic syndrome and hepatosplenomegaly, in the setting of underlying plasma cell dyscrasia or chronic inflammatory disease, including rheumatic diseases. L/HCDD and AL amyloidosis should also be considered in patients with known monoclonal protein in the serum or urine, and congestive heart failure, unexplained peripheral neuropathy, weight loss, orthostatic hypotension or malabsorption. Other useful clinical clues in patients with AL amyloid are the presence of macroglossia (Fig. 771), cutaneous purpura (particularly periorbital; Fig. 772), and a history of carpal tunnel syndrome. AA amyloidosis

Figure 77-1 Macroglossia. Note the indentations on the margins of the tongue and dental erosions.

Figure 77-2 Characteristic facial appearance showing periorbital and facial purpura in a patient with AL amyloidosis.

is associated with chronic inflammatory or infectious diseases. Candidates for hereditary or familial amyloidosis are those with a family history of neuropathy with early sensorimotor dissociative neuropathy, carpal tunnel syndrome, vitreous opacities, and unexplained renal or cardiac disease. Finally, patients on chronic hemodialysis for over 5 years in whom carpal tunnel syndrome and bone lesions develop are suspect for $_2$ -m amyloidosis (A_2 m).

Primary Amyloidosis

Epidemiology

Primary (AL) amyloidosis represents the most common form of systemic amyloid deposition in the Western world, accounting for up to two thirds of cases in large series, depending on the referral pattern. [51] In Olmsted County, Minnesota, the overall age- and sex-matched annual rate of AL per million person-years was 8.9. [52] The median age at diagnosis in AL amyloidosis is about 60 years, with a 2:1 male predominance. The incidence of AL amyloid in the setting of myeloma is also variable, and at our institution, 38% of patients with myeloma entered on a prospective clinical trial had a positive screening fat aspirate for amyloid. [53]

Presenting Manifestations

The most common presenting features are weakness or fatigue and often marked weight loss in over half the cases. [51] Peripheral edema may be due to congestive heart failure or hypoalbuminemia as a result of nephrotic syndrome or protein-losing enteropathy. Paresthesias, orthostatic dizziness, and syncope are often noted in patients with peripheral or autonomic neuropathy. In some instances, hoarseness or a change in voice (frequently becoming weaker later in the day) may suggest amyloidosis. Purpura or other skin manifestations may be observed in 510 % of cases. In a recent series of 474 patients with AL amyloidosis, the clinical presentation could be classified as one of the following: nephrotic/renal failure (28%), congestive heart failure (17%), carpal tunnel syndrome (21%), peripheral neuropathy (17%), orthostatic hypotension (11%), macroglossia (9%), and hepatomegaly (24%). [51]

Organ Involvement

Cardiac

Symptomatic cardiac involvement occurs in 2550% of patients with AL amyloidosis. [51] Intractable congestive heart failure may be an initial presentation and is a major cause of disease-related mortality. Cardiac manifestations reflect predominantly myocardial amyloid deposition, and patients usually present as restrictive cardiomyopathy with diastolic dysfunction, or, less commonly, as dilated cardiomyopathy with systolic dysfunction. Rarely, endocardium, pericardium, or valves may be involved, leading in some cases to cardiac tamponade, valvular dysfunction, or rupture of myocardium. Patients may also present with ischemic heart disease due to amyloid deposition in intramyocardial arteries. [54] Electrocardiographic abnormalities include low-voltage QRS complexes in the limb leads or a pseudoinfarction pattern. Both conduction defects and atrial or ventricular tachyarrhythmias are common and may contribute to the increased incidence of sudden cardiac death in these patients. Echocardiography is the most valuable diagnostic tool in the evaluation of cardiac amyloid. [55] [56] Increased myocardial echogenicity with a granular sparkling appearance is almost pathognomonic, but is rarely seen. The major echocardiographic features are increased ventricular wall thickness, left atrial enlargement, and diastolic dysfunction. Early cardiac amyloidosis is characterized by abnormal relaxation, whereas in more advanced cases, restrictive hemodynamics with a shortened deceleration time (<150 msec) is noted. [56] In patients with cardiac amyloid and no serum or urine M component, it is critical to distinguish between AL and ATTR because the latter is associated with a much better prognosis. [57] Vascular involvement in AL may be manifest as arm, calf, or in some instances as jaw claudication due to involvement of the temporal artery.

Renal

Renal involvement with consequent nephrosis or renal failure is a major clinical problem in systemic AL. More than half the patients with AL amyloidosis have significant proteinuria exceeding 1 g/day. [58] Nephrotic range proteinuria (>3 g/day) with consequent hypoalbuminemia and dyslipidemia is present in approximately one third of patients at diagnosis. [51] Frank renal insufficiency (serum creatinine >2 mg/dl) was present in 16% of patients at onset in our series. Hypertension is rare, as is gross hematuria. Renal vein thrombosis, nephrogenic diabetes insipidus, renal tubular acidosis, abnormalities in renal vessels, and priapism have been described. The kidneys are usually enlarged, but a great variation in size has been reported. Bence Jones protein of the type are associated with greater renal impairment and degrees of proteinuria than light chains. [59] Most renal amyloid affects glomeruli and vascular structures, although blood vessels or the interstitial area are involved in a small number. Prognosis correlates more with serum creatinine than the level of proteinuria. [59] Adrenal insufficiency due to amyloid deposition may coexist in a significant proportion of patients with renal failure requiring dialysis.

Neurologic

Sensorimotor peripheral neuropathy may be a presenting manifestation in approximately 15% of patients with AL amyloidosis

and may eventually be manifest in up to a third. It is usually a distal, symmetric, progressive, predominantly sensory neuropathy that generally involves the lower extremities.[51] [60] Autonomic dysfunction is often associated with peripheral neuropathy and may present as gastrointestinal motility disturbances, orthostatic hypotension, bladder dysfunction, impotence, abnormalities of sweating, or a chronic Guillain-Barré-type syndrome. [61] Cranial neuropathy is rare, but may be the presenting manifestation.[62] Axonal degeneration with predominant involvement of small myelinated and unmyelinated fibers is seen histologically. Carpal tunnel syndrome may be the presenting feature in 20% of cases with AL, and the possibility of AL must be considered in any patient with M protein and carpal tunnel syndrome.

Gastrointestinal and Hepatosplenic

Liver involvement, clinically manifest as hepatomegaly, is a common occurrence in AL amyloidosis, present in up to two thirds of patients in autopsy studies. Hepatomegaly may be striking and is often disproportionate to the extent of liver enzyme abnormalities. Serum alkaline phosphatase and transaminases are elevated in a third of patients; however, hyperbilirubinemia is infrequent and, when present, represents an ominous prognosis. [51] [63] Portal hypertension may develop because of sinusoidal involvement. Most patients also have extrahepatic involvement. Splenic involvement may lead to functional hyposplenism and spontaneous splenic rupture.[64] Histologic involvement of the gastrointestinal tract is common, but is usually asymptomatic. Symptoms are present in a third of patients and may result from direct amyloid infiltration or autonomic neuropathy. Clinical manifestations of gut involvement include obstruction, ulceration, hemorrhage, malabsorption, protein loss, and diarrhea.[61] Malabsorption, mostly caused by autonomic neuropathy, is responsible for a reduction of the serum carotene level (<48 g/dl) in 6% of patients and, less frequently, a decrease in the vitamin B_{12} level and steatorrhea. Infiltration of the tongue may lead to macroglossia, which may be striking (Fig. 771) and cause dysphagia, dysarthria, or obstructive sleep apnea.

Pulmonary

Although involvement of the respiratory tract is common, pulmonary dysfunction is usually not a dominant clinical problem. Amyloidosis of the respiratory tract usually occurs in one of three forms: tracheobronchial, nodular parenchymal, or alveolar septal. [65] Tracheobronchial, either nodular (pseudotumoral) or diffuse (multifocal submucosal plaques), and nodular parenchymal types are usually localized forms that rarely become systemic. Alveolar septal amyloid is clinically the most significant and is commonly associated with cardiac involvement.[66] Other pulmonary manifestations include pleural effusion, diaphragmatic weakness due to myopathy, and pulmonary hypertension due to vascular involvement.

Hematologic

The serum M protein concentration is small (median concentration 0.8 g/dl in our series). Using high-resolution serum and urine electrophoresis and immunofixation, it is possible to detect a monoclonal protein in 9095% of patients with AL. [51] [67] Immunofluorescence study of bone marrow using anti- and anti- antisera reveals a monoclonal plasma cell population, even in those rare patients with AL or L/HCDD in whom a monoclonal component is not detectable. [21] [67] [68] However, the plasma cell bone marrow infiltration is usually modest, with a median of 7% in our series, and only 4% of patients had >20% plasma cells. Anemia requiring transfusion is infrequent and, when present, is usually due to renal failure. Functional hyposplenism may lead to thrombocytosis and the appearance of Howell-Jolly bodies in the blood smear.[64] Coagulation abnormalities include prolonged thrombin time in 4050%, [69] factor X deficiency (10%), abnormalities in fibrin formation, and increased fibrinolysis due to defects in antiproteases. Most cases of acquired factor X deficiency have occurred in patients with AL amyloidosis. The deficiency is associated with binding of factor X to amyloid.[70] Splenectomy in such patients can cause a dramatic improvement in the factor X level, presumably by removal of the amyloid-laden tissue that binds factor X.[71]

Miscellaneous

Extensive deposition of amyloid in skeletal muscles may lead to pseudohypertrophy or a frank myopathy. [72] AL amyloidosis can also involve the periarticular structures. The most striking appearance is the well known shoulder pad sign from symmetric swelling of the shoulder joints. [73] Large amyloid deposits in the bone may produce osteolytic lesions and cause pathologic fractures. Amyloid is identifiable in the skin in over half of patients with AL or AA amyloidosis, regardless of whether clinically apparent lesions are present. [74] The lesions vary widely from waxy papules to nodules, tumors, and scleroderma-type changes and may be associated with purpura. Hemorrhagic bullous lesions, ear lesions, lichenoid lesions, dystrophic nail changes, alopecia, and breast lesions have also been reported.

Secondary Amyloidosis

Secondary (AA) amyloidosis (secondary systemic amyloidosis, reactive amyloidosis) is associated mainly with long-standing infectious or noninfectious inflammation and less frequently with cancer, mainly renal cell carcinoma and Hodgkin disease. [75] In many countries, where the incidence of chronic infections such as tuberculosis and leprosy has declined, AA amyloidosis is mostly caused by chronic rheumatic diseases. AA amyloidosis occurs in 410% of patients with rheumatoid arthritis, juvenile rheumatoid arthritis, ankylosing spondylitis, and occasionally Reiter disease and psoriatic arthropathy, but is rare in diseases such as lupus erythematosus and systemic sclerosis, which are associated with a much lower serum concentration of SAA. [76] Other conditions associated with the development of AA amyloidosis include chronic infections such as tuberculosis, leprosy, osteomyelitis, and bronchiectasis; cystic fibrosis, heroin abuse, and Castleman disease. Amyloidosis usually develops as a late complication in long-standing, often poorly controlled rheumatoid arthritis. The most common clinical manifestation is nephropathic, manifest as proteinuria or renal insufficiency. Indeed, the development of proteinuria in patients with rheumatoid arthritis should always raise the suspicion of AA amyloidosis. Involvement of the gastrointestinal system and liver or spleen is common, but clinically significant cardiac involvement is rare. AA amyloidosis may occur in familial Mediterranean fever, an autosomal recessive disease affecting people of Mediterranean extraction, characterized by painful febrile attacks of serositis and gradual development of amyloidosis.[77] [78] It has also been identified in Muckle-Wells syndrome, a familial amyloid nephropathy with urticaria and deafness. [79]

Treatment of AA amyloidosis depends on the underlying disease.[75] Immunosuppressive therapy with low doses of alkylating agents and prednisone may lead to improvement in nephrotic syndrome and prolong survival. Colchicine may also be useful, and is particularly effective in the treatment of acute attacks and prevention of amyloidosis in patients with familial Mediterranean fever. [77] The efficacy of colchicine in this setting appears to be limited to higher doses (>1.5 g/day) and the absence of renal insufficiency (serum creatinine <1.5 mg/dl). [80]

Hereditary Amyloidosis

Hereditary amyloidoses represent diseases in which a genetically inherited variant form of a normal protein constitutes the

amyloid fibril. Systemic forms of hereditary amyloidosis may be associated with variant forms of TTR (ATTR), apolipoprotein A-I (A ApoAI), gelsolin (A Gel), fibrinogen A 1 (A Fib), or lysozyme (A Lys). Hereditary amyloidosis is a late-onset disease with clinical symptoms beginning in most kindreds in the third to seventh decades of life. The clinical disease usually progresses over 515 years, leading to progressive neuropathy, renal failure, or cardiac disease.

Transthyretin Amyloidosis

Most of the autosomal amyloidoses characterized thus far are associated with (over 50) variants of plasma TTR. [27] Clinical presentations of these pathogenic variants can be classified as predominantly neuropathic, cardiopathic, mixed neuropathic and cardiopathic, and carpal tunnel syndrome. [81] However, ATTR may be associated with normal TTR (as in some cases of senile systemic amyloidosis), and not all TTR variants are associated with amyloidosis.

Neuropathic Syndromes

These disorders (previously termed FAP, type I) are characterized by a progressive sensory peripheral neuropathy, mostly involving the lower extremities, with loss of thermal and pain sensations. Autonomic and gastrointestinal disturbances are prominent, and varying degrees of cardiac and renal involvement are noted. Vitreous deposits of amyloid have been reported, as has been the scalloped pupil abnormality due to involvement of the ciliary nerves. [82] The prototype and most common TTR variant involved is methionine 30 (Met30), characterized by substitution of methionine for valine at position 30. [83] Although the largest numbers of kindreds have been described from Portugal and Japan, the Met30 gene has the highest frequency in northern Sweden, where as many as 3% of the population may be heterozygous for this variant.[84]

Mixed Neuropathic and Cardiopathic

In these variants, both neuropathy (predominantly autonomic) and cardiomyopathy are dominant features. Incapacitation is related to bowel disease, which is due to autonomic neuropathy, and death is due to cardiac involvement. The prototype variant, TTR-alanine, [60] was originally described in a large kindred of Irish descent from West Virginia,[85] and is quite common in Northern Ireland, affecting 1% of the population.

Carpal Tunnel Syndrome

In some variants (originally termed FAP type II), carpal tunnel syndrome is a prominent feature.[86] Vitreous opacities are common and cardiomyopathy is the usual cause of death. The variant seen in the original Indiana/Swiss kindred is isoleucine-to-serine mutation at position 84 (Ser84). [87] Since then, other mutations have been described, with some differences in clinical presentations (e.g., lack of vitreous opacities in the histidine 58 variant).

Cardiopathic

A particular group of TTR mutations is characterized by familial cardiomyopathies in which neuropathy is absent. One of these, the isoleucine 122 (Ile122) TTR mutation is present in nearly 4% of the black population in the United States, and may be a cause of increased prevalence of heart failure among older blacks. [88] Another notable variant is the methionine 111 variant described in a Danish kindred with familial amyloid cardiomyopathy. [89]

Senile Systemic Amyloidosis

Small deposits of amyloid in the brain, heart, aorta, and pancreas of elderly patients (>70 years of age) are common. [90] Amyloid deposition in the heart may occur as isolated atrial amyloid consisting of atrial natriuretic peptide, [91] or as senile systemic amyloid, involving normal or variant TTR (ATTR), of which the Ile122 variant is the most notable.[92] Senile cardiac amyloid has been noted in 25% of patients 80 years of age. Extracardiac involvement is seen, but is clinically insignificant. Antemortem recognition in the setting of congestive heart failure is important because the prognosis with ATTR (median survival, 5 years) is much superior to that in AL amyloid (median survival, 6 months).[57]

Miscellaneous Hereditary Forms

Apolipoprotein AI amyloidosis (A ApoAI) has been described in a kindred with lower extremity neuropathy, prominent renal disease, and striking incidence of peptic ulcer disease.[93] Gelsolin amyloidosis (A Gel) has been reported largely from Finland, and is characterized by progressive cranial neuropathy, lattice corneal dystrophy, and skin changes.[94] Mutations in the fibrinogen A and human lysozyme genes have been identified in people with familial autosomal dominant amyloidoses (A Fib A and A Lys, respectively) characterized by dominant renal involvement and absence of neuropathy. [35] [95]

2-Microglobulin Amyloidosis

In 1980 it was reported that carpal tunnel syndrome in patients on long-term hemodialysis was associated with amyloid deposition in the soft tissues of the wrist. [96] These patients experience diffuse arthralgias, and plain radiographs reveal areas of cystic radiolucency in the juxta-articular bones. Although initially described in patients on prolonged (>10 years) hemodialysis, the syndrome has also been noted in patients on peritoneal dialysis and undialyzed patients with chronic azotemia. The fibril subunit protein was characterized as $_2$ -m.[97] Clinical manifestations of $_2$ -m amyloidosis are mostly articular and skeletal, and although systemic deposition does occur, it is usually of little clinical consequence. In established forms, therapy is mostly supportive. Renal transplantation often leads to dramatic improvement in joint symptoms and, when performed early, represents the most effective preventive measure.[98]

Alzheimer Disease and Prion Disorders

Both of these neurodegenerative disorders are characterized by localized amyloid deposits in the brain leading to progressive dementia. Alzheimer disease, the most common form of senile dementia, is characterized by neurofibrillary tangles, senile plaques, and cerebral amyloid angiopathy consisting of amyloid protein (-A4). [99] Clinically, Alzheimer disease is manifest as gradual loss of memory, followed by progressive deterioration of higher cognitive functions and behavior. The major risk factors for Alzheimer disease are age and genetic loci on chromosome 21 and 14, linked to the presence of early-onset disease.

The human prion disorders/spongiform encephalopathies, including kuru, (occurring in Fore tribesmen of New Guinea), Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, and familial fatal insomnia, can occur at any age and display a remarkable spectrum of clinical abnormalities that include cognitive impairments, myoclonus, motor dysfunction, and ataxia.[99] These symptoms reflect spongiform changes, neuronal loss, and gliosis, particularly in the hippocampus, neocortex, subcortical nuclei, cerebellum, and brain stem. A large body of work by Prusiner and others has shown that these illnesses are transmitted by an abnormal cellular protein, termed the scrapie prion protein (PrP^{SC}).[26]

Other Localized Forms

Type II diabetes is associated with amyloid deposition in the pancreatic islets consisting of islet amyloid polypeptide. [100] In Iceland, the amyloid associated with congophilic angiopathy leading to premature strokes and intracranial hemorrhage in the third or fourth decade has been shown to consist of variant cystatin C. [101] A similar disorder in Dutch cohorts is due to variant protein, analogous to the fibril subunit found in Alzheimer disease. [102] In medullary thyroid carcinoma, the amyloid (A Cal) is composed of peptides derived from precalcitonin, and is limited to the thyroid and tumor metastases. Nonfamilial forms of localized amyloidosis commonly involve the lungs, larynx, urinary tract, and skin. Although the amyloid fibrils consist of or light chains, no M protein is seen and the disease almost never becomes manifest systemically.[103] Therapy is thus directed at local symptoms.

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DIAGNOSIS

Histologic

Diagnosis relies on tissue biopsy and microscopic demonstration of typical deposits. The most common diagnostic strategy is to take the biopsy from an easily available tissue known to contain amyloid deposits in most patients with systemic amyloidosis. Small amyloid deposits occur in subcutaneous tissue in most people with AA and AL amyloidosis,[104] and for this reason the preferred initial diagnostic procedure is to obtain an abdominal fine-needle fat aspirate, which was positive in 80% of patients with AL in the Mayo Clinic series [51] and in 93% of our series. This procedure is innocuous, fast, and inexpensive, but requires experience in the staining technique and interpretation before it can be used routinely. This simple procedure has proven useful in AA, AL, and familial ATTR, but not in $_2$-m amyloidosis. [105] Because bone marrow biopsy is usually performed to determine the number and clonality of plasma cells, it should be stained for amyloid because positive findings have been reported in 56% of patients. [51] If both abdominal fat and bone marrow biopsy results are negative, a rectal biopsy, including the submucosal vessels, should be done because the gastrointestinal tract is involved in most cases. This biopsy is positive in 75% of patients with AL. If these three sites are negative, tissue should be obtained from an organ suspected of being affected such as the kidney, liver, or heart, which give a positive result in 94%, 97%, and 100% of cases, respectively.[51] [106] Before doing so, it is advisable to undertake a coagulation screen to exclude an acquired deficiency of clotting factors or other coagulation abnormalities and to consider that patients with systemic amyloidosis are at increased risk of bleeding because of amyloid infiltration of small blood vessel walls. In patients with neuropathy, sural nerve biopsy should be performed only if other, less invasive approaches are negative and the neuropathy is severe, because this procedure results in permanent local sensory loss.

Amyloid is currently defined as fibrils that stain with Congo red and show apple-green birefringence in polarized light. The alkaline-alcoholic Congo red method [107] gives specific and consistent results and remains the reference histochemical test for amyloidosis. It is more reliable than metachromatic stains such as methyl violet and crystal violet, or fluorescent stains such as thioflavin T or S. Congo red staining can be affected by several variables: thickness of the section (optimal is 510 m), the fixative used (more intense in alcohol or Carnoy solution than in formalin), and the freshness of the Congo red solution (which should be prepared every 2 months),[108] and it is therefore recommended that positive and negative controls be included in every stain run. Characterization of the amyloid deposit is necessary for selecting the appropriate treatment and for assessing the prognosis. Chemical (potassium permanganate or alkaline guanidine) treatment of Congo red-stained sections was proposed to subclassify amyloidosis,[108] but these techniques were unreliable and have been replaced by immunochemical methods that use specific antisera against the known amyloidogenic proteins. Antisera to most fibrillar precursor proteins are commercially available and those against AA and $_2$-m give reliable positive results. Antibodies may fail to identify the amyloid protein because the antigenic determinants have been masked or altered during the process of fibril formation or during the tissue fixation and processing phases. Antigenicity is preserved much better in frozen sections than in formalin-fixed, paraffin-embedded tissues. However, structural details are better defined with the latter procedure. In our experience, optimal tissue immunostaining depends in part on the fixation and processing phases, for which short intervals are likely to provide better antigen preservation. [109] Even in optimally processed tissue samples, antigenic epitopes may still be masked, **and** in these cases microwave pretreatment may enhance immunostaining.[109] Immunohistochemical staining of TTR, -protein, and prion protein amyloids may require pretreatment of tissue sections with formic acid or alkaline guanidine or deglycosylation to enhance immunoreactivity. Antiserum to amyloid P component reacts with all amyloid types and may be used to exclude other immunoglobulin deposition diseases.

Electron microscopy allows detection of typical fibrils, which are rigid, nonbranching, and 7.510 nm wide; however, as stated previously, the final diagnosis requires Congo red staining with apple-green birefringence. Ultrastructural immunolabeling may provide definite and reliable results even in cases not otherwise characterized,[110] and it can also be successfully applied to abdominal fat aspirate. [109] In our experience, it has also proven useful in the rare instances of mixed deposits constituted by two amyloidogenic proteins (e.g., AA and light chains). Electron microscopy is useful in distinguishing typical amyloid fibrils from nonamyloid Congo red-negative fibrillar immunoglobulin deposits that occur mainly in the kidney, determining so-called immunotactoid glomerulopathy, [111] or, rarely, systemically. L/HCDD presents several clinical features common to amyloidosis and should be considered in the differential diagnosis. This condition is usually diagnosed on kidney biopsy because of its predominant renal involvement. Light microscopy frequently shows nodular glomerulosclerosis with thickening and wrinkling of the tubular basement membranes.[21] [112] [113] However, morphologic findings at light microscopy are often noncharacteristic, and the use of both electron microscopy and immunohistochemistry has been proposed for a reliable diagnosis of L/HCDD. [110] [113] Immunohistochemical studies using antisera to and light chains and immunoglobulin heavy chain reveal characteristic linear deposits along the tubular and glomerular basement membranes, in the mesangium, and along the basement membrane of Bowman capsule. Electron microscopy demonstrates finely or coarsely granular electron-dense deposits delineating the inner aspect of the glomerular and the outer aspect of the tubular basement membrane. Combinations of amyloid and L/HCDD have been reported. Amyloid typing can also be performed through extraction of fibrils from diagnostic biopsy tissues (even after formalin fixation) and subsequent chemical and immunologic characterization. Several methods suitable for the extraction and characterization of microgram amounts of amyloid deposits have been reported. [114] [115]

Nonhistologic

Radiolabeled human SAP component has been shown to localize rapidly and specifically in amyloid deposits, allowing their visualization and quantification by scintigraphic imaging and metabolic analyses. [116] Scans determine the organ distribution and quantity of amyloid, and turnover studies provide an estimate of the whole-body amyloid burden.[117] [118] However, major limitations are limited availability and poor sensitivity for detection

DIAGNOSTIC WORK-UP OF A PATIENT WITH SUSPECTED AMYLOIDOSIS

The presence or development of nephrotic syndrome, restrictive cardiomyopathy, peripheral and autonomic neuropathy, muscle weakness, carpal tunnel syndrome, giant hepatomegaly, malabsorption, rapid and important weight loss, cutaneous purpura, especially periorbital purpura, and macroglossia in a patient with serum or urinary M protein, should alert the clinician to the possibility of AL amyloidosis. Other forms of amyloidosis have slightly different clinical presentations. For example, in reactive AA amyloidosis, the finding of nephrotic syndrome, occasionally associated with hepatosplenomegaly in a patient with a chronic inflammatory disorder, is the most indicative clinical sign. Peripheral or autonomic neuropathy in combination with any of the following conditionsproteinuria, renal failure, restrictive cardiomyopathy, carpal tunnel syndrome, or vitreous opacitieswarrants consideration of hereditary amyloidosis. Taking an accurate family history is most important. However, a negative history should not rule out hereditary amyloidosis, given the incomplete penetrance of most transthyretin variants and the possible late onset of the disease.

Clinical suspicion must be confirmed by biopsy documentation of amyloid deposits. The site of biopsy should be chosen according to the accessibility and presence of amyloid deposits. For AL amyloidosis, the recommended strategy is as follows: abdominal fat and bone marrow should be sampled first. If the results are negative, a rectal biopsy, including the submucosa, should be performed; if this is negative, a biopsy of a suspected involved organ should be made after checking the coagulation screen. Congo red staining is the reference histochemical method for documenting amyloid.

Typing of amyloid deposits is of utmost importance for treatment and prognosis assessment. In AL, it is necessary to document the presence of an M protein in the serum or urine, or a monoclonal bone marrow plasma cell population, or the presence of light chains as the major constituents of amyloid deposits by immunohistochemical methods. Light and electron microscopic immunohistochemical studies allow amyloid protein characterization in most tissue samples. Micromethods for the extraction and characterization of amyloid protein from diagnostic biopsy specimens are also available. In patients with hereditary amyloidosis, the pathogenic mutation(s) should be identified through DNA testing to optimize treatment and allow genetic counseling.

Once the diagnosis and type of amyloid have been established, it is necessary to determine the extent of systemic damage caused by amyloid deposition. In AL amyloidosis, evaluation of renal function (serum creatinine, creatinine clearance, and 24-hour urinary protein) and cardiac involvement (echocardiogram, with measurement of interventricular septal thickness and ventricular deceleration time, and a baseline 24-hour Holter recording) should be undertaken even in absence of specific symptoms. In patients with peripheral neuropathy, an electromyogram is indicated. In patients with liver involvement, a computed tomography scan or ultrasound of the liver and spleen is obtained to quantitate hepatomegaly. Patients with diarrhea or malabsorption should have a determination of fecal fat and serum carotene. Patients with suspected autonomic neuropathy should be evaluated with orthostatic blood pressure, sudomotor axon reflex test, and gut motility studies, depending on symptoms. If available, radiolabeled SAP can be used for locating and monitoring the extent of systemic amyloidosis.

of cardiac involvement. More recently, it has been reported that radiolabeled aprotinin (an inhibitor of serine proteases) [119] specifically targets amyloid deposits in vivo and shows particular sensitivity for detecting heart involvement. Echocardiography is a valuable technique for the detection and monitoring of amyloid heart deposits.[55] [120] [121] If combined with electrocardiography, which shows low-voltage complexes, it can provide reliable diagnostic hints. [122] High-resolution echography and magnetic resonance imaging are useful in evaluating amyloid involvement in soft tissues, in organs (heart, gastrointestinal tract, liver, lung, thyroid), [123] and especially in bones (with magnetic resonance imaging). [124] In patients and families with hereditary amyloidosis, analysis of leukocyte DNA using restriction fragment length polymorphism analysis, polymerase chain reaction-based methodology, or direct sequencing is valuable for genetic counseling and identification of carriers. [27]

THERAPY

Principles

Recent advances in our understanding of the pathogenic mechanisms of amyloid formation have provided the basis for a more rational and, it is hoped, more effective treatment strategy for systemic amyloidoses. From the pathogenic cascade outlined in Table 772 , it is possible to identify several potential targets and possibilities for intervention. The obvious primary goal of therapy should be the inhibition or suppression of the synthesis of the amyloidogenic precursor protein or, if protein synthesis is constitutive, enhancement of its clearance. This approach has proven feasible and effective in several types of amyloidosis. In reactive AA amyloidosis, aggressive treatment of the underlying chronic inflammatory disorder can dramatically reduce the level of the SAA precursor and results in amyloid regression and restoration of damaged organ function.[125] Timely surgical excision of localized Castleman disease producing IL-6stimulated SAA synthesis results in complete recovery of severe nephrotic syndrome.[126] In hereditary TTR amyloidosis, liver transplantation, even partial liver transplantation from living donors, [127] eliminates the production of the variant pathogenic protein, improves the polyneuropathy, and offers the hope of arresting the progression of the disease. [128] [129] [130] In AL amyloidosis, more aggressive therapies against the amyloidogenic plasma cell clone with high-dose chemotherapy or dexamethasone have resulted in dramatic clinical improvement in a few patients (reviewed in Dhodapkar et al [131]). In constitutively synthesized protein such as $_2$ -m in chronic hemodialysis patients, the aim should be to reduce its plasma concentration by increasing its clearance. In fact, successful kidney transplantation lowers the plasma concentration of $_2$ -m to normal levels and is associated with rapid improvement of osteoarticular symptoms. [132]

In some types of amyloidosis, natural proteolytic modification of the precursor is required to render it amyloidogenic. This process appears particularly relevant in Alzheimer disease because of the putative activity of - and -secretases. Inhibitors of the activity of these proteases are being intensively pursued. [133] Another potential target is represented by structural modifications of the amyloidogenic precursor and its interactions with tissue components during the process of amyloid fibril formation and tissue deposition. Drugs that can stabilize the structure of the precursor, thereby reducing its tendency to abnormal aggregation, and ones that interfere with fibril growth are being

TABLE 77-2 -- Therapeutic Strategies for Amyloidosis: The Potential Targets and Possibilities for Intervention Are Derived from the Pathogenic Cascade

Pathogenic Event	Possible Target (Compounds Tested)	Available Intervention
Synthesis of precursor	Inhibit or suppress synthesis	In AL: chemotherapy (including high-dose therapy) dexamethasone
		In ATTR: liver transplantation
		In AA: suppress the chronic inflammation
Clearance of precursor	Promote the clearance of the precursor	In A_2 -m: kidney transplant
Processing of amyloid precursor	Inhibit the enzymes involved (putative inhibitors of - and -secretases in A)	
Abnormal self-aggregation	Interfere with self-aggregation (cyclodextrin, rifampicin, specific peptides, and other compounds in A)	
Interaction with common components and other enhancers of amyloid formation and deposition (AEF)	Inhibit amyloid precursor/common component interactions	
	(Congo red in prion diseases, IAPP, and A)	
	(Aliphatic sulphonates and sulphates in A and AA)	
	(I-DOX in AL and prion diseases)	
	Inhibit generation of AEF	Colchicine in AA caused by familial Mediterranean fever
Persistence of amyloid deposits	Promote mobilization of amyloid deposits (I-DOX in AL and prion diseases?) (Putative compounds displacing serum amyloid P component or proteoglycans from amyloid fibrils)	Dimethylsulfoxide (topical application)
Organ structural and functional damage	Preserve organ function	Supportive therapy
	Replace organ function	Dialysis
		Kidney and heart transplantation

AA, amyloid A protein; A, amyloid A4 protein; A_2 -m, amyloid $_2$ -microglobulin; AEF, amyloid-enhancing factors; AL, primary (immunoglobulin light chain) amyloidosis; IAPP, islet amyloid polypeptide; I-DOX, 4-iodo-4-deoxydoxorubicin; TTR, transthyretin.

actively sought (reviewed in Kisilevsky [133]). Studies on putative inhibitors of amyloid fibril formation have shown that different compounds (e.g., cyclodextrins, [134] Congo red,[135] and rifampicin[136]) may have these properties. It has also been reported that a peptide that binds specifically to amyloid protein precursors (amyloid -peptides) inhibits their polymerization into amyloid fibrils. [137] Drugs that can inhibit the interaction of amyloid precursor with basement membrane components and with other common components, and thus inhibit the process of amyloid formation and deposition, have been designed and tested successfully in vitro and in animal models.[133] [138]

Contrary to commonly held belief, amyloid is not an inert deposit. Both experimental and clinical data have shown that deposits can be reabsorbed once the supply of precursor is stopped.[117] [126] [139] [140] Thus, agents capable of promoting amyloid resorption may have an important role in the combined treatment of amyloidosis. Displacement of some protective components, such as SAP or proteoglycans, from amyloid fibrils may render them more susceptible to proteolytic degradation. [43] [141] Amyloid deposition causes severe organ damage and dysfunction, ultimately leading to death; for this reason, supportive and replacement therapy for damaged organs represents the mainstay of management of amyloidosis because it has an important impact on quality of life and survival.

Supportive Therapy

Supportive therapy is of crucial importance in the management of patients with amyloidosis because it preserves or palliates vital organ function, improves the quality and duration of life, and buys time while specific therapy is acting. Nephrotic syndrome should be managed with salt restriction and careful administration of diuretic agents. Furosemide is the first-choice diuretic; and may need to be combined with spironolactone or metolazone. [142] Albumin can be combined with diuretics to improve diuresis in patients with pleural and pericardial effusions. Great care should be taken to avoid too rapid or excessive volume depletion, which can aggravate postural hypotension and renal function. Symptomatic azotemia requires dialysis. Hemodialysis and peritoneal dialysis are equally effective and can be interchanged if needed.[143] [144] Patients with slow disease progression and those without multisystem involvementof the heart in particularobtain long-term benefit from dialytic treatment.[58] [143] Patients with these characteristics also represent the best candidates for kidney transplantation. [142] This procedure has been performed mostly in patients with AA type (familial Mediterranean fever or reactive), whereas a limited number of patients with AL have received transplants. Although amyloid recurrence in the grafted organ is well documented, in general it has modest clinical impact, [145] making renal transplantation a viable option in the treatment of young patients with amyloid end-stage renal disease without cardiac and gastrointestinal involvement.

Amyloid congestive heart failure should be managed with salt restriction and very careful administration of diuretics, such as furosemide, to avoid excessive volume contraction, which results in syncope and worsening of renal function. Afterload reduction with low-dose angiotensin-converting enzyme inhibitors is useful, if orthostatic hypotension is not present. Digitalis should be used with caution only in the rare patients with atrial fibrillation and rapid ventricular response, because patients with amyloidosis are unusually sensitive to the drug, which may cause arrhythmias or heart block. Calcium channel blockers may worsen congestive heart failure. In patients with atrial systolic failure due to amyloid infiltration, atrial standstill may predispose to systemic arterial embolization, and prophylactic anticoagulation should be carefully considered. [146] Ambulatory electrocardiographic monitoring is useful because pacemaker installation for severe conduction abnormalities and antiarrhythmic agents for ventricular arrhythmias may be life-saving

PREFERRED APPROACH TO THERAPY IN AL AMYLOIDOSIS

The aim of treatment is the elimination of the cause of the disease, the amyloidogenic bone marrow plasma cell population, and all efforts should be made to reach this goal. However, any reduction in the concentration of the amyloid precursor can be beneficial because this leads to less amyloid deposition and may shift the balance between amyloid deposition and resorption in favor of the latter. Early diagnosis and rapidly effective therapy are the two most important factors. These patients have a narrow window of therapeutic opportunity in view of limited survival, and all efforts must be made to extend this window through supportive therapy and to exploit fully the limited time through rapidly effective therapies.

We treat patients in acceptable general medical condition initially with pulsed high-dose dexamethasone or dexamethasone-based combination therapy for three cycles, with salt restriction, H_2 blocker, and antibiotic prophylaxis. Subsequently, patients (usually <65 years of age) with acceptable performance status (02) undergo stem cell collection for possible high-dose chemotherapy and autologous stem cell transplantation. Patients responding to the dexamethasone induction (particularly those with hepatomegaly and nephrotic syndrome) are switched to maintenance interferon- and dexamethasone. Patients who fail to respond to this regimen and who are candidates for high-dose therapy receive high-dose melphalan with autologous peripheral blood stem cell support. Selected patients without severe multiorgan involvement may be considered for allogeneic bone marrow transplantation. All other patients should be treated with melphalan and prednisone. Patients who fail initial anti-plasma cell approaches are considered for other experimental therapy, such as I-DOX. Supportive therapy is of crucial importance in the comprehensive treatment of patients with AL amyloidosis. Dialysis support is necessary in patients with end-stage renal failure. Kidney and heart transplantation should be offered to eligible patients (see the Supportive Therapy section). All other available means, from pacemakers to total parenteral nutrition, should be used as indicated.

(reviewed by Merlini [142]). Cardiac transplantation should be considered in patients younger than 60 years of age with severe heart involvement, preserved renal function, and no evidence of other symptomatic organ involvement or multiple myeloma.[147] [148] Although survival after heart transplantation in amyloidosis in one report was inferior to that in patients transplanted for other indications, [149] transplantation may significantly prolong life in properly selected patients and allow initiation of specific therapy.

Orthostatic hypotension may be helped by simple measures such as rising slowly and sitting for a few minutes before standing up, and wearing elastic stockings. Orthostatic hypotension in FAP benefits from a norepinephrine precursor (e.g., L-threo-DOPS). [150] [151] No trial results have been reported in patients with AL. Fludrocortisone may improve hypotension but often induces fluid retention.

Peripheral neuropathy may be relieved somewhat by amitriptyline and codeine. [142] Median nerve entrapment at the carpal tunnel may be relieved by surgery. Pain from periarticular deposition of amyloid in the shoulders and knees is usually refractory to local steroid injections, but dramatic improvement has been reported after radiosynovectomy.[152] Nodular cutaneous amyloidosis can be treated with satisfactory cosmetic results by dermabrasion. [153] Macroglossia may produce airway obstruction requiring tracheostomy. Obstructive sleep apnea may be improved by applying nasal continuous positive airway pressure. [154] Macroglossia can hamper chewing and swallowing, and necessitate a liquid diet. Gastroparesis can be effectively treated with metoclopramide or domperidone. Diarrhea may be controlled with a long-acting somatostatin analog, octreotide. [142] Severe pseudo-obstruction caused by amyloid autonomic neuropathy may require total parenteral nutrition support. In general, surgery should be avoided in patients with amyloidosis because it may have catastrophic effects by exacerbating organ failure and bleeding. Bleeding in amyloidosis is multifactorial, and its treatment should be individualized. Parenteral administration of vitamin K may be beneficial in patients with malabsorption. Splenectomy may resolve bleeding due to factor X deficiency and multiple hemostatic defects. [71] Patients with bleeding due to increased fibrinolysis can be treated with epsilon-aminocaproic acid or tranexamic acid. [142]

Specific Therapy for AL Amyloidosis and L/HCDD

Current therapeutic approaches for AL amyloid and L/HCDD are largely targeted to the underlying plasma cell clone. Conventional alkylating agent-based chemotherapy has been shown to effect a response, although the proportion of patients who benefit is still unsatisfactory. Results of the first randomized, double-blinded trial of melphalan and prednisone (MP) versus placebo involving 55 patients suggested that chemotherapy was of some clinical benefit, although survival rates did not differ significantly between the two groups. [155] A subsequent study performed by the same group on 153 patients with AL treated with MP reported a response rate (defined as evidence of regression of organ dysfunction caused by amyloidosis) of 18%. [156] Responses were attained in a median time of 1 year. Responders experienced a survival benefit, with a median survival of 89 months, versus 14 months in nonresponders. Patients with nephrotic syndrome benefited most (39%), and 15% of patients with amyloid cardiomyopathy, usually associated with a very poor prognosis, also responded. [156] Prolonged treatment with alkylating agents can induce cytogenetic abnormalities. In 10 of these 153 patients (6.5%), myelodysplastic syndrome or acute myeloid leukemia developed, and 8

died as a direct result of pancytopenia. The actuarial risk for development of myelodysplastic syndrome in patients surviving 3.5 years was estimated to be 21%. [157]

Small series of patients with L/HCDD treated with MP-based chemotherapy have indicated that this therapy is effective in a small proportion of cases. A retrospective study on 19 patients with L/HCDD showed stabilization or improvement of renal function after chemotherapy in 5 of 8 patients whose serum creatinine concentration was <4 mg/dl, whereas in patients with more advanced renal failure, treatment did not prevent the progression to end-stage renal disease. [158] In a subsequent retrospective study, only 5 of 18 patients treated (10 of whom had multiple myeloma) showed improvement in renal function. [159] The results of this latter study are worse than those reported in other small groups of patients, [112] [160] [161] [162] and may be due to patient selection. This indicates that MP may be most helpful early in the course of disease, when only moderate histologic damage is present. [162] Colchicine has been shown to prevent reactive AA in laboratory mice, to prevent or abort the painfully disabling febrile attacks, and to prevent secondary amyloidosis in patients with familial Mediterranean fever. [80] In view of these data, its use was advocated in patients with AL as well. However, the results of three large studies comparing the effectiveness of MP versus colchicine in patients with AL have consistently shown better outcomes in patients treated with MP. [163] [164] [165] In all three studies, the patients were stratified

according to their dominant clinical manifestation (i.e., renal disease, cardiac involvement, peripheral neuropathy, and other manifestations). In a prospective cross-over study involving 101 patients that compared MP with colchicine, there was no significant difference in median survival between the two groups (MP, 25 months; colchicine, 18 months). [165] However, significant differences in favor of MP were found when the survival of patients who received only one of the treatments was analyzed, or when survival was calculated from the time of entry into the study to the time either of death or disease progression. A subsequent report on a randomized trial involving 100 patients with primary amyloidosis that compared MP combined with colchicine versus colchicine alone indicated longer survival with the regimen containing melphalan (12 vs. 7 months). [164] In this study, better survival, regardless of treatment, was noted in patients receiving satisfactory supportive treatment such as transplantation or dialysis for their organ failure. A significant therapeutic advantage in terms of objective responses and prolonged survival for MP versus colchicine was also shown in a randomized trial comparing MP, MP plus colchicine, and colchicine alone in 220 patients with primary amyloidosis. [163] In this study, patients who showed serum or urinary M-protein responses (disappearance or a reduction >50%) by the end of 12 months of treatment survived significantly longer than the ones who did not respond (median 50 months vs. 36 months, $P = 0.03$). Overall, the results of these controlled trials indicate that colchicine provides no additional beneficial effect over MP.

Dimethylsulfoxide can be effective when used topically on primary cutaneous amyloidosis and when given by local instillation in urologic amyloid lesions (reviewed by Merlini [142]). The efficacy of vitamin C and E supplementation has not been proven in patients with AL amyloidosis.

Newer Approaches to AL Amyloidosis

Current treatment with traditional alkylating agent-based chemotherapy produces significant reductions of light chain amyloidogenic precursors and consequent clinical benefits in an unsatisfactory proportion of patients with AL. Furthermore, MP-based therapy requires a long time to achieve a response (median of approximately 1 year, with 21% of patients requiring an additional year of therapy to show a response), [156] and, unfortunately, the survival of many patients with amyloidosis is less than 1 year. In L/HCDD as well, alkylating agent-based chemotherapy benefits only a minority of patients. [158] [159] This stresses the importance of earlier diagnosis, and the development of more rapid and effective therapies. It appears rational to investigate all available therapeutic tools to maximize inhibition of the synthesis of the offending protein, with the aim of eradicating the amyloidogenic clone. Preliminary results with such approaches (e.g., with high-dose therapy) in AL amyloidosis and L/HCDD indicate that this is both feasible and effective.

High-Dose Dexamethasone and Combination Therapy

Taking the lead from myeloma therapy, we recently reported on nine patients with biopsy-proven AL amyloidosis treated with an induction regimen of pulsed high-dose dexamethasone followed by maintenance therapy with interferon-. [166] Improvement in organ function was noted in six of seven patients with nephrotic syndrome and two of two patients with hepatomegaly. The response to therapy was rapid, with a median time to response of 34 months in patients with nephrotic syndrome. Improvement in organ function following dexamethasone (DEX)-interferon or DEX-based combination therapies such as vincristine, Adriamycin, and DEX has been confirmed. [167] However, others have noted only modest benefit with DEX as single agent. [168] Results from a recent study also failed to demonstrate superiority of M_2 regimen over MP. [169]

Dose Intensive Therapy

High-dose therapy, particularly with high-dose melphalan, has been used in a number of patients with myeloma over the last decade and effected a high proportion of complete responses in this disease. However, until recently, this approach had not been attempted in patients with AL amyloidosis/L/HCDD, primarily because of the common occurrence of renal and cardiac dysfunction in these patients. With recent advances in supportive care and the availability of growth factors and mobilized stem cells, the morbidity and mortality rates with these treatments have been substantially diminished, and the feasibility and safety of high-dose melphalan in patients with renal dysfunction has been documented. This has prompted a number of investigators to attempt high-dose therapy in these patients. [170] [171] [172] [173] In two recent studies with >20 patients, clinical improvement in organ function was noted in up to two thirds of patients with <3 organs involved. [170] [171] In one study the 4 year estimated overall survival in patients with <3 clinical manifestations was 91%, as compared to 11% in those with 2 clinical manifestations. However, early mortality, even in these studies with highly selected patients, has been 1540%. The results in patients with limited organ involvement are quite promising and suggest that dose intensive therapy may be preferable, if these patients meet the functional criteria. Other approaches that hold promise include Total Therapy approach combining DEX and dose intensive therapy [173] and anti-angiogenesis agents.

Iododeoxydoxorubicin

It has been reported that a new derivative of doxorubicin, 4-iodo-4-deoxydoxorubicin (I-DOX) may be effective in AL amyloidosis. [174] [175] The precise mode of action of this compound is not known; however, it has a very high affinity for amyloid fibrils of any sort, which likely plays a key role in its mechanism of action. [176] It has also been reported that treatment with I-DOX significantly delayed the clinical signs of disease and prolonged survival time in Syrian hamsters with experimental prion infection. [177] The results of a phase I trial showed that 5 of 8 patients treated with 16 administrations of I-DOX at dosages of 15100 mg/m ^{2} obtained clinical improvement, concomitant with evidence of amyloid resorption. [175] The benefits appeared some weeks after treatment and persisted for a median of 6 months. The efficacy of I-DOX was not related to cytotoxicity since no significant or persistent reduction of the serum or urinary concentrations of the monoclonal protein was noticed in any of the patients. The results observed in 14 more patients treated with I-DOX at our institution (GM) and in 28 patients treated on a compassionate basis in several European countries have been recently reported. [178] Six of the 13 evaluable patients treated at our institution were responsive and four showed stable disease. Median duration of clinical benefit was 7 months. Best results were obtained in patients with soft tissue involvement, while those with heart, kidney, or liver involvement did not experience recovery of organ function. Of the 28 patients treated on a compassionate basis a response was observed in 5 patients, in 11 patients I-DOX stabilized the disease, 11 patients did not respond and the disease progressed to death in most of them. Serious adverse events were reported in three patients: two sudden deaths and an onset of acute renal failure. These data indicate that only a minor proportion of all patients responded to I-DOX as used and that clinical effects were transient and insufficient to affect the course of the disease. Further clinical investigation is necessary to define the role of I-DOX in the treatment of AL amyloidosis. At present, it appears that this drug should be used as an adjunct to more specific and effective therapy for AL amyloidosis.

Prognostic Factors

In most patients, AL amyloidosis is a fatal disease, with median survivals ranging from 1 to 3 years. [51] [142] In 171 patients seen at our institution, the median survival is 34 months (range, 1149 months; 5-year survival rate of 15%; Fig. 773A). In L/HCDD, the overall survival rate is quite variable, ranging at 5 years from 20 to 70%. [21] [158] [159] The primary determinant of prognosis in both disorders is the extent of dysfunction of a vital organ. Thus, the prognosis depends on the clinical syndrome at presentation, and the number of organ systems involved. In a series of patients with AL amyloidosis, patients with congestive heart failure had a median survival of 8 months (5-year survival rate, 2.5%), whereas those with peripheral neuropathy as the sole manifestation had the best outcome (median survival, 40 months; 5-year survival rate, 32%). [61] In a multivariate analysis of 168 patients with AL amyloidosis, the presence of congestive heart failure, urinary light chain, hepatomegaly, and

multiple myeloma adversely affected survival during the first year, whereas after the first year, increased serum creatinine, presence of myeloma, orthostatic hypotension, and serum M protein were predictors of poor survival. [179] In our experience using high-dose therapy, however, the survival of patients with myeloma and AL amyloidosis appears to be quite comparable to

Figure 77-3 (A) Overall survival of 171 patients with primary amyloidosis (dotted lines denote 95% confidence intervals). **(B)**

that of myeloma patients without amyloidosis. [53] In a large cohort of patients with AL amyloidosis followed at our institution, the presence of congestive heart failure was one of the major prognostic determinants (Fig. 773). Few studies have specifically addressed the prognostic variables in a large cohort of patients with L/HCDD, but the extent of renal and cardiac dysfunction appears similarly to affect the survival rates of these patients. [158] In approximately half the patients with AL amyloidosis, the cause of death is cardiac related. [164]

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FUTURE DIRECTIONS

Amyloidosis causes progressive organ damage that is ultimately fatal. Early diagnosis is of paramount importance to establish timely therapy and recover the function of the organs involved by the disease. The hematologist should maintain a high level of alertness, in particular in patients with known M protein. Diagnosis is now rapid and reliable; traditional and ultrastructural immunohistochemistry allow rapid typing of the amyloid, which is necessary to choose the appropriate treatment. The management of these patients is and will be increasingly articulated. Supportive therapy should be applied with full intensity, and specific therapy will become more comprehensive as the unraveling of the complex pathogenesis discloses new potential therapeutic targets. The recent advances in our understanding of the nature of the different amyloid forms have been remarkable. The genetic defects of the hereditary amyloidoses have been identified, thus allowing patients with these diseases to benefit from diagnostic testing, genetic counseling, prenatal testing, and treatments, such as liver transplantation. The molecular mechanisms involved in the transformation of a native protein into a polymeric and fibrillar structure, and the clarification of the role of other tissue components in the formation and persistence of amyloid deposits, offer potential for the development of novel approaches to therapy.

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ACKNOWLEDGMENTS

This work was supported in part by a Career Development Award from the American Society of Clinical Oncology (MVD), and grants from AIRC, from the Italian Ministry of Health (GM), and Telethon Italy E-793[VB].

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Chapter 78 - Atypical Immune Lymphoproliferations

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INTRODUCTION

Development of modern classification systems for hematologic malignancies resulted from the recognition that specific histologic and cytologic patterns correlated with prognosis.[1] [2] [3] [4] The Working Formulation for Clinical Usage predicts prognosis in many of the subtypes of lymphoma, but is limited by the lack of immunophenotype and molecular data that the Revised European and American classification proposal provides. [1] [2] The Rye classification scheme for Hodgkins disease correlates prognosis with histologic features such as the number of Reed-Sternberg cells, the composition of the background lymphoid cells, and the degree and type of sclerosis.[3] The French-American-British (FAB) scheme for leukemias relies on cytologic features for lymphocytic leukemias and a combination of cytologic and cytochemical features for myeloid leukemias.[4] These classification systems can be reliably applied to most clinically relevant neoplastic lesions, but some lymphoproliferations are not addressed because they do not meet the pathologic definition of malignancy. No unifying system exists for classifying atypical or preneoplastic lymphoproliferations that provides prognostic information to predict whether a patient will have a self-limited illness or progress to lymphoma or death.

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WHAT CONSTITUTES AN ATYPICAL LYMPHOPROLIFERATION?

The tremendous complexity of the immune system and its variable responses to challenges has led to recognition of certain hyperplastic responses that are clearly benign and manifest specific histologic patterns when biopsied. [5] Examples of benign hyperplastic immune responses include follicular hyperplasia, in which B-cell areas of the lymph node increase in size and number. As a nonspecific diagnosis, follicular hyperplasia may be seen in a wide range of clinical presentations, from bacterial infections to generalized lymphadenopathy of acquired immunodeficiency syndrome (AIDS). Sinus histiocytosis, in which benign histiocytes distend lymph node sinuses, [5] is seen in reaction to particulates and organisms in hilar lymph nodes and in lymphoid drainage sites of carcinomas. Clearly benign or malignant lesions can be easily diagnosed by biopsy, clinicopathologic correlation, and the exclusion or establishment of clonal rearrangements.

A significant group of patients present with unusual clinical features, and their biopsies result in pathologic diagnoses that prompt use of terms such as atypical or suspicious for malignant lymphoma, leaving the physician with incomplete information

on how to treat the condition. Some disorders present with clinical features that suggest a malignant process but show characteristic histologic features that correlate with a benign outcome. The majority of these processes are related to an abnormal immune response to some inciting stimulus. Others may sometimes result in the death of the patient, either by progression to malignancy or by damage to the immune system. Accurate diagnosis requires careful correlation of immunohistologic, karyotypic, virologic, and genotypic analyses with the clinical findings. The more commonly encountered atypical lymphoproliferations related to viruses, drugs, genetic abnormalities, and unknown causes will be discussed.

VIRUS-ASSOCIATED ATYPICAL LYMPHOPROLIFERATIONS

Epstein-Barr Virus: Fatal Infectious Mononucleosis

Biological Aspects

The prototypical cause of atypical lymphoproliferations is the ubiquitous Epstein-Barr virus (EBV). [6] A knowledge of the mechanisms by which EBV induces lymphoproliferation provides a model for understanding other virus-associated lymphoproliferations. [6] [7] EBV causes the well-characterized clinical syndrome of infectious mononucleosis (IM) discussed in Chapter 45 . This disscussion will not specifically address IM but will cover those lesions that fall outside the usual clinical spectrum of self-limited IM.

EBV, a large herpesvirus (172,000 base pairs), preferentially infects B cells in humans and causes lifelong infection. [6] [8] The key to understanding atypical responses to EBV involves the relationship that is established between the virus and the immune system after primary infection. [6] [8] Primary infection by EBV results in two main responses, depending on the age of the individual or the maturity of the immune system. [6] [8] If the primary infection occurs in early childhood, the immune response and clinical symptoms are almost always silent, but about two-thirds of infected older children and adults develop IM. [6] [8] Once infected by EBV, an individual maintains a lifelong relationship with the virus in which a virus-driven B-cell proliferation is kept in check by the host immune surveillance. A low level of virus production occurs in the oropharyngeal region by unknown mechanisms; about 1 in 10^6 B cells in peripheral blood carries EBV. [6] [8] The intimate association of oropharyngeal epithelium with oropharyngeal lymphoid tissue results in a constant cycle of circulating infected B cells. Disease results when the immune system is depressed, either by iatrogenic means or by concurrent infections with other agents (Fig. 781).[6] The resulting immune suppresion allows escape of EBV-infected B cells and resultant lymphoproliferation. [6] [7] [8] [9] [10] [11] The self-limited course of IM contrasts with the atypical responses that can occur, such as fulminant infectious mononucleosis (FIM).[6] FIM is characterized by extensive infiltration of lymphoid and parenchymal organs by polyclonal T and B cells in varying degrees of transformation, resulting in a polymorphous pattern (Plate 78-1A), which contrasts with the monomorphic pattern of malignant lymphoma (Plate 78-1B).[6] [8] [9] [10] [11] The extent of T-cell and histiocytic reaction in FIM is often dramatic. Hemophagocytosis is common. [7]

Epidemiology

FIM occurs in approximately 1 in 3,000 IM cases, or about 40 cases annually in the United States. [6] [7] [8] [10] [11] The explosive lymphoproliferation that occurs in FIM mimics that in certain hematologic neoplasms, such as lymphoid leukemia, and can obscure recognition of FIM as a cause of death. [6] [7] [10] [11] The median age at presentation is 13 years, with a 1:1 male/female ratio. [10]

Clinical Manifestations

Patients who progress to FIM initially present with the usual signs and symptoms of IM, including fever, sore throat, malaise, anorexia, nausea, vomiting, and a maculopapular rash, but

Figure 78-1 The EBV-immune system relationship and the development of lymphoproliferations associated with various immune deficits or diseases.

these symptoms are usually more extensive than those of IM.[10] Patients initially manifest an atypical lymphocytosis but subsequently develop severe, persistent pancytopenia, hepatic dysfunction resulting in fulminant hepatitis, meningoencephalitis, and varying degrees of myocarditis. [6] [7] [8] [9] [10] [12]

Laboratory Diagnosis

The diagnosis of EBV-associated atypical lymphoproliferations and of FIM can be difficult because the usual EBV antibody responses may be lacking or unusually high, and the clinical picture may resemble an acute leukemia, another malignancy, or overwhelming sepsis. [6] [10] [11] [12] [13] [14] Reactive lymphocytes may be seen in the peripheral blood. An accurate diagnosis may require performing serologic studies of the EBV-specific antibodies to the viral capsid antigen (VCA), early antigen (EA), and EBV nuclear-associated antigen (EBNA); staining of lymphoid tissue for latent membrane protein (LMP)-1; establishment of lymphoblastoid cell lines; and in some cases, molecular analyses including in situ and Southern blot hybridization or polymerase chain reaction studies. [6] [10] [11] [12] [13] [14] (Table 781). Because FIM is rare, a search for a heritable immune deficiency in family members is mandatory. [6] [10] [11] [12] [13] [14] Although FIM is the most common manifestation of males affected with X-linked lymphoproliferative disease (XLP), FIM can occur outside the context of XLP. [6] [8] [10] [11] [12] [15] Clinically, FIM is indistinguishable from other viral associated hemophagocytic syndromes (VAHS), familial hemophagocytic lymphohistiocytosis (HLH), and the accelerated phase of Chediak-Higashi disease. [7] [16]

TABLE 78-1 -- EBV Diagnostic Procedures

Parameter Assayed	Method
Infectious (viable) EBV virions	
EBV cytopathic effect	Conventional tissue culture
EBV early antigen (EA) detection	Centrifugation culture and direct immunofluorescence
Establishment of EBNA and/or EBV genome-positive lymphoblastoid cell lines	Culture of lymphocytes from peripheral blood or biopsy material; cord-blood lymphocyte transformation by throat washings
Circulating EBV antibodies	
VCA: IgG, IgM, IgA	Indirect immunofluorescence
EA-D: IgG, IgA	Indirect immunofluorescence
EA-R: IgG	Indirect immunofluorescence

EBNA: IgG	Anticomplement immunofluorescence; enzyme-linked immunoabsorbent assay
Heterophil: IgM	Paul-Bunnell-Davidson test
Monospot	Rapid slide test
EBV protein(s) in tissue	
EBNA	Anticomplement immunofluorescence; Western blot analysis
LMP-1	Immunoperoxidase
EBV-specific cytotoxic T-cell functions	Lymphocytotoxicity assay; regression assay
EBV nucleic acid analysis	
EBV DNA	Dot blot hybridization; Southern blot analysis; in situ hybridization; polymerase chain reaction
EBV RNA	Dot blot hybridization; Northern blot analysis; in situ hybridization
EBV terminal repeat (clonal marker)	Southern blot analysis

EBV, Epstein-Barr virus; EBNA, Epstein-Barr nuclear antigen; VCA, viral capsid antigen; EA, early antigen; LMP-1, latent membrane protein-1.

A GENERAL APPROACH TO THE DIAGNOSIS OF ATYPICAL LYMPHOID HYPERPLASIAS

Accurate diagnosis of atypical lymphoproliferations requires a systematic approach. The patients history and physical examination often suggest a malignant process, which requires a biopsy for confirmation. If the clinical features and biopsy findings are suggestive of a viral process, serology and virologic assays may be confirmatory, and the patient can be observed without further workup. If the family history and clinical features suggest a heritable immune defect, then pedigree and immunologic analysis of the patient and family members is mandatory. Conditions that have a characteristic histologic appearance (e.g., Castlemans disease) can be accurately diagnosed with histologic examination alone, but most patients will require application of more sophisticated diagnostic procedures on appropriately prepared specimens.

To obtain optimum results from a lymph node biopsy in the settings described in this chapter, close cooperation between the referring physician, surgeon, and pathologist is required. In our practice within the Nebraska Lymphoma Study Group, we sometimes see patients who have had one or more biopsies that have had improper handling of the tissue, negating the opportunity to perform adjunctive studies. These procedures require fresh tissue. Thus the practice of placing the entire lymph node biopsy specimen in formalin is to be condemned, for it is a frequent cause of a second biopsy. Therefore, a protocol to handle lymphoid tissue is mandatory to evaluate these difficult lesions. It is imperative for the referring physician to communicate the differential diagnosis of lymphoma, atypical immune response, transplant status, and recent drug history to the surgeon and pathologist so that fresh tissue is appropriately partitioned. At the Nebraska Lymphoma Study Group, fresh lymph node tissue is routinely frozen for possible immunohistochemical studies and molecular gene rearrangement analyses after routine histology. Other laboratories choose to form cell suspensions for flow cytometry. In addition, cytogenetic analysis is performed on a portion of the fresh tissue. Case records are maintained for each patient in our lymphoma, XLP, and PTLD registries. In situ hybridization for EBV is performed in all cases of suspected immune deficiency. After a diagnosis of EBV-positive lymphoproliferation, new masses are biopsied to rule out transformation to non-Hodgkins lymphoma.

Therapy

All these diseases are characterized by uncontrolled T lymphocytes and macrophages, and immunosuppression with corticosteroids, cyclosporin A, and/or anti-thymocyte globulin (ATG) has been effective. The use of etoposide (VP-16) to quench macrophage activation and to down-regulate T-cells, in conjunction with immunosuppression, can be effective even in the most severe cases. Occasionally, the central nervous system is involved and intrathecal chemotherapy is required to resolve the symptoms.[7] [17] [18]

Prognosis

Patients who develop FIM succumb to severe and progressive multiorgan failure, apparently brought on by anomalous cytotoxic T-cell and natural killer cell activity, which nonselectively destroys uninfected hepatocytes, bone marrow elements, skin,

and other organs during the unrestricted polyclonal immune response initiated by the EBV infection. [6] [7] [8] [9] [10] [17] The median survival time is approximately 4 weeks, and splenic rupture is conspicuously rare as a cause of death in these patients. [6] [10]

Other Presentations of EBV Lymphoproliferations

Rarely, elderly patients, malnourished patients, and those with cancer also develop EBV-associated atypical lymphoproliferation. [6] [11] [13] These patients have a secondary immunodeficiency. The majority of them will have had a prior infection with EBV and will have achieved a virusimmune system balance prior to acquiring an immune deficiency, which then allows the virus to escape the defective immune surveillance mechanisms. Lymphoproliferations ranging from IM to FIM to overt lymphoma (Plate 781A & B) can then occur.[6] [10] [11] [13]

Patients with the acquired immunodeficiency syndrome also experience virus reactivation because the T-cell arm of the immune system is selectively attacked by the AIDS virus, resulting in loss of control over the persistent EBV infection. [6] Various lesions, including explosive follicular hyperplasia, hair leukoplakia of the tongue, and lymphoma have all been associated with EBV.[13] [14] Children with AIDS often develop lymphoid interstitial pneumonitis resulting from EBV-induced polyclonal B-cell proliferation.[19] Adults with AIDS have a 1,000-fold increased risk of developing malignant lymphomas, most of which probably begin as polyclonal EBV-associated (50%) lymphoproliferations and progress to monoclonal lymphomas. [6] [14] This is secondary to the development of cytogenetic events that occur

during the lack of adequate control of EBV by the damaged T-cell systems. [6] [11] [14]

Cytomegalovirus

Cytomegalovirus (CMV), also a herpesvirus, can cause the mononucleosis syndrome but is usually latent until unmasked by immune deficiency, pregnancy, multiple drug exposures, or immune suppression. CMV causes less of a lymphoproliferative response than EBV, and activation often results in more extensive inflammation and tissue necrosis. [20] Diagnosis is made by appropriate serologic studies, by identification of characteristic nuclear inclusions in cytologic or tissue biopsy specimens (Plate 78-2A), immunoperoxidase studies (Plate 78-2B), or by molecular techniques, including in situ hybridization and polymerase chain reaction (PCR). [20] [21] Therapy includes the use of current antiviral agents such as acyclovir and gancyclovir.

Virus-Associated Hemophagocytic Syndrome

Introduction and Epidemiology

Virus-associated hemophagocytic syndrome (VAHS) is also included in the spectrum of atypical lymphoproliferations and usually occurs in the setting of immunodeficiency. The syndrome was originally described in patients with viral infections but has subsequently been encountered in patients with fungal, bacterial, and parasitic infections; in immunodeficient patients; and in patients with T-cell lymphomas. [22] VAHS has now been classified as a secondary form of type II histiocytic diseases, termed histiocytic lymphohistiocytosis (HLH). [16] A familial form of HLH (FHLH) also occurs, called familial erythrophagocytic lymphohistiocytosis (FEL), which is an autosomal recessive condition, that usually presents before 5 years of age with hepatosplenomegaly and fever. [23] [24] Patients exhibit multiple defects in cellular immunity. [25] [26]

Clinical Manifestations

Approximately 80% of patients with FIM exhibit clinicopathologic findings consistent with VAHS. [15] [17] [27] [28] Patients with VAHS present with hepatosplenomegaly, abnormal liver function tests, fever, rashes, lymphadenopathy, coagulopathy, and pancytopenia, but may also present with central nervous system and pulmonary abnormalities. [7] [16] [22] [27]

Laboratory Diagnosis

Early in the course of VAHS the bone marrow appears normal, but myeloid hyperplasia, lymphocytic infiltration, cellular necrosis, and increased macrophages subsequently appear. [22] [27] Marked sinus histiocytosis with prominent erythrophagocytosis [22] [27] (Plate 783) is evident throughout the reticuloendothelial system of affected patients, and hyperplasia of the spleen can result in splenic weights in excess of 1 kg. [27] Histologic examination of lymph nodes, especially in FEL, shows lymphoid proliferation early in the disease, which is followed by lymphoid depletion at later stages. At necropsy activated macrophages, exhibiting phagocytosis, can be seen infiltrating areas of hemorrhagic necrosis. [15] [16] [27] The disease often terminates in a hemophagocytic syndrome with pancytopenia, jaundice, and marked erythrophagocytosis.

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RETROVIRUSES AND LYMPHOPROLIFERATION

Human Immunodeficiency Virus

During the recent decade, the RNA retroviruses have been implicated in a spectrum of lymphoproliferations. [29] The AIDS retrovirus, human immunodeficiency virus (HIV), does not transform lymphocytes but selectively destroys helper T cells. [29] During acute HIV infection, a mononucleosis-like syndrome often occurs, and the virus can cause chronic persistent lymphadenopathy. [29] [30] In situ hybridization of lymph nodes will demonstrate HIV sequences. Often EBV positive cells will be increased 5- to 10-fold in lymphoid tissues of HIV-infected patients compared to those non-HIV-infected patients. The T-cell immunodeficiency caused by HIV permits opportunistic viral, bacterial, protozoal, and fungal infections.

Human T-Cell Leukemia/Lymphoma Virus Type I

The most extensively studied transforming retrovirus is the human T-cell leukemia/lymphoma virus type I (HTLV-I), which is implicated in the endemic form of adult acute T-cell leukemia/lymphoma (ATL) in Japan and the Caribbean basin. [29] This virus has the capacity to selectively immortalize T cells in vitro, but HTLV-I infection alone is insufficient to cause malignancy, as only one in 2,500 chronic viral carriers develop the T-cell malignancies after a long latent period. [29] HTLV-I infection is transmitted during sexual intercourse or by blood transfusion. The viral infection probably increases the likelihood of further cytogenetic events by stimulating T-cell lymphoproliferation. [29] Seropositive asymptomatic carriers of the virus exhibit subtle signs of immunodeficiency. [29] Those who progress to ATL have HTLV-I-positive serology, generalized lymphadenopathy, hepatosplenomegaly, skin lesions, and distinct involvement of peripheral blood by a spectrum of malignant T cells ranging from small lymphocytes to bizarre hyperlobulated forms. [29] Not all patients manifest a leukemic phase. The prognosis is poor, as patients tend to present with stage IV disease; the median survival is less than 6 months. [29] Of the many HTLV-infected patients who do not develop ATL, most are asymptomatic, but a small group develops an early benign transient lymphocytosis; if subacute or chronic lymphocytosis persists, the risk of progression to ATL increases.

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DRUG-ASSOCIATED LYMPHOPROLIFERATIONS

While some drugs permit activation of latent viral infections such as EBV by causing immunosuppression, other drugs cause

TABLE 78-2 -- Drug-Associated Lymphadenopathy

EBV-associated
Corticosteroids
Cyclosporine
FK506
Methotrexate
Antithymocyte globulin
Azathioprine
Unknown mechanism
Dilantin
Carbamazepine

atypical lymphoid responses by unknown mechanisms (Table 782). Diphenylhydantoin (Dilantin) is a rare but well-documented cause of such responses. [31] [32] The clinical presentation is similar to that of a viral infection and includes fever, rash, lymphadenopathy, and eosinophilia. [31] [32] Acquired immunoglobulin A deficiency may develop. The symptoms usually abate when the drug is stopped. [31] [32] The lymph node pathology is similar to that in infectious mononucleosis, with a florid follicular hyperplasia or paracortical expansion by a polymorphous immunoblastic infiltrate that is sometimes mistaken for lymphoma. [31] [32] Focal necrosis and Reed-Sternberg-like cells may be evident. Hodgkins disease and non-Hodgkins lymphoma have both been reported in association with diphenylhydantoin therapy. [32] Other hyperplastic lymphoid responses to drugs have been reported, including pseudoperipheral T-cell lymphoma in association with carbamazepine. [33] The immunosuppressive drugs, including cyclosporine, steroids, antilymphocyte globulin, tacrolimus, and cytotoxic agents usually unmask latent viral infections.

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POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS

Introduction

Bone marrow and solid organ transplant recipients are exposed to a variety of immunosuppressive agents, including azathioprine, prednisone, cyclophosphamide, antilymphocyte globulin, anti-T-cell monoclonal antibodies, cyclosporine, and tacrolimus FK506. These patients are prone to developing a post-transplant lymphoproliferative disorder (PTLD), which includes predominantly atypical B-cell lymphoproliferations and, rarely, Hodgkins disease and T-cell lymphomas. [34] [35] [36] [37] [38] [39] [40] This discussion will be largely confined to the EBV-positive B-cell PTLDs.

Epidemiology

Over 90% of early (<6 months post-transplantation) PTLDs are EBV-positive, whereas late (>2 years) PTLDs tend to be EBV-negtive. [40] [41] The incidence of PTLD is highest in the first year after transplantation. The prevalence and the post-transplantation interval that precedes development of PTLD vary with the type of organ transplanted and the immunosuppressive agents administered. [37] Renal transplant patients have a low incidence (<2%) of PTLD. [34] [35] [36] [37] [42] The majority (78%) of cases are extranodal, with the brain and spinal cord being involved in 39% and the allograft itself in 15%. [37] The incidence of PTLD in heart transplant recipients ranges from 513% and in heart-lung recipients is 9%. [42] [43] [44] The organ transplant group at greatest risk for PTLD is the liver/small bowel transplant group in children treated with FK506, with up to a 22% incidence. [45] This is partially due to the low incidence of prior EBV infection in these young children. The use of FK506 appears to have increased the risk of PTLD, due to the enhanced immune suppression achievable with less toxicity. [45]

Among bone marrow transplant recipients, those receiving T-cell-depleted bone marrow allografts appear to be at particularly high risk for EBV-associated lymphoproliferation. [46] [47] [48] [49] EBV-genome-positive lymphomas have occurred after intervals of 249 months and do not respond well to acyclovir or conventional chemotherapy. [47] [48]

Biology and Molecular Aspects

These atypical lymphoproliferations can be aggressive with continued immunosuppression, even when polyclonal or oligoclonal, as defined by immunoperoxidase and gene rearrangement studies. The clonal ambiguity has led to difficulties in diagnosis, nomenclature, and therapy. [36] [50] [51] The most recent classification of PTLD, by Knowles, describes three main subgroups: plasmacytic hyperplasias, polymorphous PTLDs (Fig. 78-2), and monomorphous PTLDs, which include lymphoma and multiple myeloma. The monomorphous PTLDs have a higher incidence of clonal heavy chain or light chain immunoglobulin gene rearrangements. This subgroup also has a low incidence of translocations and mutations in p53. [52]

The pathogenesis of PTLD is related to a disruption of the hosts EBV immune surveillance by immunosuppression (Fig. 78-1). Impairment of T-cell-mediated responses to EBV leads to a serologic pattern of acute infection, and increased oropharyngeal shedding of the peripheral blood. During the first 6 months after transplant, cytotoxic T-lymphocyte response to EBV is often absent. [53] In addition, patients on immunosuppressants such as cyclosporine have an imbalance of T-cell-secreted cytokines, favoring B-cell proliferation. [54] Therefore, if immunosuppression is continued, EBV-infected B cells proliferate explosively, resulting in multiple independent, or oligoclonal, EBV-driven B-cell proliferations. Subsequent additional cytogenetic events then allow a dominant clonal population of B lymphocytes to emerge, resulting in the development of lymphoma. [52] The B-cell of origin appears to be predominantly host cells in solid organ transplant patients, but may be of donor origin in bone marrow transplant patients. [47] [48] [55] Donor and host sources of EBV both occur. [56] [57]

Clinical Manifestations

Patients with PTLD present several ways: single mass lesions like lymphoma, infectious mononucleosis with tonsillar enlargement, cervical lymphadenopathy and/or hepatitis, or a

Figure 78-2 Post-transplant lymphoproliferative disorder, polymorphous, expanding the perivascular space (hematoxylin and eosin × 200).

septic appearance with fevers but no radiologically detectable mass. [34] [35] [36] [37] [38] [39] [40] [41] [42] [43] [44] [45] [46] [47] [48] [49] [50] [51] [52] [53] [58]

Laboratory Evaluation

Radiology studies may reveal a mass, a miliary pattern if PTLD is in the lungs, or no lesion at all. Peripheral blood will sometimes reveal circulating plasmacytoid lymphocytes or plasma cells. Quantitative measurements of EBV load in the peripheral blood often rise prior to the rapid development of PTLD lesions. [59] Tissue biopsy should be performed for immunophenotyping and in situ hybridization for EBV sequences as LMP-1 protein will be negative in 25% of cases. [60] Typically, the lymphoid tissue is composed of plasmacytoid B cells, with few T cells, and often regional areas of necrosis. Distinguishing between a polyclonal and a monoclonal PTLD often requires lymphoid receptor gene analysis, since up to 50% of PTLDs do not express surface immunoglobulin.

Therapy and Prognosis

Mortality among patients with PTLD is as high as 5090%. [36] [37] [42] [47] [48] [49] [50] [51] [55] [61] [62] [63] [64] Successful treatment of PTLD is a therapeutic challenge, in part because of the patients increased toxicity from chemotherapy, increased susceptibility to life-threatening infections, and the necessity to maintain the allograft, especially with vital organs (heart, lung, or liver). Reduction of immunosuppression is the most widely used approach. [65] Many times this is sufficient in controlling the disease, especially in localized, polyclonal cases or cases that present like infectious mononucleosis. Patients who do not tolerate reduction of immunosuppression (i.e., graft rejection), or do not respond to immunosuppression reduction, require more aggressive therapy and have a much poorer prognosis. Anti-viral agents (acyclovir or ganciclovir), and/or intravenous immunoglobulin (IVIG) have been used extensively for prophylaxis and treatment of PTLD. The efficacy of anti-virals and IVIG is difficult to assess because reduction of immune suppression is almost always initiated simultaneously.

Surgery or radiotherapy are very effective in curing localized disease, but this represents only a small percentage of patients. [42] [61] [62] [64] Non-cytotoxic approaches

such as interferon-alpha (-IFN) and anti-B-cell monoclonal antibodies have been attempted. Responses are often seen using -IFN (83%), but these responses are often not durable. In the largest reported series (18 patients) using -IFN, in addition to relapse, infection and rejection were significant problems and the median survival was 6 months.[63] Response rates with anti-B-cell monoclonal antibodies were also good (73%), but no response was observed in monoclonal disease. Again, death resulting from progressive disease, infection, and rejection was significant with a 50% 1-year survival. [64]

Because virus-specific cytotoxic T cells (CTL) are critical in the control of EBV-driven B-cell proliferations, donor leukocyte infusion (DLI) has been used to treat PTLD.[66] DLI has been successful in the treatment of PTLD following T-cell-depleted bone marrow transplantation (BMT), but has been associated with severe graft-vs.-host disease (GvHD) and a lethal shock-like syndrome. [67] [68] The use of EBV-specific CTLs generated ex vivo has been very successful in preventing and treating PTLD following BMT without significant GvHD. [69] [70] However, the issue of using DLI in solid organ transplant patients with PTLD is complex. First, cadaveric organs are most widely utilized; therefore, donor leukocytes are often not available, and the use of closely matched relatives leukocytes runs the risk of both rejection and GvHD.

The use of chemotherapy for PTLD does not require waiting for the progression to the standard pathologic criteria for diagnosis of lymphoma, as the polymorphous hyperplasias subgroup of PTLD can be equally lethal. Chemotherapy has been efficacious in treating PTLD in some patients. The intensive, multi-drug (i.e., anthracycline-based) regimens traditionally used to treat high-grade lymphomas are usually immunosuppressive enough to maintain the organ graft, but death resulting from toxicity and infection has been a significant problem and 1-year survival is only 4045%. [61] [62] [71] AIDS patients with non-Hodgkins lymphoma also have increased toxicity to chemotherapy and susceptibility to life-threatening infections. Because it has been shown that the overall survival is improved with less aggressive therapy in AIDS patients treated for non-Hodgkins lymphoma,[72] a similar approach using low-dose chemotherapy may be more efficacious for treating PTLD patients.[71] [73]

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ATYPICAL LYMPHOPROLIFERATIVE DISEASE IN PATIENTS WITH AUTOIMMUNE DISORDERS

Other disorders of immune regulation have an increased prevalence of lymphoid neoplasia. Patients with autoimmune collagen-vascular disease (i.e., rheumatoid arthritis) can develop non-Hodgkins lymphomas, which often arise in the setting of reactive lymphadenopathy. [74] [75] Rheumatoid arthritis patients have a specific defect in T-cell inhibition of EBV-induced lymphocyte proliferation and a five-fold increase in the rate of spontaneously transforming B-cell clones in vitro. [76] These patients develop atypical lymphoid hyperplasia and non-Hodgkins lymphoma while on methotrexate or azathioprine therapy. [77] [78] [79] Case reports describe spontaneous resolution upon discontinuation of methotrexate. [78] [79] Individuals with Sjögrens syndrome also show a progression of lymphoid hyperplasia to frank neoplasia, with a risk 44 times that observed in the general population. [80] Patients treated for Hodgkins disease have an increased prevalence of secondary lymphoid malignancies.[81] These lymphomas show a predilection for extranodular involvement, particularly of the gastrointestinal tract. [81]

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GENETICALLY LINKED IMMUNODEFICIENCY AND LYMPHOPROLIFERATIONS

Introduction

In 1952 Burton recognized that recurrent and persistent pyogenic infections in a young male patient were due to lack of antibodies. [14] Subsequently, the triad of autoimmunity, immunodeficiency, and lymphoproliferation has been defined in an increasingly large group of patients with inherited and acquired immunodeficiency disorders.[6] [11] [14] About six well-defined X-linked immunodeficiency disorders are known, and many persons with uncontrolled lymphoproliferations other than these well-defined disorders probably have subclinical or undefined inherited immunodeficiencies. [11] [14] The prevalence of lymphoproliferations ranges from about 2% in patients with Burtons agammaglobulinemia to 25% in males with the X-linked lymphoproliferative (XLP) disease. [11] [14] [15] [28]

The X-Linked Lymphoproliferative Disease

Introduction

XLP disease illustrates the spectrum of lymphoproliferations that can occur in hereditary immune deficiencies, ranging from benign or fatal IM to non-Hodgkins lymphoma.[6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [28] Following EBV infection, chronic or fatal IM, acquired hypogammaglobulinemia or agammaglobulinemia, pure red cell aplasia, necrotizing lymphoid vasculitis, and/or non-Hodgkins lymphoma ensues. [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [28]

Biological and Molecular Aspects

The mutation responsible for the XLP syndrome is genetically linked to a restriction fragment length polymorphism detectable

with the DXS42 probe on chromosome Xq25.[82] The XLP gene has been identified and sequenced, and the mutational pattern in an SH2DIA domain of the gene in numerous XLP families has been characterized. Current research is focused on analyzing the protein and its function. [83]

Clinical Manifestations

The disease should be suspected in families in which maternally related boys develop FIM, acquired hypogammaglobulinemia, agammaglobulinemia, or malignant lymphoma (Fig. 78-3).[6] [7] [8] [9] [10] [11] [13] [14] [15] [28] [84] The main differential diagnosis in these patients involves lymphoma versus florid IM. Patients who develop malignant lymphoma usually present with discrete mass(es), often extranodal, with or without dissemination at the time of diagnosis. [28] [84] In contrast, patients with severe or fatal IM have a disseminated lymphoproliferation involving generalized lymphadenopathy, as well as multiple organ sites, including skin, liver, spleen, lung, bone marrow, and brain.[7] [12] [15] [28] [84] Males with XLP do not usually have clinical evidence of immunodeficiency prior to EBV infection, though approximately 10% will have manifestations, including lymphoma, without evidence of EBV infection, and they are not unduly vulnerable to other infectious agents. [15] [28] [84] [85]

Laboratory Evaluation

Though affected males can be identified by DNA linkage analysis, a definitive diagnosis of XLP in the family must be present, i.e., two or more maternally related males manifest phenotypes of XLP.[15] [28] Once linkage is established identification of carrier females and prenatal diagnosis may be performed. The defect gene was localized to the Xq24-26 region of the X chromosome in 1987. [82] A 2-megabase pair deletion was found in 1989. [83] This region has been mapped and a small deletion (100 kilobases) has been found, which led to the discovery of the XLP gene. [83] [87] Identification of the gene now allows direct mutational analysis for definitive diagnosis in families in which a single male has symptoms consistent with XLP, and promises to provide us with a much greater insight on the control of EBV infections and the immune system in general.

Therapy and Prognosis

Complete remissions (CR) at rates as high as 80% can be achieved in XLP boys presenting with lymphoma utilizing standard lymphoma protocols; however, relapses or other manifestations,

Figure 78-3 Frequency of phenotypes in X-linked lymphoproliferative disease. FIM, fulminant infectious mononucleosis; Dysgam, dysgammaglobulinemia; AA, aplastic anemia; LYG, lymphoid granulomatosis/vasculitis.

i.e., hypogammaglobulinemia, are very common.[28] Boys with dysgammaglobulinemia receive monthly IgG injections, but this does not prevent EBV infection and its sequelae. Several boys have developed FIM and died while receiving IgG supplementation. [15] [28] Many treatments have been used to treat FIM of XLP, including antibiotics, steroids, intravenous IgG, plasma infusions, acyclovir, interferon (both and), chemotherapy, and/or cyclosporin A. Because FIM is essentially indistinguishable from other hemophagocytic syndromes, i.e., hemophagocytic lymphohistiocytosis (HLH) and the accelerated phase of Chediak-Higashi, it is now recommended that boys receive aggressive chemotherapy to quell the immunologic anarchy of uncontrolled T lymphocytes and macrophages, with etoposide (VP-16) to quench macrophage activation and steroids and/or cyclosporin A to down-regulate T cells. [7] [17] [28] Eight of 10 boys treated in this fashion have had complete response. Again, relapse is common (50%); therefore, the recommended therapy for FIM is now aggressive intervention with etoposide and immunosuppression, and initiation of a search for a suitable bone marrow donor, with transplantation performed once the patient is stabilized.

Allogeneic hematopoietic stem cell transplantation (HSCT) remains the only curative therapy for XLP. [88] [89] Data from the XLP Registry show 13 boys having undergone HSCT; the only non-survivors were all over 15 years of age. This survival advantage observed in younger patients is reflective of the findings regarding the use of HSCT in other immunodeficiencies, especially with alternative donor sources. [88] [89]

Other Heritable Immunodeficiency Diseases with Lymphoproliferations

Clinical Manifestations

A complete review of genetically linked diseases manifesting lymphoproliferation is beyond the scope of this discussion, but certain conditions have been sufficiently characterized to allow recognition. Wiskott-Aldrich syndrome, also X-linked in inheritance, is characterized by chronic eczema, chronic suppurative otitis media, anemia, and thrombocytopenic purpura.[14] Patients have defective cell-mediated immunity and poor antibody responses to polysaccharide antigens. Males with Wiskott-Aldrich syndrome are more immunocompetent than males with XLP, and FIM has not been reported in association with this syndrome. [14] EBV-specific antibody responses are less commonly abnormal in Wiskott-Aldrich patients; however, these patients are more likely to develop widely disseminated malignant lymphoma (50%) than XLP males with the malignant phenotype.[6] [14]

Ataxia-telangiectasia is an autosomal recessive trait characterized by severe progressive cerebellar ataxia associated with oculocutaneous telangiectasia, sinopulmonary infections, and abnormal eye movements.[6] [14] The immune defects involve both T and B cells, and decreased levels of IgA and IgG are frequent. [6] [14] EBV reactivation occurs in ataxia-telangiectasia patients but has not been extensively studied. Lymphocytosis associated with chromosome abnormalities may persist for years.[14] Chromosome 14 abnormalities in these patients are associated with a high incidence of chronic T-cell leukemia, and preleukemic clonal expansions of cells and full-blown leukemias in these patients often demonstrate the inv(14q)(q11q32) or the t(14;14)(q11;q32) translocation. [90] [91] Molecular analysis has shown that the cytogenetic breakpoint at 14q11 occurs in the joining region of the T-cell receptor chain. [90] [91] Hybrid recombinations between T-cell receptor and occur, indicating genome instability.[92] The 14q32 breakpoints appear to be more heterogenous and include breaks near the immunogobulin heavy chain locus and at 14q32.1, the site of TCL1, an oncogene. [90] [91] Identical cytogenetic and molecular findings have been detected in T-cell

neoplasms not associated with ataxia-telangiectasia. [90] [91] The chromosome 14 abnormality involves both the immunoglobulin heavy chain locus and the T-cell receptor chain locus.[90] [91] The chromosomal location of many of the gene defects in these hereditary illnesses will be mapped in the future and will provide further molecular evidence of the etiology of these diseases.

Epidemiology

Many genetically linked diseases can manifest with lymphoproliferations. An increased incidence of lymphoproliferative disease is observed in individuals with antibody deficiencies, i.e., X-linked agammaglobulinemia, IgA deficiency, as well as cellular or combined deficiencies, i.e., severe combined immunodeficiency (SCID), XLP, Wiskott-Aldrich syndrome, ataxia-telangiectasia, hyper Ig-M (CD40 ligand deficiency), common variable immunodeficiency (CVID), and autoimmune lymphoproliferative syndrome (fas deficiency). [93] [94] [95] [96] [97] Data from the Immunodeficiency Cancer Registry show that 50% of all cancer seen were non-Hodgkins lymphoma, but Hodgkins disease (8.6%), leukemia (12.6%), adenocarcinomas, mainly gastric carcinomas (9.2%), and others (19.2%) have also been observed.

Laboratory Evaluation

Diagnosis of lymphoma can be difficult in these patients, who frequently have reactive lymphoid hyperplasia or chronic granulomatous inflammation. Demonstration of EBV in lesions is helpful in evaluating a lymphoid lesion, but is not synonymous with lymphoma. EBV-positive cells can be found in greater than normal numbers in benign nodes, and EBV is not found in all the lymphomas. [94] [96]

Therapy and Prognosis

Although complete remissions can be achieved in these patients, the outcome for patients with primary immunodeficiencies and lymphoma compared to that for immunocompetent patients with lymphoma has been poor. One reason is death due to chemotherapy toxicities, especially in ataxia-telangiectasia. Death due to infections and sepsis is also frequent, and relapses are common. [94] [96] Therefore, induction of remission with low-dose or standard-dose chemotherapy and correction of the underlying immune defect (i.e., allogeneic stem cell transplant, if a suitable donor can be identified) offers the greatest chance of long-term survival.

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ATYPICAL LYMPHOPROLIFERATIONS OF UNKNOWN CAUSE

Angiofollicular Lymph Node Hyperplasia (Castlemans Disease)

Introduction and Epidemiology

Angiofollicular lymph node hyperplasia (AFH), or Castlemans disease, has many other names, including giant lymph node hyperplasia, angiomatous lymphoid hamartoma, lymph nodal hamartoma, and lymph node hyperplasia of Castleman.[98] There is no sex or age preference, and most patients present with asymptomatic mediastinal masses, which are discovered on routine chest radiographic examination. There are two types of localized AFH, the more common hyaline-vascular (90%) and the plasma cell type (10%).[98]

Multicentric lesions have been identified in a subgroup of patients and may herald a higher risk of subsequent development of overt lymphoma. [98] [99] Most reports in the literature favor the concept that the hyaline vascular type of AFH is a reactive chronic lymphoid hyperplasia. [100] [101] The plasma cell type is considered to have an inflammatory pathogenesis, either through chronic antigenic stimulation (i.e., infection) or via an autoimmune mechanism. The plasma cell group tends to affect younger persons and has a higher rate of mesenteric and retroperitoneal tumors. [98]

Clinical Manifestations

When symptomatic, the hyaline vascular subgroup presents with mass-related symptoms such as pain, chronic cough, and obstruction. Clinical symptoms associated with the plasma cell type include fever, sweats, weight loss, and fatigue. Patients with multicentric disease can be extremely ill, with malaise and cytopenias. [98] [99]

Laboratory Evaluation

Associated abnormalities include anemia, an elevated erythrocyte sedimentation rate, hypergammaglobulinemia, hypoalbuminemia, hyperferremia, and hypertransferrinemia.[98] The hyaline-vascular type has a histologic pattern characterized by follicular hyperplasia distributed evenly thoughout the lymph node. [98] The follicles are unusually small, and their germinal centers are often penetrated by radial capillaries, which are surrounded by collagen or hyaline material, hence the name (Plate 78-4). [98] [99] The germinal centers are surrounded by multiple concentric layers of lymphocytes (Plate 78-4A). [98] [100] In the plasma cell type, which is less common (10%) and may represent an earlier stage of the lesion, [98] the germinal centers are larger, the peripheral cuffs of mature lymphocytes are not as prominent, and their interfollicular areas are occupied by extensive sheets of plasma cells, occasionally including some atypical forms (Plate 78-4B). Immunohistochemical and gene rearrangement studies have recently identified clonal cell populations in some cases of multicentric AFH. [102] [103] The human herpesvirus-8 (HHV-8), also called Kaposis sarcoma herpesvirus (KSHV), has been associated with the multicentric form of Castlemans disease in 25% of cases, but not with the more common subtypes.[104]

Therapy

Localized Castlemans disease is a self-limited process, curable with local therapy. [98] [105] [106] Following local excision, there is frequently total disappearance of systemic symptoms with only rare local recurrences. Multicentric Castlemans disease is a systemic disease associated with an aggressive, usually fatal course associated with infectious complications, and a risk of developing malignancies such as lymphoma or Kaposis sarcoma. Experimental and clinical data indicate that IL-6 plays a pivotal role in the biogenesis of multicentric Castlemans disease. [107] The hyperplastic germinal centers of these patients have been shown to produce excessive quantities of IL-6, and transient alleviations of the systemic manifestations of this disorder have been achieved with an anti-IL-6 monoclonal antibody. [107] [108] The median survival of patients with multicentric Castlemans disease has been reported to be 29 months. [106] Twenty-six patients in one series died within the first year of diagnosis. A limited body of information is available regarding the treatment of multicentric Castlemans disease. [104] [106] [108] [109] A reduction in the quantity of involved tissue either surgically or by means of radiation therapy may be an effective strategy to control the systemic signs and symptoms. [106] High doses of corticosteroids have been reported to result in sustained unmaintained remissions, although continuous high-dose prednisone therapy is frequently required. [106] Chemotherapy with single agents or combination therapy used to treat non-Hodgkins lymphoma has resulted in partial responses although occcasionally patients may enjoy durable complete remissions.[106] [105] Occasionally, patients may enjoy spontaneous remissions and not require any therapeutic interventions. [105] [106]

Angioimmunoblastic Lymphadenopathy with Dysproteinemia

Clinical Manifestations

Angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) is the preferred term for a condition that occurs more often in elderly patients and is characterized by generalized lymphadenopathy, hepatosplenomegaly, fever, night sweats, weight loss, anemia frequently due to antibody mediated hemolysis, and hypergammaglobulinemia and pruritic skin rashes. [110] [111] Other terms for AILD include immunologic aberrations in idiopathic reticuloses, atypical lymph node hyperplasia with fatal outcome (immunodysplastic disease), diffuse plasmacytic sarcomatosis, chronic pluripotential immunoproliferative syndrome, immunoblastic lymphadenopathy, and lymphogranulomatosis X.[110] [111]

Laboratory Evaluation

Biopsies of lymph nodes affected by AILD show complete effacement of the architecture, a prominent vascular proliferation, a florid immunoblastic proliferation, abundant plasma cells in clusters or sheets, burned-out germinal centers, focal necrosis, eosinophilic material, a background of mature lymphocytes, and, occasionally, eosinophils and neutrophils [110] [111] (Plate 78-5). Serum protein electrophoresis reveals a polyclonal hypergammaglobulinemia, and a complete blood count shows mild to moderate anemia. The Coombs test is frequently positive. Clonal T-cell gene rearrangements are present in many cases. Trisomy 3, trisomy 5, and an additional X chromosome are the most frequent chromosomal aberrations in AILD. Although AILD is considered a malignancy, a large number of cytogenetically unrelated clones exist that cannot originate from a common cell of origin because of their completely different karyotypes. There unrelated clones have been hypothesized to be the consequence of increased genetic instability and an immune defect resulting in impaired clearing of damaged cells. [112] [113]

Therapy

The median survival of patients with AILD is 30 months.[110] Most patients are treated with prednisone or combination chemotherapy.[111] [114] [115] [116] [117] [118] The most important prognostic factor in AILD is achievement of complete remission.[114] [115] [116] [117] [118] Randomized prospective treatment trials are needed to determine optimum treatment.

Although aggressive courses are observed in most patients, some patients experience a more indolent course or even spontaneous remissions. A prospective evaluation of standardized treatment in patients with AILD has been reported.[118] In this study patients initially received prednisone therapy followed by five cycles of COPBLAM (cyclophosphamide, vincristine, prednisone, bleomycin, doxorubicin, procarbazine) followed by 2 cycles of ifosfamide methotrexate (IM)VP-16. The combination chemotherapy was administered to patients who did not achieve a complete remission with prednisone or relapsed after prednisone therapy. Prednisone therapy was skipped in patients who presented with rapidly progressive life-threatening disease. Initial prednisone therapy led to resolution of B symptoms and reduction in tumor load in most patients.[118] The complete remission rate with prednisone only was 20%, and over 60% of these patients eventually relapsed. In contrast, the complete remission rates after primary and secondary chemotherapy were 56% and 64%, respectively. Siegert et al. have concluded that primary chemotherapy is superior to sequential therapy with prednisone with or without chemotherapy. In their series, the projected event-free survival was 35% at 36 months, and no relapses occurred after 12 months.[118] Because of the high rate of death resulting from *Pneumocystis carinii* and aspergillosis during treatment with prednisone or combination chemotherapy, strong consideration should be given to prophylactic therapy with trimethoprim-sulfamethoxasole and oral antifungal agents.[118]

Prognosis

AILD progresses to malignant T-cell lymphoma. It is defined by the presence of clusters, islands, or diffuse infiltrates of monomorphic immunoblasts and has a much graver prognosis than AILD alone.[111] [119] [120] Many apparent cases of AILD may actually represent early T-cell lymphomas; clonal populations of T cells have been identified in several cases studied by Southern blot analysis and PCR.[121] Cytogenetic analysis and fluorescent hybridization demonstrate the clonal nature in many instances.[112] [122]

Histiocytic Necrotizing Lymphadenopathy

Histiocytic necrotizing lymphadenitis (Kikuchis disease) is a pseudolymphomatous lesion, which presents as cervical lymphadenopathy and occurs predominantly in young women. Patients usually present with a cervical mass, but are otherwise asymptomatic and do not show evidence of IM.[123] [124] [125] The process usually resolves spontaneously in 1 to 4 months without further therapy.[123] [124] [125] These patients do not have an increased risk of lymphoma, and surgical excision is curative. Although the etiology of histiocytic necrotizing lymphadenitis is unknown, its histologic and clinical features are similar to those of systemic lupus erythematosus,[126] and the disease may, in fact, reflect a self-limited autoimmune condition resembling systemic lupus erythematosus. Therefore, an autoimmune disorder such as lupus must be ruled out when a diagnosis of Kikuchis disease is received.

Sinus Histiocytosis with Massive Lymphadenopathy

Clinical Manifestations

Patients with sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease) usually present with bilateral lymphadenopathy predominantly affecting the cervical region, and they usually exhibit some systemic symptoms, including fever, polyclonal hypergammaglobulinemia, and increased erythrocyte sedimentation rate.[127] [128] The disease occurs most commonly in black children.[127] [128] The etiology of the disease remains obscure. A relationship to an underlying immunodeficiency has been postulated.[129]

Laboratory Evaluation

Biopsies reveal pericapsular fibrosis, dilation of sinuses, numerous intrasinusoidal histiocytes, and abundant plasma cells. The most striking histologic feature is the presence of lymphocytes and other hematopoietic cells within the cytoplasm of the sinus histiocytes, called emperipoiesis.

Therapy and Prognosis

Clinically most of the patients are thought to have a non-Hodgkins lymphoma. However, the disease has a protracted but benign course; spontaneous regression of the lymphadenopathy and total recovery will occur in most cases.[126] [127] Administration of antibiotics, radiation, antituberculin therapy, and/or steroids does not have a major impact on the course of the disease.[126] [127]

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CONCLUSION

Atypical lymphoproliferations occur in a variety of clinical settings in response to a variety of stimuli. Accurate diagnosis of these processes is made by careful clinicopathologic correlation and in some cases by genetic, immunologic, virologic, and molecular techniques. In many of these conditions emphasis should not be placed on determining whether this is a malignant lymphoma but, rather, whether it is a lethal clinical condition requiring optimally staged therapy.

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Chapter 79 - Clinical Approaches to Infections in the Compromised Host

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INTRODUCTION

Patients with hematologic disease may be predisposed to the development of infections as a result of a wide array of potential defects in host defenses. Just as the clinical spectrum of hematologic diseases is broad, so is the range of associated abnormalities in host defense. Accordingly, the types of infection encountered in this population as well as their relative severity will vary dramatically.

Good medical care of immunocompromised patients demands a broad base of factual knowledge, but optimal management mandates evaluation of each patient on an individual basis. Although it is useful, and often appropriate, to establish general guidelines for the diagnosis and treatment of infectious complications, decisions should always reflect each patients specific, often unique, clinical setting.

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TYPES AND SOURCES OF PATHOGENS ENCOUNTERED IN COMPROMISED PATIENTS

The list of predominant pathogens encountered in the compromised host is extensive and includes bacteria, fungi, viruses, and protozoa. The range of potential pathogens in any given patient will depend on the specific perturbations in host defense present in that individual, which in turn will vary according to the underlying disease and its management (Table 79-1). For example, the bacterial pathogens encountered in neutropenic patients with acute myeloid leukemia (AML) will differ dramatically from those commonly encountered in a patient with sickle cell anemia and splenic dysfunction. This chapter presents a detailed analysis of the pathogens associated with specific hematologic disorders.

The epidemiology of most pathogens is well established, but it is often difficult to determine the precise environmental or endogenous origin of organisms isolated from individual infectious episodes. It is clear, however, that most pathogens have colonized a body site at some point before causing infection in compromised patients. Many of these, such as the enteric gram-negative bacteria or *Candida albicans*, may be found in the normal flora of healthy individuals. Others, such as *Pseudomonas aeruginosa* or *Aspergillus* spp., are usually acquired from exogenous sources but cause transient colonization before the development of invasive disease. Still others, such as varicella-zoster virus and *Pneumocystis carinii*, may be present in a latent or subclinical stage for years before significant infection develops. Ultimately the presence or absence of serious invasive disease will be determined both by the relative virulence of the resident or colonizing organism and by the severity and type of host impairment (Fig. 79-1). Clinically, it is crucial that potential sources of infection and mechanisms of colonization be identified in order to develop rational strategies for infection prevention in high-risk populations.

Bacteria

Bacteria are responsible for most infections encountered in compromised patients, accounting for the greatest morbidity and mortality. The predominant bacterial pathogens encountered will vary substantially, depending on the population at risk. Patients with neutropenia are at high risk of serious infections due to gram-negative pathogens such as *Escherichia coli* and *Klebsiella pneumoniae. E. coli* and *K. pneumoniae* are Enterobacteriaceae present in the normal gastrointestinal (GI) flora, while *P. aeruginosa* is more often acquired from exogenous sources. Encapsulated gram-negative bacteria, such as *Hemophilus influenzae and Neisseria* spp., are more often encountered in patients who have undergone splenectomy. *Salmonella* spp. infections occur with increased frequency in those with dysgammaglobulinemia or AIDS. Gram-negative bacteria of increasing importance include other Enterobacteriaceae (e.g., *Citrobacter* spp., *Enterobacter* spp., or *Serratia marcescens*), *Acinetobacter* spp., non-*aeruginosa Pseudomonas*-like organisms (e.g., *Stenotrophomonas maltophilia*, *Burkholderia cepacia*), and *Legionella* spp.

Patients with serious illness may show a dramatic change in the pattern of their colonizing microbial flora after admission to the hospital. Indeed, the most consistent and clinically important change observed is a relative decrease in the organisms making up the normal flora and a concomitant increase in potentially pathogenic aerobic gram-negative bacteria. These changes have been observed in the noncancer population [1] as well as in patients with leukemia. [2] In the study by Fainstein et al.,[2] the oropharyngeal and fecal flora of 33 leukemic patients was examined serially during the course of hospitalization for intensive chemotherapy. Although most patients had a

TABLE 79-1 -- Predominant Pathogens in Compromised Patients: Association with Selected Defects in Host Defense

Host-Defense Impairment	Bacteria	Fungi	Viruses	Other
Neutropenia	Gram-negative Enteric organisms (*E. coli, K. pneumoniae, Enterobacter* spp. *Citrobacter* spp.) *Pseudomonas aeruginosa* Gram-positive Staphylococci (coagulase-negative, coagulase-positive) Streptococci (group D, -hemolytic) Anaerobes (anaerobic streptococci, *Clostridium* spp. *Bacteroides* spp.)	*Candida* species (*C. albicans, C. tropicalis,* other species) *Aspergillus* species (*A. fumigatus, A. flavus*)		
Abnormal cell-mediated immunity	*Legionella* *Nocardia asteroides* *Salmonella* spp. Mycobacteria (*M. tuberculosis* and atypical mycobacteria) Disseminated infection from live bacteria vaccine (BCG)	*Cryptococcus neoformans* *Histoplasma capsulatum* *Coccidioides immitis* *Candida*	Varicella-zoster virus Herpes simplex virus Cytomegalovirus Epstein-Barr virus Herpesvirus 6 Disseminated infection from live virus vaccines (vaccinia, measles, rubella, mumps, yellow fever, live polio)	*Pneumocystis carinii* *Toxoplasma gondii* *Cryptosporidium* *Strongyloides stercorali*
Immunoglobulin abnormalities	Gram-positive *Streptococcus pneumoniae, Staphylococcus aureus* Gram-negative *Hemophilus influenzae* *Neisseria* spp., enteric organisms		Enteroviruses Disseminated infection from live virus vaccines (vaccinia, measles, rubella, mumps, yellow fever, live polio)	*Giardia lamblia*
Complement abnormalities				

C3, C5	Gram-positive *S. pneumoniae*, staphylococci Gram-negative *Hemophilus influenzae, Neisseria* spp., enteric organisms			
C5C9	*Neisseria* spp. (*N. gonorrhoeae, N. meningitidis*)			
Anatomic disruption				
Oral cavity	-Hemolytic streptococci, oral anaerobes (*Peptococcus, Peptostreptococcus*)	*Candida*	Herpes simplex virus	
Esophagus	Staphylococci, other colonizing organisms	*Candida*	Herpes simplex virus Cytomegalovirus	
Lower gastrointestinal tract	Gram-positive Group D streptococci Gram-negative Enteric organisms Anaerobes (*Bacteroides fragilis, Clostridium perfringens*)	*Candida*		*Strongyloides stercoralis*
Skin (IV catheter)	Gram-positive Staphylococci, streptococci *Corynebacteria, Bacillus* spp. Gram-negative *P. aeruginosa,* enteric organisms Mycobacteria*M. fortuitum, M. chelonei*	*Candida Aspergillus*		
Urinary tract	Gram-positive Group D streptococci Gram-negative Enteric organisms *P. aeruginosa*	*Candida*		
Splenectomy	Gram-positive *S. pneumoniae* *DF2 bacillus* *Capnocytophaga canimorsus* Gram-negative *S. pneumoniae* *H. influenzae* *Salmonella* (sickle cell disease)			*Babesia*

Figure 79-1 Sources of nosocomial infection in high-risk patients. Interactions between colonization and infection.

normal pattern of microbial flora on admission, by the completion of the hospital stay 68% of the initial throat isolates and 57% of the fecal isolates had changed. The main shift was in the acquisition or increasing prominence of aerobic gram-negative bacilli (mostly *E. coli*, *K. pneumoniae*, *Enterobacter* spp., and *P. aeruginosa*). Although these changes were initially seen even before antibiotics were administered, they were clearly accentuated after courses of antibacterial therapy.

In another study addressing this issue, 48 patients with leukemia were followed for 2.5 years with extensive microbiologic surveillance. [3] Again, the culture data and clinical observations indicated that most infections caused by gram-negative bacteria originated in the endogenous flora. However, 47% of infections were caused by organisms that had colonized the patients only after admission to the hospital. Studies have also suggested that differences may exist among the gram-negative bacteria, not only in their propensity to colonize but also in their ability to produce invasive infection after colonization. For example, certain biotypes of Enterobacteriaceae species have been associated with a particularly high incidence of both colonization and infection in leukemic patients. [4] Nevertheless, most patients who become colonized with enteric gram-negative rods do not develop clinically significant infections. By contrast, other organisms such as *P. aeruginosa* appear to be intrinsically more virulent and are much more likely to cause invasive infection after colonization, especially during periods of severe host impairment such as profound granulocytopenia. [5]

Over the past 15 years, the incidence of infections due to gram-positive organisms has increased significantly in both compromised and noncompromised patients. [6] [7] [8] [9] [10] [11] [12] [13] The most common gram-positive organisms causing infection in almost all groups of immunocompromised patients are the coagulase-positive and coagulase-negative staphylococci (usually *Staphylococcus aureus* and *S. epidermidis*, respectively), streptococcal species, and enterococci, including vancomycin-resistant enterococci. Although relatively uncommon overall, *Streptococcus pneumoniae* is seen with increased frequency in dysgammaglobulinemia or splenectomy patients. Serious infections due to -hemolytic streptococci may be seen more commonly in patients who have received high doses of cytosine arabinoside (Ara-C) and who have mucositis. Streptococcal bacteremia in these patients may be complicated by pulmonary involvement, including adult respiratory distress syndrome.[14] The use of prophylactic fluoroquinolones appears to be associated with an increased incidence of staphylococcal and streptococcal infection. [15] [16] Other potentially important gram-positive bacteria that are encountered less frequently include *Corynebacterium* spp., *Bacillus* spp., and *Listeria monocytogenes*. Some of these organisms, such as *S. pneumoniae*, enterococci, and *S. epidermidis*, are found in almost all healthy individuals as part of the normal GI or skin flora. Others, such as methicillin-resistant *S. aureus* or *Corynebacterium jeikeium*, are not commonly found as part of the normal flora but are more often acquired from exogenous sources, particularly in hospitalized patients. [17] [18] The increase in bacteremias due to organisms that colonize the skin (e.g., *C. jeikeium* and *S. epidermidis*) is often attributed to the increased use of indwelling intravenous catheters, although not all studies have corroborated this finding. [12] [19] One study by Khabbaz and co-workers[20] has suggested that GI tract colonization with *S. epidermidis* may correlate better with subsequent bacteremia than does skin colonization.

The major anaerobic bacteria causing infection in compromised patients are *Clostridium* spp. (both *C. perfringens* and non-*perfringens* clostridia), *Bacteroides fragilis*, and other *Bacteroides* spp. In addition, a variety of other anaerobic organisms have been reported, including *Fusobacterium* spp., *Peptostreptococcus*, *Peptococcus*, *Eubacterium*, *Veillonella*, and *Bifidobacterium*. [21] [22] [23] [24] Most of these anaerobic organisms are normal residents of the human GI tract, with anaerobes accounting for 90% of fecal organisms. Infections due to these anaerobes are most often seen in patients with disruption of GI mucosal barriers, often as a result of chemotherapy or tumor invasion. Anaerobic infections are relatively uncommon, accounting for only 510% of bacteremias, even in profoundly neutropenic patients. [25] Clearly, they play a more prominent role in localized intra-abdominal infections or in certain soft tissue infections such as perirectal cellulitis.

From an epidemiologic perspective, infection due to *C. difficile* may differ from infection caused by most other anaerobic organisms. *C. difficile*-related disease (ranging from mild GI symptoms to pseudomembranous colitis) often develops as a result of overgrowth of endogenous toxin-producing organisms that have colonized the GI tract, particularly after surgical procedures or exposure to antibiotics. Studies addressing epidemiologic issues have suggested an important role for nosocomial acquisition and transmission of this organism. McFarland and colleagues [26] reported that one of five patients with stool cultures positive for *C. difficile* acquired the organism while hospitalized, having been culture negative on admission. Of these patients with newly acquired positive stool cultures, approximately one-third developed a diarrheal illness, while two-thirds remained asymptomatic. Patient-to-patient transmission of *C. difficile* was strongly suggested by the temporal and spatial clustering of incident cases with identical immunoblot types and by the significantly more frequent and earlier acquisition of *C. difficile* among patients exposed to roommates with positive cultures. Almost two-thirds of the hospital personnel caring for these patients had positive cultures for *C. difficile* isolated from

their hands, and significant contamination of the rooms of both symptomatic and asymptomatic patients was found. Clearly, these data may have important implications for the prevention of *C. difficile*-related disease.

Anaerobic bacteria of the GI tract may assist in protecting against infections caused by other organisms. This theory of colonization resistance suggests that the large quantity of anaerobic bacteria normally present in the gut produces unfavorable conditions for colonization or overgrowth by other more pathogenic organisms, such as the Enterobacteriaceae or *P. aeruginosa*.[27] Indeed, this concept has been supported by a number of preclinical studies. For example, Van der Waaij and colleagues[28] showed that GI colonization could be established in germ-free mice by oral administration of small inocula (approximately 10^3 organisms) of gram-negative bacteria. By contrast, >10[7] organisms were required to establish colonization in mice with their normal flora intact. Accordingly, recent antibiotic prophylaxis regimens have been designed to suppress aerobic gram-negative organisms while preserving endogenous anaerobic flora. Antibiotics such as trimethoprim-sulfamethoxazole and the fluoroquinolones have antimicrobial activity that is almost exclusively against aerobic organisms, and so these agents have been widely employed for prophylaxis.

In the past, mycobacterial infections were relatively uncommon in most immunocompromised populations. A striking increase has been documented in the number of infections due to *Mycobacterium tuberculosis*, and a particular concern is the emergence of multi-drug-resistant tuberculosis in the United States. [29] [30] Infections due to *M. tuberculosis* as well as *M. avium-intracellulare* complex are seen primarily in patients with human immunodeficiency virus-1 (HIV-1) infection. Patients with hairy cell leukemia appear to be at increased risk for the development of infection with atypical mycobacteria such as *M. kansasii*, *M. fortuitum*, *M. chelonei*, and *M. avium-intracellulare*. [31] [32] [33] In rare cases, certain rapid growers, such as *M. fortuitum* and *M. chelonei*, cause significant infections around exit sites of indwelling intravenous catheters.

The community and the hospital environment have many exogenous sources that can lead to colonization or infection, or both. In addition to patient-to-patient and staff-to-patient transmission,

exogenous sources include food, air, water, and specialized equipment such as catheters, respirators, and humidifiers; as well, contamination may result from a variety of invasive procedures.[34] [35] For example, *P. aeruginosa* is not part of the normal GI flora but flourishes in moist environments, particularly in soil or on certain fruits and vegetables. Accordingly, it might be prudent for certain patients at risk, particularly those receiving antibiotics, to restrict direct contact with potential sources such as potted plants or uncooked foods.[36] [37] [38] Knowledge of the common sources of certain organisms can also help prevent the spread of infection or prevent an outbreak. For example, a clustering of infections due to *Legionella pneumophila* should direct attention to potential water sources such as air-conditioning cooling towers,[39] [40] an outbreak of infection due to *Burkholderia cepacia* in an intensive care unit should raise the possibility of contamination of equipment used for respiratory care,[41] and a clustering of infection due to *C. difficile* or methicillin-resistant *S. aureus* should raise suspicion of a staff or patient carrier or might point to the need for more rigorous hand washing by patient care staff.

Fungi

The major fungal species that cause serious infection in compromised patients are *Candida* spp., *Aspergillus* spp., and *Cryptococcus neoformans*. Other less frequently encountered but nevertheless potentially important fungal pathogens include the Zygomycetes (e.g., *Mucor* spp. or *Rhizopus* spp.), *Fusarium* spp., *Trichosporon beigelii*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Malassezia furfur*, and the dematiaceous fungi, such as *Bipolaris* species.[42] [43] [44] [45] [46] [47] The predominant fungal pathogens will vary according to the nature of host impairment in any given patient. For example, invasive infections due to *Candida* or *Aspergillus* are most commonly encountered in patients with prolonged episodes of neutropenia. Infections due to *C. neoformans*, *H. capsulatum*, or *C. immitis* are more often seen in patients having an underlying disease (e.g., lymphoma, HIV infection) or receiving a therapeutic intervention (e.g., corticosteroid treatment) associated with impaired cell-mediated immunity.

Overall, *Candida* spp. represent the most common fungal pathogens responsible for serious infection in immunocompromised patients. *C. albicans* may be found as part of the normal human GI and cutaneous flora,[48] but certain factors may predispose patients to a change in the patterns of colonization with this fungus. This change is often marked in those receiving broad-spectrum antibiotics, requiring extensive hospitalization, or receiving immunosuppressive agents. Indeed, widespread and even visibly apparent GI overgrowth can occur in these settings. [49] GI colonization and overgrowth can then provide an ideal setting for the development of invasive disease, which often occurs secondary to intercurrent mucosal disruption as a result of surgery, tumor invasion, or chemotherapy. Certain fungal species appear to be intrinsically more pathogenic than others; colonization with these organisms is more frequently associated with invasive infection. For example, in one series, 16% of cancer patients colonized with *Candida* (*Torulopsis*) *glabrata* developed serious invasive infection. [50] In another study, 14 of 25 patients colonized by *Candida tropicalis* developed an infection with this organism. [51]

Aspergillus spp. constitute the second most common fungal pathogens in immunocompromised hosts. *Aspergillus* spp. are not part of the normal human flora but are usually acquired from exogenous sources in the hospital setting, such as unfiltered air, contaminated ventilation systems, sites of renovation or construction, food, and ornamental plants.[44] [52] Air is the principal route of transmission of *Aspergillus* spp. within the hospital environment, and the respiratory tract is the most common entry portal. Upper airway colonization with *Aspergillus* probably precedes most cases of invasive infection; some authorities believe that documentation of nasal carriage of *Aspergillus* may predict the development of aspergillosis in certain high-risk settings. [53] Also, smokers appear to have an increased incidence of colonization with *Aspergillus* spp., but in some studies they do not appear to have an increased incidence of invasive *Aspergillus*, even in the setting of severe immunosuppression.[54] [55]

C. neoformans, the third most common pathogenic fungus in the compromised population, is an encapsulated yeast that is ubiquitous in nature. Although pigeon excreta are the classically implicated environmental source, this fungus can be found widely in many types of soil. [56] [57] *C. neoformans* does not normally colonize humans. Primary infection is usually not hospital acquired and is often subclinical. Disseminated infection in high-risk patients usually represents reactivation of latent infection.

Viruses

The most significant viral infections seen in immunocompromised patients belong to the herpes group and include herpes simplex virus (HSV), varicella-zoster virus (VZV), and cytomegalovirus (CMV). Serious infections due to these viruses are seen with increasing frequency in certain subgroups of immunosuppressed patients, particularly those with impaired cell-mediated immunity. Infection due to HSV, VZV, or CMV can result either from primary infection or from reactivation of latent infection. Most serious infections in adults occur as a result of reactivation of infection initially acquired before the onset of impaired immune function.

Other viruses can cause serious infection in this population, but their incidence does not appear to be substantially increased compared to a normal population. These include Epstein-Barr virus (EBV), human herpesvirus type 6 (HHV-6), influenza and parainfluenza viruses, respiratory syncytial virus (RSV), measles virus, adenoviruses, and the enteroviruses. Infection with some viruses, such as parvovirus or HIV-1, may, in certain hosts, result in secondary hematologic abnormalities that further compromise immune function.[58]

Both HSV-1 and HSV-2 can lead to significant morbidity in the compromised host. As in immunocompetent individuals, HSV-1 is encountered with greater frequency. Primary infection with HSV-1 usually occurs in childhood between the ages of 2 and 10 years and is most often transmitted by contact with oral secretions. HSV-2 infection is usually spread by genital contact and is generally acquired after puberty. In adults, the incidence of antibodies to HSV (presumably reflective of prior infection) has been 30100% in various studies and appears to be higher in groups of lower socioeconomic status. [59] [60] [61] [62] In a retrospective review of sera from patients with hematologic diseases presenting for bone marrow transplantation, 51 of 93 had elevated titers of anti-HSV antibody. [63] High titers of anti-HSV-1 have been correlated with the development of subsequent infections in immunocompromised patients. [64] [65] In addition to reactivation of latent disease, seronegative individuals may acquire primary infection from contact with either asymptomatic execretors or overtly infected persons, although virus titers are higher and transmissibility is greater in the latter group. [59]

In the healthy population, VZV is a highly contagious virus that causes primary infection predominantly in children. Primary varicella infection, or chickenpox, occurs in 50% of children before they enter school, and >90% of adults have VZV antibodies as a result of prior clinical or subclinical infection. In immunocompromised patients, both primary disease and reactivation of latent virus are clinically important. In accordance with its prevalence in the healthy population, primary varicella in compromised patients tends to affect children and is responsible for most serious infections in those with hematologic malignancies. [66] [67] Adult patients who are

seronegative are also at risk.

Person-to-person transmission to other patients at risk of

primary varicella infection can occur after close contacts both in and out of the hospital. Primary varicella is transmitted predominantly by the respiratory route. Secondary varicella infection, or herpes zoster (also known as shingles), can occur in otherwise healthy patients, particularly in the aged, but it is more common and usually more severe in immunosuppressed patients.[68] [69] [70] [71] In virtually all cases, herpes zoster represents reactivation of latent disease. Little concrete evidence has been found to support its acquisition by exposure to other patients with either primary varicella infection or zoster. [71] [72] However, patients at risk of the development of primary varicella infection may acquire the infection through contact with people who have zoster or chickenpox.

CMV can cause serious infection in a variety of immunocompromised populations, but most notably in the HIV-infected population and in those undergoing allogeneic bone marrow transplantation. Most studies indicate a 6070% prevalence of antibody to CMV by adulthood in both compromised and noncompromised patients. [73] [74] [75] [76] As with HSV, the prevalence of antibodies will vary with the socioeconomic condition, geography, and age of the population studied. A study from Houston found that only 25% of healthy white subjects aged 1822 had positive titers, compared with 50% of nonwhite individuals. [77]

In the compromised host, serious CMV infection can occur either by reactivation of latent disease as a result of immunosuppression or through primary infection. Among patients undergoing bone marrow transplantation, the incidence of significant CMV infection (usually pulmonary) is substantially higher for patients who are initially seropositive for anti-CMV antibody than for those who are seronegative. Meyers et al. [74] found that 69% of seropositive patients developed CMV, compared with 36% of seronegative patients. Presumably, most of the seropositive patients experienced reactivation of latent disease, while the seronegative patients acquired a primary infection. For this latter group, it was subsequently established that the source of virus was often transfused blood products or bone marrow from seropositive donors. The exclusive use of seronegative blood products for seronegative patients has nearly eliminated serious CMV infection in these patients. [78]

Increasing age may be associated with reactivation of CMV. McVoy and Adler [79] used IgM positivity as a marker of reactivation in patients with known positivity for IgG and found an increase in positivity from 15% for those <20 years old to 63% for those >60 years old. Consequently, older patients with hematologic diseases could be at higher risk than younger patients for the development of CMV infection.

The development of CMV in high-risk seropositive patients has been assumed to occur largely as a result of reactivation of endogenous previously acquired virus. Accordingly, blood products for patients who are already seropositive have generally not been screened. However, studies using restriction enzyme digest profiles and strain-specific neutralizing antibody measurements indicate that even seropositive patients can develop symptomatic CMV as a result of reinfection with different donor strains.[80] [81] [82] In light of these findings, further studies may be warranted to address the value of exclusive use of seronegative blood products even for seropositive patients.

The respiratory viruses, including RSV, influenza, parainfluenza, and adenovirus, have been increasingly recognized as pathogens in immunocompromised patients over the past decade. RSV is the leading cause of pneumonia and bronchiolitis in young children and is an important cause of nosocomial infection in childrens hospitals. A study by Hall et al.[83] found a higher incidence of nosocomial acquisition of RSV, of RSV pneumonia, and of deaths due to RSV in children with cancer than in children with normal immune function.[83] Another study has described severe RSV pneumonia in adults with lymphoma and in adults who underwent bone marrow or solid organ transplantation.[84] Prompt identification and isolation of infected patients are mandatory to prevent nosocomial transmission of RSV infection.

Parasites

With the exception of *P. carinii*, parasites are relatively uncommon causes of infection in compromised patients with non-HIV-related diseases. The parasitic infections most commonly encountered include those due to *P. carinii*, *Toxoplasma gondii*, *Cryptosporidium*, and *Strongyloides stercoralis* and occur most commonly in patients with altered cell-mediated immunity. When infection develops as a result of these organisms, it usually represents the reactivation of a latent or asymptomatic infection previously acquired outside the hospital setting. Gastroenteritis due to *Cryptosporidium* is an exception, with person-to-person transmission (both in and out of the hospital) and large water-source outbreaks having been clearly documented. [85] [86] [87] [88] [89] [90]

Symptomatic infection due to *P. carinii* likely results from reactivation of latent cysts acquired early in life rather than from new acquisition of disease. One study supporting this concept evaluated serum from 600 normal volunteers and used an indirect fluorescent antibody test for detection of anti- *P. carinii* IgG.[91] Of the children tested in this series, significant titers were found in nearly all by the age of 2 years, which implies asymptomatic infection early in life. In addition, the largest published study of a so-called institutional clustering of *P. carinii* infection, which occurred at St. Jude Childrens Cancer Research Hospital, failed to corroborate, or even suggest, any patient-to-patient spread.[92]

By contrast, studies of clusters of *P. carinii* pneumonia have suggested the possibility of person-to-person transmission. [93] [94] [95] In a study from Memorial Hospital, 11 cases of *P. carinii* pneumonia were seen in a 3-month period, 10 of them involving patients with leukemia or lymphoma. Epidemiologic analysis indicated that most of the patients either had had extensive contact with each other or had been treated by the same physician before the onset of disease. [94] In a study by Ruebush et al.,[93] 10 cases of *P. carinii* pneumonia were seen in a 10-month period, all in children with acute lymphocytic leukemia (ALL). Although their risk of infection appeared to be related to the intensity of chemotherapy, members of the hospital staff who had close contact with these children had significantly higher titers of anti- *P. carinii* antibody than other staff members or the parents of the children, suggesting that transmission could have occurred within the hospital environment. In addition, animal data suggest airborne transmission from infected to uninfected rats.[96] Although these studies are suggestive, person-to-person transmission of *Pneumocystis* has not been clearly documented, even in centers with a large number of cases.

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ALTERATIONS IN HOST DEFENSE-ASSOCIATED PATHOGENS AND MAJOR CLINICAL CORRELATES

The range of abnormalities in host defense that can occur in patients with hematologic diseases and the major associated pathogens are listed in Table 79-2 . For any given patient, only rarely is a specific defect in host defense seen in isolation. Nevertheless, certain types or patterns of defects are more often associated with specific underlying diseases or particular therapeutic interventions.

Anatomic Alterations

The skin and mucosal surfaces represent an important primary defense against both endogenous and exogenous sources of infections.

Compromised patients frequently have alterations in integumentary and mucosal barriers. Disruption of skin and mucosa may result from invasion by malignant cells, from the effects of chemotherapy or radiation therapy, from the use of invasive diagnostic or therapeutic procedures (e.g., intravenous catheters), and from the effects of locally disruptive infections such as oral HSV. Such mucosal alterations may provide a nidus for microbial colonization, a focus for localized infection, and a portal of entry for systemic invasion.

The types of organisms associated with specific alterations in skin or mucosal surfaces will depend on the site of breakdown as well as the presence or absence of other associated factors. For example, isolated disruption of the skin associated with insertion of an indwelling intravenous catheter primarily increases the risk of infection with certain gram-positive organisms, usually coagulase-negative staphylococci. However, in patients who have been hospitalized, who are neutropenic, or who have previously received broad-spectrum antibiotics, the normal flora of the skin can change, and serious catheter-related infections due to a variety of other organisms can occur. These organisms include other gram-positive bacteria, such as *C. jeikeium* or *Bacillus* spp.;[18] [97] [98] a variety of gram-negative organisms, including *P. aeruginosa*, non-*aeruginosa Pseudomonas*-like organisms, or enteric gram-negative rods; [99] [100] [101] [102] fungi such as *C. albicans* or *Aspergillus* spp.;[103] [104] [105] and atypical *Mycobacterium* spp. such as *M. chelonei* or *M. fortuitum*.

Because the GI tract is normally colonized by a wide array of aerobic and anaerobic bacteria as well as by some fungi, disruption of its mucosa can lead to infection by a variety of pathogens, and sometimes to polymicrobial infection. Identification of a specific site of mucosal damage in the GI tract may help predict which pathogens play a role, based on a knowledge of normal flora in that area. Oral ulceration, for example, not only will suggest the possibility of HSV reactivation but may also predict bloodstream infection with -hemolytic streptococci. Alternatively, isolation of certain organisms in blood cultures can help direct diagnostic or therapeutic intervention toward specific sites, even in the absence of clinically evident disease. For example, isolation from the blood of organisms normally found in the mouth, such as *Peptococcus* or *Capnocytophaga*, should focus attention on the oral cavity, while cultures yielding *B. fragilis* or *Streptococcus bovis* can be clues to pathology originating in the lower GI tract. [22] [106] [107] [108]

One of the most common causes of disruption of the GI mucosal integrity is administration of chemotherapeutic agents to patients with malignancy. Those that lead to the most serious problems include Ara-C, the anthracyclines (daunorubicin and doxorubicin), methotrexate, mercaptopurine, and fluorouracil. Although stomatitis is usually the most clinically recognizable manifestation of GI toxicity, the clinician should remember that significant oral mucositis is often only the tip of the iceberg and that diffuse GI involvement is likely present.

The urinary tract may also be affected by tumor, invasive procedures, or cytotoxic therapy, with subsequent colonization and the potential for local or invasive infection. The urinary tract pathogens most commonly encountered in these settings are the enteric gram-negative rods, enterococci, and *C. albicans*.

In addition to actual mucosal breakdown, other anatomic alterations can occur in compromised patients, increasing their risk of infection. Mechanical obstruction of body passages by tumor, for example, can greatly increase the risk of serious localized infection. This may be due in part to stasis of local body fluids, with resultant overgrowth of potentially pathogenic colonizing organisms. Common sites of secondary infection due to obstruction include the lung, urinary tract, biliary tract, and auditory tube. An obstructive process should be considered when infection at any of these sites fails to respond to appropriate antibiotics.

Anatomic alteration can also predispose to infection simply by providing a nidus for growth of organisms. It is likely that most healthy individuals experience periodic but uneventful episodes of transient bacteremia (e.g., secondary to toothbrushing). In the setting of altered anatomy or foreign bodies such as prosthetic devices, however, even transient bacteremia can lead to persistent localized infection. Important examples of hematologic diseases associated with such anatomic alterations include sickle cell anemia and hemophilia. In patients with sickle cell disease, macrophage and splenic dysfunction predispose to the development of certain bacteremias, especially caused by *S. pneumoniae* and *Salmonella* spp.[109] [110] [111] Many of these patients have underlying anatomic abnormalities of bones and joints as a result of vaso-occlusive crises causing infarction of bone marrow, bony cortex, or synovium. In turn, these changes may predispose to the development of infection at the sites of anatomic alteration, such as osteomyelitis or arthritis. [112] [113] Decreased blood flow to these areas and increased adherence of organisms may be additional contributing factors. Similarly, hemophiliacs may develop anatomic alterations of joints as a result of repeated episodes of hemarthrosis. Although suppurative arthritis has been less frequently described in hemophiliacs than in patients with sickle cell disease, it can occur and should be considered in the differential diagnosis of any hemophiliac whose articular signs and symptoms fail to improve quickly after administration of appropriate coagulation factors. [114] Patients with chronic hemolytic states may develop gallstones, which can also provide a nidus for infection.

Phagocyte Defects

Quantitative and qualitative defects affecting polymorphonuclear leukocytes (PMNs) and monocytes can occur in compromised patients. Although both cell types are important, the contribution of granulocyte abnormalities to the pathogenesis of infectious diseases has been better described and more completely elucidated, primarily because isolated defects in monocytes are relatively rare.

Quantitative Abnormalities

Significance of Neutropenia in Predisposing to Infection

Of the many abnormalities in host defense that potentially affect compromised patients, granulocytopenia is perhaps the most important, for two reasons: (1) it predisposes patients to a wide array of pathogenic organisms, and (2) it may be associated with rapid progression of infection, often in the absence of classic signs

and symptoms.

Most clinicians associate granulocytopenia with malignant disease, with which it can occur as a result of either a myelophthisic process or, more commonly, cytotoxic therapy. Indeed, cancer patients account for most cases of neutropenia encountered in the clinical setting. However, it is important to recognize that many other pathologic processes may be associated with granulocytopenia. Unfortunately, very few data specifically address the relative risks, types, or management of infections in neutropenic patients with nonmalignant diseases. In the absence of these data, the clinician is often forced to extrapolate from studies in the cancer population.

For cancer patients receiving cytotoxic therapy, the relationship between the development of granulocytopenia and serious infection has been established unequivocally. Bodey et al.[115] followed the course of 34 patients with ALL and 29 patients with AML (age range, 177 years) and correlated the development of infection with granulocyte counts over the course of treatment (Table 79-3). The salient findings of this study were as follows. First, the incidence of proven infection and the absolute granulocyte level showed a definite relationship. The risk of infection

TABLE 79-2 -- Selected Hematologic Diseases, Associated Host Defense Impairment, and Common Pathogens

Disease/Condition	Factors Contributing to Infection						Most Common Pathogens
	Neutropenia	Phagocyte Dysfunction	Abnormal Cell-Mediated Immunity	Abnormal Humoral Immunity	Anatomic Disruption	Splenic Dysfunction	
Acute myeloid leukemia	Major factor	Abnormalities described	May result from therapy (e.g., bone marrow transplantion)	May result from therapy	Often significant, may be caused by therapy, tumor invasion, localized infection	Not prominent	Bacteria Gram-positive (staphylococci, streptococci) Gram-negative (enteric organisms, *P. aeruginosa*) Fungi *Candida* *Aspergillus* Viruses Herpes simplex Varicella-zoster Other *P. carinii*
Acute lymphocytic leukemia	Major factor	Abnormalities described	May result from disease or therapy	May result from therapy	Often significant (see AML)	Not prominent	See AML. *P. carinii* more common in some centers
Hairy cell leukemia	Major factor (also monocytopenia)	Probably contributes	Probably contributes	Not prominent	Not prominent unless aggressive therapy	Major factor if therapeutic splenectomy	Gram-positive and gram-negative bacteria Atypical mycobacteria
Chronic lymphocytic leukemia	Not prominent unless aggressive therapy or end stage	Not prominent	May result from therapy (e.g., steroids)	Major factor	Not prominent unless aggressive therapy	Not prominent	Bacteria *S. pneumoniae* *H. influenzae* *Neisseria* spp.
Chronic myeloid leukemia	Not prominent unless aggressive therapy or end stage	Abnormalities described	Not prominent	Not prominent	Not prominent unless aggressive therapy	Not prominent	Few infections in stable chronic phase Blast crisis (see AML)
Multiple myeloma	Not prominent unless aggressive therapy or end stage	Abnormalities described	Not prominent	Major factor	Not prominent	Not prominent	Bacteria *S. pneumoniae* *H. influenzae* *Neisseria* spp. Enteric gram-negative
Hodgkin disease	May be major factor, depending on therapy	Abnormalities described	Major factor	May result from therapy	May be significant, depending on therapy	Major factor if diagnostic splenectomy or radiation to splenic bed	See AMLviral and parasitic infections may occur with higher frequency
Myelodysplastic syndromes	Variable	Abnormalities described	Not prominent	Not prominent	Not prominent	Major factor if therapeutic splenectomy	Gram-positive and gram-negative bacteria
Aplastic anemia	Major factor	Abnormalities described	May result from therapy (e.g., steroids, antithymocyte globulin, cyclosporine, bone marrow transplantation)	Not prominent	Not prominent unless aggressive therapy	Not prominent	Gram-positive and gram-negative bacteria*Candida, Aspergillus* Other depends on therapy
Chronic granulomatous disease	Not prominent	Major factordefective oxidative metabolism	Not prominent	Not prominent	Not prominent	Not prominent	Catalase-positive organisms*S. aureus,* enteric gram-negative bacteria, *P. cepacia, Nocardia, Aspergillus*
G6PD deficiency	Not prominent	Major factor if severe	Not prominent	Not prominent	Not prominent	Not prominent	Catalase-positive organisms if severe

Myeloperoxidase deficiency	Not prominent	Major factor (usually with few clinical manifestations)	Not prominent	Not prominent	Not prominent	Not prominent	Infections rare; possible increase in *Candida* infections
Paroxysmal nocturnal hemoglobinuria	May contribute	Potentially contributorydeficient Fc receptor	Not prominent	Not prominent	Thromboembolic disease may contribute (intra-abdominal infections)	Not prominent	Bacterial infections
Thalassemia factor	Not prominent	Abnormalities described	Abnormalities described	Abnormalities described	Gallstones may serve as nidus	Major if therapeutic splenectomy	Bacteria Staphylococci Streptococci Enteric gram-negative *Salmonella*
Sickle cell disease	Not prominent unless aplastic crisis	Abnormalities described	Not prominent	Probable major factorcomplement activation and opsonization	Bone infarctions may serve as nidus	Major factor	Bacteria *S. pneumoniae* *H. influenzae* *Salmonella*

began to increase significantly when granulocyte counts fell to <1,000/mm ^{3} and was most marked when the counts were 100/mm^3 . Second, in addition to the absolute granulocyte count, an important factor in predicting the risk of infection at any level was the duration of granulocytopenia. Thus, counts in the range of 1,000/mm ^{3} for a short duration (e.g., <1 week) were associated with little risk of infection, compared with a significant risk if the counts stayed within that range for 14 weeks. By contrast, neutropenia in the range of 100/mm^3 , even for a few days, was associated with a significant risk. Third, the incidence of infection was also related to a falling granulocyte count, but the magnitude of the fall was less important than the final granulocyte levels. Fourth, at any granulocyte level, the severe infectious episodes occurred more frequently during relapse than during remission, suggesting that other factors played a role in predisposing to infection.

For practical purposes, granulocytopenia is usually defined as a cell count of <500 PMNs/mm ^{3} and band forms/mm^3 . The absolute granulocyte count, the duration of granulocytopenia, and whether the neutrophil count is falling or rising must all be considered when assessing the risk to any individual patient. Granulocytopenia primarily predisposes to the development of bacterial and fungal infection but does not per se appear to increase the incidence or severity of viral and parasitic infections.

Types of Bacterial Infections in Neutropenic Patients

General trends in the frequency of infection caused by certain types of organisms have occurred since the 1950s and 1960s, when aggressive cytotoxic regimens were in early development. In those years, gram-positive bacteria were encountered most commonly. [116] [117] [118] However, by the 1970s, gram-negative organisms had emerged as the predominant pathogens isolated in neutropenic patients. Interestingly, during the 1980s and 1990s,

TABLE 79-3 -- Association of Granulocyte Level and the Chance of Developing Significant Infection

Granulocyte Level (per mm^3)		Percentage of Serious Infections for Different Durations of Granulocytopenia (in wk)							
Initial	**Change**	**1**	**2**	**3**	**4**	**6**	**10**	**12**	**14**
Any level	Any fall	12							
Any level	Fall to 2,000	2							
Any level	Fall to 1,500	5							
Any level	Fall to 1,000	10	30	45	50	65	70	85	100
Any level	Fall to 500	19							
Any level	Fall to <100	28	50	72	85	100			

Adapted from Bodey et al.,[115] with permission.

gram-positive organisms re-emerged as common bacterial isolates at many centers; at some centers they are now the most frequently encountered organisms. [8] [10] [119] [120] [121] Although an appreciation of these fluctuations can be helpful for the clinician, the potential for institutional variation in the patterns of infection cannot be overemphasized. Accordingly, clinicians treating granulocytopenic patients should have a working knowledge of the specific bacterial isolates encountered at their own facility.

Gram-Negative Bacteria.

The relative frequency of infections due to gram-negative organisms has decreased over the past 15 years. [120] In particular, the National Cancer Institute (NCI) has reported a dramatic decrease in infections due to *P. aeruginosa*, often an extremely virulent pathogen in neutropenic hosts. [120] *E. coli* and *K. pneumoniae* are now the most common gram-negative isolates at most centers. These organisms are frequently isolated from the blood; the most common endogenous source is the GI tract. Other common sources include the urinary tract and the respiratory tract, although a precise source of gram-negative bacteremia is identified in only a few cases. In neutropenic patients, gram-negative organisms may be isolated from cultures done on specimens obtained at the onset of fever or may cause breakthrough secondary infections in patients already receiving antibiotics. *Enterobacter* spp., *Citrobacter* spp., and *S. marcescens* are increasingly encountered Enterobacteriaceae that can rapidly become resistant to a broad range of -lactam antibiotics through the induction of chromosomally mediated -lactamases. [122] Other less common gram-negative organisms include *Acinetobacter* spp., *Hemophilus* spp. (usually nontypable *H. influenzae*),[123] and non-*aeruginosa Pseudomonas*-like organisms (often catheter related and antibiotic resistant). [101]

Gram-Positive Bacteria.

The gram-positive organisms most frequently encountered are the coagulase-negative staphylococci (most commonly *S. epidermidis*), coagulase-positive staphylococci (*S. aureus*), enterococci (most commonly *E. faecalis*), and -hemolytic streptococci (e.g., *S. mutans* or *S. viridans*). They can cause either primary or secondary infection in neutropenic patients. Both coagulase-positive and coagulase-negative staphylococci are most commonly isolated from the blood, and often from patients with indwelling intravenous catheters or skin breakdown from other causes. In addition, both have been well documented to cause infections around catheter sites or foreign bodies, such as prosthetic heart valves or orthopedic implants. *S. aureus* tends to be significantly more virulent and may cause a picture of rapidly developing septic shock as well as serious deep-seated infections. Most infections due to coagulase-negative staphylococci tend to be relatively indolent compared with *S. aureus* infection. A picture of septic shock is rarely, if ever, seen with most coagulase-negative staphylococcal bacteremias, and it has been debated whether these organisms can cause invasive visceral infections (e.g., pneumonia). Enterococci are part of the normal GI flora, are frequent components of intra-abdominal infections, and are a relatively common cause of urinary tract infections (second to the enteric gram-negative rods). Compared with streptococci, they are relatively -lactam resistant. Of additional concern, isolates of *Enterococcus* that are resistant to vancomycin have become a significant problem at some centers. Vancomycin-resistant enterococci are frequently resistant to aminoglycosides and -lactam drugs as well, posing a serious therapeutic dilemma. [124] Most -hemolytic

streptococci are part of the normal oral flora and may be seen with increased frequency after chemotherapy that produces significant oral mucosal disruption (e.g., high-dose Ara-C).[125] [126] Most -hemolytic streptococci are sensitive to a variety of -lactam antibiotics. However, recent reports have documented particularly virulent strains of -hemolytic streptococci that do not respond to -lactam antibiotics. Other gram-positive bacteria encountered in neutropenic patients include *Bacillus* spp. (often catheter related), *Corynebacterium jeikeium* (often catheter related and relatively antibiotic resistant), *Enterococcus faecium*, which may be resistant to vancomycin and other antibiotics, and *Lactobacillus,* which may also be resistant to vancomycin.

Anaerobic Bacteria.

Infections due to anaerobic bacteria may also be encountered in neutropenic patients, although less commonly than those due to aerobic organisms. [127] [128] [129] The anaerobic organisms most frequently encountered are *B. fragilis* and *Clostridium* spp., both normal inhabitants of the human GI tract. Infections caused by these organisms are often associated with a concomitant abnormality in GI mucosal integrity. Although bacteremia may occur, only rarely are anaerobes isolated from cultures done on specimens obtained at the discovery of initial fever in neutropenic patients. More commonly they present as secondary infections. Bacteremia due to *C. perfringens* can be a particularly fulminant and rapidly lethal process. [125] By contrast, when bacteremia due to *B. fragilis* occurs, it is often relatively indolent. [129] These anaerobes are most commonly isolated as components of necrotizing gingivitis or of intra-abdominal infections, including peritonitis, intra-abdominal abscesses, and perirectal cellulitis or abscesses. [129] Although *B. fragilis* and *C. perfringens* are the most common infecting organisms, other species may be clinically important. These include other species of *Bacteroides* [22] and *Clostridia* (e.g., *C. tertium* or *C. septicum*, which are often clindamycin resistant).[24] *C. difficile* is also a common cause of morbidity in neutropenic patients. It is not associated with bacteremia or invasive disease; rather, it produces a wide range of GI pathology due to elaboration of toxins.

Mycobacteria.

Infections attributable to *Mycobacterium* spp. are only rarely encountered in neutropenic patients and for the most part are not seen with increased frequency in this population. With the emergence of *M. tuberculosis* in a number of American cities, however, it seems likely that an increase in this infection will be observed in cancer patients. Patients with hairy cell leukemia appear to have an increased risk of developing infections with certain atypical mycobacteria. Hairy cell leukemia is often associated not only with neutropenia but also with profound monocytopenia, which may be an important risk factor for these infections. [130] Dysfunction of cell-mediated immunity may also be a contributing factor. In addition to the more common bacterial infections seen in other neutropenic hosts, patients with hairy cell leukemia may develop significant infections due to *M. kansasii*, *M. fortuitum*, *M. chelonei*, and *M. avium-intracellulare* complex.[31] [32] [33] Fast-growing mycobacteria (*M. fortuitum* and *M. chelonei*) may also cause persistent exit-site or tunnel infections in patients with indwelling intravenous catheters or wound infections after surgery.

Types of Fungal Infection Seen in Neutropenic Patients

The next most common type of organism encountered in neutropenic patients is fungus. Although neutropenia per se may predispose to the development of fungal infection, certain subsets of neutropenic patients appear to be at heightened risk,

most notably (1) those with prolonged neutropenia (>2 weeks) and (2) those receiving broad-spectrum antibacterial therapy. In contrast to the bacterial infections often associated with the onset of fever in neutropenic patients, fungal infections only rarely cause primary infection (i.e., initial infection in patients not yet receiving antibiotics). More commonly, fungal infections occur as secondary processes in patients receiving antibacterial drugs. Although a variety of fungal infections can be encountered in the neutropenic host, *Candida* spp. and *Aspergillus* spp. predominate. Emerging pathogens, such as azole-resistant *Candida* spp., *Fusarium* spp., Zygomycetes, *Trichosporon* spp., and dematiaceous molds, pose new threats to immunocompromised hosts, particularly those with neutropenia. Patients treated with glucocorticosteroids, particularly for postengraftment graft-versus-host disease (GvHD), are also at high risk for the development of invasive aspergillosis.

Qualitative Abnormalities

A variety of hematologic diseases may be associated with pronounced functional defects in effector cells, which in turn may contribute to the development of infectious complications. The microbicidal activity of granulocytes and monocytes involves complex interactions between the cell and the organism or inflammatory site. Some of the major functions important for microbicidal activity that have been elucidated include migration of the cell to the inflammatory site (chemotaxis), cell activation, phagocytosis, and intra- or extracellular killing by both the oxygen-dependent and oxygen-independent pathways. Clinically important defects in granulocyte function have been more completely delineated than have abnormalities in monocytes, with specific abnormalities described in nearly every measurable function of neutrophils. Problematic are patients with borderline granulocyte counts (e.g., absolute granulocyte counts in the range of 5002,000/mm ^{3} that are not falling precipitously), which occur most commonly with the myelodysplastic syndromes or newly diagnosed leukemias.

Previously it was thought that only cells less mature than the metamyelocyte were deficient in microbicidal activity, whereas more mature cells were fully competent. This supposition was based on early data on white blood cells (WBCs) obtained from healthy individuals. Subsequently, however, a number of studies documented significant functional defects in mature PMNs from patients with AML. [131] [132] [133] [134] In addition, one study initiated in patients with AML before treatment found a correlation between abnormalities in myeloperoxidase activity of the PMNs and the subsequent development of infectious complications. [135] Although fewer data are available addressing this issue for patients with ALL, studies have shown evidence of functional deficits in PMNs from these patients. [136] [137] Accordingly, it is prudent to approach all patients with acute leukemia and borderline granulocyte counts as if they had an absolute neutropenia.

Significant defects in granulocyte function have also been found in PMNs from patients with myelodysplastic syndromes and preleukemic states; [138] [139] [140] in some studies, these have correlated with infectious complications. [141] Although it is likely that these functional defects contribute to the increased infections seen in these populations, their effects per se are difficult to establish. The clinician should probably assume that neutrophils from patients with myelodysplastic syndromes or preleukemia are functionally defective. As in the case of AML or ALL, from a clinical perspective, patients with borderline granulocyte counts should be approached as if they had an absolute neutropenia.

Phagocyte dysfunction can also be due to the therapeutic use of pharmacologic agents and radiation for the treatment of the underlying hematologic disease. Again, because most patients receiving these therapies have multiple defects in host defense, the clinical significance of these in vitro findings is often difficult to establish. In addition to pronounced effects on phagocyte function, many of the drugs themselves (e.g., cyclophosphamide) can impair other immune parameters, including the production of quantitative defects, and can cause abnormalities in humoral responses and cell-mediated immunity.

In vitro studies have demonstrated inhibition of a variety of functional granulocyte parameters by antineoplastic agents, including inhibition of hexose monophosphate shunt activity, superoxide production, phagocytosis, chemotaxis, expression of Fc receptors, and microbicidal activity. The implicated agents have included methotrexate, 6-mercaptopurine, vincristine, vinblastine, the anthracyclines, cyclophosphamide, carmustine, and platinum compounds. [142] [143] [144] [145] [146] [147] [148] [149] [150] [151] [152]

It is widely accepted that exogenous administration of glucocorticoids leads to increased susceptibility to infection. Glucocorticoid therapy has been associated with the development of fulminant varicella infections in cancer patients and has been correlated with an increased risk for deep fungal infections in immunocompromised hosts.[153] [154] The major effect of steroids on in vitro measurement of granulocyte function appears to be confined to a decrease in chemotactic activity, which inhibits accumulation of PMNs at the site of infection and decreases the localized inflammatory response. [155] This may in large part account for the clinical observation that the signs and symptoms of even severe infections may be masked or greatly reduced in patients receiving steroids. In vitro, steroids may also impair PMN phagocytosis, microbicidal activity, and antibody-dependent cytotoxicity. [156] [157] In addition to their effects on granulocytes, corticosteroids may have important effects on the function of circulating monocytes, although the available data are limited. Documented effects have included in vivo monocytopenia as well as in vitro defects in monocyte chemotaxis, phagocytosis, and killing of bacteria and fungi. [158] In addition to their action on granulocytes and monocytes, steroids may enhance susceptibility to infection through other mechanisms, including negative effects on wound healing, skin fragility, lymphocyte function, production of cytokines, and humoral immune responses. The use of recombinant cytokines may reverse or prevent the corticosteroid-induced immunosuppression of phagocytic cell function against pathogenic fungi. For example, granulocyte colony-stimulating factor (G-CSF) and interferon- (IFN-) prevent corticosteroid-induced suppression of human PMN-induced damage of *Aspergillus fumigatus* hyphae.[159]

Various biologic agents are increasingly employed in the management of hematologic disorders, among which the colony-stimulating factors (predominantly

granulocyte-macrophage colony-stimulating factor [GM-CSF] and G-CSF) and interleukin-2 (IL-2) are most notable. A number of functional parameters may be enhanced by GM-CSF or G-CSF, including oxidative metabolism, phagocytosis, microbicidal activity, and antibody-dependent cytotoxicity. In vitro, recombinant human G-CSF has been shown to partially restore respiratory burst and bactericidal activity in neutrophils from patients treated with chemotherapy. [160] However, several pieces of data underscore the potential for inhibition of cell function as well. For example, in patients undergoing autologous bone marrow transplantation, impaired in vivo migration of granulocytes during GM-CSF administration has been documented. [161] Additionally, G-CSF has been shown to significantly decrease motility and bacteria-induced chemotaxis of neutrophils from patients undergoing cancer chemotherapy. [162] Impaired granulocyte function has been noted in some patients receiving high doses of IL-2. Patients receiving high-dose IL-2 therapy appear to have an increased incidence of significant infections due to *S. aureus*.[163] [164] Granulocyte function studies in patients receiving IL-2 demonstrated decreased production of superoxide, decreased chemotaxis, and decreased Fc receptor gamma-III expression.[164] Although the clinical significance of any of these effects on functional parameters has yet to be established, one

must keep an open mind to their potential to impair, as well as improve, cell function.

Many other commonly employed drugs have had documented in vitro or in vivo effects on phagocyte function, including nonsteroidal anti-inflammatory drugs, [165] [166] [167] [168] [169] opiates and other narcotic analogues, [170] [171] [172] ethanol,[173] benzodiazepines,[174] methylxanthines,[175] -blocking agents, [176] phenytoin,[177] [178] inhaled and intravenous anesthetics,[179] [180] nicotine,[181] allopurinol,[182] cyclosporine,[183] [184] heparin,[185] and calcium channel-blocking agents. [186] Even antibiotics, such as the tetracyclines, macrolides, and amphotericin B, have been shown to inhibit neutrophil function in vitro. [187] [188] [189]

Radiation therapy has also been associated with granulocyte dysfunction both in animal studies and in humans. Baehner and colleagues [190] studied granulocyte function in children with ALL. They found significant impairment of bactericidal activity when the cells were obtained during periods of craniospinal irradiation as compared with values before radiation therapy. The effect was transient, and cell function returned to normal 24 weeks after completion of radiation therapy.

Defects in Cell-Mediated Immunity

Cellular immune dysfunction may either be primary, as in a number of congenital immunodeficiency states, or secondary to other disorders or therapeutic interventions. Secondary deficiency in cell-mediated immunity is most commonly seen in patients with lymphoid malignancies, those with acquired immunodeficiency syndrome (AIDS), recipients of organ transplants, and patients receiving steroids or radiation therapy. The specific pathogens encountered and the spectrum of clinical findings in patients with defects in cell-mediated immunity vary with the underlying disease, the degree of immunosuppression, and the presence or absence of other host defense abnormalities. Defective cell-mediated immunity can lead to infections caused by bacteria, fungi, viruses, and protozoa. For the most part, however, the organisms are distinct from those that cause serious infection in patients with phagocyte abnormalities (Table 79-1). The most frequently encountered pathogens are often characterized as intracellular organisms because they can survive and even replicate inside macrophages in a nonimmune individual or in the absence of T-cell immunity. T-cell function appears to be required for macrophage activation and subsequent microbicidal activity. [191] Among the pathogens are (1) bacteria, including mycobacteria (both *M. tuberculosis* and atypical mycobacteria), *Legionella*, *Nocardia asteroides*, and *Salmonella* spp.; (2) fungi, including *C. neoformans*, *H. capsulatum*, and *C. immitis*; (3) viruses, including VZV, HSV, CMV, and EBV; and (4) parasites, including *P. carinii*, *T. gondii*, *Cryptosporidium*, and *S. stercoralis*. Patients with a deficiency in cell-mediated immunity are also at risk for the development of disseminated infection due to live vaccines, even with attenuated organisms. Accordingly, these patients should not receive bacillus Calmette-Guérin (BCG), vaccinia, measles, rubella, mumps, yellow fever, or live polio vaccines. The one exception is that all HIV-infected children, regardless of symptoms, should receive the measles vaccine. Although severe measles infection with high mortality does occur rarely, little morbidity from the measles vaccine has been reported in this patient population. [192]

Hodgkin disease, followed by the non-Hodgkin lymphomas, is the best-characterized and most commonly encountered malignant disorder associated with impaired cell-mediated immunity.[193] [194] [195] While the degree of immune impairment may correlate with the extent of disease and is often compounded by the administration of immunosuppressive therapy, both Hodgkin disease and non-Hodgkin lymphoma are associated with an intrinsic impairment of cell-mediated immunity that can persist even after apparent cure. Patients in remission are still at increased risk for the development of certain infections, particularly disseminated VZV infection; the identification of primary chickenpox or even localized zoster in these patients mandates prompt therapy with intravenous acyclovir (see the discussion under Clinical Approach to Viral Infections in Immunocompromised Patients).

Clinically significant intrinsic impairment of cell-mediated immunity has not been well established for other malignancies. Defects in cell-mediated immunity have been postulated to help explain the incidence of atypical mycobacterial infections in patients with hairy cell leukemia and also occur in patients with the relatively rare T-cell malignancies such as mycosis fungoides or T-cell chronic lymphocytic leukemia (CLL). Defects in cell-mediated immunity clearly exist in children with ALL, as evidenced by their increased susceptibility to the development of *P. carinii* or disseminated VZV infection, but it is likely that concurrent therapy plays a major role. Similarly, while defects in cell-mediated immunity may occur in patients with a variety of other malignancies, they are often explained by the use of therapeutic agents or by concomitant nutritional deficiencies.

Impaired cell-mediated immunity is not a prominent feature of nonmalignant hematologic disorders unless it is associated with therapy or the acquisition of HIV-1 infection. Abnormalities in cell-mediated immunity have been described in patients with hemophilia who have received factor VIII concentrates. [196] [197] Clearly, hemophiliacs who have acquired infection with HIV-1 from factor replacement therapy may have severe impairment of cell-mediated immunity. However, some studies have shown that HIV-1-seronegative hemophiliacs who have received factor VIII concentrates, as well as previously untreated hemophiliacs, have immune impairment.[198] Patients with sickle cell anemia have been found to have anergy in association with zinc deficiency and decreased nucleoside phosphorylase activity;[199] cell-mediated immunity defects have been described in patients with thalassemia. [200] Overall, however, the clinical significance of these findings is uncertain.

Pharmacologic agents and irradiation are major causes of impaired cell-mediated immunity. Corticosteroids are the pharmacologic agents most often associated with abnormalities in cell-mediated immunity, although they may also cause immune suppression through their effects on other host defense mechanisms. [201] The degree of immunosuppression and the relative risk of infection will depend on the dose and duration of corticosteroids as well as on the underlying disease. Even when these factors are known, however, infectious complications in patients receiving corticosteroids may be highly variable and difficult to predict on an individual basis. [155] Nevertheless, whether corticosteroid therapy is being used should always be taken into account when one is confronted with potentially infected patients. Those patients about to embark on corticosteroid-containing treatment regimens who have a known history of tuberculosis or positive purified protein derivative (PPD) should be treated with prophylactic isoniazid (INH) to prevent reactivation and potential dissemination of disease.

A number of cytotoxic agents may also be associated with impaired cell-mediated immunity, notably methotrexate, cyclophosphamide, 6-mercaptopurine, and azathioprine.[202] [203] Infections due to impaired cell-mediated immunity, such as *P. carinii* pneumonia, have also been associated with certain combination chemotherapeutic regimens more often than with others.[204] In patients with hematologic malignancies, cytotoxic agents are frequently administered in combination with other immunosuppressive therapies, such as corticosteroids or radiation therapy, which may further impair cell-mediated immunity and increase infection risk for diseases such as *P. carinii* pneumonia.

Cyclosporine is an immunosuppressant used to suppress transplant rejection and also as a therapeutic modality in aplastic

anemia. It is associated with alterations in T-helper cells, effector T cells, and natural killer (NK) cells. It has not been established, however, that the use of cyclosporine is associated per se with an increased risk of infection. Although there are anecdotal reports of unusual infections in patients receiving cyclosporine, evidence shows that its use can decrease certain infections as compared with other immunosuppressive regimens used for allograft recipients. [205] [206]

Radiation therapy can result in impaired cell-mediated immunity, especially when used in combination with other immunosuppressive agents or for the treatment of patients with underlying diseases associated with intrinsic cell-mediated immunity defects (e.g., as a component of the preparatory regimen for bone marrow transplantation or for the treatment of Hodgkin disease). Defects in cell-mediated immunity may persist for >1 year after intensive radiation therapy or after bone

marrow transplantation.[207] Accordingly, patients should be considered at risk for the development of certain infections during this period.

A number of infections may themselves result in impaired cell-mediated immunity. Viral infections may affect cell-mediated immunity either directly (e.g., by infecting crucial cellular components such as T lymphocytes or macrophages) or by affecting other immunoregulatory mechanisms.[208] The key viral infection associated with impaired cell-mediated immunity is HIV-1. Patients with AIDS have profound defects in cell-mediated immunity and are susceptible to a wide array of infectious complications. Other viral infections associated with cell-mediated immunity defects include CMV, EBV, RSV, HBV, and influenza. Other nonviral infections that have been variably associated with impaired cell-mediated immunity by in vitro testing have included tuberculosis, leprosy, bacterial pneumonia, *Brucella*, typhoid fever, coccidioidomycosis, syphilis, and a variety of parasitic diseases.[209]

Noninfectious disorders linked to abnormal cell-mediated immunity include chronic protein-calorie malnutrition, uremia, diabetes mellitus, sarcoidosis, cystic fibrosis, and other conditions resulting from surgery and the administration of anesthesia.

Abnormalities of Humoral Defense MechanismsImmunoglobulins and Complement

Immunoglobulins and complement are among the most important components of the humoral immune system; defects or deficiencies in either may be associated with serious infections. Other proteins classified as part of the humoral defense system include lysozyme and lactoferrin, tuftsin, and fibronectin. Abnormalities in these proteins have been variably associated with infectious complications. Immunoglobulins and complement each have associated opsonic, lytic, and neutralizing activities and function predominantly against bacterial infection. Patients with either primary or secondary defects or deficiencies in these proteins are at highest risk for the development of serious infection from the encapsulated bacteria and, to a lesser extent, from the enteroviruses and *Giardia lamblia*.

Multiple myeloma is frequently associated with a defect in humoral immunity.[210] The degree of humoral impairment in multiple myeloma is related to the stage of the disease. Malignant plasma cells induce the production of a protein that is synthesized by macrophages and that selectively suppresses B-cell function. Cell-mediated immunity remains intact unless patients are treated with corticosteroids or cytotoxic therapy. In addition, myeloma patients with IgG paraprotein have an increased rate of catabolism of both normal and clonal IgG.

Patients with myeloma are most susceptible to recurrent pyogenic infections from high-grade polysaccharide-encapsulated bacteria such as *S. pneumoniae*, *H. influenzae*, or *Neisseria* spp. However, infections due to enteric gram-negative rods and staphylococci are also frequently encountered. In the series reported by Doughney et al.,[211] gram-negative bacilli accounted for most isolates. Savage et al.[212] found a biphasic pattern of infection, with infections due to *S. pneumoniae* and *H. influenzae* occurring earlier in the course of disease (i.e., within the first 8 months of diagnosis) or in patients responsive to therapy, and those due to gram-negative bacilli occurring more frequently in patients with refractory or advanced disease. The sites of recurrent infection are most often the upper respiratory tract, urinary tract, or skin. Repetitive bouts of infection may herald the diagnosis of multiple myeloma; infection is the major cause of death.

Because pneumococcal infections are frequent, some studies have explored the potential role of pneumococcal vaccine administration to these patients. In patients with multiple myeloma antibody titers may rise after administration of pneumococcal vaccine but, because preimmunization titers are frequently low, postimmunization titers often fail to reach a protective level.[210] In addition, postimmunization antibody levels fall rapidly in this population, in part because of the increased catabolism of immunoglobulins.[207] This is of practical importance, because revaccination with pneumococcal vaccine may be associated with Arthus reactions or systemic reactions.[213] Nevertheless, it is currently recommended that patients with multiple myeloma receive pneumococcal vaccine and that revaccination be considered in patients sustaining a rapid decline in pneumococcal antibody levels.[214] Revaccination should be done no sooner than 6 years after administration of the initial vaccine.[215] [216] The efficacy of prophylaxis with passive administration of immunoglobulins has not been established and cannot be recommended.

Patients with B-cell CLL also appear to have an intrinsic defect in the clonal B cells that leads to unbalanced immunoglobulin chain synthesis and resultant hypogammaglobulinemia. The incidence of infection correlates with the duration and stage of the disease as well as with the serum levels of immunoglobulins (particularly IgG). Infection is a major cause of morbidity and mortality and may account for as much as 60% of deaths in certain series.[217] The organisms causing serious infection are often the encapsulated bacteria, although patients are also at risk of infection due to staphylococci and enteric gram-negative bacilli.[218] Upper and lower respiratory tract infections are encountered most commonly, although septicemia and other sites such as the urinary tract and skin are frequently involved. Therapeutic administration of corticosteroids or cytotoxic drugs will increase the risk of infection and may dramatically expand the list of potential pathogens.[218] [219] It is recommended that patients with CLL receive polyvalent pneumococcal vaccine, although its efficacy in this population has not been demonstrated unequivocally. A multicenter trial demonstrated that prophylaxis with pooled intravenous immunoglobulin (IVIG) decreased the incidence of bacterial infection; however, the decrease was primarily seen in minor or moderately severe infections.[218] In addition, the cost-effectiveness of immunoglobulin prophylaxis and its effect on the quality of life are questionable.[220]

The precise role of abnormalities in humoral defenses in other malignancies has not been well defined. Observed defects may be due in part to the components of therapy, as opposed to intrinsic defects associated with the underlying disease. Patients with acute leukemia have been found to have lower levels of antibody to the core glycolipid of the Enterobacteriaceae than noncancer patients, and their antibody levels fall after cytotoxic therapy.[221] Theoretically, this decrease in antibody levels could contribute to an increased risk of infection with enteric gram-negative bacteria, but this has not been established. Cytotoxic chemotherapy, radiation therapy, and steroids all adversely affect B-cell as well as T-cell functions, which may result in diminished opsonizing activity, inadequate agglutination and lysis

of bacteria, and deficient neutralization of bacterial toxins. Cytotoxic therapy can blunt the humoral response to vaccine administration,[222] [223] but adequate responses have also been documented for patients in remission on maintenance chemotherapy.[224]

Role of Splenectomy and Splenic Dysfunction

Abnormalities in splenic function may be a prominent part of a number of hematologic disorders owing to either intrinsic impairment or to therapeutic or diagnostic splenectomy. Splenic dysfunction in the absence of splenectomy is crucial in patients with sickle cell disease, who lose function presumably as a result of repeated infarctions. Splenectomy can be performed either as a part of staging or as a therapeutic intervention.

The precise reason for enhanced susceptibility to infection in asplenic patients or patients with splenic dysfunction is not entirely clear. The spleen probably plays an adjunctive role in fighting infection in a number of ways. First, it appears to function as a sieve, removing organisms from the blood that have been ineffectively opsonized by complement. In addition, it participates in the primary immunoglobulin response and is involved in the regulation of the alternate complement pathway, with low levels of immunoglobulins and properdin reported in patients after splenectomy.[225] [226] A decrease in the opsonic peptide tuftsin has also been reported after splenectomy,[227] and alternate pathway defects may be important in patients with sickle cell disease and splenic dysfunction.[228]

The risk of the development of serious infection and the type of infections that develop vary somewhat, depending on the cause of the abnormal splenic function and the presence or absence of other immunologic abnormalities. For example, patients who have undergone splenectomy after trauma may be at lower risk of infection than other groups. Overwhelming infection after splenectomy occurs in 6.9% of post-splenectomy patients, with 50% of infection-related deaths occurring in the first 3 months.[229] An increased risk for *Salmonella* infection appears to be unique for the sickle cell population. In general, however, most asplenic patients or splenectomized patients are at increased risk of serious bacterial infection, primarily infection due to *S. pneumoniae* and *H. influenzae* as well as *Neisseria* spp. and *Capnocytophaga canimorsus*. The initial presentation of even overwhelming infection can be deceptively subtle, with fever often the only sign. Accordingly, all asplenic patients with underlying hematologic disease who present with fever should be managed initially as potentially septic. In addition to a detailed physical examination, the evaluation should routinely include at least two blood cultures and a urine culture, a culture of any other potentially infected site, and chest radiography. For all such patients we first give empirical intravenous antibiotics (even for those who are non-neutropenic) and continue a course of antibiotics for a minimum of 72 hours until the preantibiotic culture results are known. If the cultures are found to be negative and the patient is clinically stable, we often discontinue antibiotics and observe. We do not recommend routine prophylactic antibiotics for asplenic patients. However, most authorities do recommend penicillin prophylaxis for small children or for those with increased immune impairment from malignant disease.

Because asplenism predisposes to the development of pneumococcal infection, pneumococcal vaccine should be administered l2 weeks before elective splenectomy.[230] The rationale for this timing of vaccination is twofold. First, evidence shows that splenectomized patients respond less well to pneumococcal polysaccharides than do patients with intact splenic function.[231] However, a 1986 study in patients with Hodgkin disease showed that the antibody response to

pneumococcal vaccine was not affected by the timing of immunization relative to splenectomy.[222] Second, and perhaps more important, presplenectomy immunization can result in protective titers immediately after splenectomy. Finally, it should be remembered that, although pneumococcal vaccine is effective in these patients, it does not eliminate the risk of serious pneumococcal disease, the possibility of which should be kept in the forefront even in vaccinated patients. Because asplenic patients are at risk of infection with all encapsulated bacteria, they should also be immunized with *H. influenzae* b and the meningococcal vaccines, which can be given with pneumococcal vaccine.[232]

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PROPHYLAXIS OF INFECTIONS IN HIGH-RISK PATIENTS

Prophylaxis of Bacterial Infections

With bacterial organisms accounting for most infections in compromised patients, trials designed to assess prophylactic strategies have appropriately focused on these pathogens. Strategies assessed fall into four major categories: (1) the use of mechanical techniques to prevent acquisition of new pathogens; (2) the use of various absorbable or nonabsorbable oral antibiotic regimens either to prevent acquisition or to decrease the number of potentially pathogenic colonizing organisms; (3) the use of passive or active immunization; and (4) the use of prophylactic granulocyte transfusions (Tables 79-4 and 79-5). In addition, a number of biologic agents (e.g., the CSFs) are under evaluation for the prevention of infection.

However, the single strategy that is applicable in every setting involving immunocompromised patients is hand washing. Although stressing the need for rigorous hand washing may appear somewhat trivial and seems intuitively obvious to many, its importance cannot be overemphasized, particularly in the hospital setting. Organisms of all types can be transmitted on the hands of health care workers or patients. If questioned, most health care workers avidly affirm the importance of hand

TABLE 79-4 -- Methods for Preventing Infection in High-Risk Patients

Prevent Acquisition and/or Suppress or Eliminate Microbial Flora	Methods to Improve or Modify Host Defenses
Isolation	Immunization
Simple or reverse isolation	Active
Isolation with HEPA air filtration	*Hemophilus influenzae*
Prophylactic antibiotics	*Pseudomonas*
Nonabsorbable antibiotics	*Pneumococcus*
Trimethoprim-sulfamethoxazole, erythromycin	VZV
Selective decontamination	Passive
Quinolones	J-5 core glycolipid
Prophylactic antivirals	Pooled immunoglobulins
Acyclovir	Hyperimmune globulins
Amantadine	Monoclonal antibodies
Prophylactic antifungals	Cell component replacement
Nystatin	Leukocyte transfusions
Imidazoles	Accelerate granulocyte recovery
Triazoles	Lithium
Amphotericin B	CSF-G
Prophylactic antiparasitics	CSF-GM
Thiabendazole	
Trimethoprim-sulfamethoxazole	
Combination-comprehensive	
Total protective isolation	

TABLE 79-5 -- Relative Merits and Deficiencies of Various Strategies to Prevent Infection in Granulocytopenic Patients

	Total Protected Environment	Nonabsorbable Antibiotics	Trimethoprim-Sulfamethoxazole	Selective Decontamination	Quinolones
Efficacy					
Reduce infection	Yes	No	±	±	Yes
Decrease in fever	Yes	No	No	No	No
Decrease or shorten need for antibiotics and antifungals	No	No	No	±	Yes
Contribute to survival	No	No	No	No	No
Compliance					
Well tolerated?	No	No	±	±	Yes
Impact on efficacy	Yes	Yes	Yes	±	No
Liabilities					
Emergence of resistant organisms	Yes	Yes	Yes	Yes	Yes
Organ side effects					
Interference with other drugs	Yes	Yes	Yes	No	No
Bone marrow suppression	No	No	Yes	Yes	No
Specific organ toxicity	No	No	Yes	Yes	Yes
Cost					
For the drugs or regimens	Yes	Yes	No	Yes	Yes
For surveillance or monitoring	Yes	Yes	Yes	Yes	Yes

Reducing need for hospitalization or for drugs	No	No	No	±	?

washing, yet only a few adhere to a strict policy. In one study of an intensive care unit, health care personnel washed their hands after fewer than one-half of patient contacts, and physicians specifically did so after fewer than one-third of contacts. [233] In the high-risk setting, all health care personnel should wash their hands immediately after every patient contact, no matter how brief the contact. In addition, high-risk patients (or their parents or guardians) should be instructed in the importance of hand washing and urged to remind health care workers who inadvertently forget. Physicians, nurses, and other health care workers should not be embarrassed or intimidated by gentle but firm reminders from patients or colleagues that hand washing has been overlooked.

Methods

Mechanical Techniques

A number of so-called mechanical techniques or intervention strategies to prevent infections have been assessed in granulocytopenic patients. Typical reverse isolation, after the onset of neutropenia, has no documented efficacy in preventing infection. First, colonization by potential pathogens is likely to have already occurred by the time granulocytopenia develops. In addition, requiring patients to wear a surgical mask outside of their own rooms will do little to protect against subsequent infection. Likewise, perhaps the only value to having medical personnel gown, glove, and mask before entering a patients room is to heighten their awareness of the importance of other less expensive and more effective measures, such as hand washing. [234] On mostly theoretical grounds, some authorities have recommended that all foods be thoroughly cooked and that fresh fruits or vegetables be avoided to avoid transmission of gram-negative bacteria, which can colonize raw or unprocessed food.[36] [37] However, the actual value of these measures in preventing infection is unproved.

A total protective environment is a comprehensive anti-infective regimen designed to reduce the patients endogenous microbial burden while preventing the acquisition of new organisms. A sterile environment is created in a clean-air room with constant positive air flow and is maintained by an aggressive hygiene program, including surface decontamination, sterilization of all objects that enter the room, and an intensive regimen to disinfect the patient that includes the use of oral nonabsorbable antibiotics, skin antiseptics, antibiotic sprays and ointments, and a low microbial diet. A number of studies have documented that a total protective environment can reduce infections in profoundly granulocytopenic individuals. [235] However, a total protective environment is expensive and, because of improvements in treating established infections and in shortening the duration of neutropenia, it does not offer a survival advantage to most patients. Thus, a total protective environment is not necessary for the routine care of most granulocytopenic patients.

Oral Antibiotic Regimens

The value of oral antibiotic regimens designed to protect the patient against the development of bacterial infections during the course of granulocytopenia has been subject to ongoing debate. The rationale for this approach is based on the observation that most organisms ultimately implicated in infection (particularly the gram-negative bacteria) can be isolated from the patients own flora. [3] Since the mid-1970s, numerous studies have evaluated a variety of prophylactic oral regimens. These have included nonabsorbable antibiotics (e.g., gentamicin, vancomycin, polymyxin, and colistin), [235] [236] [237] [238] [239] [240] [241] [242] as well as antibiotics absorbed from the GI tract (e.g., trimethoprim-sulfamethoxazole, erythromycin, or quinolones). [237] [243] [244] [245] [246] [247] [248] [249] [250] [251] [252] [253] [254] [255] [256] The goal of many of the earlier studies employing nonabsorbable agents was total decontamination of the alimentary tract, which met with only marginal success in the absence of strict isolation procedures. [236] The agents often tasted bad and noncompliance was a major problem, potentially leading to rebound overgrowth of pathogenic organisms. [236]

The use of trimethoprim-sulfamethoxazole and the fluoroquinolones for the prophylaxis of bacterial infections is meant primarily to result in selective decontamination of the GI tract, i.e., to eliminate the potentially pathogenic aerobic flora (mostly the enteric gram-negative bacteria) while preserving most anaerobic organisms.

Despite the voluminous literature on the subject, a final answer on the efficacy of trimethoprim-sulfamethoxazole in this setting has not been established, and debate as to its value continues. [244] [250] [257] [258] [259] Potential pitfalls associated with the use of trimethoprim-sulfamethoxazole include bone marrow suppression,

[244] [250] [257] [258] [259] the development of resistant organisms,[236] [260] rash,[261] and an increase in fungal colonization. [250]

Fluoroquinolones offer the advantage of gram-negative coverage, including coverage of *Pseudomonas aeruginosa*, with preservation of the anaerobic flora. In clinical trials of immunocompromised patients, fluoroquinolones have shown better efficacy in the prevention of gram-negative bacteremia than either placebo [261] or trimethoprim-sulfamethoxazole;[260] [262] they have also been well tolerated. However, studies have demonstrated colonization with resistant gram-negative organisms during the prophylactic use of fluoroquinolones. [263] [264] In addition, there has been an increase in gram-positive infections in patients receiving prophylactic fluoroquinolones.[16] [262] [265] [266] [267] Kotilainen et al.[266] documented a small outbreak of ciprofloxacin-resistant coagulase-negative staphylococcal infections in neutropenic patients after the introduction of ciprofloxacin prophylaxis onto their ward. In another study, Cruciani et al. [262] compared 21 children given prophylactic norfloxacin to 23 given trimethoprim-sulfamethoxazole; there were four documented streptococcal infections in those treated with norfloxacin, compared to only one gram-positive infection in the trimethoprim-sulfamethoxazole group. The multicenter Italian GIMEMA study is one of the few studies comparing the different fluoroquinolones.[264] In this study, 619 patients were randomized to receive either ciprofloxacin or norfloxacin. Although more benefits were seen with ciprofloxacin, that was true only for those patients who were severely neutropenic for <1 week. Because current information does not definitively support the use of prophylactic antimicrobial agents and because of the disastrous consequences of bacterial resistance, we do not support the routine use of prophylactic antimicrobial agents.

In the bone marrow transplant setting, fluoroquinolone prophylaxis is widely employed, particularly for those undergoing allogeneic transplantation, although there are few data to solidly support this practice. A large randomized study comparing ciprofloxacin with trimethoprim-sulfamethoxazole prophylaxis was performed in transplant patients at the Dana-Farber Cancer Center. [265] Among 145 evaluable patients there were no gram-negative bacillary infections in those receiving ciprofloxacin, versus four gram-negative infections in trimethoprim-sulfamethoxazole recipients. This difference was not statistically significant. However, of the ten documented bacteremias in patients taking ciprofloxacin, nine were due to *Streptococcus viridans*, an organism that may cause shock and adult respiratory distress syndrome in neutropenic patients. In contrast, only four patients in the trimethoprim-sulfamethoxazole group developed bacteremia due to *S. viridans*. Again, these data underscore the trend toward an increased rate of gram-positive infections in patients who receive fluoroquinolone prophylaxis.

Growth Factors and Immune Mediators

The development of cytokines in the past decade is allowing clinicians both to influence the most critical factor in susceptibility of infection and neutropenia and to manipulate the hematopoietic and immune systems. The cytokines that have been most intensively studied and widely employed are G-CSF and GM-CSF, which stimulate the proliferation and maturation of bone marrow progenitor cells. Although G-CSF and GM-CSF do not prevent neutropenia, multiple studies have demonstrated an increase in the neutrophil count and a decrease in the duration of neutropenia after their use. [268] [269] [270] [271] There are many reports of G-CSF and GM-CSF use, but these studies have used multiple doses and schedules, patients have had varied underlying malignancies and received different chemotherapeutic regimens, and few studies have been randomized or placebo controlled. Crawford et al. [269] randomized 104 patients to receive placebo and 95 patients to receive G-CSF at 230 g/m^2 /day after chemotherapy for small cell lung cancer, prior to the onset of neutropenia. Patients treated with G-CSF had a significant decrease in the number of days with a neutrophil count of <500 cellsmm^3 (3 days, compared to 6 for placebo patients) and in depth of neutropenia. G-CSF-treated patients also had significantly fewer episodes of fever and neutropenia, and the treatment group had 51% fewer culture-confirmed infections. Other advantages were a 45% reduction in days of hospitalization and a 47% decrease in days of intravenous antibiotic use for the G-CSF cohort. Other studies have shown fewer significant differences with the addition of G-CSF or GM-CSF. Accordingly, the American Society of Clinical Oncology has published guidelines for the use of cytokines, indicating that they should be employed prophylactically only if there is an expectation of a >40% incidence of febrile neutropenia. [272]

More recently (1997), a large randomized double-blind placebo-controlled trial of G-CSF in afebrile outpatients with severe chemotherapy-induced neutropenia showed that although adjunctive G-CSF significantly accelerated recovery from neutropenia, it did not result in a decreased rate of hospitalization for febrile neutropenia or in fewer documented infections.[273] Based on these data, there is no current recommendation to initiate G-CSF once a patient has developed deep neutropenia but is still afebrile. It appears that the greatest benefit of cytokine prophylaxis occurs when the CSF is started immediately after chemotherapy, when the

patients peripheral neutrophil count has not yet decreased.

A randomized double-blind placebo-controlled trial of GM-CSF was performed by Nemunaitis et al. [274] in patients with leukemia and lymphoma undergoing autologous bone marrow transplantation. Sixty-five patients were treated with GM-CSF (250 g/m^2 /day), while 63 were given placebo. The neutrophil count of GM-CSF-treated patients rose more rapidly to 500 cells/mm^3 . Although no difference was noted between the two groups in number of days with fever or percentage of patients with fever, patients receiving GM-CSF did have a decrease in number of days of antibiotic therapy and number of days of hospitalization.

Because of their stimulation of neutrophil and macrophage function, these growth factors may also have a role in the treatment of fungal infection; a study conducted by Roilides et al.[275] found that incubation of neutrophils with G-CSF in vitro produced an increase in the oxidative burst of the neutrophils in response to *C. albicans*.

G-CSF has also been demonstrated to be of benefit in patients with aplastic anemia, [276] congenital or acquired neutropenia, [277] and HIV infection.[278] However, in addition to their expense, side effects are noted with both agents. [279] Either drug can cause bone pain or an increase in hepatic transaminases. The use of G-CSF may rarely result in Sweet syndrome, while the use of GM-CSF has been more often complicated by fever, chills, hypoxia, skin rash, fluid retention, and pericarditis.

Other cytokines currently being studied include M-CSF, which stimulates macrophage and monocyte cell lines and augments the immune response to *C. albicans* in vitro. IL-1 affects the immune system at multiple levels, including the stimulation of other cytokines and increasing the number of polymorphonuclear leukocytes. [279] Animal models have shown an increase in survival in mice infected with *Pseudomonas* or *Klebsiella* and then treated with IL-1.[280] IL-3 not only stimulates all cell lines but also enhances the effect of monocytes and eosinophils. [281] IFN- enhances the activity of macrophages against intracellular organisms and protozoa and increases the activity of monocytes and PMNs against some bacteria and fungi.[279] [282] It has been shown to decrease the frequency of infection and the need for antibiotics in patients with chronic granulomatous disease.[283] These cytokines are promising agents for future use in bone marrow recovery and in the treatment of bacterial or fungal infection.

Prophylaxis of Fungal Infections

Because of the increasing incidence of invasive mycoses in immunocompromised patients, antifungal prophylaxis has been studied fairly extensively (Table 79-4). The most frequently evaluated antifungal agents have included nystatin, miconazole, clotrimazole, ketoconazole, fluconazole, and orally administered amphotericin B. Prophylactic regimens have generally been aimed at reducing invasive infections due to *Candida* spp. and by virtue of the antifungal activity of the aforementioned agents, they have a significant impact on *Aspergillus* infections or mucormycoses. Strategies designed to prevent infection by *Aspergillus* have largely concentrated on preventing its acquisition from environmental sources.

Until recently, several problems with antifungal prophylaxis were noted. First, when an adequate dose of an antifungal agent such as amphotericin B, ketoconazole, or clotrimazole was administered, there was a consistent decrease in fungal colonization but not necessarily a concomitant decrease in invasive fungal infections. [260] Second, several studies employing prophylactic and empirical antifungal regimens reported a shift in the colonization pattern of fungal organisms. In general, these drifts were toward more resistant fungi. Thus, prophylactic regimens can successfully eradicate the susceptible fungi (particularly *C. albicans*) but can lead to overgrowth and ultimately invasion by more resistant species, especially non- *albicans Candida* species. However, a double-blind randomized study of bone marrow transplant recipients reported by Goodman et al. [284] found a decrease in both fungal colonization and infection rates after antifungal prophylaxis with fluconazole. One hundred seventy-nine patients were treated with fluconazole, 400 mg/day, from initiation of chemotherapy until neutrophil counts of 1,000 cells/mm ^{3} were achieved or until drug toxicity or an invasive fungal infection was suspected. Compared with patients given placebo, fluconazole-treated patients had a significant decrease in rates of fungal colonization (67.2% vs. 29.6%), superficial fungal infection (33.3% vs. 8.4%), and systemic fungal infection (15.8% vs. 2.8%). In addition, the number of deaths due to invasive fungal disease in the fluconazole-treated group showed a significant decrease. Although the number of fluconazole-treated patients eliminated from the study because of hepatotoxicity was not significant, the mean increase in alanine aminotransferase level was significantly higher in the fluconazole-treated group. Although this study showed no significant increase in *C. krusei* infection in the fluconazole-treated group as compared with the placebo group, Wingard et al.[285] subsequently revealed a sevenfold increased risk of *C. krusei* infection after fluconazole prophylaxis in marrow transplant recipients and acute leukemia patients. During the time that Wingard et al. used fluconazole as antifungal prophylaxis, *C. krusei* became the most commonly isolated fungal pathogen in patients receiving fluconazole. The topic of antifungal prophylaxis is extensive and has been reviewed in depth elsewhere. [286]

Overall, the potential benefits of prophylactic antifungal therapy must be balanced against the toxicity and relative efficacy of the regimen employed, as well as epidemiologic considerations. Although the data are clear that antifungal prophylaxis with fluconazole reduces the frequency of disseminated candidiasis in allogeneic bone marrow transplant recipients, the use of this strategy in other populations is not recommended. Until a clear benefit can be proved, widespread chemoprophylaxis against fungi should not be attempted.

Prophylaxis of Viral Infections

Herpes Simplex Virus

HSV can be a frequent cause of morbidity in compromised patients, particularly those undergoing bone marrow or renal transplantation or intensive chemotherapy for acute leukemia. In these settings the incidence of reactivation is high (80%) in seropositive patients not receiving prophylaxis. [64] [65] [287] Higher antibody titers (1:16) appear to be most predictive of subsequent development of clinical infection. Studies employing prophylactic intravenous acyclovir [64] [65] [288] or oral acyclovir[289] [290] in these settings have demonstrated nearly 100% efficacy in the prevention of clinically significant reactivation of HSV. The placebo-controlled study by Saral and co-workers[64] in bone marrow recipients used intravenous acyclovir at a dose of 250 mgm^2 t.i.d. beginning 3 days before the transplant. None of ten patients receiving acyclovir developed HSV infection, whereas seven of ten patients receiving placebo did. In the study by Wade et al., [289] oral acyclovir (400 mg five times per day) was begun 1 week before bone marrow transplantation. Among patients who had a minimum of 40% compliance with the prescribed dose, the prophylaxis was 100% clinically effective. The study by Gluckman and colleagues [290] also showed complete clinical efficacy, but at a lower dose of oral acyclovir (200 mgm^2 q.i.d.). All the studies employing prophylactic acyclovir have demonstrated the absence of significant toxic effects in these settings.

Studies of the prevention of CMV disease with ganciclovir in recipients of allogeneic marrow transplants have also addressed the prophylaxis of HSV infection. [291] [292] In one study, high-dose acyclovir was given to patients until marrow engraftment, after which the regimen was changed to ganciclovir. [292] None of the ganciclovir recipients excreted HSV, compared to 14 of 31 controls. In the study by Winston et al., [291] ganciclovir (2.5 mg/kg t.i.d.) was begun before transplantation and continued until day 120 after transplantation but held during periods in which the neutrophil count was <1.0 × 10^9 /L. In this study, acyclovir was not given. Only 5% of ganciclovir recipients excreted HSV compared with 29% of those receiving a placebo; however, there were cases of localized, clinically evident HSV disease. No trials have been conducted to compare acyclovir versus ganciclovir in the prevention of both HSV and CMV in marrow transplant recipients. It seems prudent to use either oral or intravenous acyclovir in a prophylactic regimen for several subgroups of high-risk patients: (1) those who are seropositive (with titers of 1:16 or higher) undergoing bone marrow transplantation (on engraftment, these patients may be changed to ganciclovir if CMV positive); (2) those who are seropositive and receiving intensive therapy for acute leukemia; and (3) those with a previous clinical history of HSV infection undergoing these therapies. In addition, it is reasonable to consider prophylactic acyclovir in conjunction with cytoreductive therapy of any type if previous cycles of similar therapy have resulted in clinically significant HSV infection.

Varicella-Zoster Virus

One of the most important and simple methods of preventing VZV infection is isolation of infectious individuals from other high-risk immunosuppressed patients. Infectious patients include any with a diagnosis of either primary VZV (chickenpox) or secondary VZV (zoster). Of these groups, those with chickenpox are more highly infectious, the respiratory route being the major mode of transmission. In addition to those with diagnosed VZV infection (i.e., the presence of characteristic lesions), individuals at risk of chickenpox who have had known exposure to the virus (mostly young children) should be considered potentially infectious and kept away from susceptible immunosuppressed patients. The incubation period for chickenpox is 1021 days, and patients may spread the infection from 2 days before the appearance of lesions until the time of crusting of all lesions. Therefore, any individual who has not had chickenpox but who has been exposed should not be allowed contact with

susceptible immunosuppressed patients from 1 week after the initial exposure until at least 3 weeks after the exposure or until complete crusting of any lesions that appear.

Passive immunization with zoster immunoglobulin (ZIG) has been shown to reduce the incidence of pneumonitis and encephalitis and decrease the mortality of chickenpox from 7% to 0.5% in immunocompromised patients. Immunosuppressed children or adults who are seronegative or possess low-titer antivaricella antibody should receive ZIG (1 vial/10 kg) within 72 hours after exposure to a potentially infectious source. One dose should be protective for about 4 weeks.

A live attenuated varicella vaccine is commercially available, although it is not currently licensed for use in immunocompromised children. [293] Although 95% of normal children seroconvert after receiving the vaccine, 80% of immunosuppressed children will produce antibodies to VZV and 25% will be antibody negative at 1 year. [294] The vaccine has been shown to prevent transmission of chickenpox from normal children to children with leukemia in 85% of cases. [295] Varicella vaccine has also been given to healthy siblings of children with malignancies; of 30 children vaccinated, six contracted chickenpox and only four transmitted it to their immunocompromised siblings.[296] Despite the effectiveness of the varicella vaccine, there are still concerns about its use. Most immunocompromised patients have had their chemotherapy interrupted for 1 week before and after vaccine administration. Little data regarding the efficacy and safety of vaccine administration during chemotherapy exist. Arbeter et al.[297] compared results in 24 children who continued to receive 6-mercaptopurine while the vaccine was administrated with 20 children whose chemotherapy was suspended. Although the number of patients studied was small, there was no difference in antibody response or in vitro lymphocyte proliferation, no difference in side effects, and no difference in breakthrough infection. Approximately 40% of immunocompromised children develop a rash at the vaccine site after immunization. The child is contagious with this rash, and may require isolation and therapy with acyclovir. Additionally, rash, viremia, and elevated hepatic transaminase levels were documented in a vaccinated 4-year-old child with leukemia. [298] Because the clinical picture of varicella is milder in breakthrough infection, there is some concern that vaccine recipients may have an asymptomatic infection and may be infectious without exhibiting physical signs of varicella. The concern that VZV vaccine would increase the risk of the development of herpes zoster infection in immunocompromised patients has been dismissed by Hardy et al., who demonstrated a lower incidence of zoster in vaccinated children with leukemia (13 children with zoster of 548 vaccinated) than in children who have had natural infection (15 of 96).[299] The varicella vaccine seems better suited to providing herd immunity in the normal population than in protecting those who are immunocompromised.

Cytomegalovirus Infection

Because of the high morbidity and mortality of CMV disease, especially in recipients of marrow and solid organ transplants, attention has focused on its prevention. CMV infection develops either from exogenous sources (i.e., transfused blood or transplanted organs) or from reactivation of endogenous, latent virus. The seroprevalence of CMV is >50% in the United States, and because reactivation disease occurs in most latently infected patients, there is a large population at risk for the manifestations of CMV infection. The three preventive strategies that have been most successful in preventing CMV disease are (1) the use of seronegative blood products, (2) passive immunization with IVIG, and (3) chemoprophylaxis with acyclovir or ganciclovir.

Transplantation of bone marrow and solid organs from seronegative donors into seronegative recipients results in a markedly decreased incidence of CMV infection post-transplant. Furthermore, the sole use of seronegative blood products in seronegative recipients can virtually eliminate primary CMV infection. [78] However, blood product screening is not effective in preventing CMV infection in seronegative patients if the organ or marrow donor is seropositive. [300] The efficacy of immunoglobulin is somewhat more controversial. In a number of studies,[301] IVIG prophylactically, usually on a weekly basis, had little effect on the incidence of CMV infection (as measured by viremia or virus shedding) in CMV allogeneic marrow transplant recipients. However, two studies have shown a dramatic decrease in the incidence of CMV interstitial pneumonitis in those patients given IVIG prophylactically, despite the lack of a significant reduction in CMV infection rates. [302] [303] [304] Consequently, IVIG is generally given in the allogeneic marrow transplant setting from about 1 week prior to transplantation to approximately day 100, on a weekly or biweekly basis. It is thought that the immunoglobulin may act by reducing the incidence of GvHD. [304] Prophylactic high-dose acyclovir also appears to decrease the occurrence of CMV disease and significantly lowers patient mortality in some studies. [305] It is now widely used to prevent CMV disease as well as for HSV prophylaxis in this population, at 500 mg/m^2 every 8 hours. Ganciclovir given prophylactically can also decrease the incidence of interstitial pneumonitis, but bone marrow toxicity limits its utility.[306] The primary adverse event associated with ganciclovir use has been neutropenia. In one study, over half of the marrow transplant patients who received prophylactic ganciclovir had to have the drug interrupted owing to the development of neutropenia. Others have noted an increased rate of bacterial infection during periods of ganciclovir-induced neutropenia.

The preemptive use of ganciclovir is an interesting strategy that has been developed to specifically target ganciclovir therapy to those asymptomatic patients who are at highest risk for CMV disease following bone marrow transplantation. Seropositive post-transplant patients are screened periodically for CMV antigenemia, or for positive CMV cultures from urine, throat, or bronchoalveolar lavage washings. Ganciclovir preemptive therapy of patients with evidence of active CMV replication has been shown to prevent serious CMV disease, which decreased from 43% in patients without prophylaxis to only 3% in those who did receive preemptive ganciclovir.[307] Early ganciclovir use in a preemptive fashion was also shown to markedly decrease mortality in bone marrow transplant recipients, from 17% in untreated patients who were shedding CMV to 2.7% in treated patients. [307] However, this approach does not prevent CMV infection in the small number of patients in whom CMV reactivation is not detected until the onset of severe symptoms.

Weekly screening for CMV antigenemia through day 100 is now routine in most allogeneic bone marrow recipients. Detection of the CMV coat protein, pp65, by a specific monoclonal antibody yields a positive test, indicating active replication of CMV. A positive antigenemia test is clearly predictive for the development of CMV invasive disease and is more sensitive than routine culture because it typically becomes positive approximately 710 days before shell-vial culture detection of CMV. One problem with this approach is the tremendous variability in the performance and interpretation of the antigenemia test. Polymerase chain reaction (PCR) tests are becoming increasingly common as the assay of choice for detection of CMV replication following transplantation, and as a cue to initiate preemptive ganciclovir therapy.

Amantadine and rimantadine, antivirals with activity against influenza A virus, can prevent illness in 7090% exposed to the virus. They can also reduce the severity of symptoms if begun within the first 48 hours of influenza A illness. [308] The use of amantadine or rimantadine should be considered in persons at high risk of severe influenza infection during a

community outbreak. However, amantadine causes mild central nervous system (CNS) disturbances that may be more severe in the elderly or in those with diminished renal function. Rimantadine has slightly fewer neurologic side effects. Neither drug has activity against influenza B virus. [308]

Prophylaxis of Parasitic Infections

Strategies aimed at preventing parasitic diseases in immunosuppressed patients have primarily targeted *P. carinii* (Table 79-4). The agent studied most extensively has been trimethoprim-sulfamethoxazole, which has proved effective in preventing *P. carinii* infection in children with leukemia, in patients undergoing bone marrow transplantation, and in AIDS patients. An intermittent (i.e., two or three times weekly) dosage schedule is both effective and less toxic. [309] Although trimethoprim-sulfamethoxazole is the drug of choice for *P. carinii* prophylaxis in AIDS patients, up to 65% of adults with AIDS have adverse reactions (primarily dermatologic and hematologic) to this drug combination. [310] [311] Recent studies have shown aerosolized pentamidine to be less effective but better tolerated. [312] Dapsone may be a useful alternative in preventing *P. carinii* infection, but only small numbers of patients have been studied. [313] [314] Other agents given for prophylaxis include atovaquone, pyrimethamine with sulfadoxime, and pyrimethamine with dapsone, but data on their efficacy are limited. [311] The value of these agents in high-risk groups other than AIDS patients is only speculative at present.

Active and Passive Immunization for the Prevention of Infections in High-Risk Groups

Active Immunization

In the United States, few eligible adults are actually immunized. Shapiro et al. [315] conducted an investigation which showed that only 21% of those with an indication for the pneumococcal vaccine received it, despite the increasing incidence and severity of pneumococcal infection due to (1) the AIDS epidemic, (2) the growing

population that is >65 years of age, and (3) the increase in penicillin-resistant *S. pneumoniae*. The pneumococcal vaccine contains 23 of the serotypes responsible for >88% of pneumococcal bacteremias in the United States; however, it provides effective protection in only 61% of immunocompetent recipients and is less protective in immunocompromised hosts.[316] Despite its varying efficacy, the pneumococcal vaccine is recommended for patients who are asplenic, undergoing transplantation, have certain malignancies (Hodgkin disease, lymphoma, multiple myeloma), or are infected with HIV. [232] Another inactivated vaccine against encapsulated bacteria is the *H. influenzae* b vaccine. Although few data are available in adults, it is recommended for patients with anatomic or functional asplenia and its use should be considered in HIV-infected patients. [232] Yearly vaccination with the influenza vaccine, an inactivated viral vaccine, is recommended for those at increased risk of severe disease with influenza infection, including the elderly, persons with chronic cardiac or pulmonary disease, persons immunosuppressed through malignancy or drug therapy, and those with symptomatic HIV infection, although the protective value in severely immunocompromised patients is suboptimal.[192] [308] It is important for health care workers and family members of immunocompromised persons to receive the influenza vaccine in order to prevent transmission to those at risk of severe disease.

As a general rule, live attenuated viral or bacterial vaccines should not be administered to patients who are immunosuppressed as a result of leukemia, lymphoma, generalized malignancy, symptomatic HIV infection, or therapy with alkylating agents, antimetabolites, radiation, or large amounts of corticosteroids. [216] The only live bacterial vaccine currently available in the United States is BCG. The live viral vaccines that are available include varicella, measles, mumps, and rubella vaccines, oral poliovirus vaccine, and yellow fever vaccine. The latest recommendations of the Centers for Disease Control (CDC) state that patients with leukemia in remission whose chemotherapy has been terminated for 3 months may be given live virus vaccines. With respect to corticosteroid usage, low- to moderate-dose short-term systemic corticosteroid therapy (<2 weeks), topical steroid therapy, long-term alternate-day treatment with low to moderate doses of short-acting systemic steroids, and intra-articular, bursal, or tendon injection of corticosteroids should not be considered contraindications to live viral vaccine administration. [216] A 1989 study in bone marrow transplant recipients documented the safety and efficacy of administering measles-mumps-rubella (MMR) vaccine to patients in remission 2 years after transplantation who do not have active GvHD.[317] Live vaccines should generally be avoided in patients with HIV infection. The CDC recommends that MMR vaccine be administered to all HIV-infected children, even if they have symptomatic HIV infection, because of the recent resurgence of measles and the potential severity of measles in this population.[192] In general, varicella and oral polio vaccines should not be given to severely immunocompromised patients.

Passive Immunization

Passive immunization involves administration of preformed antibodies to high-risk patients, with the goal of either replacing qualitatively or quantitatively defective immunoglobulins or of attenuating the virulence of pathogenic organisms or their toxic components. Studies have examined the use of a variety of antibody preparations, including pooled immunoglobulins, immunoglobulins collected from individuals with high titers of specific antibodies (hyperimmune preparations), and monoclonal antibodies.

Passive immunization with VZIG is effective in preventing infection and decreasing the morbidity and mortality associated with primary chickenpox in susceptible hosts. Another hyperimmune preparation that has been investigated in high-risk patients consists of the so-called J-5 antisera collected from patients with high titers of antibody directed against the core glycolipid of Enterobacteriaceae. In a clinical trial, J-5 antiserum was not shown to reduce the mortality of neutropenic patients with gram-negative infections.[318] A randomized double-blind trial of HA-1A, a human monoclonal IgM antibody to the lipid component of endotoxin, demonstrated improved survival in patients with gram-negative bacteremia.[319] However, subsequent follow-up data showed unanticipated toxic effects in the HA-1A-treated patients, precluding its current use.

Immunoglobulin preparations with high titers against CMV have been used as adjuncts in the prevention and treatment of CMV-associated disease in bone marrow transplant recipients.[78] [301] [302] [303] [320] [321] Prophylactic CMV-enriched immunoglobulin has been shown to reduce the incidence of CMV clinical disease in renal transplant recipients by 5060%.[301] Immunoglobulin that has not been made from CMV hyperimmune plasma has also been shown to decrease CMV disease in bone marrow transplant recipients.[302]

Pooled immunoglobulin preparations have also been assessed in a number of immunocompromised populations. Both intramuscular and intravenous routes have been studied. In patients with hypo- or dysgammaglobulinemia not associated with malignancy or cytotoxic therapy, administration of pooled immunoglobulins has been effective in preventing infections due to encapsulated bacteria and in the treatment of certain enteroviral infections. [322] In patients with malignancy, the effects of immunoglobulin preparations have been less compelling, with one large randomized trial in allogeneic bone marrow transplant recipients showing a marginal benefit of IVIG in reducing bacterial sepsis.[302] A randomized placebo-controlled

trial of IVIG in patients with CLL showed that it was effective in decreasing the incidence of bacterial infections. [219] Although some early reports suggest a potential benefit of IVIG in patients with multiple myeloma, its value in this population remains controversial, and further studies will be needed before firm conclusions can be drawn.[322]

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MANAGEMENT OF FEVER AND INFECTION IN NEUTROPENIC PATIENTS

Initial Evaluation of the Febrile Neutropenic Patient

The goal of the initial evaluation of a newly febrile neutropenic patient is to identify potential sources of infection in order to optimize therapy. Ideally we would like to be able to distinguish at the outset those febrile patients with an infection from those who are infection free. In a prospective study from the NCI evaluating >1,000 consecutive episodes of fever and neutropenia, there were no features identified on presentation that reliably distinguished bacteremic patients from those with unexplained fever.[323] Physical examination was helpful only if a specific site of infection (e.g., cellulitis or abscess) was readily detectable. Other measurements that might be predictive in noncompromised patients, including the degree of fever, the presence of chills or rigors, or a toxic appearance, were not helpful in this population. Indeed, significant and potentially life-threatening bacteremias were not infrequently present, even in the absence of any symptoms or signs on physical examination. Of the patients with proven bacteremia, 55% had no evidence of infection on physical examination aside from fever.

For neutropenic patients with a new fever, the standard initial evaluation should include the following:

1. *Careful physical examination*. Particular attention should be devoted to areas that may hide a site of infection unless closely examined, notably the oral cavity and the perianal area. There is some debate as to whether neutropenic patients should have an initial rectal examination because manipulation in this area may lead to bacteremia. It is our policy to first perform a complete external examination of the perirectal area, including deep palpation. Then, only if there are findings suggestive of a localized inflammatory site (e.g., pain or fluctuance) do we perform a careful digital examination. We have found that virtually all perianal or perirectal sites of infection can be detected with this approach.
2. *Blood cultures*. A minimum of two sets of blood samples should be obtained for cultures. If the patient has an indwelling intravenous catheter, at least one set should be drawn through the catheter and another drawn from a peripheral vein. For patients with multilumen intravenous catheters, a sample should be drawn through each lumen, as well as from the specific lumen clearly identified on the culture bottle. This is important because catheter infection may be limited to a single lumen. It must be emphasized that a peripheral culture is essential in addition to catheter cultures, in order to make the diagnosis of a catheter infection.
3. *Urine culture*. Because of the absence of granulocytes, microscopic examination of the urine may be normal even in the presence of a urinary tract infection. Nonetheless, a urine culture should be performed, and the urine may be considered a source of infection in the neutropenic patient even if pure growth is <10 [5] of a single organism.
4. *Chest radiography*.
5. In addition, accessible sites of potential infection should be aspirated or biopsied and appropriate material sent for Gram stain, culture, and histologic examination. Other diagnostic tests directed at certain sites, such as cranial computed tomography (CT) or lumbar puncture, should be reserved for patients who have symptoms or signs referable to those areas.

Even with a comprehensive evaluation, an infectious cause of the initial fever is demonstrated in only 3050% of patients. Moreover, the definitive diagnosis may take days, presumably because of the relatively low microbial inoculum. This probably reflects the short period that elapses between the onset of fever and the evaluation and initiation of empirical therapy. Even subtle inflammation must be considered as indicating a site of infection in the presence of granulocytopenia. For example, minimal perirectal erythema and tenderness may be the harbinger of a perirectal cellulitis. Minimal erythema or discharge at the exit site of an indwelling intravenous catheter may signal a tunnel or exit-site infection.

Colonization with microorganisms often precedes the development of significant infection. Logic has dictated that identification of a specific pathogen during the colonization phase might help guide the clinician toward earlier use of appropriate antimicrobial therapy at the first signs of infection. Accordingly, some investigators have recommended routine serial surveillance culturing in neutropenic patients. To evaluate the clinical value of this concept, an NCI study of 652 febrile granulocytopenic episodes was conducted.[45] Nose, throat, urine, and stool specimens were cultured serially during the neutropenic episode. Of those patients who became septic and from whose blood a specific causative pathogen was isolated, 62% were found to be colonized with the infecting organism. However, the results of the surveillance cultures provided little clinically useful information, for the following reasons: (1) no single body site was predictive; (2) other potential pathogens were usually isolated in addition to the organism responsible for infection; and (3) the actual pathogens isolated from the blood cultures were usually known prior to their isolation from the surveillance cultures. Furthermore, the cost of routine surveillance is enormous, and knowledge of the colonizing flora is unlikely to have a significant impact on initial antibiotic management, because most clinicians routinely employ broad-spectrum antibiotics for the empirical treatment of febrile granulocytopenic patients. For these reasons, we do not recommend routine surveillance cultures in neutropenic patients. They can be helpful, however, in certain subgroups of patients, such as those with protracted neutropenia (>34 weeks) and those treated at centers that have a high incidence of particularly virulent or resistant organisms, such as *P. aeruginosa* or *Aspergillus* spp.

Antibiotic Strategies

Empirical Antibiotics

The management of neutropenic cancer patients as compared with nonimmunocompromised patients more frequently involves the empirical use of antibiotics directed against a wide array of potential pathogens. It is now well accepted that when a neutropenic patient develops a new fever (fever is usually defined as one oral temperature reading of 38.8°C or two successive readings of 38°C during a 4-hour period), an empirical antibacterial regimen should be started expeditiously. This holds true even in patients with no clinically evident source of infection. The rationale for this approach evolved from the observation that bacteremias in neutropenic patients are often rapidly lethal, especially those due to gram-negative organisms. [5] [324] [325] [326] [327] A delay of even 2448 hours for the results of initial blood cultures to become available can substantially increase morbidity and mortality. In addition, no reliable way has been found to determine whether an isolated fever in a neutropenic patient is due to an underlying infection. Within that context, even fevers that are temporally associated with the administration of blood products or with fever-producing antineoplastic drugs should be considered potentially infectious in origin and treated as such. In sum, virtually all new fevers in the neutropenic population warrant careful clinical and microbiologic

Box 79-1. APPROACH TO THE NEUTROPENIC PATIENT WITH FEVER AND INFECTION

We define clinically relevant neutropenia as <500 polymorphonuclear leukocytes and band forms per microliter or any count that is falling and expected to be <500 l within 24 hours. Any fever in this population should be interpreted as potentially indicating serious infection (including fevers following administration of blood products). Conversely, even in the absence of fever, any subtle indication of infection should be considered potentially serious and treated as such. Fever is defined as one oral temperature reading >38.5°C or more than two successive readings of >38°C in a 12-hour period.

Routine workup should include a complete history and physical examination, with particular attention directed toward sites that may hide infection, such as the mouth and perirectal area. At least two sets of blood samples should be obtained (including one from a peripheral vein) for culture, with samples drawn through each lumen of any multilumen intravenous access device. A urine sample for culture and a chest radiograph should be obtained. Further workup is determined by the patients clinical presentation.

Empirical antibacterial therapy should be administered to all neutropenic patients with fever of new onset or clinical evidence of infection even in the absence of fever. There is no single best regimen; however, there are many inappropriate regimens. At a minimum, regimens should have broad-spectrum activity against gram-positive and gram-negative organisms (including *P. aeruginosa*). Aminoglycosides or extended-spectrum penicillins (e.g., ticarcillin, mezlocillin, piperacillin, azlocillin) should never be used as single agents or as the only agents with anti-gram-negative activity.

For most patients, we initiate single-agent empirical therapy (monotherapy). Of the antibiotics that are potentially useful for monotherapy, ceftazidime has been studied most extensively and has the longest track record of efficacy. The development of resistance to these agents, although not reported frequently, must be monitored if they are used alone. Imipenem appears to be equally efficacious but is often associated with intolerance or toxic effects. Nausea and vomiting with higher doses and seizures in susceptible patients have been reported. Cefepime is also effective.

The monobactam aztreonam is useful for patients with significant -lactam allergies who still require the use of an antipseudomonal antibiotic in addition to an aminoglycoside. Accordingly, for patients with significant -lactam allergies who present with fever and neutropenia, we begin a combination of aztreonam plus an aminoglycoside plus vancomycin. Further study is warranted to see if aztreonam can suffice as the only anti-gram-negative component of empirical regimens (thus avoiding the use of an aminoglycoside).

Because the incidence of infection due to gram-positive organisms is increasing, the question of whether vancomycin should be included in the initial empirical regimen has been the subject of considerable debate. Because of the emerging resistance to vancomycin and the relatively lower morbidity with a number of gram-positive organisms, empirical vancomycin is not recommended for routine use.

For certain patients who present with a defined site of infection, the standard regimen should be individually modified. For example, for a neutropenic patient presenting with a perirectal abscess, coverage might be extended to include an antianaerobic agent (e.g., metronidazole or clindamycin) as well as an agent active against enterococci. For a neutropenic patient with Hickman catheter exit site infection, vancomycin might be added to extend gram-positive coverage.

For most neutropenic patients who present with fever, a microbiologically or clinically documented infectious source is not identified (i.e., they have unexplained fever). For this group we prefer to continue empirical antibiotics until the neutropenia resolves. However, this is not always practical, especially for those with prolonged neutropenia. Therefore, for persistently neutropenic patients with unexplained fever who undergo defervescence and remain afebrile from days 7 to 14 (i.e., they do not need empirical amphotericin B or other modifications), we discontinue antibiotics on day 14 and carefully observe. Depending on the amount of additional time they remain neutropenic, a substantial number may experience recrudescence and require prompt resumption of empirical therapy. In patients with hematologic evidence of recovery, antibiotics can be discontinued sooner.

For patients who present with a documented infection, we continue therapy for a minimum of 1014 days, even if the granulocytopenia resolves before this time. If the infection has been identified microbiologically, we switch to narrow-spectrum therapy or oral therapy to complete the course at the resolution of granulocytopenia.

Common modifications in antibacterial regimens include changes in gram-negative coverage for potentially resistant organisms and the addition of intravenous vancomycin for improved gram-positive coverage or of oral vancomycin for *C. difficile*, and the addition of clindamycin or metronidazole to improve anaerobic coverage.

For patients with a persistent or new fever after >17 days of empirical antibacterial therapy, we begin empirical amphotericin B and continue it along with the antibacterial agents until the neutropenia resolves. Following an initial test dose of 1 mg given over 1 hour, we proceed directly to 0.5 mg/kg/day (without dose escalation). Adequate hydration and diuresis (saliuresis) are important in limiting the resultant azotemia. However, patients needing long courses of amphotericin B will invariably have some rise in BUN and creatinine levels, which usually stabilize.

Acyclovir is the most common antiviral agent added to regimens for neutropenic patients and may be directed toward HSV or VZV. Ganciclovir provides activity for the treatment or prevention of CMV. Trimethoprim-sulfamethoxazole is the most common antiparasitic agent added, with activity directed primarily against *P. carinii*.

Although gram-positive organisms will cause most infections associated with central venous catheters, gram-negative organisms are not infrequently encountered. Neutropenic patients who present with even a mild exit-site infection should receive broad-spectrum coverage directed at gram-positive and gram-negative organisms, including *P. aeruginosa*.

More than 90% of catheter-related bacteremias can be successfully treated with antibiotics alone without the need for catheter removal. Initial specimens for culture should be drawn through all lumina or any multilumen device, and antibiotic administration should be rotated among the ports. Persistently positive cultures or the progression of infection after 2448 hours of appropriate antibiotics mandates prompt catheter removal. Other indications for initial catheter removal are infection caused by *Bacillus* spp. or *Corynebacterium jeikeium*, any tunnel infection (extending to >2 cm from the exit site), and candidemia.

TABLE 79-6 -- Essential Properties of Empirical Regimens

Broad spectrum of activity that includes *Pseudomonas aeruginosa*
Ability to achieve high serum bactericidal levels
Effective in the absence of neutrophils
Low potential for the emergence of resistance
Acceptable toxicity profile

evaluation, followed by prompt initiation of empirical antibiotic therapy. Conversely, any clinically evident site of potential infection mandates expeditious broad-spectrum therapy, even in the absence of fever.

The goal of empirical antibiotic therapy is to protect against the early morbidity and mortality that result from untreated bacterial infections. Accordingly, regimens have been formulated to maximize activity against organisms that are commonly encountered or particularly virulent. One must remember that empirical regimens cannot realistically be designed to cover every potential bacterial pathogen. Likewise, no regimen is capable of completely eliminating the risk of subsequent development of infections in persistently neutropenic patients. Empirical antibiotic regimens should be individualized at each institution to cover organisms seen at

that hospital and to cover organisms associated with certain chemotherapeutic regimens.

Traditionally, gram-negative bacteria have been the most frequently isolated pathogens in the neutropenic population, with *E. coli*, *K. pneumoniae*, and *P. aeruginosa* the most common. Although gram-negative bacteria may still predominate at some institutions, the trend in the past 20 years has been toward more gram-positive infections, which now account for most isolates at many centers.[8] [10] [119] [328] [329] [330] [331] [332] [333] In general, gram-negative infections tend to be more virulent; early empirical regimens were formulated to provide protection primarily from these organisms while maintaining a broad spectrum of activity against other potential pathogens. Indeed, adequate coverage of these gram-negative organisms is still an essential property of any empirical regimen (Table 79-6).

In order to achieve the desired properties of empirical therapy, it has been necessary to employ combinations of antibiotics. Early experience combining aminoglycosides and an expanded-spectrum penicillin such as carbenicillin resulted in an improved clinical outcome.[334] This combination appeared to obviate both the poor clinical efficacy of aminoglycosides when used alone and the emergence of resistance to the expanded-spectrum penicillins often encountered when used as single agents. In addition, a body of in vitro data emerged showing a synergistic effect of such combinations against the gram-negative bacilli, particularly *P. aeruginosa*.[211] [335] [336] [337] [338] [339] [340] [341] [342] [343] [344] Such combination regimens are still widely used and represent a standard against which newer regimens are tested.

Many variations of the initial gentamicin-carbenicillin combination have been studied (Table 79-7). These consist of an aminoglycoside at the core, usually in combination with an extended-spectrum (or antipseudomonal) penicillin and often with an antistaphylococcal -lactam antibiotic (e.g., nafcillin or a first-generation cephalosporin). Third-generation cephalosporins can also be combined with aminoglycosides, provided that the third-generation cephalosporin has good antipseudomonal activity (e.g., ceftazidime or cefoperazone).[345] In general, no specific combination has been shown to have consistently superior efficacy. If an aminoglycoside-containing combination regimen is to be employed, the choice of specific antibiotics should be based primarily on the institutions antibiotic sensitivity patterns and secondarily on toxicity and cost differences.

In order to avoid the use of aminoglycosides yet retain the theoretical advantages of combination antibiotics, various non-aminoglycoside-containing combination regimens have been devised and studied. For the most part these have consisted of combinations of two -lactam antibiotics (so-called double -lactam regimens), usually consisting of an expanded-spectrum carboxy- or ureidopenicillin and a third-generation cephalosporin (e.g., piperacillin and ceftazidime).[346] [347] Although double -lactam combinations may offer some of the potential advantages of other combinations, they also have some of the inherent disadvantages of any combination regimen (see later). In addition, increased -lactamase induction is a theoretical disadvantage when certain of these agents are combined (thus accelerating the development of resistant organisms).[347]

Single-agent coverage with an aminoglycoside or an extended-spectrum penicillin is not a viable option because of the rapid emergence of pathogens when either of these agents are used alone. The combination of an aminoglycoside and a -lactam antibiotic has demonstrable efficacy, but in the past decade, a number of potent cephalosporins and carbapenems have been developed that offer the option of effective antibiotic monotherapy for the febrile neutropenic patient. Ceftazidime has been the most extensively studied of the third-generation cephalosporins; most other agents in this class exhibit inadequate activity against *P. aeruginosa* for use as monotherapy (*P. aeruginosa* is among the most virulent of the gram-negative organisms).[348] [349] Cefoperazone also has activity against *P. aeruginosa*, but its clinical value in this setting has not been as extensively evaluated. In the carbapenem class, imipenem and meropenem are the currently available agents; several trials support the efficacy of both drugs as monotherapy.[350] [351] [352]

Ceftazidime has a broad spectrum of activity that encompasses the vast majority of bacterial pathogens encountered in neutropenic hosts, including *P. aeruginosa* (Table 79-8). Standard dosing results in serum and tissue levels that are bactericidal for these organisms, and the toxicity profile is similar to that of most -lactams.

TABLE 79-7 -- Aminoglycoside-Based Combination Regimens

Aminoglycoside +	Antipseudomonal -Lactam ±	Additional Anti-Gram-Positive
Gentamicin Tobramycin Amikacin	Extended-spectrum penicillin Carbenicillin Ticarcillin Azlocillin Mezlocillin Piperacillin OR Third-generation cephalosporin Ceftazidime Cefoperazone OR Aztreonam	Isoxozolyl-penicillin Nafcillin Oxacillin OR First-generation cephalosporin Cephalothin Cefazolin OR Vancomycin

TABLE 79-8 -- Activity of Newer Antibiotics Against Pathogens Commonly Encountered in Cancer Patients

Antibiotic	Enteric Gram-Negative	*P. aeruginosa*	Coagulase-Positive Staphylococci	Coagulase-Negative Staphylococci	Enterococci	Streptococci	Anaerobes
Ceftazidime	Good	Good	Moderate (poor against methicillin-resistant strains)	Poor against the majority (most are methicillin-resistant)	Poor	Good	Poor
Cefepime	Good	Moderate to good	Moderate (poor against methicillin-resistant strains)	Poor against the majority	Poor	Good	Poor
Other third-generation cephalosporins	Good	Poor to moderate	Moderate (poor against methicillin-resistant strains)	Poor against the majority	Poor	Good	Poor to moderate (moxalactam and ceftizoxime have some activity)
Imipenem	Good	Good	Good (poor against methicillin-resistant strains)	Poor against the majority	Good	Good	Good
Quinolones	Good	Good	Good (including some methicillin-resistant strains)	Good (limited clinical experience)	Poor to moderate	Poor to moderate	Poor
Aztreonam	Good	Good	Poor	Poor	Poor	Poor	Poor

A large randomized study evaluating 550 consecutive episodes of fever and neutropenia conducted at the NCI compared a combination of antibiotics (i.e., cephalothin, gentamicin, and carbenicillin) with ceftazidime as a single agent.[349] The overall results showed that monotherapy compared favorably with a standard combination regimen (Table 79-9 (Table Not Available)). Approximately two-thirds of the episodes in both groups were successfully treated for the entire duration of the granulocytopenia without requiring any changes in the initial regimen. Another one-third of the episodes required some change or modification (e.g., addition of an antibacterial, antifungal, or antiviral drug) to ensure a successful outcome (Table 79-10) (see also discussion under Modifications of Antibiotic Therapy During the

Course of Granulocytopenia), and an equally low number in both groups (5%) died of infection. None of the deaths was attributable to

TABLE 79-9 -- Outcome of 550 Febrile Neutropenic Episodes Randomized to Monotherapy or to Combination Antibiotic Therapy

(Not Available)

Modified from Pizzo et al.,[323] with permission.

a specific deficiency in one regimen that was not present in the other (i.e., an organism sensitive to one regimen but resistant to the other). In addition, the average time to initial defervescence was equivalent for those receiving monotherapy and those treated with combination antibiotics.

Two subgroups of patients identified in this study appeared to need more frequent modifications of the initial regimen in order to achieve a successful outcome: (1) those presenting with a documented source of infection to account for the initial fever, and (2) those having relatively protracted periods of granulocytopenia (>1 week). However, the need for modification in these subgroups was identical for those episodes treated with monotherapy and those treated with combination therapy. In this study, these modifications did not represent a failure of either regimen per se but instead reflected the limitations of any regimen in treating patients at high risk for the development of subsequent infections.

In a 1991 review of 12 randomized, controlled trials comparing ceftazidime as single-agent therapy for febrile, neutropenic patients with combination therapy, Sanders et al.[353] studied 1,077 episodes of fever and neutropenia and 248 episodes of bacteremia. In this meta-analysis, no advantage of combination therapy over monotherapy with ceftazidime could be detected.

Cefepime is a new extended-spectrum cephalosporin with excellent activity against gram-negative and gram-positive bacteria. It is comparable in efficacy to ceftazidime against *P. aeruginosa* but it has greater stability against degradation by certain -lactamases, particularly those produced by *Enterobacter* and *Serratia* spp. Additionally, it has better in vitro activity than ceftazidime against gram-positive organisms such as methicillin-sensitive staphylococci and viridans streptococci. Despite its improved antimicrobial profile, cefepime was found to be equivalent to either ceftazidime monotherapy or the combination of piperacillin plus gentamicin for the empirical coverage of febrile neutropenia in cancer patients. [353A] In a randomized study, 109 patients received cefepime and 107 received either of the comparator regimens. In 75% of febrile episodes, patients

TABLE 79-10 -- Modifications of Therapy During the Course of Granulocytopenia

Clinical Event	Possible Modifications of Therapy
Breakthrough bacteremia	If gram-positive isolate (e.g., *S. epidermidis*), add vancomycin If gram-negative isolate (i.e., presumably resistant), switch to regimen containing non-cross-resistant antibiotics (e.g., aminoglycoside plus a carbapenem or extended-spectrum penicillin)
Catheter-associated infection	Add vancomycin (as well as gram-negative coverage if not already being given)
Severe oral mucositis or necrotizing gingivitis	Add specific antianaerobic agent (e.g., clindamycin or metronidazole) or change to antibiotic with improved anaerobic coverage (e.g., imipenem)
Esophagitis	Trial of oral clotrimazole, fluconazole, IV amphotericin B, or acyclovir
Pneumonitis, diffuse or interstitial	Trial of trimethoprim-sulfamethoxazole and erythromycin (plus broad-spectrum antibiotics, if patient is granulocytopenic)
New infiltrate in a granulocytopenic patient also receiving antibiotics	If granulocyte count is rising, watch and wait If granulocyte count is not recovering, biopsy to establish diagnosis, add amphotericin B empirically
Perianal tenderness	If patient is already receiving broad-spectrum antibiotics, add a specific antianaerobic agent If patient is not on antibiotics, begin broad-spectrum therapy with anaerobic coverage
Persistent fever and neutropenia	Continue antibiotics after 1 week of persistent fever and neutropenia; add systemic antifungal therapy empirically

had a satisfactory response at the completion of therapy and eradication rates were similar for all pathogens treated by the three regimens. In an era of increasing antibiotic resistance, however, cefepime may assume a more important role in populations in whom -lactamase-producing gram-negative rods are a particular problem.

Imipenem is a member of the carbapenem class of antibiotics and offers another option for empirical monotherapy. Overall it has the broadest spectrum of activity of any available antibiotic (Table 79-8). Of note is its good in vitro activity against many enterococci, staphylococci, and many anaerobes, in addition to its excellent gram-negative activity. A large randomized study from the NCI compared monotherapy with ceftazidime to monotherapy with imipenem.[350] Both drugs showed success without modification in slightly >60% of episodes of fever without a known source. In patients with a documented infection, the majority of patients in both groups required antibiotic changes or additions. Among those receiving imipenem, 70% needed antibiotic modification, while 83% of those receiving ceftazidime needed a change in antibiotics. Often the anaerobic coverage was added to the regimen of patients on ceftazidime. However, more side effects were seen in patients on imipenem, most notably nausea (21% vs 3% in ceftazidime recipients) and *C. difficile* colitis (11% vs 4% in ceftazidime recipients). In addition, imipenem could not be used in patients with brain tumors or with a decreased creatinine clearance rate because of the increased risk of seizures. [354]

Several authorities have raised concerns regarding the use of single-agent therapy for fever and neutropenia. Some of these concerns are purely theoretical; others are based on unique experiences limited to specific centers. However, these issues underscore the appropriateness of maintaining a flexible approach with regard to selection of an empirical regimen, which should be based on individual and institutional experience. Also, regardless of the specific regimen chosen, the clinician must be acutely aware of the potential need for changes in that regimen during the course of granulocytopenia (Table 79-10). This mandates frequent and meticulous clinical evaluation by an experienced supportive care team.

During the late 1980s, gram-positive organisms re-emerged as the most frequently encountered isolates at many centers. [331] [332] [333] Although the precise reason for this shift is unclear, it is evident that many of these gram-positive bacteria (e.g., the enterococci and the coagulase-negative staphylococci) are either resistant to, or poorly covered by, most standard empirical regimens. Some authorities have therefore recommended the addition of vancomycin to empirical regimens. Conversely, it has been argued that because many of these organisms are of relatively low virulence and are often inhibited by the antibiotics (even suboptimal antibiotics), vancomycin may be safely withheld until the gram-positive isolate has been identified microbiologically.

In a randomized study of a vancomycin-containing versus a non-vancomycin-containing regimen conducted by Karp et al., [119] the incidence of secondary gram-positive infections was reduced in the vancomycin-containing group. However, no difference in morbidity was related to gram-positive infections between the two groups, and all the gram-positive infections in the non-vancomycin-treated group were successfully treated by its addition after the organism had been identified and reported by the microbiology laboratory. There also appeared to be less need for amphotericin B in the group that did not receive vancomycin initially. Subsequently, a study conducted by the European Organization for Research and Treatment of Cancer (EORTC) looked at results in 747 patients who were randomly assigned to receive ceftazidime and amikacin or ceftazidime, amikacin, and vancomycin.[355] No difference was found in the number of febrile days between the two groups. The group receiving ceftazidime and amikacin did need more antimicrobial modifications, most commonly the addition of vancomycin, but the group receiving the vancomycin-containing regimen required the addition of antifungal therapy more frequently than those without vancomycin in the initial regimen. Notably, no deaths due to gram-positive infection occurred in the first 3 days of therapy, regardless of the antibiotic regimen.

In addition, a retrospective analysis from the NCI indicated that no excess morbidity resulted when the institution of vancomycin was delayed by waiting for either a microbiologic or clinical indication for its use (i.e., a positive culture for a resistant gram-positive organism or a clinical infection developing in the presence of other antibiotics).[8] All the primary infections with gram-positive isolates (from cultures done before the empirical institution of therapy) and 82% of the secondary infections with gram-positive isolates (from cultures done after the institution of antibiotics) were treated successfully with this pathogen-directed approach. These data strongly

support the practice of not including vancomycin in the initial empirical regimen. However, the most compelling reason to avoid routine vancomycin use is the emergence of vancomycin-resistant enterococci (VRE) in recent years. Between 1989, when VRE were first identified in the United States, and 1993 there was a 20-fold rise in the proportion of nosocomial isolates of enterococci showing resistance to vancomycin. [356] The severe infections that are caused by VRE frequently occur in patients with cancer, and there is an attributable mortality of nearly 40% due to VRE bacteremia in this population. [357] Because the emergence and spread of VRE in hospitals are related to the use and overuse of vancomycin, the CDC has published guidelines for prudent vancomycin

use. Specifically, these guidelines indicate that vancomycin should not be used for empirical antimicrobial therapy for febrile neutropenia and should not be used prophylactically to prevent indwelling catheter infections. [358]

The development of newer broad-spectrum antibiotics has expanded the available options for the empirical treatment of fever and neutropenia; both aztreonam and ciprofloxacin are useful additions to the antibiotic armamentarium. Aztreonam, a monobactam active against gram-negative aerobes, including *P. aeruginosa*, should not be used as monotherapy because of its limited spectrum. At the NCI, aztreonam is used in combination with vancomycin in patients with allergies to -lactam antibiotics. Ciprofloxacin, the most commonly used quinolone, has a distinctive mechanism of action: inhibition of bacterial DNA gyrase. Its spectrum of activity includes some staphylococci and gram-negative organisms, including multiply resistant *P. aeruginosa*, *Enterobacter*, *Serratia*, and *Klebsiella*. However, it has poor streptococcal coverage and is inactive against anaerobes. The use of ciprofloxacin as monotherapy for fever and neutropenia has been complicated by an increase in breakthrough bacteremias with streptococci. [359] [360] Although ciprofloxacin can be combined with vancomycin or a penicillin as empirical therapy, we prefer to reserve its use for multiply resistant gram-negative infections.

Approach to the Low-risk Patient with Fever and Neutropenia

The excellent bioavailability and broad spectrum of the oral fluoroquinolones, particularly ciprofloxacin and ofloxacin, make them attractive for use in the management of low-risk patients with fever and neutropenia. Low-risk patients may be defined as those who have fever in the setting of an anticipated short duration of neutropenia (e.g., <10 days) and no medical comorbid conditions (e.g., hypotension, abdominal pain, vomiting, mental status changes, respiratory compromise) on presentation. To explore the use of oral antibiotic therapy, Rubenstein et al. randomized 83 episodes of low-risk febrile neutropenia to treatment with either oral ciprofloxacin plus clindamycin or intravenous therapy consisting of aztreonam plus clindamycin, with both regimens administered in an outpatient setting. [361] Overall responses were good in both groups, with no mortality. All therapy was accomplished in the outpatient setting, following a 24- to 36-hour initial treatment and observation period in the clinic. However, six patients in the oral regimen cohort were readmitted for persistent fever or for nephrotoxicity that appeared to be associated with ciprofloxacin use in dehydrated patients. This study was relatively small and did not include a comparison group managed as inpatients, but it does suggest that outpatient therapy is a feasible alternative for low-risk patients with febrile neutropenia.

In a larger study performed in Pakistan (n = 122 patients), Malik et al. found that ofloxacin monotherapy, 400 mg given orally every 12 hours, appeared to be comparable in efficacy to combination parental antibiotics for the management of low-risk fever and neutropenia in adults. There were no differences in rates of successful therapy, either with or without modifications of the initial regimen, between the two treatment groups. The overall success rate for patients in each cohort was 75%. [362] Significantly lower response rates were seen in both groups in patients with prolonged and profound neutropenia, and in those with documented infections compared to those with fever of unknown origin. Two *P. aeruginosa* bacteremias were treated with ofloxacin in this trial, although the outcomes were not specifically reported. As the antimicrobial activity of ofloxacin is less potent than that of ciprofloxacin against *P. aeruginosa*, ofloxacin is theoretically less reliable for empirical therapy in cancer centers where this organism is more prevalent. However, ofloxacin may be more potent than ciprofloxacin against *S. aureus* and less prone to breakthrough drug-resistant isolates. These features may amount to an overall benefit as gram-positive organisms are increasingly isolated in the setting of febrile neutropenia. The same investigators also reported a prospective, randomized trial comparing inpatient and outpatient management of low-risk patients with fever and neutropenia with oral ofloxacin. [363] There were no differences in overall outcome, and most patients were treated without modifications of the empirically employed oral fluoroquinolone. However, 21% of those originally assigned to outpatient therapy were readmitted for persistent fever or unresolved infection. Mortality was very low and was comparable in both groups (2% for inpatients vs 4% for outpatients). However, a single early death in an outpatient who developed vomiting and difficulty with oral intake and who subsequently refused to be admitted to the hospital emphasizes the need for good patient compliance as well as close patient contact and strict vigilance by health care workers when treating even low-risk patients outside of the hospital setting.

The importance of these studies was the demonstration of efficacy, excellent tolerability, decreased expense, and low toxicity of oral fluoroquinolone therapy when compared with parental combination antibiotics for the management of fever and neutropenia in low-risk patients. At the NCI, we have paired oral ciprofloxacin with amoxicillin/clavulanate in a double-blind randomized comparison of this oral combination with intravenous ceftazidime monotherapy among low-risk febrile neutropenic inpatients. We have found no difference in overall efficacy between the oral regimen and intravenous ceftazidime in patients hospitalized with fever and short-duration (<10 days) neutropenia (unpublished observations). The use of oral empirical antibiotic therapy in the outpatient setting is becoming more acceptable for low-risk patients. However, patients must be monitored frequently in the outpatient setting and be taught to return to the hospital when any signs of decompensation emerge.

Approach to the Patient with Prolonged Granulocytopenia

Length of Antibiotic Therapy

A question of practical importance is the length of time antibiotics should be continued in persistently neutropenic patients. Should they always be continued until the granulocyte count recovers, or can they be safely discontinued before that time? Our overall approach is illustrated in Figure 79-2 .

Operationally, it is easiest to approach the question of duration of therapy by placing patients in two categories: (1) those whose initial workup did not reveal the source of infection (i.e., patients with fever of undetermined origin), and (2) those whose initial workup revealed a documented infection to account for the fever. Most patients (60%) at most centers will fall into the former category, although this will vary with the institution, therapy, and patient population.

Limited data specifically address the issue of duration of empirical therapy in neutropenic patients presenting with fever of undetermined origin. In a randomized NCI study, patients with fever of undetermined origin and persistent granulocytopenia were assigned either to discontinue antibiotics on day 7 of therapy or to continue them until the resolution of the neutropenia (Table 79-11). [364] A large percentage of afebrile patients who stopped taking antibiotics developed recurrent fever, and an alarmingly high percentage of persistently febrile patients whose antibiotics were discontinued had hypotensive episodes. It was concluded that day 7 was too early to discontinue antibiotics in this group.

In a subsequent randomized study conducted at the NCI, persistently neutropenic afebrile patients were assigned either to continue or to discontinue antibiotics on day 14. Analysis

Figure 79-2 Algorithm for antibiotic management in febrile neutropenic patients. F+ N+, febrile, neutropenic; F N+, afebrile, neutropenic; FUO, no source of fever on preantibiotic evaluation; AMPHO B, amphotericin B.

TABLE 79-11 -- Outcome of Patients with Unexplained Fever Who Were Randomized Either to Continue or to Discontinue Empirical Antibiotics When They Remained Persistently Granulocytopenic

	Continue Antibiotics		Discontinue Antibiotics	
	F G+ (%)	F+ G+ (%)	F G+ (%)	F+ G+ (%)
Remained afebrile or improved	100	62	59	44
Became febrile or worsened	0	38	41	56
Became hypotensive	0	0	12	38

Abbreviations: F, reduction of fever; G+, granulocytopenia; F+, continuation of fever.

showed no difference between the two groups; approximately one-third of patients became febrile again, regardless of whether they stopped or continued taking antibiotics. However, those whose fevers recurred after discontinuation of antibiotics responded to a re-institution of their initial regimen, while those remaining on antibiotics required the addition of amphotericin B. Currently, for patients with fever of unknown origin who are predicted to have a long duration of neutropenia and who have remained afebrile at day 14, we discontinue antibiotics and cautiously observe. Additional studies have suggested that antibiotics can also be safely discontinued in afebrile patients who have evidence of bone marrow recovery, even though their neutrophil count is still <500 cells/mm ^{3} . [365] [366]

In addition, for persistently neutropenic patients who have had clinical and microbiologic resolution of their infection and who are afebrile at day 14 we consider discontinuing antibiotics. The final decision as to whether to continue or discontinue antibiotics rests on a number of clinical parameters, such as the degree of or potential for antibiotic toxicity, the predicted duration of neutropenia, the seriousness of the initial infection, and the presence or absence of other factors predisposing to subsequent infection. It should be emphasized that any neutropenic patient whose antibiotics are discontinued requires meticulous follow-up evaluation so that any new fevers or infection can be quickly detected.

Modifications of Antibiotic Therapy During the Course of Granulocytopenia

Empirical antibiotics have their greatest impact early in the course of neutropenia. It is during these early days, after the initial febrile episode and before results of the initial cultures are known, that the antibiotic regimen is truly empirical. One must not assume that the efficacy of an empirical regimen implies that it will suffice as the sole antimicrobial therapy throughout a protracted course of neutropenia. Indeed, it is during a prolonged granulocytopenic episode that the patient is at highest risk for multiple types of secondary infections or superinfections, as well as for changing clinical parameters, many of which dictate specific types of modifications of the initial regimen to ensure a successful outcome. Only a few secondary infections actually represent a true failure of the initial antibiotic regimen. Rather, they should be viewed as part of the natural history of most patients with prolonged neutropenia, and the need for modifications of the initial regimen should be expected and planned for.

Modifications for Resistant Bacterial Isolates.

Bacterial isolates resistant to the initial empirical regimen are invariably encountered when one is managing neutropenic patients. Although their occurrence can be minimized if the initial regimen is selected on the basis of regional sensitivity patterns, the overall incidence of resistant organisms will vary dramatically, depending on the institution, characteristics of the patient population, selection of antibiotics, and unexplained ecologic fluctuations. Clinically, it is important to distinguish between organisms in the environment with intrinsic or de novo antibiotic resistance (primary resistance) and organisms that develop resistance while the patient is receiving antibiotics (secondary resistance). Organisms with primary resistance may cause either initial infection or breakthrough infections, while organisms developing secondary resistance cause infections in patients already being treated with antibiotics.

Primary resistance is predominantly dependent on the institution and/or location and is reflective of a regional pool of organisms with similar sensitivity patterns. In addition, individual patients may be colonized with resistant organisms in the absence of an environmental reservoir. For a patient whose site of infection is believed likely to harbor a resistant organism (e.g., coagulase-negative staphylococci from a catheter exit-site infection) or for a patient with a prior history of recurrent infections due to a rare but resistant organism, appropriate modifications of the initial empirical regimen are warranted.

Organisms with intrinsic resistance to most components of standard empirical regimens may also cause secondary or breakthrough infection. Commonly encountered examples include breakthrough infections caused by methicillin-resistant coagulase-negative staphylococci, enterococci, or anaerobes in patients whose initial regimens do not include antibiotics active against these organisms. Rarer examples include secondary infections due to non- *aeruginosa Pseudomonas*-like organisms, *C. jeikeium*, or atypical mycobacteria. For patients receiving non-vancomycin-containing empirical regimens, reports from the microbiology laboratory of gram-positive cocci growing from a culture should prompt the addition of vancomycin, with subsequent modifications made on the basis of a final sensitivity determination. If anaerobes are reported, clindamycin or metronidazole should be added.

The appearance of secondary resistance is seen more frequently with certain organisms. For example, *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. harbor inducible -lactamases; a 1989 report noted the appearance of a clinically significant clustering of resistant *Enterobacter* in a neutropenic population.[367] Accordingly, when these organisms are isolated from a patient, careful observation for the emergence of resistance is warranted, and an aminoglycoside should be added to the regimen of patients receiving monotherapy with a broad-spectrum -lactam. *P. aeruginosa* may become resistant to imipenem through a relatively novel mechanism involving a change in porins. Hence, for patients receiving single-agent therapy with imipenem who have a documented infection with *P. aeruginosa*, an aminoglycoside should be added to their regimen. Secondary development of resistance among the gram-positive organisms is somewhat rarer, although it has been described. Most notable is the increasing incidence of highly resistant enterococci in patients receiving vancomycin. [356] [357] [367]

Modifications Based on Changing Clinical Parameters.

For neutropenic patients receiving antibiotics, the appearance of a new clinically documented infection (e.g., cellulitis, pneumonia) or the progression of a previously documented infection should raise suspicion of the presence of resistant organisms. Attempts should be made to obtain microbiologic confirmation with appropriate tissue samples. In addition, the empirical regimen should be changed to cover the potential for resistant bacteria. The site of infection can be helpful in guiding certain antibiotic choices (Table 79-10).

The development of marginal or necrotizing gingivitis is relatively common in patients who have received intensive cytotoxic therapy (Fig. 79-3). Anaerobic organisms contribute to this process, and an antianaerobic agent such as clindamycin or metronidazole should be added to the empirical regimen if gingivitis is diagnosed.

Figure 79-3 Marginal gingivitis. Note well-demarcated red (dark) border on gingiva.

The most common pathogens contributing to perianal cellulitis (which may manifest only with localized pain) are the aerobic gram-negative bacilli, enterococci, and bowel anaerobes.[129] Therefore, the appearance of this infection in a patient already receiving broad-spectrum antibiotics mandates the addition of antianaerobic agents as well as a change in broad-spectrum coverage. Similarly, any suspected intra-abdominal site of infection should prompt the addition or inclusion of antibiotics active against aerobic gram-negative bacilli, enterococci, and bowel anaerobes.

In febrile and neutropenic patients with abdominal pain, the possible development of typhlitis or pseudomembranous colitis should be considered. Typhlitis, a necrotizing inflammation of the cecum, presents as right lower quadrant pain and fullness but may progress to an acute abdomen with decreased bowel sounds, abdominal distention, and rebound.[369] Mortality is high, and the optimal therapy is controversial, but all patients should receive broad-spectrum antibiotics, specifically with coverage of *Enterococcus*, anaerobes, and gram-negative bacteria. Antibiotic-associated colitis has long been associated with the administration of a variety of antibiotics; the risk of its development may also be increased by antineoplastic agents. Consequently, neutropenic patients receiving broad-spectrum antibiotics are at high risk. Most cases are thought to be caused by a toxin produced by *C. difficile*.[370] The spectrum of disease caused by *C. difficile* may range from asymptomatic colonization or mild diarrhea to overt pseudomembranous colitis with peritoneal signs and extensive mucosal erosion. [371] The diagnosis should be made by

documenting toxin production, and therapy should be instituted with either oral vancomycin (125 mg q.i.d.) or oral metronidazole (250 mg q.i.d.). The treatment of *C. difficile*-associated disease in patients who cannot take oral medications is problematic. Intravenous vancomycin does not achieve sufficient levels within the colon to be effective, but intravenous metronidazole does achieve such concentrations, and successes with the latter approach have been reported. [372]

The development of an exit-site infection or cellulitis around an indwelling intravenous catheter is often caused by a gram-positive organism; if such an infection is noted, vancomycin should be added to the regimen, if not already included. However, one must remember that neutropenic patients may be infected at any site by atypical organisms. Accordingly, for most new or progressive clinically documented infections, empirical changes should usually include modification of the components directed at the gram-positive as well as those directed at the gram-negative bacteria.

The development of a new site of infection may also warrant the addition of other antibiotics directed at fungi, viruses, or parasites. Burning retrosternal pain is frequently an indication of esophagitis, most often caused by cytotoxic therapy, *Candida* infection, or HSV infection. Accordingly, empirical therapy with amphotericin B or acyclovir, or both, may be indicated (see the sections on herpes simplex and *Candida* infections). The development of pulmonary infiltrates might raise suspicion not only of resistant bacteria but also of *P. carinii*, fungi, or a viral pneumonia. In a neutropenic patient receiving broad-spectrum antibiotics who has a new localized infiltrate, perhaps the first question to ask is whether the granulocyte count is rising. If so, the appearance of the new infiltrate may simply represent an inflammatory reaction at a previously unrecognized site of infection, and close observation without any modification may be appropriate. [373] If the granulocyte count is not rising and the patient has been neutropenic for only a short time (i.e., <1 week), a bacterial process is most likely, and changes in the antibacterial coverage should be considered. If the patient has been persistently neutropenic for a more prolonged period, a fungal pneumonia should also be strongly considered and amphotericin B added while a diagnostic workup is initiated. The development of an interstitial infiltrate in a neutropenic patient should raise suspicion of a variety of infectious and noninfectious diagnoses. The most likely infectious causes of interstitial infiltrates are *P. carinii* and CMV, although a variety of other organisms, including bacteria (e.g., *Legionella*), other viruses, and fungi, should be considered. Appropriate modifications of therapy will depend on the presence or absence of other risk factors (e.g., bone marrow transplantation or administration of corticosteroids) and on the availability and feasibility of diagnostic tests such as sputum induction, bronchoalveolar lavage, or open lung biopsy (see Fig. 79-4 for approach to the cancer patient with pulmonary infiltrates).

Patients in whom hypotension develops while they are receiving broad-spectrum antibiotics should be presumed septic

Figure 79-4 Algorithm for the management of the cancer patient with a pulmonary infiltrate.

with a resistant organism or breakthrough infection. In these cases, changes in the empirical regimen should be made expeditiously and continued for the duration of treatment if an organism is not recovered. So-called culture-negative sepsis may occur when the growth of resistant organisms is suppressed by marginally effective antibiotics or when the specimens for culture are not drawn during the bacteremic episode. In addition, it is likely that a variety of endogenous mediators (e.g., tumor necrosis factor) play a role in the associated cardiovascular changes, which often persist in the absence of identifiable bacteremia.

Empirical Antifungal Therapy

The rationale for empirical utilization of antifungal compounds is based on several observations. First, the diagnosis of even a disseminated fungal infection is difficult in an immunocompromised patient. Withholding antifungal therapy until a definitive clinical or microbiologic diagnosis is established frequently permits widespread dissemination. Second, it is now possible to identify subgroups of immunosuppressed patients who are at greatest risk of mycotic disease. Thus, neutropenic patients who remain persistently febrile despite a 5- to 7-day trial of broad-spectrum antibacterial therapy are particularly susceptible to fungal infection. [374] [375] [376] Finally, an accumulating body of data suggests that the early institution of effective antifungal agents will improve the therapeutic outcome in immunocompromised patients with established mycoses.[377] Thus, use of an empirically based antifungal regimen might be expected to have a dual effect: suppression of the fungal overgrowth that inevitably accompanies the administration of broad-spectrum antibacterial agents, and early treatment of subclinical localized mycotic disease before locally invasive infection or dissemination develops.

Two prospective randomized studies were the first to demonstrate the efficacy of empirical amphotericin B in persistently febrile neutropenic patients. [374] [378] In the NCI study,[374] patients who were granulocytopenic and persistently febrile without an identifiable infectious source were randomly assigned after 7 days of broad-spectrum antibacterial therapy to three study arms. In group I, all antibiotic therapy was discontinued; in group II, the broad-spectrum antibacterial agents were continued; and in group III, the antibacterial agents were continued and amphotericin B was added (at a dosage of 0.5 mg/kg/day). Clinically or microbiologically proved infections (bacterial and fungal) occurred in six of the 16 patients in group I, in six of the 16 patients in group II, but in only two of the 18 patients in group III. Documented fungal infections occurred in one patient in group I (esophageal candidiasis), in five patients in group II (invasive candidiasis in three, disseminated aspergillosis in one, and a disseminated mixed *Candida* and *Aspergillus* infection in one), and in one patient in group III (a fatal pulmonary infection with *Petriellidium boydii*, an amphotericin B-resistant organism). Further confirmation of the beneficial role of empirical antifungal therapy was provided by consideration of the mean time to defervescence after day 7 randomization. Patients receiving empirical amphotericin B (group III) defervesced within 35 days. Patients remaining on antibiotics alone (group II) required 78 days to defervescence, while those randomized to discontinue all therapy (group I) required 1112 days. Thus, this trial strongly suggested a beneficial role for empirical amphotericin B among high-risk patients.

The other randomized trial of empirical amphotericin B was performed by the European International Antimicrobial Therapy Cooperative Group. [378] This randomized multicenter trial examined the use of empirically administered amphotericin B in granulocytopenic patients who remained persistently febrile for 4 days despite administration of antibacterial antibiotics. Patients were divided into two groups: 68 were randomly assigned to receive empirical amphotericin B and 64 were assigned to continue the protocol antibiotics. Amphotericin B was administered at 0.6 mg/kg/day or 1.2 mg/kg every other day. Abatement of fever occurred in 69% of the amphotericin B-treated group and in 53% of the other group ($P = 0.09$). Six documented fungal infections, four of which were deemed severe, occurred in the 64 patients randomized not to receive empirical amphotericin B, in comparison with only 1 patient with fungemia among the 68 treated empirically with amphotericin B ($P = 0.01$). No deaths occurred as a result of fungal infections in those receiving empirical amphotericin B, whereas four deaths due to fungal infections occurred in those not receiving it ($P = 0.05$). Although the mortality from fungal infections was reduced, no difference in overall survival between the two groups was observed. These studies are consistent with retrospective evaluations, which have also reported a benefit for amphotericin B. [375] [379]

Other strategies for empirical antifungal therapy using antifungal azoles have been studied. A study at the NCI attempted to avoid the toxicity of amphotericin B yet provide empirical antifungal therapy with ketoconazole. [380] Seventy-two patients who remained neutropenic and febrile after 7 days of antimicrobial therapy were randomized to receive amphotericin B (0.5 mg/kg/day) or ketoconazole (800 mg/kg/day). The results were similar in terms of duration of fever after antifungal randomization, number of documented fungal infections, number of patients requiring crossover to the alternate regimen due to intolerance, and overall outcome scored as success (i.e., survival) or failure (i.e., death due to fungal disease). However, almost one-third of patients eligible for this study could not be enrolled because of their inability to tolerate oral medications, including ketoconazole. The use of ketoconazole was also complicated by its erratic absorption; only eight of 12 patients with ketoconazole peaks measured had adequate levels. Once the diagnosis of an invasive fungal infection was made, however, disease progression was likely unless the patient received amphotericin B. In a similar randomized prospective study from the M.D. Anderson Hospital, 172 neutropenic patients received either amphotericin B (at a dose of 0.61.0 mg/kg/day) or ketoconazole (200 mg PO q.i.d.); these patients had remained febrile for 7296 hours after institution of empirical antibiotic therapy.[381] Overall, these investigators also reported equivalent efficacy for both regimens. However, the frequency of aspergillosis was too low in this study to permit evaluation of the impact on this infection. Thus, conventional amphotericin B desoxycholate has remained the first-line agent of choice for empirical antifungal therapy.

Unfortunately, the current use of conventional amphotericin B is complicated by nephrotoxicity and infusion-related toxicity. Moreover, breakthrough fungal infections continue to occur despite the use of conventional amphotericin B. Despite further support and clinical evidence for the efficacy of empirical therapy with amphotericin B,[376] [380] [382] some clinicians remain reluctant to administer a potentially toxic agent to patients in whom a definitive diagnosis of a fungal infection has not been made. Fever, nephrotoxic effects, chilling reactions, and electrolyte imbalances (especially hypokalemia) are frequently associated with amphotericin B; anemia,

thrombocytopenia, and anaphylaxis have been noted less frequently.

Lipid formulations of amphotericin B may provide an effective avenue by which to reduce the nephrotoxicity and infusion-related toxicity of the parent compound. In order to test this hypothesis, liposomal amphotericin B was compared to conventional amphotericin B for empirical antifungal therapy in a randomized, double-blind, multicenter trial. This study, which enrolled 687 patients and was powered to a delta of 10%, found that liposomal amphotericin B was equivalent to conventional amphotericin B in overall therapeutic success and was superior in reducing proven breakthrough fungal infections, infusion-related

toxicity, and nephrotoxicity.[383] We therefore now recommend liposomal amphotericin B for empirical antifungal therapy in persistently febrile neutropenic patients, particulalrly bone marrow transplant recipients and patients with acute leukemia.

Management of Indwelling Intravenous Catheters

While infections due to gram-positive bacterial infections (especially staphylococci) are the most frequent in patients with indwelling catheters, other bacterial and nonbacterial species are also encountered, particularly in the neutropenic patient. These include resistant *Corynebacterium* spp., *Bacillus* spp., gram-negative bacteria, mycobacteria, and fungi. In approaching management issues for the patient with a catheter-related infection, it is important to process several key pieces of information: (1) the specific type of infection, (2) the location (i.e., bacteremia versus exit site versus tunnel), (3) the duration of infection, (4) any history of recurrent or previous infections and responses to antibiotics, and (5) overall clinical status.

The vast majority of simple catheter-related bacteremias and exit-site infections can be cleared by using appropriate antibiotics and do not necessitate catheter removal (Table 79-12). This applies to both neutropenic and non-neutropenic patients. If multilumen devices are used, the antibiotics must be rotated among the ports when infusing the antibiotics, since infection may be limited to one lumen (failure to follow this procedure can cause persistent infection despite antibiotics). Specimens for diagnostic culture should also be drawn through all ports of any multilumen device. Persistent bacteremia after 48 hours of appropriate therapy warrants removal of the catheter. Failures of therapy are more common when the infections are due to such organisms as *Bacillus* spp., *C. albicans*, *Mycobacterium chelonei*, or *Mycobacterium fortuitum*; in these cases, we recommend prompt catheter removal as well as antibiotic therapy.

In addition, specific clinical parameters may dictate the approach to therapy. For example, infections that extend to involvement of the tunnel of a catheter usually mandate prompt removal of the device, as antibiotics alone rarely cure this closed-space infection, particularly in the granulocytopenic host. Likewise, infections around the reservoir of an implantable subcutaneous device can be difficult to eradicate if the catheter is still in place. Patients with recurrent catheter infections

TABLE 79-12 -- Recommended Approaches to Central Intravenous Catheter-Related Infections

Type of Infection	Management
Most bacteremias	Appropriate antibiotic(s), depending on cultures and granulocyte status Rotate antibiotics through all lumina of the catheter if multilumen device used Removal of catheter if persistent cultures after 48 hr of therapy, or sooner if clinical deterioration
Exit-site infections	Appropriate antibiotics; obtain cultures (remembering that not all exit-site infections are gram-positive) Catheter removal if no response or progression after 48 hr
Tunnel infections	Immediate removal of catheter and appropriate antibiotics
Bacillius spp. or *Corynebacterium jeikeium* infections	Removal of catheter and appropriate antibiotics
Fungemia	Removal of catheter and appropriate antibiotics

(despite a history of appropriate therapy) are also candidates for prompt catheter removal.

Another question of practical importance is how to approach a febrile non-neutropenic patient with a central intravenous catheter who has no identifiable source of infection on examination. Should empirical antibiotics be started? Our policy in this setting is to obtain material for culture and start appropriate antibiotics; stable patients are treated with intravenous ceftriaxone and followed daily as outpatients pending culture results. This approach protects against the progression of undetected yet virulent infections (e.g., *S. aureus*), can minimize the need for ultimate catheter removal, and decreases hospital costs while maximizing outpatient time. If by 72 hours the cultures are negative and the patient is stable, we discontinue antibiotics.

Granulocyte Transfusions

Since granulocytopenia is the predominant factor predisposing to infection in cancer patients, many investigators have hypothesized a therapeutic role for granulocyte transfusion in infected patients. Early data from small trials were encouraging, but they have not been corroborated in other studies, including prospective controlled trials.[384] [385] [386] [387] The efficacy of granulocyte transfusions has been hindered by the low yield of granulocytes obtained from donors and the resultant disappointing increase of white cells in the recipient. The result has been the need for multiple donors, increasing the risk of alloimmunization [388] and of transmission of infections (e.g., CMV,[389] [390] toxoplasmosis).[391] In a recent study by Bensinger et al.,[392] eight WBC donors were given subcutaneous G-CSF daily, and granulocytes were harvested on a mean of 7.6 occasions. Not only was the mean number of granulocytes collected (41.6×10^9) sixfold higher than in historical controls, but mean granulocyte levels in the bone marrow recipients receiving these cells were significantly higher than those seen in historical controls (0.95×10^9 at 24 hours post transfusion).[392] Although these data are preliminary, this method of collection could improve the efficacy of granulocyte transfusion and might permit more reliable and successful administration of cell component therapy to profoundly granulocytopenic patients. [393] [394]

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CLINICAL APPROACH TO FUNGAL INFECTIONS IN IMMUNOCOMPROMISED PATIENTS

The most common fungal infections encountered in immunocompromised patients are those due to *Candida* spp., *Aspergillus* spp., and *C. neoformans*. Serious invasive *Candida* and *Aspergillus* infections are seen most often in granulocytopenic patients, although other populations may also be at risk. Infections due to *C. neoformans* are more common in patients with altered cellular immunity, and HIV-infected patients now constitute the largest group at significant risk for this infection.

Infections Due to *Candida* Species

Oropharyngeal Candidiasis

Of all *Candida* infections seen in the immunosuppressed population, oral candidiasis, or thrush, is most often encountered. Patients with cell-mediated immune deficits are particularly susceptible to the development of oropharyngeal and esophageal candidiasis. The clinical importance of oropharyngeal *Candida* infections in immunocompromised patients is threefold. First, locally invasive disease may be painful, especially when it occurs in association with mucositis or oral HSV infection. Second, oropharyngeal infection may be a harbinger of more serious mucosal disease affecting other sites, including the epiglottis, esophagus, or lower GI tract. Finally, it may serve as a nidus for the development of fungemia and disseminated

Box 79-2. APPROACH TO FUNGAL INFECTIONS

***CANDIDA* INFECTIONS**

In neutropenic patients we first treat symptomatic, proven oropharyngeal candidiasis with clotrimazole (usually clotrimazole troches, administered five times per day). If there is no improvement, we discontinue clotrimazole and begin fluconazole, 100200 mg/day. If there is still no improvement, we discontinue fluconazole and begin amphotericin B, 0.5 mg/kg/day. For suspected or diagnosed *Candida* esophagitis, we begin fluconazole. For patients with persistent fever and neutropenia with or without esophagitis, we begin liposomal amphotericin B, 3.0 mg/kg/day. Because of the risks associated with esophagoscopy and biopsy in the neutropenic population, we usually elect to treat empirically and not to perform this procedure.

Isolation of *Candida* from blood cultures has been increasingly achieved owing to changing characteristics of the neutropenic population and improved microbiologic techniques. In a neutropenic patient even a single colony of *Candida* isolated from the blood should be viewed as significant and not discarded as a contaminant. When candidemia occurs, we take the following approach: (1) begin amphotericin B; (2) remove and/or change all intravenous lines; and (3) begin a diagnostic workup to look for disseminated disease, which at a minimum should include follow-up evaluation of blood cultures, measurement of alkaline phosphatase levels, an ophthalmologic examination, and CT of the liver and spleen. Abnormalities on CT may not become apparent until the granulocytopenia resolves, and imaging studies should be repeated at this time if disseminated disease is suspected. Should the organism be germ-tube positive (*C. albicans*), we use fluconazole, 800 mg/day.

Hepatosplenic candidiasis is the most common manifestation of disseminated fungal disease in the neutropenic population. Patients with neutropenia of prolonged duration who are receiving broad-spectrum antibacterial agents are at highest risk. Clues to the diagnosis include abdominal pain, rebound leukocytosis and persistent fever following recovery from granulocytopenia, and elevated alkaline phosphatase levels. US, CT, and MRI may all be useful in making a presumptive diagnosis. The definitive diagnosis requires histologic and/or microbiologic demonstration of *Candida* in hepatic tissue. However, even when organisms are demonstrated histologically, they rarely grow in culture. We initiate therapy with a combination of amphotericin B (0.5 mg/kg/day) and 5-flucytosine (initially 100 mg/kg/day in four divided doses, assuming normal renal function), carefully monitoring 5-FC levels. Alternatively, patients can be successfully treated with liposomal amphotericin formulations, especially when there is evidence of impaired renal function. When the patient is stable, we change therapy from amphotericin B to fluconazole. Therapy should be continued until radiographic abnormalities resolve.

***ASPERGILLUS* INFECTIONS**

In most non-neutropenic patients with aspergillomas, we favor a conservative approach, reserving surgical resection for those with recurrent or significant hemoptysis. Surgery should also be considered for patients with isolated aspergillomas who are about to receive cytotoxic therapy that will place them at increased risk of bleeding.

Invasive aspergillosis occurs most commonly in patients with prolonged periods of granulocytopenia (average time of onset is 3 weeks in most studies). Fever, cough, and pleuritic chest pain are clues to the diagnosis and may have their onset before the appearance of pulmonary infiltrates. The lungs and sinuses are the sites most commonly affected, and lesions are often multifocal. When invasive aspergillosis is suspected, CT of the lungs and sinuses should be performed and may reveal widespread disease in the face of minimal findings on plain radiographs. In the appropriate clinical setting, positive sputum or nasal cultures are highly suggestive of the diagnosis. Surgery has little role in therapy because the disease is usually multifocal and because many patients have concomitant thrombocytopenia. In the absence of granulocyte recovery, invasive aspergillosis is nearly always fatal. The goal of therapy is to stabilize the disease and buy time until the granulocytopenia resolves. We treat patients with high-dose amphotericin B (1.01.5 mg/kg/day). Itraconazole, 600800 mg/day, with careful monitoring of levels to ensure bioavailability, may be used if patients have recovered from neutropenia and are afebrile, and lesions are resolving. Therapy should be continued until the radiographic abnormalities resolve.

disease, particularly in granulocytopenic patients with concurrent mucosal disruption.

The presumptive diagnosis of oropharyngeal candidiasis is often relatively easy to make by visual examination. In most cases, characteristic creamy white patches appear on the mucosal surfaces, which can be friable and which bleed easily when scraped. Wet-mount examination of scrapings will show typical yeast forms. However, the definitive diagnosis is often elusive because other local processes such as HSV or noninfectious mucositis may be clinically and visually indistinguishable. Oral swabs for microscopic examination or fungal culture should be performed to confirm mucosal candidiasis.

Therapeutic approaches include a number of options. First, for asymptomatic patients at relatively low risk for disseminated *Candida* (e.g., those who are nongranulocytopenic or who have a short expected duration of granulocytopenia), specific therapy may not be warranted. Many of these infections will clear spontaneously after the return of the granulocytes or discontinuation of antibiotic or immunosuppressive therapy, or both. Therapy should be considered for patients who are chronically immunosuppressed (e.g., AIDS patients), who have significant symptoms (usually pain), or who are at relatively high risk of dissemination (e.g., granulocytopenic). Potential therapeutic drugs include oral nonabsorbable antifungal agents (e.g., nystatin or clotrimazole), oral absorbable antifungal agents (e.g., fluconazole, ketoconazole), and amphotericin B. Both nystatin and clotrimazole can be effective in the treatment of thrush, although clotrimazole appears to be better tolerated, and prophylactic studies suggest its superior efficacy. [395] [396] [397] [398] Nystatin is usually administered in doses of 100,000 units three to six times per day, and clotrimazole troches are administered five times per day.

If no symptomatic response occurs after a trial of nonabsorbable agents, or if the condition of the patient precludes administration of oral nonabsorbable agents, treatment with systemic antifungal agents is warranted, depending on the severity of the symptoms and the degree of immunosuppression. In addition, failure to respond to nonabsorbable agents with symptomatic improvement should alert the clinician to the potential existence of more serious or more extensive *Candida* infection, as well as

to the possibility of nonfungal processes, such as HSV infection or mucositis induced by cytotoxic therapy. Accordingly, appropriate diagnostic studies, including culture of oral lesions for HSV, should be initiated (see the discussion under Clinical Approach to Viral Infections in Immunocompromised Patients). Certain cases may warrant concomitant empirical antiviral therapy with acyclovir.

Oropharyngeal candidiasis refractory to nonabsorbable agents may be treated with fluconazole or amphotericin B. Fluconazole at a dose of 100200 mg q.d. has been shown to be as effective as ketoconazole but has the advantages of absorption independent of gastric pH and of availability in oral and intravenous formulations. [399] For oropharyngeal candidiasis in granulocytopenic patients that is either severe or unresponsive to other agents, amphotericin B in relatively low doses (e.g., 0.3 mg/kg/day) may be effective. We prefer to administer 0.6 mg/kg/day in neutropenic patients and to continue therapy until the granulocyte count has resolved.

Esophageal Candidiasis

Esophageal candidiasis is a locally invasive infection of the esophagus caused by *Candida* spp. The infection usually remains localized in nongranulocytopenic patients.[400] Significant granulocytopenia may predispose patients with esophageal candidiasis to submucosal vascular invasion as disseminated disease. The most common site of infection is the distal third of the esophagus, perhaps because *Candida* grows especially well in acidic environments. Many of the principles of diagnosis and management of thrush apply to esophageal candidiasis.

The diagnosis of esophageal candidiasis requires a high index of suspicion in susceptible hosts. Burning pain localized to the retrosternal or epigastric areas is the most commonly reported symptom. Its presence in the appropriate host should alert the clinician to the possibility of this diagnosis. The pain is often aggravated by swallowing and may also be associated with dysphagia localized to the site of maximal involvement. However, even significant esophageal candidiasis may be asymptomatic.[401] [402] [403]

The physical examination is usually not helpful in establishing the diagnosis of esophageal candidiasis. Although patients often have either visibly apparent or symptomatic oropharyngeal involvement, or both, the absence of thrush does not rule out the diagnosis, since the distal esophagus may be the only focus of disease. Conversely, the presence of even severe thrush in a patient with burning retrosternal pain does not establish the diagnosis of esophageal candidiasis, since other processes could be operative. Signs suggestive of oral HSV infection or of chemotherapy-related mucositis may suggest other causes but are not diagnostic and do not rule out the presence of a mixed process. For example, patients may present with severe oral HSV infection and concomitant esophageal candidiasis, or vice versa.

In terms of diagnostic tests, oral smears or cultures for fungus or HSV may be suggestive but are not diagnostic. Mouth cultures for *Candida* are virtually never helpful because of the high rate of colonization even in the absence of infection. If oral lesions suggestive of HSV infection are apparent, cultures should be done. Importantly, oral cultures positive for HSV do not establish the diagnosis of HSV esophagitis, nor do negative cultures rule it out. A barium swallow examination or upper GI tract series may be helpful in establishing or ruling out certain noninfectious processes, such as gastroesophageal reflux or peptic ulcer disease. They may also reveal mucosal irregularities suggestive of *Candida* esophagitis (e.g., cobblestoning) but are not helpful in establishing the definitive diagnosis. [404]

Only endoscopic biopsy with culture of the specimen and histologic tissue examination can establish the diagnosis of esophageal candidiasis with certainty and at the same time rule in or out other processes. Endoscopic examination without biopsy may suggest a diagnosis on the basis of visual appearance and culture results. However, similarities in the visual appearance of different lesions, coupled with the nonspecificity of culture results from specimens obtained noninvasively, detract from the clinical value of this procedure. Unfortunately, not all patients are able to tolerate an endoscopic biopsy. The most clinically relevant complications of esophagoscopy with biopsy are bacteremia and bleeding. Both complications are more common in patients who have received cytotoxic therapy and who are granulocytopenic or thrombocytopenic, or both.

Accordingly, in granulocytopenic patients showing symptoms and signs consistent with esophageal candidiasis, we initiate empirical therapy directed at *Candida* or HSV (or both) and reserve endoscopic biopsy for patients whose disease is refractory to therapy or in whom empirical therapy with amphotericin B is relatively contraindicated (e.g., patients with renal insufficiency or amphotericin B intolerance). For noncytopenic patients, the decision to perform endoscopic esophageal biopsy should be predicated on the relative risks and benefits of the procedure within the context of the degree of immunosuppression, ease of empirical therapy, and response or lack of response to selected agents. Patients who are granulocytopenic, who are receiving other aggressive immunosuppressive regimens (e.g., corticosteroids, total body irradiation, antithymocyte globulin), or who otherwise have a high propensity for dissemination of *Candida* and who have documented esophageal candidiasis or have failed an initial empirical trial of oral nonabsorbable agents are candidates for therapy with intravenous amphotericin B. Amphotericin B should be administered intravenously at a dosage of 0.6 mg/kg/day. For neutropenic patients, it should be continued at least until the neutropenia resolves. In patients in whom the granulocytopenia is unlikely to resolve (e.g., patients with aplastic anemia who have not responded to therapy), we administer amphotericin B for 2 weeks (approximately 500 mg total dose for most adults or 10 mg/kg for children). If symptoms have resolved and the patient is afebrile and clinically stable, we discontinue amphotericin B and watch closely for the return of symptoms. In high-risk patients, particularly those with fever and granulocytopenia, the clinician should also consider the potential presence of clinically occult diffuse GI or disseminated candidiasis.

Granulocytopenic patients with clinical symptoms of infective esophagitis who are not candidates for endoscopy are often candidates for presumptive or empirical therapy. At the NCI, if such patients have only mild to moderate symptoms and are clinically stable, a 4-day trial of fluconazole, 400 mg/day, is initiated. In patients with more severe symptoms or symptoms that fail to respond to, or worsen with, clotrimazole, amphotericin B, 0.6 mg/kg/day, is initiated. In addition, in patients in whom definitive diagnostic procedures are not feasible, treatment with acyclovir is often initiated as a logical extension of empirical amphotericin B therapy. In patients with active herpetic stomatitis, we usually initiate acyclovir (250 mg/m ^{2} q.8h., intravenously) simultaneously with empirical antifungal therapy. For patients without clinical evidence of HSV infection, we begin acyclovir if there is no response to initial antifungal agents.

Nongranulocytopenic symptomatic patients who are clinically stable and who have no apparent foci of *Candida* beyond the esophageal mucosa may benefit from fluconazole, 200400 mg/day given intravenously. [405] For nongranulocytopenic symptomatic patients with organisms resistant to fluconazole, amphotericin B (0.6 mg/kg/day) may be used. Most patients can be treated successfully with 12 weeks of therapy.

Most ambulatory patients with AIDS, solid tumors, or other non-neoplastic diseases who have esophageal candidiasis respond favorably to fluconazole in oral dosages of 100200 mg once daily. [406] Nonabsorbable agents such as nystatin and clotrimazole

also may be effective for this indication, but they are not as effective and are best utilized for prevention of recurrent esophageal candidiasis. In AIDS patients,

esophageal candidiasis is often a chronically recurrent process, as are many of the fungal and protozoal infections. Accordingly, some form of chronic suppressive antifungal therapy is often desirable in this population. Suppressive regimens that can be effective in selected patients include oral fluconazole (100200 mg/day), nystatin suspension (500,0001.5 million units, 515 ml, orally q.i.d.), or clotrimazole troches (10 mg five times a day, dissolved slowly in the mouth). Relapse of esophageal candidiasis during maintenance oral therapy may require institution of intravenous amphotericin B and should prompt the physician to consider other causes of infective esophagitis. As the emergence of resistance to fluconazole may complicate such a chronic suppressive strategy, [407] we employ long-term fluconazole suppression only in those patients in whom recurrences are no longer tolerated or successfully treatable.

Approach to the Patient with Candidemia

Candidemia is associated with a high morbidity and mortality and may be a harbinger of disseminated *Candida* infection. Candidemia is seen almost exclusively in patients with significant impairment in host defenses. Those at highest risk are neutropenic patients receiving broad-spectrum antibacterial agents. Other populations in whom candidemia and disseminated candidiasis occur include transplant recipients, postsurgical patients, burn victims, low-birth-weight neonates, patients with indwelling intravenous catheters, and those receiving hyperalimentation. [408]

During the past two decades, there has been a progressive increase in the frequency of candidemia in most medical centers. [409] [410] [411] [412] For example, Beck-Sague and colleagues [410] analyzed secular trends in the incidence of nosocomial fungal infections in U.S. hospitals between 1980 and the end of 1990. The rate rose from 2.0 to 3.8 infections per 1,000 discharges. *C. albicans* was the most frequently isolated fungal pathogen (59.7%), followed by other *Candida* spp. (18.6%). Bloodstream infections due to *Candida* spp. increased nearly tenfold. Patients with bloodstream infections who had a central intravascular catheter were more likely to have a fungal pathogen isolated than were other patients with bloodstream infection (relative risk = 3.2; $P < .001$): 29% of fungemia patients and 17% of patients with bloodstream infection due to other pathogens died during hospitalization ($P < .001$). Fungi are emerging as important nosocomial pathogens, and control efforts should target fungal infections, especially fungemia. Additional studies found that *Candida* spp. are the third to fourth most common bloodstream isolates in hospitalized patients. Estimates from large teaching hospitals have been as high as 8.5 per 10,000 admissions, with candidemia accounting for up to 10% of nosocomial bloodstream infections.[411] The mortality attributable to candidemia has been 2152%. [411] [413] [414] Candidemia as an independent event directly increases the average length of hospital stay by 30 days. [411]

Candidemia usually occurs in patients who already have extended hospitalizations. In the study by Horn et al., [412] the average period of hospitalization before the onset of candidemia was 20 days. In addition, the first positive blood cultures were obtained after a mean of 11.7 days of neutropenia and a mean of 11.8 days of antibiotics. Fever is often the only sign; its presence in a susceptible host, in the absence of other causes, should always raise the possibility of fungal infection. Other signs that can occur with early candidemia include embolic skin lesions, which in some series have occurred more commonly with *C. tropicalis*, and diffuse myalgias, which may be indicative of *Candida* myositis.[412] [415] A large percentage of immunocompromised patients with candidemia will have disseminated candidiasis; signs referable to involvement of specific organ systems (e.g., eye, CNS, heart valves, liver, and spleen) should also be sought.

In an immunosuppressed patient in whom a blood culture is positive for *Candida* spp., the following clinical approach seems prudent on the basis of current knowledge. A positive blood culture should rarely, if ever, be considered due to a contaminant. Culture contamination probably occurs far less frequently than does true candidemia in a susceptible host. In addition, even viscerally disseminated candidiasis is associated with a low incidence of positive blood cultures. Accordingly, there is danger in waiting for a second confirmatory culture before taking action, as it can yield negative results in the face of significant disease.

Central venous catheters, arterial catheters for monitoring blood pressure, and hyperalimentation lines have all been associated with an increased risk of candidemia and may be considered independent risk factors for disseminated infection. [416] [417] [418] If the patient does not have a catheter in place, an endogenous source should be assumed, and the likelihood of disseminated disease can be even higher. These patients should receive antifungal therapy as outlined below. If the patient does have a catheter, it is difficult to determine whether a positive blood culture represents an infection limited to the lumen or to the surface of the catheter itself, or whether the organisms were drawn from the circulation. If quantitative culture techniques are available (e.g., the lysis-centrifugation method) and simultaneous specimens have been obtained peripherally and through the catheter, a higher number of colonies in blood obtained through the catheter (or a positive culture of blood drawn exclusively through the catheter) may indicate an infection localized to the catheter. However, because *Candida* adheres avidly to plastic surfaces, an infection that appears to be originating from the catheter could simply represent locally proliferating organisms that adhered to the catheter during a period of candidemia.

The decision as to whether to remove intravascular catheters from patients with candidemia is both difficult and controversial. Although some recommend catheter removal only in cases of thrombophlebitis or disseminated infection, the catheter may represent a nidus for infection and a source for dissemination. In one study, only 11 patients of 155 with fungemia and central intravascular lines were treated without removal of the line; of these, nine were treatment failures despite receiving >500 mg of amphotericin B.[414] In addition, it is difficult to predict which patients can be successfully treated with the catheter in place, and such treatment delays can be costly. Therefore, we favor prompt removal of all indwelling intravenous catheters in this setting. Another study found that in the subset of non-neutropenic patients with candidemia and a catheter in place at the time of their first positive blood culture, removal and replacement of all intravascular catheters without exchange over a guidewire from a preexisting line on or before the first day the study drug was administered was associated with a reduction in the subsequent mean duration (± SE) of candidemia, from 5.6 ± 0.8 days to 2.6 ± 0.5 days ($P < .001$).[416]

All patients with impaired host defenses and documented candidemia should undergo evaluation for dissemination of disease. Documentation of disseminated disease mandates aggressive and often protracted therapy. The most common sites of dissemination are the eye (endophthalmitis), skin, liver, and spleen. Evaluation for disseminated disease should include careful ophthalmologic examination, examination of the skin, and imaging studies of the liver and spleen. It is important to realize that in the neutropenic host, assessment of the eyes, liver, and spleen can show negative results, even in the face of widespread dissemination. [419] [420] This presumably reflects that the lesions visualized on direct examination or imaging studies largely represent an inflammatory reaction. Accordingly, meaningful assessment of disseminated disease in neutropenic patients

with candidemia is probably best accomplished after recovery of the granulocytes. There is a direct relationship between duration of persistent fungemia and the risk of disseminated candidiasis.

The next question to address is whether a patient with candidemia and an indwelling intravenous catheter (but without evidence of dissemination) should receive antifungal therapy after removal of the catheter or whether catheter removal alone will suffice. Most authorities recommend that all episodes of candidemia in this population be treated with a course of antifungal therapy. [421] [422] Data to support this rationale and approach have been reported in retrospective series that have documented an increased incidence of disseminated *Candida* infection at autopsy in patients with prior candidemia who were not treated with amphotericin B, as compared with those who were so treated.[414] In another study, four of 33 patients with candidemia that was believed to be catheter related and who were treated only by catheter removal (and not with amphotericin B) developed *Candida* endophthalmitis, diagnosed 866 days after the last positive blood culture.

Intravenous amphotericin B is effective in the treatment of candidemia due to most *Candida* spp. in neutropenic patients. In uncomplicated candidemia in neutropenic patients without evidence of disseminated disease, we initiate a dose of 0.6 mg/kg/day without a preceding test dose. If the organism is germ-tube positive, indicating *C. albicans*, we change to fluconazole, 800 mg/day in adults or 12 mg/kg/day in children. Uncomplicated candidemia due to *C. albicans* in non-neutropenic patients may be treated with fluconazole.[422] There are also compelling in vivo and clinical data to support the use of fluconazole for treatment of candidemia due to *C. albicans* in neutropenic hosts.[423] [424] Because *C. tropicalis* and *C. parapsilosis* may be more amphotericin B resistant than other *Candida* spp., if either is isolated we administer amphotericin B at a dose of 1 mg/kg/day and consider the addition of flucytosine. There is no evidence that a stepwise escalation of dose to the targeted daily dose decreases toxicity. Indeed, such an approach may delay the attainment of effective plasma and tissue concentrations that may otherwise be more readily achieved at full dosages. No data are available on the most appropriate duration of therapy. We currently administer a minimal course for a total of 10 mg/kg or 500 mg (2 weeks of therapy in most adults). If the patient is neutropenic at the time the isolate is obtained, we continue amphotericin B at least until recovery of the granulocyte count to >500mm^3 , even if the 500 mg total dose is achieved before that time. Neutropenic patients should also be evaluated for disseminated disease within 1 week after recovery of the granulocyte count. Persistent fever, localizing symptoms, or elevation on liver function tests (particularly of the alkaline phosphatase level) after recovery of the granulocyte count should suggest the possibility of disseminated *Candida* infection.

If candidemia continues despite amphotericin therapy, we add flucytosine (100 mg/kg/day) with careful monitoring of blood levels. [425] There is little evidence that the addition of an azole antifungal agent will be of benefit in this situation, but this may be considered after other treatment options have failed. However, fluconazole may

have a role as monotherapy in the treatment of patients with candidemia but with a low probability of disseminated *Candida* infection.

Candida Endophthalmitis

Candida endophthalmitis is a relatively common complication of candidemia. Montgomerie and Edwards[426] found that five of 25 patients receiving hyperalimentation developed ocular lesions clinically consistent with *Candida* endophthalmitis. The lesions of *Candida* endophthalmitis are white, resemble cotton balls in appearance, and involve the chorioretina, from which they rapidly extend into the vitreous. In the appropriate setting, they often indicate not only infection of the eye but also widespread dissemination to other organs, the number of ocular lesions observed funduscopically correlating with the extent of disseminated visceral infection. [427] It must be remembered that eye lesions may not be recognized in granulocytopenic patients until recovery from neutropenia. Of the various *Candida* species, *C. albicans* appears to have a predilection for eye involvement. [428] Recognition of these lesions mandates a search for other locations of disseminated disease and expeditious treatment with amphotericin B, since *Candida* endophthalmitis can cause irreversible loss of vision or complete blindness in the involved eye. In selected cases, partial vitrectomy may also be helpful both diagnostically and therapeutically. [408] Adult patients with *Candida* endophthalmitis should probably receive an amphotericin B dose of 0.751.0 mg/kg/day for 68 weeks, where possible, with continuation of therapy at least until resolution of the ophthalmologic findings. Strong consideration should be given to the addition of flucytosine, especially in cases with perimacular involvement. If coexisting disseminated disease is found at other sites, even more prolonged therapy may be required.

Hepatosplenic Candidiasis

Only since about 1980 has hepatosplenic candidiasis or chronic disseminated candidiasis been recognized as an important clinical entity in the immunosuppressed host.[429] [430] Although this disease may infect any organ site and is most appropriately designated as chronic disseminated candidiasis, the term hepatosplenic candidiasis has been used most extensively in the literature and will be used here. It is most commonly seen in patients who have had prolonged or recurrent episodes of neutropenia. Presumably it results from hematogenous dissemination, and therefore all patients with documented candidemia should be evaluated for hepatosplenic involvement. However, only a few patients with documented hepatosplenic candidiasis will have had preceding candidemia. One must be aware of the signs and symptoms suggestive of this diagnosis, even in the absence of positive fungal blood cultures.

Four findings should raise the suspicion of hepatosplenic candidiasis in patients who have, or have recently recovered from, episodes of neutropenia, whether seen alone or in concert: (1) persistent fever after recovery of the granulocyte count, which is unresponsive to standard antimicrobial therapy, (2) rebound leukocytosis, (3) elevated serum alkaline phosphatase levels, and (4) abdominal pain.

Thaler and colleagues[429] reviewed their experience with eight cases of hepatosplenic candidiasis seen at the NCI, as well as 65 other cases that were diagnosed ante mortem and reported in the literature. Of the 73 patients, 68 had been neutropenic. In 87%, the underlying diagnosis was acute leukemia, with only three patients having neutropenia due to aplastic anemia. The clinical findings and laboratory abnormalities became apparent only after recovery of the granulocytes to normal levels, and none of the patients was diagnosed while neutropenic. Fever was a presenting symptom in 85% of patients and abdominal pain in 57%. An elevated serum alkaline phosphatase concentration was the most consistent biochemical abnormality. Serum transaminase and bilirubin concentrations were usually normal. Leukocytosis was present in 31%, and 50% had received prior antifungal therapy. With regard to imaging studies, US and CT were both helpful in demonstrating the lesions, and there was a suggestion that CT might be more sensitive and US more specific (with the appearance of the characteristic bulls-eye pattern, Fig. 79-5). Magnetic resonance imaging (MRI) may prove more helpful, as even smaller lesions may be detectable.

The definitive diagnosis can be established only by histologic or microbiologic methods. Grossly, the liver and spleen are usually studded with white to yellow nodules of 12 cm. The earliest histologic appearance of the invasive *Candida* lesion is an

Figure 79-5 CT scan showing typical lesions of hepatic candidiasis.

abscess composed of a necrotic center surrounded by an intense acute inflammatory infiltrate. Because the yeast forms and pseudohyphae often are only in the center of the abscess, serial sections and special stains are frequently needed to show their presence. As the lesion ages, the polymorphonuclear inflammatory cells mix with mononuclear cells, and a peripheral fibrous proliferation begins to wall off the lesion. As healing occurs, this proliferation progresses to a well-defined granuloma with occasional giant cells. Because resolved or resolving lesions may coexist with more acute lesions, the absence of organisms on a liver biopsy section should not be considered evidence that invasive candidiasis has resolved if there is clinical evidence to the contrary. It is important to realize that the culture of the liver specimen will often be negative even if yeast and pseudohyphae are visualized. In the review by Thaler et al., [429] biopsy material was cultured in 45 patients and was negative in 26. Of the 26 patients who received antifungal therapy before the biopsy, cultures were negative in 18. Of the 18 patients who did not receive antecedent antifungal therapy, fungal cultures were negative in seven. Of the 31 *Candida* isolates in this review, 20 were *C. albicans*, six were *C. tropicalis*, one was *C. pseudotropicalis*, and one was *C. stellatoidea*, and in three patients the species of *Candida* isolate was not determined. The decision of whether to perform a liver biopsy depends on the ability of the patient to withstand the procedure safely and on the degree of clinical suspicion for the diagnosis of hepatosplenic candidiasis as opposed to other possible diagnoses. In general, we prefer to make a histologic or microbiologic diagnosis, if at all possible.

We treat all patients initially with amphotericin B with or without 5-fluorocytosine (5-FC), depending on the presence of renal lesions and severity of infection. Following a 2-week course and stabilization of the patient, we attempt to convert therapy to fluconazole. There are both experimental and clinical data to support the use of fluconazole for treatment of this infection. [422] [431] [432] Although experience is limited, fluconazole has been useful in the treatment of hepatosplenic candidiasis that was refractory to amphotericin B therapy. For example, six patients with leukemia and hepatosplenic candidiasis who had received more than 4 g of amphotericin without resolution of infection were treated with fluconazole (200400 mg/day). In all patients fever and other symptoms resolved within 28 weeks and CT showed improvement within 48 weeks. A second study of patients who either did not improve with or were intolerant of amphotericin demonstrated response to fluconazole in 14 of 16 cases. The most favorable outcome in the study of Anaissie et al. indicated that patients treated initially with amphotericin B followed by fluconazole had the best therapeutic response.[411] In the absence of clearly defined end points, we continue antifungal therapy until resolution of all radiographic evidence of disease, performing follow-up studies at monthly intervals or more frequently if clinically indicated. Fever, leukocytosis, and alkaline phosphatase levels can also be helpful in assessing response in individual cases. We routinely employ a daily dose of 0.51 mg/kg of amphotericin B, with the higher doses used for documented *C. tropicalis*, failure of response to lower doses, or cases in which amphotericin B is used as a single agent (i.e., without concomitant 5-FC). We routinely add 5-FC to the therapy for *C. tropicalis* infection at least during the first month, carefully monitoring serum levels. In patients with normal renal function, we begin with a total daily dose of 100 mg/kg and carefully monitor serum levels to avoid toxicity (aiming for a serum level in the range of 50100 g/ml). More recently, studies have suggested that the use of lipid formulations of amphotericin B may allow for delivery of larger doses with less toxicity. [433] [434] The lipid formulations of amphotericin B are indicated for patients who are intolerant of or refractory to conventional antifungal therapy. [435]

A practical question that often arises with many patients diagnosed as having hepatosplenic candidiasis is whether chemotherapy can be safely continued during the course of antifungal therapy. Recognizing the critical importance of treating the underlying neoplastic disease without delay of chemotherapy, we continue antifungal therapy and chemotherapy cycles simultaneously, watching closely for progression of fungal disease. This approach has resulted in no breakthrough fungemias or progression of hepatosplenic candidiasis during neutropenia. [436]

Candida Infections of the Urinary Tract

Candida infections of the urinary tract are classified as upper (involving the kidneys) and lower (involving the urinary bladder). This distinction has important pathophysiologic and therapeutic implications. Renal candidiasis in immunocompromised patients is usually due to hematogenous dissemination; ascending urinary candidiasis is unusual as a cause of renal candidiasis unless there are preexisting anatomic abnormalities of the collection system. The risk factors for hematogenous renal candidiasis are the same as those for candidemia and disseminated candidiasis. The kidney is the most frequently involved organ in most series of disseminated *Candida* infection,[437] and candiduria may be the harbinger of disseminated disease in neutropenic and other immunocompromised patients. Treatment consists of systemic antifungal chemotherapy directed toward deep visceral candidiasis. By comparison, the major risk factors for the development of *Candida* infection of the lower urinary tract are the presence of an indwelling urinary catheter, the administration of broad-spectrum antibiotics, and diabetes mellitus.

Successful management of candidiasis of the lower urinary tract is most readily accomplished by simple removal of the indwelling urethral catheter.

A commonly encountered clinical situation is a urinalysis showing yeast or a urine culture positive for *Candida*. In immunocompetent patients without predisposing conditions, this often represents contamination from *Candida* colonizing the external urogenital tract. In addition, *Candida* spp. were isolated from the urine of 8% of normal males and 12% of normal females in one series.[437] In patients with urinary tract symptoms, an indwelling catheter, or immune impairment, the finding of candiduria may be more significant. The most difficult problem is in differentiating between bladder or catheter colonization and true infection. As a general rule, pyuria in non-neutropenic patients or symptoms of urinary tract infection in the absence of other causes should indicate the potential for true infection due to *Candida*. Quantitative colony counts are of little value in determining the significance of candiduria.[438] There are few laboratory tests that definitively distinguish between upper and lower tract infection. The presence of renal *Candida* casts in a urinalysis is highly specific for renal candidiasis; however, they

are not reliably found in neutropenic hosts.[439] Abdominal CT, MRI, or US can be helpful and should be performed in patients with candiduria and at risk for disseminated disease.

The simplest and most effective means of eradicating the *Candida* from the lower urinary tract in patients who have indwelling urethral catheters is removal of the catheter. However, certain groups of non-neutropenic patients may benefit from additional therapy aimed at local eradication, including (1) those with symptoms that fail to resolve with catheter removal or for whom catheter removal is not feasible; (2) those with upper urinary tract abnormalities (e.g., history of papillary necrosis or ongoing urinary tract obstruction), in whom there is a more significant risk of the development of ascending infection; (3) those with candiduria who will require urinary tract procedures such as transurethral resection of the prostate or chemotherapy for bladder cancer; and (4) those with persistent candiduria who are about to receive aggressive systemic cytotoxic therapy for ensuing neutropenia. For these patients with disease believed to be confined to the lower urinary tract, several strategies may be effective: fluconazole; intravenous amphotericin B; and amphotericin B bladder irrigations. For amphotericin B bladder washes, doses of 100 mg in 500 ml of D_5 W or in sterile water may be instilled and removed once or twice daily. The potential use of a single intravenous dose of amphotericin B in these settings has been suggested, but there are insufficient data to support this approach. The use of 5-FC alone is not recommended because of the potential for the rapid emergence of resistance. Approximately 70% of a fluconazole dose is excreted unchanged into the urine and can provide a less toxic alternative to amphotericin in non-neutropenic patients. In addition, the ease of administration and relative lack of toxicity make fluconazole an attractive alternative for chronic suppression of candiduria; however, physicians must be alert for the potential for *C. glabrata* and *C. krusei* superinfection because of their resistance to fluconazole.

Other groups of patients may need systemic amphotericin B. Clearly, patients (usually neutropenic) with renal involvement attributed to hematogenous spread need treatment with intravenous amphotericin B or high-dose fluconazole, depending on the *Candida* species. Refractory cases of renal candidiasis may be effectively treated with the combination of amphotericin B plus 5-FC. Neutropenic patients with persistent or symptomatic candiduria should also be considered for systemic therapy. In granulocytopenic patients, persistent candiduria may pose a risk of subsequent dissemination or, rarely, may be the first sign of established visceral involvement.

Infections Due to *Aspergillus* Species

In non-HIV-infected immunocompromised patients, *Aspergillus* spp. are the second most commonly encountered fungal pathogens. *A. fumigatus* and *A. flavus* account for the vast majority of human infections, with infections due to *A. terreus*, *A. niger*, and other species reported infrequently. Serious invasive infections due to *Aspergillus* spp. occur most frequently in patients with prolonged granulocytopenia or patients receiving high doses of corticosteroids.[47] [52] [440] [441] [442] Aspergillosis may be classified as allergic, saprophytic (as exemplified by aspergilloma), and deeply invasive infection. The latter two conditions are most relevant to the immunocompromised patient.

Aspergillomas

The term aspergilloma refers to colonization of a pulmonary cavity by *Aspergillus* spp. Aspergillomas can be either primary or secondary. Primary aspergillomas tend to follow granulocyte recovery in patients who have had an episode of invasive pulmonary aspergillosis during a period of granulocytopenia. Pathophysiologically, this results from a walling off of the infection associated with the inflammatory response generated by PMNs.

The most common symptom encountered is hemoptysis, which occurs to some degree in up to 75% of patients.[443] It can range in severity from intermittent blood-tinged sputum to massive pulmonary hemorrhage with exsanguination. In most patients who have not developed the initial infection in the setting of granulocytopenia, the lesions are usually solitary, with radiographs typically demonstrating a round or oval mass overlaid by a crescent of air (Monads sign)[444] (Fig. 79-6). Sputum cultures may be positive for *Aspergillus* in 50% of cases and may be highly suggestive of the diagnosis in the appropriate setting, but they are neither sensitive nor specific. In patients suspected of having aspergilloma, serum should be sent for determination of *Aspergillus* precipitins, which were positive in 92% of 66 patients tested in one review of ten reported series.[443] However, serologic tests for the diagnosis of *Aspergillus* remain experimental and are not commercially available.

Available therapeutic options include conservative management (with no surgery and no medication), intracavitary installation of amphotericin B, systemic administration of amphotericin B, and surgical removal of the lesion. Currently, most authorities recommend a conservative approach, when possible.[443] [445] Several arguments support conservative management. First, life-threatening bleeding is relatively rare, especially with the first episode of hemoptysis.[446] Second, many patients have

Figure 79-6 Typical radiographic appearance of aspergilloma.

underlying diseases associated with significant pulmonary compromise, making them relatively poor operative risks. Third, many patients sustain relatively long asymptomatic periods. In addition, resolutions of aspergillomas without therapy have been reported.[439] Most authorities agree that surgery is indicated for significant recurrent hemoptysis or severe hemoptysis. Clearly, each patient must be approached on an individual basis, and the decision with respect to surgical intervention should be made within the context of the severity of the symptoms, the prognosis of the underlying disease, and the relative operative risks.

Because aspergillomas primarily represent colonization within a preexisting cavity, systemic administration of anti-fungal therapy is rarely effective.[447] However, in a small percentage of patients who have localized invasive disease surrounding the aspergilloma and also have systemic symptoms (so-called chronic necrotizing pulmonary aspergillosis), intravenous amphotericin B has reportedly been effective.[439] [448] Oral administration of itraconazole may be used for long-term ambulatory management. In order to further investigate the utility of itraconazole in the management of this infection, there is currently a randomized, placebo-controlled trial of itraconazole in non-neutropenic patients with aspergilloma. Intracavitary amphotericin B has been reported to be effective in stabilizing or improving radiographic findings in small series of patients, but more data will be needed to assess its ultimate value.[443] [449]

Invasive *Aspergillus* Infections

Of the infections encountered in compromised patients, invasive *Aspergillus* infections are among the most serious because they are often multifocal, are associated with extensive tissue invasion and destruction, and, in the absence of appropriate host defenses, respond poorly to available antimicrobial therapy. Among cancer patients, those with acute leukemia who are undergoing aggressive cytotoxic therapy appear to be at highest risk.[54] However, in a study by Weinberger et al.,[450] fungal infections were responsible for 72% of deaths from infection, and more than one-half of these fungal infections were due to *Aspergillus*. Overall, prolonged neutropenia is clearly the most important risk factor predisposing to invasive *Aspergillus* infection.[451]

With rare exception, *Aspergillus* infection is acquired by inhalation of *Aspergillus* conidia. Consequently, invasive infection occurs primarily in the lung, frequently with

upper respiratory sites (usually the sinuses) affected as well. [452] The disease is often multifocal, with many patients having concomitant involvement of sinuses as well as multiple lung sites. Within the respiratory tree *Aspergillus* has a predilection for invading blood vessels as well as tissue parenchyma. Tissue infarction and subsequent necrosis often occur and may lead to rapidly progressive extensive disease (Fig. 79-7). The process often behaves in a malignant fashion, extending predominantly by local invasion, with little respect for tissue planes and anatomic boundaries. Extension of primary disease in the sinus or orbit through bone to involve the roof of the mouth or the brain is not uncommon. Disease originating in the lung can extend through the pleura and even involve the chest wall. Hematogenous dissemination to distant organ sites such as the CNS, liver, and skin is relatively rare but has been reported. [453]

Since early diagnosis and prompt institution of therapy have been associated with improved outcome, [377] knowledge of the early signs and symptoms of invasive aspergillosis is critical for the clinician caring for patients with prolonged episodes of granulocytopenia. The most common symptom is pleuritic

Figure 79-7 (A) Autopsy specimen of lung from patient with invasive *Aspergillus*. **(B)** Histologic specimen from lung showing invasion of *Aspergillus* around blood vessels.

chest pain, which occurs in 100% of patients; in 50% of patients, it develops an average of 6 days before the appearance of pulmonary infiltrates. [449] Pathophysiologically, pleuritic pain probably occurs as a result of tissue infarction, which often extends to involve the pleural surfaces. Cough occurs in 93% of patients and shortness of breath in 27%. With respect to pulmonary signs, rales are most common and are heard in 100% of patients with invasive aspergillosis.

The sinuses are also frequently involved in invasive aspergillosis; they may be the initial or sole site of disease, or sinus involvement may occur concomitantly with pulmonary infection. Signs or symptoms include sinus pain, nasal ulceration or eschar, periorbital edema, epistaxis, and discharge. In a study by Gerson et al., [451] one-third of patients with invasive pulmonary aspergillosis had microbiologic or pathologic documentation of *Aspergillus* sinus infection. Accordingly, all granulocytopenic patients with suspected or proven invasive aspergillosis should undergo clinical and radiologic evaluation of the sinuses. In addition, *Aspergillus* infection in the maxillary sinuses may invade inferiorly into the roof of the hard palate, and initial symptoms may be minimal, even in the presence of bone destruction. A small ulceration of the hard palate may be the first sign of widespread disease (Fig. 79-8A). Sinus infection may also extend directly to involve the CNS or the orbit.[452]

A high index of suspicion is critical to the early diagnosis of aspergillosis; this infection should be considered with the appearance of any new abnormality in the chest radiograph of a persistently neutropenic patient with fever. The radiographic appearance of invasive pulmonary aspergillosis can range from discrete or multifocal nodular lesions to patchy densities to diffuse consolidation or even cavitation (Fig. 79-8).[451] [454] The appearance can vary dramatically from patient to patient or can change dramatically at granulocyte recoverythe ensuing inflammatory response can even give the appearance of worsening disease when in fact the condition is improving clinically. In our experience at the NCI, the most characteristic finding on chest radiography is a localized infiltrate extending toward the pleural surface. Standard chest radiographs often do not show the full extent of pulmonary disease, especially if obtained early in the course or while the patient is neutropenic. CT scans of the chest are more accurate at defining the extent of disease and often show multifocal lesions when single or no lesions are visible on plain chest radiographs (Figs. 79-8C & D).[455] The radiographic appearance of *Aspergillus* sinusitis can range from minimal mucosal thickening to sinus obliteration and extensive bony erosion or destruction.

The only way to achieve a definitive diagnosis of invasive aspergillosis is by biopsy of the involved area, with either histologic or microbiologic confirmation, or both. Unfortunately, invasive procedures such as open lung biopsy or even transbronchial biopsy or sinus aspiration are often impractical or contraindicated in patients with suspected aspergillosis, owing to thrombocytopenia, other bleeding abnormalities, or concurrent medical problems. Even a highly directed open biopsy may fail to disclose the fungal elements and yield only necrotic or infarcted tissue. [456] Therefore, a less invasive means of establishing the diagnosis would be of great practical value. Accordingly, the clinical value of culture material obtained noninvasively (from sputum or nares or from fiberoptic bronchoscopy with brushings or lavage) has been examined in a number of studies.[53] [55] [457] [458] [459] Cumulative data in these studies would suggest that, in a high-risk patient with signs or symptoms consistent with invasive *Aspergillus*, positive cultures of material from these sites are often predictive of invasive disease. In these patients, directed therapy is often indicated, even in the absence of a definitive diagnosis. However, these data also indicate that negative cultures do not rule out the diagnosis. False-negative culture results may occur in >50% of histopathologically proven cases, even when the specimens are obtained bronchoscopically. Conversely, because *Aspergillus* may colonize the respiratory tract, positive cultures in the absence of signs or symptoms should be interpreted with caution, and directed therapy should rarely be based solely on these results.

Blood cultures are rarely, if ever, positive in invasive aspergillosis. The growth of *Aspergillus* spp. in a blood culture is much more likely to represent a contaminant than a clinically significant isolate. Immunodiagnostic assays for the detection of *Aspergillus* antigens in serum and body fluids are promising but are not widely available and require further study.

For granulocytopenic patients with a diagnosis of invasive aspergillosis, therapy with amphotericin B should be initiated promptly, and a workup to determine the extent of disease should be begun simultaneously. This should include a careful physical examination, with special attention to the lungs, hard palate, nares, sinuses, and orbits. A minimal radiographic evaluation should include chest and sinus radiographs. Any suspicious findings or documented abnormalities should be followed by either CT or MRI in order to define the extent of disease more accurately and to provide better guidelines for assessment of subsequent therapeutic response. Aspergillosis may become evident as a focal neurologic deficit as the first manifestation of disseminated infection. Unlike CNS candidiasis or cryptococcosis, CNS aspergillosis has a high propensity for angioinvasion, infarction, and focal neurologic deficits. [460]

In order to detect invasive aspergillosis while still limited to the lung, the combined strategies of bronchalveolar lavage, CT, and galactomannan antigen detection may afford earlier detection in neutropenic patients. Illustrating this concept, Caillot et al. found that early CT coupled with galactomannan antigen detection by ELISA improved the diagnosis in these patients.[461] Among 23 histologically proven and 14 highly probable cases of invasive pulmonary aspergillosis, the most frequent clinical signs associated with invasive pulmonary aspergillosis were cough (92%), chest pain (76%), and hemoptysis (54%). The bronchoalveolar lavage fluid assay was positive for *Aspergillus* spp. in 22 (69%) of 32. Galactomannan antigen was positive in 83% of cases when tested on bronchoalveolar lavage fluid. Early CT scans routinely performed in febrile neutropenic patients with pulmonary infiltrates on chest radiograph suggested CT halo signs in 92% of patients. Using this strategy, the mean time to the diagnosis of invasive pulmonary aspergillosis was reduced from 7 to 1.9 days compared to historical controls. As increasing attention has been directed to the halo sign as a radiologic diagnostic marker for invasive aspergillosis, our laboratory studies have found that this finding correlates with a zone of reversible ischemia and organism-mediated pulmonary injury.[462]

Amphotericin B should be administered at a dose range of 0.51.5 mg/kg/day. Empirical treatment with amphotericin does not preclude the development of aspergillosis; 22 of 24 patients who were receiving amphotericin empirically developed progressive pulmonary infiltrates ultimately shown to be due to *Aspergillus*. If a persistently neutropenic patient is receiving empirical amphotericin B and has either a clinical picture compatible with aspergillosis or evidence of documented infection, the dosage of amphotericin should be increased to 1.01.5 mgkg.[463] Patients should continue amphotericin B until clinical and radiographic evidence of disease resolves. Itraconazole may be administered to patients who are stable but should be accompanied by careful monitoring of plasma levels in order to ensure bioavailability. Peak plasma levels of itraconazole by bioassay of >5 g/ml or by HPLC of >3 g/ml should be sought. The response to therapy and the ultimate survival of patients with invasive aspergillosis will be critically dependent on recovery from granulocytopenia, decreased dosage of any steroids, and removal of other immunologic deficits, and may be dependent on the dosage of amphotericin B administered.

Figure 79-8 (A) Invasive aspergillosis extending from the maxillary sinuses to involve the hard palate. **(B)** Chest radiograph showing classic appearance of invasive aspergillosis, with wedge-shaped infiltrate extending to the pleura. **(C)** Chest radiograph from a granulocytopenic patient with invasive aspergillosis. Note subtle appearance of round densities in the right lung field. **(D)** CT scan of the chest from the patient whose chest radiograph is shown in B. Note expensive involvement despite minimal abnormalities on plain radiograph. **(E)** MRI showing invasive aspergillosis in the maxillary

sinus extending through bone to involve the roof of the mouth.

In the absence of bone marrow recovery, virtually all granulocytopenic patients with invasive aspergillosis will die despite receiving maximal antifungal therapy. The objectives of therapy are to delay progression and allow time for bone marrow recovery. Adjunctive therapy with granulocyte-stimulating factors such as G-CSF and GM-CSF should be considered to hasten bone marrow recovery. Many studies using standard dosages of amphotericin B (in the range of 0.5 mg/kg/day) still reported mortality rates of 75100%. Although encouraging findings indicate improved survival with higher dosages of amphotericin B (1.01.5 mg/kg/day), [463] the overall prognosis remains dismal, as reflected in the recent report by Pannuti et al., [464] in which *Aspergillus* was the cause of 36% of nosocomial pneumonias after bone marrow transplantation and resulted in the death of 19 of 20 infected patients.

Three lipid formulations of amphotericin B are indicated for immunocompromised patients with invasive aspergillosis who are refractory to or intolerant of conventional amphotericin B: ABLC (amphotericin B lipid complex, Abelcet), ABCD (amphotericin B colloidal dispersion, Amphotec), and liposomal amphotericin B (AmBisome). [465] [466] [467] While controversy exists concerning equivalent dosages, we recommend administration of 5 mg/kg/day as a starting dosage for any of the formulations used.

In general, surgical resection of lesions has little role in treating severely immunocompromised patients with invasive aspergillosis when the disease is often multifocal, and other medical conditions frequently preclude even less invasive procedures. However, resection may be considered in selected patients who are considered good surgical candidates, who are not responding to antifungal therapy, and whose noninvasive evaluation indicates disease confined to a single accessible area. Surgery may also have a role in the treatment for bone and soft tissue extension of infection that develops during antifungal therapy. [468]

A question that often arises is how to approach patients with a previous history of invasive aspergillosis who subsequently need myelosuppressive chemotherapy. That issue was addressed in a study by Karp et al. [469] conducted in 1988, in which 10 patients with AML and a prior history of invasive aspergillosis underwent 14 subsequent courses of chemotherapy. In nine of these patients, prophylaxis with amphotericin B (1 mg/kg/day) plus 5-FC was begun before institution of chemotherapy. All these patients survived the ensuing granulocytopenic episode without evidence of reactivation of invasive aspergillosis. By contrast, the one patient who did not receive prophylactic therapy suffered reactivation of aspergillosis and died. On the basis of these data, prophylaxis with amphotericin B with or without 5-FC seems reasonable in certain high-risk patients. However, more data are needed before firm recommendations can be established.

Infections Due to *Cryptococcus neoformans*

Cryptococcal infection can cause disease ranging from mild or asymptomatic to life-threatening and affects both immunocompetent and immunosuppressed individuals. Of patients with cryptococcosis in the pre-AIDS era, >50% had no predisposing factors, and white males predominated. [470] [471] The recent increase in cases of cryptococcal infection is largely due to the increasing numbers of patients with HIV infection, among whom it is one of the most common life-threatening infections. Other immunosuppressed populations at risk include patients receiving therapeutic doses of corticosteroids, those with lymphoid malignancies (particularly Hodgkin disease), patients with sarcoidosis (even if not receiving corticosteroids), patients with organ transplants, and patients with diabetes mellitus. [470] [471] The most common site of serious *Cryptococcus* infection is the CNS, followed by the lungs. Other sites, such as skin, bone, kidneys, and other viscera, have been reported less frequently. Interestingly, when CNS cryptococcosis occurs, there is no apparent pulmonary involvement in most cases. Conversely, respiratory involvement frequently occurs in isolation, although it often coexists with extrapulmonary disease, particularly in the immunosuppressed population. [472] The onset of CNS cryptococcal infection is frequently insidious, although heavily immunosuppressed populations may have an abrupt or even fulminant onset. Typically, symptoms are subtle, evolve over weeks to months, and most frequently include waxing and waning fevers, headaches, dizziness, somnolence, and signs of cognitive impairment. In addition, cranial nerve abnormalities are seen in 20% and papilledema in 30% of cases. Classic meningeal signs, such as nuchal rigidity, are rare. Symptoms and signs associated with pulmonary cryptococcosis are often mild or may be entirely absent. Typically, symptoms, if they occur, may include mild aching pain, scant hemoptysis, and, rarely, pleuritic pain, rales, or friction rubs. [472] Chest radiographs may show an isolated pulmonary nodule, an interstitial infiltrate, or a consolidative process.

In patients with significant deficiencies in cell-mediated immunity, any unexplained CNS or pulmonary signs or symptoms should raise the suspicion of infection with *Cryptococcus*. Subtle or intermittent symptoms should not be disregarded. Fever may be absent but may also be the only clue to infection, even with significant CNS disease; unexplained fever in patients with AIDS or other causes of impaired cell-mediated immunity should prompt a workup for cryptococcal infection. Immunosuppressed patients are at increased risk of disseminated disease, and this should be investigated even if initial symptoms or signs indicate an isolated site. The appropriate workup includes, at a minimum, cultures of suspected sites, tests for cryptococcal antigen in CSF and serum, and CSF examination. If tissue specimens are obtained, the diagnosis may also be established histopathologically.

CSF examination is helpful in establishing the diagnosis of CNS disease. In non-AIDS patients, abnormalities may include elevated opening pressure, low glucose concentration (50%), elevated protein concentration, and a mononuclear cell pleocytosis (usually with <500 cells/mm ^{3}).[472] [473] Importantly, AIDS patients or patients receiving high doses of corticosteroids may have little or no inflammatory response and consequently have little or no pleocytosis in the face of significant CNS disease. This is considered a poor prognostic sign. Overall, mass lesions due to *Cryptococcus* are relatively rare but may occur in 1025% of patients. If suspected, mass lesions should be ruled out before lumbar puncture.

The latex agglutination test for detection of cryptococcal polysaccharide antigen is >90% sensitive and specific for the diagnosis of cryptococcal disease and should be performed on both serum and CSF of patients in whom the diagnosis is suspected. [473] False-positive results may occur in the presence of rheumatoid factor (which may be eliminated by digestion with pronase or addition of reducing agents), in some malignancies, or by cross-reactivity with the rarer fungus *Trichosporon beigelii*.[474] [475] [476] Approximately 25% of HIV-infected patients will have a false-negative antigen in the CSF; the yield is often improved with repeated sampling. Specimens for India ink staining and for culture should always be obtained when possible. When the meninges are involved, *Cryptococcus* will usually grow from CSF specimens. Results of sputum cultures in

pulmonary cryptococcosis are more variable, however. Sputum cultures are frequently negative, particularly if disease is limited to a solitary nodule. A retrospective review from Duke University has shown excellent results from the culture of bronchoalveolar lavage fluid; however, only five patients were sampled. [477] In addition, positive sputum cultures for *Cryptococcus* are occasionally encountered in the absence of any evidence of parenchymal disease. [478]

The only appropriate therapy for CNS cryptococcosis is amphotericin B, with or without 5-FC. The combination of amphotericin B and 5-FC for 6 weeks is generally considered the treatment of choice for cryptococcal meningitis in non-AIDS patients. In a 1979 study comparing 0.4 mg/kg/day of amphotericin B alone for 10 weeks with a combination of 0.3 mg/kg/day of amphotericin B plus 5-FC for 6 weeks, no difference in mortality was found between the two regimens, but the combination regimen resulted in higher cure rates, fewer relapses, more rapid sterilization of the CSF, and decreased nephrotoxicity. [479] More recent data (in a 1987 report) indicate that in selected patients with mild meningitis in the absence of risk factors, 4 weeks of combination therapy might suffice. [480] If 5-FC is used, care must be taken to monitor serum 5-FC levels closely, to avoid toxicity (diarrhea, hepatitis, and bone marrow suppression), maintaining the peak serum concentration at 4080 gml. We and others have found that in patients with normal renal function, initial dosages of 5-FC of 25 mg/kg q.i.d. are adequate (as opposed to 37.5 mg/kg per dose, which is often recommended).

In patients in whom 5-FC is contraindicated or cannot be tolerated, successful therapy with amphotericin alone at dosages of 0.50.75 mg/kg/day can be effective and should be continued for at least 6 and perhaps 10 weeks (for immunosuppressed patients, a minimum of 10 weeks is recommended). Patients with lymphoreticular malignancies or those requiring continuation of corticosteroid therapy may need more protracted antifungal regimens; in these cases, the duration of therapy is adjusted according to the results of clinical and laboratory response.

The most appropriate therapy for CNS cryptococcosis in AIDS patients has not yet been defined, but it is clear that eradication of the organism is rarely achieved and that long-term suppressive therapy is usually indicated. A retrospective review of treatment of cryptococcosis in AIDS patients, published by Chuck and Sande [481] in 1989, indicated that the addition of 5-FC to amphotericin B neither enhanced survival nor prevented relapse in this population. In addition, AIDS patients appear to be particularly susceptible to the toxicities of 5-FC, with >50% requiring its discontinuation because of secondary cytopenias, according to this study. [481] Because this study was uncontrolled and flucytosine levels were not measured, and because other groups have reported success with the combination of amphotericin B and

flucytosine, the role of flucytosine remains controversial. [477] Additional studies prospectively comparing fluconazole and amphotericin B with and without flucytosine have shown varying results; the optimal therapy for cryptococcal infection in HIV-infected patients is unknown. [482] [483] The 4060% rate of relapse after cessation of treatment mandates lifelong maintenance therapy for HIV-infected patients. Initially, most patients received weekly amphotericin B, but maintenance with fluconazole has proved more effective with the advantages of fewer side effects and available oral preparations. [484]

After therapy, patients should have repeat lumbar punctures performed at least every few months for the first year, as most relapses occur within the first 6 months; a reasonable schedule is at 1, 3, 6, and 12 months after the conclusion of therapy. The CSF should be followed even if the original disease did not involve the CNS, as relapses have been found to occur there. Moreover, CNS disease can relapse at extraneural sites.

Other Fungal Infections in Immunosuppressed Patients

Zygomycosis (Mucormycosis)

Infections due to members of the Mucoraceae family are the fourth to fifth most commonly encountered mycoses in compromised patients. The Mucoraceae include the genera *Rhizopus*, *Rhizomucor*, *Mucor*, and *Absidia*. Patients at risk for zygomycosis include those with granulocytopenia, diabetic ketoacidosis, kwashiorkor, and those receiving deferoxamine. The clinical and radiologic manifestations of zygmycosis may resemble those of aspergillosis. The two most common clinical presentations are rhinocerebral and pulmonary zygomycosis, most commonly seen in patients with uncontrolled ketoacidosis or with granulocytopenia. Early and aggressive surgical debridement, intravenous amphotericin B, and correction of underlying metabolic abnormalities or immunologic deficits are essential for the successful treatment of rhinocerebral zygomycosis. Treatment of pulmonary and disseminated disease requires early initiation of intravenous amphotericin B therapy and reversal of immunologic impairment.[485] Lipid formulations of amphotericin B have been especially useful in the management of zygmomycosis, where patients such as those with diabetes mellitus may already have some preexisting renal insufficiency.

Pseudallescheria boydii

Pseudallescheria boydii is a soil saprophyte and a common cause of mycetoma. However, various medical centers have increasingly reported *P. boydii* as a cause of lethal disseminated infection in immunocompromised patients. [486] The populations at risk and the clinical and histopathologic manifestations of disseminated pseudallescheriosis are similar to those of disseminated *Aspergillus* infection. However, *P. boydii* is usually resistant to amphotericin B but is susceptible to antifungal azoles, including itraconazole and miconazole. [487] Given the dismal prognosis of infection due to this pathogen, combination therapy with amphotericin B and an antifungal triazole in profoundly compromised patients may enhance therapeutic efficacy. Although intravenous miconazole has been invoked as the preferred azole, this compound may cause fatal ventricular arrhythmias, hepatitis, and phlebitis. Other antifungal azoles, including ketoconazole, itraconazole, and saperconazole, have been used with varying degrees of success. [488] [489] Thus, itraconazole, 812 mg/kg/day administered orally, may provide a better therapeutic index if adequate plasma levels can be achieved. Whether the new third-generation antifungal triazoles will be effective against *P. boydii* infections awaits further investigation.

Fusarium Species

Disseminated infections with *Fusarium* spp. usually occur in granulocytopenic patients. They are often associated with prominent erythematous maculopapular to nodular skin lesions. Biopsy will reveal branching septate hyphae invading blood vessels, and the organism can be cultured from the lesions. Amphotericin B is the drug of choice for therapy, although resistance has been reported and mortality is as high as 75%.

Trichosporon beigelii

Disseminated disease due to the yeast *T. beigelii* has been increasingly documented in severely immunosuppressed patients and may resemble disseminated candidal disease. [46] Important risk factors for infection include granulocytopenia, corticosteroid use, and the presence of an intravascular catheter. [490] The

lungs, kidneys, skin, and eyes are the main sites affected. In untreated patients, serum and CSF may be falsely positive for cryptococcal antigen. [476] [490] Bone marrow recovery is the most important factor for a favorable outcome. Most isolates of *T. beigelii* are resistant to the fungicidal effects of amphotericin B. Laboratory and clinical studies demonstrate that fluconazole is safe, effective, and superior to amphotericin B in the successful treatment of invasive trichosporonosis. [490] [491]

CLINICAL APPROACH TO VIRAL INFECTIONS IN IMMUNOCOMPROMISED PATIENTS

Viral infections cause a wide range of disorders in patients with impaired immunity, ranging from subclinical infection to overwhelming disease and death. Of the viruses causing disease in this population, those belonging to the herpes group are encountered most commonly. The human herpesviruses that are most frequently encountered clinically include HSV, VZV, CMV, EBV, and HHV-6. Almost all these viruses are encountered with greater frequency in patients with immune dysfunction than in normal hosts. Patients with defects in cell-mediated immunity are the major population at risk. Impairment of cell-mediated immunity that may lead to serious viral infection is seen in patients with hematologic malignancies (particularly Hodgkin disease and the non-Hodgkin lymphomas), those with HIV-1 infection, patients undergoing organ transplantation (bone marrow, kidney, liver, heart), patients receiving corticosteroids or other aggressive immunosuppressive agents (including many cytotoxic antineoplastic regimens), severely malnourished individuals, and those with primary congenital or acquired defects in cell-mediated immunity.

A characteristic shared by each of these viruses is that they can cause disease either at initial exposure (i.e., primary infection) or after reactivation of latent disease. Indeed, latency is the hallmark of herpesvirus infections and, by application of current molecular biologic techniques, the mechanisms by which these viruses establish and maintain latency as well as undergo reactivation from the latent state are being elucidated. [492]

Infections Due to Herpes Simplex Virus

Infection with HSV may cause significant morbidity but is only rarely fatal in the compromised host. HSV infection usually represents reactivation of latently established disease.

When HSV infection occurs even in the most severely immunocompromised patients, it tends to remain localized and only rarely disseminates to distant sites. Although disseminated HSV infection can lead to death, the morbidity associated with localized HSV infection in compromised patients is a far more clinically relevant issue. The most common sites at which HSV occurs in non-AIDS patients are the mouth and perioral area, the nares, and the esophagus. In addition, a patient with a previous history of genital or perianal HSV may experience reactivation at these sites. However, the absence of a history for recurrent herpes does not preclude the presence of latent virus, since >50% of latent HSV infections in normal hosts are asymptomatic. Pain is the hallmark of lesions attributable to HSV. As compared with its effects in healthy individuals, the infection in compromised patients causes more symptoms (predominantly pain), is associated with larger and more numerous lesions, and persists for a longer period (Fig. 79-9). Chronic ulcerated lesions in the oral or anogenital region may persist for weeks to months in certain subgroups, such as those with hematologic malignancies or AIDS. [493] [494] [495] Esophagitis due to HSV typically manifests with burning pain on swallowing with or without associated dysphagia, and may or may not be associated with visibly apparent oral lesions. HSV esophagitis may be clinically indistinguishable from other causes of similar symptoms in this

Box 79-3. APPROACH TO VIRAL INFECTIONS HERPES SIMPLEX VIRUS

HERPES SIMPLEX VIRUS

In compromised hosts, HSV is rarely fatal but is a significant cause of morbidity. For treatment of symptomatic infection, either oral acyclovir (200 mg five times per day) or intravenous acyclovir (750 mg/m^2 /day in three divided doses) is effective. Patients with persistently compromised immune status (e.g., those with AIDS) may need chronic suppressive treatment. Acyclovir resistance may develop.

VARICELLA-ZOSTER VIRUS

Those at risk of primary infection (chickenpox) who are exposed to an infectious source should receive zoster immune globulin (ZIG) and be isolated from other susceptible patients. ZIG can decrease both the morbidity and the mortality associated with subsequent infection. In seriously compromised cancer patients who develop primary chickenpox (and who do not receive ZIG or early antiviral therapy), visceral dissemination will occur in about one-third, and 20% may die. Prompt institution of intravenous acyclovir (1,500 mg/m^3 /day in three divided doses) can decrease the morbidity and mortality. Acyclovir should be continued for 7 days and until complete crusting of the lesions.

Secondary VZV infection (zoster) can also cause significant morbidity in compromised patients, although dissemination and death are less frequent than with primary disease. Among seriously compromised cancer patients who do not receive antiviral therapy, cutaneous dissemination occurs in 2550% and visceral dissemination in 819%. The lungs and liver are the most common visceral sites of dissemination. Intravenous acyclovir (1,500 mg/m^2 /day in three divided doses) can decrease dissemination and hasten healing of lesions and should be administered as soon after the appearance of lesions as possible (preferably within 72 hours). We begin all high-risk patients on intravenous acyclovir. (High-risk patients we define as those undergoing neutropenia-producing cytoreductive therapy, those who have received total body irradiation or bone marrow transplantation within the previous year, those with Hodgkin disease or non-Hodgkin lymphoma, those with AIDS, and other patients with significant depression of cell-mediated immunity, such as those receiving high doses of corticosteroids.) Therapy should continue for 7 days or until the lesions have crusted. We discourage the use of oral acyclovir for prophylaxis or treatment because of the low serum levels attained and the development of resistance. Patients with acyclovir-resistant HSV or VZV infection may be treated with foscarnet.

CYTOMEGALOVIRUS (CMV)

Ganciclovir is the drug of choice for patients with evidence of CMV infection. Foscarnet serves as second-line therapy and, on occasion, can be used in combination with ganciclovir when there is evidence of disease progression on monotherapy.

Figure 79-9 Extensive oral herpes simplex in a granulocytopenic patient with chemotherapy-induced mucositis and oral candidiasis.

population, including chemotherapy-induced mucositis, *Candida* esophagitis, and CMV esophagitis.

Mucocutaneous HSV infection may also cause secondary complications such as bleeding or infection. Mucosal disruption may lead to localized secondary bacterial infection and may also provide a portal of entry for bacteria into the bloodstream.

Dissemination beyond the oral, anogenital, nasal, or esophageal mucosa occurs rarely. The incidence of dissemination is not well established but is probably <1%. The most common sites of dissemination are the skin, lungs, liver, CNS, and adrenals. The diagnosis of disseminated HSV should be considered in any patient with cell-mediated immunity defects and unexplained findings referable to these sites.

The diagnosis of localized oral, nasal, or anogenital HSV infection is rarely problematic. Early lesions usually have a characteristic vesicular appearance, and cultures are usually positive within 48 hours. When obtaining material for culture for HSV, it is important to remember the following steps. False-negative results may be obtained from lesions that appear either very early (i.e., before vesicle formation) or late in the course of evolution (after crusting). Ideally, culture material should consist of serous fluid from vesicles that is obtained after gentle unroofing. Also, specimens should be promptly inoculated into tissue cultures and should not be kept at room temperature for prolonged periods. It is helpful to store frozen culture medium on the hospital floors so that it is readily available to the clinician. Techniques such as direct immunofluorescence assay, ELISA, and monoclonal antibody staining allow for rapid diagnosis.

The definitive diagnosis of HSV esophagitis or disseminated disease may be more elusive. Accordingly, for patients with defects in cell-mediated immunity in whom the diagnosis is suspected on clinical grounds, empirical therapy is often instituted. In patients with esophagitis, the simultaneous presence of characteristic oral lesions may point toward the diagnosis but neither confirms nor rules out other contributing factors. In the absence of oral lesions, positive oral cultures may indicate esophageal infection, but they may also reflect asymptomatic excretion or infection at another site. In these settings, empirical therapy with acyclovir or amphotericin B, or both, may be indicated, and in selected patients esophagoscopy and biopsy may be performed (see also discussion of *Candida* esophagitis).

The diagnosis of HSV pneumonitis presents similar problems. Positive cultures of respiratory secretions usually reflect asymptomatic excretion or excretion from another infected site (e.g., oral cavity), but occasionally they may indicate lung or tracheobronchial involvement. Lavage fluid showing multinucleated giant cells may be helpful but is not specific. The diagnosis of HSV meningoencephalitis is made by use of PCR technology for HSV DNA in CSF. The PCR test is extremely sensitive, specific, and rapid, becoming positive as early as 1 day after disease onset. [496] Even in PCR- or biopsy-confirmed cases, HSV is seldom cultured from the CSF.

Acyclovir is the drug of choice for the treatment of HSV infections in immunosuppressed patients. For mucocutaneous disease, either oral or intravenous acyclovir is effective in shortening the length of symptoms, the time of virus shedding, and the time to healing. Because many patients with oral or esophageal HSV infection have difficulty swallowing, intravenous administration is often more useful. The appropriate intravenous dosage is 250 mg/m ^{2} (or 5 mg/kg) t.i.d. Therapy should be continued for 1 week or until lesions have crusted. Patients with diagnosed or suspected visceral or CNS dissemination should receive intravenous acyclovir at a dosage of 500 mg/m^2 (or 10 mg/kg) t.i.d., and therapy should be continued for 10 days. During the past several years, treatment of HSV infections has been increasingly complicated by the emergence of acyclovir-resistant virus. Resistant HSV has primarily been isolated in the most immunosuppressed patients, such as those with AIDS or who have undergone bone marrow transplantation. [497] [498] In a review by Englund et al., [497] seven patient isolates of 207 were found to be resistant to acyclovir; all seven of these patients were immunocompromised from marrow transplantation (four cases), solid organ transplantation (two cases), or AIDS (one case). Although resistance to acyclovir has been associated with chronic low-dose acyclovir in both animal models and case reports, no specific regimen or dose has been implicated. [499] [500] Spontaneous mutations in the viral thymidine kinase lead to a mixed population of HSV, and chronic acyclovir therapy may permit the emergence of the acyclovir-resistant viral population. Resistant HSV is usually susceptible to foscarnet, which is used for successful treatment of these patients. [498]

Antivirals

Recently, two new orally available antiherpes drugs have become available, famciclovir and valaciclovir. The former is a

prodrug of penciclovir, an agent with potent activity against both VZV and HSV types 1 and 2. Famciclovir is effective for the treatment and suppression of genital herpes in normal hosts and is a promising agent for use in immunocompromised patients with mild HSV recurrences because of its oral bioavailability and its twice daily schedule of administration, which make it much simpler to take than acyclovir, which requires dosing five times daily. [501] Valaciclovir, a prodrug of acyclovir, is also highly active against herpes simplex and zoster viruses but is currently contraindicated in immunocompromised patients because of reports of thrombotic thrombocytopenic pupura/hemolytic uremic syndrome (TTP/HUS) in patients with advanced HIV and bone marrow and renal transplant recipients. TTP/HUS has not been reported in association with famciclovir, but clinical trials in immunocompromised patients are ongoing. [502]

Infections Due to Varicella-Zoster Virus

In contrast to HSV infection, VZV infection in compromised patients may be associated with significant morbidity and mortality. Both primary disease (chickenpox) and reactivation of latent disease (zoster or shingles) are clinically important in this population.

Primary Varicella Infection (Chickenpox)

In noncompromised patients, chickenpox is a highly contagious but relatively benign, self-limited disease that requires no therapy. Primary infection usually occurs during childhood (usually by the age of 3 years) and may result in either subclinical or clinically apparent disease. Approximately 90% of individuals have acquired antibodies to VZV (implying primary infection) by the age of 15 years. [503] In healthy children, chickenpox is usually limited to the skin. When it occurs in adults, 15% also have radiographic evidence of pulmonary involvement, although fewer have pulmonary symptoms, which are usually mild in nature. [503] [504] In immunocompetent patients, dissemination is rare, with a self-limited cerebellar ataxia occurring in 1 in 4,000 children and encephalitis in 0.10.2%. [503] By contrast, primary varicella frequently disseminates and can cause serious morbidity and mortality in patients with impaired cell-mediated immunity. Children with immune impairment represent the highest-risk group for primary varicella, which parallels its age distribution in the normal host, with only 10% of adults at risk of primary disease. However, for patients of any age with a newly diagnosed disease associated with defective cell-mediated immunity (e.g., Hodgkin disease) or for those about to receive therapy that can result in impaired cell-mediated immunity (e.g., intensive chemotherapy or corticosteroids), it is prudent to check for a clinical history of chickenpox or to check serum titers in the absence of clinical information. The absence of VZV antibody titers indicates a significant risk of the acquisition of primary disease, if exposed, and has important implications for management after potential exposure.

Of those seriously immunocompromised children who contract primary varicella and who do not receive either ZIG or early therapy with acyclovir, about one-third will experience visceral dissemination; among these children mortality is in the range of 720% (usually due to progressive pulmonary insufficiency). [66] The lung is the major site of dissemination, which occurs 37 days after the onset of skin lesions, with chest radiographs classically showing diffuse nodular infiltrates. The CNS and liver are two other frequent sites of dissemination. VZV hepatitis in the absence of CNS or pulmonary involvement rarely causes death.

Among children, those at highest risk for serious disseminated VZV are those with malignancy who are undergoing intensive cytoreductive therapy, those receiving organ transplants, and those with primary disorders of cell-mediated immunity. Among children being treated for malignancy who develop VZV, those with leukemia and lymphoma represent the largest group, probably because hematologic malignancies are the most common in this age group. Dissemination is more likely in children with 500 lymphocytes/mm^3 at the onset of disease and in those in whom chemotherapy was continued after the onset of skin lesions. [66] Disseminated VZV occurs more often in patients with advanced disease, although this may be related to the use of more aggressive therapy. [67]

The diagnosis of primary cutaneous chickenpox is usually made relatively easily on clinical grounds from the appearance of the lesions. However, immunosuppressed patients, particularly those with AIDS, occasionally develop atypical lesions such as papules or hyperkeratosis. A high index of suspicion must be maintained in susceptible hosts. Knowledge of antecedent VZV antibody titers or of a clinical history of prior infection can be helpful in this setting. A specimen should be obtained for viral culture from any suspicious vesicular lesion by unroofing the vesicle and scraping cells from the base of the lesion (not vesicular fluid) in order to capture this highly cell-associated virus. Cultures of skin lesions often yield positive results, with shell-vial cultures for VZV taking only a few days to become positive. Immunofluorescent stains on scrapings from skin lesions (the direct fluorescence antibody test) are very helpful in early diagnosis and are often more sensitive than

culture. Acute and convalescent serum antibody measurements are rarely helpful in confirming the diagnosis retrospectively.

All immunosuppressed patients with suspected primary VZV infection should undergo chest radiography and measurement of liver transaminases in order to establish a baseline and to detect clinically silent disseminated disease. Examination of the sputum in VZV pneumonia may show multinucleated giant cells, and culture may yield positive results. CNS involvement may be very difficult to diagnose with certainty ante mortem; PCR may be a useful technique in the diagnosis of CNS disease due to VZV.[505]

Patients with confirmed or suspected primary varicella should be placed in immediate isolation to avoid exposure of other susceptible patients. Other immunocompromised patients at risk of acquisition of primary varicella who had contact with the patient during the infectious stage (beginning 48 hours before the appearance of the lesions and lasting until crusting of the lesions) should be contacted and promptly given varicella-zoster immunoglobulin. The incubation period is 1021 days after exposure; exposed patients should consequently avoid contact with other patients for that period.

Early institution of intravenous acyclovir will dramatically reduce the incidence of visceral dissemination of primary varicella. In a randomized double-blind placebo-controlled trial of acyclovir in immunosuppressed children with primary varicella, pneumonitis developed in five of 11 placebo recipients but in none of 11 patients who received acyclovir.[506] Vidarabine therapy can also be effective, although it is rarely used, as acyclovir is widely available. [507] Intravenous acyclovir should be instituted at a dose of 500 mg/m^2 (or 10 mg/kg) t.i.d.; therapy should be continued for at least 7 days, or until all lesions have crusted. Patients receiving this dose of acyclovir should be well hydrated in order to maintain urine flow and avoid renal precipitation and toxicity. Therapy for disseminated disease is identical, although more protracted courses may be required.

Herpes Zoster

Herpes zoster, or shingles, is believed to represent reactivation of latent disease. Although some reports have suggested that zoster can be acquired exogenously, this has not been firmly substantiated, and the vast majority of cases are not temporally associated with antecedent exposure. [69] [71] [508] Although zoster can

occur in healthy individuals, it is clearly more common in certain immunosuppressed populations. Patients with malignancy, especially those with Hodgkin disease, are at highest risk (15%), followed by patients with non-Hodgkin lymphoma (8%), and patients with solid tumors (2%). [67] [69] [505] [509] [510] [511] [512] [513] [514] Zoster is more common in patients with advanced disease, as opposed to those with limited disease or in remission. However, in certain subgroups (e.g., patients with Hodgkin disease or lymphomas), an increased risk continues even after cure of the malignancy. [515] Zoster is more likely to develop at skin sites that have been previously damaged by tumor or radiation therapy. More intensive immunosuppression is associated with a higher risk of zoster (e.g., patients who receive combinations of chemotherapy and radiation therapy are at greater risk than those who receive either modality alone). Following significant immunosuppressive therapy such as for bone marrow transplantation, the increased risk for zoster may last 1 year.[515] Zoster usually appears unilaterally and follows a dermatomal distribution even in immunosuppressed patients. Early lesions may be very subtle (Fig. 79-10). Rarely, it may present with cutaneous dissemination without a prior history of dermatomal involvement (so-called atypical disseminated zoster or varicelliform zoster, which resembles primary varicella). [69] [513] [516] Visceral dissemination of zoster occurs less frequently than does dissemination of primary varicella infection. Prior to the prompt, routine use of acyclovir in all immunosuppressed patients with zoster, the incidence of dissemination was 2450%, while visceral dissemination (to the lungs, CNS, liver, and uvea) occurred in 819%. [517] [518]

Figure 79-10 Early lesions of zoster in an immunosuppressed patient.

In the current era, the liberal use of acyclovir has dramatically reduced the incidence of disseminated zoster.

Guidelines for the diagnosis of zoster are similar to those for the diagnosis of primary varicella. When an immunosuppressed patient with suspected or diagnosed zoster is encountered, several important questions arise. First, has the patient had significant contact with other patients who may be at risk for the development of primary varicella? Although patients with zoster are less infectious than those with primary varicella, direct contact may spread infection to susceptible individuals, who should receive ZIG. Second, is the disease localized, or is there any evidence of cutaneous or visceral dissemination? All patients at risk of dissemination should undergo a baseline physical examination, chest radiography, and measurement of liver transaminases. Third, is the patient a candidate for intravenous therapy? Clearly, any patient with disseminated disease is a candidate for therapy with intravenous acyclovir.

In addition, patients with localized disease who are at significant risk for dissemination should be treated with acyclovir. This includes (1) patients with an underlying diagnosis of either Hodgkin disease or non-Hodgkin lymphoma, whether or not undergoing therapy (those receiving therapy will be at higher risk of dissemination); (2) patients with any malignancy who are undergoing aggressive cytotoxic therapy (defined as therapy that produces significant leukopenia); (3) patients who have received total body irradiation or bone marrow transplantation, for 1 year after the procedure; (4) patients with AIDS; and (5) other patients with severe congenital or acquired defects in cell-mediated immunity (including those receiving systemic corticosteroids or other immunosuppressive drugs). Intravenous acyclovir is recommended for immunosuppressed patients with dermatomal zoster.

Attempts should be made to treat patients as soon after the appearance of the lesions as possible (preferably within 72 hours), even if the lesions are confined to a single dermatome, since earlier therapy may result in improved outcome. However, the results of studies summarized following indicate that patients with older lesions that have not healed should also receive therapy.

Although both vidarabine and acyclovir have been shown in controlled trials to be effective for the treatment of zoster in immunocompromised patients, acyclovir has been more effective.[517] [518] [519] [520] A 1986 study by Shepp and colleagues [520] compared intravenous acyclovir with vidarabine in compromised patients. Acyclovir was found to be significantly better in preventing disease progression or dissemination, shortening culture positivity, decreasing new lesion formation, and decreasing time to pain resolution and crusting of lesions. In addition, acyclovir is extremely well tolerated and lacks the neurologic side effects seen occasionally with vidarabine therapy.

The first randomized, double-blind, placebo-controlled trial of acyclovir for zoster in immunosuppressed patients was reported in 1983 by Balfour and co-workers. [517] A 1-week course of acyclovir, 1,500 mg/m^2 /day given intravenously, halted significant progression of disease in patients presenting with either localized or cutaneous disseminated disease. For patients who presented with lesions confined to a single dermatome, acyclovir did not prevent local skin progression (i.e., either increase in the rash within the initial dermatome or extension to a new contiguous dermatome). However, it did decrease wider cutaneous spread, as well as visceral dissemination in patients who presented with either localized or disseminated cutaneous disease. Of the four patients randomized to placebo in whom visceral disease developed, three died. Two patients who presented with initial visceral dissemination and received acyclovir both survived. Acyclovir was effective at halting progression of disease even if begun in patients whose rash was >72 hours old at entry.

On the basis of these data, intravenous acyclovir at 1,500 mg/m^2 /day, given on an every-8-hours basis, is the treatment of choice for zoster in immunosuppressed patients. It should be continued for a minimum of 7 days, or until crusting of the lesions is noted. Oral acyclovir, even at high doses, is not appropriate therapy for zoster infections. The bioavailability of oral acyclovir is only 15%, and systemic concentrations achieved following oral dosing are not sufficient to inhibit VZV replication (VZV is 1020 times more resistant to acyclovir than is HSV). [521] Famciclovir, which has excellent oral bioavailability and activity against VZV, has been shown to be effective in treating zoster in the normal host, in whom it decreased postherpetic neuralgia. Famciclovir may also be useful in immunocompromised hosts.[501]

Clinically significant acyclovir-resistant VZV has become an increasing problem in the AIDS era. Chronic infection, often with atypical lesions and pain, is not uncommon in AIDS patients. As in acyclovir-resistant HSV, resistant VZV is often associated with chronic low-dose acyclovir use. [522] [523] In order to decrease the risk of developing resistance, it is best to aggressively treat the original VZV infection with high-dose intravenous acyclovir. Since viral susceptibility patterns are not

readily available, treatment decisions must often be based on the clinical response to therapy. Foscarnet is an alternative in most patients with resistant VZV infection.[523]

Infections Due to Cytomegalovirus

CMV causes a variety of syndromes in both immunocompetent and compromised hosts. The virus is ubiquitous, and most of the population is eventually infected, although clinical disease is relatively rare in healthy individuals. In noncompromised hosts, in addition to subclinical infection it may cause heterophil-negative mononucleosis and, rarely, has been associated with more serious complications, including interstitial pneumonitis, hepatitis, Guillain-Barré syndrome, meningoencephalitis, myocarditis, and hemolytic anemia.

In immunosuppressed patients CMV may cause significant morbidity and mortality. Of patients with hematologic disorders, those who present the highest risk of acquiring serious infection are bone marrow transplant recipients, particularly those undergoing allogeneic bone marrow transplantation, those who receive total body irradiation as part of the ablative regimen, and those who develop acute GvHD after transplantation.[524] [525] Other patients with hematologic malignancies (e.g., leukemia, lymphoma) are also at risk, although the incidence is lower. Other groups at risk include AIDS patients and recipients of solid organ transplants. In immunocompromised populations, disease may represent either acquisition of primary infection or, more commonly, reactivation of latent infection. Transfusion of seronegative blood products into seronegative recipients can dramatically decrease the acquisition of primary disease in selected populations.

CMV may infect many organ systems in compromised patients. In the bone marrow transplant population, interstitial pneumonia is the most common and most serious complication, associated with a high mortality rate. However, clinically important infection of other organ sites has been recognized with increasing frequency, most notably in AIDS patients but in other populations as well. The GI tract may be infected at any site from the esophagus to the colon. Submucosal ulcerations are common, and symptoms may range from isolated fever to severe hemorrhage or explosive diarrhea. CMV has also been implicated in the development of pancreatitis and cholecystitis.[526] [527] CMV retinitis may progress to blindness if untreated and occurs in 1020% of adult AIDS patients, although it has also been described in other immunosuppressed populations.[528] CNS involvement in non-AIDS patients is probably rare and has been reported only anecdotally. Neurologic complications in HIV-infected patients include subacute encephalitis, dementia, and polyradiculopathy.[529] This discussion focuses on CMV pneumonitis, the most commonly encountered serious CMV infection in non-AIDS patients and the most thoroughly studied.

Cytomegalovirus Pneumonitis

Depending on the presence or absence of specific risk factors, up to 50% of allograft bone marrow recipients develop interstitial pneumonia, and 70% of these cases will be associated with CMV. Typically, this infection occurs within the first 3 months after allogeneic marrow transplantation, with a median onset of about 60 days. Cases developing later than 100 days have also been well documented, primarily in patients with chronic GvHD.[530] Diffuse infiltrates are most common, but localized or nodular infiltrates have been described. Pleural effusions are rare.

CMV pneumonia is clinically indistinguishable from other causes of diffuse infiltrates in the compromised host, especially *P. carinii* infection and respiratory viruses such as influenza and RSV. While positive cultures from blood, urine, or respiratory secretions may raise suspicion of infection, they do not necessarily correlate with pulmonary infection. Serology for CMV may not be helpful because of false-negative results seen with immunosuppression. To establish the diagnosis, direct examination of pulmonary specimens is required. Although only open lung biopsy can be considered definitively diagnostic, bronchopulmonary lavage may be of value, particularly in bone marrow transplant patients. In examining specimens, use of the shell-vial culture system with immunofluorescent antibody staining frequently yields culture results positive for CMV within a few days.[531] [532] Classic histologic examination of tissue is the reference standard for the diagnosis of interstitial pneumonitis due to CMV. It will reveal typical intranuclear inclusions of CMV (Fig. 79-11). It should be stressed that even if diagnosis of CMV pneumonia is made or strongly suspected, other potential pathogens should still be sought, as CMV has been identified in association with other organisms.

Therapeutic options for treating serious CMV infection are limited and complicated by serious toxic effects. Both foscarnet and ganciclovir appear to be effective in treating CMV infections in patients with AIDS; however, the use of both therapies is limited by toxic effects, the need for intravenous dosing, and the emergence of resistant strains of CMV.[533] [534] Foscarnet not only has activity against CMV, it also shows in vitro inhibition of HIV reverse transcriptase; some studies have suggested improved survival of AIDS patients with CMV retinitis treated with foscarnet.[535] [536] [537] CMV isolates from both AIDS patients and marrow transplant recipients have been found to be ganciclovir resistant. The clinical significance is unclear. Resistance has been associated with progressive clinical disease. However, patients may be infected with multiple strains, and the virus isolated may not have the same antiviral susceptibilities as the actual infecting strain. It is impossible to make a clinical distinction of progressive CMV disease due to a virulent strain from disease due to a resistant one. Although foscarnet has been used to successfully treat patients with ganciclovir-resistant CMV, therapy with both foscarnet and ganciclovir has been beneficial with particularly aggressive disease.[538] [539] The neutropenia and thrombocytopenia that often accompany ganciclovir therapy are problematic in patients with bone marrow suppression from chemotherapeutic agents, zidovudine, or their underlying disease.[533]

Ganciclovir alone has not been effective in altering the outcome of CMV pneumonia in patients who have received bone marrow transplants.[540] [541] Studies have documented improved outcome in marrow transplant recipients with CMV pneumonia

Figure 79-11 Pulmonary biopsy specimen with cell showing typical intranuclear inclusion of cytomegalovirus (arrow).

who were treated with combinations of ganciclovir (2.5 mg/kg t.i.d. or 5 mg/kg b.i.d.) and high intravenous doses of immunoglobulin; overall survival improved to 5070%, as compared to <15% survival for historic controls.[542] It should be noted that although AIDS patients frequently appear to respond to ganciclovir or foscarnet therapy, relapse is almost certain when therapy is discontinued. Accordingly, continuous prophylaxis with ganciclovir or foscarnet is required. Immunocompromised patients with CMV disease should also receive maintenance therapy until immune system recovery. Potential therapies now being investigated include immunotherapy with CMV-specific CD8-positive lymphocyte subsets and treatment with CMV-specific monoclonal antibody.[543] [544] [545]

Infections Due to Respiratory Viruses

The respiratory virusesRSV, influenza, parainfluenza, and adenoviruscommonly cause upper and lower respiratory tract infections in immunocompetent children and adults. These viruses are becoming increasingly recognized as important community-acquired and nosocomially acquired pathogens in immunocompromised patients, particularly those undergoing intensive therapy for leukemia or recipients of bone marrow or solid organ transplants. RSV is a frequent cause of lower respiratory tract infection in young children and it is associated with mild upper respiratory infections in older children and adults, but is not considered a significant pathogen in the older age group. However, a number of studies have recognized that life-threatening respiratory disease due to RSV occurs in moderately to severely immunocompromised adults.[84] [546] [547] Englund et al.[84] described 11 immunocompromised adult patients with RSV infection, most of whom presented with cough, fever, and rhinorrhea. Lobar or interstitial infiltrates were observed in eight patients, and four died of progressive interstitial pneumonia despite treatment with ribavirin. Among bone marrow transplant patients, those who develop RSV pneumonia prior to engraftment appear to have a worse prognosis than those in whom engraftment has already occurred.[546] In a study of adult patients with acute leukemia who were hospitalized for acute respiratory illness, RSV was isolated from 10%. Among the nine patients with RSV infection, five died of interstitial pneumonitis.[547] In addition, the same group at the M.D. Anderson Cancer Center reported that one-third of adult leukemia patients who were hospitalized with acute respiratory illness had influenza A isolated from respiratory secretions, and 80% of those had an associated viral pneumonia.[548] The high rate of pneumonia in this selected group of patients hospitalized for respiratory symptoms is probably an overrepresentation of those with more severe infections. In a study of immunocompromised outpatients, including renal and bone marrow transplant patients and those with leukemia, 25 patients with influenza A were identified, of whom 22 had a clinical course indistinguishable from that of an immunocompetent host with influenza.[549] Unlike other respiratory viruses, adenovirus may disseminate, resulting in pneumonitis, hepatitis, colitis, or hemorrhage cystitis. In one study of adenoviral infections in immunocompromised patients, 12 of 15 had pneumonia and eight had hepatitis.[550] Shields et al.[551] reported a 5% incidence of adenoviral infection after bone marrow transplantation. In both studies, the adenoviral infection, often a pneumonia, was shown to increase mortality.

A limited number of antiviral agents are available for treatment of respiratory viral infections in immunocompromised patients, and none has been formally evaluated in clinical trials in this population. Ribavirin, a synthetic nucleoside analog with activity against RSV, influenza, and parainfluenza viruses, has been used anecdotally

for treatment of RSV in immunocompromised patients. In a study of 19 bone marrow transplant recipients with acute respiratory illness due to RSV, 16 patients developed pneumonia. Mortality was 100% in those who were either untreated or treated late, i.e., after the onset of respiratory failure, whereas it was 22% in those who received therapy with ribavirin plus IVIG prior to the onset of profound respiratory compromise. [552] Although early diagnosis and treatment appear to be beneficial in severely immunosuppressed bone marrow transplant recipients, it is not clear that those who have RSV infection in the setting of less immune dysfunction still require drug therapy. Amantadine is effective therapy for influenza A (not B) in immunocompetent adults, in whom it shortens the duration of fever and systemic symptoms if given within 48

hours of the onset of symptoms. It has not been formally evaluated in immunocompromised patients but it is strongly recommended for those who do develop influenza A infection.

CLINICAL APPROACH TO PARASITIC INFECTIONS IN IMMUNOCOMPROMISED PATIENTS

Pneumocystis carinii Pneumonia

P. carinii pneumonia is the most common parasitic disease affecting immunocompromised patients. The populations most commonly affected are those with hematologic malignancies (particularly ALL, Hodgkin disease, and non-Hodgkin lymphomas) or AIDS. In addition, however, *P. carinii* pneumonia can occur in patients with solid tumors, transplant recipients, and other immunosuppressed populations, particularly in those receiving therapy associated with impaired cell-mediated immunity (e.g., corticosteroids). Of all the subgroups at risk for *P. carinii* pneumonia, adult AIDS patients have by far the greatest relative risk.

In non-AIDS patients the most common clinical manifestations of *P. carinii* pneumonia include fever, cough, and tachypnea, generally with intercostal retractions and the absence of detectable rales. A chest radiograph typically shows a hazy bilateral alveolar infiltrate, which often begins at the hilus and spreads to the periphery. Arterial blood gas measurements reveal a low Pa_{O_2} , normal Pa_{CO_2} , and alkaline pH. The clinical presentation can be indolent (12 months) but is more often rapidly progressive (45 days). Chest radiographic findings are occasionally atypical (e.g., lobar consolidation, effusion, or nodular or unilateral pneumatocoeles); in rare cases, the radiograph may appear normal despite the presence of pneumatocoeles on biopsy. *P. carinii* pneumonia in cancer patients differs from that in AIDS patients; the median duration of symptoms is 28 days in AIDS patients but 5 days in non-AIDS patients. [553] In AIDS patients, disseminated *P. carinii* infections have recently been reported: although the lungs are still the most common site of infection, the lymph nodes, spleen, liver, bone marrow, adrenal glands, kidneys, and GI tract are also commonly involved. [554]

The diagnosis of *P. carinii* pneumonia requires demonstration of cysts or trophozoites in pulmonary material from patients with a clinically compatible course; cysts have been found in asymptomatic, previously healthy individuals autopsied after traumatic death. Classically, material obtained from bronchoalveolar lavage or open lung biopsy and stained with Gomori methenamine-silver nitrate or toluidine blue has been the most reliable for diagnosis. At the NCI, immunofluorescent monoclonal antibody stains on induced sputum specimens have given rapid and reliable results (Fig. 79-12).[555]

In clinical situations in which the likelihood of *P. carinii* pneumonia is great, the choice is between proceeding with a diagnostic procedure and administering an empirical course of therapy with trimethoprim-sulfamethoxazole. If techniques for examining induced sputum or bronchoalveolar lavage fluid are readily available, these are initially the procedures of choice for establishing the diagnosis. However, if they are not available, or if the patients clinical or hematologic status does not permit bronchoalveolar lavage, an empirical trial of trimethoprim-sulfamethoxazole (20 mg/kg/day of trimethoprim) plus erythromycin (for *Legionella*) is recommended, rather than proceeding directly to open lung biopsy. This approach is based on the results of a randomized NCI trial demonstrating that in

Figure 79-12 (A) Cluster of *P. carinii* obtained from bronchoalveolar lavage (stained with toluidine blue). **(B & C)** *P. carinii* from induced sputum specimen stained with immunoflurescent monoclonal antibody.*(Courtesy of Drs. V. Gill and J. Kovacs, Clinical Center, National Institutes of Health.)*

non-neutropenic patients with diffuse infiltrates, empirical therapy is as safe and effective as open-lung biopsy. [556] The clinician should realize that a response to therapy may not be apparent for 45 days. Indeed, progression over the first few days does not necessarily imply inappropriate or ineffective therapy. It is theorized that this delay in improvement is due to the hosts inflammatory response; for this reason, steroids are recommended during the first 72 hours for patients with an arterial oxygen pressure of <70 mm Hg or an arterial-alveolar gradient of <35 mm Hg. [557] If the patient fails to improve (e.g., continued fever, depressed Pa_{O_2} , or progressive infiltrates) after 4 days of therapy, modification is then appropriate. In this setting we prefer to add pentamidine (4 mg/kg/day) rather than discontinue the trimethoprim-sulfamethoxazole, since progression may represent slow response rather than true failure.

If a histologic diagnosis is necessary and not achievable by bronchoalveolar lavage, not all procedures, such as transtracheal aspiration, transbronchial biopsy or aspiration, or open lung biopsy, are of comparable diagnostic accuracy. Burt and colleagues [558] examined each of 17 patients who had undergone open lung biopsy to evaluate whether the diagnosis of a diffuse interstitial infiltrate could be established with a transthoracic needle aspirate and a transbronchial brush and biopsy. The patients in this unique study served as their own controls. The diagnosis was established from only 30% of the aspirates and from 59% of the transbronchial biopsy samples, suggesting that open lung biopsy is the procedure of choice. Open lung biopsy provides the soundest guidance for patient management, especially if the patient is neutropenic and requires multiple antimicrobial agents.

Because patients who are candidates for open lung biopsy are often thrombocytopenic, appropriate hematologic support for surgery is vital. Elevation of the platelet count to a surgically safe level of >60,000/mm^3 can usually be accomplished by infusion of 812 units of platelet concentrates 1 hour before surgery. Maintenance of the platelet count at this level for 24 hours after surgery with additional platelet concentrates minimizes any postoperative bleeding complications.

Because of the importance that *P. carinii* has assumed in patients with AIDS, the search for new therapeutic agents has intensified. Alternative regimens studied have included dapsone alone, trimethoprim with dapsone, clindamycin with primaquine, atovaquone, eflornithine, trimetrexate, and piritrexim. [311] The efficacy of trimethoprim-sulfamethoxazole as primary prophylaxis against both *P. carinii* pneumonia and toxoplasmosis in AIDS patients is unknown. A French study found that the combination of dapsone and pyrimethamine was as effective as aerosolized pentamidine in preventing *P. carinii* pneumonia and was also effective in preventing toxoplasmosis in *Toxoplasma*-antibody-positive patients. [559]

Another clinical dilemma is the treatment of patients who develop *P. carinii* pneumonia while on a preventive regimen. Although it is logical to assume drug failure and change regimens for treatment, there are no data to support this, and simply switching to a parenteral route of administration may suffice for treatment. [311]

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Chapter 80 - Nutritional Support of Patients with Hematologic Malignancies

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INTRODUCTION

Patients with hematologic malignancies are highly heterogeneous in terms of their nutritional needs and requirement for nutritional intervention. Patients with aggressive leukemias and lymphomas receive among the most intensive of oncologic therapies and frequently experience significant toxicities involving the gastrointestinal tract as well as other organ systems, decreased dietary intake, and weight loss. Patients with good-risk acute lymphocytic and chronic leukemias less commonly encounter significant nutritional problems.

The advent and widespread use of central venous access devices has helped obviate iatrogenic malnutrition in cancer patients. The use of total parenteral nutrition (TPN), however, has come under increased scrutiny, and among patients with solid tumors has not prolonged survival and may actually increase morbidity. [1] [2] This chapter addresses the appropriate use of TPN and other dietary intervention strategies, such as tube feedings and low microbial diets, in patients with hematologic malignancies.

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NUTRITIONAL PROBLEMS

Protein-Calorie Malnutrition

Significant weight loss at diagnosis is not a prevalent finding. DeWys and colleagues [3] reported that only 4% of patients with acute myeloid leukemia (AML), 10% with favorable-prognosis non-Hodgkin lymphoma (NHL), and 15% with unfavorable-prognosis NHL presented with more than 10% weight loss during the 6 months before initiation of chemotherapy. Among pediatric patients with newly diagnosed acute leukemia, very few present with significant weight loss. [4] [5] [6] [7] [8] The risk of the development of malnutrition subsequent to diagnosis depends on the toxicity of the therapy administered and the progression of the disease. Rickard and coworkers [9] identified several types of pediatric patients at high risk for nutritional depletion, including patients with NHL involving the gastrointestinal tract, AML, poor-prognosis acute lymphocytic leukemia (ALL), and patients who have experienced multiple relapses of their leukemia. [9] By the time patients with hematologic malignancies become candidates for marrow transplantation, malnutrition is more prevalent. Deeg and coworkers categorized a large cohort of adult and pediatric marrow graft recipients by weight status and found that except for chronic myelogenous leukemia (CML) in chronic phase and low-risk pediatric ALL, 828% of patients have mild to moderate malnutrition pretransplant (Table 80-1).[10]

Some limited data are available about the effect of protein-calorie malnutrition on outcome in hematologic malignancies. DeWys and investigators [3] found that weight loss before diagnosis had no influence on survival in patients with AML but was associated with a significant decrease in survival in patients with favorable- and unfavorable-prognosis NHL. During marrow transplantation, underweight patients are at an increased risk of early death. Among adults, survival to day 150 post-transplant is significantly worse for patients at 8595% ideal body weight (p = 0.004) or <85% ideal body weight (p = 0.0001) than for patients at 95140% ideal body weight status. Underweight children had a less marked but similar decrease in survival when compared to well-nourished children (p = 0.22 for <85% vs. 95140% ideal body weight and p = 0.01 for 8595% vs. 95140% ideal body weight). [10]

Weight loss of 1015% correlates with a 20% loss of protein mass and clinically significant impairment of physiologic function. [11] The adverse consequences of malnutrition on immunity, especially T-cell function, phagocytosis, and complement-mediated defenses, are well described. [12] [13] The effects of malnutrition on gastrointestinal function and immunity may be particularly relevant in hematologic malignancies. The thinning of mucous membranes, decrease in secretory IgA, and altered gut flora observed in malnutrition have been linked to an increase in the incidence of gastrointestinal infection. [14] [15] Because the gastrointestinal barrier can be breached during intense oncologic therapy, enteric infections are common and have been implicated as a source of bacteremias and sepsis. [14] [16] [17] Inadequate protein intake during an insult such as endotoxin as well as lack of luminal nutrition (e.g., during TPN) are associated with bacterial transfer across the intestinal mucosa and impairment of gut-associated lymphoid function and morphology in experimental models. [18] [19] [20] [21] [22] [23] [24] The role of dietary variables in bacterial translocation and gut immunity has not been established in humans. [25] [26] Nonetheless, the adequate nourishment of major organs, especially the gastrointestinal tract, seems prudent in immunocompromised patients.

Altered Metabolism

The metabolism of malignant bone marrow is markedly accelerated. Energy expenditure averages 3550% higher than predicted in both pediatric and adult patients with leukemia. [27] [28] [29] [30] Rates of whole-body protein synthesis and breakdown are almost twice normal. [28] [30] Similar derangements in energy and protein metabolism have not been found in patients with

TABLE 80-1 -- Patients with Malnutrition Prior to Marrow Grafting

Diagnosis	Adults		Children	
	Total N	N <95% IBW (%)	Total N	N <95% IBW (%)
AML, low risk	114	15 (13.2)	43	7 (16.2)
AML, high risk	307	40 (13.0)	105	24 (22.8)
ALL, low risk	35	4 (11.4)	21	0
ALL, high risk	158	28 (25.9)	217	25 (11.5)
CML, chronic phase	372	16 (4.3)	23	3 (13.0)
CML, other	206	32 (15.5)	33	8 (24.2)
Lymphoma, remission	92	7 (7.6)	18	4 (22.2)
Lymphoma, relapse	159	23 (14.5)	25	7 (28.0)

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; IBW, ideal body weight.

Data from Deeg HJ et al. [10]

lymphoma.[30] Energy expenditure decreases in patients with acute leukemia within days of starting chemotherapy [29] [31] further evidence that the leukemia itself induces the high metabolic rate. Analogously, maintenance chemotherapy in children with ALL in remission appears to exert no significant adverse effects on energy metabolism.[7] [32] During induction chemotherapy[29] or marrow transplantation, [33] energy expenditure and protein losses increase presumably due to the complications that arise during neutropenia.

Toxicities of Oncologic Treatment

The nutritionally relevant toxicities of oncologic treatment are summarized in Table 80-2 . Aggressive multimodal treatment often produces multiple toxicities; when prolonged, it can lead to significant nutritional deficits, particularly for children. The nutritional consequences of biologic response modifiers are associated with anorexia and mild to moderate gastrointestinal disturbances. [34] [35]

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ASSESSMENT OF ALTERED NUTRITION STATUS

Basic Evaluation

Initial and serial nutritional evaluation and screening are indicated in all patients (see Algorithm for Nutrition Assessment and Intervention). Early identification of depleted weight status (either rapid weight loss or inadequate reserves when compared to an ideal body weight estimate) and poor oral intake may avert the morbidity associated with moderate to severe malnutrition. In a survey of 173 pediatric oncology patients of whom 45% had leukemia or lymphoma, Tyc and colleagues found that poor oral intake, even as a subjective report, was the single best predictor of need for intervention with TPN or enteral tube feeding. [36] The mean length of time after diagnosis for nutrition support intervention was 33 weeks in this cohort, suggesting that as soon as poor oral intake is identified there may often be sufficient time to implement earlier intervention programs to increase caloric intake, including nutritional counseling and behavioral therapies. Earlier intervention may potentially avert the need for more invasive and costly nutrition therapies. [36]

Weight assessment as an index of body composition is of less value in states of fluid imbalance (edema, dehydration, iatrogenic fluid overload). Weight must be interpreted with caution in metabolically stressed patients, such as those experiencing infectious complications or organ dysfunction with induction therapy or marrow grafting.[37] Actual nutrient intake as documented by the dietitian often provides the best guide for judging nutritional adequacy during periods of stress. In many institutions, bedside metabolic monitors are available that measure energy expenditure and help establish the energy goals of nutrition therapy.

Other measures of nutrition assessment, including immune function tests and serum protein data, have not added to clinical judgment in predicting nutrition-associated complications or survival in surgical patients. [38] [39] Similar studies have not been reported in oncology patients. However, measurements of serum albumin, prealbumin, and transferrin seem to reflect metabolic stress, not the adequacy of recent nutrient intake, in patients with leukemia undergoing induction chemotherapy[29] [40] or marrow transplantation.[41] [42]

Pediatric Assessment

Accurate serial weight and height measurements are essential in the assessment of growth and development in children and adolescents. Lack of weight gain in growing children is indicative of nutritional deterioration; weight loss flags the need for

TABLE 80-2 -- Nutritional Problems Associated with Treatment of Hematologic Malignancies

Treatment	Complications
Chemotherapy	
Alkylating agents[a]	Nausea, vomiting
Antibiotics and antimetabolites[b]	Anorexia, nausea, vomiting, stomatitis Esophagitis: doxorubicin, cytarabine, floxuridine, idarubicin, thioguanine
	Gastrointestinal ulceration: doxorubicin, methotrexate, dactinomycin, floxuridine
	Hepatotoxic: lomustine, methotrexate, carmustine, mitromycin C, 6-mercaptopurine, streptozocin, floxuridine, idarubicin, thioguanine
	Nephrotoxic: cisplatin, busulfan, carmustine, cyclophosphamide, 6-mercaptopurine, thioguanine, floxuridine, fludaribine phosphate, lomustine, methotrexate, mitromycin C, streptozocin
	Diarrhea: doxorubicin, hydroxyurea, methotrexate, carboplatin
	Constipation: hydroxyurea
	Taste disturbances: carboplatin, cisplatin, streptozocin thioguanine, cladribine
Plant alkaloids[c]	Anorexia, nausea, vomiting
	Diarrhea: etoposide, vinblastine
	Hepatotoxic: etoposide
	Stomatitis, constipation, ileus: vinblastine, vincristine
Nitrogen mustards[d]	Anorexia, nausea, vomiting
	Esophagitis: mechlorethamine, thiotepa
	Hepatotoxic: chlorambucil, melphalan
	Gastrointestinal: chlorambucil, mechlorethamine
	Metallic taste: mechlorethamine
Others[e]	Anorexia, nausea, vomiting
	Stomatitis: mitoxantrone, procarbazine, hydroxyrurea, pentostatin
	Xerostomia: procarbazine
	Nephro- and/or hepatotoxic: L-asparaginase, carboplatin, cisplatin, pentostatin, procarbazine
	Diarrhea: levamisole, procarbazine, pacletaxel
Hormones[f]	Muscle and bone loss, hypokalemia, fat deposition and weight gain, fluid retention, glucose intolerance, hyperphagia, increased thirst, nausea, vomiting, anorexia, hypercalcemia, hyperlipidemia, gastrointestinal toxicity, negative nitrogen balance
Radiation	
Head and neck	Stomatitis, xerostomia, odynophagia and dysphagia, anorexia, taste alterations, dysosmia, osteonecrosis
Esophageal	Esophagitis, esophageal stricture

Stomach	Anorexia, gastritis, nausea, vomiting
Abdominal	Enteritis, diarrhea, steatorrhea, malabsorption, stenosis, obstruction, fistula formation, protein-losing enteropathy, fluid and electrolyte imbalances
Total body	Stomatitis, nausea, vomiting, diarrhea, taste alterations, xerostomia, growth failure
Biologicals[g]	Anorexia, nausea, vomiting: interferon, interleukin-2
	Fluid and electrolyte imbalances, diarrhea: interleukin-2, interferon
	Taste disturbances: interferon
Marrow transplantation	Stomatitis, esophagitis, gastrointestinal ulceration, diarrhea, taste alterations, xerostomia
	Veno-occlusive disease; renal and pulmonary dysfunction; multiple organ failure
	Acute and chronic graft-versus-host disease
Infectious enteritis	

Data from Cheney and Aker; [53] *Charuhas and Aker;* [35] *and Baltzer and Berkery.* [166]

[a]Busulfan, carboplatin, carmustine, cisplatin, cyclophosphamide, dacarbazine, ifosfamide, lomustine, mechlorethamine, streptozocin.
[b]Bleomycin, cladribine, cytarabine, dactinomycin, daunorubicin, doxorubicin, floxuridine, fludarabine phosphate, hydroxyurea, idarubicin, lomustine, mercaptopurine, methotrexate, metoxantrone, mitomycin C, plicamycin, thioguanine.
[c]Etoposide, vinblastine, vincristine.
[d]Chlorambucil, mechlorethamine, melphalan, semustine, thiotepa.
[e]L-Asparaginase, hydroxyurea, leucovorin, mitoxantrone, pacletaxel, pentostatin, procarbazine.
[f]Diethylstilbestrol, fluorymesterone, methylpregnisolone, prednisone, tamoxifen, megestrol acetate.
[g]Interferons, interleukins, colony-stimulating factors, including granulocyte and granulocyte/macrophage.

ALGORITHM FOR NUTRITION ASSESSMENT AND INTERVENTION SCREEN FOR NUTRITIONAL RISK

Step 1Obtain height, weight, and weight history. Compare current weight with usual and ideal weight.

Step 2Ask about changes in dietary intake compared to normal and any oral or gastrointestinal symptoms (diarrhea, constipation, nausea, vomitting, xerostomia, oral or esophageal pain, dysgeusia)

Step 3Does patient have:

10% weight loss in last 6 months (5% in children)?
90% ideal weight (<10th percentile weight/height in children)?
or
Poor appetite or physiologic or metabolic problem interfering with intake/assimilation of an adequate diet?

immediate dietary evaluation and intervention. Weight-for-height assessment is the appropriate tool to assess adequacy of body reserves, using standardized charts in prepubertal children [43] and tables in postpubertal adolescents. [44] Van Eys [45] identifies children who are <90% of the standard weight/height ratio (50th percentile) as at risk and those who are <80% as in need of vigorous intervention, whereas Kibirige and coworkers [46] suggest that patients below 95% of standard height/weight ratio may require supplemental nutritional support. Rickard and colleagues [9] define pediatric oncology patients as malnourished if weight-for-height falls below the fifth percentile when height is above the fifth percentile for age, while patients who are below the fifth percentile in both weight and height for age are chronically malnourished.

Serial heights should also be plotted on velocity charts. [47] Decreased height velocity has been observed in children receiving 18 or 24 Gy whole-brain irradiation within 6 months of therapy, [48] [49] [50] as well as in those undergoing marrow transplantation prepared with total-body irradiation [51] or chemotherapy only. [52] The potential role of chronic nutrient deficits has not been evaluated in poor growth velocity. However, nutrition evaluation is always indicated in children with growth rates below the fifth percentile.

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NUTRITIONAL INTERVENTION

Dietary Recommendations and Modifications

Dietary recommendations during periods of treatment toxicities and prolonged anorexia have been extensively reviewed. [35] [53] [54] Modifications in consistency, texture, and taste of food are required when patients experience mucositis, esophagitis, xerostomia, or dysgeusia. [55] [56] Reduction of fat and lactose content may decrease nausea or diarrhea. A dietitian can recommend appropriate nutritional supplements in patients who exhibit malabsorption or show an inability to gain weight. During hospitalization,

a food service that is individualized and that includes frequent follow-up by trained nutrition staff best meets the needs of patients undergoing intense oncologic therapy. [9] [57] [58] [59]

Diet Guidelines for Immunosuppressed Patients

Hospitals treating oncology and peripheral stem or marrow transplant patients routinely provide some form of low microbial food service, particularly for those undergoing gastrointestinal decontamination and treated in ultraisolation laminar air flow (LAF) or HEPA-filtered environments. [60] [61] [62] [63] Diets historically used in these types of protective environments can vary in the level of microbial restriction ranging from sterile to cooked food only diets. [60] [61] [64] Sterile diets consist of commercially canned, steam autoclaved, or oven-baked food and require aseptic food and tray preparation and distribution from separate kitchen facilities. A sterile diet is used in few institutions because of high production costs. [62]

Low microbial or cooked food diets vary widely in terms of food choices (e.g., the exclusion or inclusion of fresh fruits and vegetables) and bacterial content and are generally empirically formulated, although some institutions culture foods and assess acceptability when developing such diets. [61] [65] [66] Moe[61] cultured 198 foods prepared by conventional methods. Although 80% of beverages, starches, cooked fresh meats and entrees, and frozen vegetables were acceptable, only 36% of pasteurized dairy products and 42% of dessert and snack items met minimal microbiologic criteria. Up to 17 different species of gram-negative rods in concentrations as high as 10^6 /cfu/ml in milk, pudding, and ice cream were identified. Ayers et al. [67] found that serving foods containing only *Bacillus* resulted in transient colonization with *Bacillus* spp. in 41% of patients studied, but no infections occurred.

The protective benefit of low microbial diets against infection has not been firmly established, owing to lack of controlled trials. [60] [61] Nonetheless, the possibility that food may serve as a source of contamination in immunosuppressed patient populations including marrow transplant recipients has been shown by reports of pathogenic organisms implicating food and other sources. [67] [68] [69] [70] [71] [72] [73] [74] Case reports of *Lysteria monocytogenes* and *Clostridium botulinum* infections in marrow transplant recipients have proven difficult to attribute to contaminated foodstuffs. [72] [73] [74] The incubation period (e.g., up to 30 days for *L. monocytogenes*) for these as well as other specific organisms may be such that suspect foodstuffs have been discarded and an accurate food intake history is difficult. Furthermore, the majority of the cases of *Listeria* infection in marrow transplant patients have been detected in the ambulatory care setting, making it more difficult to trace to specific food intake. [72] Fungal infections are also common in the immunosuppressed host, but difficult to link with food-originated infection. Numerous foods, such as aged cheeses and others (e.g., miso and tempeh, which are fermented products), as well as naturopathic substances, [75] may serve as sources of fungal infections.

Fresh fruits and vegetables have historically been excluded from low microbial diets. Organisms routinely found on washed raw fruits and vegetables do not appear to be common sources of infection in immunosuppressed patients. At the Fred Hutchinson Cancer Research Center, 29 cultures of assorted raw, washed fruits showed gram-positive cocci and gram-negative rods (<10^1 /cfu/ml) of common origin in foods (S.N. Aker unpublished data, 1997). One of three strawberry cultures showed <2002,000/cfu/ml of three mold types. Thirty-six samples of raw, washed vegetables resulted in a broader variety of organisms at levels of <10^1 /cfu/ml of gram-positive and gram-negative organisms with few yeasts or molds. The organisms cultured from these fruits and vegetables are not representative of common infections routinely seen in the immunosuppressed cancer and marrow transplant patient populations.

TABLE 80-3 -- Diet Guidelines for Immunosuppressed Patients

These diet guidelines restrict high-risk foods as *potential* sources of organisms known to cause infection in immunosuppressed persons. It is recommended that patients follow the diet guidelines during treatment with immunosuppressive therapy (e.g., steroids, cyclosporine, chemotherapy, radiation, etc.) and/or for a minimum of 3 months following marrow or peripheral stem cell transplantation.

Food Restrictions
Raw and undercooked meat (including game), fish, shellfish, poultry, eggs, hot dogs, tofu, sausage, bacon
Cold smoked fish and lox; pickled fish
Unpasteurized and raw milk products including cheese and yogurt
Aged cheese (e.g., brie, camembert, bleu, roquefort, sharp cheddar, Stilton)
Refrigerated cheese-based salad dressings, (e.g., bleu cheese), not shelf-stable
Mexican hot pepper (e.g., hot chili pepper), farmers and feta cheeses
Unwashed raw vegetables and fruits and those with visible mold; all raw vegetable sprouts (e.g., alfalfa, mung bean)
Commercial unpasteurized fruit and vegetable juices
Raw and unpasteurized honey
All miso products (e.g., miso soup); tempeh; Maté tea
Raw, uncooked brewers yeast
All moldy and out-dated food products
Well water, unless tested yearly and found safe

Courtesy of the Clinical Nutrition Department, Fred Hutchinson Cancer Research Center, Seattle, WA.

Except for infrequent reports such as cyclosporiasis associated with imported fresh raspberries [76] and *Escherichia coli* 0157:H7 contamination of unpasteurized apple cider and other fruit juices [77] and raw vegetable sprouts, food outbreaks associated with raw, washed domestic fruits and vegetables are rare.

The use of strict isolation environments has been largely replaced with single hospital rooms with strict handwashing only for severely neutropenic patients. Furthermore, the trend of using the ambulatory care setting whenever possible to manage neutropenic patients has made obsolete past approaches to dietary precautions with immunocompromised patients. Immunosuppressed patients with hematologic malignancies, wherever their care environment, may best be counseled to follow a low-risk diet as summarized in Table 80-3 . The goals of a low-risk diet are both to minimize the introduction of high-risk foods and potential pathogenic organisms via food and to promote healthy food options in patients who historically exhibit poor oral intake. Such diet guidelines should ideally be accompanied with education on safe food handling.

Tube Feedings

Published literature on tube feedings in patients with hematologic malignancies is sparse, although there is renewed interest in the use of enteral nutrition because of the gut atrophy, greater expense, and infection risk associated with TPN. The risks of tube feeding are intuitively recognized as those associated with neutropenia, thrombocytopenia, mucosal and esophageal ulceration, vomiting, diarrhea, and decreased intestinal motility, which may predispose to hemorrhagic problems and sinusitis from the irritation of a feeding tube, aspiration pneumonia, and nutrient malabsorption.

Diarrhea was the primary complication observed in adults with acute leukemia receiving nasogastric tube feedings because of nausea and vomiting during administration of cytosine arabinoside, 6-thioguanine, and daunorubicin. [78] Diarrhea and poor gastric emptying also limited the ability to deliver adequate volumes of tube feeding to patients receiving high-dose chemotherapy and autologous marrow rescue for treatment of

solid tumors, necessitating supplementation with parenteral nutrition. [79] Tube feedings were otherwise tolerated in both reports. Tube feedings were less successful in patients with leukemia undergoing allogeneic marrow transplantation with more intensive conditioning, including total-body irradiation. [57] Most patients randomized to an enteral feeding program were crossed over to TPN or required supplemental intravenous amino acids, and very few patients had successful tube placements because of nausea, vomiting, and diarrhea.

These studies raise concerns about the microbiologic safety of enteral nutrition. There was a trend toward more bacteremias in the autologous patients on tube feedings.[79] *Pseudomonas* sepsis was associated with a hospital-made pasteurized formula in one patient undergoing induction therapy despite selective gastrointestinal decontamination to eliminate gram-negative rods and yeast. [78] Significant contamination can also occur with commercially sterile formulas in the clinical setting, owing to open feeding systems, low osmolar formulas, inadequate handwashing, and prolonged hang times at room temperature. [80] [81] [82] [83] [84] Patients receiving antacids, gastric acid inhibitors, H_2-antagonists, antibiotics, steroids, or immunosuppressive therapy may be at increased risk of bacterial colonization during tube feedings. [85]

Several studies of gastrostomies in pediatric cancer patients, which included patients with hematologic malignancies, have reported high success rates in achieving body weight goals and overall low rates of significant complications, [86] [87] although wound infections are higher in patients with cancer compared to patients with a non-cancer diagnosis.[88] Acquino and coworkers[86] reviewed the use of permanently placed gastrostomy tubes in 25 children with cancer; infections at the tube site occurred during periods of severe neutropenia at a rate of only 1.58 episodes per 1,000 days of use compared with rates of 5.0/1,000 days reported with TPN. [89] In another review of surgical or percutaneous endoscopic gastrostomies in 33 pediatric cancer patients, 14 developed infection or colonization with bacteria or fungi at the insertion site, but only 1 patient developed a bacteremia. [87] The authors also reported bleeding complications at the tube site in eight patients, one of whom required surgical exploration to control bleeding after the tube was accidentally dislodged. [87]

For the patient who has a functional gastrointestinal tract and requires nutritional intervention, tube feedings should be considered, as noted in the algorithm. If the anticipated duration of enteral feeding is less than several weeks, then nasoenteric placement is recommended. If a longer period of feeding is expected, a gastrostomy or jejunostomy placement should be considered. Only commercially sterile products should be administered, preferably in a closed feeding system, for a hang time not to exceed 8 hours. In patients with unexplained fever or diarrhea, the enteral feeding should be cultured. Proper tube insertion, formula selection and administration, and monitoring of complications are extensively reviewed by Rombeau and Caldwell. [90]

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TOTAL PARENTERAL NUTRITION

Infectious Complications

Most patients with hematologic malignancies have central catheters placed for central venous access. In patients provided with TPN, an increase in infection rates has been observed compared to patients with central catheters not on TPN. [41] [89] [91] In 310 children with cancer and central venous lines (including 244 patients with acute leukemia), administration of TPN was associated with a 2.4-fold increase in the risk of infection ($p < 0.001$), defined as documented catheter-related septicemia, catheter exit site, port, or tunnel infection, or sepsis of unknown origin. [89] Among 104 adult and pediatric recipients of allogeneic marrow grafts, Weisdorf and coworkers documented bactermias in 72% of patients randomized to TPN versus 48% randomized to hydration therapy during the first post-transplant month ($p < 0.001$).[41] Lough and associates demonstrated similar findings in a smaller study of 29 allogeneic and autologous transplant patients in which 57% of the patients randomized to TPN developed positive blood culture compared to <7% randomized to maintenance hydration for 14 days ($p < 0.05$). [91] No deaths were attributed to the infectious complications described in these reports.

The etiology of the association of TPN and infectious complications remains to be established. Suggested hypotheses for this phenomenon are overfeeding of dextrose or lipid, deficiency of glutamine in TPN formulas, and lack of enteral stimulation of gut immunity. Elevated serum glucose impairs neutrophil and complement function and in particular predisposes patients to *Candida* infections.[92] [93] Maintenance of serum glucose below 200 mg/dl during TPN by targeting energy provision to metabolic needs, administration of insulin as necessary, and substitution of a greater proportion of dextrose energy with lipid may help reduce infection. Analogous to the potential dangers of overfeeding dextrose, it appears that lipids in only large doses (up to 4 g/kg) or at very fast infusion rates (100200 ml/hr of 20% emulsions) demonstrate any adverse effect on neutrophil, phagocyte, and reticuloendothelial system function. [94] There is no evidence that lipids affect humoral immunity or the complement system, although their impact on T-cell lymphocyte activities is not clearly established. [95] In general, an infusion of moderate amounts of lipids (2530% of total calories) over a minimum of 12 hours is considered prudent and safe practice. Indeed, a large randomized trial failed to show an association between such a moderate dose of lipid (2530% of total energy) versus a low dose (68% of total energy to prevent essential fatty acid deficiency) and bacterial and fungal infections during the first hospitalization for marrow grafting in patients with hematologic malignancies. [96]

The addition to TPN of glutamine, a non-essential amino acid oxidized by stimulated lymphocytes and macrophages and intestinal mucosal cells, has been reported to reduce infectious complications[97] and hospitalization time[97] [98] following marrow grafting. It is postulated that pharmacologic doses of intravenous glutamine may maintain the gut mucosal barrier during periods of gut rest, as has been described in animal models of methotrexate or radiation-induced intestinal injury. [99] [100] [101] The adverse effects of non-glutamine-enriched TPN on human intestinal immunity have yet to be confirmed. [102] Data are also lacking on the effect of glutamine supplementation on long-term survival in patients with hematologic malignancies; because glutamine enhances the synthesis of the intracellular antioxidant glutathione, it is possible that pharmacologic doses given at the same time as antitumor therapy could be protective of the tumor.

Finally, the role of enteral stimulation on infection outcome deserves consideration. Numerous trials in surgical patients have demonstrated reduced infectious morbidity and improved survival in patients randomized to tube feeding compared to TPN. [103] [104] Similar trials have not been reported in cancer patients. In the absence of any major gastrointestinal dysfunction, transition to tube feeding or encouragement of oral feeding, even in minimal amounts, are appropriate low-risk interventions in patients requiring TPN until more data are available to guide nutrition therapy in this area.

Efficacy

Do the benefits of weight maintenance and provision of macronutrient and micronutrient substrates outweigh the infectious risks of TPN? Limited studies are available to guide the decision to intervene with TPN. Popp and coworkers [105] found no difference

in long-term survival among patients with advanced diffuse lymphoma undergoing multidrug chemotherapy randomized to adjunctive TPN or to oral nutrition. A few studies suggest TPN may promote hematopoietic recovery during intensive induction therapy for AML [106] or following marrow grafting.[107] In marrow graft recipients, implementation of TPN during the conditioning regimen in well-nourished patients results in improved long-term survival. [41] Weisdorf and colleagues [41] randomized allogeneic and autologous marrow graft recipients to either TPN or hydration through the first month post-transplant. Among control patients, 60% were crossed over to TPN (median day 21 post-transplant) because of nutritional depletion. Overall survival (Fig. 80-1) and time to relapse were significantly improved in the patients receiving prophylactic TPN. When the autograft patients were analyzed separately, significant differences in survival and relapse rates were not found, but the small numbers of autograft patients limited the power of the study to detect true differences. Other studies have not confirmed improved survival with TPN during marrow transplantation but, again, small study populations may have limited the ability to detect differences. [57] [91]

Other patient populations may benefit from TPN. Rickard and coworkers [108] found that children with advanced malignant disease, including relapsed leukemia and advanced NHL, required TPN to reverse malnutrition. Additionally, these clinical investigators identified those children with AML and poor-prognosis ALL as appropriate candidates for TPN.[109] Although improved survival with adjunctive TPN during therapy has not been adequately investigated, maintenance of major organ system function and, in children, growth maintenance and restoration of play are important considerations when oral or enteral feedings fail.

Implementation and Formulations

Safe and appropriate TPN may be initiated and monitored in consultation with a hospital nutrition support service or with a nutrition support-certified or oncology dietitian. Adequate delivery of TPN has been enhanced by the use of multilumen central venous access devices. [110] Iatrogenic fluid overload poses a greater risk to patients receiving multiple intravenous therapies than does inadequate nutrition. Table 80-4 outlines an approach to implementation of TPN.

Research on the pharmacologic applications of nutritional support have intensified as evidence accumulates about the modulation of physiologic, immune, and endocrine functions by nutrients. TPN solutions enriched with branched-chain amino acids have been investigated as a fuel source designed to blunt the muscle catabolism and obligatory nitrogen losses associated with marrow transplantation with mixed success. [42] [111] Taurine, another non-essential amino acid not contained in adult TPN solutions, may also be deficient following marrow grafting. [112] Taurine conjugates hepatotoxic bile acids and improves bile acid secretory rates. [113] Clinical trials may be warranted to determine the benefits of taurine in marrow graft patients who experience a high incidence of liver dysfunction and biliary sludge. [114] Glutamine-supplemented TPN is discussed earlier under infectious complications.

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NUTRITIONAL SUPPORT IN MARROW AND PERIPHERAL STEM CELL TRANSPLANTATION

Standard supportive care during the first month post-transplant has included TPN, [41] [42] [97] [110] and many patients with slow gastrointestinal healing or graft-versus-host disease (GVHD) have relied on TPN to some degree for extended periods in the ambulatory setting. [115] As other supportive measures diminish the toxicity associated with transplantation, the role of TPN as well as its substitution with enteral feedings will require re-examination.

Early initiation of TPN should be considered in patients with impaired pretransplant nutritional status, oral intake less than basal needs for 3 to 4 days, or gastrointestinal toxicities that are anticipated to interfere with recovery of oral intake within 1 week. Among patients who are at an increased risk of GVHD, early TPN intervention helps prevent significant weight loss before the onset of acute or chronic GVHD when metabolic needs appear higher and may be more difficult to achieve.[116] The decision to terminate TPN may depend on a constellation of clinical factors, including nutritional status, severity of gastrointestinal toxicities, ability to sustain or progress with oral intake, and the physiologic and psychological impact of TPN. In a large, double-blind trial that included adult and pediatric allogeneic and autologous transplant patients, Charuhas and colleagues compared the effect of continuing TPN or changing to hydration fluids on the time to resume adequate oral intake in patients who were not able to eat 70% of estimated maintenance energy needs at time of hospital discharge. [117] Patients randomized to hydration fluids met oral calorie goals a median of 6 days sooner than patients on TPN (p = 0.049) without evidence of adverse consequences, such as increased hospital readmissions or clinically significant weight loss, when TPN was withheld for up to 1 month. It appears safe to discontinue TPN when patients are eating 30% of estimated energy needs at hospital

Figure 80-1 Influence of TPN on overall survival in marrow graft recipients. Life-table curves (Kaplan-Meier plot) show overall survival in TPN prophylaxis versus control groups, p = 0.011. Tick marks indicate length of survival at analysis. *(From Weisdorf et al.,[41] with permission.)*

TABLE 80-4 -- Approach to TPN Management

1. Determine macronutrient requirements (protein, dextrose, lipid, and fluid) .

Nutrient	Adult	Adolescent	Children
Protein (g/kg) *Available as crystalline amino acids 4 kcal/g protein*			
Maintenance	1.0	1.0	1.2 (710 yr)
			1.5 (46 yr)
			1.8 (13 yr)
Stress	1.52.0	2.0	2.4 (710 yr)
			3.0 (16 yr)
Energy (kcal/kg)			
Maintenance 1.21.3 × basal needs	30	4050	5060 (710 yr)
			6070 (46 yr)
			7085 (13 yr)
Rehabilitation/stress 1.5 × basal needs	4045	4565	6575 (710 yr)
			7590 (46 yr)
			90100 (13 yr)
Carbohydrate (mg/kg/min)[a] *Available as dextrose monohydrate 3.4 kcal/g*	<5	<710	<1015
Lipid *Available as long chain triglycerides (soybean or safflower oil, glycerol, egg-yolk phospolipid) in 10% (1.1 kcal/ml), 20% (2 kcal/ml), and 30% (3 kcal/ml) solutions; 20% and 30% lipids preferred because of lower phospholipid content*	% of total kcal: Minimum 68% Maximum 60%		14 g/kg
Maintenance fluid	1,500 ml/m^2		100 ml/kg up to 10 kg
			+50 ml/kg for each kg 1120 kg
			+20 ml/kg for each kg 2140 kg

2. Provide electrolytes, vitamins, and trace elements daily, altering requirements based on organ function .

Electrolyte or Micronutrient	Adult	Adolescent	Children
Potassium[b] *Available as phosphate, chloride, and acetate salts. Increased needs with amphotericin, thiazide diuretics, steroids, anabolism, GI losses. Decreased needs with spironolactone, renal dysfunction, tumor lysis syndrome.*	60120 mEq		23 mEq/kg
Sodium[b] *Available as chloride, acetate or phosphate salts. Increased needs with GI losses. Decreased needs with pulmonary edema, congestive heart failure, VOD*	50100 mEq		24 mEq/kg
Calcium *Available as gluconate salt. Increased needs with foscarnet. Decreased need with renal dysfunction, tumor lysis syndrome*	1025 mEq	0.250.5 mEq/kg	1030 mEq

Phosphorus *Available as potassium or sodium salts. Increased needs with diuretics, cyclophosphamide, cisplatinum, foscarnet, anabolism. Decreased needs with renal dysfunction*		1020 mM	0.51.0 mM/kg	12 mM/kg
Magnesium *Available as sulfate salt. Increased needs with amphotericin, cyclosporine, cisplatinum, foscarnet, GI losses, anabolism. Decreased needs with renal dysfunction, tumor lysis syndrome*		1632 mEq		0.250.5 mEq/kg
Zinc *Available as chloride or sulfate salt in both single entity or combination preparation. Increased needs with GI losses, wound healing*		2.54.0 mg		100 g/kg[c]
Copper *Available as sulfate salt in both single entity or combination preparation. Increased needs with GI losses. Decreased needs with cholestasis.*		0.51.5 mg		20 g/kg[c]
Manganese *Available as chloride salt in both single entity or combination preparation. Decreased needs with cholestasis.*		150800 mcg		210 g/kg[c]
Chromium *Available as chloride salt in both single entity or combination preparation*		1015 mcg		0.2 g/kg[c]
Selenium *Available as selenious acid in both single entity or combination preparation*		4080 mcg		3 g/kg
Electrolyte or Micronutrient		**Adult**	**Adolescent**	**Children**
Molybdenum *Available as ammonium molybdate as single entity.*		20120 g		0.25 g/kg
Vitamins *Available as adult and pediatric multivitamin combinations of water- and fat-soluble vitamins or single infusions of cobalamin, folate, thiamine, pantothenic acid, pyridoxine, ascorbic acid, and vitamin K.*	A	3,300 IU (990 RE)		2,300 IU (690 RE)
	D	200 IU (5 RE)		400 IU (10 RE)
	E	10 IU (6,7 RE)		10.4 IU (7 RE)
	B_1 (thiamine)	3.0 mg		1.2 mg
	B_2 (riboflavin)	3.6 mg		1.4 mg
	Niacin	40 mg		17 mg
	Folic acid	400 g		140 g
	B_6 (pyridoxine)	4 mg		1.1 mg
	Panthothenic acid	15 mg		5.0 mg
	Biotin[d]	60 g		20 g
	B_{12} (cobalamin)	5 g		1.0 g
	C	100 mg		80 mg
Vitamin K *Available as a component of pediatric but not adult multivitamin combinations*		12 mg (or 10 mg once weekly)		200 g (as part of multivitamin combination)
Iron *Available as iron-dextran*		Contraindicated in patients with hematologic malignancies		

***3. Monitor for special managements issues*.**	
Refractory hyperglycemia	Limit dextrose to <3 mg/kg/min in adults, <710 mg/kg/min in children; increase lipids to 5060% total energy
Hypertriglyceridemia	>500 mg/dl: limit lipids to 48% total energy to provide essential fatty acids
	>2000 mg/dl: discontinue lipids to decrease risk of pancreatitis
Fluid overload	Provide concentrated dextrose, lipid, amino acid solutions to maximize nutrient support
4. Monitor ability to eat or transition to tube feeding, or both, as soon as feasible.	

Abbreviations: GI, gastrointestinal; TPN, total parenteral nutrition, VOD, veno-occlusive disease.

Recommended daily trace element supplementation for adults from American Medical Association: Guidelines for essential trace element preparations for parenteral use. JAMA, 241:2051, 1979.

Recommended daily vitamin supplementation for adults and adolescents from Multivitamin preparations for parenteral use: A statement by the nutron advisory group. JPEN J Parenter Enteral Nutr 3:258, 1979.

Recommended daily vitamin, mineral and trace element supplementation for children from Greene HL, Hambridge KN, Schanler R et al: Guidelines for the use of vitamins, trace elements, calcium, magnesium, and phosphorus in infants and children receiving total parenteral nutrition: Report of the Subcommittee on Pediatric Parenteral Nutrient Requirements from the Committee on Clinical Practice Issues of the American Society for Clinical Nutrition. Am J Clin Nutr 48:1324, 1988.

[a]To calculate carbohydrate dose, divide total mg dextrose per day by 1,440 min/day/wt in kg. For example, 1,700 ml 25% dextrose in a 70 kg patient = 425 g dextrose × 1,000 mg/g 1,440 min/day/70 kg = 4.2 mg/kg/min dextrose.
[b] After determining phosphate needs, remainder provided primarily as chloride salt (except in presence of metabolic acidosis) to meet chloride needs.
[c]Up to 5 years; older than 5 years, use guidelines for adolescent and adult.
[d]Currently available multivitamin combinations do not contain biotin.

discharge unless a clinical condition, such as malnutrition, malabsorption, or significant gastrointestinal toxicities, warrant otherwise.

Total energy and protein (oral plus TPN) support is recommended at stress levels during the neutropenic period based on the well-described losses of nitrogen and body cell mass.[37] [42] [118] Patients who still require TPN after engraftment and are otherwise without infectious problems or GVHD may be supported at maintenance needs. Few studies have been reported to characterize micronutrient status following marrow grafting. Serum levels of the antioxidants vitamin E and -carotene in patients on TPN are markedly depressed, but the clinical significance is unknown. [119] Preliminary reports suggest that depleted antioxidant status is associated with increased free radical activity post-transplant. [120] [121] No correlations have been established between these findings and the degree of toxicities observed with cytoreductive therapy nor the effect on tumor cell killing. In experimental models, vitamin C at therapeutic doses protects bone marrow cells, [122] suggesting cautious use of antioxidants with our current state of knowledge. Manganese toxicity that manifested as a Parkinson-like syndrome with brain magnetic resonance imaging suggestive of manganese accumulation has been described in a patient with cholestatic liver disease who was supplemented with 0.3 mg/day of manganese during 2 months of TPN.[123] These investigators found elevated serum manganese levels in eight other transplant patients with liver disease, suggesting the need to restrict manganese in the presence of cholestasis. [123]

Gastrointestinal Complications

Histologic and functional abnormalities of the intestinal tract result from cytoreduction therapy, infectious complications, and GVHD and have been reviewed by Beschorner[124] and McDonald and colleagues.[125] Chemoradiotherapy results in moderate to extensive necrosis of intestinal crypts and diffuse mucosal abnormalities, which resolve histologically by day 20 post-transplant. [125] Normal digestion and absorption may not recover as quickly. Both proximal and terminal small intestinal absorption is impaired for prolonged periods, even in the absence of GVHD, as evidenced by abnormal D-xylose and Schilling tests at 4 months post-transplant. [126] [127] [128] Permeability studies suggest that mucosal damage is equivalent for conditioning with chemotherapy only and chemoradiotherapy and is greater in patients over 30 years of age.[129]

Mucosal competence is also impaired. Salivary IgA concentrations

are one-half normal levels as late as day 90 post-transplant. [130] Whether IgA secretions at other intestinal sites show similar prolonged immunodeficiency is unknown. The number of immunoglobulin-containing plasma cells in the small intestine decreases after chemoradiotherapy but appears to recover within weeks, provided intestinal GVHD does not develop. [131]

Nausea, vomiting, diarrhea, and oropharyngeal mucositis inhibit oral intake for 1 month (Fig. 80-2). The severity of mucositis is increased in patients treated with total-body irradiation[132] or methotrexate as prophylaxis against GVHD.[133] Limited data are available on the way in which growth factors might influence recovery from mucositis. Nemunaitis and coworkers[134] described no difference in the severity of mucositis in a randomized, double-blind trial of granulocyte-macrophage colony-stimulating factor (GM-CSF) in autologous marrow graft recipients, most of whom received total-body irradiation as part of cytoreduction therapy. When TPN was used as a surrogate for gut toxicity, Sheridan and colleagues found decreased use in patients who received GM-CSF after busulfan and cyclophosphamide and autologous marrow grafting when compared with historical controls, [135] whereas Bruemmer and coworkers observed no difference in length of TPN support in patients undergoing unrelated allogeneic marrow grafting with or without GM-CSF. [136] The effect of peripheral versus marrow as the source of stem cells on recovery of oral intake has not been reported.

The use of pharmacologic doses of oral glutamine to reduce the severity of oral mucositis following BCNU, etoposide, and melphalan and autologous grafting for hematologic malignancies has been explored by Jebb and coworkers.[137] No differences in patient or observer mucositis score, diarrhea, or days of TPN required were observed in a double-blind trial that randomized patients to 16 g glutamine or placebo daily from day 1 post-transplant until mucositis resolved.

Organ Complications

Veno-occlusive Disease

Hepatic veno-occlusive disease (VOD) is characterized by occlusion of small hepatic veins, damaged hepatocytes, fluid retention, ascites, hepatomegaly, and jaundice. The pathophysiology, diagnosis, and treatment of this disease have been reviewed by Shulman and Hinterberger. [138] Insidious weight gain is the first sign of the disorder, and if it progresses, more severe symptoms of liver failure occur, including encephalopathy, coagulopathy, and renal failure. [139] [140] [141] Fluid and TPN management are controversial because limitation of fluids to minimize edema and ascites is frequently complicated by intravascular volume depletion and deterioration of renal function. Conversely, repleting the intravascular space often results in pulmonary edema and massive ascites.

Daily weights and thrice weekly serum bilirubins in the immediate post-transplant period facilitate early detection of disease in patients at increased risk. Nomograms for six time intervals between day 1 pretransplant and day +16 post-transplant that estimate the probability of developing severe VOD using total serum bilirubin and percent weight gain above baseline have been published. [142] In patients with weight gain and decreased urinary sodium excretion, restriction of total fluid and sodium may decelerate fluid accumulation. Concentrated TPN solutions are indicated if renal perfusion is adequate. The capacity to eliminate intravenous lipids from the bloodstream should be monitored in patients with severe VOD. If encephalopathy develops, the benefits of Hepatamine (an amino acid solution with lower aromatic amino acids, tryptophan, and methionine content) have not been established. [143] If hyperbilirubinemia persist longer than 1 week, the biliary-excreted trace elements, copper and manganese, should be removed from TPN. Measurement of energy needs with indirect calorimetry if available may avert additive hepatotoxicity associated with overfeeding, as well as the risks of debilitation with prolonged underfeeding.

Renal Disease

Renal insufficiency is typically multifactorial and associated with total-body irradiation, chemotherapy, drug toxicities, sepsis, and especially liver toxicity. [144] Renal damage is suspected when serum creatinine level is twice baseline. An elevated blood urea nitrogen, however, may be partially due to nonrenal factors, including increased protein intake, gastrointestinal bleeding, or hypercatabolism. Prolonged protein restriction to minimize a rise of blood urea nitrogen should be avoided to ensure that adequate calorie and protein support are provided.

Figure 80-2 Average daily oral calorie intake in adult marrow transplant recipients (n = 295).

Renal complications are managed by maintaining intravascular volume and correcting electrolyte imbalances. The large fluid load necessitated by TPN is problematic in the oliguric patient and requires daily manipulation, depending on urine output and clinical signs of fluid overload. Hypervolemic hyponatremia is treated with water restriction to prevent congestive heart failure. General indications for hemodialysis are extracellular fluid volume expansion, acidemia, hyperkalemia, and azotemia.

The primary goal of nutritional therapy in acute renal failure is to minimize uremia toxicity and other metabolic derangements and yet prevent malnutrition. Protein levels are typically restricted before dialysis by the TPN volume tolerated. During dialysis, protein intake should meet stress needs as defined in Table 80-4 . Serum triglycerides need to be monitored weekly because the clearance of intravenous lipids may be reduced. [145] Water-soluble vitamins are lost in the dialysate and should be provided instead of standard intravenous multivitamins, in an effort to prevent excessive serum vitamin A levels. [146]

Pulmonary Disease

Pulmonary edema due to increased capillary permeability following cytoreduction therapy may be compounded by iatrogenic fluid overload. Management includes reducing total sodium from oral intake, TPN, and medications, and using concentrated TPN solutions. During ventilator dependency, adequate TPN or tube feeding should be provided to preserve muscle reserves.

Graft-versus-Host Disease

Acute GVHD

Minimal to life-threatening skin, liver, and intestinal GVHD can occur as early as the first week post-transplant, but more typically after evidence of donor engraftment. Approximately 30% of patients with human leukocyte antigen-identical sibling donors develop clinically significant GVHD. Both the incidence and severity are higher in patients who are older, undergo transplants from an unrelated or human leukocyte antigen-incompatible family donor, or fail to tolerate sufficient prophylactic drug therapy.[147]

In the most severe form of acute intestinal GVHD, voluminous diarrhea is a prominent manifestation, with the volume corresponding to the extent of mucosal damage.[148] The diarrheal fluid is green and watery, with ropy strands of mucus, protein, and cellular debris, and often contains occult blood. Protein content is high, as evidenced by falling plasma protein levels or by measurements of fecal -antitrypsin in fecal water. [149] Associated symptoms include anorexia, nausea, vomiting, and crampy abdominal pain, which may be related to food ingestion or may occur spontaneously owing to the secretory nature of the diarrhea. Other causes of diarrhea should always be ruled out because enteric pathogens and late-onset conditioning toxicities may occur with similar signs and symptoms of acute intestinal GVHD. [150] Biopsy findings range from necrosis of individual intestinal crypt cells to total mucosal denudation. [151] [152] Intestinal function has received limited study, and the characteristics of malabsorption have yet to be determined. No evidence suggests that early post-transplant restriction of food protein as potentially antigenic stimulants decreases the risk of acute GVHD.[153] Fluid and electrolyte management may be problematic in patients with large volume diarrhea (>2.53.0 L/day). Antidiarrheal agents are generally contraindicated because of the risk of ileus and abdominal distention. The use of octreotide acetate has been reported to control

diarrhea with variable success, but may lower serum cyclosporine levels. [154] [155] [156]

Weisdorf and colleagues [157] described a syndrome of upper intestinal GVHD presenting clinically as anorexia, dyspepsia, food intolerance, nausea, and vomiting. The clinical picture frequently progressed to symptomatic lower gastrointestinal involvement after failure of immunosuppressive therapy, suggesting that this syndrome may be an early manifestation of a unique intestinal immunopathology. Prolonged nausea and vomiting constitute indication for endoscopic evaluation to establish a diagnosis of stomach or upper intestinal GVHD. [158]

The oral mucosa may also be involved in acute GVHD. Oral examination reveals erythema and lichenoid changes that cannot be differentiated from conditioning-induced mucositis until approximately 3 weeks post-transplant. [159] [160] It may evolve into a chronic form, and patients may develop taste fatigue with prolonged use of oral supplements and bland foods, experiencing significant weight loss unless supported with TPN or tube feeding.

Acute liver GVHD is characterized by abnormal liver function tests, jaundice, and mild hepatomegaly. [125] Hepatic synthesis and enterohepatic circulation of bile salts may be diminished or inhibited, resulting in steatorrhea.

Adequate nutrition is a vital adjunct to immunosuppressive drugs in the treatment of acute GVHD. Energy needs during acute GVHD have been predicted at 4550 kcal/kg in adults and 65 kcal/kg in children. [116] In intestinal GVHD, the patient is initially dependent on TPN because complete bowel rest is the only means of decreasing diarrhea. [148] Additional daily zinc is recommended at 10 mg/L of stool volume in excess of 1 liter. When diarrheal volumes diminish and abdominal pain subsides, isotonic oral liquid supplements are introduced to stimulate intestinal regeneration and assess absorption. Guidelines for the introduction of oral intake have been empirically derived using a five-phase regimen, which emphasizes foods low in lactose, fat, fiber, and total acidity. [161] Severe ileal involvement or liver GVHD may necessitate prolonged fat restriction.

Drug therapy for GVHD often has a profound effect on nutritional status and may require nutritional intervention as summarized in Table 80-5 .

Chronic GVHD

Chronic GVHD is a multisystem disorder that affects 3540% of allogeneic marrow graft recipients, presenting 70400 days post-transplant. [162] Clinical manifestations that may adversely affect nutritional status include anorexia, mucositis, xerostomia, dysphagia, esophageal stricture, cholestatic liver disease, diarrhea or steatorrhea, dyspnea and limited exercise tolerance, restricted joint mobility, and generalized wasting (Table 806). Even skin disease can affect nutritional therapy in that adequate sources of vitamin D must be achieved through diet or multivitamin supplementation because the use of sun-blocking agents diminish photosynthesis of vitamin D in the epidermis. Because immunosuppressive therapy invariably includes steroids, vitamin D and calcium status must be optimized to prevent clinically significant osteoporosis. Loss of vertebral bone mass at 1 year post-transplant has been described by Stern and investigators in a small series of patients treated with cyclosporine and corticosteroids. [163] In prescribing a multivitamin/mineral supplement, iron-free formulas are recommended because iron overload is a frequent finding in long-term survivors. [164] When skin disease is treated with psolaren plus ultraviolet A radiation, supplemental -carotene, which protects against ultraviolet radiation, [165] should be discouraged.

Oral disease is common, with frank stomatitis occurring in a significant portion of patients. Pain, burning, and loss of taste have been described as prodromes of chronic GVHD. [166] In severe cases, only bland liquids and soft foods may be tolerated. Complete nutritional supplements and nutrient-dense carbohydrate polymers are important dietary adjuncts in patients experiencing

TABLE 80-5 -- Nutritional Consequences of GVHD Immunosuppression

Immunosuppressive Agent	Nutritional Effects	Recommended Interventions
Corticosteroids	Muscle wasting	Early intervention with physical therapy if exercise level low; high protein intake (twice normal requirements)
	Sodium and fluid retention	Low sodium diet if edema limits mobility
	Hyperphagia and weight gain	Regular exercise, dietary counseling
	Hyperglycemia	Insulin therapy, blood glucose monitoring, dietary counseling to limit overfeeding and excessive total carbohydrate intake. Oral agents have not been shown to be safe or efficacious
	Hyperlipidemia	No intervention (lipid-lowering agent if patient with history of pancreatitis); reassess plasma lipids several months after discontinuation of drug to assure return to baseline level
	Bone loss, fracture risk	Vitamin D: 400800 IU/day
		Calcium: 8001,200 mg/day children, 151,600 mg/day adolescents and adults
		Bone density studies, endocrinology consult in patient with suspected osteoporosis
Cyclosporine, FK-506	Renal insufficiency	Maintenance fluid or more via oral ± intravenous; routine monitoring serum creatinine
	Magnesium wasting	Magnesium replacement via oral ± intravenous route; oral dosing often limited by diarrhea (protein complex forms may be better tolerated); routine monitoring serum magnesium
	Hyperglycemia	May exacerbate hyperglycemia observed with corticosteroids (see corticosteroids)
	Hyperlipidemia	Usually not to same degree as with corticosteroids (see corticosteroids)
	Hyponatremia	Limit free water; encourage fluid with solute
Mycophenolate mofetil	Diarrhea, vomiting	Rule out other causes of diarrhea; otherwise antiemetic therapy and adequate hydration status

weight loss. If significant weight loss occurs, gastrostomy feeding may be indicated.

Esophageal webbing or stricture can result in severe swallowing difficulties. [125] Typical symptoms include pain and difficulty in swallowing food and pills, as well as retrosternal pain caused by esophageal thinning. Webs and strictures are managed with periodic dilation. Diet tolerance varies widely and may be limited to liquids in patients with extensive webbing. Gastrostomy tubes may be indicated when the passage of food is obstructed.

TABLE 80-6 -- Nutrition-Related Problems 1 Year After Bone Marrow Grafting

Sign/Symptom	Chronic GVHD Status[a]		
	None (%)	Limited (%)	Extensive (%)
Weight loss	27	19	33
Weight gain	14	28	34
Oral sensitivity	7	14	41
Xerostomia	10	11	27
Stomatitis	3	3	14

Anorexia	3	6	13
Reflux symptoms	1	6	12
Diarrhea	3	3	12
Steatorrhea	1	0	9
Dysgeusia	0	0	6
Esophageal stricture	0	0	2
Dyspnea	0	0	7
Contractures	0	0	2

Adapted from Lenssen P, Sherry ME, Cheney CL et al: Prevalence of nutrition-related problems among long-term survivors of allogeneic marrow transplantation. With permission from Journal of the American Dietetic Association, 90:835, 1990. Copyright the American Dietetic Association.

[a]Among 192 allogeneic transplant recipients. Percentage of total number of patients is shown.

Diffuse intestinal involvement is a rare manifestation of chronic GVHD. [125] Bacterial overgrowth, medications, chronic liver GVHD, and pancreatic insufficiency may contribute to diarrhea or steatorrhea. When weight loss occurs in chronic liver GVHD, the relative contributions of deficient energy intake and malabsorption need to be determined. Patients are often anorectic, yet others have good dietary intake but significant stool nutrient losses. A malabsorption work-up may include quantitative stool fat collection, Sudan stain for stool fat, serum carotene, serum D-xylose or Schilling test, or serum vitamin D. Vitamin D status is of special concern because inadequate hepatic and renal hydroxylation to the active metabolite may occur. In patients with malabsorption, a moderate fat restriction, calorie supplementation with medium-chain triglycerides, and mineral and fat-soluble vitamin supplementation may be necessary interim measures. Some patients respond to pancreatic enzymes. In patients who fail to gain weight, a gastrostomy feeding should be considered given the known slow resolution of the disease.

Children with extensive chronic GVHD represent a special risk group. Decreased height velocity has been related to pretransplant cranial irradiation, both chemotherapy-only and total-body irradiation conditioning regimens, and chronic GVHD. [51] [52] Although prednisone therapy has been incriminated in growth stunting, catch-up growth has not been observed following cessation of treatment. [51] The contribution of poor nutrient intake as a factor in growth failure after transplantation has not been investigated. Children displaying weight loss, inappropriate weight gain, or growth failure deserve thorough nutritional evaluation to rule out treatable dietary deficiencies. Height should be monitored every 3 months post-transplant for the purpose of early detection of growth failure. When growth velocity falls below the third percentile, treatment with growth hormone is recommended to optimize growth potential. [167]

ACKNOWLEDGMENT

Preparation of this work was supported in part by grant #DK35816 from the Clinical Nutrition Research Units, US Department of Health and Human Services.

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Chapter 81 - Psychosocial Aspects of Hematologic Disorders

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Major changes in the understanding and treatment of cancer have led to a wider range of treatment options and increasing length of survival from time of diagnosis. Although such advances are quite positive, their impact on the emotional lives of patients and families faced with complex treatment decisions as well as the emotional consequences of chronic illness cannot be overlooked.

Hematologic malignancies often carry a large burden of emotional consequences attributable to the chronic nature of the diseases. Involvement with a complicated and fragmented health care delivery system; the need for episodic and aggressive treatment; remissions and exacerbations of acute and uncomfortable symptoms; family separation; financial burden; functional limitations and role disruptions are but a few of the

issues that characterize the life of patients with hematologic malignancies, not to mention the threat to life that these diagnoses impose.

Because hematologic malignancies are known to be fatal when untreated, their diagnosis often leads to fear and intense psychological concerns. Psychosocial issues associated with all cancers may be intensified in patients with hematologic malignancies because of their association with a poor prognosis, a prolonged treatment course often involving numerous hospitalizations, and the systemic nature of the diseases. [1] Death, discomfort from painful medical procedures, body image disturbances secondary to hair loss, central line catheters, sexual dysfunction related to fatigue and fertility, and role and relationship disruptions resulting from lengthy hospitalizations are just a sampling of the issues that confront the patient with hematologic malignancies, highlighting the necessity for monitoring of and attention to the psychosocial needs that accompany all aspects of the illnesses.

The goal of this chapter is to provide information on factors that affect psychosocial adjustment among patients with chronic illness, the wide range of psychological responses that are possible throughout the illness trajectory, and the efficacy of various modes of psychosocial intervention in minimizing distress and promoting adaptation. Some practical guidelines regarding patient management and identification of patients who may require formal psychiatric consultation are offered.

CHANGES IN TREATMENT AND SOCIETAL ATTITUDES: ACCOMPANYING TRENDS IN PSYCHOSOCIAL ISSUES

The secrecy that prevailed in the 1960s and prohibited disclosure of a cancer diagnosis by most physicians [2] [3] has given way to the practice of imparting the particulars of diagnosis, treatment options, and prognosis on a routine basis. Despite this change, it has been suggested that ongoing fears and concerns among health care providers about cancer have led to a discrepancy between attitude and action, resulting in communication of emotionally laden information in a fashion ranging from overprotective and paternalistic to blunt and matter-of-fact. [4] [5]

Discrepancies between attitude and practice have also been demonstrated by clinicians who avoid clear, open discussions of topics such as prognosis and death despite consistently expressed beliefs regarding the importance of openness and honesty with all mentally competent patients. Therefore, although the prevailing attitude in health care supports the disclosure of medical information and active involvement of patients in decisions that affect them, the actual behavior of health care providers likely reflects a more limited improvement in patient care. [5]

Clinicians should be mindful of how their personal views and concerns about cancer affect communication with patients, especially at key decision-making and transition points along the continuum of care. The use of peers and alternate providers to assist patients with issues and concerns that are particularly upsetting for primary clinicians are useful strategies for ensuring maximum support for patients faced with the complex decisions inherent in the treatment of hematologic malignancies.[6]

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CLINICAL COURSE OF HEMATOLOGIC MALIGNANCIES

In contrast to many solid tumor cancers, treatment of hematologic malignancies often involves numerous and lengthy hospitalizations and ongoing outpatient monitoring of the patients condition. The clinical course of cancer typically follows one of several possible trajectories (Fig. 81-1). A number of patients respond to the curative attempt with long-term remission, remain well, and are, after a period, considered cured. Some patients

Figure 81-1 Clinical course of a patient with a hematologic malignancy. *(Adapted from Levenson JA, Lesko LM: Psychiatric aspects of adult leukemia. Semin Oncol Nurs 6:76, 1990, with permission.)*

have a positive response to a curative attempt but then relapse. Other patients begin treatment with a hope for cure, but do not respond and progressively decline. In some patients, the disease is too far advanced when diagnosed, and they experience a progression of their disease.

The acute stress response[7] has been described as a usual response to the diagnosis of cancer, occurring at each transitional point of illness (beginning treatment, relapse, treatment failure, disease progression). The response is characterized by shock, disbelief, anxiety, depression, sleep and appetite disturbance, and difficulty performing activities of daily living. The acute stress response usually subsides when the medical condition stabilizes and the patient knows what to expect in terms of a treatment plan. Because of the protracted hospitalization associated with the hematologic malignancies and often life-threatening complications such as neutropenia and sepsis, the acute stress response may occur periodically, especially during times of medical crisis or periods associated with excessive physical symptomatology, or both. Emotional support, giving clear information, controlling physical symptoms such as pain and nausea, and enhancing patient control over the environment can enhance resolution and inhibit more severe and long-standing emotional sequelae.

Diagnostic Phase

The prediagnostic and diagnostic phases are the time of initial symptom discovery. Systemic symptoms such as weakness and fatigue are often ignored initially; [1] medical advice is usually sought when symptoms persist and worsen. The period of suspect symptoms associated with a definitive diagnosis is often characterized by fear, shock, and disbelief.

The period from time of diagnosis through initiation of treatment is characterized by medical evaluation, the development of new relationships with unfamiliar medical personnel, and the need to integrate a barrage of information that is at best frightening and confusing. Within the context of this anxiety-provoking situation, a decision must be made regarding treatmentas one man aptly stated, a decision upon which my very life or death might be based. This statement illustrates the tremendous responsibility, concern, and isolation that many people experience during this period. [8]

Because of the particularly poor prognosis often associated with hematologic malignancies by the general public, patients and their families are often anxious when receiving initial information regarding diagnosis and treatment. Consequently, care should be taken to repeat information at several sessions and to inquire about the patients and familys understanding of facts and options.

TABLE 81-1 -- Factors That May Predict Poor Coping in Patients with Cancer

Past psychiatric history
Limited social support
Alcohol or drug abuse
Recent losses
Advanced disease
Uncontrolled symptoms
Pessimistic outlook on life
Multiple obligations

Adapted from Rowland,[45] with permission.

Weissman and Worden[9] described the first 100 days after diagnosis as the period of existential plight in cancer. Patient concerns focused on existential issues of life and death more than on concerns related to health, work, finances, religion, self, or relationships with family and friends. Although it is unusual to observe extreme and sustained emotional reactions as the first response to a cancer diagnosis, it remains important to assess the nature of the patients reaction carefully. Initial reactions are often predictive of later adaptation. [10] [11] Early assessment by clinicians can help to identify people at risk for later adjustment problems and in greatest need of ongoing psychosocial support [12] [13] (Table 81-1).

Initial response to diagnosis may be profoundly influenced by a persons prior association with cancer. [1] Those with memories of close relatives with cancer often demonstrate heightened distress, particularly if the relative died or had negative treatment experiences. During the diagnostic and early treatment period, patients may search for explanations or causes for their cancer and may struggle to give personal meaning to their experience. [14] Because many clinicians are guarded about disclosing information until a firm diagnosis is established, patients may develop highly personal explanations that can be inaccurate and provoke intensely negative emotions. Ongoing involvement and accurate information minimize uncertainty and the development of maladaptive coping strategies based on erroneous beliefs.

Although the literature substantiates the devastating emotional impact of a cancer diagnosis, it is also well documented that many patients cope effectively. Positive coping strategies such as taking action and finding favorable characteristics in the situation have been reported as effective. Maintaining optimism [15] and having an active determination to recover have been associated with positive adjustment. Contrary to the beliefs of many clinicians, denial has also been found to assist patients

in coping effectively with a diagnosis of cancer, unless used to an excessive degree.

With the firm establishment of the cancer diagnosis, planning for treatment begins. If patients have been given a clear explanation of their condition while encouraged to maintain hope, the initial reaction of shock, fear, and desperation can give way to a sense of optimism. [7] Health care providers have an important role in monitoring and possibly mediating psychosocial adjustment. Keeping patients informed and actively involved in their care and being aware of the unique meaning that people may associate with a diagnosis of cancer are vital. Those with pervasive and unyielding negative affect that persists long after that crisis of diagnosis may require ongoing psychosocial intervention throughout treatment and the disease course. [16]

The Treatment Decision

Psychosocial factors are critical parameters in considering which treatment is best for a particular patient. [17] The development of a treatment plan should include information about all aspects of the medical/surgical treatments as well as what is known about the psychosocial sequelae.

Often, patients react to a diagnosis of cancer with feelings of fear and helplessness. The patient looks to the primary physician for a curative treatment that will preserve quality of life. The patient may feel threatened and think nothing can be done but rely on the doctors. Combating feelings of helplessness during this period can help to alleviate painful anxiety. This is best done by a member of the health care team who has established a treatment alliance with the patient. The health care provider should make the patient feel like a partner in everything that takes place. This is especially true regarding decisions about treatment choice.

Active treatment for cancer usually initiates another acute phase of the cancer experience. It can occur while a patient is receiving treatment, or as a complication of treatment. An important standard of clinical practice, based on extensive research, is to provide patients with information to prepare them for treatment. These studies provide convincing evidence that patients who receive specific information about the nature, pattern, and timing of treatment side effects report less disruption in activity than those who are not given this detailed information. [18] Some providers wait until patients complain about potentially expected side effects. When this happens, patients may become skeptical about the completeness or accuracy of any future information given by the person. This potential distrust has important implications for decision making, for patient choice of care-setting in the future, and for recommendations made by patients to others who are seeking a source of cancer care.

The Decision for Bone Marrow Transplantation

Bone marrow transplantation (BMT) is used increasingly for the treatment of malignant and hematologic disorders. [19] The decision to undergo a BMT is a major life crisis for the patient and family. In addition to the very real threat of death, patients experience social isolation, bodily discomfort, major body image changes, and a sense of loss of control. These issues lead to a myriad of emotions, including hope, anger, depression, anxiety, anticipation, guilt, and joy. [20]

Every aspect of the patients personal and professional life is disrupted with BMT. [21] The financial burden is tremendous, including medical expenses for the patient and marrow donor in the case of an allogeneic BMT, in addition to potential travel expenses and loss of income for other family members. [22] Patients and their immediate families are often far from usual support systems because of the distance to the BMT center. In some cases, family members who have not been close in the past may be forced by the situation to interact with each other, leading to additional stress.

Grant and colleagues[23] recently reported that BMT patients seem to experience more fatigue than other patients in studies done on quality of life. The effects of treatment for cancers of all stages can include transient or permanent physical changes, distressing physical symptoms, and functional impairments. Increasingly, intensive cancer therapies, given in the hospital, produce severe and at times life-threatening side effects. The somatic symptoms often experienced by hospitalized patients with cancer include chills, fever, stomatitis, pain, nausea, vomiting, alterations in mobility, inability to tend to self-care needs, and, at times, total dependence on caregivers. There are a number of studies that have been done on BMT recipients, and a range of physical, psychological, and social problems have been identified.

Some preliminary studies have linked negative emotions such as depression with long-term survival among BMT patients. [24] [25] Although such studies have been small and inconclusive and recognize the multiple factors contributing to outcome,

further research in this area is needed. Several BMT centers use routine psychosocial screening [26] and follow-up of BMT patients because of the length of the hospitalization, the aggressiveness of the treatment protocols, and the high incidence of complications and physical discomfort. Interventions that maximize patient control have proven useful.[16] Fear of the unknown can be decreased through patient education about procedures and potential complications. Participation in support groups during hospitalization has proven to be helpful to both patients and family members. [27] In addition to routine psychosocial assessment and follow-up evaluation, routine social work referrals are recommended to assist patients in dealing with the commonplace financial and insurance issues. [28] [29]

Cancer is well recognized as a chronic disease, requiring patients to undergo treatment and long-term surveillance months and years after diagnosis. Strauss [30] has identified critical problems needing attention if patients and families are to be helped to normalize life with chronic illness. These include management of regimens and symptoms, redesigning lifestyles, minimizing financial stress, and confronting psychological, marital, and family problems.

Survivorship

The successful treatment of the hematologic malignancies has resulted in cure for many patients and progressively longer lives for others. Longer survival, however, is not without significant emotional sequelae. [11] [31] [32] [33] [34] [35] Innovative and new treatments may produce long-term physiologic consequences, such as infertility and organ system failure, that can magnify and exacerbate the psychological issues initially associated with diagnosis and treatment. [36] [37] Many patients lack information about what to expect once they are discharged from the hospital. Clinicians need to establish ongoing mechanisms to monitor patients symptoms, psychosocial status, and the problems they are having in terms of resuming activities and responsibilities. The clinician is then in a position to help the survivors to find the best solution for their situation.

The overwhelming evidence from the literature involving survivors with different cancer diagnoses is that, on the average, most do very well after the initial adjustment in the first 12 years post-treatment. Psychological aspects of survivorship may include concern over the termination of treatment, fear of relapse, preoccupation with somatic symptoms, re-entry into previous roles, lingering affinity with death, and financial, job, and insurance difficulties. These issues may manifest in a variety of ways, including denial of past illness, leading to medical compliance issues; ongoing problems with anxiety, panic, and depression; and inability to re-enter previous roles. There is considerable evidence that the wide range of surgical, chemotherapeutic, and radiation therapies can leave permanent damage to organs and physiologic functioning, and disfigurement across the different hematologic diagnoses (Table 81-2). Health care providers should be mindful of psychological sequelae among patients, even within the context of remission and a hopeful prognosis, and refer to a mental health specialist, if necessary.

Progressive Disease

The psychosocial issues experienced by the person with cancer depend in part on the clinical course of the disease process. As the disease progresses, the person often reports an upsetting scenario that includes frequent pain, disability, increased dependence, and diminished functional ability. [7]

The development of a relapse after a disease-free interval can be especially devastating for patients and those close to them. The medical work-up is often difficult and anxiety provoking,

TABLE 81-2 -- Long-term Consequences of Therapies for Hematologic Cancers

Anxiety
Depression
Fear of recurrence

Disfigurement
Conditioned nausea and vomiting
Unemployment
Denial of life insurance
Denial of health benefits
Increase in life insurance rates
Difficulty changing health care coverage
Breakdown of marriage/relationship
Decline in participation in leisure activities
Diminution of support from others
Disruption in sexual functioning
Infertility

and psychosocial problems experienced at the time of diagnosis frequently resurface, often with greater intensity. [7] Shock and depression often accompany relapse and require the patient and family to re-evaluate the future. In spite of the overwhelming nature of the psychosocial responses, however, most patients do indeed cope effectively with progressive illness, and it is important to recognize that intense emotions do not necessarily equate with maladaptive coping.

Investigators studying quality of life in patients with cancer have demonstrated a clear relationship between a persons perception of quality of life and the presence of discomfort.[37] [38] As uncomfortable symptoms increase, perceived quality of life diminishes. Thus, an important goal in the psychosocial treatment of patients with advanced cancer centers around symptom control.

An issue that repeatedly surfaces among patients, family members, and professional care providers deals with the use of aggressive treatment protocols [39] in the presence of progressive disease. Often, patients and families request participation in experimental protocols, even when there is little likelihood of extending survival. Controversy continues about the efficacy of such therapies and the role health professionals can play in facilitating patients choices about participating.

The need for health care professionals to establish structured dialogue between patients, family members, and health care providers regarding treatment goals and expectations is essential. That certain patients respond to investigational treatment with increased hope, despite progressive disease, should be a consideration in treatment planning. The need to separate and clarify the values, thoughts, and emotional reactions of care providers, patients, and families to these delicate issues is important if individualized care with attention to the particular patients psychosocial needs is to be provided.

It is beyond the scope of this chapter to present a comprehensive overview of the psychosocial reactions of patients to the process of dying. Once the terminal period has begun, it is not usually the fact of dying, but the quality of dying, that seems to present the overwhelming concern. [40]

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FACTORS THAT INFLUENCE PSYCHOSOCIAL ADJUSTMENT

Psychosocial responses to cancer vary widely and are influenced by several factors that clinicians should bear in mind when considering the responses of individual patients. A review of the literature points to key factors that may have an impact on psychosocial adjustment. These include (1) previous coping strategies and emotional stability, and (2) the existence of social support.

Previous Coping Strategies and Emotional Stability

One of the key predictors of psychosocial adjustment to cancer is the coping strategy and emotional stability of the person before diagnosis. People with a history of poor psychosocial adjustment before development of cancer are at highest risk of emotional decompensation and should be monitored closely throughout all phases of treatment.[40] This is particularly true of people with a history of a major psychiatric syndrome or psychiatric hospitalization, or both.

Because a persons coping style is determined relatively early in life and remains stable over time and across situations, it serves as a useful predictor of adjustment to cancer. Several investigators have identified specific personality characteristics, coping strategies, or life experiences that either enhance or inhibit positive adjustment to cancer. Those coping strategies found to be most effective have included a fighting spirit and having a feeling of control over events, resulting in active participation in treatment. By contrast, poor adjustment has been associated with avoidant coping strategies, prior negative sexual experiences, body image problems, and inhibition in discussing personal and sexual problems. [41]

The Existence of Social Support

Social support has consistently been found to influence a persons psychosocial adjustment to cancer. The ability and availability of significant others in dealing with diagnosis and treatment can significantly affect the patients view of himself or herself. Those diagnosed with all types of life-threatening chronic disorders experience a heightened need for interpersonal support. Those who are able to maintain close connections with family and friends during the course of illness are more likely to cope effectively with the disease than those who are not able to maintain such relationships.

Living with a chronic illness often requires continuing care and management by a team of specialists. Care is usually provided through follow-up visits to ambulatory or outpatient clinics and consulting rooms, rather than through hospitalization. Historically, patients often are not referred to home nursing care once they are discharged from the hospital. An initial home nursing visit can be invaluable in assisting the patient and family with the transition, in addition to identifying areas in which ongoing assistance is needed. Home care referral can assist families, who are increasingly relied on within the current health care system, to be the major providers of care outside the hospital. The phenomenon of caregiver burden acknowledges that cancer affects not only the patient, but the members of the family. [42] The burden that caregiving places on the family highlights the familys needs and the importance of targeting support information that can help reduce family caregiver burden. Helping to arrange respite care for the patient also aids in relieving caregiver burden.

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DIFFERENTIATING PSYCHIATRIC COMPLICATIONS FROM EXPECTED PSYCHOLOGICAL RESPONSES

Most patients manifest transient psychological symptoms that are responsive to support, reassurance, and information about what to expect regarding the cancer course and its treatment. Some require more aggressive psychotherapeutic intervention, such as pharmacotherapy and ongoing psychotherapy. The following guidelines can assist the clinician in identifying those patients who exhibit behavior suggesting the presence of a psychiatric syndrome.

Most patients do not react to a diagnosis and treatment for cancer by development of a clinically diagnosable psychiatric condition. In some cases, however, a psychiatric syndrome does occur. If the patients problems become severethat is, if the provider believes that supportive measures are insufficient and ineffective in controlling emotional distressreferral to a psychiatric clinician is indicated. Factors that can prevent adjustment to cancer and its treatment include a history of significant depression, manic-depressive illness, schizophrenia, neuroses, organic mental conditions, personality disorders, lack of social support, or inadequate control of physical discomfort.

Because transient symptoms of anxiety and depression are common in patients with cancer, the ability of health care providers to distinguish expected reactions from more severe psychiatric complications is crucial. Anxiety and depression are common symptoms that are particularly evident at transition points during the clinical course of cancer. These symptoms usually subside within 24 weeks and are responsive to supportive reassurance and information regarding what to expect during the course of treatment. For a proportion of patients, psychological distress does not subside with usual interventions. Unfortunately, clinically relevant and severe psychiatric syndromes are often missed by nonpsychiatric care providers. It can be difficult to detect serious psychiatric reactions in patients because several of the diagnostic criteria used to evaluate the presence of severe depression (i.e., lack of appetite, insomnia, decreased sexual interest, diminished energy) may overlap with usual disease and treatment effects. In addition, it is not unusual for health care providers to confuse their own fears about cancer with the emotional reactions of their patients (i.e., I too would be extremely depressed if I were in a similar situation).

General guidelines designed assist in distinguishing patients who should be referred for evaluation by a trained psychiatric clinician include the following:

1. Past history of psychiatric hospitalization or significant psychiatric/personality disorder
2. Persistent refusal, indecisiveness, or noncompliance with regard to needed treatment
3. Persistent symptoms of anxiety and depression that are unresponsive to usual support from health care providers or family members; symptoms may present in the form of constant fear associated with treatment and procedures or excessive crying and hopelessness that worsen rather than improve with time
4. An abrupt, unexplained change in mood or behavior
5. Insomnia, anorexia, diminished energy out of proportion to expected treatment effects
6. Persistent suicidal ideation
7. Unusual or eccentric behavior or confusion (may be indicative of an organic mental disorder)
8. Excessive guilt and self-blame for illness
9. Evidence of dysfunctional family coping or complex family issues

After referral to a psychiatric specialist, one or a combination of several therapeutic modalities may be used. Cancer and its treatment may precipitate an exacerbation of an underlying mental illness to which the patient was already predisposed and that may require extensive treatment (e.g., hospitalization for a psychosis, ongoing pharmacotherapy or psychotherapy). A discussion of these specialized forms of treatment is not warranted here, but the interested reader can consult appropriate standard texts.[41]

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MANAGEMENT OF PSYCHOSOCIAL PROBLEMS

Increased length of survival from time of diagnosis has highlighted the need for psychopharmacologic, psychotherapeutic, and behaviorally oriented interventions to reduce distress, promote adjustment, and improve quality of life for patients with cancer. Numerous studies have documented the efficacy of a

variety of modalities in managing psychosocial problems for such patients. Problems that can be managed effectively include emotional distress such as anxiety and depression; sexual dysfunction; body image disturbances; marital and family difficulties; noncompliance, pain, and neurologic complications such as delirium and dementia induced by brain metastasis or treatment, anticipatory and post-treatment nausea and vomiting; and anorexia and feeding problems.

Pharmacologic Interventions

Pharmacotherapy, as an adjunct to one or more of the psychotherapies, can be an important aid in bringing psychological symptoms under control. Psychopharmacologic agents for the treatment of psychiatric complications associated with cancer are reviewed only briefly here. For a thorough review, as well as expanded treatment guidelines, the reader is referred to a comprehensive chapter by Massie and Lesko. [43]

For patients with excessive anxiety, factors other than a psychological state must first be evaluated. Metabolic abnormalities, pain, hypoxia, and drug withdrawal states can all present as anxiety. Medications such as steroids and antipsychotics, often used to control nausea, can also cause anxiety characterized by agitation and motor restlessness. After medical or drug-induced causes for anxiety are ruled out, an anxiolytic agent is the treatment of choice, with the exception of patients who present with panic episodes, in which case tricyclic antidepressants are most efficacious. Anxiolytic agents are often fast acting and effective (Table 81-3). These drugs are most effective when used at adequate dosages and as standing orders. Use of these medications may also assist the patient to participate in psychotherapy, which can provide more permanent control over psychological symptoms. When anxiety develops in the context of the terminal stages of cancer, it is often secondary to hypoxia or an untreated pain syndrome. Intravenous morphine sulfate is usually an effective palliative treatment. [40]

There are a number of anxiolytics available to treat patients with cancer. These drugs are similar in their overall clinical effects. Because of their shorter half-lives, alprazolam and lorazepam have advantages for elderly patients because toxicity from sedating medications is more common and withdrawal reactions may occur on abrupt discontinuance unless the drug is tapered gradually.

It is common for patients with cancer to demonstrate transient depressive symptoms at various points in the illness trajectory, particularly when a hope for cure is no longer realistic. In patients who exhibit prolonged or severe depressive symptomatology, a major depressive illness must be considered. Depression can be related to recurrence of a past depressive disorder; stress associated with cancer treatment; or disease or treatment effects. Certain medications and cancer treatment agents can produce severe depressive states. A diagnosis of major depression in patients with cancer relies heavily on the presence of affective symptoms such as hopelessness, crying spells, guilt, preoccupation with death or suicide, diminished self-worth, and loss of pleasure in most activities, such as being with friends and loved ones. The neurovegetative symptoms that usually characterize depression in physically healthy people are not good predictors of depression in the medically ill because the cancer and its treatment can also produce these symptoms. A combination of psychotherapy and antidepressant medication often proves useful in treating major depression in patients with cancer (Table 81-4).

The primary medical contraindication to the use of tricyclic antidepressants is significant cardiac conduction delay, which should be ruled out before initiating treatment. These medications are started in low doses (2550 mg) and are increased slowly, over days to weeks, until symptoms improve. Peak dosages are usually substantially lower than those tolerated by physically healthy people. Antidepressant medications may take 26 weeks to produce their desired effects. Patients may need ongoing support, reassurance, and monitoring before experiencing the antidepressant effects of medication. Patients require close monitoring during the initiation and modification of psychopharmacologic regimens by a consistent provider.

Selective serotonin reuptake inhibitors (SSRIs) are a more recently discovered class of antidepressants than the tricyclics and are widely precribed in the general population (Table 81-4). These antidepressants are often desirable because fewer anticholinergic, cardiac, or cognitive adverse effects occur, compared with tricyclic antidepressants. [44] Undesirable side effects for a cancer population may include agitation and anorexia. [44]

Psychostimulants such as dextroamphetamine and methylphenidate (Table 81-4) have been useful in the treatment of depression in medically ill patients. Advantages include rapid onset of action and rapid clearance if side effects occur. They can also counteract opioid-induced sedation and improve pain control through a positive action on mood. Common side effects of psychostimulants include insomnia, anorexia, tachycardia, and hypertension, although incremental increases in dosage allow adequate monitoring of therapeutic versus side effects. In patients with cardiac conduction problems, stimulants may be the treatment of choice. In patients with cancer, a 1- to 2-month trial can provide remission from depression even after discontinuation of the drug.

Psychotherapeutic Modalities

Depending on the nature of the problem, the treatment modality may take the form of individual psychotherapy, group therapy, family therapy, marital therapy, behaviorally oriented therapy, or some combination. [16] Table 81-5 outlines the major psychotherapeutic modalities and the advantages, goals, and indications for each.

TABLE 81-3 -- Commonly Prescribed Anxiolytics in Patients with Cancer

Drug	Starting Dosages	Absorption	Half-Life	Comments
Alprazolam (Xanax)	0.250.5 mg po tid	Intermediate	Intermediate	Generalized anxiety; panic attacks; mixed anxiety; depression; detoxification of patient may be difficult.
Lorazepam (Ativan)	0.51.5 mg po tid	Intermediate	Intermediate	Similar to alprazolam; can produce transient amnesia; given before procedures and chemotherapy. Agent of choice with hepatic impairment.
Diazepam (Valium)	2.55 mg po tid	First	Long	Because of long half-life, not ideal for patients with organic neurologic syndromes.

Adapted from Levenson JA, Lesko LM: Psychiatric aspects of adult leukemia. Semin Oncol Nurs 6:76, 1990, with permission.

TABLE 81-4 -- Antidepressants Commonly Used in Oncology Settings

Drug	Starting Dose	Therapeutic Range	Sedation	Anticholinergic Effect	Hypotension	Cardiac Arrhythmia
Tricyclics						
Amitriptyline (Elavil)	25 mg	75150 mg	High	High	High	Yes
Doxepin (Sinequan)	25 mg	75150 mg	High	Intermediate	Intermediate	Yes
Desipramine (Norpramin)	25 mg	75150 mg	Low	Low	Intermediate	Yes
Nortriptyline (Pamelor)	1025 mg	50125 mg	Intermediate	Low	Low	Yes
Specific serotonin reuptake inhibitors						
Fluoxetine (Prozac)	5 mg	1020 mg	Low	Low	Low	Rare
Sertraline (Zoloft)	10 mg	3050 mg	Low	Low	Low	Rare
Trazodone (Desyrel)	25 mg	150250 mg	High	None	Intermediate	Yes
Psychostimulants						
Dextroamphetamine and methylphenidate	5 mg	520 mg	None	None	None	Rare

Adapted from Levenson JA, Lesko LM: Psychiatric aspects of adult leukemia. Semin Oncol Nurs 6:76, 1990, with permission.

CONCLUSIONS

The psychosocial issues faced by people diagnosed and treated for cancer are influenced by sociocultural, medical, family, and individual factors. Although involvement in decision making is clearly a positive aspect of current cancer therapies, great care should be taken to ensure the communication of timely, repeated, and relevant information consistent with the patients needs, tolerance, and comprehension. A multidisciplinary approach is essential to guarantee the communication of comprehensive information to all patients. Patients should be given the opportunity to speak with multiple members of the treatment team and other patients who have experienced similar management and treatment protocols. Care should be taken to provide needed information from a variety of expert perspectives while respecting the unique characteristics, psychosocial profiles, needs, and desires of each individual. In treatment settings with limited resources, every effort should be made to enlist the help and support of providers and services that can assist patients

TABLE 81-5 -- Psychotherapeutic Modalities in the Oncology Setting

Modality	Selected Indications	Goals and Advantages	Comments
Individual psychotherapy	Prolonged adverse reactions to diagnosis, treatment, and other aspects of chronic illness (i.e., anxiety, depression)	Support patient and enhance ability to cope with distressing feelings. Short-term therapy; focused and goal directed.	Pharmacotherapy and family involvement are useful adjuncts in some cases.
Support groups	Patients desire exposure to others who are experiencing chronic illness	Support patient and enhance coping ability. Usually does not involve a fee. Patients benefit by observing the coping strategies of others.	Expands social network of patients with limited support systems.
Family and marital therapy	Relationship problems secondary to illness (i.e., family tension, role changes, conflict, sexual problems)	Assists couples to clarify problems and solve them together. Addresses role changes in family system.	Problems, issues, and concerns about children can be addressed.
Progressive muscle relaxation and guided imagery	Patients desire assistance with control of pain; anxiety; anticipatory and post-treatment nausea and vomiting; fears associated with medical procedures	Increase sense of control and participation in treatment. Individualized to meet patients preferences and circumstances. Time limited and goal directed. Evaluated in terms of observable changes in symptoms.	Realistic goals should be stated explicitly (some patients view relaxation and guided imagery as a cancer cure).

with their complex treatment decisions. Referral to community resources and support services after discharge from the hospital is often helpful, even for patients who cope well with initial treatment. [45]

Most patients undergoing cancer treatment, as well as their families, experience expected periods of emotional turmoil that occur at transition points along the clinical course of cancer. For a small proportion of patients, more severe psychiatric complications may occur, warranting referral to a psychiatric specialist. A variety of psychotherapeutic modalities are useful in helping patients work through the expected emotional responses to cancer, as well as more severe responses. Supportive psychotherapeutic measures should be used routinely because they minimize distress and enhance feelings of control and mastery over self and environment. For these reasons alone, their value in the care of patients with cancer is paramount. Again, for patients who are not responsive to routine support and information, referral to a psychiatric specialist may be indicated.

Throughout the clinical course of cancer, the patients relationship with health care providers as well as the presence of a supportive social network are important factors in ensuring successful negotiation of the many physical and psychosocial demands imposed by a cancer diagnosis and treatment. Further investigation regarding the utility of systematically tested interventions aimed at promoting psychosocial adjustment to cancer are needed. Investigation aimed at enhancing our understanding of the behaviors of people experiencing the crisis of cancer and identification of those in need of intensive psychosocial support is also needed.

As scientific inquiry continues to produce vast, although sometimes conflicting information regarding etiology and treatment for cancer, concurrent investigation regarding the psychosocial aspects of the disease is crucial. This line of inquiry will, at the very least, assist in promoting emotional well-being in patients faced with an extreme and unexpected life crisis. At best, expanding the knowledge base relative to the psychosocial aspects of cancer may provide some missing links regarding psychosocial adaptation and quality of life, and their impact on survival.

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Chapter 82 - Pain Management and Antiemetic Therapy in Hematologic Disorders

Janet L. Abrahm

INTRODUCTION

Relieving the pain of patients who have hematologic disorders requires a multifaceted approach. [1] [2] An understanding of the transmission of the pain signal and a thorough evaluation of the pain complaint provide a rational basis for treatment decisions. Once the source of the pain has been identified, both nonpharmacologic and pharmacologic therapies can be employed. The neurosurgical methods of pain control are not discussed in this chapter; those interested in these techniques are referred to several excellent reviews. [1] [2] [3]

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TRANSMISSION OF THE PAIN SIGNAL

For a patient to feel pain,[1] a signal arising from a noxious stimulus in the periphery must be transmitted to the centers in the brain that create the experience of pain. Following activation of peripheral receptors by chemical, thermal, or mechanical stimuli, the signal passes along myelinated A- or unmyelinated A- or C fibers and enters the spinal cord via the dorsal root ganglion. Chemicals released at the site of tissue injury initiate or increase the transmission of the signal. Histamine, serotonin, and bradykinin activate local fibers directly, and prostaglandins, which do not themselves activate these fibers, sensitize them to lower levels of mechanical and chemical stimulants.[4]

The dorsal horn of the spinal cord is where the peripheral signals are received and modulated. The excitatory amino acids (glutamate and aspartate) and neuropeptides (substance P and calcitonin gene-related peptide) enhance transmission of the peripheral signal. [1] [5] But a complex interaction of other neurotransmitters (-aminobutyric acid [GABA], glycine, adenosine, bombesin, cholecystokinin, dynorphin, enkephalin, neuropeptide-Y, neurotensin, substance P, somatostatin, and vasoactive intestinal peptide) determines whether the signal will proceed up the spinothalamic tract. [1] Those signals that are transmitted ascend in one of the two contralateral spinothalamic tracts: the paleospinothalamic tract, which mediates the suffering and autonomic reactions to pain, or the neospinothalamic tract, which localizes the pain and records its intensity. [1]

Transmission of the pain signal can be inhibited at several sites along this course. Inhibitory signals arise from the somatosensory cortex, the paraventricular hypothalamic nucleus, the pontine lateral tegmental field, and the nucleus raphe magnus. They travel down the spinal cord via the dorsal longitudinal fasciculus to the dorsal horn to inhibit transmission of the pain signal. [6] The neurotransmitters involved in this inhibition include serotonin, norepinephrine, and the endogenous opiates -endorphin, enkephalin, and dynorphin. [6] Opiate receptors for both endogenous and exogenous opioids are present in the dorsal horn of the spinal cord, in those ventromedial thalamic nuclei that transmit signals from the paleospinothalamic tract, in the periaqueductal gray, the periventricular diencephalon, and the amygdyla, but not in the neospinothalamic tract. [6] Thus, exogenous opioids can provide relief from suffering without affecting the localization of the source of pain, and, when they are applied directly to the spinal cord (e.g., after surgery), low-dose opioids provide effective pain relief without systemic side effects. [7]

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EVALUATION OF THE PAIN COMPLAINT

Initial Evaluation

In evaluating the complaint in patients with hematologic disorders, efforts are initially directed toward determining the cause(s) of the pain: (1) the disease itself, (2) the specific therapy for the disease, and (3) unrelated disorders. Splenomegaly, bone injury (infarction, infection, hemarthroses, infiltration), leptomeningeal infiltration, and spinal cord compression frequently accompany hematologic diseases. Chemotherapy and radiation therapy can cause mucositis, typhlitis, hemorrhagic cystitis, and peripheral neuropathy, and myalgia can follow steroid withdrawal. [8] Immunosuppression, caused by the diseases themselves or by the therapies used to treat them, leads to painful infections such as perirectal abscesses, herpetic or candidal esophagitis, and herpes zoster.

Distress, however, may arise from nonanatomic sources. The pain complaint may represent the patients only means of expressing nonspecific feelings of distress to the physician. Chapman[9] recognized three categories of this distress: (1) anxiety, arising from fear of disfigurement or of uncontrollable pain, fear of loss of social position or of self-control, or fear of death; (2) anger at the failure of the physicians to provide a cure; and (3) depression from loss of physical ability, a sense of helplessness, and the impact of financial problems. In addition to these psychological, social, and financial contributions, spiritual concerns may exacerbate any concomitant painful sensations. [10] Alleviating them can significantly reduce distress and decrease the need for pain medications or other interventions.

Furthermore, for the patient whose complaint of pain does not seem to have a straightforward explanation or who has not responded well to the therapeutic maneuvers outlined below, additional questions must be asked to uncover the source of the distress. Patients may deny their disease and use the complaint of pain to justify their incapacity. Their denial is demonstrated by answers to the following types of questions. *How would life be different without the pain?* Such patients will give unrealistic assessments of the extent of their abilities if only the pain were gone. Hidden fears, resentments, and distrust of the physician may be revealed by answering the question, *What do you think is causing the pain?* Unrealistic expectations of pain treatment may be revealed by answers to the question, *To what extent do you expect your pain to be relieved?* The patient may expect that taking one pill a day will provide total relief, while the physician expects that even a multimodality regimen will relieve only 75% of the pain. A contract detailing the expectations of both patient and physician may be formulated once these unspoken assumptions are understood. Finally, other sources of confusion in the way the patient communicates the type of pain experienced can be clarified by the answer to the question, *Do you seem to show your feelings about the pain to the same extent that your family and friends do?* Patients may come from cultural backgrounds different from the person reporting the pain, and people with cultural differences often report pain very differently. [11] [12] [13] For example, patients of stoic stock may not communicate their distress to their spouse, who may then question the patients need for opioid pain medication (i.e., if they

themselves were in that much pain, they would be sure to tell their spouse). Unless the cultural differences are explored, adequate pain control might not be achieved.

Continued Assessment

Effective pain management requires repeated comprehensive assessment of the patients pain. Pain reports by patients should be believed. The clinician may not be aware that the clinical presentation of a patient suffering from chronic pain is very different from that of a patient in acute pain. If a patient does not manifest the common autonomic manifestations of acute pain (e.g., tachycardia, sweating, elevated blood pressure) or facial grimacing, the clinician might doubt that the patient is suffering from severe pain. It must be understood that a patient with severe but chronic pain will not manifest these autonomic findings but will often be withdrawn, quiet, depressed, or irritable, and will move very little spontaneously but complain of discomfort when moved. When the pain is relieved, these patients often exhibit completely different behaviors, becoming mobile, engaged, and involved with other people. Therefore, the first component in the assessment is to believe the patients complaint.

Patient reports of pain are valid, reliable, and reproducible. [1] These reports should be used, much as a blood sugar measurement is used in diabetic patients, to monitor the efficacy of the therapy. A variety of assessment tools that can be completed within 510 minutes are available. [1] [14] [15] They should be used to determine a number of aspects about the pain, including the pain location, intensity, quality, onset, and duration; what relieves or exacerbates it; and its functional consequences, including how the pain affects the patients ability to sleep or eat and how it affects physical activity, relationships with others, emotions, and concentration. The goal is to lower the pain *to a level acceptable to the patient.* Communication between nurse and physician is greatly improved by using these tools rather than mere qualitative descriptors, such as the pain is better. Using, for example, a scale of 0 (no pain) to 10 (the worst pain one can imagine), a decrease in pain intensity from 10 to 8 indicates that the patient needs a stronger pain medication, while a decrease from 10 to 3 suggests that the current medication regimen is effective.

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THERAPY

A major component of pain therapy is an attempt to ameliorate the underlying cause of the pain. Surgery, chemotherapy, radiation therapy, and antibiotics may all be employed. However, the pain can still be treated effectively during diagnostic testing to define the cause, during specific therapy, or after all disease-related therapies are exhausted.

The goal of pain management is to relieve pain while preserving the patients ability to perform normal activities. This requires expertise on the part of health care personnel, who seek to maximize the relief obtained while minimizing side effects and alterations in daily routines. A multifaceted approach is required that includes nonpharmacologic approaches, anesthetic techniques, correct use of a variety of medications so that pain will be relieved with a minimum of side effects, and patient and family education at each step to foster communication and cooperation with the therapeutic plan.

Nonpharmacologic Methods of Pain Management

Cognitive-Behavioral Interventions

Education and Reassurance

Patients with serious hematologic disorders are often required to undergo extensive diagnostic testing, which can include painful procedures. A rehearsal of the planned test or procedure, including a discussion (or view) of the appearance of the room and the length of time to be spent in the test apparatus, can minimize the patients anxiety. Such explanations, offered preoperatively, lessen the need for postoperative medication and shorten the patients hospital stay. [16] To divert attention from certain procedures (e.g., bone marrow aspiration or biopsy) that take place in the physicians office or in the patients room, a pleasant distraction can be helpful.[17] For example, the physician might encourage the patient to bring in a portable tape player with earphones so that the patient can listen to a favorite piece of music or a book on tape while the procedure is taking place. Alternatively, the physician might turn on the television or radio in the patients room and encourage the patient to pay attention to that, rather than to the procedure. Patients with a good imagination can pretend to be in a place they have previously enjoyed (e.g., at the beach or in the mountains). They can dissociate themselves [18] from the procedure by concentrating on those pleasant memories and thereby diminish the painfulness of the procedure.

Hypnosis

Practitioners with formal training in hypnosis can use more elaborate hypnotic techniques to help their patients deal with painful procedures or conditions. [17] [18] Hypnosis takes advantage of the natural ability to enter a trancelike state. An athlete playing through the pain is an example of the spontaneous induction of such a state. Patients who are trained to enter a trance at will can modify their perception of pain and diminish sleeplessness, anxiety, and the anticipation of discomfort. [19] Hypnotic training of patients with sickle cell anemia or hemophilia decreases the frequency and pain intensity of painful crises [20] or bleeding episodes, [21] [22] respectively.

Even in the absence of a formal hypnotic induction, the words used by the practitioner to describe procedures are very important. For example, the suggestion that hand or arm coolness and numbness will persist after application of an alcohol swab may markedly diminish the discomfort of starting an intravenous line. Using the phrase You will feel something; Im not sure what you will feel, as everyone feels this a little differently in place of This is going to hurt a lot! gives the patient permission to alter the sensation and may also diminish the experience of pain.

Counseling

Psychological counseling provides education, support, and skill development for patients with pain. [23] It can improve patients ability to communicate their pain to care personnel and may be effective in overcoming anxiety and depression. Spiritual counseling aids patients who have lost hope, can find no meaning in their lives, or feel they are being punished or have been forsaken by God. [24] They may interpret their pain in the light of these feelings. Through counseling, they can regain a sense of worth and belonging. As they recast the pain in its true light, it is often diminished.

Cutaneous Techniques

Acupuncture, massage, vibration, and applying cold or heat to the skin over injured areas are often very effective. Cold wraps, ice packs, or cold massage using a cup filled with water that has frozen into a solid piece of ice relieve the pain of muscles that are in spasm from nerve injury. Heat from heating pads, hot wraps, or paraffin treatments can soothe injured joints but should not be used over areas of vascular insufficiency. [25]

Transcutaneous Electrical Nerve Stimulation

Transcutaneous electrical nerve stimulation (TENS) devices are indicated for patients with dermatomal pain, such as postherpetic

neuralgia (see discussion under Postherpetic Neuralgia) or radiculopathy from spinal cord compression. [26] For optimal effect, a physiatrist or physical therapist familiar with the device should train the patient in its use.

Anesthetic Techniques

EMLA, a cream containing two topical anesthetics (lidocaine 2.5% and prilocaine 2.5%) is used, especially in children, to decrease the pain of superficial cutaneous procedures (e.g., venous cannulation or skin anesthesia prior to lumbar puncture, bone marrow aspiration, or biopsy). [27] [28] [29] To achieve anesthesia, the EMLA cream must be applied 11 1/2 hours before the planned procedure in a mound under a semipermeable dressing such as Opsite or Tegaderm. [30] [31] When EMLA is used as directed, methemoglobinemia has not been a problem even in infants as young as 3 months old; [32] [33] skin blanching occurs, sometimes exceeding, but in other studies equaling, the frequency of that found with placebo moisturizing cream placed under the occlusive dressing. [31] [34]

Trigger-point injections, nerve blocks, and neurolytic procedures are useful for both acute and chronic localized pain. After excisional biopsy of an axillary lymph

node, for example, a burning, constricting pain in the posterior arm and chest wall may develop; this pain is often promptly relieved by trigger-point injection. [35] [36] Lymphoma or myeloma may involve the spine and lead to vertebral collapse or pain from progressive disease that is refractory to antineoplastic therapy. Such pain is often particularly difficult to manage. Insertion of temporary or permanent indwelling epidural or intrathecal catheters to deliver either opioids or local anesthetic agents, or both, can be very effective, especially in relieving lower thoracic or lumbar spine pain, as well as pelvic and lower extremity pain. [37] [38] Those interested in the indications for and techniques of the anesthetic and neurolytic procedures are referred to several reviews. [39] [40]

Drug Therapy

Drugs useful for pain relief include nonopioid analgesics, opioids, and adjuvant drugs that either potentiate the actions of opioid analgesics or treat their side effects. Most patients require a combination of medications for optimal pain relief (Fig. 821).

Nonopioid Analgesics

Nonopioid analgesics should be given to patients with mild to moderate pain. Aspirin, acetaminophen, and nonsteroidal anti-inflammatory drugs (NSAIDs) are especially useful in treating bone pain as they decrease local prostaglandin release and may thereby reduce the sensitization of pain receptors. [41] It is important to prescribe an adequate dose of each drug at regular intervals, switching to another nonopioid analgesic only when maximal doses of the first have become ineffective.

Tramadol, which binds opioid receptors and shares opioid-induced side effects but is not an opioid, also relieves mild to moderate pain. A dose of 100 mg is more effective than 60 mg of codeine.[42] Ketorolac tromethamine (Toradol) is an NSAID of particular value in relieving moderate to severe acute pain. A dose of 30 mg of ketorolac given intramuscularly equals the pain-relieving potency of 15 mg of morphine intramuscularly, and acute toxicity is minimal if the total daily dose is under 100 mg.[43] However, ketorolac has all the side effects of the NSAIDs and is not recommended for long-term use. If that degree of pain relief is needed chronically, an opioid agent should be substituted.

Since the NSAIDs can cause renal insufficiency in a significant number of patients, renal function should be assessed 1 or 2 weeks after initiation of any of these agents. NSAIDs should

Figure 82-1 Strategy for pharmacologic management of pain. Multiagent therapy is usually required for optimal pain management. Patients with mild to moderate pain should be started on a nonopioid analgesic or on a combination of nonopioid analgesic and a short-acting opioid. If this combination does not produce adequate relief, or if patients present with severe pain, stronger opioids, in either short-acting or sustained-release form, should be begun immediately. These patients should also receive nonopioid analgesics, if indicated. Laxatives are given to all patients receiving opioids (even for mild pain), and adjuvant tricyclic medications for sleep are offered. Other adjuvant agents should be included as indicated (see text). The drug names given are meant to be illustrative; they are drugs commonly used.

, adjuvant drug;

, an opioid;

, a short-acting opioid;

, a long-acting opioid;

, a nonopioid analgesic (ASA, aspirin; ACET, acetaminophen; NSAID, nonsteroidal anti-inflammatory drug);

, a tricyclic antidepressant;

, combination drugs Percodan, Percocet, and Tylox all include both an opioid and a nonopioid analgesic.

be used with caution in patients with a history of aspirin allergy or asthma because they will precipitate bronchospasm in as many as 20%. [44] Significant edema can occur in patients with cirrhosis or congestive heart failure. [44] If NSAIDs are required in patients with a history of significant gastritis or ulcer disease, or who are over 70, concomitant omeprazole should be considered. [45]

The nonopioid analgesics should be continued in appropriate cases when opioid analgesics are added, as they will potentiate the pain-relieving effect of the opioid (Fig. 821).[46] However, when aspirin is included in a fixed drug combination (e.g., Percodan), salicylate toxicity may develop if the patient takes the pills more often than the prescription indicates. The metabolism of salicylates is limited by the capacity of the hepatic microsomal system. Once that is saturated, salicylate levels are dependent on renal clearance. Thus, small increases in maintenance doses can lead to serious salicylism. [47] Patients with low albumin levels or acid urine are particularly susceptible to the development of salicylate toxicity. [48]

Adjuvants for Neuropathic and Bone Pain

Tricyclic antidepressants (e.g., amitriptyline, nortriptyline), anticonvulsants (phenytoin, carbamazepine, gabapentin, and clonazepam), and steroids are nonopioid analgesics that demonstrate particular efficacy in relieving pain from nerve injuries. The tricyclic antidepressants are the drugs of choice for painful

MANAGEMENT OF SEVERE PAIN

Narcotic therapy is the cornerstone of management of the patient presenting with severe pain. Our practice is to begin with reassurance of patients and their families. We tell them that to relieve the pain as quickly as possible, we will initiate the use of intravenous opioid medications immediately, but that we will begin oral pain medication as soon as the pain is well controlled. Without this explanation, patients have misinterpreted the morphine drip as an indication that they were considered terminal. The starting dose is calculated from the patients baseline opioid requirement or weight (e.g., 0.05 mg/kg/hour of morphine). With continuous patient monitoring, a morphine bolus is given, the drip is begun, and every 2030 minutes the degree of pain relief is reassessed. If the relief is inadequate, another bolus is given; in patients without severe underlying chronic obstructive pulmonary disease, the drip is adjusted upward every 812 hours based on the number of boluses required. There is no maximal morphine dose; we give whatever is required to relieve the pain. If the patient falls asleep, this is usually an indication that pain relief has been achieved, not that the dose should be lowered. We lower the dose if the respiratory rate falls to below 1012 breaths per minute.

Agents to prevent side effects are begun along with the opioid (Fig. 821). All patients are given Senokot or Senokot S, one or two tablets orally daily, up to a maximum of 8 pills per day. If more laxative effect is needed, lactulose is added. The starting dose is 1530 ml PO at bedtime, repeated if necessary in the morning. Lactulose is effective and, unlike other agents, does not produce either cramping or a feeling of fullness. Amitriptyline (10 or 25 mg PO) is usually ordered as a patient may refuse sleep medication. In opioid-naive patients, Compazine (10 mg PO tid or bid) is given for the first 4872 hours to prevent the otherwise common development of nausea. In patients with bone or nerve pain, appropriate adjuvants are added (see text).

When pain relief is adequate, the patient is converted to an equivalent dose of oral morphine. Three times the parenteral dose is required (Table 821). For example, a patient who requires 10 mg of morphine per hour (i.e., 240 mg/24 hours IV) will need 720 mg/day of the oral long-acting agent (240 mg × 3). This can be given as 360 mg PO every 12 hours. Two hours after the oral opioid is begun, the drip is discontinued. Short-acting immediate-release morphine should be available for rescue dosing (see text for dosing recommendations). If the amount of opioid taken as a rescue dose is significant (>25% of the daily dose), the total dose of long-acting agent is adjusted upward accordingly.

peripheral neuropathies and for postherpetic neuralgia. They have anticholinergic side effects of varying intensity and must be used with caution in those who are elderly or who have cardiac conduction abnormalities or bladder outlet obstruction. Phenytoin and carbamazepine, drugs used to treat trigeminal neuralgia, can also be effective for postherpetic neuralgia.[49] [50] Corticosteroids given epidurally, intravenously, or orally are useful for tumor cell lysis (e.g., in leukemia, lymphoma, and myeloma) and can also provide nonspecific relief for patients with spinal cord compression and plexus infiltrations. Doses of 16100 mg of dexamethasone are needed to reduce vasogenic edema in spinal cord compression,[51] but lesser doses (620 mg/day) can be helpful in patients with plexus injuries.[8] Patients must be monitored for the development of oral or esophageal candidiasis and steroid-induced delirium. Steroids are also effective for bone pain, as are pamidronate (90120 mg),[52] calcitonin (100200 IU bid),[53] and strontium chloride (^{89} Sr).[54]

Opioid Analgesics

Patient Education

Opioid analgesics are the mainstay of therapy for moderate to severe pain of malignant or nonmalignant origin. To ensure patient compliance with an opioid prescription, however, education of other members of the health care team, the patient, and the family is often required to dispel the many misconceptions associated with opioid therapy. Even physicians who are cancer specialists hesitate to prescribe opioids as needed for patients with severe pain.[55]

Fear of addiction is a common cause of inadequate dispensing of opioids[56] and a barrier to their acceptance by patients.[55] The physician can increase compliance by providing a full explanation of the differences between addiction and physical dependence, along with reassurance that research has repeatedly indicated that patients with malignancies who take opioids do not become addicts.[57] [58] Patients may also fear that if they take opioid medications for moderate pain, these medications will no longer be effective if more severe pain occurs. Since this fear, if unexpressed, can lead to undertreatment, the topic should be addressed even if the patient does not raise the question. A functional goal of therapy, such as returning to a favorite hobby or reinstituting normal activities of everyday life, may enable the patient and the family to accept the opioid. Finally, misconceptions about religious teachings may prevent health care personnel, patients, and their families from giving or accepting adequate pain medication. Catholics, for example, may not be aware of the Churchs position, as stated in the current catechism, that opioids may be used at the approach of death, even if their use ultimately shortens the patients life.[59] [60] The Church does not consider this use of pain medication to be a means of suicide or euthanasia.[61]

Choice of Medication

Since a wide variety of medications are available, pharmacokinetic considerations and side-effect profiles should be considered when choosing opioid agents. Intermittent moderate to severe pain lasting hours to several days is amenable to oral analgesics with short half-lives (34 hours) with appropriate potency (e.g., immediate-release oxycodone or morphine, hydromorphone [Dilaudid]). Severe pain of relatively constant intensity should be treated with oral sustained-release morphine or oxycodone (taken every 8 or 12 hours[62] [63]) or transdermal fentanyl (renewed every 4872 hours). In addition, a 1224-hour formulation of oral morphine (Kadian, Kapanol) is available; for patients unable to take pills, the capsule can be opened and the pellets sprinkled on food or suspended in water and given through a feeding tube.[64]

Drugs with short half-lives should also be used for rescue doses, given for pain with movement (incident pain) and for between-doses pain exacerbations. The dose of the rescue medication should be 10% of the total 24-hour dose.[65] For example, if a patient is receiving 180 mg of oral sustained-release morphine twice a day, the rescue dose should be 10% of 360 mg, which is 36 mg of short-acting morphine. Agents with short half-lives should also be used in the elderly and in patients with impaired renal or hepatic function.[65] In patients with a previous drug abuse history, agents with longer half-lives, such as methadone or levorphanol, are preferred.

The side-effect profiles of the opioid agents differ widely. It is often useful, therefore, to switch to another agent if a patient

TABLE 82-1 -- Relative Potencies of Commonly Used Narcotics

Drug	Epidural	IM (mg)	PO (mg)

Morphine	1	10	30 (repeated dose)
			60 (single dose)
Codeine		130	200
Oxycodone (in Percodan, Tylox)		N/A	20
Meperidine (Demerol)		75	300
Levorphanol (Levo-dromeran)		2	4
Hydromorphone (Dilaudid)		1.5	7.5
Methadone		10	20
Fentanyl		0.1	

Adapted from Foley,[1] with permission.

is experiencing dose-limiting side effects with the initial opioid chosen. [66] For example, if the patient receiving morphine experiences disabling nausea, the substitution of hydromorphone at an equianalgesic dose should be considered (Table 821). Because of incomplete cross-tolerance, the initial dose for patients on higher doses of opioids should be only one-half to two-thirds the calculated equianalgesic dose. [65] For example, in the same patient taking morphine (360 mg/day PO), hydromorphone should be begun at 45 or 60 mg/day (one-half or two-thirds the 90-mg dose that would be equianalgesic). For patients receiving even higher doses of opioid, as little as 20% of the replacement opioid may be appropriate. [67] The short-acting rescue medication (5 or 6 mg hydromorphone) will provide relief if this initial dose does not prove adequate.

Meperidine is not indicated for chronic use in patients with long-lasting moderate to severe pain. [1] It provides pain relief for only about 12 hours [8] and gives rise to an active metabolite, normeperidine, which induces dysphoria, is excitatory to the central nervous system, and can cause seizures. [68] Normeperidine has a half-life of 1324 hours, which can lengthen with renal failure. [69] Thus, the frequent administration of meperidine required for pain control causes normeperidine levels to rise and increases the likelihood of seizures even in patients with normal renal function. [1] The seizure incidence is further increased if the opioid antagonist naloxone (Narcan) must be given.[70] Other opioids are therefore better choices for patients with moderate or severe pain who will require days of sustained analgesia. In sickle cell disease, for example, intravenous or oral sustained-release morphine has been shown to be very effective in both inpatient and outpatient pain management. [71] Should hyperirritability or a seizure occur in a patient receiving meperidine, intravenous diazepam (Valium) rather than phenytoin should be used to control the seizure,[72] and morphine should replace the meperidine for pain control.

Routes of Delivery

Opioids can be delivered noninvasively (orally, rectally, or transdermally) or invasively (subcutaneously/intravenously or by spinal infusion). For patients switched from oral or rectal to parenteral or spinal medication, or vice versa, the dose must be altered accordingly to avoid overdose or undertreatment (Table 821). No matter which route is chosen, patients experiencing continuous pain should receive the analgesics regularly and be awakened, if necessary, to administer medications that will prevent recurrence of the pain. [1]

Oral.

Most patients will be able to achieve excellent pain relief with either short-acting or sustained-release oral opioid preparations. Oral transmucosal fentanyl citrate induces rapid analgesia, relieves anxiety, and produces sedation. [73] It is especially useful in children and can relieve breakthrough pain in adults.

Rectal.

Rectal opioids (morphine, oxymorphone, and hydromorphone) replace IM injections for patients who are suddenly unable to take oral medications. They have about the same potency and half-life as orally administered agents [74] [75] [76] [77] and must therefore be administered frequently. In single-dose bioavailability studies of sustained-release morphine preparations, despite delayed absorption from the rectal route, total morphine absorption over 24 hours was equivalent, whether the drug was given orally or rectally. [74] [75] [76] [77]

Transdermal.

The transdermal fentanyl patch (Duragesic) delivers the lipophilic fentanyl into the fat-containing areas of the skin. The drug diffuses continuously from the patchs reservoir through a rate-controlling membrane and is absorbed from the skin depot into the bloodstream, where it is rapidly metabolized (Figure 822). [78] The onset of pain relief is delayed about 12 hours and a relatively constant plasma concentration of fentanyl is not reached until about 1420 hours after the initial patch is placed.[78] Rescue medication must therefore be provided during the first 48 hours of use of the patch. [79] Similarly, if a patient develops signs of fentanyl overdose, naloxone (Narcan) must be given until the skin reservoir has become depleted. [80] About 50% of the drug is still present 24 hours after patch removal. [81] Converting patients from oral or parenteral medication to the patch is easily accomplished using the table provided in the package insert. [82] Most patients will require dose escalations, as the conversion is a conservative one. A new patch is applied every 72 hours, although a few patients require a new patch every 48 hours.

The transdermal system is an effective method of delivering pain relief for patients with (1) moderate to severe pain, (2) no oral route available or no desire to take pills, and (3) a stable level of chronic pain. Side effects include those due to the contact adhesive, along with those commonly associated with other opioids, but may be better tolerated than those caused by morphine. [81] [82] [83]

The transdermal system should not be used in septic patients, those experiencing acute pain, or those with markedly fluctuating opioid requirements. When the patients temperature rises to 40°C, drug absorption from the skin can increase by as much as 35%. [78] If hepatic function is impaired or sepsis or shock develops and blood flow to the liver decreases, plasma concentrations may rise sharply. [81] Lower doses may also be required

Figure 82-2 Duragesic is a rectangular transparent unit comprising a protective liner and four functional layers. Proceeding from the outer surface toward the surface adhering to the skin, these layers are (1) a backing layer of polyester film, (2) a drug reservoir of fentanyl and alcohol USP gelled with hydroxyethyl cellulose, (3) an ethylenevinyl acetate copolymer membrane that controls the rate of fentanyl delivery to the skin surface, and (4) a fentanyl-containing silicone adhesive. Before use, a protective liner covering the adhesive layer is removed and discarded.

TABLE 82-2 -- Adverse Effects from Spinal Infusions

Narcotic	Anesthetic	Catheter-Related	
		Epidural	Subarachnoid
Respiratory depression	Hypotension	Insertion related (e.g., hematoma)	Insertion related (e.g., hematoma)
Sedation		Fibrosis	CSF leak
		Skin abscess	Meningitis

Data from Paice and Williams.[38]

in elderly patients or in those with respiratory insufficiency. [84]

Subcutaneous and Intravenous.

Continuous subcutaneous or intravenous administration of opioids can provide pain relief in the shortest amount of time, with a minimum of oversedation. The drugs can be delivered by portable infusion pump and initiated or continued in the home. [85] [86] [87] Guidelines for their use are available. [88] Patient-controlled analgesia (PCA) systems for subcutaneous or intravenous drug delivery have the advantage of responding to the individual patients threshold for pain while eliminating delays when nurses must administer supplemental medication.[89] The pumps administer a continuous fixed infusion of the opioid chosen and allow the patient to self-administer boluses of additional medication at frequencies chosen by the physician. By recording the additional amounts of self-administered medication, the devices also facilitate the adjustment of the continuous dose required for pain relief.

Spinal.

Epidural or intrathecal opioid infusions, which include the option of patient-controlled epidural analgesia, can be helpful for selected patients with pain below the midthoracic area.[37] [90] The infused opioids block pain transmission by binding to receptors in the dorsal horn of the spinal cord. [6] [7] Since the drug is being infused in close proximity to the receptors, only a small amount of opioid is needed and the systemic side effects are reduced. Problems with this delivery system in patients who are not opioid naive[38] are listed in Table 822 . If tolerance to the opioid develops and higher doses are required for relief, the incidence of side effects will approach that of systemically administered opioids. Addition of local anesthetic or alpha-adrenergic agent (e.g., clonidine) [91] to the epidural opioid infusion allows for fairly rapid lowering of the opiate concentration and reestablishment of opioid sensitivity. [38] [39]

Adjuvant Medications

Adjuvant medications are used both to add to the analgesic effect of the opioids and to treat the complications they induce.

Adjuvant Analgesics

Adjuvant analgesics enhance the pain-relieving properties of opioids. Tricyclic antidepressants are among the most effective psychotropic analgesics. [50] It has been suggested that they produce these effects by raising the concentration of the endogenous pain inhibitors serotonin and norepinephrine [92] [93] and increase the bioavailability of morphine. [94] These tricyclics act more promptly and at lower levels as opioid adjuvants than they do as antidepressants (e.g., amitriptyline is effective within 23 days at 50100 mg/day).[93]

The addition of dextroamphetamine or methylphenidate (2.57.5 mg PO or 10 mg IM bid[95] [96]) permits lowering of the opioid dose by one-third to one-half while maintaining equivalent analgesia and less sedation. [95] Pemoline is a chewable psychostimulant that is chemically unrelated to amphetamines but that may be as useful in reversing opioid-induced sedation. [97] Patients are started on one tablet (18.75 mg) in the morning and at noon, and the dose is gradually increased to a total of 75 mg/day. These combinations are very useful, for example, in treating neutropenic patients with perirectal abscesses whose severe pain generally occurs as the neutrophil count rises. Tolerance to the amphetamine effect does not develop before 23 weeks of therapy, by which time the abscess pain has usually resolved.

Hydroxyzine (25100 mg PO or IM)[98] [99] also has opioid-potentiating abilities, diminishes opioid-induced nausea and vomiting, [99] and is especially useful in patients who might benefit from an anxiolytic agent.

Laxatives

Constipation is the most common opioid-induced side effect (Fig. 821);[53] laxatives should therefore be given routinely, not on a PRN basis, [1] [53] [100] to patients treated with any of the drugs listed in Table 821 . Detailed bowel preparation recommendations can be found,[8] [100] [101] but none has been studied in a controlled fashion. If bowel obstruction occurs, pain relief can be maintained with methylphenidate or dextroamphetamine. Alternatively, methotrimeprazine, a phenothiazine, can be substituted for a short time.[102] Single-dose studies of 15 mg IM of methotrimeprazine have shown this drug to be equivalent to 15 mg IM of morphine. The recommended parenteral starting dose is 510 mg. Both clonidine[103] and continuous subcutaneous infusion of metoclopramide (to 60 mg/day) [104] reverse the opioid bowel syndrome,[103] enabling patients to return to oral opioids.

Nausea

Prochlorperazine (10 mg bid or tid) will prevent the nausea that occurs in most patients during the first days of opioid therapy. Relieving constipation, or changing the opioid (e.g., from morphine to oxycodone) will often eliminate the later development of nausea. Rarely, patients will need oral or intravenous ondansetron (8 mg bid to tid).[105]

Sleep Medications

Sleep medications that produce sedation (benzodiazepines, barbiturates, chloral hydrate) are not good choices for patients receiving opioids, as these drugs will produce excessive daytime sedation. Tricyclic antidepressants (e.g., 1050 mg of amitriptyline) are preferred as they produce only nighttime sedation and act as opioid adjuvants (Fig. 821).[50] [93]

Naloxone

Naloxone (Narcan) reverses opioid-induced respiratory depression, although repeated doses are often required. [8] Respiratory depression can occur in patients with mild to moderate pain during the initial use of opioids, although it is rare in patients with severe pain or in those chronically receiving opioids. Caution should be exercised before administering naloxone to the patient who is chronically receiving opioids, to avoid precipitation of severe withdrawal. In such cases, it is inadvisable to administer the usual 0.4 mg/ml dose; rather, 0.4 mg of naloxone should be diluted with 10 ml of saline and only enough given to reverse respiratory depression. [1] In a comatose patient, endotracheal tube placement is recommended to prevent aspiration from the salivation and bronchial spasm that will be induced. [1]

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SPECIFIC CLINICAL PROBLEMS

Oral Complications

Oral complications of chemotherapeutic and marrow transplant regimens can be a frequent cause of pain. A thorough dental evaluation and prompt treatment of infections can minimize the discomfort arising from underlying periodontal disease and caries, secondary bacterial, viral, and fungal infections, and mucositis. [106] Preventive regimens include saline, sodium bicarbonate,

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or chlorhexidine gluconate rinses, acyclovir, amphotericin, and ice. [107] Sucralfate,[108] capsaicin,[109] and granulocyte-colony-stimulating factor [110] diminish the incidence, duration, and intensity of chemotherapy-induced mucositis. Viscous lidocaine (Xylocaine) or a slurry of sucralfate, diclonine, or Kaopectate in diphenhydramine provides symptomatic treatment of mucositis pain.[111] For individual lesions, benzocaine in Orabase can be helpful. Milk of magnesia, which dries the mucosa, is not recommended.[106] The more severe cases, occurring in marrow transplant recipients, usually require infusional morphine therapy, delivered by standard drip or PCA.[111] Pilocarpine (57.5 mg tid or qid 1 hour before meals) reverses xerostomia from neck irradiation [112] and opioids. Sugar-free hard candy is also useful for opioid-induced xerostomia.

Coagulation Disorders

Patients with inherited or acquired disorders of coagulation may have excessive risk of bleeding if aspirin-containing pain relievers are used. [113] [114] If acetaminophen is not effective, these patients may obtain significant relief from ibuprofen or from the nonacetylated salicylates, such as salsalate (Disalcid) or choline magnesium trisalicylate (Trilisate), which do not prolong the bleeding time. [114] [115] [116] Since these agents share aspirins ability to compete with warfarin for albumin binding, [48] careful monitoring of patients coagulation parameters is recommended when these drugs are started or stopped.

Postherpetic Neuralgia

Postherpetic neuralgia can be a difficult problem for patients with hematologic disorders and has been the subject of several reviews. [49] [117] Phenytoin (300 mg/day) or carbamazepine (started at 100 mg/day and slowly titrated to 400800 mg/day) has some efficacy, but amitriptyline is the agent of choice, having been effective in 6070% of patients in a randomized double-blind controlled trial. [118] [119] Elderly patients, however, do not tolerate the anticholinergic side effects well. Nortriptyline (Pamelor) may be useful in these patients. Topical lidocaine gel or ointment (510%) [120] and EMLA[121] can also be effective. Topical capsaicin (0.075%), which depletes substance P, has shown efficacy in a multicenter, double-blind, randomized placebo-controlled trial. [122] Capsaicin (0.075%) is also used for musculoskeletal discomfort. TENS devices provided relief for 1 year in 50% of postherpetic neuralgia patients, and 30% were pain free at 2 years. [26] Acute herpes zoster pain may be diminished by a combination of acyclovin and prednisone. [123]

Sickle Cell Anemia

Patients with sickle cell anemia suffer from both chronic and episodic pain. Chronic arthritic pain can be treated with physical therapy and full doses of antiarthritic medication, but some patients require low doses of chronic opioid therapy to maintain independent functioning. Several studies have confirmed the safety and efficacy of long-term opioids in the treatment of pain of nonmalignant origin. [71] [124] [125] [126] In some cases, joint replacement may be required.

When a patient with sickle cell anemia experiences a severe episode of pain, it is always important to attempt to define the precise cause of the pain before attributing it to a vaso-occlusive crisis. To manage crisis pain, except in patients with renal failure, nonopioid analgesics that treat bone pain, a tricyclic adjuvant such as amitriptyline, and laxatives should be added to opioid therapies (Fig. 821). Oxygen is not required in the absence of documented hypoxia. [127]

Because intravenous access is often difficult in these patients, opioids such as morphine can be given orally [128] on a fixed schedule, with a patient may refuse provision. Meperidine should be avoided in this population for the reasons mentioned above, and has been associated with seizures in between 1% and 12% of sickle cell patients.[129] Furthermore, patients with sickle cell anemia absorb intramuscular meperidine poorly. [130] In that minority of patients who need large doses of medication, intravenous morphine delivered by standard drip or PCA is usually effective. [71] [131] Unfortunately, morphine has been noted to cause rash, pruritis, nausea, and vomiting in a significant number of patients.[132] To dispel any atmosphere of distrust between patient and health care provider, [128] a contract agreed to by both provider and patient can be established. [133] The contract describes the dose, duration, and type of analgesics to be used and is updated yearly. When the crisis is resolved, the dose is tapered daily, without lengthening the interval between doses; if necessary, the patient is begun on equivalent doses of an oral opioid medication.

Problems of the Elderly

Pain management in the elderly is complicated by difficulties in pain assessment as well as by the altered pharmacokinetics of opioids and of psychotropic adjuvant medications.[134] Elderly patients may under-report pain. [135] Physicians may ascribe observed limitations in social contact and physical activity to age-related changes when in fact they are pain-induced limitations. [136] Family members can be crucial to success in managing an elderly patients pain.

Elderly patients are particularly susceptible to the side effects of NSAIDs, and patients taking them should be monitored closely. [134] In elderly patients (ages 7089), the volume of distribution for opioids is generally smaller, the drugs have a longer plasma half-life, and renal and hepatic clearance is decreased, all of which lead to a prolonged duration of effect.[136] Therefore, the effective doses for these patients are one-half to one-quarter of those needed in younger patients. [134] [136] Drugs with short half-lives should be used (e.g., oxycodone or hydromorphone), and initial doses should be low. [136] Patients should be monitored carefully for the development of sedation or confusion, especially if they are receiving antihistaminic agents (cimetidine, diphenhydramine) or drugs with anticholinergic activity.

The acute urinary retention due to opioids (especially in patients with prostatic hypertrophy) and the hypotension and tachycardia caused by tricyclic compounds can be more frequent and of more clinical severity in this population. The starting dose should be low (usually 10 mg at bedtime), and the dose should be slowly increased as tolerated. Doxepin, which does not share these side effects, may be a better tolerated opioid adjuvant. [92]

Patients with Opioid Addiction

A team approach, including the patients family and drug counselor, maximizes the coordination of care for these patients. Opioid requirements may be significantly higher, and dosing intervals are shorter in the addict. [137] [138] In patients on methadone maintenance, therapeutic dosing must be provided over and above their baseline dose.[137] In all cases, the goal should be to deliver adequate medication to relieve the patients pain. It has been recommended that (1) the physician always work from a written treatment plan, (2) one physician prescribe all psychotropic medication, (3) information about the patients drug use be obtained from sources in addition to the patient, and (4) when the question of addiction first arises, consultation be obtained from an addiction medicine specialist. [138] If opioids are needed, patients should be given limited quantities of long-acting medications on a scheduled basis.

Both former opioid abusers themselves and the physicians caring for them hesitate to use opioids for pain relief, as they fear inducing a relapse of the addiction. [139] This concern is based

on the observation that once a receptor has been habituated to the opioid, it retains its avidity for opioids, even after a long period of abstinence. [137] In addition to those noted above, suggested techniques to minimize these risks include discussion of the individuals concerns and increased involvement by the patients in their recovery programs.[137]

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ANTIEMETIC THERAPY

A number of effective antiemetic therapies are used to prevent the nausea and vomiting induced in patients by treatment of their hematologic disorders with chemotherapy or radiation therapy. These antiemetic therapies markedly improve patients quality of life. Most patients can expect complete control of nausea and vomiting.[140]

Pathophysiology of Vomiting

The means by which chemotherapy agents induce vomiting are still incompletely understood but are thought to include stimulation of the chemoreceptor trigger zone, which is probably the most common mechanism; gastrointestinal injury; stimulation of the cerebral cortex; inner ear processes; and changes in taste and smell. [140]

Although the sites of emetic action of the chemotherapeutic agents have not been identified, blocking agents directed against type 3 serotonin receptors (5-HT_3 receptors) have been effective in inhibiting chemotherapy-induced nausea and vomiting. Higher centers in the brain, such as the cortex, are also thought to be involved in producing anticipatory nausea and vomiting. Thus, cognitive therapy, as well as antianxiety and amnesic agents, may provide effective antiemesis.

Therapy

Anticipatory Nausea and Vomiting

Anticipatory nausea and vomiting (ANV) is thought to be a classic conditioned response. [141] Chemotherapy administration (the unconditioned stimulus) results in nausea and vomitingthe unconditioned response. Clinic sights, smells, and sounds are the conditioned stimulus. After frequent pairings of chemotherapy administration and the clinic sights, smells, and sounds, the response (nausea and vomiting) can be triggered in the absence of any chemotherapy by clinic sights, smells, or sounds or simply by seeing clinic personnel, even at a location distant from the site of treatment.

Patients in whom post-treatment nausea or vomiting never develops do not develop ANV either. In those who do experience post-treatment nausea and vomiting, the risk of developing ANV increases with the increasing frequency, severity, and duration of the symptoms. [138] Other possible predisposing factors include susceptibility to motion sickness,[142] [143] awareness of tastes or odors during infusions, younger age, lengthier infusions, greater autonomic sensitivity, and general anxiety or emotional distress.[140] [141]

The best way to prevent ANV is to give aggressive antiemetic therapy and to treat anxiety with the agents described below. However, in patients for whom this approach has not been successful and in whom ANV develops, a variety of behavioral techniques have been helpful. These include hypnosis, [144] progressive muscle relaxation with guided imagery,[145] systemic desensitization, [146] and distraction.[147] Results may be optimized if a therapist trained in these techniques works with the patient, but a recent study of progressive muscle relaxation with guided imagery suggests that patients can apply this technique on their own. [141] [145]

Two different drug regimens have been shown to reduce ANV. Clonidine can be given for 5 days before chemotherapy. [148] Alternatively, alprazolam can be given the night before, the morning of, and just before chemotherapy, and then qid for 2 days after therapy. [149] Alprazolam significantly reduced ANV compared with placebo, even though it had no effect on anxiety levels.

Antiemetic Agents

Most studies of the potent antiemetic agents have been conducted in patients receiving therapy with cisplatin, a drug of high emetic potential. Although cisplatin is not generally used to treat patients with hematologic malignancies, the data are included in the following discussion, as the findings can probably be extrapolated to other drugs of equivalent or less emetogenic potential. When available, data regarding efficacy in patients receiving radiation therapy or emetogenic drugs commonly used to treat hematologic disorders are reviewed.

Choice of Agent

The choice of antiemetic agent(s) depends on (1) the emetogenic potential of the chemotherapy or radiation therapy regimen, (2) the side-effect profile of the antiemetic agent(s), and (3) patient preferences and characteristics.

The emetogenic potential of the drugs is shown in Table 823 . Although most drugs produce emesis 12 hours after they are given (in patients who have never before received

TABLE 82-3 -- Emetogenic Potential of Drugs Commonly Used to Treat Hematologic Malignancies

Emetogenic Potential	Drug
High (>90%)	Carmustine (>250 mg/m^2) Cyclophosphamide (>1,500 mg/m^2) DTIC Mechlorethamine
Moderately high (6090%)	Cyclophosphamide (>750 mg/m^2 1,500 mg/m^2) Cytarabine (>1,000 mg/m^2) Doxorubicin (>60 mg/m^2) Methotrexate (>1,000 mg/m^2) Procarbazineoral

Moderate (3060%)	Azacytidine Cyclophosphamide (<750 mg/m^2) Cyclophosphamideoral Cytarabine (1,000 mg/m^2) Doxorubicin (2069 mg/m^2) Epirubicin (90 mg/m^2) Idarubicin Methotrexate (2501,000 mg/m^2) Mitoxantrone (<15 mg/m^2)
Moderately low (1030%)	Asparaginase Etoposide Melphalan Mercaptopurine Methotrexate (>50 mg/m^2 <250 mg/m^2) Pentostatin Thiotepa
Low (<10%)	Bleomycin Busulfan (regular dose) Chlorambuciloral 2-Chlorodeoxyadenosine Fludarabine Hydroxyurea -Interferon Methotrexate (50 mg/m^2) Thioguanineoral Vinblastine Vincristine

Data from Berger and Clark-Snow,[140] Bradbury,[203] and Hesketh et al.[204]

COMBINATION ANTIEMETIC REGIMENS*

High to Moderately High Emetogenic Potential

Ondansetron, 24 mg PO × 1 or 8 mg IV bid
OR

Granisetron, 1 mg IV or 2 mg PO
OR

Dolasetron, 1.8 mg/kg IV or 200 mg IV or 100 mg PO qd, plus

Dexamethasone, 1020 mg IV × 1 over 15 minutes, ± lorazepam, 1 mg qd and pm

May replace dexamethasone with metopimazine, 35 mg/m^2 24 continuous infusion, then 30 mg PO qid × 4 days[162]

For treatment failures

Dexamethasone, 1020 mg IV/PO q1224h, plus

Metoclopramide, 2 mg/kg/dose IV/PO q6h, plus

Diphenhydramine, 2550 mg IV/PO q6h, plus

Lorazepam, 1 mg qd IV/PO

Moderately High to Moderate Emetogenic Potential

Ondansetron, 8 mg IV (<60- to 75-kg patient) or 8 mg PO bid
OR

Granisetron, 2 mg PO qd or bid, plus

Dexamethasone, 1020 mg IV

Moderately Low or Low Emetogenic Potential

Dexamethasone, 1020 mg PO or IV over 15 minutes
OR

Prochlorperazine, 10 mg PO or 25 mg parenterally q6h prn
OR

Thiethylperazine, 10 mp PO q46h
OR

(If control is not achieved, or there is past history of inadequate control)

Ondansetron, 812 mg IV (<60- to 75-kg patient) or 8 mg PO bid or tid, or

Thiethylperazine, 10 mp PO q46h
OR

(If control is not achieved, or there is past history of inadequate control)

Ondansetron, 812 mg IV (<60- to 75-kg patient) or 8 mg PO bid or tid, or granisetron, 2 mg PO qd, plus

Dexamethasone, 1020 mg IV
OR

Dexamethasone, 10 mg IV/PO q12h, plus

Metoclopramide, 2 mg/kg/dose IV/PO q6h, plus

Diphenhydramine, 25 mg IV/PO q6h

For Prevention of Delayed Nausea and Vomiting

Ondansetron, 8 mg PO bid × 3 days, ±

Dexamethasone, 8 mg PO bid × 3 days
OR

Dexamethasone, 46 mg PO bid × 2 days, then 24 mg PO bid × 2 days, ± lorazepam
OR

Metoclopramide, 0.5 mg/kg PO qid × 4 days, plus

Dexamethasone, 8 mg bid PO × 2 days, then 24 mg bid × 2 days, plus

Diphenhydramine, 50 mg PO q68h, plus

Lorazepam, 1 mg PO q68h

Breakthrough Nausea and Vomiting

Prochlorperazine, 10 mg q46h IV or PO, or 25 mg PR
OR

Thiethylperazine, 10 mg q46h PO or PR
OR

Metoclopramide, 2040 mg PO q46h, or 12 mg/kg q34h IV, plus

Diphenhydramine, 2550 mg PO/IV q46h

* Data from Johnson et al., [155] Bradbury[203] , and NCCN.[205]

chemotherapy), the onset of emesis from high-dose intravenous cyclophosphamide is delayed until 918 hours after treatment, [150] and from high total doses of cisplatin until 2472 hours later.[151]

The side-effect profiles for the antiemetic agents are discussed below. Past history of alcohol intake and patient age modify certain aspects of the profiles. Patients who have a history of alcohol intake of more than five alcoholic drinks per day (>100 g of alcohol) tend to have less nausea and vomiting. This has been studied carefully in patients receiving high-dose cisplatin therapy [152] [153] but has been anecdotally observed in patients receiving other agents.

Younger patients have a higher incidence of chemotherapy-induced nausea and vomiting and of acute dystonic reactions after receiving metoclopramide therapy: trismus or torticollis was seen in only 2% of patients over 30 years of age but in 27% of younger patients. [154] Even if they are receiving only moderately emetogenic therapy, they should be given an antiemetic regimen for high-emetogenic drugs. [155] Younger patients find cannabinoids more effective than do older patients because they can better tolerate the side effects associated with the therapeutic doses of these agents. [156] Elderly patients also have a high risk of extrapyramidal side effects and are more susceptible to anticholinergic and sedating side effects. [155] 5-HT_3 receptor antagonists are therefore preferred to regimens containing metoclopramide and diphenhydramine.

Patient preferences regarding degree of alertness can help the health care provider choose an antiemetic regimen, as can the level of anxiety the provider observes in the patient. Some patients do very well with regimens that include no antianxiety agents; others may benefit from the inclusion of those agents, despite the somnolence that accompanies their use.

5-HT_3-*Receptor Antagonists (Ondansetron, Granisetron, Dolasetron)*

Ondansetron and granisetron are the best studied of the 5-HT_3 -receptor antagonists; this class also includes the drugs dolasetron, tropisetron, and RG 12915. Several studies of patients receiving regimens that include cyclophosphamide, methotrexate, or doxorubicin showed significant efficacy and superiority of ondansetron over placebo.[157] [158] [159] Ondansetron also demonstrates efficacy in patients with emesis (induced by non-cisplatin-containing chemotherapy) that has been refractory to standard antiemetics, with complete control achieved in 50% of these patients. [160] [161] When dexamethasone is added, control rises to as high as 91%.[162]

Ondansetron effectively prevents nausea and vomiting in patients treated with highly emetogenic agents for acute leukemia, [163] [164] for multiple myeloma (i.e., high-dose melphalan), [165] or who are being prepared for bone marrow transplant with cyclophosphamide and total-body irradiation. [166] [167] In the transplant patients, 83% of patient-days were without any vomiting or retching, and in a further 10% there were no more than two emetic episodes. Ondansetron is also effective in preventing emesis induced by single- or multiple-fraction radiation therapy. [168] [169] Ondansetron is as effective as metoclopramide in preventing nausea induced by cisplatin[170] [171] [172] or by cyclophosphamide, alone or with doxorubicin chemotherapy. [173] [174] [175]

Oral granisetron has shown equivalence to ondansetron in studies of patients receiving cisplatin- or carboplatin-based regimens. [140] [176] [177] In patients receiving moderately emetogenic therapy, oral granisetron with dexamethasone was as effective as intravenous ondansetron and dexamethasone. [178] [179] Oral or intravenous granisetron, and dexamethasone were effective in a bone marrow transplant program in which patients were treated with Cytoxan and total-body irradiation [180] and, along with oral prochlorperazine, were also effective in patients undergoing peripheral blood stem cell transplants for which patients received Cytoxan, VP-16 or thiotepa, and cisplatin. [181]

Ondansetron and the other 5-HT_3 -receptor antagonists have

far fewer side effects than metoclopramide. Reports of extrapyramidal reactions are very rare, [182] and sedation, dystonic reactions, akathisia (severe restlessness, ants-in-pants feeling), and tardive dyskinesias do not occur; patients develop only constipation, mild headache, and elevations in transaminases. [140]

The therapy of choice for moderately or highly emetogenic chemotherapy is therefore a 5-HT $_3$ -receptor antagonist plus a corticosteroid (usually dexamethasone). [140]

Metoclopramide

Metoclopramide is a substituted benzamide capable of blocking the emetic activity of chemotherapy agents by blocking 5-HT $_3$ receptors in the chemoreceptor trigger zone. Delivery of the drug on a schedule that maintains adequate levels during expected emesis appears to be important. [140]

Metoclopramide is also effective in radiation therapy-induced emesis, possibly because this type of emesis may be induced by serotonin release from the small bowel or by vagal nerve stimulation. Metoclopramide is effective in preventing nausea induced by chemotherapeutic agents, even of high emetogenic potential. [183] [184] [185] [186] [187]

However, the side effects, which may be caused by the interaction of metoclopramide with dopamine receptors, can be quite troublesome. They include akathisia, dystonic reactions (age related), sedation, and diarrhea. Benzodiazepines such as lorazepam can prevent or reverse the akathisia, and diphenhydramine or Cogentin can prevent or reverse the dystonias. [188] However, these agents induce additional side effects, including dry mouth and sedation. Furthermore, short-term high-dose metoclopramide or long-term use of the drug has been associated with persistent and disabling movement disorders, especially tardive dyskinesias. [189] [190]

Corticosteroids

The mechanism of the antiemetic action of corticosteroids remains undefined. Corticosteroids are effective used alone to prevent emesis induced by agents of moderate or low emetogenic potential. [191] [192] [193] They are also a useful component of antiemetic therapy regimens that include either ondansetron, granisetron, or metoclopramide, and add efficacy in randomized controlled trials. [140] [176] [178] [180] [181] [194] Dexamethasone and methylprednisolone are the best-studied agents, but no trials have demonstrated the superiority of one agent over another.

Benzodiazepines

The benzodiazepine lorazepam has only mild antiemetic activity on its own. [188] It markedly decreases the akathisia and anxiety associated with metoclopramide therapy, however, and induces a dose-related memory loss and marked sedation. [188] [195] [196] Alprazolam is useful in preventing ANV.

Other

Other agents that are more active than placebo include the butyrophenones haloperidol and droperidol, [197] the phenothiazine prochlorperazine, [198] and the cannabinoids dronabinol (THC) and nabilone. [140] [156] [199] [200] These agents are less effective drugs than the agents mentioned above, and all but prochlorperazine are associated with significant side effects. All cause sedation. In addition, the butyrophenones produce dystonic reactions, akathisia, and occasionally hypotension. The cannabinoids cause ataxia, dry mouth, orthostatic hypotension and dizziness, euphoria or dysphoria, and a feeling of being high. [201>]

Combination Antiemetic Therapy

For drugs of low or moderately low emetogenic potential, no antiemetic drug or dexamethasone alone may be sufficient. For drugs of higher emetogenic potential, however, standard antiemetic treatment usually includes combinations of several antiemetic agents along with agents designed to treat anxiety, provide amnesia, or prevent known side effects. For drugs with high emetogenic potential, it is very important to give antiemetic therapy for an adequate period before administering chemotherapy agents, and to continue to prevent emesis for about 2472 hours after the drugs have been given. Other agents useful in combination include transdermal scopolamine and metopimazine, a new dopamine D_2 antagonist, which is a phenothiazine derivative. Transdermal scopolamine adds efficacy when added to a standard regimen of metoclopramide and dexamethasone. [202] Metopimazine significantly enhances the efficacy of ondansetron when used twice a day in patients receiving moderately emetogenic drugs. [162]

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Chapter 83 - Indwelling Access Devices

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INTRODUCTION

Indwelling devices that permit chronic access to the venous and central nervous systems have permitted novel and more comfortable forms of treatment for both children and adults. Indwelling central venous access devices are useful for patients who require frequent administration of blood or blood products, peripheral stem cell apheresis, or infusional therapy with medications such as chemotherapeutic agents, desferoxamine, amphotericin, or pain medications. Similarly, through the use of indwelling epidural or intrathecal catheters and Ommaya reservoirs, prolonged access to the cerebrospinal fluid (CSF) may be obtained for the delivery of chemotherapy, antibiotics, antifungal agents, or pain medications. The choice of the appropriate device and application of careful maintenance procedures can minimize the associated complications and maximize the patients quality of life.

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INDWELLING CENTRAL VENOUS ACCESS DEVICES

Chronic venous access devices can minimize the discomfort of repeated venipuncture; prevent venous thrombosis, phlebitis, and vesicant infiltration; maintain patient mobility; and minimize hospital stays. Certain patients with hematologic diseases are at increased risk for the development of thrombophlebitis from standard (peripheral) intravenous lines and may therefore be best served by an indwelling access device. Factors associated with this increased risk include (1) age of the patient (>60 years); (2) certain characteristics of the solutions being infused (e.g., hypertonicity of the solution, irritating diluents such as alcohol, highly acidic or alkaline pH, particulate matter in the solution); (3) type of drugs infused (vesicant chemotherapy, certain antibiotics, dexamethasone, furosemide, phenytoin); (4) factors associated with the catheter itself and its insertion (size and composition, traumatic insertion, microbial contamination); and (5) duration of infusion (85% of cases of phlebitis occur 2448 hours after placement of the intravenous line). [1] [2] [3] [4] [5] Since patients with these risk factors or with visibly poor peripheral venous access can usually be identified before therapy begins, early placement of an indwelling device is reasonable. To minimize the occurrence of septic episodes, the devices should be placed before the administration of agents that induce neutropenia. [6] [7] [8] With intraoperative platelet transfusions, catheters can be safely placed in patients whose thrombocytopenia is not due to disseminated intravascular coagulation. [8]

Device Types

Catheters

In all indwelling catheters used for prolonged central venous access, the proximal capped portion of the catheter exits from a subcutaneous tunnel on the chest or abdominal wall while the distal tip is indwelling in a central vein (Fig. 83-1).[9] All catheters are composed of either radiopaque elastomeric hydrogel, polyurethane, or silicone elastomer but differ in type of opening and internal diameter. [10]

Catheters with a simple opening at the distal tip include those placed at the bedside (midline catheters and peripherally inserted central catheters [PICCs]) and the tunneled Hickman, Broviac, and apheresis catheters, which are surgically inserted. Midline catheters, which do not extend into the veins beyond the arm itself, come in 24 to 16 gauge and are 38 inches long. [5] [10] The PICCs, which terminate in the superior vena cava, vary in size from 1.9 to 4.8 F (23 to 16 gauge) and are 1527 inches long. [10] [11] The tunneled catheters range from 2.7 to 12.5 F and are 29.542.7 inches long. [10] For apheresis (e.g., for peripheral stem cell harvest, or for preoperative exchange transfusions or vaso-occlusive crisis in patients with sickle cell disease [12]), 8.4 to 11.5-F catheters are available and are 5.418 inches long. [10]

The Groshong catheter, available in a variety of internal diameters, has a three-position valve adjacent to a closed distal tip. The valve remains closed at rest but opens outward for infusion when positive pressure is applied and opens inward for aspiration when negative pressure is applied. [13] Catheters with a Groshong-type opening are available as peripherally or surgically inserted devices.

For patients requiring nutritional support or infusional therapy along with blood sampling and blood product administration, all the above-mentioned catheters are available with double lumens, and there is a triple-lumen tunneled catheter available. [10] [14] Double-lumen devices have either 1.3-mm internal diameters each or 1.0- and 1.6-mm internal diameters; those with triple lumens have 1.0-, 1.0-, and 1.25-mm internal diameters (Fig. 83-2).

Figure 83-1 Schematic diagram of an indwelling central venous catheter in place. Insertion site refers to the insertion of the proximal end of the catheter into the central vein; the exit site denotes the exit of the distal end of the catheter at the chest wall. Clamp attached to the catheter is shown next to the Luer-Lok cap at the distal end. *(From Hickman Subcutaneous Port, Use and Maintenance, and How to Care for Your Hickman or Broviac Catheter. Davol Inc., Cranston, RI, with permission.)*

Implantable Central Venous Devices (Ports)

Totally implantable central venous access devices consist of single- or dual-lumen ports that may be round, square, oval, or hexagonal [10] and are connected to a radiopaque silicone elastomer or polyurethane catheter (Fig. 83-3).[9] [10] The port includes a self-sealing silicone septum with a body of plastic or metal, both of which will cause an artifact on magnetic resonance imaging (MRI). [10] The artifact effect is greater with stainless steel than with plastic. No portion exits onto the chest wall. This port is surgically placed in a subcutaneous pocket, and the catheter is inserted into a central vein. The single or double port is connected to a catheter with either a standard opening or a Groshong-type valve. Attached catheters range from 4 to 12 F and from 19.7 to 34.5 inches long. [10] For continuous infusions, these devices require an external infusion pump.

Ports that can be placed in the antecubital fossa are also available (the Port-A-Cath P.A.S. Port and the CathLink). [7] [10] [15] [16] [17] The P.A.S. Port reservoir is much smaller than the standard port, and the septum has only a 6.6-mm internal diameter. The attached 5.8-F polyurethane catheter has a 1.0-mm internal diameter, the same size as the Broviac. The CathLink catheter is made of a new polyurethane material (Chronoflex), and a series of panels with centrally located openings replaces its silicone septum. CathLink is accessed by standard peripheral intravenous catheters, such as angiocatheters. [8]

Other implantable devices have self-contained pumps. These devices and guidelines for their use are discussed elsewhere. [18] [19] [20] [21]

Device Choice

Patient characteristics and preference as well as the duration of use, purposes for which the device is required, and relative complication rates all aid in deciding among the types of

Figure 83-2 Central venous catheters with built-in clamps. **(A)** Hickman and Broviac single-lumen catheters. **(B)** Hickman and Leonard round dual-lumen catheters. **(C)** Hickman triple-lumen catheters. *(From Hickman Subcutaneous Port, Use and Maintenance, and How to Care for Your Hickman or Broviac Catheter. Davol Inc., Cranston, RI, with permission.)*

catheters and in choosing between catheters and ports (Table 83-1).[5] [7] [9] [14] [22] [23] [24] [25] [26] [27] [28]

Patient Characteristics and Preference

For female patients, or for any patient being treated in the outpatient setting, peripherally accessed devices may be the devices of choice, as the patient need not disrobe. The peripheral devices are also helpful in patients with chest wall abnormalities that would preclude the use of centrally placed catheters or ports (e.g., subcutaneous carcinoma of the chest wall, open wounds, tracheostomies, or fibrosis induced by radiation therapy). [8] [14]

Catheters with Groshong-type valves, which require less care, are preferred for patients unable to care for a Hickman or Broviac catheter at home. [13] [25] [30]

Children and adolescents have a lower risk of complications with ports than they do with catheters. [24] Four prospective nonrandomized studies indicated a significantly lower complication rate for the ports than for the catheters, especially in the

Figure 83-3 Totally implanted Hickman subcutaneous (central venous access) port with a noncoring needle in place. *(From Hickman Subcutaneous Port, Use and Maintenance, and How to Care for Your Hickman or Broviac Catheter. Davol Inc., Cranston, RI, with permission.)*

youngest children. [22] [24] [31] Ports are also useful for adult patients who are unable to care for a catheter or who do not wish to do so, and for those who prefer a less visible access device.

In obese patients and in patients with thrombocytopenia, the subcutaneous location of the ports poses a problem. In the obese patient, it may be difficult to find a port placed on the chest wall. For the patient with chronic thrombocytopenia (platelet count < 20,000/mm ^{3}), neither type of port may be satisfactory because of the risk of hematoma associated with the recurrent needle punctures required for access. Ports have been successfully used, however, to give prophylactic factor VIII therapy to children with severe hemophilia. [32] [33] [34]

Duration of Use

Because a surgical procedure is not needed, midline catheters and PICCs are useful for patients needing only short-term access, usually several weeks to months. The average PICC is in place for 210 weeks, but they have functioned for more than 300 days. [5] [11] [35] [36]

In patients needing a longer-duration of infusion and more extensive supportive care, surgically placed catheters and ports are generally preferred. Both catheters and ports have remained in place safely for months to years. [37] [38] Although the initial installation cost is greater for the ports than for the tunneled or nontunneled catheters, the replacement and maintenance costs of the catheter make it more expensive after 6 months of use. [24] [25] [26] The P.A.S. Port is even more cost-effective than a standard port because it does not require fluoroscopy, an operating room, or a physician for placement; physician assistants can place them safely. [10] [39] [40] [41]

Purpose for Which the Device Is Required

PICCs are used for antifungal therapy, hyperalimentation, or infusion of chemotherapy regimens that may include vesicant

TABLE 83-1 -- Choice of Indwelling Venous Access Device

	PICC[a]	P.A.S. Port	Tunneled Catheter	Port
Patient characteristics				
Chest wall problem	X	X		
Outpatient (especially female)	X	X		
Age				
Child or adolescent				X
Obese	X	X	X	
Thrombocytopenic	X		X	
Duration of use				
<3 mo	X			
<6 mo		X	X	
>6 mo				X
Purpose				
Frequent access			X	
Rapid infusion			X	X
Vesicant infusion	X		X	

[a] Peripherally inserted central catheter.

agents. [11] [30] [42] [43] Their smaller bore makes them less useful for blood drawing or transfusion. The P.A.S. Port is not recommended when fluids or blood products must be given rapidly, because the flow rate is only 610 ml/minute, which is less than that of standard central lines or ports placed in the chest. [41]

In general, surgically implanted catheters are more useful than ports or PICCs in patients in whom frequent access to the device or frequent blood drawing or blood product administration is needed. [7] The larger-bore catheters are more useful for blood drawing and blood product administration because the incidence of clotting after such use is lower with them than with smaller-bore devices. [37] Groshong-type catheters, however, may be less useful in patients from whom blood must be drawn frequently because the valve may make blood aspiration more difficult. If a port or a PICC is chosen, the catheter with the largest bore is recommended. In the larger-gauge PICCs, which collapse easily, blood drawing is usually more successful if a syringe is used instead of a vacuum tube collection system. [42]

Continuous vesicant infusion may be more safely accomplished with catheters, as they avoid the danger of needle dislodgment and disconnection of the catheter from the septum associated with the implanted ports. [44] However, the use of a needle-containing device called a Gripper, which holds the needle in the port, may decrease the incidence of needle dislodgment.

For support during autologous or allogeneic bone marrow transplantation, double- or triple-lumen catheters are used. [45] [46] In those transplant patients undergoing peripheral stem cell apheresis, percutaneously placed translumbar silicone apheresis catheters are preferred, because the internal diameter of the Hickman catheter

is often too small.[47]

Relative Complication Rates

Midline catheters are associated with lower rates of phlebitis than short peripheral catheters and with lower infection rates and lower cost than PICCs or tunneled catheters.[5] PICCs cause more phlebitis than tunneled catheters, and earlier reports indicated that the infection rates associated with their use were also higher than those for tunneled catheters. Recent studies, however, suggest that the infection rates may be the same for PICCs and tunneled catheters. [5] [8] Conflicting data have been reported regarding the relative complication rates seen with tunneled catheters and ports, although the overall complication rate is probably less for the ports than for the catheters. Five of six prospective randomized trials and all retrospective studies, including one cohort study and one matched study, found a significantly higher rate of complications with tunneled catheters than with ports. [5] [7] [48] [49] Only catheter occlusion is no more likely with catheters than with ports. [7] Patients with ports also reported fewer restrictions in activity and hygiene. [48]

In human immunodeficiency virus (HIV)-infected patients, retrospective studies demonstrate that catheters [50] [51] [52] appear to cause more infectious complications than ports (0.47 to 0.97 per 100 days, versus 0.17 to 0.39 per 100 days). [52] [53] Patients receiving infusional ganciclovir through catheters for cytomegalovirus retinitis who had neutrophil counts less than 1,000/mm^3 were at particular risk for early septicemia. [52]

Device Insertion

Catheters

Catheters can be inserted nonsurgically or surgically. Midline catheters and PICCs are placed nonsurgically in the hospital or in the home by appropriately trained physicians or nurses. [10] [30] [42] Insertion failure can occur in 822% of attempts, although the failure rate decreases with experience. [11] After careful skin preparation using sterile technique and, for large-gauge introducers, local anesthesia, the catheter is inserted in the antecubital fossa into the basilic vein. PICC catheters are advanced into the superior vena cava (or, less frequently, the axillary, innominate, or subclavian vein) and secured by sutures or sterile tape. If chemotherapy, total parenteral nutrition solutions, or certain hyperosmolar solutions are to be infused, radiologic verification of the catheter tip position in the superior vena cava is recommended.[10]

Tunneled Hickman, Broviac, and Groshong catheters (and ports; see below) are inserted surgically in an operating suite or minor procedure room, physicians office, radiology suite,[54] or at the bedside. [8] All catheter insertion techniques include (1) creating a subcutaneous tunnel on the anterior chest wall, (2) pulling the catheter up through the tunnel, and (3) positioning the catheters Dacron cuff(s) in it, leaving its remaining proximal portion, with the Luer-Lok tip, exiting from the tunnel on the chest wall (Fig. 83-1). [7] [13] [22] [55] The distal catheter tip is then usually placed percutaneously into the central circulation through the axillary, subclavian, or cephalic vein[20] [56] and threaded into the superior vena cava under fluoroscopic guidance. [10] Its position in the superior vena cava at the right atrial junction [8] should be confirmed by chest radiography. In patients expected to need crutches for a prolonged period of time, catheters should be inserted via the internal or external jugular vein or in the subclavian vein lateral to the midclavicular line to prevent pinching the catheter in the costoclavicular space, which leads to catheter pinch-off and fracture.[28]

No significant difference has been found in time to failure or in infection or obstruction rate of catheters inserted percutaneously rather than under direct visualization.[20] [57] [58] Catheters should be inserted by experienced personnel, however, as complication rates fall with increasing experience in catheter insertion. [37] [59] [60] If the chest wall or the superior vena cava and subclavian veins are not usable, the best alternative site is the inferior vena cava. [20] [47] [61] [62] Apheresis catheters for peripheral stem cell harvest are preferentially inserted into the inferior vena cava. Techniques for their insertion are reviewed elsewhere. [47]

Ports

Ports are surgically inserted into the antecubital fossa or chest or abdominal wall with the patient under local anesthesia. [63] If possible, they should be placed in the nondominant arm or in the side of the chest near it to minimize the probability of needle dislodgment or coring of the port septum during continuous access. [19]

The P.A.S. Port can be inserted at the bedside by physicians or physician assistants. [8] [39] [40] [41] It consists of an implantable port with an attached polyurethane radiopaque catheter, the distal tip of which contains an electromagnet. The port is placed percutaneously in a pocket in the antecubital fossa. The venous catheter is introduced using the same techniques as described above for PICCs without attached ports. [39] [40] [41] [42] Fluoroscopy is unnecessary, as the progress of the catheter toward the right atrium can be externally tracked with a hand-held sensor wand (Cath-Finder) that detects the electromagnet in the distal tip. [10] A chest radiograph to verify catheter position is still recommended prior to infusion of certain solutions. [39]

The chest wall ports are placed into a subcutaneous pocket in the infraclavicular space, and the attached catheter is placed under fluoroscopic guidance into the subclavian, jugular, or cephalic vein and threaded through the superior vena cava to the junction with the right atrium (Fig. 83-3). No portion of the device remains visible.

Device Management

Catheters

Care of nontunneled and tunneled catheters is begun by the nurses and later taught to patients and their families. To minimize

infectious complications, it is preferable to assign this responsibility (as well as infusions and blood sampling through the catheter) to nurses expert in catheter care and use.[5] [22] [64] [65] [66] Standard procedures are available and should be adopted by each institution, to be followed by all who care for the patient. [14] [30] [42] [67]

Patient and family education in catheter care is required to maintain patency, to prevent damage to the external portion of the catheter, to prevent air embolization, and to reduce the risk of infection. [13] [23] [30] [42] Instructions should cover permissible activities, techniques of dressing change, heparin instillation, and changing the Luer-Lok plug, as well as emergency care in the event of damage to the external portion of the catheter. This educational program should be started well in advance of patient discharge, to ensure patient and family competence, and may be reinforced by an educational booklet, [68] by attendance at a hands-on class, or by nurses sent into the home, at least initially, to verify compliance with procedures. Patients with catheters or ports in place should be given written information about the device[19] as well as a medical alert card, necklace, or bracelet indicating its location and type of access and a person to be contacted if problems arise. [30]

Dressing changes for surgically inserted catheters require sterile technique for 2 weeks after insertion, until fibrous growth into the Dacron cuffs is complete. [9] [23] [64] The catheter exit site is cleaned with a povidone-iodine solution. Hydrogen peroxide is also sometimes used, as it prevents scab formation at the site. Sterile dressing changes are done every other day or every third day for 23 weeks after insertion, depending on the dressing materials used. [9] [14] [23] The type of dressing, if any, needed to cover the site is controversial. [14] [23] [30] One prospective randomized study indicated that when a rigorous cleaning protocol was followed, the incidence of site infection was not different whether standard gauze and povidone-iodine, a transparent polyurethane dressing (e.g., Opsite and Tegaderm) that is permeable to water vapor, or no dressing was applied. [69] Another study, however, found an increased risk of infection when transparent dressings were used. [70] The advantages and disadvantages of transparent dressings, gauze, or no dressing have been reviewed. [30] Moisture vapor-occlusive dressings are strongly recommended for patients with tracheostomies or open wounds near the catheter site. After 23 weeks, clean technique is used in some institutions for non-neutropenic patients, and a Band-Aid or small gauze dressing may be used to cover the exit site. [9] [71] More complete guidelines for access and maintenance of surgically inserted catheters are available. [5] [14] [67]

The catheter is not usually considered to be securely anchored in place until 1021 days after catheter placement, although insertion site stitches are usually removed after 10 days. Even then, catheters should not be allowed to hang freely; instead, they should be taped to the chest wall or inserted into a brassiere cup. [30] A clamp should be immediately available in the event of any breaks in the line. Some manufacturers include a clamp placed on the catheter (Fig. 83-2). Patients should be

advised to keep the catheter out of the reach of pets or small children, who could inadvertently dislodge it by pulling on it.

Since a thrombus on the catheter tip can be the nidus for a bacterial or fungal infection, [72] [73] every effort should be made to prevent one from forming. In a prospective randomized trial, low-dose coumadin (1 mg/day) begun 3 days before catheter insertion and continued for 90 days significantly decreased the rate of venogram-proved thromboses from 38% in control patients to 9.5% in those treated with warfarin. [74] In addition, heparin should be instilled in PICC and Hickman- and Broviac-type catheters after each episode of blood drawing or blood product administration and when the catheters are not being used for intravenous therapy. A minimum of 5 ml of heparin in saline (100 U/ml) should be instilled in each lumen [14] because such flushes reduce bacterial growth on catheter tips. [75] In the outpatient setting, PICCs require 12 ml daily, [14] while for tunneled catheters, twice weekly flushes may be adequate if the catheter is not being used. [30] A further decrease in intraluminal infection rate in immunocompromised, non-neutropenic patients with tunneled catheters may be provided by adding vancomycin (25 g/ml) to the heparin flush. [76] [77] The PICC or tunneled line is then plugged with a Luer-Lok injection plug, which is taped in place and changed once a week. [9] [14]

It should be remembered, however, that the use of the heparin flush will alter coagulation tests if blood is drawn through the catheter. [78] The first 10-ml sample will show spurious elevations in levels of fibrin degradation products, prothrombin times, and partial thromboplastin times, and spuriously low fibrinogen levels. Elevations in the prothrombin time and partial thromboplastin time will persist in the second 10-ml sample.

Catheters with Groshong-type valves require less maintenance than do Hickman or Broviac-type catheters. However, they do require a 5-ml normal saline flush weekly or after use [9] [13] [14] [29] because the special valve can be kept in the open position by small clots or solid residues from medications, and blood will flow back into the catheter, forming a clot.

For apheresis catheters used for peripheral stem cell harvest, a regimen of aspirin, 325 mg/day, begun the day after catheter placement led to a greater number of thrombosis-free apheresis procedures than was noted with historical control subjects. [79] More detailed guidelines for nursing education and practice regarding maintenance of catheters are available. [67]

Ports

Ports can be used immediately after insertion. However, because postoperative edema and discomfort often delay any attempts at access for several days, patients should be sent from the operating room with the Huber needle in place and ready for use. [19] [30] [80]

To access a port that has no needle in place, the skin is prepared with povidone-iodine solution [81] and a freezing agent (ethylene dichloride) or topical anesthetic cream (EMLA) [82] is applied, followed by insertion of a 19- to 22-gauge Huber needle (depending on the product to be infused) (Fig. 83-3). [19] This steel needle has a deflected point and side opening, designed to prevent coring of the septum. [18] The needle is primed with saline, attached to a saline-containing syringe and connecting tubing, and then inserted perpendicular to the septum. [83] Correct placement in the port can be confirmed by aspiration for blood return. However, aspiration for blood is often unsuccessful, usually because the needle is misaligned or a fibrin sheath has formed, creating a ball-valve effect. In the absence of swelling after a 10- to 20-ml saline infusion, the needle can be assumed to be correctly placed and the port safely used. [9] Detailed accessing procedures for drawing blood and for administering drugs and blood products are available. [19] [83]

During use, the port is covered with a transparent occlusive sterile dressing, further reinforced by a window frame of paper tape. [18] [30] [83] The dressing is changed when the needle is changed, as well as when the dressing becomes nonocclusive or wet or evidence of infection or skin irritation develops. After each use or every 4 weeks, implanted access devices also require heparin instillation (35 ml of 100 U/ml heparinized saline solution) or a 20-ml saline flush. [9] [18] [30] Use and maintenance of the peripheral port are essentially the same as for ports implanted in the chest wall. [15] More detailed guidelines for nursing education and practice regarding the maintenance of implanted ports and reservoirs are available. [80] [84]

Device Removal

Nontunneled catheters are removed nonsurgically by applying steady tension. If the catheter appears to be stuck, measures

should be taken to reverse vasospasm (e.g., application of warm compresses, flushing with normal saline). [10] If the catheter remains fixed, a cutdown on the venous insertion site to remove thrombotic material is usually effective. [8] Tunneled catheters should be removed by surgical cutdown around the cuff, which should be entirely removed. [8] For cases in which bacterial studies of the internal catheter tip are desired, blunt dissection of the cuff, with transection of the catheter above it and sterile removal of its inner portion, can be performed under local anesthesia. [44] [85] Ports, including the reservoir, catheter, and all suture materials, are removed surgically with the patient under local anesthesia. [8]

Complications

Complications of indwelling vascular access devices include those occurring during initial placement, cutaneous reactions to standard care materials, mechanical problems, phlebitis and infiltration, infection, hemorrhage, catheter occlusion of nonthrombotic or thrombotic origin, and vesicant drug extravasation.

Initial Placement

Rarely, patients who have had a midline catheter composed of a hydrogel elastomer inserted and flushed experience an anaphylactoid reaction (5% of which are complicated by cardiac arrest). [5] [86] This reaction was not seen when silicone midline catheters were used. [87] Some 14% of patients in whom a central catheter is inserted percutaneously develop a pneumothorax. [8] Other surgical complications include hemorrhage into the thoracic space, venous or cardiac perforation, and pericardial tamponade. [8]

Cutaneous Reactions

Approximately 5% of patients develop skin reactions to the products used in caring for the devices. [88] These reactions include erythema, urticaria, exanthematous or purpuric eruptions, and skin peeling or abrasion. These skin abnormalities must be distinguished from exit-site infections by appropriate culturing of the site for bacteria. In uninfected patients, change of the dressing material, tape, and local skin care regimen is effective in reversing the skin abnormalities. [88]

Skin erosion over the implanted ports, either from malnutrition, separated wound edges, local infection, or carcinoma metastatic to the skin, occurs in 310% of patients, and removal of the port is always required. [30] [89] P.A.S. Ports may cause this complication more frequently than ports placed on the chest or abdominal wall. [8]

Mechanical Problems

Catheters

Damage to the external segment of the tunneled catheter includes (1) separation between the Luer-Lok connection and the tubing; (2) cracks caused by repeated cross-clamping, if rubber-tipped forceps are not used; and (3) cuts made by scissors mistaken for clamps. [9] Catheter repair kits should therefore be available. Patients should also be instructed to contact their physicians or the oncology nursing staff immediately if the catheter is damaged at home.

Catheter fractures and emboli occur in about 0.2% of subclavian catheter insertions. [8] The internal portion of the catheter usually fractures at the junction of the clavicle and first rib, [90] and angiographic studies reveal the pathognomonic catheter pinch-off sign. [91] Catheter pinch-off occurs when catheters inserted medially to the midclavicular line become compressed by shoulder movements. [91] The resulting fragments migrate to the right heart or pulmonary artery and cause thromboses, cardiac arrythmias, and fatal emboli; extravasation of fluids, lymph, or vesicant agents may also ensue. [8] [90] Patients complain of pain or swelling at the port or vein

insertion site, chest pain, cough, or palpitations. [90] Catheter fragments are usually removed percutaneously. [90]

PICC lines can also embolize if they are sheared by needles, sutures, or surgical instruments while they are being inserted. If the fragment cannot be prevented from migrating more proximally by applying local pressure at the break site or by placing a tourniquet proximal to the site, it can be removed by venous cutdown. [90] [92] If it has migrated, percutaneous techniques or a thoracotomy will be required, [90] [92] but some fragments are never retrieved.

Apheresis catheters placed in the inferior vena cava can also develop fractures of the external segment, with visible leakage, [47] and they can be repaired using the kits mentioned above. Fractures of the internal portion of the catheter, however, require catheter removal. Most of these fractures can be detected by injecting radiologic contrast material through them and observing the flow of the dye fluoroscopically. [47]

Ports

The port may flip within its pocket because of defective suturing or excessive arm movement, or because of manipulation by the patient. [30] [93] This malposition can be surgically corrected.

Phlebitis and Infiltration

Phlebitis and infiltration are uncommon complications associated with properly positioned centrally placed access devices but are noted with the P.A.S. Port [8] and in 2.223% of patients with PICCs. [5] [16] [42] [94] [95] A randomized controlled trial compared the complications associated with steel needles, small-bore short Teflon catheters, and PICCs. [96] PICCs were associated with the highest incidence of phlebitis (27%) and the lowest incidence of infiltration (8.1%), steel needles were associated with the lowest incidence of phlebitis (8%) and the highest incidence of infiltration (45%), and Teflon catheters fell in between, with a 1920% incidence of both phlebitis and infiltration. The aseptic phlebitis usually occurs within the first week after PICC insertion. It can be prevented by administration of nonsteroidal anti-inflammatory agents [39] or treated with warm compresses applied for 4872 hours. [42] Catheter removal is usually not required.

Infection

Definitions

The Centers for Disease Control have established the following definitions for catheter- and port-associated infections: [5]

Colonized catheter: growth of 15 colony-forming units (semiquantitative culture) or >10^3 (quantitative culture) from a proximal or distal catheter segment in the absence of accompanying clinical symptoms.

Exit-site infections: erythema, tenderness, induration, or purulence within 2 cm of the skin at the exit site of the catheter.

Pocket infection: erythema and necrosis of the skin over the reservoir of a totally implantable device, or purulent exudate in the subcutaneous pocket containing the reservoir.

Tunnel infections: erythema, tenderness, and induration in the tissues overlying the catheter and >2 cm from the exit site.

Catheter-related bloodstream infection: isolation of the same organism (i.e., identical species, antibiogram) from a semiquantitative or quantitative culture of a catheter segment and from the blood (preferably drawn from a peripheral vein) of a patient with accompanying clinical symptoms of bloodstream infection and no other apparent source of infection. In the absence of laboratory confirmation, defervescence after removal of an implicated catheter from a patient with bloodstream infection may be considered indirect evidence of catheter-related bloodstream infection.

Infusate-related bloodstream infection: isolation of the same organism from infusate and from separate percutaneous blood cultures, with no other identifiable source of infection.

Incidence

Both retrospective and prospective studies showed exit-site infections to be responsible for 3945.5% and tunnel infections for 20.322% of all catheter-related infections. [37] [97] Skin contaminants are the likely source of infections for catheters in place for less than 10 days, while the catheter hub is the more likely source if the catheter has been in place for more than 30 days. [98] Prophylactic antibiotics have not consistently proved beneficial in preventing these infections, and their use is not recommended. [5] [23] [37] Similarly, silver-impregnated cuffs do not decrease infection rates. [99] Infections are generally prevented by meticulous insertion and maintenance techniques. [64] [65] With maximal barrier precautions, catheter insertion at the bedside is associated with no higher an infection incidence than placement in the operating room. [5]

The incidence of device-associated infection varies depending on the type of device inserted and the medical disorder of the patient. Overall, the incidence of midline catheter-associated infections is 0.8 per 1,000 days of patient use [86] and that of tunneled catheters is about 13 per 1,000 days of patient use. [23] The use of multiple-lumen catheters probably increases the infection rate significantly. [5] The infection rate with implanted ports is lower, 0.3 per 1,000 patient-days for ports inserted on the chest wall and 0.8 per 1,000 patient-days for P.A.S. Ports. [23] [100] [101] Patients with HIV infection [50] [51] [52] [53] or those receiving a bone marrow transplant [45] [46] [102] have significantly higher rates of both catheter- and port-related infections. Patients with hemophilia [33] and sickle cell anemia [103] do not (0.60.86 per 1,000 days of patient use).

Organisms

Staphylococcus, Streptococcus, and *Candida* species are the most common infecting organisms. [23] [104] Both staphylococcal and candidal organisms adhere to the fibrin or fibronectin sheaths surrounding indwelling catheters, and the staphylococcal slime further promotes bacterial proliferation on the catheter surface. [73] [104] In patients not infected with HIV, an average of 28% of infections are due to coagulase-negative staphylococci, 16% to *Staphylococcus aureus,* and 8% to enterococci. [5] Other gram-negative organisms, including *Pseudomonas,* also cause infection. [8] [105] *Pseudomonas* is particularly problematic when showering or swimming has been allowed. *Xanthomonas* infections can be particularly troublesome.

A reported 5.49.9% of infecting organisms are fungal, three-quarters of which are *Candida* species, [4] [23] [37] [106] [107] although nosocomially acquired cutaneous infections with *Aspergillus flavus* have been noted at the sites of insertion and along the subcutaneous tract of Hickman catheters. [108] [109] [110] Liposomal amphotericin B was successful in eradicating a port-associated fungemia from *Fusarium oxysporum* in an HIV-infected patient. [111] Septicemia due to catheter infection by either typical or atypical *Mycobacterium* species has also been noted. [22] [112] [113] [114] [115]

In HIV-positive patients, staphylococci are the most common infecting organisms, [50] [51] [52] but *Pseudomonas* species causing fatal septicemias, [50] [52] [116] [117] [118] [119] other gram-negative organisms, [120] and lactobacilli [121] are increasingly reported. *S. aureus* is the most common organism infecting ports of patients with hemophilia, especially those with inhibitors. [33]

Indications for Removal

Exit-Site and Tunnel Infections

Exit-site infections caused by bacteria rarely require catheter removal for resolution, as most (69100%) will respond to antibiotics

TABLE 83-2 -- Indications for Catheter Removal

Venous access devices
Exit-site infection caused by atypical mycobacteria
Tunnel infections
Bacteremia caused by *Staphylococcus aureus, Xanthomonas, Pseudomonas* spp.
Fungemia
Central nervous system access devices
Deep catheter track or epidural space infection

alone.[37] [59] [60] [97] [103] These infections are most often caused by *S. epidermidis.*[20] [23] Similarly, with implanted ports, infections of the skin pocket have been found to resolve in about 70% of cases without removal of the device.[22] [23] [38] [63] [103]

Tunnel infections, by contrast, usually do not respond to antibiotics; they have been reported to resolve without catheter removal in only 2550% of cases. [37] [59] [60] [97] Catheters were removed from all patients with cutaneous *Aspergillus* infection, six of whom recovered after antifungal therapy and local wound care (Table 83-2).[108] Resolution of leukopenia was required for infection resolution. Similarly, *Mycobacterium* [22] and atypical mycobacterial infection of the tunnel or exit site requires catheter removal as well as excision of infected tissue.[115]

Septicemia

In a patient with septicemia and a catheter or port in place, it is often difficult to determine whether the catheter itself is truly infected or whether the bacteremia is from another source. Quantitative culture is the most sensitive technique,[5] but the semiquantitative roll-plate method is most often used to diagnose catheter-related infections.[122] [123] Both, however, require that the catheter be removed. Quantitative blood culture techniques can be used with a catheter in place. If the colony count in cultures of blood drawn from the catheter is 5 to 10 times greater than the count in cultures of peripherally drawn blood, the infection is very likely to be catheter related.[5] For presumed *Candida* infection, it has been suggested that molecular analysis of material from various anatomic sites may help determine the true source of the infection (e.g., sputum versus blood) and prevent needless device removal.[124]

In assessing the cause of infection in a patient with a port, care must be taken not to draw blood for culture through a possibly infected port pocket unless the Huber needle is already in place. Accessing a port through an infected port pocket could introduce organisms into the reservoir, and from there into the systemic circulation.[30] [103] [125] To determine whether a port is truly infected, after it is removed, material within the port should also be cultured.[126]

When another source is definitely identified, however, the catheters can usually be left in place, because the incidence of hematogenous colonization is low (1%). [37] [55] When there is no thrombophlebitis, even when the catheter is believed to be the source of the infection, or when no source is clearly identified, resolution of bacterial septicemia occurs in 75% of episodes without removal of the catheter.[23] [127]

The possibility of clearing the infection without catheter removal, however, is much lower in patients with septic thrombophlebitis, occluded catheters, exit-site infections, or bacteremia due to *S. aureus, Xanthomonas,* or *Pseudomonas* or fungal septicemia.[8] [23] [52] [128] [129] In one retrospective review, adult cancer patients with candidemia did just as well with catheters left in place as those whose catheters were removed,[130] and in one pediatric study, *Candida* fungemia was treated successfully with the line left in place.[6] In other studies, however, children with catheter-related candidemia had higher rates of treatment failure, secondary complications, and mortality if the catheters were left in place during antifungal treatment.[131] [132] In general, catheter

removal is recommended in patients with bacteremia due to *S. aureus, Xanthomonas,* or *Pseudomonas* and in those with fungemia due to either *Aspergillus* or *Candida* (Table 83-2). Successful surgical removal of right atrial thrombi superinfected with *C. tropicalis* and *S. epidermidis* has been reported in several patients, including those with sickle cell anemia.[133] [134] Replacement of catheters on the contralateral side within 13 days after removal is usually not associated with recurrent catheter infection.[55] [63]

Hemorrhage

Despite the frequent occurrence of thrombocytopenia in patients with indwelling access devices, few bleeding complications are associated with their placement or with the placement of the larger apheresis catheters.[55] [55] Capillary fragility from prolonged steroid therapy, however, may contribute to perioperative hemorrhage.[59] Pressure dressings and platelet transfusions given pre- and postoperatively usually control local oozing.[55] However, in patients with uncontrolled disseminated intravascular coagulation, excessive bleeding has occurred with catheter insertion; many groups consider disseminated intravascular coagulation an absolute contraindication to catheter placement.[23] [55] [59] In contrast, catheters and ports have been placed without excessive bleeding in patients with hemophilia when factor levels were maintained at 100% preoperatively and for 5 days postoperatively.[33] [34]

Catheter Occlusion

Nonthrombotic Causes

Although clot is the most common cause of occlusion,[104] inability to aspirate blood from the port or catheter does not necessarily mean that it has clotted. Other causes include a malpositioned Huber needle, catheter abutment against the wall of the vein, catheter kinking, catheter pinch-off, precipitation of drug solutions in the catheter lumen, development of fibrin sheaths, and catheter migration with resultant malposition of the tip.[30] [104] [135] Algorithms for the evaluation and treatment of catheter occlusion are available.[104]

Catheter abutment may resolve by changing the patients position or by performing a Valsalva maneuver.[14] [19] [30] Repositioning patients with catheter pinch-off will often relieve the compression and re-establish catheter function.[90]

Precipitation of incompatible solutions occurs with etoposide, calcium, diazepam, phenytoin, heparin, and TPN infusions.[104] Catheter blockage due to precipitation of calcium carbonate was reported at from 8 to 24 weeks in 50% of patients with metastatic colon carcinoma receiving once weekly 24-hour infusions of high dose 5-fluorouracil (2,600 mg/m2) and leucovorin (500 mg/m^2).[136] Although precipitates may occasionally resolve with warm soaks over the tunnel site, dilute solutions of hydrochloric acid have successfully cleared precipitates from TPN solutions alone, or with lipids, etoposide, calcium salts plus sodium bicarbonate, and heparin with incompatible antibiotics.[8] [104] The 0.20.5 ml solution of 0.1M HCl is instilled and allowed to stand for 20 minutes.[104] Febrile reactions may occur. Sodium bicarbonate has, in one study, resolved a phenytoin precipitate.[137]

Occlusion by a fibrin sheath, leading to a ball-valve effect,[9] [20] occurs in 1020% of cases.[135] [138] The fibrin sleeve itself may embolize as well.[139] [140] Techniques using nonlytic and lytic agents to remove the sheaths have been described.[19] [30] Lytic therapy is discussed below.

Maintaining correct position of the catheter tip can help prevent numerous complications. Catheter migration occurs in 5.529% of insertions when the subclavian approach is used.[141] Review of the patients chest radiograph will most commonly show the malpositioned catheter tips to be in the ipsilateral jugular vein or in the contralateral brachiocephalic vein. Venous thrombosis can occur as a result of damage to the endothelium, turbulence created by the tip at venous branching points, or insufficient dilution of infusates that cause thrombophlebitis. Catheter erosion through the vessel or cardiac wall can produce extravasation, fistula formation, and pericardial tamponade.[90] Catheters inserted into the left internal jugular or subclavian veins have also eroded into the bronchi; the ensuing venobronchial fistulas

were associated with cough, pneumonia, and respiratory failure. [142] Repositioning the catheter can usually be done without catheter removal, using angiographic techniques. [135] [141]

PICC malposition ranges from 2155%. [90] The catheters can usually be repositioned by trained nurses employing simple bedside techniques. [90] Migration of the apheresis catheter in the subcutaneous space also manifests as access failure, since the catheter tip is pulled back to the wall of the inferior vena cava or out of the intravascular space. [47] Intravascular malposition can be corrected by tip deflectors or J-wires. [143]

Thrombosis

Thrombosis can occur in the catheter itself or in the superior vena cava or veins of the upper extremity. The thrombus usually results from failure to follow standard flush procedures. Thromboses were seen in 16% of implanted ports. [38] An incidence of 18% is reported with PICCs, [144] but the incidence of thrombosis in patients with properly cared for centrally inserted catheters is only 010%. [27] [38] [145] [146] Retrospective studies of sickle cell patients indicated no increased incidence of thrombosis necessitating catheter removal as compared with patients with cancer or AIDS. [103] The incidence is equally low in syngeneic and allogeneic marrow transplant patients, despite the use of multiple-lumen catheters. [45] [46] [47] In patients undergoing autologous marrow transplantation, however, the incidence of thrombosis is 1320%. [146] This higher incidence may be due to the increased number of patients in this group who have platelet counts greater than 150,000/mm ^{3} and to the frequent placement of two catheters at bone marrow harvest. [146]

Noninvasive methods used for diagnosis, including duplex ultrasound, [145] [147] MRI, [148] Doppler imaging, [145] [149] and plethysmography, [145] have low sensitivity or have not been reliable, and nuclear scans have been falsely normal because of the development of collateral circulation. [145] Venography is recommended to support the clinical diagnosis. [38] [47] [145] In patients with prior subclavian catheterization, however, duplex scans may be useful to predict the success of repeat catheter placement. [150]

Therapy

The course of action in patients undergoing high-intensity antineoplastic therapy and in whom asymptomatic subclavian or innominate vein thromboses develop is unclear. Asymptomatic occlusion is seen in 25% of patients undergoing autologous marrow transplantation; partial occlusion develops in an additional 34%. [151] Because the incidence of complications caused by these thrombi is unknown, no therapeutic recommendations can be made.

Patients with symptomatic upper extremity deep venous thrombosis (DVT), however, probably do require therapy. They may develop septic thrombophlebitis, [23] [37] superior vena cava syndrome, major long-term upper extremity disability, venous gangrene, and pulmonary emboli. In one retrospective study and review, 12% of patients with catheter-induced upper extremity DVT developed pulmonary emboli, 40% of which occurred in patients receiving anticoagulant therapy. [145] A 16% incidence of pulmonary emboli was found in a prospective study of patients with upper extremity DVT in whom lung scans were performed within 24 hours of venographic diagnosis of the thrombosis. [152] Patients with polyurethane and siliconized catheters had a significantly lower incidence of emboli than those with polyvinyl chloride or polyethylene catheters (7% vs. 26%). [152]

There is no consensus about the best therapy for patients with upper extremity DVT. Some reports suggest that conservative therapy (heat and elevation) is efficacious, producing resolution of edema and pain and minimal chronic venous insufficiency. [146] Other investigators have recommended anticoagulation with heparin, thrombolysis, or surgical clot removal. [38] [47] [145] [153]

Anticoagulation with heparin, however, has not been consistently successful in preventing pulmonary emboli from subclavian vein thrombi. [141] [152] [154] Therefore, bolus or infusional thrombolytic therapy with streptokinase, urokinase, or tissue plasminogen activator (t-PA) is usually considered. Bolus thrombolytic therapy has reopened occluded catheters in 8590% of episodes, and removal of the catheter is not usually required. [64] [153] [155] [156] [157] [158] One commonly used procedure is to instill 13 ml of a solution of streptokinase (75010,000 IU/ml) or urokinase (5,000 IU/ml) into the catheter or port, leave it in place for 20 minutes to 1 hour, and then aspirate at 5-minute intervals until the catheter reopens. [67] If the first instillation is ineffective, the procedure can be repeated or the other solution used. [47] [104] [155] [157] [159] No excessive bleeding has been noted, even in patients with hemophilia. [33] A randomized trial comparing bolus urokinase (10,000 U) with t-PA (2 mg) suggested marked superiority for t-PA. Twenty-five of 28 t-PA-treated versus 13 of 22 urokinase-treated catheters returned to normal function, 17 versus 7 were normal radiographically, and 13 versus 4 needed only one drug instillation. [158] Similarly, fibrin sheaths have been dissolved in 87% of patients in whom streptokinase was infused (3,000 IU/h) into each catheter lumen for 1224 hours. [135] No fevers, coagulation abnormalities, or other adverse reactions have been noted with urokinase, but streptokinase can cause mild to moderate systemic reactions (fever and chills) in patients with recent streptococcal infections or in those who have previously received the drug for thrombolysis. Once patency is restored, heparin is usually given for 57 days.

For thrombi refractory to the bolus administration of streptokinase or urokinase, options to dissolve the thrombus include infusions of urokinase or streptokinase through a catheter placed at or within the clot. [153] [160] High-dose streptokinase therapy is effective [160] but expensive and is associated with a high incidence of bleeding. It is not recommended for patients with such bleeding risks as thrombocytopenia or mucositis. Infusion of urokinase for 2472 hours restored catheter patency in 74% of patients (81% of those with clots present for less than 7 days and 56% of those with clots present for more than 7 days). [153] Infusion into the superficial venous circulation of the involved extremity was not effective. Even though a low infusion concentration was used (5,000 IU/h of streptokinase or 5002,000 IU/kg/h of urokinase), a systemic lytic state was documented. Therefore, urokinase infusion should not be undertaken in patients with contraindications to systemic fibrinolytic therapy. [161] Another regimen for refractory clots involves an infusion of urokinase (40,000 U/h) for 6 hours. This regimen led to dissolution of thrombi in 90% of patients; a repeat 6-hour infusion raised the dissolution rate to 95%. [162] [163] Infusions as short as 13 hours were successful in half the cases studied. [164] Addition of heparin to the 1- to 12-hour infusion did not improve the results. [164] No bleeding complications were seen. In patients with catheter tips placed below the carina and not adherent to the venous wall, there is a low risk of reocclusion; these catheters need not be removed. [164] The same urokinase instillation technique is recommended for patients with occluded ports. [80] Successful clot resolution with urokinase was reported in 5087% of patients. [22] [63]

Vesicant Drug Extravasation

It is very unusual for a spontaneous leak to form in a large-bore catheter, but an attempt to irrigate an occluded catheter with a small syringe can cause a rupture through which the drug can extravasate. [9] [55] Occlusion of the catheter tip by a fibrin sheath may force drug back up the sheath and through the exit site of the catheter. [38] [165] This so-called back-tracking appears to be more common with percutaneously placed access devices. [90]

Leaks may develop if the catheter is disconnected from the reservoir or if the catheter is punctured by mistake by the Huber needle. [30] In addition, outpatients using a port for continuous infusion of chemotherapy can experience drug extravasation if the Huber needle is dislodged from the septum. [44] [166] [167] [168] Even usually nonvesicant drugs can produce skin necrosis severe enough to warrant removal of the port. [44] Use of the Port-a-Cath port, which has a thicker septum than the MediPort or Infus-a-Port devices, has been associated with only a 34% incidence of extravasation. [63] [166] [167] [168] The thickness of the septum has been postulated to be responsible for the low incidence of needle displacement noted with the Port-a-Cath, [168] but no randomized trials have compared complications associated with the three devices. Securing the needle to the chest wall with tape has been recommended, [166] [167] but use of a Gripper needle provides a much more secure needle placement and may lower the incidence of this complication. If infusion pumps are used, attention must be paid to minimizing tension between the needle and the infusion tubing.

Treatment protocols for vesicant drug extravasation, as recommended by the Oncology Nursing Society, [169] [170] [171] [172] [173] [174] [175] [176] are outlined in Table 83-3 . In studies of their use (excluding the recommended therapy with dimethyl sulfoxide [DMSO]), 89% of vesicant extravasations were reported to resolve without additional

TABLE 83-3 -- Management of Vesicant Drug Extravasation

Drug Therapy	Antidote Preparation	Method of Administration

Antibiotics[170] [171] [176] Dactinomycin Mitomycin Plicamycin Daunorubicin Doxorubicin	None	Apply 12 ml of 1 mmol DMSO (50100%) to an area twice that of the affected area once. Air dry without a dressing. Apply *cold* packs for 20 minutes qid for 2448 hours, then use normally
Vinca alkaloids[171] [172] [173] Vincristine Vinblastine Vindesine Etoposide	Add 1 ml preservative-free normal saline to 1 vial containing 150 U hyaluronidase	Inject 5 1-ml doses subcutaneously into site with multiple injections. Apply *warm* compresses for 20 minutes, qid for 72 hours
Alkylators[170] [174] [175] Mechlorethamine	Mix 1.6 ml of 25% sodium thiosulfate with 8.4 ml sterile water for injection	Inject 5 ml IV through existing line. Apply *cold* packs to the site for 20 minutes, qid for 72 hours

therapy. [170] However, 30% of anthracycline extravasations progressed to ulceration. A prospective uncontrolled study of anthracycline extravasation showed that the application of DMSO four times a day for 4 days prevented progression to ulceration in all patients. [176]

At many centers, it is recommended that an extravasation kit be kept in units in which vesicant drugs are given. The kit includes the appropriate medications, as well as order sheets preprinted for immediate use. In general, if an antidote is to be administered through the device, as much of the residual drug as possible should be aspirated from the needle, tubing, and tissues before the antidote is given. If swelling or pain persists for 7296 hours after drug administration, a plastic surgeon should be consulted.

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CENTRAL NERVOUS SYSTEM ACCESS DEVICES

In patients with hematologic disorders, devices that permit chronic access to the CNS are useful for a variety of purposes. The available devices include temporary or permanent epidural catheters, ports with attached silicone elastomer catheters, implanted pumps with attached silicone elastomer catheters, and Ommaya reservoirs. The choice of device is determined by a number of factors, including available routes, duration of therapy, cost, efficacy for the therapy planned (e.g., antineoplastics versus pain medication), and the ability of the patient and family to care for the device. [177]

Catheters

Local anesthetics alone or combined with fentanyl, given through temporary epidural catheters (in place for less than 5 days), have successfully managed the pain and improved oxygenation in children with sickle cell crisis. [178] Chronic epidural access has become widely available since the development of a permanent epidural catheter that can be placed percutaneously. [179] The catheters are most commonly used for pain control. The catheter consists of three pieces: two radiopaque silicone rubber cathetersan epidural segment (1.3-mm outer diameter, and an exteriorized line (3.1-mm outer diameter, 0.68-mm inner diameter) with an external Luer connector and a subcutaneous Dacron cuffand a splice segment that joins the two catheter segments. [179]

Insertion

Catheter insertion is done under local anesthesia. [179] A paravertebral incision is made at the level of the L-2 dorsal spine and the epidural portion of the catheter is inserted through a 14-gauge Hustead or Touhy needle to the desired spinal cord level. Epidural placement is verified by fluoroscopy and sensory blockade. The exteriorized line is then tunneled from a subcostal location on the mid-nipple line around to the lower end of the paravertebral incision, and the splice segment is secured to the two catheters and then to the supraspinous tissue to avoid kinking. A Millex-OR 0.22-micron filter is attached to the Luer connector, and a locking Luer injection cap is connected to the filter.

Access and Management

The catheter can be used immediately. Bolus doses can be given from a syringe, or the catheter can be connected to external pumps that deliver continuous infusion opioid with bolus rescue doses or combinations of opioids and local anesthetics (see Chap. 82). Only preservative-free solutions can be used. Wound and catheter exit-site cleaning and dressing changes are recommended until the last sutures are removed, usually 3 weeks. After that, the exit site is cleaned every other day with hydrogen peroxide and povidone-iodine wash, and the filter and injection port are changed every 4 days using sterile technique. [179] More detailed nursing protocols for accessing and managing these catheters and for monitoring patients receiving epidural opioids are available. [67] Patient and family education in catheter care can be effectively supplemented by referral to a home health or hospice agency. [177]

Complications

The catheters can remain safely in place for months, [180] but even temporary catheters can cause serious infections if left in place for days to weeks. [181] Complications include those attributable to the opioids being infused, pain during injection, [182] myoclonus, [183] epidural fibrosis, [184] obstruction and dislocation, [182] [185] and infection. [180] [182] [186] [187] Patients whose catheters take longer to insert have higher infection rates. [187] Exit-site and superficial catheter track infections occur infrequently (in about 10% of patients). [180] [186] The epidural space infection rate has been reported as 1 in 1,702 days of catheter use, similar to the infection rate of 1 in 1,045 per days of use associated with the Hickman catheter. [181] *S. aureus* and *S. epidermidis* account for two-thirds of all the infections. [180] [181] Exit-site and superficial catheter track infections may be cleared without catheter removal, [180] [186] but catheter removal is required for patients with deep catheter track or epidural infections (Table 83-2). [180]

Epidural Ports/Implanted Pumps

Epidural ports (e.g., Port-a-Cath) and implanted pumps (e.g., Infusaid) have also been used to deliver bolus or continuous infusions of opioids into the epidural or intrathecal space to relieve pain of malignant or nonmalignant origin. [183] [188] Subarachnoid infusions using the implanted pumps are recommended as efficacious and cost-effective for patients with a relatively long life expectancy (>3 months). [183] The use of implanted pumps has been reviewed elsewhere. [189]

Complications include both those noted above for implanted ports, and pain on injection of morphine, pump pocket seromas, CSF leaks, CSF hygromas, and postspinal headache. [183] [190] But the use of injection ports appears to reduce the rate of complications associated with percutaneously inserted epidural catheters. A retrospective comparison of catheters with or without associated injection ports indicated that those attached to ports became dislodged much less frequently and were associated with half the infection rate per 1,000 patient-days. [191] Port removal rates for infection were similar to removal rates of ports used for vascular access (10%). [190] Because the seromas act as growth media for bacterial contamination, they should be monitored carefully. The management of hygromas and postspinal headache is reviewed elsewhere. [183] Detailed access and management procedures for epidural ports and implanted pumps and for monitoring patients receiving opioids through them are available. [21] [80]

Ommaya Reservoir

The Ommaya reservoir device was first described in 1963 by Ommaya, [192] and with minimal changes it is still used for access to the spinal fluid within the cerebral ventricle. The reservoir is used to remove CSF for culture, cytology, or measurement of drug levels; to drain cysts in craniopharyngiomas and astrocytomas; to administer antibiotics or antifungal agents; to administer intraventricular chemotherapy to treat leukemic or carcinomatous meningitis; to administer interferon or lymphokineactivated killer cells directly into a tumor; and to administer opioid pain medications. [177] [193]

The device consists of a dome- or mushroom-shaped capsule with a top made of a self-sealing silicone elastomer that can be punctured numerous times without leaking. The flat base of the

capsule, by contrast, is composed of firm polypropylene and is not easily punctured. An outlet arm connected to a ventricular catheter is attached at the base, either laterally or in the center, extending downward. Capsules range in size from 12 mm in diameter (for babies) to 30 mm. Those commonly used for adults have an internal volume capacity of 1.452.4 ml.

Insertion

The Ommaya reservoir is placed subcutaneously by a neurosurgeon. [194] Prior to insertion, computed tomography (CT) is generally required to evaluate ventricular size and placement. Presoaking the device in Bacitracin to prevent subsequent infection has been advocated. [195] For placement, the ventricular catheter is passed through a bur hole into the frontal horn of the right lateral ventricle or, if necessary, into that of the left lateral ventricle or the ventricle body. The catheter end is connected to the base or side arm of the capsule, which then is fitted into the bur hole or a subgaleal pocket. The Silastic skirt of the reservoir may be sutured to the periosteum. A postoperative CT scan is suggested to verify the catheter tip position.

Accessing the Device

The Ommaya reservoir can be used immediately postoperatively for sampling CSF or for injection of drugs. Usually, however, it is not accessed until the third postoperative day. [193] The thoroughly cleaned, gently shaved scalp is prepared with three iodine scrubs and, with use of sterile technique, the reservoir is accessed obliquely with a 23- or 25-gauge butterfly needle inserted with the bevel downward. [193] The CSF can be directly aspirated, or antibiotics or chemotherapeutic agents can be administered through a second syringe attached to the butterfly needle. After removal of the needle, the injected medication can be gently pumped into the spinal fluid by emptying the capsule using repeated pressure, but, to allow CSF to refill the reservoir, one should avoid steadily compressing the device. [196] Once the incision has healed and the stitches have been removed, no special local care or flushing is required. The device can remain in place for months or years. More detailed accessing and management guidelines are available. [80]

Complications

Infection

In general, the Ommaya reservoir has proved very safe, with a complication rate of approximately 1020%, [194] [195] [197] [198] although higher rates were reported in the past. [199] The most common complication is infection, which occurs in about 1015% of patients, [198] especially those who have undergone radiation therapy or who required a second surgical procedure for revision of the catheter. [194] Most infections have been due to *S. epidermidis,* but infections due to numerous other gram-positive and gram-negative bacteria as well as fungal organisms have been documented. [198] [200] In general, the device is not removed, and patients are treated as though they had meningitis. For infections with *S. epidermidis,* vancomycin is given intravenously [201] or, in refractory cases, instilled into the reservoir. [202] Removal of the reservoir is sometimes required.

Miscellaneous

Neurologic complications are rare when the catheter is placed into the nondominant ventricle, but a variety of other complications, which occur infrequently, have been reported. These include (1) intraventricular hemorrhage or subdural hematoma shortly after catheter placement; (2) leakage of CSF around the catheter, primarily in patients with increased intracranial pressure, which caused backflow of fluid along the catheter and produced a subgaleal collection; [199] [203] (3) reservoir leaks after repeated use; [197] (4) occlusion by cellular debris or, when a catheter is placed directly into a tumor, by very proteinaceous tumor fluid; [197] (5) obstruction by lodging of the catheter in brain tissue or abutment against a ventricle wall; [197] (6) seizures immediately after injection of medications; [197] (7) white matter disease (leukoencephalopathy or brain necrosis), most often due to methotrexate injection via the Ommaya device, although found with systemic administration of methotrexate as well; [197] [204] and (8) tumor growth around the cannula. In one case, the catheter may have permitted the spread of Burkitt lymphoma cells from the meninges into the cerebral tissue, where the tumor was found. [205]

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Part VII - Transplantation

Chapter 84 - Overview of Stem Cell Transplantation

Philip McGlave

INTRODUCTION

Hematopoietic stem cell transplantation has come of age. Over the last four decades this therapy has been used to correct a variety of marrow failure states, inborn errors of metabolism, immune deficiencies, hematologic malignancies, and even solid tumors. [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] This chapter provides a brief overview of principles underlying clinical human stem cell transplantation. Various aspects of human hematopoietic stem cell transplantation are discussed in Chapters 85 , 86 , 87 , 88 , 89 , 90 , 91 , 92 , 93 , 94 , 95 , 96 , 97 .

The first successful transplants were performed with stem cells derived from the marrow of identical twins (syngeneic transplantation); however, application of transplantation therapy broadened with the use of stem cells obtained from either related or unrelated donors (allogeneic transplantation) suitably matched at the human leukocyte antigens (HLA), or even with a patients own stem cells (autologous transplantation). [16] [17] The term bone marrow transplantation (BMT) is historically applied to the field; however, stem cell transplantation may be a more appropriate term since peripheral blood, umbilical cord blood, and even fetal liver are also used as sources of hematopoietic stem cells for human transplantation therapy. [18]

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ALLOGENEIC STEM CELL TRANSPLANTATION

Donor-Recipient HLA Matching

In distinction to transplantation of either syngeneic or autologous stem cells, transplantation of allogeneic stem cells requires careful matching of donor and recipient. Failure to do so is associated with both graft rejection and graft-versus-host disease (GVHD). Either rejection or GVHD can occur when immunocompetent cells of host or donor respectively respond to alloantigens encoded by the major histocompatibility complex (MHC) and peptides presented in association with these MHC antigens (reviewed in Martin[19]).

The MHC is located on chromosome 6 in humans and encodes two distinct human HLA families implicated in alloreactivity. HLA class I antigens are encoded by three loci termed HLA-A, HLA-B, and HLA-C. HLA class II antigens are encoded by three loci termed HLA-DR, HLA-DP, and HLA-DQ (reviewed by Dupont and Yang [20]).

In both the related and unrelated donor transplant setting, optimal clinical results are achieved when donor and recipient are matched at both HLA class I and HLA class II. Successful allogeneic transplantation can occur, however, even with some degree of HLA mismatch. Both rejection and GVHD can occur even in cases where apparent MHC matching is achieved, suggesting that in some cases mismatch of antigens not recognizable with current serologic or molecular testing methods, or perhaps not encoded by the MHC, may have clinically relevant consequences.

Transplant Principles

Successful allogeneic stem cell transplantation depends on three principles demonstrated first in animals then in the human clinical setting. First, hematopoietic stem cells obtained from donor marrow or other sources can be infused into the venous blood system and engraft in the recipients hematopoietic microenvironment. [21] Second, the recipients immune system will tolerate engraftment of donor stem cells so that rejection does not occur. [22] Third, immune effector cells from the donor will tolerate host tissue so that fatal GVHD does not occur. [23]

Preparative Regimen

To ensure engraftment, recipients are treated with a preparative regimen usually consisting of a combination of cyclophosphamide and total body irradiation or busulfan and cyclophosphamide (discussed in Chap. 91). The use of these agents in human clinical transplantation is predicated on early animal experiments demonstrating their immunosuppressive effects in the stem cell transplant setting. [24] [25] [26] [27] In some cases, such as the hemoglobinopathies, the preparative regimen arguably creates space for donor stem cell engraftment, while in other cases, such as the leukemias, it provides an antitumor effect. In all cases, however, the preparative regimen immunosuppresses the recipient so that donor stem cells (the graft) will be tolerated.

Graft-versus-Host Disease

GVHD occurs when immune effector cells from the donor engraft and attack host tissue (discussed in Chap. 93).[28] Acute GVHD occurs in the first several months after engraftment, while a somewhat different clinical syndrome termed chronic GVHD occurs later. Chronic GVHD often appears as a sequela of acute GVHD and can be a source of chronic illness. GVHD may be exacerbated by mismatch of major or minor donor-recipient histocompatibility antigens, infection, prior host tissue damage inflicted by the underlying disease, previous therapy, or the preparative regimen. [29] [30] [31] Cyclosporine has become the backbone of in vivo GVHD prophylaxis and treatment. This compound has a specific suppressive effect on T-lymphocyte function and is often used in combination with other immunosuppressive agents such as steroids, methotrexate, and antithymocyte globulin as well as with a variety of novel immunosuppressive agents. [32] [33] [34]

Acute GVHD can also be prevented or ameliorated by ex vivo depletion of T lymphocytes (T-cell depletion) from the stem cell inoculum. Ex vivo T-cell depletion can be accomplished by negative selection of T cells with monoclonal antibodies, antibodies conjugated to toxins (immunotoxins), or other innovative approaches. Alternatively, positive selection of hematopoietic stem cells based on their physical characteristics provides an inoculum relatively depleted of T cells. [35]

Graft-versus-Tumor Effect

Early investigators postulated that effective antitumor therapy with stem cell transplantation might require not only an ablative effect of the preparatory regimen, but also an ongoing, allogeneic graft-versus-tumor or graft-versus-leukemia effect. [16] [36] The graft-versus-tumor effect plays a role in the long-term suppression of malignant clones of host origin, which

sometimes survive even the most rigorous preparative regimens (discussed in Chap. 96).

In certain conditions such as transplant therapy for chronic myelogenous leukemia (CML), the graft-versus-tumor effect is very important. Efficient prevention of GVHD with ex vivo T-cell depletion may result in eventual relapse in the majority of CML transplant recipients, presumably because donor cells necessary for the graft-versus-tumor effect are also damaged or removed by a process designed to remove GVHD effector cells. In other hematologic malignancies and solid tumors, a graft-versus-tumor effect may have varying clinical relevance. [37]

Clinical Application

The first successful use of human hematopoietic stem cell transplantation was reported in the late 1950s by E. Donnall Thomas and colleagues, who demonstrated that marrow cells from identical twin donors could reconstitute hematopoiesis after supralethal irradiation therapy for acute lymphocytic leukemia. [16] Subsequently, investigators used marrow transplants to restore immune function in children with immune deficiencies and to attempt correction of inborn errors of metabolism.

By the late 1970s Thomas and his group had ushered in the era of successful antitumor therapy with stem cell transplantation when they reported that HLA-matched sibling transplantation could be used to correct refractory cases of acute leukemia. [17] A similar story unfolded in the treatment of CML[38] and other hematologic malignancies, as well as severe aplastic anemia and other marrow failure states. [39] [40] Dramatic improvement in transplant outcome occurred when investigators realized that stem cell transplant therapy was most effective when used early in the disease course rather than as a last resort in patients with advanced or

therapy-refractory disease (discussed in Chaps. 85 and 86).[4] [6] [8]

Alternative Donors

Application of donor stem cell transplantation was broadened when investigators demonstrated that alternative donors could be used as a source of stem cells. Alternative donors include related donors mismatched at one or more HLA antigens, and HLA-matched (or one-antigen-mismatched) volunteer unrelated donors located through registries such as the National Marrow Donor Program (discussed in Chap. 89).[41] [42] [43] [44] [45] [46] Perhaps the most exciting recent development has been the successful use of placental umbilical cord blood derived either from related or unrelated donors for successful stem cell transplantation (discussed in Chap. 90).[47] [48]

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AUTOLOGOUS STEM CELL TRANSPLANTATION

Transplant Principles

Autologous transplantation is most commonly used to re-establish hematopoiesis after high-dose cancer chemotherapy or radiation therapy. This approach permits the use of antitumor agents in doses much higher than can be provided in a conventional therapy setting. Autologous stem cell transplantation is most useful in circumstances where a correlation exists between increased therapy dose and tumor response, and where the dose-limiting feature of therapy is hematopoietic suppression (discussed in Chaps. 87 and 88). [49] [50]

In selected cases autologous stem cell transplantation may have advantages over allogeneic transplantation. There is no need to identify HLA-matched related or unrelated donors. Immunologic complications of allogeneic transplantation such as rejection and GVHD are avoided. The use of immunosuppressive agents for transplant preparation, GVHD prophylaxis, and GVHD treatment is unnecessary. Not surprisingly, peritransplant mortality is lower. Hospital stay and convalescence periods are shorter. Older patients tolerate the autologous stem cell transplant procedure relatively well.

Unfortunately, autologous stem cell transplantation has important drawbacks. The benign stem cell population may be reduced or damaged by previous therapy, resulting in delayed or partial engraftment. The stem cell inoculum, whether derived from marrow or blood, may be contaminated with clonogenic malignant cells capable of re-establishing tumor in the transplanted recipient. [51] [52] [53] Finally, autologous stem cell transplantation does not provide an ongoing graft-versus-tumor effect analogous to that observed after allogeneic transplantation. [37]

Clinical Application

Autologous stem cell transplantation has proven useful in a variety of circumstances. Autologous transplant therapy for selected subsets of patients with acute nonlymphoblastic leukemia in first remission as well as for patients with poor-prognosis intermediate- or high-grade lymphoma can result in long-term disease-free survival in the majority of cases.[5] [54] [55] [56] Best results are achieved when transplantation is performed early in the disease course. In distinction to allogeneic transplantation, older recipient age is not necessarily correlated with poorer outcome.

The use of autologous stem cell transplantation in the treatment of a variety of other hematologic malignancies, including CML, multiple myeloma, myelodysplastic syndromes, and low-grade lymphomas, is being assessed.[57] [58] [59] [60] Similarly, the efficacy of autologous stem cell transplantation following high-dose combination chemotherapy is being tested in solid tumors, including breast cancer, ovarian cancer, lung cancer, brain tumors, and other neoplasms. [61] [62] [63] [64] [65] [66] [67] [68] [69] [70] [71] [72] [73]

Priming and Stem Cell Mobilization

The use of priming with growth factors and, in some cases, chemotherapy prior to stem cell harvest has been a major advance in autologous transplantation (discussed in Chaps. 92 and 94). The priming process facilitates the collection by pheresis of a mobilized peripheral blood mononuclear cell population enriched for hematopoietic progenitors.[74] Chemotherapy priming may add an antitumor effect in some cases.[75] The use of progenitors mobilized by priming as well as the peritransplantation use of hematopoietic growth factors can provide engraftment of white blood cells and platelets in less than 2 weeks. The shortened time to engraftment associated with stem cell mobilization and judicious peritransplant use of hematopoietic growth factors, the aggressive prophylactic and empirical use of antimicrobial agents and transfusion products, and other advances in the field have resulted in a growing shift to outpatient-based autologous transplantation (discussed in Chap. 97).[76]

FUTURE DIRECTIONS

The future of stem cell transplantation seems assured. The development of internationally linked unrelated adult donor and umbilical cord blood registries continues to increase the likelihood that a suitable unrelated donor can be found for patients lacking a related donor. New molecular methods for testing of donor and recipient antigens clinically relevant to allogeneic transplantation will allow refinement of the donor selection process (reviewed by Petersdorf et al. [77]). Heightened understanding of the cellular and molecular events underlying GVHD will probably result in improved prophylaxis and treatment of this stubborn clinical problem.

Advances in autologous stem cell transplantation may depend on the use of this procedure as a platform for further innovation. An enriched population of benign stem cells can be

obtained by ex vivo methods such as purging of malignant cells, or by positive selection based on the physical characteristics of the benign progenitor. [12] [61] The benign progenitor population can be expanded ex vivo and perhaps transduced with a variety of genes designed to provide an antitumor effect (discussed in Chap. 95).[78] Endogenous effector cell populations can be stimulated by administration of IL-2 or other cytokines to provide post-transplant antitumor immunotherapy. [79] Finally, autologous antigen-presenting cells such as dendritic cells, or effector cells themselves such as cytotoxic T lymphocytes or natural killer cells, can be isolated, stimulated, and expanded ex vivo, then reinfused to provide additional antitumor therapy. [80] [81]

Even as the stem cell transplant field advances, a variety of innovative, non-transplant-based approaches to human marrow failure states, hematopoietic malignancies, and solid tumors are emerging. It is likely that optimal patient care will be predicated on an open-minded approach to disciplined, clinical testing and the comparison of transplant-based and non-transplant-based therapy.

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Chapter 85 - Hematopoietic Stem Cell Transplantation for Nonmalignant Diseases

H. Joachim Deeg

Administration of bone marrow cells for the treatment of anemia associated with leukemia or infections was used first approximately a century ago (reviewed in Santos[1]). However, only after the development of the atomic bomb and the observations of the medical effects of radiation exposure at Hiroshima and Nagasaki did systematic research into the possibility of marrow transplantation begin. [2] Studies on total body irradiation (TBI) in animal models revealed three levels of TBI-related injury: a marrow syndrome (hematopoietic failure) at approximately 500700 cGy, an intestinal syndrome (severe enteritis) at 1,20010,000 cGy, and a cerebral syndrome (coma) at even higher doses.[1] [3] Shielding of the spleen during TBI, implantation of autologous (patients own) or syngeneic (identical twin) spleen cells, or infusion of syngeneic marrow cells after TBI rescued animals from the marrow syndrome.[1] [3]

Initially, humoral factors released by the spleen or by other hematopoietic cells were thought to be responsible for marrow recovery, a concept certainly of interest in view of the current use of hematopoietic growth factors. Subsequent studies showed, however, that hematopoietic reconstitution derived from the infused or implanted cells,[4] the most effective way being intravenous (IV) injection. By a mechanism now termed homing and mediated by a series of receptors and counter-receptors on cells and vascular endothelium, injected hematopoietic cells reached the marrow cavity and other hematopoietic organs. Studies in a rat model showed that hematopoiesis in lethally irradiated animals was restored by parabiosis with a normal animal, [5] proving in principle the presence of circulating stem cells in peripheral blood, subsequently confirmed in dogs, nonhuman primates, and other species. These observations,

TABLE 85-1 -- Nonmalignant Diseases Treated by Stem Cell Transplantation

Acquired	Congenital	
Aplastic anemia	Hematologic defects	Mucopolysaccharidoses
	Fanconis anemia	Hurlers disease
Pure red cell aplasia	Thalassemia	Hunters syndrome
Paroxysmal nocturnal hemoglobinuria	Sickle cell disease	Maroteaux-Lamy syndrome
	Blackfan-Diamond anemia	Others
Acquired immunodeficiency syndrome	Dyskeratosis congenita	Others
	Glanzmanns thrombasthenia	Mucolipidoses
Autoimmune disorders	Chronic granulomatous disease	Metachromatic leukodystrophy
	Congenital neutropenia	Adrenoleukodystrophy
Langerhans cell histiocytosis	Congenital amegakaryocytosis	Other lipidoses
	Thrombocytopenia-absent radius syndrome	Other lysosomal diseases
	Congenital erythropoietic porphyria (Gunthers disease)	Lesch-Nyhan syndrome
	Others	Type IIA glycogen storage disease
	Immunodeficiencies	Gauchers disease
	Severe combined immunodeficiencies	
	Wiscott-Aldrich syndrome	
	Familial erythrophagocytic lymphohistiocytosis	
	Chediak-Higashi syndrome	
	Combined immunodeficiencies	
	Omenns syndrome	
	Leukocyte adhesion defects	
	Griscellis syndrome	
	Hyper-IgM syndrome (CD40L)	
	X-linked lymphoproliferative syndrome	
	Others	
	Osteopetrosis	

particularly with parabiotic animals, are of central importance to the current clinical work on the use of mobilized peripheral blood progenitor cells (PBPC): quite likely, the irradiation treatment of the recipient animal triggered a rise in various growth factors that, by way of cross-circulation, were able to release early hematopoietic precursors from the marrow into the peripheral circulation.

Although the infusion of autologous or syngeneic cells rescued marrow-ablated animals without complication, animals given cells from a nonidentical twin (allogeneic) donor acquired secondary disease, now known as graft-versus-host disease (GVHD). [3] These observations showed convincingly that transplantation of spleen or

marrow cells comprised not merely a transfer of hematopoietic stem cells, but that such infusions also mediated immune effects. [6] [7] The development of GVHD was detrimental because it resulted in significant morbidity and mortality. Although moderate GVHD was associated with improved survival in transplanted mice with leukemia because of what has been termed a graft-versus-leukemia effect, [8] no such benefit could be expected in recipients transplanted for a nonmalignant disorder.

The first modern clinical transplantation attempts, beginning in 1957, mostly in patients with end-stage leukemia, [1] [9] [10] were discouraging: patients transplanted from a syngeneic donor did well immediately post-transplantation, but died from progressive leukemia. Patients transplanted from an allogeneic donor usually died with severe GVHD. Work by Dausset and others beginning in the late 1950s showed that, similar to earlier observations in mice, human patients differed from each other and from their donors in regard to major histocompatibility complex (MHC) antigens. [11] With the use of appropriate typing sera and cellular assays, matched individuals suitable as marrow donors could be distinguished from mismatched individuals. [12] With these insights, and driven by the pioneering work of Thomas, Good, Santos and others, hematopoietic stem cell transplantation has evolved as the treatment of choice for diseases such as severe aplastic anemia, severe combined immunodeficiency (SCID), other congenital diseases, and numerous malignant disorders.

Table 85-1 lists nonmalignant disorders that are accepted as indications for transplantation therapy. These indications are continuously under revision as nontransplantation modalities, including hematopoietic growth factors, cytokines, immunotoxins continue to evolve. Although the number of separate disease entities amenable to transplantation is considerable, most transplantations are performed to cure malignancy, and a smaller proportion are carried out for nonmalignant disorders.

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RATIONALE FOR TRANSPLANTATION

Hematopoietic stem cells for transplantation can be obtained from various donors and different sources (Table 85-2). Depending on the patients underlying disease and the objectives

TABLE 85-2 -- Potential Stem Cell Donors

Stem Cell Source		Histocompatibility Barrier	
		Minor	**Major**
Autologous	Patients are their own donors		
Syngeneic	Monozygotic (identical) twin		
Allogeneic	HLA genotypically identical donor	+	
	HLA phenotypically identical donor		
	Related	+	/+
	Unrelated	+++	/+
	HLA-nonidentical donor		
	Related	+	++
	Unrelated	+++	++
Xenogeneic	Different species donor	+++	+++

HLA, human leukocyte antigen.

of therapy, however, only some of these possibilities may be useful and available for a given patient. [13] Myelosuppression is often the dose-limiting toxicity of chemoradiation therapy used for treatment of malignancies. In those patients, infusion of hematopoietic stem cells capable of hematopoietic reconstitution serves as a rescue procedure. A second use for transplanted hematopoietic stem cells is as replacement therapy in patients with congenital or acquired marrow failure or immunodeficiencies. Along these lines, hematopoietic stem cells or more mature precursors may also be important as vehicles for gene therapy [14] to replace defective or missing enzymes (e.g., adenosine deaminase; glucocerebrosidase). Finally, it was noted that patients with aplastic anemia or other disorders who also had an autoimmune disease (e.g., rheumatoid arthritis), when transplanted with normal allogeneic marrow, were not only cured of their hematologic disease but had sustained improvement or resolution of their autoimmune disorder (reviewed in Marmont [15] [16]). Results obtained in animal models of spontaneously occurring autoimmune disorders are compelling.[17] [18] These findings suggest, therefore, that marrow transplantation can also be used to correct autoimmune diseases.

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DONOR SELECTION

The choice of a source of hematopoietic stem cells depends on several factors, the most important of which is the patients disease. Although autologous stem cells obtained from marrow or peripheral blood should be available for every patient except, possibly, patients with severe aplastic anemia, the use of autologous cells would not be useful for genetically determined disorders. A haploidentical donor (matched at one half of the HLA loci, such as a parent, sibling, or child) would be available for most patients, but significant human leukocyte antigen (HLA) differences still present major problems for transplantation because of GVHD and the risk of rejection. Thus, the preferred donor is a genotypically HLA-identical sibling.

Approximately 2530% of patients who have siblings can be expected to have an HLA genotypically identical donor. In addition, approximately 1% of phenotypically matched donors among closely related family members can be identified, and a rare patient will have an identical twin donor. These statisticsthat is, the lack of a matched related donor in 7075% of patients who could benefit from transplantationhave stimulated the development of transplantation in two directions: (1) the establishment of large data banks of volunteer unrelated allogeneic donors, and (2) the development of techniques to purge autologous marrow or PBSCs.

Thanks to the efforts of the National Marrow Donor Program (NMDP) in the United States as well as other donor registries in the United States and worldwide, more than 2 million volunteer donors have been typed for HLA-A and -B antigens, and more than 500,000 are also typed for HLA-DR (MHC class II) antigens. [19] Similar registries have been established in the United Kingdom, France, Germany, Japan, and other countries. If an unrelated donor search is initiated, computer inquiries quickly generate the probability of identifying such a donor. In North America, the probability of finding an HLA-A, -B, -DR match during the initial search is approximately 5055%; an additional 1215% of patients find a match when available HLA-A and -B matched donors are typed for DR, and 20% of patients have a one-HLA-locus-incompatible unrelated donor available. [20]

As discussed previously, autologous or syngeneic hematopoietic stem cells would not be an option for the treatment of genetically determined diseases because the same defects as those to be corrected would also be present in the transplanted cells. An autologous stem cell approach may be possible, however, with acquired disorders. Granulocyte colony-stimulating factor (G-CSF) mobilized PBSCs have been harvested and transplanted even in patients with severe aplastic anemia. A benefit is expected only if residual normal stem cells are present in the patient and no stromal defect exists in the marrow. The possibility also has to be considered that the treatment given in preparation for transplantation might have an effect similar to that of immunosuppressive therapy in patients with aplastic anemia, as discussed elsewhere. In patients with paroxysmal nocturnal hemoglobinuria (PNH), phenotypically normal (CD50+, CD59+) hematopoietic precursors have been demonstrated, and the possibility of using those for transplantation has been suggested. [21] The experience in patients with autoimmune disorders is too limited to permit comment. However, experiments in rodents (e.g., with experimental allergic encephalomyelitis) suggest that transplantation of autologous stem cells depleted of T lymphocytes can restore neurologic function by eliminating V-8positive autoreactive T cells. [22] This is a rapidly developing area of research, and new insights are likely to be gained in the near future.

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PREPARATIVE REGIMENS FOR TRANSPLANTATION

Rationale for Conditioning

In preparation for hematopoietic stem cell transplantation, it is necessary for several reasons to condition the patient:

1. To eradicate the patients disease or at least reduce the number of abnormal or defective cells below detectable levels; this applies to allogeneic, syngeneic and autologous donors.
2. To suppress the patients immunity, natural or acquired through allosensitization, to prevent rejection of donor cells; this applies to allogeneic but not to autologous cells or to syngeneic cells, which should not encounter a histocompatibility barrier. However, immunosuppression is needed in preparation for some syngeneic transplants in patients with aplastic anemia, apparently to eliminate autoimmune reactivity that may interfere with sustained hematopoietic reconstitution.
3. To create space so that donor cells can establish themselves in the recipient marrow, an issue that has been discussed predominantly for diseases where the patients marrow may be very cellular, as seen for example in patients with thalassemia.[23] This concept of space creation, however, is somewhat controversial, and recent research in animal models has shown that engraftment is possible without recipient conditioning, albeit with extremely high cell doses. [24]

Exceptions to the requirement of conditioning exist in children with SCID in whom HLA-identical and occasionally even HLA-haploidentical marrow engrafts without conditioning. This is apparently related to the nature of the underlying disease, namely, a T-cell defect that does not allow for rejection of transplanted donor cells, and in which partial donor engraftment may completely correct the genetic defect. [25] [26]

Modalities of Conditioning

Therapeutic modalities used to prepare patients for transplantation have been reviewed extensively elsewhere. [13] [27] In principle, they comprise the following.

Irradiation

Irradiation is given either in the form of TBI, total lymphoid irradiation, or modifications thereof. Exposure rate, fractionation schedule, and total dose are important parameters. The optimum regimen has probably not been defined yet and may

differ for different diseases. Many investigators attempt to avoid the use of irradiation in patients who are transplanted for nonmalignant disorders, at least in patients transplanted from an HLA-identical related donor. Others have used 300 cGy TBI or 400600 cGy of thoracoabdominal irradiation, usually delivered at exposure rates of 725 cGy/min and combined with cyclophosphamide. Low-dose TBI is also frequently given to patients with lysosomal storage diseases. In patients transplanted from an HLA-nonidentical or unrelated donor, TBI is often used at higher total doses, such as 11 × 120 or 6 × 200 cGy given over 34 days. For some indications, usually malignant disorders, bone-seeking (e.g., holmium) or other isotopes (e.g., ^{131} I) conjugated to monoclonal antibodies directed at lymphoid or myeloid antigens (e.g., CD20, CD45) are used.

Chemotherapy

Chemotherapy, particularly cyclophosphamide (120200 mg/kg over 24 days), although quite toxic, is included in most regimens. In patients with aplastic anemia and Fanconis anemia (FA), cyclophosphamide has been used successfully as a single agent for transplant conditioning. For other indications, such as for thalassemia, sickle cell anemia, and some storage disorders, cyclophosphamide is frequently combined with busulfan (±antithymocyte globulin [ATG]).

Biologics

Biologic reagents such as ATG (a polyclonal antibody preparation) or monoclonal antibodies directed at T-cell antigens or adhesion molecules are used to suppress recipient immunity. These reagents are in general noncytotoxic and associated with little acute clinical toxicity.

Cellular Therapy

Viable donor buffy coat cells have been used to facilitate engraftment in patients with severe aplastic anemia. The observation that broad T-lymphocyte depletion of donor marrow (see later) increased the probability of graft failure has led to protocols of selective T-cell addback to ensure engraftment. It has recently been suggested that a combination of low-dose (well tolerated) TBI and repeated donor cell infusion may be effective in achieving sustained engraftment.

Other procedures include plasmapheresis of the patient to remove isoagglutinins directed at the donors ABO blood group or, conversely, removal of plasma from the donor marrow to remove isoagglutinins directed at recipient cells. Alternatively, the donor red blood cells with which recipient antibodies may react can be removed, thus minimizing the risk of transfusion reactions. [13]

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TRANSPLANTATION PROCEDURE

Sources of Hematopoietic Stem Cells

Hematopoiesis evolves through three embryologic stages: mesodermal (yolk sac), hepatosplenic, and medullary. At the time of birth, the bone marrow has usually become the only site of hematopoiesis. In contrast to other species such as mice, in which splenic hematopoiesis persists throughout life, in adult human beings extramedullary hematopoiesis occurs only under pathologic conditions (e.g., chronic myelogenous leukemia, myelofibrosis). Accordingly, hematopoietic stem cells can be obtained from fetal (but not adult) livers, whereas an adult donor usually can donate stem cells only in the form of bone marrow or from peripheral blood wherein some stem cells circulate (Table 85-3). As discussed previously, the presence of circulating stem cells in blood was previously proven in animal models by

TABLE 85-3 -- Sources of Stem Cells

Bone marrow
Peripheral blood
Cord blood
Fetal liver

the fact that transplantations with cells exclusively obtained from peripheral blood resulted in complete lymphohematopoietic reconstitution. This finding was not necessarily surprising (but reassuring nevertheless) because bone marrow cells are usually transplanted through transfusion into a vein, from which they cross the pulmonary vascular bed and into bone arteries to home to the marrow cavity.

Bone Marrow

For the marrow harvest, the donor receives anesthesia and, under sterile conditions, multiple small-volume aspirates of marrow are obtained from both posterior iliac crests.[28] Additional potential aspiration sites are the anterior iliac crests, the sternum, and, in children, the tibia head. Every aspirate consists of a mixture of marrow cells and peripheral blood (owing to perfusion of the marrow space and damage inflicted by needle placement and aspiration). It appears that the larger the volume aspirated, the greater the relative contribution of blood, including T lymphocytes. Therefore, with a single aspirate, no more than 35 ml should be removed. Approximately 1015 ml/kg donor weight are collected. The marrow is then passed through filters, either manually or in closed systems, to break up cell aggregates and remove bone particles. If no ABO incompatibility exists and if the marrow is not to be subjected to any in vitro purging procedure, the resulting cell suspension is infused intravenously, usually through an indwelling IV catheter (e.g., Hickman line). If the plan is to process the aspirated marrow before infusion, peripheral blood contamination need not be a concern. Treatment of marrow with monoclonal antibodies and complement, magnetic beads, or other techniquesthat is, those aimed at removing marrow T lymphocytesalso removes T lymphocytes contributed by blood. The marrow cell yield declines somewhat with donor age (Table 85-4).

Peripheral Blood Stem Cells

Hematopoietic stem cells are present at low concentrations outside the bone marrow.[29] One such alternative source, peripheral blood, contains dramatically increased frequencies of early hematopoietic precursors during the recovery phase after cytotoxic therapy.[30] The administration of recombinant hematopoietic

TABLE 85-4 -- Marrow Cell Yield by Donor Age[a]

Age (y)	Allogeneic		
	Volume[b]	Cells[c]	Concentration[d]
9	13.0	4.5	3.2
10-19	10.8	2.8	2.6
2059	9.5	2.2	2.4
60	8.5	2.0	2.2

Adapted from Buckner CD, Clift RA, Sanders JE et al: Marrow harvesting from normal donors. Blood 64:630, 1984; and Jin NR, Hill RS, Petersen FB et al: Marrow harvesting for autologous marrow transplantation. Exp Hematol 13:879, 1985.

[a]Only first harvests are considered.
[b]Milliliters per kilogram donor weight (median).
[c]Nucleated marrow cells × 10^8 /kg donor weight (median).
[d]Nucleated marrow cells × 10^7 /mL marrow (median).

growth factors, such as G-CSF or c-kit ligand (Steel factor), has a similar effect, and the peak of stem cell yield at 56 days after initiation of treatment with growth factor is more predictable than with chemotherapy. It would also be difficult to justify administration of chemotherapy to normal donors, whereas growth factors appear to be tolerated with very few side effects. A single leukapheresis on a normal donor may be sufficient to harvest the numbers of cells required for a transplant; the goal is usually at least 5 × 10^6 CD34+ cells/kg of recipient weight.[31] As discussed elsewhere, the transplantation of mobilized PBPCs rather than normal marrow cells is associated with a very rapid hematopoietic recovery in the recipient.

Umbilical Cord Blood

Umbilical cord blood, representing a segment of the peripheral circulation of the fetus, is another rich and easily accessible source of hematopoietic stem cells. [32] [33] [34] Because there is also evidence that cord blood cells are less immunocompetent than adult cells, cord blood cells might carry a lower risk of inducing GVHD than adult marrow cells. Although the concentration of stem cells is high (frequency of long-term culture-initiating cells approximately 1:100, compared with 1:500 in

peripheral blood), the small volume usually available (100150 mL) may limit the use of these cells for transplantation.

Fetal Liver

For several months during human fetal development, the liver is physiologically part of the hematopoietic tissues. It is during this time, from the second to the seventh monthand ideally before the onset of lymphopoiesisthat fetal liver cells, a mixture of hepatocytes and hematopoietic stem cells, can be used for transplantation. Fetal liver cells have been studied extensively in experimental models and have been shown to reconstitute both hematopoietic and immunologic systems in children with congenital immunodeficiencies.[35]

Fetal liver cells can be obtained only from aborted fetuses, and are not routinely available. Because of ethical concerns and the development of alternative approaches (HLA-nonidentical donor, T-cell depletion), fetal liver cells have recently been used only by a few investigators and for very selected indications.

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PERITRANSPLANTATION AND POST-TRANSPLANTATION CARE

Hematopoietic stem cell transplantation is intensive therapy. Complications are related to the underlying disease, therapy given before transplantation, the preparative regimen (regimen-related toxicity), the interactions of donor cells with recipient tissue, and prophylactic and therapeutic measures administered after transplantation.

Many of the patients coming to transplantation have received nontransplantation therapy for extended periods of time. For example, although patients with aplastic anemia who have an HLA-identical related donor and are no older than 45 years of age usually receive a transplant as first-line therapy, all others are initially treated with immunosuppressive therapy, transfusions, and hematopoietic growth factors. Only when these attempts fail and an alternative donor is identified will a transplant be considered. As a result, almost all these patients become allosensitized and transfusion refractory. Because of prolonged neutropenia and immunosuppression, many are infected, often with fungal organisms, including *Candida*, *Aspergillus*, and *Mucor* species. Only hematopoietic reconstitution can eradicate these organisms. However, it is necessary to give aggressive antibiotic therapy before conditioning is initiated. Similarly, patients with FA or thalassemia may have received multiple transfusions. In addition to allosensitization, iron overload develops. In patients with thalassemia, the presence of hepatic fibrosis is a significant risk factor for transplantation outcome. Pretransplantation infections are also common in patients with severe combined immunodeficiency, dyskeratosis congenita, and other immunodeficiencies.

With currently used conditioning regimens, all patients experience severe pancytopenia before hematopoietic reconstitution from the transplanted (donor-derived) stem cells occurs. Pancytopenia may last 24 weeks with marrow cells, but possibly only 1012 days with the use of mobilized PBPCs. With cord blood cells, recovery may take 56 weeks. The administration of growth factors such as G-CSF, granulocyte/macrophage colony-stimulating factor (GM-CSF), or c-kit ligand early after transplantation accelerates granulocyte recovery after marrow infusion. It is not clear whether post-transplantation growth factors further accelerate recovery after transplantation with mobilized PBPCs; there is a minimum time interval required for cell replication and differentiation. [31] [36] Virtually all marrow recipients need transfusion support with platelets and red blood cells. Erythropoietin administration post-transplantation has been shown to speed reticulocyte recovery and moderately reduce the red blood cell transfusion requirements in some patients. [37] Conceivably, the recently cloned thrombopoietin [38] will be able to enhance platelet recovery.

Infection prophylaxis consists mostly of systemic broad-spectrum antibiotics. [39] Granulocyte transfusions are warranted only in granulocytopenic patients in whom bacterial or fungal septicemia is not controlled by antibiotics. Treatment of patients in laminar air flow rooms and with gastrointestinal decontamination may reduce the frequency of infections and the duration of febrile episodes. [40] Because of an 8090% risk of reactivation of herpes simplex virus, prophylaxis with acyclovir is given for the first month post-transplantation. Some protocols also include the infusion of IV immunoglobulins on a weekly basis. Trimethoprim-sulfamethoxazole is given primarily for *Pneumocystis carinii* prophylaxis, but also reduces the incidence of other infections, in particular in patients with chronic GVHD. Fluconazole may be administered as antifungal, and ganciclovir as anticytomegalovirus prophylaxis.

Because of mucositis and gastroenteritis, most patients are unable to eat in the early post-transplantation period and require parenteral nutrition and hydration.

After allogeneic transplantation, all patients receive some form of GVHD prophylaxis. [41] [42] Modalities involve in vivo administration of immunosuppressive agents such as methotrexate (MTX), cyclosporine (CSP), glucocorticoids, or FK506, either alone or in combination. [43] The most widely used regimen is a combination of MTX, 10 mg/m^2 IV on day 1, 3, and 6 (or 15 mg/m^2 on day 1, and 10 mg/m^2 on days 3, 6 and 11), and CSP, given daily starting on day 1, usually at doses of 35 mg/kg/day IV in the early postgrafting period and subsequently at 12 mg/kg/day orally, at gradually tapering doses for 6 months. [44] [45] Alternatively, T lymphocytes, responsible for triggering GVHD, are depleted from the donor marrow in vitro before infusion (see later).

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CLINICAL RESULTS

Acquired Disorders

Aplastic Anemia

The original description of aplastic anemia as *myelophthisis* very aptly indicates the fact that not only is erythropoiesis defective, but that the marrow fails in all lineages. Nevertheless, the differential diagnosis may at times be difficult, and the distinction from a myelodysplastic syndrome (MDS; refractory anemia) may be impossible.[46] However, patients in whom a cytogenetic abnormality is identified should be treated as MDS because transplantation regimens used for aplastic anemia, when

applied to these patients, are unsuccessful and are followed by early relapse and evolution into leukemia. [47]

TREATMENT DECISIONS FOR PATIENTS WITH SEVERE APLASTIC ANEMIA

Severe aplastic anemia has served as an important model for marrow transplantation. [48] Mechanisms involved in severe marrow failure include stem cell damage, immune-mediated inhibition of hematopoiesis, functional inadequacy of the marrow microenvironment, and others. [49] Some patients respond to immunosuppressive therapy with ATG, CSP, glucocorticoids, or combinations thereof. [50] However, even in patients who respond to this treatment, hematopoiesis usually is *not* normal, and both responding and nonresponding patients are at risk for development of a clonal hematopoietic disorder (i.e., PNH, MDS, or myeloid leukemia). In various series, an incidence of 1545% has been observed. [51] [52] [53]

Transplantation from a Matched Related Donor

The only definitive treatment of aplastic anemia is allogeneic stem cell transplantation. In patients with an HLA-identical, healthy sibling donor, marrow transplantation is the treatment of choice. Opinions about the upper age limit of patients differ, although 45 years is accepted by most transplantation centers.

In early trials, 3060% of previously transfused (allosensitized) patients conditioned with cyclophosphamide failed to achieve sustained engraftment, and only 4050% became long-term survivors. Conversely, among untransfused patients, only 5% failed to engraft, and 75% survived with normal hematopoiesis (Fig. 85-1 (Figure Not Available)). With the addition of viable donor leukocyte (buffy coat) infusions to the marrow or the use of TBI (e.g., 300 cGy) or total lymphoid irradiation (400750 cGy) given in combination with cyclophosphamide, the probability of graft failure was reduced to 515% even in transfused patients, and 7080% of patients survived with sustained engraftment.[48] [54] One disadvantage of the addition of donor buffy coat cells (which contain immunocompetent peripheral blood T lymphocytes) was an increase in the incidence of chronic GVHD to as high as 50% even in children. [48] The use of irradiation, in particular limited-field irradiation, has been associated with an increased risk of post-transplantation (i.e., apparently treatment-related) malignancies. [55] Furthermore, radiation exposure is associated with impaired growth and development in children and infertility in adults. [56] [57] Therefore, alternative immunosuppressive approaches of comparable efficacy but fewer side effects were sought.

Studies in patients who had rejected a first marrow graft

Figure 85-1 (Figure Not Available) Effect of transfusion status on survival in patients with aplastic anemia. Patients were transplanted between 1978 and 1991 using cyclophosphamide-based conditioning. The transfused group of patients (n = 115) had an increased incidence of graft rejection relative to untransfused patients (n = 53), presumably due to sensitization to the donors non-HLA antigens resulting from prior blood transfusions. *(Modified from Doney et al.,[52] with permission.)*

after preparation with cyclophosphamide had shown that a combination of ATG and cyclophosphamide was successful in most in achieving sustained engraftment of a second marrow inoculum.[58] Based on these results, this regimen (cyclophosphamide 4 × 50 mg/kg plus ATG 3 × 30 mg/kg) was introduced as conditioning for the initial transplant. Recent results show that this approach allows for engraftment in 95% of patients and survival in excess of 90% [59] (Fig. 85-2). Furthermore, for reasons that are not completely understood, the probability of chronic GVHD, still a major problem after transplantation (Fig. 85-3), was reduced.[59] Results of these and other representative trials are summarized in Table 85-5 .

Transplants from Alternative Donors

Only 2530% of patients have an HLA-matched donor in the family. As discussed, first-line treatment for the remaining 70% of patients is usually immunosuppressive therapy, and transplantation is considered if that approach fails. The reason to delay transplantation is that results with HLA-nonidentical

Figure 85-2 Survival of 39 patients with aplastic anemia given an HLA-identical marrow transplant and postgrafting graft-versus-host disease prophylaxis with methotrexate/cyclosporine after conditioning with cyclophosphamide plus antithymocyte globulin. Surviving patients are indicated by tick marks. *(Courtesy of R. Storb, Fred Hutchinson Cancer Research Center, Seattle, WA.)*

Figure 85-3 Prevalence of chronic graft-versus-host disease (GVHD) in 78 patients with aplastic anemia transplanted at the Fred Hutchinson Cancer Research Center and given HLA-identical marrow transplants after conditioning with cyclophosphamide only (Cy) or Cy combined with antithymocyte globulin (Cy + ATG), followed by GVHD prophylaxis with methotrexate/cyclosporine. *(Courtesy of R. Storb, Fred Hutchinson Cancer Research Center, Seattle, WA.)*

marrow donors are inferior.[60] [61] [62] An analysis of results at the Fred Hutchinson Cancer Research Center shows that 3045% of patients who receive a transplant from an HLA-nonidentical donor and are conditioned without the use of TBI fail to achieve engraftment, and only 1535% become long-term survivors. Acute GVHD occurs in 7080% of patients and chronic GVHD develops in 50%. With the use of TBI (e.g., 6 × 200 cGy) in addition to cyclophosphamide for conditioning, engraftment is usually achieved, and 50% of patients have long-term survival (Table 85-6).

The establishment of registries of volunteer, normal, unrelated donors (e.g., NMDP; Anthony Nolan Foundation; Stefan Morsch Foundation) typed for HLA has provided the option of stem cell transplantation to additional patients. Some data are summarized in Table 85-6 . Overall results of unrelated donor transplants are inferior to those obtainable with HLA-identical sibling donors but comparable with early results achieved with sibling donors. Retrospective analyses show a probability of survival of 3035%. Data from a recent study using low-dose TBI, ATG, and cyclophosphamide suggest that survival in the range of 5055% can be achieved with transplantation from an unrelated donor matched for HLA-A, -B, and -DRB1. [63]

Syngeneic Transplants

Of interest with regard to disease mechanisms are the results in patients with aplastic anemia transplanted from a syngeneic (monozygotic twin) donor. In this donorrecipient combination, no histocompatibility barriers exist and the infusion of normal stem cells should lead to prompt hematopoietic reconstitution. However, this approach is not uniformly successful, and approximately 50% of patients require an immunosuppressive conditioning regimen. These observations support the notion that in some patients aplastic anemia is immunologically mediated, as was suggested by in vitro studies. [64]

If aplastic anemia is a stem cell disorder, autologous stem cell transplantation is in principle not an option. However, if residual normal stem cells are present, it is possible that treatment with G-CSF to mobilize stem cells, with conditioning treatment given before the reinfusion, modifies the patients immune status and the marrow microenvironment such that the

TABLE 85-5 -- Severe Aplastic Anemia: Results of Transplantation with Marrow Cells from an HLA-Identical Sibling

Center	Number of Patients	Median Age (range) in Years	Conditioning	GVHD Prophylaxis	Rejection (%)	GVHD (%)		Survival (%)	Follow-up (median) in Years
						Acute	Chronic		
UCLA[197]	46	19 (244)	Cy + TBI (300 cGy)	MTX	2	70	NA	63	0.754.5 (2)
FHCRC[198] [a]	50	17 (332)	Cy	MTX	10	23	37	82	112 (7)
Minneapolis[199]	58	18 (245)	Cy + TLI (750 cGy)	MTX + ATG + Pred	5	38	1254	70	<0.58
EBMT[200]	218	150	Cy ± TLI, TAI, or TBI	MTX or CSP	NA	NA	NA	63	<16
Hammersmith[201]	49	22 (347)	Cy	CSP	17	50	37	69	1.87.8 (5.8)
UCLA[202]	29	19 (141)	Cy + TLI (300 cGy)	MTX/CSP	23	22	NA	78	0.55 (2)
FHCRC[203]	26	10 (118)	Cy	MTX + CSP	26	12	27	92	210 (5.7)
St. Louis[204]	107	19 (546)	Cy + TAI (600 cGy)	MTX, CSP, or MTX + CSP	3	32	55	62	110 (3.7)
Johns Hopkins[205]	24	21 (453)	Cy	CSP	29	4.5	0	79	0.88 (5)
Memorial Sloan-Kettering[206]	23	13 (2.532)	Cy + TLI (600 cGy)	CSP, MTX, or CSP + MTX	13	31	17	65	310 (5.6)
IBMTR[207]	186	19 (256)	Cy or Cy + TBI or Cy + TLI/TAI	MTX ± Other, CSP ± Other	20	39	37	48	(6)
	648	20 (157)	Cy or Cy + TBI or Cy + TLI/TAI	MTX ± Other, CSP ± Other, MTX + CSP	11	37	47	61	(6)
	471	20 (151)	Cy or Cy + TLI/TAI or Cy + ATG	CSP ± Other, MTX + CSP	16	19	32	66	(5)
FHCRC[208]	39	24 (252)	Cy + ATG	MTX + CSP	5	15	8	92	3.68.2 (5.2)

HLA, human leukocyte antigen; GVHD, graft-versus-host disease; NA, not available; UCLA, University of California, Los Angeles; FHCRC, Fred Hutchinson Cancer Research Center (Seattle); EBMTG, European Bone Marrow Transplant Group; IBMTR, International Bone Marrow Transplant Registry; Cy, cyclophosphamide; TBI, total-body irradiation; TLI, total lymphoid irradiation; TAI, thoracoabdominal irradiation; ATG, antithymocyte globulin; MTX, methotrexate; CSP, cyclosporine; Pred, prednisone; MTX ± Other, methotrexate alone or with a second agent (not CSP).

[a] Only nontransfused patients in study.

TABLE 85-6 -- Transplants for Severe Aplastic Anemia Using HLA-Nonidentical Related or Unrelated (Alternative) Donors

Center	Number of Patients	Median Age (range) in Years	Donor(REL/URD)	Conditioning	GVHD Prophylaxis	Rejection (%)	GVHD (%)		Survival (%)	Follow-up (median) in Years
							Acute	Chronic		
EBMTG[209]	46[a]	35	REL (26)	Cy (±Pro)	CSP	30	35	NA	45 (=)	1.37
		11	URD	Cy + TBI/TLI	MTX		(III/IV)		25 (1 Ag)	
				Other	TCD				11 (>1 Ag)	
Five centers[210]	40[b]	19 (141)	URD	Cy ± TBI/TLI	MTX; CSP	18	86	NA	28 (=)	0.713 (4.5)
IMUST	15[c]	13 (538)	URD		MTX + CSP	28	50	NA	50 (=)	
					TCD					
Milwaukee[61]	13	9	URD	Cy	MTX	23	10	15	53	0.78

		4	URD	Cy + TBI	CSP					
				Cy + AraC + TBI + MP	CSP + TCD					
FHCRC[211]	40	15 (244)	REL (9 =)	Cy	MTX	0	33	67	89	318
			(15)	Cy	MTX + CSP	71	100	100	0	
			(16)	Cy + TBI	Pred + MTX (± CSP) TTX + CSP	14	79	75	50	1.511.3
NMDP[63]	141	18 (0.947)	URD (91 =)	Cy ± Chemo	MTX + CSP ± Other	18	53	31	42 (=)	18 (3)
			(50)	TBI ± Chemo	CSP ± Other				20 ()	
				Cy ± LFI	TCD Other					
NMDP[63]	34	18 (346)	URD (27 =) (7)	Cy + ATG + FTBI	MTX + CSP	0	55	41	55	0.33.5 (2)

HLA, human leukocyte antigen; GVHD, graft-versus-host disease; EBMTG, European Bone Marrow Transplant Group; FHCRC, Fred Hutchinson Cancer Research Center (Seattle); NMDP, National Marrow Donor Program; Cy, cyclophosphamide; Pro, procarbazine; TBI, total-body irradiation; FTBI, fractionated TBI; TLI, total lymphoid irradiation; TAI, thoracoabdominal irradiation; ATG, antithymocyte globulin; REL, related donor; URD, unrelated donor; =, HLA match; , HLA-nonidentical; NA, not available; MTX, methotrexate; CSP, cyclosporine; Pred, prednisone; MTX ± Other, methotrexate alone or with a second agent (not CSP); TCD, T-cell depletion.

[a]Includes 14 patients with Fanconis anemia.
[b]Retrospective.
[c]Prospective.

reinfused cells encounter growth conditions that allow for hematopoietic repopulation. [65]

Other Acquired Disorders

Pure Red Cell Aplasia

Some patients who present with red cell aplasia of undetermined etiology and who do not respond to treatment with glucocorticoids or other nontransplantation regimens aimed at the underlying disorder are referred for marrow transplantation. These patients are transplanted with conditioning regimens as used for aplastic anemia. No large series has been reported. Available data suggest that problems with sustained engraftment may be encountered. [66]

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria is an acquired clonal disorder of hematopoiesis due to somatic mutations in the X-linked *PIG-A* gene. This gene is responsible for a protein that affects the synthesis of glycosylphosphatidyl-inositol, an anchor for numerous cell surface antigens. Cells arising from the mutated clone gain a survival advantage. Patients present either with marrow aplasia or with hemolytic or thrombotic complications while maintaining a cellular marrow. As discussed previously, PNH develops in 2030% of patients with aplastic anemia treated with immunosuppressive therapy. [51] Some patients show evolution into acute leukemia.

Allogeneic stem cell transplantation is effective therapy for patients with either aplastic or cellular marrows. Approximately 80% of patients survive in the long term after conditioning with cyclophosphamide, given alone or combined with busulfan, TBI, procarbazine, or ATG. Transplantation corrects hematopoiesis and resolves thrombotic complications.[67] Observations in patients infused with marrow cells from a syngeneic donor without receiving conditioning confirm the assumption that the PNH clone has a survival advantage and requires cytotoxic therapy for elimination. [68]

Investigators of the International Bone Marrow Transplant Registry recently summarized information on 41 patients with PNH transplanted in 19781992 (unpublished report). Two transplants were from monozygotic twin donors, 35 from an HLA genotypically or phenotypically identical family member, and 4 from an HLA-matched unrelated donor. The time interval from diagnosis to transplantation was 2240 (median, 34) months, and patients were 1047 (median, 27) years of age at the time of transplantation. Conditioning regimens included cyclophosphamide alone or combined with TBI, limited-field irradiation, or busulfan. One identical twin recipient received no conditioning. Two-year survival among HLA-identical sibling transplants was 47%. Among five recipients of marrow from alternative donors (one parent; four unrelated), one is surviving. Both twin recipients are surviving, one after failing the initial marrow infusion and receiving conditioning followed by a second marrow infusion. The most common causes for treatment failure were lack of sustained engraftment and infections.

One group of investigators reported that normal CD34+, CD38 hematopoietic precursors can be obtained from the marrow of patients with PNH and separated from CD34+ cells that do not express CD59 or decay accelerating factor. [21] This would suggest the possibility of performing transplantations with normal autologous cells in patients with PNH.

Acquired Immunodeficiency Syndrome

Because of the decline in CD4+ lymphocytes and the severe immune defects associated with acquired immunodeficiency syndrome (AIDS), it was thought that transplantation of normal hematopoietic cells, particularly when combined with antiviral therapy, might allow for immunologic reconstitution and control human immunodeficiency virus (HIV). Holland and colleagues reported the case of an HIV-1infected patient in whom an undifferentiated lymphoma had developed. [69] The patient was conditioned with TBI and cyclophosphamide followed by allogeneic marrow and high-dose zidovidine. The patient engrafted, and marrow (and other) cultures became HIV-1 negative, and antibodies to HIV disappeared. The patient died of lymphoma relapse on day 47. At autopsy, all tissues were negative for HIV-1 by polymerase chain reaction, suggesting that HIV-1 had been suppressed to nondetectable levels. Additional reports on transplantation for AIDS appeared subsequently, but no long-term success was reported. [70] [71] Vilmer and colleagues reported on three patients in whom AIDS had developed at 856 months after marrow transplantation for aplastic anemia or leukemia. [72] These patients were given six sequential lymphocyte infusions from the original marrow donor combined with interferon (IFN)- and later IFN-. All three patients showed hematologic and immunologic improvement, although CD4 levels did not normalize.

More recently, Ho et al. proposed to transplant stem cells transfected with retroviral vectors containing the gene for a hairpin ribozyme capable of cleaving HIV-1 RNA. In vitro studies showed, indeed, that transfected cells were resistant to infection by HIV. [73] Ongoing studies are exploring the possibility of combining a hematopoietic stem cell transplant with cytotoxic T cells specific for HIV-1. [74]

Autoimmune Disorders

Anecdotal observations in patients who received a stem cell transplant for various indications, but who also had an autoimmune disorder (e.g., rheumatoid arthritis), show that with a successful transplantation, the autoimmune disorder usually also improved. [75] Marmont recently summarized results on nine patients with rheumatoid arthritis, of whom four survive in unmaintained remission from arthritis after allogeneic transplants; one patient relapsed after two years. One patient with CREST (calcinosis, Raynauds phenomenon, esophageal motility disorder, scleroderma, and telangiectasis) had a transient response after autologous transplantation, whereas one patient with myasthenia gravis achieved a complete remission. [16] Other groups have carried out stem cell transplantations for multiple sclerosis [5] [76] and scleroderma (P. McSweeney et al., Fred Hutchinson Cancer Research Center, Seattle, unpublished data), and early results suggest improvement of symptoms. Data on more than 40 patients with various disorders transplanted under the auspices of the European Bone Marrow Transplant Group were presented at a recent meeting

(A. Gratwohl, Kantos Spital, Basel, Switzerland, personal communication). Early results are encouraging. In addition, several animal models (e.g., of immune-mediated diabetes or experimental allergic encephalomyelitis) show that allogeneic transplants, and in some instances even T-cell depleted autologous transplants, result in complete remission of the immune disorder.

Based on these data, additional clinical protocols have been activated to test the toxicity and efficacy of stem cell transplantation in patients with systemic lupus erythematosus, systemic sclerosis, multiple sclerosis, and other indications.

Langerhans Cell Histiocytosis

Langerhans cell histiocytosis, formerly called histiocytosis X, is an uncommon disorder of childhood. Children whose disease progresses to the disseminated form have a poor prognosis. Because the originator cell of the disease is thought to be marrow derived, marrow-ablative therapy and transplantation should provide effective therapy. Both autologous and allogeneic approaches have been taken. [77] [78] [79] [80] [81] The numbers of patients reported are small. However, after conditioning with chemotherapy (e.g., etoposide and cyclophosphamide) and TBI, engraftment is

achieved, with approximately half of the patients surviving free of disease. [78] [82]

Congenital Disorders

Several congenital disorders such as Wiskott-Aldrich syndrome or dyskeratosis congenita are characterized by both hematologic and immunologic defects. They are discussed under sections on either hematologic or immunologic disorders, although this assignment may appear arbitrary.

Hematologic Defects

Fanconis Anemia

Fanconis anemia is a syndrome of chromosome fragility that is associated with various malformations, hematopoietic failure, and the development of MDS, leukemia, and nonhematopoietic malignancies. The disease is heterogeneous; at least five complementation groups have been recognized, and one of the genes (*FAC*) has been cloned. [83] The only definitive treatment available to correct the hematopoietic abnormalities is hematopoietic stem cell transplantation. Results are summarized in Table 85-7 . Because of toxicity associated with commonly used conditioning regimens [84] and the documented susceptibility of FA cells to alkylating agents, [85] many conditioning regimens have used limited-field irradiation and rather low doses of cyclophosphamide (approximately 10% of the dose used to condition patients with aplastic anemia). With this approach, survival in patients transplanted from an HLA-identical sibling has increased to 8090%. [86] [87] [88] The success rate with HLA-nonidentical and unrelated transplantation is approximately 30%. [89] Although failure of engraftment with sibling transplants has been rare, nonengraftment is observed in 2030% of patients transplanted from an alternative donor. [88] [89]

Patients with FA who are transplanted when they contract MDS or leukemia have a probability of survival in remission of approximately 25%.

Nonhematologic complications are not prevented by transplantation. In fact, a recent analysis of transplantation results in patients with marrow failure identified FA as the major risk factor for the development of solid tumors after transplantation. [90]

Thalassemia

Anecdotal reports in the early 1980s showed that, in principle, thalassemia was curable by marrow transplantation. [91] However, not all patients were treated successfully; outcome appeared to depend on disease duration and prior therapy. Based on those findings, Lucarelli and colleagues subsequently analyzed their results and developed a classification system for patients with thalassemia. [23] Considering hepatomegaly, portal fibrosis, and inadequacy of iron chelation therapy as criteria, these investigators categorized patients as class 1 if none of these factors was present, class 2 if one or two, and class 3 if three factors were present. [23] [92] In 1993, they reported results in 89 class 1 patients transplanted in Pesaro from 1982 to 1992. [93] The most frequently used conditioning regimen employed busulfan plus cyclophosphamide and CSP as GVHD prophylaxis. The probabilities of survival, rejection-free survival, nonrejection causes of death, and rejection were 92%, 85%, 6%, and 8%, respectively. Outcome in class 2 and class 3 patients, many of whom were older, was inferior. These results have been confirmed in several hundred patients transplanted in Pesaro and reported subsequently: [94] overall survival and disease-free survival rates were 82% and 75%, respectively. Results from a recent review are summarized in Figure 85-4 . An analysis of results in 215 patients in class 3 transplanted from an HLA-identical sibling donor showed age and conditioning regimen to be critical factors for outcome. [95] Cyclophosphamide doses of <200 mg/kg were associated with 5-year survival probabilities of 0.74 and 0.63 for patients younger than 17 years of age and older patients, respectively. Outcome was inferior at 200 mg/kg. In addition, and similar to earlier observations in patients with aplastic anemia, pretransplantation transfusion with more than 100 units of red blood cells decreased the 5-year survival probability from 0.53 to 0.24. Clearly, therefore, transplantation early in the disease course (i.e., in young and not heavily transfused patients) yields optimum results.

The U.S. experience with transplants for thalassemia was summarized by Walters and colleagues. [96] Thirty patients were identified who had been transplanted in 19811992. Patients were 0.514 years of age; 27 received marrow from an HLA-identical sibling, and 3 from an alternative donor. Most patients were conditioned with busulfan, 1224 mg/kg in combination with cyclophosphamide, 120240 mg/kg; some received TBI, 720 cGy plus cyclophosphamide, 120 mg/kg. Among HLA-identical transplant recipients followed from 0.2 to >10 years, the event-free survival rate was 54%; the actuarial rate of recurrence of thalassemia was 24%. It is not entirely clear why survival was lower than seen in Pesaro; however, it is important to note that American patients were not classified according to the Pesaro classification. Similar results have been reported from Thailand. [97]

Sickle Cell Disease

In 1994, Johnson and colleagues summarized results in five patients with sickle cell disease who had been transplanted in the United States. [98] Three of the patients had experienced cerebrovascular accidents or severe vaso-occlusive crises. Two patients were transplanted for other indications (acute leukemia and Morquios disease, respectively). Conditioning consisted of cyclophosphamide plus busulfan or TBI. All patients acquired the donors hemoglobin pattern. With a median follow-up of approximately 1.5 years, all patients were surviving and apparently had benefited from the transplantation.

Results of a multicenter study involving 22 patients <16 years of age were presented by Walters et al. [99] The indications for transplantation included a history of stroke (n = 12), recurrent acute chest syndrome (n = 5), or recurrent pain crisis (n = 5). Patients were conditioned with busulfan plus cyclophosphamide plus ATG. Twenty patients survived with a follow-up of 1051 (median, 24) months, and 16 of these have donor engraftment, with mixed donorhost chimerism being present in 1. Four patients rejected their graft and had recurrence of sickle cell disease (n = 3) or acquired marrow aplasia (n = 1). The two deaths were related to intracranial hemorrhage and GVHD, respectively. Probability of survival (event-free survival) at 4 years was 91% (73%). Lung function and central nervous system vasculopathy stabilized.

Vermylen and Cornu reported on 42 patients transplanted in Belgium. [100] [101] Engraftment was sustained in 36 patients, and all became symptom free. Five patients rejected their graft. Of these, two were retransplanted successfully, and three had autologous recovery. One patient died with GVHD, and 41 are alive 175 months after transplantation, 6 with chronic GVHD. Similar results have been reported from France. [102]

At least one study indicates that even patients who fail to engraft and experience autologous recovery show improvement of their disease because of increased levels of fetal hemoglobin. [103]

Walters and colleagues also carried out an analysis to determine why many patients are not referred to transplantation. Among 4,848 patients <16 years of age, 315 (6.5%) were found to meet protocol entry criteria. Among these, 128 (41%) had HLA typing performed, and 44 (i.e., 14% of those meeting entry criteria) had an HLA-identical sibling. Reasons for not proceeding with transplantation included a lack of a suitable donor

TABLE 85-7 -- Fanconis Anemia: Results of Marrow Transplantation from Related and Unrelated Donors

Reference	Year of Transplant	Number of Patients	Median Age (range) in Years	Conditioning	GVHD Prophylaxis	GVHD (%)		Survival (%)	Follow-up (median) in Years
						Acute	Chronic		
Hows et al.[212]	19771989	10 (=) 9 (; URD)	9 (523)	Cy 20/TBI, 3 × 200	CSP ± TCD	69	45	47	0.35 (3.4)
Kohli-Kumar et al.[86]	NA	18 (=)	7.6 (1.510)	Cy 20/TAI 400/ATG	CSP/Pred/ATG	0	16	94	0.56.3 (2.3)
Zanis-Neto et al.[87]	19831993	22 (=) 2 ()	10 (432)	Cy 200 (n = 10) Cy 140/± ATG (n = 12) Cy 20/TBI 400 (n = 2)	MTX or CSP, MTX + CSP	40	18	58	0.35.8 (2)
Gluckman et al. (IBMTR)[88]	1978-1992	151 (=)	7(<1-35)	Cy > 100 ± ATG (n = 25) Cy 1525 + TBI/TLI ± ATG (n = 102)	MTX + CSP MTX ± Other CSP ± Other	42	44	66 (2y)	0.3-13.5 (2.8)
		48 ()	6 (127)	Other (n = 24) Cy > 100 ±ATG (n = 2) Cy 15-25 + TBI/TLI ± ATG (n = 24)	TCD	51	46	29 (2 y)	
Seattle/Curitiba[213]	19731994	38 (=) 3 ()	10 (432)	Cy (200-120) Cy + ATG	MTX CSP MTX + CSP	36	38	61	222 (3.1)
Solh et al.[84]	19851990	10 (=) 1 ()		Cy 20/TBI 600		62	NA	54	3.57 (6)
Davies et al.[89]	19901994	7 (; URD)	(7.528)	Cy 40/TBI 400-450	MTX + CSP MTX + Other	70	50	42	0.83
Pasquini et al. (unpublished)	19831996	49 (=) 4 () 5 (URD)	9 (332)	Cy 200-100	MTX MTX + CSP	850	067	60	0.18.8 (3.5)
Socie et al. (unpublished)	19811996	49 (=)[a] 1 ()	11 (426)	Cy 20/500 TAI (n = 45) Cy 40/500 TAI (n = 5)[b]	CSP	55	70	74 (4 y)	116

=, matched related; , mismatched related or unrelated donor; URD, unrelated donor; IBMTR, International Bone Marrow Transplant Registry; ATG, antithymocyte globulin; CSP, cyclosporine; Cy, cyclophosphamide; GVHD, graft-versus-host disease; MTX, methotrexate; Pred, prednisone; MTX ± Other, methotrexate alone or with a second agent (not CSP); TCD, T-cell depletion; TAI, thoracoabdominal irradiation; TBI, total-body irradiation; NA, not available.

[a]Four patients received cord blood from an HLA-identical newborn sibling.
[b]The Cy dose was increased in patients who showed evidence of transforming into myelodysplastic syndrome.

Figure 85-4 Results in patients with thalassemia. The Kaplan-Meier estimates of survival to December 1997 for 649 patients with thalassemia younger than 17 years who received HLA-identical transplants using regimens containing busulfan 14 mg/kg and cyclophosphamide 200 mg/kg through 1996. The categorization into risk classes is described in Lucarelli et al. [214] *(Courtesy of G. Lucarelli Hospital of Pesaro, Pesaro, Italy, and R.A. Clift, Fred Hutchinson Cancer Research Center, Seattle, WA.)*

(24%), lack of financial or psychological support (10.5%), and parental or physician refusal (14%); history of medical noncompliance and others accounted for the remainder.

Blackfan-Diamond Anemia

Blackfan-Diamond or congenital hypoplastic anemia is a rare disorder of erythropoiesis presenting with normochromic, macrocytic anemia. Malformations such as triphalangeal thumb or cleft palate may be associated. The disease occurs sporadically or in an autosomally inherited form. Standard treatment is glucocorticoids. Other agents used for immunosuppressive treatment of aplastic anemia such as ATG and CSP have little effect. [104] If a suitable donor is identified, those patients usually receive a marrow transplant.[105] Although no large series have been presented, case reports show that transplantation is successful in correcting the hematopoietic defect.[106] [107] [108]

Dyskeratosis Congenita

Patients with dyskeratosis congenita are referred for transplantation because of pancytopenia and marrow failure. Results on small groups of patients have been reported from several centers. Langston and colleagues described eight patients, six of whom were transplanted from an HLA-identical sibling after conditioning with cyclophosphamide, and two from an unrelated donor after conditioning with TBI (6 × 200 cGy) plus cyclophosphamide. [109] Two patients died early and could not be evaluated for engraftment, and six patients achieved sustained engraftment. Three patients died from infections, one patient from GVHD, and three patients from pulmonary fibrosis at 70 days, 8 years, and 20 years, respectively. One surviving patient was recently diagnosed with colon carcinoma. Observations by other teams are similar.[110] [111] [112] [113] [114] Thus, as observed in patients with FA, marrow transplantation effectively corrects hematopoietic defects but does not affect other disease manifestations. Of particular note is the late development of pulmonary fibrosis. [109] [114]

Familial Erythrophagocytic Lymphohistiocytosis

Familial erythrophagocytic lymphohistiocytosis is a rare, nonmalignant disorder associated with pancytopenia and hemophagocytosis. Some patients are treated successfully with immunosuppression.[115] [116] Some patients without response have been treated successfully by allogeneic marrow transplantation. [117]

Chediak-Higashi Syndrome

Chediak-Higashi syndrome in its accelerated phase has some similarity to Langerhans cell histiocytosis and familial erythrophagocytic lymphohistiocytosis (see earlier). Although the disease responds to treatment with etoposide, remissions are usually short. Preliminary results suggest that at least half of these patients are cured by marrow transplantation if applied early in the course. [118]

For all other diagnoses listed in Table 85-1 , very few or single cases treated with marrow transplantation have been reported, [119] and no general recommendations

can be made at present.

Immune Defects

The treatment of immune defects with transplantation and nontransplantation modalities is described in detail in textbooks of clinical immunology and pediatric hematology/oncology. Only some diagnoses and principles are discussed in the following sections.

SCID

Marrow transplantation is the treatment of choice for SCID. [25] With HLA genotypically identical transplants, approximately 90% of children survive, usually with donor T-cell engraftment. B cells, monocytes, as well as red blood cells usually remain of host origin. Even HLA-haploidentical marrow grafts (from a parent), depleted of T cells to prevent GVHD, have been successful in 5060% of patients. Although engraftment is achieved in many patients without prior conditioning, an immunosuppressive regimen is required in patients with SCID and associated adenosine deaminase deficiency. [25] [35]

Filipovich recently summarized results of an international review of transplants from unrelated donors that included 100 children treated in the United States and 7 in Europe for 12 different immunodeficiencies. [120] Among these, 27 had SCID or one of its variants (22% X-linked SCID; 3% SCID with adenosine deaminase deficiency; 22% with Omenns syndrome; 53% with other variants, including ZAP-70 deficiency), the distribution being rather different from that in children transplanted from an HLA-identical sibling (35% X-linked; 32% lymphogenic). Patients were 6 weeks to 3.7 years (median, 9 months) of age at the time of transplantation. The survival rate at 3 years was 61%. Very encouraging was the observation that the incidence of acute GVHD was not different from that with HLA-identical sibling transplants. As illustrated in Figure 85-5 , outcome was inferior to that observed with HLA-identical sibling transplants

Figure 85-5 Probability of survival after marrow transplantation for severe combined immunodeficiency dependent on marrow stem cell source and marrow manipulation. Data on unrelated donor transplants were provided by the National Marrow Donor Program; all other data were collected by the International Bone Marrow Transplant Registry. BMT, bone marrow transplantation. *(Reproduced with permission from Filipovich.[120])*

but superior to that achieved with unmodified or T-cell-depleted haploidentical marrow from related donors. A major complication after T-cell-depleted haploidentical transplantation was the development of a post-transplantation lymphoproliferative disorder, which occurred in 32% of these patients. [121] The diagnoses of Omenns syndrome and pulmonary infection were associated with poor outcome. [122] [123]

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome is a rare disease, and transplantation experience at any given institution is limited. A recent report presented data on 26 patients transplanted at a single center. [124] Among 10 patients given unmodified marrow from an HLA-genotypically identical donor, 8 are surviving at 1.516.5 years with normal hematologic and immunologic parameters. Sixteen patients were given an HLA partially incompatible transplant (T-cell depleted in 15) from a related donor after conditioning with busulfan and cyclophosphamide; 12 patients engrafted, and 6 patients became long-term survivors. The addition of monoclonal antibody (MAB) against LFA-1 and CD2 appeared to enhance engraftment. Complications included delayed immunoreconstitution, infections, and the development of Epstein-Barr virus-associated lymphoproliferative disorders. These authors suggested that the transplantation indication should be liberal in patients with an HLA-identical donor, but should be restricted to patients with severe disease if an HLA-identical donor is not available.

In the international survey cited previously, 30 patients with Wiskott-Aldrich syndrome were identified who had received a transplant from an unrelated donor. Patients were 0.519.7 (median, 2.1) years of age at transplantation. Conditioning usually included busulfan, cyclophosphamide, and ATG. The actuarial survival rate at 3 years was 67%. As shown in Figure 85-6 , this was inferior to results with matched siblings (92%) but better than outcome with haploidentical related transplants (45%). The incidence of acute GVHD grades IIIV was 50%. A major prognostic factor was age, with 20 of 23 boys transplanted at younger than 5 years of age surviving, compared with 1 of 8 older than 5 years. The age effect appeared to correlate with disease progression.

With various other defects, T-cell deficiencies, and phagocytic disorders, post-transplantation survival rates range from 20 to 80%, and, as expected, the outcomes tend to be better with HLA-identical than with nonidentical donors. Chief causes of morbidity and mortality are GVHD, severe infections in the early post-transplantation period, and Epstein-Barr virus-associated lymphoproliferative disorders.

Figure 85-6 Probability of survival after transplantation for Wiskott-Aldrich syndrome dependent on stem cell donor. Sources of data as in Figure 85-5 . *(Reproduced with permission from Filipovich.[120])*

Osteopetrosis

Osteopetrosis is a disease of abnormal bone formation and hematopoietic failure. The genetic defect involves osteoclast function and may occur in a dominant (usually milder) and a recessive form (usually more severe). In 1980, Coccia et al. reported a successful allogeneic marrow transplant in a patient with osteopetrosis. [125] The patient showed improved hematopoiesis, bone restructuring, hearing, vision, and development. Gerritsen et al. summarized results of transplantation for autosomal recessive osteopetrosis in 69 patients treated in 19761994. [126] Four patients infused with marrow without prior conditioning experienced no lasting benefit. Sixty-five patients were prepared with myeloablative therapy. Among those transplanted from an HLA-identical sibling donor, the 5-year survival rate was 79%; among patients transplanted from an alternative (related or unrelated) donor, the 5-year survival rate was 38%. Osteoclast function developed in all patients with engraftment. Particularly in children 2 years of age or older, recovery tended to be associated with hypercalcemia. Among 15 patients with visual impairment at the time of successful transplantation, two showed subsequent improvement. Similar results have been obtained at other centers. [127]

In patients transplanted from HLA-haploidentical donors, graft failure has been a problem. [126] [128] The use of MAB against CD18 as part of the conditioning regimen has improved results. [128] [129]

Thus, even though success is not achieved uniformly, marrow transplantation after marrow-ablative conditioning remains the treatment of choice for patients with osteopetrosis.

Storage Diseases

Although transplantation success has in general been excellent with immunologic and hematopoietic disorders, results in patients with mucopolysaccharidoses, mucolipidoses, and other lysosomal storage diseases have been less consistent. [130] Dramatic improvements have been observed with adrenoleukodystrophy, globoid cell leukodystrophy, and Hurlers disease. Results with Hunters disease, however, have not been encouraging, and Sanfilippos disease may be a contraindication. [131] For all diagnoses in these disease categories, transplantation should be carried out only at specialized centers.

Hurlers Disease

Hurlers disease is a disease caused by -L-iduronidase deficiency and associated with progressive cerebral atrophy and declining intellectual function. Because the defective enzyme is expressed in hematopoietic cells, transfer of cells from a normal donor may provide the enzyme level required to maintain intellectual function. In 1993, Whitley et al. reported results in 11 consecutive patients who had been followed for 3.88.9 years after transplantation. [132] Nine patients (82%) were surviving, seven with complete donor engraftment, and two as mixed chimeras. Iduronidase levels reflected the donor genotype and the level of engraftment. Urinary glycosaminoglycan excretion had declined to normal levels. All surviving children achieved normal or near-normal intracranial pressure within 1.5 years of

engraftment, and there was no progression of cerebral atrophy. Among nine survivors, four children with a developmental quotient >80 pretransplantation maintained scores above that level post-transplantation. In the five survivors with lower pretransplantation scores, significant cognitive defects have developed post-transplantation.

In 1996, Peters et al. summarized results in 40 children transplanted from volunteer unrelated donors. [133] Patients were 0.93.2 (median, 1.7) years of age at transplantation and were conditioned with high-dose chemotherapy alone or combined with irradiation. Twenty-five patients achieved engraftment, and

50% were alive at 2 years, two-thirds with sustained allografts, and one-third with autologous hematopoiesis. The marrow cell dose was correlated with engraftment and survival. T-cell depletion and the type of conditioning did not appear to affect engraftment. Baseline and post-transplantation neuropsychological data were available in 11 surviving patients with engraftment. Among six children with pretransplantation mental developmental indices >70 who have been followed long enough for evaluation, none has shown a decline in age-equivalent scores. Among children with a baseline score <70, post-transplantation scores have continued to decline in five, whereas they have remained stable or improved in three.

These data suggest that in children with Hurlers disease, early marrow transplantations may stabilize or improve mental development, although this conclusion has been challenged.[134] Despite these concerns and despite considerable transplant-related problems, transplantation remains the only effective treatment option for these patients. Gene transfer experiments show encouraging in vitro results, but additional work is required before clinical application can be considered. [135]

Hunters Syndrome

Hunters syndrome results from a defect in the iduronate-2-sulfatase gene on the X chromosome. The disease typically progresses to severe mental retardation and death in adolescence. In an attempt to correct the enzyme deficiency, allogeneic marrow transplantation has been carried out in some of these patients. At least one patient, transplanted at the age of 2.7 years, has been reported who showed a regression of hepatosplenomegaly and a stabilization of his neuropsychological profile at 2 years after transplantation.[136] Other reports, however, show disease progression after transplantation, [137] and the current assessment is that patients with Hunters syndrome in general do not benefit from transplantation. [131]

Gauchers Disease

Gauchers disease is another lysosomal storage disease related to a defect of glucocerebrosidase (cerebroside--glucosidase). Gauchers disease may present in various forms at various stages in life.[138] Bone marrow transplantation can completely correct the defect.[139] [140] Because this disease can be traced to a single enzyme (gene) defect, it has become an attractive target for gene therapy. Attempts to date have not been successful, however, basically because of the very low transfection efficiency in transplanted cells. [141]

The recognition that the success rate with transplantation is in general better the younger the patient, has led to attempts at intrauterine transplantation. If a firm diagnosis can be established by amniocentesis, this approach may indeed yield superior outcomes. Very promising results have been reported in patients with SCID or Wiskott-Aldrich syndrome.[142] [143]

Others

Patients with adrenoleukodystrophy, metachromatic leukodystrophy, and globoid cell leukodystrophy and others may also benefit from allogeneic stem cell transplantation.[144] [145] [146] [147]

An interesting by-product of transplantation for storage disorders has been the documentation of donor-derived microglial cells in the recipients central nervous system, and, based on these findings, the speculation that a GVH reaction may occur in the brain. [148]

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POST-TRANSPLANTATION COMPLICATIONS AND THERAPEUTIC INTERVENTIONS

Complications associated with current regimens used for hematopoietic stem cell transplantation are frequent and depend on diagnosis, pretransplantation therapy, conditioning regimen, type of transplant, patient age, patient immune status, and other factors. By definition, these problems are iatrogenic and, as such, they should be amenable to treatment modifications that will result in therapeutic gains.

GVHD

Graft-versus-host disease is a major complication of allogeneic marrow transplantation, [41] [42] and occurs in a proportion of patients despite the use of GVHD prophylactic regimens. With a combination of CSP (given daily) and MTX (given on days 1, 3, and 6 or 1, 3, 6, and 11), acute GVHD is observed in 1050% of patients given an HLA-identical transplant and in 5090% of patients transplanted from an alternative donor. The corresponding figures for chronic GVHD are 2050% and 3060%, respectively. Preceding acute GVHD is the single most important risk factor for the development of chronic GVHD. [149] [150] Acute GVHD may occur within 56 days of transplantation, particularly among HLA-nonidentical transplant recipients, or as late as 5060 days post-transplantation, but usually develops within a month of transplantation. The main target organs of GVHD are the immune system, skin, liver, and intestinal tract, although other organs may be involved. [151] Only skin, liver, and intestinal tract are currently considered in GVHD grading systems. [152] The basic requirements for the development of GVHD as summarized by Billingham are (1) transfer of immunocompetent cells; (2) histoincompatibility of donor and recipient (for major or minor antigens), and (3) inability of the recipient to destroy the donor cells.[6] These conditions are met after allogeneic marrow or PBPC transplantation, unless donor T lymphocytes are removed from the transplanted cells (T-cell depletion) before infusion. This approach, however, carries the risk of engraftment failure (see later).

The result of interactions between donor and host cells is an activation and clonal expansion of donor T cells (afferent phase of the graft-versus-host reaction/GVHD). Activation leads to a complex pattern of cytokine release, recruitment of secondary effector cells, and destruction of recipient target cells and tissues (efferent phase).[42] These interactions occur not only in recipients who differ from the donor for MHC class I or II antigens but even between HLA (MHC)-matched people, because of differences in non-HLA or minor antigens encoded by genes outside of but presented by the MHC. GVHD-like reactions have also been observed after syngeneic or autologous transplantation, triggered apparently by inappropriate reactions to self-antigens. [153] [154]

The immunopathophysiology underlying GVHD is complex. Detailed discussions have been presented elsewhere. [41] [42] [155] As described previously, transplant recipients are usually prepared for transplantation with cytotoxic therapy. [156] This results in tissue damage and release of cytokines, including IFN-, tumor necrosis factor (TNF)-, and interleukin (IL)-1, [157] which, among other effects, lead to enhanced expression of MHC antigens and critical adhesion molecules, such as LFA-1 and CD44.[42] The observation that the incidence of GVHD is in general somewhat lower in patients with aplastic anemia (and other nonmalignant disorders) is consistent with such a notion, insofar as conditioning regimens for these diseases are less intense than those for malignant disorders. [158]

In allogeneic transplantation, mature donor T cells recognize alloantigens in the host and become activated. This recognition is facilitated by the milieu generated by the initial tissue damage and cytokine-induced increases in host cell surface receptors. Both CD4+ cells, which recognize MHC class II antigens, and CD8+ cells, which react primarily with MHC class I antigens and their associated peptides (in the case of GVHD, minor antigen peptides) can be involved. Activated donor T cells then proliferate and secrete IL-2. Activated T cells also express CD40 ligand, which provides a signal for CD40 on dendritic cells, thereby enhancing their maturation and function as

antigen-presenting cells. Furthermore, activated T cells express Fas-ligand, and are involved in Fas-mediated apoptotic cell death, a hallmark of GVHD. If the principal T-cell response is a T-helper $_1$ or inflammatory response (IL-2 and IFN- release), these cytokines activate additional donor (and residual host) mononuclear cells and macrophages to secrete IL-1 or TNF-. The result is further release of cytokines that amplify local tissue injury, at least in part through active nitrogen intermediates. If the principal T-cell response is a T-helper $_2$ response in which IL-4 and IL-10 predominate, the overall result, at least in murine models, is a chronic or stimulatory form of GVHD with increased IgE synthesis and exaggerated lymphoproliferation. [159] [160] [161] [162]

The chronic GVHD syndrome has prominent features of autoreactivity, [41] [155] and T lymphocytes with abnormal cytokine profiles (secreting, e.g., IL-4 and IFN-) may be present.[163] Experimental evidence suggests that thymic damage, inflicted by the conditioning regimen and acute GVHD, results in a failure of intrathymic selection and an escape of autoreactive cells to the periphery. [164] Such a mechanism also readily explains the occurrence of syngeneic or autologous GVHD.

Methods of GVHD prophylaxis are summarized in Table 85-8 . Combination regimens of MTX and CSP with or without the addition of IV immunoglobulins offer the most effective currently available prophylaxis. [45] [165] The value of added prednisone is controversial. [165] [166] Ongoing trials with FK506 are encouraging and are expected to yield results similar to those obtained with CSP. [167] In vivo administration of MAB (murine or the humanized form) against the IL-2 receptor or MAB neutralizing TNF- have been of only transient benefit. [168] [169] [170] [171] Soluble IL-1 receptor or IL-1 receptor antagonist has yielded encouraging preliminary results in prevention and treatment of GVHD.[159] [172] Although these agents are antigen nonspecific and may not interfere with the initial steps of allogeneic activation, they may be able to prevent the manifestation of the graft-versus-host reaction (i.e., GVHD), and possibly interfere with other disease processes, such as septicemia or capillary leak syndrome, associated with diffuse tissue damage.

T-cell depletion of donor marrow by 24 log, clearly effective in reducing the incidence of GVHD, has been complicated by an increased probability of graft failure (1030% with HLA-identical, 3060% with HLA-nonidentical transplants). [173] [174] [175] Because failure of sustained engraftment is a potential problem in patients with aplastic anemia even with unmanipulated marrow, T-cell depletion has been applied only very cautiously to this patient group. However, promising results have been reported in patients transplanted with marrow from alternative donors, T-cell depleted with MAB T10B9 and complement (Table 85-6).[61] Of note is the observation in several studies that the incidence of GVHD was lowest in patients who showed mixed

TABLE 85-8 -- Graft-versus-Host Disease Prophylaxis

T-lymphocyte depletion of donor marrow in vitro
Complete
Selective (CD4, CD8)
With partial addback

In vivo treatment of patient
Methotrexate, cyclosporine, FK506
Polyclonal anti-T-cell antibodies
Monoclonal anti-T-cell antibodies
Murine
Humanized
Cytokine blockade
Gnotobiosis
Induction of mixed chimerism

chimerism the coexistence, at least transiently, of donor and host lymphohematopoietic cells postgrafting. [176] The mechanism is not clear. However, recent data from studies aimed at inducing mixed chimerism by design have confirmed the clinical observations. [177]

Graft-versus-host disease is the most important risk factor for long-term survival, and in patients with nonmalignant disorders is not associated with any beneficial effect. Therefore, if GVHD develops, it requires aggressive therapy, not only for the acute but for the chronic form. Current therapy for acute GVHD includes glucocorticoids, CSP, monoclonal antibodies, humanized monoclonal antibodies (e.g., anti-Tac), immunotoxins, and cytokine blockade. [43]

Graft Failure

The problem of graft failure has been introduced previously. The depletion of T cells from the donor marrow removes an important element of GVHD that also counteracts residual host cells, thus rendering T-cell-depleted marrow more susceptible to rejection. The probability of rejection is further exacerbated by recipient sensitization to HLA antigens, predominantly through blood transfusions given pretransplantation. [178] [179] These problems have usually been countered by intensification of the conditioning regimen, which in turn has resulted in increased transplant-related toxicity. There is evidence that certain rat anti-human MABs (e.g., Campath I G) or an antibody directed at LFA-1 given as part of the conditioning regimen, or ATG or MAB/immunotoxin conjugates administered postgrafting, may reduce the graft failure rate without increasing toxicity. [180] [181]

In some patients, graft failure is not due to frank immunologic rejection but to a failure to thrive of transplanted cells. This may be the case in approximately 5% of patients with aplastic anemia. Recombinant growth factors (e.g., G-CSF, GM-CSF, IL-3) given prophylactically or therapeutically may be beneficial in some patients,[182] including those with marrow microenvironmental defects or with cytomegalovirus infection. Other factors, such as c-kit ligand (Steel factor) or flt-3 ligand, are being tested.[183] [184]

Infections

The principles of infection prophylaxis have been described previously. [39] One additional modality is the use of IV immunoglobulin (IVIG). Patients who receive prophylactic IVIG after transplantation experience fewer infections while IVIG is administered. However, after discontinuation of IVIG administration, these patients appear to have a higher rate of infection than patients not given IVIG. [185] Apparently, the passive immunity provided by IVIG suppresses and delays recovery of the patients active immune response.

The use of IVIG is also of interest in the context of GVHD. Although IVIG was administered originally with the aim of infection prophylaxis, results suggested that patients so treated had a reduced incidence of GVHD. This was confirmed in a prospective, randomized study. [186] The effect was most prominent in HLA-identical transplant recipients older than 20 years of age. The mechanism is not well understood, but may involve Fc receptor-mediated effects on inflammatory cytokine production (e.g., secretion of IL-1ra is enhanced by macrophages when their Fc receptors are cross-linked), the presence of neutralizing antibodies to cytokines, the presence of anti-idiotype antibodies in the IVIG preparation, or a combination of these processes.

To what extent immunity toward herpes virus (in donor, recipient, or both) affects GVHD has remained controversial. [187] Studies in Europe have suggested that increased viral burden in the recipient contributes to GVHD, and the presence of processed

TABLE 85-9 -- Delayed Complications of Bone Marrow Transplantation

Chronic graft-versus-host disease
Infections
Airway and pulmonary disease
Autoimmune dysfunction
Impaired growth and development
Endocrine dysfunction
Sterility
Cataracts
Dental problems
Osteoporosis
Aseptic necrosis of the bone
New malignancies
Psychosocial dysfunction

viral antigens, which are known to elicit vigorous T-cell responses under certain conditions, could act as additional minor histocompatibility antigens. [188]

Delayed Complications

The longest surviving patients have now been followed for up to 25 or 30 years post-transplantation, and most are leading normal lives; some, however, have experienced late complications (Table 85-9). Complications are related either to pretransplantation events, the conditioning regimen (irradiation), or transplant-associated events (chronic GVHD, immunodeficiency). [189] [190] Potentially life-threatening problems include infections and pulmonary dysfunction, particularly bronchiolitis obliterans, which is usually associated with chronic GVHD. [191] Autoimmune disorders may be due to adoptive transfer (from a donor who is affected), may be related to GVHD, or may be idiopathic. [192] Conversely, as discussed earlier, patients with aplastic anemia or leukemia who also had an autoimmune disorder noted improvement or complete resolution of their autoimmune disease after transplantation. These observations provide a further illustration of the effect of

transplantation on immunity in general and autoimmunity in particular, an insight that is now being applied to the treatment of autoimmune disorders. [193] [194]

The use of fractionated rather than single-dose TBI as part of the conditioning regimen has reduced the frequency and severity of transplant-related complications such as interstitial pneumonitis, cataracts, and impairment of growth and development. [56] It is desirable to avoid irradiation completely, at least in very young children, where growth and development of the skeleton, as well as the central nervous system, occur at an exponential rate.

Systematic psychosocial studies investigating the effects of transplantation on personal development, family dynamics, and partner relationships are only now being pursued. Active rehabilitation is necessary in many instances. [195] [196]

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SUMMARY

Hematopoietic stem cell transplantation has been developed into curative therapy for many nonmalignant disorders. Results are best with HLA-identical sibling (or syngeneic) transplants, whereas outcome may be inferior with transplants from HLA-nonidentical related or from unrelated donors. A major problem is GVHD, especially with its chronic manifestations, which are also responsible for systemic or organ-specific late complications. Because, in contrast to malignant diseases, there is no potential benefit of GVHD in the form of a graft-versus-tumor effect, every effort must be directed at preventing GVHD. To what extent the application of gene transfer to transplantation for nonmalignant disorders will affect long-term outcome remains to be seen.

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Chapter 86 - Results of Allogeneic Stem Cell Transplantation for Malignant Disorders

Mary M. Horowitz

The first successful allogeneic hematopoietic stem cell transplantations (allogeneic stem cell transplants or allotransplants) were done in 1968, using bone marrow from human leukocyte antigen (HLA)-identical sibling donors, in three children with congenital immune deficiencies. [1] [2] [3] [4] In the 1970s, most transplantations were done for nonmalignant disorders. However, in the mid-1970s, Thomas and colleagues reported long-term leukemia-free survival (LFS) after HLA-identical sibling transplantation in some patients with refractory acute leukemia. [5] [6] Better outcome was subsequently demonstrated in patients transplanted in first or second remission.[7] [8] [9] [10] Durable cytogenetic remissions and long-term LFS after identical twin and allogeneic transplants for chronic myeloid leukemia (CML) were reported in the late 1970s and early 1980s. [11] [12] [13] Use of allogeneic stem cell transplants (SCT) to treat leukemia increased dramatically in the 1980s. By 1985, approximately 75% of transplantations were performed for therapy for leukemia, with approximately equal numbers for CML, acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL); over 90% received stem cells from HLA-identical sibling donors. Allogeneic transplantation is now increasingly applied in other hematologic and nonhematologic cancers. An estimated 15,00020,000 allogeneic SCTs were done in 1997, based on data reported to the International Bone Marrow Transplant Registry (IBMTR). The most common indications are shown in Figure 86-1 . These are consistent with data reported for Europe by the European Blood and Marrow Transplant Group (EBMTG).[14] Seventy-five percent of allogeneic SCTs are performed or therapy for leukemia or preleukemia: 25% for CML, 25% AML, 15% for ALL, 7% for myelodysplastic syndromes (MDS), and 3% for other leukemias. Twelve percent are for other cancers, including non-Hodgkin lymphoma (NHL; 7%), multiple myeloma (4%), and Hodgkin disease (1%). The remainder are for aplastic anemia, immune deficiencies, and other nonmalignant disorders. Use of allogeneic SCTs for cancer treatment is usually restricted to young patients. The median age of patients receiving transplants for malignancies in 1997 was 33 years; only 6% were older than 50 years.

GRAFT-VERSUS-LEUKEMIA EFFECTS

Allogeneic SCT involves administration of high (myeloablative) doses of chemotherapy with or without radiation followed by infusion of hematopoietic stem cells from a healthy donor. Stem cells may be collected from bone marrow or from blood.[15] [16] [17] [18] The purpose of high-dose therapy (conditioning) is twofold: to eradicate malignant cells and to eliminate host immune cells capable of rejecting donor stem cells. The ability to restore hematopoiesis with donor stem cells allow administration of substantially higher doses of cytotoxic therapy than is otherwise possible. Dose-response effects of various anticancer agents can thus be exploited (see Chap. 87).

Donor cells also play an important antitumor role. This was first suggested more than 30 years ago by Barnes et al. [19] They studied leukemic mice treated with high-dose total-body irradiation (TBI) and compared those receiving syngeneic (identical

Figure 86-1 Indications for allogeneic blood and marrow transplantation, worldwide, in 1997. CML, chronic myeloid leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndromes; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin lymphoma; HD, Hodgkin disease; MM, multiple myeloma.

twin) and allogeneic bone marrow infusions. The mice receiving syngeneic marrow died quickly of leukemia. Those receiving allogeneic cells survived longer but eventually acquired fatal graft-versus-host disease (GVHD). Importantly, they had no evidence of leukemia at death. Mathé and colleagues [20] proposed the term adoptive immunotherapy for the antitumor effect of allogeneic cells. Antitumor effects could be specific (i.e., after sensitization of the donor [21] or the donors cells[22] to antigens present on malignant cells) or nonspecific (i.e., associated with GVHD, an immune reaction of donor lymphocytes against normal and malignant host cells presumably triggered by differences in major or minor histocompatibility antigens [23] [24] [25]). The term graft-versus-leukemia (GVL) was coined by Bortin and coworkers[26] [27] to indicate the adoptive immunotherapeutic effect of transplanted allogeneic hematopoietic cells against leukemia cells. In some animal models, the GVL effect could be distinguished from GVHD, whereas in others, the two were inseparable. [26] [27] [28] [29] [30] [31] Clinical evidence for the importance of GVL in eradicating leukemia includes (1) lower incidence of leukemia relapse in allograft recipients with acute or chronic GVHD than in those without GVHD; [23] [24] [25] [32] [33] [34] [35] [36] [37] (2) higher relapse rates after identical twin versus allogeneic stem cell transplantations; [25] [38] [39] (3) high relapse rates after T-cell-depleted transplants; [25] [40] [41] [42] [43] [44] and (4) durable cytogenetic and molecular remissions induced after post-transplantation relapse by infusion of donor leukocytes without other antileukemia chemotherapy.[45] [46] [47] [48] [49] [50] [51]

Clinical evidence for graft-mediated antitumor effects is strongest for leukemia, but is also supported by data reported in NHL, Hodgkin disease, and multiple myeloma.[52] [53] [54] [55] [56] [57] Investigators at Johns Hopkins report lower relapse rates in patients in whom GVHD developed than in those in whom GVHD did not develop after allotransplantations for lymphoma. [52] Complete remissions can be effected by donor lymphocyte infusions in patients relapsing after allogeneic SCTs for lymphoma and multiple myeloma.[50] [51] [55] [56] [57] Some data suggest lower relapse rates with allogeneic than autologous SCTs for lymphoma and multiple myeloma. However, in the latter setting, relapse from reinfused malignant cells in autografts cannot be excluded. [53] [58]

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PROGNOSTIC FACTORS FOR TRANSPLANTATION OUTCOME

Although allogeneic SCT has efficacy in several cancers, the procedure carries a substantial risk of morbidity and mortality. Transplant-related mortality may result from toxicity of pretransplantation conditioning to lung, liver, and other organs, infection or bleeding related to cytopenias, GVHD, and infection related to delayed immune reconstitution, especially in the setting of GVHD or its treatment. In addition, many patients have recurrence of their primary disease despite intensive conditioning and GVL. Causes of death after allogeneic transplantation for malignancies are shown in Table 86-1 . The prognosis of transplant recipients is influenced by several factors associated with risk of transplant-related mortality or cancer recurrence. Some of these are disease specific and discussed in the following section on Results in Specific Malignancies. A few generalizations are possible. First, transplant-related mortality is lower with HLA-identical sibling transplants than with alternative related or unrelated donor transplants because of less graft failure and less GVHD. [59] [60] [61] [62] [63] [64] [65] [66] [67] Among alternative donor transplants, those from more closely matched donors tend to have lower risks of GVHD and transplant-related mortality. [61] [62] [63] [66] [67] Second, outcome is better when transplantation is done early in the course of a malignancy. Transplantations done for advanced disease are associated with higher risks of both relapse and transplant-related mortality. High transplant-related mortality in this setting probably reflects poorer clinical status and more extensive prior treatment of patients transplanted for advanced disease. This does not mean, necessarily, that transplantation should always be done early. Some patients with newly diagnosed cancers, such as children with standard-risk ALL, have an excellent prognosis with nontransplantation therapy. Appropriate timing of transplantation requires consideration of likely outcomes with transplantation and nontransplantation therapies. However, even when not used as first-line therapy, transplantations should not be inordinately delayed, because patients with refractory disease or severe complications from extensive prior therapy are unlikely to benefit. The possibility and appropriate timing of transplantation should be considered early in planning management strategies for patients with diseases potentially treatable by allografting. This includes determining the availability of suitable related or unrelated donors. [68] Third, factors affecting the risk of transplant-related mortality influence the outcome of transplantation for all malignancies. Transplant-related mortality is lower, and consequently survival is higher, in patients who are young, cytomegalovirus negative, and have good performance scores and no active infection. Finally, in most but not all settings, factors associated with increased likelihood of relapse or progression after conventional therapy also predict increased risk of post-transplantation relapse or progression. Importantly, patients whose disease does not respond to conventional therapy are significantly less likely to have durable remissions after transplantation than those with responsive disease.

RESULTS IN SPECIFIC MALIGNANCIES

Chronic Myeloid Leukemia

The incidence of CML is 12/100,000 population; the median age at onset is >60 years. [69] Approximately 35% of patients are <55 years of age at diagnosis. With nontransplantation treatment, complete remissions occur in a minority of patients, and

TABLE 86-1 -- Causes of Death After Allogeneic Stem Cell Transplantations Done in 19901996 and Reported to the International Bone Marrow Transplant Registry

Indication for Transplantation	No. Transplanted	No. of Deaths	Primary Cause of Death					
			Primary Disease	GVHD	IPn	Infection	Organ Toxicity	Other
CML	5,503	1,664	14%	25%	9%	22%	13%	17%
AML	5,290	1,811	37%	13%	5%	18%	12%	15%
MDS	1,309	499	21%	18%	5%	20%	16%	20%
ALL	3,808	1,435	37%	12%	7%	16%	14%	14%
CLL	220	72	21%	17%	8%	29%	8%	17%
Lymphoma	1,412	573	29%	11%	7%	17%	19%	17%
Multiple myeloma	750	285	20%	15%	9%	17%	21%	18%

GVHD, graft-versus-host disease; IPn, interstitial pneumonitis; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndromes; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia.

few, if any patients are cured. Reported median survivals range from 3 to >8 years. [70] [71] [72] [73] [74] Eighty to 90% of patients die of causes related to CML. Allogeneic stem cell transplantation using a related or unrelated donor can cure a substantial proportion of patients with CML. [12] [13] [33] [40] [41] [42] [66] [75] [76] [77] [78] [79] Follow-up of survivors transplanted >20 years ago confirms the durability of posttransplantation morphologic and cytogenetic remissions.

A study of 10 countries in Europe, North America, Australia, and New Zealand, covering the years 19891991, suggested that approximately 3040% of people with CML younger than 55 years of age receive allografts. [80] This proportion likely increased in subsequent years with more widespread use of transplants from unrelated donors. According to data reported to the IBMTR, there are approximately 3,500 allogeneic SCTs for CML yearly. Seventy to 75% of these are from related and the rest from unrelated donors. Eight-five percent of HLA-identical sibling transplantations are done in the first chronic phase, 15% in the second chronic or accelerated phase, and 5% in the blast phase. Corresponding figures for unrelated donor transplants are 70% in chronic phase, 5% in accelerated or second chronic phase, and 25% in blast phase, reflecting time needed for donor identification and work-up and reluctance to risk the higher transplant-related mortality of an unrelated donor transplantation early in the course of CML. The median interval from diagnosis to transplantation is 9 months for HLA-identical sibling transplantations and 21 months for unrelated donor transplantations. The median age of transplant recipients is 36 years; 10% are <20 years of age and 10% are >50 years of age.

The most common conditioning regimen used before transplantation for CML is high-dose busulfan (416 mg/kg) and cyclophosphamide (120200 mg/kg), accounting for approximately 55% of CML transplants reported to the IBMTR in 1997. Studies comparing busulfan and cyclophosphamide to TBI and cyclophosphamide in CML suggest similar relapse and transplant-related mortality with the two approaches. [81] [82] The former is in general simpler and less expensive to administer. A randomized trial addressing the question of whether intensifying pretransplantation conditioning reduces relapse and increases survival after allografts for CML found fewer relapses but more transplant-related mortality with 15.75 versus 12 Gy TBI; LFS was similar. [83] Transplantations for CML using unrelated donor transplants are more likely to include TBI in the conditioning regimen, as are transplantations using T-cell-depleted bone marrow. Post-transplantation cyclosporine and methotrexate is the most frequent GVHD prophylaxis regimen, accounting for approximately 70% of transplantations for CML. [84] [85] [86]

The high-dose therapy given for pretransplantation conditioning likely cures some patients with CML, accounting for long-term LFS of 3050% of patients receiving identical twin transplants. [38] [39] However, there is strong evidence for the importance of GVL effects in preventing CML relapse after transplantation. Patients with GVHD, particularly chronic GVHD, after HLA-identical sibling transplantation for CML, have significantly lower risks of relapse than those without GVHD. [24] [25] Recipients of identical twin transplants for CML in whom GVHD does not develop also have significantly higher risks of post-transplantation relapse than recipients of allogeneic transplants. [25] [39] Development of clinically evident GVHD is apparently not necessary for a GVL effect in CML, because patients receiving allogeneic grafts in whom acute or chronic GVHD does not develop have lower relapse rates than those receiving identical twin transplants. [25] [39] GVL effects of allografts in CML are substantially reduced by removing T lymphocytes from the donor bone marrow, an effective strategy for reducing GVHD that was introduced in the 1980s. [40] [41] [42] [44] [87] Relapse rates after T-cell-depleted HLA-identical sibling transplantation are similar to rates after identical twin transplantation. This is not fully explained by a lower incidence of GVHD because relapse rates are high in T-cell-depleted transplant recipients in whom GVHD

Figure 86-2 Actuarial probabilities of relapse after HLA-identical sibling transplantation for chronic myeloid leukemia.

develops. [25] The high relapse rate after T-cell-depleted transplantation for CML is one reason there has not been widespread adoption of this strategy, although it is very effective in preventing GVHD. Approximately 5% of HLA-identical sibling transplantations and approximately 10% of unrelated donor transplantations for CML use T-cell-depleted bone marrow. Some data suggest that GVL effects may be preserved despite T-cell depletion in unrelated donor transplantation. [88] Techniques suggested to reduce relapse risk after T-cell-depleted transplants are intensification of the conditioning regimen and selective depletion of T-cell subsets. [44] [89] [90] [91] [92] [93] [94] [95] An effective GVL response can be established by infusing donor lymphocytes in many patients who relapse after an HLA-identical sibling transplantation for CML. The subsequent hematologic, cytogenetic, and molecular remissions appear durable. [45] [46] [47] [48] [50] [51] Some strategies being explored for minimizing transplant-related mortality (especially GVHD-related mortality) while maintaining antileukemia efficacy of allotransplants for CML include T-cell depleting donor bone marrow with subsequent infusion of donor lymphocytes, either at the time of a post-transplantation relapse or prophylactically after toxicity from the conditioning regimen has resolved. [90]

The strongest determinants of outcome after allogeneic SCTs for CML are donor type and phase of disease at time of transplantation. Among 4,114 recipients of HLA-identical sibling transplantations done between 1990 and 1996, reported to the IBMTR, 3-year actuarial probabilities of relapse (95% confidence intervals) are 17% (1519%) for 3,359 patients transplanted in the first chronic phase, 42% (3747%) for 583 in the accelerated phase, and 44% (3553%) for 172 in the blast phase (Fig. 86-2). Three-year probabilities of LFS are 58% (5660%), 34% (3038%) and 29% (2236%), respectively (Fig. 86-3). Patients relapsing

Figure 86-3 Actuarial probabilities of leukemia-free survival (LFS) after HLA-identical sibling transplantation for chronic myeloid leukemia.

Figure 86-4 Actuarial probabilities of survival after HLA-identical sibling transplantation for chronic myeloid leukemia.

after an HLA-identical sibling transplantation for CML often survive for long periods with conventional treatment. [96] Many achieve durable remissions with infusion of donor lymphocytes (see earlier). Consequently, 3-year survival rates after transplantation are somewhat higher than LFS rates: 67% (6569%) in the chronic phase, 41% (3745%) in the accelerated phase, and 29% (2236%) in the blast phase (Fig. 86-4). Unrelated donor transplantation for CML carries a higher transplant-related mortality rate than HLA-identical sibling transplantation. However, a significant proportion of patients become long-term survivors. Among 1,338 unrelated donor transplantations done between 1990 and 1996 reported to the IBMTR, 3-year survival rates were 45% (4149%) for 909 patients transplanted in the chronic phase, 28% (2234%) for 337 transplanted in the accelerated or second chronic phase, and 30% (2040%) for 92 transplanted in the blast phase. The survival rates of patients receiving an unrelated donor transplant in the blast phase may be misleading because many patients with transformation to the blast phase die before an unrelated donor transplantation can be performed or are deemed ineligible based on the refractory nature of their disease. Survival rates as high as 74% are reported with HLA-matched (using molecular HLA typing techniques), unrelated donor transplantation done early after diagnosis. [67] The timing of transplantation influences the outcome of related and unrelated donor transplantations for CML. Survival rates are significantly higher when transplantation is done <1 year after diagnosis (Fig. 86-5).

Several disease-related features at diagnosis, such as white blood cell count, percentage circulating blasts, hemoglobin, and spleen size, predict survival duration in patients with chronic-phase

Figure 86-5 Actuarial probabilities of leukemia-free survival (LFS) after HLA-identical sibling and unrelated donor transplantation for chronic myeloid leukemia, by interval between diagnosis and transplantation.

CML who receive nontransplantation therapy. [97] [98] [99] [100] These factors are associated with the rate of transformation to the acute or blast phase, which is the cause of death in most patients receiving nontransplantation treatment. In contrast, relatively few deaths after HLA-identical sibling transplantation for chronic-phase CML result from leukemia, particularly if the transplants are not T-cell depleted. Most deaths are from transplant-related complications such as regimen-related toxicity, GVHD, and infection (see Table 86-1). Consequently, prognostic factors for survival after allogeneic transplantation for chronic-phase CML are those that influence the risk of transplant-related complications, such as donor type, older age, donor parity, and prior treatment. [101] Splenomegaly is not associated with more relapses, although it may lead to delayed engraftment. [102] Studies of pretransplantation splenic irradiation or splenectomy do not show a survival benefit. [103] [104] [105]

The type of initial therapy given for CML can affect the outcome of a subsequent transplantation. Patients receiving busulfan, once frequently used to control leukocyte counts and symptoms, have higher risks of transplant-related mortality after HLA-identical sibling transplantation than patients receiving hydroxyurea. [76] Whether interferon- affects transplantation outcome is uncertain. Conflicting observations are reported. [106] [107] [108] [109] Recent data in over 209 patients receiving interferon- before an HLA-identical sibling transplantation suggest no adverse effect on outcome. [108] Another study of 184 patients receiving unrelated donor transplants shows higher risks of severe GVHD and GVHD-related death in those receiving interferon for 6 or more months. [109]

Timing of transplantation, even among patients who remain in the chronic phase, also affects transplantation outcome. Several studies demonstrate a better outcome of HLA-identical sibling transplantation when done within 1 year of diagnosis [13] [67] [76] (see Fig. 86-5). The adverse effects of delaying transplantation include both increased transplant-related mortality and increased relapse. Survival after unrelated donor transplantation is also lower when transplantation is delayed. [66] [67] This makes decisions about initial therapy for CML difficult. CML has a long natural history, with few patients dying in the first 12 years after diagnosis; patients with major cytogenetic responses to interferon have long expected survivals. Consequently, delaying transplantation to determine the interferon response is an appealing strategy. This is especially so for patients without an HLA-identical sibling donor or with risk factors for transplant-related complications. However, the delay, and possibly interferon exposure, may adversely affect transplantation outcome. Recent studies comparing large databases of nontransplant and transplant recipients suggest that early transplantation (transplant <1 year after diagnosis) yields the largest number of long-term survivors and longest average survivals for patients younger than 50 years of age. [110] [111] An exception might be patients 4050 years of age without an HLA-identical sibling donor and with features predicting good prognosis with nontransplantation treatment. [110]

Acute Myeloid Leukemia

The incidence of AML is 23/100,000 population in the United States. [69] The median age at onset is >60 years; approximately 35% of patients are <55 years of age at diagnosis. [65] Using current induction regimens, 6080% of patients achieve complete remission, and 2050% of these become long-term leukemia-free survivors after further consolidation therapy. [112] [113] Patients failing to achieve remission with primary induction therapy or relapsing after an initial remission have a poor prognosis, with almost all dying of causes related to AML. Allogeneic transplantation is an effective treatment for AML, with LFS rates as high as 80% in young patients transplanted in first remission. [114] [115] [116]

The international survey cited previously indicated that approximately 25% of patients diagnosed with AML in 19891991 in the 10 countries studied received allotransplants. [117] The survey also revealed wide variation in this proportion among countries, reflecting different strategies for applying transplantation (i.e., as consolidation vs. salvage therapy). IBMTR data indicate approximately 3,500 allogeneic SCTs for AML yearly. Approximately 80% of these are from related and the rest from unrelated donors. Five to 10% of HLA-identical sibling transplantations are done in patients never achieving first remission, 60% as consolidation therapy in first remission, 1015% in first relapse, 1015% in second remission, and <5% in a subsequent relapse or remission. Few unrelated donor transplantations are done in patients never achieving remission. Twenty-five percent of unrelated donor transplantations are done in first remission, 20% in first relapse, 30% in second remission, 10% in a subsequent relapse, and 5% in a subsequent remission. The median interval from diagnosis to transplantation is 6 months for HLA-identical sibling transplantation and 12 months for unrelated donor transplantation. The median age of transplant recipients is 31 years; 30% are <20 years of age and 5% are >50 years of age.

As for CML, the most common conditioning regimen used before transplantation for AML is busulfan and cyclophosphamide, accounting for 45% of AML transplantations reported to the IBMTR in 1997. Randomized trials comparing busulfan and cyclophosphamide to TBI and cyclophosphamide in AML suggest similar relapse and similar or somewhat higher transplant-related mortality rates with the busulfan regimen. [118] [119] [120] [121] One randomized trial in AML demonstrated decreased relapse risk with intensification of the pretransplantation conditioning regimen. [122] In this study, an increase in the TBI dose from 12 to 15.75 Gy decreased the relapse rate from 35 to 12%. However, the potential survival benefit was offset by a comparable increase in transplant-related mortality. Post-transplantation cyclosporine and methotrexate is the most frequent GVHD prophylaxis regimen, and is used in approximately 65% of transplantations for AML.

The GVL effect is important in preventing relapse after transplantation for AML. As in CML, patients in whom GVHD develops, particularly chronic GVHD, after HLA-identical sibling transplantation for AML have significantly lower risks of relapse than those without GVHD. [24] [25] [37] [123] Recipients of identical twin transplantation

for AML also have significantly higher risks of post-transplantation relapse than recipients of allogeneic transplants, with or without GVHD, demonstrating an important GVL effect even in the absence of clinically detectable GVHD. [38] [39] The effect of T-cell depletion of donor bone marrow on relapse risk is not as dramatic in AML as in CML. Although relapse rates are higher after T-cell-depleted transplantation than non-T-cell-depleted transplantation for AML, the decrease is entirely consistent with reduction of GVHD and GVHD-associated GVL effects. [44] Donor lymphocyte infusions are effective in reinducing durable remissions in a minority (approximately 25%) of patients relapsing after receiving allografts for AML. [50] [51]

As in CML, the strongest determinants of outcome after allogeneic transplantation for AML are donor type and phase of disease at time of transplantation. Patients in first remission have the best transplantation outcomes. Those with more advanced disease have lower probabilities of LFS because of higher risks of both relapse and transplant-related mortality. Among 4,171 recipients of HLA-identical sibling transplantations done between 1990 and 1996, reported to the IBMTR, 3-year actuarial probabilities of relapse were 24% (2226%) for 2,651 patients transplanted in first complete remission, 41% (3646%) for 571 transplanted in second or subsequent remission, and 58% (5462%) for 949 transplanted in relapse (Fig. 86-6). Three-year probabilities of LFS were 59% (5761%), 40%

Figure 86-6 Actuarial probabilities of relapse after HLA-identical sibling transplantation for acute myeloid leukemia. CR, complete remission.

(3545%), and 27% (2434%), respectively (Fig. 86-7). Salvage therapy infrequently produces durable remissions after post-transplantation relapse, although some patients may be successfully treated with chemotherapy or donor lymphocyte infusions. [50] [51] Unrelated donor transplantations have higher transplant-related mortality but lower relapse rates than HLA-identical sibling transplantations. Among 743 unrelated donor transplantations done between 1990 and 1996 reported to the IBMTR, 3-year LFS rates were 51% (4755%) for 188 patients transplanted in first remission, 35% (3139%) for 249 transplanted in second or subsequent remission, and 18% (1620%) for 306 transplanted in relapse (Fig. 86-8).

Several disease-related features such as high white blood cell count at diagnosis, M4b-7 FAB classification, cytogenetic abnormalities such as t(9;22) and abnormalities of chromosomes 5 and 7, and first remission durations of <1 year, known predictors of poor response to conventional therapy, also predict poorer transplantation outcome. [116] [124] [125] [126] In addition, transplantation outcome is influenced by factors associated with risk of transplant-related complications, such as age and cytomegalovirus status. Among patients transplanted in first remission, transplantation outcome is not affected by the type or amount of prior consolidation therapy. [127]

There is general agreement that most patients younger than approximately 55 years of age who fail conventional therapy and who have good performance status should be treated with allogeneic SCT if an appropriate related or unrelated donor is available. Although there are no formal comparisons of transplantation versus conventional therapy in this setting, transplantation can salvage 1530% of patients with advanced

Figure 86-7 Actuarial probabilities of leukemia-free survival (LFS) after HLA-identical sibling transplantation for acute myeloid leukemia. CR, complete remission.

Figure 86-8 Actuarial probabilities of leukemia-free survival (LFS) after unrelated donor transplantation for acute myeloid leukemia. CR, complete remission.

disease, including those never achieving remission with conventional therapy. [128] [129] [130] [131] A study comparing two observational databases, one of chemotherapy recipients and the other of patients receiving HLA-identical sibling transplants for AML in second remission, found a significant advantage for transplantation in younger but not in older patients. [129] The use of allogeneic transplantation in first remission is common but still somewhat controversial. Between 20 and 50% of patients achieving first remission have long-term LFS with conventional consolidation therapy. Several randomized and nonrandomized studies indicate significantly higher LFS for patients receiving HLA-identical sibling transplants, although others indicate no advantage. [132] [133] [134] [135] [136] [137] [138] [139] [140] [141] [142] [143] [144] [145] [146] [147] Few demonstrate overall survival advantages because some patients relapsing after conventional therapy achieve long-term survival with a transplant in first relapse or second remission. [129] [130] A strategy of performing HLA-identical sibling transplantation in first remission only for patients with adverse prognostic factors is advocated by some, with transplantation reserved for first relapse or second remission in other patients, but a differential benefit of transplantation by prognostic factors is not proven. On the other hand, there are currently few transplantations done in first remission of M3-AML, presumably because of the good results possible with all-trans retinoic acid. [148] Studies directly comparing unrelated donor transplantation with conventional chemotherapy for AML in first remission are lacking. Most unrelated donor transplantations in first remission are in patients with adverse prognostic factors such as high leukocyte counts, adverse cytogenetic abnormalities, or requiring more than two cycles of induction therapy to achieve remission. Long-term LFS rates between 40 and 60% are reported in young patients. [59] [66] [149] [150] Among children (<16 years of age), there is little difference in reported results of unrelated donor and HLA-identical sibling transplantation.

Myelodysplastic Syndromes

Myelodysplastic syndrome (MDS) is a clonal hematologic disorder with a high likelihood of transformation to acute leukemia. [151] [152] De novo MDS, the more common form, is a disease of older adults, with a median age of onset in the eighth decade of life. Less than 10% of patients are younger than 55 years of age at diagnosis. MDS may also develop after treatment for other cancers, particularly in patients receiving alkylator agents or epipodophyllotoxins. [153] Treatment depends on the clinical syndrome. Patients with cytopenias are usually treated conservatively with transfusions and hematopoietic growth factors; those with transformation may receive standard antileukemia regimens. Response to the latter is poor, with fewer than 60% achieving remissions, and the median remission duration is <12 months. [154] Allogeneic SCT is not extensively used in MDS because most patients are older than the generally accepted upper age limits for allografts. However, long-term disease-free survival, and presumably cure, is possible with allogeneic SCT, with reported 5-year LFS rates ranging from 10 to 60%. [151] [154] [155] [156] [157] [158] [159] [160] [161] [162] [163] [164] [166]

Data from the IBMTR indicate approximately 1,000 allogeneic SCTs for MDS yearly. Approximately 75% of these are from related donors. Thirty percent of HLA-identical sibling transplantations are done in patients with refractory anemia (RA) and 70% in those with refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEBT), or chronic myelomonocytic leukemia (CMML). Corresponding figures for unrelated donor transplantations are 20% and 80%. The median interval from diagnosis to transplantation is 6 months for HLA-identical sibling transplants and 11 months for unrelated donor transplants. The median age of transplant recipients is 36 years. Twenty percent are younger than 20 years of age, and 14% are older than 50 years. This is in striking contrast to the age distribution of all patients with MDS.

The most common conditioning regimen used before transplantation for MDS is busulfan and cyclophosphamide, accounting for approximately half of MDS transplantations reported to the IBMTR in 1997. The need for intensive pretransplantation conditioning was demonstrated by Appelbaum and colleagues, who showed higher risks of graft failure and relapse when transplantations for RA were done with less intensive regimens, similar to those used for aplastic anemia. [162] The relative benefits of TBI versus non-TBI regimens are uncertain, although one retrospective study suggested an advantage for TBI. [163] Post-transplantation cyclosporine and methotrexate is the most frequent GVHD prophylaxis regimen, and is used in approximately 65% of transplantations for MDS.

The importance of graft-mediated immune effects (i.e., GVL) in preventing relapse after allotransplantation for MDS is uncertain. Donor lymphocyte infusions are sometimes effective in reinducing durable remissions after post-transplantation recurrence. [50] [51]

Outcome of allotransplantations for MDS depends in part on FAB classification. Patients with RA or RA with ringed sideroblasts (RARS) have lower recurrence rates and better survival rates than those with RAEB or RAEBT. Among 619 recipients of HLA-identical sibling transplants done between 1990 and 1996 and reported to the IBMTR, 3-year actuarial probabilities of relapse were 8% (313%) for 180 patients with RA or RARS and 39% (3246%) for 439 with RAEB, RAEBT, or CMML (Fig. 86-9). Three-year probabilities of disease-free survival were 58% (5064%) and 38% (3343%), respectively (Fig. 86-10). Salvage therapy

Figure 86-9 Actuarial probabilities of relapse after HLA-identical sibling transplantation for myelodysplastic syndromes. RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEBT, refractory anemia with excess blasts in transformation; CMML, chronic myelomonocytic leukemia.

Figure 86-10 Actuarial probabilities of disease-free survival (DFS) after HLA-identical sibling and unrelated donor transplantation for myelodysplastic syndromes. RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEBT, refractory anemia with excess blasts in transformation; CMML, chronic myelomonocytic leukemia.

infrequently produces durable remissions after post-transplantation relapse. Among 170 unrelated donor transplantations done between 1990 and 1996 and reported to the IBMTR, 3-year disease-free survival rates were 15% (030%) for 30 patients with RA or RARS and 27% (1836%) for 135 with RAEB, RAEBT, or CMML (Fig. 86-10). Survival rates as high as 4050% are reported in some series. [161] Prognosis is better in younger than in older patients and in those transplanted sooner rather than later after diagnosis. Results appear similar whether the MDS is de novo or secondary to prior anticancer treatment, assuming similar morphologic, cytogenetic, and patient characteristics. [164]

Because patients with MDS tend to be older and may have prolonged survival with conservative therapy, especially if presenting only with RA and not requiring transfusions, the role and timing of transplantation is controversial. However, allogeneic SCT offers the only chance for cure and should be considered for young patients with disease features predicting short survival or with evidence of transformation to acute leukemia. These include patients with therapy-related MDS, platelet transfusion dependence, severe neutropenia, excess or increasing percentages of marrow blasts, or clonal cytogenetic abnormalities. There is little evidence that intensive nontransplantation chemotherapy improves the outcome of subsequent allografting.

Acute Lymphoblastic Leukemia

The incidence of ALL is 12/100,000 population. The median age at onset is 11 years. However, the age distribution is bimodal, with peaks in childhood and older adulthood. Approximately 60% of patients are younger than 20 years and 20% are older than 55 years of age at diagnosis. [69] Most patients with ALL, approximately 70% of children and 35% of adults, are cured with conventional chemotherapy. [166] [167] [168] Consequently, transplantation is usually reserved for patients failing conventional therapy (i.e., in relapse or second or subsequent remission) or for patients in first remission with prognostic factors predicting a high risk of failure with conventional therapy. [116] [166] [167] [168] [169] [170] [171] [172] [173] [174] The most frequent indications for transplantation in first remission are older age, high leukocyte count at diagnosis, poor-prognosis chromosome abnormalities such as t(9;22) or t(4;11), extramedullary leukemia, and difficulty obtaining a first remission. Allogeneic SCT is an effective treatment for ALL, with LFS rates 60% in young patients transplanted in first remission. [171] [172] [174] [175]

The international survey cited previously indicated that only 510% of patients diagnosed with ALL in 19891991 received allotransplants. [117] As in AML, there was wide variation among countries, reflecting different strategies for timing of transplantation. IBMTR data indicate approximately 2,500 allogeneic SCTs for ALL yearly. Approximately 70% of these are from related and the rest from unrelated donors. Fewer than 5% of HLA-identical sibling transplantations are done in patients never achieving first remission, 40% as consolidation therapy in first remission, 10% in first relapse, 35% in second remission, 5% in a subsequent relapse, and 10% in a subsequent remission. Few unrelated donor transplantations are done for patients never in remission. Approximately 20% of unrelated donor transplantations are done in first remission, 10% in first relapse, 40% in second remission, 10% in a subsequent relapse, and 20% in a subsequent remission. The median interval from diagnosis to transplantation is 10 months for HLA-identical sibling transplants and 24 months for unrelated donor transplants. The median age of transplant recipients is 18 years; 45% are <16 years of age, 15% are 1619 years, and <5% are >50 years of age. Most (75%) patients receiving transplants in first remission are >16 years of age.

The most common conditioning regimen used before transplantation for ALL is TBI and cyclophosphamide, with or without other drugs, accounting for 60% of ALL transplantations reported to the IBMTR in 1997. Although there is concern about the effect of TBI on growth and development in young children and on the risk of second cancers, some studies suggest that transplant-related mortality is higher and LFS lower with non-TBI regimens, particularly busulfan and cyclophosphamide, in ALL. [176] [177] Other studies show no difference. [178] [179] Post-transplantation cyclosporine and methotrexate is the most frequent GVHD prophylaxis regimen and is used in approximately 60% of transplantations for ALL.

Graft-versus-leukemia effects are important in ALL, although not as striking as in CML and AML. [25] [44] [180] Although GVHD decreases relapse risk, relapse rates are not as markedly increased after identical twin or T-cell-depleted transplantation as they are in CML. [25] [44] Donor lymphocyte infusions are only rarely effective in reinducing durable remissions in patients relapsing after allografts for ALL. [50] [51]

As in other leukemias, LFS after allogeneic SCT for ALL depends on the phase of disease at time of transplantation. Patients in first remission have the best transplantation outcomes. Those with more advanced disease have lower probabilities of LFS because of higher risks of both relapse and transplant-related mortality. Among 2,894 recipients of HLA-identical sibling transplants for ALL done between 1990 and 1996 and reported to the IBMTR, 3-year actuarial probabilities of relapse were 29% (2632%) for 1,213 patients transplanted in first remission, 55% (5258%) for 1,212 in second or subsequent remission, and 66% (6171%) for 469 not in remission (Fig. 86-11).

Figure 86-11 Actuarial probabilities of relapse after HLA-identical sibling transplantation for acute lymphoblastic leukemia. CR, complete remission.

Figure 86-12 Actuarial probabilities of leukemia-free survival (LFS) after HLA-identical sibling transplantation for acute lymphoblastic leukemia. CR, complete remission.

Three-year probabilities of LFS were 53% (5056%), 40% (3743%), and 21% (1725%), respectively (Fig. 86-12). Unrelated donor transplantation has a higher transplant-related mortality rate than HLA-identical sibling transplantation. Among 939 unrelated donor transplantations for ALL done between 1990 and 1996 and reported to the IBMTR, 3-year LFS rates were 42% (3846%) for 215 patients transplanted in first remission, 34% (3236%) for 537 transplanted in second or subsequent remission, and 16% (1318%) for 187 transplanted in relapse (Fig. 86-13). Among patients <20 years of age, available data suggest that LFS is similar with HLA-identical sibling and unrelated donor transplantation. [149] [181]

Several disease-related features such as age at onset, high white blood cell count at diagnosis, non-T-cell phenotype, cytogenetic abnormalities such as t(9;22) and t(4;11), a prolonged time to achieve first remission, and first remission durations of <1 year, all of which predict poor response to conventional therapy, also predict poorer transplantation outcome. [116] [170] [171] [172] [173] [175] Allogeneic transplantation is an effective and accepted treatment for relapsed ALL. A matched-pair analysis comparing 255 children (18 years of age) receiving HLA-identical sibling transplants with 255 children on chemotherapy protocols of the U.S. Pediatric Oncology Group for ALL in second remission, demonstrates a significant advantage for transplantation in this setting. [182] An advantage is seen in children with short and long first remissions. Although formal comparisons of transplantation and chemotherapy are not available in adults relapsing after first remission, transplantation can salvage 1545% transplanted in first relapse or second remission, substantially more than would be expected with conventional therapy. Long-term LFS in patients receiving HLA-identical sibling transplants after

Figure 86-13 Actuarial probabilities of leukemia-free survival (LFS) after unrelated donor transplantation for acute lymphoblastic leukemia. CR, complete remission.

failing to achieve a first remission with conventional therapy is also well documented. [128] [183] The role of transplantation in first remission for patients with high-risk ALL is controversial. Long-term LFS rates of 3040% in patients with Philadelphia chromosome-positive ALL after allogeneic SCT in first remission are reported in several

series, and transplantation in these patients seems appropriate because long-term remissions are unusual with conventional therapy. [184] [185] Some prospective and retrospective comparative studies fail to show a significant LFS advantage for adults receiving HLA-identical sibling transplants for ALL in first remission, with lower relapse rates offset by high transplant-related mortality rates. [186] [187] [188] Two other studies suggest an advantage for some patients, one in patients with high-risk features (i.e., t[9;22], null or undifferentiated phenotype, high leukocyte count, age >30 years at onset, and >4 weeks to achieve first remission), and the other in patients <30 years of age at time of treatment. [189] [190] There are no published comparisons of unrelated donor transplantation with conventional therapy for ALL. A comparison of unrelated donor transplantation with autologous transplantation showed superior LFS with unrelated donor transplantation in some groups of patients, particularly those beyond first remission. [191] Unrelated donor transplantation is usually reserved for patients failing conventional therapy or with poor prognosis cytogenetic abnormalities. The ability to use this approach effectively depends on the efficiency of identifying and collecting cells from unrelated donors because many patients with advanced or high-risk ALL have rapid disease progression. [68]

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a common hematologic malignancy affecting 34/100,000 population in the United States. [69] Like MDS, CLL is primarily a disease of older adults. Median age at onset is 70 years, with approximately 15% of patients <55 years of age at diagnosis. [69] Treatment and prognosis depend on the presenting stage. [192] [193] Many patients have asymptomatic lymphocytosis and require no immediate treatment. Their survival may be similar to that of an age-matched normal population. Those with cytopenias, lymphadenopathy, or splenomegaly have a worse prognosis. Allogeneic SCT can cure some of these patients, although experience with the procedure in CLL is not nearly as extensive as with the other leukemias. [194]

Data from the IBMTR indicate approximately 150 allogeneic SCTs for CLL annually. Eighty-five percent are from related donors. The median interval from diagnosis to transplantation is approximately 40 months. The median age of transplant recipients is 46 years; 25% are older than 50 years of age.

The most common conditioning regimen used before transplantation for CLL is TBI and cyclophosphamide, accounting for 70% of CLL transplantations reported to the IBMTR in 1997. Post-transplantation cyclosporine and methotrexate is the most frequent GVHD prophylaxis regimen and is used in approximately 60% of transplantations for MDS. The importance of graft-mediated immune effects (i.e., GVL) in preventing relapse after allografting for CLL is unknown, although one case report suggests an anti-CLL effect of GVHD. [195]

A review of 54 HLA-identical sibling transplantations for CLL reported to the IBMTR or the EBMTG showed 46% (95% confidence interval, 3260%) survival at 3 years. [196] Transplant-related mortality was high; 46% of patients died of treatment-related causes. Most of these patients had long-standing or advanced disease. Details of prior treatment were not available for all patients, but most had received multiple courses of chemotherapy. The authors concluded that transplantation should be considered for young patients with a poor prognosis receiving conventional chemotherapy. A more recent study suggested that transplant-related mortality may be reduced and survival

increased by prior treatment with fludarabine. [197] Among 138 recipients of HLA-identical sibling transplants between 1990 and 1996 and reported to the IBMTR, 3-year actuarial probabilities of relapse and survival were 20% (1525%) and 41% (3646%), respectively. Causes of death are shown in Table 86-1 .

Because patients with CLL tend to be older and may have prolonged survival with conservative therapy, especially if presenting with stage 0 or 1 disease, the role and timing of transplantation are controversial. Symptomatic patients can achieve good responses and prolonged survival with alkylating agents or fludarabine; however, neither of these approaches is curative. Allogeneic SCT should be considered for younger patients with disease features predicting short survival, that is, patients with Rai stages IIIV disease or those failing to respond to or relapsing after fludarabine.

Non-Hodgkin Lymphoma

The incidence of NHL is approximately 15/100,000 population in the United States. [69] The median age at onset is 65 years, with approximately 35% of patients younger than 55 years of age at diagnosis. [69] Prognosis depends on histologic subtype, age at onset, and other patient- and disease-related features. [198] [199] Approximately 60% of patients achieve complete remission with combination chemotherapy. Patients with low-grade histologic types may have prolonged remissions or survival but rarely are cured with conventional chemotherapy. Approximately 40% of those with intermediate- and high-grade histologic types are cured with intensive chemotherapy, with or without radiation, but most eventually have recurrence and die of their disease. Most hematopoietic stem cell transplantations for NHL use autologous cells collected from the blood or bone marrow. [194] Autografting cures a substantial proportion of patients with high-risk intermediate-grade and immunoblastic lymphoma, but is less effective for low-grade and lymphoblastic lymphomas (see Chap. 87). Only 10% of transplantations for NHL use allogeneic donor cells. Centers usually reserve allogeneic SCT for patients with a high likelihood of relapse after autografting or from whom an adequate autologous graft cannot be obtained. [194] [200] [201] This includes patients with low-grade or lymphoblastic NHL, tumors refractory to conventional chemotherapy, extensive marrow involvement, co-existing myelodysplasia, or poor marrow reserve or prior pelvic irradiation. Some patients relapsing after an autograft for NHL can achieve long-term disease-free survival with allogeneic SCT. [202] IBMTR data indicate approximately 1,000 allogeneic SCTs for NHL are done yearly. Approximately 90% of these are from related donors. Eighty percent of the HLA-identical sibling transplantations and 15% of the unrelated donor transplantations are for low-grade lymphoma. Twenty-five percent of HLA-identical sibling transplantations are done in patients never achieving first remission, 25% as consolidation therapy for high-risk NHL in first remission, 20% in first relapse, 20% in second remission, and 10% for more advanced disease. Thirty percent of unrelated donor transplantations are done for patients never in remission, 10% are done in first remission, 20% in first relapse, 25% in second remission, and 20% for more advanced disease. The median interval from diagnosis to transplantation is 13 months for HLA-identical sibling transplantation and 18 months for unrelated donor transplantation. Median age of transplant recipients is 33 years; 20% are <20 years of age and 5% are >50 years.

The most common conditioning regimen used before allogeneic SCT for NHL is TBI and cyclophosphamide, accounting for 45% of NHL transplantations reported to the IBMTR in 1997. Patients who received local radiation therapy for their lymphoma are often not candidates for TBI. A variety of high-dose chemotherapy regimens are also used, including busulfan and cyclophosphamide and regimens commonly used for autografting, such as cyclophosphamide, carmustine, and etoposide (CBV),

Figure 86-14 Actuarial probabilities of survival after HLA-identical sibling transplantation for low-grade non-Hodgkin lymphoma.

and carmustine, etoposide, cytarabine, and cyclophosphamide (BEAC). Data on relative efficacy of different conditioning regimens are lacking. Post-transplantation cyclosporine and methotrexate is the most frequent GVHD prophylaxis regimen, and is used in approximately 60% of transplantations for NHL.

Graft-versus-lymphoma effects may play a role in preventing relapse after transplantation for NHL. One observation supporting this is the lower relapse rate after allogeneic versus autologous transplantation. [52] [53] [203] It is possible that some of this difference results from reinfusion of lymphoma cells in autologous grafts. [53] However, among allograft recipients, patients with GVHD have lower relapse rates than those without GVHD. [53] Unfortunately, lower relapse rates after allografts do not result in higher survival rates because of offsetting higher rates of transplant-related mortality. As in ALL, donor leukocyte infusions are only occasionally effective in patients with recurrent NHL after an allograft. [51] [204]

Because of the selection practices noted previously, results of allogeneic SCT for NHL must be interpreted with the understanding that these patients represent a group with an extremely poor prognosis with nontransplantation treatment. Allogeneic SCT can produce durable remissions and possibly cure in patients with low-grade lymphoma. [200] [201] [205] [206] Several small studies and one review of over 100 patients suggest long-term disease-free survival in 5060% of patients receiving HLA-identical sibling transplants for low-grade lymphoma, despite the fact that many of the patients in these series had failed prior chemotherapy regimens. Among 189 recipients of HLA-identical sibling transplants for low-grade lymphoma done between 1990 and 1996 and reported to the IBMTR, 3-year actuarial probabilities of survival are 46% (3755%) for 36 patients transplanted in remission and 43% (3848%) for 153 patients not in remission (Fig. 86-14). Among 220 recipients of HLA-identical sibling transplants for intermediate and immunoblastic lymphoma, 3-year survival rates are 38% (3046%) for 43 patients in remission at time of transplantation and 29% (2533%) for 177 not in remission (Fig. 86-15). Causes of death are shown in Table 86-1 . Factors associated with better prognosis include low-grade histologic type, chemotherapy-sensitive disease, and absence of B symptoms. [201]

Which patients with NHL might best benefit from allogeneic SCT versus autografting or chemotherapy is uncertain. Good candidates might be young patients with low-grade or lymphoblastic lymphoma. Patients with advanced refractory disease are unlikely to benefit.

Hodgkin Disease

The incidence of Hodgkin disease is 23/100,00 in the United States; the median age at onset is 35 years, and 75% of patients are <55 years of age at diagnosis. [69] Most patients are cured with

Figure 86-15 Actuarial probabilities of survival after HLA-identical sibling transplantation for intermediate- and high-grade non-Hodgkin lymphoma.

conventional chemotherapy or radiation therapy. However, for the 2030% who fail conventional therapy, hematopoietic stem cell transplantation is effective salvage therapy. Autografts are commonly used in patients with advanced Hodgkin disease. [194] Five-year disease-free survival rates are 4070% for patients transplanted in remission and 1020% for those with advanced disease (see Chap. 87). This contrasts with survival rates of <550% with salvage chemotherapy. [207] [208] [209] [210] In both Hodgkin disease and NHL, there are several possible advantages of allogeneic SCT in advanced or recurrent disease: (1) advanced donor hematopoietic cells have not been exposed to chemotherapy, (2) there is no risk of lymphoma cells in the graft, and (3) there may be a graft-versus-lymphoma effect. However, relatively few allogeneic SCTs for Hodgkin disease are done, approximately 150 yearly. Ninety percent are from related donors. As in NHL, most are in extensively treated, poor-risk patients.[211] The median interval between diagnosis and transplantation is 30 months.

A recent study from the IBMTR examining outcome of 100 HLA-identical sibling transplantations for Hodgkin disease done between 1982 and 1992, showed high transplant-related mortality rates (61%) in large part due to a high incidence of pulmonary complications. [211] Most received their transplants after extensive prior nontransplantation treatment. The 3-year disease-free survival rate was 15%. A matched-pair analysis of autologous and allogeneic SCT for Hodgkin disease showed fewer relapses but more transplant-related deaths with allografts; survival was similar. [212] Among 108 HLA-identical sibling transplantations for Hodgkin disease done between 1990 and 1996 and reported to the IBMTR, the 3-year probability of disease-free survival is 16 ± 7% (Fig. 86-16).

Figure 86-16 Actuarial probabilities of survival after HLA-identical sibling transplantation for Hodgkin disease.

Multiple Myeloma

The incidence of multiple myeloma is approximately 4/100,000 in the United States. [69] It is a disease of older adults, with a median age at onset of approximately 70 years.[69] Only 15% of patients are younger than 55 years of age at diagnosis and therefore candidates for allogeneic SCT. The most common treatment strategy for multiple myeloma is melphalan and prednisone; response is common but complete remissions unusual. [213] The median survival is approximately 3 years. Most hematopoietic stem cell transplants for multiple myeloma use autologous cells; a recent prospective, randomized trial demonstrated prolonged survival with this approach compared with standard chemotherapy.[214] However, complete remissions after autografting are unusual and cures, if any, are rare. Some studies using polymerase chain reaction (PCR) techniques to ascertain presence of minimal residual disease suggest that although myeloma cells may persist for months or years after allogeneic SCT, at least some patients eventually become PCR negative and remain so for years. [215] Long-term (>4 years) disease-free survival is estimated to occur in 1040% of allograft recipients, although few patients have been followed this long posttransplantation. [216] [217] [218] [219] [220]

Relatively few allogeneic SCTs for multiple myeloma have been performed. IBMTR data indicate approximately 500 allogeneic SCTs for myeloma in 1997. More than 90% of these are from related and the rest from unrelated donors. Most patients who receive allotransplants for myeloma have advanced disease and have failed multiple prior chemotherapy regimens.[216] [217] [218] [219] [220] According to IBMTR data, the median interval from diagnosis to transplantation is 13 months for HLA-identical sibling transplantation and 16 months for unrelated donor transplantation. Median age of transplant recipients is 45 years; 20% are >50 years.

Transplantation regimens used for multiple myeloma are heterogeneous. Approximately 50% of patients receive TBI. The relative benefits of various conditioning regimens are unknown, although reported results do not differ dramatically with different regimens. Post-transplantation cyclosporine and methotrexate is the most frequent GVHD prophylaxis regimen, and is used in approximately 65% of transplantations for multiple myeloma.

The importance of graft-versus-myeloma effects in preventing relapse after allografting for multiple myeloma is uncertain. No decreased relapse rates in association with GVHD or increased relapse rates with T-cell-depleted transplants are reported. However, relapse rates after allografting are lower than after autografting, and there are several reports of donor lymphocyte infusions successfully inducing remissions after post-transplantation relapse. [55] [56] [57] [58]

Between 40 and 70% of patients receiving allografts for multiple myeloma achieve a clinical complete remission. [216] [217] [218] [219] [220] Many of these patients relapse. Transplant-related mortality is high, between 20 and 45%. The 3-year probability of survival after 674 HLA-identical sibling transplantations done between 1991 and 1996 and reported to the IBMTR is 37% (3539%). The 3-year survival rate after 39 unrelated donor transplantations for multiple myeloma is 24% (1632%). Factors associated with better transplantation outcomes are stage 1 disease at diagnosis, no more than one course of previous treatment, lower-stage disease at transplantation, low $_2$ -microglobulin levels, IgG rather than IgA or Bence-Jones myeloma, shorter time from diagnosis to transplantation, and female sex. [218] [219] [220] Not surprisingly, patients who achieve a complete remission after transplantation have a higher probability of long-term survival. Few patients have received unrelated donor transplants for multiple myeloma. There are some long-term survivors, but the role of alternative donor transplantation is uncertain.

The relative benefit of autologous and allogeneic SCTs for

multiple myeloma was studied in a matched-pair fashion by several investigators. Although relapse rates after allografts are lower than after autografts, they are offset by high transplant-related mortality. Long-term survival rates are similar. [58] [221] High-dose therapy with stem cell rescue should be considered for most young patients with myeloma. The choice of allogeneic versus autologous SCT depends on evaluation of individual risk factors for disease progression versus transplant-related toxicity, availability of a suitable donor, and development of allograft approaches with reduced toxicity.

Other Cancers

Allogeneic SCT has been used in patients with various other cancers, each accounting for <0.5% of cases reported to the IBMTR yearly. These include neuroblastoma, soft tissue and bone sarcomas, melanoma, and, recently, breast cancer. Although successful outcomes are reported in some patients, the role of allogeneic SCT in these diseases is difficult to assess because of the small number of patients treated. Extrapolating from experience with the leukemias, it may be rational to apply allogeneic SCT to diseases sensitive to chemotherapy but unlikely to be cured with chemotherapy in patients at low risk of transplant-related mortality.

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LATE EFFECTS OF ALLOGENEIC STEM CELL TRANSPLANTATION

There are now over 20,000 people surviving 5 or more years after allogeneic SCT, and that number will grow rapidly. Most of these survivors lead normal lives, but allograft recipients remain at risk for late complications long after transplantation. [222] [223] These include late infections, abnormalities of growth and development, infertility and sexual dysfunction, thyroid disorders, chronic lung disease, cataracts, and avascular necrosis. Some of these are related to high-dose preparative regimens, some to immunosuppressive effects of GVHD and its treatment, and some to prior nontransplantation treatment. There is also an increased incidence of lymphoproliferative disorders and solid tumors in transplant recipients compared with the general population. [224] [225] [226] [227] These second cancers may not all be due to the transplant per se but to the chemotherapy or radiation preceding transplantation. Regardless, life-long surveillance is necessary, as is increased awareness of late complications among the many nontransplant physicians who will care for these patients.

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Chapter 87 - Results of Autologous Stem Cell Transplantation for Hematologic Malignancies

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Since the late 1960s, combination chemotherapy regimens have been developed for treatment of most hematologic malignancies. These regimens use agents with relatively nonoverlapping toxicities and are based on the concept of superior disease control with combinations of single agents to decrease the emergence of drug resistance.[1] These chemotherapy regimens were designed to maximize objective clinical responses while minimizing toxicity. Conventional-dose chemotherapy regimens vary based on the tumor being treated.

Although conventional-dose chemotherapy produces frequent responses in patients with a wide variety of hematologic malignancies, for many patients these chemotherapy regimens produce responses that last only for short intervals, with relapse ensuing, ultimately leading to the death of the patient. For these reasons, the development and evaluation of new treatment strategies have focused on intensification of dose as a means of overcoming resistance to chemotherapy. In laboratory models of a variety of tumors, delivery of the highest possible doses of chemotherapy is a requirement for achieving long-term control. [2] These studies also indicate that resistance to a variety of cytotoxic agents can be overcome with a 5- to 10-fold escalation in the dose of drugs used to treat the malignancy.

Delivery of dose-intensive therapy is facilitated by the harvest of either marrow or peripheral blood stem cells to provide autologous hematologic stem cell support after delivery of high-dose chemotherapy or radiation and chemotherapy (see Chap. 92). Autologous hematopoietic stem cell support or autologous stem cell transplantation (SCT) shortens the duration of hematologic toxicity as a limiting step in the chemotherapy or radiation dose intensification and makes other organ toxicities the major limitation to increasing the dose. Table 87-1 shows the list of chemotherapy drugs commonly used in autologous SCT regimens and their dose-limiting organ toxicity.

TABLE 87-1 -- Agents Used in Autologous Transplant Regimens and Their Dose-Limiting Toxicity

Agent	Toxicities
Etoposide	Mucositis, skin
Cyclophosphamide	Cardiac failure, pulmonary failure, hemorrhagic cystitis
Cisplatinum	Renal failure, hearing loss
Carmustine	Interstitial pneumonitis, seizures
Total-body irradiation	Interstitial pneumonitis, enteritis
Melphalan	Mucositis
Busulfan	Veno-occlusive disease of liver, interstitial pneumonitis, seizures

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RATIONALE FOR HIGH-DOSE THERAPY

Hryniuk and Bush introduced the concept of dose intensity (drug dose administered in mg/m ^{2} /week) for the purposes of being able to quantify the dose response effect.[3] The dose response effect is defined as the correlation of the effect of a drug with increasing doses. It was their hypothesis that dose intensity correlated with response, which in turn correlated with survival. Several assumptions are inherent in this hypothesis and include:

1. All drugs in a given regimen are therapeutically equivalent.
2. Synergy and cross-resistance between drugs do not play an active role.
3. The area under the curve is more important than peak drug concentrations.
4. Scheduling has no importance other than what is reflected in total dose intensity.

This suggests that chemotherapy regimens should be designed to maximize dose intensity and focus on the overall dose administered over time rather than achieving the highest peak dose. Laboratory models and clinical observations have demonstrated a dose-response relationship against a variety of tumors, leading to the concept that the highest possible doses should be incorporated into the design of the regimen. [4] [5] Where possible, combinations of active, non-cross-resistant agents should be used to decrease the emergence of drug resistance. This latter consideration is based on studies by Skipper that estimate a spontaneous rate of mutation conferring resistance to a single drug to be on the order of 1 in 10 ^{6} or 1 in 10^7 cancer cells.[2] The implication of this is that spontaneous mutations are highest in patients at diagnosis or at relapse (high tumor burden). The agents used in the regimen need to have different dose-limiting toxicities to avoid dose reduction and prevent additive toxicity. This would facilitate the delivery of full, single-dose therapies of the drug. To minimize the rapid emergence of resistance, combinations of drugs in high-dose regimens with different mechanisms of action would limit the emergence of cross-resistance.

Table 87-2 lists the malignant hematologic diseases that have been treated using high-dose chemotherapy or chemoradiation therapy and autologous SCT. Unlike allogeneic SCT, where the preparatory regimen provides both cytoreduction to reduce or eliminate the tumor burden and immunosuppression to facilitate engraftment, the regimen used for autologous SCT is designed to provide maximal antitumor activity limited only by the nonhematologic toxicity of the regimen.

CONTRIBUTION OF THE GRAFT TO RELAPSE

The major cause of relapse in patients undergoing autologous SCT for hematologic malignancy is persistent disease in the host. Even under the best of graft conditions, namely, a syngeneic (identical twin) graft, the cure rate is not 100%, and emphasizes

AUTOLOGOUS TRANSPLANTATION THERAPY FOR THE HEMATOLOGIC MALIGNANCIES

Autologous stem cell transplantation therapy for the hematologic malignancies offers several advantages over donor transplantation for the same disorders. Identification of a willing, human leukocyte antigen-matched donor is unnecessary. Graft-versus-host disease and its attendant problems of organ damage, immunosuppression, and infection do not occur. Consequently, hospitalization and convalescence are shortened and transplant-related mortality is diminished. On the other hand, the autologous stem cell inoculum may be contaminated with clonogenic malignant cells capable of engraftment and shown by several molecular marker studies sometimes to be responsible for persistent or recurrent disease. The autologous graft also lacks effector cells capable of mounting an allogeneic graft-versus-tumor response. These allogeneic effector cells engraft and circulate in the recipient after transplantation, suppressing emergence of malignant hematopoietic clones of recipient origin that have survived the preparative regimen. The graft-versus-tumor effect is most notable in donor transplantation therapy for chronic myeloid leukemia, but has been observed in acute nonlymphocytic leukemia (ANLL) and acute lymphocytic leukemia, and may occur in multiple myeloma and the lymphomas.

Nevertheless, autologous stem cell transplantation can be a powerful antitumor therapy, yielding up to 50% long-term, disease-free survival rates in selected populations such as patients with first-remission ANLL and patients coming to autologous transplantation with therapy-responsive lymphomas. Major reduction of tumor burden accomplished by autologous stem cell transplantation may also set the stage for additional innovative antitumor therapy, such as transplantation of stem cells transduced with chemotherapy-resistant genes, or use of interleukin-2-stimulated effector cells in the post-transplantation interval.

the contribution of the body burden of disease to relapse. [6] However, the high likelihood of reinfusing tumor cells, even from a remission marrow of a hematologically based tumor, has led to the development of methods designed to eradicate tumor cells from the autologous graft with the goal of achieving clinical results comparable with syngeneic transplantation. Intuitively, it would seem that all sources of autologous stem cells from patients with hematologic malignancy might contain cells that contribute to relapse, and studies of stem cell products have documented the presence of tumor cells in the grafts, including leukemia, myeloma, lymphoma, and chronic myeloid leukemia (CML).[7] [8] [9] [10] The success of autologous SCT for hematologic malignancy depends in part on whether any clonogenic cells remaining

TABLE 87-2 -- Autologous Transplantation for Hematologic Malignancies

Acute myelogenous leukemia: first and subsequent remission
Lymphoma: intermediate, high and low grade; high risk, relapse
Chronic lymphocytic leukemia
Hodgkin disease: induction failure, second remission and relapse
Multiple myeloma: chemosensitive, responsive disease
Chronic myeloid leukemia: chronic phase
Acute lymphoblastic leukemia: high-risk first remission, second remission

in the reinfused stem cell product can cause relapse. Although laboratory studies show the efficacy of purging to reduce tumor cell contamination, the benefit of purging of tumor cells in human transplant studies has remained controversial. Although retrospective analyses give some indication as to the value of purging, no prospective, randomized trials addressing the efficacy of ex vivo purging have been reported. [11]

Most trials have tested either chemotherapeutic agents or monoclonal antibodies against differentiation antigens on B-hematopoietic tumor cells as purging methods. These analyses suggest that purging reduces the number of clonogenic cells but often delays engraftment. [12] [13] The most direct evidence of the contribution of the infusion of autologous tumor cells to relapse resulted from an approach using gene therapy. Brenner et al. incubated a third of harvested marrow mononuclear cells from two children undergoing autologous stem cell transplantation for acute myeloid leukemia (AML) with the L and L-6 retroviral vector. [14] This vector contained the selectable neomycin resistance gene that marked approximately 5% of cultured progenitor cells. Patients underwent transplantation with infusion of both marked and unmarked marrow cells. At the time of the relapse, some of the leukemia cells contained the neomycin-resistant gene, demonstrating that these leukemia cells were present in the reinfused graft and could be detected at relapse. This has also been observed in patients with CML undergoing autologous transplantation. [15] Studies in patients with lymphoma have correlated the ability to deplete contaminating malignant lymphoma cells from the graft with overall disease-free survival after transplantation.[16] Thus, tumor cell contamination of the stem cell graft can, under some circumstances, be the cause of treatment failure in patients undergoing autologous stem cell transplantation for hematologic malignancy.

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ACUTE MYELOID LEUKEMIA

The first successful case of autologous SCT for AML was reported in 1977 for a patient with relapsed AML with a chloroma. [17] Initially, autologous transplantation applied the same clinical approach previously used for patients undergoing allogeneic transplantation. Patients with advanced disease were treated with high-dose chemotherapy and then received infusion of their own marrow, usually collected during remission instead of marrow from a human leukocyte antigen (HLA)-identical sibling. Although this approach produced a high remission rate, there were few, if any, cures. Similar to the strategy with allogeneic transplantation, autologous transplantation was used earlier in the clinical course of the disease to enable high-dose consolidation therapy for patients who were in complete remission, usually in patients who lacked a histocompatible sibling donor or were of older age. The high-dose chemoradiation therapy regimens that have been used for autologous transplantation for AML are generally derived from those used for allogeneic transplantation. The most common regimens used for the treatment of AML include total-body irradiation (TBI) and cyclophosphamide, TBI with etoposide and cyclophosphamide, and busulfan and cyclophosphamide. [18] [19] [20] [21] [22]

Studies using unpurged marrow, purged marrow, and peripheral blood as a source of hematopoietic stem cells have reported disease-free survival rates for patients with AML transplanted in first remission of 3470%, 4176%, and 3580%, respectively. [23] Although each trial demonstrates the efficacy of autologous transplantation, they have been criticized for including patients with varying subtypes of disease (e.g., cytogenetics, French-American-British [FAB] classification type, white blood cells [WBC] at diagnosis) who had received widely varying induction therapies and types and numbers of consolidation cycles before hematopoietic cell transplantation, and had different durations of complete remission (CR) before transplantation. In addition, there are differences in the stem cell product manipulation and in preparative regimens. Nevertheless, numerous phase II and phase III autologous transplantation trials for AML have shown autologous SCT to be a useful alternative for patients who lack a sibling donor or are of older age. Most trials show a similar outcome for autologous SCT compared with allogeneic SCT, and a higher cure rate compared with chemotherapy. [24] The most common cause of treatment failure after autologous SCT for AML is relapse. Risk features for relapse in first remission include cytogenetic subtype, elevated WBC at diagnosis, and number of chemotherapy cycles required to achieve a first remission. [23] Figure 87-1 (Figure Not Available) shows a survival curve from a randomized trial comparing allogeneic and autologous SCT to standard chemotherapy, demonstrating the relative efficacy of autologous SCT in the treatment of this disease.

The role of autologous transplantation in second remission is better defined than in first remission because the cure rate with conventional therapy for such patients is very low. Under these circumstances, most patients have undergone transplantation using either chemically or immunologically purged marrow cells. Trials using marrow purged with 4-hydroperoxycyclophosphamide, maphosphamide, or monoclonal antibody have demonstrated a disease-free survival rate of 1956% and a relapse rate of 2575%. [12] [25] A study comparing autologous transplantation with unrelated donor transplantation for advanced leukemia has been conducted. [26] The relapse rates were lower with unrelated donor transplantation; however, the disease-free survival rate was not significantly different between the two types of transplant. These studies indicate that autologous transplantation is a reasonable alternative for patients who do not have a suitable donor and for older patients who achieve a second remission and may not be candidates for allogeneic transplantation.

As described in Chapter 54 , patients with newly diagnosed AML have significantly higher chances of long-term disease-free survival than two decades ago, largely because of better induction chemotherapy regimens, supportive care, and the selective use of allogeneic transplantation. Studies with autologous transplantation indicate that this option is a reasonable

Figure 87-1 (Figure Not Available) Kaplan-Meier estimates of disease-free survival for patients in first remission of acute myeloid leukemia assigned to allogeneic bone marrow transplantation (BMT; dotted line, n = 168), autologous BMT (dashed line, n = 128), or intensive consolidation chemotherapy (solid line, n = 126). Percentages represent estimated disease-free survival rates ± standard error. *(Reprinted with permission from Zittoun RA, Mandelli F, Willemze R et al: Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. European Organization for Research and Treatment of Cancer [EORTC] and the Gruppo Italiano Malattie Ematologiche Maligne dellAdulto [GIMEMA] Leukemia Cooperative Groups. N Engl J Med 332:217, 1995. Copyright © 1995 Massachusetts Medical Society. All rights reserved.)*

Figure 87-2 Survival in patients with Hodgkin disease with refractory disease or in first relapse treated with either high-dose therapy and autografting (n = 60, solid line) or conventional salvage therapy (n = 103, dashed line). **(A)** Overall survival. Log-rank *P* = 0.25. **(B)** Event-free survival. Log-rank *P* < 0.01. *(Reprinted with permission from Yuen AR, Rosenberg SA, Hoppe RT et al.* [28] *)*

therapeutic choice, particularly for those patients who have poor-prognosis cytogenetic abnormalities in whom chemotherapy is unlikely to have a major impact on disease-free survival. Importantly, knowledge of the molecular biologic characteristics of the patients leukemia can be used to determine the appropriate therapy and whether the transplantation should be performed during first or subsequent remission. For those patients who do not undergo a transplantation in first remission and suffer a relapse, the opportunity to undergo transplantation requires that stem cells were collected during first remission or that a second remission is achieved and stem cells can be procured.

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HODGKIN DISEASE

High-dose therapy followed by autologous SCT can produce long-term disease-free survival in selected patients with advanced refractory Hodgkin disease. [27] Reports from a number of single institutions and cooperative group trials indicate that 3065% of selected patients with Hodgkin disease can become long-term disease-free survivors when treated with autologous transplantation. [27] The primary indications for transplantation have been patients who had a relapse or failed to achieve a remission after treatment with multiagent chemotherapy. This includes patients who never achieved a CR with chemotherapy or were in untreated first relapse, sensitive relapse, or later CR. Therefore, most reports contain a heterogeneous group of patients with variation in the extent of disease at presentation, duration of response to therapy, use of reinduction therapy, prior use of radiation therapy, and the presence of B-cell symptoms.

Although several trials have examined the role of autologous transplantation in patients who failed to achieve a remission or had a relapse after chemotherapy, there have not been good prospective trials directly comparing salvage second-line therapy to chemotherapy followed by high-dose chemoradiation therapy and autologous transplantation. Recent investigations have compared a group of patients who received salvage chemotherapy and were matched for disease and treatment characteristics to a group of patients receiving an autologous transplant. Patients were matched for risk factors, including initial advanced disease, age, failure to achieve a CR with initial chemotherapy, and first relapse after chemotherapy-induced remission. [28] Overall survival, event-free survival, and freedom from progression at 4 years favored patients who received an autologous transplant compared with conventional salvage therapy (Fig. 87-2). This study, along with others, has identified numerous prognostic factors that influence the cure rate after transplantation [29] [30] [31] [32] [33] [34] (Table 87-3).

A recent international effort analyzing a large number of patients with Hodgkin disease suggested that there are disease characteristics that predict a high likelihood of relapse after conventional therapy. Several factors were identified, each of which reduced tumor control by 78%. [35] Among these poor prognostic factors at diagnosis are anemia (hemoglobin <10.5 g/dl), stage IV disease, male sex, age >45 years, albumin <4 mg/dl, WBC >15,000/l, and lymphocyte count <600/l or <8%. Patients with four or more of these features have a poor prognosis and

TABLE 87-3 -- Prognostic Factors for Transplantation in Hodgkin Disease

Program	Regimen	Event-Free Survival (%)	Prognostic Factors
Nebraska/M.D. Anderson	CBV	25	Performance status, failure of prior regimens
University College (London)	BEAM	50	Large mediastinal mass, female sex, failure of multiple chemotherapy regimens, induction failure
Washington/St. Louis, Western Reserve, Cleveland Clinic, Duke	CY/TBI	27	Performance status, disease duration
Vancouver	CBV ± cisplatin	64	Disease duration, stage IV disease at diagnosis, B-cell symptoms at relapse
City of Hope	CBV, FTBI/VP/CY	52	No. of prior regimens, extranodal disease at time of BMT
Stanford	CBV, FTBI/VP/CY	48	Systemic symptoms at relapse, stage IV disease at relapse (lungs, bone marrow), bulk of disease at time of BMT

BEAM, BCNU, etoposide (VP-16), ara-C (cytosine arabinoside), melphalan; BMT, bone marrow transplantation; CBV, carmustine, cyclophosphamide, and etoposide; CY, cyclophosphamide; FTBI, fractionated total-body irradiation; VP, VP-16 (etoposide).

could benefit from early autologous transplantation. Pilot trials for the use of high-dose chemotherapy and transplantation as part of the initial management of Hodgkin disease have been conducted with very encouraging results. [36] Randomized trials comparing early versus late transplantation are being developed to answer this question in this group of patients at high risk for relapse.

The preparative regimens used in autologous transplantation for Hodgkin disease have focused on combination chemotherapy, most commonly carmustine, cyclophosphamide, and etoposide (CBV). [29] [30] [31] [32] [33] [34] Augmented CBV regimens have been shown to improve long-term disease control in phase II trials. Given its known effectiveness in the treatment of Hodgkin disease, some programs have also used TBI or total lymphoid irradiation. [33] However, many patients who require transplantation have had prior mediastinal mantle radiation, thus precluding the use of TBI as a component of the treatment regimen. The combination of TBI and prior mantle radiation increases prohibitively the risk of cardiac and pulmonary toxicity. In some cases, irradiation directed to site of bulky or refractory disease can be used after transplantation.

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NON-HODGKIN LYMPHOMA

Like other tumors, the basis for high-dose chemotherapy and autologous stem cell transplantation in non-Hodgkin lymphoma (NHL) is that resistance to standard therapy can be overcome by escalation of doses of drugs or radiation beyond limiting marrow toxicity. The initial reports of successful incorporation of high-dose therapy with autologous transplantation were from the National Cancer Institute, where BACT (carmustine, cytarabine, cyclophosphamide, and thioguanine) chemotherapy followed by autologous SCT eradicated lymphoma in some patients with high-grade lymphoma previously considered incurable. [37] These observations led to the development of several trials, including a randomized trial documenting the efficacy of autologous transplantation in patients with lymphoma.

Several investigations have reported a cure rate of 3540% in patients with chemotherapy-sensitive, diffuse, aggressive non-Hodgkin lymphoma with high-dose therapy or autologous transplantation. [38] These studies were received with great enthusiasm because, under ordinary circumstances, <10% of patients with relapsed lymphoma can be cured with conventional salvage therapy (see Chap. 70). Eventually, an international randomized trial demonstrated that second-line chemotherapy with cisplatin, cytarabine, and decadron (DHAP) followed by consolidation with high-dose chemotherapy with carmustine, etoposide, cytarabine, and cyclophosphamide (BEAC) and autologous transplantation was more effective than continued conventional chemotherapy alone. [39] The 5-year event-free survival rate was 46% for transplantation patients versus 12% for chemotherapy-only patients (Fig. 87-3). This study was limited to patients younger than 60 years of age who had achieved a CR after primary therapy, had no known marrow or central nervous system involvement, and had responded to second-line therapy.

The issue of chemosensitivity appears to play an important part in the overall long-term disease-free survival of patients with lymphoma who undergo autologous transplantation. Patients refractory to initial therapy often responded to transplantation but had a relapse relatively quickly. [40] In addition, those patients with lymphoma who had responded to primary treatment but proved resistant to second-line treatment also had a relatively low response rate. In contrast, patients responding to conventional therapy did well after subsequent autologous transplantation. Figure 87-4 (Figure Not Available) shows the long-term disease-free survival rates for patients with advanced lymphoma depending on their remission status and sensitivity to chemotherapy.

Some investigators have attempted to identify patients who

Figure 87-3 Event-free survival in 109 patients with recurrent, chemotherapy-sensitive non-Hodgkin lymphoma randomized to high-dose therapy and autologous transplantation (n = 55) or conventional treatment (n = 54). Tick marks represent censored data. *(Reprinted with permission from Philip T, Guglielmi C, Hagenbeek A et al: Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkins lymphoma. N Engl J Med 330:1540, 1995. Copyright © 1995 Massachusetts Medical Society. All rights reserved.)*

had poor prognostic features at diagnosis and who were at high risk for relapse even with achievement of a CR. These phase II studies showed that early transplantation while in first remission resulted in high rates of disease-free survival in patients who would be expected to have had a greater chance of relapse with standard therapy[41] (Fig. 87-5). A large phase III trial comparing chemotherapy with chemotherapy followed by transplantation in patients with intermediate high-grade lymphoma who had one or more unfavorable prognostic features did not show a significant difference in event-free overall survival rates in the study arms.[42] However, an analysis limited to these poor-risk subsets demonstrated significant benefit in disease-free survival for patients with high-dose therapy and autologous transplantation.[43] The 5-year disease-free survival rate after autologous transplantation was 59% and superior to that with chemotherapy (39%; $P = 0.01$) in the poor-risk patient subsets.

Those patients who achieve only a partial remission with primary chemotherapy are rarely cured by further chemotherapy. Dose escalation theoretically may result in a durable response in drug-sensitive subjects and has been shown to be an effective approach. Therefore, patients who achieve only a partial

Figure 87-4 (Figure Not Available) Progression-free survival (PFS) of all adult patients with non-Hodgkin lymphoma after high-dose chemotherapy by status at time of high-dose chemotherapy. CR, complete remission; PR, partial remission. *(Reprinted with permission from High-Dose Therapy for the Treatment of Non-Hodgkins Lymphoma. In Armitage JO, Antman KR (eds): High-Dose Cancer Therapy, 2nd ed, Ch. 47, p. 763. Philadelphia, Lippincott-Williams & Wilkins, 1992.)*

Figure 87-5 Kaplan-Meier product limit estimate of the cumulative probability of disease-free survival and relapse for 52 patients with poor-risk, intermediate- and high-grade lymphoma who underwent high-dose chemotherapy and autologous stem cell transplantation while in first complete or partial remission at City of Hope National Medical Center. The black boxes indicate censored data points. *(Reprinted with permission from Nademanee A, Molina A, ODonnell MR et al.[41])*

remission but are sensitive to chemotherapy are appropriate candidates for transplantation before tumor regrowth.

A number of phase II trials have been conducted to determine the curative potential of high-dose therapy and autologous transplantation in patients with low-grade lymphomas.[44] [45] [46] [47] Even if the approach was noncurative, the prolongation of remission itself would be a worthwhile goal for this group of patients. Similar to diffuse, aggressive, high-grade lymphoma, selection of appropriate patients is crucial for the success of high-dose therapy and autologous transplantation in low-grade lymphoma. The long natural history of patients with low-grade lymphoma makes it difficult to determine if a proportion of malignant patients are cured or had their survival prolonged by high-dose therapy and autologous transplantation. Figure 87-6 illustrates what is considered to be the best available historical comparison for patients with recurrent low-grade lymphoma. This analysis from St. Bartholomews Hospital in London

Figure 87-6 Freedom from progression in 41 patients with follicular lymphoma in second remission treated with cyclophosphamide and total-body irradiation plus autologous bone marrow transplantation (ABMT) compared with 34 historical control patients treated with chemotherapy at St. Bartholomews Hospital. *(Reprinted with permission from Rohatiner AZ, Johnson PW, Price CG et al.[44])*

showed that freedom from recurrence and survival rates for patients treated with cyclophosphamide and fractionated TBI plus purged autologous marrow cells were improved compared with chemotherapy control patients matched for remission status. Although there was a statistically significant advantage in disease control with a high-dose approach, this did not translate into a survival benefit. [47]

Similar to large cell lymphoma, several groups have attempted to identify patients with low-grade lymphoma with poor-risk features who should undergo

transplantation during first remission. Disease-free survival rates in these studies were extended compared with remission durations expected with chemotherapy, and overall survival rates to date have been excellent. [48] [49] Based on available data, patients with low-grade lymphoma with brief initial remissions or in complete second remissions with standard treatment who represent groups of patients with very short survivals after relapse are the most appropriate candidates for high-dose therapy and autologous transplantation. In addition, those patients who present with poor prognostic features, similar to the criteria used for intermediate-grade lymphoma, might also be suitable candidates for clinical trials.

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OTHER TYPES OF LYMPHOMA

Autologous stem cell transplantation has been used for patients with various other subtypes of poor-prognosis lymphoma, including lymphoblastic lymphoma, Burkitt lymphoma, and mantle cell lymphoma.[38] The success of transplantation with these histologic subtypes is related to the extent of disease at the time of diagnosis and the response to conventional therapy.

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CHRONIC LYMPHOCYTIC LEUKEMIA

Although a large number of patients have undergone autologous transplantation for acute leukemia, CML, myeloma, lymphoma, and Hodgkin disease, there have been relatively few studies addressing the issue of autologous transplantation in chronic lymphocytic leukemia. [50] [51] [52] This subject has been limited by the older age of the population and the higher degree of contamination in the blood and marrow needed to procure stem cells for support of high-dose therapy. Therefore, patients undergoing transplantation represent a highly select group, namely, those patients who had a very good response to therapy and for whom stem cells could be collected. Although the complete response rate was high, the overall impact on disease-free survival is difficult to ascertain. Nevertheless, the studies suggest that it is feasible to perform the procedure in selected patients. The most important issue to pursuing autologous transplantation for chronic lymphocytic leukemia would be better induction therapies that increase the CR or near-CR rate, similar to what has been seen in other hematologic malignancies.

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CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia is a malignancy of the hematopoietic stem cell that is caused by the Philadelphia chromosome, resulting from a translocation between the *c-bcr* gene located on chromosome 22 and the *c-abl* gene located on chromosome 9 (see Chap. 62). The resulting p210 oncoprotein is sufficient for malignant transformation of hematopoietic stem cells.[53] CML is invariably lethal and, with the exception of allogeneic transplantation, there has been no curative approach developed for this disease. Conventional therapy has been focused on the use of agents to control blood counts and reduce spleen size, improving symptoms and quality of life.[54] Recently, therapy with interferon- alone or in combination with cytosine arabinoside induced hematologic remissions in up to 80% of patients. [55] [56] Approximately 30% of patients treated with interferon- achieve a complete or major cytogenetic response. [55] [56] These

cytogenetic responses are associated with superior survival rates. [55] [56] Studies also indicate that acquisition of the hematopoietic response without a concomitant major cytogenetic response does not, in and of itself, significantly affect survival. Therefore, the ability to achieve partial or complete reconstitution of normal hematopoiesis is a requirement for improved disease-free survival in this disease.

There is considerable evidence that normal, Philadelphia chromosome-negative (Ph) stem cells coexist in the marrow of patients with CML. [57] In some patients at presentation, both a Ph+ and Ph population can be detected. [57] Patients who have had higher doses of busulfan or induction chemotherapy regenerate with Ph hematopoiesis, albeit transiently. [58] [59] [60] These clinical observations have also been corroborated by a number of in vitro studies showing that Ph progenitors coexist with Ph+ cells, and long-term culture accentuates this kinetic difference (see Chap. 70).

A large number of studies have focused on approaches to enrich the benign progenitor population by negative or positive selection. The relative difference in the compartment size between the diseased cells and normal cells has made this difficult and has limited the success of autografting patients with CML. Initial transplantations were performed in blast crisis using marrow that was cryopreserved during the chronic phase. The re-establishment of the chronic phase after transplantation was usually short lived, with recurrence of blast crisis. [61] Subsequently, other investigators, using unmanipulated autologous peripheral blood stem cells in patients with CML, showed that major cytogenetic responses could be achieved and that the actuarial survival rates were higher than expected with conventional chemotherapy.[62] [63] The encouraging results seen after transplantation with these unmanipulated, highly contaminated cell populations led to the development of stem cell manipulations to augment the ratio of normal to abnormal cells. These have included the use of 4-hydroperoxycyclophosphamide, interferon, ex vivo marrow culture, antisense oligonucleotides directed against *bcr-abl,* antisense oligonucleotides directed against *c-myb,* as well as in vivo selection by priming and mobilization of stem cells after recovery from chemotherapy.[64] [65] [66] [67] [68] [69] [70] For those patients treated in the early chronic phase, the pilot data appear encouraging for obtaining a graft that is less contaminated. As noted previously, studies of genetically modified stem cells in CML have documented the contribution of the graft to relapse after transplantation.[15] Improvements in long-term disease-free survival using autologous transplantation will require attention both to the body burden of leukemia as well as to the contaminating cells in the stem cell graft. Newer approaches to modify the graft include the use of genetically modified antisense sequences, ribozymes, and tyrosine kinase inhibitors, as well as exploitation of abnormal in vitro growth characteristics of malignant CML progenitor cells. [71] [72] [73] [74] [75] Some investigators have focused on phenotypic separation of benign progenitor cells based on the concept that the normal hematopoietic stem cell is present in the CD34 fraction of marrow or blood.[76] [77] Interestingly, once the disease progresses, this stem cell compartment decreases in size, possibly explaining the benefit of early transplantation for improved disease-free survival in patients treated with aggressive chemotherapy. Because CML is one of the foremost examples of disease for which a graft-versus-tumor effect is effective, other investigators have focused on the identification of immunologic approaches to purge CML marrow, including interleukin-2, the co-incubation of natural killer and cytokine-induced killer (CIK) cells, as well as the development of antigen-specific T cells recognizing tumor-derived immunogenic peptide.[78] [79] [80] [81]

Studies to date indicate that autologous transplantation for CML, although not curative, is probably capable of improving survival. Figure 87-7 (Figure Not Available) compiles the results of 200 consecutive autologous transplantations performed with purged or unpurged

Figure 87-7 (Figure Not Available) Survival by disease stage with autologous transplantation for chronic myeloid leukemia: chronic phase (n = 141); accelerated phase (n = 30), blast crisis (n = 27). *(Update provided by Verfaillie CM, Bhatia R, McGlave PB: Autologous hematopoietic cell transplantation in chronic myeloid leukemia. In Thomas ED, Blume KG, Forman SJ [eds]: Hematopoietic Cell Transplantation. 2nd ed. Blackwell Science, Boston, 1999, pp. 9901002. With permission from Blackwell Science, Inc.)*

marrow or blood grafts at 8 different institutions in Europe and North America between June, 1984 and June, 1992. [82] The median survival of 142 patients with chronic-phase CML has not been reached, suggesting that autografting may provide prolonged survival that may exceed that associated with conventional therapy. The results are clearly improved if the patient undergoes transplantation in the early course of the disease rather than awaiting acceleration or blast crisis. Because most patients eventually show hematologic or cytogenetic relapse, this improved survival is probably not due to elimination of the Philadelphia clone but to a reduction in the overall tumor cell burden and delay of progression of the disease. It is likely that in future trials the use of intensive therapies earlier in the course of the disease will have the best chance of improving disease-free survival, an observation that has also been seen with interferon therapy, where the drug is less effective after the patient has had the disease for more than a year.

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MULTIPLE MYELOMA

Multiple myeloma is part of a spectrum of disorders referred to as *plasma cell dyscrasias,* which include benign conditions such as monoclonal gammopathy (see Chap. 76) and account for approximately 10% of hematologic tumors, with a median age at diagnosis of approximately 65 years. Symptomatic multiple myeloma requires initiation of systemic therapy using either melphalan and prednisone or combination chemotherapy, most commonly vincristine, doxorubicin (Adriamycin), and decadron (VAD). Very few patients achieve a true CR and the median survival is approximately 36 months.

High-dose cytotoxic therapy combined with autologous transplantation was first considered for multiple myeloma in 1986, resulting in some patients having prolonged survival with minimal disease activity.[83] In 1983, investigators from the Royal Marsden Hospital explored high-dose melphalan alone at a dose of 140 mg/m ^{2} .[84] The observation of true CRs after this procedure performed without hematopoietic growth factor and stem cell support led to the introduction of autologous transplantation in the mid-1980s, initially in support of higher-dose melphalan or melphalan combined with TBI. [85] [86] An underlying hypothesis in these trials was that the reinfusion of typically hypoproliferative tumor cells with low in vitro clonogenic potential would not contribute significantly to disease recurrence

despite their presence in the graft. This has led to studies focused on high-dose therapy given during the early phase of disease as well as the exploration of multiple cycles of high-dose therapy, each supported by stem cells.

A number of phase II studies indicated a high response rate as well as apparent improved disease-free survival rates in patients undergoing high-dose therapy for multiple myeloma.[87] [88] [89] [90] These studies have achieved a 4050% CR rate with a median duration of progression-free survival of 2436 months. [91] Patients with chemosensitive tumor cells or who are less heavily pretreated have the most favorable outcome. The major favorable prognostic features associated with event-free survival and overall survival after autologous transplantations include low levels of $_2$ -microglobulin and CR before transplantation; the absence of unfavorable cytogenetics (i.e., abnormalities of chromosomes 11 and 13); <12 months of standard therapy preceding transplantation; and non-IgA myeloma. Combinations of these important prognostic variables allow a risk-based therapeutic approach to patients with multiple myeloma. Most important, a recently completed national French trial of 200 patients with multiple myeloma demonstrated the efficacy of high-dose therapy in improving the outcome of these patients. [92] In this trial, patients received two courses of vincristine, melphalan, cyclophosphamide, and prednisone (VMCP) alternating with vincristine, carmustine, doxorubicin, and prednisone (VBAP), and were then randomized to receive either conventional chemotherapy (eight additional courses of VMCP/VBAP) or high-dose therapy (melphalan and TBI) followed by autologous transplantation. The transplantation patients demonstrated significantly higher response rates, event-free survival, and overall survival rates compared with those treated with conventional therapy. Response rates in the high-dose and conventional arms were 81% and 57%, respectively. The 5-year probabilities of event-free survival and overall survival was 28% and 52%, respectively, in recipients of high-dose therapy, and only 10% and 12% in patients treated with conventional therapy: The treatment-related mortality was comparable between the two groups (Fig. 87-8). Attempts to improve this outcome include the use of autologous BMT, either depleted of tumor cells or processed to select normal hematopoietic progenitor cells, as well as the use of multiple autologous transplantations and biologic therapy. [93] [94] [95]

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ACUTE LYMPHOBLASTIC LEUKEMIA

Improvements in induction and consolidation therapy for both adults and children with acute lymphoblastic leukemia (ALL) have resulted in improved disease-free survival, particularly in those with certain subgroups of disease. [96] [97] [98] For those patients with high-risk features, including the Philadelphia chromosome, high WBC count at diagnosis, and longer time to achieve remission, allogeneic transplantation has also been successfully used to prevent disease recurrence. [99] However, most patients with high-risk ALL or those with second CR do not have a readily available histocompatible related or unrelated donor. The experience of autologous transplantation, both in single institutional studies as well as in larger, randomized trials, has been less than in patients with AML and has focused in general on children with the disease, usually in second remission. One controlled trial has compared autologous transplantation with allogeneic transplantation in ALL. [100] Kersey and colleagues transplanted 91 patients with high-risk, refractory leukemia; 45 patients without a matched donor received an autologous purged transplant, whereas 46 patients who had a donor underwent allogeneic transplantation after preparation with fractionated TBI and cyclophosphamide. [100] It was noteworthy that although the overall 4-year disease-free survival rates did not differ significantly in the two groups, the causes of failure differed. Allogeneic recipients experienced a high mortality rate

Figure 87-8 Overall survival according to treatment group in patients with multiple myeloma, 60 years of age or younger, who were treated with either conventional-dose or high-dose chemotherapy. *(Re-printed with permission from Attal M, Harousseau JL, Stoppa AM et al: Autologous bone marrow transplantation versus conventional chemotherapy in multiple myeloma: a prospective randomized trial. N Engl J Med 335:91, 1996. Copyright © 1996 Massachusetts Medical Society. All rights reserved.)*

from transplantation complications, but few relapses. Autologous transplant recipients experienced a very high relapse rate.

Most centers that have pursued autologous transplantation for ALL have used in vitro purging with antibodies to B- or T-cell early differentiation antigens. [101] [102] [103] Transplantation in first remission has been used primarily in children with very high-risk features. [104] In this group, disease-free survival rates for transplantation range from 30 to 60% with variable follow-up. The European Cooperative Group for Bone Marrow Transplantation has reported the results of autologous transplantation for over 500 patients with ALL.[105] Disease-free survival rates for patients with standard risk (n = 173) and high risk (n = 41) autografted in second remission were 31% at 5 years and 23% at 30 months, respectively. For children who underwent autologous transplantation in second remission, the survival rate was 42%. In the analysis of different patient groups and treatment, disease-free survival was better in patients receiving purged versus unpurged marrow and in patients receiving fractionated versus single-dose TBI. Others have noted that the single most important predictor of outcome after autologous transplantation was the duration of the initial remission.[106] In one series, the event-free survival rate was 44% for patients with a long first remission of at least 24 months, compared with 5% among those patients who had a shorter first remission but who received the same conditioning regimen. Some investigators recently have reported more encouraging results with the addition of etoposide to a conventional regimen of cyclophosphamide and TBI. [107] In most series, adult patients had a higher relapse rate compared with pediatric patients, resulting in inferior disease-free survival for adult patients. [108]

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LONG-TERM COMPLICATIONS OF AUTOLOGOUS TRANSPLANTATION

Most patients recover from the acute effects of the high-dose regimens used in the treatment of their disease. This has been

facilitated by the use of stem cells obtained from the peripheral blood after mobilization with chemotherapy and/or hematopoietic growth factors, peritransplant growth factor support, antifungal agents, and transfusions.

Cataracts

Cataracts are a complication that usually results from the use of TBI in the preparatory regimen. An analysis from the European Bone Marrow Transplant Registry indicated that cataracts developed only in those patients who received TBI. [109] [110] Depending on the degree of impairment of visual acuity, lens replacement can be performed with excellent results.

Secondary Malignancies

Most surveys of patients undergoing autologous transplantation for treatment of malignancy have not documented an increase in the incidence of secondary malignancies. The follow-up for those patients is shorter than for allogeneic recipients, in whom an increase in solid tumors has been observed, so all long-term transplantation survivors should be followed for the development of new malignancies.

The most important long-term complication of autologous SCT is the development of myelodysplastic syndrome (MDS) or secondary leukemia. [111] [112] [113] The incidence of secondary acute leukemia and MDS, although difficult to assess, appears to be substantially lower in patients undergoing transplantation for AML than in those receiving similar preparative regimens for lymphoma and Hodgkin disease. This finding has been attributed to the type of prior therapy that is used in patients with Hodgkin disease, lymphoma, and myeloma (e.g., alkylating agents, topoisomerase I and II inhibitors), as opposed to patients with acute leukemia whose therapy is built around the use of anthracycline and cytosine arabinoside. MDS is reported to occur in 615% of patients undergoing autologous SCT for lymphoma or Hodgkin disease.[114] The most significant factor for the development of this problem is the amount of therapy the patient has received before SCT. In principle, patients who receive marrow grafts incubated with cytotoxic agents may be at risk for secondary leukemia. Despite this concern, the experience to date is probably sufficient to indicate that there is not a dramatic increase in secondary hematopoietic tumors in the recipients of chemotherapy-treated autologous grafts. [115]

Infertility

One of the major complications of high-dose chemoradiation therapy for the treatment of hematologic malignancy is infertility, a frequent concern expressed by young patients who are being evaluated for transplantation. [116] With current methods, men are sometimes able to collect viable sperm for future use and women are able, under some circumstances, to cryopreserve fertilized ova for implantation after transplantation. New techniques involving the harvesting of and storage of ova before fertilization will further expand the possibilities for female patients. Despite these technical advances, the expense is often limiting, and there is not always sufficient time to collect sperm or fertilized eggs before proceeding to urgently needed antitumor therapy. Prior therapy can reduce the quality and quantity of viable sperm necessary for storage.

Impairment of ovarian function after radiation depends on the radiation dose and the patients age. [117] Patients who are young and receive TBI can, on rare occasions, recover ovarian function and fertility. Nevertheless, for most patients receiving high-dose radiation therapy-containing regimens, infertility is a long-term problem.

More than 90% of men given TBI are azoospermic and have elevated follicle-stimulating hormone levels. There is less effect on testosterone levels, with some patients having normal levels of luteinizing hormone and testosterone. However, some patients demonstrate hypogonadism requiring replacement therapy (transdermal testosterone patches or intramuscular injections). Women in whom an early menopause develops can usually be treated with hormone replacement therapy to avoid secondary problems of osteoporosis, coronary artery disease, and sexual dysfunction. The effect of these therapies on sexual satisfaction is an important one. As the number of long-term survivors undergoing autologous transplantation increases, the issues of fertility and normal sexual function are becoming increasingly important problems.[116]

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Chapter 88 - Clinical Results of Autologous Stem Cell Transplantation for Solid Tumors in Adults

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Elizabeth J. Shpall

INTRODUCTION

Autologous bone marrow or peripheral blood progenitor cells (PBPCs) are used as hematopoietic stem cell support in patients with solid tumors after the administration of high-dose chemotherapy (HDC). This process is also known as autologous stem cell transplantation (ASCT). The HDC regimens that are responsible for the eradication of tumor cells typically use two or three drugs that meet the following criteria: known activity at conventional doses in the tumor in question, a steep dose-response effect, and often myelosuppression as the major standard dose-limiting toxicity. When doses are further escalated, the myelosuppression is surmounted by the infusion of the patients own stem cells, which are collected and cryopreserved prior to HDC administration. Thus, ASCT allows a 210-fold increase in the doses of the drugs used. In this setting, extramedullary organ toxicities become dose limiting. [1] The goal of this strategy

is to achieve more tumor cell killing than standard-dose therapy, which will hopefully result in improvements in long-term disease-free survival (DFS).

A potential obstacle to the successful use of ASCT in diseases that often involve the bone marrow, such as breast cancer, is the possibility of infusing an autograft contaminated with tumor cells. This has prompted research efforts directed at effectively purging cancer cells from the stem cell product before transplant using alternative sources of stem cells, such as peripheral blood, and identifying patients for treatment who are less likely to have marrow metastases.

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BREAST CANCER

Metastatic Breast Cancer

Metastatic breast cancer is considered incurable for the majority of patients who receive standard chemotherapy. [2] Although in many cases the tumor responds initially to chemotherapy, additional cycles become substantially less active and eventually the tumor ceases to respond.

Retrospectively, Hryniuk and Bush showed that within the conventional range, dose intensity correlates with antitumor activity and clinical outcome. [3] Prospective studies testing dose reduction below the standard range, in an attempt to reduce toxicity, produced less antitumor effect and compromised palliation. [4] A minor increase of dose intensity, however, does not improve results. [5] To overcome tumor cell resistance to the degree that would have a significant clinical impact, a substantial dose escalation beyond normal bone marrow tolerance is needed. This clinically relevant dose escalation, which exceeds normal bone marrow tolerance, requires subsequent autologous stem cell support.

The first HDC trials for patients with breast cancer were initiated in the mid 1980s. As with all other therapies that have a curative intent, minor dose reductions result in a critical compromise of the antitumor efficacy; [6] therefore, the maximally tolerated doses of the drugs were used. Alkylating agents constituted the backbone of the HDC regimens based on their activity against breast cancer, steep dose-response effect, non-cross-resistance, and nonoverlapping extramedullary toxicities. [7] The use in these trials of multiple drug combinations was supported by in vitro data and results from standard-dose treatment in other more chemosensitive diseases [7] (Table 88-1).

A sequential series of trials demonstrated that ASCT as salvage treatment for patients with refractory metastatic breast cancer produced a high response rate. [8] [9] [10] However, the responses were short-lived and had no impact on survival. Results improved when ASCT was used as initial therapy for patients with metastatic disease. [11] High-dose chemotherapy was then used as consolidation after aggressive standard-dose induction chemotherapy. [12] [13] [14] Induction regimens, such as AFM (Adriamycin, 5-fluorouracil, and methotrexate), [15] were designed to provide substantial cytoreduction prior to ASCT. Phase II trials testing the strategy of induction followed by

TABLE 88-1 -- Initial High-Dose Combinations of Alkylating Agents

Regimen	Composition	Author
CCB	Cyclophosphamide 5,625 mg/m^2 Cisplatin 165 mg/m^2 BCNU 600 mg/m^2	Peters WP et al. [8]
CTCb	Cyclophosphamide 6,000 mg/m^2 Thiotepa 500 mg/m^2 Carboplatin 800 mg/m^2	Eder JP et al. [10]
CT	Cyclophosphamide 7,500 mg/m^2 Thiotepa 675 mg/m^2	Williams SF et al. [143]

Figure 88-1 Relapse-free survival curve of the whole population of stage IV breast cancer patients treated with HDC at the University of Colorado Bone Marrow Transplant Program.

ASCT consistently demonstrated that 1525% of patients remained free of disease long-term as shown in Figure 88-1 . [12] [13] [14] Autologous SCT was most effective at a time of minimal tumor burden. Adequate local treatment (surgery or radiotherapy) to pre-transplant sites of bulky disease was added as part of the overall strategy. The mortality rate in these HDC trials has decreased from 20% initially to a current 25%, similar to what is often reported for standard-dose regimens (Table 88-2).

New High-Dose Chemotherapy Regimens

Current research efforts are directed at improving the antitumor activity of ASCT. An important strategy to deliver a single potent treatment pre-transplant is the optimization of ASCT regimens by adding more active drugs. The most active single agents against breast cancer are doxorubicin and the taxanes, paclitaxel and docetaxel. [2] Doxorubicin is an essential component of the pre-HDC induction combinations, but because of the potential for cardiotoxicity, it is not included in most ASCT regimens. Paclitaxel, while not an alkylator, has an in vitro and clinically established dose-response effect, [16] [17] [18] and, at standard doses, produces dose-limiting myelosuppression. [19] A phase I study conducted at the University of Colorado escalated paclitaxel with fixed doses of cyclophosphamide and cisplatin, up to a maximum tolerated dose of 775 mg/m^2 . [20] The resulting regimen of cyclophosphamide, cisplatin, and paclitaxel, is presently under evaluation in a phase II trial of patients with metastatic breast cancer. [21] High-dose paclitaxel has been incorporated into other ASCT regimens. [22] [23] Dose escalation of docetaxel with ASCT is being explored at the University of Colorado BMT Program.

Tandem or Multiple Transplants

A different strategy assumes that more than one cycle of ASCT is needed to eradicate the tumor. Dunphy et al. [24] used two cycles of high-dose myelosuppressive, but not myeloablative, therapy with DFS rates of 25% reported at 2 years, which is comparable to those reported with a single cycle of myeloablative HDC. Multiple rapid cycles of chemotherapy with ASCT are being investigated by workers at a number of institutions. [25] [26] The feasibility of delivering tandem cycles of myeloablative chemotherapy has been demonstrated in several studies. [27] [28] [29] The value of administering two cycles of the same HDC regimen is unclear because antitumor activity seems to decrease substantially after the first cycle. [29] The use of two different non-cross-resistant combinations administered sequentially has been explored.

TABLE 88-2 -- Sequential Strategies Used in Phase II Trials of High-Dose Chemotherapy for Metastatic Breast Cancer

Strategy	Regimens Used	No. of Patients	Complete Response (%)	Long-term Disease-Free Survival (%)
Refractory disease	CCB [9]	23	26	0
	CTCb [10]	16	6	0
Up-front Rx for MBC	CCB [11]	22	54	14
Consolidation after induction	AFM CCB[12]	45	57	22[a]
				26[b]
	AFM CTCb[13]	29	45	17
	LOMAC/FCAP CT[14]	62	32	13[a]
				17[b]

MBC, metastatic breast cancer; LOMAC/FCAP, cyclophosphamide, Adriamycin, vincristine, methotrexate with leucovorin rescue/fluorouracil, cisplatin, Adriamycin, and cyclophosphamide.

[a]DFS based on intent-to-treat analyses.
[b]DFS for patients actually treated with HDC.

Ayash et al.[30] treated chemosensitive breast cancer patients with high-dose melphalan (140180 mg/m ^{2}) followed, within a median of 35 days, by high-dose cyclophosphamide, thiotepa, carboplatin (CTCb) with ASCT after both cycles. With a median follow-up of 16 months after the second cell reinfusion, the DFS of 34% appears similar to results achieved with a single ASCT treatment. The reverse sequence of cyclophosphamide and thiotepa (CT) followed by melphalan, reported by Bitran et al.[31] with longer median intervals between cycles (median, 105 days vs. 35 days in the Ayash study), produced a DFS rate of 56% at a median follow-up of 25 months from the first reinfusion. Whether the Bitran and Ayash studies produced significantly different results is unclear, given the small size of the studies with DFS ranges that overlap. Theoretically, the longer interval between cycles of the Bitran study could potentially allow the acquired drug resistance to revert after the first cycle.

Is High-Dose Chemotherapy with Autologous Stem Cell Transplantation Better Than Standard-Dose Chemotherapy for Metastatic Breast Cancer?

Autologous stem cell transplant consistently yields a 1520% rate for long-term DFS for patients with chemosensitive metastatic breast cancer. This seems substantially higher than the long-term results, ranging from 03%, for standard-dose chemotherapy in this patient population. [32] [33] [34] However, the effect of patient selection bias, based on more extensive staging for patients on ASCT trials, age, performance status, and chemosensitivity, cannot be excluded, underscoring the need for prospective randomized trials.

Bezwoda et al.[35] randomized patients with metastatic breast cancer to receive, as first-line therapy for metastatic disease, two cycles of high-dose cyclophosphamide, mitoxantrone, and etoposide (HD-CNV) with ASCT support or six to eight cycles of standard-dose cyclophosphamide, mitoxantrone, and vincristine (CNV). Patients on the high-dose arm had significantly higher response, complete response (CR), DFS, and overall survival (OS), as well as a longer median duration of response than patients on the standard-dose arm of the trial. Although neither arm of the study used typical chemotherapy regimens, this trial showed, in a prospective fashion, higher antitumor activity and a survival advantage for the ASCT arm.

Peters et al.[36] reported the preliminary results of a large randomized trial that compared the immediate versus the delayed use of ASCT in metastatic breast cancer patients in CR following standard-dose induction chemotherapy. Four hundred twenty-three patients with metastatic breast cancer received AFM; 98 (23%) achieved a CR and were randomized to either immediate ASCT with cyclophosphamide, cisplatin, and BCNU (CCB) or observation. Patients relapsing during observation were transplanted, if feasible, with CCB. The median DFS was longer for patients immediately transplanted compared with those randomized to observation (11 vs. 3.6 months, $p < 0.01$).

However, patients transplanted at relapse had superior OS than those who underwent an immediate transplant (38 vs. 23 months, $p < 0.05$). Although this trial was not designed to directly compare ASCT and standard-dose therapy, its results indicate that ASCT may improve DFS and OS in patients with chemosensitive metastatic breast cancer.

Which Metastatic Breast Cancer Patients Are Most Likely to Benefit from Autologous Stem Cell Transplantation?

Dunphy and colleagues [37] identified liver metastases, soft tissue site, and prior adjuvant chemotherapy as independent negative predictors of OS after ASCT. Ayash et al.[38] reported that a single metastatic site and attainment of a CR to induction chemotherapy were the most significant positive predictors for DFS. Doroshow and colleagues[39] obtained the longest DFS rates in patients transplanted in CR, without a history of liver metastases, with fewer number of prior chemotherapy cycles and fewer sites of disease. A univariate analysis by Antman et al. [40] reported that age <40 was significantly associated with shorter DFS and OS rates. Patients treated with ASCT at the University of Colorado for a relapse within a previously irradiated field consistently have a poor outcome (University of Colorado Bone Marrow Transplant Program, unpublished observations). In summary, patients with metastatic breast cancer with a minimal tumor burden, no liver involvement, and chemosensitive disease are those who most likely benefit from ASCT.

A prospective phase II study was initiated in 1990 at the University of Colorado for breast cancer patients with no evidence of disease, defined as only one site of disease, that can either be resected before or encompassed within a radiation field after ASCT (University of Colorado Bone Marrow Transplant Program, unpublished observations). The latest review demonstrated that 55% of 40 patients remain free of disease at a median follow-up of 45 months. The minimal tumor burden and the potential benefit of local therapy to the site of macroscopic disease may be responsible for an improved outcome in that group of stage IV patients (Fig. 88-2).

High-risk Primary Breast Cancer

Once the high activity of ASCT regimens was seen in metastatic breast cancer, the next step was to test this strategy in high-risk primary breast cancer, where the majority of patients will eventually relapse, despite conventional adjuvant therapy. Dose intensity has a greater impact on survival in the adjuvant setting compared with the metastatic setting.[41] [42]

Peters et al. [43] at Duke University conducted the first trial of

Figure 88-2 Relapse-free survival curve of clinical trial conducted at the University of Colorado Bone Marrow Transplant Program on stage IV patients with no evidence of disease. Wedges indicate censored patients.

ASCT in patients with 10 or more (10+) involved axillary lymph nodes. At a median follow-up of 6 years, 65% of 85 evaluable patients remain free of disease. [44] Gianni and colleagues from the National Cancer Institute in Milan used a sequential high-dose single-agent approach in the same patient population. After a median follow-up of 4 years, DFS is 57%.[45] The results of both trials appear superior to standard adjuvant therapy where relapse rates of 6085% are commonly reported for 10+ node patients. Two large cooperative group randomized trials that compare conventional-dose chemotherapy to ASCT are underway to confirm the efficacy demonstrated in the single-arm ASCT trials.

The University of Colorado has piloted the use of ASCT earlier in stage II/III patients with four to nine involved axillary nodes, whose long-term DFS ranges from

4576% with standard-dose adjuvant chemotherapy. Fifty-four patients received four cycles of standard-dose chemotherapy followed by CCB with stem cell support. An actuarial 4-year DFS of 71% was demonstrated at a median follow-up of 31 months.[46] These results stimulated the initiation of a randomized trial that is presently comparing ASCT in four cycles of conventional adriamycin-containing chemotherapy followed by CCB or CTCb versus a dose-intensive nonmyeloablative sequential approach with three cycles each of adriamycin, taxol, and cyclophosphamide plus granulocyte colony-stimulating factor support, developed at the Memorial Sloan-Kettering Cancer Center.[47]

Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is a very aggressive type of presentation, with a high incidence of local and systemic recurrences, following standard therapy. With the use of multimodal therapy, including doxorubicin-based neoadjuvant chemotherapy, surgery, and radiotherapy, approximately 30% of the patients remain free of disease at 5 years.[48] Systemic relapses still occur in the majority of those with IBC.[49] High-dose chemotherapy has recently been included in the treatment, in varying schedules. Single-institution phase II ASCT trials conducted at the Dana Farber Cancer Institute [50] and the University of Colorado[51] tested CTCb before and CCB after mastectomy, respectively. In both studies patients were initially treated with neoadjuvant Adriamycin-based standard-dose chemotherapy and radiotherapy was added at the end. Preliminary results demonstrate that >70% of patients in both studies remain recurrence free at a median 2 years following transplant. The DFS curves of patients treated at the University of Colorado BMT Program in the different clinical trials of HDC for high-risk primary breast cancer are shown in Figure 88-3 .

Predictive Factors for Relapse After High-Dose Chemotherapy for Primary Breast Cancer

Somlo et al.[52] studied prognostic indicators of relapse in 114 patients with high-risk breast cancer treated with ASCT. In their univariate analyses, the risk of relapse was lower for progesterone receptor (PR)-positive (but not estrogen receptor [ER]-positive) tumors and higher for patients with IBC. PR positivity was the only prognostic factor independently associated with improved DFS in multivariate analyses.

Retrospective review of 176 high-risk patients treated with ASCT at the University of Colorado shows that tumor size, tumor grade, clinical inflammatory presentation, ER and PR negativity, and both the absolute number of positive lymph nodes and the ratio between the number of positive nodes and the total number of lymph nodes sampled (nodal ratio) were statistically associated with relapse in the univariate analyses. In a multivariate analysis, tumor size, ER/PR combined status (only considered negative if both ER and PR are negative), and the nodal ratio remained as independent predictors of relapse. A predictive model with these three tumor-related parameters was subsequently developed to identify a subset of patients with a high probability of relapse after ASCT. [53]

Purging of Stem Cell Grafts

Hematopoietic progenitor cells obtained from the peripheral blood after chemotherapy and/or growth factor priming (peripheral blood progenitor cells or PBPCs) have replaced bone marrow as the primary source of hematopoietic progenitors for ASCT. Although tumor burden may be lower in PBPCs than in bone marrow fractions, [54] current immunocytochemical techniques detect breast cancer cells in PBPC fractions of 1040% of stage IV patients and 520% of patients with stage II disease. [55] Higher relapse rates have been reported for breast cancer patients who received autologous PBPC grafts contaminated with malignant cells, identified in a long-term culture assay, compared to those with no identifiable tumor cells in their graft. [56] [57] Because the majority of patients with stage IV disease relapse in sites of previous bulk disease, the contribution to relapse of

Figure 88-3 Relapse-free survival curves of patients who have received high-dose chemotherapy at the University of Colorado Bone Marrow Transplant Program, included in the 10+ (black curve), 49 positive nodes (solid color curve), and inflammatory breast cancer (IBC) (shaded curve) groups. The number of censored patients within each time interval is indicated.

tumor in the stem cell graft cannot be evaluated in that setting. Whether breast cancer cells in the graft contribute to relapse after transplant remains unclear and will have to be determined in the adjuvant setting. Studies addressing this issue are being designed.

Negative Purging Procedures

Negative purging procedures, either pharmacologic [58] or immunologic,[59] have been used primarily to purge the bone marrow of patients with breast cancer. These procedures can damage or deplete the normal progenitors, delay engraftment, and thereby increase the patients risk of myelosuppressive complications. When immunologic PBPC purging was studied in breast cancer patients, tumor cell removal was achieved without prolongation of the engraftment rates compared with historical controls.[60] However, pharmacologic purging of the marrow,[58] as well as the combination of pharmacologic plus immunologic purging, [59] produced marked delays in engraftment of both neutrophils and platelets. Because of its potential toxicity to the normal hematopoietic cells, negative purging of PBPCs, particularly with chemotherapy, is not commonly performed.

Positive or CD34 Selection

The CD34 antigen is expressed on 0.53% of normal bone marrow cells and PBPCs, including both the committed and probably the long-term reconstituting progenitor cells. CD34 does not appear to be expressed on breast cancer cells. [61] The University of Colorado Bone Marrow Transplant Program has reported data on a series of patients with breast cancer who received ASCT with HDC and a CD34-selected marrow or PBPC graft. [62] The stem cells were CD34 selected with an immunoadsorption device and effectively reconstituted immediate and long-term hematopoiesis in 155 patients. An average two-log (range 1 to >4 logs) tumor cell depletion was achieved following the procedure. The patients who received CD34+ PBPCs, with or without marrow, experienced neutrophil and platelet recovery rates that were identical with those of patients who received unmanipulated stem cell grafts. Long-term follow-up showed that the durability of engraftment, immune reconstitution, and progression-free and OS, were also comparable to those parameters in patients who received unmanipulated hematopoietic cell fractions. [63]

Because the majority of patients with breast cancer still had detectable cancer cells present in their stem cell grafts following CD34 selection, maximally effective purging may require a combination of positive and negative selection procedures.

Future Directions

Ongoing clinical trials of allogenic stem cell transplantation for breast cancer are presently exploring its potential graft-versus-tumor effect. If such an immune-mediated effect can be demonstrated, it could enhance the therapeutic potential of HDC.

Radioimmunotherapy, active in low-grade B-cell non-Hodgkin lymphoma, [64] [65] holds promise for patients with breast cancer. At the University of Colorado a phase I PBPC-supported trial is presently exploring the dose escalation of yttrium 90, conjugated to humanized BrE-3 monoclonal antibody, directed to an epitope of the human milk fat globulin (MUC-1). Preliminary results suggest a high anti-breast cancer activity with this therapy.

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OVARIAN CARCINOMA

Improvements are needed in the treatment of advanced ovarian cancer. After initial optimal debulking surgery followed by conventional-dose chemotherapy with paclitaxel and cisplatin, 50% of stage III patients will be rendered disease free when evaluated by second-look laparotomy. Only half of these patients are ultimately cured of their disease. Therefore, optimal treatment cures only 25% of women with stage III diseases. For patients with residual disease at second-look surgery and for those who relapse, standard-dose salvage therapy, while prolonging survival, is not curative. Women with stage IV disease have a 5-year survival rate of <5%. [66]

Rationale for Dose Intensity in Ovarian Cancer

In vitro models suggest that a fivefold escalation in dose is required to overcome drug resistance in ovarian cancer. [67] Alkylating agents exhibit a steep dose-response relationship and synergistic cytotoxicity against ovarian cancer cell lines. [68] Retrospective dose-intensity analysis of 33 different clinical trials of platinum-containing chemotherapy for ovarian carcinoma demonstrated that response and survival correlate with dose intensity. [69]

Moderate dose escalation of the platinum compounds in patients refractory to standard-dose cisplatin can produce a 2030% response rate. However as with patients with metastatic breast cancer, most randomized trials that have explored minor cisplatin dose escalations have failed to show a survival advantage. [70] Because alkylators are among the most active and widely used agents for the treatment of ovarian cancer, the exploration of ASCT with major escalation in chemotherapy doses seemed warranted.

High-Dose Chemotherapy and Autologous Stem Cell Transplantation for Patients with Relapse

In a phase I/II trial of ASCT with intravenous chemotherapy plus intraperitoneal cisplatin conducted at Duke University in those with refractory ovarian carcinoma, 75% of the patients experienced pathologically documented partial response of median 6-month duration. [71] At the University of Colorado, that regimen was subsequently modified, by changing the route of cisplatin administration from intraperitoneal to intravenous and then increasing the doses of chemotherapy. [72]

Stiff et al.[73] from Loyola University, developed an ASCT regimen that includes mitoxantrone, cyclophosphamide, and a 120-hour continuous infusion of carboplatin. In 30 relapsed patients, 20 of whom were refractory to cisplatin, the response rate was 89%. Complete clinical responses occurred in 88% of the cisplatin-sensitive patients and in 47% of the cisplatin-resistant-group (p = 0.06). Median OS was 29 months for the whole population, with a 3-year DFS rate of 23%. [74] A subsequent analysis of those 30 relapsed patients identified cisplatin sensitivity and low tumor burden at the time of HDC as the most significant prognostic factors for survival after ASCT.[75] Patients with <1 cm disease at high-dose study entry, had a 10.3-month median progression-free survival versus 5.2 months for those with >1 cm (p = 0.04). Median DFS was 10.1 months for cisplatin-sensitive patients versus 5.1 months for those in refractory relapse (p = 0.03). At a median follow-up of 12 months, 80% of cisplatin-sensitive patients were alive versus <50% of those with resistant disease. The authors concluded that survival for patients with resistant relapse is similar to that for patients treated with conventional chemotherapy and HDC. Results from the universities of Duke/Colorado and Loyola studies in relapsed/refractory patients are summarized in Table 88-3 .

A Southwest Oncology Group randomized phase II trial for stage III/IV patients with residual disease (up to 3 cm) after cisplatin-based chemotherapy, conducted to compare the toxicity of the Duke/Colorado and Loyola regimens, has completed accrual. Analysis of this ambitious study will help shape future

TABLE 88-3 -- Summarized Results of High-Dose Chemotherapy for Relapsed/Refractory Ovarian Carcinoma

Author	No. of Patients	No. of Prior Chemotherapy Lines	Regimen	Toxic Death Rate	Response Rate	Complete Response	Median Response Duration
Shpall[71]	19	3	CPA, Thio, IP CDDP	10%	75%	NS	6 mo
Shpall[72]	13	2	CPA, Thio, IV CDDP	0%	70%	50%	6 mo
Stiff[74]	20	3	CPA, Mito, Cb	3.3%	89%	47%	5.1 mo

CPA, cyclophosphamide; Thio, thiotepa; IP, intraperitoneal; CDDP, cisplatin; IV, intravenous; Mito, mitoxantrone; Cb, carboplatin.

efforts in ASCT therapy of advanced ovarian carcinoma patients.

Autologous Stem Cell Transplantation as Front-Line Therapy for Advanced Ovarian Carcinoma

The Europeans have pioneered the use of ASCT as part of the front-line treatment for stage III/IV ovarian cancer. In most studies, patients are initially surgically debulked, treated with cisplatin-based chemotherapy (generally six cycles), followed by second-look surgery. High-dose chemotherapy with stem cell support is then used as consolidation.

The French Melphalan-based Trials

Viens et al.[76] reported their results in 28 ovarian cancer patients (17 with stage III and 11 with stage IV disease) with initial cytoreductive surgery. Residual tumor was <2 cm in 6 patients and >2 cm in 22 patients. All patients received six courses of cisplatin-based chemotherapy. Twenty-two patients (78%) had second-look surgery performed. Pathologic complete responses (pCR) were documented in 10 patients, microscopic disease in 2 patients, and persistent macroscopic tumors in the remaining 10 patients. Subsequently, patients received ASCT with a melphalan-containing HDC combination. The toxic death rate was 4%. The expected OS and DFS rates at 3 years are 72.5% and 52.5%, respectively.

Legros et al.[77] treated 53 patients (38 with stage III and 15 with stage IV disease) with similar ASCT consolidation after surgical debulking, six courses of conventional cisplatin-based chemotherapy, and second-look surgery. Nineteen patients had a pCR at second-look, 7 patients had microscopic disease, and 22 had macroscopic disease, most of whom underwent complete redebulking. Intensive chemotherapy consisted of melphalan 140 mg/m^2 (23 patients) or carboplatin at

1,0001,500 mg/m^2 plus cyclophosphamide at 6 g/m^2 (30 patients). With a median follow-up of 80 months, reported OS and DFS rates are 60% and 23%, respectively.

A retrospective analysis of 35 patients treated at both institutions correlated the status at transplant, following second-look surgery, with progression-free survival. [78] Although patient-selection bias may have influenced results, ASCT seems to improve OS and DFS when compared to historical controls (Table 88-4).

TABLE 88-4 -- Patient Outcome According to Status at Second-look Surgery

Status at Second Look	No. of Patients	PFS (8+ to 35+ mo FU)
Residual disease >2 cm	16	31%
Residual disease <2 cm	10	30%
No residual disease (pCR)	9	66%

PFS, progression-free survival; FU, follow-up; pCR, pathologic complete remission.

Other Studies with Autologous Stem Cell Transplantation as Consolidation for Up-Front Therapy

Benedetti-Panicci et al. [79] treated 20 stage III/IV patients, having 0.52 cm residual tumor, with two cycles of a cisplatin-based regimen, followed by ASCT with cisplatin (100 mg/m^2), etoposide (650 mg/m^2), and carboplatin (1,800 mg/m^2). Second-look surgery revealed a 37% pCR rate, whereas 26% of the patients had microscopic and 21% macroscopic disease. With a median follow-up of 4 years, OS and DFS rates for the whole group are 62% and 57%, respectively. Eighty-five percent of patients in pCR and 60% of those who had their disease completely resected at second-look surgery remain free of disease (Table 88-5).

In summary, ASCT may prolong DFS and OS, both in chemosensitive relapses and as up-front therapy for advanced disease, particularly in patients with minimal disease or negative second-look after the standard therapy. A cooperative group study that compares ASCT with high-dose therapy to the best conventional treatment for patients with advanced disease has been initiated in the United States. After four to six cycles of a platinum compound plus taxol and a second-look procedure, patients with at least a partial response and no tumor nodule >1 cm without surgical cytoreduction are randomized to six additional cycles of carboplatin-taxol or ASCT with the Loyola regimen of cyclophosphamide, mitoxantrone, and carboplatin.

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NONSEMINOMATOUS GERM CELL TUMORS (NSGCT)

First-line standard-dose cisplatin-based combinations yield long-term DFS rates of 90% in good-risk patients with advanced-disease, which decreases to 5560% in poor-risk patients.[80] Standard-dose salvage regimens, including ifosfamide and cisplatin, cure about 25% of patients in first relapse. Patients in second relapse are incurable with standard treatment. Therefore, in parallel studies directed at reducing the toxicity of therapy for good-risk patients, ASCT was explored for those indications where conventional chemotherapy offered less satisfactory results.

Autologous Stem Cell Transplantation for Refractory or Relapsed Nonseminomatous Germ Cell Tumors

Nichols and colleagues at Indiana University developed a highly active combination of carboplatin at 1,500 mg/m ^{2} and etoposide at 1,200 mg/m^2 (CE), that was used as a tandem transplant with stem cell support after each of the two cycles.[81] Long-term follow-up showed that 15% of 40 refractory patients were free of disease.[82] These two drugs became the core of ASCT regimens for NSGCT. A subsequent Eastern Cooperative Oncology Group trial confirmed their curative potential and feasibility within a multicenter setting.[83]

Autologous stem cell transplant was then administered earlier in the course of disease, to patients in first relapse. Motzer

TABLE 88-5 -- Summarized Results of the Main Studies Using High-Dose Chemotherapy as Part of the Front-Line Therapy for Advanced Ovarian Cancer

Author	No. of Patients	Pre-HDC Second-look Results	High-Dose Regimen	Toxic Death Rate	FU (mo)	DFS	OS (mo)
Viens[76]	28	pCR: 10/22 Mic. dis: 2/22 Mac. dis: 10/22	Melphalan-containing combinations	4%	>12	(Actuarial at 3 yr) 52.5 %	(Actuarial at 3 yr) 72.5%
Legros[77]	53	pCR: 19/46 Res. dis: 29/46	Mel (23 pts), Car + Cy (30 pts)	2%	80	35%	60%
Benedetti-Panici[79]	20	NS	Cis, Eto, Car	5%	60	45%	60%
Lotz[2]	30	NS	Tandem Ifo, Car, Thio ×2	8%	60	N/A	32% mOS: 24 m
Langleben[3]	25	pCR: 8/25 Mic. dis: 8/25 Mac. dis: 4/25 PD (no HDC): 5/25	Eto, Ifo, Car	5%	22	30%	mOS not reached
Extra[4]	37	pCR: 8/36 Res. dis: 28/36	Combinations of Cy, Mel, Car, and abd-pelv ×RT	2.7%	NS	43%	mOS: 47 m
Murakami[5]	42	Mac. dis: 17/42 Nonmac. dis: 23/42	Tandem Cy, Adr, Cis 2	0%	60	N/A	Stage III/IV: 26% Adjuvant: 78%

FU, follow-up; pCR, pathologic complete response; Mic. dis, microscopic disease; Mac. dis, macroscopic disease; Res. dis, residual disease (either microscopic or macroscopic); Nonmac. dis, nonmacroscopic disease (either microscopic disease or pathologic complete response); Eto, etoposide; Ifo, ifosfamide; Car, carboplatin; Cis, cisplatin; Mel, melphalan; Thio, thiotepa; ×RT, radiotherapy; Cy, cyclophosphamide; Adr, adriamycin; N/A, not available; mOS, median overall survival; DFS, disease-free survival.

and colleagues from Memorial Sloan-Kettering showed that ASCT is better tolerated when refractory patients are treated early in the course of their disease, rather than when disease is progressing.[84] Subsequent trials confirmed improved outcome for ASCT used in first relapse, with a 38% long-term DFS rate.[85] [86] As with other solid tumors, improved supportive measures progressively decreased the toxic death rate from 21% in the initial study by Nichols et al. [81] to 03% in these last trials.

The addition of a third drug to the two-drug CE combination has been explored. While short infusions of high-dose ifosfamide are often nephrotoxic, [87] a protracted 22-hour infusion of this drug at 10 g/m ^{2} can be administered safely with CE, as shown by Siegert et al.[88] Motzer et al.[89] gave 60150 mg/kg of cyclophosphamide, 1,500 mg/m^2 of carboplatin, and 1,200 mg/m^2 of etoposide (CEC) to 58 refractory patients. The CR rate attained was 40%, and the DFS rate was 21% at a median follow-up of 28 months; the toxic death rate in that trial was 12%.

In an attempt to increase their activity, doses of carboplatin and etoposide were escalated further. Their respective maximally tolerated doses were established at 2,100 mg/m^2 and 2,250 mg/m^2 per cycle.[90] Dose-limiting toxicities were neurologic and mucosal. In the phase II trial of this tandem two-drug regimen, 52% of 25 patients treated in first relapse remained disease-free at a median follow-up of 26 months.[91]

A randomized trial for relapsed patients that compares conventional salvage chemotherapy with ASCT as consolidation after standard chemotherapy is presently underway in Europe.

Up-front Autologous Stem Cell Transplantation for Poor-risk Nonseminomatous Germ Cell Tumors

A European randomized trial, conducted from 1988 through 1991 in 115 patients, failed to show a therapeutic benefit for early high-dose therapy for poor-risk patients.[92] Previously untreated patients were randomized to receive three or four cycles of vinblastine, etoposide, bleomycin, and high-dose cisplatin (200 mg/m ^{2}) (PveBV), or two cycles of PveBV followed by one cycle of a stem-cell-supported HDC regimen that included cisplatin at 200 mg/m ^{2} , etoposide at 1,500 mg/m^2 , and cyclophosphamide at 6,400 mg/m^2 (PEC). This study has been extensively criticized. The patients on the high-dose arm received less total cisplatin than those on the standard-dose arm. PEC, which was a state-of-the-art high-dose regimen in 1986, when the trial was initiated, is considered substandard by modern criteria. The cisplatin dose of 200 mg/m^2 is therapeutically similar to 100 mg/m^2 .[93] It is possible that a higher dose escalation of the platinum compound is necessary to overcome resistance in NSGCTs. Long-term follow-up suggests that carboplatin, which can be escalated more than four-fold, may be more efficacious in refractory tumors than

cisplatin-based combinations, such as PEC, where only moderate dose escalations of cisplatin can be achieved.

Motzer and colleagues administered ASCT as initial treatment to patients with advanced poor-prognostic disease, per Memorial Sloan-Kettering criteria, who had a reduced clearance of tumor markers with standard-dose chemotherapy that included vinblastine, cyclophosphamide, dactinomycin, bleomycin, and cisplatin (VAB-6).[94] Reduced clearance of tumor markers, defined as prolonged half-life of either -fetoprotein or human chorionic gonadotropin (HCG), >7 days and >3 days, respectively, was used as an early indicator of cisplatin resistance. A total of 28 patients received VAB-6 as initial therapy, and 22 of them were switched, after two cycles, to ASCT with two cycles of CE. The CR rate for patients in this trial was 56%, and 46% of patients were recurrence free at a median follow-up of 31 months. The toxic death rate was 5%.

In a continuation study,[95] these investigators substituted VIP (etoposide, ifosfamide, and cisplatin) for VAB-6 as initial therapy for 30 patients. Fourteen of them experienced slow decline

of markers after two cycles of VIP and were treated with two cycles of high-dose CEC. With a 0% toxic death rate, 57% of patients achieved a CR, and 50% remained free of disease at median follow-up of 30 months. Administering ASCT to poor-risk patients showing a slow decline in tumor markers yielded longer survival when retrospectively compared with historical controls treated with conventional-dose chemotherapy at the same institution (p = 0.001). [95]

An ongoing Intergroup randomized trial is addressing the use of up-front ASCT. Untreated poor-prognosis patients, by the International Classification, [96] are randomized to a standard-dose arm that includes four cycles of BEP (bleomycin, etoposide, and cisplatin), or the high-dose arm, which includes two cycles of BEP followed by two cycles of high-dose CEC with ASCT.

Predictive Factors for Nonseminomatous Germ Cell Tumors Treated with Autologous Stem Cell Transplantation

Beyer et al.[97] constructed a prognostic model for relapsed or refractory NSGCT treated with ASCT, based on 310 patients treated with CE-based high-dose regimens in several European and American institutions. Chemorefractoriness was defined as tumor progression while on cisplatin, progression within 4 weeks after prior response, or disease stabilization. Multivariate analysis identified chemorefractoriness, mediastinal primary tumor; and HCG >1000 U/L at ASCT, as independent negative risk factors for relapse and survival. The model assigns patients to three risk categories, according to the presence of those variables, with DFS at 2 years after ASCT of 51%, 27%, and 5%, respectively.

Broun et al.[98] of Indiana University reported that none of 12 patients with a primary mediastinal tumor who received ASCT remained free of disease long-term. Motzer et al.[89] reported that in their series of 58 patients with refractory disease, those with pretreatment levels of HCG <100 times the upper normal limit, and those with retroperitoneal as opposed to nonretroperitoneal metastases, experienced a longer median OS rate.

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SMALL-CELL LUNG CANCER

Small-cell lung cancer (SCLC) is an aggressive tumor, which is typically responsive to initial conventional-dose chemotherapy. In patients with limited SCLC, 80100% respond and 5070% achieve a CR.[99] For patients with extensive disease, the response rate is 6080%, with a CR rate of 2040%. However, despite this chemosensitivity, the overwhelming majority of patients relapse. Only 10% of patients with limited and <1% of those with extensive SCLC remain free of disease long-term.[100] Different treatment strategies, within the conventional-dose range, have been evaluated in patients with SCLC. The use of alternating non-cross-resistant combinations is not more active than the use of only one regimen. [101] Moderate increases of dose produce a higher response rate at the expense of significant toxicity and with no impact on survival. [102] [103]

These discouraging results and the fact that SCLC appeared to be chemosensitive prompted the investigation of ASCT in that disease in the early 1980s. The initial studies used ASCT as front-line therapy. Farha et al. [104] treated 14 patients with limited and extensive disease with two cycles of high-dose cyclophosphamide, etoposide, vincristine, and Adriamycin with ASCT. The CR, DFS, and OS rates were no better than those achieved with standard therapy. Souhami and colleagues conducted a series of studies in an attempt to identify a superior ASCT regimen. Initial treatment with single-agent cyclophosphamide, [105] cyclophosphamide and etoposide,[106] cyclophosphamide as consolidation of prior standard-dose induction therapy, [107] and finally, initial high-dose combination chemotherapy with carboplatin, etoposide, and cyclophosphamide or melphalan, were evaluated in consecutive phase II trials, with no improvement in clinical outcome compared to historical controls.

The next generation of studies tested late intensification to consolidate a response obtained with conventional induction treatment. The most encouraging results were achieved by Spitzer et al., [108] who administered two cycles of high-dose cyclophosphamide, Adriamycin, etoposide, vincristine, and methotrexate to 32 limited SCLC patients. At a median follow-up of 4 years, the OS was 19%. Thirty-eight percent of the patients transplanted in CR, and 11% of those who were converted from partial response to CR after ASCT, remained disease free at the same time point.

Humblet et al.[109] reported the only prospective, randomized trial using late intensification in limited- and extensive-stage SCLC. Patients who responded to induction chemotherapy were randomized to an additional cycle of the same conventional-dose combination or to high-dose cyclophosphamide, BCNU, and etoposide with ASCT. Although patients received cranial irradiation, no chest radiotherapy was delivered. The transplant arm had significant higher response and DFS rates, but OS was not significantly different between both groups, in part due to a high transplant-related mortality rate of 17%. The majority of relapses occurred in sites of previous disease, particularly in the lungs. All patients in both arms with extensive disease relapsed following therapy.

The third generation of studies addressed the problem of chest relapses, which was the major site of failure after ASCT. Bulky chest disease at diagnosis may imply intrinsic drug resistance, the presence of a chemorefractory non-small cell subpopulation (10% of patients), and poor drug perfusion. Because chest radiotherapy was included as part of standard of care for limited stage disease, [110] subsequent ASCT studies also included intensive thoracic radiotherapy, to a total dose 50 Gy, along with prophylactic cranial irradiation as an essential component of the therapeutic strategy for limited-stage disease. Another refinement in most recent studies is an improvement of ASCT regimens. Finally, only patients with limited-stage disease were considered for ASCT because there seems to be no benefit from ASCT for patients with extensive disease.

Elias et al.[111] used CCB for 19 patients who had responded to induction therapy (6 CR, 13 partial response). After HDC, 15 of 19 patients were in CR. Once patients recovered their marrow function, chest and cranial radiotherapy was delivered. Median DFS was 12 months, and OS was 53% at 2 years. The toxic death rate was 5%. The degree of response to induction chemotherapy (CR vs. partial response) was a critical factor because 57% of the patients transplanted in CR remained recurrence free at 2 years.

Brugger et al.[112] shortened induction chemotherapy cycles in an attempt to prevent the appearance of cell resistances. Fifteen of 18 patients with limited disease who responded to two cycles of etoposide, ifosfamide, cisplatin, and epirubicin were consolidated with higher doses of a similar combination, with carboplatin replacing cisplatin, plus ASCT. Subsequently, chest and cranial radiotherapy were delivered. At a short follow-up of 14 months 69% of the patients were disease free. Future research will confirm whether or not results can truly be improved when effective induction is combined with modern concurrent chest radiotherapy, followed by optimal ASCT in patients with limited-stage disease.

Contamination of Stem Cell Products with Malignant Cells in Small-cell Lung Cancer

Bilateral bone marrow aspirations and biopsies diagnose bone marrow involvement in 1734% of patients with extensive SCLC. [113] Fewer than 5% of patients present with bone marrow

metastases as the only site of extrathoracic disease. [114] However, the use of monoclonal antibodies has demonstrated marrow involvement in 50% and 77% of patients with limited and extended disease, respectively. [115] Reinoculation of clonogenic tumor cells is a potential source of relapse in SCLC, although most series show the lung as the primary site of failure. It is, however, unclear whether local relapses are entirely due to insufficient cytoreduction that cause local regrowth or if infused cancer cells contribute by homing to chest sites. If future strategies improve control of the bulky local and micrometastatic disease, the issue of seeding viable tumor will be of utmost importance.

Although the widespread use of PBPCs as the source of stem cells decreases the risk of tumor contamination, it does not ensure the reinfusion of a tumor-free cell product. Large-scale immunomagnetic purging can remove up to 3.6 logs of SCLC cells, as shown by Ball et al. [116] Clinical studies are warranted to explore the impact of in vitro purging of the stem cell product, when used in conjunction with highly effective systemic and local therapy.

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MELANOMA

As a consequence of the disappointing results using standard therapy for the treatment of metastatic melanoma, HDC with ASCT has been investigated intermittently for over 30 years in patients with this disease. [117] Early phase II clinical trials with high-dose single agents in advanced melanoma identified BCNU, melphalan, and thiotepa as the drugs where dose escalation provided the greatest increase in antitumor activity; response rates of 4055%, and CR rates of approximately 10% were reported, which appeared to be superior to responses obtained with conventional-dose therapy. [118] [119] [120] Responses from high-dose single agents, however, were brief, with a median duration of 3 months, and 15% of the patients had responses that lasted for >1 year. These trials identified patients with more limited disease, confined to skin or lymph nodes, as the ones more likely to respond, as opposed to those with visceral disease.

Subsequent studies with different drug regimens, including melphalan-BCNU, [121] CCB,[122] or diethyl-triazeno-imidazole-carboxamide (DTIC)-melphalan or ifosfamide,[123] demonstrated high response rates, but did not substantially improve long-term DFS or OS rates.

Because melanoma is one of the most immunogenic human tumors, the combined use of immunotherapy with ASCT is an attractive strategy for this disease. The use of PBPCs, which produce a more rapid immune reconstitution than bone marrow, may be necessary for subsequent biologic therapy to be effective. The use of adoptive immunotherapy has proved to be tolerable after ASCT, [124] and clinical trials evaluating this strategy are underway.

Because metastatic melanoma patients with limited disease appeared to benefit most from ASCT, it seemed logical to explore its use in the adjuvant setting for high-risk patients. A small randomized trial conducted at Duke University compared immediate treatment with CCB plus ASCT to observation with CCB at relapse, in 39 patients with melanoma involving a primary skin site and five or more proximate lymph nodes at initial diagnosis. [125] Time to progression was 35 weeks for patients on the HDC arm versus 16 weeks for those on the observation arm, which was not statistically significant. No differences in OS were noted, which was partly due to the successful salvage with HDC of relapsed patients, with a 66% response rate and a 33% CR rate.

Future directions include exploring the use of ASCT in poor-prognosis melanoma patients with less extensive disease. Examples include patients with recurrent disease that can be rendered disease free by standard local or systemic treatment. Although ASCT cannot be considered standard treatment for melanoma, it remains a useful tool to cytoreduce the tumor and attain a minimal residual disease state that may allow biologic therapies to be effective.

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BRAIN TUMORS

After surgery and radiotherapy for malignant tumors of the central nervous system (CNS), most patients experience a median survival of <1 year and die within 18 months of diagnosis. The addition of chemotherapy offers a modest survival advantage, especially for younger patients and for those with anaplastic astrocytoma, as opposed to glioblastoma multiforme.[126] Still, the majority of patients die within the first 2 years. Standard salvage therapy is ineffective for relapsed brain tumors. The existence of the blood-brain barrier limits the therapeutic armamentarium to small, lipid-soluble and nonpolar drugs. Of these, BCNU has the highest activity against glial tumors and constitutes the mainstay of standard chemotherapy for brain neoplasms. [127]

Initially ASCT for brain tumors used BCNU, [128] [129] [130] as well as other drugs with adequate CNS penetration and evidence of antitumor activity at standard doses, such as thiotepa.[131] Subsequently, drugs such as cyclophosphamide, etoposide, or carboplatin, suitable for ASCT and exhibiting good CNS penetration at high doses, were also tested.

In the first trials using high-dose BCNU for patients with refractory and progressive high-grade glioma, close to 50% of the patients responded, although most responses were short-lived.[128] [129] Based on these results, high-dose BCNU was subsequently tested as part of first-line therapy, along with surgery and radiotherapy, which initially seemed to improve survival when compared to historical controls. [130] However, when patients treated with ASCT were compared to matched patients from historical series treated with conventional therapy, the outcomes were similar. [132]

More recent studies have used combination chemotherapy. Thiotepa-etoposide has produced response rates of 2030% and 1428% long-term DFS in patients with recurrent brain tumors.[133] [134] Survival was significantly improved in patients treated with minimal residual disease. Innovative approaches include the combination of ASCT with intra-arterial chemotherapy, which in single-institution studies has shown promising results for selected patients. [135]

Since the initial trials, minimal data have been reported using ASCT for brain glial tumors in adults. Future prospects will require more active agents and the possible synergy between ASCT and biologic therapies that, as with melanoma, will require major prior cytoreduction.

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OTHER SOLID TUMORS

A few single-institution series with small numbers of patients have been reported on the use of ASCT for metastatic soft tissue sarcoma. When used as initial therapy for untreated patients[136] or salvage treatment for refractory disease,[137] CRs are reported, but they do not correlate with a survival benefit. However, when used as consolidation for selected patients who have responded to induction treatment, particularly those in CR, approximately 30% achieve long-term DFS. [138] [139] [140]

More than 100 patients with metastatic colorectal cancer have been treated with ASCT, most commonly with single-agent alkylators, and in particular melphalan. [141] [142] Although the overall response rate is approximately 50%, the CR rate is low, and there is neither an OS or DFS benefit for these patients.

Patients treated with ASCT for gastric, pancreatic, nasopharyngeal, uterine, cervical, head and neck, renal cell, and bladder carcinomas have been reported. The majority of those patients

TABLE 88-6 -- Summarized Results of High-Dose Chemotherapy with Autologous Stem Cell Transplantation for Breast Cancer, Ovarian Carcinoma, and Nonseminomatous Germ Cell Tumor

Tumor Setting	Long-term DFS	References
Breast		
High-risk 49 nodes	>70%	[46]
High-risk 10+ nodes	5665%	[44] [45]
High-risk inflammatory	>70%	[50] [51]
Metastatic, chemosensitive disease	1525%	[12] [13] [14]
Ovary		
Relapsed refractory disease	<5%	[72] [74]
As part of multimodal first-line therapy for poor-risk disease	2555%	[76] [77] [79]
NSGCT		
Refractory	15%	[83]
First relapse	3540%	[84] [85]
Second relapse	15%	[82]
Up-front for poor-risk disease	50%	[95]

DFS, disease-free survival; NSGCT, nonseminomatous germ cell tumor.

were included in phase I ASCT trials. Response rates are typically low, and the responses are short-lived.

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CONCLUSIONS

Over the past 10 years, ASCT for the treatment of patients with solid tumors has been characterized by a steady decrease in treatment-related mortality, as well as a steady improvement in the activity of high-dose regimens. The use of ASCT in combination with standard-dose induction chemotherapy, radiotherapy, and surgery has also been increasing. The addition of effective immunotherapy to ASCT holds promise, not only for the demonstrably immunogenic tumors like melanoma, but also for breast cancer, where clinical trials are in progress.

Encouraging data from ASCT trials conducted in patients with solid tumors, are summarized in Table 88-6 . These were primarily phase II trials conducted at single institutions.

Several ongoing prospective randomized trials comparing the best standard therapy to widely used high-dose regimens are actively accruing patients in North America and Europe. The next few years will likely bring exciting advances in use of HDC for patients with the solid tumors discussed in this chapter.

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Chapter 89 - Unrelated Donor Stem Cell Transplantation Therapy

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INTRODUCTION

Allogeneic stem cell transplantation (SCT) is an accepted form of treatment that can provide a cure for patients with hematologic cancers, syndromes of bone marrow failure, and congenital disorders of the lymphohematopoietic system. [1] [2] Before the establishment of several large donor registries, however, this approach was largely restricted to patients with an HLA-identical or HLA one-antigen mismatched family member who was willing to be a donor. [3] [4] During the 1980s and 1990s, investigators explored the use of other sources of hematopoietic stem cells (HSC) including haploidentical HLA-matched family members and phenotypically HLA-matched placental cord blood or marrow from unrelated donors. Stem cell transplants from haploidentical family members with more than an HLA one-antigen mismatch are associated with a very high risk of transplantation-related complications such as graft rejection or graft-versus-host disease (GVHD). The introduction of T-cell depletion for prevention of GVHD permitted successful transplantations in children with severe combined immunodeficiency, [5] but was largely unsuccessful in patients with leukemia. More recently published studies indicate that graft rejection can be prevented by additional immunosuppression for leukemic recipients of marrow grafts derived from haploidentical family members.[6] [7] However, these observations await confirmation in larger multicenter studies.

The first successful transplantations using stem cells obtained from unrelated donors were reported in the mid-1970s. [8] [9] [10] [11] [12] Despite these successes, the use of marrow from closely HLA-matched unrelated donors was limited by the lack of accessible donor registries of sufficient size to permit identification of a donor. The worlds oldest registry of volunteer unrelated marrow donors, the Anthony Nolan Bone Marrow Trust, was established in 1974 by the mother of a child with Wiskott-Aldrich syndrome. By 1983 more than 50,000 HLA typed volunteers were available through this registry. [13] In 1986 the National Marrow Donor Program (NMDP) was established in the United States to facilitate donor searches and marrow procurement for patients lacking an HLA-identical related donor. [14] The NMDP is a nonprofit corporation and holds a contract from the U.S. Department of Health and Human Services to operate the congressionally mandated National Bone Marrow Donor Registry. As of early 1998 the cumulative number of HLA-A and -B typed donors registered with the NMDP through U.S. and international donor centers exceeded 3.1 million (Table 89-1) and the NMDP could facilitate approximately 110 transplants per month. Worldwide there are now more than 4.5 million unrelated donors listed in 43 registries in 34 countries. The majority of donors has been typed only for HLA-A and -B. HLA-DR typing is available on about 1.8 million donors worldwide, of which, 1.3 million are registered in the NMDP (Table 89-1).

In the late 1980s, Bone Marrow Donors Worldwide (BMDW) was organized to provide a centralized capacity for international identification of volunteer unrelated donors.[15] BMDW maintains a database of summary HLA typing data on donors from around the world and also provides a search process for rapid identification of donors with specific HLA types. The entire BMDW database is currently available on-line via the World Wide Web (http://bmdw.leidenuniv.nl/).

In general, unrelated donors are volunteers between the ages of 18 and 61 years who are in good health, not pregnant, and not excessively overweight. Although transplant physicians may search multiple registries, most recipients of unrelated stem cells in the United States have their donor identified and their marrow procurement facilitated by the NMDP. At present,

TABLE 89-1 -- Donors in the National Marrow Donor Program Registry

Racial or Ethnic Group	Number HLA-A, -B and -DR Typed	Total Number
American Indian/Alaska Native	28,730	41,262
Asian/Pacific Islander	127,311	177,563
Black, including African American	173,494	240,545
Caucasian	627,390	1,812,630
Hispanic/Latino	151,716	220,988
Multiple race	17,802	26,461
Other/declines/unknown	197,584	581,865
Total donors	1,324,027	3,101,314

Source: National Marrow Donor Program, February 28, 1998.

Figure 89-1 Steps in the National Marrow Donor Program search process. The search process begins with preliminary searches that are available at no charge to any licensed physician. Formal searches are initiated and managed by NMDP Transplant Centers. Donors are identified through confirmatory typing performed on donors who are HLA-A, -B, and -DR matched with the proposed recipient. Once the optimal donor is identified, a request for donor work-up is submitted.

the unrelated donor transplant approach is limited by logistical difficulties in identifying a suitably HLA-matched donor and by the relatively high rate of morbidity and treatment-related mortality associated with the unrelated donor stem cell transplant procedure.

Any physician can request a free preliminary search of the NMDP Registry (Fig. 89-1). However, a formal search must be initiated by a member of the NMDP Transplant Center. The purpose of the formal search is to narrow the list of prospective donors to the single donor who is deemed most suitable for the proposed recipient. When HLA-A, -B and -DR matched potential donors are available, the Transplant Center will repeat the HLA typing on selected donors to confirm that the Registry typing is correct and to obtain the typing at a higher level of resolution (as described in the following sections). If the proposed recipients only potential matches reside among the HLA-A, -B typed donors, it is first necessary to complete HLA-DR typing in an attempt to identify an HLA-DR matched donor. In the NMDP

experience, it is uncommon that HLA-DR typing leads to identification of a suitable donor and subsequent transplantation; only 5% of NMDP transplants use donors selected from the HLA-A, -B typed pool.

If the formal search leads to identification of an acceptable match and the patient remains eligible for SCT, a work-up of the donor begins. This includes a detailed medical history and physical examination, laboratory testing to assess the health of the donor and the potential for transmission of infectious diseases, as well as thorough counseling of the donor. If the medical evaluation confirms the donors good health, and the donor agrees to proceed, the donor signs an Intent to Donate form and a marrow collection date is scheduled. For the convenience of the donor and in order to maintain confidentiality, the marrow is harvested at a member Collection Center that is located near the donors home. The freshly collected marrow is transported by courier to the recipients institution for transplantation, usually within 24 hours.

Currently 80% of all preliminary searches submitted to the NMDP identify at least one potential HLA-A, -B, and -DR identical match. Among patients who initiated a formal search in 1995 and 1996 and subsequently received an SCT facilitated by the NMDP, 5% of SCTs occurred within 1.9 months of the initiation of a formal search and 50% were within 4.0 months (Table 89-2). One must consider the length of time involved in the search process in deciding to search for an unrelated donor. Although

TABLE 89-2 -- Time from Formal Search to Transplant (Searches Begun in 1995 and 1996)

5% were within 1.9 months
50% were within 4.0 months
95% were within 16.8 months

Percentiles adjusted by Kaplan-Meier method based on transplants occurring within 24 months of formal search.

the time interval may be acceptable for patients with stable phase chronic myeloid leukemia (CML), patients with advanced leukemia or unstable medical conditions are likely to require alternative therapy before a donor is identified and may be too ill to undergo a transplantation at the time a donor is ready for harvest.

The decision to undertake an unrelated donor stem cell transplantation is complex and involves many factors that are independent of donor availability. These include the quality of the HLA match, recipient factors, and donor factors. The patients diagnosis, stage of disease, age, and major organ function at the time the donor is ready for harvest are the major factors. Secondary factors include the donors age, donors prior exposure to cytomegalovirus, and the donors pregnancy and transfusion history.

HISTOCOMPATIBILITY AND DONOR SELECTION

The major human histocompatibility complex, the HLA antigen system, is the primary immunologic barrier to successful stem cell transplantation. The HLA antigens are effective stimulators and targets of GVHD and graft rejection. Clinical outcomes of stem cell transplantation, particularly those involving unrelated donors, are dependent on optimizing the histocompatibility matching between the patient and the donor. The HLA genes are clustered in a short region on chromosome 6. The cluster of genes constitutes an HLA haplotype and tends to be inherited en bloc, which accounts for the 25% likelihood of HLA matching between any two siblings (Fig. 89-2).

Figure 89-2 Inheritance of HLA haplotypes. The HLA genes, clustered in a short stretch on chromosome 6, are inherited en bloc. Mothers two HLA haplotypes (arbitrarily labeled I and II) can combine with Fathers (arbitrarily labeled A and B) in only four ways: I-A, I-B, II-A, and II-B. Thus, between any two siblings, the likelihood of a match is 25%. With n siblings, the likelihood of a match is 1 the likelihood of no match, or 1 $(0.75)^n$.

TABLE 89-3 -- Patients Proceeding to Unrelated Marrow Transplantation, 19871998

Racial or Ethnic Group	Formal Searches	Transplantation
American Indian/Alaska Native	103	35 (34%)
Caucasian	16,282	5,604 (34%)
Hispanic/Latino	1,209	349 (29%)
Black, including African American	1,229	245 (20%)
Asian/Pacific Islander	700	147 (21%)
All others	2,011	341 (17%)
Total patients	21,534	6,721 (31%)

Source: National Marrow Donor Program, February 28, 1998.

The emergence of unrelated donor SCT as a therapeutic option for many patients has had an important influence on the development of HLA typing methods to identify histocompatibility alleles. [16] In the 1980s and early 1990s, HLA compatibility was determined by serology for HLA-A, -B, and -DR, and by the mixed lymphocyte culture (MLC). The MLC proved not to predict the development of GVHD or transplantation outcome, [17] and the test is no longer used for donor selection in most transplant centers. Techniques such as one-dimensional isoelectric focusing and DNA sequencing demonstrated that there are several polymorphisms within a single serologic specificity. Unrelated donors who are selected to be phenotypically HLA-identical by serology, may be HLA-non-identical when tested using DNA-based techniques. There is marked genotypic polymorphism for both HLA-A and HLA-B. As many as 83 HLA-A alleles and 186 HLA-B are represented by 29 HLA-A and 59 HLA-B serotypes.[18] These genotypic polymorphisms are unimportant for transplantation between HLA-identical siblings. HLA-matched siblings, who inherit identical parental chromosomes and thus, polymorphisms, are always genotypically identical (Fig. 89-2). In unrelated donor transplantation, however, these differences are biologically important because donor-recipient pairs mismatched for HLA-DR at the allelic level correlate with a higher incidence of severe GVHD and a higher risk of transplant-related mortality. [19] [20] One study has suggested that allelic disparities at the HLA-A and -B loci detected by DNA-based techniques may also be associated with a higher incidence of GVHD. [21] In a recently completed study, it was demonstrated that only 53% of donors matched by serology for HLA-A and -B and DNA typing for HLA-DR remained HLA-A, -B, and DRB1 matched when tested by DNA techniques for each of these loci. [22]

HLA genes are not randomly distributed. Rather, linkage disequilibrium occurs, resulting in common (frequently observed) and uncommon HLA haplotypes within and between various racial and ethnic groups. Patients with one or two common haplotypes are far more likely to identify a matched unrelated donor than a patient with two uncommon haplotypes, and an individual is most likely to find a donor within his or her own racial and ethnic group. An analysis of the NMDP Registry disclosed the highest rate of HLA polymorphism among black donors and the lowest rates among Asian/Pacific Islander and Caucasian donors. [23] Among minority racial and ethnic groups, the HLA polymorphism and limited numbers of registered donors (Table 89-1) reduce access to unrelated donor transplantation. The percentage of formal searches of the NMDP resulting in a transplantation procedure is outlined in Table 89-3 . Higher percentages of Caucasian and Hispanic patients undergo transplantation than African Americans and Asian/ Pacific Islanders.

To date, most reports of clinical outcome have included patients and donors typed by serology at HLA-A, -B, and -DR. In the more recent reports patients and donors have been typed by serology for HLA-A and HLA-B, and DNA-based typing techniques for HLA-DRB1. If retrospective studies confirm improved clinical outcome for patients matched at HLA-A, -B, and -DRB1 with their donors as determined by DNA-based methods, it is likely that future donors will be selected based on their DNA genotypes rather than by phenotypes determined by serology. It is unlikely that fully DNA-matched donors will be identified for many patients; therefore, it will be necessary to determine which mismatched allele combinations may be tolerated. Alternatively, transplantation physicians may need to devise approaches such as T-cell depletion for prevention of GVHD to be used when the donor and recipient are not HLA-identical.

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OUTCOME OF UNRELATED DONOR STEM CELL TRANSPLANTATION

Factors Affecting Transplant Outcome

Many factors affect transplantation outcome (Table 89-4). As reviewed previously, unrelated donor recipient pairs are usually phenotypically but not genotypically matched at HLA class I (HLA-A, -B, -C) and HLA class II loci (HLA-DR and -DQ). Additionally, multiple mismatches exist for minor histocompatibility antigens. [24] These are not currently easily defined and are not routinely identified for either family member or unrelated stem cell transplant donor-recipient pairs. Although disease stage at transplant is the dominant prognostic factor, within a homogeneous group of patients transplanted with the same disease category, the age of the patient and the level of histocompatibility between the patient and the donor are probably the most important factors affecting outcome of the transplant.

The probability of graft failure after an infusion of stem cells from a family member correlates with the degree of HLA incompatibility [4] [25] [26] and the use of T-cell depletion to prevent acute and chronic GVHD. [17] [26] [27] [28] All studies involving unrelated donor SCT have reported a higher incidence of nonengraftment and secondary graft failure following an unrelated donor transplant as compared to an HLA-identical sibling donor transplantation. [6] [17] [29] In most studies the probability of engraftment is 9095%. [17] [27] [30] HLA disparity between the patient and the donor is the single most important factor affecting the probability of engraftment. [17] [31] [32] [33] [34] The rates of primary and secondary graft failure are highest in recipients of unrelated donor stem cells with serological mismatch at HLA-A or -B. Little evidence exists concerning the role of HLA-C locus antigens in SCT. However, one study suggests that HLA-C mismatch is associated with a higher incidence of graft failure after an unrelated transplant [33] and others have demonstrated that disparities at HLA-C are associated with both graft rejection and GVHD following unrelated donor SCT. [34] [35]

The major effect of donor-recipient HLA disparity is on the incidence and severity of GVHD and the risk of transplant-related mortality associated with this complication. The probability of developing severe acute GVHD is 6595%. [17] [30] [31] The level of disparity in the class II region (HLA-DR and -DQ)

TABLE 89-4 -- Factors Affecting Transplant Outcome

Diagnosis and stage of disease
HLA match grade
Age of recipient
Age of donor
Time from diagnosis to transplant
Prior cytomegalovirus (CMV) exposure

TABLE 89-5 -- Transplants by Diagnosis (Malignant Diseases as of July 1997)

Disease	Number (%)
Chronic myelogenous leukemia	2,078 (35%)
Acute lymphoblastic leukemia	1,179 (20%)
Acute myelogenous leukemia	1,126 (19%)
Myelodysplastic and related syndromes	503 (9%)
Non-Hodgkin lymphoma	141 (2%)
Other leukemia	137 (2%)
Plasma cell disorders	68 (1%)
Hodgkin disease	18 (<1%)
Other malignancy	6 (<1%)

is the single most important variable affecting the development of GVHD. [19] [20] Matching HLA-DRB1 alleles of the donor and recipient decreases the risk of acute GVHD and improves survival after unrelated donor SCT. [19] [20] [30] Matching for HLA-DRB1 and -DQB can reduce the risk of Grade II to IV acute GVHD following an unmodified stem cell allograft from 7338% among recipients mismatched for either allele. [36] Interestingly, this effect was not observed among recipients of T-cell depleted allografts, as all recipients experienced a low level of GVHD (10%) in this study. [36] The overall incidence of chronic GVHD in most studies is approximately 50%. [17] [30] [31] The extent of HLA matching has had no detectable association with the risk of clinical chronic GVHD. However, the risk is independently associated with grafts derived from women with a history of pregnancy and is increased in older patients, especially those with CML. [30] [37]

Several reports indicate that both recipient and donor age correlate directly with the risk of GVHD, transplantation-related mortality, or both. [17] [30] [31] [37] [38] Younger patients tolerate GVHD and its treatment better than older patients. There is a significant association between graft failure and increasing donor age. [32] The explanation for this observation is unclear. But the impact of graft failure is significant as only 25% of individuals with graft failure survive long-term. [39]

Even when patients have engrafted without significant GVHD, recipients of unrelated allografts are at very high risk of developing serious infectious complications. [17] [40] [41] [42] Death from serious infections is significantly more frequent following unrelated donor stem cell allografts; cytomegalovirus (CMV) is the most common viral infection observed. [41] Before the use of ganciclovir prophylaxis at the time of engraftment, systemic CMV infections were almost uniformly fatal.

Outcome for Patients with Malignant Disorders

The majority of unrelated donor stem cell transplants are performed for patients with a malignant disorder. The diagnoses requiring transplant therapy reported to the

NMDP are outlined in Table 89-5 . The timing of the procedure in relation to disease phase or stage correlates strongly with outcome. [17] [43] [44]

Chronic Myeloid Leukemia

The majority of unrelated allografts have been performed for treatment of CML. Multiple studies have shown this approach to be curative for CML. [17] [30] [37] [45] [46] The Kaplan-Meier survival estimates for NMDP recipients with CML transplanted between 1987 and July 1997 are shown in Table 89-6 and Figure 89-3 . These include all donor recipient pairs regardless of their level of HLA disparity. The best results reported to date have been observed for patients in primary chronic phase, less than 50 years of age who received an allograft from an HLA-matched donor defined by serologic methods for HLA-A and -B and DNA typing methods for HLA-DRB1. The Kaplan-Meier estimate of survival at five years for these patients is 74% (95% confidence interval of 6280%). [30] An unmodified unrelated allograft is probably not indicated for patients in blastic phase CML. However, in one report approximately 50% of recipients of marrow depleted of T cells with the monoclonal antibody T10B9 and complement while in accelerated or blastic phase survive long-term. [45] Preliminary analysis of the first 196 unrelated donor BMTs for CML facilitated by the NMDP indicated that T-cell depletion of the stem cell graft reduces early mortality, but has not yet been shown to improve long-term survival

TABLE 89-6 -- NMDP Transplant Outcomes for Chronic Myelogenous Leukemia (July 1997)

Disease	Number of Transplants	2-Year Survivala[a] with 95% CI
CML 1st chronic phase (CP)	1,106	46% ± 3%
CML 2nd CP and accelerated phase	451	26% ± 5%
CML blast phase	106	11% ± 6%

[a]Kaplan-Meier estimate.

Figure 89-3 Kaplan-Meier survival estimates of unrelated donor stem cell transplant therapy for chronic myeloid leukemia. Results are shown for 1,663 patients transplanted between 1987 and July 1997. Patients are stratified by stage of disease. Survival of patients with less-advanced disease is superior. (Tick marks represent the time of most recent follow-up for surviving patients.)

TABLE 89-7 -- NMDP Transplant Outcomes for Acute Leukemias (July 1997)

Disease	Number of Transplants	2-Year Survivala[a] with 95% CI
AML 1st complete remission (CR)	158	39% ± 9%
AML 2nd CR	241	33% ± 7%
AML 3rd or greater CR or relapse	522	15% ± 4%
ALL 1st CR	187	43% ± 8%
ALL 2nd CR	312	40% ± 6%
ALL 3rd or greater CR	192	28% ± 7%
ALL relapse	253	12% ± 5%

[a]Kaplan-Meier estimate.

(*P* = 0.1).[37] For children with CML, unrelated allografts are clearly curative with disease-free survival estimates of 5080% depending upon the stage of disease and level of HLA disparity between the patient and the donor. [47] [48] [49]

Following an unrelated allograft for CML, the probability of relapse is very low (<10%). [30] [37] [45] A low relapse rate is observed even with the use of partial T-cell depletion for prevention of GVHD.[45]

As treatment with an unrelated allograft can be curative for patients with CML, but is associated with 2030% early mortality, timing of the procedure is now the major issue. Results of transplants performed early in the course of the disease, particularly in chronic phase patients within the first year of diagnosis, are superior to those performed later.[30] [37] However, present treatment approaches using interferon have produced clinical and sometimes cytogenetic remission, which can delay the progression of disease and prolong survival. Despite these advances, a recent decision analysis reported by Lee et al. supports the use of early transplantation from unrelated donors for most patients with CML. [50]

Acute Leukemia

Analysis of the first 156 patients with acute leukemia whose unrelated allograft was facilitated by the NMDP, revealed that among patients with acute leukemia in first or second remission, the probability of disease-free survival was 0.45 ± 0.13, which was superior to that among patients with more advanced disease (0.19 ± 0.08), *P* < 0.001.[17] Since that publication, additional patients within each category of disease and stage have received unrelated allografts (Table 89-7).

The Kaplan-Meier survival estimates for NMDP recipients with acute lymphoblastic leukemia (ALL) transplanted between 1987 and July 1997 are shown in Figure 89-4 . Survival is stratified by disease. In virtually every series, younger patients treated in first or second remission experience superior survival rates. [47] [51] [52] [53] [54] In fact, several series have demonstrated results identical to those expected for children with ALL transplanted with stem cells derived from a sibling donor. [55] [56] [57] [58]

The Kaplan-Meier survival estimates for NMDP recipients with acute myelogenous leukemia (AML) are depicted in Figure 89-5 . Remission at the time of transplantation is the most favorable prognostic factor. In one series, a higher cell dose was found to be favorable especially in patients transplanted in remission. [44] Patients receiving a mononuclear cell dose greater than the median (3.65×10^8 cells/kg) experienced a more rapid engraftment, less severe GVHD, and a lower non-relapse mortality.

An unrelated donor allograft is not usually the first line therapy for patients with acute leukemia. Improvements in conventional induction and maintenance chemotherapy have increased the numbers of patients cured by these therapies. Certain prognostic factors, however, can identify patients likely to fail front-line therapy. Patients with Philadelphia positive ALL and patients induced into a first remission with difficulty should be considered for an unrelated donor allograft. [52]

Outcome for Nonmalignant Disorders

Myelodysplasia (MDS) and Aplastic Anemia (AA)

Transplants performed for nonmalignant disorders are outlined in Table 89-8 . Figure 89-6 and Table 89-9 depict the Kaplan-Meier survival estimates for NMDP patients with MDS and AA. These results are representative of other published series of patients, even children, with these diseases. [59] [60] [61] [62] [63] The probability of survival after an unrelated allograft is probably superior to that with other available therapies, since in qualifying for transplantation protocols involving unrelated

donors, it is likely that these patients had not responded to immunosuppression or cytokine therapy.

Other Nonmalignant Disorders

The most frequent diagnoses encountered in this group of patients are immunodeficiency disorders including severe

Figure 89-4 Kaplan-Meier survival estimates of unrelated donor stem cell transplant for acute lymphoid leukemia. Results are shown for 921 patients transplanted between 1987 and July 1997. Patients are stratified by stage of disease. Survival of patients in first or second remission is superior to that of patients with more advanced disease. (Tick marks represent the time of most recent follow-up for surviving patients.)

Figure 89-5 Kaplan-Meier survival estimates of unrelated donor stem cell transplant for acute myeloid leukemia. Results are shown for 944 patients transplanted between 1987 and July 1997. Patients are stratified by stage of disease. Survival of patients in first or second remission is superior to that of patients with more advanced disease. (Tick marks represent the time of most recent follow-up for surviving patients.)

TABLE 89-8 -- Transplants by Diagnosis (Nonmalignant Diseases, July 1997)

Severe aplastic anemia	263 (4%)
Inherited metabolic disorders	157 (3%)
Inherited immune system disorders	120 (2%)
Inherited erythrocytic disorders	73 (1%)
Histiocytic disorders	54 (1%)
Other nonmalignant diseases	23 (<1%)
Inherited platelet disorders	6 (<1%)

TABLE 89-9 -- NMDP Transplant Outcomes for Nonmalignant Diseases

Disease	Number of Transplants	2-Year Survival[a] with 95% CI
Severe aplastic anemia	212	39% ± 7%
Myelodysplastic and related syndromes	386	34% ± 5%
Other nonmalignant diseases	332	45% ± 6%

[a]Kaplan-Meier estimate.

Figure 89-6 Kaplan-Meier survival estimates of unrelated donor stem cell transplant for severe aplastic anemia, other nonmalignant diseases, and for myelodysplastic syndromes and related disorders. Results are shown for patients transplanted between 1987 and July 1997. The number of patients transplanted in each category is shown. (Tick marks represent the time of most recent follow-up for surviving patients.)

combined immunodeficiency (SCID) and Wiskott-Aldrich syndrome (WAS), Fanconi Anemia (FA) and metabolic disorders including Hurlers syndrome and adrenoleukodystrophy. Kaplan-Meier survival estimates for these disorders are presented in Figure 89-6 and Table 89-9 . The results of unrelated donor allografts have been particularly promising for patients with SCID and WAS. Up to 75% of patients survive disease-free long-term. [64]

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SUMMARY

The majority of people who are candidates for allogeneic stem cell transplantation lack HLA-matched sibling donors. For these individuals, alternative stem cell sources include autologous stem cells, partially matched related donors, unrelated donor cord blood, and volunteer unrelated stem cell donors. The last of these has been made feasible through the emergence of internationally active registries of HLA-typed volunteers. Transplants using unrelated stem cell donors produce successful outcomes, but not without significant risks of transplant-related morbidity and mortality. Improvements in the availability and matching of donors, advances in HLA typing technologies, and refinement of transplantation procedures are the keys to improved safety and effectiveness of unrelated donor SCT.

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ACKNOWLEDGMENTS

The authors thank Nancy Morgan and Cynthia Sangeorgio for assistance with the preparation of the manuscript.

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Chapter 90 - Placental and Umbilical Cord Blood Transplantation

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INTRODUCTION

Transplantation of allogeneic hematopoietic stem cells (HSC) derived either from bone marrow or placental-umbilical cord blood (P-UCB) has been successfully used in the treatment of patients with high-risk or recurrent hematologic malignancies, bone marrow failure syndromes, selected hereditary immunodeficiency states, and metabolic disorders. A major limitation to the successful use of hematopoietic stem cell transplant (SCT) therapy has been the unavailability of suitable donors. Although there are currently more than 1.5 million human leukocyte antigen (HLA)-A-, B-, and DR-typed marrow donors registered in marrow donor registries worldwide, 50% of all patients requiring transplant therapy are still unable to find a suitably HLA-matched donor. [1] Furthermore, patients of racial and ethnic populations of non-Northern European descent [1] have an even lower probability of finding a suitable donor. To alleviate the shortage of suitable donors and reduce the length of the marrow donor search process, Rubinstein et al. (New York), Bertolini et al. (Milano), and Wernet et al. (Dusseldorf) initiated Placental Blood Banking Programs almost simultaneously in 1993. [2] As of December 1997, approximately 10,000 HLA-A-, B-, and DR-typed P-UCB grafts have been collected, tested, and cryopreserved for clinical use in transplantation worldwide.

In 1996, Kurtzberg et al. [3] and Wagner et al. [4] reported preliminary clinical results of unrelated donor P-UCB transplantation in 25 and 18 patients, respectively. Together, the clinical data suggested that banked unrelated donor P-UCB contained sufficient numbers of HSC and progenitor cells to achieve engraftment with lower than anticipated risk of acute graft-versus-host disease (GVHD) at least in children and smaller or younger adults. More recently, Gluckman et al. [5] reported the results of unrelated donor P-UCB transplantation in 65 patients, as reported to the EuroCord Registry. Gluckman et al. [5] reported that (1) higher graft nucleated cell dose and HLA identity predicted more rapid rate of neutrophil engraftment, (2) recipients cytomegalovirus (CMV) seropositive status predicted higher risk of acute GVHD, and (3) recipients CMV seronegative status and higher graft nucleated cell dose predicted better survival after unrelated donor P-UCB transplantation.

As a result of these observations, numerous laboratory investigators have initiated studies to understand the biologic characteristics of the primitive hematopoietic progenitors in P-UCB as well as the immune system in the neonate. Ontologically, hematopoiesis begins in the ventral aspect of the fetal aorta [6] and primitive yolk sac early after conception. Following a brief hepatic phase, it enters the bone marrow space at the end of the second gestational trimester where it remains almost exclusively throughout adulthood. [7] [8] It has long been known that human P-UCB contains hematopoietic progenitor cells at high frequency. The frequency of granulocyte-macrophage progenitor cells equals or exceeds that of adult bone marrow. [9] [10] [11] [12] Within hours of birth, however, the progenitors abruptly leave the neonatal circulation [11] and are present only at very low frequency as in unmobilized adult peripheral blood.

As of March 1998, P-UCB from sibling and unrelated donors has been used to reconstitute hematopoiesis in an estimated 600 patients with malignant and nonmalignant disorders. What still

remains to be defined is the breadth of applicability of this HSC source. Specifically:

1. Are there adequate numbers of HSC and progenitor cells to routinely engraft adult-size recipients?
2. Is the neonatal immune system sufficiently different from that of the adult to allow greater HLA disparity between donor and recipient without concomitant increases in graft rejection, GVHD, or opportunistic infection?

This chapter reviews the current state of knowledge regarding properties of the hematopoietic cells present in P-UCB and the neonatal immune system as well as the clinical results with P-UCB transplantation. Factors potentially influencing the probability of hematopoietic recovery and engraftment, risk of GVHD, and survival are also discussed.

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HISTORICAL BACKGROUND

Use of human P-UCB as a source of transplantable HSC was first suggested in 1983 by Professor Edward A. Boyse in conversations with Dr. Hal Broxmeyer and Ms. Judith Bard. In 198485, the hypothesis that P-UCB contained long-term reconstituting HSC was first tested in a murine model. [13] [14] In the first of four experiments, 7-week-old (B6 × A-T1a^{b})F1 hybrid males were irradiated with 862.8 cGy and then transplanted with heparinized whole blood obtained from near-term (B6-T1a ^{a} × A)F1 mouse embryos. Thirty-day survivors were subsequently typed for the T1a markers distinguishing donor from recipient lymphohematopoietic cells. Animals demonstrated repopulation with donor cells, showing that lethally irradiated mice could be reconstituted with blood from near-term embryos. Three additional experiments evaluated the engraftment potential of (1) smaller volumes of blood from near-term embryos, (2) blood from neonatal donors <24 hours of age, and (3) smaller volumes of blood from neonatal donors. Together, these studies demonstrated that near-term and neonatal blood contained sufficient numbers of stem and progenitor cells to allow early lymphohematopoietic recovery and engraftment of donor cells.

While Boyse et al. [14] provided data supporting the hypothesis that small quantities of neonatal blood were sufficient for at least short-term hematopoietic recovery of donor origin in a murine model, Broxmeyer et al. [11] [12] established practical and efficient methods of collecting and storing P-UCB for clinical use. Broxmeyer et al. [11] [12] evaluated the nucleated cell and progenitor content and sterility of >100 P-UCB specimens before and after cryopreservation. Hematopoietic progenitors from P-UCB remained viable at 4°C or 25°C for at least 3 days after collection. This observation confirmed that cell viability during transport between hospitals would not be compromised before cryopreservation. Moreover, the collection of P-UCB proved to be remarkably simple. Obstetricians and nurse midwives previously untrained in the collection of P-UCB learned the collection technique in a matter of minutes. These early results suggested that P-UCB could be easily obtained and would be a sufficient source of HSC and progenitor cells for an autologous or allogeneic recipient.

An international collaboration, including Drs. Eliane Gluckman, Hal Broxmeyer, Arleen Auerbach, Gordon Douglas, Joanne Kurtzberg and others, led to the first human P-UCB transplant in Paris on October 6, 1988. [15] These investigators were the first to demonstrate unequivocally that the pluripotential HSC existed in human P-UCB. This conclusion was based on the observation that in a child with Fanconi anemia, complete chimerism was sustained after transplantation in multiple lymphohematopoietic lineages.

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CHARACTERIZATION OF P-UCB PROGENITOR CELLS

Characterization of the hematopoietic progenitor cell population circulating in P-UCB has revealed similarities and differences with those in adult marrow. Saeland et al.[16] extensively characterized the CD34+ population in P-UCB. Besides the lack of a distinct subpopulation of CD10+/CD34+ B-cell precursors typically found in adult marrow, expression of adhesion receptors (e.g., leukocyte function-associated antigens 1 and 3, intercellular adhesion molecule-1, hematopoietic cell adhesion molecule, and leukocyte adhesion molecule) and other differentiation antigens (e.g., CD33, HLA-DR) were remarkably similar between P-UCB and adult marrow.

Through physical and immunologic parameters, it has been possible to separate primitive from more committed progenitors. It has long been known that immunofluorescent staining with anti-CD34 and anti-HLA-DR allows separation of more committed hematopoietic progenitors (CD34+/HLA-DR+ cells) from a more primitive (CD34+/HLA-DR) subpopulation in adult marrow. Traycoff et al. [17] demonstrated that there is a higher proportion of HLA-DR cells expressing the CD34+ phenotype in P-UCB. Notably, high proliferative potential colony-forming cells (HPP-CFC) and long-term culture-initiating cells (LTC-IC) in P-UCB did not segregate with the HLA-DR subpopulation of CD34+ cells. In contrast to adult marrow, the majority of HPP-CFC were detected in the CD34+/HLA-DR+ subpopulation (ninefold greater than the number found in the HLA-DR subpopulation) and the quantity of LTC-IC, as measured by production of burst forming unit-erythroid (BFU-E) and colony forming unit-granulocyte, macrophage (CFU-GM) after 5 weeks in culture, was consistently higher in the CD34+/HLA-DR+ subpopulation. Similarly, Dugan et al.[18] have demonstrated that the frequency of LTC-IC by limiting dilution analysis is identical between the two subpopulations of CD34+ cells segregated on the basis of HLA-DR expression (i.e., 1:100). In summary, these findings indicate that the primitive hematopoietic progenitor cells in P-UCB express both CD34 and HLA-DR suggesting that neonatal primitive progenitor cells do not share the same phenotypic properties of adult marrow primitive progenitors.

In contrast to the results with HLA-DR, Hao et al.[19] and Cardoso et al.[20] found that CD38 distinguishes subpopulations of hematopoietic progenitor cells. The CD34+CD38 immunophenotype defines a rare, quiescent CD34+ subpopulation in P-UCB as in adult marrow that can be distinguished functionally from the CD34+CD38+ population by sustained clonogenicity in extended long-term culture assay (8 weeks). In contrast to CD34+CD38 cells in adult marrow, Hao et al. [19] showed that CD34+CD38 P-UCB cells continue to proliferate well beyond 8 weeks. Importantly, the extended LTC-IC population (5%) was less efficiently transduced by retrovirus vectors as compared to the standard 5-week LTC-IC (4060%). P-UCB CD34+CD38 cells apparently proliferate more rapidly in response to cytokine stimulation in vitro and each CD34+ cell generates a significantly greater number of progeny as compared to adult marrow counterparts.

The proliferative response of purified candidate stem cell populations in adult marrow, fetal liver, and P-UCB were evaluated by Lansdorp et al. [21] When cells with the $CD34+CD45RA^{lo}$ -$CD71^{lc}$ phenotype were cultured in serum-free medium supplemented with a mixture of cytokines that include interleukin (IL)-3, IL-6, stem cell factor (SCF), and erythropoietin striking differences were observed between the number of CD34+ cells recovered from each of the cultures every 710 days. Throughout the culture period, the number of CD34+ bone marrow cells remained relatively constant at input values. In marked contrast, CD34+ P-UCB cells increased several hundred-fold (and several thousand-fold for CD34+ fetal liver cells). The purified cells also differed markedly in their response to various growth factors. PKH26-labeled CD34+ cells from bone marrow remained brightly labeled after 7 days in culture, whereas CD34+ cells from P-UCB demonstrated markedly decreased fluorescence over the same period, indicating that marrow CD34+

cells are relatively quiescent during this period, whereas P-UCB CD34+ cells are highly proliferative. Additionally, Carow et al. [22] demonstrated P-UCB multipotential CFU-granulocyte, erythrocyte, monocyte, macrophage (GEMM) progenitors had extensive replating capacity, which contrasts with that of adult marrow. Importantly, the addition of P-UCB plasma to the culture medium resulted in extensive secondary replating potential suggesting that other factors were present in P-UCB plasma not found in adult blood plasma or in artificial media supplemented with recombinant growth factors. These differences may have important implications for the use of P-UCB over marrow stem cells in gene therapy and ex vivo HSC expansion protocols.

Broxmeyer et al. [12] have attempted to determine the suitability of P-UCB as a graft for adult recipients. They reported that the total numbers of CFU-GM in a typical P-UCB graft and unpurged autologous marrow graft are remarkably similar. Although the numbers of nucleated cells and progenitors in P-UCB specimens are lower than that expected for allogeneic marrow grafts, Broxmeyer et al.[12] showed that progenitors in P-UCB had a greater capacity for expansion than marrow in short-term liquid culture in the presence of specific cytokines. Moore et al. [23] also found that a marked expansion of P-UCB progenitors could be achieved without expenditure of the primitive, LTC-IC compartment.

Numerous investigators have shown that P-UCB CD34+ cells have a greater proliferative potential and have different growth factor requirements compared to adult marrow CD34+ cells. Traycoff et al.[17] reported that SCF, IL-3, IL-6, and erythropoietin resulted in 2,500-fold increase in cell number at 9 weeks with peak production of CFU-GM and BFU-E at weeks 3 and 4 of long-term culture, respectively. Cardoso et al. [20] evaluated CD34+/CD38 P-UCB cells in liquid suspension culture containing IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), SCF, and anti-transforming growth factor (TGF-). As reported with adult marrow, the CD34+/CD38 subpopulation was significantly more effective in generating CFU-GM, BFU-E, and CFU-GEMM after long-term culture than the CD38+ subpopulation. Notably, the total CFU-GM production of the CD34+/CD38 subpopulation of P-UCB was 7.6-fold greater than the corresponding population in adult marrow.

In the severe combined immune deficiency (SCID-Hu) mouse model, P-UCB appears to have a higher engrafting capability than cells from adult marrow. These SCID repopulating cells (SRC) are found only in a fraction of cells expressing high levels of CD34 antigen and lacking CD38 antigen on their cell surface. Although there is no proven in vitro assay for the true pluripotential HSC, the SRC appears to identify a primitive progenitor. Through the use of limiting dilution analysis, the concentration of SRC in P-UCB is 1 in 9.3×10^5 cells, which is significantly greater in concentration than the 1 in 3×10^6 found in adult marrow or 1 in 6×10^6 found in G-CSF mobilized adult peripheral blood. Notably, there is 1 SRC per 600 CD34+/CD38 P-UCB cells. [24]

In summary, all assays used thus far appear to point to an increased concentration of primitive hematopoietic progenitors in P-UCB as compared to adult marrow. Moreover, recent studies suggest that P-UCB progenitors may be less mature by virtue of telomere length [25] and have a greater capacity for extensive proliferation. Analysis of cell cycle kinetics demonstrates that P-UCB progenitors are in a quiescent state but respond rapidly to stimulation by hematopoietic growth factors in sharp contrast to adult marrow progenitors that are highly proliferative.

How these characteristics potentially affect transplant outcome, capacity for ex vivo progenitor cell expansion, or efficiency of retrovirus-mediated transduction remain to be determined. Various investigators have demonstrated that culture of adult marrow CD34+ cells in the presence of multiple cytokines results in expansion of hematopoietic progenitors even in the absence of stroma. Although primitive progenitors can be induced to differentiate in such cultures, Verfaillie et al. [26] [27] showed that maintenance and extensive proliferation of LTC-IC from adult marrow is poor in stroma-free conditions but markedly improved by the presence of stroma-conditioned media supplemented by IL-3 and macrophage inhibitory protein-1a (MIP-1a). Using various starting populations and ex vivo culture conditions potentially useful for the expansion of primitive and committed hematopoietic progenitor cells in P-UCB, Han et al. [28] evaluated the ability to expand the number of colony-forming cells (CFC) and LTC-IC. As with adult marrow, the use of stroma-conditioned media supplemented by IL-3 and MIP-1a was superior to stroma-free

conditions supplemented with various cytokines with regard to LTC-IC maintenance and progenitor expansion. In contrast, fold expansion of CFU-GM was greatest for CD34+ P-UCB cells cultured without stroma in the presence of SCF, IL-3, IL-6, IL-1, G-CSF, and MIP-1a. Although these data suggest that it is possible to expand both primitive and committed progenitors in P-UCB, additional work is required using clinically available reagents as well as determining the effect of prior cryopreservation. Transplants using P-UCB cells expanded ex vivo under clinical study conditions, however, must be performed to document safety of the expansion procedure and efficacy with regard to hematopoietic recovery and engraftment.

The data presented in the preceding section suggest that methods are available for increasing the number of hematopoietic progenitor cells in P-UCB and for potentially reducing the time to hematopoietic recovery after transplantation. Because it is not known how few HSC are required for engraftment in any size recipient, only guesses can be made using surrogate markers (i.e., number of CFU-GM, SRC, or CD34+ cells). Although it appears that P-UCB grafts may contain sufficient numbers of HSC for adult recipients, time to neutrophil recovery is markedly prolonged (see Clinical Results). This observation clearly represents one of the most compelling arguments for investigating ex vivo expansion strategies with P-UCB. Early clinical trials with ex vivo expanded P-UCB HSC are underway (E. J. Shpall, personal communication).

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NEONATAL IMMUNE SYSTEM

Hematopoietic SCT is limited both by the lack of suitable donors, particularly in specific ethnic and racial subpopulations, and high risk of severe, life-threatening complications that arise when donor and recipient are not immunologically identical, namely, GVHD and opportunistic infection. In the past 30 years, research has focused on the development of new immunosuppressive drugs and treatment regimens and strategies for engineering the marrow or peripheral blood graft that will selectively eliminate alloreactive T-cell clones while preserving the short- and long-term engrafting cells. [29] [30] Although it is clear that a severe reduction in T-cell number can effectively prevent severe acute GVHD, it is also clear that T-cell depletion has increased the risks of primary and secondary graft failure, Epstein-Barr virus (EBV)-lymphoproliferative disease and relapse. Additionally, some data suggest that T-cell depletion may increase the risk of opportunistic infection. Together, it remains to be proven whether this approach will translate into improved survival.

Graft-versus-host disease results from the activation of donor-derived T cells that recognize host-specific alloantigens. The clinical syndrome is the culmination of a complex series of events that involves not only triggering of the T-cell receptor and various co-stimulatory molecules but also the release of proinflammatory cytokines. The clonal proliferation of donor T cells and activation of other effector cells (e.g., cytotoxic T cells, natural killer [NK] cells) and release of various cytokines result

in tissue damage and subsequently the manifestations of the disease. Are there qualitative and quantitative differences between the neonatal and adult immune systems that would predict or explain the low risk of GVHD observed after P-UCB transplantation, and do those differences allow greater HLA disparity between the neonatal P-UCB donor and recipient?

Thus far, the clinical observation is that patients transplanted with P-UCB may indeed have a lower than expected incidence of significant acute GVHD as compared with patients who receive adult marrow. Although case-controlled comparisons or randomized trials have not been performed to verify this conclusion, the low rate of GVHD has led to an interest in studying the neonatal immune system. Qualitative and quantitative differences between the adult and neonatal immune system have already been demonstrated. Rainaut et al. [31] have extensively investigated fetal and neonatal blood with regard to cell surface antigen expression. Relative to adult peripheral blood, P-UCB has (1) a significantly greater absolute number of lymphocytes per milliliter (two- to threefold greater), (2) a significantly lower percentage of CD8+ T cells, and (3) a significantly greater CD4:CD8 ratio. Hannet et al. [32] further characterized P-UCB lymphocytes using two-color flow microfluorometric analysis. P-UCB lymphocytes had phenotypic characteristics suggestive of T-cell immaturity. The majority of CD4+ P-UCB lymphocytes co-expressed CD45RA (91% as compared to 40% of adult CD4+ lymphocytes), fewer CD3+ T cells expressed IL-2 receptors (8% versus 18%), and fewer CD3+ T cells expressed the activation marker HLA-DR (2% versus 10%). Clement et al.[33] found that virtually all P-UCB CD4+ T cells co-expressed CD38 (95%) and CD45RA (>90%). They demonstrated that CD4+/CD45RA+ (CD38+) P-UCB T cells had no detectable helper function and their dominant immunoregulatory activity was suppression.

Investigations of the functional immunologic properties of P-UCB lymphocytes have been reported by Roncarolo et al. [34] Purified P-UCB T cells proliferated vigorously when activated by allogeneic antigens in primary mixed lymphocyte reactions (MLR) indicating that P-UCB cells respond normally to activation by alloantigens. In addition, strong proliferative responses were observed when the P-UCB T cells were activated by cross-linked anti-CD3 monoclonal antibodies. Together, these data suggest that T cells in P-UCB can be normally activated via their T-cell receptor and that their proliferative response is normal. Importantly, P-UCB cells had a reduced capacity to stimulate allogeneic cells in primary MLR. The data suggest that this defect may be related to reduced antigen-presenting capacity. P-UCB monocytes express lower levels of HLA-DR, B7, and intercellular adhesion molecule-1 compared to adult monocytes and produce lower levels of IL-10. The exact mechanism underlying the defect in the antigen-presenting capacity of P-UCB, however, remains to be clarified. In addition, P-UCB cells are also impaired in their capacity to generate allogeneic cytotoxic activity in primary MLR. Whether this defect is intrinsic to the cytotoxic T cells or due to other cells or factors preventing the generation of alloantigen-specific cytotoxic T cells is not yet known.

Interestingly, some of these data suggest that P-UCB T cells are capable of an alloreactive response and that their capacity to effect GVHD equals or exceeds that of adult peripheral blood T cells. Therefore, is the low risk of GVHD due to differences in T-cell dose or other factors? Most P-UCB T cells have the naive CD45RA+ phenotype. The alloreactive T-cell repertoire present in P-UCB is composed of unprimed cells, as compared to the repertoire of T cells in adult marrow or peripheral blood in which >50% of the alloreactive T cells are of the primed memory phenotype. On the basis of recent studies, naive, unprimed T cells are highly susceptible to tolerance induction. Studies by Roncarolo et al. [34] indicate that repeated activation of P-UCB T cells with alloantigens in vitro results in the inability of these cells to proliferate or produce cytokines following restimulation with the same antigens. This state of antigen-specific unresponsiveness may be due to an intrinsic property of P-UCB T cells not observed in adult T cells. Roncarolo et al. [34] also showed that NK activity of purified CD56+ P-UCB NK cells against NK-sensitive targets is comparable to that observed with adult NK cells.

As in BMT recipients, most circulating lymphocytes in the early post-transplant period express the CD56 phenotype. Roncarolo et al. [34] further demonstrated that IL-2, IL-6, and tumor necrosis factor- production by P-UCB mononuclear cells following activation was comparable to that observed with adult peripheral blood mononuclear cells. In contrast, interferon- and IL-10 production was significantly decreased and IL-4 and IL-5 were absent. Levels of GM-CSF were in general higher in the supernatants of P-UCB cells. Thus, P-UCB mononuclear cells differ from adult peripheral blood cells at several levels: (1) decreased capacity to stimulate an allogeneic response, (2) impaired cytotoxic effector function, and (3) unique cytokine profile (Table 90-1). Whether these properties account for the reduced capacity of transplanted P-UCB cells to modulate GVHD remains to be determined. However, it is also clear that T-cell dose in an unrelated donor P-UCB graft is lower than that in an unmodified marrow allograft. The median CD3 cell dose in recipients of P-UCB thus far is approximately 8×10^6 CD3+ cells/kg as compared to 3040×10^6 CD3+ cells/kg recipient body weight in a typical marrow allograft. Nonetheless, <2 log reduction of T cells in a marrow graft (i.e., T cell dose $>5 \times 10^5$ /kg) would be considered insufficient for preventing GVHD, particularly in the mismatched or unrelated donor setting. Most studies in SCT would suggest that a CD3 cell dose $<0.1 \times 10^6$ CD3+ cells/kg is required to eliminate the risk of severe acute GVHD. Therefore, it is unlikely that CD3 cell dose alone accounts for the low rate of severe GVHD currently observed after P-UCB transplantation.

Harris et al.[35] analyzed the development of alloreactivity in the infant over the first year of life and assessed the immunologic reactivity of P-UCB cells with lymphocytes from various family members. At birth, P-UCB cells were observed to be immunologically unreactive with cells from the mother and minimally reactive with cells from the father. Moreover, lymphocytes from the mother demonstrated a decreased ability to mediate NK lysis shortly after the time of delivery as well as a depressed alloreactive response. Although the neonatal blood was capable of mediating NK lysis, the infant did not develop the ability to generate an alloantigen-specific cytotoxic response until sometime between birth and 6 months of age. The inability of P-UCB to respond to parental lymphocytes as measured by

TABLE 90-1 -- Neonatal Lymphocyte Function

	Compared to Adult Peripheral Blood

Cytoxic T lymphocyte precursor frequency	Normal
T-cell activation	Normal
T-cell proliferation to alloantigen	Normal
Cytotoxic response to alloantigen	
Suppressor cell activity	
Antigen-presenting cell activity	
Cytokine production	
IL-2	Normal
IL-4	
IL-5	
IL-10	
Interferon	

MLR and by the inability to generate alloantigen-specific cytotoxic T cells suggests that P-UCB might be partially tolerant particularly with maternal antigens. The idea that the fetus may become partially tolerant to the non-inherited maternal allele (NIMA) was first proposed on the basis of outcomes observed in recipients of parental renal allografts.[36] [37] How such tolerance develops is unknown, but such results are particularly intriguing in view of the clinical results observed in haploidentical sibling donor-recipient pairs (see Clinical Results). If the immune cells in the P-UCB graft are tolerant to HLA antigens on the NIMA, this finding would have wide-reaching implications in defining an acceptable donor in both the related and unrelated donor setting.

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SIBLING DONOR P-UCB TRANSPLANTATION

Since the first report of a successful P-UCB transplant in 1989, [15] investigators worldwide have explored the potential of P-UCB as a source of HSC for transplantation. Early on, however, many questions in the form of editorials were raised. Would this HSC source engraft larger recipients [38] or recipients with diseases other than Fanconi anemia? Would lethal GVHD occur because of maternal lymphocyte contamination, previously shown to occur in some neonates with congenital immunodeficiency?[39] Alternatively, would P-UCB lymphocytes be less likely to cause a graft-versus-host reaction because of immunologic naivite and therefore lead to a greater risk of leukemic relapse?

In 1992, the International Cord Blood Transplant Registry was established as a repository of clinical data on outcomes observed in patients transplanted with P-UCB in an attempt to more quickly discern the true risks and benefits of this new HSC source. In 1993, a similar registry was developed in Europe as part of the European Research Project on Cord Blood Transplantation (EuroCord Transplant Registry). This section summarizes reports from both registries on outcomes observed in recipients of P-UCB transplantation from sibling donors.

Clinical Results

Data on patients receiving P-UCB transplants from sibling donors for the treatment of malignant and nonmalignant disorders have been reported previously. [40] [41] [42] As of March 1997, transplant outcomes in 74 patients were reported to the International Cord Blood Transplant Registry by 22 transplant teams. Patients were aged a median of 4.9 years (range, 0.516.3 years). Fifty-six patients received HLA 01 antigen mismatched grafts and 18 HLA 23 antigen mismatched grafts. Prophylaxis for acute GVHD was variable but most often consisted of cyclosporine alone or in combination with methylprednisolone or an anti-T-cell antibody. Hematopoietic growth factors were used early after the infusion of P-UCB in half the patients by study design.

For recipients of HLA 01 antigen mismatched sibling donor P-UCB grafts (n = 62), the actuarial probability of hematopoietic recovery at 60 days after transplantation was 0.91 ± 0.02 (Fig. 90-1). The median times to neutrophil recovery (defined as time to achieve an absolute neutrophil count [ANC] >5 × 10^8 /L) and platelet recovery (defined as platelet count >5 × 10^{10} /L untransfused for 7 days) were 22.0 days (range, 946) and 51 days (range, 15117), respectively. Four patients never had signs of hematopoietic recovery and one patient had early recovery but cells were entirely host in origin. Of the five patients without donor cell engraftment, four had undergone P-UCB transplantation for the treatment of a bone marrow failure syndrome and one for the treatment of Hunter syndrome. Similar results have been reported by Gluckman and colleagues for the EuroCord Transplant Registry. [5] [42] Although the engraftment rate is high for the group as a whole, there is a trend toward greater risk of graft failure in recipients with a prior history of a bone marrow failure syndrome, hemoglobinopathy, or storage disease (Table 90-2). Multivariate analyses with larger patient

Figure 90-1 Probability of neutrophil engraftment (i.e., ANC 5 × 10^8 /L) after HLA 01 antigen mismatched sibling donor P-UCB transplantation.

numbers are required to determine what factors are important in engraftment after sibling donor P-UCB transplantation.

Notably, no correlation between nucleated cell count or hematopoietic progenitor cell content of the P-UCB graft and time to neutrophil recovery or probability of engraftment has been discerned in the sibling donor setting. Moreover, the use of hematopoietic growth factor early after transplantation has not shortened the time to neutrophil recovery after P-UCB transplantation. Whether these observations are due to unique attributes of the neonatal HSC or simply due to small patient numbers or patient selection bias is unknown at this time.

Remarkably, acute GVHD has occurred only rarely in recipients of HLA 01 antigen mismatched P-UCB transplants. The actuarial probability of grade II-IV and grade III-IV GVHD at 100 days after transplantation remains 0.03 ± 0.02 and 0.02 ± 0.02, respectively. Despite the fact that the risk of acute GVHD is lower in young children, the incidence of acute GVHD appears to be lower as compared to similarly aged patients (ages 07 years) transplanted with HLA-identical marrow from sibling donors. Similar results have been reported by Gluckman et al. [5] Case-controlled studies will be required to determine if there is a significantly lower risk of acute GVHD after P-UCB transplantation as compared to BMT using sibling donors. Of the entire cohort of patients with a HLA 01 antigen disparate sibling donor, chronic GVHD has been reported in only three patients to date with no patient having had extensive disease.

Interestingly, moderate to severe GVHD has also been observed infrequently in 15 evaluable patients with haploidentical sibling donors (3 recipients of HLA 23 antigen disparate sibling donor P-UCB were not evaluable due to graft failure or early death). Of the 15 evaluable patients, 2 were mismatched at two antigens and 13 were mismatched at three antigens. Although patient numbers are small, donor-recipient pairs mismatched at the NIMA did appear to be less likely to develop

TABLE 90-2 -- Graft Failure After Sibling Donor UCB Transplant[a] (HLA 01 antigen mismatched)

	Proportion
Severe aplastic anemia	1/7
Fanconi anemia	1/10
Thalassemia	3/10
Sickle cell disease	1/5
Hunter syndrome	2/7

[a]Data from EuroCord Transplant Registry and International Cord Blood Transplant Registry.

grade II-IV GVHD than donor-recipient pairs mismatched at the noninherited paternal allele. This observation supports the hypothesis that partial tolerance may develop to the NIMA during gestation.

At a median follow-up of 2.0 years, the actuarial probability of survival for recipients of HLA 01 antigen mismatched grafts is 0.61 ± 0.12. Causes of death were multifactorial, including graft failure, relapse, interstitial pneumonitis/adult respiratory distress syndrome, veno-occlusive disease, intracranial hemorrhage, and early bacterial sepsis. For the entire cohort, GVHD was listed as a cause of death in only one patient with an HLA-3 antigen mismatched donor graft. The actuarial probability of disease-free survival in those treated for malignancy is 0.41 ± 0.11. Relapse was observed in two patients with relapsed neuroblastoma and in one patient with relapsed acute myelocytic leukemia after prior autologous marrow transplantation, in two patients with juvenile myelomonocytic leukemia, in three patients with acute lymphocytic leukemia (1 in complete response-1 but with the 9;22 translocation; 2 in complete response-2) and in one patient with adult type chronic myelogenous leukemia.

In summary, analysis of registry data and other case reports in the literature [13] [15] [43] [44] [45] [46] [47] [48] [49] [50] [52] [53] [54] demonstrates that engraftment occurs in most patients with greater risk of graft failure possibly in patients with bone marrow failure syndromes, hemoglobinopathy, and storage disease. Moreover, the data suggest that GVHD occurs infrequently in recipients of P-UCB. Results in haploidentical P-UCB transplants support the postulate of partial tolerance to NIMA. Although patient age has clearly been shown to be an important predictor of GVHD, [55] [56] it does not fully explain the very low incidence of grade II-IV disease observed in patients transplanted with P-UCB. Properties of the neonatal immune system that might account for the relative absence of a graft-versus-host reaction are currently being explored and have already been outlined. Importantly, these data also suggest that maternal cell contamination of the P-UCB at the time of collection may be of limited clinical importance. Kurtzberg et al. (presented by Broxmeyer et al. [13]), Wagner et al., [43] and Vilmer et al. [44] have failed to demonstrate maternal cells in the P-UCB grafts by cytogenetic or DNA techniques. Although maternal cell contamination is probably present in most P-UCB grafts, [57] [58] [59] [60] the very low incidence of GVHD observed in transplant recipients suggests that carefully collected P-UCB either contains insignificant numbers of maternal T cells or that maternal T cells are not immunologically competent in this context.

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UNRELATED DONOR P-UCB TRANSPLANTATION

As a result of the early successes with P-UCB transplantation from sibling donors, pilot programs for the banking of unrelated donor P-UCB were initiated in many countries around the world. Known benefits of banked P-UCB include rapid availability, absence of donor risk, absence of donor attrition, and very low risk of transmissible infectious diseases, such as CMV and EBV. Although this HSC source may allow us to expand the available donor pool in targeted ethnic and racial minorities, this remains to be proven.

To date, few reports have been published on the use of unrelated donor P-UCB transplantation in the literature. The largest series to date has been reported by Gluckman et al.[5] who summarized the data in the EuroCord Transplant Registry. The clinical results with unrelated donor P-UCB transplantation at Duke and the University of Minnesota, the two largest single center experiences, are also summarized. [61]

Clinical Results: EuroCord Registry

Between 1994 and December 1996, 65 patients were transplanted with unrelated donor P-UCB in Europe and reported to the EuroCord Registry. [5] P-UCB was provided by the Placental Blood Program at the New York Blood Center (n = 45) or a European P-UCB bank (n = 20). HLA typing was performed by serology for class I HLA-A and -B antigens and by low-resolution generic oligotyping for DRB1. Of the 65 patients, only 11 received HLA-matched P-UCB grafts. The clinical results are summarized in the following sections.

Hematopoietic Engraftment

Of the 49 patients with malignant disease, 42 were evaluable for engraftment. Neutrophil recovery of donor origin was documented in 88% by day 56. Similarly, of the 10 patients with bone marrow failure or genetic disease, engraftment occurred in 80%. Among the 65 recipients of unrelated donor P-UCB, factors associated with more rapid neutrophil recovery in multivariate analysis were higher cell dose (>3.7 × 10^7 nucleated cells/kg recipient body weight) and recipient weight <20 kg. Although HLA identity was not associated with neutrophil recovery, there was an association with more rapid platelet recovery. The effect of prophylactic hematopoietic growth factor on neutrophils was not addressed in this report.

GVHD

Grade II-IV acute GVHD was observed in 21 of 46 patients with engraftment after unrelated donor P-UCB transplantation. A lower rate of acute GVHD was observed in CMV-seronegative recipients (p = 0.04); however, there was no association with the degree of HLA disparity.

Survival

With a median follow-up of 10 months, the probability of survival in this group of patients was 29%. Nucleated cell dose (>3.7 × 10^7 /kg [p = 0.07]) and CMV-seronegative status (p = 0.03) were favorable risk factors for survival in multivariate analysis.

Clinical Results: Duke/University of Minnesota

Between August 1993 and August 1997, 144 patients were transplanted with unrelated donor P-UCB at Duke University and the University of Minnesota. For the purpose of this analysis, patients with <42 days follow-up (n = 25), history of prior allogeneic SCT (n = 6), or an immunodeficiency state not requiring lymphomyeloablative therapy (n = 2) were excluded. The remaining 111 patients were treated for various malignant (n = 73) and nonmalignant (n = 38) disorders. Although the median age of recipients was 7.2 years (range, 0.258), 19 patients were >18 years of age. The median weight of patients was 21.6 kg (range, 4.892) and median cell dose was 3.5 × 10^7 /kg (range, 0.733.8).

In this analysis, all unrelated donor P-UCB grafts were identified at the Placental Blood Program of the New York Blood Center (NYBC). Prior to transplantation, confirmatory HLA typing of patient and cryopreserved donor specimens was performed using standard serologic techniques identifying all World Health Organization-recognized specificities for HLA-A and -B antigens and serologic level or high-resolution DNA techniques for HLA-DR antigens. Units were selected to deliver the highest cell dose with the closest matched unit, prioritizing matching at HLA-DR over HLA-A or -B antigens. For purposes of this analysis (in contrast to that reported for the EuroCord Transplant Registry), HLA typing was based on high-resolution oligotyping of DRB1.

Hematopoietic Recovery and Engraftment

For the 111 recipients eligible for this analysis, the median volume collected, nucleated cell dose, and CFU-GM dose in the

Figure 90-2 (A)>5 × 10^8 /L) and **(B)** probability of platelet recovery (i.e., platelet count >5 × 10^{10} L) after HLA 03 antigen mismatched unrelated donor P-UCB transplantation.

P-UCB graft at the time of collection was 84 mL (range, 40214), 3.5 × 10^7 /kg (range, 0.733.8), and 1.6 × 10^4 /kg (range, 0.123), respectively, as reported by the NYBC. The median CD34 and CD3 doses, as determined at the transplant centers at the time the P-UCB graft was thawed, were 1.6 × 10^5 (range, 0.132.9) and 8.0 × 10^6 /kg (range, 0.4101), respectively.

The overall probability of neutrophil recovery by day 42 and platelet recovery by 6 months were 0.83 (range, 0.760.90) and 0.76 (range, 0.690.88), respectively (Fig. 90-2). The median time required to achieve an ANC >5 × 10^8 /L and platelet count >5 × 10^{10} /L was 23 days (range, 1259) and 2.4 months (range, 18), respectively. For both neutrophil and platelet recovery, there was no difference in probability of recovery for patients with 01 versus 23 HLA antigen-disparate grafts. In contrast to previous reports of sibling donor P-UCB transplantation, time to neutrophil recovery strongly correlated with the dose of cryopreserved nucleated cells (correlation coefficient, 0.40 [p < 0.01]) and CFU-GM and dose of thawed CD34 cells. Of note, secondary graft failures have not been observed to date; all patients engrafting

after unrelated donor P-UCB transplantation without relapse continue to have complete chimerism (i.e., persistent mixed chimerism has not been observed).

In univariate analysis, a significantly faster rate of neutrophil and platelet recovery was seen in recipients of a non-total body irradiation (TBI) containing preparative regimen or higher graft cell dose content (nucleated cells, CD34, CFU-GM). A trend could be discerned for more rapid recovery in recipients of prophylactic G-CSF. In Cox regression analysis, among continuous variables, nucleated cell dose, CFU-GM dose, and CD34 cell dose were the only factors significant for predicting neutrophil recovery by day 42 and platelet recovery by 6 months.

GVHD

The overall probabilities of grade II-IV and grade III-IV acute GVHD for the entire group of patients was 0.35 (range, 0.260.44) and 0.12 (range, 0.060.18) by day 100 after unrelated donor P-UCB transplantation, respectively. In univariate analysis, no factor was significantly associated with risk of acute GVHD, including CD3 cell dose or degree of HLA disparity. Probability of acute GVHD was 0.29 (range, 0.160.42) and 0.40 (0.270.53) in recipients of HLA 01 and HLA 23 antigen-disparate grafts, respectively. Moreover, there was no difference in the probability of acute GVHD between patients treated with cyclosporine plus high-dose methylprednisolone (n = 54, 0.34 [range, 0.210.47]) versus lower dose methylprednisolone (n = 38, 0.36 [range, 0.200.52]) versus other regimens (n = 19, 0.36 [range, 0.130.60]) (p = NS). The only factor that approached significance in univariate analysis was nucleated cell dose (<3 vs. >3 × 10^{7}/kg, p = 0.09). In Cox regression analysis, no risk factor was identified for acute GVHD. Notably, the probability of extensive chronic GVHD was 0.04.

Relapse

The probability of relapse in the patients with malignant disease was 0.10 (range, 0.030.17) at 1 year and 0.14 (range, 0.050.23) at 2 years after P-UCB transplantation. In univariate analysis, only prophylactic use of G-CSF was associated with a lower risk of relapse (p < 0.01). Notably, risk of relapse was not significantly different between standard- and high-risk patients (0.16 vs. 0.10, respectively). In Cox regression analysis, however, no factor predicted higher risk of relapse.

Survival

With a median follow-up of 1 year, the probability of survival at 2 years after unrelated donor P-UCB transplantation was 0.44 (range, 0.320.56). In univariate analysis, younger recipient age (<2 years, p = 0.05), harvested graft nucleated cell dose (>3 × 10^{7}/kg, p < 0.01) and thawed graft CD34 dose (>2 × 10^{5}/kg, p < 0.01) were associated with improved survival. However, in Cox regression analysis, CD34 cell dose and age were the most favorable risk factors for survival. Causes of death are shown in Figure 90-3 .

At these two centers, 19 patients >18 years of age have been transplanted with unrelated donor P-UCB. Thirteen had malignant disease (9 high risk) and six had nonmalignant disease. Eight patients had 01 HLA-disparate grafts and 11 had 2-antigen HLA-disparate grafts. The median age and weight of this subset of patients were 26 years (range, 2058) and 68 kg (range, 4392), respectively. The overall probability of neutrophil recovery was 0.86 (range, 0.691.00) at a median of 28 days (range, 1345). Graft failure occurred in 4 patients (2 had Fanconi anemia and 2 had acute leukemia in relapse). The probability of grade II-IV acute GVHD was 0.21 (range, 0.030.39) and probability of survival was 0.62 (range, 0.390.85) with a median follow-up of 6 months (range, 0.228).

Figure 90-3 Causes of death after unrelated donor P-UCB transplantation.

TABLE 90-3 -- Unrelated UCB Transplantation Cinical Results

	NEJM[5] (n = 65)	Blood[62] (n = 272)	Duke/Minnesota (n = 111)
Engraftment			
Probability at 60 days	0.87	0.89	0.83
Days to ANC 500/L		24	23
Days to platelet 50,000/L		72	72
GVHD			
Probability at 100 days (IIIV)	0.39		0.35
(IIIIV)		0.23	0.12
Survival			
Probability at 1 year	0.29		0.50

Data from Gluckman et al. [5] and Rubinstein et al.[62]

Summary

The results reported by Gluckman et al.[5] for the EuroCord Registry and Rubinstein et al. [62] for the NYBC database are consistent with the Duke/University of Minnesota database in terms of rates of hematopoietic recovery and engraftment and risks of grade II-IV acute GVHD (Table 90-3). The reasons for differences in survival between the EuroCord Registry and Duke/University of Minnesota are not readily apparent but may be due to differences in patient selection. Importantly, both the EuroCord Registry and Duke/University of Minnesota have independently documented the importance of cell dose in predicting engraftment and survival (Table 90-4). Although degree of HLA-A, -B and -DRB1 disparity has not had an impact on survival according to the EuroCord Registry and Duke/University of Minnesota, this may reflect limited patient sample sizes. More recent data from the NYBC (P. Rubinstein, personal communication) suggest that degree and class of HLA disparity may affect survival with poorer survival in patients with greater degrees of HLA disparity or disparities at HLA-DR.

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AUTOLOGOUS P-UCB TRANSPLANTATION

Transplantation of autologous P-UCB has thus far been linked to gene therapy. Review of the literature would suggest that P-UCB HSC may be transduced more efficiently by retroviral vectors than adult marrow HSC. To date, four infants with adenosine deaminase (ADA) deficiency SCID have been transplanted with genetically modified autologous P-UCB HSC collected and genetically modified shortly after birth. [63] [64] No myeloablative regimen was administered to these four infants reasoning that the T-cell progeny of the corrected HSC would have a selective survival advantage over time. In each case, engraftment was documented albeit at low levels with some indication that the proportion of T cells containing the corrected ADA gene is increasing over time.

TABLE 90-4 -- Unrelated Donor UCB Transplantation: Analysis of Risk Factors

Outcome	Predictive Factor	
	NEJM (n = 65)[5]	**Duke/Minna[a] (n = 111)**
Engraftment	Nucleated cell dose	CFU-GM dose
	HLA identity	CD34 dose
Acute GVHD	Recipient	None
	CMV status	
Survival	CMV status nucleated cell dose (p = 0.07)	Nucleated cell dose (p = 0.05)

Data from Gluckman et al. [5]

[a] Unpublished.

To date, there have been no other reports on the use of autologous P-UCB. Clinical protocols, however, currently exist for its use in the treatment of patients with Fanconi anemia (FAC complementation group). As in patients with ADA deficiency, it has also been reasoned that corrected Fanconi HSC will have a survival advantage with eventual replacement of the abnormal Fanconi hematopoiesis. Although several candidate patients exist, such therapy has yet to be used.

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ALLOGENEIC PLACENTAL BLOOD BANKING

Documentation of hematologic reconstitution after myeloablative therapy and P-UCB transplantation has resulted in considerable interest in the techniques of P-UCB collection and storage. Although the individual procedures used are not novel, the collection and processing procedures for P-UCB are not the same as those used for bone marrow. Unlike the typical bone marrow collection, the obstetrician or hematologist cannot collect a predetermined volume of P-UCB but rather is restricted to the finite amount available.

A variety of collection methods have been proposed to optimize the collection volume and reduce the risks of microbial and maternal cell contamination. [65] [66] [67] [68] [69] [70] Although no single method has been proven to be substantially better than another, the collection of free-flowing P-UCB into an open collection jar has largely been abandoned. The most important advantage of open collection was ease of collection, requiring no training and little set-up time; however, the most important disadvantage was the greater potential for microbial and maternal cell contamination. In contrast, closed systems using catheters and needles are technically more challenging and not uniformly transferable to obstetricians or midwives who have not had the opportunity to evaluate the system prior to the umbilical cord blood collection. Closed collection systems have been principally used by designated P-UCB collection centers with trained staff.

Different procedures for P-UCB collection, separation, and cryopreservation have been evaluated and reported in anticipation of large-scale banking projects proposed in the United States and Europe. Bertolini et al. [69] have reported on one of the most extensive evaluations of P-UCB collection procedures thus far. Open and closed collection systems, the effect of vaginal versus cesarean section delivery, and the recoveries of CFC and HPP-CFC after density-gradient centrifugation and gelatin sedimentation of both fresh and cryopreserved cell samples were compared. Bertolini et al. [69] failed to demonstrate any statistical differences in the collection volumes of P-UCB recovered during vaginal delivery (in utero, n = 445) or after cesarean section deliveries (ex utero, n = 82). The median volume of blood collected was 72 ± 34 ml and 62 ± 19 ml, respectively. Furthermore, no significant difference in collection volume could be discerned between open and closed collection systems. Expectedly, there appeared to be a lower risk of bacterial contamination for samples collected by venipuncture into a blood collection bag as compared to the open collection method (4% vs. 14%, respectively).

In an attempt to reduce the complication of dimethyl sulfoxide (DMSO) toxicity and ABO incompatibility reaction, red blood cell (RBC) depletion of the P-UCB graft has been evaluated. Broxmeyer et al.[11] first reported significant losses in progenitor recovery with P-UCB after density-gradient centrifugation (Ficoll-Hypaque, 1.077 g/ml, Sigma, St. Louis, MO). Broxmeyer et al.[11] found that CFC were lost by a variety of RBC separation techniques, suggesting that RBC depletion prior to clinical transplantation, even if the recipient and donor were ABO incompatible, should be carefully reconsidered. Others, [68] [69] [70] [71] however, failed to observe the same substantial losses of progenitor cells as assessed by in vitro CFC assays. Harris et al. [68] described a double Ficoll-Hypaque procedure in which the final preparation was virtually devoid of RBC and polymorphonuclear leukocytes but contained virtually all CFC. Bertolini et

al.[69] compared the double Ficoll-Hypaque method proposed by Harris et al., [68] and a 3% gelatin sedimentation method proposed by Nagler et al. [72] P-UCB separation using either Ficoll-Hypaque or gelatin sedimentation resulted in only 814% loss of CFC and HPP-CFC. However, Bertolini et al. [69] also found that the effectiveness of either separation procedure was markedly reduced when P-UCB was stored for more than 12 hours before the procedure. Although the gelatin procedure took less time (1.5 vs. 2.5 hours) relative to the Ficoll-Hypaque method, in one-third of instances, the gelatin procedure failed to result in RBC depletion when performed at room temperature. This technical issue, however, was corrected simply by performing the procedure at 4°C. Rubinstein et al. [73] suggest that the P-UCB graft be placed in a sterile bag and then thawed in a 38°C water bath with gentle agitation. With an equal volume of dextran/albumin solution added after thawing, the cells are centrifuged gently and the supernatant is removed. Together this procedure removes the bulk of RBC ghosts, free hemoglobin, and DMSO, thus reducing some of the risks associated with the procedure. These data suggest that RBC depletion by either density-gradient centrifugation or gelatin sedimentation results in only modest losses of hematopoietic progenitor cells.

Interest in the creation of large-scale unrelated donor P-UCB banks worldwide makes RBC separation a particularly important issue. Cryopreservation of mononuclear cell preparations would (1) reduce the risk of ABO-incompatible reactions (i.e., anaphylaxis and effects secondary to the infusion of free hemoglobin), (2) reduce the volume of the P-UCB graft and thus significantly reduce the space required for banking, (3) potentially improve the ability to manipulate these cells after thawing (e.g., CD34+ selection and ex vivo progenitor cell expansion), and (4) reduce the risk of DMSO-related reactions. Importantly, both density-gradient centrifugation with Ficoll-Hypaque and gelatin sedimentation [46] [47] of P-UCB have been successfully used prior to clinical transplantation without deleterious effects on hematopoietic recovery and engraftment.

As of March 1998, 10,000 P-UCB grafts have been collected, HLA typed, tested for transmissible infectious diseases, and cryopreserved. Recently, three additional P-UCB banks at Duke University, University of California at Los Angeles, and Childrens Hospital of Orange County have been designated and funded by the National Institutes of Health (NIH). These banks have developed a standardized P-UCB banking procedure. In close cooperation with transplant centers, these NIH-sponsored P-UCB banks have developed standardized quality assessment procedures (i.e., quantification of hematopoietic progenitors, sterility, detection of genetic and transmissible infectious diseases); streamlined histocompatibility testing using restricted volumes of the sample from the P-UCB graft; established repositories of viable cells, serum, and DNA on donor and mother for future testing; optimized the cryopreservation and thawing procedure to reduce cell loss and minimize infusion of DMSO and RBC debris; and established a computer network for efficient data storage and retrieval. Currently, P-UCB searches are most commonly accomplished by direct communication with individual P-UCB banks around the world or via NE CORD (consortium of banks in Milano, Dusseldorf, Barcelona). Recently, the National Marrow Donor Program (NMDP) has initiated discussions with various P-UCB banks worldwide to serve as the search coordinating center. Programs, such as the NMDP, could also serve as repositories of outcome data for the P-UCB banks. Clearly, such a construct offers multiple advantages for the P-UCB banks and transplant centers. Searches of both the marrow and P-UCB donor registries simultaneously will allow us to determine how much time is saved in the search process as well as the costs of using banked P-UCB rather than fresh marrow. It is hoped that a uniform search algorithm will be developed. Once it has been demonstrated that unrelated P-UCB reduces the risks of allogeneic stem cell transplant therapy or that it can supplement the pool of unrelated marrow donors, particularly for certain ethnic or racial minorities, it is likely that these repositories of P-UCB will be greatly expanded.

With the creation of unrelated donor P-UCB banks and the increasing awareness of P-UCB transplantation in general, there are increasing requests to store P-UCB for parents of children with leukemia, lymphoma, neuroblastoma, marrow failure syndrome, immunodeficiency state, inborn errors of metabolism, or other diseases potentially treatable by stem cell transplantation. Such a request for storing HLA-matched or partially HLA-matched P-UCB for a specific family member is appropriate and should be considered by the family and the hematologist or oncologist. Although not available at every transplant center, P-UCB testing and storage under such circumstances is widely available and is usually covered by third-party payers.

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AUTOLOGOUS STORAGE OF P-UCB

On the basis of the results with allogeneic P-UCB, repositories of autologous P-UCB have also developed at least in the United States and Canada. It has been suggested that the storage of the infants own P-UCB might be useful in several ways:

1. It might be an optimal source of tumor-free, virus-free HSC if the child should develop a disease amenable to stem cell transplantation later in life.
2. It might be a source of HLA-matched or closely matched HSC for a sibling requiring transplant therapy at some point.
3. It might be an optimal source of HSC for gene therapy should such therapy be needed either for the treatment of a genetic disorder in that child.

It has been suggested that autologous storage of P-UCB HSC is a form of biological insurance.

Biocyte Corporation was founded on the concept that P-UCB would be useful in the treatment of a variety of disorders, including cancers, genetic disorders, and immunodeficiency states. The company holds a US patent[74] for the collection and cryopreservation of P-UCB. The validity of the patent, however, is being considered at this time.

Several companies currently offer this service for a fee to expectant parents as shown in Table 90-5 . The routine collection of autologous P-UCB, as biologic insurance is controversial. The need for such a service is not clear. Several unknown factors need to be considered regarding autologous P-UCB, including the effect of long-term storage on the graft, the absence of graft-versus-leukemia/tumor effect, and preferences of the transplant physician. However, autologous P-UCB may be useful in specific situations, such as a vehicle for gene therapy. It may be worthwhile collecting and storing P-UCB from neonates with a high risk of disease possibly amenable to gene therapy in the future (e.g., Fanconi anemia, ADA deficiency) or in a family with familial cancer risk.

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ETHICAL CONSIDERATIONS

The collection of P-UCB poses a number of ethical issues. If it is considered to be like any other organ or tissue, then consent must be obtained by the tissue donor. In the case of P-UCB, the donor is always a minor, and therefore, consent must be obtained from the infants mother. The questions that arise include:

TABLE 90-5 -- **Commercial UCB Banks**

Cord Blood Registry San Bruno, CA	International Cord Blood Foundation San Francisco, CA
CorCell Philadelphia, PA	Stem Cell Sciences, Inc. New York, NY
Cryo-Cell International Safety Harbor, FL	United States Center for Cord Blood Altamonte Springs, FL
CryoTech, Inc. Roseville, MN	Viacord, Inc. Boston, MA

When should consent be obtained from the mother, and secondly, will the infant donor at 21 years of age have any rights to the P-UCB that was previously given to the unrelated P-UCB registry? Although the second issue must be addressed by the legal agencies, current practice would dictate that consent from the donors mother should be obtained before labor or at least at some finite period of time after delivery.

Alternatively, P-UCB may be considered to be discarded tissue. If so, consent is not required. The collection of P-UCB from the delivered placenta poses no risk to mother or infant. However, if P-UCB is to be considered discarded tissue and consent is not required, what do we do about the issue of testing for human immunodeficiency virus and how do we protect the rights of individuals whose religious and cultural practices would not allow the collection and transplantation of P-UCB? Although P-UCB offers several real and potential advantages as an alternate source of HSC, it also opens a new set of issues that need to be considered before large-scale collection of this tissue worldwide.

Other related issues that need to be addressed include the commercial aspects of P-UCB collection and storage, and the ability to back track and perform additional donor testing before the use of a P-UCB graft. By necessity, these and other issues need to be considered by medical ethicists and the physicians offering this treatment option.

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REGULATORY ISSUES

With the development of numerous P-UCB banks around the world, some form of regulation is necessary. The transplant physician is often not able to assess the quality of the banking practices at an individual site. Over the past 5 years P-UCB has moved from the status of biologic waste to a potentially important source of HSC. Clinical experience has already demonstrated that P-UCB contains sufficient numbers of HSC to engraft at least small recipients consistently. As a result, banks have been developed or are being considered throughout the United States and Europe as well as many countries in South America and Asia. Moreover, the potential for storing the childs own P-UCB as a form of biologic insurance has also been considered, resulting in the establishment of commercial banks. Hence, there has been an explosion of banking activity, making the need for standard policies and procedures particularly acute. It is likely that P-UCB will be the first source of HSC to be regulated by the US Food and Drug Administration (FDA). [75] [76] Although it is generally agreed that some form of regulation of P-UCB banking is important, the optimum mechanism for such regulation is being debated. In December 1996, the Center for Biologics Evaluation and Research of the US FDA issued a draft document in the *Federal Register* entitled Concerning the Regulation of Placental/Umbilical Cord Blood Stem Cell Products Intended for Transplantation or Further Manufacturing into Injectable Products, which defined cells in P-UCB as biologic products subject to Investigational New Drug regulations during clinical development. This incited considerable debate within the HSC transplant field and was formally opposed by various groups, including the Foundation for the Accreditation of Hematopoietic Cell Therapy, the International Society of Hematotherapy and Graft Engineering, American Association of Blood Banks, the American Society of Bone Marrow Transplantation, and the American Society of Clinical Oncology because of the potential of such a process for restricting research and development in the field of HSC and because of an inability to characterize the essential components of the HSC product.

In February 1997, the US FDA issued a document entitled Proposed Approach to Regulation of Cellular and Tissue-Based Products, which proposes a new regulatory framework based on a tiered approach to the regulation of traditional and new products that both protect the public as well as allow for the development of new therapies and products with minimal regulatory burden. However, central to the proposal is that all banks involved in P-UCB HSC therapy be registered with the US FDA. Products that are more than mimimally manipulated or experimental treatment plans will be subject to more regulation. Key elements of the regulation of P-UCB include use of good manufacturing practice, adequate testing for transmissible diseases, and development of product standards. The US FDA states that it intends to invite professional groups and individuals to submit data and standards to the FDA Tissue Reference Group that would define the essential elements of the HSC product for safety and effectiveness. Once sufficient data are available to develop processing and product standards that ensure safety, purity, and potency, the US FDA would issue a product license on the basis of certification by the applicant that such applicable standards have been met.

Although there will be debate on the optimal procedures for handling P-UCB, there is already extensive experience in handling blood and marrow. Many of the procedures for obtaining consent, collection, testing, cryopreservation, RBC depletion, histocompatibility testing, and sample labeling are likely to be extracted from existing operating manuals for blood. A major problem, however, will be in the definition of suitable product. Moreover, the definition of suitable product may vary depending on the type of recipientself, sibling, or unrelated.

Although it is clear that P-UCB should be considered as a viable source of HSC for transplantation, we are still in the learning phase. Just as the transplant physician requires assurances that the P-UCB graft product has been processed and stored properly, the director of P-UCB bank may require assurances that the transplant team will use the graft properly. Clinical outcome data may affect the P-UCB banks methods of processing and graft characterization. Policies for defining the qualifications of transplant teams and the appropriate use of P-UCB should not be overlooked. In the past, similar issues had to be addressed by the NMDP. Perhaps existing societies, such as the International Society of Hematotherapy and Graft Engineering and the American Society of Bone Marrow Transplantation, will aid in addressing these difficult issues.

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ALLOCATION OF P-UCB

A decision algorithm for choosing P-UCB as an appropriate source of HSC for a particular patient must be based on what is known and not known about this and other HSC sources. What do we know about P-UCB?

1. P-UCB contains sufficient numbers of HSC and progenitor cells for engraftment in most recipients weighing <50 kg.
2. P-UCB collection inflicts no risk to the mother or infant donor. [77]
3. P-UCB banking eliminates the risk of donor attrition observed by all marrow donor registries.
4. P-UCB is rarely contaminated by CMV[78] [79] and EBV.[80]
5. P-UCB can cause severe GVHD.

What do we think we know about P-UCB based on existing clinical data?

1. P-UCB transplantation appears to be associated with a lower rate of acute GVHD as compared to transplant using stem cell obtained from marrow.
2. P-UCB banking appears to shorten the interval between search initiation and time of donor cell acquisition by about 1 month, as compared to marrow collection.
3. Targeted P-UCB collection should reduce the shortage of racial and ethnic minority representation.

What dont we know about P-UCB because of lack of clinical data?

1. Is the P-UCB stem cell content sufficient for successful engraftment of adult-sized recipients?

2. Is P-UCB transplant associated with a low risk of relapse as with adult unrelated donor marrow transplant?
3. Will P-UCB increase the number of stem cell transplants for patients of racial and ethnic minority heritage?

TREATMENT ALGORITHM

Although other attributes could be added to these lists of what we do and do not know, the attributes described will help guide the creation of a decision algorithm for appropriately choosing P-UCB over adult marrow or peripheral blood.

When deciding whether P-UCB is more or less appropriate for a particular patient, several factors need to be assessed (Fig. 90-4). What is known about P-UCB transplantation and marrow SCT results for the disease being considered? Young patients with chronic myelogenous leukemia in chronic phase [81] and Wiskott-Aldrich syndrome (age <5 years[82]), for example, do well after adult unrelated donor marrow SCT. In contrast, older patients (age >35 years), regardless of disease, do poorly after adult unrelated donor marrow SCT.[83] [84] It could be argued that patients with chronic myelogenous leukemia or Wiskott-Aldrich syndrome, who are predicted to do well after marrow SCT, should be offered P-UCB only if no suitable marrow donor can be

Figure 90-4 Advantages and disadvantages of transplantation of P-UCB versus adult marrow.

found. In contrast, older adults should be reasonable candidates for such phase I-II trials evaluating new approaches to reduce the proven risks of unrelated donor marrow SCT in these populations. Other factors that are potentially involved in the decision process might include urgency of the transplant and cost.

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SUMMARY

Placental-umbilical cord blood is now considered to be a clinically useful source of HSC for transplantation. Multiple reports clearly document that donor-derived multilineage hematopoiesis after P-UCB transplantation is sustained with the longest follow-up of nearly 10 years. Unfortunately, it is still unknown what dose of nucleated cells or CD34+ cells is the absolute minimum required for engraftment and the absence of well-controlled trials prevent any conclusion regarding which HSC source is optimal for a given patient. Future studies are planned that will retrospectively compare transplant outcomes after P-UCB and adult marrow SCT.

It is clear that P-UCB is immediately available and risk of herpes virus contamination is low. Furthermore, the available clinical data suggest that the risks of grade III-IV acute GVHD and extensive chronic GVHD are remarkably low. Recent studies repetitively demonstrate the importance of graft cell dose in terms of engraftment, nonrelapse mortality, and survival. Whether the degree or class of HLA disparity has any impact on outcome has yet to be determined. Although early results fail to demonstrate an association, limited numbers of patients prevent any definitive statement on this issue. Nonetheless, these data at least suggest that P-UCB banks should focus on the collection of grafts containing larger cell doses and choice of P-UCB graft should be based primarily on cell dose and secondarily on HLA match.

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Chapter 91 - Preparative Regimens for Stem Cell Transplantation

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INTRODUCTION

Recurrence of malignancy and death due to complications of the preparative regimen are the major causes of treatment failure following hematopoietic stem cell transplantation (SCT). Limitations of current preparative regimens are largely responsible; improvement is needed. This chapter describes the development of current preparative regimens, assesses their virtues and liabilities, and describes potential directions for research.

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PRECLINICAL STUDIES

Animal Studies Using Irradiation

Initial studies of SCT evolved from animal investigations that had been designed to analyze organ damage following irradiation exposure. These studies were undertaken in the hope that methods for protection from and treatment of radiation injury could be developed. Animals exposed to very high doses of radiation developed lethal cutaneous and gastrointestinal toxicities. At lower but still lethal doses, sustained marrow impairment resulted in death from infection or bleeding. Studies in mice demonstrated that otherwise lethal exposure to irradiation could be circumvented by lead protection of the spleen. [1] The subsequent demonstration that infusion of syngeneic spleen or marrow cells could protect mice and guinea pigs from lethal doses of irradiation [2] led to canine studies and later human studies using marrow as a source of stem cells.[3] [4] [5] [6] Canine studies helped elucidate the problems of graft-versus-host disease (GVHD) and graft rejection and helped to define histocompatibility antigens.

In animal models as well as in humans, SCT proved a feasible treatment for whole-body radiation exposures of 300 to 1,500 Gy. Donnall Thomas envisioned, from these studies of radiation toxicity, a radically different approach to the treatment of hematologic malignancies. This approach would not depend on an increased susceptibility of malignant cells (compared to normal hematopoietic cells) to drug therapy. Advantages of total-body irradiation (TBI) responsible for its continued use in animal and human studies of transplantation include speed of delivery, lack of metabolites that interfere with the function of transplanted cells, and potent immunosuppressive effects in multiple animal models and in humans. Characteristics favoring its use in the treatment of leukemia include its antileukemic effectiveness, the lack of cross-resistance with chemotherapeutic agents, the ability to reach privileged sites (including the central nervous system, where drugs may not penetrate well), its effectiveness regardless of blood supply, and the potential for shielding or boosting specific areas of the body. [8]

Preparative regimens for allogeneic SCT in disease states should immunosuppress the recipient sufficiently to prevent rejection of donor cells and destroy malignant or defective hematopoietic progenitor cells as well as normal hematopoietic progenitors. They should also cause minimal short- and long-term toxicity. The usefulness of a preparative regimen in a specific disorder depends on its ability to fulfill these functions.

Total-body irradiation remains the most important conditioning agent for patients with hematologic malignancies receiving allografts. When dogs are exposed to TBI doses of 4 Gy or more, they generally die from complications of marrow failure. [7] However, when dogs receive infusions of autologous stem cells following similar or higher radiation doses, they consistently survive. [7] [8] [9] Successful engraftment of stem cells from allogeneic litter mates that are dog leukocyte antigen-identical requires substantially higher TBI doses of approximately 9 Gy. Transplantation from unrelated or mismatched donors requires doses >15 Gy. [10] In histocompatible rats, dogs, and monkeys, substantially higher doses of irradiation (up to 15 Gy) are required when split into fractions over 4 days, compared to that necessary to ensure engraftment when irradiation is administered as a single dose (in which case 9 Gy is sufficient). [11]

Animal Studies Using Chemotherapy

Studies using chemotherapy agents alone as preparation for allogeneic SCT proceeded in parallel with investigations using TBI. The initial stimuli for the development of radiation-free regimens were lack of adequate facilities for administration of TBI and concern over the short- and long-term toxicity of radiation. Animal models, which had been essential to the development of transplantation using TBI, were also crucial in the development of preparation without irradiation.

In 1962, Cree reported successful allogeneic SCT in rabbits using aminochlorambucil as a single agent. [12] Cyclophosphamide (Cy) was subsequently shown to permit engraftment of allogeneic stem cells in mice and rats.[13] [14] Two hundred mg/kg of Cy was needed to ensure consistent and permanent engraftment of stem cells from Lewis rats into histoincompatible ACI donors.[15] Subsequent studies in rhesus monkeys demonstrated engraftment with cyclophosphamide doses of 180 mg/kg over 2 days. Larger total doses were required when the Cy was administered over 4 days.[16] Thus, high doses of Cy administered over short periods of time proved sufficiently immunosuppressive to ensure engraftment; similar doses over shorter periods of time were much more effective than those spread over longer intervals.

In addition to the immunosuppressive requirement, injury to normal hematopoietic cells is important for permitting engraftment in animal models. Infused marrow cells preferentially engraft in irradiated body parts versus those that have been shielded. [17] Drugs such as busulfan (Bu) and dimethylbusulfan cause profound granulocytopenia and thrombocytopenia without substantial immediate effect on lymphocyte levels or humoral antibody response. [18] [19] [20] These agents are not sufficiently immunosuppressive to permit allogeneic engraftment. However, they helped achieve engraftment with the addition of agents such as antilymphocyte serum.[21]

In rat studies, 200 mg/kg of Cy was needed to permit engraftment; however, with the addition of 30 mg/kg of Bu, 150 mg/kg of Cy proved sufficient. Further, the combination resulted in more rapid and complete engraftment than Cy alone. [15] [22] [23]

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EARLY HUMAN STUDIES

Initial Studies Using TBI and Cyclophosphamide

Two patients with acute lymphoblastic leukemia (ALL) prepared for syngeneic transplantation with only TBI developed a

recurrence of leukemia after the transplant.[24] Cyclophosphamide was added to TBI to deliver additional antileukemia therapy. Thomas et al [25] published their results using a combination of TBI and Cy in 1977. In that study, 100 patients with advanced leukemia were transplanted using marrow from HLA-identical siblings following preparation with TBI and Cy. Thirteen percent became long-term leukemia-free survivors. This innovative study set the stage for the application of SCT to a number of malignant and nonmalignant disorders over the last 20 years.

Thomas [26] used dual 60_{co} sources to achieve homogeneous TBI. Sustained engraftment required a single exposure of 10 Gy. Administration of TBI in a single exposure, followed shortly by the infusion of marrow, minimized the duration of neutropenia and thrombocytopenia. However, when high-dose TBI is administered in a single dose, it must be given at a low dose rate to prevent the development of interstitial pneumonia, [27] [28] the dose-limiting toxicity of single-dose TBI. Increasing the number of fractions in which TBI is administered reduces lung damage.[26] Hematopoietic cells repair DNA damage less effectively than other tissues. Thus, administration of TBI in multiple fractions accentuates marrow toxicity and attenuates the injury to other organs, a circumstance advantageous to the treatment of hematologic disorders.

Fractionation of irradiation over several days was considered in patients who were in better clinical condition and could tolerate a longer period of neutropenia and thrombocytopenia. Using Cy and fractionated irradiation, Thomas et al [29] [30] achieved leukemia-free survival in 50% of patients in first remission from acute myelogenous leukemia (AML). Patients who received 2 Gy/day for 6 consecutive days had a better outcome than patients who received 10 Gy in a single exposure (Fig. 911). Using a hyperfractioned regimen given 3 times per day over a 4-day period, investigators from Memorial-Sloan Kettering reported a lower incidence of interstitial pneumonia and of relapse of acute leukemia than they previously achieved with a single-dose regimen. [31] [32] These observations established the advantages of a fractionated schedule of TBI. Techniques of administration, dose rates, fractionation schedules, and total doses have varied widely. Variations in technique are frequently related to the equipment available and the preference of the radiation therapist. With rare exceptions, direct comparisons of different approaches have not been performed. Further complicating interpretations of results with different techniques are other important

Figure 91-1 Kaplan-Meier product limit estimates for event-free survival of patients with acute myelogenous leukemia in first remission, given Cy 60 mg/kg on each of 2 days, randomized to receive 2 Gy on each of 6 days (n = 26) or 10 Gy (n = 27) in one exposure followed by an infusion of stem cells from an HLA-identical sibling. *(From Thomas ED: Total body irradiation regimens for marrow grafting. I. J Radiation Oncology, Biology, Physics 19:1286, 1990, with permission from Elsevier Science.)*

variables, such as the underlying disease and stage, the condition of the patient, the method for prevention of GVHD, and other supportive care measures.

Initial Studies in Leukemia Using Busulfan and Cyclophosphamide

In 1969, Santos et al[33] reported fatal cardiac toxicity in patients undergoing allogeneic marrow transplantation after a preparative regimen of Cy, 60 mg/kg, on each of 4 consecutive days. The dose was then lowered to 50 mg/kg daily for 4 consecutive days. Twelve of 24 patients with advanced leukemia prepared with this regimen died from transplant-related complications; the other patients died from leukemia recurrence. [34] Santos et al subsequently performed a series of dose-finding studies with busulfan in patients with AML. At 20 mg/kg of Bu, mucositis and hepatic veno-occlusive disease (VOD), occurred frequently and were dose-limiting. [35] Others had also demonstrated significant toxicity with 20 mg/kg of Bu as a single agent. [36] Bu, 16 mg/kg, was the dose chosen to be used with Cy, 200 mg/kg, in patients with AML for initial studies at Johns Hopkins. This proved to be an extremely effective regimen; however, the incidence of transplant-related complications was high. [37] More effective supportive care measures, such as the use of cyclosporine to prevent GVHD, later led to improved results. [38]

Reasoning that the huge dose of Cy added little therapeutic effectiveness but was responsible for significant toxicity, investigators at Ohio State chose to decrease the dose of Cy to 60 mg/kg on each of 2 consecutive days.[39] This dose was identical to that traditionally used in Cy/TBI regimens. This little BuCy (or BuCy2) regimen was studied in a variety of diseases. Following pilot studies at Ohio State, several multi-institutional trials were performed in AML, [40] chronic myelogenous leukemia (CML),[41] and ALL.[42] Multiple myeloma,[43] Hodgkins disease, [44] and non-Hodgkins lymphoma[45] were also investigated.

In the series of multi-institutional trials, BuCy2 consistently permitted engraftment of HLA-genotypically identical stem cells in patients with hematologic malignancies.[40] [41] [42] Only 3 of 281 patients undergoing such transplants failed to engraft. Intriguingly, all 10 patients with hematologic malignancies given busulfan, 16 mg/kg, and only 90 mg/kg of Cy engrafted with HLA-identical sibling allografts, [46] further demonstrating the important role of Bu in permitting engraftment. Clearly, however, BuCy2 is not as immunosuppressive as Cy/TBI regimens. An increased incidence of graft failure occurs following BuCy preparation for transplantation using T-depleted marrow from HLA-nonidentical family members, or from unrelated donors.[47] In the setting of unmanipulated marrow, failure to engraft using nonT-depleted marrow from unrelated donors is rare after preparation with BuCy, but probably more frequent than with TBI-containing regimens. [48] [49]

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TOXICITY OF PREPARATIVE REGIMENS

The incidence of complications reported following SCT appears to be more closely related to the criteria for diagnosis of that complication at a given center than to the regimen used. Accordingly, one must interpret with care comparisons of published series of patients receiving different preparative therapies at different institutions. However, this type of comparison is often the only information available. Also, because of patient numbers and thoroughness of study or reporting, these data may be superior (for this purpose) to data obtained in randomized trials.

Attempts to analyze the toxicity resulting from specific preparative regimens have been complicated by a variety of factors, most prominently the development of injury to multiple organs that occurs following transplantation for reasons other than the

preparative regimen. These include GVHD (and its prevention and treatment), the underlying disease and its prior treatment, and the administration of drugs with the potential to damage organs.

Bearman et al[50] developed a grading system in an attempt to distinguish toxicity related to the preparative regimen from that due to other transplant-related complications. Toxicity directly related to the preparative regimen is labeled regimen-related toxicity. The system was designed for high-dose preparation for transplantation, not for lower-dose chemotherapy. Such a system permits comparison of different preparative regimens. It attempts to grade the effect of the preparative regimen on eight organs: gut, liver, lungs, kidney, mucosa, heart, bladder, and central nervous system (Table 911). Toxicity is graded weekly for 4 weeks and then on day 100 for the lungs. Grade I toxicities are mild and reversible without intervention. Grade II toxicities are moderate but generally require intervention or interfere with therapy. Grade III represents life-threatening toxicity, which often requires intensive supportive care (e.g., mechanical ventilation). Patients who die from regimen-related toxicity on or before day 28 (for pulmonary toxicity, before day 100) are labeled grade IV. Toxicity that can be attributed to other transplant-related complications (e.g., GVHD, infection, bleeding, or drugs) is excluded. Cumulative toxicity is the sum of the highest toxicity scores observed in each organ system.

In Bearmans analysis, severe regimen-related toxicity was most common in the liver, kidneys, and lungs. All but 2 of 19 patients with Grade III toxicity died within the first 100 days. For these 2, the highest-grade toxicity was mucositis. Half of the patients who developed Grade III toxicity died directly from toxicity. Less cumulative toxicity was associated with better survival. The total dose of irradiation administered was the most significant risk factor identified for development of regimen-related toxicity. Recipients of allografts showed a significantly increased incidence of Grade III or IV toxicity compared with autograft recipients. Those with advanced disease also had a significantly increased incidence of severe toxicity and death within 100 days of transplantation [51] (Fig. 912). The use of methotrexate

TABLE 91-1 -- Regimen-Related Toxicity According to Organ System[a]

	Grade I	Grade II	Grade III
Cardiac toxicity	Mild EKG abnormality, not requiring medical intervention; or noted heart enlargement on CXR with no clinical symptoms	Moderate EKG abnormalities requiring and responding to medical intervention; or requiring continuous monitoring without treatment; or congestive heart failure responsive to digitalis or diuretics	Severe EKG abnormalities with no or only partial response to medical intervention; or heart failure with no or only minor response to medical intervention; or decrease in voltage by more than 50%
Bladder toxicity	Macroscopic hematuria after 2 days from last chemotherapy dose with no subjective symptoms fo cystitis and not caused by infection	Macroscopic hematuria after 7 days from last chemotherapy dose not caused by infection; or hematuria after 2 days with subjective symptoms of cystitis not caused by infection	Hemorrhagic cystitis with frank blood, necessitating invasive local intervention with installation of sclerosing agents, nephrostomy, or other surgical procedure
Renal toxicity	Increase in creatinine up to twice the baseline value (usually the last recorded before start of conditioning)	Increase in creatinine above twice baseline but not requiring dialysis	Requirement for dialysis
Pulmonary toxicity	Dyspnea without CXR changes not caused by infection or congestive heart failure; or CXR showing isolated infiltrate or mild interstitial changes without symptoms not caused by infection or congestive heart failure	CXR with extensive localized infiltrate or moderate interstitial changes combined with dyspnea and not caused by infection or CHF; or decrease of P_{O_2} (>10% from baseline) but not requiring mechanical ventilation or >50% O_2 on mask and not caused by infection or CHF	Interstitial changes requiring mechanical ventilatory support or >50% oxygen on mask and not caused by infection or CHF
Hepatic toxicity	Mild hepatic dysfunction with 2.0 mg% bilirubin 6.0 mg%; or weight gain >2.5% and <5% from baseline, of noncardiac origin; or SGOT increase > 2-fold but < 5-fold from lowest preconditioning	Moderate hepatic dysfunction with bilirubin >6 mg% <20 mg%; or SGOT increase >5-fold from preconditioning; or clinical ascites or image documented ascites >100 ml; or weight gain >5% from baseline of noncardiac origin	Severe hepatic dysfunction with bilirubin >20 mg%; or hepatic encephalopathy; or ascites compromising respiratory function
CNS toxicity	Somnolence but the patient is easily arousable and oriented after arousal	Somnolence with confusion after arousal; or other new objective CNS symptoms with no loss of consciousness not more easily explained by other medication, bleeding, or CNS infection	Seizures or coma not explained (documented) by other medication, CNS infection, or bleeding
Stomatitis	Pain and/or ulceration not requiring a continuous IV narcotic drug	Pain and/or ulceration requiring a continuous IV narcotic drug (morphine drip)	Severe ulceration and/or mucositis requiring preventive intubation; or resulting in documented aspiration pneumonia with or without intubation
GI toxicity	Watery stools >500 ml but <2,000 ml every day not related to infection	Watery stools >2,000 ml every day not related to infection; or macroscopic hemorrhagic stools with no effect on cardiovascular status not caused by infection; or subileus not related to infection	Ileus requiring nasogastric suction and/or surgery and not related to infection; or hemorrhagic enterocolitis affecting cardiovascular status and requiring transfusion

CXR, chest x-ray; IV, intravenous.

From Bearman S, Appelbaum F, Buckner C et al: Regimen-related toxicity in patients undergoing bone marrow transplantation. J Clin Oncol 6:1564, 1988, with permission.

[a]Grade IV regimen-related toxicity is defined as fatal toxicity.

Figure 91-2 Actuarial probability of survival 100 days after transplant for good-risk and poor-risk lymphoma patients (p = 0.003). *(From Bearman S, Appelbaum F, Back A et al: Regimen-related toxicity and early post-transplant survival in patients undergoing marrow transplantation for lymphoma. J Clin Oncol 7:82, 1989, with permission.)*

in addition to cyclosporine resulted in a higher incidence of Grade III or IV toxicity, indicating that drugs used after the transplant can contribute to regimen-related toxicity. This study established a scoring system that may be useful in attempting to compare toxicities for preparative regimens. It also emphasizes the influence of factors other than the preparative regimen itself on what is defined, even under rigorous scrutiny, as regimen-related toxicity.

Nevill et al[52] from Vancouver used this grading system to analyze the regimen-related toxicity of Bu, 16 mg/kg, and Cy, 120 mg/kg, in patients undergoing allogeneic transplantation for hematologic malignancies. Stomatitis and hepatic toxicity were the most frequently observed toxicities. Sixty-three percent of patients developed Grade II or higher stomatitis and 44% Grade II or higher hepatic regimen-related toxicity. Grade II or greater toxicity was rare in other organ systems, occurring in <10% of patients. Only 17% of patients developed Grade III or IV regimen-related toxicity. Hepatic regimen-related toxicity of grade II or higher was significantly more common in patients who received methotrexate for prevention of GVHD. Amphotericin B administration and prolonged administration of antibiotics were associated with Grades II to IV hepatic toxicity. This is especially interesting in view of reports demonstrating that the use of growth factors to accelerate hematopoietic recovery is associated with a reduction in hepatic regimen-related toxicity. [53] The extent of the hepatic toxicity seen in this study was similar to that observed with a TBI-containing regimen at the same center.[54] The incidence of hemorrhagic cystitis of 21% in the same study group is approximately twice that reported with radiation and Cy. Pulmonary and cardiac toxicity occurred only rarely with BuCy, consistent with reports by other investigators using the same BuCy regimen. [40] [41] [42]

Hepatic Veno-occlusive Disease

Hepatic VOD results from obstruction of intrahepatic venules and surrounding centralobular hepatocytes and sinusoids. [55] [56] [57] [58] [59] It is the most common life-threatening regimen-related toxicity. It usually occurs within 4 weeks of transplantation. Diagnosis is made by the development of hepatomegaly, right upper quadrant pain, jaundice, and ascites. [60] A variety of risk factors for the development of hepatic VOD exist. The preparative regimen is critical. Single-dose irradiation is associated with a much higher incidence of VOD than fractionated regimens. [61] [62] [63] A higher risk of VOD is similarly associated with higher total doses of radiation.[50] [57] [64] [65]

Most, but not all, centers using both BuCy regimens and TBI-containing regimens report a higher incidence of hepatic VOD with BuCy. [56] [65] [66] Most studies find an approximately 4050% incidence of hepatic VOD using BuCy, an incidence increased by prior use of Bu [66] or nitrosurea[67] in addition to those factors previously mentioned.

To determine the incidence and clinical course of VOD, McDonald et al [65] reviewed 355 consecutive patients undergoing allotransplant and found a 54% incidence of VOD. Interestingly, this study found that multiorgan failure was significantly more common among patients with VOD, and half of the patients who developed severe VOD developed renal failure, cardiac failure, and pulmonary failure. In addition to BuCy, the use of BCV (BCNU, Cy, and VP16) was associated with a high incidence of severe VOD.

While few deaths are directly attributed to hepatic VOD, one study of Bu-treated patients demonstrated that those whose bilirubin rose to >10 mg/dl within 100 days of transplantation had a 91% treatment-related mortality rate. [68] The treatment-related mortality rate for all others was 20%. Of 29 such patients who came to autopsy, 18 had hepatic VOD and 4 had massive hepatic necrosis. Thus, in the early post-transplant period, a high bilirubin level was associated with hepatic VOD and was an extremely poor prognostic sign.

Data from the Pesaro team indicate the pivotal importance of underlying hepatic disease in the development of regimen-related toxicity following transplantation in patients with thalassemia. The consequences of iron overload and hepatitis have been well described in these patients. [69] [70] Patients who undergo SCT for leukemia also commonly have iron overload. Data are much more limited in defining the role of iron overload in hepatic or other complications of transplantation. It appears likely, however, that iron overload contributes significantly to hepatic injury following transplantation. [71] [72] [73] Toxicity attributed to the preparative regimen may be strongly related to an underappreciated or unexplored underlying condition, such as iron overload.

Interstitial Pneumonia

Interstitial pneumonia occurs less frequently with BuCy than with Cy/TBI. In a study from Seattle of patients at high risk of interstitial pneumonia because of prior chest radiation, 32% of individuals transplanted with Cy/TBI experienced interstitial pneumonia, while only 5% of patients receiving BuCy developed interstitial pneumonia.[74] Factors present before transplant clearly predispose individuals to develop specific toxicities after transplantation; the actual risk results from a complex relationship between predisposing factors and the preparative regimen.

Other Regimen-Related Toxicities

Similar complexities in other organ systems have led some to conclude that assessment of toxicity due to the preparative regimen is too difficult to draw meaningful conclusions from the limited number of patients generally studied for this purpose. While grading systems are clearly imperfect, attempts at objective analysis are essential. They have led to useful conclusions regarding differences between regimens and in determination of optimal dosing of new regimens. Ideally, future randomized trials of preparative regimens will include uniform grading of regimen-related toxicities.

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DELAYED TOXICITY OF PREPARATIVE REGIMENS

Chronic GVHD is the best-studied delayed complication of allogeneic SCT. More intense regimens have been associated with a higher incidence of GVHD, [75] [76] [77] but the mechanism is not clear.

The frequency and severity of infections following allogeneic SCT is related to GVHD and sometimes to immunodeficiencies. IgG2 and IgG4 subclass deficiencies may occur in the presence or absence of chronic GVHD. Subclass deficiency has been reported in irradiated as well as nonirradiated transplant recipients. [78] [79]

A variety of other problems, many of which are multifactorial in etiology, appear to be strongly related to the preparative regimen used. Chronic pulmonary disease, neuroendocrine dysfunction, impaired growth and development, cataracts, infertility, and secondary malignancies are among the most important delayed effects that appear to be effected by the preparative regimen used. [80] [81] Unfortunately, delayed effects have not been well reported for many preparative regimens. The most recognized and best-studied delayed effects have developed following TBI, which clearly causes profound late complications.

Chronic Pulmonary Disease

Chronic pulmonary disease appears in 10% of patients who have undergone allogeneic transplantation. Both restrictive and obstructive disease occurs. Obstructive airway disease is less reversible following allogeneic transplantation and is frequently associated with GVHD. It occurs after radiation-free as well as radiation-containing regimens. [82] [83] Few long-term studies have been performed. A study of 34 allogeneic transplant patients compared those receiving Cy alone, Bu and Cy, or Cy plus TBI. [83] Unlike patients who received Cy alone, patients who received Bu or TBI demonstrated a progressive decrease in diffusing capacity following allogeneic transplant. The extent of the deterioration was greater in the group receiving TBI, although the difference did not reach statistical significance in this small group of patients. Many patients demonstrated gradual improvement of lung function over several years following their initial deterioration.

Endocrine Dysfunction

Hypothyroidism is observed in >30% of individuals receiving single-dose TBI and in >15% of those receiving fractionated TBI. It does not occur commonly following Cy or BuCy. [84] [85] [86]

Future growth is an important concern following high-dose therapy in children. Deficiency in growth hormone production is observed in roughly 50% of children receiving high-dose TBI and up to 90% of those who receive cranial irradiation in addition to TBI. Together with direct effects of radiation on bone, growth factor deficiency leads to impaired longitudinal growth. [86] In contrast, children receiving only Cy show normal longitudinal growth. The effect of Bu on growth is controversial. It was first reported not to affect growth or other endocrine functions. [87] However, others found growth impairment similar to that in children who received Cy/TBI. [86] [88] One recent analysis found that in very young children who do not develop GVHD and/or receive high-dose corticosteroids, which impair growth, Bu does not significantly impair growth. [89] Studies of thalassemia patients indicate decreased growth velocity in children aged 1011 prepared for transplantation with BuCy, while younger children had normal growth velocity. [90]

Children who receive only Cy show normal sexual development. [91] Girls prepared with single-dose TBI have a significant delay in menarche, and boys commonly fail to produce sperm. [92] [93] [94] [95] [96] Men and women almost universally develop primary gonadal failure following TBI. There are only rare reports of women who have become pregnant following TBI. In contrast, following Cy alone, many younger women recover ovarian function and deliver normal children. [91] Similar doses in older patients (where the number of oocytes is less) may result in ovarian failure. Only rare patients have recovered sperm production 68 years following transplantation with TBI, [96] and few have fathered children. Only 5 of 323 men evaluated for 12 years following TBI demonstrated return of spermatogenesis; none of these 5 patients had received fractionated TBI. [97] Infertility is generally not a problem in Cy-conditioned patients, and fathering of children appears to be much more frequent in males given Bu [98] than in those receiving irradiation. Aspermia occurs following transplantation, but recovery was observed in two of six men evaluated >2 years out from transplant in one study and one of five in another. [97] [98]

Cataracts

Cataracts occur in 80% of patients who receive single-dose TBI and >30% of those who receive fractionated TBI. [99] The incidence of cataracts in patients receiving only Cy appears to be <20%. [99] Busulfan can cause cataracts; the incidence appears to be approximately 20%. [47]

Second Malignancies

Several recent analyses have attempted to determine the incidence of second malignancies following SCT. [100] [101] [102] Some studies have demonstrated a high risk of lymphoproliferative disorders following allogeneic transplantation. [103] [104] The use of potent immunosuppressive treatments is an important risk factor. A lower incidence of lymphoproliferative disorders occurs in patients conditioned with non-TBI regimens. [100]

A recent analysis of nearly 20,000 stem cell transplant recipients, including 3,200 who survived for 5 years, demonstrated a high incidence of cancers following transplantation. [102] The risk increased to 6.7% at 15 years and was highest in children whose transplants occurred prior to age 10 (Table 912). Melanoma and cancer of the buccal cavity, brain, liver, thyroid, bone, and connective tissue occurred with significantly increased frequency. The risk of cancer rose with the dose of radiation; the risk for those receiving the highest dose of radiation approached four times that for those who did not receive radiation (Table 913). The incidence of lymphoproliferative disorders, in the same cohort, was <1.5%, and no cases were observed 10 or more years following transplantation.

TABLE 91-2 -- Ratio of Observed to Expected Cases and Absolute Excess Risk of New Invasive Cancers According to Age at Transplantation

Age at Transplantation	No. of Patients	PersonYears at Risk	OBS	EXP	OBS:EXP (95% CI)	Excess Risk[a]
<10 yr	2,745	7,989	22	0.6	36.6 (22.9554)	26.8
1019 yr	4,178	12,008	8	1.7	4.6 (2.09.1)	5.2
2029 yr	4,948	11,996	21	4.6	4.6 (2.87.0)	13.7

3039 yr	4,474	8,914	13	9.4	1.4 (0.72.4)	4.1
40 yr	2,884	4,457	16	13.5	1.2 (0.71.9)	5.7

OBS, observed cases; EXP, expected cases; CI, confidence interval.

From Curtis R, Rowlings P, Deeg H et al: Solid cancers after bone marrow transplantation. N Engl J Med 336(13):900, 1997, with permission.

[a]The absolute excess risk is the number of observed cases minus the number of expected cases per 10,000 patients per year.

TABLE 91-3 -- Multivariate Analysis of Risk Factors for New Invasive Solid Cancers Among Patients Who Survived for at Least 1 Year After Transplantation

Variable	Cases (N = 71)	Relative Risk (95%CI)
Model 1: General risk factors[a]		
TBI, single fraction	25	2.1 (0.67.5)
TBI, mutliple fraction	35	2.1 (0.67.1)
Limited-field irradiation	5	18.4 (3.790.5)
Acute GVHD (grades IIIV)	19	0.8 (0.41.3)
Chronic GVHD	18	1.1 (0.62.0)
T-cell depletion	6	1.0 (0.42.5)
HLA mismatched or unrelated donor	4	0.6 (0.21.8)
Twin donor	3	0.5 (0.21.9)
Male sex	41	1.2 (0.71.9)
Seattle cohort	28	1.3 (0.72.3)
Model 2: Radiation dose[b]		
None	6	1.0
TBI, single fraction		
<10 Gy	3	0.9 (0.24.4)
10 Gy	22	2.7 (0.710.1)
TBI, multiple fractions		P for trend, 0.006[c]
<12 Gy	4	1.2 (0.35.7)
1212.9 Gy	15	1.8 (0.56.6)
1313.9 Gy	6	4.1 (1.017.4)
14 Gy	10	4.4 (1.117.7)
Limited-field irradiation	5	P for trend, 0.001[c]
		16.1 (3.280.7)

CI, confidence interval; TBI, total body irradiation; GVHD, graft-versus-host disease.

From Curtis R, Rowlings P, Deeg H et al: Solid cancers after bone marrow transplantation. N Engl J Med 336(13):901, 1997, with permission.

[a]The model used for general risk factors was a Poisson regression model stratified according to the primary disease and time since transplantation and adjusted for age at transplantation.
[b]The radiation-dose model was a Poisson regression model stratified and adjusted as indicated for the general model but including the radiation-dose variables. The model excludes data on patients with unknown radiation doses.
[c]P values for trend are for the comparison between TBI-dose categories and no radiation.

Recipients of transplants, in particular those who underwent transplantation at a young age and those who received high doses of TBI, have an increased incidence of cancer. The incidence increases sharply with time. Stem cell transplant recipients should be followed carefully to detect premalignant lesions and early cancer. Furthermore, patients who have undergone SCT should avoid carcinogenic exposures, such as tobacco.

MODERN PREPARATIVE REGIMENS

Cy/TBI

Bearman identified TBI as the most significant risk factor for regimen-related toxicity. Patients who receive Cy alone experience less stomatitis, enteritis, infection-related deaths, life-threatening GVHD, and interstitial pneumonia than patients who receive regimens containing Cy/TBI. [105] [106] [107] When TBI doses are escalated, the incidence of transplant-related mortality goes up, [26] [51] [108] [109] [110] while the incidence of leukemic relapse declines. Randomized studies by the Seattle group in AML[109] and CML[110] yielded similar results. Patients with AML in first remission (Fig. 913) and CML in chronic phase (Fig. 914) were randomized to receive preparation for allotransplant with Cy, 120 mg/kg, combined with either 2 Gy per day on each of 6 consecutive days or 2.25 Gy per day for 7 days, a total dose of 12 Gy versus 15.75 Gy. In each trial, patients who received the higher irradiation dose demonstrated a substantially lower incidence of recurrent leukemia but a higher frequency of transplant-related complications and death. They exhibited a higher incidence of interstitial pneumonia, hepatic VOD, renal failure and mucositis. Attempts to intensify preparative regimens have consistently

Figure 91-3 (A) Mortality in patients not relapsing after stem cell transplantation (p = 0.04). Patients who relapsed were censored at the time of relapse. **(B)** Probability of relapse (p = 0.06). **(C)** Probability of surviving relapse-free after transplantation. *(From Clift R, Buckner C, Appelbaum F et al: Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens. Blood 76:1869, 1990, with permission.)*

been associated with less leukemic relapse but an increased frequency and severity of transplant-related complications.

The toxicity of Cy/TBI regimens is significantly greater in end-stage allotransplant recipients, such as those studied initially by Thomas et al, [25] than in patients receiving allotransplants in first remission, studied later by Thomas et al. [29] [30] Similar results have been widely supported by the International Bone Marrow Transplant Registry, as well as a variety of single institutions and multi-institutional groups. [37] [40] [41] [51] [111] [112]

Figure 91-4 (A) Probability of relapse after transplantation during the chronic phase of CML for patients receiving 12 Gy or 15.75 Gy of total body irradiation (p = 0.008). **(B)** Probability of developing Grade II or worse acute GVHD after transplantation in the chronic phase of CML for patients receiving 12 Gy or 15.75 Gy of total body irradiation (p = 0.15). **(C)** Probability of survival after transplantation during chronic phase of CML for patients receiving 12 Gy or 15.75 Gy of total body irradiation. *(From Clift R, Buckner C, Appelbaum F et al: Allogeneic marrow transplantation in patients with chronic myeloid leukemia in the chronic phase: a randomized trial of two irradiation regimens. Blood 77:1663, 1991, with permission.)*

Patients in first remission from AML who receive HLA-identical stem cell grafts from siblings have leukemia-free survival rates of approximately 5060% they have been as high as 70% in some studies.[111] [112] [113] [114] Patients in second remission or in an untreated first relapse appear to have an approximately 30% leukemia-free survival rate at 5 years.[38] [47] [112] Those beyond second remission or with refractory disease have a 1020% leukemia-free survival rate, similar to that originally described by Thomas et al in a similar patient population. [25] [47] [112]

Patients with CML in the chronic phase of disease transplanted within a year of diagnosis (thus having generally received relatively minimal prior chemotherapy) have a mortality rate of approximately 15% when Cy/TBI preparation is used. The sustained disease-free survival rate is approximately 70% in patients under age 40. Patients with more advanced disease, including those who have had the disease for >1 year or those with accelerated or blastic phase disease, have a substantially lower incidence of leukemia-free survival. [110] [115]

Thomas et al [116] demonstrated that patients with ALL who underwent transplantation in second or subsequent remission had a lower incidence of transplant-related toxicity and leukemic relapse and improved survival compared to patients transplanted in relapse. Transplantation in ALL is now most frequently performed in second remission and most frequently using Cy/TBI regimens. Disease-free survival has ranged from approximately 3050%, depending on patient age, specific cytogenetic abnormalities, and other risk factors.[116] [117] [118] [119] [120] [121] [122] [123] [124] In patients with more advanced disease, disease-free survival is usually around 20%; leukemic relapse is the most common cause of failure.

Allogeneic SCT has been performed with increasing frequency and effectiveness is well-documented in selected patients with ALL in first remission who are at high risk for relapse (e.g., those with the Philadelphia chromosome). [125] [126] [127] The effectiveness of allogeneic transplantation results from the preparative regimen and the antileukemic activity of donor cells.[124] The superior effectiveness of Cy/TBI and transplantation is balanced by regimen-related toxicity, delayed effects of the preparative regimen, and GVHD. Leukemia-free survival rates of 4060% have been reported. [124] [128] [129] [130] The International Bone Marrow Transplantation Registry observed a decrease in treatment-related mortality following allotransplant in patients in first remission from 39 ± 19% to 27 ± 8% between 1980 and 1989. [131] Leukemia-free survival improved from 42 ± 20% to 57 ± 8% over the same period. Throughout, Cy/TBI remained the most frequently administered regimen.

The Cy/TBI regimen has been effective and widely used in non-Hodgkins lymphoma, Hodgkin disease, multiple myeloma, thalassemia, and other nonmalignant disorders. It has served as the standard to which all other regimens should be compared.

While preparation with Cy, 120 mg/kg, and TBI (9201575 cGy) consistently permits engraftment of stem cells from HLA-identical siblings, >10% of HLA-haplo-identical partially matched donors rejected grafts.[132] Patients given single doses or high total doses had lower rejection rates than those who received fractionated lower doses (e.g., 1,200 Gy) over intervals (e.g., 6 days).

TBI with Agents Substituted for Cyclophosphamide

Drugs other than Cy have been added to TBI, such as cytosine arabinoside, melphalan, and etoposide. TBI is a potent immunosuppressive agent, as is Cy. Immunosuppression provided by Cy/TBI regimens is effective but exceeds what is sufficient for consistent engraftment in many circumstances. The antitumor effect provided by this combination might be improved by substitution of an agent with a more potent antitumor effect. This has been attempted where the high level of immunosuppression is unnecessary, such as in transplants involving HLA-identical siblings.

Melphalan may be more effective than Cy in the treatment of many hematologic malignancies. A variety of trials have used melphalan in doses up to 180 mg/m ^{2} .

have been combined with TBI up to 1,485 cGy.[133] [134] [135] In a randomized study of patients with AML in first remission, those receiving melphalan, 110 mg/m ^{2} , and TBI had a lower relapse rate than patients receiving Cy, 120 mg/kg, plus TBI. Unfortunately, transplant-related deaths occurred more frequently in the melphalan group, and leukemia-free survival rates were similar.[135] Stomatitis and hepatic VOD were dose-limiting toxicities of melphalan and TBI.

High doses of cytosine arabinoside have also been substituted for Cy. Initial studies of cytosine with fractionated TBI reported excellent results in children with ALL.[136] Other studies have reported results similar to those achieved with Cy/TBI. [137] [138] The use of cytosine arabinoside and TBI has been associated with increased toxicity compared to Cy/TBI and has not improved results in AML.[139] Skin and central nervous system toxicity have limited dose escalation of cytosine arabinoside and TBI.

The most promising results involving the substitution of another drug for Cy have occurred with etoposide. Etoposide-plus-TBI regimens have yielded favorable results in a number of hematologic disorders. A phase I/II study using a total dose of 1,320 cGy TBI delivered in 11 fractions over 4 days achieved a maximum tolerated etoposide dose of 60 mg/kg.[140] Stomatitis and hepatic and pulmonary toxicity were dose-limiting. In the initial study, 43% of patients with advanced acute leukemia achieved sustained disease-free survival. The relapse rate was only 32 ± 20%. A phase II trial using this regimen in 69 patients with AML and ALL in remission and CML in chronic phase demonstrated that 55% of patients were leukemia-free survivors at 3 years. [141] The City of Hope and Stanford reported a disease-free survival of 61% for 57 high-risk patients with ALL who underwent allogeneic transplantation in first remission. [130]

A confounding factor in the etoposide-and-TBI trials is that the majority of patients received cyclosporine and corticosteroids for prevention of GVHD. In comparable studies performed with Cy/TBI, the majority of patients received cyclosporine and methotrexate. The combination of cyclosporine and corticosteroids does not have a long-term beneficial effect on disease-free survival; this is largely attributable to an increased incidence of chronic GVHD and of fungal infections. [42] [142] However, when evaluating acute toxicity, it would appear that substitution of corticosteroids for methotrexate results in fewer and less severe acute toxicities, including VOD. Thus, comparisons of regimen-related toxicity for Cy/TBI versus TBI/etoposide are difficult.

Cyclophosphamide Plus Antithymocyte Globulin

In aplastic anemia, there is no requirement for myeloablation, only for immunosuppression. Storb et al [143] [144] performed allogeneic SCT in patients with severe aplastic anemia after the administration of 200 mg/kg of Cy. While engraftment consistently occurred in patients who had not been sensitized by prior transfusion, graft failure rates were high in patients who had received transfusions (as had a vast majority of patients). [143] [144] [145]

Additional immunosuppression has been achieved by adding TBI, total lymphoid irradiation (TLI), or thoracoabdominal irradiation. [146] [147] [148] [149] The addition of irradiation has been associated with an increased incidence and severity of transplant-related complications. Other concerns include the increased incidence of second malignancies and other long-term sequelae following radiation.

The addition of antithymocyte globulin (ATG) to Cy has been associated with survival rates >90% in patients receiving stem cell transplants from HLA-identical siblings.[150] Unfortunately, in the setting of matched unrelated donors, the combination of antithymocyte globulin and Cy is not sufficiently immunosuppressive to permit consistent engraftment.[151] Radiation in a variety of doses and delivery formats has been used; the best approach is uncertain.

Busulfan Plus Cyclophosphamide in Thalassemia

Successful treatment of aplastic anemia with allotransplant in the 1970s led to consideration of this approach in patients with other nonmalignant marrow disorders, such as thalassemia. In 1982, the first successful allograft in a patient with thalassemia was published. [152] Lucarelli and his team in Pesaro have had the most extensive experience with the use of SCT in thalassemia.[69] [70] [153] [154] [155] The Pesaro group described the results of transplantation in 805 thalassemia patients aged 135. Patients were treated with Bu, 14 mg/kg, and Cy, 200 mg/kg, and cyclosporine alone. The following risk factors were defined: poor quality of previous iron chelation therapy, hepatomegaly, and portal fibrosis on liver biopsy. Patients with none of these risk factors were categorized as class 1, and those with three risk factors class 3; all others were class 2. Class 1 patients had a very high probability of cure and very low morbidity and mortality. The long-term event-free survival rate in patients aged <17, transplanted from HLA-identical donors, was 90%. Class 3 patients aged <17, transplanted from HLA-identical siblings, had a success rate of 50%. Severe cardiac and liver toxicity occurred commonly in class 3 patients. When the Cy dose was decreased from 200 mg to 160 mg and short-course methotrexate was used, results were more encouraging, although additional patients and longer follow-up are needed. These results emphasize the very high probability of cure with minimal morbidity and mortality when transplantation is performed early in the course of disease. The Bu dose of 14 mg/kg is lower than that generally used in hematologic malignancies.

Busulfan Plus Cyclophosphamide in Hematologic Malignancies

The effectiveness of BuCy regimens has now been evaluated in a number of malignant diseases. Studies of AML in first remission using Bu, 16 mg/kg combined with either Cy, 120 mg/kg or Cy, 200 mg/kg,[38] have generally resulted in leukemia-free survival rates of about 60% and relapse rates of approximately 20%, similar to results reported from studies using Cy/TBI. However, the only randomized study of first-remission patients with AML was the GEGMO study by Blaise et al, [114] which demonstrated increased relapse rates and decreased survival in patients receiving BuCy compared to those receiving Cy/TBI. Proponents of BuCy have not yet established that it is as effective as Cy/TBI in patients with AML in first remission; in particular, patients with M4 or M5 FAB subclasses appear to have higher relapse rates following BuCy.[40]

The original multi-institutional study of BuCy2 in CML in 1992 described leukemia-free survival rates similar to those reported for Cy/TBI in chronic, accelerated, and blastic phase disease.[41] Chronic-phase patients had an approximately 58% 3-year leukemia-free survival. Relapse rates appeared lower than were generally reported with Cy/TBI. The relapse rate was reported as 3% at 3 years in chronic-phase patients, 12% in accelerated-phase patients, and 27% in blastic-phase patients. A single study of BuCy, 200 mg/kg, demonstrated a lower survival in chronic-phase patients, but these patients were generally older and received only one drug for GVHD prophylaxis.[156] The relative effectiveness of big BuCy versus little BuCy remains unknown and unstudied.

Randomized trials of BuCy versus Cy/TBI in CML in chronic-phase patients have been published from Seattle in 1994 [157] and from the French Society of Bone Marrow Grafts in 1995.[158] The Seattle study compared 73 patients in chronic phase transplanted following BuCy2 to 69 patients transplanted following Cy/TBI. Three-year probabilities of survival were identical at 80%; relapse rates were identical at 13%; event-free survival was 71% in the BuCy group and 68% in the Cy/TBI group (Fig. 915). This study concluded that survival and relapse rates were equivalent. There was no difference in speed

Figure 91-5 The probabilities of event-free survival and of developing persistent cytogenetic relapse for patients transplanted after the Cy/TBI or BuCy regimens are shown. *(From Clift R, Buckner C, Thomas W et al: Marrow transplantation for chronic myeloid leukemia: a randomized study comparing cyclophosphamide and total body irradiation with busulfan and cyclophosphamide. Blood 84:2040, 1994, with permission.)*

of engraftment or the incidence of VOD, but the TBI group had an increased incidence of creatinine elevation and an increased frequency of acute GVHD (Fig. 916). Thus, BuCy was better tolerated and was recommended. The French Society of Bone Marrow Graft reported results in 120 patients randomized to Cy/TBI or BuCy. Similar survival and disease-free survival rates were observed in the two groups. Like the original investigators, however, they found low relapse rates with BuCy (4.4 ± 6.7%), a significantly lower rate than that observed with Cy/TBI.

Because of widespread interest in avoiding radiation-related complications in children, there is a high level of interest in BuCy and other radiation-free regimens in

childhood malignancies in general, particularly in ALL. Bu does not appear to be very immunosuppressive and does not immediately, dramatically affect lymphocyte count.[18] [19] [20] Many investigators have reasoned that BuCy would not be an effective regimen in ALL. However, following BuCy, the kinetics of reconstitution, both lymphoid and hematopoietic, in animals and humans is similar

Figure 91-6 The probabilities of developing Grade II or worse acute GVHD for patients transplanted after the Cy/TBI or BuCy regimens are shown. *(From Clift R, Buckner C, Thomas W et al: Marrow transplantation for chronic myeloid leukemia: a randomized study comparing cyclophosphamide and total body irradiation with busulfan and cyclophosphamide. Blood 84:2039, 1994, with permission.)*

to that following Cy/TBI transplants.[159] [160] Multi-institutional studies have generally demonstrated results with BuCy similar to those reported with Cy/TBI in adults with ALL.[40] [161] Sufficient data are not available, however, to make a meaningful conclusion. Expression of CD3 surface antigens in T-lineage ALL is associated with resistance to radiation.[162] Whether a radiation-free regimen might be more effective than TBI in this group is worthy of investigation.

The Nordic BMT Group reported the results of a prospective randomized study of HLA-identical sibling transplants in 167 patients with leukemia. [163] They found a higher incidence of VOD and hemorrhagic cystitis in patients receiving BuCy compared to Cy/TBI and a significant difference in transplant-related mortality in patients with advanced disease, with a rate of 62% in BuCy patients and 12% in those receiving Cy/TBI. Interestingly, in low-risk patients BuCy was very well tolerated and effective; disease-free survival was superior (though not significantly so) among BuCy patients in this category. Disease-free survival did not differ when all patients were considered.

Blume et al reported somewhat contrasting results in a Southwest Oncology Group trial comparing BuCy to VP16/TBI in a heterogeneous group of patients undergoing allogeneic transplantation from HLA-identical siblings. [164] Overall outcome was similar in these two groups. In contrast to the Nordic study, however, the low-risk patients appeared to fare better with the radiation-containing regimens and the high-risk patients fared better with BuCy. Thus, even with well-conducted, prospective, randomized studies, the relative merits and limitations of these three regimens are not fully understood.

The heterogeneity of results achieved with BuCy (as well as other regimens) may result from a variety of circumstances, including different dosing (e.g., ideal versus real body weight), dosing schedules of other drugs (e.g., dilantin, which affects Bu metabolism), as well as the learning curve in using a new regimen.

Mephalan and etoposide have also been used in combination with Bu. However, because of the limited immunosuppressive capacity of Bu, the immunosuppressive capability of these regimens is limited and their application is likely only to patients with HLA-identical sibling donors. Five patients who had relapsed after prior allotransplants were prepared for second transplants with Bu and etoposide. All engrafted and obtained complete remission. [165] Extensive study of this regimen in the autologous setting has yielded favorable results. [166] Its use in the allogeneic setting merits further study.

A pilot study of Bu and melphalan prior to allogeneic transplantation in advanced hematologic malignancies demonstrated engraftment in 24 of 25 patients receiving stem cells from HLA-identical siblings. Antileukemic activity in this study was promising. [167]

Cyclophosphamide and TBI Plus Additional Agents

For patients with advanced leukemia, relapse remains the most frequent cause of failure following allogeneic transplantation. Higher doses of radiation or other agents have been used; however, reports of favorable antileukemic effectiveness have been offset by increased toxicity. Attempts have been made to add combinations of chemotherapy agents without significant overlapping toxicity to TBI. Peterson et al [168] performed a careful phase I study designed to determine the maximum tolerated dose of Bu and Cy to be combined with 12 Gy of fractionated irradiation. Doses of 8.7 mg/kg of Bu and 69 mg/kg of Cy in conjunction with TBI led to severe regimen-related toxicity in 5 of 18 patients, while none of 15 patients treated with 6.9 mg/kg of Bu and 47 mg/kg of Cy developed severe regimen-related toxicity. A phase II study of Bu, 7 mg/kg, Cy, 50 mg/kg, and TBI, 12 Gy, in patients with morphologically advanced myelodysplasia demonstrated trends toward lower relapse rates but

higher transplant-related mortality compared to historical controls prepared with Cy, 120 mg/kg, and TBI. [169]

Similarly, it was found that 44 mg/kg of etoposide could be combined with 103 mg/kg of Cy and 12 Gy of fractionated TBI. [170] [171] A separate study indicated that etoposide administered as a continuous infusion was well tolerated. At present, the relative merits of these regimens have not been adequately examined.

The Stanford group developed a different regimen of 1,320 cGy TBI in 11 fractions, 60 mg/kg of etoposide, and 60 mg/kg of Cy followed by allotransplant from matched sibling donors.[172] Preliminary studies have demonstrated the regimen to be well-tolerated and effective. The long-term effectiveness of this preparative regimen is currently being evaluated.

Busulfan Plus Cyclophosphamide and Etoposide

Based on data in nontransplant settings where combinations of chemotherapeutic agents have been more effective than single agents, investigators have explored combinations of multiple drugs as preparative therapy for transplantation. Combinations of Bu, etoposide, and Cy have been used in various doses and schedules. This is a logical combination because of the synergistic activity of etoposide and Cy. Vaughan et al [173] reported that 11 of 24 (46%) of patients with advanced leukemias or lymphoma died of transplant-related complications using Busulfan, 16 mg/kg, Cy, 120 mg/kg, and etoposide, 60 mg/kg. While this degree of transplant-related mortality would be considered prohibitive by many investigators, the relapse rate in this study was only 20% and disease-free survival was 40% at a median follow-up of >1 year. Although the follow-up is too short to make a significant conclusion, this study raises a critical issue: what degree of transplant-related mortality is tolerable if disease-free survival with a given regimen is favorable? Investigators at John Hopkins used Bu, 16 mg/kg, etoposide, 30 mg/kg, and Cy, 150 mg/kg, over 8 days in patients with advanced lymphoid and hematologic malignancies. In a phase I trial, stomatitis and VOD were dose-limiting at etoposide doses of 40 mg/kg.[174] At 30 mg/kg, most patients developed grade I or II mucositis. VOD was diagnosed in 30% of patients and was the major factor contributing to death. These investigators reported a trend toward improved disease-free survival with this new regimen compared to historic controls. Based on dose escalation trials indicating markedly lower toxicity of 14 mg/kg of Bu compared to 16 mg/kg, investigators at Ohio State have used Bu, 14 mg/kg, etoposide, 50 mg/kg, and Cy, 120 mg/kg, in >100 patients who have undergone allogeneic transplantation from sibling or matched unrelated donors with favorable preliminary results. [48] [175]

While selected initial data are promising, there has been no clear demonstration that the addition of etoposide to BuCy or Cy/TBI represents a significant improvement. Another multidrug combination used in allogeneic transplantation is Cy, BCNU, and etoposide. This regimen has generally been used in patients with Hodgkin disease, non-Hodgkin lymphoma, and acute lymphoblastic lymphoma. [176] Good results in allogeneic transplants have been reported. Reports of late pulmonary fibrosis have dampened enthusiasm for this regimen, [177] but this requires more extensive study.

T-Cell Depletion

Removal of T cells from the donor graft is associated with an increased risk of graft failure. More intense preparative regimens have been required to prevent rejection. This has been accomplished by the administration of higher doses of radiation, adding additional chemotherapy (e.g., thiotepa), adding additional immunosuppression (e.g., ATG), or combinations of the above. Regimens such as BuCy2 provide inadequate immunosuppression in this situation.

PROMISING AREAS OF CLINICAL RESEARCH

In general, Cy/TBI, BuCy, and TBI/VP16 are associated with tolerable regimen-related toxicity and acceptable results in allograft recipients with acute leukemia in first remission and CML in chronic phase. Patients with more advanced disease, however, develop more transplant-related complications and have higher relapse rates. Shortcomings of these preparative regimens limit the proportion of patients who can be cured. Dramatic improvement in preparative regimens is needed. Investigators are attempting to improve the effectiveness of existing regimens without producing more toxicity, a difficult task in view of prior experiences. The following section will address selected areas of current research that may well advance the field.

Targeting Plasma Levels of Drugs

In multiple studies, Bu has shown wide inter- and intrapatient variability in its disposition, leading to varying plasma concentrations. [178] [179] Age, hepatic function, underlying disease, and drug interactions have all been shown to play important roles. Pharmacokinetic studies in children have shown lower plasma levels for a fixed mg/kg dose in children than in adults. Children <age 6 have demonstrated higher plasma levels and greater success when doses are based on body surface area. [180] [181] Interestingly, graft rejection, acute complications, and relapse rates have all been related to plasma Bu levels. In a multivariate analysis of 42 patients transplanted in Seattle, only the average steady-state ($\mathbf{C}_{ss}$) level of Bu was shown to be significant in predicting graft rejection. [179] Forty-two patients were administered 1630 mg/kg of Bu and Cy. Bu levels were analyzed using gas chromatography. When $\mathbf{C}_{ss}$ levels of Bu were <200 ng/ml, all 4 such patients rejected their grafts. At levels of 200600 ng/ml, 4 of 11 patients rejected, and at levels >600 ng/ml, only 1 (a one antigen mismatched, unrelated donor) of 23 patients rejected the graft. In addition, the data from Seattle indicated that partially matched, unrelated donors required higher $\mathbf{C}_{ss}$ levels of Bu (i.e., >600 ng/ml) than HLA-matched siblings, who required a level of only 200 ng/ml. The higher $\mathbf{C}_{ss}$ Bu levels required in the HLA partially matched setting strongly suggest that Bu has immunosuppressive activity.

Significant regimen-related toxicity was seen at higher Bu levels. Only 1 of 31 patients who had an average Bu $\mathbf{C}_{ss}$ <900 ng/ml had Grade III or IV regimen-related toxicity, while 4 of 11 patients who had a $\mathbf{C}_{ss}$ >900 ng/ml had Grade III or IV regimen-related toxicity (Fig. 917). [179] Regimen-related toxicity was

Figure 91-7 Relationship between regimen-related toxicity score and busulfan average concentration at steady-state P = 0.717; P = 0.0001. *(From Hutchinson F: Graft rejection and toxicity following bone marrow transplantation in relation to busulfan pharmacokinetics. Bone Marrow Transplant 16:36, 1995, with permission.)*

chiefly VOD and adult respiratory distress syndrome. The group at Johns Hopkins demonstrated a significantly higher incidence of VOD when Bu levels are high. [180] [181] [182] Lastly, patients with a low $\mathbf{C}_{ss}$ of Bu had statistically higher relapse rates. [183] Maintaining Bu $\mathbf{C}_{ss}$ within a narrow range, defined according to the underlying disorder, stage, or source of donor cells, could lower the incidence of rejection, severe toxicity, and relapse. Limiting toxicity might also permit the addition of other agents, such as directed irradiation. The use of phenytoin has complicated attempts to target specific Bu plasma levels and adjust future doses based on first-dose pharmacokinetics. Patients receiving Bu and phenytoin appear to have increased Bu clearance and decreased area under the curves for Bu following the eighth or ninth dose compared to the first dose. [183]

Cyclophosphamide is converted to 4-hydroxycyclophosphamide (HCY), a reaction responsible for its toxicity. This reaction is catalyzed by peroxidases and cytochrome P450. HCY enters cells spontaneously and is metabolized to acrolein and phosphoramide mustard, a DNA alkylator. [184] [185] [186] Analysis of Cy and HCY by high-performance liquid chromatography demonstrates extensive variability in the disposition of Cy and HCY. Slattery et al [183] reported 3.7-fold variability in the range of exposure to HCY in seven patients receiving Cy, 120 mg/kg, and TBI. A sevenfold difference was observed in the ratio of the area under the curve for HCY compared to the area under the curve for Cy, an expression of the ability to form and eliminate HCY. In patients treated with Bu followed by Cy, a greater clearance of Cy and a greater area under the curve of HCY compared to Cy was seen. The use of Bu prior to Cy results in a greater exposure to HCY (Fig. 918). [186] Further, the area under the curve of HCY was shown to correlate with the average $\mathbf{C}_{ss}$ of Bu. In part, the immunosuppressive capability of Bu may result from its effect on Cy metabolism. In addition, a relationship was seen between exposure to Cy metabolites and hepatic VOD. [186] Greater attention and understanding of the pharmacokinetics of Bu and Cy, as well as other agents, promises safer and more effective

Figure 91-8 Relationship between AUC_{HCY} and AUC_{CY} in BuCy patients (, R^2 = .171, P = .356) and Cy/TBI patients (, R^2 = .116, P = .455 with UPN 8807 and R^2 = .740, P = .028 without UPN 8807). *(From Slattery J, Kalhorn G, McDonald K et al: Conditioning regimen-dependent disposition of cyclophosphamide and hydroxycyclophosphamide in human marrow transplantation patients. J Clin Oncol 14:1484, 1996, with permission.)*

preparation for transplantation. Better understanding of specific metabolic pathways in individual patients could lead to dose adjustment and might also influence the choice of agents.

Directed Irradiation

Hematologic malignancies appear to have steep dose-response relationships to irradiation, as has been previously presented. Doses of TBI are limited by toxicity to the lungs, liver, and gastrointestinal tract.

Investigators have used a variety of techniques to deliver higher doses of radiotherapy to the marrow than to normal organs. Radioactive iron (Fe) is taken up by the erythropoietic marrow, where it irradiates adjacent cells. The marrow of patients treated with ^{52} Fe demonstrated nearly twice the radiation absorbed as did the liver. [187] In combination with standard preparative therapy, this offers a method for selective delivery of radiation to the marrow.

A logical approach to ensure selective delivery of irradiation to malignant cells is to use monoclonal antibodies directed against antigens on tumor cells, conjugated to drugs, toxins, or radionuclides. Unconjugated monoclonal antibodies have demonstrated limited capacity to kill malignant cells. [188] Conjugates of toxins with monoclonal antibodies to lymphoid or myeloid antigens have demonstrated safety and occasional complete responses in patients with malignancies. [189] [190] [191] The delivery of radiation to sites of disease using monoclonal antibodies conjugated to radionuclides has been successful in animal and human studies. [192] [193] [194] [195] [196] Studies in acute leukemia have used I-131-labeled anti-CD33 antibody [197] and most recently I-131-labeled anti-CD45 antibody. [198] CD45 is present in greater amounts on leukemic blasts and does not modulate (as does CD33). Preliminary studies have demonstrated that roughly threefold higher doses of irradiation can be delivered

to leukemic sites than to the liver and lungs. [198] While this technique has been used predominately with standard Cy/TBI, it may be more logical to combine it with a chemotherapy-only regimen. Targeting areas of distribution of malignant cells, without significant exposure of other organs, is a logical and promising approach to improving preparative regimens.

Nonmyeloablative Regimens

Storb et al[199] achieved stable mixed hematopoietic chimerism in DLA-identical litter mates using a sublethal irradiation dose of 200 cGy TBI with the immunosuppressive agents cyclosporine and mycophenolate mofetil. [199] This regimen is not myeloablative, is relatively nontoxic, and does not cause prolonged pancytopenia. A regimen including 200 cGy of TBI, cyclosporine, and mycophenolate with infusion of peripheral blood stem cells is under investigation in Seattle. Sequential lymphocyte infusions at successfully higher doses will be used. The study will initially be performed in patients with CML who are >65 years, or in patients <66 years with organ dysfunction precluding conventional allografting. This novel approach will use nonablative, immunosuppressive therapy to attain hematopoietic chimerism, then rely on a graft-versus-leukemia effect of donor lymphocytes.

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Chapter 92 - Practical Aspects of Stem Cell Collection

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INTRODUCTION

Peripheral blood stem cells (PBSC) have virtually replaced bone marrow as the hematopoietic stem cell (HSC) component for autologous transplantation, and their use is undergoing phase III studies in allogeneic transplantation. The ease of collection and the rapid engraftment kinetics of PBSC compared to bone marrow are widely recognized. Median times to achieve an absolute neutrophil count (ANC) >500/l are typically about 1214 days, and platelet recovery is even faster. [1] [2] [3] [4] [5] [6] Although graft-versus-host disease (GVHD) prophylaxis with methotrexate will slow engraftment, the kinetics of engraftment for the allogeneic PBSC recipient are similar to that experienced by the recipient of autologous PBSC. [7] [8] [9] [10] [11] [12] [13] The rapid engraftment kinetics are widely recognized despite the paucity of direct comparative trials between marrow and PBSC as sources of HSC. Beyer et al.[14] reported such a phase III study of 47 patients treated for germ cell tumor. Although all patients received granulocyte colony-stimulating factor (G-CSF) after transplantation, and despite the small number of patients enrolled, the recipients of PBSC achieved an ANC of >500/l 1 day faster, and a sustained platelet count of >20,000/l 7 days faster than the patients receiving marrow. [14]

There are also disadvantages to the use of PBSC components as a source of HSC for autologous or allogeneic transplantation. These include the usual need for multiple days of collection (especially for autologous transplantation), the current need for sophisticated flow cytometric analysis of the components (or hematopoietic cell cultures) to ensure adequacy of HSC content, the inability to collect adequate components from all patients and donors, the risks associated with administration of hematopoietic cytokines and the apheresis procedures, and the risks of infusion if the components are cryopreserved.

The presence of HSC in the peripheral circulation was suggested by animal studies as early as 1951. [15] Although the nature of the survival agent was not recognized at that time, parabiosis experiments demonstrated that some factor in the blood of a healthy animal was able to rescue another animal from the effects of lethal irradiation. Subsequently, the presence of HSC in the peripheral blood and the use of these cells to rescue animals from the marrow-lethal effects of radiation were demonstrated in a number of animal models.[16] [17] Experience in human PBSC transplantation began for patients with chronic myelogenous leukemia because of the high number of clonogenic cells circulating in the peripheral blood of those patients. [18] The concentration of HSC in the peripheral blood is normally very low. Thus, very large quantities of blood must be processed to collect a quantity of HSC equivalent to what could be collected in a bone marrow harvest. For this reason, PBSC transplantation was limited initially to a few centers that explored this source of HSC for patients who were otherwise ineligible for marrow harvesting. Some transplant centers used the transient increase in circulating HSC during recovery from marrow hypoplasia-producing chemotherapy. [19] [20] [21] Others collected cells from the patient or donor without any preceding perturbation of the host. [22] [23] [24] These early reports noted that engraftment could be faster after infusion of PBSC components compared to marrow cell transplantation. However, because of the occasionally limited quantity of HSC collected from the peripheral blood, the kinetics of engraftment for some patients was considerably slower (Table 92-1). Moreover, in a significant proportion of patients the desired quantity of cells could not be collected. The effective mobilization of HSC into the peripheral blood and the reliability of flow cytometric technique to assess quality of the collection are the direct bases for the rapid and widespread adoption of PBSC.

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MOBILIZATION OF HSC INTO THE PERIPHERAL BLOOD

The discovery that administration of hematopoietic cytokines caused a transient increase (mobilization) of HSC in the peripheral blood enabled the collection of much greater numbers of PBSC, or peripheral blood progenitor cells (PBPC) as they are sometimes called when collected after mobilization with growth factor and/or chemotherapy.[1] [25] [26] [27] No randomized studies comparing the engraftment of cytokine-mobilized HSC to those collected during steady state or after chemotherapy mobilization have been reported although retrospective studies demonstrated

TABLE 92-1 -- Relationship Between Mobilization Therapy, Dose of Progenitor Cells, and Engraftment Kinetics for Autologous PBSC Transplantation

Author	No. of Patients	Mobilization Therapy	Progenitor Cell Dose		Engraftment Kinetics	
			CFU-GM (left×10^4 /kg)	CD34+ (left×10^6 /kg)	ANCleft >500/leftl	Plateletleft >20,000/leftl
To	43	Chemotherapy	86.6	ND	11 (917)	13.5[a] (9NR)
Fermand	8	Chemotherapy	5.5	ND	16 (1025)	34 (1090)
Juttner	8	Chemotherapy	127.2	ND	11 (914)	12[a] (1028)
Kessinger	10	Steady state	8.0[b]	ND	22 (1158)	23 (1436)
Nademanee	30	Steady state	ND	1.2	20 (9458)	31 (8441)
Nademanee	39	G-CSF	ND	6.2	10 (740)	15.5 (763)
Sheridan	29	G-CSF	21.0	ND	6 (410)	11 (9136)
Weaver	692	Chemotherapy + G-CSF	30.8[c]	9.9[c]	9 (538)	9 (453[b])
Bensinger	124	Chemotherapy + G-CSF	ND	9.4	11 (420)	10 (665)

CFU-GM, colony-forming units-granulocyte/macrophage; G-CSF, granulocyte colony-stimulating factor; ND, no data.

Shown are mean values for progenitor cell quantities infused and median values for time to achieving the particular end point of engraftment.

Data from Sheridan et al.,[4] To et al.,[5] Fermand et al.,[20] Juttner et al.,[19] Kessinger et al.,[22] Nademanee et al.,[26] Weaver et al.,[30] and Bensinger et al.[29]

[a]Time to achieve >50,000 platelets/l.
[b]CFU-GM cultures performed on thawed cells.
[c]Shown are median values.

the much quicker engraftment of cytokine-mobilized HSC (Table 92-1).[28] Cytokine-mobilized PBSC components contain much greater numbers of cells expressing the CD34 antigen (CD34+ cells) that, along with myeloid progenitor cells grown in culture (colony-forming units-granulocyte/macrophage [CFU-GM]), serve as surrogate markers for the engraftment capacity of the stem cell component. It cannot be stated with certainty if it is merely the quantity of cells or some characteristic imparted by chemotherapy or cytokine mobilization that accounts for the rapid engraftment. CD34+ cells isolated from the peripheral blood differ in many characteristics from those isolated from marrow. However, the dose of CD34+ cells infused predicts the kinetics of engraftment with patient groups receiving higher quantities showing a higher probability of quicker engraftment. [29] [30] Although most transplant centers will probably accept components containing at least 12×10^6 CD34+ cells/kg recipient weight, cell doses of 2.55×10^6 CD34+ cells/kg are desirable, and earlier engraftment will be observed with even higher doses. [29] [30] Administration of G-CSF after transplantation will further speed the recovery of granulocytes, [31] [32] [33] but it may have a detrimental affect on platelet engraftment for patients receiving components containing low numbers of CD34+ cells. [29]

A variety of mobilization schemes have been developed for both patients and donors. Before the availability of hematopoietic cytokines, it was noted that marrow hypoplasia-producing chemotherapy such as that used as induction therapy for acute leukemia results in a transient increase in the number of HSC circulating in the peripheral blood. This approach was used by a variety of transplant programs to obtain PBSC for patients with leukemia or other diagnoses. Juttner and colleagues demonstrated rapid engraftment for patients receiving chemotherapy-mobilized PBSC (median 11 and 12 days to achieve an ANC >500/l and platelet count >50,000/l) as long as the dose of CFU-GM exceeded 63.0×10^6 /kg. [19] This finding that the CFU-GM quantity infused roughly correlated with the speed or success of engraftment was also found by Fermand and coworkers for chemotherapy-mobilized patients with multiple myeloma [20] and by Brice and associates for patients with non-Hodgkin lymphoma and Hodgkin disease.[21] However, Juttner also described a proportion of patients who received low doses of CFU-GM and experienced prolonged post-transplant aplasias or a secondary drop in blood counts 34 weeks after infusion. [19] Moreover, some patients, mostly those with progressive disease, marrow infiltration, or extensive prior exposure to alkylating agents or radiation therapy, failed to exhibit this response to chemotherapy and had inadequate collections for transplantation. [21] [34] The timing of apheresis was considered crucial with maximal numbers of CFU-GM in the peripheral blood when leukcocyte and platelet counts rose simultaneously during early recovery from chemotherapy. [35] [36]

It was subsequently discovered that the administration of granulocyte/macrophage colony-stimulating factor (GM-CSF) or G-CSF during the recovery from chemotherapy increased the numbers of circulating progenitor cells to levels as much as 1,000-fold higher than what was present in the blood before treatment. [1] [25] [26] [27] This started the exploration of efficacious mobilization regimens using hematopoietic cytokines, cytokine combinations, or chemotherapy plus cytokines.

Cytokine Mobilization

The ability of recombinant hematopoietic cytokines to increase the level of myeloid progenitor cells in the blood as well as mature blood cells was reported in 1988 by different groups for both G-CSF and GM-CSF. [26] [27] Subsequently, a number of different investigators reported the collection of PBSC from patients after chemotherapy and GM-CSF or G-CSF mobilization.[3] [25] [37] [38] Chemotherapy is not required to increase the numbers of HSC in the peripheral blood and many patients and all donors are treated with hematopoietic cytokines alone. Various recombinant human hematopoietic cytokines including fusion molecules increase the quantity of CD34+ cells in the peripheral blood as discussed below. Direct comparisons between cytokines of the ability to mobilize CD34+ cells are few. Furthermore, many of the single-arm cytokine studies were conducted in patient groups rather than normal donors, and the effect of prior therapy on HSC mobilization is a

confounding variable that hinders the comparison of these phase II studies.

G-CSF

At present, G-CSF is the cytokine most commonly used because of its efficacy compared to other cytokines and its relatively benign toxicity profile. Filgastrim (r-metHuG-CSF) and lenograstim (rHuG-CSF) are the two forms of this cytokine available for clinical use. [39] There is slight, if any difference between these two cytokines in the ability to mobilize PBSC. Watts et al. [40] studied 20 healthy volunteers and found that the peak levels of CFU-GM in the peripheral blood were 28% higher after treatment with the glycosylated molecule (lenograstim). In contrast, De Arriba et al. [41] treated 30 women with breast cancer in a randomized study of these two drugs using dosages containing bioequivalent units of activity and found no difference in the

mobilization of CD34+ cells. The two forms have similar biologic activity and will not be further distinguished in this discussion.

Mobilization of Hematopoietic Stem Cells Using G-CSF

Granulocyte colony-stimulating factor is the most potent cytokine currently available for the mobilization of HSC. In a randomized study of healthy volunteers comparing G-CSF, GM-CSF, and the combination of both, Lane and coworkers reported an average 0.99% CD34+ cells in the peripheral blood of healthy donors treated with 10 g/kg/day of G-CSF compared to 0.25% for donors treated with the same dose of GM-CSF. [42] The quantity of CD34+ cells in the peripheral blood before treatment averaged 1.6/l. After GM-CSF treatment, this increased to 3/l, but with G-CSF, the level increased to 61/l. Each group underwent one leukapheresis on the fifth day of treatment and the collections from donors treated with G-CSF averaged 119 × 10^6 CD34+ cells compared to 12.6 × 10^6 for the GM-CSF-treated donors.

There is a distinct time course of appearance of CD34+ cells during the administration of G-CSF, with the maximal level of CD34+ cells occurring on day 5 after 4 days of G-CSF administration. [43] [44] Smaller numbers of CD34+ are present on days 4 and 6 and the level falls rapidly on subsequent days despite a continual rise in white blood cells (WBC).

The number of CD34+ cells collected after G-CSF treatment is proportional to the number of these cells in the peripheral blood before the initiation of this cytokine. [12] Although doses as low as 5 g/kg/day have been used for allogeneic PBSC transplantation, [45] there is a dose response to G-CSF with higher average levels of CD34+ cells being achieved with 10 g/kg/day compared to 5 g/kg/day. [46] The average collection from one group of healthy donors treated with 10 g/kg/day averaged 4 × 10^8 CD34+ cells. [46] With this dose of G-CSF and appropriate apheresis technique, adequate numbers of CD34+ cells can be collected in one procedure for transplantation of most patients. Waller and coworkers studied twice daily administration of G-CSF to healthy donors and found greater yields of CD34+ cells in the apheresis component. [47] However, the total amount of G-CSF administered was also doubled so it is not clear if the twice daily administration or the higher dose is responsible for this observation. A similar dose-response relationship is observed in autologous patients and may extend to doses as high as 40 g/kg/day. [48] Patients, especially those previously treated with chemotherapy or radiotherapy (see following), will generally have lower quantities of CD34+ cells mobilized. However, previous treatment with chemotherapy does not preclude the use of G-CSF. De Luca et al. [49] noted a median 76-fold increase in CFU-GM for a population of 30 patients who were previously extensively treated for B-cell malignancies. Older donors will also have lower levels of CD34+ cells mobilized into the peripheral blood after G-CSF administration. [50]

Lymphocytes are also increased in the peripheral blood. [51] Murine studies suggest that G-CSF priming changes the cytokine response of these cells, [52] may decrease the risk of acute GVHD, [53] and may increase the graft-versus-leukemia effect. [54] The potential influence of these changes in outcome of allogeneic (or autologous) transplantation is not clear.

Toxicity and Complications of G-CSF

The toxicity of G-CSF has been most clearly defined in studies of allogeneic donors. [45] [46] [55] The autologous patient will experience a similar toxicity profile but with the added complications of the underlying malignancy and its treatment.

Virtually all recipients of G-CSF will develop somatic complaints of which skeletal pain is most prominent (Table 92-2). The somatic complaints are generally tolerable and few donors

TABLE 92-2 -- Incidence of Somatic Complaints for Normal Donors Treated with G-CSF

	Anderlini	Stroncek	Bishop
No. of donors	43	85	41
G-CSF dose	6 g/kg/d	210 g/kg/d	5 g/kg/d
Symptom			
Myalgias	82%	86%	83%
Headache	70%	28%	44%
Fatigue	20%	14%	NR
Fever	0	NR	27%
Chills	NR	NR	22%
Nausea	10%	11%	22%

NR, this particular complaint was not reported in the series.

Shown is the reported proportion of donors experiencing the somatic complaint.

Data from Bishop et al.,[45] Stroncek et al.,[46] and Anderlini et al.[55]

will require reduction in dose or discontinuation of the medicine. At present, long-term health risks for the donor appear to be minimal. Few serious complications of the mobilization regimen and donation process have been reported. [56] [57] One donor experienced splenic rupture that appeared to be temporally related to the donation process. Another donor with known coronary artery disease experienced a myocardial infarction after the first of two intended donations. A third donor succumbed to a cerebral vascular accident several days after donation and discontinuation of the cytokine. Any relationship of these events for these last two donors to the donation process is purely speculative because similar events have not been reported for autologous donors or others receiving these cytokines. Also of concern is the possibility that cytokine administration will increase the risk of marrow dysplasia or malignancy. Although this is of theoretical concern in that the cytokines used are known to stimulate the growth of leukemic cells, no clinical evidence suggests that these agents will induce abnormalities in the hematopoietic cell. There are no reports of an increased risk of myelodysplasia or hematologic malignancies in either healthy donors or patients who have been treated with short courses of G-CSF.

Administration of G-CSF results in a number of changes in blood counts and chemistries. [46] [55] Alanine aminotransferase, lactate dehydrogenase, and alkaline phosphatase levels will increase and the levels of blood urea nitrogen and bilirubin may decrease. The elevation in alkaline phosphatase is primarily of bone origin and -glutamyltransferase levels remain normal. These abnormalities of serum chemistries will resolve within 2 weeks after discontinuation of this medication. G-CSF administration will also result in a decrease in platelet count, especially if the cytokine is administered over many days. [46] WBC counts fall rapidly after discontinuation of G-CSF. In about 10% of donors, the WBC count may fall to abnormal levels (but generally remain above 1,000/l), reaching a nadir at 1014 days after discontinuation of the cytokine before stabilizing at normal levels.

GM-CSF

Much of the early experience with the use of hematopoietic cytokines for the mobilization of HSC involved GM-CSF and chemotherapy. [1] [25] [27] The increase in CFU-GM in the peripheral blood can increase by as much as 1,000-fold in this setting. [1] Moreover, at least for some patients, the combination of cyclophosphamide and GM-CSF is more effective than mobilization with G-CSF alone. [58] GM-CSF is not as potent as G-CSF. Haas et al. [59] treated 12 patients with 250 g/m^2 /day of GM-CSF and observed only an 8.5-fold increase in the number of CFU-GM in the peripheral blood (to a median of 1,347 CFU-GM/ml). [59]

As with G-CSF, there is also a dose-response relationship in mobilization of PBSC with GM-CSF over a range of 0.320 g/kg/day without a plateau being observed. [60] In this latter study, however, the average increase in CFU-GM in the blood at this highest dose level was again only 8.4-fold.

Administration of GM-CSF results in similar somatic complaints and liver function abnormalities reported after G-CSF administration. [61] In addition, 4480% of patients will experience fever, sometimes after each dose, and generalized or local skin reactions. Lieschke and Burgess noted that doses >20 g/kg/day are poorly tolerated because of fluid retention, pleural and pericardial inflammation, and venous thrombosis. [62] A first-dose reaction characterized by hypoxia and hypotension occurring within 3 hours after administration has been described for some recipients, especially after intravenous administration. [63]

Other Hematopoietic Cytokines

Recognition of the mobilization potential of G-CSF led many investigators to study other hematopoietic cytokines for their capacities to mobilize HSC into the peripheral blood. Used as single agents, these cytokines resulted in only about a 5- to 10-fold increase in circulating CFU-GM or CD34+ cells.

Macrophage Colony-Stimulating Factor

Few studies have been conducted investigating the efficacy of macrophage colony-stimulating factor (M-CSF) on the mobilization of PBSC. In one study involving six patients with acute myelogenous leukemia treated with two cycles of consolidation therapy, there was no significant difference in the number of CFU-GM in the peripheral blood after either cycle despite the addition of M-CSF at a dose of 8 × 10 ^{6} U/day during the second. [64] Although, based on this experience, M-CSF is unlikely to have a major effect on the mobilization of HSC, interpretation of this study is hindered by the fact that this was not a randomized study, all patients received the cytokine only during the second consolidation cycle, and the chemotherapy regimens differed for the two consolidation cycles.

Erythropoietin

Recombinant human erythropoietin has a mild mobilization effect. Pettengell et al. [65] treated 11 patients with malignant lymphoma with either 300 or 450 IU/kg thrice weekly for 2 weeks and found a 4.6-fold increase in CD34+ cells. The peak level of these cells was found on days 58 of administration of this cytokine.

Interleukin-3

Interleukin-3 (IL-3) has been studied primarily as a component of sequential cytokine mobilization with either GM-CSF or G-CSF. [66] [67] [68] In the nonhuman primate treated with 33 g/kg/day, IL-3 administration results in about a 12-fold increase in the number of circulating CFU-GM. [69] In contrast, Ganser and coworkers treated patients with advanced malignancies with 60250 g/m^2 /day of IL-3 for 15 days and found minimal increase in circulating CFU-GM on day 8 and a decrease for some patients on day 15. [66] Geissler et al. [68] reported similar findings.

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This fusion molecule of GM-CSF and IL-3 was studied by Bishop et al. [70] in 13 patients with malignant lymphoma in a phase I study involving doses of 250750 g/m^2 /day by continuous infusion. Peak levels of CFU-GM in the blood were found on days 46 of treatment. Although there was a mild increase in CFU-GM in the blood, there was not a consistent increase in CD34+ cells.

Stem Cell Factor

Most clinical studies of stem cell factor (SCF) report the use of this agent in combination with other cytokines (see below). Limited reports of SCF by itself are available, and this cytokine appears to result in a dose-dependent 6- to 10-fold mobilization of CFU-GM. [71] [72] Considerable dose-dependent toxicity including angioedema, urticaria, pruritis, and laryngospasm, presumably from degranulation of mast cells, has also been observed. [73] [74]

Combinations of Cytokines

The mechanisms by which any of these cytokines causes the mobilization of PBSC is not known. Multiple mechanisms are probably involved, including both an effect on proliferation of HSC as well as release of these cells from the marrow. It is conceivable, therefore, that combining different cytokines could result in additive or synergistic effect. Limited studies have been conducted to date and there is considerable potential to explore different cytokines with variations both in dose and schedule.

G-CSF Plus GM-CSF

The addition of G-CSF to GM-CSF greatly increases the number of HSC mobilized. The converse is not true. Winters et al. [75] treated patients with a variety of malignancies to regimens of GM-CSF or G-CSF (both at 5 g/kg/day) to which the other cytokine was added at day 7 of treatment and both continued through day 12. A third group received both cytokines (at 5 g/kg/day) simultaneously throughout the treatment. Apheresis was conducted on days 5, 7, 11, and 13. The simultaneous use of both cytokines achieved a much greater mobilization of CFU-GM on day 5 than either of the cytokines used singly to that point. The addition of GM-CSF on day 7 resulted in greater numbers of CFU-GM being collected in subsequent apheresis procedures than were collected on day 5, although the effect was not very great. In contrast, the addition of G-CSF on day 7 to patients treated with GM-CSF resulted in a strong release of CFU-GM 4 days later. Ho et al. [76] similarly studied the administration of GM-CSF or G-CSF at doses of 10 g/kg/day, or both cytokines together at doses of 5 g/kg/day each and found that G-CSF alone achieved greater mobilization than GM-CSF or both drugs given in combination at a lower dose.

SCF Plus G-CSF

By itself, SCF is a relatively weak mobilizing agent. Studies in dogs and baboons found that animals administered the combination of SCF and G-CSF had a 3.5- to 21.6-fold increase in progenitor cells in the blood. [77] [78] The clinical effect has been much more modest. Moskowitz and colleagues reported data on 38 patients with non-Hodgkin lymphoma randomized to SCF at 520 g/kg/day plus G-CSF 10 g/kg/day or to G-CSF alone. [79] They found no difference between the cohorts in mononuclear cells, CD34+ cells, or CFU-GM contents of the components collected by apheresis. However, patients with extensive prior therapy had increased numbers of CD34+ cells if given combination therapy, although the difference was not significant for this small number of patients. Begley et al. [80] conducted a similar phase I study of concurrent SCF and G-CSF in 62 women with early-stage breast cancer and found, in contrast, a significantly higher level of CD34+ cells in the blood of recipients treated with G-CSF plus SCF at 10 or 15 g/kg/day compared to G-CSF alone or in combination with SCF at 5 g/kg/day. The duration of mobilization for patients given the combination therapy was much broader with high levels of CFU-GM still being released into the blood several days after

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discontinuation of the cytokines. Sequential therapy with SCF given 3 days before initiation of G-CSF was even more efficacious, but even for this cohort of patients

the increase in CD34+ cell levels averaged only 2-fold greater than for recipients of G-CSF alone. The combination of SCF and G-GSF is also effective after cyclophosphamide mobilization chemotherapy.[81]

IL-3 Plus G-CSF

Geissler et al.[66] treated six patients with G-CSF at 5 g/kg/day for 5 days followed by a second course consisting of IL-3 at a dose of 5 g/kg/day for 7 days followed by G-CSF again at a dose of 5 g/kg/day for 5 days. They reported a 21-fold increase in CFU-GM in the peripheral blood after G-CSF alone, no increase after the course of IL-3, but a 56-fold increase after the combination. Peak levels of the progenitors in the blood were found on day 5 of G-CSF administration when given alone, but on day 3 if the patient was first treated with IL-3.

IL-3 Plus GM-CSF

Brugger et al.[67] treated 32 patients with etoposide, ifosfamide or cyclophosphamide, and cisplatin. The chemotherapy was followed by GM-CSF for 15 days for one group, by IL-3 for 5 days and then GM-CSF for an additional 10 days for another group, or with no cytokines for a third group. The dosages of the cytokines were not noted. The median peak number of CD34+ cells/l in the blood was 46 for the patients who did not receive either cytokine. The median for patients treated with GM-CSF alone was 426 CD34+ cells/l, and for the group treated with both IL-3 and GM-CSF, 418 CD34+ cells/l. The peak numbers of CFU-GM in the blood of the group receiving both cytokines was almost 2-fold greater than that for the group treated with GM-CSF alone.

Erythropoietin Plus G-CSF

In a retrospective comparison, Olivieri et al.[82] studied the addition of erythropoietin 50 U/kg/day to G-CSF 5 g/kg/day after mobilization chemotherapy with cyclophosphamide, etoposide, or epirubicine. The experimental group consisted of 18 patients with a variety of malignancies. The control group included 16 patients who received G-CSF alone. The groups were matched for diagnosis, previous chemotherapy, disease status, and mobilization chemotherapy agent. They found a 2.8-fold increase in the peak number of CD34+ cells for the group receiving both cytokines, and a 1.7-fold increase in the number of CD34+ cells collected during apheresis.

Chemotherapy Plus Cytokine Mobilization

The number of CFU-GM in the peripheral blood is greatly increased during the early hematologic recovery phase after marrow hypoplasia-producing chemotherapy.[5] [19] [20] [21] [34] [35] [36] A variety of chemotherapeutic regimens have been used for mobilization of PBSC including daunomycin, ara-C, L-thioguanate (DAT),[5] [19] [36] cyclophosphamide, Oncovin, methotrexate, prednisone (COMP), high-dose cyclophosphamide (CY),[34] mitoguazone, ifosfamide, methotrexate, etoposide (MIME),[21] cyclophosphamide, Adriamycin, vincristine (CAV),[20] and CY and etoposide.[83] To et al.[34] demonstrated a 14-fold increase of CFU-GM (compared to levels in healthy donors) in patients with non-Hodgkin lymphoma treated with a single dose of CY at 4 gm ^{2} . However, only 69% of treatment courses achieve the desired mobilization of CFU-GM. As with any mobilization regimen, there was considerable variation among patients in the response to this treatment.

Chemotherapy plus cytokine will generally mobilize greater numbers of PBSC than either alone.[84] Alegre et al.[58] compared the treatment of patients with multiple myeloma with CY 4 g/m^{2} followed by 8 g/kg/day of GM-CSF to a second group of patients treated with G-CSF alone at a dose of 10 g/kg/day. Although G-CSF is the more potent mobilizing cytokine, the patients treated with chemotherapy plus GM-CSF achieved greater numbers of CD34+ cells per apheresis procedure demonstrating the additive potential of chemotherapy mobilization.

A wide variety of different chemotherapy regimens have been used successfully for the mobilization of HSC into the blood. The primary consideration is that the choice of chemotherapy used must meet the treatment needs of the patient. However, evidence also indicates that the choice of chemotherapy regimens will affect the mobilization of HSC. Schwartzberg et al.[83] reported an average daily apheresis yield of 4.0 × 10 ^{7} CD34+ cells in 395 apheresis components collected from 61 patients treated with CY and average yields of 8.3 × 10 ^{7} CD34+ cells in 218 collections from 33 patients treated with both CY and etoposide.[83] (Another group of 24 patients was treated with this latter chemotherapy regimen, but also received 6 g/kg/day of G-CSF. The average CD34+ cell contents for 122 collections from these patients were significantly higher at 38.8 × 10 ^{7} .) The average quantities of CFU-GM paralleled the numbers of CD34+ cells collected. Similarly, Demirer et al.[85] studied the effect of different chemotherapy regimens for the mobilization of HSC for patients with breast cancer. Four regimens were used, all involving CY, but including etoposide with or without cisplatin, or paclitaxel. All patients also received G-CSF. The median quantity of CD34+ cells collected on the first day of apheresis after CY mobilization was 0.9 × 10^{6} /kg of patient weight. The addition of etoposide and then etoposide and cisplatin increased this to 8.1 × 10 ^{6} and 3.5 × 10^{6} CD34+ cells/kg, respectively. The median number of CD34+ cells harvested on the first day of apheresis after CY plus paclitaxel was 11.1 × 10 ^{6} /kg. Over 50% of the women mobilized with this last regimen achieved the target dose of CD34+ cells in one apheresis procedure. Of the 100 women studied, 94 achieved the target dose of >5 × 10^{6} CD34+ cells/kg. Only 4 patients failed to reach a lower but acceptable dose of 2.5 × 10 ^{6} CD34+ cells/kg.

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STRATEGIES FOR THE DIFFICULT-TO-MOBILIZE PATIENT

Most patients achieve the targeted dose of CD34+ cells after the processing of 2030 liters of blood in one to three apheresis procedures. However, about 5% to as many as 30% of patients in various series will have inadequate collections because only small numbers of HSC are present in the peripheral blood despite the administration of hematopoietic cytokines. Different chemotherapy and cytokine regimens will affect the mobilization of HSC. Patient-specific factors predictive for poor mobilization include older age, marrow disease, prior radiotherapy, and prior chemotherapy. [29] [86] [87] [88] [89] The previous administration of marrow-toxic drugs may suppress the subsequent mobilization of HSC.[90] About 50% of patients who fail to achieve the targeted dose of CD34+ cells will achieve this goal on a second attempt. High-dose (15 mg/kg twice daily) G-CSF after a 24-week drug holiday to allow marrow recovery is one strategy. Combination cytokine therapy is also of potential value in this situation, but cytokine combinations have not been tested in groups of patients who previously failed mobilization therapy. Repetition of a chemotherapy plus cytokine regimen will also work, but may be associated with increased toxicity. [89] Consideration should also be given to infusing a lower dose of CD34+ cells (as low as 1×10^6 CD34+ cells/kg) because, although the risk of delayed engraftment is incurred, [29] [30] failure of engraftment has not been reported. [85]

Consideration should be given to collection of PBSC early in the course of treatment for patients who may later be candidates for autologous HSC transplantation. HSC may be cryopreserved

and stored for years without obvious progressive loss of engraftment potential. [91]

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DEFINITION OF ADEQUATE COMPONENTS

The quantity of CD34+ cells in a PBSC component varies greatly and depends on the number in the peripheral blood at the time of apheresis, the volume of blood processed, and the efficiency of the apheresis device. Therefore, any definition of an adequate component cannot include a set number of CD34+ cells. Instead, one or more components will be collected to meet the appropriate dose of these cells for transplantation. What dose of CD34+ cells is required for infusion depends on the intended treatment regimen. For marrow-ablative regimens, increasingly higher CD34+ doses result in greater likelihood of rapid engraftment (Figs. 92-1 and 92-2).[29] [30] Lower doses of CD34+ cells appear satisfactory for nonablative regimens.[92] [93] [94] [95] For example, Bokemeyer et al.[94] infused 1×10^6 CD34+ cells per cycle for patients treated with high-dose ifosfamide and doxorubicin. Despite an overall doubling of the ifosfamide dose in this phase I study, the median times between cycles did not differ.

Quantification of hematopoietic progenitors by cell culture has been used by many transplant programs in attempts to determine the adequacy of the bone marrow or PBSC collections for transplantation.[22] [34] [96] Based on delayed engraftment failure in some patients, Juttner and colleagues recommended a minimum dose of CFU-GM of 6.3×10^5 /kg recipient weight. [19] Others have suggested lower doses of 25×10^5 CFU-GM/kg.[97] Patients who receive a dose of CD34+ cells (or CFU-GM) above a certain threshold will engraft. At lower doses of CD34+ cells there is considerable heterogeneity in engraftment speed, especially for platelet engraftment. It is not known why this heterogeneity exists, but it may reflect a weakness in the correlation between CD34+ cells and the cells responsible for engraftment or, more simply, a greater degree of error in the measurement of CD34+ cells at the lower cell concentrations. As the dose of CD34+ cells increases, the engraftment speed becomes more consistent for the population studied.[29] [30] Several investigators showed more rapid granulocyte and platelet engraftment for recipients of products containing a quantity of CD34+ cells above a dose of about 23×10^6 /kg recipient weight.[87] [88] [98] Investigators at the Fred Hutchinson Cancer Research Center found a threshold of 5×10^6 CD34+ cells/kg to achieve reliably prompt platelet engraftment,[99] and studies that include large patient populations found that higher doses of CD34+ cells will

Figure 92-1 The Kaplan-Meier probability of achieving 0.5×10^9 neutrophils/L for $<5.0 \times 10^6$ (-), >5.010.0 $\times 10^6$ (), and $>10 \times 10^6$ (---) CD34+ cells/kg (p = 0.0001). *(Reprinted from Weaver et al.,[30] with permission.)*

Figure 92-2 The Kaplan-Meier probability of achieving 20.0×10^9 platelets/L for $<5.0 \times 10^6$ (-), >5.010.0 $\times 10^6$ (), and $>10 \times 10^6$ (---) CD34+ cells/kg (p = 0.0001). *(Reprinted from Weaver et al.,[30] with permission.)*

result in even higher proportions of patients achieving quick engraftment. [30] Investigators at the Hutchinson Center noted that the dose of CD34+ cells infused was more important than the type of mobilization therapy.

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TIMING OF APHERESIS

A major problem with chemotherapy-based mobilization regimens is the difficulty in determining the optimal time to commence HSC collection. It is an obvious point that apheresis devices can only collect those CD34+ cells actually circulating in the peripheral blood. It is possible to estimate the quantity of CD34+ cells that will be collected during apheresis by multiplying the quantity of CD34+ cells in the blood by the total volume of blood processed during the apheresis procedure and by the efficiency of the apheresis device in collecting these cells. If the device has an efficiency of 50% and the patient undergoes a 10-liter exchange, about 5×10^{7} CD34+ cells will be collected for a peripheral blood level of 10 CD34+ cells/l, and 5×10^{8} CD34+ cells for a blood level of CD34+ cells that is 10-fold higher. Although many protocols call for the initiation of apheresis after chemotherapy mobilization when the WBC has recovered to >1,000/l, there is poor, if any, correlation between the peripheral blood WBC or mononuclear cell counts and the CD34+ cells in the peripheral blood. Characteristics that suggest a higher CD34+ cells level are rapidly rising WBC, a shift in differential to immature myeloid cells, circulating nucleated red blood cells (RBC), and platelet transfusion independence. However, it is much more cost effective to obtain an actual measurement of CD34+ cells in the blood and time the apheresis collection when these cells are present in adequate numbers. The published experience from many transplant centers describes a correlation between the CD34+ cells in the peripheral blood and in the harvested component. [87] [98] [100] [101] Figure 92-3 shows such a relationship for patients with lower concentrations of CD34+ cells in the peripheral blood and illustrates the very poor collections that are obtained when the peripheral blood CD34+ count is <10/l. Although there is considerable error in the enumeration of CD34+ cells at levels <5/l, this is not clinically relevant because even a doubling of the CD34+ cells in this range still results in a very poor apheresis yield.

At what level of CD34+ cells in the peripheral blood to start apheresis is a clinical decision. Although levels in the range of 50100/l or greater will reduce the number of apheresis procedures necessary to achieve a target goal of CD34+ cells, each days delay in initiating apheresis also incurs the costs of

Figure 92-3 The relationship between the quantity of CD34+ cells in the peripheral blood and the number collected by apheresis using apheresis and flow cytometry techniques previously described.[2] [29] [209] Shown are data limited to peripheral blood CD34+ cell numbers 50.0/L (n = 157, r = 0.82, p < 0.001).

additional cytokine administration and blood testing. For some patients who have been extensively treated previously and who may show a slowly rising WBC, it may be necessary to accept multiple apheresis procedures to achieve the target goal. Certainly, no patient should undergo apheresis if the peripheral blood count is <5/l (Fig. 92-3). For patients with CD34+ cell counts of 1020/l, it is possible to process more blood per day using large-volume leukapheresis (LVL) techniques and thereby reduce the numbers of days the patient is required to return to the apheresis unit. Most patients will have a rising CD34+ cell count in the peripheral blood so it is generally feasible to start apheresis the day after the patient has achieved a desirable CD34+ level. [101]

The timing of apheresis after G-CSF mobilization differs from the timing after chemotherapy and cytokine mobilization. For both patients and healthy donors, the peak concentration of CD34+ cells occurs on the fifth day of G-CSF administration (after four daily doses). [43] [44] Lower levels are present on day 4, and the concentration continues to fall after day 6 even if cytokine administration is continued and despite a continued rise in the WBC. Thus, PBSC collection should be initiated on day 4 or 5 of G-CSF administration. The kinetics of CD34+ cells in the peripheral blood for patients and donors mobilized with G-CSF alone are so reliable that it is not necessary to monitor peripheral blood levels unless there is concern that the patient has failed to mobilize cells and it is practical to obtain the cell count rapidly enough to initiate apheresis on the same day.

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APHERESIS TECHNOLOGY

Apheresis technology is widely used for the collection of platelets from healthy donors and is considered to be without major risk to the donor. The important safety considerations for PBSC collection are the same as for platelet collection and include the venous access to be used for the procedure, the extracorporeal volume of blood during the procedure, and the solutions administered to the donor. However, it must also be acknowledged that PBSC collection for autologous transplantation involves patients with underlying medical conditions who may require considerable nursing care during the procedure. In a retrospective review of >5,000 patient plasmapheresis procedures for the treatment of a variety of diseases, complications of apheresis were observed in 12% of procedures, and 40% of patients experienced at least one complication during a course of therapy. [102] Most of these were mild and transient such as fever, chills, urticaria, hypotension, and reactions to citrate anticoagulants. In contrast, patients and donors undergoing PBSC collection do not require the replacement fluids used during plasmapheresis and will not experience the toxicities associated with plasma protein infusions. However, rare anaphylactic reactions may occur for the pediatric patient who requires blood priming of the extracorporeal circuit, patients treated with angiotensin-converting enzyme inhibitors, [103] or those allergic to ethylene oxide used to sterilize the apheresis disposable set. [104] Goldberg et al. [105] studied the complications occurring during 554 PBSC collections from 75 consecutive patients, 74 of whom had subclavian or jugular venous system catheters placed for apheresis. Patient diagnoses were varied as were the mobilization treatment regimens. A median of nine collections per patient was performed using a discontinous-flow apheresis device. The most common problems were related to the venous catheters with 50% of the patients developing at least one occlusion. Hypocalcemia occurred in 14.6% of patients and hypotension in 13.3%. Sixteen percent of patients experienced infectious complications during the PBSC collection period.

Staffing of the apheresis unit should be appropriate for the medical condition of the patients undergoing apheresis. The collection of PBSC by apheresis performed in the outpatient setting should never be assumed to be a safer alternative for the donor than marrow harvesting conducted in the intensive care setting of the operating room without careful review of the medical support requirements for the individual patient or donor.

Venous Access

Adequate venous access is required for optimal apheresis technique. Continuous-flow apheresis devices require two-lumen access with a stable blood flow capacity generally >20 ml/min. Single-lumen access may be used with discontinous-flow apheresis devices, although at a much slower rate of blood processing. The great majority (90%) of adult allogeneic PBSC donors have adequate arm veins for the procedure to be conducted vein-to-vein. Some allogeneic donors, especially those with small veins and undergoing several daily procedures, may require placement of a temporary venous catheter. Venous access for the patient undergoing collection for autologous PBSC transplantation is much more heterogeneous. Vein-to-vein procedures may be performed, even on several consecutive days, with proper phlebotomy technique and postcollection care of the phlebotomy site. Most patients have received previous chemotherapy or are proceeding directly to transplantation for both of which tunneled access is commonly placed. Ideally, this venous access should be appropriate both for the apheresis procedures and the subsequent transplant. Length, lumen size, and wall stiffness all affect the blood flow that can be achieved through a catheter. For this reason, the commonly used dual-lumen Hickman or Broviac catheters are usually unsuitable for apheresis as are all subcutaneously placed ports. [106] Most triple-lumen catheters are also inadequate because of the small lumen size. If such access is already placed, consideration can be given to replacement with a shorter, stiffer tunneled catheter or to placement of a temporary percutaneous dialysis/apheresis catheter. [107] The catheters designed for dialysis and apheresis have adequate wall thickness to prevent collapse during aspiration of blood as well as a tip design to decrease local recirculation of blood and the resulting decrease in apheresis efficiency. Catheters of 10 Fr or larger size are appropriate for adult patients. Pediatric patients whose blood flow rates are considerably slower may use catheters of 57 Fr size. [108]

Problems directly related to venous access account for a considerable amount of the toxicity associated with PBSC collection. It has been suggested that mobilization with GM-CSF increases

the incidence of catheter occlusion. [109] Haird et al. [110] suggested that catheters placed in the subclavian vein in particular were associated with a high incidence of thrombosis, a complication seen less frequently with translumbar inferior vena cava placement. Goldman et al. [105] reported this complication in 50% of patients. In contrast, Alegre et al. [107] reported only a 1.8% incidence of thrombosis or clotting of catheters, possibly because of differences in cytokine or catheter type and care. Catheter thromboses are easily managed with streptokinase or urokinase instillation by the apheresis unit staff. One randomized study reported that low doses of warfarin decreased the incidence of venous thrombosis associated with indwelling catheters, but this study did not address catheter patency, nor were the patients enrolled in this study treated with hematopoietic cytokines. [111] Consultation with apheresis unit staff about venous access for an individual patient is appropriate. For example, for patients with adequate peripheral veins, a needle may be placed in one arm of the patient for blood draw with simultaneous use of both lumens of a tunneled catheter for return of the blood, thereby avoiding the need for placement of another venous access device.

Apheresis Devices

A number of apheresis devices are available for the separation of HSC from the peripheral blood. These devices may be classified as continuous-flow devices (e.g., Fenwal CS3000, COBE Spectra, Fresenius AS104) or discontinuous-flow devices (e.g., the Haemonetics family of equipment). The discontinuous-flow devices have the advantage of requiring only a single venous access. Continuous-flow devices require two access lines for aspiration and return of blood, but process much greater volumes of blood in a shorter period of time. Devices now available enrich the HSC-containing mononuclear cell fraction, reducing the quantities of granulocytes and platelets collected that may interfere with cryopreservation or other laboratory processing.

All apheresis devices will collect HSC. Which apheresis device is used by the collection center depends primarily on the needs and experience of the center. The CS3000 achieves a greater enrichment of mononuclear cells than the COBE Spectra, [112] reducing the number of granulocytes in the component, but without a difference in the collection efficiency for CD34+ cells. In constrast, the Spectra allows a much higher blood/anticoagulant ratio because additional anticoagulant solution can be added to the collect bag at the initiation of the procedure, thereby avoiding much of the citrate toxicity that may occur with the processing of the large volumes of blood during PBSC collection. Continuous-flow devices are more efficient in the collection of PBSC. In a randomized study comparing the Haemonetics MCS-3P with the COBE Spectra, Morton et al. [113] found that the latter device processed significantly more blood in a shorter period of time and collected over twice as many CD34+ cells and CFU-GM.

Anticoagulation

Anticoagulants are added to the blood during apheresis to prevent clotting of the extracorporeal circuit and clumping of cells in the component. Citrate anticoagulants have a proven record of safety in the apheresis of healthy platelet donors and are used extensively for the collection of PBSC. The major drawback is the risk of a symptomatic decrease in the level of ionized calcium (citrate toxicity), especially during the processing of large volumes of blood. [114] [115] Citrate ions chelate calcium

ions (and other divalent cations such as magnesium), making them unavailable for Ca^{2+} -dependent metabolic reactions. Acid-citrate-dextrose A contains 10.67 g citrate/500 ml volume in the form of trisodium citrate and citric acid. Citrate is diffused throughout the extravascular space and this diffusion is probably the first defense against citrate toxicity. Metabolism by liver, kidney, and muscle also reduces the concentration of citrate. Metabolism of citrate becomes more important during prolonged apheresis procedures such as LVL.

The initial signs of citrate toxicity include circumoral or acral paresthesias and may progress to nausea, vomiting, loss of consciousness, tetany, and seizures. Fatal cardiac dysrythmias have been reported (rarely) for patients undergoing plasmapheresis. [116] Citrate infusions may depress myocardial function, and it has been suggested that this may be worsened by the use of calcium channel blocking medications although reports of such complications for patients or donors undergoing apheresis have not been published. [114] Pediatric patients may not be able to relate the initial symptoms of citrate toxicity, and this should be considered for any change of behavior for these patients during the apheresis procedure.

Citrate toxicity is prevented by limiting the quantity of citrate infused either by decreasing the blood flow rate through the apheresis device or changing the blood/citrate ratio. The COBE Spectra, for example, limits the blood flow rate so that the amount of citrate infused is a maximum of 1.1 ml/min/l of patient blood volume as calculated from the patients gender and size. However, these machine parameters do not account for alterations in electrolytes, plasma protein levels, muscle mass, or renal or hepatic function that may occur for patients receiving chemotherapy. The processing of blood of patients experiencing the initial symptoms of citrate toxicity should be temporarily halted until the symptoms abate and then resumed at a slower rate.

The benefit of oral calcium supplements for these patients is not certain. Heparin may be used as a replacement for some or all of the citrate although to prevent clumping of platelets additional citrate is added to the component. [2] [117] Some centers using citrate anticoagulants will also administer intermittent or continuous infusions of calcium gluconate during the procedure, especially if large volumes of blood are being processed. [95] [118] However, excessive calcium replacement can also induce cardiac dysfunction. [114] Point of care devices that measure the ionized calcium within minutes are now available and greatly simplify obtaining this test. [119] [120]

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LARGE-VOLUME LEUKAPHERESIS

The apheresis device has a uniform and fairly reproducible efficiency of collection. Thus, for a consistent quantity of blood processed through the machine, the quantity of CD34+ cells collected is directly related to the number present in the peripheral blood. Greater quantities of CD34+ cells can be collected by increasing the number of these cells in the peripheral circulation or by increasing the volume of blood processed by the device. For those patients with lower CD34+ cell levels, multiple apheresis procedures will be required to achieve the target dose of CD34+ cells needed for transplantation. An alternate approach is to process the same total quantity of blood, but in fewer, longer procedures. Large-volume leukapheresis (LVL) is not formally defined, but in general usage refers to the processing of more than two or three times the patients blood volume.[118] [121] [122] [123] [124] [125] [126] [127] [128] [129] Typically, the quantity of blood processed is six or more times the patients blood volume, often 2536 liters of blood. The advantage of LVL is that this reduces the number of days of cytokine administration and apheresis with associated reduced costs of laboratory processing and testing. The apheresis techniques are the same as those used for the processing of smaller volumes of blood although blood flow rates may be increased to reduce the time required. The risks of LVL are the increased time required and the higher risk of citrate (or other anticoagulant) toxicity. Patients will also incur a proportional drop in platelet counts and may become profoundly thrombocytopenic.

Most reports of LVL describe the collection of more CD34+ cells than are calculated to be present in the peripheral blood at the initiation of the apheresis procedure. Obviously, there is release of cells from the marrow to replace those removed by apheresis. Apheresis of CD34+ cells is a three-compartment system consisting of the extracorporeal circuit of the apheresis device including the collection bag, the peripheral blood, and the marrow. However, it is not obvious that the apheresis technique itself consistently mobilizes CD34+ cells as suggested by the titles of some reports. Initial investigation of LVL in dog and rhesus monkey models and for human patients and healthy donors not receiving cytokine mobilization demonstrated an increase in the number of CFU-GM collected such that a doubling of these cells in the circulation must have occurred during the apheresis procedure. [123] [126] [130] [131] The experience with patients and donors treated with cytokines or chemotherapy plus cytokine is much more variable with reports describing an increase, [122] [123] [125] [126] no change,[120] [128] [129] or even an exhaustion of CD34+ cells from the peripheral blood.[128] [132] Animals receiving hematopoietic cytokine stimulation have a diminished apheresis-associated increase in circulating CFU-GM compared to those animals collected without any mobilization treatment.[131] This increase may also not be evident in patients with poor marrow function resulting from previous, extensive chemotherapy.[127]

Clinically most important is the possibility of exhaustion of HSC such that the processing of the large blood volumes exposes the patient to the risks of anticoagulant toxicity and thrombocytopenia without achieving an increased collection of HSC. Studies at the Fred Hutchinson Cancer Research Center demonstrate a continuous release of CD34+ cells from the marrow (and, presumably, return to the marrow space). Patients having higher levels of CD34+ cells in the peripheral blood appear to have a greater number of these cells circulating between the marrow and peripheral blood compartments (Table 92-3). If this is so, the apheresis device merely serves as a siphon, removing these cells from the blood as they are released from the marrow. If this is an appropriate description of CD34+ cell kinetics, it may be possible to deplete these cells from the blood and marrow by prolonged processing. Also, the model suggests that higher blood flow rates used to shorten the apheresis procedure may be counterproductive for the patient with low CD34+ cell levels in the blood.

The explanation for the increase in CFU-GM circulating in the peripheral blood during the procedure reported by some investigators is obscure. Murea et al. [125] suggested that this phenomenon

TABLE 92-3 -- Replenishment of CD34+ Cells During Large-volume Leukapheresis

UPN	CD34+Cells				
	Blood/l	Blood Total	Harvest Total	Released Total	Released/Min
10605	6.8	34.9	123.5	88.6	0.3
10698	15.6	603.3	1438.1	540.8	2.1
10849	30.7	109.7	211.1	62.6	0.3
10920	37.9	214.2	952.8	57.0	1.6
11128	66.1	280.6	1010.8	532.7	1.9

Shown are the number of CD34+ cells in the peripheral blood or apheresis component for five patients with acute myelogenous leukemia or multiple myeloma undergoing LVL after G-CSF or chemotherapy plus G-CSF mobilization treatment. Blood volumes processed were six times the calculated blood volume of the patient. PBSC collection was performed on the COBE Spectra as previously described.[2] The total number of CD34+ cells in the blood (third column) was calculated from the level of CD34+ cells in the blood and the estimated blood volume of the patient. The total number of CD34+ cells released (fifth column) was calculated from the total number in the apheresis component and the number in the peripheral blood after collection minus the total number in the peripheral blood at the start of the collection procedure. All CD34+ cell quantities (except blood levels reported per l) are $\times 10^{6}$.

may be related to a decrease in divalent cations resulting from the citrate anticoagulant. Divalent cations are necessary for the function of some but not all cell adhesion molecules.[133] A progressive decrease in ionized calcium levels for some patients during the prolonged LVL procedure could, therefore, result in an increase in circulating HSC. This may explain why some investigators using heparin-containing anticoagulation solutions or who concomitantly infuse calcium solutions have not observed this phenonmenon.[128] [129] [132]

Large-volume leukapheresis is appropriate management for the patient or donor who needs to complete apheresis in a limited number of procedures. The quality of the component collected by LVL does not differ from that collected by the processing of smaller volumes of blood, and patients experience the same rapid kinetics of engraftment after infusion of these components.[125]

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PEDIATRIC DONORS AND PATIENTS

Peripheral blood stem cells can be collected from pediatric patients including infants. The special challenges of the pediatric patient arise from the fixed extracorporeal blood volume of the apheresis device, the need for venous catheters for blood access, and the management of a patient who may be unwilling or unable to rest quietly for the period of apheresis. It is especially important in management of the pediatric patient that timing of apheresis be optimal to minimize the number of procedures required to achieve the desired quantity of PBSC. Given these considerations, a number of centers have reported successful collection of PBSC from pediatric patients and donors. [95] [117] [132] [134] [135] [136] [137] [138] [139] [140] [141]

Virtually all pediatric patients undergo insertion of a venous catheter adequate for the flow rates expected although older (>12 years) patients may tolerate vein-to-vein procedures. The whole blood flow rate for the pediatric patient is much reduced compared to adult patients and catheters as small as 5 Fr will be adequate. [108] Some centers have used arterial cathethers for blood access. [137]

Appropriate management of fluid balance during the apheresis procedure is critical for the smaller patient. The volume of RBC contained in the extracorporeal circuit of a continuous-flow apheresis device could represent 3050% of the RBC mass of a pediatric donor. Discontinuous-flow devices, although appealing because of the feasibility of performing apheresis with a single-lumen venous access, may result in even higher extracorporeal volumes and should be avoided for the smallest patients. The obvious solution to this problem is to prime the apheresis device with ABO-compatible, irradiated RBC (leukocyte-depleted and cytomegalovirus-negative blood may also be desirable) when the blood in the extracorporeal circuit is expected to exceed 15% of the patients blood volume. Packed RBC units may be diluted with saline or with albumin to reduce the loss of plasma protein that may also occur. The RBC remaining in the extracorporeal circuit at the completion of the run need not be returned (rinseback), [142] [143] [144] although if performed slowly with monitoring of vital signs, rinseback may actually increase the hematocrit after the procedure and otherwise reduce the need for RBC transfusions for these patients. Körbling et al. [139] collected and stored the RBC remaining in the machine after the first days apheresis for use in priming for the second days procedure for allogeneic donors requiring 2 days of apheresis, thereby avoiding the use of homologous blood transfusion. For the intermediate-sized pediatric patient (2550 kg), the apheresis device can be primed with a 5% albumin solution. This will reduce the albumin loss that would otherwise occur. However, clotting proteins and other proteins not contained in this solution may decrease with repetitive apheresis.

The pediatric patient may not exhibit or relate the prodromal symptoms associated with citrate toxicity. Continuous calcium

gluconate infusion may be incorporated into the procedure, or heparin may be added to the citrate anticoagulant solution or used as the sole anticoagulant. [2] [124] [125] [132] Some centers report the use of sedating medications such as chloral hydrate during the procedure. [95] [135] [139] Sedation of the pediatric patient is not necessary, however, [134] [138] [141] and hinders the ability to recognize the symptoms of citrate toxicity. (Some patients may require antihistamine premedication if the apheresis device is primed with RBC.) Centers routinely performing pediatric PBSC collection should design an environment conducive to the management of the pediatric patient and develop support procedures that recognize the unique physical and cognitive features of these patients. [145]

The range in blood volumes for pediatric donors of differing ages is greater than that for the adult donor. Therefore, most centers set a goal for volume processed based on the individuals blood volume instead of a set volume for all patients. The pediatric patient may undergo LVL to achieve the target goal of HSC with fewer procedures. [132] [140] Blood flow rates for the pediatric patient are slower than those for the adult to minimize the risk of citrate reaction. As with adults, the timing of apheresis can be optimized by monitoring the quantity of CD34+ cells in the peripheral blood. [146]

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ALLOGENEIC DONORS

Numerous centers have reported their initial experience with the use of PBSC for syngeneic or allogeneic HSC transplantation. [7] [8] [9] [10] [11] [12] [13] [147] Consistently, these reports describe more rapid engraftment kinetics than previously observed with marrow transplantation. The incidence of acute GVHD appears to be equivalent to that after marrow transplantation despite the 10-fold higher number of T lymphocytes infused. [7] [8] However, chronic GVHD seems to be much more common after PBSC transplantation,[148] especially chronic GVHD involving the skin. Phase III studies are being conducted to assess the toxicity and transplant outcome for this source of HSC compared to marrow. Peripheral blood mononuclear cell components collected from donors either with or without treatment with G-CSF are also effective in reinducing remission for patients who relapsed after allogeneic transplantation. [149] [150] [151]

The apheresis procedure is similarly well tolerated by the allogeneic PBSC donor. In contrast to the collection of PBSC intended for autologous transplantation, the collection for allogeneic transplantation is performed, usually, on a healthy donor. Virtually all allogeneic donors are treated with G-CSF for mobilization of PBSC, and the toxicities incurred with the use of this cytokine are discussed above. The risks to the allogeneic PBSC donor conferred by apheresis should not differ greatly from the risks to the platelet donor if the apheresis techniques are similar. The PBSC donor may undergo larger volume and repetitive exchanges with different anticoagulants compared to the platelet donor. The risk of the larger volume exchange is one of citrate toxicity if the donor is small or if the blood flow rate is increased to achieve this volume in a shorter period of time. Also, larger volume and repetitive exchanges will result in platelet depletion, although the platelets can be collected from the component for infusion back to the donor. [46] [55] The platelet count actually may reach its nadir several days after the completion of the apheresis collections and discontinuation of G-CSF. [46]

Most donations can be obtained using a vein-to-vein procedure. Some donors, especially pediatric donors, may require the placement of a temporary central venous catheter. The risks of catheter placement are well known and will not be repeated here.

Older donors will have more underlying medical conditions, [152] and the risks to the donor with underlying health problems must be fully considered before subjecting the donor to mobilization and apheresis. Standards are now published that describe the evaluation of the donor both for the risk of the donation process as well as the risk of transmission of disease to the recipient. [153] [154] Collection of PBSC is generally an outpatient procedure conducted in the clinic setting. In contrast, marrow harvesting has the luxury of the intensive support capability of the operating room. PBSC collection should never be viewed as a safer alternative to marrow harvesting for the donor with underlying health problems. Anderlini et al. [56] further suggested that G-CSF specifically not be administered to patients with a history of inflammatory ocular disorders, venous thrombosis, autoimmune disorders, or malignancy treated with chemoradiotherapy.

Management of RBC Incompatibility

Management of RBC incompatibility between the PBSC donor and recipient is the same as that for bone marrow transplantation. [155] Marrow processing to reduce the quantity of RBC and careful transfusion practices are used to avoid acute or delayed hemolytic transfusion reactions from the infusion of incompatible RBC or immunocompetent B lymphocytes. Eventually, recipient RBC and isoagglutinins are replaced by donor type. RBC incompatibility may be classified into two categories, one in which the recipient has antibodies directed against donor RBC with the potential of acute hemolysis during the infusion of the component (major incompatibility), and one in which the donor has antibodies against the recipient. Although the latter rarely causes difficulty during infusion of incompatible plasma, B lymphocytes carried in the component can form isoagglutinins resulting in a delayed transfusion reaction 712 days after transplantation. Apheresis devices are commonly used to remove RBC from marrow components for major RBC incompatibility. [156] Infusion of RBC quantities of 10 ml appears to be tolerable. Therefore, secondary processing of a PBSC component collected by the same technology used for the depletion of RBC from marrow grafts and already containing low quantities of RBC is not required. Minor ABO incompatibility can result in delayed transfusion reactions that may be fatal if appropriate blood transfusion support (avoiding the infusion of donor-type RBC) is not initiated before the transplant. Two case reports of severe RBC hemolysis after allogeneic PBSC transplantation have been reported. In the first, transfusion support before the event was not described. [157] The second patient received recipient-type RBC on day 7 after transplantation,[158] which is the point at which donor-derived isoagglutinins will be increasing in the recipient. Although both authors discussed the much greater numbers of B cells in PBSC components as a possible factor in these events, it is not clear from these reports that delayed-type transfusion reactions will be more likely or of greater severity in PBSC recipients.

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CELL COUNTING, PROCESSING, AND CRYOPRESERVATION

Tumor Cell Contamination

The probability that tumor cell contamination of the component could contribute to relapse was demonstrated by Brenner et al. and others in studies involving the transplantation of genetically marked marrow cells. [159] [160] [161] Sensitive immunocytostaining techniques, clonal assays, flow cytometric analysis, and polymerase chain reaction amplification of malignant genetic material detect tumor cells in the PBSC components of many patients with a variety of malignancies. [162] [163] [164] [165] In general, the incidence of contamination (number of patients with positive components) and the level of contamination (number of tumor cells per number of normal cells) is much less for PBSC than for marrow components,[162] although this has not been a consistent finding in all

studies. [163] Whether patients transplanted with PBSC have a lower relapse rate compared to those receiving bone marrow is not known, and appropriate phase III studies of this question will be difficult to design and to enroll patients onto. In a retrospective study, Sharp et al. [166] demonstrated similar probabilities of relapse-free survival for recipients of PBSC or marrow components if the components were free of lymphoma cells. In that study, patients with marrow involvement by lymphoma were assigned to transplantation with PBSC. The authors concluded that PBSC transplantation is a sensible approach to the patient with overt marrow involvement. Brugger and coworkers, however, demonstrated that patients with breast cancer involvement of the marrow at the time of chemotherapy mobilization were likely to mobilize tumor cells into the blood. [167] The most disturbing finding of this study is that the tumor cells were detected in the peripheral blood at the same time as CD34+ cells. Investigators at the Johns Hopkins Oncology Center found no difference in the incidence of tumor contamination of PBSC components between patients treated with chemotherapy and cytokines or cytokines alone. [168] The probability of tumor contamination of PBSC components decreases after induction chemotherapy.[169] Patients with marrow involvement may benefit from debulking chemotherapy before collection of PBSC with the caveat that extensive chemotherapy will decrease the yield of PBSC. It is unlikely that tumor cells in the HSC inoculum will benefit the patient, but it is also advisable that, until further data are available, reports of tumor contamination in the collections for individual patients should be interpreted with caution. Purging of PBSC collections should be limited to the research setting culminating in appropriate trials of purging efficacy.

Purging of PBSC Components for Autologous or Allogeneic Transplantation

Components of PBSC can be purged of tumor cells or T lymphocytes using the same techniques developed for marrow although the larger cell quantities collected and the multiple days of collection will increase the cost of this processing. The commercial availability of devices that enrich CD34+ cells has served to focus most purging studies involving PBSC components on this technology. The CD34 glycoprotein is a molecule of about 115-kd size found on lymphohematopoietic stem and progenitor cells including malignant cells derived from these, vascular endothelium, and, possibly, other tissues. [170] The presence of this marker on HSC and progenitor cells enables the enrichment of these cells from peripheral blood, cord blood, or marrow. Enriched populations of CD34+ cells can then be used for transplantation or for further component manufacturing such as gene therapy or cell expansion. Numerically, simple enrichment of CD34+ cells from <1% to levels of 7095% results in a 9099% depletion of CD34 cells, leading many investigators to explore CD34+ cell enrichment as a technique of T-cell depletion to prevent GVHD in allogeneic cell recipients, [171] [172] [173] [174] or tumor depletion to decrease the risk of relapse for autologous component recipients. [175] [176] [177] [178] Engraftment does not appear to be affected by the CD34+ cell enrichment unless low quantities of CD34+ cells are infused. [175]

Positive enrichment of HSC achieves a nonspecific tumor cell depletion as CD34+ cells are separated from the antigen-negative cells. Because this depletion is nonspecific, the degree of purging achieved will equal the depletion of other CD34 cells such as lymphocytes. Typically, this will range from 99.0 to 99.99% depletion. By itself, CD34+ cell enrichment will reduce the number of tumor cells below the level of detection of some assay systems. For example, Shiller et al. [175] reported that five of eight components were negative for myeloma cells after processing, but Lemoli et al. [178] found the persistence of detectable disease in five of six patients tested. The median purity of the CD34+ cells was actually greater in the latter study (77% vs. 89.5%). CD34+ cells can be successfully enriched after thawing using the same techniques used for prefreeze separations. [179] [180] [181] There is a lower cell recovery compared to prefreeze processing, but the post-thaw processed samples do not undergo the potentially damaging osmotic shock that occurs with the infusion of cells cryopreserved in dimethyl sulfoxide (DMSO). Greater depletion of tumor cells can be achieved with combinations of CD34+ cell enrichment followed by depletion of tumor cells. Clinical trials of combination purging techniques are currently being conducted so the utility of this purging cannot be assessed at this time.

Microbial Contamination of PBSC Components

The incidence of culture-positive PBSC components is considerably less than that for marrow. [182] [183] [184] In the prospective study by Attarian et al. of 1,263 PBSC components from 376 sequential patients, bacteria were detected only in 3. [182] Most of these patients were treated with chemotherapy mobilization and about 50% were still receiving antibiotics for the treatment of neutropenic fevers at the time of apheresis. None of the components collected from the patients receiving antibiotics were culture positive. The collection of cells from febrile patients appears acceptable if the patient is clinically stable for apheresis, the timing of apheresis is appropriate as determined by enumeration of CD34+ cells in the blood, and the patient is receiving appropriate therapy. The collecting facility should arrange for microbial culture of the component. Skin flora are the bacteria usually isolated and contaminated components do not need to be destroyed. Infusion of these components is generally without clinical sequelae although serious infections have occurred after the infusion of components contaminated during processing. [182] [183] [184] [185] [186] [187] Any decision regarding the disposition of culture-positive HSC must be made by the patients transplant physician after considering the type of contamination, the anticipated risks from use of the component, and the ability to replace the culture-positive component(s) in a timely manner.

Quantification of CD34+ Cells

Quantification of CD34 antigen-positive cells by flow cytometry has become the standard of care for the management of the PBSC donor because it provides a rapid and clinically relevant assessment of HSC content in the peripheral blood or the PBSC component. This antigen is found on HSC and limited populations of other blood cells,[170] and it can be identified using a variety of commercially available antibodies. If antibodies directly conjugated with dyes are used, the technique requires only about an hour of preparation time. Cell viability using propidium iodide or 7-aminoactinomycin D exclusion can simultaneously be determined if the cells are analyzed while still fresh,[188] or the cells can be fixed after staining for analysis at a later date. Other antibodies may also be added for analysis of CD34 subsets if desired (and if the flow cytometer has proper detectors to detect the different emission wavelengths of the fluorochromes used). [25] Many centers are finding strong correlations between the numbers of CD34+ cells and CFU-GM in the sample, but with a ratio of about 520:1. [10] [189] Thus, CD34 analysis will provide data similar to that obtainable with cell cultures, with the exception that the latter demonstrate the viability of the progenitor cells.

The major difficulty with the analysis of CD34+ cells is the low frequency of these cells. Clinical decisions about the initiation of apheresis are being made at levels of

CD34+ cells as low as 10/l. This may represent a cell frequency that is 0.01% of the nucleated cells in the specimen. This enumeration is possible

because of the multidimensional measurements possible with the flow cytometer. Most cytometers can measure at least five characteristics of each cell including size, granularity, and the presence of up to three different fluorochromes. Thus, the cells of interest can be separated in five-dimensional space achieving discrimination of cells as rare as 1 in 10,000. The difficulties arise from developing an adequate technique that makes optimal use of the cytometer to measure these rare cells. Sources of errors include sampling of the component, cell counting, cytometer calibration and operation, choice of antibody and fluorochrome, lysis technique, and gating strategy. Skilled operators will avoid many of these errors by selecting appropriate antibodies and properly operating the cytometer. However, cytometry provides a proportion of cells and this must be multiplied by the cell count to obtain an absolute number. The steps involved in preparing a specimen for cytometry may alter the proportion of cells in the sample and this error will be translated into an error in the absolute number. [190] The direct relationship between the number of events (CD34+ cells counted) and the precision of this count dictates that large cell numbers be analyzed. [191] Serke et al.[191] found a coefficient of variation of 30% in counting of CFU-GM colonies and of 10% in flow cytometric counting of CD34+ cells. The coefficient of variation in CD34+ cell enumeration could be as high as 65% for specimens containing few cells. This variation was decreased by the analysis of larger cell samples. Clinically, this imprecision may explain some of the range in engraftment kinetics observed for patients receiving low doses of CD34+ cells.

A variety of different cytometry techniques are used in measuring CD34+ cells. These may involve only one antibody although other techniques use two antibodies to obtain better discrimination between the CD34+ and CD34 cells. [192] [193] [194] Gating on cells that are CD45+ helps separate nucleated cells from RBC, platelets, and debris, a step that is otherwise performed using the light scatter characteristics of the cells. [194] Alternately, gating on CD14 cells allows the separation of CD34+ cells from monocytes that nonspecifically bind the CD34 antibody. However, there is considerable variation in results even between laboratories using the same cytometry technique, and no cytometer-based technique has proven to have a lower interlaboratory variability. [193]

Subset analysis will provide additional information but does not appear to be clinically useful at this time. For example, Dercksen et al. [195] reported better correlation between the number of CD34+CD33 cells and time to granulocyte engraftment and between the number of CD34+CD41+ cells and time to platelet engraftment than found with the overall number of CD34+ cells. This group reported a similar finding for the number of CD34+ L-selectin+ cells infused and platelet engraftment. [196] Given the limited range in recovery times when adequate numbers of CD34+ cells are collected and infused, however, this additional information is of limited clinical value.

There is considerable disagreement about which flow cytometry technique is best for the measurement of CD34+ cells. Any particular technique may have advantages over the others in specific settings, but no technique will be adequate if the cytometer is not calibrated and is not clean, if adequate events are not obtained for analysis, and if a uniform gating strategy is not followed. These issues will become moot with the development of automated devices that provide quick and reproducible enumeration of CD34+ cells, leaving the flow cytometer for research use. The ideal technique will provide an actual count of CD34+ cells (instead of a percentage) and be sensitive to CD34+ cell levels at least as low as 10/l (levels below this are generally not clinically relevant). Single platform devices that provide an actual count of CD34+ cells are being evaluated. [197] [198] It may be possible to adapt some hematology analyzers to this purpose. [199]

Progenitor Cell Cultures

Hematopoietic progenitor cells committed to granulocytic, erythroid, or mixed granulocytic and erythroid lineages can be identified using a variety of culture techniques. These techniques require expertise and equipment not available in many clinical laboratories and have other drawbacks that may limit their utility as routine quality control. Other than availability of equipment and expertise, the major limitation is that progenitor cell assays require 1014 days of culture before the results are available. Thus, progenitor cell cultures cannot be used in the day-to-day management of the PBSC donor. No standard culture technique has been adopted by all laboratories, complicating comparisons between laboratories of progenitor cell quantities harvested and infused. The clinical relevance of the culture technique to the transplant population must be determined if the data obtained are to be used in the management of individual patients.

Unlike other measures of component quality, progenitor cell cultures also demonstrate the functional capacity of the cells. Progenitor cell cultures are the only currently available relevant assay of HSC viability other than actual engraftment of the recipient and should be available at the PBSC processing facility for use in quality control or if questions about the viability of a particular component are raised.

Cryopreservation

Hematopoietic stem cells harvested from the peripheral blood, bone marrow, or umbilical cord blood are frozen and stored using the same techniques. The general parameters include cryopreservation in DMSO and a source of plasma protein with or without hydroxyethylstarch, cooling at 13°C/min, and storage at 80°C or colder. [200] Variations on this technique include the concentration at which the cells are frozen, the amount and source of the plasma protein, and the cooling techniques used. [201] [202] Most of these variations probably have little effect on the survival of the HSC as shown by the consistent engraftment of cryopreserved components. However, cryopreservation results in the loss of an undefined but potentially substantial proportion of HSC, and delay in engraftment can occur if the component being frozen has borderline quantities of HSC. [203] [204] There is also a considerable incidence of generally minor toxicity associated with the infusion of cryopreserved cells that must be considered when developing cryopreservation techniques. [205]

Peripheral blood stem cell components differ from marrow components because of the much larger cell quantity, frequently exceeding 4×10^{10} cells found in peripheral blood. Cryopreservation of these cells at a set cell concentration, especially if multiple days of collection are performed, may result in large volumes of cryopreserved material to be infused. DMSO itself has a variety of pharmacologic effects, [206] which may be compounded by the presence of lysed blood cells, foreign proteins from tumor cell-purging procedures, or contaminants from nonpharmaceutical grades of reagents used in the processing. The LD_{50} values (amount of DMSO required to kill 50% of test animals) reported for intravenous infusion of DMSO are 3.19.2 g/kg for mice and 2.5 g/kg for dogs. [207] The acute toxic dose of DMSO for humans has not been determined. A recent report cited two instances of encephalopathy after the infusion of cryopreserved PBSC components. [208] The components for both patients were cryopreserved with a final DMSO concentration of 10%. The volumes infused were 2,254 ml and 1,198 ml, containing 249.7 g and 132.7 g DMSO, respectively. Patient weights were not provided in this report, but the dosage of DMSO probably neared or exceeded 2 g/kg patient weight, at least for the first patient. For this reason, the volume of DMSO infused should be limited to 1 g/kg/day. Patients with larger volumes can receive the cells over >1 day.

Cryopreservation of these cells at the concentrations frequently used for marrow (510×10^{7} nucleated cells/ml) could result in component volumes in excess of 400 ml for each collection. To minimize the volume infused, PBSC may be concentrated before cryopreservation. In one series, the average cell concentration of cryopreserved PBSC was 5.59×10^{8} nucleated cells/ml. [209] High concentrations of RBC and platelets were also frozen. No detrimental effect of cryopreservation at these high cell concentrations on the recovery of nucleated cells, mononuclear cells, or CD34+ cells was found. No clinical studies have addressed the effect of cell concentration on engraftment speed or toxicity of infusion. Of concern, however, is that about 12 patients of several hundred infused at that center developed alterations in mental status including seizures after infusion of PBSC concentrated before cryopreservation (unpublished observations). It is very unlikely these events were related to the small volumes of DMSO infused.

Alternately, the cells can be washed after thawing, with removal of most of the DMSO. [210] The risk is again one of cell loss and it is preferable, if possible, that a strategy be adopted to ensure that not all cells are at risk during a single processing. An effect of post-thaw washing on the speed or success of engraftment after infusion of marrow or PBSC components has not been published. However, investigators at Duke University reported much more rapid engraftment of cord blood components if the cells are washed after thawing. [211] This observation may result from protection of the cells from the extreme osmotic shock occurring during infusion of cells contained in high concentrations of DMSO.

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Chapter 93 - Graft-versus-Host Disease and Graft-versus-Leukemia Effect

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INTRODUCTION

The ability of allogeneic stem cell transplantation (SCT) to cure leukemia is widely recognized. An important therapeutic aspect of SCT in eradicating malignant cells is the graft-versus-leukemia (GVL) effect. The importance of the GVL effect in allogeneic SCT has been recognized since the earliest experiments in stem cell transplantation. Forty years ago Barnes and colleagues noted that leukemic mice treated with a subtherapeutic dose of radiation and a syngeneic (identical twin) graft transplant were more likely to relapse than mice given an allogeneic stem cell transplant. [1] [2] They hypothesized that the allogeneic graft contained cells with immune reactivity necessary for eradicating residual leukemia cells. They also noted that recipients of allogeneic grafts, though less likely to relapse, died of a wasting syndrome now recognized as graft-versus-host disease (GVHD). Thus, in addition to describing GVL, these experiments highlighted for the first time the intricate relationship between GVL and GVHD. Since these early experiments, both GVHD and the GVL effect have been studied extensively, particularly in murine models of transplantation (reviewed by Porter and Antin [3] and Truitt et al. [4]). This chapter reviews the pathophysiology of GVHD and summarizes current understanding of the relationships between GVHD and the GVL effect.

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GRAFT-VERSUS-HOST DISEASE: CLINICAL AND PATHOLOGIC ASPECTS

Ten years after the work of Barnes and Loutit, Billingham formulated the requirements for the development of GVHD: the graft must contain immunologically competent cells, the recipient must express tissue antigens that are not present in the transplant donor, and the recipient must be incapable of mounting an effective response to destroy the transplanted cells.[5] According to these criteria, GVHD can develop in various clinical settings when tissues containing immunocompetent cells (blood products, bone marrow, and solid organs) are transferred between persons. The most common setting for the development of GVHD is following allogeneic SCT; without prophylactic immunosuppression, most allogeneic SCTs will be complicated by GVHD.

Acute Graft-versus-Host Disease

Acute GVHD can occur within days (in recipients who are not HLA-matched with the donor or in patients not given any prophylaxis) or as late as 2 months after transplantation. The incidence ranges from less than 10% to more than 80%, depending on the degree of histoincompatibility between donor and recipient, number of T cells in the graft, patients age, and GVHD prophylactic regimen.[6] The principal target organs include the immune system, skin, liver, and intestine. GVHD occurs first and most commonly in the skin as a pruritic maculopapular rash, often on the palms, soles, and ears; frequently it progresses to total-body erythroderma, with bullae formation, rupture along the epidermal-dermal border, and desquamation in severe cases. Gastrointestinal (GI) and liver manifestations often appear later and rarely represent the first and only findings. Intestinal symptoms include anorexia, nausea, diarrhea (often bloody), abdominal pain, and paralytic ileus. Liver dysfunction includes hyperbilirubinemia and increased serum alkaline phosphatase and aminotransferase values. Coagulation studies and the bleeding time may become abnormal, and hepatic failure with ascites and encephalopathy may develop in severe cases. Hepatic GVHD can be distinguished from hepatic veno-occlusive disease by weight gain or pain in the right upper quadrant in the latter. Acute GVHD also results in the delay of immunocompetence. The clinical result is profound immunodeficiency and susceptibility to infections, often further accentuated by the immunosuppressive agents used to treat GVHD.

Pathologically, the sine qua non of acute GVHD is selective epithelial damage of target organs.[7] The epidermis and hair follicles are damaged and sometimes destroyed. Small bile ducts are profoundly affected, with segmental disruption. The destruction of intestinal crypts results in mucosal ulcerations that may be either patchy or diffuse. Other epithelial surfaces, such as the conjunctivae, vagina, and esophagus, are less commonly involved. A peculiarity of GVHD histology is the frequent paucity of mononuclear cell infiltrates; however, as the disease progresses the inflammatory component may be substantial. Recent studies have identified inflammatory cytokines as soluble mediators of GVHD and have suggested that direct contact between target cells and lymphocytes may not be required for the destruction of the target cell (see following sections). There is also a histologic geography of GVHD lesions within target tissues. In the skin, damage is prominent at the tip of rete ridges; in the intestine, at the base of the crypts; and in the liver, in the periductular epithelium. These areas contain a high proportion of stem cells, giving rise to the idea that GVHD targets may be undifferentiated epithelial cells with primitive surface antigens.[8]

The histologic severity of a given lesion is at best semiquantitative,

TABLE 93-1 -- Clinical Classification of Acute GVHD[a]

Organ Involved	Symptoms
Skin	Maculopapular or diffuse erythematous rash that may progress to desquamation
Liver[b]	Elevation of serum bilirubin alone or combined with increased serum activity of hepatic enzymes
Intestinal tract[c]	Diarrhea with or without nausea and vomiting, pain, or ileus

Summary based on Thomas et al.,[25ε] Deeg and Cottler-Fox,[6] and Vogelsang.[25g]

[a]Clinical grading generally considers involvement of skin, liver, and intestinal tract along with performance status on a semiquantitative basis (1+ to 4+). Other manifestations such as conjunctivitis and mucositis are not included in current clinical classification schemes. Overall severity ranges from mild skin involvement only (grade I) to severe multiorgan involvement, usually with fatal outcome (grade IV).
[b]In the presence of veno-occlusive disease, accurate staging of liver GVHD may be difficult or impossible.
[c]Concurrent gastroenteritis of infectious origin may make the diagnosis of GVHD difficult.

and consequently pathologic findings are not used in the grading of GVHD. Standard grading systems generally include clinical changes in the skin, GI tract, liver, and performance status (Table 93-1). Although the severity of GVHD is often difficult to quantify, the overall grade correlates with disease outcome. Although mild GVHD (grade I or II) is associated with little morbidity and almost no mortality, higher grades are associated with decreased survival. With grade IV GVHD, the mortality is almost 100 percent.

Chronic Graft-versus-Host Disease

Chronic GVHD was initially defined as a GVHD syndrome presenting more than 100 days after transplant, either as an extension of acute GVHD (progressive onset), after a disease-free interval (quiescent), or with no precedent (de novo).[9] Chronic GVHD may be limited or extensive (Table 93-2). Any grade of acute GVHD increases the probability of chronic GVHD, although no singular pathologic feature of the former predicts the development of the latter. Its incidence ranges from 30% to 60%, although it may be higher after peripheral blood progenitor transplants. As with acute GVHD, the immune system appears to be affected in all patients, who are highly susceptible to bacterial, viral, fungal, and opportunistic infections. Specific abnormalities of cellular immunity include decreases in the production of antibodies against specific antigens, defects in the number and function of CD4+ T cells, and increases in the number of nonspecific suppresser cells, which further diminish lymphocyte response. Eighty percent of patients have skin changes resembling widespread lichen planus with papulosquamous

TABLE 93-2 -- Grading of Chronic Graft-versus-Host Disease

Type of Disease	Extent of Disease
Limited	Localized skin involvement, liver dysfunction, or both

Extensive	Generalized skin involvement
	Localized skin involvement or liver dysfunction plus any one of the following:
	Chronic aggressive hepatitis, bridging necrosis, cirrhosis
	Eye involvement (results on Schirmers test, <5 mm)
	Involvement of mucosalivary glands
	Mucosal involvement (on lip biopsy)
	Involvement of other target organs

Adapted from Schulman H, Sullivan K, Weiden P et al: Chronic graft-versus-host syndrome in man: a long-term clinicopathologic study of 20 Seattle patients. Am J Med 69:204, 1980. Copyright © 1980, with permission from Excerpta Medica, Inc.

dermatitis, plaques, desquamation, dyspigmentation, and vitiligo. Destruction of dermal appendages leads to alopecia and onychodysplasia. Severe, extensive chronic GVHD later resembles scleroderma, with induration, joint contractures, atrophy, and chronic skin ulcers. Chronic cholestatic liver disease occurs in 80% of patients and often resembles acute GVHD; it rarely progresses to cirrhosis. Severe mucositis of the mouth and esophagus can result in weight loss and malnutrition. GI involvement, however, is infrequent. Chronic GVHD also produces a sicca syndrome, with atrophy and dryness of mucosal surfaces caused by lymphocytic destruction of exocrine glands, which usually affect the eyes, mouth, airways, skin, and esophagus. The hematopoietic system may also be affected, and thrombocytopenia is an unfavorable prognostic factor in patients with chronic GVHD. Important predictors of unfavorable outcome are progressive onset, lichenoid skin changes, elevated serum bilirubin level, continued thrombocytopenia, and failure to respond to 9 months of therapy. [10] [11] Among patients with none of these risk factors, 70% are expected to survive, compared with less than 20% with two or more of these risk factors.

Histologic examination of the immune system reveals involution of thymic epithelium, disappearance of Hassalls corpuscles, depletion of lymphocytes, and absence of secondary germinal centers in lymph nodes. [12] Pathologic skin findings include epidermal atrophy with changes characteristic of lichen planus and striking inflammation around eccrine units. Sclerosis of the dermis and fibrosis of the hypodermis subsequently develop. GI lesions include localized inflammation of the mucosa and stricture formation in the esophagus and small intestine. Histologic findings in the liver are often similar to those that occur in acute GVHD but are more intense, with chronic changes such as fibrosis with hyalinization of portal triads, obliteration of bile ducts, and hepatocellular cholestasis. The endocrine glands of the eyes, mouth, esophagus, and bronchi show destruction focused on centrally draining ducts, with secondary involvement of alveolar components. Findings of bronchiolitis obliterans, similar to those that occur in rejection of lung transplants, are now generally considered a manifestation of chronic GVHD, although their pathogenesis is controversial.

It has become apparent in recent years that the clinical and histologic changes considered characteristic of chronic GVHD can develop 40 or 50 days after transplantation. The time frames of acute and chronic GVHD thus overlap; hence, the time of onset is an arbitrary criterion. It is more meaningful to define the disease on the basis of clinical, histologic, and immunologic findings, although the point at which therapy for chronic GVHD is initiated may be important.

Syngeneic Graft-versus-Host Disease

A GVHD-like syndrome that is usually self-limited and predominantly affects the skin can occur in recipients of syngeneic or autologous transplants. [13] Although the level of severity may be grade II or III, the disease generally resolves promptly with the administration of glucocorticoids and is not life-threatening. Virtually all patients in whom a GVHD-like syndrome develops after syngeneic transplantation have been prepared with intensive conditioning regimens, usually involving irradiation. Experimental studies suggest that such conditioning is essential for the induction of thymic dysfunction, which is necessary for the development of the disease.

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PATHOPHYSIOLOGY OF GRAFT-VERSUS-HOST DISEASE

Two important principles help to place the pathophysiology of GVHD in context. The first is that GVHD is not a disease per se; it reflects exaggerated but normal physiologic inflammatory mechanisms that occur in a setting where they are undesirable.

The donor lymphocytes that have been infused into the recipient function appropriately, given the foreign environment they encounter. The second principle is that in SCT, donor lymphocytes are infused into a host that has been profoundly damaged. The effects of the underlying disease, prior infections, and the intensity of a myeloablative conditioning regimen all result in substantial proinflammatory changes in endothelial and epithelial cells. Allogeneic donor cells rapidly encounter not only a foreign environment, but one that has been altered to promote the activation and proliferation of inflammatory cells by the increased expression of adhesion molecules, cytokines, and co-stimulatory proteins. The donor lymphocytes respond to these changes in a fashion that would usually promote the control or resolution of infection. Thus, the pathophysiology of GVHD may be considered a distortion of the cellular responses to viral and gram-negative bacterial infections. The unusual target organ distribution of GVHD supports the close relationship between infection and GVHD. Skin, gut, and liver all share an extensive exposure to endotoxin and other bacterial products that can trigger and amplify local inflammation. This exposure distinguishes them from organs that are not GVHD targets, such as the heart and kidneys. Both heart and kidneys are certainly susceptible to allogeneic cellular injury (e.g., they can be rejected), but they are virtually never target organs of GVHD. The lung is controversial in this regard. Although the lung is not a classic GVHD target, accumulating evidence suggests that it shares some degree of GVHD susceptibility with the skin, gut, and liver. [14] [15] Because of their situation as primary barriers to infection, these target organs have large populations of professional antigen-presenting cells (APCs), such as macrophages and dendritic cells, that may enhance the GVH reaction.

THE CYTOKINE HYPOTHESIS

Recent findings have demonstrated that inappropriate production of cytokines, which are the central regulatory proteins of the immune system, are a primary cause for the induction and maintenance of experimental and clinical GVHD. [16] [17] [18] Dysregulation of the complex cytokine networks that occurs in three sequential steps can be considered a framework for the pathophysiology of acute GVHD.

Phase One

The transplant conditioning regimen is an important determinant of acute GVHD because it damages and activates host tissues, including intestinal mucosa, liver, and other tissues, in a fashion that fosters the attraction and retention of white blood cells. Activated host cells secrete inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-) and interleukin-1 (IL-1). [19] The presence of inflammatory cytokines during this phase may up-regulate adhesion molecules [20] and MHC antigens, [21] [22] [23] [24] [25] thereby enhancing the recognition of host MHC and/or minor histocompatibility agents by mature donor T cells that have been infused with the stem cell inoculum. This process is in accordance with the observation that the enhanced risk of GVHD after clinical SCT is associated with intensive conditioning regimens that cause extensive injury to epithelial and endothelial surfaces, with subsequent release of inflammatory cytokines and increased expression of cell-surface adhesion molecules. [26] [27] [28] The relationship between conditioning intensity, inflammatory cytokines, and GVHD severity has recently been further supported in animal models. [29] Moreover, the risk of inducing severe GVHD appears to be less if the lymphocytes are infused well after the primary tissue injury has resolved. GVHD certainly occurs when donor lymphocytes are infused to induce GVL effects, but there appears to be a time-dependent reduction in risk, suggesting that the late infusion of cells (after 3045 days) results in less GVHD. [30] [31] This reduction may also be due to the fact that by several months after transplantation, host APCs have been replaced, at least in part, with donor APCs.

Phase Two

The second phase of acute GVHD includes antigen presentation, activation of individual T cells, and proliferation and differentiation of responding T-cell clones (Fig. 93-1). These processes have been defined at both cellular and molecular levels. [32] During antigen presentation, large proteins are digested by APCs into smaller fragments; these antigenic peptides bind to HLA (class I or class II) molecules and are displayed on the surface of the APCs as peptide-HLA complexes. T cells recognize this complex via antigen-specific T-cell receptors. In allogeneic interactions such as GVHD, mature donor T cells recognize recipient peptide-HLA complexes (alloantigens) in which both the HLA molecules and the bound peptides may be foreign. These peptides, usually eight to ten amino acids in length, represent minor histocompatibility antigens (mHA) that are largely

Figure 93-1 Pathophysiology of GVHD. During step 1, irradiation and chemotherapy both damage and activate host tissues, including intestinal mucosa, liver, and the skin. Activated cell hosts then secrete inflammatory cytokines (e.g., TNF- and IL-1), which can be measured in the systemic circulation. The cytokine release has important effects on antigen presenting cells (APCs) of the host, including increased expression of adhesion molecules (e.g., ICAM-1, VCAM-1) and of MHC class II antigens. These changes in the APCs enhance the recognition of host MHC and/or minor H antigens by mature donor T cells. During step 2, donor T-cell activation is characterized by proliferation of GVHD T cells and secretion of the Th1 cytokines IL-2 and IFN-. Both of these cytokines play central roles in clonal T-cell expansion, induction of CTL and NK cell responses, and the priming of mononuclear phagocytes. In step 3, mononuclear phagocytes primed by IFN- are triggered by a second signal such as endotoxin (LPS) to secrete cytopathic amounts of IL-I and TNF-. LPS can leak through the intestinal mucosa damaged by the conditioning regimen to stimulate gut-associated lymphoid tissue or Kupffer cells in the liver; LPS that penetrate the epidermis may stimulate keratinocytes, dermal fibroblasts, and macrophages to produce similar cytokines in the skin. This mechanism results in the amplification of local tissue injury and further production of inflammatory effectors such as nitric oxide, which, together with CTL and NK effectors, leads to the observed target tissue destruction in the stem cell transplant host. CTL effectors use Fas/FasL, perforin/granzyme B, and membrane-bound cytokines to lyse target cells.

undefined, but strategies to identify them have recently been successful. [33] [34] [35] Host APCs also provide co-stimulatory activation signals by B7/CD28. [36] In addition to the T-cell receptor, accessory molecules such as CD4, CD8, LFA-1, LFA-2, and CD44 participate in effectortarget cell interactions by intensifying cellular contact and communication. [37] More recently the role of CD40/CD40L interactions has been defined as well. [38] [39] [40]

Alloantigen composition of the host determines which T-cell subset proliferates and differentiates. MHC class II (HLA-DR, DP, DQ) differences stimulate CD4+ T cells; MHC class I (HLA-A, B, C) differences stimulate CD8+ T cells. CD4 and CD8 proteins are co-receptors for constant portions of MHC class II and MHC class I molecules, respectively. In mouse models of GVHD, where genetic differences between multiple strain combinations can be controlled, CD4+ cells induce GVHD to MHC class II differences, and CD8+ cells induce GVHD to MHC class I differences. [41] Thus, both CD4+ and CD8+ T-cell subsets can initiate the afferent phase of GVHD. In the majority of clinical marrow HLA-identical transplants, GVHD may be induced by either subset or simultaneously by both.

Antigen presentation results in the activation of individual T cells. This process involves multiple, rapidly occurring intracellular biochemical changes, including a rise in cytoplasmic free calcium and activation of protein kinase C and tyrosine kinases, [42] [43] which in turn activates transcription of genes for cytokines, such as IL-2, IL-12, interferon- (IFN-) and their receptors. Cytokines produced in response to alloantigens are predominantly secreted by T helper 1 (Th1) subset of T cells, [44] [45] particularly when APCs actively secrete IL-12. [46] Both IL-2 and IFN- have long been implicated in the pathophysiology of acute GVHD (see below), and they play central roles in further T-cell activation and induction of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cell responses. IFN- in particular is essential to the priming of donor and residual host mononuclear phagocytes to produce large amounts of IL-1 and TNF-. The T-cell activation phase is followed by clonal expansion and differentiation. DNA synthesis commences within 24 hours of antigen recognition and is maximal 35 days later. Functional differentiation then occurs as cells produce proteins required for specific effector functions, such as the protein esterases critical to CTLs. [47] The expression of many T-cell surface molecules is altered; receptors for lymphocyte homing and migration may be down-regulated, and other adhesion molecules (e.g., LFA-1) may be up-regulated, thus altering the T-cells trafficking patterns in vivo. [48]

While inflammatory effector functions of mononuclear phagocytes are stimulated by IL-2 and IFN- secreted by activated Th1 cell subsets (see below), these effector functions are inhibited by IL-4 and IL-10, cytokines that are produced by activated Th2 cells. There is now considerable evidence that the preferential expansion of Th2 T cells after allogeneic transplantation is associated with the development of a GVHD syndrome that is similar to chronic GVHD but is associated with less lethality and antibody formation in murine systems. [49] [50] [51] [52] [53]

Phase Three

The third phase of acute GVHD completes the complex cascade (Fig. 93-1). The initial hypothesis that the cytokine function of CTLs directly causes the majority of tissue damage and necrosis in GVHD targets appears to be too limited. [54] Large granular lymphocytes (LGLs) or NK cells appear to be prominent in the effector arm

of GVHD in several animal models, and they may contribute to the pathologic damage, that is, induce the changes of GVHD following the T-cell-mediated GVH reaction.[54] [55] LGLs do not recognize HLA proteins as targets, but they can be recruited and activated by cytokines secreted by T cells. The precise relationship between cytokines induced during the second step and mediators of tissue damage during this third step is an area of active investigation. Mononuclear phagocytes, which have been primed by IFN-, are triggered by a second signal to secrete TNF- and IL-1. This stimulus most frequently is lipopolysaccharide (endotoxin, LPS), which can leak through intestinal mucosa damaged by the conditioning regimen and subsequently stimulate gut-associated lymphocytes and macrophages. [56] LPS reaching skin tissues may also stimulate keratinocytes, dermal fibroblasts, and macrophages to produce similar cytokines in the dermis and epidermis. [57] [58] [59] TNF- can cause direct tissue damage through apoptosis, or programmed cell death. The induction of apoptosis commonly occurs after activation of the TNF-/Fas antigen pathway.[60] Apoptosis is a critical process in GVHD of the GI tract, [61] skin,[62] [63] and possibly endothelial cells [64] after allogeneic SCT. In addition to these proinflammatory cytokines, excess nitric oxide produced by activated macrophages may contribute to the deleterious effects on GVHD target tissues, particularly immunosuppression.[65] [66] [67] Induction of these inflammatory mediators thus synergizes with the cellular damage caused by CTLs and NK cells, [54] [68] resulting in the amplification of local tissue injury and further promotion of an inflammatory response that ultimately leads to target tissue destruction and systemic GVHD in the stem cell transplant host.

Although cytokines play important roles in the morbidity and mortality of systemic GVHD, their relative importance as mediators of damage in GVHD target organs is much less well established. The unusual cluster of GVHD target organs (skin, gut, and liver) is not adequately explained by the systemic release of cytokines. For example, intravenous infusion of TNF- and IL-1 does not cause the lymphomononuclear cell infiltration of the liver and skin that is observed in GVHD. Further, the absence of GVHD toxicity in other visceral organs, such as the kidney, argues against circulating cytokines as the sole cause of tissue-specific damage. The infiltrates seen in GVHD target organs are generally thought to contain T cells responding to alloantigens on host tissues. As mentioned above, LPS leakage through skin or mucosa may act as an adjuvant to the antigens expressed in these tissues, attracting and activating alloreactive donor T cells. A second possibility is that tissue-specific neoantigens are expressed at these sites as the result of ongoing inflammation. Such inflammation may alter the ligands for homing receptors on T cells (e.g., selectins) that enable them to traffic into specific tissues.

In experimental systems, cellular effectors contribute to GVHD (Tables 93-3 , 93-4). The application of gene knockout technology and the identification of pertinent mutant mice strains have provided important tools to dissect these effector mechanisms. Three pathways can be considered as important to GVHD effectors: the perforin/granzyme B pathway, the Fas/FasL pathway, and direct cytokine-mediated injury. Direct cellular cytotoxicity through the perforin/granzyme B pathway is mediated in two ways. Perforin causes membrane pore formation but it is not adequate to cause apoptosis on its own. [69] Granzyme B enters the cell through the perforin-derived pore and causes apoptosis. Granzyme B is particularly important to

TABLE 93-3 -- Cellular Effects of Graft-versus-Host Disease and Graft-versus-Leukemia Effect

CD4 T cells
Th1associated with GVHD/GVL
Th2do not cause acute GVHD, may suppress GVHD/GVL
CD8 T cells
Tc1less potent inducers of GVHD than Th1; important in GVL
Tc2produce little acute GVHD; important in GVL
Natural killer cells
Monocytes/granulocytes

TABLE 93-4 -- Mechanism of Graft-versus-Host Disease

Effector	Target Damage	Systemic Toxicity
Fas/FasL system	+	+/
Inflammatory cytokines[a]	+	++
Perforin/granzyme	+	
Monocytes, PMN?	+	+/
Nitric oxide, others?	+	+/

[a]Have direct effects on target or systemic tissues and indirect effects by activating effector cells and facilitating the production of other inflammatory cytokines.

GVHD induced by MHC class I mismatches, and is less critical in GVHD resulting from MHC class II mismatches, mediated by CD4+ cells. [70] Donor cells from both perforin-deficient and granzyme B-deficient knockout mice can mediate GVHD, but the onset of clinical manifestations is significantly delayed compared with that in control animals. This delay suggests that the perforin/granzyme pathway is an early-acting mechanism, but that an additional late mechanism of cytotoxicity exists. [71] [72] One candidate for a late-acting mechanism is the Fas/FasL interaction. [73] [74] [75] Fas is expressed on numerous cell types, [76] while Fas ligand (FasL) is expressed on activated T cells. Interactions between a Fas-bearing target and a FasL-bearing T cell result in apoptosis of the target cell. Thus, a setting where FasL molecules are up-regulated by cytokines may result in destruction of both specific targets and nonspecific, innocent bystanders that express Fas. When mutant mice that are deficient in FasL (*gld*) are used as stem cell donors, GVHD is attenuated; hepatic and some cutaneous GVHD occurs, but the mice develop cachexia and eventually die.[72] When Fas-deficient mice are crossed with those having perforin or granzyme B knockouts, the use of lymphocytes from these donors further diminishes, but does not eliminate, GVHD. Thus, neither FasL nor perforin is necessary for GVHD, and in the absence of both cellular effectors, GVHD can still occur, suggesting a third pathway for target cell apoptosis. [77] Cytokines such as TNF- are an attractive candidate for this pathway. [71] [78] Metalloproteinase inhibitors, which reduce shedding of both TNF- and FasL by effector cells, suppress both clinical GVHD manifestations and mortality in a murine model of acute GVHD. [79] A recent detailed analysis of a patient with transfusion-associated GVHD supports the hypothesis of multiple effectors in human GVHD. [80]

This conceptual framework of three sequential and interlocking steps helps to explain a number of unique and seemingly unrelated aspects of GVHD. For example, a number of analyses of clinical transplants have noted an increased risk of GVHD associated with advanced-stage leukemia, certain intensive conditioning regimens, and viral infections. [26] [27] [28] Similarly, the reduction in GVHD rates seen in gnotobiotic mice [81] [82] and in patients with aplastic anemia undergoing transplantation in laminar air flow environments with GI tract decontamination[83] may be explained by the reduction of bacterial LPS on the skin and gut. The beneficial effect of protective environments may be less apparent in patients undergoing transplantation for malignancies, because prior therapy and associated infections may have already resulted in an environment that facilitates GVHD. Viral infections are more frequent in patients with GVHD and may trigger the onset of GVHD or worsening of established GVHD. Cytomegalovirus has a particularly close relationship with GVHD, [84] [85] [86] [87] as does herpes simplex virus[88] [89] [90] and possibly human herpesvirus-6.[91] The precise pathophysiology of these connections remains uncertain. Although it has been hypothesized that viral antigen expression on target cells might function as mHAs, direct evidence for this association is lacking. Certainly, viral damage to the intestine or liver may increase the permeability of those organs to bacterial products such as LPS. Alternatively, GVHD targets could be innocent bystanders of virus-induced activated T-cell or NK cell attack. [92] [93] Finally, viral infections may perhaps flare as a result of the intrinsic immunosuppression of GVHD (or of additional immunosuppressive therapy) and may not cause GVHD.

Cytokines

Th1 Cytokines

Th1 cells produce IL-2 and have a pivotal role in controlling and amplifying the immune response against alloantigens, representing step two of the cytokine cascade of acute GVHD (Fig. 93-1). Experimental data show that maximal levels of IL-2 are produced by donor CD4+ T cells in the first 2 days after GVHD induction, with

lower levels observed in splenocytes from GVHD mice until 710 days post transplantation. [94] A similar early and transient increased expression of IL-2 during GVHD was demonstrated in murine GVHD models using RT-PCR techniques for detection of IL-2 mRNA. [50] [95] The addition of low doses of IL-2 during the first week after allogeneic BMT enhanced the severity and mortality of GVHD. [96] [97] [98] The precursor frequency of host-specific IL-2-producing T cells (pHTLs) in the donor predicts the occurrence of GVHD after transplantation between HLA-identical siblings. [99] [100] [101] The critical precursor frequency of host-specific pHTLs seems to be approximately 1/100,000. Patients whose donor bone marrow contained fewer host-specific pHTLs later developed only grade 01 GVHD, whereas patients whose donor bone marrow contained greater frequencies all developed significant, grade II or III GVHD. pHTL cells were detectable as early as day 20 after transplantation, often preceding the onset of acute GVHD by approximately 2 weeks, and persisted until the GVHD resolved.

The primary role of IL-2 likely includes the induction of other cytokines and recruitment of effector cells. [102] Owing to their apparent importance in initiating acute GVHD, IL-2-producing donor T cells have been the target of many experimental designs to control GVHD. The administration of cyclosporine, a powerful inhibitor of IL-2 production, is effective prophylaxis against GVHD. [103] [104] The importance of IL-2 is further underscored by experiments showing that monoclonal antibodies against the IL-2 receptor (IL-2R) are efficient in preventing GVHD in animals or in clinical GVHD when administered shortly after the infusion of T cells. [105] [106] Several studies have shown promising results of monoclonal antibodies directed against IL-2R; in those studies, between 28% and 73% of patients with steroid-resistant GVHD responded. [106] [107] [108] [109] [110] [111] However, a randomized multicenter trial of unrelated donor transplantation did not demonstrate a benefit to the use of humanized anti-Tac when given as prophylaxis. [112] Interestingly, brief administration of high doses of exogenous IL-2 early after SCT protected animals from GVHD mortality. [113] This protective effect, which was augmented by the co-transplantation of syngeneic bone marrow, was not associated with a decrease in either alloengraftment or a GVL effect. [113] [114] [115] Exogenous IL-2 may mediate its protective effect via inhibition of other cytokines, such as IFN-, [116] although this phenomenon has not been well studied in human transplantation.

IFN- is a second Th1 cytokine that is critical to the pathophysiology of acute GVHD. When a method to enumerate cytokine mRNA-containing cells that combined limiting dilution analysis (LDA) and PCR amplification of cDNA was used, IFN- secretion was found to be greatly increased during experimental GVHD. [49] [117] In several models of experimental acute GVHD, lymphocytes produce large amounts of IFN- when restimulated in vitro with mitogen or TCR cross-linking, in contrast to lymphocytes from syngeneic SCT or recipients after T-cell-depleted SCT. [117] [118] [119] [120] [121] [122] During clinical GVHD, a large proportion of T-cell clones isolated from patients also produce IFN-. [123] Increased serum levels of IFN- have also been found after SCT, but the increase was not dramatic, and in a small

number of patients studied, there were no significant differences between patients with and without GVHD. [124]

In vitro, IFN- can directly produce cytopathic effects of GVHD in skin tissue damage. [125] IFN- also mediates GVHD pathology in the GI tract, and the addition of an anti-IFN- monoclonal antibody prevents this damage. [126] Because the expression of MHC molecules is induced by IFN-, it is possible that an increase in these molecules during GVHD [127] might make epithelial target cells in the gut more susceptible to lysis by cytotoxic T cells. [128] Finally, IFN- induces the generation of the nitric oxide, which appears to mediate the suppression of lymphocyte proliferation in several experimental GVHD systems. [118] [129] [130] [131] [132]

The synergy of IFN- with LPS in the activation of mononuclear phagocytes to produce proinflammatory cytokines [133] [134] plays a central role during acute GVHD. A classic study by Nestel and colleagues showed that IFN- is sufficient to produce macrophage priming during a parental to offspring (PF_1) GVHD model. The subsequent administration of LPS to host mice then causes death owing to the increased systemic production of IFN- by macrophages. [56] Recent data have confirmed these observations and demonstrated that the inhibition of IFN- during GVHD by injection of polarized donor T cells (which secrete IL-4 but not IFN-) results in the down-regulation of LPS-triggered TNF- production and reduces GVHD-related mortality. [120] Interestingly, the induction of Th1 cells in humans as measured by expression of the $_2$ chain of the IL-12 receptor is stimulated by IFN- but not by IFN-, suggesting that IFN- may be an important signal for polarity switching. [135] [136] On the other hand, administration of IFN- after marrow grafting does not seem to increase the risk of GVHD. [137] Thus, the relative importance of various IFNs to human GVHD requires further study.

In an interesting set of experiments, injection of IFN- prevented the development of experimental GVHD. [138] These results were surprising because, according to the findings described above, one would expect IFN- to promote rather than to counteract GVHD. These unexpected results may be due to the diametrically opposing effects of local versus systemic actions of IFN-. In addition, IFN- administration early in BMT probably activates residual host elements, such as NK cells, which suppress the activation of donor T cells secreting IFN-, in a manner similar to IL-2s reduction of GVHD (detailed previously).

Th2 Cytokines

The balance of Th1 (IL-2 and IFN-) and Th2 (IL-4 and IL-10) T-cell cytokines in vivo early after BMT may be a critical factor in the development of a systemic inflammatory response in GVHD. Th1 cytokines are associated with the activation of macrophages, the secretion of inflammatory cytokines, the activation of NK and CTL cells, and enhanced production of antibodies of the IgG2a isotope, whereas Th2 cytokines are generally associated with down-regulation of cell-mediated immune responses and enhancement of IgE and IgG1 secretion. A Th1Th2 shift of T-helper cells can down-regulate cell-mediated immune responses and inflammatory processes.

In two recent studies, IL-10 was tested for its use as a prophylactic treatment for the development of severe acute GVHD, but the data have been largely negative. [119] [139] Because IL-4 is more important than IL-10 in directing the Th0 precursor T cells toward a Th2 phenotype, [140] the administration of IL-4 to prevent GVHD has also been tested. Intraperitoneal injections of rIL-4 led to an increase in the severity of GVHD and to enhanced late mortality in a fully allogeneic SCT model. [141] Recipients of IL-4 showed the same histopathologic changes in skin and liver as mice receiving no cytokine. Thus, direct administration of type 2 cytokines appears to be either ineffective or toxic.

Recently, new approaches that encompass a Th1Th2 shift in the cytokine profile of donor T cells prior to SCT have emerged to provide a new way to interrupt the amplification cascade after the allogeneic transplantation. Fowler and co-workers treated donor mice in vivo with a combination of human rIL-2 and murine rIL-4 and were able to generate CD4+ enriched splenic populations with a Th2 cytokine pattern that down-regulated subsequent GVHD. [142] [143]

The pre-incubation of donor T cells in the presence of murine rIL-4 ex vivo during a primary MLR culture to alloantigens also polarized T cells toward a Th2 cytokine phenotype. [120] Transplantation of these polarized Th2 T-cell populations failed to induce acute GVHD to MHC class I or class II antigens. Of great interest are studies of peripheral blood hematopoietic cells collected after mobilization with granulocyte colony-stimulating factor (G-CSF), which suggest that Th1Th2 polarization occurs in lymphocytes exposed to G-CSF. Pretreatment of murine blood cells with G-CSF resulted in less GVHD than in saline-treated controls. [144] [145] [146] This may reflect a change in cellular polarization or alterations in the production of other inflammatory cytokines such as TNF-. [147]

Inflammatory Cytokines

Murine BMT models have provided the strongest evidence of a link between excessive production of inflammatory cytokines and clinical GVHD, and demonstrate that activated macrophages play a key role in the process. [148] TNF- is an inflammatory cytokine that causes an extremely wide variety of biologic effects, including the induction of direct tissue injury and metabolic alterations in muscle and fat tissues leading to cachexia characterized by anorexia and weight loss that ultimately leads to death. [149] [150]

A critical role for TNF- in the pathophysiology of acute GVHD was first suggested by Piguet and colleagues, who observed that mice transplanted with mixtures of allogeneic bone marrow and T cells developed severe skin, gut, and lung lesions that were associated with high levels of TNF- mRNA. [151] [152] Subsequently, the presence of TNF- mRNA was also demonstrated in the skin of mice with GVHD induced to mHAs. [153] Target organ damage could be inhibited by infusion of anti-TNF- antibodies, and mortality could be dramatically reduced by administration of the soluble form of the TNF- receptor, an antagonist of TNF-. [19] More recent data employ the strategy of preventing macrophage shedding of TNF- with metalloproteinase inhibitors, which also reduces the manifestations of acute GVHD. [79] The role of TNF- in GVHD has been elucidated using an experimental murine GVHD model with unirradiated recipients. [56] Injury to the GI tract enables bacterial breakdown products like LPS to enter the circulation and trigger release of cytokines from mononuclear phagocytes throughout the body. It has long been known that pathogen-free mice are protected from GVHD after allogeneic SCT and that recolonization with gram-negative bacteria in the gut leads to increased GVHD severity. [81] [82] [154] The anatomic localization of macrophages to gut-associated lymphoid tissue and the ability of endotoxin to trigger inflammatory cytokine release together provide the most persuasive explanation for the ability of gut decontamination to prevent systemic GVHD.

An important role for TNF- in clinical acute GVHD has been suggested by studies demonstrating elevated levels of TNF- in the serum of patients with acute GVHD and other endothelial complications such as veno-occlusive disease. [155] [156] Importantly, the time of the first appearance of the increased levels was predictive of the severity of regimen-related toxicity and of overall survival. Monoclonal antibodies or F(ab) $_2$ fragments directed at TNF- have been studied either as therapy for steroid-resistant GVHD[157] or for prophylaxis. [158] Although several partial responses were observed, in the majority of patients GVHD flared after discontinuation of treatment. The

prophylactic trial of anti-TNF- monoclonal antibodies showed a delay in the onset of acute GVHD compared with the time of onset in historical controls, but showed only a small decrease in the rate of GVHD. These preliminary data, along with results from animal and laboratory studies, suggest that approaches to limit TNF- secretion will be an important avenue of investigation in SCT.

The second major proinflammatory cytokine that appears to play an important role in the effector phase of acute GVHD is IL-1. This cytokine is mainly produced by activated mononuclear phagocytes and shares with TNF- a wide variety of biologic activities. [159] Secretion of IL-1 appears to occur predominantly during the effector phase of GVHD in the spleen and skin, two major GVHD target organs.[160] A similar increase in mononuclear cell IL-1 mRNA has been shown during clinical acute GVHD.[161] Indirect evidence for a role of IL-1 in GVHD was obtained with administration of this cytokine to hosts in an allogeneic murine SCT model; recipients displayed a wasting syndrome and increased mortality that appeared to be an accelerated form of disease. [141] Investigations of the role of IL-1 in GVHD intensified with the discovery of IL-1 receptor antagonist (IL-1RA). [162] [163] In a murine model of GVHD to multiple minor H antigens, intraperitoneal administration of IL-1RA for 10 days after SCT prevented the development of GVHD in the majority of animals.[153] Two Phase I/II trials have studied specific inhibition of IL-1 in steroid-resistant GVHD.[164] [165] In the first trial, recombinant human IL-1RA was administered to patients with severe GVHD, with an overall response rate of 63%. Since IL-1RA has no intrinsic agonist activity and functions solely as a competitive inhibitor of both IL-1 and IL-1, the observed responses were directly related to decreased activity of IL-1. A subsequent trial using soluble recombinant human IL-1 receptor observed a 57% overall response rate. Taken together, these data strongly suggest that IL-1 is a critical mediator of GVHD.

Additional agents may have immunomodulatory properties whose mechanism of action involves altering the balance of inflammatory and anti-inflammatory proteins. The observation that -globulin administration can ameliorate GVHD [166] may be explained in part by the effects of -globulin on the up-regulation of IL-1RA and soluble TNF receptor.[167] When IgG is bound to macrophages, IL-1RA production is preferentially increased over that of IL-1. [168] Exogenously administered IgG may thus directly modulate the cytokine dysregulation during GVHD.

Interleukin-8 (IL-8), also called neutrophil-activating peptide (NAP-1), may also play a role in GVHD. IL-8 is produced in response to proinflammatory mediators such as endotoxin, IL-1, and TNF-, and its systemic administration inhibits the adhesion of leukocytes to endothelial surfaces. A recent study reports that among patients receiving stem cell transplant for -thalassemia, those with GVHD had significantly higher IL-8 blood levels than those without GVHD. [169] IL-8 may contribute to the immunocompromised status of patients who are already vulnerable to infections because of the underlying GVHD and its treatment. An interesting implication of this observation, which is consistent with the cytokine hypothesis, is that granulocytes and macrophages may contribute to GVHD pathology. Most GVHD occurs after leukocyte recovery. This fact is typically interpreted to reflect the time necessary for lymphocytes to expand to a critical mass. However, it might equally suggest that the inflammatory cytokine network has similar activation and recruitment effects on neutrophils, and that these newly produced cells follow chemotactic signals to injured epithelium, where they contribute to tissue injury by releasing activated oxygen metabolites as well as cytotoxic and proteolytic enzymes.

Chronic Graft-versus-Host Disease

Chronic GVHD is often considered an autoimmune disease because of distinctive similarities to various autoimmune disorders, especially collagen vascular disease. The relationship is difficult to prove clinically, but experimental studies have demonstrated an autoimmune aspect of chronic GVHD pathophysiology. [170] In contrast to T cells from animals with acute GVHD, which are specific for host alloantigens, T cells from animals with chronic GVHD are specific for a public (common) determinant of MHC class II molecules.[171] [172] These T cells are considered autoreactive because they recognize common class II molecule determinants rather than polymorphic major or minor histocompatibility antigens. The autoreactive cells of chronic GVHD are associated with a damaged thymus, which can be injured by acute GVHD, the conditioning regimen, or by age-related involution and atrophy. The normal ability of the thymus to delete autoreactive T cells and to induce tolerance may thus be impaired. Thymic damage may also help to explain syngeneic GVHD, where autoreactive T cells have been identified as well. The pathophysiology of chronic GVHD is generally much less well understood than that of acute GVHD and has undergone less intensive experimental modeling.

Syngeneic Graft-versus-Host Disease

In experimental models, pathologic changes of GVHD have been observed in animals transplanted with stem cells from genetically identical donors (or even with autologous stem cells), findings that substantiated reports of GVHD between identical twins described nearly two decades ago. [173] [174] In optimal models, syngeneic GVHD appears to be mediated by autoreactive lymphocytes directed at MHC class II proteins. Autoreactive T cells are thought to develop in a severely damaged thymic medulla, where MHC class II-bearing cells are deficient or absent. T cells thus escape the usual negative selection (clonal deletion) within the thymus and migrate to the periphery, where they trigger or mediate target organ damage. Other regulatory lymphocytes that would normally inactivate or eliminate such autoreactive cells have themselves been eliminated by total-body irradiation (as part of the preparative regimen). The experimental transfer of normal lymphocytes to these irradiated hosts restores the regulatory mechanism and prevents the autoaggressive process. The effects of cyclosporine are important in these models, particularly with respect to the damage that cyclosporine inflicts on the thymic medulla. [175] Syngeneic GVHD can thus be seen as an imbalance between autoreactive and autoregulatory lymphocytes, an imbalance that results from thymic dysfunction. In experimental animals, cutaneous pathologic findings of syngeneic and chronic GVHD are similar, and it appears that the efferent arms of syngeneic and chronic GVHD are similar if not identical. These observations further the perception of chronic GVHD as an autoimmune disorder.

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GRAFT-VERSUS-LEUKEMIA EFFECT

Experimental data show tight linkage between GVHD and the GVL effect, although these entities were separable under some circumstances. There is now substantial evidence that allogeneic stem cell transplantation in humans is associated with a potent GVL reaction, particularly in patients with chronic myelogenous leukemia (CML). Until recently, the evidence for this GVL effect was based on several important but indirect clinical observations. Several case reports of patients with relapse of leukemia after allogeneic SCT noted remissions of the malignancy either on abrupt withdrawal of immunosuppression [176] [177] [178] or during a flare of acute GVHD. [179] These reports provide the rationale for abrupt discontinuation of immunosuppressive therapy to induce a GVL reaction for patients who relapse after SCT, but it is likely that the majority of such patients will not re-enter remission. Further evidence that the donor graft mediates important antileukemic effect comes from observations that relapse rates for recipients of syngeneic stem cells are higher than

relapse rates for recipients of matched sibling grafts. [180] A recent update from Seattle confirms these findings in over 800 patients with advanced leukemia who underwent SCT; the relapse rate for 785 recipients of matched sibling grafts was 62%, compared to 75% in 53 recipients of syngeneic grafts ($P < 0.001$). [181] An even more dramatic effect on relapse rate was reported in a multicenter analysis of SCT recipients with acute myelogenous leukemia (AML) in first remission. In this study the relapse rate after syngeneic SCT was almost 3-fold higher than in recipients of matched sibling grafts (59 ± 20% vs. 18 ± 4%), [182] which was confirmed in a retrospective analysis by the International Bone Marrow Transplant Registry (IBMTR) (Fig. 93-2). [183] The large numbers of patients in this study demonstrated that the risk of relapse after syngeneic SCT remained higher than in recipients of matched sibling marrow grafts without GVHD. A second IBMTR analysis showed that the magnitude of this GVL effect is greater for patients with CML and AML and not statistically significant for patients with ALL in first remission. [184]

Patients who develop GVHD after allogeneic SCT experience relapse less frequently than similar patients who do not develop GVHD, confirming the experimental data showing that GVHD and the GVL effect are tightly linked. GVHD is protective against relapse both for SCT recipients with advanced leukemia [180] [185] [186] and for patients who received transplants in earlier stages of the disease. [183] [187] Initial reports suggested that chronic GVHD was most protective against relapse, [186] but other analyses demonstrate that acute GVHD is also protective. [183] [185] Results from an IBMTR analysis of 2,254 SCT recipients with less advanced leukemia (AML or ALL in first remission or CML in chronic phase) are shown in Figure 93-2 and demonstrate that the risk of relapse after SCT is lowest in patients experiencing acute or chronic (or both) GVHD. [183] As in syngeneic SCT, the magnitude of the GVL effect appears to be disease and stage specific. The Seattle group has reported that GVHD protects against relapse in patients with advanced leukemia, [185] [188] and IBMTR analyses of large patient numbers show a similar effect for patients with less advanced disease. [183]

Donor T cells included in the stem cell graft are critical for acute GVHD, and T-cell depletion of the donor graft by various strategies is one of the most successful means of reducing the incidence and severity of GVHD after allogeneic SCT. [189] [190] [191] [192] [193] [194] [195] Unfortunately, although T-cell depletion results in less treatment-related morbidity and mortality, improved overall survival rates have not been reliably demonstrated. This failure is due in large part to a reciprocal increase in the subsequent relapse

Figure 93-2 Actuarial probability of relapse after stem cell transplantation according to the type of graft and development of GVHD. *(From Horowitz et al., [183] with permission.)*

rate after T-cell depletion, as well as to graft failure and other complications. [187] [191] [196] [197] [198] T-cell depletion particularly increases relapse rates in CML. [183] [187] [196] This observation provides further strong, although indirect, evidence that donor T cells are important mediators not only of GVHD, but also of the GVL properties of the allogeneic stem cell graft.

In many experimental models donor T cells are important components of the GVL response, and cell-mediated cytotoxicity may be mediated through both MHC-restricted and unrestricted pathways. [199] [200] Most experiments implicate CD8+ cells as the vital mediators of the GVL response, [114] [199] [201] but under some conditions both CD4+ and CD8+ cells [15] [115] [200] [202] as well as NK/lymphokine-activated killer (LAK) cells [203] may be necessary for optimal GVL induction. Thus, multiple effector cells can mediate the GVL response in these experimental systems.

It is likely that important effector cells other than T cells can mediate the GVL response. For instance, various cytokines and NK cells can function without MHC restriction to enhance the GVL response in the appropriate setting. IL-2 is a potent inducer of NK cell activity and will potentiate the GVL effect in some murine models, [204] [205] [206] although NK cells are not always required for the GVL effect. [207] Donor-derived T cells appear to be primary effectors of the GVL response in clinical transplantation. The GVL effect of allogeneic SCT appears to be lost when T cells are depleted from the donor graft as GVHD prophylaxis, especially for patients with CML. [183] [187] [196] [198] [208] Many strategies for T-cell purging do not remove other potential effector cells (such as NK cells), [189] [192] [198] further supporting the role of donor T cells as primary mediators of GVL. It has also been possible to identify donor T-cell populations that possess specific cytolytic activity against allogeneic leukemia cells but not nonleukemic targets. [209] [210] After SCT, the number of circulating cytotoxic T-cell precursors directed against the leukemia identified in the peripheral blood may correlate with relapse-free survival. [211] [212]

Most available clinical data implicate CD4+ cells as important effectors of GVL. After SCT, circulating CD4+ cells that will directly lyse cryopreserved leukemic cells can be isolated in some patients. Some of these cells have been isolated and characterized as CD3+, CD4+, TCR+ cells. [210] Other cell lines have been established from patients with CML after allogeneic SCT that selectively lyse Ph+ CML cells; these cells were CD4+ lymphocytes, and specific lysis was inhibited by antibodies against MHC class II molecules. [213] In mice, immunization with a synthetic bcr-abl peptide elicited a CD4+ mediated, MHC class II-restricted response against the bcr-abl peptide. [214] This finding not only suggests that bcr-abl is a potential tumor-specific target for GVL reactivity, it also further supports the concept that CD4+ T cells are likely to be important mediators of the GVL response. For patients with leukemia undergoing allogeneic SCT, selective CD8+ cell depletion of the donor graft has reduced the incidence and severity of GVHD without yet resulting in a significant increase in relapse rates, which suggests that GVL reactivity is intact. [215] Although this finding does not directly prove a GVL effect induced by CD4+ cells, it does suggest that CD8+ cells may be more important in mediating GVHD and not the GVL effect, in contrast to some of the experimental models.

Other mononuclear cells such as NK cells may also have an important role in GVL induction in humans. NK cells routinely lyse the CML-derived cell line K652 in vitro. After SCT, the number and activity of NK cells increase in the peripheral blood, [216] [217] and NK cell isolates can be identified that will lyse cryopreserved host leukemia cells in vitro. [212] [218] Some NK cells derived from patients after SCT can also inhibit leukemic progenitor colony growth. [219] In this context, IL-2 may have a role in GVL induction because IL-2 reproducibly stimulates NK cell number and activity after allogeneic BMT, and its infusion may result in lower relapse rates. [220] [221] [222]

Data are also accumulating to suggest that some leukemic

cells may have specific antigen profiles that make them susceptible to the GVL effect. For instance, CML seems to be uniquely susceptible to T-cell-mediated GVL effects; it is known that p210 bcr-abl can bind to MHC class I [223] [224] [225] and class II molecules, and can serve as the target of T-cell cytotoxicity. [211] [213] [214] [226] Further, in vitro immunization experiments have resulted in generation of T-cell lines specific for the bcr-abl fusion peptide. [226] Similarly, in AML, the RAR- fusion peptide may be found in the MHC class I cleft, [224] and T-cell lines directed against leukemia-specific proteinase 3 have been derived. [227] It is not yet known whether fusion peptides or other leukemia-specific antigens are the target of T-cell attack in clinical situations. It is almost certain that minor histocompatibility differences between donor lymphocytes and host leukemic cells are also important targets for immunologic attack. These minor H antigen differences are clearly important for the GVL response in animal models. [228] [229] In humans, most GVL responses are associated with GVHD, suggesting that the GVL response may be an allogeneic response that is not strictly separate from GVHD. [183] [230]

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DONOR LEUKOCYTE INFUSIONS

Until recently the evidence for an important GVL effect in clinical transplantation was strong but largely circumstantial. The use of donor leukocyte infusion (DLI) to treat patients with relapse after allogeneic SCT has recently evolved. Kolb et al. reported that three patients with relapsed CML achieved a complete cytogenetic remission after treatment with IFN- and a DLI from the original donor. [231] These findings have been confirmed in several larger trials. [231] [232] [233] [234] [235] [236] [237] [238] [239] [240] [241] [242] When results from these trials are combined, the complete remission rate for patients treated for relapsed CML is consistently 6080%. [230] In addition, data from 119 patients treated with relapsed CML have been collected and analyzed by the European Group for Blood and Marrow Transplantation (EBMT) [243] and through a survey of 25 North American transplant centers.[244] Although the treatment protocols vary slightly among institutions, the results have been remarkably consistent. Complete cytogenetic and molecular responses are achieved in almost 80% of patients treated with either early relapse (cytogenetic or molecular) or hematologic evidence of chronic phase relapse. Patients with more advanced CML (accelerated phase or blast crisis) are less likely to respond. The magnitude of the GVL effect is highlighted by the finding that the majority of patients who respond achieve a complete molecular remission, which is defined as the absence of detectable cells containing bcr-abl mRNA transcripts when assayed by RT-PCR. Since RT-PCR is capable of detecting one CML cell in 10^6 normal cells,[245] clinical relapse of CML represents a minimum leukemia cell burden of approximately 10[12] cells, a molecular remission may represent a one million-fold reduction by the GVL reaction.

Adoptive immunotherapy with DLI has been associated with significant morbidity. Many patients develop pancytopenia and marrow aplasia, often associated with transfusion requirements, bleeding complications, and infections; these complications of marrow aplasia have been the immediate cause of death in several patients. Marrow aplasia presumably results from the destruction of host leukemia cells before recovery of normal donor hematopoiesis. This idea is supported by the observation that patients treated with donor MNC infusions for cytogenetic or molecular relapse rarely experience pancytopenia. [243] Occasionally, marrow aplasia has been persistent,[234] [239] [246] although this toxicity has been successfully reversed with the infusion of additional donor stem cells in some patients. [234] [239] The other major complication of DLI is acute and chronic GVHD. However, GVHD appears to be closely associated with a clinical response, [230] [247] [248] and despite the relatively high number of T cells administered without the use of GVHD prophylaxis, the severity of GVHD has generally been mild to moderate. No acute GVHD is seen in approximately 3640% of patients, and 1530% have only grade I acute GVHD. Some 2050% of patients experience grade IIIV acute GVHD, but this typically responds to immunosuppressive therapy.

The complications of DLI have resulted in treatment-related mortality rates of 20%. [230] However, GVHD has generally been responsive to immunosuppressive therapy, and the mortality rate from direct complications of GVHD is only 58%. [244] [249] Another 712% of patients have died from infectious complications, either related to marrow aplasia or to immune suppression given as therapy for GVHD. These results compare favorably with the anticipated mortality rate of 40% or higher associated with a second SCT,[250] but strategies to minimize DLI toxicity are still needed.

It is likely that similar effector cells responsible for the GVL effects of SCT also effect GVL associated with donor leukocyte infusions (see later), although this assumption has not been formally proved. Most adoptive therapy protocols administer unfractionated donor mononuclear cell preparations that contain lymphocytes, NK cells, and other leukocyte populations. There is indirect evidence suggesting that T cells are involved in GVL induction after such infusions. An increase in host-reactive T-helper precursor cells was detected in five patients who responded to donor MNC infusions for relapsed CML, while one patient who failed to respond had no detectable increase in the frequency of T-helper precursor cells. [251] There was no consistent increase in host-reactive cytotoxic T-cell precursors in the responding five patients. Jiang et al. found that two patients treated by DLI for relapsed CML had an increase in cytotoxic T-cell precursor frequency against cryopreserved host leukemia cells relative to host lymphocytes. [212] In this analysis, NK cell activity initially decreased but recovered to levels higher than preinfusion values, and this change preceded evidence of the GVL effect. The significance of these phenotypic changes is unclear, but future analysis may help determine which MNC subsets are important for GVL induction in this setting.

The administration of select subsets of donor mononuclear cell fractions is the ideal framework in which to dissect the cellular mechanisms responsible for GVL induction. Donor mononuclear cells depleted of CD8+ T cells given to patients with relapsed CML have been shown to induce a direct GVL reaction with minimal GVHD,[252] [253] further supporting the hypothesis that CD8+ T cells are not critical for GVL, at least for patients with CML. These studies are preliminary, however, and the effect of CD8+ depletion, and other novel manipulations on the duration of clinical response and the overall GVL reaction will have to await further follow-up and comparative trials.

The timing of GVHD and GVL induction in relation to SCT is of great importance to minimize toxic effects and still provide an important antileukemic effect. In dogs, allogeneic lymphocytes can be safely administered only after chimerism has been established. [254] In mice, the allogeneic donor lymphocytes given at the time of BMT result in severe GVHD; when given 21 days after the conditioning therapy, donor lymphocytes will provide a potent GVL effect without inducing severe GVHD. [30] Similar findings have been noted in other murine models. [220] Significant GVHD may be attenuated in this setting because of the separation from the cytokine phases that occurs after SCT,[16] [255] suggesting that DLI may be given safely to patients to prevent relapse if appropriately delayed from the acute toxicity of the transplant.

This strategy of delay is already in clinical trials. [255] [256] This approach may be most useful for recipients of T-cell-depleted grafts, where the high risk of relapse may be acceptable if the transplant can be performed with limited toxicity, with donor leukocytes given at a later time to restore the GVL effects. Slavin and colleagues have given incremental T-cell infusions to patients after T-cell-depleted allogeneic SCT. [255] [257] The infusions of donor T cells resulted in an increase in acute GVHD but had

no effect on event-free survival. There was a non-statistically significant trend toward lower relapse rates, but only in recipients of Campath-1M-treated marrow. Barrett and co-workers have administered varying doses of donor leukocytes at different times after T-cell-depleted SCT, and the results confirm that the timing and dose of DLI are critical determinants for the risk of GVHD. [256] Conclusions regarding the ultimate GVL effects from this strategy will require longer follow-up but will likely alter current transplant strategies, at least in patients with CML.

For patients with CML who relapse after allogeneic SCT, DLI can induce a potent GVL reaction and re-establish complete molecular remissions in the majority of patients. This is perhaps the most successful example of adoptive immunotherapy in the clinical setting and now provides indisputable evidence in humans that allogeneic donor cells mediate the GVL effect. It is now possible to manipulate the donor leukocyte product in an attempt to enhance the efficacy of GVL induction, and future clinical trials will focus on strategies designed to maximize GVL reactivity while limiting toxicity from marrow aplasia, GVHD, and other complications. This technology provides a unique opportunity to understand the detailed mechanisms of the GVL effect. Ultimately, the ability to harness the GVL potential of allogeneic donor cells will have tremendous clinical import for patients with relapsed CML, and it will allow the development of novel cancer therapeutic strategies.

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SUMMARY

Complications of SCT, particularly GVHD, remain major barriers to the wider application of allogeneic SCT for a variety of diseases. Recent advances in the understanding of cytokine networks and of the direct mediators of cellular cytotoxicity have led to improved understanding of this complex disease process. GVHD can be considered an exaggerated, undesirable manifestation of a normal inflammatory mechanism in which donor lymphocytes encounter foreign antigens in a milieu that fosters inflammation. Tissue injury related to the conditioning regimen or infection is then amplified by direct cytotoxicity via perforin/granzyme and Fas/FasL pathways, through direct cytokine-induced damage, and by recruitment of secondary effectors such as granulocytes and monocytes. Cytokine dysregulation may further result in the production of secondary mediators such as nitric oxide. The net effect of this complex system is the severe inflammatory manifestations that we recognize as clinical GVHD.

Cytokine modulation may be able to reduce the undesirable inflammatory aspects of GVHD while preserving the benefits of GVL. Multiple cellular effectors may be involved in GVL, although donor T-cell recognition of host antigens is an important element of this process. Cellular immunotherapy such as DLI offers another strategy for separating GVHD and the GVL effect. Both experimental and clinical data suggest that post-transplantation cellular immunotherapy can be performed relatively safely and effectively, and optimization of patient selection, cell dose, and timing of administration may all serve to limit toxicity and enhance the potential GVL effects.

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Chapter 94 - Graft Engineering to Enhance Engraftment, Reduce Graft-versus-Host Disease, and Provide a Graft-versus-Tumor Effect

Adrian P. Gee

INTRODUCTION

In the last few years there has been dramatic growth in the use of hematopoietic stem cell transplantation (SCT), with or without high-dose chemotherapy or radiotherapy, for the treatment of marrow failure syndromes, certain genetic disorders, and cancers that are refractory to conventional dose therapy. [1] Although the infusion of hematopoietic progenitor cells (HPC) is required to achieve normal and sustained hematopoiesis and immune function, it carries an associated risk that immunocompetent cells within the graft will recognize and react to antigenic differences expressed by normal tissues and cells of the recipient. [2] [3] The resulting graft-versus-host disease (GVHD) can be severe or fatal and is thought to be triggered primarily by antigens of the human leukocyte antigen (HLA) system, which are encoded by genes on chromosome 6 in humans.[4]

Matching of a related donor and recipient at the HLA-A, -B and -D/DR loci decreases the risk and severity of GVHD; however, fewer than one-third of potential patients have access to such a donor.[4] Several approaches have been taken to find alternative donors for such patients, including the use of HLA-matched unrelated donors obtained through various volunteer registries, [5] partially mismatched related donors,[5] [6] and grafts obtained from the patient himself or herself. [7] The use of these grafts generally carries additional risk, in the form of an increased incidence of GVHD in allogeneic SCT [8] [9] [10] and the potential for disease relapse due to reinfusion of occult viable tumor cells with autologous SCT. [7] [11] As a result, methods have been developed to manipulate or engineer HPC ex vivo, to eliminate the cells that are thought to mediate these clinical sequelae. Purging tumor cells from autologous hematopoietic progenitor cell grafts (autografts) is discussed elsewhere. In this chapter ex vivo manipulation of allogeneic hematopoietic progenitor cell grafts (allografts) is discussed.

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GRAFT-VERSUS-HOST DISEASE

A first indication of the identity of the cells mediating GVHD came from animal models in which GVHD was noticeably absent when fetal HPC were transplanted. [12] These grafts lacked mature T lymphocytes, and depletion of these cells from marrow grafts ex vivo in mice and dogs (by treatment with antithymocyte or antilymphocyte antibodies,[13] [14] or with soybean agglutinin [SBA][15]) prevented GVHD and produced stable chimeric engraftment in the recipient animals. This led to clinical studies in which T cells were depleted from HLA-mismatched grafts obtained from the parents of children with leukemia [16] or severe combined immunodeficiency. [17] T cells were removed by incubation with SBA, which agglutinates the majority of mature cells, but spares primitive hematopoietic progenitors. The SBA-negative fraction was then mixed with sheep erythrocytes, which formed rosettes with the residual T cells. These were removed by centrifugation on a density cushion.[18] This approach resulted in a reduction in the incidence and severity of GVHD compared to that previously seen in recipients of unmanipulated grafts.[16] [17] This stimulated a number of studies in which T cell-directed polyclonal and subsequently monoclonal antibodies (MAb) were used to identify and mediate the removal of T cells from grafts.[19]

Graft-versus-host disease occurs in 3050% of HLA-matched sibling transplants where it is believed to be triggered by differences between minor HLA antigens or non-HLA antigens.[9] [20] These are presented, directly or via antigen-presenting cells (APCs) such as monocytes and dendritic cells, [21] to donor T lymphocytes. It has been estimated that in any individual, approximately seven minor histocompatibility loci encode antigens that can cause GVHD, although the total number of these loci in the population is likely to be much higher. [22]

Direct presentation of these antigens in association with multiple histocompatibility complex (MHC) class I antigens (HLA-A, B, and C) predominantly triggers CD8+ T cells, resulting in their clonal expansion. If the antigen is first processed and then presented by APC, the effect is predominantly on CD4+ cells, which in turn stimulate CD8+ cytotoxic cells. Consequently, depletion of CD8+ cells from the graft would be expected to reduce or prevent GVHD, and this has been described in both murine transplant models and human clinical studies. [23] [24] [25] The complex and diverse etiology of GVHD is, however, illustrated by the observation that, in certain mouse strains, GVHD is mediated mainly by CD4+ cells, [26] whereas CD8+ cells both beneficially affect engraftment and reduce disease relapse. [27] It is likely, therefore, that various cell types and a complex array of soluble factors are responsible for the development of GVHD and that multiple interconnected pathways are involved.

This hypothesis forms the core of the cytokine storm theory of Ferrara and colleagues. [28] [29] [30] This proposes that GVHD is the result of a dysregulation of cytokines and consists of three main steps. In the first, the irradiation and chemotherapy used to condition the patient lead to damage to host tissues and cell activation, resulting in the secretion of inflammatory cytokines, for example, tumor necrosis factor-alpha (TNF-) and interleukin (IL)-1. This results, in turn, in increased expression of adhesion molecules and MHC class II antigens, which facilitates the recognition of normal host cells by donor T cells post-transplant. In the second phase, naïve T cells may be stimulated to differentiate into type 1 (Th1) or type 2 helper (Th2) T cells. Repeated stimulation in the presence of IL-12 (but not IL-4) results predominantly in the generation of Th1 cells. These secrete IL-2 and interferon (IFN)- which, in the third phase, stimulate the production of proinflammatory cytokines by accessory cells (donor and host monocytes and macrophages). These cells may also receive secondary triggering stimuli, such as endotoxin/lipopolysaccharide released from damaged intestinal mucosa, which amplify cytokine production further, resulting in the tissue damage characteristic of acute GVHD.

In contrast, stimulation of naïve T cells in the presence of

IL-4 results in the generation of Th2 cells that secrete IL-4 and IL-10. This promotes anti-inflammatory responses, resulting ultimately in chronic GVHD. A similar dichotomy of function may also hold true for CD8+ T cells. For more comprehensive descriptions of the cytokine storm hypothesis, the reader is referred to recent reviews and Chapter 93 .[29] [30]

Clinical evidence favoring cytokine dysregulation during GVHD comes from Tanaka and colleagues, [31] who described different patterns of cytokine gene expression post-transplant. They found that IL-4, IL-10, and IL-13 gene expression was suppressed in patients with severe GVHD, whereas IL-12 gene expression was increased. They felt that this indicated that severe GVHD might occur when Th1 cells were induced by a change in the balance between Th2 cytokines and IL-12. This supports the role of IL-12 as a crucial cytokine in the etiology of GVHD. Yang and colleagues [32] have reported that a single injection of this cytokine on the day of transplant markedly inhibited acute GVHD in a minor and major MHC-mismatched transplant animal model, in which CD4+ cells mediate GVHD. In contrast, CD8+-mediated graft-versus-leukemia (GVL) responses were retained. The effects of IL-12 on both of these responses appeared to be mediated by IFN-, and increased levels of this cytokine have been reported in transplant recipients suffering from GVHD. [33] There is now increasing evidence that many other factors may be intimately involved in the generation and pathophysiology of GVHD, including nitric oxide [30] [34] and IgE.[35]

Cells other than T cells also appear to be capable of mediating or exacerbating GVHD. These include natural killer (NK) cells [36] and lymphokine-activated killer (LAK) cells.[37] NK cells have been shown to promote engraftment and post-transplant immune reconstitution in mice. [38] In some animal models, however, they have been found to prevent GVHD if administered in the early post-transplant period via a pathway involving tumor growth factor (TGF)-. Later administration and the use of IL-2, however, resulted in exacerbation of acute GVHD.[38] Differences in the activities of LAK and NK cells derived from marrow versus cord blood have been implicated as an explanation for the lower level of GVHD reported in cord blood transplantation. [39] When compared to cord blood cells, marrow cells had deficient apoptotic cytotoxic activity and a lower cell surface expression of the IL-2 receptor.

Despite the undoubted complexity underlying the pathophysiology of GVHD, it is clear that a relatively simple procedure, such as T-cell depletion of the graft, is capable of achieving effective prophylaxis. This may be bought, however, at a considerable price.

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GRAFT-VERSUS-LEUKEMIA RESPONSES

A graft-versus-leukemia (GVL) effect was originally described 40 years ago in animal transplant models [40] in which it was shown that animals receiving syngeneic (identical twin) marrow primarily died from relapse of their disease, whereas those receiving histoincompatible grafts showed lower rates of relapse, but ultimately died of GVHD. The term graft-versus-leukemia was used to describe this effect 20 years later by Bortin et al. [41] Considerable effort has been expended to determine whether these effects are functionally separable, with the goal of stimulating GVL in the absence of GVHD. This has proved to be easier in animal models, where various effectors of GVL have been identified. [42] [43] These are thought to exert their effects by direct cytotoxic action toward antigens expressed specifically by leukemic cells or to minor histocompatibility antigens, expressed by both normal and leukemic host cells. Other GVL pathways may include MHC nonrestricted cytotoxicity by T-cell and nonT-cell effectors, or the release of lymphokines with direct or indirect antileukemic activity. In all cases, however, T-cell subpopulations have been implicated as the primary mediators.

The evidence for GVL in humans has come mainly from the observations that (1) the rate of leukemic relapse in patients receiving allografts is significantly lower than in recipients of syngeneic grafts; [44] (2) there is a lower incidence of relapse in recipients of allografts who develop GVHD than in those who do not; [45] and (3) there is lower incidence of relapse in recipients of allogeneic marrow who do not develop GVHD than in recipients of syngeneic grafts. [46]

The involvement of T cells as mediators of GVL in humans came initially from the observation that the incidence of relapse in patients with chronic phase chronic myelogenous leukemia (CML) was higher (50%) in recipients of T-cell-depleted grafts than in those receiving unmodified transplants (10%). [47] A retrospective analysis of data from the International Bone Marrow Transplant Registry in patients with chronic phase CML [46] also revealed that there was a significantly higher relative risk of relapse in recipients of T cell-depleted grafts who developed GVHD, than in recipients of nondepleted grafts in whom GVHD did not develop. This suggested that GVL could occur in the absence of GVHD and that this effect was dependent, directly or indirectly, on the presence of T cells within the graft. The effect is probably composed of several components. These include a direct cytotoxic activity of T cells against leukemic cells and interaction, either directly or via the release of cytokines, between T-cell populations and nonT-cell effectors, such as NK cells. Although well established initially in CML, GVHD responses have been described or evoked in a variety of diseases, including myelogenous leukemias, [45] [48] acute lymphoblastic leukemia (ALL), [49] chronic lymphocytic leukemia, [50] [51] lymphoma, [52] [53] and myeloma. [54] Passweg and colleagues reviewed International Bone Marrow Transplant Registry data on 1,132 patients with T- and B-cell lineage ALL who had received matched sibling donor transplants. [55] They were able to confirm that the development of GVHD in these patients was associated with a decreased risk of relapse that implicated a GVL response in this disease.

This immune reaction is now regarded as an important component in the development of effective therapy for a variety of leukemias and potentially also for solid tumors. [56] The exact identities and modes of action of the primary and secondary effector cells in humans remain to be determined definitively. [56] [57] Animal studies confirm that the best candidates appear to be T cells, [43] or particular T-cell subsets, [58] activated killer cells, [59] and NK cells. [60] This lack of understanding has not, however, prevented attempts or proposals to induce the effect postallogeneic transplantation to prevent or treat relapsed disease. Approaches have included tapering or sudden cessation of immunosuppression; [61] infusion of leukocytes obtained from the graft donor [62] [63] [64] (with and without use of systemic recombinant cytokines, [65] [66] or depletion of particular T-cell subsets [67]); infusion of lymphokine-activated cells and IL-2; [68] and administration of cytokines, such as IL-2, alone. [69]

There is also now a concerted effort to develop methods to generate leukemia-directed effector populations ex vivo for this type of therapy. [70] Potential effectors have included autologous T cells isolated from follicular lymphomas, [71] and CD8+ T cells primed against leukemic target cells expressing the B7 co-stimulatory molecule. [72] [73] It is possible that cell lines derived from these effectors could be used to provide a GVL effect. Irradiated MHC nonrestricted T-cell lines have shown antitumor activity when injected into tumor-bearing mice or incubated with leukemia-seeded human marrow, [74] and the use of a cytotoxic NK cell line for tumor purging of autologous grafts has been reported by Klingemann and colleagues. [75]

Any strategy for improving the outcome of allogeneic transplantation by the use of cellular manipulation, in the form of

Figure 94-1 Cellular strategies to optimize outcomes in allogeneic hematopoietic progenitor cell transplantation. Cellular conditioning: Graft recipients may be conditioned to accept grafts by the administration of enriched facilitator cell populations or to prevent graft rejection by infusion of various veto cell preparations. Seeding the marrow spaces with purified stromal cell populations in the peritransplant period may also enhance stable establishment of the hematopoietic progenitor cells. Graft manipulation: Control or prevention of GVHD can be favored by the depletion of potential effector cells (primary candidates include mature T lymphocytes, T-cell subsets, and NK populations) from the progenitor cell graft, or increasing the dose of CD34+ cells administered. Immunotherapy: Relapse of disease posttransplant (particularly in leukemias) may be abrogated or treated by infusion of tumor-directed or nonspecific alloreactive immune cells. To enhance the specificity of the intended cytotoxic response, the effector cells can be primed with tumor-associated antigen(s) ex vivo or genetically modified to provide a mechanism for deactivation if nonspecific killing is detected.

graft engineering and post-transplant immunotherapy, must therefore tread the fine line between preventing GVHD, while retaining or simulating the beneficial effects of any diseasedirected immune response (Fig. 94-1).

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THE DOWNSIDE OF T-CELL DEPLETION

Balancing Graft-versus-Host Disease, Graft-versus-Leukemia, and Engraftment

The control of GVHD by pan T-cell depletion is achieved at a price. When used to prepare grafts from HLA-matched siblings, in an attempt to reduce the GVHD seen in 3050% of the recipients of these grafts (due to mismatched minor HLA and non-HLA antigens), [4] there was about a 10-fold increase in engraftment failure and a fivefold increase in leukemic relapse. [76] This was seen predominantly in patients with CML. [47] As a result, there was no overall survival advantage over using nonmanipulated grafts. This provided powerful evidence, however, that donor T cells had a role, directly or indirectly, not only in effecting GVL but also in preventing graft rejection.

Graft Failure

The risk of graft failure in recipients of matched sibling grafts increases from about 2% to >10% with T-cell depletion. [76] [77] It may be seen as failure to achieve engraftment, which is thought to be immunologically mediated. Alternatively there can be early engraftment followed by pancytopenia or late failure of a functioning graft, both of which may be effected as a result of recurrent disease. [78] Residual immunocompetent T cells in the recipient are thought to be responsible for graft rejection. [79] The phenotype of these cells appears to be dependent on the type of transplant. Matched sibling grafts are predominantly rejected by CD3+8+57+ suppressor T cells, whereas CD3+8+57 HLA-DR+ cytotoxic T cells mediate the rejection of grafts from matched unrelated donors. [80] [81] In partially matched related transplants both pathways have been seen. [82] To overcome rejection a number of strategies have been tried. These include increasing immunosuppressive therapy and changing the composition of the graft to increase the dose of T cells, $CD34^{+}$ cells, or graft facilitator cells (see later).

The variable clinical results that have been obtained by depleting different cell populations also indicate that ex vivo graft manipulation is but one component in a more comprehensive strategy that must be used to achieve the desired outcome. Other important variables include the diagnosis, the choice of conditioning and immunosuppressive therapy, and the degree and nature of HLA incompatibility.

Lymphoproliferative Disease

The risk for post-transplant lymphoproliferative disease (LPD) is increased following use of a T cell-depleted graft. [83] Although cases of T-cell LPD and late-onset lymphoma have been described, these are relatively rare when compared to the incidence of B-cell LPD. The risk of LPD is estimated at almost 12-fold higher in patients who receive T-depleted grafts than for those who receive nonmanipulated grafts. Recipients of a Treplete HLA-matched transplant have a 0.9% ± 0.2% probability of developing such a condition at 4 years, compared to a probability of 64.8% ± 17.7% in recipients of a T-depleted HLA-mismatched transplant for a primary immunodeficiency. [84] Data from the National Marrow Donor Program for matched unrelated transplants show a 2% overall incidence of LPD, which increases to 5% with T-cell depletion. [85] Interestingly, the incidence was not uniform within this group, but varied with the method used for manipulation of the graft (see later). Depletion techniques that used highly specific T cell-directed MAb resulted in an LPD incidence of 1125%, whereas methods that depleted both T and B cells, for example, lectin-based methods and CAMPATH antibodies, were associated with an incidence of <1%. [86] [87] This must be interpreted with caution, however, because

the type and intensity of post-transplant immunosuppression substantially altered the incidence.

The explanation behind this association between T-cell depletion and B-cell LPD is the depression in specific immunity to the Epstein-Barr virus (EBV) that is seen in recipients of depleted grafts. This can be detected in vitro by the use of assays for the frequency of EBV-specific cytotoxic T-lymphocyte precursors. [88] These showed depressed immunity in 21 of 26 recipients of unmodified or T-depleted grafts at 3 months; however, by 6 months normal frequencies were in the normal range in 9 of 13 recipients. This period of depressed immunity corresponded to that in which clinical LPD was observed. Recent studies have shown that it is possible to generate EBV-specific T cells ex vivo and to use these cells adoptively both for therapy of LPD and to provide immunity to the disease for as long as 18 months. [89]

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BLOOD VERSUS MARROW AS THE GRAFT SOURCE

Attempts at engineering allogeneic grafts have predominantly focused on T-cell depletion. This presents some challenges to the cell-processing laboratory because the T-cell content of bone marrow grafts can vary widely, depending on the contamination of the harvest by peripheral blood. The switch to grafts derived from mobilized peripheral blood (peripheral blood progenitor cells, PBPCs) has not resolved this problem because this material contains approximately a 10-fold higher concentration of T cells,[90] which requires additional depletion capacity for any method that is developed for cells from both sources. Clinical data also suggest that the functional activity of T cells in blood-derived grafts is not the same as that of cells harvested with marrow. When nonmanipulated T cell-replete, HLA-identical PBPC grafts have been infused, the incidence of acute GVHD has been lower than that seen with marrow, despite the higher dose of T cells. [91] This may be at the expense of a subsequently higher incidence of chronic GVHD; the risk of developing GVHD is estimated at greater than twofold higher than that seen with marrow grafts.[92] [93]

One proposed explanation is that T cells in mobilized PBPC grafts are at a different stage of functional maturity and may, therefore, be less immunologically active in the immediate post-transplant period or may actually suppress GVHD development. Evidence for this hypothesis comes from the work of Kusnierz-Glaz and colleagues,[94] who found three different patterns of T-cell composition in normal donors mobilized with granulocyte colony-stimulating factor (G-CSF). In one group CD48+ was found in addition to the typical CD4+ and CD8+ populations. These cells were present in both the high- and low-density fractions obtained by centrifugation of the cells on discontinuous Percoll gradients. A second pattern consisted of CD48+ T cells, which collected only in the low-density fraction. In the third pattern there was no discrete population of CD48 T cells. The number of these cells in mobilized collection was about fivefold higher than that in resting blood. These double-negative cells suppressed mixed leukocyte reactions, and it was proposed that they may be used adoptively to ameliorate GVHD. [94] In a murine model system, transplantation of the low-density fraction (CD48 enriched) of G-CSF-mobilized cells resulted in the most successful reconstitution of allogeneic recipient animals. [95] These cells showed a reduced capacity to induce lethal GVHD and secreted about 10-fold more IL-4 and lower amounts of IL-2 and IFN- than unfractionated cells, suggesting that mobilization with growth factors induced a shift toward Th2 cytokine secretion in this population. The numbers of double-negative cells has, however, also been reported to increase in association with LPD, GVHD, autoimmune diseases, and liver allograft rejection, [96] suggesting that these cells may have a central role in regulation of immune responses.

If chronic GVHD continues to be a serious impediment to the use of nondepleted allogeneic PBPC grafts, it will be necessary to modify existing depletion protocols to accommodate the increased T-cell burden in these collections. Similar differences have been seen in the incidence and severity of GVHD when using cord blood in place of marrow, although the data are still somewhat confusing. The functional immaturity of cord blood T lymphocytes is usually cited as the explanation for the observations.

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TARGET T-CELL DOSE

Kernan and colleagues have proposed that to avoid GVHD, the dose of T cells should be kept <1 × 10^5 /kg recipient weight,[97] a number that was subsequently validated in a study in which the T-cell dose in HLA-matched sibling donor marrow grafts was fixed at this value. [98] At this dose, there were no graft failures and acute GVHD was limited to grade III involvement of the skin. The T-cell concentration required to achieve sustained long-term engraftment, control of GVHD, and maintenance of the potential for GVL will depend on the disease and the degree of HLA disparity. Any recommendation for a fixed number must, therefore, be interpreted with caution, especially because the identity of the effector population(s) has yet to be determined. With this proviso, it is clear that many techniques are capable of achieving levels of depletion that have been shown to be clinically effective.

METHODS FOR T-CELL DEPLETION

Figure 94-2 shows various methods of T-cell depletion and enrichment.

Sheep Red Blood Cell Agglutination

One of the most widely used methods for pan T-cell depletion has been agglutination of mature cells using sheep red blood cells (SBA), followed by a downstream additional depletion step. This lectin binds to *N*-acetyl-glucosamine, which is apparently not present on the surface of pluripotent hematopoietic progenitor cells. [99] When a cell suspension is treated with SBA, the majority of cells will clump and can be removed by sedimentation or centrifugation on a density cushion, such as 5% bovine serum albumin.[18] In a study of 87 procedures, this was found to result in a 1.26 ± 0.34 log depletion of E rosette-forming T cells (predominantly CD4+ cells) [18] [100] and enrichment of both CD34+ progenitors and committed colony-forming cells. The T-cell content of grafts treated in this way usually provides a dose >1 × 10^5 /kg, which is associated with a 50% risk of producing grade I or higher GVHD in HLA-matched recipients. [97]

To reduce the T-cell dose below 1 × 10^5 /kg it is necessary to add an additional depletion procedure, such as E rosetting, or an antibody-mediated removal (described later). In most cases, fluid-phase SBA has been added to the cells; however, Applied Immune Sciences (now part of RPR Gencell) at one time developed a panning system in which the lectin was immobilized to a plastic surface. [18] [101]

Erythrocyte Rosetting

When human T cells are mixed with a suspension of sheep erythrocytes they form rosettes with the red blood cells (E rosetting). This effectively increases the density of the T cells and allows them to be removed by sedimentation or density-gradient centrifugation. The rosettes tend to be rather fragile, and optimum rosetting is favored by bringing the cells into direct contact by gentle centrifugation to pellet them and by overnight incubation at 4°C. More stable rosettes that form rapidly can be obtained if the sheep erythrocytes are pretreated in

Figure 94-2 Manipulation of cell populations in allogeneic stem cell transplantation. Allogeneic cell populations that are infused to provide hematologic/immunologic rescue from high-dose therapy often require ex vivo manipulation to reduce the risk of producing severe or lethal GVHD, while also retaining any antidisease activity that could be exploited to prevent or reduce the risk of relapse post-transplant. Depletion: The various depletion techniques shown are primarily used to eliminate candidate GVHD effector cell populations from the graft and rely on qualitative markers that differentiate these cells from noneffector cells. A newer approach is to identify donor cells that are capable of mounting an alloreactive response to host antigens and to deplete these functionally active cells from the allograft. Enrichment: An alternative approach is to enrich the normal progenitor/stem cell populations in the graft, thereby achieving depletion of unwanted other cell populations by default. Positive selections of this type have to date been almost exclusively based on the purification of CD34+ blood or bone marrow hematopoietic progenitor cells. Other cell populations are undoubtedly important in determining the outcome of the transplant. They may enhance engraftment (stromal cells), even across histocompatibility barriers (facilitator cells), or be used as a potent means to generate disease-targeted killer cell populations for use in immunotherapy of the disease.

2-aminoethylisothiouronium bromide, washed, and irradiated before use. [18] Once mixed with the treated erythrocytes, the graft cells can be almost immediately layered onto a density-gradient material, such as Ficoll-Hypaque, and centrifuged. The T cell-depleted fraction remains at the interface, while nonrosetted sheep cells and T cells pellet to the bottom of the tubes. In an unpublished study using this technique for up-front T depletion of HLAmismatched PBPC collections, a mean 2.16 ± 0.46 log depletion (n = 25) of CD3+ cells was achieved, as determined by flow cytometry. When rosetting was combined with SBA treatment of marrow grafts, a 2.4 ± 0.5 log depletion of clonable T cells was obtained (n = 61) with an infused T-cell dose 0.632.8 × 10^4 /kg. Recoveries of nucleated cells averaged 6.6%. It has been reported that recoveries of early progenitor cells and processing times can be improved using three cycles of depletion with neuraminidase-treated E [102] and simplified rosetting techniques.[103] When this combination of methods was used to deplete T cells from 61 HLA-matched and 26 HLA-mismatched grafts, the overall incidence of grade III GVHD in the matched recipients was 3% (n = 58) versus 16% in the mismatches (n = 25).[18] This technique is, however, liable to come under increasing regulatory scrutiny as governmental agencies move to restrict the use of animal products in human therapeutic procedures.

Counterflow Centrifugal Elutriation

Agglutination and E rosetting exploit artificially induced changes in T-cell density to effect separation. T-cell populations do, however, inherently show differences in these physical properties from other blood cell subpopulations. Under normal conditions these small differences are insufficient to allow them to be used to separate the cells by sedimentation or routine centrifugation techniques. In an elutriation centrifuge, cells are pumped into a spinning chamber and will tend to sediment under the centrifugal force, based on their size and density. [104] [105] If this force is counterbalanced by the fluid flow, the cells will line up within the chamber, such that different fractions can be sequentially collected, by changing either the centrifuge speed or the fluid flow rate. When this approach is used, a T cell-depleted fraction can be obtained for transplantation. This product predominantly contains the larger committed progenitor cells. [106] In contrast, the smaller progenitor cells tend to be concentrated in the T cell-enriched fraction, [107] from which they can be recovered by positive selection (see later), and added back to the graft. [108] Grafts prepared in this way contain similar doses of CD34+ and colony-forming progenitor cells to unfractionated harvests, and a T-cell dose of approximately 6 × 10^5 /kg.[106] Add-back improved engraftment; however, post-transplant immunosuppression was still required to reduce GVHD and morbidity. [108]

Higher levels of depletion, which may be required for HLA-mismatched grafts, can be achieved using double-cycle elutriation. [109] This results in >3 log depleted graft, to which a controlled number of T cells can be added back. When this technique was used to prepare grafts for 10 HLA-matched recipients with hematologic malignancies, and which were engineered to contain 0.50.75 × 10^5 T cells/kg, all 10 patients successfully engrafted, following conditioning with total-body irradiation (TBI) and combination chemotherapy, with pre- and post-transplant immunosuppression. Fifty percent developed grade III GVHD, and 2 of the 10 went on to develop chronic GVHD. Four died of bone marrow transplant-related complications, and 2 relapsed at 46 and 1,035 days post-transplant.

Elutriation offers a number of advantages, including the use of a closed system for cell processing, a degree of automation, and the ability to recover intact all of the various cell fractions. These can be recombined, with or without addition processing, to provide a graft of prescribed composition. The disadvantages

have been the loss of smaller HPC in the T cell-depleted fraction [107] [110] (although this has not been a universal finding [111]), and the relative complexity of the

hardware.

T Cell-Directed Cytotoxic Agents

A number of chemotherapeutic drugs have been successfully used for the therapy of a variety of T-cell malignancies, [112] [113] and may be adaptable for T-cell depletion of allogeneic grafts.

Incubation of enriched mononuclear marrow cells ex vivo with 2-deoxycoformycin (Pentostatin) for 3060 minutes at 37°C in medium containing plasma, followed by the addition of 2-deoxyadenosine, and continued incubation overnight, results in a 1.52.5 log depletion of T cells. [112] This treatment spares normal myeloid and erythroid progenitors. The nucleoside analog 9-- D-arabinosylguanine inhibits DNA synthesis in T cells. [113] Overnight incubation of lymphoblastoid cell lines, or fresh leukemia cells with 100 M of the drug, results in a 6 log depletion of T cells, without significant effects on other committed HPC. [113] A more widespread toxicity, against T cells in general, allospecific T cells, NK cells, and LAK precursors has been described following the incubation of human cells with 50250 M of the lysosomotropic drug L-leucyl-L-leucine methyl ester (LLME).[114] [115] There was, however, partial or complete inhibition of colony formation by committed myeloid, monocyte, and erythroid progenitors. In a mouse transplant model, treatment of allogeneic marrow and spleen cells protected the recipients from lethal GVHD without adversely affecting engraftment. A phase 1 clinical study involved 19 patients with high-risk disease undergoing allogeneic transplantation from an HLA-identical sibling (n = 12) or a partially HLA-matched family donor (n = 7).[116] Marrow mononuclear cells were treated ex vivo with LLME concentrations of 0.250.5 mM. Marrow NK and LAK activities were essentially eliminated at concentrations 0.375 mM LLME. CD8+ cells were also reduced, and granulocyte macrophage colony-forming unit (CFU-GM) recovery was 3% at 0.5 mM LLME. The median time to an absolute neutrophil count of 500/l was 17 days. One patient with marrow treated with 0.5 mM LLME died of secondary graft failure. Complete donor chimerism was documented in each evaluable case. Grade IIIV GVHD occurred in 4 of 18 evaluable patients.

In general, T cell-directed or specific cytotoxic agents have not been widely used, partly because of their effects on some progenitor cell populations and partly because of the difficulty in achieving controllable and reproducible levels of depletion. They have found more widespread application for purging malignant T cells from grafts that are to be used for autologous transplantation.

Antibody-based Methods for T-Cell Depletion

The aim of most graft engineering procedures is to achieve reproducibly a final product with a defined cellular composition. This goal has become much more achievable with the advent of MAb technology. The high specificity of these reagents, their ever-increasing availability, and the variety of ways in which they can be used to effect cell separation have been largely responsible for much of the development of this field. In contrast, the use of polyclonal reagents required extensive adsorption of unwanted reactivities to enhance their specificity, and these reagents had to be produced by immunization of animals with intact cells, or cell extracts, expressing the target antigen to which the antibody was required. The resulting sera or peritoneal fluids were likely to contain host viruses; the purification procedures were arduous and generally resulted in poor final yields of antibody, with tremendous variability from batch to batch. In contrast, monoclonal reagents can now be prepared under controlled manufacturing conditions from defined, well-characterized cell cultures grown in serum-free media. Purification from this material is relatively simple, and the final product shows monospecificity toward the particular antigen.

Monoclonal antibodies can be used, in combination with flow cytometric analysis, for the identification of the appropriate target cell population and to effect their elimination from the cell mixture. This can be achieved by using them, together with serum complement, to lyse the cells, or to act as a link to a solid phase onto which the target cells are immobilized. Because regulatory concerns are moving the field away from the use of serum products in therapeutic procedures, physical separation techniques have now become the predominant methods for target cell elimination. These involve the use of a solid matrix material to which is attached the capture antibody. This is incubated with the cells and can then be removed together with the attached cells. In depletion techniques the antibody on the solid phase will be directed against T cells, or other potential mediators of GVHD, and the residual cells will be used for the transplant. In positive selection, the antibody on the solid phase is targeted toward HPC (usually an anti-CD34 monoclonal reagent), and these cells are then recovered from the matrix and used for grafting.

The solid phase can take several forms, from a flat plastic sheet to a variety of particles of various sizes and types. The main advantage of using a three-dimensional solid phase is that it allows a greater amount of MAb to be presented to the cells in a much smaller physical space than can be achieved with a flat configuration. The same amount of MAb can be immobilized onto a few microliters of nanoparticles, or a few milliliters of microspheres as can be attached to many hundred square centimeters of plastic sheet. These particles can be used as the matrix for column separations or, if they possess paramagnetic properties, they can be mixed as a suspension with the antibody-treated cells and then collected within a magnetic field. The limitations on these approaches now lie mainly in the identification of the appropriate target cell population rather than in the efficiency of the separation technologies.

An alternative approach is to use the MAb in a more physiologic way. This can be achieved either by allowing it to activate serum complement, either in vitro or in vivo to produce target cell lysis, or by attaching to the antibody some toxic moiety such as a plant toxin or a radionuclide.

Antibody and Complement

The cytolytic serum complement pathway is triggered by the binding of the first component of the cascade to target cell-bound MAb. Activation of the classical complement pathway ultimately results in formation of a membrane attack complex, which is composed of terminal complement components, and is responsible for colloid osmotic lysis of the target cells.[117] Activation of the cascade is achieved by binding of the first component to a single molecule of IgM antibody or to a doublet of IgG molecules, as long as the MAb is of a complement-fixing subclass.

Technically the complement lysis procedure is relatively simple. A prerequisite is a screened source of animal complement because human serum is of limited efficacy for ex vivo lysis of human cells. Some suitable MAb, such as CAMPATH-1, do fix human complement, and can, therefore, be used for both in vitro and ex vivo depletion.[118] The most widely used source is baby rabbit serum; however, batches must be carefully screened for nonspecific toxic activity toward HPC. Usually, multiple sera are screened, and suitable lots are pooled for clinical use. Such sera are now commercially available and have undergone extensive testing for possible infectious agents and pyrogens.

The graft cell suspension is first incubated with an excess

concentration of the chosen MAb(s), usually at 4°C for 30120 minutes. In some studies, the complement has then been added directly, whereas in others, excess fluid-phase antibody is first removed by extensive washing. In either case, it is important to understand the properties of the target antigen(s), for example, are they internalized, over what period, is surface expression temperature sensitive, does their binding activate the target cell? Failure to do so can result in ineffective sensitization and poor downstream lysis. The sensitized cells are then incubated with a concentration of complement shown to be in excess in preclinical studies. Because lysis is temperature dependent, it is usual to perform this incubation either at ambient temperature or at 37°C. Higher temperatures may produce increased levels of nonspecific toxicity, although the end point of the reaction will be reached faster. Because cells are lysed during this step, intracellular products, including DNA, are released. This can result in excessive cell clumping, which can be reduced by the addition of DNAse before the addition of complement. The cells are then washed extensively with infusion grade medium to remove complement, MAb, and the enzyme before infusion or cryopreservation.

This technique, although simple, has a number of disadvantages. These include the inability to control the extent and reproducibility of lysis; the escape of cells expressing low levels of the target antigen;[119] [120] nonspecific toxicity toward HPC; the presence of anticomplementary factors[121] [122] (probably predominantly decay-accelerating factors) in marrow harvests; and sensitivity to metal chelating anticoagulants (frequently used during the collection of apheresis products). Although it has found widespread clinical application (see later) and has achieved impressive results, particularly in the preparation of mismatched grafts, there is a general move toward other methods for T-cell depletion. This has been accelerated by the decision of the Food and Drug Administration that complement preparation must meet the same test standards as those set for MAb products that are intravenously infused.[123]

Immunotoxins

The problems of specificity associated with the use of complement have led investigators to examine alternative methods to use MAb to target a toxic moiety to the cells of interest. There has been considerable interest, therefore, in using various powerful plant toxins conjugate to T cell-directed MAb. Most of the early work

focused on ricin, a toxin obtained from the castor bean.[124] The molecule is composed of an A chain, which possesses the toxic activity, and a B chain, which is important in translocation of the molecule across the cell membrane,[125] where the A chain inhibits ribosome activity, which results in shutdown of protein synthesis and cell death. Unfortunately, the B chain also binds to galactosyl residues on cells, thereby counteracting some of the specificity that is introduced by conjugating intact ricin to the MAb. This can be overcome either by blocking the receptor on the B chain, by carrying out the incubation with cells in medium containing lactose, or by using immunoconjugates prepared only with the A chain. To ensure that these A chain immunotoxins are efficiently translocated into the cytoplasm, in the absence of the B chain, it is usually necessary to add lysosomotropic amines or carboxylic ionophores to the incubation medium. [126]

In an effort to circumvent some of these problems, other toxins have been tested. Pokeweed antiviral protein is a single-chain toxin (hemitoxin) that depurinates ribosomes in a similar way to ricin; however, it has mainly been used to purge leukemic cells from autologous grafts. [124] [127] Diphtheria toxin[128] and enzymes, such as glucose oxidase and lactoperoxidase, have also been conjugated to MAb.[129] [130] The two enzyme-based immunotoxins are first added to the graft preparation where the MAb targets them to the cells of interest. Excess toxin is washed out and the substrate added to the cells. The enzymes then interact in a synergistic manner with the substrate, resulting in the generation of toxic halides and hydrogen peroxide, which kill the cells, but do not harm committed normal progenitor cells. In laboratory tests, an anti-CDw52 MAb achieved a 3 log depletion of marrow cells when used in this way, without any effect on normal marrow erythroid or myeloid precursors. [129] [130]

Immunotoxins have not yet secured a major place for engineering allogeneic HPC grafts, although they have been used clinically for T-cell depletion (see later). [126] In part, this is due to the problems of efficient delivery to the point of action within the cell cytoplasm. The use of hemitoxins or molecularly engineered toxic molecules and conjugates may help to resolve this.[131] [132] Unlike complement-mediated killing, it is also necessary to prepare a toxin conjugate with each individual MAb, instead of being able to add the toxic moiety to presensitized cells. The preparation, purification, and testing of such reagents are not trivial undertakings and, with ever-increasing regulatory scrutiny, can represent an enormous cost to any cellprocessing facility.

Panning and Column Techniques

Cytolysis of cells within the graft can result in problems of clumping because of the release of intracellular components. It also complicates analysis of the efficacy of the treatment. Cells may show only partial damage after treatment, which can be difficult to detect within conventional viability stains. On prolonged incubation or after infusion, these same cells may subsequently die, which makes it almost impossible to determine the true extent of target cell elimination ex vivo (see later). Many of these problems are resolved if cell elimination is effected using physical separation techniques. In these, the antibody is linked directly or indirectly, to a solid phase that is used to collect the target population. The bound cells can then be removed from the cell mixture by withdrawing the solid phase.

In its simplest configuration the MAb can be bound to a plastic sheet, either covalently or by passive adsorption. Nonreacted surfaces on the plastic are coated with irrelevant protein, and the cell suspension is incubated on the plastic. Nontarget cells can then be recovered by gently washing them over the surface, and in some configurations, it is also possible to detach the target cells from the surface (by physical or enzymatic means) for further study. Commercially developed systems have been clinically tested for both T cell depletion [133] and for the positive selection of HPC.[101] Panning using a CD5/CD8 device resulted in higher nucleated cell recoveries than were obtained by E rosetting but a similar frequency of clonable T cells in the product. This produced a lower overall T-cell depletion and a different distribution of T-cell subsets. [18] In a pilot study,[133] there were four graft failures in 54 evaluable patients and a 30% incidence of grade III acute GVHD.

Panning techniques require relatively large two-dimensional configurations to process the number of cells present in an average HPC graft. This makes the devices somewhat cumbersome, or multiple smaller devices have to be used simultaneously or in sequence to process the cells. This can result in prolonged processing times, with a higher degree of nonspecific cell loss. Attachment of the MAb to a particle results in a more compact configuration, while allowing the same amount of antibody to be offered to the target cell population.

Affinity chromatography has long been used for the separation of fluid-phase components from complex mixtures. For example, mouse MAb can be purified over columns of Sepharose particles to which protein A or an antimouse immunoglobulin antibody has been attached. This same approach can be used for the separation of cells. The technique has been pioneered predominantly by the CellPro Company, which has developed column-based systems for the positive selection of HPC, [134] and,

more recently, for CD2+ T cells.[135] In their stem cell enrichment device a column of avidin-coated Sepharose particles is used to collect CD34+ HPC from grafts that have been treated with an anti-CD34 IgM MAb conjugated to biotin. During passage over the column the biotinylated antibody reacts with the immobilized avidin, and the HPC are retained. Nontarget cells are washed through the column, and the CD34+ population is recovered by stirring the column matrix. The same basic approach can be adapted for other separations by changing the specificity of the MAb that is used, and investigators are currently evaluating an anti-CD2-based system for the enhanced depletion of T cells from CD34-selected allogeneic grafts. [135] A similar approach has been described using supplemental depletion with anti-CD4.[136] The CellPro separation systems are automated (but allow for some user intervention) and use a closed system for cell processing. The CD34-based system has received regulatory approval in the United States and Europe.

Traditionally, one impediment to the use of columns for cell separation has been the variable yields of target and nontarget cells. This is partly due to nonspecific trapping of cells within the matrix bed. Although this may be improved by extensive washing of the column, this may also displace the captured target cells if it is too extensive or vigorous. Improved collection capacity is achieved by increasing the volume of the matrix offered to the cells, however, this can also exacerbate nonspecific cell trapping. An alternative approach is to react the cells with MAb-coated particles in the fluid phase, and then collect the particles and attached target cells from the mixture. This is the principle behind immunomagnetic separation.

Immunomagnetic Separation

The use of MAb-coated superparamagnetic particles (i.e., particles that can be collected within a magnetic field but do not become magnetized by the field) has found widespread application in HLA typing, fractionation of subcellular components and organelles, isolation of bacteria, and for the enrichment of various cell populations. Various types of particles can be used; however, these differ widely in their properties and the method in which they are used.

At one end of the spectrum there are the large (>10 m in diameter) nickel particles under development by Coulter Cellular Therapies. [137] [138] These are coated with an antimouse immunoglobulin antibody and are being clinically evaluated for the depletion of CD8+ cells from donor leukocyte preparation used in post-transplant immunotherapy. The particles sediment rapidly under gravity, which can be used to further enhance their separability from the cell mixture. The washed, MAb-treated cell suspension is slowly rotated with washed nickel particles to which the target cells attached. The beads are allowed to sediment, which occurs rapidly, and the target cell-depleted supernatant can be aspirated, either for a second incubation with particles or to be exposed to permanent magnets to remove any residual particles. The particles show extremely low levels of nonspecific cell binding, which allows them to be used in several cycles to deplete other cell types within the mixture successively, without significant loss of nontarget populations. The reaction kinetics are also rapid, which allows even large numbers of cells to be processed in a matter of a few hours.

Investigators at the Dana Farber Cancer Center and at M.D. Anderson Cancer Institute have achieved excellent levels of depletion of the target population using several rounds of treatment with the particles on a clinically relevant scale. [138] The recovery of nontarget cells has been uniformly good, and processing has been completed within 23 hours. The utility of this method must, however, wait the formal results from the clinical trial.

The most widely used paramagnetic particles have been the 4.5-m microspheres developed originally in Norway by Ugelstad and now commercially available from Dynal. These polystyrene beads are extremely uniform in size and properties and contain magnetite dispersed throughout their volume. They can be coated with antibody, either using covalent linkage or by passive adsorption. In most clinical applications an anti-immunoglobulin antibody-coated bead is used to capture a MAb-sensitized target cell (indirect separation). This tends to optimize capture efficiency because it allows fluid-phase interaction of the MAb with the target cells at saturating concentrations. In contrast, if the MAb is directly attached to the microspheres (direct separation), there is no guarantee of an excess of MAb within the system (unless extremely large numbers of beads are added), and interaction with the target antigen occurs between two solid phases. This tends to slow the reaction kinetics and generally results in the preferential separation of cells expressing large amounts of the target antigen. In our hands, however, if the target cell is a mature T lymphocyte, antigen expression is generally uniformly high. Acceptable levels of depletion can, therefore, be achieved using either the direct technique or a semidirect technique, in which anti-Ig-coated beads are first incubated with MAb, washed, and then reacted with the cells. [139] The microspheres sediment relatively

slowly under gravity, so this contributes relatively little to the separation. For most separations, the cell suspension is incubated with an excess amount of the MAb, or MAb mixture, for 3060 minutes on ice, and then washed extensively to remove any residual fluid-phase antibody, which can block anti-immunoglobulin attached to the beads. The washed cells are then incubated under slow rotation, usually at 4°C (to reduce phagocytosis of the microspheres) for 30120 minutes to allow the target cells to attach to the beads. Effective rosetting is usually achieved by offering 550 beads per target cell, depending on the system. Rosetted cells and free beads are separated from the mixture by exposure to high-strength permanent magnets, such as neodymium-iron-boron or samarium-cobalt magnets.[140]

For clinical procedures, the separations are usually achieved first using a static collection to debulk the beads and then re-exposing the residual cells to a second magnetic field to remove any remaining beads and rosettes. A number of separation devices have been described,[141] [142] although only a positive selection device, the Isolex,[143] is commercially available. Developed by Baxter Healthcare, this system has received regulatory approval in Europe and is completing the process in the United States. The company has also produced a large-scale depletion device, the MaxSep;[144] however, this is only available for nonclinical applications or for use in company-sponsored trials.

Microsphere technology has become somewhat of a gold standard for cell separation. It is capable, with careful up-front optimization, of achieving highly efficient and reproducible selective depletions or enrichments of many cell types,[140] including normal and malignant T cells and T-cell subsets.[145] [146] [147] As with almost any biologic system, effective performance depends on both understanding the characteristics and limitations of each component and systematically evaluating and optimizing each stage of the process.[140] Recovery of the target cells (be they CD34+, HPC, or T cells) requires some care. Efficient detachment by physical means is not usually possible, although rosetted cells may proliferate if placed in culture.[140] In the case of CD34+ cells, the enzyme chymopapain has been used to clip off the region of the antigen to which the MAb is bound.[143] [148] More recently, Baxter has synthesized a peptide that competes with the MAb for the binding site on the antigen and can, therefore, be used to detach the MAb/bead complex from the HPC.[149]

Although this type of immunomagnetic separation is capable of capturing target cells that are resistant to IgG/complement-mediated cytolysis by virtue of their low target antigen density,[119] it will still tend to preferentially remove cells expressing higher densities. Perhaps surprisingly, we have also shown

that certain target cells that possess extremely high levels of a target antigen can also escape capture.[150] This is presumably due to steric hindrance effects because it can be reduced, or eliminated, if the amount of MAb in the system is reduced by coupling it to the bead surface. Another restriction on the use of the Dynal microspheres has been their relatively high cost. This can become a limiting factor for large-scale separations or where multiple rounds of treatments are required. Nevertheless, the efficiency of this approach and its long history in clinical applications still make it the leading method for immunomagnetic separation.

Nanoparticles

As the size of the matrix particle is reduced further, it is possible to increase the amount of antibody used for separation, without changing the reaction volume. This can result in faster reaction kinetics because the particles now assume some of the characteristics of a fluid, rather than a solid phase, and a reduction in steric inhibition of binding to the target cells that can occur with larger particles. This is all achieved at a cost, however, because as the size of the particles is reduced it becomes increasingly difficult to collect them efficiently using simple permanent magnets. Instead, high-gradient magnetic fields are used.[151] These are generated by placing an array of metallic wires or pins in a field generated by externally placed permanent magnets. When the nanoparticle-treated cells are passed over this array, they move away from the magnets and collect on the magnetized metal. They can then be recovered by demagnetizing the array by removing it from the magnetic field. The term nanoparticles can include matrices that behave as true colloids through to discrete particles. The majority of experience has come from the use of the dextran-based nanoparticles developed by Miltenyi,[152] although other dextran-based magnetic nanoparticles have been used; for example, biotin-coated dextran-ferritin particles were linked via fluoresceinated streptavidin to antibody-coated T cells that had incubated with biotinylated $F(ab)_2$ fragments of goat antimouse IgG.[153]

Although now available for research applications in a variety of configurations, clinical applications have focused on enrichment of CD34+ cells (see later). When using most nanoparticles for positive selection of HPC, it is not necessary to remove the particles from the surface of the enriched cells. Their presence does not appear to affect either their analysis or functional activity in vitro, and coated HPC engrafted without problems in the clinical study. This suggests that if the procedure is used for T-cell depletion, the T cells may be recovered and used for immunotherapy post-transplant. The company has developed a method to remove particles from cells;[154] however, they are extremely hard to identify, even when using electron microscopy, and so validation of removal can be very difficult.

In our hands, the nanoparticles are capable of giving uniformly efficient separations at high purities and yields. Their smaller size and faster reaction kinetics have made them suitable for use under circumstances where microspheres may give problems. For example, microbead depletion of T cells using anti-CD3 MAb can give extremely variable results, due presumably to activation of the antigen by interaction with the MAb and the beads. When nanoparticles were tested they gave very reproducible and efficient depletions, perhaps due to the faster reaction times, lack of activation of the cell, and even possible internalization of the magnetic matrix by the T cell.[155] This further illustrates the importance of dissecting and analyzing each component within a separation technology.

In an initial collaboration with Amgen, Miltenyi has produced a clinical scale magnetic separator for the enrichment of CD34+ cells.[152] The system is similar in configuration to the CellPro device. It is a computer-controlled closed system, in which the antibody and particle-treated cells are passed sequentially through two columns, the first of which is designed to remove nonspecifically adherent cells from the mixture; before selective enrichment on the second column. This second column is magnetized during the collection phase by positioning it within an externally generated magnetic field. For recovery of the selected cells, the magnets are withdrawn, the metallic matrix becomes demagnetized, and the nanoparticle-coated cells are released from the column. The device has received regulatory approval in Europe and approval in the United States is pending.

Both the Amgen/Miltenyi and the CellPro devices have been predominantly used for the enrichment of CD34+ populations as a means of depleting tumor cells from autologous grafts. More recently, investigations have begun to look at positive selection as a means for depleting T cells from allogeneic grafts.[135] [143] [156] [157] [158] Depending on the ultimate clinical application, for example, use in HLA-matched or HLA-mismatched transplantation, the 23 log depletions of T cells achievable by CD34 positive selection may not be adequate when used alone to engineer an allograft. Under these circumstances, the procedure can be supplemented or preceded by a negative selection of T cells.[135] [136] In the case of the CellPro system, the company is testing a CD2 depletion column. If, however, a similar strategy is to be used for the Miltenyi device, it will be necessary to ensure that all of the nanoparticles are removed from the CD34+ cells collected during the positive selection, to avoid these cells co-collecting with the T cells during the negative selection procedure. Alternatively, the negative selection could be performed first.

Other Positive Selection Techniques

Positive selection of HPC as a means of T-cell depletion has focused on the use of CD34 antigen to target the HPC. It is clear that this antigen is present on cells other than true pluripotent hematopoietic stem cells (although recent data suggest that a population of pluripotent stem cells may even be CD34-negative).[159] [160] As the immunophenotype of these cells becomes more clearly understood, it is apparent that the pluripotent stem cell population represents probably <1% of the CD34+ population. Production of other MAb targeted more specifically at this subpopulation could, therefore, theoretically provide an additional 2 logs of T-cell depletion, if these reagents were used in place of or to supplement anti-CD34 for positive selection. Although new antibodies have been described, they apparently do not yet identify true stem cells.[161] [162] Once these become available, the questions will be whether it will become technically too difficult to isolate and handle these very small numbers of cells efficiently and with high yields, and if they can provide stable long-term engraftment in the absence of a period of very prolonged aplasia.

Additional MAb can already be used to obtain subsets of the CD34+ population. Suitable target antigens include CD38, Thy-1 and HLA-DR.[163] Practically, as successive depletions are performed, the performance characteristics of the system become crucial. Nonspecific cell losses can become critical. Perhaps the most extensive subsetting has been performed using fluorescence-activated cell sorting (FACS) by flow cytometry. In this procedure, the target populations are identified by positive or negative binding of fluorescently tagged MAb and then identified by passage within a fluid stream through laser light beams of specific wavelengths. Cells with specific binding profiles and light scattering profiles can then be deflected from the path and collected as a highly purified population. This approach has, until recently, only become practical when used by companies with access to high-speed cell-sorting capabilities because most commercially available cytometers could not

process clinically applicable cell numbers within a reasonable time.

Much of this initial work was pioneered by SyStemix in California, [164] who used the technique to prepare highly purified HPC as a means to deplete up to 7 logs of tumor cells from autologous grafts. [165] This method could be readily adapted for processing allogeneic grafts. It remains to be determined whether it would result in the depletion of cells that may facilitate engraftment (particularly across histocompatibility barriers), or provide beneficial graft-versus-disease responses. Although turbo sorting options are becoming available for laboratory flow cytometers, the economics and time considerations associated with this technology still tend to place it in the research classification rather than the mainstream. In an effort to reduce cytometer time it is usual to do some debulking of the cells by other selection technologies before performing the sort. [164] These additional steps, in T-cell depletion applications, may be adequate alone to provide the appropriate graft composition required for transplant.

Repeated negative selection of various lineage-committed cells has also been used to achieve enrichment of progenitors. [166] In these procedures, multiple MAb recognize the committed cells and the progenitors remain by default. Although this method has been used clinically for T-cell depletion, [166] it is likely to remain a research tool because it is likely to prove too expensive to produce the multiple reagents required for clinical grade standards.

Comparative Studies

Few head-to-head studies compare methods for T-cell depletion (Table 94-1). Feugier and colleagues compared complement, CD2/CD7 immunomagnetic depletion, CD5/CD8 panning, CD34 positive selection, and centrifugal elutriation in 79 procedures. [167] Depletions of 1.33 logs were obtained with negative selection giving approximately 2 logs, whereas positive selection depleted about 3 logs. There was a consistent progenitor cell recovery of about 50% from all of the methods. Specific T-cell depletions resulted in <0.5 log loss of B cells, whereas positive selection depleted more than a log and eliminated virtually all NK cells. Frame and colleagues [168] compared the use of CAMPATH plus complement, a CD5-immunotoxin (Xomazyme-H65), immunomagnetic separation, and SBA plus and minus E rosetting. They found that, with the exception of SBA alone, all techniques gave depletions in excess of 99%. The results of any comparative study must, however, be interpreted with great caution because each method must be optimized independently and performed on a clinically appropriate scale.

CLINICAL RESULTS FROM PHENOTYPIC DEPLETIONS

The choice of antibody for specifically depleting T-cell mediators of GVHD remains unresolved, but it is clear that reagents with different specificities have produced promising clinical

TABLE 94-1 -- Characteristics of Widely Used Graft Engineering Techniques

Separation Method (Commercial Systems)[a]	Target Cells	Advantages	Disadvantages
Soybean lectin	Nonstem cells	Technically easy Clinical experience	Long procedure Yield of stem cells Regulatory issues
Cytotoxic drugs	Usually leukemic cells	Technically easy	Poor drug availability for allograft treatment Target cells not available for analysis/use
Panning (AIS/RPR Gencell)	Antibody-detemined Lectin-determined	Technically easy Target cells also recoverable Lectin/antibody combinations	Devices may be cumbersome or small and multiple Regulatory issues Device availability
Antibody + complement (C)	Antibody-determined	Technically easy Second step (C) is generic Can use antibody panels Clinical experience	Regulatory issues for C Nonspecific toxicity/lot variability Variable efficacy/antigen densitydependent Target cells not available for analysis/use
Immunotoxins	Antibody-determined	Exploits antibody specificity High potency possible Clinical experience	Immunotoxin preparation required for each antibody used Specific delivery to point of action in cells
Immunoaffinity columns (CeprateCellPro)	Antibody-determined	Exploits antibody specificity Technically easy/rapid Approved in USA and elsewhere Clinical experience	Limited application for T-cell depletion Trapping of cells in matrix May require ve/+ve sequence in some separations Most experience for +ve selection only
Immunomagnetic (IsolexBaxter/Nexell) (CliniMACSMiltenyi)	Antibody-determined	Exploits antibody specificity Positive and negative separation Low toxicity/generic 2nd step Capable of good yields and purity Clinical experience	Cost Removal of particles between +ve/ve separation Some trapping of nontarget cells Some antigen density issues, less than with C Regulaory issues/one approved device
Molecular techniques	Depends on system	May offer high specificity Many possible applications	Still in early stages of development Too sophisticated for simple manipulation

[a] Not all systems are currently available or approved by the U.S. Food and Drug Administration.

results. In some cases, the method of use will have a major impact on the efficacy, both quantitatively and qualitatively, on the outcome, as will the context in which the manipulated graft was used. The data must, therefore, be interpreted with these factors in mind.

CAMPATH Antibodies

The CAMPATH series of antibodies has been extensively used in Europe for in vitro and in vivo depletion. These reagents are predominantly IgG_{2c} or IgM monoclonals, which identify a 12-amino acid antigen with a single N-linked carbohydrate and a glycosylphosphatidyl anchor, designated CDw52. [169] [170] The antibodies bind to most B and T cells, macrophages, and monocytes.[118] The IgM reagent was used together with donor serum to treat 11 HLA-matched grafts with promising control of GVHD, but three graft failures were thought to be due to rejection. [171] Subsequently a graft failure rate of 20% was reported in studies of patients with malignant disease, who showed a 17% incidence of grade I GVHD and a 7% incidence of grade IIIIV disease. Rejection was reduced to 413% by administration of cyclosporin A (CsA), with and without total lymphoid irradiation respectively. [172] Pretransplant administration of IgG_{2b} CAMPATH combined with the use of a CAMPATH-depleted graft without additional GVHD prophylaxis was studied in HLA-matched patients. The graft failure rate was 9% with a 2% incidence of severe acute GVHD and no severe chronic disease.[118] The antibody has also been used in the absence of added complement to treat grafts ex vivo, with the aim of using in vivo activation of complement-mediated lysis. This was reported to result in a low incidence of graft failure and severe GVHD, with improvements in transplant-related deaths and leukemia-free survival.[173] [174] Use of the MAb in vivo and in vitro was reported in a study of 28 patients, where it resulted in no GVHD but a 25% incidence of graft failure, attributed partly to delayed engraftment and possible destruction of graft facilitation cells. [118]

In a review of the use of CAMPATH antibodies for in vitro and in vivo T-cell depletion in matched sibling transplants, with a 10-year follow-up, the risk of relapse in acute myelogenous leukemia (AML) and ALL plateaued at 30%.[118] In CML the risk of relapse continued for at least 5 years and plateaued at 60% at 7 years, re-emphasizing the important role of GVL in this disease. In a review of 180 non-HLA-identical transplants, from matched unrelated and related partially matched donors, the incidence of graft failure was 51% for those who did not receive the antibody in vivo and 24% for those who did. [118] LPD is always a concern in patients who receive T-depleted grafts and is reported as 10 cases in 1,529 CAMPATH-manipulated grafts; however, there was a high incidence of cytomegalovirus viremia and delayed T-cell reconstitution. [87] [175]

Anti-CD3

Anti-CD3 MAb have been used with varying success. They are particularly attractive for depletion applications due to the high expression of the antigen on T lymphocytes and the commercial availability of a clinical grade preparation of the antibody. [176] Binding of the antibody blocks the function of a 20,000-dalton

component of the antigen receptor on T cells and results in a mitogenic stimulus, which leads to release of cytokines and colony-stimulating factors. [177] [178] It has been used in vivo to treat steroid-resistant GVHD. [179] In vitro, marrows from 10 patients with hematologic malignancies were incubated with anti-CD3 prior to infusion, and all engrafted in a mean of 22 days; however, 50% developed acute GVHD. [180] A subsequent study in 17 patients who received antibody-treated HLA-matched or minor mismatched grafts as part of a protocol including methotrexate as prophylaxis produced fatal GVHD in 2 recipients (with accompanying cytomegalovirus infection) and severe GVHD in a third, to give an overall incidence of 18% (compared to an incidence of 79% in historical controls). [181] These studies anticipated that the antibody-treated cells would be lysed by the patients serum complement; however, in vitro studies suggest that this may not be the case. [182]

The antibody has also been used with xenogeneic complement to treat grafts that are to be transplanted across major histocompatibility barriers. In a study of six children with juvenile CML, all engrafted granulocytes and platelets within 21 days and developed grade II or lower GVHD. [183] Three remained alive and well at 1802,400 days post-transplant, two died from infections, and one had relapsed. A larger series of marrows has been treated by Henslee-Downey and colleagues using the OKT3 preparation MAb in combination with baby rabbit complement. [184] This resulted in a 2.49 log depletion of T cells, and an infused T-cell dose of 4.63×10^4 /kg (n = 110). The probability of engraftment was 98% with a median of 15 days to a neutrophil count of 1,000/l. The incidence of grade IIIV GVHD was 32% and of grades IIIIV disease, 16% (n = 109).

Anti-CD3 MAb has also been linked to ricin A chain to form an immunotoxin, which was used to treat HLA-identical grafts in a study of eight patients. In vitro incubation produced a 3 log depletion of T cells and no acute GVHD was seen in the recipients, although there was a 25% incidence of graft failure. [185] These antibodies have also been used with Dynal microspheres for immunomagnetic depletion and produced a 1.7 log removal of T cells, resulting in an infused cell dose of 0.49×10^6 CD3+ cells/kg. This produced engraftment in 96% of HLA-matched unrelated (n = 36) and single-antigen mismatched recipients (n = 10). There were three late graft failures, and a 37% incidence of grades IIIV GVHD, which correlated with the infused dose of CD3+ cells. [186]

We have reported that anti-CD3 MAb do not work reproducibly with Dynal microspheres and have attributed this, in part, to activation of the cells during separation, with an associated attempt to internalize the bead, resulting in breakage of the link between the T cell and the microsphere. [140] When nanoparticles are used, in contrast, very reproducible results could be obtained with anti-CD3 MAb, due probably to a combination of faster reaction kinetics, and the possibility of internalization of the particles, which would not interfere with the ability to collect the T cells in the magnetic field. [139]

Anti-CD3 has also been used in the form of a tetrameric anti-CD3/antidextran complex with immunomagnetic nanoparticles to deplete T cells from 47 grafts for patients with leukemia or myelodysplastic syndromes. [153] There was a high incidence of graft failure and disease relapse after the first 27 transplants, which was attributed to overdepletion of the T cells in the graft. The protocol was amended to add back T cells to produce an infused dose of 2×10^5 /kg and CsA prophylaxis was administered. The mean level of depletion achieved was 3.64 ± 0.67 logs. Add-back of T cells reduced the incidence of graft failure from 22% to 8%; however, the incidence of grade II and grade III GVHD increased from 43% to 85% and from 18% to 54%, respectively, resulting overall in an increase in GVHD-related deaths from 15% to 46%. There was a 41% incidence of relapse in the recipients of heavily T-depleted grafts and a 0% incidence in those on the T-cell add-back protocol. Many of these problems were attributed to the activation of clonal T cells by the anti-CD3 antibody and the possible involvement of NK cells as effectors of GVHD.

Anti-CD3 reagents have also been combined with anti-CD2 to treat matched sibling grafts in 10 patients with hematologic diseases with poor prognosis in a regimen containing post-transplant methotrexate prophylaxis. [187] The levels of T-cell depletion were relatively low (88%), but all patients engrafted and no GVHD was seen. Seven (70%) were still alive, although the follow-up time was extremely short (210 months). Vartdal and colleagues with Dynal microspheres to deplete T cells in

laboratory studies used the same antibody combination. [145] This resulted in a 23 log removal, which was confirmed and reported by Gee [146] and by Knobloch et al., [188] who described 2.76 log depletion, in contrast to 3.39 logs obtained using an SBA plus rosetting technique.

Anti-CD2

Anti-CD2 MAb have also been used alone to reduce GVHD. The CT-2 MAb was used to treat grafts for 40 leukemia patients in a randomized double-blind trial. [189] This reduced the incidence of acute GVHD from 65% to 15%; however, graft failures increased from 0% to 12.5%. The incidence of disease relapse and survival was not significantly different in the two groups. When used to treat histoincompatible (n = 15) versus histocompatible (n = 8) grafts, without post-transplant GVHD prophylaxis, engraftment was seen in 7 of the 8 histocompatible recipients, but only in 4 of the 15 incompatible recipients, 2 of whom went on to develop grades IIIV GVHD. [190]

Anti-CD5

Anti-CD5 has been widely used for depletion both alone and in combination with other MAb. The T101 antibody has been used in its intact form and as an $F(ab)_2$ fragment to prepare immunotoxins with ricin A chain. [191] The fragment was particularly effective, depleting 2 log more cells than the immunotoxin prepared with intact anti-CD5. In clinical studies it achieved 99.5% depletion of T cells and the incidence of acute GVHD was 9.1% with an 18% incidence of graft failure. Anti-CD5 immunotoxins have also been used by Antin et al. to deplete 38 HLA-matched and 17 HLA-mismatched grafts for patients with acute or chronic leukemia. [126] This resulted in >95% removal of T cells, with an infused dose of $6.2 \pm 4.9 \times 10^5$ /kg. There was engraftment of all recipients within 1233 days (median 18 days) to 500 granulocytes/l, and platelet engraftment to 50,000 at a median 31 days for the matched recipients and 29 days for the mismatches. There were late graft failures in two of the matches and one of the mismatches, and the incidence of grades IIIV GVHD was 26% and 41%, respectively. Chronic GVHD was seen in 13% and 0%, respectively, of evaluable cases. There were four cases of LPD in the matched recipients, three of whom died, and one of whom responded to donor leukocyte infusions (DLI), but died from chronic GVHD.

Filipovich and colleagues used an anti-CD5 immunotoxin to treat histocompatible grafts for 29 patients with advanced leukemia and achieved complete donor engraftment in 28. [192] An increase in the dose of the reagent from 300 to 1,000 ng reduced the incidence of acute GVHD in the recipients from 100% to 34%; however, there was no direct correlation between GVHD and the T-cell dose infused. A better correlation was, however, seen between the T cells detected in the graft following 16 days in culture with IL-2.

Anti-CD6

Anti-CD6 binds to an antigen that is not expressed on myeloid precursors, nor B and NK cells, but is found on mature T cells. The antibody was used with rabbit complement to purge 112 matched sibling grafts for transplant to patients with hematologic malignancies, who received no post-transplant GVHD prophylaxis. [193] Each graft received three rounds of ex vivo treatment. Three patients (2.7%) failed to engraft, 18% developed grades IIIV GVHD, and 7.1% developed chronic GVHD. In 50 good-risk patients, the estimated probability of 3-year disease-free survival was put at 50% with a median follow-up of 44 months. A 10-year review of the use of anti-CD6 to deplete HLA-matched grafts revealed an initial engraftment rate of 98%, a 15% incidence of acute GVHD, and a 5% incidence of chronic GVHD, with 17% transplant-related mortality. [194]

Anti-CD6 has been used in combination with Dynal magnetic beads by Egeland et al. [195] in laboratory studies, where it resulted in 23 log depletions; however, residual CD2+ and CD3+ cells were detected, as was functional response to both mitogens and IL-2. This was attributed to failure of the beads to capture cells with low surface expression of CD6.

The MAb has also been combined with anti-CD8 and complement to treat HLA-matched grafts for 31 patients. [196] There was one graft failure (3.3%) and a 24% incidence of acute GVHD, of which 87.5% was grade I disease (six patients) and the remainder (one patient) grade III disease. A later review of 37 patients revealed low-level reconstitution of CD8+ cells at 60 days post-transplant, but later recovery of the CD4+ population. [197] Results using this MAb combination were compared to those obtained using T-replete matched sibling grafts and GVHD prophylaxis with methotrexate and CsA in 46 leukemia patients. [198] Forty-five patients engrafted, with slightly faster reconstitution times in recipients of T-depleted grafts. There was a 23% incidence of grades IIIII GVHD in the recipients of depleted grafts, versus 12% in those receiving T-replete transplants, and the incidence of chronic GVHD was substantially higher (51% vs. 23%, respectively). The cumulative incidence of relapse was 39% in the T-depleted group and 54% in the remainder.

Multiple Pan T Antibodies

Various antibody panels have been formulated in an attempt to increase the efficiency of T-cell depletion. Fifty-one HLA-identical grafts were treated with combinations of antibodies to CD2, CD5, and CD7 together with complement for transplant to patients with leukemia, most of whom received conditioning with TBI and cyclophosphamide, but no post-transplant prophylaxis.[199] Graft failure was seen in 19.1% and 23% died from graft-related complications. A similar antibody panel was used in HLA-identical grafts for 62 patients with poor-prognosis hematologic malignancies, 26 of whom received post-transplant GVHD prophylaxis with either CsA or methotrexate.[200] There was a 19% incidence of graft failure, but only one case of severe acute GVHD. Unlike the previous study, this one did not find any significant correlation between the infused T-cell dose and the incidence of graft failure. Wang et al. achieved a 23 log depletion of T cells using antibodies to CD2, CD5, and CD7 independently.[147] This increased to 4 logs when the three were combined. When this panel was used to purge two autologous grafts, there was complete removal of T cells, as determined by immunocytochemistry, and there was multilineage stable engraftment in the recipients.

T-Cell Subsets

An improved understanding of the T-cell subsets involved in mediating responses to histocompatibility differences (see earlier) has prompted the use of antibodies to CD4 and CD8 for allogeneic graft manipulation. Anti-CD8 has been used with both complement and immunomagnetic beads with the aim of depleting the cells responsible for mediating GVHD and retaining those with GVL efficacy. [201] In a randomized clinical trial, HLA-matched leukemia patients received grafts that were depleted of 1.52.0 logs of CD8+ cells and post-transplant CsA. This reduced the incidence of GVHD without any increase in the relapse rate. The antibody has been used by Jansen et al.[202] with immunomagnetic beads to deplete >95% of CD8+ cells from 20 matched sibling and 9 alternative donor grafts. The patients received 1.4 ± 2.7 × 10^5 CD8+ cells/kg and were given CsA prophylaxis starting the day before the infusion. There was one failure to engraft, and the median engraftment time to 500 granulocytes/l was 17 days. There was a 20% incidence of

grade II GVHD in the matched recipients, which increased to 63% in those receiving depleted grafts from alternative donors.

In an attempt to improve engraftment rates and also to decrease relapse, Gallardo and colleagues have used a combination of immunomagnetic depletion of CD4+ cells with add-back of a fixed dose of purified CD8+ cells (0.25 × 10^6 /kg).[203] Twelve patients with CML in first chronic phase were treated, and all were at a high risk of developing acute GVHD. All received CsA and methotrexate post-transplant. There were no graft failures and the incidence of grade IIIIV GVHD was 16.6% with an actuarial 3-year survival rate of 81.8%, with no observed relapses.

An antibody to the heterodimer of the T-cell receptor (T10B9.1A31) has been widely used in kidney allograft rejection [204] and also to T-cell deplete matched sibling grafts[205] and for transplant to recipients showing varying degrees of haplodisparity. [206] [207] [208] [209] In a study of 48 patients with CML who received both T10B9.1A31-treated grafts that were matched (n = 20) or mismatched at one or more major HLA loci (n = 28) and post-transplant CsA, 94% achieved engraftment. [207] The incidence of grade IIIV GVHD was 39.6%, with 8.3% developing chronic GVHD. There was no statistically significant difference in the incidence of grade IIIV GVHD in recipients of matched or mismatched grafts, and 2-year disease-free survival rates were 49% and 51%, respectively. In a subsequent analysis, the incidence of disease relapse was lower in patients who received a treated graft from an unrelated donor as opposed to an HLA-identical sibling. This was attributed to an increased opportunity for a GVL response due to a higher level of HLA disparity. [205]

Henslee-Downey et al. have used this antibody, in combination with complement, to T deplete 98 grafts from partially mismatched related donors, achieving a median 1.85 log depletion of T cells by limiting dilution analysis, and a median infused T-cell dose of 6.77 × 10^4 /kg. Engraftment was achieved in >90% of the patients in a median of 18 days to 1,000 granulocytes/l, with a 21% incidence of grade IIIV GVHD and an 11% incidence of grades IIIIV disease. [208] [209] Interestingly, Lamb et al.[210] have reported that statistically significant improved disease-free survival was seen in patients who received T10B9.1A31-treated grafts and went on to exhibit higher numbers of circulating T cells post-transplant. This could suggest a role for this subpopulation in mediating GVL activity. Kawanishi and colleagues, however, implicated this population in promoting engraftment of T10B9-treated grafts and suggested that the + population was associated with acute GVHD. [211]

Indirect Depletion by CD34-Based Positive Selection

The demonstration that long-term stable engraftment can be achieved by the transplantation of enriched CD34+ has stimulated interest in this approach as an alternative method for T-cell depletion. A number of methods have been developed, predominantly based on capturing the target cells onto a solid phase (see earlier), such as plastic plates (AISRPR Gencell), Sepharose columns (CellPro), paramagnetic microspheres (Baxter, Nexell, Dynal), or nanoparticles (Miltenyi). De Wynter and colleagues have compared techniques on a small scale. They reported that the most effective methods were fluorescence-activated cell sorting, which may be impractical for most routine clinical applications, and immunomagnetic nanoparticles. They found that the Sepharose column technology gave excellent purities but lower overall recoveries of colony-forming progenitor cells. [212]

Clarke and colleagues reported a comparison of the CellPro column and CAMPATH-1M for T-cell depletion. They achieved a 99.8% depletion of T cells with a 55 ± 12% recovery of the CD34+ cells using the CellPro laboratory scale separator and similar results with the clinical scale device. [213] This compared to a 98.4% depletion of T cells and a 50 ± 7% recovery of CD34+ cells using CAMPATH-1M. T cells, which could subsequently be used for immunotherapy, could be recovered in the unadsorbed fraction from the CEPRATE device.

Cottler-Fox and colleagues have described the use of the CellPro device to deplete T cells from marrow grafts (n = 10) and PBPC collections (n = 11). They achieved about a 3 log depletion of T cells and a CD34+ cell purity of 53.3 ± 27.9% for PBPC products and 65.4 ± 10.9% for marrow. [214] Based on these figures, an average patient would receive 3.4 × 10^4 T cells/kg in a CD34-enriched marrow graft and 1.2 × 10^5 T cells in a PBPC product. Urbano-Ispizua reported results from a study of 20 patients whose HLA-matched PBPC grafts were T-cell depleted using the CellPro system.[215] The median doses of CD34+ and CD3+ cells infused were 2.9 (range 1.58.6) × 10^6 /kg and 0.42 (range 0.12.0) × 10^6 /kg, respectively. All patients engrafted in a median of 14 days to 500 granulocytes/l and 10 days to 10,000 platelets/l. GVHD prophylaxis consisted of CsA and methylprednisolone. No patient developed grade IIIV acute or extensive chronic GVHD. Seventy-five percent of the patients were alive and in remission at a median follow-up of 7.5 months.

CellPro is currently evaluating a double-column procedure in which CD34-based positive selection is followed by negative depletion of CD2+ cells. In a case report, Martin-Hernandez and colleagues indicate that a 4.2 log depletion of a single PBPC apheresis collection could be achieved using this approach, giving a final CD3+ cell dose of 0.11 × 10^6 /kg, and a CD34+ cell dose of 5.05 × 10^6 /kg. [216]

The Isolex immunomagnetic separator (Baxter, Nexell) was used in a study of 14 patients who underwent HLA-identical sibling-matched transplants. [217] All received TBI, thiotepa, cyclophosphamide, antithymocyte globulin, and methylprednisolone before marrow infusion. No post-transplantation immunosuppressive therapy was given except for a 5-week course of steroids. The mean purity of CD34 product was 64.9 ± 6.0%, and the patients received a mean dose of 1.24 ± 0.21 × 10^6 CD34 cells/kg. A mean of 9.4 ± 1.7 × 10^4 CD3 T cells/kg was present in the CD34-enriched product, representing a 2.7 ± 0.1 log depletion. There were no graft rejections and patients achieved a sustained absolute granulocyte count of >500 in a median of 10.5 days and a sustained platelet engraftment of >20,000 in a median of 27 days. Patients were discharged a median of 21.5 days after marrow infusion. There were no instances of grade III or IV GVHD and no unexpected adverse events during the transplant hospitalization. With a median follow-up of 12 months, the estimated 100-day survival was 86 ± 9%.

There is likely to be increasing interest in this technology with the proposal from various investigators that crossing histocompatibility barriers is facilitated by the use of higher doses of stem cells. Sierra and colleagues reported that marrow-nucleated cell doses in excess of 3.65 × 10^8 /kg were associated with faster neutrophil and platelet engraftment and a decreased incidence of acute GVHD in matched or minor mismatched, unrelated marrow transplants. [218] The mechanism by which this occurs is not completely understood. It may be due to a decreased incidence of early post-transplant infections, which are thought to amplify GVHD. It may also involve veto activity by a population of cells present within the enriched stem cells, which serves to impede immunologically mediated rejection of the graft. [219] [220] [221] Doses of CD34+ cells in excess of 6 × 10^6 /kg have been recommended. [222] [223] These are difficult to achieve in routine marrow harvests. Apheresis collections from normal donors who have achieved a good mobilization may provide the requisite cell number; however, these collections contain large numbers of T cells. The use of a CD34-enrichment procedure provides the opportunity to deplete the T cells from the PBPC, and the enriched cells can be used to supplement the HPC obtained in

the T-depleted marrow harvest. This approach has been described

by Aversa and colleagues,[223] [224] who used a combination of marrow and PBPC that were T-cell depleted by a combination of E rosetting and lectin treatment. In a preliminary study of 17 leukemia patients, they were able to increase the number of colony-forming cells in the graft by 10-fold over that in the marrow alone. The patients were conditioned with TBI, cyclophosphamide, thiotepa, and antithymocyte globulin. All but one engrafted; however, 16 (56%) died of transplant-related toxicity within 18180 days of graft infusion, and a further patient died from grade IV GVHD. [223] In the subsequent 24 patients the protocol was modified to deplete the marrow using lectin and the PBPC by a combination of E rosetting and CD34 selection. [225] This resulted in a graft containing 13.7 × 10^6 CD34+ and 3 × 10^4 CD3+ cells/kg. The conditioning regimen was modified to substitute fludarabine for cyclophosphamide to reduce toxicity. Twenty-two patients (94%) engrafted and one achieved secondary engraftment.[224] At a median of 6 months (19 month range), <5% had developed acute GVHD and there was no chronic GVHD. Eight hematologic deaths (29%) and one disease relapse occurred. Interestingly, they described the appearance and persistence of a population of CD3+/8+ cells post-transplant. These cells also expressed NK receptors for HLA-C alleles and were able to lyse fresh leukemia cells.

Grafts containing the required 6 × 10^6 CD34+ cell dose and <3 × 10^4 CD3+ cells/kg can be obtained entirely from PBPC collections using a combination approach for T-cell depletion. This involves sequential use of centrifugation over light-density Percoll, E rosetting, and CD34 enrichment on a CEPRATE SC device. In a preliminary study of 29 depletion procedures, a mean 4.5 log depletion of CD3+ cells was achieved, with approximately equal contributions coming from the rosetting and CD34 enrichment steps.

Other Cell Populations in the Graft

It is likely that stem cells grafts, however they are manipulated, contain as yet poorly characterized populations of cells with potent immunologic functions. These would include a variety of veto[221] and facilitating cells.[226] One type of veto cell has been described by Thomas and colleagues in a rhesus monkey transplant model in which allogeneic $Dr^{/dim}$ marrow cells were transplanted and used to condition the animal to receive a kidney graft from the same donor. [227] The marrow cells were shown to possess veto properties, in that they could produce clonal inactivation of allospecific T-cell precursors, with minimal effects on third-party responsiveness. The veto cells were sensitive to ultraviolet-B irradiation and displayed a CD32+8+ phenotype. The authors felt that these properties may indicate that veto activity resided in a dendritic cell population. Veto activity toward allospecific T cells has also been described for lymphokine-activated killer cells when added to mixed lymphocyte cultures.[228] This was abrogated by depletion of CD56+ cells and increased by depletion of CD3+ cells. Recent results also suggest that CD34+ cells may possess veto activity in that they induce tolerance in the recipients (see later). [229] Facilitator cells, as described by Ildstad and colleagues, [226] represent about 0.4% of nucleated marrow cells, and have a characteristic CD8+/3/Thy1+/$DR^{dim/intermediat}$ / / phenotype. In a murine transplant model 3 × 10^4 of these cells could facilitate transplant of allogeneic stem cells in an MHC-specific manner. They are now being indirectly enriched by depletion of committed lymphoid and myeloid cells and the manipulated graft used for transplant across major and minor histocompatibility barriers. To date all patients are reported to have engrafted. This approach is being extended for use in solid organ transplant,[230] in which the recipient would be preconditioned using a nonmyeloablative regimen and would receive a donor-derived facilitator-enriched graft to develop a chimeric state before receipt of the allogeneic organ.

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FUNCTIONAL DEPLETIONS

The early identification of T cells as primary candidates as mediators of GVHD, combined with the empirical observations that removal of these cells from allografts did indeed result in clinical benefit, has resulted in an emphasis on the quantitative removal of these cells. To a certain extent, any antigen that was present on mature T cells was felt to be a suitable target to be used in their depletion, and, again within certain limits, this has proved to be the case. The problems of graft failure and disease relapse that have emerged as side effects of T cell depletion have, however, raised the question of whether it is more appropriate to target specific T-cell subsets. This could be achieved either by depleting T-cell subsets identified by restricted expression of an antigen(s) whose function may still not be fully understood or by targeting antigens that are more clearly identified with some T-cell function, such as the IL-2 receptor. [70]

Cavazzano-Calvo and colleagues have activated donor blood and marrow T cells by co-culture with the intended recipients cells for 2 days. [231] This results in expression of the IL-2 receptor on the alloreactive donor cells. These were then targeted using an anti-IL-2 receptor B chain MAb conjugated to ricin. This treatment abolished both primary responses in a mixed lymphocyte reaction (MLR), and also cytotoxicity toward recipient cells, but retained activity against third-party cells. Chaudhary and colleagues developed a recombinant single chain immunotoxin composed of variable regions of an anti-Tac MAb and truncated diphtheria toxin. [232] This killed cells that had been activated in an MLR with resulting expression of the p55 subunit of the IL-2 receptor. Mavroudis and colleagues used two *Pseudomonas* exotoxin-based immunotoxins with specificity toward the receptors for IL-2 and IL-4 to deplete alloreactive cells generated by co-cultivation of donor and recipient cell. This resulted in removal of >92% of reactivity against the recipients stimulator cells, with retention of >64% of reactivity toward third-party stimulators.[233] The immunotoxin had no effect on the growth of CFU-GM of normal marrow cells.

Rencher and colleagues have used fluorescence-activated cell sorting to remove donor cells that were activated by co-culture with host-derived lymphoblastoid cell lines and then stained using a panel of MAb directed against activation markers. [234] The sorted population did not contain cells responsive to HLA antigens, but retained helper and cytotoxic activity responses to foreign antigens. These techniques have mainly been used to process cells for administration as DLI post-transplant. The practicality of using this approach to process the primary graft remains to be established.

Another potential primary target is the leukocyte function antigen (LFA-1) that is expressed on cytotoxic T cells, monocytes, and NK cells. LFA-1+ cells are decreased in the peripheral circulation of animals with GVHD, suggesting that they have been recruited into the inflammatory sites. [235] When an immunotoxin was prepared using anti-LFA-1 MAb, it prevented GVHD in particular mouse donor recipient combinations. In vitro it reduced T-cell cytotoxicity by about 2 logs and also significantly impaired NK cell activity. There was, however, also a significant increase in failure to engraft, which was thought to be caused by depletion of either stem cells or graft facilitating populations. Many of these approaches are still in development or in early clinical trials; however, they provide an interesting indicator as to the future direction of the field.

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EVALUATION OF ALLOGRAFTS EX VIVO

Most allograft engineering has focused on T-cell depletion and has emphasized quantitative rather than qualitative removal. The majority of allografts are infused immediately on preparation rather than after cryopreservation and storage, and the implications of infusing large numbers of T cells can be severe or

lethal. It is, therefore, important to have available methods that can rapidly enumerate the numbers of T cells within the graft. Although early methods used detection of E rosette-forming cells (see earlier), or manual immunofluorescence following staining with pan T cell-directed MAb, most laboratories currently rely on flow cytometry. This technology is widely used in routine clinical laboratories; however, some precautions must be taken when it is used for T-depleted allografts.

Flow Cytometry

Accurate enumeration of very small numbers of target cells by cytometry requires rare event analysis. In this technique, large numbers of events must be accumulated and carefully analyzed if reliable data are to be obtained. This approach has been widely adopted for counting CD34+ cells, [236] but is still neglected often when enumerating T cells in depleted grafts. The choice of antibodies for detection of the T-cell population is also critical, when an MAb-mediated depletion technology is used. The same MAb should not be used for both depletion and analysis because cells that became coated with the antibody during the depletion phase, but which were not effectively removed, will be blocked from detection. They can, however, be detected by addition of an anti-immunoglobulin antibody conjugated to a fluorochrome different from the one conjugated to the T cell-directed MAb. The most sensitive detection is achieved by using panels of noncross-blocking antiT-cell antibodies directed against a variety of epitopes. [140]

In addition, it is important to include a viability stain in the analysis panel. Although this is less crucial when T-cell depletion is achieved by physical removal of the target cells, it is extremely important when in situ elimination methods are used. Under these circumstances, the depleted allograft may contain dead or dying T cells that will be detected by flow, but which may not contribute to postinfusion events. Suitable viability stains include propidium iodide and 7-aminoactinomycin. The analysis of cell viability after ex vivo depletion is not straightforward, however, because cell death may not be expressed immediately, but develop in the hours or days after processing and infusion. It may be possible to obtain a more accurate estimate by incubating the cells for a period before analysis. This has been particularly true in the case of depletion by immunotoxins that require the cells to divide to exert their toxic effects. [126] Stimulation of the cells with IL-2 and ex vivo culture has also been used to facilitate the detection of the residual T cells by flow cytometry. In some cases, this has provided a correlation between the numbers of T cells in the cultured sample and the development of clinical GVHD in the graft recipient. [192]

The discovery of new T-cell activation markers and the availability of MAb to these antigens may provide new tools to improve correlation between laboratory assays and the likelihood of developing clinical GVHD. The OX-40 molecule (CD134) is an early T-cell activation marker that appears on CD4+ T cells. [237] In a rat transplant model increased numbers of OX-40+ cells identified an alloreactive population of T cells that was involved in acute GVHD. In early clinical studies high numbers of positive cells appeared before clinical manifestations of the disease, and their numbers fell during effective steroid therapy. It remains to be determined whether the expression of activation markers, such as OX-40, by cells in the graft (either immediately after preparation or following ex vivo incubation) will provide a more accurate method for quality control of the T-depletion procedure. Another potentially useful assay may be levels of various cytokines, soluble cytokine receptors, and inhibitors in graft cultures.

Proliferative Assays

There is often, however, tremendous disparity between residual T-cell numbers as detected by flow cytometry, measurements of cell viability, and ultimate clinical outcome. This has prompted the development of other methods to detect residual T cells. These have focused on measuring cell function in proliferative assays.

Limiting Dilution Analysis

The most widely used has been limiting dilution analysis. In this assay, dilutions of the pre- and postdepleted graft are prepared and plated in multiple replicates in microtiter wells in medium containing growth factors (often in the presence of irradiated feeder cells). [237] [238] [239] [240] Within 23 weeks T cells in the cultures will proliferate and form small colonies, each of which is derived from a single T cell. These may be detected either by direct counting under a microscope or by uptake of a radiolabeled precursor molecule, such as tritiated thymidine. [239] For a series of replicate wells plated for a single dilution of the graft, only a certain proportion of the wells will grow out T-cell colonies. This proportion can be related to the original number of T cells present in the dilution using the Poisson distribution, and from this number the frequency of T cells in the graft may be determined. Although, at first sight, this would appear to be an ideal method for scoring T cells, the readout time is 23 weeks, meaning that, unless the graft is cryopreserved for future infusion, the results are not available at the time of the transplant. Scoring is also somewhat subjective and requires extensive intra- and interlaboratory standardization if reliable results are to be achieved. As is usual for most estimates of T-cell numbers or activity, the results obtained frequently still do not provide reliable prediction of clinical events.

T-Cell Precursor Assays

Attempts have been made to improve clinical correlations by adapting these assays to detect precursor cytotoxic and helper T cells. [240] [241] [242] In this procedure, donor cells are co-cultured with irradiated patient cells in a limiting dilution design (see earlier), and the proliferating cells are subsequently tested for cytotoxicity toward various targets, including the patients normal and leukemic cells and cells from a third-party donor.

In the helper cell assay, dilutions of donor cells are co-cultured with a constant number of irradiated patient cells at a range of ratios. The cultures medium initially contains IL-2, which is subsequently washed away. Irradiated lymphoblastoid cells produced from the recipient are then added, and 24 hours later the supernatant medium is assayed for IL-2 content. These assays are technically complex and require inclusion of appropriate control combinations of cells to be able to demonstrate the specificity of the responses. An assay that quantitates both cytotoxic and helper cell precursors has been described by van der Meer and colleagues. [242] This again uses a limiting dilution design. IL-2 production by helper cells is measured by co-culturing the proliferating cells, after irradiation, with an IL-2-dependent cell line. The response of this cell line is measured by uptake of tritiated thymidine. Cytotoxic proliferating cells are harvested and their activity can also be measured by proliferative response to phytohemagglutinin-stimulated recipient cells. Keever-Taylor and colleagues reported that the cytotoxic precursor frequency assay was most sensitive to disparities at the HLA-A and HLA-B loci that was detectable by serology or isoelectric focusing. [241] The helper cell precursor frequency assay predominantly detected class I disparity, but also responded strongly to differences at the HLA-DRB1 locus. Cytotoxic cell precursor frequency and dose was found to correlate significantly with grades III and IV GVHD and with the development of chronic GVHD. Neither assay correlated with relapse, whereas both were associated with survival. When adjustments were made for other significant covariables, however, the association between cytotoxic precursors and acute GVHD was lost,

although the association with survival was retained. Helper

precursor frequency was associated with chronic GVHD. In contrast, Lachance[243] and Weston[244] and their colleagues, found a significant association between helper precursor frequency and both acute GVHD and disease relapse in matched sibling transplants. This correlation was only weakly seen by Pei and colleagues in HLA-class II disparate transplants.[245] In the van der Meer study,[242] the assays were used to study the suitability of potential donors for two donors. For the first, three donors were equivalently matched by standard HLA typing techniques; however, there were major differences by the cytotoxic and helper cell precursor assays. For the second patient there was no HLA-matched donor by conventional typing; however, two of the donors showed a similar pattern of cytotoxic precursor responsiveness, which was different to that shown by the third donor. All of the potential donors showed different helper precursor responses. These various patterns were attributed both to differences in major HLA antigens that could be detected by conventional methods, and also to undetectable differences in some major and multiple minor antigens. Some of the differences found in the predictive value of these assays undoubtedly reflect technical variables. Methods to optimize the assay by depleting various subpopulations of the responder and stimulator preparations have been reported to improve its sensitivity, [246] accuracy, and reliability.[237] [246] [247] To a certain extent, this type of assay represents a more sophisticated reincarnation of the mixed lymphocyte reaction, in which combinations of patient and donor leukocytes were co-cultured in an attempt to detect alloreactivity by proliferative response. Although this also was thought to give promising early results, it has proved to be of very limited value in predicting clinical responses and has now been dropped by some donor registries as a required test.

As our understanding of the etiology and mediators of GVHD and GVL improves, it is possible that the existing assays can be modified by the discovery of antibodies to new antigen markers or marker combinations and by development of more sophisticated and specific culture assays. These may give results that are of true predictive value and that can be obtained within the required time frame.

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ENGINEERING-ENHANCED ENGRAFTMENT

Although GVHD occurs in 3050% of patients who receive HLA-matched grafts, the incidence and severity of the disease is higher in recipients of mismatched transplants.[5] [9] This has prompted the use of more radical T-cell depletion and investigation into what other manipulations could be used to promote the engraftment of extensively T-depleted grafts.

For many years it has been hypothesized that a single or very small number of pluripotent stem cells may be capable of eventually engrafting a recipient. Nonetheless, for practical purposes, there has also been considerable work to try to define the effective therapeutic dose of stem cells (usually defined in this context as CD34+ cells), that is required for rapid and stable hematopoietic engraftment. [248] This has been prompted largely by the switch to mobilized peripheral blood as the source of HPC because it is necessary to determine when an adequate graft has been collected and apheresis collections can be discontinued.

Despite numerous technical differences, it is now generally agreed that 15×10^6 blood CD34+ cells/kg provides an adequate dose, with faster engraftment occurring at the higher doses.[249] [250] Above a certain plateau, higher doses do not confer any particular advantage to the recipient, in terms of speed or stability of engraftment.[248] Recently, however, Aversa et al. have shown that higher doses of CD34+ cells (i.e., $>6 \times 10^6$) may be of value in promoting engraftment across histocompatibility barriers.[222] This is based on animal studies by Reisner et al., which suggested that at higher stem cell doses there may be some type of veto effect against graft rejection.[219] In laboratory experiments, Rachamim and colleagues have found that addition of purified CD34+ cells to primary mixed lymphocyte cultures leads to a marked reduction in both the frequency of alloreactive cytotoxic T-cell precursors and in their activity. [229] The tolerizing activity requires cell contact and is abrogated by irradiation. The CD34+ population producing these veto effects were MHC class I and II positive, but lacked costimulatory molecules of the B7 family. This suggests that the stem cell population, or more specifically, the CD34+ cells within an allograft may no longer simply be regarded as passengers. The total numbers, and perhaps their composition, may play a vital role in counteracting the side effects of radical T-cell depletion, to ensure the stable re-establishment of hematopoiesis by extensively T cell-depleted grafts.

Improved engraftment across histocompatibility barriers may also be achieved by administration of graft facilitator cells (described earlier). [226] [230] Infusion of marrow and PBPC preparations enriched for these cells by negative selection has resulted in 100% engraftment of one to three antigen-disparate grafts (Ildstad, personal communication). Kawanishi and colleagues have also implicated + T cells as promoters of engraftment in both matched related and unrelated transplants. [211] These cells were not found to be associated with any increase in the risk of acute GVHD, whereas + T cells produced the reverse finding. These approaches hold promise for the manipulation of cell subpopulations to both reduce the risk of nonengraftment in recipients of heavily T-depleted grafts and to permit the use of increasingly haplodisparate donors. In addition, stromal cells are believed to provide the necessary environment for the homing and proliferation of HPC. Infusion of purified and expanded stromal cells may, therefore, also provide a means to enhance engraftment. These ex vivo approaches can, of course, be supplemented by increasing immunosuppressive preconditioning of the intended recipient to reduce the numbers of functional recipient T lymphocytes.

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ENGINEERING ANTITUMOR RESPONSES

At present, most of the clinical efforts at engineering allograft have focused on pan T-cell depletion, with occasional attempts at removing T-cell subsets, with the aim of abrogating GVHD while retaining GVL activity. Subset depletions have primarily focused on the removal of CD8+ cells and have met with some success clinically.[201] [202] [203] Although depletion of donor cells that have been activated by co-culture with cells from the recipient has produced some promising results in preclinical experiments, some technical hurdles still must be overcome before it can be used routinely in the clinical setting. These include procuring the appropriate numbers of recipient and donor cells, optimizing co-culture conditions, and developing an effective separation technology that targets activation antigens, which tend to be more labile and motile.

If long-term survival is to be achieved, however, it will be necessary to maintain or boost antimalignancy responses. Many studies are, therefore, currently focused on the generation of tumor-directed immune responses.

Immunotherapy

Donor Leukocyte Infusions

The importance of the immune response in eradicating leukemic cells has, in addition, been re-emphasized recently by the success of donor lymphocyte infusions in controlling leukemia post-transplant.[64] [250] In this approach, allograft recipients who are at high risk of relapse of disease, or who have relapsed, were given small numbers of leukocytes collected from their original graft donor. These cells were infused in an unmodified form intravenously, and subsequently were shown to produce dramatic

clinical responses, particularly in patients with CML,[64] and subsequently in those with a variety of acute leukemias.[251]

The hypothesis is that the infused cells contain leukocytes that predominantly recognize antigens expressed on the recipients leukemic cells, and that after a period during which they proliferate, they will mount an immune response to these cells. Because the donor leukocytes are infused into a recipient who has already become engrafted with donor stem cells, the environment does not appear to favor the proliferation and generation of cells with GVHD activity. Such cells can, however, be produced if large numbers of donor leukocytes are infused post-transplant, or if the infusions are not properly timed. This can result in considerable severe or fatal toxicity from GVHD.[251] [252] Recent studies suggest that it is possible to use the original mobilized PBPC as the source of donor leukocytes for post-transplant immunotherapy. This would avoid the need to subject the donor to additional apheresis collections.[253] Alternatively the infusions can be manipulated to remove particular T-cell subsets. For example, CD8+ cells,[254] alloreactive cells, or clones can be selected for clinical use based on specificities measured in vitro.[255]

Based on the hypothesis that Th1 and Th2 T cells may play functionally different roles in mediating GVHD and GVL, Halverson and colleagues have generated these cells ex vivo by incubating CD8+ cells with allogeneic marrow-derived stimulator cells (predominantly CD3+ cells) in the presence of different panels of cytokines.[256] Conditions were found under which the CD8+ population developed into allospecific type 1 or type 2 cells with the typical patterns of cytokine secretion (see earlier). These cells may prove extremely useful, both to determine their roles in mediating post-transplant immune responses and for therapeutic applications.

Suicidal Lymphocytes

It is still not clear whether it will be possible to completely separate subpopulations of cells with GVL versus GVHD activity. The use of unfractionated populations carries the great risk that severe or fatal GVHD may be triggered in certain patients. In an attempt to prevent this, suicide genes can be inserted into the cells that are infused. The patients are then monitored for the earliest signs of GVHD, at which time the gene is activated to destroy the effector cells. The most common approach has been to transduce the cells with the herpes simplex thymidine kinase gene that confers sensitivity to the antiherpes drug ganciclovir.[257] Although this represents a new type of application for gene therapy, there are a number of technical hurdles. High transfection rates are required to reduce the likelihood of infusing nonsuicidal GVHD effector cells and to provide optimal numbers of suicidal cells for therapy. Secondly, it is not clear whether clinical GVHD can be detected at a time when it can still be stopped, or if irreversible proliferation of the effector cells has occurred prior to the first obvious clinical manifestation of disease.

Initial animal studies appear promising in that GVHD was prevented by administration of ganciclovir in the immediate post-transplant period; however, the animal still maintained a pool of donor T cells that responded normally to mitogens and third-party alloantigens, but were tolerant to recipient alloantigens.[257] Human clinical trials are currently in progress to evaluate this approach. Bonini and colleagues have shown that the transduced cells can persist in vivo for 12 months and were capable of mediating antitumor activity in patients with lymphoma.[258] [259] Development of GVHD was effectively controlled by the administration of ganciclovir. As further results from these studies are published they should provide useful information on the etiology, detection, and therapy of GVHD. These observations have restimulated work on immunotherapy of cancer because the clinical responses have been particularly impressive, probably because of the low initial tumor burden at the time of infusion of the leukocytes and the previously documented role of immune responses in the control of CML. They also suggest that conditions can be achieved that favor the development of GVL over GVHD, although these may still be relative rather than absolute.

Many of the questions that have remained unanswered in the field of graft engineering may be addressed more successfully in the context of DLI-based immunotherapy. Although, the strongest evidence of efficacy has come from patients with CML, there are reports of similar responses in patients with other leukemias.[251] The time course of the response may also shed light on the pathways of GVHD and GVL, by studying the sequence of events during the latency period, and even allowing us to isolate, characterize, identify, and expand potential effector populations.

Targeted Immunotherapy

These promising results from a more generic type of immunotherapy have stimulated the development of a number of specific immunotherapy protocols. This has been spurred by new work on the methods by which T cells become activated to generate cytotoxic responses toward cells bearing particular antigens. Jiang and colleagues were able to generate 122 recipient-reactive donor T-cell clones from CML recipient/donor pairs by incubating donor T cells with CML cells from the recipient.[260] They found that 32 of 78 clones recognized leukemia and normal lymphoblastoid cells, and 19 recognized only CML. The latter preferentially showed a V5, 6/7 pattern of T-cell receptor use, whereas those that recognized normal cells predominantly used V3 and 8. In the case of GVHD, very restricted and preferential expression of certain V gene segments has been described in patients with post-transfusion GVHD, although there were tremendous differences between individuals.[261] It may eventually prove possible to exploit these differences to identify and enrich subpopulations that mediate GVL for use in post-transplant

immunotherapy.

Dendritic Cell Immunotherapy

Central to the generation of specific T-cell responses is the antigen-presenting cell (APC) (described earlier). These cells process and present antigen on their surfaces in the context of MHC molecules to T lymphocytes. This triggers a series of reactions involving the proliferation of T-cell subsets, release of cytokines, and, ultimately, the generation of T cells with specific cytotoxicity toward cells expressing that antigen. Presentation of the antigen to the T cell within the appropriate context is a vital step in this process, and the ability to culture, expand, and prime APC with antigen is central to the goal of generating T cells with specific cytotoxic activity for immunotherapeutic applications. This has become more realistic with the demonstration that dendritic cells, some of the most potent APC, can be cultured and expanded from CD34+ precursors in the bone marrow, or from the peripheral blood, and that these cells can be primed ex vivo with antigen. Infusion of antigen-primed dendritic cells directly has been reported to produce antitumor responses, when the priming antigen was tumor associated. [262] Alternatively, the primed dendritic cells may be co-cultured with T cells, which can then be infused alone or together with the dendritic cells, with the aim of generating an antitumor response.

In the case of CML, the bcr-abl-positive dendritic cells can be generated from CD34+ precursors, thereby avoiding the necessity of priming the cells with a tumor-associated antigen.[263] [264] In contrast, Nieda and colleagues used a 16mer peptide that spans the b3a2 breakpoint of bcr-abl to pulse dendritic cells. [265] These were then used to generate CD8+ autologous bcr-abl-specific

cytotoxic T cells. The T cells lysed CML cells exhibiting the b3a2 breakpoint, but not those with the b2a2 breakpoint, or normal lymphoblastoid cells from the same individual. Cytotoxicity was found to be dependent on, but not restricted by, MHC class I molecules. Molldrem and colleagues reported that T cells that showed specificity toward a peptide derived from the primary granule enzyme proteinase 3 were able to inhibit CML CFU preferentially, suggesting that these cells could be used for immunotherapy.[266] These studies support the idea that it is possible to use antigen-pulsed APC to generate tumordirected effector cells ex vivo and that these cells can be specifically targeted without the risk of provoking GVHD.

Escape from Immunotherapy

Tumor cells may escape immune destruction by lacking the target antigens or certain co-stimulatory molecules required to trigger a T-cell response. Dermime and colleagues described decreased susceptibility to T- and NK-mediated cell killing and reductions in the expression of class I and II MHC in relapsed leukemias. [267] It is now possible to introduce genetic material coding for some of these missing factors into the tumor cells. This has been described by Boyer and colleagues, who have transfected murine leukemia cells with genes for the B7 co-stimulatory molecules needed to generate leukemia-reactive T cells. [73] They were able to produce cytotoxic T-cell lines that could then be used for specific immunotherapy. Cardoso and colleagues have found that optimal priming and expansion of autologous cytotoxic T cells directed toward pre-B leukemia cells is obtained when the B7 co-stimulatory molecule is expressed directly by the tumor cell. [268] They were able to transfect the B7 gene into leukemic cells to use as the source of antigen to stimulate dendritic cells and generate specific cytotoxic CD8+ effectors. These were found to kill the leukemic targets by induction of apoptosis via the perforin-granzyme pathway. Such an approach could ultimately be transferred into human trials. The complexity of these approaches is illustrated by the recent finding that T cells that lack the CD28 molecule, which binds to B7 on APC, can still mediate GVHD in a mouse model.[269]

Future Developments in Post-transplant Immunotherapy

Other potential important effector cells that could be used to mediate an antitumor effect include allogeneic T cell [74] and NK cell lines,[75] and T cells isolated from the patients tumor (tumor-infiltrating lymphocytes), and expanded ex vivo.[71] Ultimately, we may see these approaches develop into a new type of transplantation, in which high doses of T-depleted stem cells are administered with nonmyeloablative therapy to achieve chimerism. Targeted donor leukocytes, generated ex vivo, could then be used to provide a specific immune response to the patients disease, in the absence of severe or fatal GVHD. Work is already in progress to develop these types of protocols.[270] [271]

Studies such as those described above re-emphasize that a multifactorial approach may be used to address the linked issues of preventing GVHD, facilitating engraftment, and maintaining or enhancing a graft-versus-disease effect. A variety of graft and postengraftment immunoengineering techniques have been used with varying success. In addition, the regimens used to condition the patient and to provide post-transplant immunosuppression also have a major impact on the success of allograft transplantation. This is illustrated by the success of the regimens developed and applied by Henslee-Downey and colleagues for transplantation of partially HLA-matched grafts from related donors. These protocols have used graft modification by pan T-cell depletion. This by itself may be insufficient to achieve control of GVHD in the mismatched setting; however, when combined with carefully developed conditioning and post-transplant immunosuppression regimens, impressive rates of engraftment and control of GVHD have been achieved.[208] In these studies, relapse of disease has been a major complication; however, it is still difficult to determine to what extent this is the result of immune manipulation or to the very high-risk nature of the patients entered onto these protocols. The relative contributions of in vivo and ex vivo treatment can only ultimately be resolved in carefully structured and controlled clinical trials and may differ dramatically depending on factors such as the diagnosis, stage of disease, time of treatment, prior therapy, treatment regimen, and intercurrent clinical events.

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SUMMARY AND FUTURE DIRECTIONS

New biotechnologies have frequently found their first applications in the field of stem cell transplantation. The necessity of engineering both autologous and allogeneic grafts to improve outcome has provided the vehicle for some of the earliest clinical uses of MAb, immunomagnetic separation and antisense oligonucleotides.

In allogeneic transplantation it is clear that the problem of GVHD can be partially solved by ex vivo alteration of the cellular composition of the graft. This can be efficiently accomplished by a variety of different technologies; however, the identity of the target cell still remains to be unequivocally established. There have been major advances in our understanding of the etiology of GVHD, which suggest that the process is extremely complex, involving multiple cell types and humoral mediators. In parallel, however, relatively simple procedures, such as panT-cell depletion, have produced encouraging clinical results. Adding to the complexity is the close inter-relationship between GVHD, engraftment, and GVL. Achievement of the optimal balance between these has become the holy grail of allogeneic transplantation. It is still unclear whether the cellular mediators of these reactions are indeed functionally separable, and there is still conflicting experimental evidence on the identities of the effector populations. The discovery of the GVL effect has, however, reawakened the area of cancer immunotherapy, and this should, in turn, provide more information on the identity of the effector cell populations mediating graft/host interactions. The technology for separating, manipulating, activating, and expanding these various cell populations already exists and awaits an improved understanding of the appropriate cellular target populations.

A change in the nature of transplantation is already occurring. There is a gradual move away from myeloablative high-dose therapy with stem cell rescue toward using the transplanted cells to achieve other goals. Alterations of the dose of cells and enrichment of facilitating populations should improve engraftment rates of heavily T cell-depleted grafts and allow the use of donors of increasing haplodisparity, thereby extending transplantation to a larger patient population. Stem cells are now being used to induce tolerance for the transplantation of solid organs in the absence of myeloablative conditioning. They can also be used to establish a chimeric environment, into which donor lymphoid cells can be introduced to produce an antimalignancy response. Developments in gene therapy, cell culture, and molecular biology will have a tremendous impact on the direction in which we will move in the coming years. Whatever the direction, the journey promises to be an eventful and exciting one.

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Chapter 95 - Gene Therapy for Hematologic Disorders

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INTRODUCTION

Advances in recombinant DNA technology over the past several decades have offered the promise that inherited disorders may be treated by gene replacement. Acquired disorders such as AIDS and cancer may also become amenable to modulation via the introduction of selected genes that block viral replication or neoplasia. Although the concept of human gene therapy is simple, its application to clinical disorders has been slow because of the complexities inherent in achieving efficient gene transfer and appropriate gene expression. The most direct model for gene therapy is the introduction of a correctly functioning gene into the appropriate target cell of an affected individual. The newly inserted gene is transcribed and translated into a protein that alters the phenotype of the target cell. For hematologic disorders, the cells in which gene expression is required may be lymphocytes, monocytes, neutrophils, or other mature blood elements (Fig. 95-1). In the ideal case, gene transfer could be targeted to the pluripotent hematopoietic stem cell. Introduction of the genetic material into stem cells would ensure the continuous production of genetically modified blood elements over the lifetime of the patient. Theoretically, any genetic disease affecting one of the stem cell-derived lineages could be treated by gene insertion into a repopulating cell, provided that regulated, cell-specific gene expression could be maintained. A number of technical difficulties have hampered the application of gene transfer into stem cells. Other potential target cells include lymphocytes, tumor cells, muscle cells, hepatocytes, and endothelial cells. This chapter focuses on both the methods of gene delivery and target cells relevant to gene therapy of hematologic disorders, and discusses some of the unresolved issues in this rapidly changing scientific discipline.

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GENE DELIVERY

Retroviruses, parvoviruses, adenoviruses, herpes viruses, and vaccinia viruses are currently being developed for gene transfer into human cells. [1] Most methods of gene delivery use viral vectors to capitalize on the inherent efficiency of viruses to transfer and express genetic material in host cells. Typically, viruses bind specific cellular receptors and enter host cells by membrane fusion or endocytosis. Viral vectors can either lead to transient or stable transfer of the foreign gene. The process of stable transfer of foreign genetic material into the host chromosome is referred to as transduction. Alternatively, a latent viral infection may occur with the viral genome maintained in an extrachromosomal state. This may lead to high-level but transient gene expression.

Viral Methods

Retroviral Vectors

Among the retroviruses being developed for gene therapy are oncoretroviruses and lentiviruses. Vectors derived from components of the murine leukemia virus (MuLV) have been used in clinical trials to date. [2] In the future, vector systems based on the lentiviruses such as human immunodeficiency virus (HIV) and its relatives may offer advantages for many gene therapy applications.

Retroviruses are single-stranded RNA viruses that bind to a specific cell surface receptor for entry into the cells. [3] Once inside the cell, the viral RNA is converted into double-stranded DNA by the enzyme reverse transcriptase before integration into the host cell genome as provirus. The recombinant retroviral vectors in current clinical use (Fig. 95-2) contain the gene of interest flanked by the MuLV long terminal repeats (LTRs); the LTRs contain the promoter, polyadenylation signals and the sequences required for viral replication and integration. [4] The utility of retroviruses as vectors for gene therapy has been greatly enhanced by the ability to engineer packaging cell lines that secrete retroviral virion particles (Fig. 95-3). These cell lines are designed to express a helper genome encoding MuLV viral proteins. A separate vector plasmid that contains the gene to be transferred (along with *cis* elements necessary for RNA encapsidation, replication, and integration) is then stably introduced into these cell lines to generate a producer packaging line. Split function packaging cell lines separate the retroviral genes onto different transcriptional units, [5] significantly reducing the risk of generating replication-competent retroviruses by homologous recombination.

The main advantages of MuLV vectors are their ability to integrate into the host cell genome and confer long-term expression of the transferred gene. The primary concern regarding the use of retroviral vectors is the generation of replication-competent retroviruses and the possible induction of a neoplasm by insertional mutagenesis following integration. [6] The major limitation of MuLV vectors is that the target cells must be actively replicating for the vector to be able to integrate into a host chromosome. [7] [8]

Receptors for retroviral vectors have been identified recently [9] and appear to be broadly expressed in human tissues. [10] Low levels of receptors on target cells such as hematopoietic stem cells, however, may impede vector entry. [11] Pseudotyped retroviral vectors such as those containing the gibbon ape leukemia virus (GALV) envelope have been developed by using packaging cell lines that express alternative envelope proteins such as the GALV envelope. [12]

In recent years, researchers have been interested in the development of vectors based on lentiviruses such as HIV. [13] Lentiviruses are much less dependent on cell division to achieve transduction since reverse transcription results in a relatively stable intermediate that is transported by virtue of signals in associated proteins through the nuclear membrane. [13] Consequently, HIV-based vectors may be suitable for gene transfer to such targets as liver, muscle, and neurons, [13] and possibly quiescent hematopoietic stem cells.

Adenovirus Vectors

These vectors are important because of their potential for in vivo gene delivery. Human adenoviruses are non-enveloped DNA viruses. Out of nearly 50 known serotypes, only types 2

Figure 95-1 Cell targets for gene transfer and corresponding therapeutic gene products or disorders amenable to gene therapy.

Figure 95-2 Integrated proviral form of the Moloney murine leukemia virus depicting structural features. GAG, virion structural protein; POL, reverse transcriptase/integrase; ENV, envelope glycoprotein; LTR, viral RNA synthesis, integration; , encapsidation signal.

(Ad2) and 5 (Ad5) have been used for gene transduction strategies. [14] Adenoviruses exhibit two phases of gene expression during their lytic life cycle: early and late, separated by the onset of viral DNA replication. Their genome includes four distinct early regions (E1E4) and a major late region consisting of five components (L1L5). Recombinant replication-deficient adenoviral vectors have an extensive deletion of the E1 gene region that blocks transcriptional upregulation, viral replication, and cell death. In most recombinant adenoviral vectors, the gene to be transferred (referred to as the transgene) is located within the deleted E1 region.

The major advantages of adenoviral vectors include the ease with which they can be engineered by deletion of critical regulatory genes, their efficiency as in vivo gene transfer vehicles, their ability to transduce both dividing and nondividing cells, and the fact that they can be produced at high titers (10^{11} 10^{12} viral particles/ml). One limitation of adenoviral vectors is transient expression in the target cell resulting from the absence of chromosomal integration. In addition, inflammatory and immune reactions directed against viral proteins can limit their use. [15] Their most promising role in gene therapy of hematologic disorders appears to be in the transduction of cancer cells in vivo with tumor suppressor genes, cytokine genes, and drug sensitivity genes.

Adeno-associated Virus Vectors

Adeno-associated viruses (AAV) are single-stranded DNA parvoviruses. [2] The viral genome consists of *rep* genes encoding regulatory proteins and *cap* genes that encode the capsid proteins. The genome is flanked by inverted terminal repeats (ITR) that function as viral origins of replication and are necessary for encapsidation and integration of viral DNA into the cellular genome. In the absence of a helper virus such as adenovirus, AAV causes a latent infection characterized by the integration of viral DNA into the cellular genome. The wild-type virus has a property unique among viruses of integrating into a defined region of the human genome (chromosome 19q13).

Recombinant AAV vector production involves co-transfection of a permissive cell line (such as the 293 line) with a recombinant plasmid containing the gene to be transferred (with a promoter and polyadenylation signal) flanked by the AAV ITRs and a helper plasmid carrying the *rep* and *cap* genes but lacking the AAV ITRs (Fig. 95-4). The 293 cells are coinfected with adenovirus to generate AAV virion particles. These particles are released with cell lysis and are contaminated with helper adenovirus. Subsequent heating to destroy adenovirus is followed by purification and concentration of heat-stable recombinant AAV particles. The production of recombinant AAV vectors has

Figure 95-3 Generation of a producer cell line following transfection of a packaging cell line with a recombinant retroviral vector genome. The element refers to the encapsidation signal, which is provided by the transfected vector genome (see text for details).

Figure 95-4 Generation of rAAV by co-transfection of recombinant and helper genomes and co-infection with adenovirus.

improved with the development of packaging cell lines and the generation of virus stocks without an absolute requirement for helper adenovirus. [16]

The major advantages of AAV vectors include lack of pathogenicity in humans, a broad range of infectivity, and, as with lentiviral vectors, the capability of infecting nondividing cells such as muscle cells, liver cells, and neurons. [17] The packaging limitation of 5.2 kb precludes the use of AAV for transferring larger genes. In the instances in which it has been studied, recombinant AAV does not demonstrate the same site-specific integration as the wild-type virus. [17]

Nonviral Methods

These methods rely on receptor-mediated endocytosis or fusion of cell membranes. DNA delivered by nonviral methods is maintained in an extrachromosomal (episomal) state and not integrated into the cellular genome. [18]

Physical Methods, Cationic Liposomes, and Virosomes

Transfection (including calcium phosphate co-precipitation and electroporation), microinjection into the target cell nucleus, particle bombardment using a gene gun, [19] and direct intramuscular injection of purified DNA have all been used, but these methods are variably efficient and may result in transient expression of the transferred gene.[20]

Cationic liposomes are positively charged artificial lipid vesicles that incorporate negatively charged DNA. Under optimal conditions, it is often possible to transduce greater than 90% of cells in vitro with cationic liposomes. [21] Commercially available liposome preparations are routinely used for in vitro gene transfer. Cationic lipids have also been shown to be effective gene transfer agents in vivo. They have been used to successfully transfer 1-antitrypsin and cystic fibrosis transmembrane conductance regulator (CFTR) genes to the lungs. They have also been used to transfer the HLA B7 gene to tumor cells to boost antitumor immune responses. [22] The favorable features of this system include the absence of viral sequences, no cDNA size constraints in vector construction, and the ease of preparation.

Virosomes are liposomes that contain viral proteins, such as hemagglutinin, intercalated in the lipid bilayer that facilitate fusion of the liposomal bilayer with the membrane of the target cell.[23] The efficiency of gene transfer can be further enhanced by encapsulating DNA with nuclear proteins within liposomes. [24] These proteins can enhance DNA delivery to the nucleus as a result of nuclear-targeting signals.

Receptor-Mediated Gene Delivery

Receptor-mediated gene transfer involves creation of a protein ligand-polylysine-DNA complex that binds specifically to a ligand receptor. Studies with ligand-DNA complexes using transferrin and erythropoietin as ligands have demonstrated effective

in vitro gene delivery to various cell types, including hematopoietic cells. [25] Most methods of DNA delivery involve entry into the cells by endocytosis, and most of the DNA that enters the endosome is destroyed before it reaches the nucleus. In order to improve transduction efficiencies, it might be important to enhance the release of intact DNA from the endosome. In this modification, ligand-polylysine-DNA complex is coupled with inactivated adenovirus particles. The adenovirus particles disrupt lysosomes in the target cells, reducing DNA degradation and releasing DNA into the cytoplasm. In vitro studies using transferrin and folate as ligands have claimed increases in the efficiency of gene delivery by 100- to 1000-fold. [26] However, adenoviral complexes have not been shown to be effective in vivo.

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TARGET CELLS FOR GENE TRANSFER

Lymphocytes

Diseases in which genetically modified lymphocytes have been used include cancers (melanoma and Epstein-Barr virus-induced lymphoproliferative disease), inherited immunodeficiency diseases (adenosine deaminase deficiency), and infectious disorders (acquired immunodeficiency syndrome or AIDS). Lymphocytes may be good targets for gene transfer because they are easily isolated from peripheral blood and they can be induced to proliferate in tissue culture, thus facilitating transduction by retroviral vectors.[27] However, unlike pluripotent hematopoietic stem cells, lymphocytes are terminally differentiated and may persist for months but eventually will be cleared from the circulation. Some of the clinical uses of lymphocytes in gene therapy are summarized in the following sections.

Inherited Immunodeficiency Disorders

Adenosine deaminase deficiency (ADA), a rare inherited disorder caused by profound deficiency of the enzyme adenosine deaminase, was the first disease to be treated by gene replacement therapy. The first clinical protocol for ADA deficiency used peripheral blood T lymphocytes as the target cells. The cells were expanded ex vivo by exposure to interleukin-2 and anti-T-cell receptor antibodies, repeatedly infected with recombinant retroviral vector supernatant to achieve a transduction efficiency of 510%, and reinfused into the patients. T-cell number and function and serum ADA levels rose in two patients treated in this manner. [28] [29]

Neoplastic Disorders

Tumor infiltrating lymphocytes (TIL) are T lymphocytes that tend to localize in tumor masses. A gene marking trial using the neomycin resistance gene was designed to analyze trafficking, homing, and survival of TILs. [30] In this trial, TILs were obtained from biopsies of melanoma tissues, expanded in vitro in the presence of IL-2, and then returned to the patients by intravenous injection. Polymerase chain reaction (PCR) analysis demonstrated the presence of gene-marked TILs in the circulation for three months and in tumor biopsy specimens for two months. The study also demonstrated the safety of the retroviral gene marking procedure. TILs are also being used to deliver cytokine genes into the tumor bed. In a clinical protocol designed for advanced melanoma patients, TILs were transduced by a retroviral vector containing the gene for human tumor necrosis factor (TNF).[31] It is hoped that these cells will migrate to sites of tumor and achieve a high local concentration of TNF, inducing cytotoxicity without causing serious systemic toxicity.

Gene marking has been used to evaluate the efficacy of in vitro expanded, donor cytotoxic T-lymphocytes (CTLs) for the prophylaxis and treatment of Epstein-Barr virus (EBV) induced lymphoproliferative disease following allogeneic transplantation from a matched unrelated donor. Persistence of infused CTLs for several months, amplification of CTLs in the context of recrudescence of EBV infection, and infiltration of marked CTLs into EBV-induced tumors have been documented. [32] [33] Based on these important results, development of CTL infusion therapy for other forms of cancer has become an important goal.

In another application of gene transfer to lymphocytes, a herpes simplex virus thymidine kinase (HSV-TK) gene was introduced into donor lymphocytes collected for recipients of allogeneic stem cell transplantation (SCT).[34] In patients with recurrent chronic myelogenous leukemia, acute leukemia, lymphoma, and multiple myeloma following SCT, donor lymphocyte infusions have been shown to be effective in inducing remissions (as a result of graft-versus-leukemia or GVL) but can also induce severe and potentially lethal graft-versus-host disease (GVHD). Italian investigators showed that the HSV-TK-transduced lymphocytes had antitumor activity in five patients. In three patients who developed GVHD as a result of the donor lymphocyte infusions, administration of ganciclovir effectively controlled the GVHD by eliminating the transduced donor lymphocytes.[34]

AIDS

Current strategies of gene therapy against AIDS can be classified into: (1) elimination of human immunodeficiency virus (HIV)-infected cells; [35] (2) inhibition of HIV replication in infected cells;[36] [37] [38] and (3) protection of susceptible cells from HIV infection. [39] One ingenious approach involves the expression of an intracellular antibody to the HIV reverse transcriptase. Resulting inhibition of the enzyme made cells resistant to HIV infection, presumably by blocking this early stage of HIV viral replication.[40]

Hematopoietic Progenitor and Stem Cells

In principle, hematopoietic stem cells represent an ideal target for gene therapy because they can be harvested from bone marrow, peripheral blood, or umbilical cord blood and can be cultured and transduced ex vivo. Their properties of self-renewal and repopulation of progeny blood cells should result in the continuous maintenance of the transgene in blood cells of the patient. There are several biologic obstacles that limit gene transfer into hematopoietic stem cells. [41] First, no in vitro assay to identify and quantitate true stem cells exists. Second, the pluripotent cells appear to be predominantly in G_0 phase of the cell cycle, making these cells resistant to proviral integration and transduction with retroviral vectors in current clinical use. Third, the receptors for a number of vectors, including retroviruses and adeno-associated viruses, are expressed at low levels in primitive hematopoietic cells. Fourth, since chromosomal integration is necessary for delivery of the transgene to progeny cells, non-DNA integrating delivery systems will result in only transient expression.

The clinical trials of gene transfer to hematopoietic stem cells can be classified as indicated in the following sections.

Gene Marking Studies

Gene marking studies, appended to autologous stem cell transplantation protocols, have been conducted in order to determine, in cases of disease relapse, whether the source of relapse is residual endogenous disease or tumor contamination of the donor inoculum. In a study of patients with acute myeloid leukemia undergoing autologous transplantation, gene-marked tumor cells were found to contribute to relapse, thus arguing for

ex vivo purging in order to eliminate contaminating tumor cells in the autologous graft. [42]

Inherited Disorders

One of the major objectives of gene therapy is the replacement of defective or missing genes in congenital diseases affecting the hematopoietic system (Fig. 95-1). A

number of single gene disorders such as the hemoglobinopathies,[43] chronic granulomatous disease,[44] and Gaucher disease[45] are potential candidates for hematopoietic stem cell gene therapy. We will focus here on two congenital disorders involving defects in the lymphohematopoietic organ, ADA, and Fanconi anemia (FA). The hematologic deficiencies in both of these disorders can be successfully treated by stem cell transplantation, implying that normal stem cells can reconstitute hematopoiesis.

For ADA, a number of patients have been treated with autologous gene-modified progenitor cells of either bone marrow or cord blood origin. These cells were transduced ex vivo with retroviral vectors carrying the ADA cDNA. In an Italian trial, two patients were treated with repeated infusions of ADA-transduced bone marrow cells and peripheral blood lymphocytes (transduced by two different vectors).[46] There was no reported toxicity, and a small number of ADA-positive cells were detected in the peripheral lymphocyte and granulocyte population that expressed the marrow-derived ADA as opposed to the lymphocyte-derived ADA. Presumably, these results indicate that transduction of more primitive lymphohematopoietic stem cells can provide long-term reconstitution with ADA-corrected cells. In a second trial, umbilical cord blood hematopoietic progenitor and stem cells were transduced with an ADA retroviral vector and returned to three ADA-deficient infants.[47] In this study, ADA-positive T cells were found in the peripheral blood at least 2 years after gene transfer.

Another disorder that may be amenable to progenitor/stem cell transduction is FA. Fanconi anemia is a rare, genetically heterogeneous

HEMATOPOIETIC STEM CELL GENE TRANSFER: PROGRESS AND PROBLEMS

The first decade of serious effort to develop gene therapy applications in which hematopoietic stem cells were targeted focused on the use of vectors developed from murine leukemia virus (MuLV). These viruses were studied intensely because of their oncogenic potential in animal models. Knowledge of the MuLV genome permitted the development of packaging cell lines that express the viral genes. From these cells, producer clones could be developed for vectors carrying marker or therapeutic genes that remained free of potentially dangerous replication-competent retroviruses.[1] [2] In the mid-1980s, the discovery that retroviral vectors could integrate genes into murine repopulating stem cells triggered an intense effort to achieve human stem cell targeted gene transfer. Work in larger animals demonstrated a much lower efficiency of gene transfer into stem cells than can now be routinely achieved in the murine models and a similar low efficiency has been observed in early clinical trials.[45] Lymphocytes from peripheral blood emerged as an alternative target because these cells can be induced to proliferate in culture, rendering them susceptible to transduction by MuLV vectors.[29] Interesting clinical results have been obtained in patients with immunodeficiency but stem cells remain a preferred target because of the potential persistence of the therapeutic effect and the broad spectrum of diseases that could be effectively treated if efficient gene transfer into stem cells were achieved.

During the past 57 years, attention has turned from a predominant focus on MuLV vectors for stem cell targeted gene therapy to a much more diversified effort to understand the barriers to stem cell transduction and to develop strategies and alternative vectors to overcome these barriers. By purification and study of primitive hematopoietic cell populations that are more than 1,000-fold enriched in stem cells, we have come to know that the receptor for the amphotropic MuLV vectors used in human clinical trials is expressed at very low levels in such cells,[51] that their metabolically inactive cytoplasm does not support reverse transcription of the vector genome[52] and that the nuclear membrane of these quiescent cells prevents access of the viral genome to human DNA,[53] effectively preventing viral integration. These properties are more than sufficient to explain the low levels of gene transfer observed in human clinical trials to date.

Lentiviral viruses such as human immunodeficiency virus (HIV) have the capacity to transduce nondividing cells.[13] A stable reverse transcription intermediate is formed that, by virtue of signals in its protein components, can traverse the nuclear membrane without cell division. Adeno-associated viruses can also transduce nondividing cells but primary hematopoietic cells have proved refractory to this vector system. Lentiviral vectors encoding marker or therapeutic genes have been developed, and these can be packaged into pseudotyped particles having the internal proteins of HIV and the external co-protein of vesicular stomatitis virus (VSV-G).[13] The VSV-G protein facilitates cellular entry via a ubiquitously expressed membrane phospholipid. Cellular activation is still likely to be required for successful transduction of stem cells but recent data suggest that such activation may be achieved with complex cytokine mixtures that include high concentrations of stem cell factor (kit-ligand and Flk-3 ligand).[54] Harvest of stem cells after cytokine administration has also been shown to increase gene transfer efficiency even with MuLV vectors.[55] Unification of these promising leads into an integrated experimental protocol has yet to be accomplished but may well result in stem cell transduction efficiencies of up to 10%. Recent results in murine models have unequivocally established that inclusion of a gene for a drug resistance marker in the vector genome allows stable amplification of the genetically modified hematopoietic population from 10% to more than 60%.[56] All of these advances encourage optimism that therapeutically useful gene transfer into stem cells will be achieved in the next few years.

The next decade is likely to bring many applications of gene therapy. Immunodeficiency disorders in which there are powerful selective advantages for gene-corrected cells[57] are likely to be among the first diseases treated effectively by stem cell targeted gene transfer. Effective gene therapy for hemoglobin disorders is likely to require more time because high-level, regulated gene expression must be achieved in a significant proportion of bone marrow erythroblasts. Both lentiviral and AAV vectors have the capacity to transduce fully differentiated, quiescent cells in vivo including muscle and liver cells. Accordingly, the hemophilias have emerged as target diseases for gene therapy in the relatively near future.

disorder characterized by bone marrow failure, physical anomalies, and a predisposition to malignancy. At the cellular level, the phenotypic defect is marked by hypersensitivity to DNA-damaging agents. We demonstrated phenotypic correction of hematopoietic progenitor cells derived from FA patients using retroviral and adeno-associated virus vectors.[48] [49] A clinical trial using the retroviral vector is currently being conducted.[50] As in ADA, there may be a selective advantage for gene-corrected cells, since transduction of the mutant hematopoietic cells markedly improves viability in culture.

Induction of Chemoprotection

Bone marrow suppression is one of the most common toxicities of chemotherapy regimens. One approach to increasing the dose of chemotherapy administered is introduction of drug-resistance genes such as the multidrug resistance (MDR) gene into bone marrow stem and progenitor cells. The protein product of this gene, called P-glycoprotein, extrudes various chemotherapy drugs, rendering the progenitors chemoresistant. Mice with MDR-gene modified marrow cells have been shown to tolerate much higher doses of chemotherapy.[58] Clinical trials using this approach are currently in progress. Two other promising new strategies for chemoprotection involve using variants of the dihydrofolate reductase (DHFR) gene to confer resistance to antifolates[56] and using the methylguanine methyltransferase (MGMT) gene to confer protection against nitrosourea drugs such as BCNU and CCNU.[59]

Tumor Cells

In addition to the induction of chemoresistance in hematopoietic cells, gene therapy directed at cancer cells has been the focus of intensive efforts and can be divided into three major strategies: (1) immunomodulation using cytokine genes; (2) induction of chemosensitivity by suicide genes; (3) modification of oncogenes and tumor suppressor genes.

Immunomodulation

These trials are based on observations in animal models that have shown that local expression of cytokine genes in tumors leads to immune-mediated tumor regression. A number of clinical trials based on this strategy have been initiated.[60] In the first approach, tumors from patients are resected and transfected ex vivo with vectors containing different cytokine genes such as interleukin-2 or interleukin-12, TNF, or granulocyte-macrophage colony stimulating factor. Subsequently, the genetically modified cells are irradiated and returned to the patient as a form of autologous tumor vaccine. In the second approach, a single standardized transduced cell line or mixture of transduced cell lines is used as an allogeneic vaccine. In this circumstance, the vaccine cells must share antigens with the patients tumor (as has been shown for melanoma) and probably must also match at least one HLA class I allele with the patient.

Induction of Chemosensitivity

Induction of chemosensitivity involves introduction of suicide genes directly into tumors. These genes include the HSV-TK gene that confers sensitivity to

ganciclovir[61] and *E. coli* cytosine deaminase conferring sensitivity to 5-fluorocytosine. There are several ongoing clinical trials using this approach in brain tumors and intraperitoneal ovarian cancers. The brain tumor protocol involved stereotactic injection of retroviral vector producer cells containing murine HSV-TK gene into intracranial tumors. Only the transduced tumor cells should be able to convert ganciclovir to its toxic form, thereby causing cell death. Preliminary results from this trial indicated responses in only very small tumors in which a high density of vector-producing cells had been placed. [62]

Modification of Oncogenes and Tumor Suppressor Genes

In vitro interference with oncogenes in some tumor cell lines may reduce malignant potential. For example, in a lung cancer model with *k-ras* oncogene overexpression, antisense gene expression made cells less tumorigenic. [63] Conversely, in tumors with a mutated p53 gene, transfer and expression of wild-type p53 gene may result in the elimination of the malignant clone. [64] A novel variation on this idea was the development of adenovirus mutants that may replicate selectively in p53-deficient human tumor cells.[65] As alluded to earlier, adenoviruses can be genetically engineered lacking the *E1B* gene. This gene encodes for a 55-kD protein that inactivates p53. Initial studies have suggested that the *E1B*-deficient adenovirus mutants were able to selectively lyse p53-deficient human tumor cells but not cells with functional p53.

Vascular Endothelial Cells

There has been great interest in transducing vascular endothelial cells in vivo in order to develop therapeutic strategies for vascular disorders. [66] [67] [68] [69] [70] At this time, the best vector for vascular cells, based on in vitro and animal studies, may be recombinant adenovirus. However, the major limitation to adenoviral vector use remains transient expression. Several growth factor and cytokine genes including platelet-derived growth factor-, fibroblast growth factor-1, and transforming growth factor-1 have been expressed in porcine arteries in vivo. These studies help in defining the role of these growth factors in vascular injury, repair, and vasculitis.

Many proteins are associated with the cell membrane via a glycosyl phosphatidylinositol (GPI) linkage. Recently, GPI-linked proteins such as CD59 were shown to undergo transfer from erythrocytes to endothelial cells following transplantation of the erythroid cells. [71] These findings raise the possibility of specific protein delivery to the endothelium.

Solid Organs

This approach is aimed at delivering gene products to the circulation by transferring genes to keratinocytes, fibroblasts, myocytes, or hepatocytes. Muscle tissue has recently become an attractive target for constitutive expression of secretable proteins such as hematopoietic growth factors and clotting factors. While several non-viral (naked DNA) and viral (retrovirus and adenovirus) vectors have been tested using intramuscular injection, gene transfer is limited to a small percentage of cells near the injection site. Each of these gene transfer methods has some drawback: plasmid DNA remains episomal and is expressed transiently, [72] whereas MuLV-based retroviral vectors inefficiently transduce nondividing muscle cells. [73] Adenovirus that infects muscle is rapidly eliminated by the host immune response. [74] HIV-based vectors are capable of infecting nondividing tissue such as muscle, but safety issues concerning contaminating wild-type HIV have not yet been fully resolved.[75]

Stable transduction of muscle tissue with an AAV vector has been reported and carried out beyond one year. [76] A recombinant AAV vector carrying the *lacZ* marker gene and delivered by local injection transfected a greater number of muscle fibers when compared with an adenovirus vector. The number of positively staining cells did not change from 4 days to 19 months, suggesting that the majority of transduction events went on to form stable integrants or episomes. In contrast to adenovirus-mediated gene transfer, no cell-mediated immune reaction against either the virus or the gene marker was detected. Neutralizing

antibody against AAV capsid proteins did not prevent readministration of vector. [77]

Hormones, growth factors, and clotting factors are products that potentially could be delivered by muscle cells transduced with AAV vectors. Injection of an AAV vector carrying a human erythropoietin gene into murine muscle tissue increased mouse hematocrits in a dose-dependent fashion. [78] The absolute increase in red cell mass correlated with human erythropoietin levels in mouse sera. Similar experiments have explored the use of AAV for leptin treatment of obese mice [79] and proinsulin production in diabetic animals.

AAV-mediated gene transfer of the hemophilia clotting factor IX is highly promising. Portal vein infusion of an AAV vector carrying the human factor IX gene (rAAV/hF.IX) into mice resulted in transduction of hepatocytes and achieved plasma levels of human factor IX which were 20% of normal. [80] The levels of human factor IX were persistent, without any morphologic or biochemical signs of liver toxicity. Intramuscular injection of rAAV/hF.IX into mice produced a similar therapeutic effect, with plasma factor IX levels at 6% of normal.[81] Intramuscular injection of rAAV/hF.IX into hemophilia B dogs increased clotting activity in a dose-dependent manner prior to anti-human F.IX inhibitor antibody development. [82] Immunohistochemical analysis of injected muscle confirmed persistent human factor IX expression. Limited areas of focal lymphocytic infiltration and myofiber pathology correlated with staining for the helper adenovirus but not AAV itself. Based on these results, new methods for the removal of adenovirus, concentration, and scale-up of AAV vectors will be needed before clinical trials can begin.

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SUMMARY

In many respects, gene therapy has been a logical extension of the application of molecular biology to medicine. The first genetic disease elucidated at the molecular level was sickle cell anemia, and our understanding of inherited and acquired blood disorders continues to become more sophisticated. While gene therapy of the hemoglobinopathies has been more difficult to achieve than anticipated, it seems likely that at least certain genetic hematologic disorders, such as adenosine deaminase deficiency, will soon become amenable to effective gene replacement therapy. To some extent, scientific progress in this field will depend upon a better understanding of human hematopoiesis as well as the development of simple and reliable vectors for gene transfer. Whether or not complex disorders such as neoplasia can be effectively treated by gene transfer is unclear at the present time, but it seems reasonable to expect that novel and important research will continue to result from the interface between this applied science and hematology.

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APPENDIX: Glossary of Terms

Amphotropic: Term used to describe a retrovirus capable of infecting nonmurine (human) as well as murine cells.

Antisense: Nucleic acid (RNA or DNA) complementary in sequence to the mRNA transcript of a target gene. Antisense molecules are thought to inhibit translation of the target mRNA.

Ecotropic: Term used to describe a retrovirus capable of infecting murine cells.

Gene marking: The introduction of foreign viral sequences into the chromosomal DNA of a patients bone marrow to mark or track cells.

Insertional mutagenesis: Occurs when the retroviral genomes insert into the human genome at or near a gene and cause that nearby gene to behave in a deranged manner that disrupts normal control of cell growth or differentiation.

Integration: Process whereby exogenous genetic material becomes incorporated into the host cell genome. Usually refers to the latent or proviral state of certain viruses in infected cells.

Intracellular immunization: Genetic alteration of a cell to induce resistance to viral (e.g., HIV) replication or infection.

Locus control region: Control elements that flank the human -globin gene cluster and modulate globin gene expression.

Long terminal repeat: Terminal sequences of a retrovirus that usually contain the transcriptional control elements (promoters), polyadenylation signals, and sequences involved in replication and integration.

Promoter: Control element responsible for transcription of a gene.

Provirus: Integrated form of a transducing virus, such as a retrovirus.

Selectable marker: To optimize and enrich for transduced cells, genes encoding for bacterial antibiotic resistance proteins (such as neomycin or hygromycin) are incorporated into the vector. Following transduction, incubation with the antibiotic selects for transduced cells resistant to the antibiotic.

Transduction: The stable transfer of genetic material into a cell, usually implying integration of DNA into the nuclear genome.

Transfection: The uptake of foreign genetic material into a cell. Transfection is usually accomplished by physical means such as co-precipitation of the DNA with insoluble calcium phosphate. Transfected DNA can either exist in the cell as an episomal (extrachromosomal) element or be integrated within the nuclear genome. The efficiency of DNA transfer into cells depends on the particular method used.

Vector: Vehicle for gene transfer.

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Chapter 96 - Experimental Cell Therapy

Robert S. Negrin

INTRODUCTION

The use of cellular populations for therapeutic purposes is an attractive alternative to conventional treatment modalities. The exquisite functional specificity of certain cellular populations, as well as advances in our basic understanding of cell biology, hematology, and cellular immunology, has brought these concepts forward. Technical progress in cytokine biology, cellular manipulation, cell separation, and purification have made the idea of isolating a specific cell type and expanding relatively pure populations of cells that are activated and functional a reality. In addition, the prospects for inserting and controlling genetic programs, thereby using cellular populations for gene therapy delivery are improving. This chapter presents some of the basic principles, as well as potential clinical applications, of cellular therapy.

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HISTORICAL CONSIDERATIONS

The concept of using cells for therapeutic purposes in hematologic practice is not new. The best example of this is the field of stem cell transplantation (SCT). Early studies following the development of the atomic bomb led investigators toward understanding how radiation injures living organisms. In these experiments, it became clear that mice could withstand an otherwise lethal dose of radiation if their spleens were shielded before exposure. [1] Soon thereafter it was discovered that the infusion of marrow provided similar protection from lethal irradiation. [2] At first this was thought to result from a humoral factor and later it became clear that the transfer of cells was responsible for radioprotection.

Seminal studies in dogs firmly established the role of marrow cells in protecting animals from the lethal effects of radiation. [3] These studies also demonstrated the adverse effects of donor-derived cells, namely that of graft-versus-host disease (GVHD). Further investigation demonstrated that animals surviving long-term not only had a fully intact hematopoietic system but were also tolerant to skin grafts from the donor animals. [4] These studies, and many others, ushered in the modern era of stem cell transplantation. Initially, the beneficial effects of allogeneic SCT were thought to result from the high-dose chemotherapy and radiation therapy used before the transplant. Subsequent studies have demonstrated that SCT is far more complex, with much of the benefit being derived from the transfer of immunoreactive cells capable of exerting a graft-versus-tumor or graft-versus-leukemia (GVL) effect. The existence of a GVL phenomenon was initially based upon the observation that relapse rates were lower in those patients who developed some degree of GVHD, especially chronic GVHD. [5] [6] Further indications of a GVL effect were observed in clinical situations where the effect is absent, such as following an identical twin or syngeneic SCT, [7] [8] or following rigorous T-cell depletion. [9] [10] Direct demonstration of a GVL effect following the transfer of donor-derived lymphocytes has been an important advance in SCT as well as a further demonstration of the powerful therapeutic benefits of cellular therapy. [11]

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CLINICAL APPLICATIONS OF CELL THERAPY

Allogeneic and autologous SCTs are examples of cellular therapy routinely used in the clinical setting. This topic is discussed thoroughly in Chapters 85 , 86 , 87 , 88 . Other applications of cellular therapy include the use of donor lymphocyte infusions (DLI) for the treatment and prevention of relapse following an allogeneic SCT, the use of autologous cellular populations for the treatment of malignancies and to prevent relapse following SCT, the use of cytotoxic T lymphocytes for the treatment of viral infections and the use of cell therapy for a vehicle for gene delivery (Table 96-1).

The major cellular populations that have been proposed for cell therapy fall into several broad categories. For a successful SCT, the graft must contain adequate numbers of hematopoietic stem cells. Stem cells and hematopoietic progenitor cells have also been targeted for gene insertion and gene therapy protocols. This population of cells is discussed in Chapter 95 . The other cellular populations useful for cell therapy fall into the general category of immune reactive cells. These include natural

TABLE 96-1 -- Clinical Applications of Cell Therapy

Hematopoietic stem cell transplantation
Donor leukocyte infusions for treatment of relapse following allogeneic transplantation
Ex vivo activated cellular therapy for treatment of malignancies
Natural killer and lymphokine activated killer cells
Cytokine induced killer cells
Cytotoxic T lymphocytes
Dendritic cells
Cytotoxic cell lines
Cellular therapy of viral infections
Gene delivery

killer (NK) cells and cytotoxic T lymphocytes (CTLs). Some basic biologic features of these cellular populations will be reviewed before discussion of preclinical studies and clinical trials of cellular therapy.

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IMMUNOLOGIC EFFECTOR CELLS

The evaluation of immune reactive cells capable of recognizing and lysing tumor cell targets has been the subject of intense investigation. These cellular populations can be divided into two categories. The first category of cells, exemplified by NK cells, are capable of lysing a broad array of tumor cell targets without prior exposure. NK cells have been termed non-major histocompatibility complex (MHC)-restricted, although evidence suggests an important role for certain HLA alleles in NK cell function.[12] The second broad category, characterized by CTLs, are MHC-restricted and recognize cells based upon the appropriate presentation of peptide in the context of MHC proteins. Some overlap exists between NK cells and CTLs; this will be discussed in detail later in this chapter. Despite this overlap, the distinction between MHC- and non-MHC-restricted cytotoxicity continues to be useful.

NK cells have been extensively characterized in both rodent and human systems.[13] [14] [15] These cells generally lack expression of CD3 or other proteins of the T-cell receptor (TCR) complex on the cell surface.[15] [16] [17] [18] Most NK cells express CD56 (neural cell adhesion molecule) and CD16 (the Fc receptor). NK cells are capable of lysing a broad array of tumor cell lines, multidrug-resistant cell lines and autologous tumor cells. [16] NK cells have intracellular cytolytic granules that contain perforin and granzymes. Upon appropriate engagement, these cytolytic granules are secreted into the extracellular space between the NK cell and its target. [19] The perforin molecules, in the presence of Ca^{++} , induce pore formation of the target cell membrane resulting in osmotic lysis, as well as the introduction of granzymes that induce apoptosis.[20] NK cells are capable of cytolysis through a Ca^{++} -independent mechanism controlled by expression of fas-ligand (fasL) which in turn binds to fas on some target cells and induces apoptosis directly.[21] [22] NK cells also express CD16 and are therefore capable of antibody-dependent cellular cytotoxicity (ADCC) which may be important in some clinical settings. In addition to these mechanisms, which require cellular contact, NK cells are also capable of cytokine production such as interferon- (INF-), granulocyte macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor- (TNF-). [23] The production of cytokines may play a local role in cytolysis of virally infected and malignant cells, as well as in recruiting other immunoreactive cellular populations. Among these mechanisms, the granzyme/perforin mediated cytolysis is thought to be the most important as demonstrated by the marked reduction in NK cell function in knockout animals deficient in these proteins.[24] [25]

NK cells can in turn be activated by several interleukins, resulting in enhanced function. The most potent of these is interleukin-2 (IL-2), which results in modest proliferation and marked activation of NK cells. Activation of NK cells by IL-2 results in a population of cells that have been termed lymphokine activated killer (LAK) cells. LAK cells have been shown to lyse a broad array of tumor cell lines including autologous tumor cells and multidrug-resistant cell lines. Neither NK cells nor LAK cells recognize normal hematopoietic stem cells; however, NK cells do lyse endothelial cells. [26] [27] Several other cytokines have also been characterized that activate NK cells in addition to IL-2. The most potent of these cytokines was initially called natural killer cell stimulating factor and later renamed IL-12. [28] [29] IL-12 binds to a distinct heterodimer receptor on the surface of NK cells, resulting in enhanced activity. [30] [31] IL-15 is another cytokine that has been characterized and cloned and which also activates NK cells by binding to components of the IL-2 receptor. [32] [33] Both IL-2 and IL-12 have been used clinically with promising results in some clinical settings.

Recently, a number of investigators have identified specific receptors on the surface of NK cells that serve an inhibitory function. [34] These receptors, termed killer inhibitory receptors (KIRs), are present on the surface of some NK cells and upon engagement inhibit NK function. Genes for several of the KIRs are located on chromosome 19.[35] Interestingly, KIRs recognize certain HLA class I alleles that inactivate the NK cells. [36] KIRs recognizing HLA-A, -B, and -C alleles have been characterized and cloned.[37] [38] [39] [40] The role of peptide has been somewhat controversial because some KIRs do not appear to be peptide-specific; whereas others are peptide-specific. [41]

A functionally analogous but structurally dissimilar family of receptors has been identified on murine tissue. These receptors are clustered in a region designated the NK cell complex, which is found on murine chromosome 6. [42] Two families of proteins, termed NKR-P1 and Ly-49, have been identified. Like human KIRs, engagement of Ly-49 molecules inhibits NK cell function. [43]

NK cells are thought to form the first line of defense against viral infections and may also play a role in tumor surveillance. The finding of inhibitory receptors on NK cells suggests that viral infection or malignant transformation may either down-regulate certain HLA alleles or may alter peptide presentation so that these molecules no longer block NK function resulting in engagement and cytolysis. Alternatively, activatory receptors may be expressed on these abnormal cells, and these may be recognized by NK cells and overcome any inhibitory signal. [44]

In contrast to NK cells, the mechanistic aspects of CTLs are well described. CTLs recognize their targets through engagement of the TCR, which specifically binds to HLA class I and II molecules expressing an appropriate peptide. [45] In order for a productive interaction to occur, accessory and costimulatory molecules are also necessary.[46] In the absence of costimulatory molecules, anergy results. However, if the TCR is engaged along with costimulatory molecules, a productive interaction results in the synthesis of cytokines such as IL-2 and clonal expansion. [47] The best-characterized system of costimulatory molecules is the CD28-B7 system of which at least two members, B7.1 and B7.2, have been cloned and extensively studied. [46]

The expansion of CTLs occurs through the presentation of peptide antigens by accessory cells termed antigen presenting cells (APCs) These APCs not only express HLA molecules but are capable of processing antigens and presenting peptides. APCs also express costimulatory molecules and are capable of cytokine production. Several different APC populations have been described, the most potent of which are cells termed dendritic cells.

In addition to these protein molecules, adhesion molecules such as leukocyte function antigen-1 (LFA-1) and its counter-receptor, intracellular adhesion molecule-1 (ICAM-1), play important roles in both NK and CTL function.[48] Binding of the TCR

TABLE 96-2 -- Possible Mechanisms of Tumor Cell Evasion of the Immune System

Inhibition of NK cell function through the appropriate expression of HLA molecules
Lack of expression of tumor-specific antigen or peptide
Lack of costimulatory and/or adhesion molecule expression by the malignant cells
Resistance to apoptosis
Expression of fas-ligand on the tumor cell, thereby inactivating fas-expressing effector cells

results in an increased affinity of LFA-1 toward ICAM-1 for a finite period of time, which allows a stable interaction. [49] Cytotoxic granule contents are then recruited along the cytoskeleton and released into the space between the two cells, resulting in pore formation by perforin and induction of apoptosis by the granzymes. [50] [51] For perforin to function properly, Ca^{++} is required. In addition to granzyme/perforin-mediated lysis, a Ca^{++} independent pathway mediated through fasLfas interactions also plays an important role in CTL-mediated cytotoxicity. [21] [52]

These mechanistic studies suggest strategies for therapeutic intervention, many of which are discussed later in this chapter. They also demonstrate the complexity of the system and suggest mechanisms by which tumor cells may escape recognition by the immune system (Table 96-2). With respect to NK cell-mediated killing, tumor cells may merely inactivate the effector cells through the appropriate expression of MHC molecules to escape lysis. The fact that many investigators have demonstrated autologous cytolysis of tumor cell targets suggests that this inhibition can be overcome. In regard to CTL recognition of tumor cell targets, a number of mechanisms of escape are possible. First, there may not be tumor-specific antigens that can be recognized by CTLs. Investigators have searched intensively for tumor-specific Ags, which have been identified for a minority of malignant cells. [53] Second, if tumor cells do not express costimulatory molecules, anergy results. This has been shown in a number of tumor cell types.[54] Third, tumor cells may be relatively resistant to apoptotic signals. Finally, recent evidence has indicated that certain tumor cell types express fasL. FasL expression was initially demonstrated on melanoma cells, and has subsequently been found on a variety of other tumor cell types including myeloma cells. [55] [56] Therefore, tumor cells expressing fasL could inactivate CTLs by inducing apoptosis of the effector cell through engagement of fas. The significance of these various mechanisms of tumor cell escape and the development of strategies designed to overcome them is an area of intense basic and clinical investigation.

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CLINICAL APPLICATIONS OF CELL THERAPY IN HEMATOLOGY

Clinical settings in which cell therapy has been applied in hematologic practice are outlined in Table 96-1 and in the following sections.

Donor Leukocyte Infusions for Treatment and Prevention of Relapse

The use of DLI to treat patients who have suffered a relapse following allogeneic SCT has been an exciting advance that further documents the importance of GVL reactions. The first patient treated with DLI was a 2.5 year old child with ALL who was treated in 1986. [57] [58] This patient reentered a complete remission and continues to survive in CCR at least 10 years later. Kolb et al. reported on three allogeneic SCT recipients with relapsed CML who were treated with interferon- and DLI, all of whom entered complete hematologic and cytogenetic remissions. However, the clinical course of these patients was complicated by GVHD. [11] Since that time, a significant number of patients have been treated throughout the world with exciting results. A summary of these results is presented in Table 96-3 . Drobyski and colleagues treated eight patients with CML who suffered a hematologic relapse after allogeneic SCT, six of whom were in accelerated phase and two in blast crisis (BC). These patients received a predetermined T-cell dose between 2.55 × 10^8 T cells/kg.[59] Some patients were also treated with interferon-. Seven patients developed GVHD that was fatal in one, and four patients developed marrow aplasia. Both patients with BC died, whereas all six patients who received the DLI treatments in accelerated phase were alive and in cytogenetic remission at a median of 42 weeks from the infusion at the time of the report. These early studies pointed out much of the promise, as well as some of the problems of DLI therapy such as GVHD and aplasia.

An additional 11 patients with CML were reported who were treated with a combination of IFN- and DLI. Three patients developed significant GVHD and limited cGVHD developed in five patients. Six of eight patients with chronic phase CML after SCT had complete remissions, whereas none of the three patients with accelerated phase CML entered remission. [60]

Collins et al. reported on an additional 18 allogeneic SCT recipients, eight of whom had diseases other than CML. Six patients responded to DLI treatments, including two of five patients with AML. [61] GVHD was reported in seven patients; however, no patient developed marrow aplasia in this study.

The Hadassah group used DLI combined with IL-2. Seventeen relapsed allogeneic SCT recipients were reported of whom 10 were reinduced into CR. [62] Four patients with cytogenetic relapses responded to DLI alone, whereas five of six responding patients treated at the time of hematologic relapse appeared to require both DLI and IL-2.

Two large retrospective analyses of DLI for post-SCT relapse have been reported from Europe and North America. The results of the European Group for Blood and Marrow Transplantation have further documented the beneficial effects of DLI. In a series involving 27 transplant centers, 84 relapsed SCT patients had CML and of these 54 (73%) were induced into CR with DLI. Only five (29%) of the patients developed a CR, and none of the 22 patients with ALL had a CR. [63] These results are presented in Figure 96-1 . Fifty-two patients (41%) developed clinically significant GVHD and 41 patients (54%) had myelosuppression from the DLI. Seventeen patients (12.6%) died of causes other than their underlying leukemic disorder.

A similar review of 25 North American bone marrow transplant programs was performed. In this study, 140 SCT recipients with relapsed disease underwent DLI. [64] Again, CML patients

TABLE 96-3 -- Cumulative Results of Donor Leukocyte Infusions for the Treatment of Relapse Following an Allogeneic Stem Cell Transplant

Author	Number of Patients	Cell Dose × 10^8 /kg	GVHD	Myelo-suppression	CR
Kolb[12]	3	4.47.4	2	0	3
Drobyski[59]	8	25.5[a]	7	4	6
Porter[60]	11	0.98.4	3	2	6
Collins[61]	13	1.184.28	7	0	6
Slavin[62]	13	1.18	4	6	6
Kolb[63]	135	0.115	52	41	61
Collins[64]	140	0.518.08	58	26	45
Total	323		133	79	133
			(41.2%)	(25.5%)	(41.2%)

GVHD, graft-versus-host disease; CR, complete response.

[a] T cells.

Figure 96-1 Probability of survival of patients who have relapsed following an allogeneic stem cell transplant and who were subsequently treated with donor leukocyte infusions. Survival is based from the time of the donor leukocyte infusion. Results are for patients with CML (chronic myelogenous leukemia), PCV (polycythemia vera), ALL (acute lymphocytic leukemia), AML (acute myelogenous leukemia) and MDS (myelodysplastic syndrome). *(Reprinted with permission from Kolb et al.[65])*

responded the best, with 60% CR rates following DLI. Responses were better for patients with cytogenetic and chronic phase relapses, compared to patients with accelerated or blastic disease. Responses were durable, as actuarial probability of remaining in CR at two years was 89.6%. Results were not as favorable for SCT recipients with relapsed AML (15.4% CR) or ALL (18.2% CR). It is interesting to note that two of four patients with multiple myeloma responded to DLI. Complications included GVHD (60%) and pancytopenia (18.6%).

Overall, these results indicate that DLI has substantial efficacy in the control of relapse following allogeneic SCT. This is especially true for SCT recipients with CML detected in cytogenetic or early hematologic relapse. Upon progression to accelerated or blastic phase of the disease, DLI clearly has less efficacy. Studies are currently in progress to determine whether SCT recipients with only molecular evidence of recurrence detected by the polymerase chain reaction (PCR) will also benefit from DLI. Donor leukocyte infusions in SCT recipients with relapses of acute leukemia have been less successful. This may be a result of the more rapid proliferative capacity of these cells. Some groups have advocated the use of chemotherapy to gain control of the disease before DLI is performed. Importantly, the responses achieved with DLI in a variety of diseases, especially CML, have been durable.

Donor leukocyte infusion has also been used to treat patients who develop Epstein-Barr virus-induced lymphoproliferative disease (EBV-LPD), which occurs following allogeneic SCT, especially in patients who have undergone T-cell depleted SCT. [65] [66] Anti-EBV cytotoxic T-lymphocyte precursors have been assessed and found to be deficient in the time period when patients develop EBV-LPD. [67] Papadopoulos and colleagues performed DLI treatment for five patients with EBV-LPD. These tumors were of B-cell origin, and in at least four of the patients were of donor origin. In this study, patients were treated with DLI at a dose calculated to provide 1×10^6 CD3+ T cells/kg of recipient body weight. In all five patients, there were complete pathological or clinical responses first documented within 821 days following the infusion. [68] In at least three of these patients, long-term durable remissions were achieved, and the other two patients died of respiratory failure without evidence of lymphoma.

Despite these encouraging results, both DLI and myelosuppression complicate therapy. Timing of therapy following DLI is complicated by the fact that GVHD has been related to tumor response with DLI. Some groups have explored the concept of a defined dose of T cells for infusion. In one study, a T-cell dose of 1×10^7 T cells/kg resulted in a low incidence of GVHD with a clear antitumor cell response. [69] Other groups have attempted to deplete CD8+ cells in an effort to reduce the incidence of GVHD while retaining GVL activity. [70] [71] These studies are difficult to interpret because of patient and disease heterogeneity. Additional studies are required to determine whether this approach improves results.

Another approach involves genetically modifying the donor lymphocytes so that they are susceptible to certain drug treatments that will allow for their eradication if the patient develops GVHD. Ganciclovir treatment of cells containing the herpes simplex virus thymidine kinase (HSV-TK) gene results in cell death. Donor lymphocytes have been transfected with the HSV-TK gene by retroviral mediated gene transfer, and those transfected donor lymphocytes have been infused into patients who have relapsed or developed an Epstein-Barr virus-induced lymphoma. In one study of eight patients, the transferred lymphocytes survived for up to 12 months and resulted in anti-tumor activity in five of the patients. Three patients developed GVHD, which could be controlled by treating the patients with ganciclovir. [72] These exciting data pave the way for engineering cell types with specific functions whose activities can be controlled with certain drug treatments.

The other major complication associated with DLI has been myelosuppression, and in some instances severe pancytopenia requiring retransplantation. The more severe cases have been observed in patients who have no evidence of donor-derived hematopoiesis at the time of DLI. [73] Work is needed to define the optimal approach to DLI and to determine which population(s) of cells is responsible for the observed clinical effect.

Ex Vivo Activated Cellular Therapy for Treatment of Malignancies

Natural Killer Cell-based Cellular Immunotherapy

The observation that effector cell populations are capable of recognizing autologous tumor cell targets and that these cells can be activated with ex vivo treatment such as IL-2 has generated enthusiasm for clinical application. Treatment with IL-2 and IL-2-activated NK cells or LAK cells has met with some success; however, the requirement for IL-2, which has severe toxicities, has dampened enthusiasm. [74] In addition, many of the patients treated with LAK cells had advanced and/or refractory disease. Clinical responses have been observed, some of which are dramatic; however, most patients have not responded or have progressed.

Improvements in cell selection and cell culturing techniques, as well as basic advances in cytokine biology and cellular immunology, have renewed interest in the use of ex vivo activated cellular immunotherapy. Cellular immunotherapy could be used in a variety of ways; however, following SCT appears to be an ideal clinical time to test the effectiveness of this approach. The majority of patients are in a state of minimal residual disease following the transplant, and cellular immunotherapy is more likely to be effective. In addition, the precursor populations required, as well as the expertise in cellular manipulation, is readily available.

Autologous LAK cells have been obtained from patients with hematologic disorders such as lymphoma, acute leukemias, and CML. One approach has been to culture marrow cells in the presence of IL-2 for 24 hours, which results in tumor cell purging and a graft-versus-tumor effect in murine model systems. [75] [76] Similar results have been obtained using human marrow

cells purposefully spiked with tumor cells. Incubation with IL-2 resulted in effective purging of the malignant cells without excessive toxicity against normal hematopoietic stem cells. [77] [78] This approach has been extended to human phase I/II clinical trials with the hope that the in vitro activation of marrow cells with IL-2 will provide effective tumor cell purging and transfer of a graft-versus-tumor effect. [79]

Several studies have been performed using LAK cells with IL-2 following autologous SCT. Benyunes and colleagues performed a study of 16 patients with malignant lymphoma and five patients with AML who underwent apheresis to obtain peripheral blood lymphocytes (PBLs) following autologous SCT. The apheresis products were incubated with IL-2 at 300 U/ml for 5 days and then reinfused. The infusion of the cells was associated with transient dyspnea requiring O_2 therapy and which resolved within several hours. Four of five patients with AML were in continuous complete remission (CCR) from 1325 months at the time of the initial report. [80] Seven of the 16 lymphoma patients were relapse-free at the time of a subsequent report two years later. [81] In these studies, it was technically difficult to obtain the PBLs by apheresis following autologous SCT due to thrombocytopenia, especially in AML patients in whom prolonged thrombocytopenia is common following an autologous SCT using marrow as a source of stem cells. Presumably, the use of peripheral blood as a source of stem cells for transplant would make it easier to perform apheresis following transplantation due to the more rapid recovery of platelets. In addition, IL-2, which may have substantial biologic activity of its own, was also administered to these patients. [82] Therefore, it is difficult to know whether the cellular component or the IL-2 itself was responsible for any positive results in these studies. A further difficulty has been the relatively poor expansion of NK cells ex vivo. However, advances have been made in the cultivation of NK cells. [83] [84]

Another population of adherent NK (ANK) cells has also been used for immunotherapy. ANK cells are selected by binding to plastic surfaces and cultured in the presence of IL-2 and concanavalin-A activated allogeneic feeder cells. After several days in cultures, these cells develop enhanced cytotoxicity against a variety of target cells and have a phenotype similar to that of NK cells. ANK cells have been used to treat patients with hematologic malignancies following autologous transplantation. These studies are in an early stage, and to date minimal toxicity has been encountered. [85] IL-2 was also used following the infusion of the cells.

Lymphokine-activated natural killer cells have been utilized outside of the transplant setting for the treatment of solid tumors. Responses have been noted in some patients with malignant melanoma and renal cell carcinoma. [86] [87] [88] Relatively few patients with hematologic malignancies have been treated with LAK cells outside the SCT setting. In one study of 117 patients, an overall response rate of only 9% was observed. Of six patients with lymphoma, none responded. [89]

In addition to the toxicities noted previously, immunotherapy with IL-2 and LAK cells has also been associated with a number of hematologic toxicities. In one study of 42 patients, the majority (88%) of patients developed anemia that required >4 units of red cell transfusions, and 43% developed thrombocytopenia with platelet counts <50,000/l. Mild neutropenia was also observed. [90]

There is much room for improvement in defining biologically active cell populations, in activation ex vivo, and in careful selection of patients to improve upon current results.

Cytokine-Induced Killer Cells

Other approaches have been developed to expand populations of effector cells with antitumor cell activity. In addition to CD3-CD56+ NK cells, certain populations of CD3+ cells have also been shown to have antitumor cell cytolytic activity. [91] [92] One population of CD3+ T cells that co-express the NK cell marker CD56 has

demonstrated activity against a number of tumor cell lines.[93] CD3+CD56+ cells are rare in peripheral blood and represent only 15% of T cells from normal individuals.

Novel protocols have been developed by combining T cell mitogenic stimuli with cytokines such as IL-2. This results in the expansion of T cells that are further activated and are capable of recognizing tumor cell targets.[94] One approach has been to pretreat cells with interferon- followed one day later with anti-CD3 monoclonal antibody (MAb) and IL-2. Cells expand rapidly under these culture conditions, and the resultant effector cells have been termed cytokine-induced killer (CIK) cells.[95] [96] In these cultures, the cells with the greatest cytolytic activity have the phenotype CD3+CD16CD56+.[97] The CD3+CD56+ cells are derived from CD3+CD56 T cells and not from CD3CD56+ NK cells. CD3+CD56+ cells do not express CD16 and are therefore not capable of ADCC, yet do contain granzymes and perforin, which are released in the presence of tumor cell targets, or by crosslinking of CD3.[98] The expansion of CD3+ CD56+ T cells is augmented by other cytokines in addition to IL-2, with IL-12 being the most important.[99] Under these cultures, endogenous IL-12 is released by monocytes upon activation with IFN-.[100]

The in vivo activity of CIK cells has been tested using mice with severe combined immunodeficiency (SCID) that lack functional B and T cells and accept human tumors.[101] [102] In one series of experiments, human PBLs were purposefully contaminated with SU-DHL-4 tumor cells, which are a B cell NHL cell line with a t(14;18) chromosomal translocation that can be sensitively amplified using the PCR.[103] The mixed cells were incubated with or without CIK cells for 24 hours and then injected into SCID mice. The CIK-treated cultures did not result in tumor cell growth and animal death whereas the control cultures resulted in animal death after approximately 40 days (Fig. 96-2).[95] Further experiments were performed in which SU-DHL-4 tumor cells were injected into SCID mice and were followed one day later by CIK cells or LAK cells. In these experiments, CIK cells were found to have significantly greater antitumor activity compared to the LAK cells.[97] Approximately 3040% of the animals became long-term survivors and were completely free of disease as assessed by t(14;18) PCR. Using CIK cells in this animal model, IL-2 is not required for in vivo activity in contrast to LAK cells. CIK cells have also been expanded from a number of patients with hematologic diseases. Notably, the cells expanded from patients with CML are Philadelphia

Figure 96-2 Effect of treating SCID (severe combined immunodeficiency) marrow cells purposefully contaminated with SU-DHL-4 cells (human B-cell lymphoma) with cytokine-induced killer cells. *(Reprinted with permission from Schmidt-Wolf IGH, Negrin RS, Kiem H et al: Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. J Exp Med 174:139, 1991. Copyright The Rockefeller University Press.)*

chromosome-negative and are capable of exerting antitumor cell activity against autologous CML cells engrafted in SCID mice.[104] The ability of the CIK cells to expand ex vivo and their biologic activity observed in the absence of IL-2 makes this population of cells attractive for clinical application.

Cytotoxic Cell Lines

A problem with the use of ex vivo activated cell populations has been the variability in expansion and biologic activity of the effector cells. One approach to overcoming this limitation is to isolate stable cell lines with cytotoxic activity. Several such cell lines developed from patients with leukemia and lymphoma have been adapted to tissue culture. These cell lines have phenotypic and biologic characteristics that are very similar to those of NK and CIK cells.

The cell line TALL-104 was developed from a child with acute T-cell lymphoblastic leukemia. TALL-104 cells lyse a broad panel of tumor cell lines yet do not lyse normal tissues.[105] These cells are CD3+, TCR+, CD4, CD8+, CD16, CD56+, a phenotype similar to that of cells expanded in CIK cultures. TALL-104 cells have been used in vivo in SCID mice experiments using the human myelomonocytic leukemia cell line U937. In this model system, the TALL-104 cells protected the mice from an otherwise lethal challenge of U937 cells.[106] Irradiated TALL-104 cells have been used to treat dogs with a variety of different malignancies without toxicity. A number of the animals whose malignancies were refractory to other therapies responded to this form of cellular immunotherapy.[107]

The NK-like cell line NK-92 has been used for in vitro purging studies. NK-92 cells are CD3CD16CD56+ and react against a wide variety of target cells.[108] [109] Another cell line termed NKL, which is CD3CD16+CD56+, has morphology similar to that of a typical NK cell.[110] These cells are capable of lysing target cells via a typical granzyme-perforin mediated mechanism, as well as by ADCC resulting from the presence of CD16.

These cell lines have the theoretical advantage that a continuous source of cellular material is available. However, the cell lines are genetically dissimilar to those of the recipient and therefore would be rapidly cleared if used in vivo. If they are not cleared, they may result in GVHD.

Cytotoxic T Lymphocytes

The specificity of CTLs has made their clinical application an attractive goal. As discussed previously, CTLs recognize their target cells through the appropriate expression of a peptide associated with the MHC molecule in the context of a specific T-cell receptor expressed on the surface of the T cells. Other molecules such as costimulatory molecules and adhesion molecules must also be present in order for a productive interaction to occur. Considerable effort has been made to identify and expand CTL populations capable of lysing target cells. This has proceeded in two areas, namely the recognition of virally infected cells and the recognition of tumor cell targets. The recognition of virally infected cells is theoretically a simpler proposition due to the exogenous nature of the viral infection and the ability to replicate such interactions in vitro. The clinical settings that have been explored for CTL therapy most extensively are those of EBV and CMV infection.

Human EBV-specific cytotoxic T lymphocytes have been selected from PBLs isolated from seropositive donors and expanded ex vivo. These CTLs exhibit strong EBV-specific and HLA-restricted activity in vitro. In order to study their in vivo activity, SCID mouse models have been developed in which animals are inoculated with PBLs from EBV seropositive donors or with EBV-transformed lymphoblastoid cell lines. These animals develop lethal human EBV+ B-cell lymphoproliferative disorders that are similar to those that arise in immunodeficient patients. Using this model system, EBV-specific CTLs were capable of enhancing SCID mouse survival.[111] The EBV-specific CTLs were active when injected either intraperitoneally or intravenously. In addition, SCID mice that were tumor-bearing with large subcutaneous EBV+ tumor cells had regression following the injection of 10^7 EBV-specific CTLs. Prior experiments with adoptive transfer of peripheral blood mononuclear cells, purified T cells, IL-2 activated PBLs, or anti-CD3 activated T cells derived from EBV seropositive donors into EBV-infected SCID mice did not result in improved survival of the mice.[111]

Studies have also been performed using EBV-specific CTL cell lines in patients. In one report, 10 allograft recipients who were at risk for EBV-LPD received infusions of EBV-specific CTLs. The infusions were well-tolerated without significant toxicity. Three patients showed signs of EBV reactivation with an increase in EBV-DNA copy number. In these three individuals who received CTL injections, all three showed a marked decrease in EBV copy number following the infusion (Fig. 96-3).[112] In addition, one patient, who received four infusions, had resolution of an immunoblastic EBV-associated lymphoma. These CTLs had been gene-modified before infusion, and these investigators showed, using PCR analysis, that the cells persisted for 10 weeks after administration.[112]

CD8+ cytotoxic T-cell clones have also been generated against CMV-infected cells. These CD8+ CTLs were isolated from the blood of the patients donor. In 14 patients, the CTLs were infused into recipients beginning at 3040 days after SCT. In these patients, cellular immunity against CMV was monitored before, during, and after the infusions of the CTLs.[113] The infusion of the cells did not result in toxicity. In vitro studies of blood samples isolated from these individuals demonstrated that cytotoxic activity against CMV was significantly increased after the infusions in 11 of the patients who were deficient in this activity before therapy (Fig. 96-4). By performing PCR analysis of T-cell receptor genes in the individuals, it was demonstrated that the transferred cells persisted for up to 12 weeks. None of these individuals developed CMV disease.[113]

These striking results underscore the potential clinical efficacy of CTL-mediated immunotherapy. The positive results demonstrate not only feasibility but also that these effects can be persistent and have clear biologic activity. The isolation and expansion of CTLs is laborious and costly, and despite these successes, there are considerable obstacles that need to be overcome to make CTL therapy a treatment option for patients with malignancies. Another major drawback has been the lack of tumor-specific antigens that can be recognized by T cells. Significant advances have been made in this field, and a number

Figure 96-3 Treatment of patients with anti-Epstein-Barr virus (EBV) cytotoxic T cells. The time course and response to CTL (cytotoxic T lymphocyte) infusions as measured by EBV DNA copy

number is shown. *(Reprinted with permission from Rooney et al.[112] Copyright 1995 by The Lancet, Ltd.)*

Figure 96-4 Lytic activity against cytomegalovirus (CMV)-infected targets before and after infusion of anti-CMV CTLs. Filled boxes show lytic activity of peripheral blood lymphocytes after each infusion. *(Reprinted with permission from Walter et al.[113])*

of human tumor antigens have been identified that are recognized by T cells. [114] [115] These have primarily been identified on the surface of melanoma cells, particularly the MAGE system. Tumor-specific HLA A2 restricted CTLs have also been developed against metastatic breast cancer cells that showed tumor-specific recognition of the tumor cells yet no detectable lysis of normal cells or of K562 tumor cells. [116] The cytotoxicity of these CTLs against HLA A2 positive allogeneic targets was significantly higher than that of HLA A2 negative tumor cell lines. In this particular study, the target antigen was not identified. CTLs have been isolated and expanded that recognize tumor-associated mucins, which are frequently expressed on breast cancer cells. [117] Other researchers have developed CD3 + + CTLs from peripheral blood lymphocytes of breast cancer patients that also had cytolytic activity against autologous tumor cells yet lacked lysis of NK-sensitive lines. [118] The cytotoxic activity of these clones could be inhibited with anti-LFA-1 MAbs but could not be abolished by MAbs against CD3, class I, or class II MHC molecules. This suggests that these CTLs will recognize their tumor cell targets independent of the T-cell receptor by a not-yet-defined mechanism. [118]

A number of groups have attempted to generate CTLs against leukemic cells. In one study, irradiated leukemic cells from a patient with AML were used as stimulator cells, and the peripheral blood from the HLA matched identical sibling donor was used as responder cells. Several CTL lines were generated that had specific lysis against the recipient leukemic cells in chromium release cytotoxicity assays. Two such CTL lines were cloned by limiting dilution and were found to be HLA class I and II restricted.[119] These studies demonstrate the feasibility of generating CTLs against these types of malignancies; however, they also underscore the difficulty in obtaining and expanding such clones to a sufficient number of cells for clinical use. An alternative approach has been to use CTLs that are reactive against minor histocompatibility antigens and determine whether or not these cells will also recognize clonogenic leukemic cells. Faber and colleagues have performed such studies and demonstrated that the CD8+ CTL clones were able to recognize minor histocompatibility antigens HA-1 and HA-2. [120] However, these minor histocompatibility antigens are also expressed on immature hematopoietic progenitor cells.

An alternative approach has been to extract and culture lymphocyte infiltrating tumors (tumor-infiltrating lymphocytes [TILs]). The cells with the majority of activity in these cultures are typical CTLs with the phenotype of CD3+CD8+CD56 and are MHC-restricted. [121] [122] Tumor infiltrating lymphocytes are reported to be more potent than LAK cells; however, the generation of sufficient numbers of TIL cells with antitumor activity is difficult. In addition, TILs are often difficult to obtain or are not available from patients with hematologic malignancies.

A number of reports have suggested that tumor cells may lack the appropriate costimulatory molecules necessary for a productive interaction between tumor cells and CTLs. In an attempt to overcome this, follicular lymphoma cells were preactivated with CD40, which is known to upregulate expression of costimulatory molecules including the B7 proteins. Using this approach, autologous TILs could be expanded by the addition of IL-2 and further expanded in the presence of IL-4, IL-7, or gamma interferon.[123] These activated T cells showed cytotoxicity against follicular lymphomas in four of five patients tested. These studies suggest that CTLs may not be stimulated by the appropriate microenvironment of the tumor. This may occur because tumor cells lack expression of the appropriate costimulatory molecules that may be upregulated ex vivo to enhance the generation of CTLs. In fact, many tumor cells do not express the costimulatory molecules B7-1 and B7-2. [124] [125] Another approach has been to introduce these costimulatory molecules into the tumor cells by transfection. This has been performed successfully and results in enhanced recognition or rejection of transfected tumor cells using in vivo model systems. [126] These observations have also been extended to hematopoietic tumors such as murine AML cells that have been found not to express B7-1, which was then transfected into the tumor cells with a B7-1 containing retrovirus. The resulting transduced AML cells were used in murine models that induced immunity against the wild type AML cells. [127] However, these studies were performed in animals that had not been previously exposed to the leukemic cells. It remains to be seen whether the injection of B7-expressing tumor cells into diseased hosts or patients will overcome anergy.

Antigen-Pulsed Dendritic Cells

Antigen-presenting cells (APCs) present peptide antigens to T cells and thereby initiate a cellular immune response. These professional APCs also express critical accessory molecules required in this process and produce cytokines. The most potent APCs identified to date are a population of cells termed dendritic cells (DCs). Dendritic cells are characterized by the morphologic appearance of large cells with an elongated stellate process. Dendritic cells express high levels of MHC molecules B7-1, B7-2, CD40, ICAM-1, and LFA3. Dendritic cells lack expression of surface markers typical for T, B, and NK cells, as well as monocyte markers, and have been identified and purified based on their density characteristics. [128] [129] [130] [131] [132] Dendritic cells can be isolated from PBLs or expanded from CD34+ progenitor cells by culture in the presence of cytokines such as TNF, IL-4, c-kit ligand, GM-CSF, and CD40 ligand. [133] The hematopoietic growth factor flt3 ligand has been reported to increase the number of functional DCs in the bone marrow, gastrointestinal lymphoid tissue, liver, lymph nodes, peripheral blood, spleen, and thymus of treated animals.[134]

Cultured cell lines, such as the T2 line, have also been developed that can serve as APCs under the appropriate culture conditions. T2 cells lack the transporter proteins required for peptide presentation of endogenous proteins and are unable to present endogenous antigens. T2 cells, however, when pulsed exogenously, can present peptide to T cells. [135] This approach has been used to generate CTLs specific for a peptide derived from proteinase 3, and these CTLs preferentially recognize and lyse

human myeloid leukemia cells expressing HLA A2.[136] In this particular instance, the T2 cells were pulsed with a 9-mer peptide termed PR1 that was synthesized and tested for binding to HLA A2. A similar approach has been performed using peptide antigens that span the breakpoint of the *bcr-abl* gene of CML and bind with high affinity to HLA molecules. These peptides could elicit specific class I restricted CTLs in vitro from HLA-matched healthy donors. In this initial study, these peptides were specific for HLA A3 and HLA A11.[137] A second group has identified a 17 amino acid peptide that again spans the *bcr-abl* infusion region and which resulted in specific CD4+ T cell lines that were HLA DR4-restricted. [138] An alternative approach has been to use CD34+ cells isolated from patients with CML that are then cultured in vitro under conditions that stimulate their differentiation into DCs. These cells are then able to present antigen to T cells in vitro. [139]

The in vivo application of DCs that were pulsed in vitro has been demonstrated in murine models. Murine DCs pulsed with tumor antigens induced tumor resistance even after a single treatment in vivo. [140] [141] [142] The protective tumor immunity is antigen-specific and CTL-mediated as shown in Figure 96-5 . In this study, a peptide antigen corresponding to the amino acid sequence of ovalbumin residues 257264 was synthesized and used to pulse murine DCs. The DCs were expanded from lymphocyte-depleted marrow cells in the presence of GM-CSF and IL-4. These DCs were then pulsed for 2 hours with or without the ova peptide plus beta-2 microglobulin and then washed extensively before being injected into mice. The animals were then challenged with a syngeneic melanoma cell line (B16) or a second cell line formed by a transfection of B16 with an ovalbumin-containing plasmid (MO5). The animals treated with DCs rejected the MO5 but not the B16 tumors, and this required CD8+ T cells for activity. [143]

Pulsed DCs have been used to treat patients with follicular lymphoma by Hsu and colleagues. In these studies, human DCs were harvested from PB by differential centrifugation and pulsed with patient specific idiotype antigen. The pulsed DCs were then infused into patients with non-Hodgkin lymphoma. In this study, four patients with follicular B-cell lymphoma were treated with a series of idiotype pulsed autologous DC infusions followed by subcutaneous injections of soluble idiotype. Antitumor immunity was demonstrated in vitro, and significant tumor regressions were noted in two patients with the resolution of molecular disease in a third patient (Fig. 96-6).[144] This powerful approach is limited by the requirement for a purified tumor-specific protein or peptide for each patient that is currently available only in a minority of tumor types. In addition, these peptides react with certain HLA alleles. Currently clinical trials are ongoing using antigen-pulsed DCs to treat patients with low grade non-Hodgkin lymphoma and multiple myeloma following autologous and allogeneic SCT.

Figure 96-5 Tumor immunity induced by peptide-pulsed dendritic cells. C57 BL/6 mice were immunized with either PBLs (open symbols) or peptide-pulsed dendritic cells using an ovalbumin peptide.

The MO5 cell line (panels A and D) has been transfected with ovalbumin whereas the B16 cell line (panels B and E) is the parental non-transfected line. CD8+ cells are responsible for the biological effect because depletion of this population of cells in vivo with an anti-CD8 MAb results in increased tumor size (panel C) and loss of the protection afforded by the peptide pulse dendritic cells (panel F). *(Reprinted with permission from Celluzzi CM, Mayordomo JI, Storkus WJ et al: Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. J Exp Med 183:283, 1996. Copyright The Rockefeller University Press.)*

Figure 96-6 Computed tomography scans of two patients before and after immunization with idiotype pulsed dendritic cells. *(Reprinted with permission from Hsu et al.[144])*

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CONCLUSION

The use of cellular therapy to treat a variety of conditions is under intense laboratory and clinical investigation. Some forms of cellular therapy are part of routine clinical care, whereas others are only now being developed. The rapid expansion in basic biologic understanding of the types of effector cells and the role of accessory cells and molecules, as well as the cytokines that expand and activate these cells, has resulted in renewed optimism. Ongoing and future clinical studies will determine where this novel therapeutic approach will be best applied in clinical medicine.

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Chapter 97 - Complications After Stem Cell Transplantation

Daniel J. Weisdorf

The high-dose therapy used in hematopoietic stem cell transplantation (SCT) results in clinical toxicities induced directly by the treatment and secondarily by the prolonged immunodeficiency and extended recovery process. Recognition of clinical risk factors for particular complications allows risk-specific supportive care regimens to be designed that can reduce the overall morbidity and mortality accompanying transplantation (Table 97-1).

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EARLY TRANSPLANT-ASSOCIATED COMPLICATIONS

High-dose chemotherapy and radiation regimens are used before transplantation for both antineoplastic and immunosuppressive effects. However, these treatments may also damage host tissues, resulting in significant clinical morbidity. [1] These toxicities may include oropharyngeal mucositis, [2] hepatic veno-occlusive disease (VOD), interstitial pneumonitis (IP), and hemorrhagic cystitis. [3] Each of these toxicities may simulate infections or be compounded by concurrent infections. In addition, because epithelial tissue repair may be delayed by ongoing neutropenia and local microinvasive infection, delay in hematopoietic engraftment can exaggerate and prolong these toxicities.

Graft Failure After Stem Cell Transplantation

Failure to establish hematologic engraftment (primary graft failure) and loss of an established graft (late graft failure) are serious complications of both autologous and allogeneic marrow or blood stem cell transplantation. Delayed or poor graft function can exaggerate and prolong the risks of infection and can increase peritransplantation mortality. Failure to engraft can occur if insufficient stem cells are infused. Approximately 2×10^4 colony-forming cells from bone marrow per kilogram recipient weight are needed to establish autologous engraftment. This is accomplished by infusing a minimum of approximately 1×10^8 autologous marrow mononuclear cells/kg. Most investigators recommend infusion of a minimum of 2×10^8 cells/kg to ensure establishment of an allogeneic graft. Stem cells may be damaged by cryopreservation or by ex vivo purging, and additional cells are required if intensive purging, especially with alkylators such as 4-hydroperoxycyclophosphamide, is used.

Recently, hematopoietic stem cells and progenitors harvested from the blood by apheresis have become widely used for autologous and, to a lesser extent, allogeneic transplantation. Grafts are collected after mobilization of marrow-derived progenitors into the blood by cytokine (granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF]) therapy or during recovery from myelosuppressive chemotherapy, often in combination with growth factors. [4] [5] Progenitor content in these grafts is now assayed by quantitation of mononuclear cells expressing the stem cell-associated surface marker, CD34.

Graft failure is uncommon if 2×10^6 CD34+ cells/kg are collected, cryopreserved, and later reinfused as an autologous graft. [6] [7] The minimum CD34 content for an allogeneic graft is less well defined, but $>5 \times 10^6$ CD34 cells/kg is frequently cited as a target collection for a sibling donor allograft. [8] [9] In general, these mobilized blood stem cell transplants yield satisfactory and rapid trilineage hematologic recovery, but the risks of graft failure or ongoing poor hematopoietic function (especially thrombocytopenia) are uncertain. [10] [11]

Recipient myelofibrosis or splenomegaly may also interfere with engraftment. Splenomegaly may delay hematologic recovery, [12] presumably because both progenitors and mature blood

TABLE 97-1 -- Major Noninfectious Complications of Stem Cell Transplantation

Time After Transplant	Complication	Incidence
Early (030 days)	Regimen-related toxicity	
	Mucositis	6075%
	Cyclophosphamide/radiation myocarditis or pericarditis	Rare
	Hemorrhagic cystitis	10%
	Veno-occlusive disease	540%
	Pneumonitis	1020%
	Alveolar hemorrhage	510%
	Infections	75%
	Graft failure	210%
	Adverse drug reactions from polypharmacy	Common
Intermediate (13 months)	Acute GVHD	2050%; more frequent with nonsibling donors
Late (beyond 3 months)	Chronic GVHD	2040%
	Hypothyroidism	3050%
	Growth disturbances	Common in prepubertal children
	Sterility/hypogonadism	Common
	Cataracts	2540%
	Avascular necrosis of bone or demineralization of bone	515%
	Malignant relapse	Variable
	Second malignancy	210%, but total incidence is uncertain

GVHD, graft-versus-host disease.

cells are sequestered in the spleen. Prior splenectomy is associated with a shorter interval to engraftment. [13] The presence of moderate or severe myelofibrosis also delays engraftment, perhaps because of faulty homing of stem cells in the marrow microenvironment. [14] [15]

Post-transplantation therapy may also jeopardize engraftment. Graft failure or poor graft function with continuing pancytopenia has been associated with use of methotrexate, antithymocyte globulin (ATG), acyclovir, ganciclovir, trimethoprim-sulfamethoxazole (TMP-SMX), 5-fluocytosine, as well as histamine blockers.

Post-transplantation complications such as cytomegalovirus (CMV), mycobacterial, or fungal infection, acute and chronic graft-versus-host disease (GVHD), or, rarely, Epstein-Barr virus-associated lymphoma may also compromise successful engraftment.

Allogeneic SCT, especially using unrelated or mismatched donors, poses unique problems with engraftment. Transplants between siblings matched at human leukocyte antigen (HLA)-A, -B, and -DR loci are rarely (15%) associated with graft failure; however, the probability of graft failure in the related donor transplantation setting increases up to 1020% with greater degrees of HLA incompatibility. [16] [17] [18] [19] [20] [21] The problem is frequent (515%) in the unrelated donor setting, where graft failure may occur even after transplantation from donors serologically matched at the HLA-A, -B, and -DR loci. [22] [23] [24] In some cases, failure of unrelated donor stem cells to engraft may result from reactivity against important histocompatibility determinants not recognized by current HLA typing methods. [25] Early failure of an allogeneic graft may be accompanied by emergence of cytotoxic T lymphocytes of host origin, [26] presumably representing immune-mediated graft rejection.

T-lymphocyte depletion of donor marrow performed as GVHD prophylaxis can also adversely affect engraftment, even from matched sibling donors. [16] [20] [27] [28] [29] Ex vivo marrow manipulation may deplete stem cells. Alternatively, T-cell depletion may render the graft immunoincompetent and functionally less capable of preventing host rejection of the graft. In addition, T-cellderived soluble factors that support engraftment may not be available after T-lymphocyte depletion. [30] Correction of this deficiency by preservation of specific T-cell subsets in the graft is under study. [31] [32]

The availability of recombinant human growth factors such as G-CSF and GM-CSF, which stimulate myelopoiesis, has revolutionized the treatment of graft failure. [33] Fifty to 60% of patients experiencing poor graft function have a myelopoietic response within 1421 days after initiation of growth factor therapy, although patients receiving purged autografts may be less likely to respond. Clinical trials testing the relative merits of different growth factors, growth factor combinations, and dose schedules are underway.[34] These studies may resolve the possibilities that growth factors may stimulate either residual host cells (augmenting mixed chimerism) or residual myeloid leukemia cells (increasing the risks of relapse). Available growth factors increase peripheral blood leukocytes but have no major effect on platelet recovery.[35] Recombinant human thrombopoietin has been tested in conjunction with myelosuppressive chemotherapy and in limited trials for stimulating platelet recovery after marrow or stem cell transplantation.[36] Limited experience suggests the possible value of erythropoietin in reducing red cell transfusion needs. [37]

A second stem cell infusion may be useful if graft failure occurs.[38] In the case of a failed autograft, infusion of previously harvested and frozen marrow or peripheral blood cells frequently reestablishes a graft. Procurement and storage of back-up stem cells should be considered in the case of autograft protocols in which a high rate of graft failure is anticipated. In the case of marrow failure after related donor transplantation, a second infusion of donor marrow or cytokine-stimulated peripheral blood stem cells, sometimes after reconditioning with reduced doses of cytotoxic agents or after further immunosuppression with ATG, corticosteroids, or cyclosporine, may allow successful engraftment.

The incidence of graft failure after unrelated donor transplantation is high and poses special problems. [16] [22] [24] [39] Unrelated donors may not be available for a second donation or may not have recovered sufficiently from the first harvest to undergo marrow extraction. Even if the unrelated donor is willing to undergo a second harvest, delays of more than 4 weeks from the time of request may occur. For these reasons, it is prudent to store autologous stem cells from patients undergoing unrelated donor transplantation.

Host Defense Defects After Stem Cell Transplantation

Multiple immunologic deficiencies are induced by SCT (Table 97-2). Mucocutaneous barriers to invasion of colonizing pathogens are disrupted. Early neutropenia and hypogammaglobulinemia further compromise the ability to eliminate these organisms. [40] Severe cellular immune dysfunction, including T-cell lymphopenia and anergy, persists for some months after engraftment

TABLE 97-2 -- Host Defense Defects After Stem Cell Transplantation

Time After Transplant	Immune Defects	Major Infections
Early (030 days)	Mucosal + skin injury Neutropenia	Aerobic bacteria Gram-positive, especially coagulase-negative staphylococci, *viridans* streptococci Gram-negative bacilli Fungi *Candida* sp. Molds (*Aspergillus* sp.) Viruses Herpes simplex virus Respiratory viruses (respiratory syncytial virus, parainfluenza virus)
Intermediate (13 months)	T-cell dysfunction Hypogammaglobulinemia Acute GVHD	Fungi: molds, *Candida* sp., PCP Viruses CMV, respiratory viruses Encapsulated bacteria pneumococci, *Hemophilus influenzae*
Late (312 months)	Slow T-cell reconstitution Chronic GVHD	PCP Varicella zoster virus Encapsulated bacteria CMV

CMV, cytomegalovirus; PCP, Pneumocystis carinii pneumonia.

INFECTION PROPHYLAXIS FOR STEM CELL TRANSPLANT RECIPIENTS

A typical comprehensive regimen for SCT recipients has all patients supported in single, HEPA-filtered, positive-air-pressure, sealed rooms with strict hand washing as the primary protective isolation measure. All patients should receive trimethoprim-sulfamethoxazole (TMP-SMX) or ciprofloxacin plus fluconazole daily. Fevers occurring during the initial conditioning and neutropenic period are first treated with a semisynthetic penicillin plus an aminoglycoside, or with ceftazidime for patients receiving cyclosporine. Vancomycin or alternate gram-positive bacterial coverage is indicated if coagulase-negative staphylococci or *viridans* streptococci are identified. After 72 hours of unexplained fever, patients should be treated with empiric amphotericin (0.5 mg/kg/day or 0.3 mg/kg/day in patients receiving cyclosporine), but amphotericin should be initiated earlier and in higher doses in those colonized with yeasts or with lesions suspect for invasive mycotic infection (sinusitis or nodular pulmonary lesions). Lipid amphotericin preparations may be as effective and less nephrotoxic, but need further study as antifungal prophylaxis.

Herpes simplex virus-seropositive patients require acyclovir prophylaxis to prevent viral reactivation. CMV-seronegative patients receive seronegative or leukocyte-depleted (by filtration) blood products, and CMV-seropositive patients should receive antiviral suppression with ganciclovir and perhaps with intravenous immunoglobulin (see later).

Anti-infection prophylaxis later in the course includes oral fluconazole or mycostatin until day 100 for all patients and ongoing anticandidal therapy for patients being treated for continuing GVHD. Heightened fungal risks in unrelated donor or mismatched donor allograft recipients may indicate a need for ongoing protection against *Aspergillus*. However, the value of itraconazole or continuing low-dose amphotericin (conventional or lipid) is unproven. All patients should receive *Pneumocystis* prophylaxis for 1 year post-transplant (TMP-SMX double strength bid 2 days per week, or aerosolized pentamidine 300 mg once monthly).

Patients with ongoing GVHD receive continuing *Candida* and *Pneumocystis* prophylaxis until 1 month after discontinuation of all immunosuppression, and also receive daily penicillin or erythromycin for protection against pneumococcal bacteremia. Intravenous immunoglobulin for those with documented hypogammaglobulinemia and infections can be beneficial as well.

and is usually prolonged in patients with ongoing GVHD. Interestingly, natural killer cell activity regenerates much earlier, often by 48 weeks post-transplantation. [41] [42]

In the early post-transplantation period, nearly all patients have fever, although infectious pathogens are identified in only 50% of patients. Fevers may also be due to tissue inflammation (oropharyngeal or enteric mucositis), transfusions, or amphotericin or other drug fever. During initial neutropenia, the most common infections identified are due to aerobic bacteria (frequently coagulase-negative staphylococci, *viridans* streptococci, and enteric gram-negative bacilli). Colonizing yeasts also invade because of neutropenia and disruption of normal host flora, thus leading to systemic mycotic infections in 1015% of patients. [43] [44]

Prophylactic strategies[45] may include suppressive antibiotics such as vancomycin, quinolones, or TMP-SMX. In some centers, a pathogen-free environment with laminar flow isolation, gut sterilization, special diets, and broad-spectrum prophylactic antibiotics is used to reduce risks of infection. However, these intensive isolation measures are both cumbersome and expensive, and have not been proven helpful in preventing early post-transplantation mortality. Newer antifungal agents, including fluconazole, have been strikingly effective in preventing systemic candidiasis, although resistant species (*Candida krusei* or *Torulopsis glabrata*) have emerged as secondary pathogens and may require additional attention. [46]

Treatment of fever in the neutropenic transplant recipient requires strategies similar to the ones that have been effective in patients with leukemia. [47] Broad-spectrum antibacterial coverage should be initiated early. Patients with persistent unexplained fever, particularly those colonized with yeast, should receive empiric amphotericin. Repeated vigorous investigation to identify sources of infection, even for fever recurring after initial defervescence, is essential. In many centers, third-generation cephalosporins and aminoglycosides have been effective in treating the most common gram-negative enteric bacilli (*Escherichia coli*, *Klebsiella* sp., *Enterobacter* sp., and *Pseudomonas* sp.).[48] However, resistant gram-negative pathogens, including *Citrobacter* sp., *Acinetobacter* sp., and *Xanthomonas* sp., have emerged in recent years as secondary pathogens in the antibiotic-exposed neutropenic population. Institutional antibiotic sensitivity profiles must guide empiric and therapeutic antibiotic choices and should, of course, be individualized based on specific pathogenic isolates and their sensitivities. Extensive use of vancomycin and broadly active cephalosporins may also promote colonization or enteric infection with vancomycin-resistant enterococci, which can be highly virulent in the compromised or neutropenic host. Isolation measures to reduce nosocomial acquisition of vancomycin-resistant enterococci may become more important as these organisms become more prevalent.[49]

Infection with invasive fungal organisms remains a significant hazard despite the use of protected environments, HEPA (high-efficiency particulate air) filters, and reverse isolation.[50] [51] Up to 10% of transplant recipients may have invasive fungal infections, usually with *Aspergillus* sp. but *Fusarium* sp., *Alternaria* sp., and other, less common isolates have been reported. Therefore, aggressive and repeated evaluation for mycotic infection, particularly in the persistently febrile neutropenic host, is essential along with early initiation of amphotericin. Liposomal amphotericin preparations, itraconazole, or growth factors designed to accelerate neutrophil recovery or activate inflammatory phagocytes (i.e., GM-CSF, G-CSF, macrophage colony-stimulating factor [M-CSF]) have all been proposed as newer therapies for fungal infection, but their specific role and value are as yet unclear. [52] [53]

All categories of infection are seen more frequently in allogeneic transplant recipients in whom immune reconstitution (even after resolution of neutropenia) is delayed. Chronic GVHD (see later) is accompanied by persistent hypogammaglobulinemia, cellular immune dysfunction, and a continuing risk of infection with bacterial, fungal, and viral pathogens. Although intravenous immunoglobulin supplementation has been given [54] to reduce risks of infection, one prospective trial suggested that its prophylactic use does not prevent later post-transplantation infection and could impair humoral immune reconstitution. [55] Late infections may include varicella zoster virus (usually dermatomal shingles, which may disseminate); encapsulated bacterial infections (pneumococci or *Hemophilus influenzae*), suggesting an immunologic defect similar to that seen with hyposplenia or asplenia; and *Pneumocystis carinii* pneumonia. These all are common in the late (312 months) post-transplantation period and require additional prophylaxis or prompt therapy, especially in allograft recipients with chronic GVHD. [56] [57] [58] [59] [60] [61] [62] Augmented infection risks in recipients of unrelated

donor allografts have been recognized, even beyond the reportedly higher infection risks associated with acute and chronic GVHD. [63] [64] More intensive prophylaxis and surveillance measures may be required for this subgroup.

Veno-occlusive Disease

Veno-occlusive disease (VOD) is a serious liver disorder complicating up to 50% of marrow transplants. [65] [66] [67] Signs of VOD usually occur within 1 month of stem cell infusion, but may be recognized much sooner, even during administration of the preparative regimen. [68] Clinical evidence of VOD includes hyperbilirubinemia,

hepatomegaly, ascites, and weight gain.[69] [70] VOD is a hepatotoxic lesion involving obstruction of small, intrahepatic venules and damage to the surrounding centrilobular hepatocytes and sinusoids.[71] Concentric fibrosis with obliteration of the hepatic venules, fibrosis of the centrilobular zone, and atrophy of adjacent hepatocytes may occur.[72] Deposition of fibronectin and factor VIII/von Willebrand factor at the site of the damaged endothelium may be associated with activation of the coagulation system and subsequent postsinusoidal obstruction.[69] [73] [74] Such changes are often associated with depressed plasma protein C levels,[75] and may be associated with other evidence of procoagulant activity, including depressed factor VIII and antithrombin III levels as well as elevated fibrinogen levels.[71] [76] [77] [78] Tumor necrosis factor- (TNF) levels are elevated in VOD,[79] [80] and may be causally related to endothelial damage.

Veno-occlusive disease can result in encephalopathy, renal failure, pulmonary failure, or multiorgan failure.[70] [71] [81] The mortality rate of VOD is highly variable in different series, perhaps due to differing stringency of diagnostic criteria,[70] [71] [82] but severe VOD is often fatal within several weeks of onset.[66] [70] [81]

Establishing a definite diagnosis of VOD is difficult. Most investigators require two of three clinical criteria, including jaundice, hepatomegaly, or ascites occurring within 2 weeks of stem cell infusion.[65] [69] [70] [82] However, other causes of hyperbilirubinemia and weight gain early after transplantation (e.g., drugs [especially cyclosporine, TMP-SMX, methotrexate, or estrogens], capillary leak, salt and colloid overloading) complicate the differential diagnosis. Percutaneous or transabdominal needle biopsy of the liver is extremely hazardous in thrombocytopenic transplant recipients, and should be avoided.[83] Transvenous biopsies may provide sufficient histologic material for diagnosis and may allow determination of hepatic wedge pressure product (greater than 10 mm is associated with VOD),[84] but may be associated with hemorrhagic complications as well.[84] [85] [86] Ultrasound Doppler flow studies demonstrating reversal of portal flow or a higher portal vein resistive index have been suggested as a noninvasive means of confirming the diagnosis, but their validity has recently been questioned.[87] [88] [89] [90] [91]

Risk factors associated with development of VOD include history of pretransplantation hepatitis, intensive preparative regimens, increased radiation dose and dose rate, and increased busulfan dose.[1] [65] [68] [70] [81] [92] [93] [94] [95] [96] VOD may also be more frequent after mismatched related or unrelated donor transplantations.[65] [68]

Effective methods for prevention and treatment of VOD have not been defined. Promising approaches include preventive therapy with low-dose heparin,[97] [98] [99] prostaglandin E,[100] pentoxifylline (a TNF blocking agent),[101] [102] or ursodiol.[103] [104] Recombinant tissue plasminogen factor has been used successfully to treat established VOD,[105] [106] [107] [108] [109] as has administration of urokinase;[109] however, both agents are associated with substantially increased risks of hemorrhage. Transjugular portosystemic shunts have also been used.[110]

Interstitial Pneumonitis

Interstitial pneumonitis (IP) is a common and frequently fatal complication, affecting up to 35% of allogeneic transplant recipients,[111] [112] although recent advances in supportive care may substantially reduce this risk.[40] It is less common after autografting.[113] It is characterized by diffuse, nonbacterial interstitial inflammation. Risk factors associated with development of IP include (1) prolonged methotrexate therapy for GVHD prophylaxis; (2) age >21 years; (3) severe GVHD; (4) >6-month interval from diagnosis of hematologic disease until transplantation; (5) <100% pretransplantation Karnofsky performance score; and (6) radiation dose rate >4 cGy/minute.[112] Remarkably, when none of these factors was present, the reported risk of IP was 8%, and that risk rose to 94% when all six were present. It has been hypothesized that unrelated donor transplantation is more immunosuppressive and thus associated with more severe opportunistic infections and greater risks of IP, but this has not been rigorously investigated.

The course of IP is often catastrophic, presenting with rapidly progressive tachypnea, dyspnea, hypoxemia, and hemodynamic compromise. Therefore, therapeutic intervention must frequently occur before the return of definitive diagnostic tests, and must be initiated based on the assessment of clinical risk factors, associated clinical clues, and the clinical setting (especially the type of transplant and the time of onset).

Infectious Causes of Interstitial Pneumonitis

CMV Infection

Prevention of CMV Infection.

Interstitial pneumonia due to CMV is the most common cause of IP, responsible for 50% of all cases.[114] Prevention of CMV pneumonia as well as other manifestations (enteritis, hepatitis, retinitis) depends on understanding of risk factors and institution of specific preventive measures.

Seropositive Recipients.

The most powerful risk factor predisposing to CMV infection is seropositivity of the recipient.[115] [116] Clinical observations strongly suggest that reactivation of latent CMV is the most important mechanism resulting in CMV disease; however, virologic and molecular confirmation of this suspicion has been difficult. The approach to CMV prevention in the seropositive patient must include chemoprophylaxis or immunoprophylaxis as well as aggressive surveillance to allow prompt detection of infection. An additional role for preventing exogenous exposure (through choosing seronegative bone marrow or blood donors) is not proven. Chemoprophylaxis has been very useful in preventing CMV pneumonia among seropositive recipients. The use of acyclovir was associated with a decrease in CMV pneumonia incidence from 40 to 19% among seropositive recipients,[117] whereas ganciclovir given prophylactically[118] [119] or when administered at the time of viremia, CMV antigenemia,[120] [121] [122] [123] [124] or bronchoalveolar lavage (BAL) recovery of virus[125] [126] [127] [128] [129] from asymptomatic patients has reduced the incidence of CMV pneumonia to under 5%. Ganciclovir induced significant neutropenia in these trials and sometimes secondary sepsis as well. Intravenous immunoglobulin may also be useful in reducing CMV infections, perhaps through its effect in reducing risks of GVHD.[40] [111]

Seronegative Recipients.

It is clear that the nearly all CMV infections in seronegative recipients are the result of exogenous exposure (primary CMV infection), either from a seropositive stem cell donor or from cellular blood products from seropositive donors. Noncellular blood products do not transmit CMV.[130] Delivery of CMV-seronegative blood products only to seronegative recipients reduces the risk of CMV infection to less than 5% and nearly eliminates the risk of CMV pneumonia.[131] [132] Unfortunately, the demand for CMV-safe blood is greater than the supply of seronegative blood donors at most centers. Excellent clinical and laboratory data point to leukocytes as the carriers of infectious CMV in blood products. Several recent studies, including one large, prospective trial, have shown that removal of white blood cells from transfused unscreened products by sedimentation or filtration is an effective alternative in prevention of primary CMV infections.[133] [134] [135] [136] The frequent use of

intravenous immunoglobulin for CMV prevention,[137] [138] which has limited efficacy, has largely been supplanted by delivery of CMV-safe blood products to seronegative patients.

Autologous Recipients.

A second powerful risk factor for CMV pneumonia is allogeneic (compared with autologous) SCT.[139] [140] Enright et al. reported CMV pneumonia complicating the course of 12% of over 400 allogeneic recipients, but only 3% of 229 autologous recipients.[139] However, CMV pneumonia was as severe in autologous as in allogeneic recipients, with a high-case fatality rate. Other studies have also defined a similar incidence of CMV pneumonia among autologous recipients,[141] or have shown delayed engraftment among autologous recipients in whom CMV infection developed.[121] [142] Thus, prevention of CMV infection through use of noninfective blood products (for seronegative recipients) or chemoprophylaxis (for seropositive recipients) is still indicated.[143] Viremia or recovery of virus from BAL fluid should likewise be treated aggressively.

Treatment of Viremia.

Post-SCT CMV viremia is a strong predictor of CMV pneumonia[144] and should probably be treated regardless of the patients previous risk group. CMV pneumonia follows 60% of asymptomatic viremias within 14 days (median). Treatment of viremia with ganciclovir can reduce the incidence of CMV pneumonia to 5%.[120] [121]

Surveillance for CMV pp65 antigenemia or polymerase chain reaction assay of CMV DNA has markedly augmented the sensitivity of blood testing for early infection.[121] [122] [127] The clinical consequences and best pre-emptive therapeutic intervention after detection of antigenemia are still under study. The cost and myelosuppression associated with ganciclovir or foscarnet therapy must be weighed against the risks of inadequately treated antigenemia progressing to active CMV disease.[121] [123] [124] [127] [129]

Diagnosis of CMV Pneumonia.

Cytomegalovirus pneumonia usually presents as an IP associated with fever and hypoxia. Although lung biopsy showing CMV inclusions is the gold standard for diagnosis of CMV pneumonia, shell vial culture of BAL fluid is positive in most cases of CMV pneumonia [145] and is a less risky approach to diagnosis. Shell vial culture of CMV from BAL is not, however, specific to CMV pneumonia, and is frequently positive in asymptomatic seropositive people who are shedding CMV in oral or respiratory secretions without pneumonia.[146] [147] [148] Despite this lack of specificity, even in asymptomatic people, finding CMV in BAL is a strong predictor for development of CMV pneumonia and should be treated. The practical approach is to use the combination of a compatible clinical syndrome (especially in the presence of CMV antigenemia) plus either BAL direct staining, shell vial cultures, or polymerase chain reaction to diagnose CMV pneumonia. [126] [127] [128] [149] If BAL is undertaken for other indications and CMV is found coincidentally, it should be treated to prevent development of clinical CMV pneumonia.

Treatment of CMV Pneumonia.

Cytomegalovirus pneumonia should be diagnosed and treated as promptly as possible. If patients are already respirator dependent at the time of institution of therapy, therapy is nearly always ineffective and the mortality rate is nearly 100%. [139] The combined use of ganciclovir and immunoglobulin has been the most successful treatment for CMV pneumonia, with resolution in 5075% of patients (nonrespirator dependent). [139] [150] [151] [152] Prolonged therapy (2 months) with the combination is indicated because shorter treatment regimens have been associated with recurrence of CMV pneumonia. [141] Foscarnet can be effective in clinical settings where ganciclovir fails or is associated with excess toxicity, usually myelosuppression. [143]

Fungal Infections

Pulmonary fungal infections may be associated with an interstitial pattern, alveolar infiltrates, or nodules on chest radiography. [50] [51] [153] *Aspergillus* pneumonia has been very difficult to treat, partly because of the difficulty in making the diagnosis

APPROACH TO PREVENTION AND TREATMENT OF CMV INFECTION

PREVENTION

1. Seronegative recipient with seronegative donor (allogeneic and autologous): Transfuse only CMV-safe blood products. Leukocyte depletion by filtration and blood from CMV-seronegative donors are clinically equivalent alternatives.
2. Seronegative recipient with seropositive donor (allogeneic): Deliver only CMV-safe blood products (seronegative or leukocyte depleted), but administer chemoimmunoprophylaxis as well to prevent reactivation of endogenous virus.
3. Seropositive recipients: Prophylaxis with acyclovir appears to be useful. Use of immunoglobulin may be beneficial (especially by modulation of GVHD). There is no proven role for seronegative blood products. Ganciclovir is highly effective when given prophylactically, but is toxic to marrow cells. Patients who must interrupt the course of ganciclovir because of leukopenia are at risk for development of CMV pneumonia. Ganciclovir is the current best prophylaxis in high-risk patients, but probably is not indicated for autologous recipients.

TREATMENT

1. Asymptomatic infections (all types of transplants): Ganciclovir treatment of asymptomatic CMV infection detected by either bronchoalveolar lavage (BAL) antigenemia or blood culture is recommended to prevent development of CMV pneumonia. Intensive treatment followed by a maintenance phase of 5 days/week therapy is necessary. The requisite duration of treatment for CMV antigenemia or viremia is uncertain, but a minimum of 3 weeks of therapy with resultant negative testing is advised, along with close surveillance of therapy. Asymptomatic viruria suggests a need for close follow-up and for serial blood viral testing, but does not usually require therapy.
2. CMV pneumonia: Ganciclovir in combination with immunoglobulin is the recommended regimen. This should be instituted as promptly as possible. Once the disease has progressed to cause respiratory failure and ventilator dependence, therapeutic success is rare.

NO TREATMENT

The following situations are important negatives in which ganciclovir is not indicated for empiric therapy of interstitial pneumonitis; in these circumstances, CMV pneumonia is unlikely to be present:

1. Seronegative recipients with seronegative marrow and blood donors.
2. Any patient in whom BAL is negative by direct staining and shell vial culture. However, BAL CMV studies do have a small (<5%) false-negative rate, so CMV interstitial pneumonitis cannot be absolutely excluded.

antemortem. [50] *Aspergillus* infections may occur early post-SCT (during the neutropenic phase) or later, especially complicating the immunosuppression associated with acute or chronic GVHD.[50] [154] [155] Allogeneic (vs. autologous) SCT, prolonged neutropenia, and GVHD all predispose to *Aspergillus* infections.[155] [156] [157] Exposure to construction sites has also been associated with *Aspergillus* infections.[158] Measures to prevent aspergillosis include prophylactic low-dose amphotericin, inhaled amphotericin,

early empiric use of amphotericin, itraconazole, and close attention to air filtration in patient care areas, although the relative effectiveness of these approaches has not yet been defined. More recent approaches include prophylactic hemopoietins (GM-CSF, G-CSF) for their prophagocytic as well as neutrophilic potency.

Nasal and bronchial washings for *Aspergillus* may be inadequately sensitive,[159] [160] and lung biopsy may be required to obtain a definitive diagnosis. The pattern of the infiltrate may be helpful diagnostically and can sometimes be better defined by computed tomography scan of the chest. [161] Persistent high-grade fever that does not improve on antibacterials is often assumed to be due to fungal infection.

Although unquestionably the treatment of choice for fungal infections, the use of high-dose amphotericin may be limited by renal dysfunction. Promising but costly new approaches to treatment of fungal pneumonia include several newly licensed lipid formulations of amphotericin, itraconazole, and GM-CSF or M-CSF. [162] [163] [164]

Pneumocystis carinii Pneumonia

Pneumocystis carinii causes IP, typically bilateral with a butterfly pattern on radiography. [59] Although the 515% risk of *P. carinii* pneumonia (PCP) has largely been eliminated by the use of trimethoprim-sulfamethoxazole (TMP-SMX) given 2 consecutive days/week,[165] TMP-SMX is not tolerated by all patients because of allergy or marrow depression by the drug. Recently, use of inhaled pentamidine (300 mg every 4 weeks) or dapsone has offered effective alternatives. [60] [62] [166] TMP-SMX therapy virtually eliminates PCP from the differential diagnosis, but only if patient compliance with the prophylaxis is certain. Inhaled pentamidine or dapsone, although partially effective, does not totally eliminate the risk of PCP. PCP can be diagnosed by cytologic evaluation of silver-stained preparations of BAL cells or sputum. It is effectively treated by high-dose TMP-SMX or parenteral pentamidine. Supplemental steroid therapy has improved therapeutic results in patients with acquired immunodeficiency syndrome,[167] and may be helpful after SCT as well. Prophylaxis against PCP should be continued during the period of immunosuppression (6 months to 1 year after SCT) and for the duration of any therapy for chronic GVHD. PCP has been recognized as late as 13 years after BMT, especially after relapse of a malignancy that is not rapidly fatal (e.g., chronic myeloid leukemia or low-grade lymphoma). [59] Ongoing prophylaxis for such patients should be considered.

Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is a potentially fatal cause of IP [168] [169] that is seasonal, occurring most frequently in the fall and winter months. RSV should be suspected if the patient has had nasal congestion and if RSV has been frequently recognized either in the community or in the hospital. Diagnosis may be made by rapid antigen testing on nasal washings or BAL specimens. Because of the possibility of horizontal transmission, patients with RSV should be isolated. Treatment is with inhaled aerosolized ribavirin (usually 6 g/day) for 37 days. [168] [170] A similar but nonseasonal syndrome due to parainfluenza virus has been described. [171] [172] No rapid antigen test for parainfluenza is available, and its response to ribavirin therapy is uncertain.

Noninfectious Causes of Interstitial Pneumonitis

Idiopathic Interstitial Pneumonitis

Idiopathic interstitial pneumonitis (IIP) is a diagnosis of exclusion based on typical findings and ruling out infectious causes. Its timing is somewhat earlier than other causes of IP,[111] [114] [172] typically occurring within the first 27 weeks post-SCT. Interestingly, the recognized clinical risk factors for IIP have been similar to those for IP, and include (1) extensive pretransplantation chemotherapy; (2) high-dose cyclophosphamide; (3) total-body irradiation (TBI; increased by higher total dose and dose rate); (4) blood transfusions; (5) administration of methotrexate; and (6) GVHD. [173]

The observation that IIP is as frequent among syngeneic as among allogeneic recipients [174] suggests that immunosuppression is less of a risk factor for IIP than it is for infectious IP. Clinical observations support a toxic cause of IIP, [173] and radiation-induced lung damage appears to be the major contributor. [175] These clinical observations are supported by animal studies suggesting that irradiation is the major contributor to post-SCT lung damage, [176] whereas cyclophosphamide makes only a minor contribution.[177] In addition, in controlled trials, higher-dose TBI has increased the incidence of IIP. [96] Effective therapy has not been established, although high-dose corticosteroid therapy is often administered.

Diffuse Alveolar Hemorrhage

Diffuse alveolar hemorrhage (DAH) has been recently recognized primarily, but not exclusively, in autologous SCT recipients. [178] [179] [180] It is likely that some allogeneic SCT recipients acquire a similar complication that, because of its early timing, has been included with the diagnosis of IIP. Diagnostic criteria include (1) diffuse consolidation on chest radiography, (2) hypoxia requiring oxygen supplementation, and (3) progressively bloody BAL on aliquots from more than one lung segment.[178] Although alveolar hemorrhage is the critical diagnostic criterion, the most typical radiologic findings at presentation are bilateral interstitial infiltrates, with alveolar infiltrates developing rapidly through the course. [181] DAH presents early (within the first 2 weeks post-SCT) with dyspnea, hypoxia, and cough, but usually not overt hemoptysis. This syndrome is very serious, with a reported mortality rate of 77100%. Treatment includes correction of any coagulopathy and aggressive ventilatory support. Limited, uncontrolled experience suggests that high-dose corticosteroids may be effective therapy for this syndrome. [182] [183]

It seems likely that DAH is a subcategory of IIP, and is due to lung injury from chemotherapy and irradiation. Two observations suggest that neutrophils may contribute to its pathogenesis by causing inflammatory damage to injured lung cells. First, DAH often appears at the time of neutrophil recovery. Second, when BAL was undertaken before autologous SCT for 123 patients (of whom 14 eventually acquired DAH), the presence of >20% polymorphonuclear neutrophils or >0% eosinophils before SCT both predicted development of DAH (43% if both were present, 4% if neither). [184]

Graft-versus-Host Disease

Acute GVHD develops in the first 13 months after allogeneic transplantation and can involve the skin, the liver, or the gastrointestinal tract. It manifests in the skin as a maculopapular dermatitis that, when severe, can lead to large bullae or even resemble toxic epidermal necrolysis. GVHD can produce cholestatic hepatitis with marked elevation of serum bilirubin and alkaline phosphatase, but usually only mild transaminase alterations. Hepatocellular function (protein synthesis, coagulation factor production) is not impaired. In the intestine, upper gastrointestinal tract GVHD can produce nausea, vomiting, and anorexia, [185] whereas small bowel and colon GVHD produce large-volume secretory diarrhea.[186] Histologically, acute GVHD is manifest as single-cell degeneration and necrosis in the stem cell compartment of the skin epidermis or the intestinal or biliary epithelium. This yields basal epidermal cell vacuolization with dyskeratosis, exocytosis, and, when more advanced, dermal-epidermal clefting with bullae formation. These changes

APPROACH TO INTERSTITIAL PNEUMONITIS

The presentation of interstitial pneumonitis (IP) after SCT should be considered an urgent medical situation, and empiric, broad-spectrum therapy must be initiated. The choice of therapy is influenced by the following:

1. Timing: Within the first 3 weeks after SCT, IP is more likely to be idiopathic (including diffuse alveolar hemorrhage) or fungal than due to CMV infection. Beyond 6 weeks, idiopathic pneumonitis is unusual and the etiology is more likely infectious. PCP is rare beyond 1 year after transplant. RSV infections are seasonal (fall and winter), and community outbreaks may be prevalent.
2. CMV serology and prophylaxis: If a seronegative recipient has received seronegative graft and noninfective (seronegative or leukocyte-depleted) blood, CMV pneumonia is unusual. Seropositive recipients are at higher risk, although with ganciclovir prophylaxis the risk is markedly reduced. Other prophylactic regimens for CMV, such as acyclovir or intravenous IgG, have still been associated with significant risk for serious CMV infection in the seropositive recipient.
3. Prolonged neutropenia: This factor is associated with infectious etiologies, particularly with fungal pneumonias.
4. Type of transplant: Diffuse alveolar hemorrhage (early post-SCT) has been reported more frequently, although not exclusively in autologous recipients. CMV pneumonia is unusual (23%) in autologous recipients, but it still has a high case fatality rate. All infectious causes are more common after allogeneic SCT. More intensive conditioning regimens (e.g., higher-dose TBI, carmustine) are associated with more frequent pneumonitis.
5. Compliance and prophylaxis: A thorough assessment of what the patient has actually been receiving (e.g., TMP-SMX, penicillin, CMV prophylaxis, transfusions outside the SCT center, etc.) is critical to assess risk.
6. Chest radiograph: The pattern and distribution of the infiltrate may narrow the differential diagnosis. Cardiac enlargement or pleural effusions may suggest pulmonary edema. A chest computed tomography scan may be useful, especially if nodularity or cavitary lesions (possibly fungal) are suspected.
7. Epidemiology: What are the etiologies of other recent cases of IP? This may be most helpful with infections that are horizontally transmitted (RSV) or have common environmental risk factors (*Aspergillus* infection associated with construction).
8. Bronchoalveolar lavage: This can be extremely useful establishing a specific diagnosis or excluding others. CMV rarely causes pneumonia without positive BAL findings (either direct staining of CMV-associated antigens in BAL cells or shell vial culture). BAL also usually detects RSV and *P. carinii*, and can identify alveolar bleeding. It is less sensitive for fungal pneumonias.
9. Lung biopsy: Although this is the gold standard for definitive diagnosis of most of the possible causes of IP, it can often be avoided through use of the clinical data listed previously. It may be necessary for definitive diagnosis of fungal pneumonias, pulmonary changes associated with chronic GVHD (lymphocytic bronchitis or bronchiolitis obliterans), or idiopathic interstitial pneumonitis.
10. Ventilator therapy: Progressive respiratory failure after SCT is rarely (<5%) reversible, especially in adults. Although aggressive diagnostic and therapeutic measures are essential, some centers offer patients (and their families) the option of foregoing mechanical ventilatory support if survival is unexpected. Preliminary discussion of this possible complication in pretransplantation patient counseling may facilitate decision making if respiratory failure does occur.

are accompanied by a modest lymphoid infiltrate, typically of CD8+ T lymphocytes. Similarly, the basal cells of the intestinal glands or the colonic crypts show single epithelial cell degeneration, satellite (lymphocyte) cell necrosis, and sometimes crypt abscesses progressing to mucosal sloughing. In hepatic portal tracts, single cells of bile ducts are affected first with eventual disruption or disappearance of the bile ducts.

Current understanding of the pathogenesis of GVHD suggests that alloreactive donor T lymphocytes recognize histocompatibility antigens on host cells and initiate secondary inflammatory injury, leading to the clinical symptoms of GVHD.[186] The process may be primed or accelerated by conditioning regimen-induced tissue injury and by proinflammatory cytokines (e.g., interleukin-1, TNF), which can augment the alloreactive response.[187] [188] [189] [190] The antigenic targets of GVHD are not well defined. Developing even after major histocompatibility complex (MHC)-identical sibling donor allografts,[191] [192] the frequency of GVHD suggests that minor histocompatibility antigens may be involved. In addition, antigens expressed after tissue damage from chemoradiation therapy conditioning or by environmental pathogens can serve as immune targets for GVHD. GVHD is more frequent and more severe in recipients of partially matched or histoincompatible transplants,[22] [193] suggesting that MHC-encoded molecules may be the prime antigenic targets initiating the alloreactive T-cell response. However, autologous recognition of host tissue antigens may also occur (autologous GVHD) because of poor immune regulation, which can be exaggerated by cyclosporine therapy.[194] This initiating T-cell response leads to a modest lymphoid infiltrate of primarily cytotoxic (CD8+) T cells within the injured tissue targets. However, recent evidence strongly suggests that secondary cytokine release (possibly including TNF and interleukin-1) may amplify and exaggerate the clinical severity of GVHD.[188] [189] [195] [196] [197]

Acute GVHD develops more commonly in older patients, in those with previously alloimmunized donors (usually parous women), or in recipients of marrow from other than a histocompatible related donor. Even with current immunoprophylaxis, 2550% of patients receiving HLA-identical sibling donor marrow[191] [192] and 7090% of patients receiving unrelated donor marrow acquire GVHD.[17] [22] [23] [198] [199] [200] [201] [202] The diagnosis is established by the clinical symptomatology (diffuse maculopapular skin rash, cholestasis, or watery diarrhea), but frequently requires histologic confirmation to distinguish it from other frequent toxicities in the early post-transplantation period (e.g., hypersensitivity drug rash, drug-induced cholestasis, or infectious enteritis). Severe GVHD (clinical grades IIIIV) is associated with poor survival because of the ongoing morbidity and the debility of chronic illness, because of progression to chronic GVHD, and, most important, because of secondary opportunistic infection with bacterial, viral, and fungal pathogens.[203] [204] [205] GVHD of lesser severity (grades III) is less often life threatening and may be accompanied by a graft-versus-leukemia effect that offers protection against malignant relapse.

Prophylaxis of GVHD

The most effective techniques for GVHD prevention have involved depletion of donor T lymphocytes from the donor marrow inoculum, most often coupling immunologic recognition (monoclonal anti-T-cell antibodies) with depletion techniques (immunomagnetic beads, complement cytotoxicity, or toxin immunoconjugates). Although vigorous T-cell depletion prevents acute GVHD, it also increases the risk of graft failure and neoplastic relapse after transplantation (because of absence of the graft-versus-leukemia effect).[206] [207] [208] [209]

Pharmacologic immunosuppression administered in the first several months after transplantation can prevent or blunt the initiating T-cell recognition and proliferative response that triggers GVHD and can allow the development of immune tolerance and complete lymphohematopoietic chimerism. Methotrexate, corticosteroids, ATG, cyclosporine, and, recently, tacrolimus have been used for prophylaxis of GVHD and have successfully reduced both the frequency and severity of clinical GVHD.[193] [210] [211] [212] [213] [214] The balance of complications has made it impossible to demonstrate a clear survival advantage for the use of either T-cell depletion or pharmacologic GVHD prophylaxis. It is clear, however, that some form of GVHD prophylaxis is needed after allogeneic SCT.

Treatment of Acute GVHD

Therapy of acute GVHD requires both immunosuppression to blunt the T-cell-induced inflammatory tissue injury and appropriate supportive care. Therapy should be intensified to achieve a complete or partial response to GVHD therapy, because effective treatment of acute GVHD can both protect against chronic GVHD and improve survival. Limited area (<50%) skin GVHD can be treated with topical corticosteroids alone. Corticosteroids (prednisone 12 mg/kg/day), ATG, and cyclosporine (for those not receiving it as prophylaxis) have been the mainstays of immunosuppressive therapy for GVHD.[203] [204] [205] [206] [215] Steroids produce a complete or partial response in approximately 40% of patients, whereas salvage therapy with ATG can control GVHD in 2540% of the remainder. GVHD of lesser severity and involving only single organs responds to immunosuppressive therapy in 5060% of patients, whereas only 2530% of patients with multiorgan GVHD, especially involving the liver, respond to therapy.[203] [204] [216] GVHD after unrelated donor SCT may also be relatively more resistant to therapy.[213] [214] [217]

Newer immunosuppressive agents, including monoclonal antibodies or immunotoxins directed against T cells or inflammatory cytokines, may be used to limit the severity of GVHD.[195] [196] However, in addition to effective immunosuppression, successful management of acute GVHD involves attention to supportive care to reduce the morbidity and opportunistic infections that accompany GVHD. Adequate nutritional support and fluid replacement for severe diarrhea are required. Avoidance of unnecessary medications can simplify the therapeutic regimen and lessen the chances of adverse drug interactions or hypersensitivity skin rashes. Most important, infection prophylaxis directed against fungi, encapsulated bacteria, CMV, and PCP is an essential element of GVHD treatment.

Chronic GVHD

A related syndrome that occurs later after allotransplantation is called chronic GVHD.[218] [219] This syndrome develops in 3040% of allograft recipients[220] [221] [222] and

usually manifests between 50 and 200 days after transplantation. It occurs most frequently in patients with preceding acute GVHD, but may also occur in 10% of sibling allograft recipients de novo, without any preceding acute GVHD. Chronic GVHD may develop more frequently after mismatched or unrelated donor grafts, or after peripheral blood stem cell transplantation. [9] [223] The manifestations of chronic GVHD resemble autoimmune disease, somewhat similar to scleroderma. An inflammatory dermatitis may progress to severe dermal and periarticular fibrosis with loss of skin appendages (hair and sweat glands) as well as significant skin tightness and loss of joint flexibility. Additional manifestations may include dry eyes and dry mouth, which can resemble Sjögrens syndrome clinically and histologically; enteritis with anorexia, early satiety, malabsorption, weight loss, and wasting;

GRAFT-VERSUS-HOST DISEASE AND STEM CELL TRANSPLANTATION

1. Donor selection: Lower risks of GVHD follow SCT from a genotypically identical sibling donor versus a closely matched related or an unrelated donor. Given equivalent donor: recipient histocompatibility, GVHD risks are lower with younger donors; those who are not previously alloimmunized (by prior pregnancy or transfusion); and gender pairings other than female donormale recipient. If the recipient is CMV seronegative, a seronegative donor is also a better choice.
2. GVHD prophylaxis: Near-complete T-lymphocyte depletion (to $<10^5$ T cells/kg recipient weight) offers the best protection against acute GVHD, although outcome is compromised by greater risks of graft failure and leukemia relapse. Short-course methotrexate (15 mg/m^2 intravenously [IV] day +1 and 10 mg/m^2 +3, +6, +11) plus cyclosporine 6 mg/kg IV from day1 to day +50, then taper); or cyclosporine/steroids; or methotrexate/cyclosporine/steroids have been the most effective chemoprophylaxis regimens.[211] [212] [213] [214]
3. GVHD diagnosis: Early recognition of GVHD symptoms is important in successful management. Skin biopsy to distinguish a GVHD rash from a drug eruption is advised. Nausea, vomiting, or diarrhea suggest gastrointestinal tract GVHD, and endoscopy is necessary to distinguish gut GVHD from infectious or peptic upper gastrointestinal tract symptoms or from infectious, toxic, or osmotic diarrhea.
4. Acute GVHD treatment: Corticosteroids (usually methylprednisolone 12 mg/kg/day) for several weeks and a slow tapering schedule are the usual initial therapy.[203] [204] [205] [215] [216]
5. Chronic GVHD treatment. Initial therapy with modest dose prednisone (0.51 mg/kg/d) and cyclosporine followed by 912 months of maintenance therapy with alternate-day prednisone + cyclosporine has been widely used. Chronic GVHD induction therapy with high-dose methylprednisolone (15 mg/kg IV weekly × 68 weeks) can be added to this regimen. Azathioprine (1 mg/kg/day) can be used, but may enhance the risks of infection without better symptom control. Adequate nutrition, vitamin and mineral supplementation, and liberal fluid intake are also important.
6. Infection prevention during GVHD treatment: Prophylaxis against fungi (*Candida* sp.: fluconazole 100 mg po daily or mycostatin); encapsulated bacteria (penicillin VK 250 mg bid or erythromycin 250 mg bid if allergic); *P. carinii* (TMP-SMX DS bid 23 days/week or pentamidine 300 mg by aerosol once monthly); and CMV (dependent on serostatus) is essential during immunosuppressive therapy of GVHD (acute or chronic). Consider IV IgG replacement if hypogammaglobulinemia persists. Prompt and aggressive treatment of all infections is important.

or severe cholestatic jaundice. Bronchiolitis obliterans occurs in up to 10% of patients with chronic GVHD. [224] [225]

The pathogenesis of chronic GVHD may differ from acute GVHD. Autoimmune manifestations (the clinical similarity to scleroderma) and autoantibody formation (antinuclear antibodies, anti-erythrocyte antibodies, rheumatoid factor, lupus-like anticoagulants) are frequent. In addition, the major complications of chronic GVHD include secondary infection due to hypogammaglobulinemia, severely impaired cellular immune responses (skin test anergy and hypoproliferative T-cell mitogen responses), and splenic dysfunction. This chronic inflammatory syndrome may also be accompanied by stable lymphohematopoietic engraftment, but poor myeloid function with leukopenia, anemia, and, often, an immune complex or autoimmune consumptive thrombocytopenia.

The treatment of chronic GVHD, even more than acute GVHD, demands particular attention to prophylaxis and aggressive therapy of secondary opportunistic infection in addition to specific immunosuppressive treatment. Consequently, ongoing antibacterial, antifungal, and antiviral prophylaxis should be continued as well as intravenous immunoglobulin support for patients manifesting persistent hypogammaglobulinemia. After the onset of chronic GVHD, 2540% of patients die within 2 years, often of secondary infections.

Like acute GVHD, the specific immunosuppressive therapy of chronic GVHD is most often corticosteroids. [226] [227] However, the ongoing and long-lasting nature of the syndrome demands that reduced doses and, if possible, alternate-day steroid therapy be used to minimize the chronic complications of corticosteroid treatment, including osteoporosis, avascular necrosis of bone, [228] [229] cataracts, muscle wasting, hyperglycemia, and cutaneous atrophy. In addition to corticosteroids, azathioprine, cyclosporine, or thalidomide have been used to blunt the T-cellmediated proliferative responses that sustain the autoimmune syndrome. [230] [231] In addition, investigational therapies using ultraviolet light as photoimmunotherapy have shown some, albeit limited activity. [232] [233] Most successful strategies for chronic GVHD, however, incorporate reduced-dose immunosuppressive treatment, aggressive antimicrobial prophylaxis, and long-term therapy for 69 months beyond any active GVHD symptoms, followed by slow withdrawal of therapy. Much longer therapy may be required for some patients, and early withdrawal of therapy has been frequently accompanied by flares of chronic GVHD.

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LATE COMPLICATIONS

Beyond the initial post-transplantation period, subsequent complications result from cumulative organ injury induced by the pretransplantation conditioning, from residual ill effects of any acute complications, and from ongoing immunodeficiency leading to late infections. [234] TBI, alkylating agents, and corticosteroids all contribute to the formation of cataracts, which develop in over one-third of patients by 5 years post-transplantation and often require surgical therapy. [235] Hypothyroidism (3050% of patients) and hypogonadism (6090%) are also common. [236] [237] [238] Therefore, appropriate follow-up care should include monitoring of thyroid and gonadal function, with particular attention to menopausal symptomatology and sexual dysfunction, which may be ameliorated by appropriate hormone replacement. Permanent sterility is expected in nearly all men and most women undergoing transplantation, [238] although transplantation without TBI may be fertility sparing in nearly one-third of men and women. Prepubertal children may retain fertility, although secondary sexual development may be delayed.

Growth retardation is frequently seen in children receiving TBI, [239] with 4050% of children having markedly depressed growth rates for 2 years after SCT. This may be complicated by nutritional deficiency and chronic illness, and especially by chronic GVHD. Regimens without TBI have been proposed as less likely to cause the late growth and development complications in children, [240] but definitive evidence of lesser toxicity is not available. Neurologic complications, including toxic encephalopathy, peripheral neuropathy, and seizures, may result from damage due to previous high-dose therapy and radiation, neurotropic viral infections (e.g., herpes group viruses), or from cyclosporine. Destructive or inflammatory arthropathy may develop. Radiation, steroid therapy, and GVHD may produce avascular necrosis of bone [228] [229] or periarticular contractures that limit mobility.

Finally, pretransplantation chemoradiaton therapy conditioning may be carcinogenic. Secondary neoplasia may develop after SCT, especially if extensive antineoplastic therapy was administered before transplantation. [241] [242] [243] [244] A six- to eightfold higher risk of cancer for SCT recipients has been reportedmostly non-Hodgkin lymphomas, but recently secondary myelodysplastic syndromes and leukemia have been seen, as well. [245]

Stem cell transplantation survivors eventually return to full and complete functional status. Several quality-of-life studies have shown that by 1 year after transplantation, more than three-fourths of patients are back to work, and nearly 90% of autologous SCT recipients report an above-average to excellent quality of life. [246] Allograft recipients recover more slowly, especially those older than 30 years of age and those with chronic GVHD, in whom ongoing physical and psychological problems delay a return to good health. [247] [248] [249] [250]

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Part VIII - Hemostasis and Thrombosis

Chapter 98 - Megakaryocyte and Platelet Structure

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INTRODUCTION

The megakaryocyte is not only the most conspicuous and largest cell in mammalian bone marrow, but arguably, it is also unique among species. First, it is polyploid, a term not to be confused with polynucleated. Polyploidy connotes multiple reduplications of chromosomes (from the usual 2N to 64N or even 128N) contained within the same nuclear envelope. Secondly, megakaryocytes generate their progeny, the blood platelets, not by the usual process of cell division, but by cytoplasmic fragmentation. Thus platelets are anuclear pieces of cytoplasm, have no DNA, and have little, if any, biosynthetic machinery. Their indispensible hemostatic functionwhich during normal homeostasis is maintained at levels ranging from 150,000 to 450,000/mm ^{3} is almost entirely conferred to them by substances synthesized in the mother cell. However, platelets are metabolically extremely active using glycolysis as well as oxidative phosphorylation to support their endocytic and secretory activities. Under normal circumstances, they survive in the circulation for 710 days. Platelets are not migratory and do not diapedese into tissues. Megakaryocytes, on the other hand, are motile cells. Most of them appear to leave the bone marrow intact and shed their platelets in the capillary bed of the lungs (see following). Some fragmentation occurs in the marrow sinusoids, where elongated extensions of megakaryocyte cytoplasm consisting of many platelet fields have been referred to as "proplatelets." Such proplatelets can also be observed to form in vitro, when megakaryocytes are allowed to settle on a surface. Although they are motile, megakaryocytes are not known to leave the vasculature to migrate back into tissues as is the case for most leukocytes. The finely tuned mechanism whereby the number of megakaryocytes in the bone marrow and the number of platelets released into the circulation is maintained is largely attributable to a newly characterized hormone, thrombopoietin, which is described in greater detail elsewhere in this volume (see Chap. 17).

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MEGAKARYOCYTES

Space restriction permits communication of only the most basic information necessary to understand how the structure of megakaryocytes subserves their main function, which is the production of platelets. More detailed accounts have been published elsewhere. [1] [2] [3] Megakaryocytes are believed to derive from the same pluripotential stem cells that generate other blood cells. Their precursors are morphologically indistinguishable from mononuclear cells, even after their commitment to the megakaryocyte/platelet lineage has occurred. Lineage commitment becomes immunologically detectable, by virtue of the lineage-specific epitope GpIIb/IIIa [4] (Fig. 98-1). In rodents, acetyl cholinesterase can be used as an early marker for this cell series. [5] From thence on, morphologic differentiation of megakaryocytes deviates from all other bone marrow elements and the cell is, therefore, easily recognized.

Nucleus

The round or slightly indented diploid (2N) nucleus of the megakaryoblast undergoes a series of endomitosesusually from 2N up to 64N (with an average of 16N)increasing the number of chromosomes from 46 to 368 or even 1,472. During endomitosis the nuclear membrane disappears (Fig. 98-2) and depending on the previous ploidy attained, several mitotic spindles form each with its own centrosome. The number of centrioles seen during interphase probably correlates with the ploidy of the cell (Fig. 98-3). (For a review see reference 6 .)

After endomitosis, the nuclear membrane reforms and the reduplicated DNA disperses. The result is a very large, multilobed nucleus, which distinguishes the cell from an osteoclast, also a large cell, but with many separate nuclei. The nucleoli of megakaryocytes are small and the abundant biosynthetically inactive heterochromatin is distributed along the nuclear membrane. Thus the megakaryocyte nucleus resembles that of a mature cell rather than a "blast" (Fig. 98-4). After the platelets have been shed, there remains a naked or denuded nucleus, which on light microscopy of marrow smears or sections looks like an oversized pyknotic erythroblast (Fig. 98-5). Regeneration of cytoplasm from a denuded nucleus has never been observed. It is noteworthy, however, that a physiologically denuded megakaryocyte nucleus does not have the appearance of a nucleus that has undergone apoptosis as is the case when normal thrombopoiesis has been artificially stimulated with cytokines, such

Figure 98-1 A "mononuclear" cell seen in the inset was isolated from human umbilical cord blood with anti-CD34-coated beads. Subsequently, the specimen was incubated with anti-GpIIb/IIIa. The epitope was detected by the immuno-gold technique (arrow indicating black particles), which revealed that this cell had been committed to the megakaryocyte/platelet lineage. (For details see reference [69] .) (Magnification of whole cell in inset × 6000; detail × 22,000.

Figure 98-2 Megakaryocyte in the process of endomitosis. Note absence of nuclear membrane and arrangement of chromosomes in the configuration of several metaphases. (× 3,000.)

as interleukin-6. [7] Denuded megakaryocyte nuclei are difficult to find in normal bone marrow, which supports the contention that most megakaryocytes leave the medullary space intact.[8] On the other hand, numerous denuded megakaryocyte nuclei are seen in the bone marrow of individuals infected with human immunodeficiency virus (HIV), where their presence has diagnostic significance. [9] This phenomenon may be attributable to impaired migration of the infected cells or inability of the phagocytic system to remove the effete material.

Figure 98-3 Section through the pole of a megakaryocyte in interphase (nucleus is not within the plane of section). There are at least 14 centrioles (arrow). Diploid cells have only two centrioles. (× 10,500.)

Figure 98-4 Mature, fully granulated megakaryocyte exhibiting the extensive membrane system demarcating platelet territories (arrows). Note multilobed nucleus with inconspicuous nucleoli and peripherally distributed heterochromatin. The peripheral zone (PZ) is devoid of organelles. On light microscopy, blebbing of the peripheral zone is often mistaken for "budding" platelets. However, platelets are not released by budding (see text). (× 4,000.)

Cytoplasm

Conventionally, maturation of the megakaryocyte cytoplasm has been divided into three stages, which is of little relevance to the thrust of this chapter. Suffice it to say that maturing megakaryocytes, like other granulated cells, have an abundance of ribosomes and profiles of rough endoplasmic reticulum, where protein synthesis occurs. They have several Golgi stacks from

Figure 98-5 Section of a bone marrow aspirate stained with hematoxylin and eosin, obtained from an HIV-infected patient illustrates two mature megakaryocytes (M) and a denuded megakaryocyte nucleus (arrow). (× 1,000.)

where the synthesized proteins are packaged into granules. The largest granules, which are referred to as -granules, measure 200500 nm in diameter and contain the lineage-specific proteins, such as -thromboglobulin, platelet factor 4 (PF4), thrombospondin, von Willebrand factor, as well as endocytosed plasma proteins, such as fibrinogen, albumin, IgG, factor V, and others. [10] [11] [12] Recently, the amyloid precursor protein APP has been identified in DAMI cells, a megakaryocyte cell line, which differs from primary megakaryocytes in many respects.[13] The author has been unable to detect APP in freshly isolated megakaryocytes by immunoelectron microscopy. In addition to the -granules, there are smaller vesicles and granules measuring 50100 nm in diameter. These are considered lysosomes because they contain acid phosphatase, aryl sulfatase, and cathepsin. [14] [15] The mechanism whereby sorting of these substances from the Golgi to different organelles is

accomplished can only be deduced from studies carried out on other cell types, but has not yet been fully elucidated in megakaryocytes.

By far the most interesting feature of megakaryocyte cytoplasm is a system of membranes referred to as the demarcation membrane system (DMS). Its main function appears to be the demarcation of cytoplasmic areas likely to delineate future platelets. The literature dealing with the formation of the DMS is confusing because most investigators have focused on a single mechanism.[16] [17] Yet, it is evident, that the DMS is derived from multiple sources, including Golgi vesicles, coated vesicles (Fig. 98-6), and components of invaginated plasma membrane (Fig. 98-7). As holds true for circulating platelets, the peripheral zone of the megakaryocyte cytoplasm is devoid of membranes or organelles (Fig. 98-4). Conceptually, one may assume that some trigger is necessary to induce invagination of the plasma membrane and its fusion with the DMS. This would result in extrusion of cytoplasmic fragments of variable size and in essence constitute platelet release. Much has been made of the term proplatelet, (reviewed in reference [19]), , when this signifies nothing more than protrusion of megakaryocyte cytoplasm or a pseudopod, which, in the case of the megakaryocyte, comprises many platelet fields extending into a sinusoidal space and destined

Figure 98-6 Detail of a megakaryocyte that had been incubated with cationized ferritin (CF) before fixation. Ferritin coats the surface membrane and, subsequently, is seen to line the entire DMS. The arrow indicates fusion of a coated vesicle with the DMS. The arrowhead indicates a segment of the DMS that did not react with CF. (× 60,000.) *(From Behnke,[17] with permission.)*

Figure 98-7 Detail of a megakaryocyte that had first been incubated with cationized ferritin like the cell depicted in Fig. 98-6 . Subsequently, latex particles (L) were added to the suspension. Coalescence of invaginated membrane compartments is clearly evident. Also note that some segments of the DMS did not react with CF (arrowheads). (× 34,000.)

to fragment into individual platelets at a later time in the circulation (Fig. 98-8). When maturing megakaryocytes are fixed in suspension culture, fragmentation of cytoplasm occurs randomly into pieces comprising one or more platelet fields (Fig. 98-9). On the other hand, when freshly collected bone marrow is placed on a glass surface, megakaryocytes will spread.

Figure 98-8 "Pseudopod" or "proplatelet" extending into the sinusoidal space. The nucleus of the cell is not in this plane of section. Arrows indicate sinusoidal endothelium. L, leukocytes; E, erythrocyte. (× 3,000.)

Figure 98-9 Megakaryocyte grown in a suspension culture system clearly shows fragmentation process. Putative platelets are of variable size and the peripheral zone of the megakaryocyte (PZ) does not necessarily form part of the platelet envelope. (× 3,000.) *(Reproduced with permission from Zucker-Franklin D, Petursson S: Thrombocytopoiesis: analysis by membrane tracer and freeze-fracture studies on fresh human and cultured mouse megakaryocytes. J Cell Biol 99:420, 1984. Copyright The Rockefeller University Press.)*

Under such in vitro conditions, the development of long filiform processes showing constrictions presumed to demarcate platelet territories has been recorded by time-lapse cinematography[20] (Fig. 98-10). Although interesting, these conditions can hardly be equated with those prevailing in vivo. The author encourages use of the term "compound" platelet denoting a large platelet consisting of multiple platelet territories (Fig. 98-11A), rather than the terms descriptive of size or shape, such as proplatelet, giant platelet, or megathrombocyte.

PLATELETS

Platelets circulate as lenticular pieces of membrane-bound cytoplasm measuring 23 m in diameter and having an average volume of 68 femtoliter (fL). These measurements are important because, in clinical situations, platelet number may not always reflect total platelet volume. [2124] Yet, very large compound platelets (see previous discussion) may partially compensate for a shortage in their numbers. Figures 98-12 and 98-13 depict equatorial and cross sections, respectively, of a resting "unit" platelet as opposed to a giant or "compound" platelet, consisting of many platelet territories depicted in Figure 98-11A . The lenticular shape of the platelet is maintained by a microtubule coil, which in most sections appears as a bundle of microtubules. Any shape change, no matter what the stimulus, be it mechanical or receptor mediated, causes depolymerization of the microtubules. As in other cells, the microtubules are also sensitive to low temperature, colchicine, and vinca alkyloids, whereas they are stabilized by Taxol and its derivatives. [25] Microtubules reform in pseudopod-like structures when shape change is complete, i.e., after platelets have participated in the stabilization of a clot. No band of microtubules seems to exist in compound platelets, although microtubules can be seen to be oriented in various directions.

Organelles

Practically all platelet organelles have been preformed in megakaryocytes. The most conspicuous among these are the -granules (Figs. 98-11 and 98-13). They contain the lineage-specific proteins, mentioned above, as well as endocytosed substances. [26] [27] Because platelets continue to endocytose plasma proteins, platelet -granules may be larger than those of megakaryocytes, though this has not yet been precisely measured. The granule proteins have been identified mostly by immunologic means (e.g., Fig. 98-14). Some of them, e.g., P-selectin (PADGEM, GMP-140, CD62) and GpIIb/IIIa, are located almost exclusively on the granule membrane.[28] [29] [30] Others, like von Willebrand factor, appear to be associated with intragranular tubular structures [31] or in vaguely delineated regions, only defined on the basis of varying electron opacity. [32] Whether such stratification is due to a substructure within the granule matrix, not preserved by currently used fixatives, or whether there is a differential effect of embedding materials on various substances contained in the granules is not known (Figs. 98-13 , 98-15 , and 98-16). In addition to the aforementioned proteins, the presence

Figure 98-10 Scanning electron photomicrograph of a megakaryocyte that has been allowed to spread on glass. The cell unravelled into many filamentous processes showing constrictions, which are presumed to separate platelet territories. N, nucleus. *(From Haller and Radley,[20] with permission.)*

Figure 98-11 (A) A compound platelet, which customarily has been referred to as a giant platelet, when examined by light microscopy, is seen to consist of numerous platelet territories separated by membranes akin to the DMS in megakaryocytes. At higher magnification, many structures, usually not seen in mature platelets, such as rough endoplasmic reticulum and clustered ribosomes, can be resolved. (× 8,000.) **(B)** Detail of the area indicated by the arrow in **(A)**

of protease Nexin II (amyloid -protein precursor) in -granules has also been well established, [33] even though it is not yet clear whether this enzyme is endocytosed or synthesized in this cell line. Lastly, there are a few extremely electron-opaque granules, also called dense bodies, which contain the nonmetabolic pool of adenine nucleotides, serotonin, pyrophosphate, and calcium ions, [34] [35] [36] the content of which is also released during platelet activation.

As in other cells, glycogen particles and mitochondria are the organelles subserving the platelets' metabolic activities (Figs. 98-15 and 98-16), whereas small vesicles are representative of lysosomes discussed before. [14] [15] [37] As would be expected, compound platelets, or even single platelet units, which may be prematurely released during accelerated thrombocytopoiesis, such as following hemorrhage or stimulation with thrombopoietin, [38] may show ribosomes, rough endoplasmic reticulum,

Figure 98-12 Equatorial section through a platelet showing the microtubule coil with a "loose" end to good advantage (arrow). G, -granule; M, mitochondrion. (× 22,000.)

or even other organelles not seen in mature platelets during normal homeostasis (Fig. 98-11B). Another structure, the dense tubular system is usually not enumerated among the organelles, but this designation would be justified because it consists of a membrane-bounded sac containing platelet-specific peroxidase [39] and glucose-6-phosphate dehydrogenase. [40] By analogy with the sarcoplasmic reticulum, it has also been postulated to be a major site of prostaglandin synthesis. [41] [42]

The Canalicular System (Endocytosis and Secretion)

The system of membrane-bound channels, which is likely derived from the DMS of the megakaryocyte, is often referred to as the open or surface connected canalicular membrane system. It is by no means "open" because energy is required for even solutes to be taken into these channels. [43] However, as illustrated in the megakaryocyte (Fig. 98-7), large objects, such as latex particles, bacteria, and viruses (Fig. 98-18), can be engulfed by invagination of the plasma membrane, which subsequently coalesces with the canalicular system. Fusion of the invaginated membrane containing endocytosed objects with -granules, akin to what occurs between lysosomes and phagocytic vacuoles in granulocytes, [44] has never been observed in platelets. On the other hand, the uptake of plasma proteins, such as fibrinogen and IgG, is likely to be accomplished by specific receptor-mediated vesicular transport. [45] Apart from a storage function, the endocytotic activity of the platelet appears to be of only minor physiologic significance. In this context, it should be remembered that the platelet has little, if any, proteolytic or bactericidal ability and that, therefore, microorganisms, including HIV (Fig. 98-18), might be stored or transported by platelets to other sites, such as the macrophage/phagocytic system, with impunity.[46] This could have adverse consequences.

The so-called release phenomenon or the mechanism whereby platelets secrete much of their granule content is still controversial. Even before the advent of electron microscopy,

Figure 98-13 Resting platelet seen in cross section. The peripheral band of microtubules is indicated by arrows. Note compartmentalization of substances in several -granules as evidenced by different electron opacity. C, canaliculus. (× 30,000.)

it was noted that, almost immediately on stimulation by any aggregating agent, be it adenosine diphosphate (ADP), thrombin, epinephrine, collagen, or others, the granules move to the center of the platelet (Fig. 98-19). This is in marked contrast to what happens in any other secretory cell where, as a rule, the granule membranes fuse with the surface membrane when their content is discharged. In the case of platelets, there is little doubt that coalescence of granules occurs within the cytoplasm (Fig. 98-20A and B), and with diligent search, images suggesting that the granule content exits via the surface connected canalicular system can be found (Fig. 98-20C).[47] However, during this process, the canalicular membrane does not seem to become incorporated into the plasma membrane as is the case for other exocrine cells (e.g., basophils and mast cells). [48] On the other hand, several granule membrane proteins (e.g., P-selectin) appear to be translocated to the surface.[28] This occurs even before degranulation is conspicuous. The determination of P-selectin or activated GpIIb/IIIa [49] by flow cytometry is rapidly becoming one of the most sensitive and efficient methods for the detection of platelet activation (Fig. 98-21).[50] This is of particular interest because

Figure 98-14 Detail of a platelet that was embedded in Lowicryl and reacted with an antibody to fibrinogen by the immuno-gold method. The gold particles (black) indicate the presence of fibrinogen in the -granules. (× 91,000.)

subtle forms of in vivo platelet activation may have significant clinical implications in the area of cardiovascular and thrombotic diseases. [51] [52] It is likely that such membrane proteins are laterally translocated by flow within the plane of the membrane by means of a process akin to the "capping" phenomenon, [53] rather than by physical evagination of entire membrane regions. It has also been suggested that the pores seen by freeze fracture on the external membrane leaflet of resting platelets may widen during the secretory process.

Adhesion/Cohesion, Contraction

A chapter meant to cover structure cannot do justice to the most important function of plateletstheir role in hemostasis and clot retraction. What is there to be seen that makes platelets sticky, allows them to adhere to each other as well as to tissue matrices denuded of endothelium? Obviously, this capacity must be vested in their surface membrane.

To begin with, the so-called glycocalyx of the platelet surface membrane is much thicker than that of other circulating blood cells. This can be shown most dramatically, when the cells are fixed in the presence of ruthenium red (Figs. 98-15 , 98-16 , and 98-17). During aggregation, "bridges" form between adjacent platelets, which do not appear to alter the structural integrity

Figure 98-15 Detail of a platelet fixed in the presence of ruthenium red to show the thickness of the glycocalyx (arrow) as well the variation in electron opaqueness within -granules. M, mitochondrion; MT, microtubules. (× 38,000.)

Figure 98-16 Detail of a platelet taken from the same specimen as the platelet depicted in Figure 98-15 showing glycogen particles to good advantage. Gl, glycogen. (× 45,000.)

of the membrane itself (Fig. 98-22). The bridges can be resolved during all phases of adhesion, aggregation, and release, even after the platelets are completely degranulated and have undergone what used to be called "viscous metamorphosis" because of the refractility of the thrombus by light microscopy. [54] [55] Fibrinogen as well as fibrin have been detected at the site of the bridges by immunohistochemical means, [56] but the chemical nature of these structures remains to be more completely defined.

Even more intriguing than the thickness of the glycocalyx is the observation that, on freeze fracture, the partition coefficient of the intramembranous particles (IMP) of the platelet plasma membrane has been observed to be the reverse of that seen in any other mammalian cell, including the megakaryocyte, [43] i.e.,

Figure 98-17 Detail of an erythrocyte that was present in the same specimen as the platelets depicted in Figures 98-15 and 98-16 to illustrate the difference in comparative thickness of the glycocalyx between the cells. (× 44,000.)

Figure 98-18 Platelet from a specimen of platelet-rich plasma prepared from a healthy donor and incubated with HIV-infected cultured lymphocytes. Numerous virus particles (arrow) were seen to have been endocytosed. (× 29,000.) *(From Zucker-Franklin et al.,[46] with permission.)*

twice as many particles remain associated with the external than with the internal leaflet of the bimolecular membrane. Although little progress has been made in recent years to identify the biochemical nature of the IMP, it is likely that they represent integral membrane proteins. Therefore, proteins intimately associated with the external leaflet may be assumed to be more susceptible to external triggering. By teleologic reasoning, this may preclude activation and degranulation of megakaryocytes, when platelets are activated to function in hemostasis.

The contractility of platelets and their role in clot retraction were noted more than 100 years ago. Bettex-Galland and Lüscher extracted the contractile protein, which they called thrombosthenin.[57] It was also shown that thrombosthenin constitutes more than 20% of the platelet's protein. [57] [58] Subsequent advances in biochemical and ultrastructural techniques permitted more definitive recognition of actin and myosin. [59] [60] [61] [62] [63] When

Figure 98-19 Platelet from a specimen of platelet-rich plasma fixed 1 minute after addition of ADP. The granules are crowded in the center. The peripheral band of microtubules has depolymerized. No granules are seen in contiguity with the surface membrane. (× 34,000.)

Figure 98-20 (A) and **(B)** Details of platelets from a specimen of platelet-rich plasma fixed 30 seconds after the addition of 0.1 U thrombin. Although no granules are seen at the plasma membrane, coalescence of granules evidenced by size, contour, and differing electron opacity, has clearly occurred. (A × 52,000; B × 35,000.) **(C)** Platelet from the same specimen as **(A)** and **(B)**

platelets are subjected to hypotonic media, an amazing network of cytoskeletal filaments can be resolved (Fig. 98-22B). Such filaments are much more prominent in platelets than in other mammalian nonmuscle cells, which are also known to contain actin and myosin.

Even more significant may be the observation that these cytoskeletal filaments are closely associated with the plasma membrane as well as with the membranes constituting the canalicular system, but they do not appear to be associated with the membranes of the -granules. [62] Although the role of the

Figure 98-21 Flow cytometric analysis of platelet activation using two monoclonal antibodies: PAC 1, an antibody that recognizes the activation-dependent epitope of GpIIb/IIIa (horizontal axis) and an antibody recognizing P-selectin (vertical axis). The left panel shows unstimulated platelets; the right panel shows platelets stimulated with 5 M ADP. *(Courtesy of Dr. Kenneth Ault, Main Medical Center Research Institute.)*

Figure 98-22 (A) Bridges (B) between two adjacent platelets in a platelet aggregate. The bridges span the extracellular space, but they do not appear to alter the trilaminar structure of the platelet membrane. (× 120,000.) **(B)**

cytoskeletal proteins in platelet contractility is beyond question, the mechanism whereby a transmembrane connection between surface receptor/ligand complexes (GpIIb/IIIa/fibrinogen), located on the external aspect of the membrane, and the contractile elements (e.g., actin) located in the cytoplasm, remains to be elucidated. Only a very small portion of membrane actin has been shown directly associated with GpIIb/IIIa. [64] Other cytoskeletal proteins, such as vinculin, fibulin, and talin may be involved. Among these, talin has emerged as the most important, because it has been proven to be instrumental in the formation

Figure 98-23 (A) Talin appears to be uniformly distributed in the cytoplasm of resting platelets. **(B)**

Figure 98-24 Platelet aggregate formed on enzyme-treated cartilage to uncover collagen fibrils (arrow). As a rule, platelets do not adhere to cartilage because proteoglycan precludes interaction with underlying collagen. (× 13,000.) *(From Zucker-Franklin D, Rosenberg L: Platelet interaction with modified articular cartilage. J Clin Invest 59:641, 1977, with permission.)*

of adhesion plaques between fibroblasts and substrates to which they attach. [65] Talin is a 235,000 d protein, which is very abundant in human platelets, making up more than 3% of total platelet protein. [66] [67] In the resting platelet, talin is diffusely distributed throughout the cytoplasm, but on stimulation, either in suspension or when adherent, talin assumes a submembranous location [68] (Fig. 98-23). This relocation is mediated by calcium-dependent proteolysis of talin [69] and facilitates colocalization of talin with -chains of integrins. [69]

Following adhesion, cohesion, and contraction, there remains a refractile thrombus, referred to as a "hemostatic plug" in earlier literature. [70] The pathophysiologic function of this structure in hemostasis is so well recognized that it requires no comment. It is, however, remarkable, that within this aggregate, individual platelet territories remain distinct (Figs. 98-24 and 98-25). Mechanical trauma would be just as likely to rupture adjacent tissue as renting the thrombus along cleavage planes between platelet membranes.

For the sake of completeness, the mechanism whereby thrombi are removed to permit revascularization and wound healing should be briefly addressed. This process appears to be accomplished by phagocytic cells. The event can be simulated in vitro by adding an aggregating agent, such as ADP or collagen, to a suspension of platelets and leukocytes. As illustrated in Figure 98-25 , granulocytes appear to be attracted to the thrombus and can be seen in the process of ingesting platelet debris. It is not difficult to extrapolate this image to conditions prevailing in vivo. It may be assumed that substances released

Figure 98-25 Platelet aggregate following the addition of collagen to a suspension of leukocytes and platelets in heparinized plasma. Monocytes and neutrophils are seen in the process of ingesting platelet debris. (× 2,800.) *(From Zucker-Franklin, with permission.)*

by aggregating platelets are chemotactic for leukocytes. These still remain to be identified.

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Chapter 99 - The Molecular Basis for Platelet Function

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Platelets ordinarily circulate in blood vessels as individual entities that do not interact with other platelets or other cell types. A transition from this nonadhesive to an adhesive state can be rapidly initiated if platelets are exposed to an appropriate stimulus. A scenario of the platelet adhesive reactions initiated in response to an injury to a blood vessel wall, as exemplified by rupture of an atherosclerotic plaque, is illustrated in Figure 99-1 . Disruption of the endothelial cell lining of the vessel exposes constituents within the subendothelial matrix, including a variety of adhesive proteins that can support initial platelet attachment. After attachment, platelets may undergo a spreading reaction that permits formation of multiple and tight contacts between the cell surface and the matrix. These additional contacts may be critical in stabilizing the association of the platelets with the matrix in flowing blood. In conjunction with these adhesive reactions, the cells often encounter agonists in the microenvironment that can trigger platelet secretion. The secretory response results in the release of the contents of intracellular storage granules from within the platelet. Granule constituents include substances that can stimulate circulating platelets and cause them to acquire new adhesive properties. These stimulated platelets interact with one another, during platelet aggregation, to form an effective plug to seal the injured vessel wall and prevent excessive blood loss. This series of platelet responsesattachment, spreading, secretion, and aggregationis essential for the hemostatic function of platelets. Nevertheless, these same events, occurring on an injured endothelial cell or on an atherosclerotic plaque, may result in the formation of a platelet-rich thrombus, which can compromise the patency of the blood vessel and lead to thrombosis. At the other extreme, abnormalities in platelet adhesive reactions of either a genetic (e.g., Glanzmanns thrombasthenia or Bernard-Soulier syndrome) or an acquired origin can result in bleeding episodes. Thus, platelet adhesive reactions and secretion are central events in health and disease processes; bleeding, hemostasis,

Figure 99-1 Hemostatic response of platelets to injury. **(A)** Disruption of the endothelial cell lining of the blood vessel exposes constituents of the subendothelial matrix. **(B)** Platelets attach to, and spread on, the matrix constituents. **(C)** Platelet secretion may be initiated. **(D)** Released platelet constituents can activate additional platelets, which aggregate with one another to form a thrombus.

and thrombosis are delicately balanced on these platelet functions.

This chapter addresses the molecular basis of platelet adhesive reactions and secretory responses. Great strides have been made in defining the mechanisms that govern these functional responses. At the heart of these platelet responses are ligand-receptor interactions. Indeed, the platelet has often served as a model cell type for studying ligand-receptor interactions and establishing basic mechanisms of cell adhesion and secretion.

MOLECULAR BASIS OF PLATELET ADHESION

Substrates for Platelet Attachment and Spreading

Some of the major subendothelial matrix proteins that support platelet attachment or spreading reactions are listed in Table 99-1 . From the extent of this list, it is clear that the platelet can adhere to a variety of substrates once the endothelium has been disrupted. The endothelium must also create an effective barrier to prevent circulating platelets from reaching the matrix and initiating thrombus formation. In addition to serving as a physical barrier, endothelial cells synthesize and elaborate components, notably prostaglandin I_2 , nitric oxide, and an enzyme that degrades adenosine diphosphate (ADP), which prevent platelet activation and impart a nonthrombogenic character to the normal endothelium (see Chaps. 100 and 101).

Several considerations have an impact on the role of the individual matrix constituents in mediating platelet adhesion:

1. Not all of the adhesive proteins listed in Table 99-1 support the same spectrum of platelet adhesive responses. For example, under some conditions, platelets attach to, but do not spread on, laminin,[1] whereas von Willebrand factor (vWF) and fibronectin support cell attachment and spreading. [2] [3] [4] Collagen not only supports the attachment and spreading of platelets but can provoke a secretory response. [5] Of the multiple forms of collagen, types I, III, and VI are regarded as being particularly important in supporting platelet adhesion.

2. There is growing evidence that the phenotypic properties of endothelial cells from different blood vessels vary; it also is likely that the composition of the subendothelial matrix varies. Thus, certain proteins may play a dominant role in supporting platelet adhesion in certain blood vessels. Moreover, even at the same anatomic location, the nature of the injury can expose different substrata.
3. Shear rate developed by flowing blood varies with vessel caliber and greatly influences platelet adhesion. Shear is particularly important in defining the contribution of vWF to platelet adhesion. Patients with von Willebrand disease (see Chap. 114) have a bleeding diathesis, attesting to the importance of vWF in supporting platelet function. Yet, in in vitro experiments, a role of vWF in platelet adhesion is demonstrable at high but not at low shear rates. [3] [6] By contrast, a role for fibronectin in supporting platelet adhesion can be demonstrated at both high and low shear. [7] [8]
4. A variety of these adhesive proteins interact with one another. For example, vWF, fibronectin, and thrombospondin all bind to collagen [9] [10] [11] (although they exhibit differential reactivity with different collagen types). These interactions may bridge the platelet to a matrix protein or may modulate the adhesive properties of a matrix protein.
5. Several of the adhesive proteins are present in platelet secretory granules or in plasma, or both, as well as being matrix constituents. vWF and fibronectin are present in all three locations, and thrombospondin is a major platelet granule constituent. [12] [13] Proteins from all three sourcesthe matrix, the platelet, and the plasmacontribute to platelet adhesion. The adhesive proteins derived from these different sources may be functionally distinct because they are not structurally identical. For example, the degree of vWF and fibronectin multimerization differs for molecules derived from plasma and the matrix.
6. Matrix proteins are subject to degradation by a variety of proteolytic enzymes. Such proteolysis can modulate the adhesive properties of the matrix proteins. In some cases, cryptic adhesive sequences can be exposed that interact with additional sets of platelet adhesion receptors. [14] [15]

TABLE 99-1 -- Subendothelial Matrix Constituents That Support Platelet Adhesion

Matrix Constituent	Comment
Collagens	Large family of proteins with certain members supporting platelet adhesion, aggregation, and secretion
von Willebrand factor	Large multimeric protein critical for the hemostatic function of platelets
Fibronection	Dimeric or multimeric protein that supports attachment and spreading of platelets
Thrombospondins	Trimeric proteins exhibiting both adhesive and antiadhesive properites
Laminins	Proteins supporting platelet attachment
Microfibrils	A fibular bundle of protein constituents found in certain matrices

Overall, the subendothelial matrix should be viewed as a dynamic and mutable interface that provides multiple substrates to support platelet adhesion.

Platelet Adhesion Receptors

The individual adhesive proteins come in contact with the platelet by serving as ligands for specific cell surface receptors (Table 99-2). Because several nomenclature systems have been used to identify the membrane proteins of the platelet, the same receptor may have multiple designations. One of the most widely used nomenclatures is based on electrophoretic mobility of the membrane proteins on polyacrylamide gel systems, in which the highermolecular-weight proteins move more slowly. This separation gave rise to glycoprotein (GP) I, II, III, and so on, with GPI having the highest molecular weight. As the gel systems used became more discriminating, several proteins were discerned in the GPI position, hence GPIa, GPIb, and GPIc, and so forth. [16] Further refinement is still required; for example, there are at least three distinct polypeptides in the GPIc position. Several of the membrane proteins exist on the platelet surface as noncovalent complexes; thus, GPIb-IX, GPIc-IIa, and GPIIb-IIIa can be regarded as single-membrane proteins. Other nomenclature systems have arisen from the fact that several platelet membrane proteins are present and have been assigned different names on other cell types. This is the basis for the VLA (very late antigens) designations for some of the platelet membrane proteins. Similarly, certain proteins may also have extracellular matrix (ECM) and leukocyte differentiation antigens

TABLE 99-2 -- Platelet Receptors for Adhesive Proteins

Ligand	Receptor(s)	Other Designations

Collagen	GPIa-IIa	VLA-2, $_{2\ 1}$
	GPIIb-IIIa	$_{IIb\ 3}$
	GPIV	GPIIIb, CD36
	GPIV	
Fibrinogen	GPIIb-IIIa	$_{IIIb\ 3}$
Fibronectin	GPIc-IIa	VLA-5, $_{5\ 1}$
	GPIIb-IIIa	$_{IIIb\ 3}$
Thrombospondin	Vitronectin receptor	$_{V\ 3}$
	GPIV	GPIIIb
	Integrin-associated protein	IAP
Vitronectin	Vitronectin receptor	$_{V\ 3}$
	GPIIb-IIIa	$_{IIIb\ 3}$
von Willebrand factor	GPIb-IX	$_{IIIb\ 3}$
	GPIIb-IIIa	
Laminin	GPIc-IIa region	VLA-6, $_{6\ 1}$

VLA, very late antigen.

(CD) designations. These latter nomenclature systems are used inconsistently in the platelet literature. Some receptors have been named on the basis of their function (e.g., the vitronectin and the fibronectin receptors). Although functional designations seem highly appropriate from a descriptive standpoint, at least two membrane proteins on platelets can serve as receptors for vitronectin and fibronectin (see Table 99-2), and several platelet constituents have been referred to as collagen receptors. Beyond creating a nomenclature complexity, the redundancy of the platelet receptors enables the cell to establish multiple contacts with a single matrix constituent; thus, a single ligand may initiate several distinct functional responses by engaging different receptors.

The Integrin Family of Adhesion Receptors

Many of the adhesive protein receptors on platelets are members of the integrin family. The integrins are broadly distributed heterodimeric (two subunits) cell surface molecules that share certain structural, immunochemical, and functional properties. [17] [18] [19] [20] [21] [22] [23] [24] [25] [26] The -subunits, of which eight are known, are highly homologous to one another, exhibiting at least 3545% identity at the primary amino acid sequence level. The -subunits, of which 14 are known, are also similar to one another but exhibit less extensive sequence identity. [21] [27] The -subunits are synthesized as single-chain polypeptides; some, such as GPIIb, are proteolytically processed to a two-chain form.[28] [29] [30] [31] Each -subunit combines in a noncovalent complex with an -subunit to form an adhesive protein receptor. A single -subunit can combine with several -subunits. Platelets express two major -subunits, $_{1}$ and $_{3}$ ($_{2}$ has been reported to be present at low levels[32]), and five -subunits (Table 99-3). Of the integrins expressed

TABLE 99-3 -- Platelet Integrins

Integrin	Ligand
GPIa-IIa (VLA-2, $_{2\ 1}$)	Collagen
GPIc-IIa (VLA-5, $_{5\ 1}$)	Fibronectin
GPIIb-IIIa ($_{IIb\ 3}$)	Fibrinogen, fibronectin, vitronectin, von Willebrand factor
Vitronectin receptor ($_{V\ 3}$)	Fibrinogen, fibronectin, vitronectin, von Willebrand factor, thrombospondin
GPIc-IIa region ($_{6\ 1}$)	Laminin

VLA, very late antigen.

on blood cells, GPIIb-IIIa is the most narrowly distributed and is restricted predominantly to platelets/megakaryocytes. GPIIb-IIIa may contribute to the association of platelets with tumor cells, an interaction important to the metastatic dissemination of neoplastic cells. [33] [34] [35]

Role of GPIb-IX in Platelet Adhesion

GPIb-IX is a notable example of a cell surface molecule involved in platelet adhesion that is not a member of the integrin family. Platelets from patients with Bernard-Soulier syndrome lack GPIb-IX; these patients have a marked bleeding diathesis [36] [37] [38] (see Chap. 130). A major role of GPIb-IX in hemostasis can be traced directly to its function as a receptor for vWF. vWF is made up of multiple functional domains, including domains involved in binding to GPIb and to matrix constituents such as collagen and microfibrils [9] [39] [40] [41] [42] [43] [44] [45] (see Chap. 113). Thus, vWF in the matrix may directly mediate platelet adhesion, and the plasma or platelet forms of the molecule may bridge the cells to other matrix components. In vitro, vWF does not interact directly with GPIb on platelets. An interaction can be measured in the presence of ristocetin, an antibiotic, or botrocetin, a snake venom peptide. Altered forms of vWF, asialo-vWF (the protein treated to remove the sialic acid residues from its carbohydrate moieties) and bovine vWF can interact directly with platelets. [46] [47] [48] It appears that high shear serves as the physiologic counterpart of ristocetin or botrocetin in humans. High shear alters the conformation of vWF or GPIb-IX, permitting their high-affinity interaction. [45] Such high shear stress can be attained in the microcirculation or in narrowed arteries.

GPIb is multifunctional because it can also serve as a binding site for thrombin. [50] [51] It is also a target for certain of the drug-induced platelet antibodies. [52] The full primary structures of both GPIb[53] [54] and GPIX[55] have been determined from cDNA cloning approaches. GPIb is composed of a heavy chain () and a light chain (), and both span the platelet membrane. GPIb associates with the cytoskeleton within the platelet. [56] [57] [58] This chain is highly susceptible to proteolysis, and a large proteolytic fragment, glycocalicin, can be detected in the plasma of some patients with thrombocytopenic disorders. [59] GPIX is a small, single-chain polypeptide with a molecular weight of 20,000 d. The contribution of GPIX to the function of GPIb-IX is uncertain. GPV also is deficient in Bernard-Soulier platelets and forms a loose association with GPIb-IX.[36] [37] An important consequence of occupancy of GPIb-IX by vWF is the induction of intracellular signaling events that ultimately lead to activation of GPIIb-IIIa and then platelet aggregation [60] (see later discussion). Such communication between adhesion receptors is a repeating theme for platelets as well as other cells.

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PLATELET SECRETION

The foregoing discussion has emphasized the adhesive functions of platelets in mediating the physical closure of breaks in blood vessels. Another important function of platelets is the release of a variety of substances that stimulate or inhibit platelets or other blood and vascular cells, which can covalently modify the thrombus to affect its mechanical properties, as well as regulate coagulation, contribute to cell adhesive events, and modulate the growth of cells of the vessel wall. Thus, the platelet is not simply the stop leak of the vasculature, but also has the capacity to signal its presence in a thrombus in a variety of ways.

The majority of these substances are actively and selectively secreted from platelets either from preformed storage granules or by being synthesized de novo from membrane phospholipids. There are also factors in the platelet cytoplasm that can have effects on thrombus structure or on vascular cell growth; they are released as a result of minor degrees of platelet lysis during hemostasis. Some of these factors and their intraplatelet sources are illustrated in Figure 99-2 . The dense bodies (see Chap. 98)

Figure 99-2 Substances released by platelets and their intraplatelet sources. Illustrated are some of the bioactive substances released from dense bodies, -granules, lysosomes, the cytoplasm, and the platelet membrane. PDECGF, platelet-derived endothelial cell growth factor; PAF, platelet-activating factor; HETEs, hydroxyeicosatetraenoic acids; HMWK, highmolecular-weight kininogen; C1 INH, C1 inhibitor; PAI-1, plasminogen activator inhibitor 1; vWF, von Willebrand factor; PDGF, platelet-derived growth factor; CTAP, connective tissue activating peptide; TGF-, transforming growth factor .

and platelet membranes are a source of a group of rapidly secreted mediators that are short acting; that is, they are either rapidly inactivated or rapidly diffuse away from the site of the thrombus. Their effects are rapid on surrounding cells, and they serve to modulate both the behavior of platelets as well as the behavior of vessel wall cells, particularly as regards vascular tone. The platelet dense bodies, the most rapidly secreted of platelet organelles, [61] release ADP, which is a potent agonist to recruit other platelets, as well as adenosine triphosphate, which is an agonist for other cells of the blood. [62] [63] They also release biogenic amines such as serotonin, which can influence vascular tone. The dense bodies are also rich in divalent cations. The physiologic role of released dense body calcium is not clear; however, one could speculate it serves to ensure adequate calcium for some of the calcium-dependent enzymes involved in coagulation or cross-linking of the thrombus. Finally, dense body, but not -granule membranes, contain granulophysin, [64] a tetraspanin membrane protein of unknown function, that is deficient in Hermansky-Pudlak syndrome.[65]

Arachidonate-derived mediators, such as thromboxane A_2 , are generated as a consequence of hydrolysis of membrane phospholipids; [66] their role in recruitment of other platelets and as vasoactive substances is extensively discussed in Chapter 91 . Mention should also be made of platelet activating factor [67] (alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), which was originally described as platelet activator released from stimulated mast cells. It is clear that its biologic effects are much more widespread than on platelet activation, [68] [69] although its effects on platelets clearly play a role in some of the platelet sequestration that occurs during allergic injury. Similarly, it is produced by a variety of cells in addition to platelets. Is it possible that this important mediator, produced locally, may contribute either to recruitment of other platelets or to some of the vascular phenomena associated with hemostasis.

Platelets contain lysosomes [24] whose enzymes are released, but platelets are probably a minor source of lysosomal hydrolases in the blood compared with neutrophils. Nevertheless, mention should be made of platelet-associated heparatinase, [70] probably a lysosomal enzyme, which can cleave vascular endothelial cell surface glycosaminoglycans to produce an antiproliferative fragment. Factor XIII, a major transglutaminase, which catalyzes the formation of isopeptide bonds between the -glutaminyl residues and the amino groups of lysines, forming stable covalent cross-links between proteins, is contained in the platelet cytosol. [71] Factor XIII functions in the cross-linking of fibrin, as well as in the cross-linking of other components of a thrombus, such as fibronectin [72] or $_2$ -antiplasmin,[73] to fibrin. Moreover, platelet factor XIII has been suggested to play a role in cross-linking and stabilizing cytoskeletal elements. [74] [75] It is likely that small quantities of platelet factor XIII are released into thrombi, where it may also affect their stability.

Platelets contain a wide variety of peptides, primarily in alpha granules, that can modulate the growth and patterns of gene expression of cells of the vessel wall. The first of these to be described was the platelet-derived growth factor [76] (PDGF), which has three isoforms and two distinct receptors on smooth muscle cells and fibroblasts.[77] PDGF probably plays an important role in the smooth muscle cell proliferation that may occur consequent to platelet interaction with the vessel wall. Its receptors are transmembrane tyrosine kinase-type receptors, and signal transduction from the PDGF receptor [78] is similar to that from other tyrosine kinase receptors such as the insulin and epidermal growth factor receptors. Another -granule growth factor is connective tissue activating peptide (CTAP) III, which stimulates fibroblast proliferation. CTAP III is a probable precursor of -thromboglobulin, [79] and its structure is related to another -granule protein, platelet factor 4. Both platelet factor 4 and CTAP III are members of a large protein family involved in growth control and inflammation. [80] [81] Interleukin-1 is expressed on the surface of activated platelets, [82] but the mechanism whereby it is found there is unclear. Transforming growth factor (TGF)- was first isolated from platelets, [83] and platelets are a rich source of this peptide mediator, which is a potent stimulus for the biosynthesis of matrix molecules and their receptors. TGF- has complex effects on cell proliferation, in some cell systems stimulating and in other systems inhibiting cellular proliferation. In addition, thrombospondin, of which platelets are the major source in blood, is a large (450 kd) protein that, as noted previously, may play a role in platelet aggregation, regulation of angiogenesis, and activation of TGF-. [84] Thrombospondin is produced by endothelial and smooth muscle cells [85] [86] and its mRNA is inducible by growth factors such as PDGF. [86] Indeed, thrombospondin appears to play a role in regulating the proliferation of smooth muscle cells [87] and angiogenesis. [88]

Multimerin is a novel platelet granule and endothelial protein that exists as massive disulfide-linked multimers of a 155-kd subunit. [89] [90] Multimerin has a repeating structure and its sequence contains the adhesive motif Arg-Gly-Asp-Ser, central coiled-coil sequences, several epidermal growth factor-like motifs, and a globular domain that is similar to a protein binding domain found in complement C1q and in collagens type VIII and X. [91] Multimerin binds the coagulation protein factor V and its activated form, factor Va. In platelets, but not in plasma, all of the biologically active factor V is complexed with multimerin. [92] Multimerin may also have functions as an extracellular matrix or adhesive protein. Members of two Canadian families with autosomal dominant bleeding disorders are deficient in platelet multimerin. [93] This novel granule protein may play important roles in platelet procoagulant activity and in platelet adhesion.

A large number of plasma proteins involved in coagulation and cell adhesion are present in platelet -granules. It is clear that during thrombopoiesis, plasma proteins are taken up by megakaryocytes and stored in -granules [94] [95] (see Chap. 98). Thus, it is important to have some basis to evaluate the significance of literature reporting a variety of plasma proteins in -granules. Three points should be borne in mind in reading any work that describes the presence of a plasma protein in platelets: (1) Is the plasma protein enriched in platelets relative to plasma? A simple way to calculate this is to determine the concentration of the plasma protein in plasma per milligram of protein, and compare it to its concentration in platelets per milligram of protein. (2) Is the plasma protein synthesized by megakaryocytes? (3) Is there a structural or functional difference between the platelet protein and the plasma protein? Thus, it is clear that plasma proteins such as albumin and IgG are present in -granules; [71] [96] however, their concentrations do not appear to be greater than those in plasma. Moreover, clear structural differences between the plasma

and platelet proteins have not been demonstrated, and they are probably not synthesized in megakaryocytes. Evidence [95] has been presented that they arrive in -granules strictly by transport from the plasma. Special note should be made of the IgG component in -granules, because measurement of platelet-associated IgG has in the past been used as an attempt to classify patients with thrombocytopenia. Clearly, its presence predominantly in -granules, rather than on the cell surface, has required a re-evaluation of the significance of this measurement (see Chap. 126).

With respect to coagulation proteins present in and released by platelets, a wide variety of procoagulant and anticoagulant enzymes and cofactors have been reported in platelets. It is clear that megakaryocytes synthesize factor V, [97] [98] and more factor V is present in platelet -granules [99] than can be accounted for by uptake from plasma. Moreover, platelet factor V plays a clear role in assembling the platelet prothrombinase, [100] which is involved

in the final step in blood coagulation (i.e., the generation of thrombin). Megakaryocytes take up fibrinogen, [95] and its biosynthesis by megakaryocytes remains controversial. Platelets also contain cofactors protein S [101] and plasminogen activator inhibitor-1 [102] (PAI-I). Protein S is a cofactor for the action of activated protein C (see Chap. 104) and PAI-1 is an inhibitor of urokinase and tissue plasminogen activators (see Chap. 103). Thus, the concentration of these proteins in platelets suggests that platelets may be a favored site for the anticoagulant action of protein C, a tempting idea considering the local concentration of factor V, a protein C substrate, on the platelet surface. Similarly, the local release of PAI-1 from platelets may play a role in modulating the fibrinolytic events in the vicinity of thrombi. [103] [104]

Platelets are the major peripheral blood source of -amyloid precursor protein (APP). [105] APP is a membrane protein, thought to be localized to -granules, [106] that serves as the precursor of the 40 residue peptides found in amyloid deposits in the brains of patients with Alzheimers disease. APP is a protease inhibitor, and is processed by proteolytic cleavage. [107] Its inhibition of factors Xa, XIa, and IXa [108] [109] suggests a possible role as a natural anticoagulant. The effects of platelet APP on the spectrum of coagulation and fibrinolytic proteases will clearly be of interest, in addition to its potential role in deposits of amyloid in the brain.

P-Selectin [110] [111] [112] [113] (GMP-140, GPIIa, PADGEM, CD62) is an -granule membrane protein that is absent from the surface of resting platelets. It is structurally similar to E- and L-selectins, [114] a family of carbohydrate binding proteins involved in adhesive interactions of circulating leukocytes (see Chap. 5). Platelet P-selectin mediates interactions of monocytes and neutrophils with platelets. [114] These interactions are important in cross-talk between these cell types [115] and in the recruitment of leukocytes to thrombi. [116] Some of the speculated pathogenetic roles of platelet P-selectin are in promoting thrombogenesis [116] and platelettumor cell interactions, [117] and in monocyte recruitment. It may play a physiologic role in the leukocyte phagocytosis of platelets. In addition, P-selectin plays an important role in leukocytevessel wall interactions [118] and hemostasis. [119]

As noted earlier, platelet -granules [13] contain the adhesive plasma proteins fibrinogen, fibronectin, vWF, and vitronectin, as well as the platelet and cellular adhesive protein thrombospondin. Platelet fibrinogen has been previously discussed. Platelet vWF is concentrated in the platelets, megakaryocytes synthesize it, [120] and the platelet form is enriched in the larger, presumably more hemostatically effective multimers. [121] Platelet fibronectin appears to be enriched in alternatively spliced forms that are relatively lacking in plasma, [122] suggesting a possible role for platelet fibronectin in events such as matrix assembly. Vitronectin is present in platelets; [123] however, its concentration suggests that it is probably passively taken up from the plasma. PAI-1 binds vitronectin, [124] and these two proteins are released as a complex. [125]

Secretory Mechanisms

Platelet secretion is triggered by a variety of strong agonists such as thrombin. Induction of secretion by weak agonists (e.g., ADP) occurs when the cells are brought into close contact such as occurs during aggregation. [126] The latter secretory mechanism is clearly dependent on thromboxane A2 generated as a consequence of arachidonic acid release. Discussion of signaling pathways involved in secretory events is in Chapter 91 .

As noted previously, the two morphologically prominent platelet storage granules, -granules and dense bodies, contain a variety of substances important in platelet function. Since these granules have a limiting membrane, it would seem likely that the final secretory event would involve exocytosis [127] (i.e.,

Figure 99-3 Three possible exocytotic pathways for -granule contents. **(A)** Simple exocytosis in which the granule membrane fuses with the plasma membrane. **(B)** Fusion of the granule with an invagination of the plasma membrane, the open canalicular system. **(C)** Fusion of granules with each other to form a compound granule that then fuses with the plasma membrane. Note that the final morphologic appearance of **(B)** and **(C)** are quite similar.

fusion of the secretory granule membrane with the plasma membrane). This has been observed in dense body secretion. [128] [129]

In -granules, the incorporation of a membrane marker into the plasma membrane [130] [131] [132] directly established that exocytosis occurs, [133] and simple exocytosis occurs occasionally as depicted in Figure 99-3A . Nevertheless, in human platelets, most of the -granules are seen to move centripetally during secretion, and are thus not in a locale that would permit fusion with the peripheral plasma membrane. [61] [133] [134] [135] [136] Two hypotheses, illustrated in Figure 99-3B and C, have been proposed to account for exocytosis when the granules are centralized. The mechanism illustrated in Figure 99-3B suggests that there are invaginations of the plasma membrane deep into the center of the platelet and that -granule membranes fuse with these invaginations, and the secretory products then are moved to the outside of the cell through a conduit termed the open canalicular system or surface-connected canalicular system. [137] [138] [139] The other mechanism, illustrated in Figure 99-3C , suggests that -granules fuse with each other or with another cellular compartment to form a compound granule that is morphologically distinct from the -granule. It is the compound granule that then moves toward and fuses with the plasma membrane. [61] [133] [140] Whatever the route by which granule components reach the cell surface, it is clear that there is substantial inward membrane traffic in platelets as well. [130] [141] [142] [143] This inward traffic can serve to clear adhesive and procoagulant proteins from the cell surface, and hence limit prothrombotic events. [144]

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MOLECULAR BASIS OF PLATELET AGGREGATION

The Aggregation Response of Platelets

Platelets in blood, in plasma, or as an isolated cell population do not interact with one another. If an appropriate agonist is added to the cells, however, rapid aggregation of the cells can ensue. Thus, a key element of this response is that platelet aggregation is a stimulated event. Some agonists that can initiate this response are listed in Table 99-4 . Of particular physiologic relevance are the platelet-derived agonistsADP, serotonin, platelet activating factor, and arachidonate metaboliteswhich provide a means for stimulated platelets to recruit additional cells; collagen, which supports platelet adhesion and secretion as well as aggregation; thrombin, which links platelets and the

TABLE 99-4 -- Common Platelet Aggregating Agonists

Agonist	Comment
Adenosine diphosphate	Released from platelet -granule; acts synergistically with many other agonists
Thrombin	Formed by activation of the coagulation system
Collagen	In subendothelial matrix
Epinephrine	May allow for hormonal regulation of hemostasis
Calcium ionophore	Not naturally occurring; mobilizes calcium in platelets
Arachidonate + metabolites	Active metabolites are formed and released form stimulated platelets
Serotonin	Released from platelets, may primarily sensitize platelets to other agonists
Platelet-activating factor	Lipid mediator produced by other cells that can activate a variety of cells, including platelets

blood coagulation system in thrombus formation; and epinephrine, which permits hormonal regulation of platelet function. These agonists activate platelets by interacting with specific receptors. Receptor occupancy triggers a complex series of intracellular reactions (detailed in Chap. 91) that ultimately converge to a set of common steps that permit the cells to aggregate. Platelet aggregation is energy dependent and can be distinguished on this basis from platelet agglutination induced by ristocetin or certain platelet antibodies.

At blood concentrations of 13×10^8 /ml, platelet suspensions are opalescent. On addition of an agonist, a stirred suspension of normal platelets aggregates, and a visible decrease in turbidity can be observed. Dedicated instruments, platelet aggregometers, for measuring the changes in light transmission through platelet suspensions, are used extensively in clinical laboratories to evaluate platelet function (see Chap. 106). Certain instruments also provide simultaneous measurements of other platelet functions, such as secretion.[126] Although the information gained from aggregometry can be extremely useful, aggregation in vitro does not necessarily reflect platelet function in vivo. In particular, clinical bleeding and the aggregation response of platelets need not precisely coincide.

A typical aggregometer tracing obtained with a suspension of isolated human platelets is shown in Figure 99-4A . From this pattern, the three essential components required for this functional response can be identified.

1. *Platelet agonist.* The agonist used in Figure 99-4 is ADP. This agonist induces platelet shape change, from discoid to a more spherical form, a transition that can be detected in the aggregometer as a slight decrease in light transmission. This transformation is not a prerequisite for platelet aggregation; epinephrine aggregates platelets but does not cause a shape change that is recordable in most conventional aggregometers. At the molecular level, shape change reflects a reorganization of the platelets actin cytoskeleton.
2. *Divalent cations.* Calcium and magnesium as well as some other, but not all, divalent cations, support platelet aggregation. Trace amounts of calcium may be a prerequisite for platelet aggregation under all circumstances, and divalent cations can influence the specificity of GPIIb-IIIa for its ligands. [145]
3. *Fibrinogen.* Fibrinogen is not only a major plasma protein but is present in, and secreted from, platelets [146] [147] (see Chaps. 102 and 112). By virtue of its capacity to form fibrin and support platelet aggregation, fibrinogen plays a dual role in thrombus formation. These activities, as well as the contribution of fibrinogen to blood viscosity, are believed to account for the increased risk of cardiovascular disease associated with elevated levels of fibrinogen. [148] The dependence of platelet aggregation on fibrinogen concentration is evident from the tracings illustrated in Figure 99-4B .

In certain circumstances, vWF may substitute for fibrinogen in supporting platelet aggregation. It is postulated that, at the high shear rates encountered in certain blood vessels, vWF may play a dominant role in aggregation. [149] Certain pathologic circumstancesplatelet-type von Willebrand disease and type IIB von Willebrand diseasemay also favor a role for this protein in platelet aggregation. [150] [151] [152] [153] [154]

The Molecular Mechanisms Involved in Platelet Aggregation

The basis for the requisite roles of the agonist, calcium, and fibrinogen in platelet aggregation becomes evident when the direct interaction of fibrinogen with the cell is examined. Little fibrinogen binds to nonstimulated platelets (Fig. 99-5). If an agonist is added, however, time-dependent binding is observed. Stimulated platelets can bind 40,000 or more fibrinogen molecules per cell, a considerable number, in view of the small size of platelets. [155] [156] [157] If the system is depleted of divalent ions, fibrinogen no longer binds to the stimulated platelets. Thus, the role of the agonist is to induce fibrinogen receptors, and calcium is required to support fibrinogen binding to its receptors.

These series of events define the fibrinogen-dependent pathway of platelet aggregation, [158] [159] [160] [161] [162] [163] schematically depicted in Figure 99-6 . All the platelet agonists listed in Table 99-4 can initiate this pathway of platelet aggregation. The common event triggered by these platelet agonists is receptor inductionthe conversion of a latent cell surface receptor to a state in which it can bind fibrinogen. Receptor induction is very rapid, and the cell is fully competent to bind fibrinogen within seconds after its initial encounter with an appropriate agonist.

Reversible binding of fibrinogen to the stimulated platelet is

Figure 99-4 Aggregation response of human platelets. **(A)** To aggregate, isolated human platelets require divalent ions (calcium, Ca^{++}), an agonist (adenosine diphosphate [ADP]), and an adhesive protein (fibrinogen [Fg]). Shape change is observed as a slight decrease in light transmission induced by ADP and is followed by an increase in transmission as the platelets aggregate. **(B)** Both the rate and extent of platelet aggregation depend on the concentration of fibrinogen. The concentration of fibrinogen (in micrograms per milliliter) is indicated below each tracing.

Figure 99-5 Binding of fibrinogen to human platelets. ^{125}I-fibrinogen binds minimally to platelets in the absence of an agonist but does bind when an agonist such as adenosine diphosphate (ADP) is added. A divalent ion, calcium, must be present for fibrinogen binding to occur.

a typical equilibrium interaction. The dissociation constant, K_d, of fibrinogen for the stimulated platelet is approximately 0.3 M.[155] [156] [158] [159] [160] Although this value indicates a relatively low-affinity interaction (hormone receptors can often bind their ligands with 10^3 to 10^6-fold higher affinities), the plasma concentration of fibrinogen (10 M) exceeds its K_d by approximately 30-fold. These interrelationships indicate that the fibrinogen binding sites on stimulated platelets can become saturated with the ligand under physiologic circumstances. The reversible binding of fibrinogen to its platelet receptor requires divalent cations. Millimolar concentrations of calcium or magnesium can meet these requirements.[155] [156] [159] [164]

After reversible fibrinogen binding, the interaction undergoes a time-dependent stabilization.[160] [161] [164] [165] [166] This transition is termed *irreversible fibrinogen binding* and correlates closely with irreversible platelet aggregation. Conditions that initially dissociate fibrinogen from the cell and disaggregate platelets, such as the removal of divalent ions or the agonist, are no longer effective in removing the bound ligand or dissociating the platelets after a period of time. The molecular basis for irreversible fibrinogen binding remains controversial. Either bound fibrinogen remains on the cell surface, but its exposure changes considerably,[167] or the ligandreceptor complex is internalized.[141] [142] The stability of platelet aggregates imparted by irreversible fibrinogen binding may be important for maintaining a thrombus in a microenvironment in which agents that prevent additional platelet activation and thrombus growth are being generated.

GPIIb-IIIaThe Platelet Receptor for Fibrinogen

A single-membrane protein, GPIIb-IIIa, serves as the platelet receptor for fibrinogen.[155] [168] [169] [170] Occupancy of this receptor is essential for platelet aggregation. It is the most abundant cell surface protein of platelets, accounting for approximately 15% of the protein mass of the platelet membrane. GPIIb-IIIa is also a constituent of platelet -granule membranes;[130] this internal pool can become surface expressed and functional during platelet activation.[147] [148] The resting platelet has 40,00080,000 copies of GPIIb-IIIa on its surface, and platelet activation can increase this number by 0100%. Each GPIIb-IIIa is capable of binding one fibrinogen molecule.

GPIIb-IIIa is a member of the integrin family of cell adhesion receptors (see earlier). GPIIb, the -subunit, is largely specific for cells of the platelet/megakaryocyte lineage. GPIIIa, the -subunit, has been designated $_3$. This -subunit is known to complex with a different -subunit to form the vitronectin receptor, $_v{}_3$.[171] $_v{}_3$ is expressed by a variety of cell types, including platelets, megakaryocytes, and endothelial cells.[172] [173] [174] These two sister receptors bind many of the same ligands and antagonists, but their specificities are not identical.[175] The complete amino acid sequences of both GPIIb and GPIIIa subunits have been deduced from their cDNAs;[29] [176] the carbohydrate side chains and disulfide linkages have also been placed.[177] [178] [179] [180] A schematic model illustrating some of the important structural features of the two subunits is illustrated in Figure 99-7. GPIIb is synthesized in megakaryocytes as a single chain, but becomes proteolytically processed to the two-chain form during its transit to the cell surface.[181] The two subunits interact noncovalently in a 1:1 stoichiometry to form the GPIIb-IIIa complex.[182] Complex formation occurs soon after biosynthesis of the individual subunits and is required for efficient cell surface expression.[181] [183] Micromolar calcium concentrations are required to maintain the subunits in a complex,[184] [185] [186] [187] [188] as compared with the millimolar requirements for fibrinogen binding. Platelet activation is necessary to transform GPIIb-IIIa to a state in which it is competent to bind ligand. The biochemical basis for this transformation is unknown. At extremes, access of ligand to the receptor could be regulated by a change either directly in GPIIb-IIIa or in its microenvironment. Although there is support for both possibilities,[189] [190] [191] [192] most evidence favors a direct conformational change within the receptor to induce ligand binding function.[193] [194] The process by which a signal is transmitted from within the platelet to convert the extracellular domain of GPIIb-IIIa from a ligand incompetent to a ligand competent state is referred to as inside-out signaling.[194] [195] [196]

Figure 99-6 Fibrinogen (Fg)-dependent pathway of platelet aggregation. Stimulation of platelets with an agonist causes induction of fibrinogen receptors. The reversible binding of fibrinogen to the competent receptor requires divalent ions. Irreversible fibrinogen binding occurs with time as the ligandreceptor interaction becomes nondissociable.

Figure 99-7 Schematic model illustrating structural features of GPIIb-IIIa, the platelet fibrinogen receptor.

Several mechanisms can be envisioned to explain how fibrinogen binding to GPIIb-IIIa can result in platelet aggregation. The simplest possibility is that a single fibrinogen molecule, by virtue of its dimeric structure, bridges two GPIIb-IIIa molecules symmetrically on adjacent platelets. Indeed, evidence can be cited to support this possibility.[197] [198] [199] Because multiple sites within each fibrinogen molecule can be recognized by GPIIb-IIIa (see later), asymmetric variations of direct bridging can be envisioned. Still another possibility is that changes subsequent to fibrinogen binding are required for platelet aggregation. Irreversible fibrinogen binding,[161] conformational changes in bound fibrinogen[200] [201] and in occupied GPIIb-IIIa,[202] clustering of fibrinogen/GPIIb-IIIa,[203] and additional interactions of the receptor with the cytoskeleton of the cells[204] [205] [206] occur after the initial binding of fibrinogen to the cells. Moreover, a series of intracellular signaling events are initiated and propagated, including tyrosine and serine/threonine kinase and phosphatase activation, as a consequence of receptor occupancy and platelet aggregation.[194] [195] These events are referred to as outside-in signaling. Whether such postreceptor occupancy events play a direct role in platelet aggregation is unknown, although circumstances have been described in which fibrinogen is bound to GPIIb-IIIa without induction of platelet aggregation.[194]

Although the exact mechanism of platelet aggregation is not fully resolved, it is clear that occupancy of GPIIb-IIIa by ligand is essential for eliciting the cellular response. Accordingly, antagonists of ligand binding to GPIIb-IIIa provide a means of blocking platelet aggregation and thrombus formation. Antagonists of ligand binding include monoclonal antibodies, small peptide ligands (see later), and nonpeptidic ligand mimetics. Representatives of these classes of antagonists are being evaluated in animal models and in patients as antithrombotic agents.[207] [208] [209] [210] [211] [212] [213] The first of these GPIIb-IIIa antagonists to receive U.S. Food and Drug Administration approval was a humanized chimeric Fab fragment of a monoclonal antibody to GPIIb-IIIa.[214] Intravenous lowmolecular-weight antagonists have had promising antithrombotic effects in clinical trials,[215] and orally active GPIIb-IIIa antagonists loom on the horizon. Together, these inhibitors of platelet aggregation will undoubtedly signal a new era of antithrombotic therapy.[213] [216]

Recognition Specificity of GPIIb-IIIa

Discrete amino acid sequences establish the way in which GPIIb-IIIa recognizes fibrinogen and its other ligands (see later). Two peptides define this recognition specificity of GPIIb-IIIa (Table 99-5). One peptide corresponds to the extreme COOH-terminus of the -chain,[197] [217] one of the three constituent chains of fibrinogen. The recognized amino acid sequence may be as small as the extreme six amino acid residues.[218] The second peptide is as small as four amino acids and contains the following three amino acids in sequence: arginyl-glycyl-aspartic acid (RGD).[219] [220] [221] [222] RGD sequences occur in two sites within the A chain of fibrinogen.[223] RGD sequences are also found in a variety of other proteins, including several that bind to GPIIb-IIIa. In addition, several other integrin receptors recognize RGD sequences within their ligands.[25] [224] [225] [226] [227] [228] [229] Thus, the RGD amino acid sequence is a broadly used recognition code in cellular adhesive reactions. By contrast, the -chain sequence appears to be unique to fibrinogen. Synthetic peptides containing either the -chain peptide sequence or the RGD sequence interact

directly with GPIIb-IIIa and bind to the same or mutually exclusive sites within the receptor. [230] [231] However, the preponderance of evidence indicates that it is the COOH-terminus of the -chain that is critical for fibrinogen binding to GPIIb-IIIa. [232] [233] [234] Nevertheless, in the design of GPIIb-IIIa antagonists, the RGD sequence has often served as the starting compound because of its smaller size. [235]

Fibrinogen is not the only ligand that binds to GPIIb-IIIa. Several other adhesive proteins that can serve as ligands are noted in Table 99-2 . All these adhesive proteins contain at least one RGD sequence, and RGD peptides interfere with the binding of fibronectin, [219] vWF, [220] and vitronectin [236] to platelets. Because plasma fibrinogen concentrations are 10-fold higher than its dissociation constant for GPIIb-IIIa, under physiologic conditions, the receptor is nearly saturated with fibrinogen. Thus, fibrinogen is the dominant ligand for GPIIb-IIIa in this environment. However, all these ligands, except fibrinogen, are present in subendothelial cell matrices, and these other interactions may dominate in this microenvironment. In addition to its role in platelet aggregation, GPIIb-IIIa may also be involved in platelet adhesion, particularly in stabilizing cellmatrix interactions. It may be in this latter function that these other GPIIb-IIIa ligands play a key role. Thrombospondin associates with the surface of resting and stimulated platelets. [237] [238] [239] On the surface, it plays an auxiliary role in platelet aggregation by stabilizing platelet aggregates. [240] This activity may arise from its capacity to bind to fibrinogen. [241] Several candidate receptors for thrombospondin have been proposed, [242] [243] [244] [245] [246] and the interaction may be mediated by a complex of several of these candidate receptors, including CD36, integrin-associated protein, and GPIIb-IIIa. [247] Complexation of GPIIb-IIIa with other platelet membrane proteins also may play a role in controlling its activation and ligand binding functions.

TABLE 99-5 -- Recognition Peptides of GPIIB-IIIa[a]

Peptide Designation	Structure[b]
Fibrinogen -chain	-XXKQAGDV
RGD	RGDX

[a]The naturally occurring sequences in human fibrinogen; S (serine) or F (phenylalanine) at the COOH-terminus of the two RGD sequences within the -chain; and H (histidine) -H-L (leucine) -G-G-A at the NH_2 -terminus of the -chain peptide.

[b]Amino acids: K, lysine; Q, glutamine; A, alanine; G, glycine; D, aspartic acid; V, valine; X, one of several amino acids.

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Chapter 100 - The Molecular Basis for Platelet Activation

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Broadly speaking, platelets support hemostasis in three ways. First, by sticking to collagen and then to each other, platelets form part of the barrier that physically limits the loss of blood at sites of vascular injury. Second, by accelerating the rate at which coagulation proteins are activated, platelets accelerate fibrin deposition; and third, by releasing the contents of their storage granules, platelets cause local vasoconstriction and promote eventual wound healing. This chapter summarizes current information about the molecular basis for these events, focusing in particular on the earliest events of platelet activation.

In general, cells are activated when they encounter agonists that can activate receptors on their surface. This generalization applies to platelets as well. Platelet activation begins when vascular injury exposes collagen fibrils and causes the local generation of thrombin, both of which activate platelets (Fig. 100-1). Platelets are normally shielded from collagen by a continuous barrier of endothelial cells, but when the endothelial cells are damaged or removed, platelets adhere to the exposed collagen fibrils, forming a discontinuous carpet. In vitro adhesion to collagen can occur without the help of accessory molecules, but in vivo, the shear forces and turbulence caused by blood flow would strip platelets away from collagen were it not for the stabilizing effects of von Willebrand factor. Having adhered to

Figure 100-1 Platelet activation at the vessel wall. Platelet activation begins when vascular damage exposes collagen and tissue factor. Circulating platelets adhere to the collagen, either directly or through von Willebrand factor (vWF), and are activated by locally generated thrombin. These events are followed by a change in the platelets shape, storage granule secretion, and the formation of multicellular aggregates as additional platelets are recruited into the growing platelet plug. The recruitment of nonadherent platelets is facilitated by the release of soluble factors from the platelets, such as adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2). This phase of hemostasis is completed when the platelet plug is stabilized by a meshwork of fibrin.

the collagen with the help of von Willebrand factor, platelets change their shape, spreading along the fibrils and releasing into the circulation thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP). The released TxA_2 and ADP recruit additional platelets, activating them in turn and causing them to stick to each other and to the platelets directly adherent to collagen. This growing mound of adherent platelets is stabilized by a cross-linked fibrin clot, but the critical contact between adjacent platelets depends on the binding of fibrinogen or fibrin to $_{IIb\ 3}$ on the platelet surface, and this occurs only after platelets have been activated. Because fibrinogen is a symmetric molecule, it can bind to two platelets. Repeated as many as 50,000 times per platelet, this allows platelets to stick to each other. Like collagen, soluble platelet agonists cause platelets to change their shape, losing the discoid appearance characteristic of resting platelets and transforming them into an irregular sphere with pseudopodia. Underlying this change of shape is a rapid reorganization of the platelet cytoskeleton as actin filaments are uncapped, severed, and rebuilt.

Collagen, ADP, and TxA_2 are not alone in their ability to activate platelets. At the same time that collagen is exposed, so is tissue factor, and formation of the tissue factor/factor VIIa complex leads to the local generation of thrombin from prothrombin (Fig. 100-1). Thrombin is a potent agonist, activating platelets at concentrations in the picomolar range by interacting with receptors on the platelet surface. Platelets facilitate this process by providing procoagulant phospholipids that accelerate thrombin generation. As a result, platelet activation and fibrin deposition are intimately associated with one another, maximizing the growth and strength of the hemostatic plug. The platelet receptor for thrombin was identified relatively recently and provided a fresh insight into the interaction of extracellular proteases with cells in general, much as the interaction of $_{IIb\ 3}$ with fibrinogen on the surface of activated platelets has historically provided insights into the interaction of other types of cells with adhesive proteins. It also provides another potential target for pharmacologic intervention in platelet activation.

The process of transforming a freely circulating platelet into an adherent platelet is one of the most fascinating aspects of platelet biology. It is also the final common denominator in platelet responses to most agonists, making it a frequent target for drug development. Stated succinctly, the central issue is this: although resting platelets are surrounded by plasma fibrinogen, they normally do not stick to each other to form circulating aggregates that would lodge in the arterial and capillary circulation. The reasons for this are multiple, but it is ultimately due to the inability of the resting conformation of $_{IIb\ 3}$ to bind fibrinogen or fibrin. As long as the resting conformation is maintained, platelet aggregation does not occur. Left to themselves, platelets pass each other by without sticking to each other. However, even in the absence of substantial vascular injury, it is likely that the buffeting that platelets withstand as they move through the circulation, perhaps encountering low concentrations of thrombin or being smashed against exposed atherosclerotic plaques, would push them toward activation. Working against this tendency are a number of internal and external controls that dampen the intracellular signals that would otherwise

promote platelet activation. These include tight regulation of the cytosolic Ca^{2+} concentration, intracellular phosphatases that defeat signaling through kinase-dependent pathways, extracellular ADPases that hydrolyze released ADP, and the increase in platelet cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate levels produced, respectively, by prostaglandin I_2 (PGI_2) and nitric oxide (NO) released from endothelial cells. Collectively, these provide a threshold that prevents spontaneous platelet activation.

PLATELET AGONISTS AND THEIR RECEPTORS

With the exception of collagen, most platelet agonists trigger platelet activation by binding to G-protein-coupled receptors on the platelet surface. The agonists whose receptors fall into this category include thrombin, TxA_2 , ADP, epinephrine, platelet-activating factor, and vasopressin (Table 100-1). The number of copies of each of these receptors per platelet at first glance appears small, but given the limited surface area of human platelets, the density of the receptors is actually substantial. Platelet activation by collagen is thought to work somewhat differently. Collagen binds directly or indirectly to at least four different molecules or molecular complexes on the platelet surface, glycoprotein (GP) Ib/IX, $_{2\ 1}$, $_{IIb\ 3}$, GP IV, and GP VI. These receptors range in number to as high as 50,000 per platelet.

G-protein-coupled receptors are formed by a single polypeptide with an extracellular N-terminus, an intracellular C-terminus, and seven membrane-spanning domains (Fig. 100-2). The sites on the receptor for interactions with agonists and antagonists are located in the extracellular and transmembrane domains. The cytoplasmic domains determine which G proteins interact with which receptor. G proteins are molecular switches consisting of a guanosine triphosphate (GTP)-binding subunit plus a heterodimer. G proteins are inactive when guanosine diphosphate (GDP) is bound to the subunit and active when GTP is bound. Activated receptors promote the replacement of GDP with GTP, switching on the G protein. G-protein-coupled receptors typically have a limited duration of activity, after which they are turned off by phosphorylation and, in some cases, cleared from the cell surface by endocytosis. The sites involved in phosphorylation and endocytosis are also located in the cytoplasmic domains of the receptor, particularly the cytoplasmic tail.

Platelet agonists are sometimes classified as strong or weak, although the distinctions are often blurred. By one definition, strong agonists are those that can trigger granule secretion even when aggregation is prevented. Thrombin and collagen are examples of strong agonists. In contrast, weak agonists, such as

Figure 100-2 Signaling through G proteins and G-protein-coupled receptors. There is only one mechanism known for signaling through G-protein-coupled receptors: agonist binding to the receptor causes the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the guanine nucleotide binding site of the G-protein subunit. This causes the dissociation of G from G , both of which can activate intracellular effectors and ion channels. Each activated receptor can activate multiple G proteins before desensitization of the receptor and hydrolysis of the G -bound GTP eventually shuts down signaling.

ADP and epinephrine, require aggregation for secretion to occur. Presumably the real differences between strong and weak agonists reflect differences in the sets of intracellular effectors that are coupled to their receptors. Strong agonists potently stimulate phosphoinositide hydrolysis and TxA_2 formation, and are relatively unaffected by inhibitors of cyclooxygenase such as aspirin. The weaker agonists, on the other hand, have little or no ability to cause phosphoinositide hydrolysis and are more dependent on TxA_2 formation for their effects.

Thrombin and the Family of Protease-Activated G-Protein-Coupled Receptors

Thrombin is arguably the most potent activator of platelets in vivo. When added to platelets in vitro, it causes phosphoinositide hydrolysis, TxA_2 formation, protein phosphorylation, and an increase in the cytosolic free Ca^{2+} concentration, as well as shape change, granule secretion, and aggregation. These responses can be detected at thrombin concentrations as low as 0.1 nM. Thrombin also suppresses cAMP synthesis in platelets by inhibiting adenylyl cyclase. All of these effects require thrombin to be proteolytically active. In 1991, two laboratories independently cloned thrombin receptors from the human megakaryoblastic Dami cell line [1] and from hamster fibroblasts.[2] The predicted sequences of these receptors resembled others that interact with G proteins, with the notable presence of an extended N-terminus containing a potential cleavage site for thrombin between residues Arg41 and Ser42 [1] (Fig. 100-3). Mutations at this

TABLE 100-1 -- Agonist and Antagonist Receptors on Platelets

Agonist/Antagonist	Receptor(s)	Type	Examples of Effectors	No. per Platelet	References
Thrombin	PAR1, PAR4	GPCR	PLC ; PI3K ; AC()	2,000 (PAR1)	[11A] [11B]
TxA_2	TxA_2 R	GPCR	PLC	1,000	[188] [189] [190]
Epinephrine	$_2$ -AR	GPCR	AC()	300	[191] [192] [193]
PAF	PAF R	GPCR	PLC	2002,000	[194] [195]
Vasopressin	V_1 R	GPCR	PLC	100	[196] [197] [198] [199]
PGI_2 [a]	PGI_2 R	GPCR	AC(+)		[200]
Adenosine diphosphate	1) P_{2Y1} ? 2) P_{2X1}	1) GPCR 2) Ion channel	1) PLC ; AC() 2) Ca^2 + influx; shape change	1) 5001,000 2) ?	[31] [34]
Collagen	$_{2\ 1}$ GP VI GP IV (?)	Integrin Glycoprotein Glycoprotein	Syk, PLC		[45] [46] [47] [48] [49] [53] [54] [201]

AC, adenylyl cyclase; GP, glycoprotein; GPCR, G-protein-coupled receptor; PAF, platelet-activating factor; PAR, protease-activated receptor; PGI_2 , prostaglandin I_2 ; PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; TxA_2 , thromboxane A_2 .

[a] PGI_2 receptors are an exception on this list because they stimulate adenylyl cyclase and cause an increase in cyclic adenosine monophosphate formation, antagonizing platelet activation.

Figure 100-3 Protease-activated receptor 1 (PAR1), a typical G-protein-coupled receptor. The predicted sequence of the human PAR1 is shown. [1] Potential sites are indicated for cleavage by

thrombin and cathepsin, N-linked glycosylation, and phosphorylation by protein kinases, including protein kinase C (PKC), protein kinase A (PKA), and -adrenergic receptor kinase (ARK).

site prevent receptor activation by thrombin,[1] [3] and synthetic peptides containing at least the first five residues beyond the cleavage site (SFLLRN) were found to mimic the effects of thrombin. These and subsequent observations suggest that thrombin receptors are activated by cleavage at a specific site, exposing a new N-terminus (the tethered ligand) that interacts with residues in the second extracellular loop and proximal N-terminus of the receptor initiating signaling [1] [4] (Fig. 100-3). Once activated, each receptor generates signals only briefly before it is turned off and desensitized by the phosphorylation of serine and threonine residues located in the cytoplasmic domains of the receptor. The kinases responsible are members of the family of G protein receptor kinases (GRKs). [5] [6]

The thrombin receptor that was cloned first is now known as protease-activated receptor 1 (PAR1) to distinguish it from two similar receptors, PAR2 [7] and PAR3[8] (Table 100-2). PAR1 is expressed on human platelets, endothelial cells, vascular smooth muscle cells, fibroblasts, and neurons. Knockouts of the PAR1 gene in mice are notable for the death in utero of approximately half of the homozygous (/) mice at a point in development at which thrombin is not thought to be present [9] [10] (Table 100-3). The surviving mice appear normal. However, although their fibroblasts lack the ability to respond to thrombin, their platelets do notan observation that led to the eventual identification of PAR3. PAR2 is expressed on human endothelial cells, keratinocytes, vascular smooth muscle, and gastrointestinal epithelium. It is not expressed on human platelets and is not activated by thrombin, but can be activated by trypsin or, with less efficiency, tryptase, a protease secreted by mast cells.[11] In contrast, PAR3, like PAR1, is a thrombin receptor. PAR3 is expressed in mouse and rat platelets (which do not express PAR1).[8] mRNA encoding PAR3 has been detected in human cells and tissues, but it remains to be seen whether PAR3 contributes to platelet activation by thrombin. PAR4 is the most recently identified member of this family. PAR4 appears to be expressed in both human and murine platelets. Its only known activators are thrombin and peptides, corresponding to the tethered ligand domain. PAR4 is 12 logs less sensitive to thrombin than are PAR1 and PAR3. [11A] [11B] Whether there are other proteases that activate PAR4 at lower concentrations or whether there is an important role for a low sensitivity thrombin receptor in normal platelet biology remains to be determined.

Based on binding studies with monoclonal antibodies and radiolabeled thrombin, there are approximately 2,000 copies of PAR1 on the surface of human platelets. Additional copies of PAR1 are initially located in the surface connecting membrane system and become exposed when platelets are activated. [12] Cleavage of PAR1 can be detected with antibodies directed N-terminal to the cleavage site [13] or with assays for the released N-terminal fragment.[14] [15] Because platelets synthesize very little protein, they have no real ability to replace cleaved receptors with new ones. As a result, human platelets usually respond to thrombin only once. This is in contrast to endothelial cells, which possess a large intracellular pool of PAR1 that can replace cleaved receptors over 23 hours. [16] [17]

Epinephrine and $_2$ -Adrenergic Receptors

In several respects, epinephrine is unique among platelet agonists because it causes aggregation and secretion, but not the cytoskeletal reorganization that underlies shape change. Phospholipase C (PLC) activation by epinephrine appears to depend on TxA $_2$ formation and can be suppressed with aspirin.[18] Aspirin also blocks second-wave, or secretion-dependent, platelet aggregation in response to epinephrine. This gives rise to a characteristic aggregometer tracing in which epinephrine-induced primary aggregation is followed by disaggregation of the initially formed platelet clumps (Fig. 100-4). Dependence on TxA_2 formation does not adequately account for epinephrines ability to aggregate platelets, however, because epinephrine can still cause fibrinogen receptor expression in aspirin-treated platelets.[19] [20] These observations suggest that there are other, as yet unknown, mechanisms involved in platelet responses to epinephrine. What might those be? Epinephrine, like thrombin, is a potent inhibitor of cAMP formation, but it does not appear that this alone can trigger platelet activation. Epinephrine has also been reported to increase the rate of Na^+/H^+ exchange across the platelet plasma membrane,[21] although this may not occur until after fibrinogen receptor exposure. [22] Platelet responses to epinephrine are mediated by $_2$ -adrenergic receptors and are both labile and variable among individuals. Some otherwise normal platelets fail to respond to epinephrine, or do so only after a prolonged delay. [23] The reasons for this variability are not entirely clear, although there are reports of families in which a mild bleeding disorder was associated with impaired epinephrine-induced aggregation and reduced numbers of $_2$ -adrenergic receptors[24] [25] (Table 100-3).

TxA_2 and TxA_2 Receptors

Thromboxane A_2 is produced from arachidonate in platelets by the aspirin-sensitive cyclooxygenase pathway. A number of stable endoperoxide/thromboxane analogs have been synthesized, including the Upjohn compound, U46619. When added

TABLE 100-2 -- Protease-Activated Receptors (PARs)[a]

	PAR1	PAR2	PAR3	PAR4
Proteases (activating)	Thrombin Trypsin	Trypsin Tryptase	Thrombin	Thrombin
Proteases (disabling)[b]	Cathepsin G Chymotrypsin Plasmin Chymase	?	?	?
Cleavage sequence and tethered ligand	LDPR/SFLLR	SKGR/SLIGK	LPIK/TFRGA	PAPR/GYPGQV
Peptide agonists	SFLLRN	SLIGKV SFLLRN	?	GYPGQV
Present in platelets?	Yes (human) No (mouse)	No (human) ? (Mouse)	? (human) Yes (mouse)	Yes (human) Yes (mouse)
Present in endothelial cells?	Yes	Yes	?	?

[a] See reference Brass and Molino[158] for individual citations to PAR1, PAR2, and PAR3. For PAR4, see references [11A] [11B]

[b] Disabling proteases prevent PAR-1 activation by cleaving its N-terminus at the wrong site, amputating the tethered ligand and disabling the receptor. [202] [203]

TABLE 100-3 -- Inherited and Imposed Disorders of Platelet Activation in Humans and Mice

System Affected	Species	Disorder or Gene	Cause	Phenotype	References
Receptors	Human	Bernard-Soulier syndrome	Loss of a von Willebrand factor receptor due to decreased functional GP Ib/IX/V complex	Variable bleeding and bruising due to decreased adhesion to collagen.	[204]
Receptors	Human	$_2$ $_1$ (GP Ia-IIa) deficiency	Loss of a collagen receptor	Variable bleeding and bruising due to decreased responses to collagen.	[48] [49]
Receptors	Human	GP VI deficiency	Loss of a collagen receptor	Mild bleeding and decreased adhesion to collagen.	[46]
Receptors	Mouse (gene knockout)	PAR-1	Absence of PAR-1	No effect on platelet function because of presence of PAR-3. Absent thrombin response in fibroblasts. Fifty percent mortality on E9.	[9] [10]

Receptors	Human	$_2$ -AR	Diminished numbers of $_2$ -adrenergic receptors	Usually no apparent phenotype, but mild bleeding has been reported.	[23] [24] [25]
Receptors	Mouse (gene knockout)	PGI_2 R	Failure to increase cAMP levels in response to endothelial cell PGI_2	Increased aggregation in response to ADP. Increased thrombosis in a carotid artery injury model.	[205]
Receptors	Mouse (gene knockout)	Adenosine A_{2a} receptor	Failure to increase cAMP levels in response to A_{2a} receptor agonists, including adenosine and caffeine	Increased aggregation with ADP.	[33]
Signaling	Human	G_q	Decreased G_q	Mild bleeding disorder.	[60]
Signaling	Mouse (gene knockout)	G_q	Absence of G_q	Loss of aggregation (but not shape change) in response to thrombin, ADP, U46619, and collagen. Improved survival in an acute thrombosis model.	[55]
Signaling	Mouse (gene knockout)	G_{i2}	Absence of G_{i2}	No reported effect on platelet function.	[206]
Signaling	Mouse (gene knockout)	12-Lipoxygenase	Absence of 12-lipoxygenase	Increased aggregation in response to ADP.	[207]
Signaling	Mouse (gene knockout)	Src	Absence of Src tyrosine kinase	No apparent effect on platelet function.	[133]
Signaling	Mouse (gene knockout)	Syk	Absence of Syk tyrosine kinase	Full knockout is lethal. Syk (/) platelets from chimeric mice show decreased response to collagen, but not thrombin.	[52] [134] [135]
Aggregation	Human	Glanzmanns thrombasthenia	Decreased fibrinogen binding due to decreased functional $_{IIb\ 3}$	Variable bleeding and bruising due to absent aggregation and decreased adhesion to fibrin.	[208] [209]
Secretion	Human	Storage pool disorders	Decreased or absent - or -granules	Variable bruising and minimal bleeding due to a failure of secondary aggregation.	[210]
Procoagulant activity	Human	Scotts syndrome	Reduced procoagulant activity	Hemorrhagic disorder with potential for life-threatening bleeding.	[211] [212] [213]
ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; GP, glycoprotein; PAR, protease-activated receptor.					

Figure 100-4 Aggregation tracings. The four sets of tracings shown in the figure reflect changes in light transmission through suspensions of platelets that have been activated by adenosine diphosphate (ADP), epinephrine, arachidonic acid, or the stable thromboxane A_2 analog, U46619. The characteristic sequence of primary aggregation followed by secondary aggregation is best seen in the response to epinephrine shown in black. The shaded tracings were obtained using platelets from a donor who had taken aspirin, which irreversibly inhibits thromboxane A_2 formation. The effects of the aspirin are reflected in the failure to respond fully to ADP, epinephrine, or arachidonate. The response to U46619, on the other hand, is normal, because U46619 serves as a substitute for the missing thromboxane A_2 .

to platelets in vitro, U46619 causes shape change, aggregation, secretion, phosphoinositide hydrolysis, protein phosphorylation, and an increase in cytosolic Ca^{2+}, while having little effect on cAMP formation. Similar responses are seen when platelets are incubated with exogenous arachidonic acid. [26] Because the effects of arachidonate can be inhibited with aspirin, they are thought to be largely due to thromboxane formation. Once formed, TxA_2 can diffuse across the plasma membrane and activate other platelets [27] (see Fig. 100-1). Like ADP, this amplifies the initial stimulus for platelet activation and helps to recruit additional platelets. [28] This process is effective locally, but is limited by the brief (30 sec) half-life of TxA_2 in solution, helping to confine the spread of platelet activation to the original area of injury.

ADP and P_2 -Purinergic Receptors

In contrast to other platelet agonists, ADP is stored in platelet dense granules and released on platelet activation. When added to platelets in vitro, ADP causes TxA_2 formation, protein phosphorylation, an increase in cytosolic Ca^{2+}, shape change, aggregation, and secretion. ADP also stimulates GTP binding to G-protein subunits and inhibits cAMP formation. These responses are half-maximal at approximately 1 M ADP. However, ADP is at most a weak activator of PLC in human platelets, and the increase in cytosolic Ca^{2+} seen in ADP-stimulated platelets is mostly due to Ca^{2+} influx rather than the release of Ca^{2+} from intracellular stores. At least two patients with bleeding defects have been described whose platelets show greatly diminished responsiveness to ADP and reduced numbers of binding sites for ADP analogs [29] [30] a phenotype not dissimilar to that produced by ADP antagonists such as ticlopidine. Efforts to identify a single receptor that can account for all of the effects of ADP on platelets have been unsuccessful. The hypothetical receptor was originally designated P_{2T} to indicate that it was a purinergic receptor. [31] P_2 receptors have been divided into P_{2Y} receptors coupled to G proteins and P_{2X} receptors, which are ligand-gated ion channels. Recent studies suggest that human platelets express P_{2Y1} [32] and P_{2X1} [33] receptors, and it has been proposed that shape change and Ca^{2+} influx are mediated by the P_{2X1} receptors, whereas the P_{2Y1} receptors are responsible for PLC activation, inhibition of adenylyl cyclase, fibrinogen receptor exposure, and aggregation (reviewed in Mills [31] and Gachet et al. [34]). Other ADP-binding proteins on the platelet surface have also been identified, including aggregin. [35] It is unclear what role they play.

Collagen: Binding Proteins and Signaling Receptors

Collagen causes platelets to change shape, secrete, and aggregate. [36] These responses are accompanied by phosphoinositide hydrolysis, TxA_2 formation, protein phosphorylation, and an increase in cytosolic Ca^{2+}. [37] [38] [39] [40] [41] As with thrombin, cyclooxygenase inhibitors retard, but do not eliminate, platelet responses to collagen, suggesting that TxA_2 formation is not essential. Human collagen exists in a number of forms. Several of these, including connective tissue collagen (types I and III) and basement membrane collagen (types IV and V) can activate platelets. Type I collagen has a fibrillar structure in which three 100-kd polypeptides are arranged in a triple-helical array referred to as *monomeric collagen* or *tropocollagen*. These monomers polymerize to form a larger parallel array that becomes covalently cross-linked by enzymes in the connective matrix. Platelets are able to adhere to at least some forms of monomeric collagen, [42] but require the quaternary structure presented by polymeric collagen for secretion and aggregation. [43] Recently, a collagen-related, cross-linked triple helical peptide (CRP) has also been shown to activate platelets. [44]

Along with activation by thrombin, it is the initial adhesion of platelets to collagen that is believed to lead to aggregate formation in vivo (Fig. 100-1). This process can occur in vitro in the absence of additional protein co-factors. However, under the conditions of rapid flow and high shear that are normally

present in vivo, von Willebrand factor is required for platelet adhesion to the subendothelial connective tissue matrix, accounting for the hemostatic defect in von Willebrand disease. Von Willebrand factor supports the binding of platelets to collagen by forming a bridge between collagen and glycoproteins on the platelet surface, particularly GP Ib. In addition, platelets also express several other surface proteins through which they can bind to and be activated by collagen. These include the integrin $_{2\ 1}$ (GP Ia-IIa) [45] and GP VI. [46] [47] Two patients with hemorrhagic disorders and defective platelet responses to collagen had reduced expression of $_{2\ 1}$ [48] [49] (Table 100-3). Presumably, the binding of $_{2\ 1}$ to collagen initiates outside-in signaling, much as it does for other integrins. Notably, the collagen-related

peptide, CRP, activates platelets independently of $_{2\,1}$, by a mechanism that involves PLC and the tyrosine kinase, Syk.[50] Watson and co-workers have proposed that this also occurs with native collagen and is mediated by a pathway within the platelet that involves phosphorylation of platelet Fc receptors followed by the binding of Syk and the subsequent phosphorylation and activation of PLC$_2$.[51] [52] GP IV (CD36) has also been proposed to be a collagen receptor,[53] but platelets from healthy donors lacking GP IV appear to have normal responses to collagen.[54]

Finally, a recent report shows that collagen-induced platelet aggregation is abolished in platelets from mice that do not express G $_q$.[55] This suggests that collagen, either directly or indirectly, activates a G-protein-dependent mechanism in mouse platelets. This could be mediated by a hitherto unsuspected G-protein-coupled receptor or it could be due to release of TxA$_2$ and ADP and their subsequent activation of TxA$_2$ and purinergic receptors. However, aspirin does not abolish platelet activation by collagen, so this remains to be established. Recent reviews of this topic can be found in Moroi and Jung [56] and Sixma et al.[57]

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INTRACELLULAR SIGNALS

In general, extracellular agonists cause platelet activation by triggering intracellular signaling pathways. Two of these pathways that are particularly central to platelet activation are the phosphoinositide hydrolysis pathway and the arachidonate or eicosanoid synthesis pathway (Fig. 100-5). The phosphoinositide

Figure 100-5 Pathways for platelet activation. Agonists such as thrombin and thromboxane A_2 (TxA_2) activate PLC through G_q and G derived from the G proteins in the G_q and G_i subfamilies. This leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI[4,5]P_2$) to yield inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), which raise the cytosolic Ca^{2+} concentration by releasing sequestered Ca^{2+} from the dense tubular system and activate protein kinase C (PKC). This promotes phospholipase A_2 (PLA_2) activation and, by a mechanism that is still poorly understood, leads to activation of the IIb_3 integrin, fibrinogen binding, aggregation, and outside-in signaling through the integrin. PLA_2 has been shown to be a substrate for phosphorylation by the membrane-associated protein kinases, ERK1 ($p42^{MAPK}$), ERK2 ($p44^{MAPK}$), and, possibly, $p38^{MAPK}$, but it appears that phosphorylation does not regulate arachidonate release in platelets. Activation of adenylyl cyclase by prostaglandin I_2 (PGI_2) raises cyclic adenosine monophosphate (cAMP) levels in platelets, which inhibits platelet activation. AA, arachidonic acid; ATP, adenosine triphosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; P13K, phosphatidylinositol 3-kinase.

hydrolysis pathway begins when PLC cleaves membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, both of which serve as second messengers. The eicosanoid pathway begins when phospholipase A_2 (PLA_2) releases arachidonate from membrane phospholipids to form TxA_2. Because so many of the agonists that activate platelets do so through G-protein-coupled receptors, the next section of this chapter begins with the G proteins that are present in platelets and then continues with a brief consideration of other participants in platelet signal transduction.

Heterotrimeric G Proteins

G proteins are heterotrimers that mediate the interaction of cell surface receptors with intracellular effectors (Figs. 100-2 and 100-5). In the inactive state, GDP is bound to G . Activated receptors promote the release of GDP and its replacement with GTP present in the cytosol. At the same time, G undergoes a conformational change, dissociating it from G and leaving both in their active states. After an interval that is determined in part by the intrinsic GTPase activity of the subunit and in part by extrinsic factors, including GTPase-activating proteins, G hydrolyzes the bound GTP to GDP and recombines with G until the next cycle of receptor-mediated activation. Originally it was thought that G was the exclusive mediator of signaling in G-protein-coupled pathways. However, it is now clear that G also serves a primary role, independently activating some forms of PLC, ion channels, G-protein-coupled receptor kinases (GRKs), and PI 3-kinase().

The G proteins that have been identified in human platelets are shown in Table 100-4 . Except for G_z and G_{16}, all of these are relatively ubiquitous. Expression of G_z is prominent in platelets and some neuronal cells, although it can also be detected elsewhere. Peptide-directed antisera show that G_{12} and G_{13} are present in platelets. G_{16} has been detected by reverse transcriptase polymerase chain reaction. The role of G_z is unknown, although one report has suggested that it can inhibit cAMP formation.[58] When expressed in Sf9 cells, G_z can couple to PAR1 among other receptors.[59] Genetic approaches are starting to be applied to understanding G-protein function. Knockout of G_q in mice results in a prolonged bleeding time and increased risk of bleeding [55] (Table 100-3). Platelets from G_q (/) mice aggregated normally when stimulated with a Ca^{2+} ionophore or phorbol esters, but failed to respond to thrombin, ADP, collagen, or the TxA_2 analog, U46619. The inhibition of the response to collagen is notable because it implies that it is completely dependent on G_q, which had not been previously suspected. Unlike mouse platelets, human platelets express G_{11} as well as G_q. However, despite this, there is a recent report of a patient with platelet dysfunction and diminished expression of G_q.[60]

Phospholipase C, Phosphoinositide Hydrolysis, and PI 3-Kinases

One of the earliest responses of platelets to most agonists is the activation of PLC. Platelets contain and forms of this enzyme. The forms are activated by G proteins, whereas the forms are regulated by tyrosine phosphorylation.[61] [62] PLC is thought to be primarily responsible for the rapid burst of phosphoinositide hydrolysis that occurs during platelet activation by agonists such as thrombin and TxA_2 analogs. In general, PLC_1 and PLC_3 respond best to G , particularly members of the G_q family,[63] [64] [65] [66] [67] whereas PLC_2 responds best to G .[67] [68] [69] [70] [71] [72] Based on studies with pertussis toxin, PAR1 in platelets is thought to be coupled to PLC_2 by G derived from G_i ,[73] [74] whereas TxA_2 receptors are coupled to $PLC_{1/3}$ by G derived from G_q or G_{11} (Fig. 100-5).

Once PLC has been activated, it hydrolyzes PI-4,5-P_2 to diacylglycerol plus 1,4,5-IP_3.[75] [76] [77] Diacylglycerol activates protein kinase C and contributes to protein phosphorylation, granule secretion, and fibrinogen receptor exposure.[78] [79] [80] IP_3 binds to receptors in the dense tubular system and releases sequestered Ca^{2+}.[81] [82] [83] [84] The most abundant of the inositol-containing phospholipids in the platelet membrane is PI (phosphatidylinositol). A 4-kinase phosphorylates the inositol ring of PI to form PIP (phosphatidylinositol 4-phosphate), which in turn is phosphorylated by a 5-kinase to form PIP_2. PLC is able to hydrolyze all three forms of the phosphoinositides. However, only PI-4,5-P_2 hydrolysis yields 1,4,5-IP_3. Production of 1,4,5-IP_3 and its subsequent conversion to 1,3,4-IP_3 takes only a few seconds in thrombin-stimulated platelets.[76] [85] Because 1,3,4-IP_3 has little, if any, second messenger activity, this effectively limits the duration of the signal carried by 1,4,5-IP_3.

Platelets also contain the p85/p110 and p101/p110 forms of PI 3-kinase and produce 3-phosphorylated phosphoinositides.[86] [87] Their involvement in platelet aggregation is suggested by the ability of inhibitors such as wortmannin and LY294002 to inhibit platelet aggregation [88] and fibrinogen receptor exposure,[89] and by the inhibitory effects of the Rho inhibitor, C3 toxin, on PI 3-kinase activation [90] and platelet aggregation.[91]

Phospholipase A_2 and the Formation of TxA_2

The second major pathway for signaling in platelets is the arachidonate or eicosanoid pathway. Eicosanoids are formed from the arachidonate released from membrane phospholipids by PLA_2 during platelet activation (Fig. 100-6). Because the availability of arachidonate is the rate-limiting step in this process, PLA_2 is tightly controlled. The cytoplasmic form of PLA_2 ($cPLA_2$) is an 85100-kd enzyme that is optimally active at micromolar and sub-micromolar Ca^{2+} concentrations. In cells other than platelets, $cPLA_2$ has been shown to increase its activity

TABLE 100-4 -- Heterotrimeric G Proteins in Human Platelets

G Protein	kd	Toxin	Phosphorylated?	Effector	Function	References

G_{i2} >> G_{i3} > G_{i1}	4041	Pertussis	No	Adenylyl cyclase	cAMP	[151]
G from G_i [a]				PLC PI3K()	IP_3 /DAG 3-PPIs	
G_z	41	Neither	Yes	?	?	[214] [215] [216]
G_q , G_{11}	42	Neither	No	PLC	IP_3 /DAG	[217] [218]
G_{12} , G_{13}	44	Neither	Yes	?	?	[218] [219] [220]
G_s	45	Cholera	No	Adenylyl cyclase	cAMP	[221]
G_{16}			?	?	?	[222]

cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IP_3 , inositol 1,4,5-triphosphate; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; 3-PPI, 3-phosphorylate polyphosphoinositides.

[a]The involvement of the G_i family members as the source of the G is presumptive, but is based on the absence of any other pertussis toxin-sensitive G proteins in platelets.

Figure 100-6 Eicosanoid pathways. The formation of arachidonate and its subsequent metabolism to prostaglandins, thromboxanes, and leukotrienes is illustrated. Arachidonate is released from membrane phospholipids such as phosphatidylcholine (PC) and phosphatidylserine (PS) by the direct action of phospholipase A_2 , or is formed from phosphatidylinositol (PI) by the sequential action of phospholipase C and diacylglycerol (DG) lipase. In platelets, arachidonate is metabolized by the aspirin (ASA)-sensitive cyclooxygenase pathway to form thromboxane A_2 (TxA_2). In endothelial cells, prostaglandins such as PGI_2 are formed. In white cells, 5-lipoxygenase directs arachidonate metabolism toward leukotriene formation. Platelets also contain 12- and 15-lipoxygenase. The biologic significance of the molecules formed by this pathway is less clear, but platelet 12-hydroxyeicosatetraenoic acid (HETE) can be metabolized to 12,20-DiHETE in neutrophils, where it may competitively inhibit the metabolism of leukotriene (LT) B_4 .[223] HPETE, hydroperoxyeicosatetraenoic acid.

upon phosphorylation on Ser505 by p42/44 mitogen-activated protein kinase (MAPK), leading to the suggestion that Ca^{2+} plus phosphorylation regulates arachidonate release.[92] In platelets, phosphorylation of $cPLA_2$ has been shown to occur. However, arachidonate release was unaffected by the MAP kinase inhibitor, PD98059, at concentrations that blocked $p42/44^{MAPK}$ activation.[93] It has been proposed that $p38^{MAPK}$, rather than $p42^{MAPK}$ or $p44^{MAPK}$, regulates $cPLA_2$ in platelets, but the finding that the $p38^{MAPK}$ inhibitor, SB203580, also does not affect arachidonate release leaves this an open issue. [93] [94] [95] [96] [97] Therefore, all that can conservatively be said at this point is that platelet $cPLA_2$ is likely to be regulated by the increase in cytosolic Ca^{2+} that accompanies platelet activation with an incompletely defined contribution by phosphorylation. A recent review of the regulation of $cPLA_2$ can be found in Leslie.[98]

Once released from membrane phospholipids, arachidonate can be metabolized to prostanoids, leukotrienes, and epoxides (Fig. 100-6). Which of these metabolites are formed appears to depend in part on which enzymes are present in any particular cell. The prostanoids are formed by the cyclooxygenase pathway (COX-1 in platelets) and include endoperoxides and thromboxanes, as well as the prostaglandins. The leukotrienes are formed through the lipoxygenase pathway; the epoxides, by the cytochrome P-450 epoxygenase pathway. In platelets, the predominant route for arachidonate metabolism leads to TxA_2 [99] as opposed to endothelial cells, which synthesize PGI_2 (Fig. 100-6). COX-1 and thromboxane synthetase have been localized to the platelet dense tubular system, the apparent site of TxA_2 formation[100] (Fig. 100-5). COX-1 is irreversibly inactivated by aspirin, [101] [102] which acetylates a serine residue near the C-terminus. [103] [104] Because platelets lack the ability to synthesize meaningful amounts of protein, inactivation of COX-1 by aspirin effectively halts TxA_2 synthesis until new platelets are formed. Indomethacin and other nonsteroidal anti-inflammatory agents also inactivate COX-1, but without covalently modifying the enzyme. [105] [106]

The synthesis of leukotrienes from arachidonate begins with lipoxygenase (Fig. 100-6). Three different forms of this enzyme have been described. Each adds oxygen to arachidonate at a different site, producing 5-, 12-, or 15-hydroperoxyeicosatetraenoic acid (HPETE). Platelets express 12-lipoxygenase and 15-lipoxygenase and are able to pass 12-hydroxyeicosatetraenoic acid (HETE) to neutrophils and to receive, in turn, leukotriene A_4 for metabolism to biologically active leukotriene C_4 . Exposure of platelets to aspirin or indomethacin increases HPETE and HETE formation, but the significance of this effect is unknown.

Ca^2 +: Inside and Outside

Platelet activation is typically accompanied by a dramatic increase in the cytosolic free Ca^{2+} concentration. Ca^{2+} ions serve as intracellular second messengers and, like protein kinases, affect enzyme activity and proteinprotein interactions. Based on studies with intracellular probes such as Fura-2, the cytosolic free Ca^{2+} concentration in resting platelets is 0.1 M. Strong agonists, such as thrombin or collagen cause an increase to 1 M. Weaker agonists, particularly epinephrine, may have little or no effect on cytosolic Ca^{2+} .

The ability of resting platelets to maintain a low cytosolic Ca^{2+} concentration is due primarily to the limited permeability of the platelet plasma membrane, which restricts Ca^{2+} influx (Fig. 100-7A). In addition, platelets are able to expel Ca^{2+} from the cytosoleither back across the plasma membrane or into the dense tubular system, a structure derived from megakaryocyte endoplasmic reticulum. Like similar structures in other

Figure 100-7 Ca^{2+} homeostasis in platelets. **(A)** Resting platelets maintain their cytosolic free Ca^{2+} concentration at approximately 10^7 M by pumping Ca^{2+} out of the cell across the plasma membrane and by sequestering Ca^{2+} within the dense tubular system. Ca^{2+} uptake into the dense tubular system is driven by an adenosine triphos-phate (ATP)-dependent Ca^{2+} pump that resembles that present in muscle. The mechanism that pumps Ca^{2+} out of the cell is unknown.**(B)** When platelets are activated by agonists such as thrombin, collagen, and adenosine diphosphate (ADP), but not epinephrine, the cytosolic free Ca^{2+} concentration increases to $>10^6$ M. This is due partly to the inositol 1,4,5-trisphosphate (IP_3)-mediated release of Ca^{2+} from the dense tubular system and partly to increased Ca^{2+} influx across the plasma membrane. The mechanism that triggers Ca^{2+} influx is unknown.

cells, the dense tubular system lies close to the plasma membrane and possesses an inwardly directed Ca^{2+} -Mg^{2+} -ATPase pump that allows it to sequester Ca^{2+} . The capacity of the dense tubular system to store Ca^{2+} is finite.[107] To compensate for the steady-state influx of Ca^{2+} across the plasma membrane, platelets also need an outwardly directed Ca^{2+} efflux. In other cells, this is accomplished by either a Ca^{2+} -Mg^{2+} -ATPase or by an Na^+ /Ca^{2+} exchanger.

When platelets are activated, the cytosolic Ca^{2+} concentration increases because of a combination of Ca^{2+} release from the dense tubular system and Ca^{2+} influx across the plasma membrane (Fig. 100-7B). Platelets do not have voltage-gated Ca^{2+} channels. Instead, Ca^{2+} entry is linked to the opening of ligand-gated Ca^{2+} channels, including the P_{2X} -purinergic receptors thought to respond to ADP. The trigger for Ca^{2+} release from the dense tubular system is IP_3 .

How does the increase in cytosolic Ca^{2+} contribute to platelet activation? Part of its effect is exerted through enzymes that are not optimally active at the Ca^{2+} concentration present in resting cells, but which become active when the Ca^{2+} concentration rises. These include $cPLA_2$, PLC, phosphorylase kinase, and myosin light chain kinase. Myosin light chain kinase phosphorylates the 20-kd light chain of myosin and enhances its interaction with actin. Ca^{2+} also affects actin polymerization directly. Finally, platelets contain two proteases, calpains I and II, that are activated by Ca^{2+} . Known platelet substrates for calpain include actin-binding protein and P235 (talin). [108] [109] [110] The nature of these substrates and the high concentrations of Ca^{2+} required for calpain activity suggest that calpain

participates in the late events of platelet activation, those which follow the agonist-induced increase in the cytosolic free Ca^{2+} concentration.

Protein Kinase C and Pleckstrin

Protein kinase C is a family of ubiquitous, serine- and threonine-specific protein kinases that play an important, but still incompletely defined, role in signal transduction in platelets. At least eight members of the family have been identified and divided into groups based on structure and their interactions with Ca^{2+} and lipids. Platelets contain at least the , , and forms.[111] In platelets, as in other cells, the usual stimulus for the common forms of protein kinase C is the formation of diacylglycerol and the increase in cytosolic Ca^{2+} that occur when PLC is activated[79] (Fig. 100-5). Together, these activate protein kinase C and cause it to associate with the plasma membrane.[112] Protein kinase C can also be activated in platelets by phorbol esters and synthetic membrane-permeable diacylglycerols.[79] [80] [113] Once activated, protein kinase C appears to be involved in a variety of responses in platelets, some of which seem contradictory. These include positive effects, such as aggregation and secretion, as well as negative effects, which emerge when platelets are preincubated with phorbol esters before the addition of an agonist. The inhibitory effects are discussed later in this chapter. Several of the proteins that are phosphorylated by protein kinase C during platelet activation have been identified, including the subunits of the G proteins, G_z , G_{12} , and G_{13} , myosin light chain (P20), pleckstrin (p47), actin binding protein,[114] myosin light chain kinase, and GP180, a transmembrane glycoprotein whose function is unknown.[115] Phosphorylation of G_z appears to uncouple it from G .[116] Phosphorylation of myosin light chain occurs at a site distinct from myosin light chain kinase and is believed to affect the interaction of myosin with actin.

Arguably, the best known substrate for protein kinase C in platelets is pleckstrin, the platelet and leukocyte C-kinase substrate, a protein with an apparent molecular weight of 47 kd.[117] [118] Pleckstrin, or p47, is a 350-residue polypeptide with an internal homology between the first and last 100 residues.[119] These regions are now commonly referred to as pleckstrin homology or PH domains[120] [121] because recent analysis has shown them to be present in more than 100 proteins, forming a major structural motif for proteinprotein or proteinlipid interactions. Targets that have been identified for pleckstrin include G and PIP_2 .[122] [123] [124] [125] Protein kinase C phosphorylates pleckstrin on Ser[113] , Thr[114] , and Ser[117] [126] three residues clustered between the N-terminal PH domain and the recently described DEP domain.[127] The role of pleckstrin is still under investigation. However, when overexpressed in COS-1 cells, pleckstrin causes the formation of microvillous projections on the cell surface.[125] This process appears to require the N-terminal PH domain and phosphorylation of the protein. It suggests that pleckstrin, like other proteins that can bind to PIP_2 , plays a role in regulation of the actin cytoskeleton. In addition to this role, pleckstrin may also participate in feedback inhibition of agonist-induced phosphoinositide hydrolysis. This role is discussed later in the chapter in the section on inhibitory pathways.

Protein Tyrosine Kinases and Their Substrates

In addition to serine/threonine kinases such as protein kinase C, platelets contain a large number of tyrosine kinases, some of which become active during platelet activation. A detailed consideration of the role of tyrosine kinases in platelets is beyond the scope of this chapter, and readers are referred to several excellent recent reviews.[128] [129] [130] However, several general points are worth making. First, like other cells, human platelets contain tyrosine kinases that are receptors for extracellular ligands, such as platelet-derived growth factor receptors and Eph

receptors. They also contain a large number of nonreceptor tyrosine kinases, including Src, Fyn, Lyn, Hck, and Syk. When platelet lysates are probed with anti-phosphotyrosine antibodies, a number of proteins appear phosphorylated for the first time or show increased phosphorylation on tyrosine. Some of these turn out to be the kinases themselves. Others are substrates for the kinases. However, many of the proteins have not yet been identified. The extent of tyrosine phosphorylation of platelet proteins is determined in part by whether platelet aggregation has occurred. Some tyrosine kinases in platelets are activated by platelet agonists even when platelet aggregation is blocked or when the process is observed in thrombasthenic platelets that lack $_{IIb\ 3}$. Others, such as the focal adhesion kinase (FAK), are active only when aggregation has occurred or show enhanced activity after aggregation compared with agonist-stimulated platelets that have not aggregated. The nonreceptor tyrosine kinase Syk is a good example of a kinase whose activity is enhanced by platelet activation and further enhanced when aggregation occurs.[131] According to current models, aggregation-dependent kinase activation and tyrosine phosphorylation in platelets are due at least in part to outside-in signaling through $_{IIb\ 3}$ when it binds to fibrinogen.[128] [129] Outside-in signaling involves the formation of complexes of signaling proteins centered on the cytoplasmic domains of the integrin.[132] Some of those proteins are tyrosine kinases, others are substrates for the kinases; still others are both.

The role of most tyrosine kinases in platelets is still incompletely understood. Some of the kinases and substrates have been studied in detail, but their place in platelet signaling pathways is not necessarily clear. Platelet activation can be inhibited by some broadly specific inhibitors of tyrosine kinases, such as genistein or the tyrphostins. Inhibitors of phosphotyrosine phosphatases, such as vanadate, promote platelet activation. Techniques that are now commonly used in transfectable or microinjectable cells to understand the contribution of a particular kinase or kinase substrate have been less successfully used in platelets. Murine knockouts of kinases that are present in human platelets either produce no obvious platelet-related phenotype or are lethal for unrelated reasons. For example, deletion of Src, which is abundantly expressed in platelets, has no apparent effect on platelets or megakaryocytes, presumably because of the presence of several related kinases in the Src family[133] (Table 100-3). Knockout of Syk, which has been strongly implicated in platelet responses to a number of agonists, including collagen, proved to be lethal,[134] [135] and until recently, dissecting out the role of Syk in platelets rested on the use of doubtfully selective inhibitors such as picceatannol. However, the development of chimeric mice that lack Syk only in their hematopoietic cells has made it possible directly to observe the consequences of Syk deficiency in platelets. The initial results show impairment of collagen responses, but not thrombin responses.[52] In general, tyrosine phosphorylation can serve two roles: it can have a regulatory effect on the phosphorylated protein, perhaps by causing a conformational change, or it provides a binding site for modular domains located in other proteinssuch as SH2 and PTB domains. Either way, it is fair to say that tyrosine kinases and their substrates are among the important missing links in the pathways that couple agonist receptor occupation to downstream events in platelets such as aggregation and secretion.

Ras Family Members and MAPK Activation

In addition to heterotrimeric G proteins, platelets also contain members of the $p21^{ras}$ or Ras family. Like the heterotrimeric G proteins, the Ras family members are switches that bind GTP in their on state and GDP in their off state. The ratio of active to inactive Ras is determined in part by accessory proteins, including guanine nucleotide exchange factors, GDP dissociation inhibitors, and GTPase activating proteins. Some of the Ras family members that have been identified in platelets include RhoA, Rac1, cdc42, Ral, Rap1, and Rap2b. In cells other than platelets, members of this family are involved in the regulation of proliferation, growth, cytoskeletal reorganization, vesicular transport, nuclear transport, endocytosis, and, possibly, integrin activation. Their roles in platelets are not always entirely clear. Proteins involved in nuclear transport or proliferation serve no obvious role in platelets, but may be important in megakaryocytes. This part of the chapter ends by briefly considering some of the possible pathways for Ras activation in human platelets leading to the eventual activation of MAPK and other downstream effectors.

The best-known pathway begins with a growth factor receptor that phosphorylates itself on one or more tyrosine residues when a ligand binds. This creates site(s) on the receptor for binding the Grb2/Sos complex. Sos is an exchange factor for Ras, causing it to exchange GDP for GTP and bind to the protein kinase, Raf-1. Raf-1 in turn phosphorylates MEK, causing it to phosphorylate the p42 and p44 forms of MAPK. Pathways that begin with G-protein-coupled receptors, which are more typical in platelets, are somewhat different. Figure 100-8 illustrates these pathways with PAR1, but the same principles apply to other receptors as well. Activation of PAR1 makes G and G available and activates PLC . The subsequent

Figure 100-8 Pathways that can lead to Ras and membrane-associated protein kinase (MAPK) activation in platelets. Three pathways have been described that may lead to Ras and MAPK activation in platelets. Two are the immediate consequence of G-protein-coupled receptor activation: the first arising from the Ca^{2+} -dependent activation of PYK2 (RAFTK), the second from the PI 3-kinase() (PI 3K)-dependent activation of Shc. The third pathway is an indirect consequence of receptor activation because it results from outside-in signaling after integrin engagement, which is itself a consequence of receptor activation. DG, diacylglycerol; FAK, focal adhesion kinase; IP_3 , inositol 1,4,5-trisphosphate; MEK, MAP kinase; P. tox, pertussis toxin; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC , phospholipase C ; PMA, phorbol myristate acetate; Tyr k, tyrosine kinase.

rise in the cytosolic Ca^{2+} concentration causes the tyrosine phosphorylation of the FAK-related protein kinase, PYK2 [136] [137] (originally identified as RAFTK in megakaryocytes[138] [139] [140] [141]). The tyrosine kinase that phosphorylates PYK2 has not been identified, although Src family members can associate with PYK and

become activated.[136] [137] PYK2 is a large (1,009-residue) tyrosine kinase that lacks a transmembrane domain and SH2 and SH3 domains. [136] It includes two proline-rich domains that may interact with SH3 domains in other proteins, and has at least two tyrosine residues that become phosphorylated: Y882, which appears to be a Grb2 binding site, and Y402, which is a binding site for members of the Src family. [136] The association of Grb2 brings Sos into the complex and is one way that Ras can become activated.

A second method for coupling Ras activation to G-protein-coupled receptors in platelets involves Shc, which has been shown to become tyrosine phosphorylated and to associate with the Grb2/Sos complex in response to G .[142] This process is sensitive to inhibitors of PI 3-kinase such as wortmannin, [143] suggesting that the G -responsive form of this enzyme, PI 3-kinase(), is involved. [86] [87] [144] Finally, late in the process of platelet activation, agonist-induced integrin engagement can also contribute to and perhaps sustain the activation of Ras. Once $_{IIb\ 3}$ binds fibrinogen, it can act as a nidus for the assembly of signaling complexes that are known to include FAK[145] [146] [147] [148] and possibly PYK2.[140] [141] In this sense, activated integrins are much like activated growth factor receptors, and outside-in signaling through the integrin parallels the signals initiated by the original agonist with FAK substituting for PYK2 (Fig. 100-8). Once Ras is activated by any of these three pathways, it causes the phosphorylation and activation of MAPK in platelets, [149] particularly p42^{MAPK} , and presumably through Raf-1 and MEK.

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PREVENTING OR LIMITING PLATELET ACTIVATION

Although platelet aggregation and secretion are necessary to maintain hemostasis at sites of vascular injury, inappropriate platelet activation can cause irretrievable damage. In the process of moving through the heart, arterial circulation, microcirculation, and spleen, platelets are subjected to shear forces, turbulence, and collisions with other cells. None of these should cause platelet activation; ideally, a balance should exist so that platelets activate rapidly when exposed to true vascular injury, but otherwise resist activation. The mechanisms that accomplish this are nearly as diverse as those that cause platelet activation, and play an important role in the prevention of thrombosis. The list includes processes that (1) minimize platelet contact with agonists, (2) limit platelet responsiveness to agonists, (3) limit the duration of agonist receptor activity, and (4) provide negative feedback during platelet activation. For example, contact with collagen is normally prevented by a barrier of endothelial cells. Endothelial cells also produce PGI_2 and NO, which raise platelet cAMP and cyclic guanosine monophosphate levels, and have on their surface a recently described ecto-ADPase (CD39) that metabolizes secreted ADP before it can cause platelet activation. [150] Prolonged contact between platelets and thrombin is limited by natural inhibitors such as antithrombin III and protease nexin I, and by the tendency of blood flow to dilute and wash out unbound thrombin. The effects of TxA_2 are limited by its brief half-life. Receptor activity is limited by phosphorylation and clearance. Other mechanisms exist as well, including the tight controls on cytosolic Ca^{2+} concentration that were described earlier in this chapter and, most basic of all, the inability of $_{IIb\ 3}$ to bind plasma fibrinogen until it has undergone the conformational switch that accompanies platelet activation. Collectively, these processes provide a threshold that must be exceeded for platelet activation to occur, much as minor activation of the coagulation cascade by tissue factor is limited by the extrinsic pathway inhibitor.

cAMP

Platelets contain both of the G proteins that are known to regulate cAMP formation by adenylyl cyclase in most mammalian tissues: G_s and G_i . G_s is present in at least its 45-kd form. G_i is present in all three of its known forms, with an order of prevalence $G_{i2} >> G_{i3} < G_{i1}$. [151] Traditionally, agents that increase cAMP levels in platelets, such as PGI_2 , are described as having receptors that are coupled to G_s , whereas agonists that suppress cAMP formation, such as thrombin and epinephrine, have receptors coupled to G_i . Preincubating platelets with forskolin, PGI_2 , or a membrane-permeable cAMP analog effectively increases the intracellular cAMP concentrationas does inhibiting cAMP phosphodiesterase, the enzyme normally responsible for metabolizing cAMP. Drugs such as dipyridamole (Persantine) exert weak antiplatelet effects by inhibiting phosphodiesterase. The mechanism by which cAMP dampens platelet responsiveness is incompletely understood. In general, the effects of cAMP are thought to be mediated by cAMP-dependent protein kinase, also known as protein kinase A. Platelet substrates for this enzyme include the 24-kd chain of glycoprotein Ib, actin-binding protein, and myosin light chain, as well as several unidentified proteins and the Ras-related protein, Rap1B. Agents that increase cAMP levels have been demonstrated to cause a number of specific changes in platelet function. These include a reduction in agonist binding, impaired phosphoinositide hydrolysis, a smaller increase in the cytosolic free Ca^{2+} concentration in response to agonists, and accelerated uptake of Ca^{2+} into the dense tubular system. The mechanism by which cAMP inhibits phosphoinositide hydrolysis has not been established conclusively, but data from several laboratories suggest that cAMP may inhibit the resynthesis of PI-4-P and $PI\text{-}4,5\text{-}P_2$.[152] [153] Because the PIP_2 pool in platelets is relatively small and turns over rapidly when platelets are stimulated, anything that limits its replenishment would be expected to limit IP_3 and diacylglycerol formation.

Receptor Desensitization

As a general rule, G-protein-coupled receptors, including those present on platelets, are rapidly desensitized once they have been activated. This limits the amount of time that the receptor is in the active state and can reduce or entirely prevent a second response to the same agonist. Desensitization is usually due to phosphorylation of the receptor on cytoplasmic serine and threonine residues, particularly those in the cytoplasmic tail of the receptor. Phosphorylation is accomplished by members of a family of protein kinases that have come to be called G-protein-coupled receptor kinases, or GRKs. [154] The first of these kinases to be described were rhodopsin kinase (GRK-1) and two -adrenergic receptor kinases (ARK-1 and ARK-2), but at least four others have now been cloned. ARK-2 (GRK-3) has been shown in overexpression systems to phosphorylate the human PAR1 and is presumed to be responsible for the brief period that this receptor remains active once it has been cleaved by thrombin.[5] At least some of the GRKs are stimulated by G , which provides a rational means to link receptor activation with activation of the kinase. Phosphorylation of G-protein-coupled receptors uncouples them from their associated G proteins and also facilitates the binding of arrestin family members to the tail of the receptor, leading to receptor internalization. [155] [156] Once internalized, G-protein-coupled receptors can be dephosphorylated before being recycled back to the cell surface. Recently published evidence suggests that this is accomplished by members of a newly discovered family of receptor-directed phosphatases.[157] In the case of PAR1, dephosphorylation and recycling

does not restore responsiveness because thrombin requires an intact N-terminus to cause PAR1 activation. [158]

Protein Kinase C and Negative Feedback

In addition to contributing to platelet activation, protein kinase C can inhibit platelet activation. The inhibitory effects emerge when platelets are preincubated with phorbol esters or diacylglycerol under conditions in which platelet activation does not occur. If an agonist is added several minutes later, the responses of the platelet to that agonist, especially those dependent on the products of phosphoinositide hydrolysis, are either attenuated or abolished. This phenomenon may represent a form of negative feedback. Several different mechanisms have been proposed to underlie this effect. Protein kinase C has been shown to phosphorylate platelet IP_3 -5-phosphomonoesterase, the enzyme that hydrolyzes $1,4,5\text{-}IP_3$ to $1,4\text{-}IP_2$.[159] Phosphorylation accelerates the rate of $1,4,5\text{-}IP_3$ hydrolysis and may limit the duration of the signal for Ca^{2+} release. It has also been shown that phorbol esters can block the ability of platelet agonists to stimulate phosphoinositide hydrolysis and attenuate cAMP formation.[76] [160] Additional targets for protein kinase C that may affect signaling through G-protein-coupled receptors include the receptors themselves, pleckstrin (see later), and the G proteins G_z , G_{12} , and G_{13} . In the case of G_z , phosphorylation has been shown to affect its interaction with G . [116]

Pleckstrin as an Inhibitor

The biology of pleckstrin in platelets is still not completely understood, but there is some evidence that phosphorylated pleckstrin may act as a brake on platelet activation by interfering with phosphoinositide hydrolysis and the activation of PI 3-kinase(). When overexpressed in COS-1 cells, pleckstrin has been shown to inhibit phosphoinositide hydrolysis initiated through several different G-protein-coupled receptors and the growth factor receptor, Trk-A. [161] This inhibition was dependent on the presence of an intact N-terminal PH domain, and appeared to be regulated by the phosphorylation of pleckstrin at the protein kinase C sites. Pleckstrin variants that substituted charged amino acids at the sites of phosphorylation also inhibited phosphoinositide hydrolysis, whereas neutral substitutions were relatively inactive.[126] This suggests that pleckstrin may inhibit phosphoinositide hydrolysis by virtue of its ability to bind directly to $PI\text{-}4,5\text{-}P_2$.

The other inhibitory effect of pleckstrin that has been documented is the ability of recombinant pleckstrin to inhibit PI 3-kinase activity in platelet lysates. This inhibition is specific for the phosphorylation of PI-4,5-P_2 to PI-3,4,5-P_3 by the G -activated form of the enzyme, PI 3-kinase(), and can be overcome by the addition of purified G .[162] Pleckstrin appears to have no effect on growth factor-activated, p85-associated PI 3-kinase activity. These observations suggest that pleckstrin, once it has been phosphorylated by protein kinase C, acts as a brake on further PI-4,5-P_2 hydrolysis and PI-3,4,5-P_3 formation by binding directly to PIP_2 and possibly G . Because PI-3,4,5-P_3 has been shown to activate several isoforms of protein kinase C,[163] [164] the appearance of phosphorylated pleckstrin during platelet activation may also serve to limit protein kinase C activity, although this has not been tested directly.

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ONCE IT BEGINS, HOW DOES IT END?

Once an agonist binds to its receptor and initiates second messenger formation, shape change, secretion, and aggregation follow rapidly. In contrast to the initial steps of signal transduction described earlier in this chapter, comparatively little is known about the events that occur just before secretion and aggregationthat is, those events that follow the generation of second messenger molecules, but precede secretion and fibrinogen receptor expression. Clearly, there are changes in the cytosolic Ca^{2+} concentration and in the phosphorylation state of numerous platelet proteins. Previously quiescent enzymes become activated. Precisely how these changes lead to membrane fusion, granule exocytosis, and fibrinogen receptor expression is still unknown.

Cytoskeletal Arrangements

One of the more dramatic events during platelet activation is the metamorphosis that occurs when platelets adhere to and spread on exposed collagen fibrils or become activated in the circulation by soluble factors such as thrombin or ADP. In either case, platelets lose their distinct discoid shape and acquire an irregular morphology with multiple filopodial projections (illustrated in Chap. 98). This transformation is associated with and largely due to cytoskeletal rearrangements within the platelet. The platelet cytoskeleton includes at least 14 different proteins, most of which have counterparts in other cells. [165] The proteins are arranged in three major structures: a cytoplasmic meshwork, the membrane-associated cytoskeleton, and a microtubule coil. Together, these lend support to the platelet plasma membrane and give shape to both resting and activated platelets. Although each of these is considered separately, it should be remembered that in the intact cell they form an integral unit.

The cytoplasmic meshwork is composed of actin filaments and associated proteins. Actin is a globular, 42-kd protein that accounts for as much as 20% of total platelet protein. In resting platelets, 4050% of the actin is present as filamentous F-actin. This increases to 7080% during platelet activation [166] in a coordinated sequence of events in which the actin filaments present in resting platelets are severed and the resultant smaller fragments used as the nidus for new, longer actin filaments. This process is thought to be regulated in part by the increase in PI-4,5-P_2 levels that accompanies platelet activation. [167] At the same time, myosin is phosphorylated by myosin light chain kinase[168] and becomes associated with F-actin, forming filaments that are anchored to the platelet plasma membrane by attachment (by actin-binding protein) to the GP Ib/IX complex. [169] [170] [171]

Tension is generated by contraction of the anchored filaments. Anchorage of platelets to extracellular matrix proteins by way of cell surface glycoproteins also has parallels with the formation of focal adhesion plaques in fibroblasts. Recent evidence shows that activation of the $_{IIb\ 3}$ complex leads to the phosphorylation on tyrosine of a number of proteins, including the tyrosine kinase, $pp125^{Fak}$.[145] [146] [147] In fibroblasts, $pp125^{Fak}$ is localized at sites called focal adhesions where transmembrane glycoproteins of the integrin family bind to extracellular matrix proteins, on the one hand, and intracellular cytoskeletal proteins, on the other. These sites are thought to help anchor cells to the surrounding matrix and provide sites at which tension can be applied by contractile elements within the cell, but it is clear that in platelets these events also generate a new round of signaling molecules whose significance is just beginning to be understood. [172]

The membrane-associated cytoskeleton is composed of actin, actin-binding protein, P235 (talin), vinculin, spectrin, -actinin, and several membrane glycoproteins. Actin-binding protein is an elongated, 250-kd protein that is present in platelets as a dimer. [173] [174] Actin-binding protein is able to bind to both actin and the membrane GP Ib/IX complex, allowing it to link actin filaments to the platelet plasma membrane. [169] [170] [171] In resting platelets, actin-binding protein is part of a semirigid array that helps to maintain the platelets discoid shape and limits the lateral movement of GP Ib. This role is analogous to that performed by spectrin in erythrocytes. [175] When platelets are activated, actin filaments form and attach to actin-binding protein. Later,

the rising cytosolic Ca^{2+} concentration activates calpain, which cleaves actin-binding protein, severing the link to GP Ib. [108] [110] Calpain also cleaves P235, a 235-kd protein that is antigenically cross-reactive with talin. The third major structural element in platelets is the microtubule coil. [176] This coil is a single, tightly wound polymer of tubulin that encircles the platelet perimeter and helps to maintain its discoid shape. [177] During platelet activation, the microtubule coil contracts. At the same time, platelet granules move toward the center of the cell.

Secretion

Secretion occurs from at least three morphologically distinguishable types of storage granules: dense granules, -granules, and lysosomal granules (see Chaps. 98 and 99). Several of the - and dense granule constituents are capable of supporting platelet activation, including ADP, Ca^{2+} , von Willebrand factor, and fibrinogen. Therefore, the release of stored materials, like the formation of TxA_2 , helps to recruit additional platelets into expanding platelet aggregates. This phenomenon is readily demonstrated in a platelet aggregometer, where an initial primary response triggers secondary aggregation. This phenomenon is seen particularly well with weak agonists such as epinephrine or with low concentrations of ADP. In general, a maximal platelet response to injury requires granule secretion. Therefore, a defect in either signal transduction leading to secretion or the storage granules themselves adversely affects platelet function (Table 100-3). The mechanism by which platelet storage granule membranes are fused with the membranes of the surface connecting system has not been explored in detail, but is presumed to resemble the process that has been mapped out in other cells in which membrane proteins (v-SNAREs) on the granules dock with protein complexes in the target cellular membranes (t-SNAREs), allowing fusion and exocytosis to occur.[178] A recent study has shown that human platelets express at least some of the required docking proteins, lending support to this hypothesis. [179]

Aggregation

The hallmark of platelet activation is aggregation. Platelets can form multicellular aggregates because fibrinogen binds to the platelet surface and acts as a bridge between adjacent platelets. The mechanism by which fibrinogen receptors become activated on the platelet surface remains one of the great unsolved mysteries of platelet biology, although a solution to this problem seems to be drawing closer. This issue is covered in detail in Chapter 99 and is only summarized here. The platelet fibrinogen receptor is formed by the integrin $_{IIb\ 3}$. There are 4050,000 copies of this integrin per platelet. Based on the ability of complex-dependent monoclonal antibodies to bind to resting platelets as well as activated platelets, $_{IIb\ 3}$ is present on resting platelets, but unable to bind fibrinogen. In other words, platelet activation evokes a change in $_{IIb\ 3}$ that renders it competent to bind fibrinogen. [180] [181] In support of this model, other monoclonal antibodies have been developed that bind to $_{IIb\ 3}$ only after the addition of agonist, suggesting that new antigens have become available as a consequence of activation. This change occurs within seconds of agonist addition. The significance of the fibrinogen receptor to normal platelet function is reflected in the substantial bleeding disorder seen in patients with Glanzmann thrombasthenia, whose platelets lack functional $_{IIb\ 3}$ (see Chap. 130 ; Table 100-3).

The complete primary sequences of glycoproteins IIb and IIIa have been deduced from full-length cDNA clones and the proteins expressed in naive cells. The results suggest that both glycoproteins cross the platelet plasma membrane and have short cytoplasmic extensions. It is reasonable to suppose that intracellular events in the platelet signal fibrinogen receptor expression by interacting with the cytoplasmic domains of the integrin. Various strategies have been used to identify proteins that can bind to the cytoplasmic domains of $_{IIb\ 3}$ and other integrins, including $_{3}$ -endonexin,[182] CIB,[183] and the cytohesin-1.[184] However, with the exception of the cytohesin, which binds to 3-phosphorylated phosphoinositides,[185] it is unclear how these proteins might regulate integrin function. When co-expressed with $_{IIb\ 3}$, $_{3}$ -endonexin activates the integrin.[186] Whether this occurs in platelets, and how interactions between this and other proteins that interact with the cytoplasmic domains of $_{IIb\ 3}$ are hooked into the signaling pathways described earlier in this chapter, are the subjects of intense investigation.

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Chapter 101 - The Blood Vessel Wall

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The vasculature plays a major role in conveying and distributing hematopoietic cells, nutrients, gases, metabolites, and various chemical mediators. The interior of the vessel wall is lined by the endothelium, comprising more than 10^{12} endothelial cells, covering a surface of about 500 m^2 and weighing approximately 1 kg in total.[1] [2] The endothelium forms a continuous monolayer at the interface between blood and tissue and thus contributes significantly to the sensing and transducing of signals between blood and tissue, the trafficking of hematopoietic cells, and the maintenance of a nonthrombogenic surface permitting the flow of blood. Normally quiescent, with cell turnover measured on the order of years, endothelial cells have a remarkable capacity to proliferate and vascularize tissues in physiological (menstrual cycle) and pathological (tumorigenesis, diabetic retinopathy) situations. [3] The endothelium is critical for initiating and potentiating the inflammatory response. The pathogenesis of several disorders, such as atherosclerosis, hypertension, diabetic angiopathy, and microangiopathic hemolytic anemias, involves dysfunction of the endothelial lining. The complexity and the vast array of its functional responses has led to the description of the endothelium as a distributed organ. [4] In this chapter we will provide a conceptual framework of the structure and development of the vessel wall and the physiological functions of the endothelium as it relates to the hematopoietic system.

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STRUCTURE OF THE VESSEL WALL

The circulatory system has traditionally been divided into the macrovasculature (vessels >100 m in diameter) and the microvasculature. [5] The arterial system transports blood to tissues, resists changes in blood pressure proximally, and regulates blood flow distally. Veins return blood to the heart and act as capacitance vessels, as they contain approximately 70% of the total blood volume. Venules with luminal diameters less than 50 m are structurally similar to capillaries. [5] Capillaries, and microvessels in general, are particularly important in the exchange of gases, macromolecules, and cells between blood and tissue. Although large vessels play an important role in maintaining vascular tone, a significant proportion of peripheral resistance arises from the capillaries. [6] Capillary endothelial cells also have a metabolic role, such as in the conversion of angiotensin and hydrolysis of lipoproteins. Finally, the sprouting of new vessels is initiated in the microvasculature.

Macrovasculature

Large vessels are composed of three layers: intima, media, and adventitia. [5] [7] [8] The intima comprises the endothelium and the subendothelium. The endothelial cells of large vessels contain a distinct rod-shaped organelle, measuring about 3 m by 0.1 m, called the Weibel-Palade body. [9] Ultrastructural studies indicate the presence of a single membrane around the Weibel-Palade body with tubular structures within. This organelle contains von Willebrand factor (vWF), and P-selectin has been reported to be present on the surrounding membrane. [10] [11] [12] The abluminal face of the endothelium rests on a basement membrane, which supports the endothelial cell and can act as a secondary barrier against the extravasation of blood. [1] The subendothelial matrix contains occasional smooth muscle cells and scattered macrophages. Both smooth muscle cells and endothelial cells contribute to the extracellular matrix of the intima. [7] In large vessels the media is separated from the intima by a layer of elastin, the internal elastic lamina. The medial layer is composed primarily of concentric layers of smooth muscle cells and their secreted matrix, which is a complex mix of glycoproteins and proteoglycans. This layer is responsible for structural integrity of the wall and for maintaining vascular tone. Mutations of the fibrillin-1 gene, a microfilament protein in elastic fibers, result in disruption of the media in Marfans syndrome. [13] Defects of type III collagen can cause aortic rupture in patients with Ehlers-Danlos syndrome type IV. [14] An attenuated band of elastic fibers, the external elastic lamina, separates the adventitia from the media. The adventitia, composed of loose connective tissue, and the outer portion of the media contain small nerves and nutritive blood vessels, the vasa vasorum. The external limit of the adventitial layer is loosely defined and becomes continuous with the surrounding connective tissue of the organ. [5] [7]

Microvasculature

Capillaries and post-capillary venules are composed of two cell types: [15] [16] endothelial cells and pericytes. Pericytes and endothelial cells are invested with a basement membrane and, depending

upon the vascular bed, variable amounts of matrix separate the two cell types. Both cell types contribute to the secretion of basement membrane proteins. Long pericyte processes extend over the abluminal surface of the endothelial cell and reciprocal extensions of the endothelial cell make contact with the pericyte. Pericytes and endothelial cells have been shown to communicate via gap junctions. [17] A variety of functions has been ascribed to the pericyte, including: [15] [16] [18] (1) a contractile function, which regulates blood flow; (2) multipotential capabilities resulting in differentiation to adipocytes, osteoblasts, phagocytes, and smooth muscle cells; and (3) regulation of capillary growth. The best evidence probably exists for the last function. In animal models [19] [20] and human disease (diabetic microangiopathy, hemangiomata), [21] [22] a lack of pericytes is associated with microaneurysms and disordered microvasculature. In addition, there is a temporal correlation between pericyte contact and cessation of vessel growth in wound healing, [23] and pericyte contact suppresses endothelial cell growth and proliferation in vitro. [24] [25]

Endothelial Structure and Function

In contrast to circulating blood cells and vascular smooth muscle cells, but similar to epithelial cells, the endothelium exhibits polarity manifested by the asymmetric distribution of cell-surface glycoproteins and by the unidirectional secretion of some extracellular matrix proteins and chemical mediators. [26] [27] [28] Although in cultured endothelial cells, an apical-basal polarity is established prior to confluence, intercellular junctions may provide a role in maintaining the asymmetry in vivo. [26] [29]

Four types of intercellular junctions have been described between adjacent endothelial cells: [29] [30] tight junctions, gap junctions, adherens junctions, and syndesmos. Their distribution varies along the vascular tree with tight junctions being more frequent in the larger arteries, correlating with a more stringent requirement for permeability control. The molecular structure of endothelial tight junctions is similar to that of epithelium, consisting of a network of fibrils with the critical component being a transmembrane protein, occludin. The distribution of gap junctions tends to follow that of tight junctions. Connexin-40, connexin-37, and, in arterioles, connexin-43 are gap junction proteins that have been detected in endothelial cells. Communication between adjacent endothelial cells and between endothelial cells and pericytes or smooth muscle cells is mediated by gap junctions. Adherens junctions are formed by transmembrane glycoproteins called cadherins, which make the link between cell-to-cell contacts and the cytoskeleton. Several different types of cadherins are expressed in endothelial cells. Recently, an endothelial-specific cadherinVE-cadherin/cadherin-5was identified and found to be expressed on virtually all types of endothelium. [31] [32] Similar to other cadherins, VE-cadherin forms homotypic contacts with VE-cadherin on adjacent cells. Within the cell VE-cadherin complexes with catenins which, through other proteins, contact the actin cytoskeleton. Homotypic engagement of VE-cadherin has been reported to be responsible for the density-dependent inhibition of endothelial cell growth. [33] The structure of a fourth type of junction, the syndesmos, is not well-elucidated. Other membrane proteins that are located at interendothelial junctions include platelet-endothelial cell adhesion molecule-1 (PECAM-1), which may be important in directing the formation of junctions, and the integrins (particularly $_{2\,1}$ and $_{5\,1}$). [33] In addition to the functions listed previously, intercellular contacts are also important in maintaining cell survival. [34]

On the luminal side, endothelium is exposed to blood elements, and under pathological conditions, to circulating molecules such as cytokines and bacterial products. Engagement of endothelial receptors by these humoral factors activates a well-described series of responses including the recruitment and transmigration of leukocytes and changes in endothelial cell coagulant activity. More recently, biomechanical forces resulting from pulsatile blood flow have been shown to mediate striking changes in endothelial morphology and metabolism. Vessels must withstand three types of physical forces: radial distension (tension), longitudinal stretch, and tangential shear stress. In response to flow (shear stress), endothelial cells have been shown to reorganize their cytoskeletal architecture, rearrange focal contacts at the *basal* surface, and to align in the direction of flow. [35] [36] [37] Some endothelial cell responses following exposure to physical forces occur within seconds, such as activation of potassium channels and increased release of nitric oxide, resulting in vasodilation. Other endothelial cell responses to flow are related to changes in gene expression and occur following a delay of a few hours. Elements in the promoters of various adhesion molecule and growth factor genes have been shown to contain sequences that respond to shear stress (in a positive or negative fashion) and have been referred to as shear-stress response element (SSRE). [35] [36] [37]

Endothelial cells vectorially secrete certain extracellular matrix (ECM) proteins to the abluminal face. The matrix molecules that are secreted by endothelium include several types of collagen, elastin, fibronectin, laminins, and proteoglycans (e.g., heparan sulfate, dermatan sulfate). The exact composition of the subendothelium varies with location in the vascular tree, age, and disease states. [1] [7] [14] [38] [39] Endothelial cells bind to the ECM via heterodimeric cell-surface glycoproteins, the integrinswhich link and integrate matrix proteins to the cytoskeleton, at sites referred to as focal contacts. [40] The integrins detected in resting endothelium include $_{6\,1}$, $_{5\,1}$, $_{2\,1}$ and $_{v\,3}$. [41] Interestingly, endothelial cells express integrins on the luminal as well as abluminal surfaces. [41] The ECM serves several important functions: (1) it serves as a barrier to macromolecules in the event of disruption of the endothelium; (2) it sequesters growth factors and mediates their high-affinity binding to endothelial cells (e.g., heparan sulfate binds to fibroblast growth factor); (3) it acts as a counterstructure for the binding of endothelial cell integrins. [7] [14] [38] [42] This binding of endothelial cells to the ECM serves at least four purposes: (1) certain matrix molecules provide a physical scaffold, while others act as chemotactic agents to allow endothelial cells to migrate; [14] (2) clustering of integrins at focal adhesion contacts by certain matrix molecules can transduce survival or differentiation signals by causing the phosphorylation of various proteins and lipids. [42] Fibronectin and vitronectin provide survival signals while laminins appear to signal differentiation; [43] [44] [45] (3) maintenance of cell shape. Spreading of cells independently of the signaling resulting from integrin clustering provides an anti-apoptotic signal; [46] [47] (4) by anchoring the cell, the matrix provides a mechanism whereby blood flow at the luminal surface of the endothelium creates shear stress, which also transmits signals to cells. [35]

Endothelial Heterogeneity

Despite the common features, quiescent endothelial cells in vivo represent a widely heterogeneous population, with their phenotype depending on vessel caliber and location. While it is evident that the exposure to different physical forces and the different functions served by vessels of different caliber are reflected in different endothelial phenotypes, the molecular basis of the heterogeneity of these different populations has not been well studied. Within the microvasculature there is a structural heterogeneity of capillaries depending on the organ supplied. Even within a single organ endothelial cells exhibit different phenotypes depending on their functional role. When microvessels from different organs are harvested and cultured in vitro, they lose some of their distinctive characteristics with

progressive passaging. Some of the specialization of the different endothelial cells can be retained if they are cocultured with cells or matrix from the organ that they are derived from. Thus, matrix proteins, soluble factors from the organ, or heterotypic contacts with parenchymal cells or pericyte/smooth muscle cells are felt to be important factors in specifying endothelial cell phenotype. [48] [49] [50] [51] Specific examples of microvessels found in hematopoietic tissues are discussed in the following sections.

High Endothelial Venules (HEV)

Lymphocyte migration into secondary lymphoid sites such as lymph nodes, Peyers patches, as well as chronically inflamed nonlymphoid tissues, occurs at specialized postcapillary venules. [52] The endothelial cells of these venules exhibit a plump morphology (hence the name, HEV), display intense biosynthetic activity, and are encircled by a continuous basal lamina. They secrete a thick glycocalyx of which a proportion is GlyCAM-1, a ligand for L-selectin. [53] CD34 is another HEV addressin on peripheral lymph node endothelial cells. Endothelium of mesenteric lymph nodes and Peyers patches express MAdCAM-1 as a ligand for L-selectin and $_{4\,7}$ integrin. Expression of these different addressins may act to recruit specific subpopulations of lymphocytes to different lymphoid tissues (i.e., they facilitate the homing of lymphocytes). Tight junctions are present at intermittent spots, and there is extensive overlap between the membranes of adjacent cells, which prevents macromolecules from interendothelial transit. However, when lymphoid cells transit HEV, there is a temporary breach in the barrier. [53]

Bone Marrow Sinuses

Much less is known about the marrow sinuses than HEV. The marrow sinus endothelial cell is flat, in contrast to that of HEV, and the basal lamina is discontinuous. It has been suggested that hematopoietic cells traverse pores present at attenuated areas of the endothelium, rather than moving by an interendothelial route. [54] Clearly, the marrow sinus endothelial cell is specialized given the regulated egress of cells from the marrow. For example, if a red cell that is still nucleated begins to enter the circulation, the body of the cell is allowed to cross and is released as a reticulocyte, while the nucleus is retained extravascularly. The adventitial reticular cell (similar to a pericyte) is also thought to play an important role in controlling hematopoietic cell egress. [55] The adhesion molecule VCAM-1 expressed on marrow endothelial cells (and spleen endothelial cells in the mouse) has been implicated in a role as bone marrow addressin for hematopoietic progenitor cells expressing VLA-4 ($_{4\,1}$ integrin). [56] The VLA-4/VCAM-1 interaction may also be important in regulating the traffic of hematopoietic progenitors in and out of the marrow, but other adhesion molecules, which at present are undefined, are also likely to be important. [56] As will be described later, the marrow endothelium is also involved in regulating hematopoiesis.

VASCULAR DEVELOPMENT AND DIFFERENTIATION

The human embryo develops a vascular system by the third week when its nutritional needs are no longer met by diffusion. [57] Vascular development in the embryo proceeds in two ways. The first, occurring primarily in the embryo, is vasculogenesis, wherein blood vessels form de novo from the differentiation of mesodermal precursors. The second, angiogenesisthe outgrowth of new capillaries from preexisting vesselsis the major, and, in most cases exclusive, mode of new vessel development in the adult. Much of our early knowledge of vascularization was provided by studying the avian system. In recent years the generation of knockout mice has given us a unique insight into vascular development in mammalian systems.

Vasculogenesis

Vasculogenesis in the yolk sac proceeds initially by the differentiation of mesodermal cells into angioblasts. [58] [59] Angioblasts are vascular cells that express some, but not all, endothelial markers. These cells arise from mesodermal cells resting on the endoderm (splanchnopleuric mesoderm), but not from the mesoderm adjacent to the ectoderm (somatopleuric mesoderm). Thus, it is felt that the endoderm positively regulates vascular development, whereas the ectoderm negatively regulates vasculogenesis. Organs that are primarily of ectodermal origin, for instance, brain and kidney, are vascularized by angiogenesis and not by vasculogenesis. The mesodermal cells migrating outward from the endoderm form primitive structures termed blood islands. The cells at the center of the blood island are hematopoietic precursors, while those arranged peripherally are angioblastic precursors. Vasculogenesis within the embryo begins shortly after that in the yolk sac, again in close association with endoderm. [60] [61] However, except for a region on the ventral aspect of the embryonic aorta, intraembryonic vascular development occurs in solitary angioblasts rather than blood islands. Angioblasts differentiate in situ and form primary capillary plexi with lumens, or they migrate and fuse with other angioblasts or capillaries. Fusion of angioblasts or blood islands results in the formation of a capillary plexus that undergoes extensive remodeling over the developmental period. [58] [59]

Angiogenesis

In the normal adult, angiogenesis occurs primarily in the female reproductive system. In contrast, angiogenesis is a process that has a major impact in several pathological situations. Probably the best known and studied example of pathological neovascularization is that of tumor progression and metastasis. Angiogenesis is also important in chronic inflammation, ischemia, and wound healing. The formation of capillary sprouts from the existing microvasculature occurs secondary to an inciting stimulus that results in increased vascular permeability, accumulation of extravascular fibrin, and local proteolytic degradation of the basement membrane. [62] [63] [64] [65] The endothelial cells overlying the disrupted region become activated, change shape, and extend elongated processes into the surrounding tissue. Directed migration toward the angiogenic stimulation results in the formation of a column of endothelial cells. Just proximal to the migrating tip of the column is a region of proliferating endothelial cells. These proliferating cells cause an increase in the length of the sprout. In the region of proliferation, up to 20% of endothelial cells may enter the cell cycle. This is in marked contrast to quiescent endothelium, where less than 0.01% of cells are cycling. Proximal to the proliferative zone, the endothelial cells undergo another shape change, adhere tightly to each other, and begin to form a lumen. Secondary sprouting from the migrating tip results in a capillary plexus, and the fusion of individual sprouts at their tips closes the loop and circulates blood into the vascularized area. Activated macrophages, by secreting growth factors, monokines, proteases, and protease inhibitors, can influence all phases of the angiogenic process. [66] [67]

Recruitment of Peri-endothelial Cells

Whether formed by vasculogenesis or angiogenesis, maturation of new vessels requires the recruitment of smooth muscle cells or pericytes to re-establish vessel integrity. These peri-endothelial cells (PEC) provide structural support, assist in the production of extracellular matrix, provide contractile function so as

TABLE 101-1 -- Endogenous Peptide Inhibitors of Angiogenesis

Thrombospondin 1 peptides[a]
Platelet factor 4 fragment
Fibronectin (29 kDa fragment)
Prolactin (16 kDa fragment)
Epidermal growth factor peptide
Angiostatin (38 kDa plasminogen fragment)
Endostatin (20 kDa collagen type XIII fragment)

[a] The intact protein also has some anti-angiogenic activity.

to modulate vessel caliber, and maintain the cells in a quiescent state. In the embryo, PEC are derived from locally-available mesenchymal cells as endothelial cells invade organ rudiments. The local derivation of PEC may be one mechanism that allows for tissue-specific phenotype of the vasculature. [16] A recent report suggests that embryonic endothelial cells may transdifferentiate into vascular smooth muscle cells. [68] Genetically altered mice that fail to invest their vessels with pericytes develop microaneurysms. [19]

Extracellular Matrix

As previously discussed, the ECM plays a crucial role in vascular structure. During angiogenesis the provisional matrix is composed of fibronectin. [14] [69] Mice deficient in fibronectin die during embryogenesis and show vascular defects. Type I collagen is also important in vascular development, as mice that are null for this protein die of circulatory failure just before birth. ECM proteins and/or their proteolytic fragments have been shown to inhibit angiogenesis (Table 101-1).

Dissolution of the matrix allows endothelial cells to migrate at the initiation of angiogenesis. [67] [70] Matrix-bound growth factors are also released as a consequence of ECM degradation. The balance between positive and negative regulators is the basis of tight control in this process. Plasminogen activators (tPA and uPA), by

generating plasmin, can activate collagenases and other matrix metalloproteinases. Plasminogen activator inhibitors may block angiogenesis at this step. The action of the matrix metalloproteinases is required for angiogenesis, and their function is regulated by the tissue inhibitors of metalloproteinases.

Cell Adhesion Molecules

Of the various classes of cell adhesion molecules involved in angiogenesis, the integrins have been the most studied. [14] [69] [71] [72] The $_{v}$ $_{3}$ integrin appears to play a key role. Immunohistochemical studies localize this integrin to the tips of sprouting vessels and neutralizing antibodies abrogate angiogenesis and induce vascular cell apoptosis in vivo. Mice that are null for $_{v}$ show cerebral vascular defects consistent with the notion that brain vascularization in the embryo proceeds by angiogenesis rather than vasculogenesis. It is not clear whether $_{v}$ $_{3}$ requires a specific matrix protein for this angiogenic role, or whether any one of its ligands will suffice. One of the integrin receptors for fibronectin, $_{5}$ $_{1}$, has also been shown to be necessary for vascular development, while $_{2}$ $_{1}$ seems important for the formation of tubes by endothelial cells in vitro. The junctional proteins VE-cadherin and PECAM-1 are expressed early in development and may also have a role in assembling the vasculature. [59] [73]

Remodeling, Regression, and Apoptosis

Even though the vasculature is laid down before circulation begins, hemodynamic forces are important for maintenance and remodeling. Most of the vessels laid down during vasculogenesis regress or are remodeled. Following neovascularization, for instance during wound healing, the vessels regress when no longer needed. A chronic decrease in blood flow results in narrowing of the vessel lumen. This change in vessel caliber is dependent on an intact and functional endothelium. [74] Remodeling, which involves the loss of some vessels as well as changes in lumen diameter and wall thickness, requires both cell death and proliferation (in addition to remodeling of the extracellular matrix). In addition to survival signals transmitted by integrins, shear stress is important for endothelial survival and vessel healing following injury. [75] [76] Oxygen tension is also important in vascular maintenance. Hypoxia increases levels of vascular endothelial growth factor (VEGF) which provides signals for vessel maintenance and neovascularization. [77] [78] Hyperoxia, on the other hand, inhibits VEGF expression, which leads to regression and death of retinal vessels. [79] In some models, regression of vessels has been shown to occur by apoptosis of vascular cells. [80] [81] Endothelial cells express several anti-apoptotic molecules in order to maintain viability when quiescent and when stressed. [82] [83] [84] Most likely, there is an intricate balance between cell death and proliferation maintained by activators and inhibitors of both processes.

Role of Secreted Factors and Their Receptors

Numerous factors have been reported to regulate vascular development in a positive or negative fashion. However, definitive evidence is lacking in many cases. Some of the key molecules and their receptors that are involved in the process of vascular development and differentiation are discussed below, and a model for vascular development is depicted in Figure 101-1 .

Inducers of Angiogenesis

Fibroblast Growth Factors.

The role of fibroblast growth factors (FGFs) in vascular development is controversial. [58] [59] [85] Because of the possible functional redundancy in the numerous family members, it has been difficult to assign a specific role to the various members of the FGF family. There is evidence to suggest that FGF receptors signal an inductive pathway in differentiating mesoderm prior to vascular morphogenesis. [22] [86] [87] FGF has been reported to upregulate VEGF receptor-2 in avians, which may be a mechanism whereby mesodermal cells can respond to VEGF and commence vasculogenesis. [59]

Vascular Endothelial Growth Factors.

Five members of the VEGF family have been identified: [58] [64] [65] [85] [88] [89] [90] VEGF (also called vascular permeability factor), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). Three members of the receptor tyrosine kinase (RTK) family, [58] [65] [91] VEGF-R1 (flt-1), VEGF-R2 (flk-1/KDR), and VEGF-R3 (flt-4), differentially respond to individual members of the VEGF family. VEGF functions as a homodimer, but has also been shown to heterodimerize with VEGF-B and PlGF. [85] [86] VEGF binds VEGF-R1 and VEGF-R2, whereas VEGF-C binds VEGF-R2 and VEGF-R3, and PlGF activates VEGF-R1. [65] [85] [89] VEGF-C induces proliferation of lymphatic, but not vascular, endothelium. [85] On the other hand, VEGF has a crucial, dose-dependent effect on vasculogenesis. [58] [64] [65] Lack of VEGF-R2 prevents the development of endothelial cells and a hematopoietic system, because cells lacking VEGF-R2 do not reach the correct location to form blood islands. [92] Mice that have been rendered deficient for VEGF-R1 have normal hematopoietic progenitors and abundant endothelial cells, but do not form capillary tubes or functional vessels. [93] Both VEGF-R2- and VEGF-R1-deficient mice die at an early embryonic stage. In von Hippel Lindau disease, the development of hemangioblastomas may be due to the stabilization of VEGF mRNA. [94] VEGF is also felt to play a key role in propagating tumor angiogenesis. Finally, injection of VEGF is capable of relieving limb ischemia by the generation of collateral vessels. [62] VEGF appears to collaborate with the angiopoietins to stimulate vascular development.

Figure 101-1 A model for vascular development. Although this figure highlights the role of secreted proteins and membrane receptors in vascular development, other factors such as cell adhesion molecules and extracellular matrix components also contribute significantly. Ang, angiopoietin; ECM, extracellular matrix; FGF, fibroblast growth factor; PEC, peri-endothelial cell (smooth muscle cell, pericyte); PDGF, platelet-derived growth factor; TGF-, transforming growth factor-, VEGF, vascular endothelial cell growth factor; VEGF-R, VEGF receptor.

Angiopoietins.

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are two highly related, secreted glycoproteins that share about 60% amino acid identity. [95] [96] [97] [98] Both Ang1 and Ang2 bind to the RTK, Tie-2/Tek. [95] [97] Binding of Ang1 to Tie-2 results in tyrosine phosphorylation of Tie-2, but, unlike VEGF, Ang1 does not stimulate endothelial proliferation or tube-formation. [95] Early in development, Ang1 is mainly found in the myocardium surrounding the endocardium, but it also comes to be expressed in the mesenchyme surrounding developing vessels. [95] Disruption of either Ang1 or its receptor Tie-2 in the mouse results in embryonic lethality due to similar defects. [96] [98] [99] These mice die at a slightly later stage than VEGF-receptor-deficient mice. Although endothelial cells are present, there is a lack of vascular complexity and a scarcity of peri-endothelial cells. Reciprocal interactions between the endothelial cells and surrounding matrix and mesenchyme appear to be disrupted. A Tie-2 mutation in humans causes vascular malformations that show a depletion of smooth muscle cells in the vascular wall. [100] Ang2 also binds Tie-2, but rather than inducing receptor phosphorylation, acts as a natural antagonist for Ang1 and Tie-2. [97] Mice engineered to overexpress Ang2 specifically in their vasculature show embryonic lethality and vascular defects that are reminiscent of those seen in Ang1- or Tie-2-null embryos. [97] It appears then, that Ang1/Tie-2 coupling mediates vascular maturation by sustaining endothelial cell/peri-endothelial cell/matrix interactions, and may also be involved in maintaining endothelial cell quiescence. As Ang2 is only found at sites of vascular remodeling, it has been proposed that Ang2 may act to loosen matrix contacts, thus allowing access and responsiveness to angiogenic factors such as VEGF. [97] [98] In the absence of growth factors, disruption of the vessel architecture by Ang2 may result in vascular cell apoptosis and vessel regression.

Tie-1 is an RTK that exhibits structural similarities to Tie-2. A ligand for Tie-1 has not yet been identified. [58] [91] Disruption of the Tie-1 gene in mice results in lethality at a much later point in development and Tie-1-null mice may survive up to birth. [99] [101] Tie-1$^{/}$ mice die of hemorrhage and edema, implicating Tie-1 in signaling the control of fluid exchange across capillaries and in maintenance of vessel integrity under hemodynamic stress. Chimeric mice that express Tie-1 $^{/}$ and Tie-1$^{+/}$ endothelial cells show underrepresentation of Tie-1 $^{/}$ cells in vessels primarily derived by angiogenesis, but not in embryonic vessels derived by vasculogenesis, suggesting a differential function for Tie-1 in angiogenesis. [102]

Platelet-Derived Growth Factors.

The platelet-derived growth factor (PDGF) family is made up of two chains, PDGF-A and PDGF-B, which can associate in a homodimeric or heterodimeric fashion. Similarly, the receptors, and are RTKs that can form homo- or heterodimers. PDGF-BB can bind the receptors PDGFR- or PDGFR-, but the PDGFR- only binds PDGF-BB not PDGF-AA or AB. [58] Mice null for PDGF-B die perinatally with renal, hematological and cardiovascular abnormalities. [103] The large vessels and heart of

these mice are dilated and microvessels exhibit microaneurysms due to the lack of pericytes. [19] [103] PDGFR--knockout mice do not show an overtly abnormal cardiovascular phenotype, but generation of chimeric mice demonstrates that PDGFR-$^{/}$ cells are underrepresented in all muscle lineages (smooth, cardiac and skeletal).[104] [105] Thus, it appears that PDGF-BB elaborated by the endothelial cell provides a signal to recruit mesenchymal peri-endothelial cells as part of the maturation process of vascular morphogenesis.

Transforming Growth Factors-.

Members of the transforming growth factor- (TGF-) family are multifunctional homodimeric peptides with diverse effects on cell proliferation, migration, differentiation, adhesion, and expression of cell-adhesion molecules and ECM. [58] [106] [107] They are secreted as inactive precursors and once activated, transmit signals to cells by binding heteromeric complexes of type I and type II serine/threonine kinase receptors. Contact between endothelial cells and PEC is required for the production of active TGF-.[15] Although there has been confusion as to the part that TGF- plays in angiogenesis, several recent developments attest to its key role in vascular morphogenesis. Mice lacking TGF- or TGF- receptor type II exhibit similar defects in vasculogenesis and hematopoiesis. [106] [107] Endothelial proliferation is not affected, but there are poor contacts between endothelial cell and mesothelial layers in embryos of TGF- $^{/}$ mice resulting in a disorganized and reduced vascular network lacking capillary tubes. [106] Mutations that affect a cell surface TGF- binding protein, endoglin, cause hereditary hemorrhagic telangiectasia type 1. [108] Disruption of TGF- signaling likely plays a role in the telangiectasia seen in this disorder.

Coagulation Factors.

The potential involvement of fibrinolytic factors in angiogenesis has been mentioned previously. Tissue factor is a member of the cytokine receptor superfamily. As well as having a role in initiating coagulation as a cofactor for factor VII, tissue factor may also be involved in intracellular signaling. Tissue factor knockout mice have abnormalities of their large vessels and microvasculature secondary to defects in mesenchymal cell/peri-endothelial cell accumulation and function. [20] Abnormal development of the vasculature also affects 50% of mice that are deficient in factor V. The affected mice die in utero, and the 50% embryonic lethality is similar to that observed in thrombin receptor-deficient mice that die without obvious coagulation defects. [109] It is possible that factor

V-dependent generation of thrombin is important for early vascular development by signaling through the thrombin receptor. [58]

Inhibitors of Angiogenesis

As with the angiogenesis inducers, multiple factors have been reported to negatively regulate vascular morphogenesis. [62] Of particular interest is an emerging class of endogenous angiogenesis inhibitors that are fragments of larger proteins that have little or no angiogenesis-related activity in their intact form. The mechanism of action of these protein fragments is not entirely clear. Some of these fragments have been shown to inhibit proliferation or induce apoptosis of endothelial cells. [65] [110] [111] [112] A list of some of these polypeptide fragments is outlined in Table 101-1 . Other endogenous inhibitors include interferons, chemokines and interleukin (IL)-12. In addition, several compounds such as AGM-1470, thalidomide, and synthetic metalloproteinase inhibitors, are in clinical trials in an attempt to cause regression of tumors by inhibiting angiogenesis.[62] [65]

Relationship Between Vascular Development and Hematopoiesis

Hematopoietic cells and endothelial cells are intertwined in several ways. First, there is the likely existence of a common precursor. Second, the endothelium is intimately involved in hematopoiesis in a supportive role structurally and nutritionally. Finally, the endothelium organizes the controlled egress and ingress of hematopoietic cells in hematopoietic and other tissues. The last issue will be covered in the subsequent section.

As hematopoietic cells and endothelial cells both arise from the embryonic mesoderm in a common structure, the blood island, a shared precursor for these two lineages seems intuitive. But what is the evidence for a cell that can differentiate either into an endothelial cell or a hematopoietic cell? Several lines of evidence are suggestive. Studies using quail-to-chicken embryonic transplants suggest that endothelial cells arise from two separate regions. Mesoderm from the splanchnopleural region is capable of giving rise to both endothelial cells and hematopoietic precursors, [61] [113] leading investigators to call these cells hemangioblasts, i.e., having dual potential. A study using single cell injections of a tracer indicated that a single labeled cell would give rise to cells of the ventral aortic endothelium and the hematopoietic precursors that aggregate in that region. [114] Further evidence comes from the zebrafish mutant, *cloche*, which affects endothelial and blood lineages. [115] As discussed earlier, the VEGF-R2 chimeric mouse shows that only VEGF-R2-positive cells contribute to both lineages. [92] However, in this study the authors were able to show erythropoiesis arising from embryoid bodies of VEGF-R2-null mice. Most intriguing is the evidence that a circulating cell can reendothelialize an impervious Dacron graft and that a circulating mononuclear CD34+ cell can be induced to differentiate into endothelial cells in vivo and ex vivo. [116] [117] In the latter study, circulating CD34+ cells were harvested and injected into animal models of hindlimb ischemia, a procedure which results in substantial neovascularization. [117] The labeled CD34+ cells were detected in the endothelial cells of the neovascularized areas. These studies suggest the presence of a circulating endothelial cell precursor that might be of marrow origin. In contrast, in a study of sex-mismatched bone marrow transplants using in situ hybridization to detect the Y chromosome, it was concluded that marrow endothelial cells were of host derivation.[118] However, if endothelial cells are more resistant to the conditioning regimen due to their low turnover rate, then there would be less need for the donor-derived endothelial cells. Most of the marrow biopsies in this study were done less than a month following transplant, which may be too short a time to detect the few endothelial cells that might have differentiated from transplanted cells. More circumstantial evidence for the existence of a common progenitor is the array of shared cell surface markers (e.g., CD34, CD31) or transcription factors (e.g., Tal1/SCL, Gata-2) between these two cell lineages.[61] [119] [120]

Bone marrow stromal cells secrete cytokines, produce ECM, and are in direct cellular contact with hematopoietic cells, thereby providing a microenvironment suitable for hematopoietic proliferation, differentiation, and self-renewal. [121] A wealth of studies demonstrate the supportive role of endothelium in hematopoiesis. The physical proximity of endothelium and hematopoietic precursors within the marrow and the requirement of blood cells to transit marrow endothelium to reach the circulation is presumptive evidence of an important role of endothelium. Marrow endothelial cells constitutively express high levels of IL-6, stem cell factor (SCF), granulocyte-colony stimulating factor (G-CSF), and granulocyte macrophage (GM)-CSF. [122] Both yolk sac and marrow endothelial cells support long-term proliferation and differentiation of hematopoietic cells in vitro. [122] [123] However, endothelial cells have also been reported to inhibit hematopoiesis. [124] The altered hematopoiesis observed in mice that are deficient in both P- and E-selectin likely reflects a response to infection due to impaired trafficking of neutrophils to tissue rather than a direct involvement of these receptors in hematopoiesis. [125] As already discussed, endothelial VCAM-1 may also be important in trafficking and homing of progenitor cells.[56] In all likelihood, the endothelium, in concert with other stromal cells, provides a finely tuned system to modulate hematopoiesis such that differentiation, proliferation and self-renewal occur in a regulated fashion.

Human endothelial cells have also been reported to express receptors for IL-3, SCF, erythropoietin, and thrombopoietin and show functional responses to IL-3 and erythropoietin.[126] [127] [128] The shared responses to growth factors, combined with the importance of macrophages in angiogenesis and the production of cytokines by monocytes/macrophages, suggest that hematopoietic cells may play a reciprocal role in maintaining the endothelium.

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PHYSIOLOGICAL FUNCTIONS OF THE ENDOTHELIUM

The Endothelium as a Barrier

As with other functions, vessel permeability is dependent on the type of vessel and its location. The microvasculature (capillaries and post-capillary venules) act as the exchange vessels of the circulation. There is virtually unimpeded movement of lipophilic and low molecular weight hydrophilic substances between blood and tissue, but the vessels are selectively permeable to macromolecules. This semi-selective barrier is necessary to maintain the fluid balance between intravascular and extravascular compartments, yet antibodies, hormones, cytokines, and other molecules must have access to the interstitial space for the initiation and potentiation of various processes, including inflammation, immune response, and wound repair.

Permeability to Macromolecules

The movement of macromolecules across the vessel wall is governed by: (1) hydrostatic and oncotic pressure gradients; (2) physicochemical properties of the molecule such as size, shape, and charge; (3) properties of the barrier. The barrier of the vessel wall is formed by the cellular components, endothelial cells, and pericytes, as well as by the charge and compactness of the matrix components, the glycocalyx and basement membrane. Macromolecules can either pass directly through the endothelial cell (transcellular path), or between adjacent endothelial

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cells (paracellular path). Surprisingly, the mechanisms of macromolecular movement remain controversial, and data generated by physiologists, morphologists, and cell biologists have not yet been consolidated in a model that satisfies the findings of the different groups.

Functionally, physiologists have proposed the existence of two sets of pores based on experiments measuring the movement of dextran and other macromolecules: a small pore of radius 35 nm for the transport of water and small hydrophilic molecules, and a large pore of radius 2560 nm for macromolecular transport. [129] [130] [131] [132] Transport into cells may proceed by receptor-mediated systems, e.g., clathrin-coated pits in which the molecules are usually targeted to the lysosome. Alternatively, molecules may be moved across the cell by plasmalemmal vesicles or caveolae, which are abundant in capillary endothelial cells. Caveolae are 50100 nm membrane invaginations that can participate in transcytosis, as well as in the translocation of glycosylphosphatidylinositol-linked proteins into the cytoplasm and in transmembrane signaling.[133] Because of the known leakiness of tumor microvasculature, others have studied these microvessels and identified a structure designated the vesiculo-vacuolar organelle. [64] [134] These organelles are grape-like clusters of interconnecting uncoated vesicles and vacuoles that span the entire thickness of vascular endothelium, thereby providing a potential trans-endothelial connection between the vascular lumen and the extravascular space. [134] Vesiculo-vacuolar organelles are distinguished from clathrin-coated vesicles and capillary caveolae by their larger size and the functional regulation of the channel by diaphragms comprised of a single membrane. Interestingly, their function is enhanced by the injection into normal skin of VEGF which is known to increase the permeability of vessels.[64]

During inflammation, the binding of neutrophils to the endothelium results in the generation of oxidants that can mediate endothelial cell injury and increase permeability.[135] Thrombin, an inflammatory mediator, can increase endothelial permeability by several mechanisms resulting from the activation of its receptor on endothelial cells.[136] [137] [138] First, there is an increase in transcellular vesicular permeability. There is also increased paracellular permeability that results from the phosphorylation of endothelial cell nonmyosin light chains and contractile activity generated by the movement of actin and myosin filaments past each other. The contraction and retraction of endothelial cells are accompanied by the loosening of intercellular junctions and focal integrin contacts with the ECM. Finally, thrombin may also alter the repulsive effect of the negatively charged glycocalyx. Pericyte contractility has also been hypothesized as a mechanism of increasing permeability in inflammatory states.[139]

The Endothelium as a Nonthrombogenic Surface

The mechanisms of hemostasis and thrombosis are addressed in Chapters 100 , 101 , 102 , 103 , 104 . The purpose of this section is to place the endothelium in the appropriate context in these processes. An overview of endothelial cell contributions to the anticoagulant and procoagulant states is presented in Figure 101-2 . Normal unperturbed endothelium presents a nonthrombogenic surface to the circulation by preventing the activation and propagation of coagulation, enhancing fibrinolysis, and inhibiting platetet aggregation. These activities are accomplished by both passive and active processes. Conversely, when injured or under inflammatory conditions, the endothelium may become procoagulant.

Four mechanisms may be utilized by endothelial cells to inhibit coagulation. [140] [141] (1) thrombomodulin on the surface of endothelial cells binds thrombin. This coupling inhibits the coagulant properties of thrombin and also increases its affinity for protein C, which it cleaves and activates. Protein C also binds to the endothelial cell protein C receptor, which may also be essential for protein C activation; [142] (2) protein S, which is thought to be synthesized primarily by the endothelial cell, acts as a cofactor for protein C, but also has anticoagulant properties in its own right. Independent of the presence of activated protein C, free protein S is able to inhibit the prothrombinase and intrinsic tenase complexes and interact directly with factors Va and VIIIa; [140] [143] (3) heparan sulfate proteoglycans are secreted onto the luminal surface of endothelial cells and into the subendothelium. Heparan sulfates are capable of binding and activating antithrombin, thereby accelerating the inactivation of several procoagulant serine proteases including thrombin, factor Xa and factor IXa; (4) tissue factor pathway inhibitor (TFPI) is synthesized in the liver and in endothelial cells, and has been shown to be localized in apical granules of the endothelial cell. The tissue factorfactor VIIafactor Xa complex is inhibited by TFPI.[141] Other proteins such as TFPI-2, annexin V, and protease nexins 1 and 2 may also play an endothelium-dependent anticoagulant role.[140] Although the preceding discussion outlines the

Figure 101-2 An overview of endothelial function in coagulation. EDRF, endothelium-derived relaxing factor; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PGI_2, prostacyclin; PC, protein C; PS, protein S;TM, thrombomodulin; TF, tissue factor; tPA, tissue plasminogen activator. *(Adapted from Wu and Thiagarajan [141] with permission from the Annual Review of Medicine Volume 47, 1996, by Annual Reviews, Inc.)*

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major potential endothelial anticoagulant pathways, other factors are likely to be important.

Endothelial cells are the major source of tissue-type plasminogen activator (tPA). [141] [144] Although cultured endothelial cells can secrete urokinase (uPA), the in vivo significance of this finding is not clear. About 40% of tPA is bound to its inhibitor, plasminogen activator inhibitor-1 (PAI-1), also secreted by endothelial cells. Stresses such as exercise, acidosis, hypoxia, shear forces, increased venous pressure, and thrombin will release tPA, [141] [144] and presumably activate plasminogen. Receptors for plasminogen and tPA are present on the endothelial cell surface, allowing for effective localized production of fibrinolytic activity.

When in close proximity to endothelial cells, platelets become unresponsive to agonists. This inhibition of platelet aggregation is accomplished by the secretion of prostacyclin (PGI_2) and nitric oxide (NO), and by the surface expression of an ecto-ADP (adenosine diphosphate)ase recently identified as CD39. [141] [145] Prostacyclin is synthesized mainly by vascular endothelial and smooth muscle cells as a product of arachidonic acid metabolism. It inhibits platelet activation, secretion, and aggregation, and monocyte interactions with endothelial cells. It also causes vascular smooth muscle cell relaxation. Nitric oxide (NO) similarly has a wide range of functions including the inhibition of platelet adhesion, activation, and aggregation. Most of the NO released from endothelial cells is elaborated abluminally where it acts on the smooth muscle cell to cause vasodilation. However, some NO may also enter the lumen and thereby diffuse into platelets. Prostacyclin and NO can act synergistically to reverse platelet aggregation. [146] The released platelet agonist, ADP, can be inactivated by an endothelial membraneassociated ecto-ADPase. Metabolism of ADP by the ecto-ADPase eliminates platelet recruitment and returns the platelets to their resting state.

Although intact endothelium is necessary to maintain blood in a fluid state and inhibit coagulation under normal conditions, when injured the endothelium can rapidly downregulate its anticoagulant functions and become procoagulant, even without overt vascular damage such as with trauma or surgery. Further tissue injury or vascular pathology also leads to exposure of the underlying matrix, which is procoagulant by virtue of its binding to and activation of platelets. Endothelial cells that have been induced to undergo apoptosis in vitro become procoagulant. Apoptotic endothelial cells expose phosphatidylserine on their surface, and downregulate their anticoagulant properties described above. Apoptotic endothelial cells and vascular smooth muscle cells have also been shown to increase thrombin formation in recalcified citrated plasma. [147] [148] Microvascular endothelial cells undergo apoptosis following exposure to plasma from patients with thrombotic thrombocytopenic purpura, which may play a role in the formation of microthrombi seen in this disorder. [149]

Even without endothelial death or vascular injury, perturbation of the vascular lining by inflammatory mediators or altered shear stresses can tip the balance such that the endothelium converts from a nonthrombogenic surface to a procoagulant one. This is due to the downregulation of anticoagulant factors as well as the induction of procoagulant ones. For instance, tumor necrosis factor (TNF) and IL-1 have been shown to downregulate thrombomodulin as well as induce expression of tissue factor on endothelial cells in vitro. [144] [150] Tissue factor, which is not normally expressed by endothelial cells, has been shown to be present in endothelial cells overlying atherosclerotic plaque [151] and can activate the extrinisic pathway of coagulation. Tumor necrosis factor, IL-1, and endotoxin can also increase the expression of PAI-1 in endothelial cells with downregulation or no change in tPA levels, thereby impairing fibrinolysis. In the setting of acute inflammation, there is also increased release of vWF, platelet activating factor, and fibronectin, all of which may potentiate thrombus formation.

Control of Vascular Tone

The control of vascular tone is primarily orchestrated by a balance of endothelium-derived vasodilators (NO, PGI_2, an endothelium-derived hyperpolarizing factor (EDHF) and vasoconstrictors (endothelin-1, superoxide). In addition to inhibiting platelet aggregation, NO and PGI_2 also act as vasodilators. [152] [153] NO is produced by the conversion of L-arginine to L-citrulline by NO synthase (NOS). Three forms of NOS exist: a constitutive NOS in neuronal tissue; an inducible enzyme found in macrophages and other cells that plays a role in NO-induced cytotoxicity; and a constitutively active endothelial form (ecNOS). [51] The inducible form of NOS is also present in endothelial cells and may be responsible for the uncontrolled vasodilation seen in septic shock. [51] Injection into the forearm of L-arginine analogues that inhibit NOS causes substantial vasoconstriction. Conversely, ecNOS-deficient mice are hypertensive, suggesting that NO release is crucial for maintaining a basal vasodilation. [152] [153] The major physiological stimulus for the continuous production of NO in vivo is shear stress. The action of NO on platelets (anti-aggregatory), endothelial cells and smooth muscle cells (relaxation) is due to the activation of guanyl cyclase and the formation of cyclic guanosine 3:5-cyclic monophosphate (cyclic GMP). Whereas NO is quite unstable, the formation of S-nitrosothiols in the presence of oxygen and thiols provides a stable reservoir of NO. [154] Hemoglobin is an avid scavenger of NO, which may account for the vasoconstriction observed with the administration of cell-free, hemoglobin-based red cell substitute. [155] Physiologically, however, S-nitrosohemoglobin acts as a regulator of blood flow. Deoxygenation is accompanied by an allosteric change in S-nitrosohemoglobin that releases the NO group, relaxing blood vessels to bring blood flow into line with local oxygen requirements. [156]

Prostacyclin, on the other hand, does not appear to have as global a role in vasodilation as NO. PGI_2 is synthesized mainly by endothelial cells and acts locally at sites of injury, and may counterbalance the vasoconstriction induced by the platelet-produced arachidonic acid metabolite, thromboxane A_2 (TXA_2). Most PGI_2 is released luminally, where it has an anti-platelet effect. Prostacyclin transduces a cellular signal by increasing the levels of cyclic AMP, whereas TXA_2 signals via the phosphoinositol pathway and lowering of cyclic AMP levels. Synthesis of prostaglandins begins by the action of cyclo-oxygenases (COX1 and COX2) on arachidonic acid. Aspirin inhibits cyclo-oxygenase irreversibly in both platelets and endothelial cells. However, the clinical effect is seen primarily in platelets for two reasons. [51] [153] One reason is that platelets, being non-nucleated, cannot synthesize new cyclo-oxygenase, although endothelial cells can. Therefore, TXA_2 synthesis recovers only when new platelets enter the circulation whereas cyclo-oxygenase production by endothelial cells restores PGI_2 levels within a few hours. The second reason is that platelets encounter orally administered aspirin before it is deacetylated by the liver and diluted by the venous circulation.

There is evidence that endothelial cells release another relaxing factor, EDHF, that acts by increasing the membrane potential of smooth muscle cells. The nature of EDHF is unclear, but it appears to act by effecting potassium efflux. This factor is released in isolated coronary arteries, in the presence of an arginine analogue (NOS-inhibitor) and indomethacin, a COX-inhibitor, suggesting that EDHF, NO, and prostanoids contribute differentially to relaxation in human coronary arteries. [157] Recent findings suggest that EDHF is a cytochrome P450-linked, arachidonate metabolite. [158] [159] [160]

Endothelin-1 (ET-1) is a 21-amino-acid peptide, released preferentially at the abluminal surface of endothelial cells, that exhibits potent vasoconstrictor activity. [161] [162] Of the three known endothelins, only ET-1 is produced by endothelial cells. At least two receptors have been identified (ET-A and ET-B) that bind to

all three endothelins. ET-A is abundantly expressed on smooth muscle cells while ET-B is predominantly expressed on endothelial cells. The vasoconstrictor activity of ET-1 is preferentially mediated by ET-A receptors on smooth muscle cells. Engagement of ET-B on endothelial cells by ET-3 may paradoxically cause a transient vasodilation. There is little evidence that ET-1 plays a role in essential hypertension, but it might contribute to pregnancy-induced hypertension and may also play a role in reperfusion injury following ischemia. [162]

Another seemingly important regulator of vascular tone is the superoxide anion. [152] [163] The source of this free radical may be the endothelium itself or inflammatory cells that have been recruited to sites of injury or inflammation. Interaction of superoxide radicals and NO produces peroxynitrite and reduces the concentration of NO. Peroxynitrite can also oxidize low-density lipoprotein and deleteriously modify other proteins, thereby causing endothelial dysfunction. Increased production of superoxide inhibits synthesis of PGI_2, but not that of TXA_2. [163]

The endothelium also expresses angiotensin-converting enzyme at its surface which converts angiotensin I to angiotensin II, a potent vasoconstrictor. The interaction between endothelin, angiotensin II and alpha-adrenergic agonists in the pathogenesis of hypertension is believed to be complex. [164]

An altered balance of the vasoactive substances described in this section has been proposed to cause endothelial dysfunction and the attendant vascular pathology observed in atherosclerosis, hypertension, and diabetes mellitus. Furthermore, alteration of vascular function in these diseases may then perpetuate the endothelial dysfunction and, consequently, worsen disease.

The Interaction of Blood Elements with the Vessel Wall

Leukocytes

In the absence of any inflammatory stimulus, neutrophils circulate freely and do not interact with the endothelium. In contrast, there is a continuous, low-level physiologic traffic of monocytes and lymphocytes across the vessel wall. Monocytes emigrate from the bloodstream to develop into tissue macrophages that may

exhibit tissue- or organ-specific functions. To maintain immune surveillance of tissue, lymphocytes recirculate between blood and lymphatics, gaining entrance to the latter at the HEV of postcapillary venules in lymphoid tissue.

Studies by intravital microscopy have established a sequence of events involved in leukocyte emigration at extravascular sites of inflammation. [165] Under conditions of flow, leukocytes are first observed to roll along the endothelium of postcapillary venules adjacent to the site of inflammation. Subsequently, some of the rolling leukocytes stick firmly, migrate along the endothelial surface, diapedese between endothelial junctions, and then migrate through subendothelial matrix. These steps in emigrationrolling, firm adhesion, and transendothelial migrationare also involved in lymphocyte emigration at HEV. They result from the interaction of distinct leukocyte and endothelial receptors in an adhesion cascade (Fig. 101-3 ; Chap. 5).[166] [167] [168] Rolling is observed only under flow conditions and is the consequence of shear forces acting on the leukocyte and adhesive interactions between selectin receptors and their glycoconjugate counterstructures. It is initiated primarily by activation of the endothelium by extravascular stimuli such as bacterial-derived products or by endogenous mediators produced by the endothelium or cells in tissue (Table 101-2). Early on, rolling is mediated by P-selectin, which is rapidly translocated from Weibel-Palade bodies to the luminal surface, and possibly also by induced ligand(s) for L-selectin. [169] E-selectin is involved only at later time-points as it is not constitutively expressed, but rather is induced over hours by de novo synthesis.

In order for leukocytes to circulate freely, their integrin receptors must be in a low avidity, i.e., non-binding, state but able to increase avidity rapidly. Once tethered to endothelium by selectin interactions, [170] [171] the leukocyte integrin receptors are activated by endothelial membrane-expressed platelet-activating factor, endothelial membrane-bound chemokines, or locally-secreted chemoattractants. Activation of the leukocyte integrins increases their avidity for their endothelial ligands, which are

Figure 101-3 Leukocyte and endothelial adhesion molecules. Endothelial P-selectin recognizes SLe^x or related sialylated and fucosylated glycoconjugates together with sulfate residues expressed on PSGL-1. Endothelial E-selectin recognizes sialylated and fucosylated glycoconjugates expressed on PSGL-1, L-selectin, and murine ESL-1. Leukocyte L-selectin binds to an uncharacterized ligand on systemic endothelium and to sialylated, sulfated, and fucosylated moieties on GlyCAM-1 and CD34 on peripheral node HEV and MAdCAM-1 on mucosal HEV. Leukocyte $_{L\ 2}$ binds to ICAM-1 and ICAM-2; $_{M\ 2}$ to ICAM-1 and to an as yet unidentified ligand(s). Leukocyte $_{4\ 1}$ interacts with VCAM-1 and $_{4\ 7}$ with MAdCAM-1. The IgSF protein PECAM-1 is expressed on both the leukocyte and the endothelial cell and participates in transendothelial migration. SLe^x, Sialyl Lewis X; PSGL-1, P-selectin glycoprotein ligand; ESL-1, E-selectin ligand; GlyCAM-1, glycosylation-dependent cell adhesion molecule; MAdCAM-1, mucosal addressin cell adhesion molecule; IgSF, immunoglobulin gene superfamily; ICAM-1, intercellular adhesion molecule; VCAM-1, vascular cell adhesion molecule; PECAM-1, platelet-endothelial cell adhesion molecule.

TABLE 101-2 -- Endothelial Cell Activation[a]

	Agonists	Response
Rapid (protein synthesis-independent)	Thrombin, histamine	Synthesis of PAF Secretion of vWF P-selectin, tPA
	Thrombin Oncostatin M	Secretion of TFPI, PGI2 Secretion of P-selectin
Delayed (protein synthesis-dependent)	IL-1, TNF, LPS	Synthesis of ICAM-1, VCAM-1, E-selectin, IL-1, IL-6, IL-8, MCP-1, GM-CSF Synthesis of TF, PAI-1, tPA, uPA Downregulation of TM
	IFN-/, TNF, LT	Synthesis of Class I MHC
	IFN-	Synthesis of ICAM-1, Class II MHC
	IL-4	Synthesis of VCAM-1, P-selectin
	Oncostatin M	Synthesis of E-selectin, VCAM-1, ICAM-1, IL-6
	Thrombin	Synthesis of IL-8, IL-6, E-selectin

ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; LT, lymphotoxin; MCP, monocyte chemoattractant protein; MHC, major histocompatibility complex; PAF, platelet activating factor; PAI, plasminogen activator inhibitor; vWF, von Willebrand factor; TF, tissue factor; TFPI, tissue factor pathway inhibitor; tPA, tissue type plasminogen activator; TM, thrombomodulin; uPA: urokinase type plasminogen activator; VCAM, vascular cell adhesion molecule.

[a] Selected agonists and responses; many other stimuli have been reported to activate.

constitutively expressed (ICAM-1, ICAM-2), further up-regulated (ICAM-1), or induced (VCAM-1) by locally-generated cytokines (Table 101-2). Although the activation-dependent increases in integrin avidity allow shear-resistant firm adhesion, subsequent leukocyte migration over the endothelium requires reversible adhesion due to cyclic modulation of receptor avidity. [172]

Upon encountering an intercellular junction, some of the adherent, migrating leukocytes diapedese between endothelial cells to enter extravascular tissue and then migrate to the site of inflammatory or immune reaction. This process of transendothelial migration utilizes leukocyte integrin interactions with endothelial ligands and, for phagocytes, homophilic binding of PECAM-1.[173] Other leukocyte and endothelial molecules involved in transendothelial migration have not yet been fully characterized. Diapedesis also appears to involve signaling by the leukocyte to the endothelial cell, triggering a disruption of junctional integrity. [174] [175]

Leukocyte recruitment is terminated by several mechanisms. E- and P-selectin are removed from the endothelial cell surface by endocytosis, [176] while L-selectin is cleaved from leukocytes by a membrane protease.[177] Decay of cytokine, chemokine, or chemoattractant generation leads to gradual resolution of endothelial adhesion molecule expression and integrin activation. Locally-generated mediators such as nitric oxide [178] or TGF-[179] also act to inhibit further leukocyte adhesion to endothelium.

Several caveats should be considered with respect to this model of an adhesion cascade. First, selectin-mediated rolling is not a prerequisite for emigration under conditions of reduced flow, as might sometimes occur at sites of inflammation. [180] [181] Second, the model was developed from observations in the systemic microcirculation, where leukocyte emigration occurs in postcapillary venules under relatively low shear forces. Its validity in the pulmonary microcirculation where emigration occurs in the capillaries [182] or in the arterial circulation where shear forces are greater is uncertain. Third, under some conditions leukocytes are able to roll via receptors other than selectins (e.g., VLA-4 or CD44). [183] [184] Finally, a number of other adhesion molecules are involved in leukocyte binding to endothelium and their roles in this cascade remain to be defined.

Although critical for host defense and repair, under some circumstances, leukocyteendothelial interactions may lead to vascular and tissue damage. Activated leukocytes tightly adherent to endothelium may release toxic products that damage endothelium, producing permeability edema or thrombosis. Emigrated leukocytes may similarly damage adjacent tissue. Increased expression of endothelial adhesion molecules occurs in involved tissues in diverse inflammatory and immune disorders.[185] Moreover, blockade of leukocyte adhesion to endothelium by monoclonal antibodies or other antagonists has been demonstrated to reduce vascular and tissue injury in a wide variety of animal models of inflammatory and immune disease. [186] Similar protection is observed in mice with targeted disruption of adhesion molecule genes. Anti-adhesion therapy is now being tested in several clinical trials.

Platelets

Like neutrophils, unactivated platelets do not interact with unperturbed endothelium. Following a vascular injury that produces endothelial denudation or retraction, platelets rapidly adhere to the exposed subendothelium. As discussed in greater detail in Chapters 99 and 100 , at high shear rates this initial adhesion does not require platelet activation and involves platelet glycoprotein (GP)Ib/IX/V binding to vWF in the subendothelial matrix. Platelet activation occurs following adhesion,

leading to platelet spreading mediated by integrin receptors binding to matrix components and aggregation mediated by fibrinogen binding to GPIIb/IIIa.

There is evidence that unactivated platelets can bind directly to activated endothelium via endothelial P-selectin [187] and PECAM-1[188] in vivo or via vWF in vitro.[189] Also, activated platelets have been shown to bind to HEV in vivo via platelet P-selectin [190] and to endothelium in vitro via platelet GPIIb/IIIa.[191] Platelet adhesion to intact endothelium could contribute to thrombus formation in the microcirculation and may provide a link between thrombosis and inflammation.

Red Blood Cells

Only sickled red blood cells have been shown to interact significantly with endothelium. Binding of sickle cells to microvascular endothelium may be an important pathogenic factor in vaso-occlusive events (see Chap. 30). Several adhesive interactions between sickle red cells and endothelium have been described in vitro. [192] Of particular interest is the finding that sickle reticulocytes have abnormally increased expression of VLA-4 ($_{4}{}_{1}$) and CD36. VLA-4 was found to promote sickle reticulocyte adhesion to VCAM-1[193] or fibronectin[194] on activated endothelium. Thrombospondin was shown to serve as a bridge between CD36 on sickle reticulocytes and an endothelial cell receptor, likely $_{v}{}_{3}$.[195] Notably, hydroxyurea therapy, which reduces the clinical severity of sickle cell disease, was found to reduce both VLA-4 and CD36 expression on sickle reticulocytes, providing correlative evidence that these adhesion receptors contribute to the pathophysiology. [196]

Endothelial Cell Activation

Although once viewed as a passive barrier between blood and tissue, it is now evident that the endothelium is a dynamic organ that responds to diverse stimuli. In particular, activation of endothelial cells induces a proinflammatory and prothrombotic phenotype (Table 101-2). Activation responses of endothelium have been categorized as rapid (minutes), protein synthesisindependent

(type I) or delayed (hours to days), protein synthesis-dependent (type II). [197] [198] Histamine and hyperacute xenograft rejection elicit a type I response, while TNF, IL-1, most cytokines, and delayed xenograft rejection induce predominantly a type II activation pattern. [198] Some agents, such as thrombin[199] and oncostatin M,[200] trigger both types of activation.

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Chapter 102 - Molecular Basis of Blood Coagulation

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INTRODUCTION

Blood coagulation is a host defense system that maintains the integrity of the high-pressure closed circulatory system. After tissue injury, alterations in the capillary bed and laceration of venules and arterioles lead to extravasation of blood into soft tissues or external bleeding. To prevent excessive blood loss, the hemostatic system, which includes platelets, vascular endothelial cells, and plasma coagulation proteins, is called into play. Immediately after tissue injury, a platelet plug is formed through the processes of platelet adhesion and aggregation. Blood coagulation may be considered a mechanism for rapid replacement of an unstable platelet plug with a chemically stable fibrin clot. A series of interdependent enzyme-mediated reactions translates the molecular signals that initiate blood coagulation into a major biologic event, the formation of the fibrin clot.

In vitro the generation of thrombin and the formation of a fibrin clot propagate through two separate pathways, the intrinsic pathway and the extrinsic pathway. [1] [2] To generate a clot via the intrinsic pathway in a test tube, components intrinsic to whole blood are required. To generate a clot via the extrinsic pathway, components intrinsic to whole blood are required along with an activator, tissue factor, that is extrinsic to blood (Table 102-1). Tissue factor, also known as tissue thromboplastin, is a membrane protein that comes in contact with the flowing blood on blood vessel injury.

The intrinsic pathway of blood coagulation includes protein cofactors and enzymes (Fig. 102-1). This pathway is initiated by the activation of factor XII by kallikrein on negatively charged surfaces, including glass in vitro. High-molecular-weight kininogen facilitates this activation. The enzyme form of factor XII, factor XIIa, catalyzes the conversion of factor XI, a proenzyme, to its active enzyme form, factor XIa. In the presence of Ca^{2+} , factor XIa activates the proenzyme factor IX to its enzyme form, factor IXa. Factor IXa binds to the cofactor factor VIIIa bound on membrane surfaces in the presence of calcium ions to generate a complex with enzymatic activity known as tenase, a nickname for the enzymatic activity that acts on factor X. This tenase complex converts the proenzyme factor X to its enzyme form, factor Xa. In a parallel series of interactions, factor Xa binds to the cofactor factor Va bound on membrane surfaces in the presence of calcium ions to generate a complex with enzymatic activity known as prothrombinase. This complex converts the proenzyme prothrombin to its enzyme form, thrombin. Thrombin acts on fibrinogen to generate the fibrin monomer, which rapidly polymerizes to form the fibrin clot. During laboratory analysis of blood clotting, the intrinsic pathway of blood coagulation is evaluated by using the activated partial thromboplastin time (PTT). The clotting of plasma is initiated by the addition of negatively charged particles such as kaolin.

The extrinsic pathway of blood coagulation also includes protein cofactors and enzymes (Fig. 102-1). This pathway is

TABLE 102-1 -- Properties of the Genes, mRNAs, and Gene Products of the Components of the Blood Coagulation Cascade

	Molecular Weight	Gene (kb)	Chromosome	mRNA	Exons (kb)	Plasma Concentration (g/ml)	Function
Prothrombin	72,000	21	11p11q12	2.1	14	100	Protease zymogen
Factor X	56,000	22	13q34	1.5	8	10	Protease zymogen
Factor IX	56,000	34	q2627.3	2.8	8	5	Protease zymogen
Factor VII	50,000	13	13q34	2.4	8	0.5	Protease zymogen
Factor VIII	330,000	185	q28	9.0	26	0.1	Cofactor
Factor V	330,000	7.0	1q2125	7.0	25	10	Cofactor
Factor XI	160,000	23			15	5	Protease zymogen
Factor XII	80,000	12	5	2.4	14	30	Protease zymogen
Fibrinogen	340,000					3,000	Structural
A-chain	64,000		4q26q28		5		
B-chain	56,000		4q26q28		8		
-chain	47,000		4q26q28		9		
Protein C	62,000	11		1.8	8	4	Protease zymogen
Protein S	80,000			2.4		25	Cofactor
von Willebrand factor	225,000 × n[a]	175	12pterp12	8.5	52	10	Adhesion
Tissue factor	45,000	12	1pterp12	2.1	6	0.0	Cofactor/initiator
Factor XIII	320,000					60	Fibrin stabilization

[a] n, number of subunits, where the subunit M_r is 225,000.

Figure 102-1 Blood coagulation cascade. Glycoprotein components of the intrinsic pathway include factors XII, XI, IX, VIII, X, and V, prothrombin, and fibrinogen. Glycoprotein components of the extrinsic pathway, initiated by the action of tissue factor located on cell surfaces, include factors VII, X, and V, prothrombin, and fibrinogen. Cascade reactions culminate in the conversion of fibrinogen to fibrin and the formation of a fibrin clot. Certain reactions, including the activation of factor X and prothrombin, take place on membrane surfaces. Proenzymes, diamonds; procofactors, squares; enzymes and cofactors, circles; macromolecular complexes on membrane surfaces, shaded rectangles. FG, fibrinogen; F, fibrin; PT, prothrombin; T, thrombin; TF, tissue factor; HMWK, high-molecular-weight kininogen. *(Modified from Furie and Furie,[276] with permission.)*

initiated by the formation of a complex between tissue factor on cell surfaces and factor VIIa. Although the mechanism by which a small amount of factor VII is converted to factor VIIa in the absence of ongoing blood clotting is unknown, plasma contains factor VIIa at levels of 0.58.4 ng/ml. [3] When tissue factor is exposed to plasma following injury, this factor VIIa complexes with tissue factor to form an enzyme complex that activates factor X to factor Xa. In turn, factor Xa can feed back to convert more factor VII to factor VIIa,[4] thus accelerating the rate of activation of the extrinsic pathway. Factor VIIa/tissue factor complex, like the tenase complex, converts factor X to its active form, factor Xa, which binds to the cofactor factor V bound on membrane surfaces in the presence of calcium ions to generate the prothrombinase complex. This complex converts prothrombin to thrombin, which converts fibrinogen to fibrin to generate the fibrin clot. During laboratory analysis of blood clotting, the extrinsic pathway of blood coagulation is evaluated by using the prothrombin time. The clotting of plasma is initiated by the addition of exogenous tissue factor.

This scheme of blood clotting (Fig. 102-1) remains invaluable for understanding clot formation in vitro, and specifically for laboratory monitoring of anticoagulation therapy for, and diagnosis of, coagulation disorders. Yet the physiologic pathways relevant to blood coagulation in vivo are clearly different. A scheme of blood coagulation in vivo must consider the following salient features. First, since patients with hereditary factor XII, prekallikrein, or high-molecular-weight kininogen deficiency have a markedly prolonged PTT but no bleeding phenotype, these proteins are not components required to maintain hemostasis in vivo and should not be included in an in vivo model of blood clotting. [5] [6] [7] Second, tissue factor, a normal constituent of the surface of nonvascular cells and of stimulated monocytes and endothelial cells, activates blood coagulation. Third, the tissue factor/factor VIIa complex activates not only factor X but factor XI as well, suggesting a central role for factors VIII and IX in coagulation initiated by the tissue factor pathway. [8] Finally, factor XI deficiency is not invariably associated with a bleeding phenotype; as such, the position and prominence of factor IX in the blood coagulation cascade remain uncertain. Figure 102-2 presents one model of blood coagulation and depicts the pathway of sequential reactions that might lead to clot formation in vivo. [9] The key to the initiation of blood coagulation is tissue factor. The exposure of cell surfaces expressing tissue factor to the plasma proteins leads to the binding of factor VIIa to tissue factor. Either this tissue factor/factor VII complex has low but finite coagulant activity[10] or tissue factor complexed to the activated form of factor VII, factor VIIa, activates factors IX and X. [11] The protease responsible for initial activation of factor VII is unknown, but once clotting is activated, several proteases farther down the pathway can activate factor VII. Factors Xa and VIIa both catalyze the activation of factor VII, so a potential mechanism for acceleration of factor VII activation exists. [12] Furthermore, both thrombin and factor XIa in the presence of negatively charged surfaces catalyze the activation of factor XI. [13] Once factor XIa is generated, an additional mechanism augments the activation of factor IX. Factor IXa in complex with factor VIIIa on membrane surfaces activates factor X. Factor Xa in complex with factor Va on membrane surfaces activates prothrombin. Thrombin cleaves fibrinogen, yielding monomeric fibrin, which then polymerizes to form the fibrin clot.

The blood coagulation cascade proceeds on cell surface membranes. Indeed, only on the membrane surfaces of stimulated cells can the reactions involved in blood clotting proceed efficiently. Phosphatidylserine, a critical component of the cell

Figure 102-2 Physiologic pathways of blood coagulation. Blood coagulation is initiated by tissue factor (TF) expressed on cell surfaces. When plasma comes in contact with tissue factor, factor VIIa in plasma binds to this receptor. The factor VIIa/tissue factor complex activates both factor IX and factor X. The proteolytic activation of factor VII to VIIa, factor XI to XIa, factor VIII to VIIIa, and factor V to Va through feedback mechanisms (e.g., thrombin [T] or factor XIa [FXIa]) greatly accelerates the rate of blood clotting. The process culminates in the generation of fibrin and its polymerization to form a fibrin clot. *(From Furie and Furie, [5] with permission.)*

membrane that is required for assembly of blood clotting proteins on membrane surfaces, is exposed on the external membrane surface following cell activation. Phosphatidylserine is asymmetrically distributed on the phospholipid bilayer of the plasma member. An enzyme known as phospholipid scramblase catalyzes the calcium-dependent redistribution and phosphotidylserine exposure to the outer leaflet. [14] [15] [16] Tissue factor is an integral membrane protein constitutively expressed on the surface of nonvascular cells[17] [18] [19] and expressed on the surface of monocytes and endothelial cells following cell stimulation with certain agonists. This protein binds to factors VII and VIIa, generating the tissue factor/factor VIIa complex on membrane surfaces that activates factor X. [20] In another example of enzyme assembly on membrane surfaces, factors IXa and VIIIa both bind to anionic phospholipid membranes. [21] [22] It is envisioned that these two proteins skate about on the membrane, collide, and form the tenase complex, factor IXa/factor VIIIa. This complex has the enzymatic activity to convert factor X, also bound to the cell membrane, to factor Xa. Similarly, factors Xa and Va bind to anionic phospholipid membranes. [23] These two proteins also skate about on the membrane, collide, and form the prothrombinase complex, factor Xa/factor Va.[24] This complex has

the enzymatic activity to convert prothrombin, also bound to the cell membrane, to thrombin, which is released from the cell surface. In vivo, platelet membranes contribute the critical surface for blood clotting. Activated (but not resting) platelets express a factor VIIIa-binding site. [25] During platelet activation in vitro, small microparticles are released from the platelet membrane that are especially rich in receptors for factors VIIIa and Va. [26] [27]

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STRUCTURE OF THE BLOOD COAGULATION PROTEINS

Domain Structure

Each of the blood coagulation proteins contains multiple functional units, derived from common ancestral genes (Fig. 102-3). As a rule these functional units, or domains, are encoded by individual exons. Although the domains are not structurally identical, they have sufficient homology in structure to suggest homology of function as well. These domains are responsible for directing protein trafficking and post-translational processing during biosynthesis and for membrane-binding properties, protein complex formation, and enzyme function of the mature protein. In factor IX, for example, these domains are independently folded but give evidence of significant interdomain interaction. [28]

Signal Peptide

The nascent chains of secreted proteins contain a short domain that permits translocation of the growing polypeptide

Figure 102-3 Structural domains of the proteins involved in hemostasis and related proteins. Domains, identified in the key, include signal peptide, propeptide, Gla domain, epidermal growth factor (EGF) domain, repeat sequences, kringle region, fibronectin (types I and II) domains, aromatic amino acid stack, zymogen activation region, and catalytic domain. Sites of proteolytic cleavage associated with synthesis of mature protein are indicated by thin arrows and those associated with zymogen activation by thick arrows. PT, prothrombin; FIX, factor IX; FX, factor X; FVII, factor VII; PC, protein C; FXI, factor XI; FXII, factor XII; PS, protein S; tPA, tissue plasminogen activator; PUK, prourokinase; MGP, matrix Gla protein; BGP, bone Gla protein; Pmg, plasminogen; T, trypsin. *(Modified from Furie and Furie,[276] with permission.)*

chain into the endoplasmic reticulum. This domain is dominated by hydrophobic amino acids and is usually 1530 residues in length. The blood coagulation proteins found in the plasma are initially synthesized with a signal peptide, which is cleaved within the endoplasmic reticulum by the signal peptidase.

Propeptide/-Carboxyglutamic Acid-Rich Domain

The vitamin K-dependent proteins contain a propeptide domain between the signal peptide and the -carboxyglutamic acid-rich N-terminal region. This propeptide, 18 residues long in factor IX, [29] [30] contains a -carboxylation recognition site that directs -carboxylation of the vitamin K-dependent proteins after synthesis [31] and is sufficient to carboxylate glutamic acid residues regardless of their sequence context. [32] Adjacent to the carboxylation recognition site is the -carboxyglutamic acid-rich region of these proteins. Glutamic acid residues within this region serve as substrates for the vitamin K-dependent -carboxylase. After carboxylation, the propeptide is cleaved from the mature protein in a late post-translational processing step. A single exon in the vitamin K-dependent proteins encodes the propeptide (including the -carboxylation recognition site) and the -carboxyglutamic acid-rich region of 40 residues. The -carboxyglutamic acid domain (Gla domain) of the vitamin K-dependent proteins contains 1012 -carboxyglutamic acid residues and is critical to the Ca^{2+} -binding properties of these proteins. This domain is responsible for promoting the calcium-dependent interaction of these proteins with membrane surfaces, a characteristic essential for the function of these proteins.

The three-dimensional structure of the -carboxyglutamic acid-rich region in bovine prothrombin fragment 1 is disordered in the absence of Ca^{2+} .[33] In the presence of Ca^{2+} the Gla domain assumes a unique structure. [34] Of the ten -carboxyglutamic acid residues, four residues on one side and two residues on the other define a carboxylate surface that chelates five Ca^{2+} organized in a linear cluster that extend from one side of the molecule to the other (Fig. 102-4). The NH_2 -terminal alanine loops back to form an ion pair with Gla 17, Gla 21, and Gla 27. With the exception of Gla 6, all the -carboxyglutamic acid residues are critical for prothrombin function. [35] The mechanism by which this region promotes interaction of the protein with membrane surfaces involves the formation of this unique fold and the expression of a membrane-binding site. The Gla domains of factor VII and factor IX show marked three-dimensional structural homology in their calcium-stabilized conformer, compared to the Gla domain of prothrombin. [36] [37]

Epidermal Growth Factor Domain

The epidermal growth factor (EGF)-like domain is a common motif found in many proteins, including some of the proteins involved in blood coagulation. In the blood clotting proteins it is highly homologous with the EGF precursor. This domain, 4350 amino acid residues in length, contains three disulfide bonds arranged in a characteristic covalent structure. Although the EGF domain in some proteins mediates their interaction with an EGF or EGF-like receptor on cell surfaces, the EGF domains on the blood clotting proteins participate in protein complex formation. A calcium-binding site, present in some of the EGF domains of the vitamin K-dependent proteins, is defined by carboxylate groups on aspartic acid. [38] The structures of the EGF domains of factors IX and X, determined by nuclear magnetic resonance (NMR) spectroscopy, demonstrate marked homology with the three-dimensional structure of EGF. [39] [40] Factors IX, X, and VII contain two adjacent EGF domains (as does protein C, while protein S has four EGF domains). Factor XII contains two nonadjacent EGF domains, while tissue plasminogen

Figure 102-4 Three-dimensional structure of the -carboxyglutamic acid-rich region of prothrombin. This region is responsible for metal binding and membrane-protein interaction. *(Modified from Soriano-Garcia et al.,[34] with permission.)*

activator (t-PA) and prourokinase each have a single EGF domain.

Kringle Domain

Another common motif in proteins is the kringle domain. This region, with a characteristic covalent structure defined by a pattern of three disulfide bonds, is 100 amino acid residues in length. The three-dimensional structure of the prothrombin kringles reveals an oblate ellipsoid, with folding defined by close contacts between the sulfur atoms of two of the disulfide bridges. [33] Internal structures are well conserved among kringles from various proteins, but molecular surface differences relate to differences in function. The kringle domains play a role in protein complex formation. In prothrombin, the second kringle domain interacts with factor Va in the prothrombinase complex. [41] Among the proteins involved in hemostasis, kringles are found in prothrombin, [42] factor XII, [43] plasminogen, [44] prourokinase, [45] and t-PA. [46]

Catalytic Domain

A catalytic domain that is highly homologous with the structure of trypsin and chymotrypsin is common to all the blood clotting enzymes. This domain includes a site for the conversion of an inactive proenzyme to an active enzyme via cleavage of peptide bonds, a regulatory process known as zymogen activation by limited proteolysis. Furthermore, this domain contains the enzymatic machinery for cleavage of peptide bonds, the specific recognition site for protein substrates, and the site for interaction with specific protein inhibitors that regulate enzymatic activity. The blood clotting proteases are serine proteases, a class of enzymes with a common mechanism of enzymatic action that requires the catalytic triad of serine, aspartic acid, and histidine within the active site. The catalytic domain of the blood clotting proteases has an active site and an internal core nearly identical

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to that of trypsin.[47] The molecular surfaces surrounding the enzyme active site are likely responsible for defining the extended substrate-binding site of the enzyme. Indeed, solution of the crystal structure of human -thrombin has emphasized the structural homology between thrombin and other well-characterized serine proteases while simultaneously providing details of the molecular structure not available from models derived indirectly from other homologous structures. [48] Two patches of positively charged amino acids close to the C-terminal B-chain helix form the presumed heparin-binding site and a secondary fibrinogen-binding site. [49] The interaction of the A and B chains is stabilized by charged residues. A deep canyon-like active site cleft probably contributes significantly to the narrow substrate specificity of thrombin.[48] [49]

Other Domains

Other motifs that appear within the proteins involved in hemostasis include the aromatic amino acid stack domain, the repeat regions observed in factor XI, and the fibronectin type I and type II domains. The functions of these domains are unknown.

Components of the Intrinsic and Extrinsic Pathways

Factor XII

The factor XII gene, located on chromosome 5, contains 14 exons and 13 introns (Fig. 102-5).[43] [50] Exon I encodes the signal peptide, exon II encodes a segment with no structural homology with other proteins, exons III and IV code for a region homologous with the type II fibronectin structure, exons V and VII each encode EGF domains, exon VI encodes a fibronectin finger domain that intervenes between the EGF domains, and exons VIII and IX each encode a kringle domain. The trypsin-like catalytic domain, including the activation region, is encoded by the remaining exons XXIV. The gene organization of the catalytic domains parallels that of urokinase, factor XI, and t-PA. The factor XII mRNA is 2.4 kb in length.[50] [51] The mature, plasma form of factor XII is composed of 596 amino acid residues in a single polypeptide chain.[43] [51]

Factor XII, also known as Hageman factor, is the first component of the intrinsic pathway. As such, it is a component of the contact phase of activation of blood coagulation observed in vitro. This protein does not appear to have a physiologic role in blood coagulation in vivo, because patients lacking this protein do not have a bleeding disorder. The protein circulates in the blood as a single-chain proenzyme of 80,000 molecular weight. [52] [53] Factor XII is a glycoprotein, with carbohydrate attached to Asn 230 and Asn 414. Other glycosylation sites include Thr 280, 286, 309, 310, and 318 and Ser 289. The surface-binding properties of factor XII, specifically the enhancement of the rate of factor XII activation by negatively charged surfaces, may be mediated by the positively charged amino acid sequence His-Lys-Tyr-Lys, a structure common to factor XII and kininogen.[54] The factor XII concentration in plasma is 30 g/ml. Factor XII has a plasma half-life of 2 days. [55]

Factor XI

The gene for factor XI, an intrinsic pathway component, contains 15 exons and 14 intervening sequences (Fig. 102-5). [56] Exon I encodes a 5 untranslated sequence, while exon 2 encodes a signal peptide. Four repeat sequences are each encoded by two exons: repeat 1 by exons III and IV, repeat 2 by exons V and VI, repeat 3 by exons VII and VIII, and repeat 4 by exons IX and X. The trypsin-like domain, responsible for the proteolytic function of the enzyme form of the zymogen, is encoded by exons XIIXV. Factor XI has a molecular weight of 160,000[57] and is composed of two identical chains bound together by disulfide bonds. [58] Factor XI circulates in the blood at a concentration of 5 g/ml. Its biologic half-life is 3 days. [59] [60]

Factor IX

Factor IX plays a critical role in blood coagulation (see Chap. 108). Its gene, adjacent to the factor VIII gene, is located on the X chromosome. Defects in this gene, both major and minor, are the cause of hemophilia B (see Chap. 108). This gene is 34 kb in length, contains eight exons, [61] and has a structure that is highly homologous with the structures of factors VII and X and protein C genes (Fig. 102-5). These genes are sufficiently similar to suggest that they were derived ancestrally from a common related gene. Factor IX is encoded on a 2.8-kb mRNA transcript. The factor IX gene is regulated by a liver-specific cis-activating element that binds to the liver-specific transcription factors CCAAT enhancer-binding protein (C/EBP) and NF-1; the NF-1 binding site is located at 99 to 76 upstream of the transcriptional start site.[62] The EBP-binding site is located close to the transcription initiation site. An androgen-responsive promoter element in the factor IX gene explains the curious phenotype of factor IX Leyden (see Chap. 108).[63]

Figure 102-5 Gene structures among the blood coagulation and regulatory serine proteases. Exons are shown schematically according to scale. Introns (thin lines) are not drawn to scale. *(Modified from Furie and Furie,[276] with permission.)*

The mature protein, with a molecular weight of 56,000, requires vitamin K for its synthesis and contains 12 -carboxyglutamic acid residues. The fully carboxylated form of the protein binds to calcium ions and to membrane surfaces in the presence of calcium ions. Factor IX contains metal-binding sites, defined by -carboxyglutamic acid,[64] and another class of metal-binding site that is a component of the EGF domain.[65] [66] The site within the EGF domain is defined in part by Asp 64, a residue that is partially -hydroxylated. Factor IX is a glycoprotein containing N- and O-linked sugars. A tetrasaccharide is O-linked to Ser 61 through a fucose. [67] Factors IX and IXa bind to phospholipid membranes composed of phosphatidylserine-phosphatidylcholine. [22] [68] This interaction requires both Ca^{2+} and fully carboxylated factor IX.[68] The x-ray structure of porcine factor IXa in the absence of calcium demonstrates the relationship of the two EGF domains and the serine protease domain; however, the Gla domain is partially disordered.[69] The NMR-derived structure of human factor IX Gla domain in the presence of calcium supports, as a first approximation, a model of the Gla domain based upon that of prothrombin. [37] [70]

The expression of phospholipid-binding properties involves two metal-dependent conformational transitions. [68] The calcium-stabilized conformer expresses a phospholipid-binding site that is located at the N-terminus of the protein.[71] An alternative hypothesis, also pointing toward the N-terminus, is also plausible and consistent with existing data.[72] Factors IX and IXa bind to activated platelets but not to resting platelets. [73] [74] However, it is not known whether a receptor is present on the platelet surface, nor is the role played by surface phospholipid known. Factor IXa, but not factor IX, binds to factor VIIIa on the surface of activated platelets. The factor VIIIa-binding site includes regions of the second EGF domain and the serine protease domain.[75] The endothelial cell-binding site of factor IX resides in the Gla domain.[76] Collagen on the surface of endothelial cells defines this factor IX-binding site. [77] [78] However, the biologic role of this site remains uncertain. The factor IX concentration in plasma is 5 g/ml. This protein has a plasma half-life of 24 hours.[79] As with other proteins of this molecular size, it partitions between the intravascular and extravascular space. A comprehensive review of the structure of the factor IX gene and the protein it encodes is presented in Chapter 108 .

Factor VIII

Factor VIII is a critical cofactor required for normal blood clotting (see Chap. 107). Defects in the factor VIII gene that lead to a deficiency in factor VIII are the cause

of hemophilia A. The factor VIII gene is 186 kb in length and is divided into 26 exons (see Fig. 107-1). [80] [81] As such, it is one of the largest genes known. Located on the X chromosome, it is near the locus of the factor IX gene. Factor VIII is synthesized as a single polypeptide chain, including a 19-residue signal peptide; in its mature form it contains 2,332 amino acid residues. [80] [81] Although this factor is synthesized by many cell types, the liver is a major site of synthesis. [82] A molecular weight of 330,000 has been estimated for the glycosylated protein. The protein sequence of factor VIII demonstrates marked sequence homology with factor V, another protein cofactor. [83] Factor VIII circulates in the blood as a heterodimer composed of two polypeptide chains derived from the original single chain. These two chains, including one of relative molecular weight (M_r) 80,000 and one of M_l varying between 90,000 and 200,000, are derived from the COOH-terminus and the NH_2-terminus of the single-chain precursor, respectively. Their interaction is Ca^{2+} dependent. A region of the C2 domain, from residues 2,303 to 2,324, assumes an amphipathic alpha helix that is responsible for the membrane-binding properties of factor VIII. [84] Presumably, six hydrophobic residues on one face of the helix contribute to membrane binding, while two arginines penetrate the hydrophobic plane to interact with phosphate moieties in the phospholipid bilayer. Although membranes including phosphatidyl-L-serine are required for factor VIII interaction, phosphatidylethanolamine enhances membrane binding. [85] [86] In its circulating form, factor VIII is inactive or minimally active as a cofactor in blood coagulation. It circulates in the blood at very low concentration (100 ng/ml) bound to von Willebrand factor (vWF). Its plasma half-life is 812 hours. [87] Factor VIII is converted into its active cofactor form by the proteolytic cleavage of two or more peptide bonds within the protein by thrombin. The structure and function of factor VIII are described in detail in Chapter 107 .

Factor X

The factor X gene, located on the long arm of chromosome 13 adjacent to the factor VII gene, is composed of eight exons and is 22 kb in length (Fig. 102-5).[88] The organization of this gene is identical to that of the factor IX gene. Exon I encodes the signal peptide, exon II the propeptide/-carboxyglutamic acid-rich domain, exon III the short aromatic amino acid stack domain, exons IV and V the two EGF domains, exon VI the activation region, and exons VII and VIII the catalytic domain. The 5 end of the factor X gene is linked to the 3 end of the factor VII gene. [89] A liver-specific promoter element, FXP1-binding site, was located 63 to 42 bp upstream of the factor X gene, while other promoter elements, FXP2 and FXP3, span 215 to 149 and 457 to 351, respectively. [89] After transcription, the factor X mRNA of about 1.5 kb includes a short 3 untranslated region following the stop codon. [90] [91] The polyadenylation signal is located within the 3 end of the coding sequence. Factor X, with a molecular weight of 56,000, is synthesized as a single polypeptide chain. [88] [91] However, factor X as isolated from plasma is composed of two polypeptide chains, a heavy chain with a molecular weight of 38,000 [92] [93] [94] and a light chain with a molecular weight of 18,000. [95] [96] These chains are linked by a single disulfide bond. The Arg-Lys-Arg sequence at residues 139141 in the single polypeptide chain appears very susceptible to intracellular or extracellular proteolysis, yielding the predominant two-chain form. [88] [91] The light chain of factor X includes 11 -carboxyglutamic acid residues at positions 6, 7, 14, 16, 19, 20, 25, 26, 29, 32, and 39 in the human protein. [97] A single -hydroxyaspartic acid residue is located in the first EGF domain at residue 63. Bovine factor X contains both asparagine-linked carbohydrate at Asn 36 and threonine-linked carbohydrate at Thr 300. [98] Like prothrombin, the asparagine-linked sugars contain NeuAc2 3Gal1 3(NeuAc2 6)GlcNAc in the outer chain. The two EGF domains are important for binding to the cofactor, factor Va. The second EGF domain can support factor Va binding but at reduced affinity, [75] suggesting a role for the first EGF domain in this interaction as well. [99]

Factor X is a calcium-binding protein that interacts with membrane surfaces in the presence of calcium. It contains both low- and high-affinity metal-binding sites, [100] occupancy of which leads to conformational changes and the expression of membrane-binding properties. [101] Like the other vitamin K-dependent proteins, factor X binds preferentially to acidic phospholipid surfaces. [102] [103] [104] Bound factor X is an extrinsic membrane protein in that no component of it is embedded within the membrane. The three-dimensional structure of bovine factor X has been determined by x-ray crystallography and demonstrates marked structural homology with trypsin and the other serine proteases involved in blood coagulation. [105] [106] The Gla domain linked to the first EGF domain shows little interaction between the two domains, but the orientation of these domains is altered by the presence of calcium ions. [107] As is the case for factor IX, [108] the N-terminus of the Gla domain of factor X shows little stable structure in the absence of Ca^{2+} . [109]

The plasma concentration of factor X is maintained at 10 g/ml. Its half-life in plasma is 36 hours. [79]

Factor VII

The factor VII gene is 13 kb in length [110] and is located on the long arm of chromosome 13, immediately adjacent to the factor X gene. [111] The coding region is found on nine separate exons (Fig. 102-5). Preprofactor VII is synthesized via two alternative forms. In one form, incorporating exon IB encoding for the signal peptide, factor VII arises from a gene whose gene organization is identical to that of the factor IX gene. In a second form, exon IA directs the coding of the signal peptide instead of exon IB. In this form, preprofactor VII has a polypeptide extension of the NH_2-terminus that elongates the signal peptide/propeptide from 38 to 60 residues. Exon II encodes the propeptide and -carboxyglutamic acid-rich domain, and exon III encodes the short aromatic amino acid stack domain, a segment common to all the vitamin K-dependent proteins. Exons IV and V each encode one of the EGF domains. The catalytic domain is coded by exons VIVIII, with the activation peptide encoded within exon VI. The mRNA for factor VII is about 2.4 kb in length, [112] with a 3 untranslated region of 1.0 kb length and a poly(A) tail located after the stop codon.

Factor VII is a component of the extrinsic pathway of blood coagulation and forms a complex with tissue factor to generate an enzyme complex that activates factor X. Human factor VII, with a molecular weight of 50,000, circulates in plasma as a single-chain zymogen containing 406 amino acid residues. [112] The NH_2-terminal domain includes 11 -carboxyglutamic acid residues at positions 6, 7, 14, 16, 19, 20, 25, 26, 29, 34, and 35 in bovine factor VII. [113] Asp 63 is partially -hydroxylated. Factor VII is a glycoprotein, containing 13% carbohydrate. The structure of the complex of the active site-inhibited factor VIIa and extracellular domain of tissue factor has been determined by x-ray crystallography. [36] Salient general features of this complex include the extensive embracing of extended conformations of one protein by the other. This interaction involves multiple domains and multiple contact sites within each domain. The structure of the calcium-stabilized Gla domain is homologous to that of prothrombin and factor IX.

In contrast to other proenzymes involved in blood coagulation, factor VII circulates in the blood in two forms: the inactive zymogen factor VII and the enzymatic active factor VIIa. [3] Factor VII may be a true zymogen, with no enzymatic activity prior to cleavage. [114] The concentration of factor VIIa is low but sufficient to generate significant factor X-activating activity when the factor VIIa forms a complex with newly exposed tissue factor. The factor Xa formed can activate factor VII to factor VIIa, increasing the amount of factor VIIa available during tissue injury. This model would allow significant amplication of thrombin formation via the extrinsic pathway.

Factor V

Factor V is a plasma glycoprotein with a molecular weight of 330,000. This protein is a critical cofactor that in its activated form facilitates activation of prothrombin by factor Xa. Factor V is a single-chain protein that circulates in the blood in a precursor, inactive cofactor form. The factor V gene, located on chromosome 1 at 1q2125, encodes a 7-kb mRNA, which itself encodes a pre-factor V including a 28-amino acid residue signal peptide and a mature protein composed of 2,196 amino acid residues. [115] [116] The gene contains 25 exons that range in size from 72 to 2,820 bp [117] and resembles the factor VIII gene in organization. The heavy chain region is composed of two domains with notable structural homology, termed the A domain. The light chain region is composed of another A domain and of two homologous C domains. The heavy chain region and light chain region are joined by a connecting region known as the B domain. The A1-A2-B-A3-C1-C2 domain structure is also present in factor VIII. [80] Proteolytic activation of factor V by thrombin leads to the cleavage of three peptide bonds, but a single site adjacent to Arg 1,545 in human factor V is critical for the generation of biologically active factor Va. [118]

Although the liver appears to be the primary site of synthesis of factor V, megakaryocytes also synthesize this protein. In addition to its presence in plasma, factor V is a component of the -granules in megakaryocytes and subsequently in platelets [119] and is secreted on platelet stimulation with specific agonists. Factors V and Va bind to two classes of binding sites on the surface of platelets. [120] However, the higher-affinity binding sites interact specifically with factor Va, and not with factor V. The plasma concentration of factor V is 10 g/ml, and its plasma half-life is 12 hours. [121]

Prothrombin

The prothrombin gene, located on chromosome 11, [122] is 21 kb in length and is composed of 14 exons, each encoding all or part of a functional domain of prothrombin (Fig. 102-5): [123] signal peptide (exon I), propeptide/-carboxyglutamic acid-rich domain (exon II), aromatic amino acid stack domain (exon III), two kringle domains (exons IVVII), the activation region (exons VIII and IX), and the catalytic domain (exons XXIV). The introns vary considerably in size, from 84 bp for the intron between exons VIII and IX to 9,447 bp for that between exons XII and XIII. Although the structure of prothrombin is homologous with those of factors IX, X, and VII and protein C, the prothrombin gene demonstrates only partial homology with the genes of these proteins. Exons IIII are shared by all these proteins, but prothrombin contains

exons IVVII encoding the kringle domains and has a homologous serine protease domain composed of seven exons, in contrast to the two exons found in the factor IX gene family. The prothrombin mRNA is 2.1 kb in length and includes a 5 untranslated region of >150 bp, a 1.8-kb open reading frame, and a 97-bp 3 untranslated region. The prothrombin gene contains a weak promoter before the transcription initiation site and liver-specific enhancer element spanning about 900 bases upstream from the transcription initiation site. This site interacts with hepatic nuclear factor-1 (HNF-1). [124] [125]

Prothrombin is a plasma glycoprotein with a molecular weight of 72,000. [42] [126] [127] On the basis of direct protein sequence analysis and the predicted sequence based on the nucleotide sequence of the cDNA, the complete amino acid sequence of the human protein is known. [123] [126] [127] As with all the blood clotting proteins, prothrombin is synthesized with a hydrophobic signal peptide from residues 43 to 19. After translocation to the rough endoplasmic reticulum, the signal peptide is removed by a signal peptidase. The propeptide, containing the -carboxylation recognition site, [31] includes residues 18 to 1. During protein synthesis, but after -carboxylation, this peptide is removed by an intracellular propeptidase. The mature prothrombin that circulates in the plasma is composed of 579 amino acid residues arranged in a single polypeptide chain. The ten -carboxyglutamic acid residues are located in the Gla domain of human prothrombin at residues 6, 7, 14, 16, 19, 20, 25, 26, 29, and 32. Carbohydrate represents about 10% of the mass of prothrombin. *N*-Asparagine-linked carbohydrate is attached to Asn 78, Asn 100, and Asn 373 in bovine prothrombin, [128] and human prothrombin likely contains carbohydrate at the homologous amino acids. Complex asparagine-linked oligosaccharides include $NeuAc_2$ 3Gal1 3(NeuAc2 6)GlcNAc. The short aromatic amino acid stack domain has significant -helical structure and serves to link the Gla domain to two kringle domains, [33] which are similar to structures found in factor XII, plasminogen, and t-PA and are defined structurally by the pattern of disulfide bonds. The function of these domains is uncertain, but they may be important for protein complex formation with factor Va. The remainder of prothrombin, accounting for approximately one-half the protein structure, represents the

catalytic domain. This region includes the activation domain that is critical for the conversion of zymogen into the active enzyme and the trypsin-like region that possesses the protease activity. Prothrombin has no coagulant activity in its zymogen form and must be converted to thrombin in order to participate in blood coagulation.

The metal-binding properties of prothrombin are conferred by -carboxyglutamic acid residues. [129] Abnormal (des--carboxy) prothrombin, lacking -carboxyglutamic acid, does not bind to Ca^{2+} and does not interact with membrane surfaces in the presence of calcium. [129] [130] Prothrombin binds Ca^{2+} and other metal ions via two classes of metal-binding sites [131] [132] [133] [134] and, on metal binding, undergoes conformational changes leading to expression of membrane-binding properties. [135] [136] [137] Concomitantly, neoantigens are exposed on the metal-stabilized conformers of prothrombin. [138] [139] [140] [141] Prothrombin is an extrinsic membrane-binding protein. In the presence of calcium ions, a surface of the prothrombin/metal complex interacts with phospholipid vesicles; a marked preference for acidic phospholipids, specifically phosphatidylserine, has been demonstrated. The N-terminal third of the protein contains the lipid-binding domain.

The plasma concentration of prothrombin is 100 g/ml, [142] [143] and the plasma half-life of prothrombin is 3 days. [79] [144]

Fibrinogen

Fibrinogen is the most abundant plasma protein involved in blood coagulation. At a plasma concentration of 23 mg/ml, it represents 2% of the total plasma proteins. In addition, platelets contain fibrinogen within their -granules, but they do not synthesize the protein. [145] [146] Variant forms likely arise via post-translational modifications or protein degradation. Fibrinogen, unique among the blood clotting proteins, is encoded by three separate genes, each of which encodes one of the three subunits. These three genes, located on chromosome 4, are clustered on a 50-kb region. [147] The fibrinogen genes are organized with the -chain gene 10 kb upstream of the -chain gene, which in turn is 13 kb upstream of the -chain gene. The -, -, and -chain genes contain nine, five, and eight exons, respectively. The regulation of these genes is coordinated at the transcriptional level by the synchronous production of three separate mRNA species. [148]

Fibrinogen is a structural protein that circulates in the plasma in a functionally inert precursor form. Its conversion to fibrin leads to polymerization of fibrin and to formation of a fibrin clot. Human fibrinogen, with a molecular weight of about 340,000, is composed of three pairs of polypeptide chains: two A-chains, two B-chains, and two -chains. [149] The -, -, and -chains demonstrate significant structural homology within their amino acid sequence, suggesting their evolutionary origin from a common ancestral gene. [150] [151] [152] The A-chain has a molecular weight of 63,500 and contains 610 amino acid residues, the B-chain has a molecular weight of 56,000 and contains 461 amino acid residues, and the -chain has a molecular weight of 47,000 and contains 411 amino acid residues. The A-, B-, and -chains are covalently linked through disulfide bonds. The disulfide bonds near the N-terminal regions of these polypeptide chains link the three chains to each other, as well as linking the two A-chains together and the two -chains together. This region is known as the disulfide knot. Electron micrographs of fibrinogen show a trinodular molecule. [153] X-ray crystal structures of fibrinogen domains have begun to provide insight into the mechanism by which fibrin self-assembles. Crystal structures of fragment D consist of a coiled-coil region and two homologous globular regions. [154] The crystal structure of the C-terminal fragment of the -chain reveals a mechanism for electrostatic steering that guides the alignment of the fibrin monomers during polymerization. [155] The plasma half-life of fibrinogen is 35 days. [156] [157]

Factor XIII

Factor XIII is a zymogen of a cysteine transglutaminase that circulates in the blood. It is composed of two peptide subunits, the -chain (M_r 75,000) and the -chain (M_r 80,000). [158] Factor XIII, with a molecular weight of 320,000, is a tetrameric structure with two -chains and two -chains held together non-covalently. After binding to Ca^{2+}, [159] factor XIII ($_2$ $_2$) is activated to its enzyme form, factor XIIIa ($_2$ $_2$), by thrombin through cleavage of the bond between Arg 37 and Gly 38 in the -chain, thus releasing an activation peptide with a molecular weight of 4,500. Factor XIIIa contains a free sulfhydryl group at the active site on the -chain and functions as a transamidase in cross-linking glutamic acids and lysine residues. The concentration of factor XIII in plasma is 60 g/ml. Platelet factor XIII is composed of only -chains in the form of a dimer, $_2$. Enzymatically, the platelet and plasma form of factor XIIIa are equivalent.

Tissue Factor

Tissue factor is an integral membrane protein with a molecular weight of 45,000 that is located on the plasma membrane of most vascular cells. [160] [161] This protein, a receptor for factor VII, is required for the initiation of blood coagulation through the extrinsic pathway. Factor VII binding to tissue factor is calcium dependent. Tissue factor is a transmembrane protein composed of 263 amino acid residues. [18] [19] Its three-dimensional structure is known. [36] A short hydrophobic domain of 23 amino acids likely represents the membrane-spanning region (Fig. 102-6). The N-terminal domain of 219 amino acid residues is a dominant component of the protein and is oriented extracellularly. A short, 21-residue C-terminal cytoplasmic domain contains palmitate and stearate bound through a thioester bond to a cysteine residue. [162] Although specific glycosylation sites have not been established, tissue factor has multiple potential N- and O-linked sites. Tissue factor is expressed constitutively in most nonvascular cells; in monocytes and endothelial cells, its expression is associated with cell stimulation. [163] [164] [165] The tissue factor gene is 12.4 kb in length and is composed of six exons. [166]

von Willebrand Factor

A multimeric glycoprotein, vWF is present in the plasma and within the -granules of platelets and the Weibel-Palade bodies of endothelial cells (see Chap. 113). The gene for vWF is located on chromosome 12 and is 176 kb in length. [167] The gene includes 52 exons that encode a signal peptide, a large propolypeptide (M_r 80,000), and the mature vWF monomer (M_r 225,000). [168] A 9-kb mRNA encodes a precursor form of the protein, synthesized as a single polypeptide chain containing 2,813 amino acid residues. [168] [169] [170] [171] Pro-vWF undergoes post-translational modification involving initial formation of dimers, which then undergo multimerization via a process that is dependent on the presence of the intact propeptide. [172] [173] The high-molecular-weight vWF multimers vary in size up to about 10 million, the largest forms displaying the most potent biologic activity.

Two critical biologic roles are fulfilled by vWF. First, it can mediate the adhesion of platelets to the injured vascular wall. Adhesion occurs through simultaneous binding of vWf to its receptor, glycoprotein Ib, on platelet surfaces and to collagen in the subendothelium. In addition to being constitutively secreted from endothelial cells, vWF is stored in the Weibel-Palade bodies of endothelial cells and released on stimulation of granule release. [174] As a plasma carrier protein, vWF binds noncovalently to and stabilizes factor VIII and circulates in the blood as a factor VIII/vWF complex. Factor VIII binds stoichiometrically to the vWF subunit. [175] Under physiologic conditions, however, most of the factor VIII-binding sites on mature vWf are unoccupied.

Figure 102-6 Structure of tissue factor. The extracellular domain extends from residues 1 to 229. This region contains three potential glycosylation sites (CHO). A short transmembrane domain is rich in hydrophobic amino acids. The cytoplasmic domain, extending from residues 243 to 263, contains a free sulfhydryl (SH) group, which undergoes esterification to palmitate or stearate.

SYNTHESIS OF THE BLOOD COAGULATION PROTEINS

Biosynthesis of the Vitamin K-Dependent Blood Clotting Proteins

Six of the plasma proteins involved in blood coagulation or the regulation of blood coagulationprothrombin, factors IX, X, and VII, protein C, and protein Srequire vitamin K for their synthesis. These proteins contain between 10 and 12 residues of -carboxyglutamic acid [176] [177] within the first 45 residues of their NH_2 -termini (Fig. 102-7). They represent a unique class of calcium-binding proteins that assemble on membrane surfaces in the presence of Ca^{2+} . These proteins are synthesized in a precursor form, which includes a typical signal peptide and a propeptide that intervenes between the signal sequence and the mature NH_2 -terminus of the protein. Following translocation through the rough endoplasmic reticulum, specific glutamic acid residues in the prozymogen are selectively -carboxylated.

Vitamin K-dependent carboxylation is catalyzed by a membrane-bound -carboxylase located in the endoplasmic reticulum. In the presence of reduced vitamin K, molecular oxygen, carbon dioxide, and the protein precursor substrate, specific glutamic acids adjacent to the -carboxylation recognition site on the propeptide are converted to the corresponding -carboxyglutamic acids. A synthetic peptide whose sequence was based on the -carboxylation recognition site within the propeptide of prothrombin binds to the carboxylase, offering a strategy for purification of the carboxylase by affinity chromatography. [178] A highly purified carboxylase was obtained by this strategy using the propeptide of factor IX. [179] These enzyme preparations catalyze both the formation of -carboxyglutamic acid from glutamic acid and vitamin K epoxide from vitamin K, which indicates the coupling of these two processes (see Chap. 115).[180] The vitamin K-dependent carboxylase is a single-chain protein with a molecular weight of 94,000 that is composed of a single polypeptide chain of 758 amino acids. [179] [180] [181] [182] The carboxylase can be expressed in insect cells otherwise lacking carboxylase activity. [183]

The propeptide within the vitamin K-dependent proteins directs -carboxylation. This region demonstrates sequence homology among proteins that contain -carboxyglutamic acid (Fig. 102-8), [184] [185] an observation that has suggested a role for the propeptide in carboxylation. Site-specific mutagenesis has shown that factor IX species lacking the 18-residue propeptide or containing point mutations at conserved residues, 16 or 10, within the propeptide eliminate -carboxylation. [31] Analogous studies of prothrombin have demonstrated the importance of amino acids at residues 18, 17, 15, and 10 in the propeptide of prothrombin. Deletion mutants of proprotein C lacking residues in the 17 to 12 portion of the propeptide were

Figure 102-7 -Carboxyglutamic acid-rich domains of the vitamin K-dependent proteins. A one-letter amino acid code is employed (denotes -carboxyglutamic acid). Sequences have been aligned to maximize sequence homology.

Figure 102-8 Sequence comparison of the propeptide domains of the vitamin K-dependent blood coagulation proteins. Homologous regions of other vitamin K-dependent proteins are aligned. Residues that demonstrate significant sequence homology are boxed and shaded; regions with conservative amino acid substitutions are boxed. *(Modified from Furie and Furie,[276] with permission.)*

also associated with impaired -carboxylation. [186] These results demonstrate that the propeptide contains a recognition element, termed the -carboxylation recognition site, which designates the vitamin K-dependent proteins for -carboxylation. This propeptide is required for carboxylation. Furthermore, expression of a cDNA construct in which the propeptide is adjacent to a glutamic acid-rich region of thrombin leads to carboxylation of these glutamic acid residues. [32] Thus, the propeptide is sufficient to support carboxylation, and no other components within the substrate are required, except to improve efficiency of carboxylation.

Peptides containing a complete propeptide sequence and carboxylatable glutamic acid residues are efficiently carboxylated, with a K_m of about 3 M. [187] [188] By contrast, peptides without the -carboxylation recognition site or with a truncated site are poor substrates for the carboxylase. Glutamic acid, but not aspartic acid, is a substrate for the carboxylase. Based on the predicted structure of the propeptide of factor X, an 18-residue peptide stimulates the carboxylation of the pentapeptide FLEEL (Phe-Leu-Glu-Glu-Leu) by the partially purified carboxylase by about eightfold. [189]

The propeptide of prothrombin contains the carboxylation recognition site on its N-terminus and the propeptide cleavage site at the C-terminus. This peptide incorporates a ten-residue amphipathic -helix, from residues 3 to 13. [190] This helix serves as a rigid extension to expose the carboxylation recognition site (Fig. 102-9). In contrast to factor IX, profactor IX cannot be activated to its enzymatically active form and does not bind to acidic membranes even though it is fully carboxylated.[191] The propeptide plays three roles in the synthesis of the vitamin K-dependent proteins: (1) via the -carboxylation recognition site, the propeptide signals for carboxylation of adjacent glutamic acid residues; (2) the propeptide inhibits premature zymogen activation and membrane binding during intracellular processing; and (3) the propeptide activates the vitamin K epoxidase activity of the carboxylase. [192]

The propeptide contains a sequence adjacent to the sessile bond that is characteristic of many proproteins and prohormones: Arg-X-Arg/Lys-Arg at residues 4 to 1, where propeptide cleavage occurs between 1 and +1. The propeptidase enzyme responsible for cleavage is probably PACE/furin, [193] a constitutively expressed protease located in the trans-Golgi region that cleaves substrates with adjacent basic amino acid residues. Site-specific mutagenesis of profactor IX has emphasized the importance of Arg 1, the lack of importance of the side chain of residue 3, and the effect of mutation of residue 2 on the efficiency of cleavage. [194]

During synthesis of the vitamin K-dependent proteins, the signal peptide contains a recognition element that directs the partially synthesized protein to the endoplasmic reticulum (Fig. 102-10). With cleavage of the signal peptide, the carboxylation recognition site on the propeptide is expressed. The vitamin K-dependent carboxylase is anchored to this region and modifies all the glutamic acids within a given proximity to the recognition site. After carboxylation, the protein is transported to the Golgi apparatus, where the propeptide undergoes cleavage. Final processing of the protein is completed in the Golgi compartment.

Post-translational -Hydroxylation

An unusual amino acid, *erythro*--hydroxyaspartic acid, is located in the NH_2 -terminal EGF domains of protein C[195] and factors IX, X, and VII. In addition, *erythro*--hydroxyasparagine has

Figure 102-9 Vitamin K-dependent carboxylase. The precursors of the vitamin K-dependent blood clotting proteins, which contain the -carboxylation recognition site (-CRS) within the propeptide, bind to carboxylase through this recognition site. Glutamic acids are converted to -carboxyglutamic acids by carboxylase in the presence of vitamin K, oxygen, and carbon dioxide. *(Modified from Furie and Furie,[276] with permission.)*

Figure 102-10 Biosynthesis of the vitamin K-dependent blood coagulation proteins. Signal recognition particle binds to the signal peptide, thus directing this complex to the endoplasmic reticulum. Signal peptide directs translocation to the luminal aspect of the endoplasmic reticulum. After signal peptide cleavage by signal peptidase, propeptide is expressed. This region, containing the -carboxylation recognition site, binds to the vitamin K-dependent carboxylase. Specific glutamic acids are converted to -carboxyglutamic acids by this enzyme. Propeptide is removed in the trans-Golgi region. *(Modified from Furie and Furie,[276] with permission.)*

been identified in protein S.[196] These amino acids are formed by post-translational hydroxylation of aspartic acid and asparagine. Their function remains unknown, although it has been suggested that they are involved in defining the metal-binding properties of these proteins.

Unlike post-translational -carboxylation, -hydroxylation of factor IX is not directed by the propeptide, nor does this process require vitamin K or concomitant -carboxylation. -Hydroxylation occurs in domains homologous to the EGF precursor in certain vitamin K-dependent proteins,[196] as well as in proteins outside this family, including the complement proteins Clr and Cls, thrombomodulin, uromodulin, and the low-density lipoprotein receptor.[196] [197] [198] A consensus sequence encompassing the -hydroxylated aspartic acid and asparagine residues within a number of EGF domains has been noted by Stenflo et al:[196]

EGF domains that lack the consensus sequence do not contain this post-translational modification. The hydroxylation is catalyzed by aspartyl -hydroxylase, an enzyme that requires 2-ketoglutarate and Fe^{2+}.[199] This reaction is blocked by agents that inhibit 2-ketoglutarate-dependent dioxygenases.[200]

von Willebrand Factor Multimerization

vWF is synthesized as a single polypeptide chain containing a signal peptide, a large propolypeptide (M_r 80,000) and the mature vWF (M_r 225,000). During synthesis, the mature vWF forms a dimer, which multimerizes to the high-molecular-weight, biologically active forms critical for cellular adhesion. Synthesis of vWF takes place in endothelial cells and megakaryocytes. In endothelial cells vWF is secreted via a constitutive pathway involving mainly dimeric forms. In addition, the regulated pathway involves storage of vWF in Weibel-Palade bodies, within which vWF undergoes multimerization and subsequently cleavage of the propolypeptide. Endothelial cells degranulate under specific stimuli, leading to the secretion of vWF. The propolypeptide is required for multimerization[172] [173] and is further required for directing vWF to the Weibel-Palade bodies.[201]

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ACTIVATION OF BLOOD COAGULATION

Mechanism of Extrinsic Activation

Activation of blood clotting through the extrinsic pathway likely plays a dominant physiologic role in hemostasis. Tissue factor, a cellular receptor for factors VII and VIIa, is present on most cell surfaces. The expression of tissue factor activity is constitutive on most nonvascular cells and is inducible via de novo synthesis in cells within the blood or on the blood vessel wall, including monocytes and endothelial cells. On tissue injury and laceration of blood vessels, nonvascular cells become exposed to blood, leading immediately to the formation of a complex of tissue factor on the cell surface with factor VIIa from the blood. The formation of this complex initiates the tissue factor pathway, culminating in the generation of thrombin and in the formation of a fibrin clot:

Through its extracellular domain, tissue factor forms a catalytic complex with factor VIIa in the presence of Ca^{2+} .[20] [161] Low amounts (10100 pM) of factor VIIa are present in normal plasma.[3] With tissue factor as a cofactor, factor VIIa can autocatalyze

Figure 102-11 Factor VII activation. The zymogen, factor VII, is composed of a single polypeptide chain. Activation of factor VII to factor VIIa involves cleavage of the bond between Arg 152 and Ile 153 by factor Xa or factor VIIa. Light (L) chain and heavy (H) chains are linked by a disulfide bond.

the activation of factor VII to factor VIIa.[202] [203] Deletion of the membrane-anchoring region of tissue factor abolishes autoactivation of factor VII but not cofactor function in the activation of factor X or factor IX. [204] The tissue factor/factor VIIa complex acts on factor IX and on factor X, leading to the generation of factor IXa and factor Xa, respectively. Factor Xa is able to feed back to convert more factor VII to factor VIIa, thereby amplifying the initiation of clotting. [4] Activation of factor VII involves cleavage of the Arg 152-Ile 153 bond, leading to a form of factor VIIa composed of a light chain (M_r 20,000) and a heavy chain (M_r 30,000) linked by a disulfide bond (Fig. 102-11).[205] [206] [207] This reaction is greatly enhanced by the presence of tissue factor and phospholipid vesicles. [11] [208] [209] Activation of factor VII is associated with the expression of the catalytic triad within the heavy chain, His 41, Ser 192, and Asn 90, common to the active site of all serine proteases. Although the potential for factor Xa-mediated and factor VIIa-mediated amplification of this pathway is apparent, the nature of the protease that generates constitutive factor VIIa in plasma is unknown.

Activation of Factor IX

The activation of factor IX proceeds either via the tissue factor pathway or through the intrinsic pathway with factor XIa:

Factor IX can be activated through the extrinsic pathway. [210] The factor VIIa/tissue factor complex activates both factor IX and factor X in reactions that require Ca^{2+} . Although the kinetics of factor IX activation by factor XIa and by factor VIIa/tissue factor differ, both enzymes cleave the same peptide bonds and generate the same structural form of factor IXa.

The activation of factor IX by factor XIa, in contrast to the activation of the other vitamin K-dependent blood clotting proteins or the activation of factor IX by factor VIIa tissue factor (which occur on membrane surfaces), takes place in the solution phase. Membrane surfaces, including those of artificial phospholipid

Figure 102-12 Factor IX activation. The zymogen, factor IX, is composed of a single polypeptide chain. Activation of factor IX to factor IXa involves cleavage of two peptide bonds. Factor IXa contains a light (L) chain and a heavy (H) chain linked by a disulfide bond. An activation peptide (AP) is released during the activation process.

vesicles or platelets, do not accelerate the generation of factor IXa. The reaction has an absolute requirement for Ca^{2+} .[211] Factor IX is activated by the cleavage of two internal peptide bonds, the Arg 145-Ala 146 bond and the Arg 180-Val 181 bond (Fig. 102-12).[212] [213] A factor IXa light chain (M_r 18,000), from residues 1145, and a factor IXa heavy chain (M_r 27,000), from residues 181416, are generated by the proteolytic activation. These chains remain covalently attached through a single disulfide bond. An activation peptide, from residues 146180, is cleaved from factor IX during activation.

Like factor IX, factor IXa binds to phospholipid vesicles in the presence of calcium ions and binds to calcium and other metal ions. Activation of factor IX to factor IXa leads to the expression of a factor VIII-binding site and active enzyme site with full coagulant activity. Cleavage of Arg 180-Val 181 is required for at least partial expression of the factor VIII-binding site; the cleavage of Arg 145-Ala 146 in addition to Arg 180-Val 181 leads to full enzymatic and cofactor binding activity.

Activation of Factor X

Factor X, at the confluence of the intrinsic pathway and the extrinsic pathway, may be activated by the factor IXa/factor VIIIa complex (tenase complex) or by the factor VIIa/tissue factor complex.

The tenase complex, composed of factor IXa and factor VIIIa, requires factor VIII in its active cofactor form, factor VIIIa. Factor VIII expresses either no or minimal cofactor activity but is converted to factor VIIIa by the cleavage of two or possibly three peptide bonds (Fig. 102-13).[214] Factor VIII, a heterodimer composed of a heavy chain that varies in molecular weight from 90,000 to 200,000 and a light chain with a molecular weight of 76,000, is a substrate for thrombin, an enzyme that cleaves at least four peptide bonds within factor VIII. The cleavage of peptide bonds at Arg 372373 and at Arg 1,6861,689 is required

Figure 102-13 Activation of factor VIII, an inactive cofactor composed of a single polypeptide chain. Factor VIII is converted to its active cofactor form, factor VIIIa, by the cleavage of two, and possibly three, peptide bonds to generate a heavy chain and a light chain noncovalently linked in the presence of Ca^{2+} .

Figure 102-14 Factor X activation. Factor X is composed of two chains, a heavy (H) chain and a light (L) chain, linked by a disulfide bond. Activation of factor X to factor Xa requires cleavage of a single peptide bond on the heavy chain. AP, activation peptide.

for factor VIII activation.[214] The resulting factor VIIIa contains polypeptide chains of 50,000 molecular weight (derived from the heavy chain), of 43,000 molecular weight, and 73,000 molecular weight (derived from the light chain) (see Chap. 105). [215] Factors VIII and VIIIa bind tightly to phospholipid vesicles and to activated platelets.[25] [216]

Factor IXa binds to factor VIIIa that is bound to phospholipid membranes and activated platelets. The formation of this enzyme complex is required for activation of factor X on a physiologically relevant time scale. [217] Alternatively, factor VIIa in complex with tissue factor on cell surfaces can activate factor X on a physiologically relevant time scale. The formation of this complex requires Ca^{2+} .

Factor X, a zymogen with no coagulant activity, is converted to its active enzyme form, factor Xa, by cleavage of a single polypeptide bond between Arg 51 and Ile 52 in the heavy chain (Fig. 102-14). This cleavage is catalyzed either by the complex of factor IXa and factor VIIIa on membrane surfaces in the presence of Ca^{2+} or by the complex of factor VIIa and tissue factor on membrane surfaces in the presence of Ca^{2+} .[218] [219] An activator without physiologic relevance, the coagulant protein of Russells viper venom is often used for clinical laboratory measurements in a test known as the Stypven or RVV time. [220] The activation peptide remains associated with factor Xa[221] but, with the exception of the C-terminal amino acids, does not contribute to the initial formation of a complex with tenase. [222] Although zymogen activation results in a major functional change in the protein, only subtle structural changes in factor X are associated with the development of enzymatic activity. [223] The expression of enzymatic activity involves the catalytic triad common to all serine proteasesHis 93, Asp 138, and Ser 233in the heavy chain of bovine factor X.

Factor Xa is inhibited by specific plasma protease inhibitors, including antithrombin III and $_2$ -macroglobulin.[224] These inhibitors are probably scavengers in that they neutralize the potent coagulant activity of any factor Xa that flows past the site of tissue injury.

The tissue factor pathway is regulated by a protease inhibitor, tissue factor pathway inhibitor (TFPI). [225] [226] [227] [228] The gene for this protein is located on chromosome 2 and includes 9 exons.[229] [230] This inhibitor, with a molecular weight of 34,000, contains three tandem Kunitz-type protease inhibitor domains. [231] TFPI binds factor Xa directly and inhibits factor VIIa-tissue factor activity in a reaction that appears to involve the formation of a factor Xa/TFPI/factor VIIa/tissue factor complex. [232] The generation of this complex down-regulates the activity of the tissue factor pathway.

Generation of Thrombin: Assembly of the Prothrombinase Complex

The conversion of prothrombin to thrombin is mediated by the enzyme action of factor Xa and the cofactor factor Va in a complex formed on membrane surfaces. The factor Xa/factor Va complex that is formed on membranes in the presence of Ca^{2+} is known as the prothrombinase complex, since it serves to act on prothrombin as substrate (Fig. 102-15).

The factor Xa/factor Va/membrane complex has many structural and functional parallels with the factor IXa/factor VIIIa/

Figure 102-15 Model for the macromolecular complex associated with zymogen activation. A central feature of the proteins involved in blood coagulation is their assembly on membrane surfaces. Interaction of Ca^{2+} with the -carboxyglutamic acids on vitamin K-dependent proteins leads to the exposure of a membrane-binding site on these proteins. Assembly of these proteins on membranes in a geometry defined by the protein cofactor facilitates enzyme-substrate interaction. The protein cofactor likely plays an important regulatory role in this complex. Factor Xa, enzyme (L, light chain; H, heavy chain); prothrombin (PT), substrate; factor Va, cofactor; , Ca^{2+} ; , -carboxyglutamic acid; lipid-binding sites in black. *(Modified from Furie and Furie,[276] with permission.)*

Figure 102-16 Activation of factor V, an inactive cofactor composed of a single polypeptide chain. Factor V is converted to its active cofactor form, factor Va, by the cleavage of two or possibly three peptide bonds to generate a heavy chain and a light chain noncovalently linked in the presence of calcium ions.

membrane complex. Factors V and Va are extrinsic membrane-binding proteins. The interaction of these factor V forms with membranes is independent of Ca^{2+} or other metal ions. Factor V binds with high affinity to phospholipid vesicles rich in phosphatidylserine, [233] [234] [235] [236] to activated platelets,[120] [237] and to microparticles derived from activated platelets.[238] [239]

Factor V circulates in the blood as an inactive cofactor or as a cofactor with low intrinsic activity. It is converted to its active cofactor form, factor Va, by the hydrolysis of three peptide bonds, Arg 709-Ser 710, Arg 1,018-Thr 1,019, and Arg 1,545-Ser 1,546, by thrombin or factor Xa (Fig. 102-16).[240] [241] [242] [243] These cleavages generate a heavy chain (M_r 110,000) and a light chain (M_r 78,000) that are linked noncovalently in the presence of Ca^{2+} .

In the presence of Ca^{2+} , a complex of factors Xa and Va forms on phospholipid vesicles. The formation of the prothrombinase complex involves three steps: (1) the binding of factor Va to membrane surfaces, (2) the binding of factor Xa to membrane surfaces, and (3) the interaction of membrane-bound factors Va and Xa. [244] Although factor Xa can activate prothrombin directly in the absence of Ca^{2+} , factor Va, and membrane surfaces, the rate of activation is very slow and irrelevant on a physiologic time scale. By contrast, the rate of prothrombin activation by the prothrombinase complex is about 300,000 times as high as the rate of activation by factor Xa.[244]

Although phospholipid vesicles serve as a model system for study of prothrombinase complex formation on platelets, the details of formation of the complex on the two surfaces may vary. Factor Va binds to specific sites on the platelet surface, defining the formation of the prothrombinase complex. Factor Va appears to be the factor Xa receptor on platelets.[245] [246] Factor Xa binds to platelets, leading to the expression of prothrombinase activity.[247] Furthermore, factor Va binds to endothelial cells, monocytes, and lymphocytes, facilitating assembly of the prothrombinase complex.[248] [249] [250] [251]

Prothrombin is converted to thrombin by the prothrombinase complex. Although multiple fragments can be generated by the action of prothrombinase on prothrombin, two or possibly

Figure 102-17 Activation of prothrombin, which is composed of a single polypeptide chain. Its activation to thrombin involves cleavage of two or possibly three peptide bonds. Thrombin, the active enzyme, is composed of two chains, the A chain and the B chain, linked by a disulfide bond.

three peptide bonds are necessarily cleaved in generating thrombin, a two-chain enzyme with a molecular weight of 38,000 (Fig. 102-17).[252] [253] [254] [255] Cleavage of the Arg 271-Thr 272 or Arg 286-Thr 287 bond in the presence of plasma proteins yields fragment 1 2 (M_r 43,000) or fragment 1 2 3 (M_r 45,000), respectively, both derived from the NH_2 -terminus of human prothrombin.[256] [257] With these cleavages prethrombin 2 (M_r 38,000) or prethrombin 2 with a 13-residue NH_2 -terminal extension is generated. Cleavage of the Arg 322-IIe 323 bond in prothrombin generates meizothrombin and in prethrombin 2 generates both the A chain (M_r 5,000) and the B chain (M_r 32,000) of thrombin, linked by a single disulfide bond. In contrast to thrombin, meizothrombin has enzymatic activity toward small substrates but does not convert fibrinogen to fibrin.[258] The charge relay system, common to the trypsin-like serine proteases, is located at His 365, Asp 419, and Ser 527.

Effector cell protease receptor-1 (EPR-1), a receptor for factor X with a molecular weight of 65,000, is widely distributed on cells and recognizes a portion of the EGF domain exposed upon zymogen activation of factor X.[259] This receptor is exposed on the platelet surface only upon platelet activation, and participates in prothrombinase assembly on cell surfaces.[260]

Thrombin is inhibited by antithrombin III. This inhibition is greatly accelerated by heparin (see Chap. 104).

Conversion of Fibrinogen to Fibrin

Fibrinogen circulates as a plasma protein in a biologically inactive form. It is converted to fibrin as a consequence of the cleavage of peptide bonds in both the A- and the B-chain by the enzyme thrombin (Fig. 102-18). Thrombin is specific for the Arg 16-Gly 17 bond in the A-chain. Cleavage of this bond releases fibrinopeptide A, a peptide containing 16 amino acid residues from the A-chain, thereby generating a new amino terminus

Figure 102-18 Conversion of fibrinogen to fibrin monomer. Fibrinogen is composed of three chains, the A-, B-, and -chains, arranged as a heterodimer, $A_{\ 2}B_{2\ 2}$. The conversion of fibrinogen to fibrin, $_{2\ 2\ 2}$, requires the cleavage of peptide bonds to release fibrinopeptide A and fibrinopeptide B.

on the -chain. Thrombin is also specific for the Arg 14-Gly 15 bond in the B-chain, cleavage of which releases fibrinopeptide B, a 14-amino acid peptide, from the B-chain, thereby generating a new amino terminus on the -chain. Thus, the covalent structure of fibrinogen and fibrin are identical except for the removal of two fibrinopeptide A and two fibrinopeptide B fragments from fibrinogen.

The fibrin monomer, on generation, homopolymerizes to form long strands known as protofibrils (Fig. 102-19). Both fibrinogen and fibrin are characterized by a trinodal domain structure, with a linear D-E-D domain organization. After removal of the fibrinopeptides, binding sites within the central E domain of the fibrin monomer bind to sites on the D domain of the -chain of another fibrin monomer.[261] This yields a half-staggered noncovalent complex between two monomeric units.[262] Addition of a third monomer in a half-staggered orientation facilitates the end-on-end interaction of the D domains of two adjacent monomers. Through these two intermolecular interactions, E domain-D domain and D domain-D domain, two-stranded protofibrils are formed. Only after the protofibrils have become sufficiently long is their lateral associationa necessary event in the formation of thick fibrin fibrilsobserved.

The polymerized form of fibrin contains fibrin monomers that are noncovalently bound to each other. As such, the fibrin strands are unstable. Through the action of factor XIIIa, the - and -chains of adjacent fibrin strands are covalently cross-linked to yield a form of fibrin that is not easily disrupted. Factor XIIIa, a transglutaminase, catalyzes the condensation of lysine residues on one chain and glutamic acid residues on a second chain.[263] Factor XIII, an inactive precursor, is a plasma protein that is converted to its active form, factor XIIIa, by the proteolytic action of thrombin.

Although many details of blood coagulation remain unclear, there has been considerable definition of the structure and function of participating components and of the special role that cell membranes play in complex formation. The rapid generation of thrombin, with concomitant platelet activation and fibrin clot formation, is localized to the region of tissue injury.

Contact Phase of Blood Coagulation: Activation of Factors XII and XI

Patients with factor XII, prekallikrein, or high-molecular-weight kininogen deficiency have no bleeding phenotype despite prolonged PTTs. These proteins are not required for hemostasis (i.e., the rapid generation of fibrin clot). However, these proteins may play a role in fibrinolysis and in fibrin formation during inflammation and wound healing.

Prekallikrein, with a molecular weight of 100,000, is in part bound to high-molecular-weight kininogen in plasma. It is converted to kallikrein, the active enzyme form of the protein, by factor XIIa. Kallikrein, a serine protease, is composed of two subunits, a heavy chain of 52,000 and a light chain of about 35,000. The active site resides on the light chain, whereas the heavy chain binds to high-molecular-weight kininogen.[264] In plasma, kallikrein is inactivated by $_2$ -macroglobulin and by C1 inhibitor.[265] [266]

High-molecular-weight kininogen, with a molecular weight of 120,000, is a plasma protein that participates in contact activation. High-molecular-weight kininogen accelerates the rate of surface-dependent activation of factor XII[267] and the rate of prekallikrein activation by activation products derived from factor XIIa.[268]

Factor XII, a zymogen of 80,000 molecular weight, is composed of 596 amino acids in its circulating form.[43] [50] [51] It is activated to factor XIIa by plasma kallikrein (Fig. 102-20A).[269] [270] [271]

This reaction is greatly accelerated by the presence of high-molecular-weight kininogen and by contact with negatively charged surfaces such as glass or collagen.[272] [273] The activation of factor XII to generate factor XIIa involves cleavage of the peptide bond between Arg 353 and Val 354. A heavy chain (M_r 50,000) composed of 353 amino acid residues binds to negatively charged surfaces and is derived from the NH_2 -terminus of factor XII. This chain contains the EGF domains, the kringle

domain, and the fibronectin type I and type II domains. A light chain (M_r 30,000) composed of 243 amino acid residues contains the catalytic domain, which is common to serine proteases. The catalytic triad includes His 393, Asp 442, and Ser 544. The light chains and heavy chain are linked by disulfide bonds. Factor XIIa functions to convert factor XI to its activated form, factor XIa, and to convert prekallikrein to kallikrein (Fig. 102-20B).

Factor XI is composed of two identical polypeptide chains, each of 80,000 molecular weight, connected by a disulfide bond. Factor XI is converted to its enzymatic form, factor XIa, by proteolytic cleavage of the Arg 369-Ile 370 bond in each chain (Fig. 102-20B).

Figure 102-19 Formation of fibrin strands. **(A)** Fibrin forms **(B)** a staggered dimer. **(C)** The addition of another fibrin monomer end-on-end yields a trimer. **(D)** Continued addition of fibrin monomer generates the fibrin strands.

Figure 102-20 Contact phase of blood coagulation. **(A)** Factor XII, a zymogen composed of a single polypeptide chain, is converted to its active form, factor XIIa, by the cleavage of a single peptide bond. Kallikrein catalyzes this reaction, and the rate of the reaction is greatly accelerated by high-molecular-weight kininogen and by contact with negatively charged surfaces. The light chain of factor XIIa contains the enzyme-active site. **(B)** Factor XI, composed of a homodimer formed by two identical subunits, is converted to factor XIa by the enzyme factor XIIa. Each subunit is cleaved to yield a heavy chain and a light chain; the latter contains the enzyme-active site.

The heavy chain (M_r 50,000), derived from the amino terminus, and the light chain (M_r 30,000) remain attached through a disulfide bond. Recent data suggest that thrombin is the physiologically relevant activator of factor XI. [13] The catalytic domain of factor XIa resides on the light chain; the catalytic triad common to all serine proteases includes His 44, Asp 93, and Ser 188.

Factors XI and XIa bind to platelets. [274] [275] It remains uncertain whether the factor XI-platelet interaction has a physiologic role and whether factor XIa on platelets activates factor IX in the solid phase.

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Chapter 103 - Molecular and Cellular Basis of Fibrinolysis

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OVERVIEW OF THE FIBRINOLYTIC SYSTEM

The fibrinolytic system in mammalian blood plays an important role in the dissolution of blood clots and in the maintenance of a patent vascular system. The fibrinolytic system (Fig. 1031) comprises an inactive proenzyme, plasminogen, that can be converted to the active enzyme, plasmin, that degrades fibrin into soluble fibrin degradation products. Two immunologically distinct physiologic plasminogen activators have been identified in blood: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Inhibition of the fibrinolytic system may occur either at the level of the plasminogen activators, by specific plasminogen activator inhibitors (PAI-1 and PAI-2), or at the level of plasmin, mainly by $_2$ -antiplasmin. Some physicochemical properties of the main components of the fibrinolytic system are summarized in Table 1031 . Tissue-type plasminogen activatormediated plasminogen activation is primarily involved in the dissolution of fibrin in the circulation,[1] whereas u-PA binds to a specific cellular receptor (u-PAR) resulting in enhanced activation of cell-bound plasminogen. The main role of u-PA is the induction of pericellular proteolysis during tissue remodeling and repair, macrophage function, ovulation, embryo implantation, and tumor invasion. [2] A u-PARindependent function of u-PA in fibrin clearance [3] and in arterial neointima formation[4] has been demonstrated.

Regulation and control of the fibrinolytic system is mediated by specific molecular interactions among its main components, and by controlled synthesis and release of plasminogen activators and plasminogen activator inhibitors, primarily from endothelial cells. Disorders of the fibrinolytic system may result either from impaired activation (thrombotic complications) or from excessive activation (bleeding tendency). [5] [6] [7] [8]

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PROTEIN STRUCTURE OF THE MAIN COMPONENTS

The enzymes of the fibrinolytic system are serine proteases i.e., their active site consists of a catalytic triad composed of the amino acids serine, aspartic acid, and histidine. This active site is located in the COOH-terminal serine protease domain, while the NH_2 -terminal regions contain one or more functional domains, such as the finger domain (homologous to the fingers in fibronectin), the epidermal growth factor-like domain and kringle domains. The inhibitors of the fibrinolytic system are grouped into the serpin (serine proteinase inhibitor) superfamily. Serpins have in their COOH-terminal region a specific reactive site peptide bond which is cleaved by their target enzyme, resulting in the release of a peptide from the inhibitor and formation

Figure 103-1 Schematic representation of the fibrinolytic system. t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI-1 and PAI-2, plasminogen activator inhibitor-1 and -2; $_2$ -AP, $_2$ -antiplasmin.

of an inactive enzyme-inhibitor complex. The reactive site of the serpins has a basic amino acid residue (arginine or lysine) in the P1 position (Arg-X or Lys-X) (Table 1031).

Human plasminogen is a 92 kD single chain glycoprotein, consisting of 791 amino acids; it contains 24 disulfide bridges and five homologous kringles (Fig. 1032).[9] [10] Native plasminogen has NH_2 -terminal glutamic acid (Glu-plasminogen) but is easily converted by limited plasmic digestion to modified forms with NH_2 -terminal lysine, valine, or methionine, commonly designated Lys-plasminogen. The plasminogen kringles contain lysine binding sites that mediate the specific binding of plasminogen to fibrin and the interaction of plasmin with $_2$ -antiplasmin; they play a crucial role in the regulation of fibrinolysis. [11] Nuclear magnetic resonance spectroscopy studies indicate that the first kringle is a compact globular structure built around a core of hydrophobic aromatic amino acids, whereas chemical modification studies have revealed that specific arginine residues are involved in fibrin binding. [12] Kringle 4 of plasminogen mediates its binding to tetranectin. [13]

Tissue-type plasminogen activator (t-PA) is a 70 kD serine protease, originally isolated as a single polypeptide chain of 527 amino acids (Fig. 1033).[14] However, native t-PA contains an NH_2 -terminal extension of three amino acids (Gly-Ala-Arg-). [15] Tissue-type plasminogen activator is converted by plasmin to a two-chain form by hydrolysis of the Arg^{275} Ile^{276} peptide bond. The NH_2 -terminal region is composed of several domains

TABLE 103-1 -- Some Properties of the Main Components of the Fibrinolytic System

	M_r	Carbohydrate Content (%)	Number of Amino Acids	Catalytic Triad	Reactive Site	Plasma Concentration (g/ml)
Plasminogen	92,000	2	791			200
Plasmin	85,000	2	±715	His^{603} ,Asp^{646} ,Ser^{741}		
t-PA	68,000	7	530 (527)[a]	His^{322} ,Asp^{371} ,Ser^{478}		0.005
u-PA	54,000	7	411	His^{204} ,Asp^{255} ,Ser^{356}		0.008
u-PAR	55,000					
	60,000	±35	313			
$_2$ -Antiplasmin	70,000	13	464		Arg^{376} Met^{377}	70
PAI-1	52,000	ND	379		Arg^{346} Met^{347}	0.05
PAI-2	60,000	ND	393		Arg^{358} Thr^{359}	<0.005
	47,000					

t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; u-PAR, u-PA receptor; PAI-1, plasminogen activator inhibitor-1; PAI-2, plasminogen activator inhibitor-2; ND, not determined.

[a] The numbering of amino acid residues is usually based on the initially determined incorrect value.

with homologies to other proteins: a finger domain including residues 450, an epidermal growth factor (EGF) domain consisting of residues 5087, and two kringles including residues 87176 and 176262. The region constituted by residues 276527 represents the serine protease part with the catalytic site, composed of His^{322} , Asp^{371} , and Ser^{478} .[14] These distinct domains in t-PA are involved in several functions of the enzyme, including its binding to fibrin, fibrin-specific plasminogen activation, rapid clearance in vivo and binding to endothelial cell receptors. Binding of t-PA to fibrin is mediated via the finger and the

Figure 103-2 Schematic representation of the primary structure of plasminogen. Pli indicates the plasmic cleavage sites for conversion of Glu-plasminogen to Lys-plasminogen; UK indicates the cleavage site for plasminogen activators, yielding plasmin. *(Adapted from Sottrup-Jensen et al.,[c] with permission.)*

Figure 103-3 Schematic representation of the primary structure of tissue-type plasminogen activator. The arrow indicates the cleavage site for plasmin. The active site residues are indicated with an

asterisk. *(Adapted from Pennica et al.,[14] with permission.)*

second kringle domains.[16] Physicochemical characterization of t-PA suggests that the molecule is ellipsoidal and relatively compact, [17] with the individual domains folded within the molecule yielding a globular structure, stabilized by strong interactions between the proteinase domain and the finger domain or EGF domain. [18] Nuclear magnetic resonance studies of the structure of the finger domain of t-PA support this model. [19] The t-PA molecule comprises three potential N-glycosylation sites, at Asn[117] , Asn^{184} , and Asn^{448} . Tissue-type plasminogen activator preparations usually contain a mixture of variant I (with all three glycosylation sites) and variant II (lacking carbohydrate at Asn ^{184}).[14] In contrast to the single-chain precursor form of most serine proteases, single-chain t-PA is enzymatically active. On the basis of conformational similarities between single-chain and two-chain t-PA, the activity of single-chain t-PA may involve an equilibrium between an active and a zymogen conformation. The structure is shifted to the active conformation upon substrate binding. [20] Alternatively, amino acid Lys[156] was shown to contribute directly to the enzymatic activity of the single-chain molecule, by forming a salt bridge with Asp ^{194} that selectively stabilizes the active conformation. [21]

Single chain urokinase-type plasminogen activator (scu-PA) is a 54 kD glycoprotein containing 411 amino acids (Fig. 1034). [22] Upon proteolytic cleavage of the Lys^{158} Ile^{159} peptide bond, the molecule is converted to a two-chain derivative (tcu-PA). The catalytic triad is located in the COOH-terminal polypeptide chain and is composed of Asp^{255} , His^{204} , and Ser^{356} . The NH_2 -terminal chain contains an epidermal growth factor domain (residues 549) and one kringle domain. A low Mr tcu-PA (33 kD) can be generated with plasmin by hydrolysis of the Lys [135] Lys [136] peptide bond following previous cleavage of the Lys ^{158} Ile^{159} bond.

Urokinase-type plasminogen activator receptor (u-PAR), the specific cell surface receptor for u-PA, is a heterogeneously glycosylated protein of 5060 kD, synthesized as a 313-amino-acid polypeptide, anchored to the plasma membrane by a glycosyl phosphatidylinositol (GPI) moiety that is attached at amino acids 282, 283, or 284. The u-PAR molecule is composed of three related structural domains, of which the NH_2 -terminal domain is involved in binding u-PA;[23] it binds all forms of u-PA containing an intact growth factor domain.

Binding of scu-PA to u-PAR on the cell surface appears to be crucial for the activity of u-PA under physiological conditions. The binding results in enhanced plasmin generation, due to effects on both the activation of plasminogen and on the feedback activation of scu-PA to tcu-PA by generated plasmin. Both of these effects are critically dependent on the cellular binding of plasminogen. Cell-associated plasmin is protected from rapid inhibition by $_2$ -antiplasmin, which further favors activation of receptor-bound scu-PA.[23]

Alpha$_2$ -antiplasmin is a 70 kD single chain glycoprotein containing about 13% carbohydrate. The molecule consists of 464 amino acids and contains two disulfide bridges (Fig. 1035). [24] [25] Alpha$_2$ -antiplasmin is a serpin with reactive site peptide bond Arg ^{376} Met^{377} . Its concentration in human plasma is about 70 g/ml (about 1 M). Alpha$_2$ -antiplasmin is synthesized primarily in a plasminogen-binding form that becomes partially converted in circulating blood to an inactive form that lacks a 26-residue peptide from the COOH-terminal end.[26] The inhibitor is cross-linked to the fibrin chain when blood is clotted in the presence of calcium ions and factor XIIIa; Gln[14] is involved in this cross-linking. [27]

Plasminogen activator inhibitor-1 (PAI-1) is a 52 kD single chain glycoprotein consisting of 379 amino acids; it is a serpin

Figure 103-4 Schematic representation of the primary structure of single chain urokinase-type plasminogen activator. The arrow indicates the cleavage site for plasmin. The active site residues are indicated with an asterisk. *(Adapted from Holmes et al.,[22] with permission.)*

with reactive site peptide bond Arg ^{346} Met^{347} .[28] PAI-1 is stabilized by binding to S-protein or vitronectin; [29] the PAI-1 binding site on vitronectin comprises residues Lys^{348} to Arg^{370} . [30] PAI-1 also binds to heparin through positively charged amino acids in the region between residues 65 to 88. [31]

Plasminogen activator inhibitor-2 (PAI-2) exists in two different forms with comparable kinetic properties; a 47 kD intracellular nonglycosylated form with pI 5.0 and a 60 kD secreted glycosylated form with pI 4.4.[32] PAI-2 is a serpin,[33] containing 393 amino acids with reactive site Arg ^{358} Thr^{359} . The function of intracellular PAI-2 is unclear because its main target enzyme (u-PA) occurs extracellularly. It may constitute

Figure 103-5 Schematic representation of the primary structure of $_2$ -antiplasmin. *(Adapted from Holmes et al.,[24] with permission.)*

a storage pool from which PAI-2 can be secreted upon cell injury. [34]

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GENE STRUCTURE OF THE MAIN COMPONENTS

The human plasminogen gene was mapped to the long arm of chromosome 6 at band q26 or q27[35] (Table 1032). It spans 52.5 kb of DNA and consists of 19 exons.[36] Each of the five kringles is encoded by two separate exons with a single intron in the middle of each structure. The gene is closely related to that of apolipoprotein (a).[37]

The human *t-PA* gene has been localized on chromosome 8 (bands 8.p.12q.11.2)[38] and more than 36 kb of its sequence have been determined.[39] It consists of 14 exons, and the intronexon organization suggests that the assembly is an example of the exon shuffling principle whereby the distinct structural domains are encoded by a single exon or by adjacent exons.[40] The

TABLE 103-2 -- Some Characteristics of the Genes of the Main Components of the Fibrinolytic System

	Gene Length (kb)	mRNA (kb)	Exons (number)	Chromosomal Location
Plasminogen	52.5	2.7	19	6
t-PA	36.6	2.7	14	8
u-PA	6.4	2.4	11	10
u-PAR	23	1.4	7	19
$_2$ -Antiplasmin	16	2.2	10	18
PAI-1	12.2	2.4;3.2	9	7
PAI-2	16.5	1.9	8	18

t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; u-PAR, u-PA receptor; PAI-1, plasminogen activator inhibitor-1; PAI-2, plasminogen activator inhibitor-2.

proximal promoter sequences in the human *t-PA* gene contain typical TATA and CAAT boxes and several potential recognition sequences for transcription factors have been identified.[41] [42] A consensus sequence of a cyclic adenosine monophosphate (cAMP)-responsive element (CRE) and a sequence homologous with the consensus sequence of the AP-2 binding site were recently identified, which may have a cooperative effect on constitutive *t-PA* gene expression.[43]

The human *u-PA* gene is 6.4 kb long and is located on chromosome 10.[38] It contains 11 exons and the intronexon organization of the gene closely resembles that of the *t-PA* gene.[44] However, exons III, VIII, and IX of the *t-PA* gene are totally, and exon IV partially, missing in the *u-PA* gene; this accounts for the absence of a finger domain and a second kringle in u-PA. Exon II codes for a signal peptide consisting of 20 amino acids; exons III and IV code for the growth factor domain and exons V and VI for the kringle region. The 5-region of exon VII, which codes for the peptide connecting the light and the heavy chain, is 39 bp longer than the corresponding exon X of the *t-PA* gene. The 3-region of exon VII and exons VIII to XI code for the heavy chain.

The human *u-PAR* gene is located on chromosome 19 bands q13.1q13.2.[45] It consists of 7 exons spread over 23 kb of genomic DNA. The organization of the exons reflects the pattern of triplicated homologous domains observed in the mature protein. Each protein domain is coded for by a pair of exons, with the intron break within each pair always between the 5th and 6th cysteine residue. The urokinase binding domain is encoded by exons 2 and 3.[46] Besides the 1.4 kb mRNA encoding a protein of 313 amino acids (*u-PAR1*) and a signal peptide, a variant form of human *u-PAR* mRNA has been reported (*u-PAR2*) arising by alternatively splicing in the middle of the third domain.[47] The deduced amino acid sequence of this variant consists of a signal peptide and 259 amino acids, of which the 230 residue NH_2 -terminal sequence is identical to that of *u-PAR1*, whereas the 29 residue COOH-terminal sequence is unique. The variant form, *u-PAR2*, contains the u-PA-binding domain, but lacks the GPI anchor and may be a soluble secreted protein.[47] A soluble form of *u-PAR* has been detected in ascitic fluid from patients with ovarian cancer and also in human plasma.[48] [49]

The human $_2$-*antiplasmin* gene is located on chromosome 18, bands p11.1q11.2.[50] It contains 10 exons covering approximately 16 kb of DNA.[51] The NH_2 -terminal region of the protein, comprising the fibrin cross-linking site, is encoded by exon IV, whereas both the reactive site and the plasminogen-binding site in the COOH-terminal region are encoded by exon X.

The *PAI-1* gene has been mapped to chromosome 7, bands q21.3q22.[52] It is approximately 12.2 kb in length and consists of nine exons. As a result of alternative polyadenylation, two mRNA species of 3.2 and 2.4 kb occur. The *PAI-2* gene is located on chromosome 18 q2123. It spans 16.5 kb and contains 8 exons; a consensus sequence TATAAAA is found 22 bp 5 of the proposed transcription initiation site. The structure of the gene is quite different from that of *PAI-1*, but is similar to that of the chicken ovalbumin gene.[53]

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MECHANISM OF ACTIVATION OF PLASMINOGEN TO PLASMIN

All plasminogen activators convert plasminogen to plasmin by cleavage of a single Arg ^{561} Val^{562} peptide bond, using the 791 amino acid numbering system. The two-chain plasmin molecule is composed of a heavy chain containing the five kringles (NH_2 -terminal part of plasminogen) and a light chain (COOH-terminal part) containing the catalytic triad, composed of His ^{603} , Asp^{646} , and Ser^{741} . [9] [10] Activation of Glu-plasminogen in human plasma probably occurs by direct cleavage of the Arg^{561} Val^{562} peptide bond without generation of Lys-plasminogen intermediates. [54]

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MECHANISM OF INHIBITION OF PLASMIN BY $_2$ -ANTIPLASMIN

Alpha$_2$ -antiplasmin forms an inactive 1:1 stoichiometric complex with plasmin. The inhibition of plasmin (P) by $_2$ -antiplasmin (A) can be represented by two consecutive reactions: a fast, second order reaction producing a reversible inactive complex (PA), which is followed by a slower first-order transition resulting in an irreversible inactive complex (PA). This model can be represented by:

The second order rate constant of the inhibition is very high (k_1 = 2 to 4 × 10^7 M^1 s^1), but this high inhibition rate depends upon the presence of free lysine binding sites and a free active site in the plasmin molecule and upon the availability of a site complementary to the lysine binding site (plasminogen binding site) and of the reactive site peptide bond in the inhibitor (Fig. 1035). The half-life of plasmin molecules generated at the fibrin surface, which have both their lysine binding sites and active site occupied, is two to three orders of magnitude longer than that of free plasmin. [55]

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MECHANISM OF ACTION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

In the Presence of Fibrin

Tissue-type plasminogen activator is a poor enzyme in the absence of fibrin, but the presence of fibrin strikingly enhances the activation rate of plasminogen. [56] During fibrinolysis, fibrinogen and fibrin itself are continuously modified by cleavage with thrombin or plasmin, yielding a diversity of reaction products. [57] Thrombin-catalyzed formation of desA-fibrin monomer, and desA-fibrin polymerization are essential for stimulation of plasminogen activation by t-PA. Optimal stimulation is only obtained after early plasmin-cleavage in the COOH-terminal A-chain and the NH_2 -terminal B-chain of fibrin, yielding fragment X-polymer. [57] Kinetic data support a mechanism in which fibrin provides a surface to which t-PA and plasminogen adsorb in a sequential and ordered way yielding a cyclic ternary complex. [56] Formation of this complex results in an enhanced affinity of t-PA for plasminogen (Michaelis constant K_m of 0.16 M as compared to 65 M without fibrin), yielding an up to three orders of magnitude higher catalytic efficiency for plasminogen activation (Fig. 1036). In agreement with this mechanism, the increase in fibrin stimulation after formation of fibrin X-polymers is associated with an enhanced binding of t-PA and plasminogen, which is mediated in part by COOH-terminal lysine residues generated by plasmin cleavage. Interaction of these COOH-terminal lysines with lysine binding sites on t-PA and plasminogen may allow an improved alignment as well as allosteric changes of the t-PA and plasminogen moieties, thus enhancing the rate of plasminogen activation. [57]

Lipoprotein (a) (Lp(a)) competes with plasminogen for binding to fibrin, as a result of binding of Lp(a) to fibrin via its lysine-binding domains. [58] [59] [60] Binding of Lp(a) to fibrin is enhanced by partial proteolytic degradation of the fibrin surface. [58] A functional consequence of the competition between Lp(a) and plasminogen for binding to fibrin is that the fibrin-dependent enhancement of plasminogen activation by t-PA may be reduced,

Figure 103-6 Schematic representation of the molecular interactions regulating the fibrin-specific activation of the fibrinolytic system by tissue-type plasminogen activator. t-PA in circulating plasma in the absence of fibrin has a low affinity for its substrate plasminogen (Km = 65 M), and no efficient plasminogen activation occurs. t-PA binds specifically to fibrin and the bimolecular t-PA/fibrin complex has a high affinity for plasminogen (Km = 0.16 M). Plasminogen binds to the binary t-PA/fibrin complex and is activated to plasmin at the fibrin surface. In loco generated plasmin is protected from rapid inactivation by $_2$ -antiplasmin.

[59] [60] although kinetic studies suggest only a limited effect. [61] However, the inhibition constant of Lp(a) for the activation of plasminogen by t-PA is of the same order as the physiologic plasma concentration of Lp(a), suggesting that elevated levels of Lp(a) might indeed impair physiologic fibrinolysis.

A Thrombin Activatable Fibrinolysis Inhibitor (TAFI), which is activated by the thrombin-thrombomodulin complex, has recently been characterized. TAFI is a carboxypeptidase B-like enzyme that inhibits fibrinolysis by downregulating the cofactor functions of partially degraded fibrin. This effect is most likely mediated by removal of newly exposed COOH-terminal lysine residues that are generated upon plasmin cleavage of fibrin. [62]

At the Cell Surface

A striking analogy exists between the role of fibrin and that of cell surfaces in plasminogen activation. [63] Many cell types bind plasminogen activators and plasminogen, resulting in enhanced plasminogen activation [64] [65] [66] [67] and protection of bound plasmin from inhibition by $_2$ -antiplasmin. [68] [69]

Binding of plasminogen to cultured human umbilical vein endothelial cells (HUVEC) was reported with a K_D of 310 nM, and approximately 10^6 binding sites per cell. [65] Other studies report that most cells bind plasminogen via its lysine binding sites with a high capacity (>10^7 sites per cell) but a relatively low affinity (dissociation constant of approximately 1 M). Gangliosides, [70] as well as a class of membrane proteins with COOH-terminal lysine residues such as -enolase, [71] play an important role in binding of plasminogen to cells. The catalytic efficiency of t-PA for activation of cell bound plasminogen is about 10-fold higher than in solution, possibly as a result of conversion of the plasminogen conformation to the more readily activatable Lys-plasminogen-like structure. [72] Lp(a) competes with plasminogen for binding to endothelial cells, potentially resulting in downregulated activation of cell surface bound plasminogen by t-PA. Thus, Lp(a) may also play a role in the regulation of fibrinolysis at the endothelial cell surface. [73] Alternatively, it was shown that vascular cells have the capacity to regulate pericellular fibrinolysis by modulating the expression of plasminogen receptors; enhanced receptor occupancy results in enhanced plasminogen activation by t-PA. [74]

Specific, saturable and reversible binding of t-PA to HUVEC was also demonstrated. [64] [75] A high affinity binding site (K_D of 29 pM) with a low number of binding sites (3,700 per cell) and a lower affinity binding site (K_D of 18 nM) with a high number of binding sites (800,000 per cell) have been identified. A 40 kD membrane protein (related to annexin II) was proposed as the functional t-PA receptor. [76] Cell surface bound t-PA retains its enzymatic activity and is protected from inhibition by PAI-1. Assembly of plasminogen and plasminogen activators at the endothelial cell surface thus provides a focal point for plasmin generation and may play an important role in maintaining blood fluidity and nonthrombogenicity.

Cellular receptors may also play a role in the rapid clearance of t-PA from the circulation. Circulating t-PA (half-life of 56 min in man) may interact with several receptor systems in the liver. Liver endothelial cells have a mannose receptor which recognizes the high mannose-type carbohydrate antenna on kringle 1, and liver parenchymal cells contain a calcium-dependent receptor which interacts with the finger and/or EGFdomains of t-PA. [77] Parenchymal cells also contain a high affinity receptor for the uptake and degradation of t-PA/PAI-1 complexes, which also binds free t-PA albeit with lower affinity; this receptor, termed low density lipoprotein receptor-related protein (LRP) is identical to the $_2$ -macroglobulin receptor. [78]

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MECHANISM OF ACTION OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR

In the Presence of Fibrin

In contrast to tcu-PA, scu-PA displays very low activity toward low molecular weight chromogenic substrates. Scu-PA appears to have some intrinsic plasminogen activating potential, which represents less than 0.5% of the catalytic efficiency of tcu-PA. [79] [80] Other investigators, however, have claimed that scu-PA has no measurable intrinsic amidolytic or plasminogen activator activities. [81] The occurrence of a transition state of scu-PA with a higher catalytic efficiency for native plasminogen than tcu-PA has also been postulated. [82] In plasma, in the absence of fibrin, scu-PA is stable and does not activate plasminogen; in the presence of a fibrin clot, scu-PA, but not tcu-PA, induces fibrin-specific clot lysis. [79] Scu-PA does not bind to a significant extent to fibrin, although in the presence of Zn^{2+} ions some binding may occur. [83] An intrinsic activity of scu-PA toward fibrin-bound plasminogen may contribute to its fibrin-specificity. In addition, plasma $_2$ -antiplasmin prevents conversion of scu-PA to tcu-PA outside the clot and thus preserves fibrin-specificity. [84] Fibrin fragment E-2 selectively promotes the activation of plasminogen by scu-PA, mainly by enhancing the catalytic rate constant of the activation. [85] Scu-PA is an inefficient activator of plasminogen bound to internal lysine residues on intact fibrin, but has a higher activity toward plasminogen bound to newly generated COOH-terminal lysine residues on partially degraded fibrin. [86] Thus, the fibrin-specificity of scu-PA does not require its

Figure 103-7 Plasminogen activation by u-PA at the cell surface. Urokinase-type plasminogen activator (u-PA) bound to its cellular receptor (u-PAR) efficiently converts plasminogen (Plg) bound to its cellular receptor(s) (PlgR) to plasmin (Pli), which is protected from rapid inhibition by $_2$ -antiplasmin ($_2$ -AP). Plasmin converts u-PAR-bound scu-PA to tcu-PA, resulting in enhanced plasminogen activation. Receptor bound tcu-PA, but not scu-PA is inhibited by PAI-1 and PAI-2.

conversion to tcu-PA, but is mediated by enhanced binding of plasminogen to partially digested fibrin. [87]

At the Cell Surface

The binding of scu-PA to u-PAR on the cell surface was claimed to be crucial for its activity under physiological conditions. Binding results in a strongly enhanced plasmin generation, due to effects on both the activation of plasminogen [88] and on the feedback activation of scu-PA to tcu-PA by generated plasmin. [89] Both of these effects are also critically dependent on the cellular binding of plasminogen. Cell-associated plasmin is protected from rapid inhibition by $_2$ -antiplasmin, which further favors the activation of receptor-bound scu-PA. [88] This system can be efficiently inhibited by both PAI-1 and PAI-2 [90] (Fig. 1037). The mechanisms underlying these stimulatory effects are not completely elucidated. A model based on u-PAR-dependent complex formation has been proposed, which would allow initiation of plasminogen activation by the low intrinsic activity of scu-PA. [91] However, the observation that direct anchorage of u-PA to the cell surface (using a GPI-anchored u-PA mutant) leads to a potentiation of plasmin generation equivalent to that observed in the presence of u-PAR, suggests that u-PAR only functions to localize u-PA at the cell-surface. [92] Furthermore, a u-PAR-independent function of u-PA has been demonstrated in fibrin clearance [3] and in arterial neointima formation. [4]

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MECHANISMS OF INHIBITION OF PLASMINOGEN ACTIVATORS

Multiple mechanisms are involved in the rapid inhibition of t-PA in human plasma. PAI-1 is a specific rapid-reacting inhibitor of t-PA, which is present at a low concentration in normal plasma, but at higher concentrations in many clinical conditions. [93] In addition, t-PA is inhibited slowly by $_2$ -antiplasmin, $_1$ -antitrypsin and C1-inhibitor.[94] The main mechanism of removal of t-PA from the blood, however, is by clearance via the liver. In healthy volunteers and in patients with acute myocardial infarction, the initial half-life of t-PA is 4 to 6 min. [95] [96]

In human plasma, tcu-PA is also slowly inhibited by several proteinase inhibitors [97] including $_2$ -macroglobulin, $_1$ -antitrypsin, antithrombin III, $_2$ -antiplasmin, and plasminogen activator inhibitor-3 (PAI-3, which is identical to activated protein C inhibitor). More specific and rapid inhibition occurs by PAI-1 and PAI-2. In contrast to tcu-PA, scu-PA is not inhibited by plasma proteinase inhibitors. The main mechanism of removal of u-PA from the blood is by hepatic clearance. [98] [99] Rapid clearance does not involve carbohydrate receptors and is not mediated via the NH_2 -terminal region of the molecule.[99] In patients with acute myocardial infarction, scu-PA shows a biphasic disappearance rate with an initial half-life in plasma (post-infusion) of about 4 min. [100]

PAI-1 reacts with single-chain and two-chain t-PA and with tcu-PA, but not with scu-PA.[101] The second order rate constant for the inhibition of single chain t-PA by PAI-1 is about 10^7 M^1 s^1 , and inhibition of two-chain t-PA and tcu-PA is somewhat faster. PAI-1 inhibits its target proteinases by formation of a 1:1 stoichiometric reversible complex, followed by covalent binding between the hydroxyl group of the active site serine residue of the proteinase and the carboxyl group of the P1 residue at the reactive center (bait region) of the serpin. Highly positively charged regions in t-PA (residues 296304) [102] and in u-PA (residues 179184)[103] are involved in the rapid interaction. A molecular form of intact PAI-1 has been isolated that does not form stable complexes with t-PA but is cleaved at the P1-P1 peptide bond (substrate PAI-1).[104] By point mutations in the reactive site loop, PAI-1 could also be converted from an inhibitor into a substrate. [105] Examination of the structure of the cleaved substrate variant using X-rays shows that it has a new -strand (s4A) formed by insertion of the NH_2 -terminal portion of the reactive site loop into -sheet A subsequent to cleavage.[106] Thus, inhibitory PAI-1 may not only convert to latent PAI-1, which can be reactivated, but also to substrate PAI-1, which may be irreversibly degraded by target proteinases, including t-PA, u-PA, and thrombin. [104] PAI activity is very rapidly cleared from the circulation via the liver; a half-life of 7 min in the rabbit has been reported.[107]

PAI-2 inhibits tcu-PA with a second order rate constant (k_1) of 9×10^5 M^1 s^1 , which is about 10-fold slower than PAI-1. PAI-2 also efficiently inhibits two-chain t-PA (k_1 of 2×10^5 M^1 s^1), less efficiently single-chain t-PA (k_1 of 9×10^3 M^1 s^1) and it does not inhibit scu-PA.[101]

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SYNTHESIS AND SECRETION OF PLASMINOGEN ACTIVATORS

The synthesis and secretion of t-PA, u-PA, and PAI-1, mainly by endothelial cells, is highly regulated, although some mediators influence the production of plasminogen activators and plasminogen activator inhibitors in parallel.

Vascular endothelial cells synthesize and secrete t-PA into the circulating blood. [108] Human endothelial cells grown under arterial shear stress synthesize more t-PA.[109] Stimulation of vascular endothelium by venous occlusion, infusion of DDAVP or epinephrine, and physical exercise results in a rapid release (within minutes) of t-PA.[110] This response is too rapid to represent increased synthesis and may reflect release from cellular storage pools, although such a storage pool has not been conclusively identified. Agents, such as thrombin, that stimulate the release of t-PA from endothelial cells, also stimulate the secretion of PAI-1. [111] [112] Dexamethasone increases t-PA antigen levels in hepatoma cells moderately but increases PAI-1 antigen levels to a greater extent, resulting in inhibition of t-PA activity.[113]

A variety of agents have been shown to increase the synthesis of t-PA by cultured endothelial cells, including thrombin, [111] [112] histamine,[112] butyrate,[114] phorbol myristate acetate (PMA), [115] basic fibroblast growth factor,[116] activated protein C,[117] butanol and alcohol derivatives, [118] and retinoids.[115] However, only histamine, butyrate, or a combination of cAMP with protein kinase C agonists exclusively stimulate t-PA synthesis without affecting PAI-1 synthesis.

The mechanisms involved in the stimulatory effect of these

various agents on t-PA synthesis appear to be different. A functional retinoic acid response element (RARE), which consists of a direct repeat of the GGTCA motif spaced by five nucleotides (DR5), has been localized 7.3 kb upstream for the transcription start site of the human t-PA gene. This element mediates the direct regulation by retinoic acid in human fibrosarcoma, endothelial, and neuroblastoma cells. [120] Vasoactive substances, such as histamine and thrombin, bind to specific receptors and activate phospholipase C, which acts on phosphatidyl-inositol biphosphate to produce diacylglycerol. Diacylglycerol activates membrane-bound protein kinase C, which plays an important role in the regulation of t-PA synthesis. This is suggested by the findings that direct activation of protein kinase C by phorbol esters induces t-PA synthesis, whereas suppression of protein kinase C impairs the increase in t-PA synthesis by histamine and by PMA. [121] The increase of t-PA induced by histamine, thrombin, and PMA in endothelial cells is paralleled by increased levels of mRNA, as a result of enhanced transcription of the *t-PA* gene.[122] Overexpression of t-PA in endothelial cells using a retroviral expression vector did not alter the morphology, attachment, proliferation, migration, or invasion in in vitro systems. Potentially such *t-PA* transduced cells could increase local fibrinolysis and may be useful for in vivo therapeutic interventions. [123] Plasmin inhibits the biosynthesis of t-PA antigen by human umbilical vein endothelial cells in a dose-dependent manner, possibly through the signal transduction pathway involving one or more protein kinases.[124]

Whereas the synthesis of t-PA occurs mainly in endothelial cells, immunocytochemical staining of tissues indicates that many cells of different origin (i.e., fibroblasts, epithelial cells, pneumocytes, etc.) produce u-PA. [125] The scu-PA concentration in human plasma ranges between 2 and 20 ng/ml.

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SYNTHESIS AND SECRETION OF PLASMINOGEN ACTIVATOR INHIBITORS

PAI-1 mRNA has been demonstrated in a large variety of tissues, suggesting that common cells in these tissues, such as endothelial or smooth muscle cells, may be the site of production.[126] PAI-1 is found in plasma, platelets, placenta, and in the extracellular matrix. Except for platelets, which contain essentially inactive PAI-1, PAI-1 is not stored within cells, but is rapidly and constitutively secreted after synthesis. For unknown reasons PAI-1 exhibits a circadian variation; its plasma concentration is highest in the morning and lowest in the late afternoon and evening, whereas t-PA exhibits an opposite diurnal variation.

Synthesis and secretion of PAI-1 can be modulated by various agonists such as hormones, growth factors, endotoxin, cytokines, and phorbolesters. [126] [127] Although posttranscriptional regulation of *PAI-1* mRNA levels has been suggested,[128] [129] [130] most studies on the regulation of PAI-1 expression demonstrated an effect at the transcriptional level. [131] [132] [133] [134] [135] Alterations in mRNA stability may also contribute to increased PAI-1 levels in some cells. [126] In endothelial cells *PAI-1* gene expression is stimulated by lipopolysaccharide, [129] [136] [137] [138] [139] interleukin 1 (IL-1), [138] [139] [140] tumor necrosis factor-, [129] [141] [142] transforming growth factor ,[143] basic fibroblast growth factor, [143] phorbol esters, [135] [144] [145] [146] thrombin, [111] [112] [147] very low density lipoprotein, [148] Lp(a), [149] insulin, [134] or proinsulin. [150] Studies on the mechanism of the induction by phorbol esters suggest that it is mediated by two regulatory DNA sequences in the proximal promotor of the *PAI-1* gene which were called Box A (65 to 50) and Box B (82 to 65). [151] Recently, the DNA encoding a novel transcription factor which selectively interacts with Box B and which is also involved in basal expression of PAI-1 was characterized. This factor has conserved helicase and RING finger domains, and was called helicase-like transcription factor. [152]

In endothelial cells juxtaposed to thrombi, in smooth muscle cells adjacent to the neointima, and in macrophages, *PAI-1* mRNA is increased and PAI-1 protein is detectable. This augmented arterial wall expression of PAI-1 induced by thrombosis may shift the local balance between fibrinolysis and thrombosis towards the latter. [153] Only a few studies have reported a downregulation of PAI-1 synthesis in endothelial cells, either by forskolin or by endothelial cell growth factor combined with heparin. [144] [154] [155]

PAI-2 has been identified in human placenta and in pregnancy plasma; it is also secreted by leukocytes and by fibrosarcoma cells. Secretion of PAI-2 is regulated by endotoxin and by phorbol esters which stimulate the gene transcription of PAI-2. [101] [156]

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CONCLUSIONS

Fibrinolysis is regulated by specific molecular interactions between its main components. Activation of plasminogen by t-PA is enhanced in the presence of fibrin or at the endothelial cell surface. Inhibition occurs at the level of the plasminogen activators or at the level of plasmin. Fibrinolysis may also be regulated as a result of increased or decreased synthesis and/or secretion of t-PA and of PAI-1 from the vessel wall. Assembly of fibrinolytic components at the surface of many cell types via binding to specific receptors provides a mechansim for generation of localized cell-associated proteolytic activity. The physiological importance of the fibrinolytic system is demostrated by the association between abnormal fibrinolysis and a tendency toward bleeding or thrombosis.

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Chapter 104 - Regulatory Mechanisms in Hemostasis: Natural Anticoagulants

Charles T. Esmon

INTRODUCTION

Understanding hemostasis requires consideration of the mechanisms that regulate this process and thereby serve to localize the clotting response to the immediate area of injury. This chapter has three main goals: to examine the molecular mechanisms by which the natural anticoagulant complexes function to regulate coagulation, to examine how the expression of the anticoagulant complexes may be altered in response to mediators, and to review current understanding of the physiologic ramifications of these systems.

Current information suggests that there are three major natural anticoagulant mechanisms directed at the control of coagulation responses per se: the heparin-antithrombin III (AT III) system,[1] [2] [3] the protein C anticoagulant pathway,[4] [5] [6] and the tissue factor pathway inhibitor, [7] formerly referred to as lipoprotein-associated coagulation inhibitor and the extrinsic pathway inhibitor. [8] These pathways appear to complement each other and to work in concert to exert a potent in vivo anticoagulant response. Defects in these pathways, particularly the protein C pathway, are more commonly associated with familial thrombophilia than any other mechanisms.

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INHIBITION OF THE VITAMIN K-DEPENDENT PROTEASES OF THE COAGULATION CASCADE: THE ANTITHROMBIN III/HEPARIN MECHANISMS

Antithrombin III is a glycoprotein inhibitor that functions by neutralizing coagulation proteases, indicated below by the letter E for enzyme, by forming a 1:1 complex between the enzyme and AT III:

This reaction probably accounts for the major mechanism of inhibition of thrombin and factors Xa and XIa, [3] but the reaction is slow in the absence of heparin. Factor VIIa is not inhibited effectively by AT III with heparin unless the factor VIIa is complexed to tissue factor. [9] [10] Heparin accelerates inhibition of the coagulation proteases about 1,000-fold.[11] As a result, AT III must be present[12] for heparin to function as an effective anticoagulant. With thrombin, optimal rates of inactivation require the simultaneous interaction of heparin with AT III and thrombin. [13] [14] Heparin acts as a bridge between thrombin and AT III, thereby concentrating the reactants and accelerating the reaction. In addition, the interaction of heparin with AT III alters the conformation

Figure 104-1 Thrombin inhibition by AT III in the presence of heparan molecules on the endothelial cell surface. The binding of AT III to the heparan results in a conformational change in the AT III that results in rapid inactivation of thrombin. Both thrombin and antithrombin bind to the heparan. After complex formation with AT III, the complex rapidly dissociates from the heparan.

of the inhibitor, which makes the protease trap more accessible to thrombin and the other coagulation factors. [3] [15] Relative to thrombin, the interaction of heparin with factor Xa plays a much less important role in accelerating inactivation. [3] [11] This difference in the nature of the reactions has led to a new class of antithrombotic agents, the low-molecular-weight heparins. The low-molecular-weight heparins retain most of their ability to inactivate factor Xa, but because of their lower molecular weight, they are less effective in forming the bridge between thrombin and AT III and hence have reduced activity toward thrombin. As the size of the heparin fractions become smaller, the ratio of activity toward factor Xa versus thrombin increases. The pharmacologic ramifications of this remain to be fully explored, but this property would be anticipated to increase the half-life of thrombin relative to factor Xa as compared with high-molecular-weight heparins.

Little naturally occurring heparin is found in the blood. The vascular endothelium, and the microvascular endothelium in particular, is rich, however, in anticoagulantly active heparin-like proteoglycans. [3] Most of the heparin-like proteoglycans are on the abluminal side of the endothelium. [16] It has been proposed that these proteoglycans accelerate inhibition of some of the coagulation proteases. [16] [17] Whether the low concentration of heparin-like proteoglycans on the luminal surface is responsible for the acceleration of thrombin clearance or the larger pool of abluminal proteoglycan can interact with thrombin remains unclear. [16] A model of the mechanisms by which these proteoglycans may function is illustrated in Figure 1041 . Proteoglycans are endothelial cell-associated proteins with heparin/heparan side chains bearing the critical carbohydrate sequences required for AT III recognition. Both thrombin and AT III bind to sulfated carbohydrates and are brought into close proximity to each other. AT III undergoes a conformational change required for rapid inactivation of thrombin. Little evidence exists that thrombin alters its structure on binding, but thrombin interaction with heparin is clearly important. [13] [14] Heparin functions catalytically, facilitating the rapid dissociation of the thrombin/AT III complex from the proteoglycan.[14] This critical step is responsible for re-exposure of the heparin sites for binding with other thrombin and AT III molecules.

One important feature of heparin as an anticoagulant is that the molecules are heterogeneous, both structurally and functionally. Less than one-third of the heparin in commercial preparations is catalytically active in accelerating AT III-dependent inhibition of thrombin, and <10% of the naturally occurring heparin-like proteoglycans are anticoagulantly functional[16] (see Chap. 122). The critical structure responsible for the inhibition of thrombin by AT III is a specific sequence of sulfated sugars corresponding to iduronic acid-N-acetylglucosamine-6-OSO_3 -glucuronic acid-N-sulfated glucosamine-6-OSO_3 -iduronic acid-2-OSO_3 -N-sulfated glucosamine-6-OSO_3 -iduronic acid-2-OSO_3 -glucuronic acid-6-OSO_3 .[3]

The function of other forms of nonanticoagulantly active forms of heparin in commercial heparin preparations is unresolved. Some functions of heparin, such as its potential role in blocking angiogenesis, [20] are distinct from its role as an anticoagulant.

INHIBITION OF THE EXTRINSIC PATHWAY OF COAGULATION

The AT III/heparin complex accounts for inhibition of all the vitamin K-dependent proteases, except factor VIIa. Although it has long been recognized that the factor VIIa/tissue factor complex is inhibited by serum, factor VIIa alone is remarkably stable both in serum and in vivo. [7] An inhibitor of the extrinsic pathway has been described, recently renamed as tissue factor pathway inhibitor (TFPI). The available evidence suggests a remarkable and novel mechanism of action. TFPI forms a complex with factor Xa, thereby inhibiting factor Xa activity. [21] How, then, does this serve as the mechanism for inhibition of the tissue factor/factor VIIa complex? Insight into the mechanism comes from structure/function analysis. Structure/function studies, outlined below, indicate that the factor Xa/TFPI complex interacts with the membrane, raising the local concentration of the TFPI and facilitating the inhibition of the factor VIIa/tissue factor complex (Fig. 1042). The supporting evidence for this model consists of the following observations. Binding of factor Xa to TFPI is Ca^{2+} independent and does not require the Gla domain of factor Xa. [21] Both Ca^{2+} and the Gla domain are required for factor Xa-dependent inhibition of factor VIIa. Furthermore, once TFPI inhibits the tissue factor/factor VIIa/factor Xa

Figure 104-2 Inhibition of factor VIIa by tissue factor pathway inhibitor (TFPI). In this model, the binding of TFPI to factor Xa results in a conformational change in the TFPI that facilitates the inhibition of factor VIIa, which is complexed to tissue factor (TF).

Figure 104-3 (A) Formation and function of activated protein C under basal conditions. A small thrombus containing platelets and fibrin is illustrated on the lining of a small vessel. Thrombin (Th) originating on the surface of the thrombus is carried in a capillary bed by blood flow. On the endothelial cells in the capillaries, thrombin binds to thrombomodulin. The thrombin/thrombomodulin complex rapidly converts protein C (PC) to activated protein C (APC). The APC subsequently functions as an anticoagulant by enzymatically degrading clotting factors Va and VIIIa on membrane surfaces. Protein S (S) is required for these inhibitory steps to occur. Approximately 60% of the protein S is complexed to C4b-binding protein (C4BP) and is not functionally active. C4b-BP also binds serum amyloid P (SAP). APC is inhibited in plasma by protein C inhibitor, which is also known as plasminogen activator inhibitor-3, and by $_1$ -antitrypsin. *(From Esmon,* [5] *with permission, adapted from Esmon.* [157] *)* **(B)** Inflammation-induced changes in the coagulation system. During inflammation, the thrombomodulin available on the endothelial surface is decreased. This results in decreased activated protein C formation. Tissue factor (TF) is expressed on the cell surface, resulting in increased factor Xa formation, leading to more thrombin formation on the platelet surface. Binding of leukocytes to the endothelial cell surface is increased by the appearance of endothelial leukocyte adhesion molecules (E- or P-selectin) and the bound leukocytes then contribute to local vascular damage. In the plasma, inflammation results in increased levels of C4b-BP. This increase shifts the protein S to the complexed, inactive form; farther down, it regulates the protein C system. These inflammation-induced changes promote thrombus formation.

complex, the addition of chelating agents frees factor VIIa from inhibition and dissociates TFPI. [7] [22] Thus, the TFPI/factor Xa complex forms first, and this complex combines with the membrane-bound factor VIIa/tissue factor complex. The precise role played by factor Xa in factor VIIa inactivation remains unclear. Although the model shows the initial event of factor Xa/TFPI interaction as a solution phase reaction, the possibility exists that the reaction can occur on the membrane surface as well.

Some potential insights into the molecular basis for the capacity of the inhibitor to neutralize two proteases simultaneously can be derived from an analysis of the structure of TFPI. This protein has been cloned and a cDNA coding for TFPI characterized. [8] The inhibitor has three tandem repeated domains with strong homology to other basic protease inhibitors of the Kunitz type (i.e., the same class as pancreatic-trypsin inhibitor). [7] [8] Factor Xa reacts with the second domain and factor VIIa binds to the first domain. Binding of factor Xa at one site may alter the conformation of the inhibitor at a second site, thereby baiting the trap for factor VIIa. Alternatively, factor Xa may concentrate the inhibitor at the membrane surface, forcing the equilibrium toward factor VIIa neutralization.

One potential advantage of this mechanism is that the inhibition of factor VIIa may be delayed temporally until factor Xa is formed, ensuring that some product can be made when tissue factor is exposed to the blood. This may contribute significantly to the fine-tuning of the regulatory mechanisms.

The importance of this inhibitor is illustrated by the observation that gene deletion in mice results in embryonic lethality, probably due to inadequate control of the coagulation reactions and subsequent thrombosis. [23]

INHIBITION OF THE REGULATORY PROTEINS (COFACTORS) OF THE BLOOD COAGULATION SYSTEM: THE PROTEIN C ANTICOAGULANT PATHWAY

The third major regulatory mechanism involves the protein C anticoagulant pathway. This pathway differs from the other pathways by the nature of the target molecules that it inhibits. Unlike the TFPI and AT III mechanisms directed at the inhibition of the protease components of the coagulation cascade, activated protein C inhibits two of the nonproteolytic regulatory proteins (cofactors) of the blood coagulation cascade, factor Va and factor VIIIa. The components of the pathway are illustrated schematically in Figure 1043A (which depicts normal physiologic conditions) and in Figure 1043B (which depicts this pathway after an inflammatory insult).

In this model, thrombin forms at a wound site and excess thrombin moves downstream to bind to thrombomodulin on endothelial cells. On endothelial cells in larger vessels,[24] an endothelial protein C receptor (EPCR) binds to protein C and augments protein C activation by increasing the affinity of the activation complex for protein C (Fig. 1044).[25] The thrombin/thrombomodulin complex activates protein C[26] catalytically, and activated protein C rapidly dissociates from cells in the microcirculation expressing only the thrombin/thrombomodulin complex, but more slowly from endothelium of larger vessels because EPCR binds both protein C and activated protein C. When bound to EPCR, activated protein C loses anticoagulant activity and cannot inactivate factor Va, but retains the capacity to be inhibited by proteinase inhibitors.[27] Presumably, the complex between EPCR and activated protein C catalyzes cleavage of new plasma or cellular substrates that are yet to be identified. These may be involved in the anti-inflammatory activities associated with activated protein C (see later). For activated protein C to function effectively, it must interact with protein S bound to cell surfaces.[28] [29] [30] This interaction facilitates activated protein C binding to platelet [31] and endothelial cell surfaces, [32] although

Figure 104-4 A model of the function of the endothelial cell protein C receptor in protein C activation. Protein C can bind directly to the protein C receptor (EPCR) whether or not thrombomodulin (TM) is present. The complex of protein C (PC) with EPCR is activated more effectively by the thrombin (T)-TM complex than free protein C. The activated protein C (APC) that is formed does not inactivate factor Va and presumably cleaves an alternative, as yet to be identified, substrate. Once the APC dissociated from EPCR it can bind to protein S (S) and inactivate factor Va. *(Modified from Stearns-Kurosawa et al.,[25] with permission. Copyright 1996 National Academy of Sciences USA.)*

other cell types may be involved as well.[33] [34] The cell surface-bound complex can then inactivate the target proteins, factors Va and VIIIa.[35] The procofactors factors VIII and V seem relatively resistant to the action of activated protein C.[36] [37] Protein S circulates in the blood in at least two forms: (1) as the free protein, and (2) noncovalently associated with a large, multisubunit regulatory protein of the complement system, C4b-binding protein (C4b-BP).[38] [39] Only the free form of protein S functions as an active inhibitor in this pathway.[40] [41] Recently, protein S has also been proposed to inhibit prothrombin activation directly, probably by binding to factor Va or factor Xa, or both.[42] The relative importance of this direct inhibition versus the cofactor function for activated protein C is unknown, but it seems likely that the cofactor function predominates. In addition to protein S, Dahlbäck et al. [43] have identified patients whose plasma is normal in protein S, but fails to respond to addition of activated protein C. The abnormality in this plasma is due to a mutation at one of the factor Va cleavage sites (Arg 506) [44] (see Genetics of Factor V below). Factor V, the precursor of factor Va, accelerates the inactivation of factor VIIIa, [45] suggesting an even more complex regulatory mechanism than illustrated in Figure 1043A . In the presence of activated protein C, the rate-limiting steps in coagulation appear to be the formation of adequate factor Va or factor VIIIa, or both, to amplify the original coagulation stimulus. Evidence for this concept comes from the observation that prior activation of factor V to factor Va in plasma almost totally abolishes the anticoagulant activity of activated protein C. This finding is consistent with the observation that factor Va interacts with activated protein C on activated platelets with high affinity, but the catalytic rate of inactivation is relatively slow.[46] Once activated protein C is formed, the enzyme has a half-life in the circulation of approximately 15 minutes,[47] [48] in contrast to a thrombin half-life of 1020 seconds.[49] Inhibition involves two major inhibitors: a protein C inhibitor [50] and $_1$ -antitrypsin.[51] Both are relatively slow reacting. Of the two, only the protein C inhibitor activity is enhanced by heparin. Recent studies have demonstrated that both inhibitors function in vivo.[48]

Protein C, protein S, thrombomodulin, and the endothelial cell protein C receptor all interact with the membrane surface, but the mechanisms are distinct (Fig. 1045). For protein C and protein S, membrane association requires -carboxyglutamic acid residues and Ca^{2+} . Because these proteins require vitamin K for their biosynthesis, the proteins synthesized in the presence of vitamin K antagonists or in the absence of vitamin K

Figure 104-5 Schematic representation of protein C, protein S, thrombomodulin, and the endothelial cell protein C receptor. The disulfide bonds in thrombomodulin are predicted by homology. The -carboxyglutamic acid residues in protein C and protein S are indicated by small Y-shaped symbols. *(From Esmon,[5] with permission.)*

are inactive.[52] [53] Their binding to membrane surfaces is largely Ca^{2+} dependent and requires negatively charged phospholipids.[54] Thrombomodulin is an integral membrane protein and does not dissociate from the cell surface. Proteolytic modification of thrombomodulin with elastase yields a soluble protein that retains the capacity to accelerate thrombin-dependent protein C activation [55] that may be structurally similar to the soluble form of thrombomodulin described.[56] [57] The protein C receptor is also an integral membrane protein.[58] Neither thrombomodulin nor EPCR requires Ca^{2+} or negatively charged phospholipid to remain membrane bound.

Examination of the structures of protein C, protein S, and thrombomodulin shows a remarkable number of tightly knotted structures.[59] [60] [61] [62] [63] [64] These have the disulfide bond patterns characteristic of the epidermal growth factor (EGF) precursors. In thrombomodulin, this domain is all that is required for thrombin binding and for the activation of protein C.[65] [66] [67] In fact, the major thrombin binding site is located in an 80-residue stretch defined almost exclusively by the last two EGF-like regions (Fig. 1045C). Ca^{2+} is required for protein C activation. There are three classes of Ca^{2+} binding sites in protein C: (1) those involving Gla residues, (2) the site in the first EGF domain, and (3) a site in the protease domain. The Ca^{2+} dependence for activation of protein C by soluble thrombin/thrombomodulin complexes is not mediated through the Gla domain, but rather through a single Ca^{2+} -binding site in protein C[68] [69] [70] that is located in the protease domain.[71] [72] Occupancy of this site changes the conformation of protein C, so that it is not activated effectively by thrombin alone, but rather is a substrate for the thrombin/thrombomodulin complex.[73] [74] A schematic representation of how substrate recognition is defined is presented in Figure 1046 . On the membrane surface the Gla domain is important for interaction with phospholipids[75] and the protein C receptor.[76] Optimal interaction with membranes also appears to involve the Ca^{2+} binding site in the EGF domain of protein C.[69] The -hydroxy aspartic acid residue in protein C[77] and the three hydroxylated aspartic acid and asparagines in protein S[78] are located within the EGF domains. The role of these hydroxylated residues is unknown, but they appear to be located near the Ca^{2+} -binding site. Substitution of the aspartic acid with glutamic acid interferes with normal Ca^{2+} binding.[63] This observation, coupled with their known role as metal-binding residues in bacterial proteins, [79] suggested that they are involved in Ca^{2+} binding, but recent two-dimensional nuclear magnetic resonance structural studies indicate that these side chain modifications are not directly involved in ligating the Ca^{2+} .[80] The hydroxylated residues could be involved with metal-dependent recognition of other proteins. Activated protein C also

contains a monovalent ion-binding site that accelerates cleavage of synthetic substrates. [81]

Bovine thrombomodulin has also been shown to contain a -hydroxy aspartic acid residue. [82] The role in thrombomodulin function remains unknown.

The COOH-terminal regions of protein C, and protein S, demonstrate little structural similarity. In protein C, this region contains the serine protease domain that has extensive sequence

Figure 104-6 A plausible mechanism for thrombomodulin function. Thrombin (T) can interact with protein C (PC) in the absence of Ca^{2+} (upper left). In the presence of Ca^{2+}, a conformational change in protein C occurs that prevents the interaction with thrombin (upper right). A conformational change occurs in the enzymatic active site of thrombin when thrombin binds to thrombomodulin. This change prevents cleavage of protein C by thrombin in the absence of Ca^{2+} (lower left). However, the change in the binding site of thrombin induced by thrombomodulin results in the binding of the Ca^{2+} -stabilized conformation of protein C (lower right) and the subsequent formation of activated protein C. TM, thrombomodulin. *(From Johnson et al., [65] with permission.)*

homology to trypsin.[83] In activated protein C, the protease domain is covalently linked to the EGF and Gla domains by a single disulfide bridge. [83] [84] Protein C circulates both as a single-chain and two-chain molecule with a ratio of about 1:3. [85] [86] There are also two subforms, referred to as and , with the latter appearing somewhat smaller and differing specifically in the heavy chain. The different forms differ in carbohydrate content.

In contrast to protein C, protein S is a single-chain molecule. Consistent with the proposed model as a binding protein (Fig. 1043), the COOH-terminal domain has no similarity with proteases. Some sequence similarities have been noted with steroid-binding proteins. [87] The role of this domain remains uncertain, but evidence suggests that the domain is involved in C4b-BP interaction, [88] although this concept would appear to be inconsistent with site-specific mutations in this region of protein S that do not influence C4b-BP binding. [89]

In thrombomodulin, the role of the NH_2 -terminal domain is also obscure. Limited sequence similarities to some lectins has led to the hypothesis that it may be involved in carbohydrate binding, but no direct evidence for this function exists. This domain is not critical to protein C activation, leaving open the possibility that this receptor may participate in more than one control mechanism. Consistent with this possibility, thrombomodulin gene deletion in mice results in embryonic lethality prior to development of the cardiovascular system.[90]

Endothelial protein C receptor has a very short cytosolic tail, the single transmembrane spanning domain and two extracellular domains. [56] EPCR is homologous to the major histocompatibility class 1 molecules. Molecular modeling based on this homology suggests that the extracellular region forms a relatively compact structure without obvious domains.

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CONTROL MECHANISMS IN PROTEIN C ACTIVATION

Protein C activation can be controlled by a number of different mechanisms. When thrombin binds to thrombomodulin, several coagulation reactions, including fibrin generation and platelet activation, are inhibited. [91] [92] [93] [94] [95] Bound thrombin reacts with AT III more rapidly than does free thrombin, [93] an acceleration that depends on the presence of a sulfated galactosaminoglycan (chondroitin sulfate) on the thrombomodulin molecule. [96] Thrombin bound to thrombomodulin is also inhibited rapidly by the protein C inhibitor, [97] but this rapid inactivation does not require the chondroitin sulfate moiety on thrombomodulin. The rapid inactivation of thrombomodulin-bound thrombin ensures the termination of protein C activation shortly after thrombin generation has ceased. Thus, the two processes are very tightly coupled. Thrombomodulin appears to work in concert with vascular heparins to augment thrombin inhibition. The relative importance of the role of thrombomodulin and vascular heparin-like molecules in thrombin inhibition by proteinase inhibitors is uncertain and may vary within different organs. For instance, thrombomodulin is difficult to detect in the microcirculation of the human brain, [98] although low levels are present. [99]

Thrombin can also be cleared by internalization when bound to thrombomodulin, [100] but the extent of the observed internalization varies and its importance is controversial. [101] This internalization is much slower than the rate of thrombin inhibition, raising the question of the role of this process in regulation. The thrombin/AT III complex dissociates readily from thrombomodulin, so that neither thrombin nor thrombin/ antithrombin clearance via internalization is likely to contribute significantly to overall clearance of either species from the circulation. [96]

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INFLAMMATORY MEDIATORS, THROMBOMODULIN, AND THE HEMOSTATIC BALANCE

Under most circumstances, the blood clotting process is under very tight control, and the in vivo regulatory mechanisms adequately limit the extent of clotting in response to injury. Inflammatory events, such as occur in gram-negative bacterial sepsis, can shift this balance in favor of the clotting response at the expense of the natural anticoagulant mechanisms. A prime target for this modulation appears to be the protein C anticoagulant pathway. A hypothetical depiction of the vasculature following an inflammatory insult is presented in Figure 1043B . As compared with the normal vasculature, inflammatory mediators depress thrombomodulin and EPCR,[58] thereby inhibiting protein C activation. [102] [103] [104] [105] As a result, more free thrombin is formed. The levels of C4b-BP are elevated;[106] this leads to a shift toward the C4b-BP/protein S complex. Because this complex is not active in supporting activated protein C anticoagulant activity, [40] [41] the limited amounts of activated protein C that are formed are not fully functional. Thus, there is a major decrease in the anticoagulant activity of this system.

Not only is the anticoagulant pathway down-regulated, but several procoagulant events ensue essentially simultaneously. Tissue factor synthesis and cell surface expression[107] are initiated, leading to the activation of factors X and IX. This occurs not only on the endothelium (Fig. 1043B), but on the macrophage/monocytes as well. Leukocytes are also bound to the cell surface. [108] Because these cells release both proteases and active oxygen species, this may contribute to vascular injury. In particular, recent studies have shown that human thrombomodulin is especially sensitive to oxidant damage due to oxidation at Met 388. [109]

Formation of tissue factor and the down-regulation of the

protein C pathway is predicted to lead to factor Xa formation and to increased stability of the factor Xa/factor Va complex on the platelet surfaces.

These observations raise the question of why inflammatory stimuli do not lead to even more severe injury. Available evidence suggests that TFPI remains fully active.[7] Little evidence has been presented that vascular heparin-like substances are down-regulated by these stimuli.

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WHERE DO ANTICOAGULANT FACTORS FUNCTION? COORDINATED CONTROL OF A COMPLEX PROCESS

As pointed out by Busch et al.,[110] the vasculature presents an unusual feature. As blood moves from the large vessels to the capillaries, the surface area of endothelium in contact with each milliliter of blood rises dramatically. For endothelial cell surface-bound receptors, this translates directly into an increase in the concentration of the regulatory receptor. Thrombomodulin is present at approximately 100,000 copies per endothelial cell. The concentration of thrombomodulin in a vessel about the size of a coronary artery is 0.10.2 nM. Rapid clotting with thrombin occurs at concentrations of 10 nM. Thus, the concentration of regulatory proteins is insufficient to control the process. By contrast, in the microcirculation the concentration of thrombomodulin is 500 nM. Because the interaction with thrombomodulin is governed by a K_d of 0.5 nM,[111] virtually no free thrombin would exist in the microcirculation. This assumes that the concentration of the receptor is invariant throughout the vasculature. Certainly, thrombomodulin is present in capillaries, and the available evidence suggests that the concentration per cell is approximately constant.[112] [113] If one makes the same assumptions for other regulatory mechanisms of the endothelium, it would appear that the microcirculation may be a major site for the function of vascular heparins, prostacyclin, and plasminogen activator, as well as thrombomodulin.

The heparin and protein C systems do not work in isolation, but rather orchestrate a coordinated control over coagulation events (see Fig. 1041). AT III inhibits many factors in the presence of heparin, but the inactivation of platelet-bound factor Xa/factor Va complex is relatively resistant to the action of heparin and AT III. [114] This complex is also relatively resistant to inactivation by activated protein C. [29] [115] Protein S, however, is capable of at least partially overcoming this protection. [46] Thus, activated protein C may be required for factor Xa clearance by the AT III/heparin system. No comparable interplay has yet been elucidated between TFPI and these two anticoagulant systems.

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POTENTIAL ROLE OF PROTEIN C IN PREVENTION OF THROMBOSIS AND DISSEMINATED INTRAVASCULAR COAGULATION

Clinical manifestations of impaired functions of the protein C system provide important clues that it is a major inhibitor of coagulation in the microcirculation. Homozygous protein C deficiency is usually manifested by neonatal purpura fulminans that results from microvascular thrombosis. This has been effectively treated with protein C concentrate,[116] establishing an unambiguous link between the deficiency state and the evolution of the skin lesions. A second clinical manifestation of protein C deficiency appears to be warfarin-induced skin necrosis. Skin lesions appear shortly after the onset of oral anticoagulant treatment at times when protein C levels are much lower than all the vitamin K-dependent proteins except factor VII.[52] The incidence of warfarin-induced skin necrosis is increased in heterozygous protein C-deficient individuals and some patients with protein S deficiency.[117] [118] [119] [120] [121] [122] Progression of the lesion can be halted by treatment with protein C concentrate,[120] [121] [122] further linking inhibition of protein C function during oral anticoagulant therapy to the skin necrosis. The basis for localization to the skin is unknown, but the skin is an inflammatory organ, perhaps linking the down-regulation of the protein C system due to inflammation in the skin to the necrotic lesions. Like the purpura fulminans commonly seen in homozygous protein C-deficient infants, warfarin skin necrosis involves microvascular thrombosis. Both observations are consistent with the fact that protein C activation occurs largely in the microcirculation.

Another setting in which skin necrosis can occur is in gram-negative sepsis. Because the protein C system seems to be a major target of inflammatory mediators with respect to the coagulation system, activated protein C might offer protection from gram-negative sepsis. In a baboon model of *Escherichia coli* sepsis, activated protein C blocked both the fibrinogen consumption and the organ damage that ultimately lead to death.[123] Although high levels of activated protein C were required, this approach holds promise as an adjunct therapy for disseminated intravascular coagulation. Despite the bacterial challenge to vascular integrity, no overt bleeding complications were observed with activated protein C treatment of the shock process.

Protein C is also consumed in patients during septic shock.[124] Some of these patients develop severe purpura lesions resembling those of homozygous protein C-deficient infants. It follows that protein C might effectively prevent these lesions from progressing. Based on limited clinical data, protein C may be effective in treating patients with very low protein C levels and septic shock.[125] [126] [127]

Activated protein C has also been studied for its ability to inhibit fibrinogen accretion on preformed jugular vein thrombi in dogs and rhesus monkeys.[128] Fibrin deposition was decreased approximately 4-fold in dogs and 10-fold in rhesus monkeys relative to a control. Despite the obvious influence on thrombus growth, bleeding at surgical sites was not significantly increased.

Activated protein C has also been tested in a baboon model of platelet-dependent thrombosis,[129] entailing examination of platelet deposition on prosthetic vascular grafts. Under the conditions used, infusion of activated protein C reduced platelet deposition by 70%. Thus, in this model, activated protein C was an effective antithrombotic agent and did not alter primary hemostasis. Because activated protein C is generated in response to thrombin/thrombomodulin interaction, it follows that systemic infusion of thrombin, which would lead to protein C activation, might actually retard platelet and fibrin formation on the vascular grafts. Indeed, infusion of thrombin into baboons at 1 or 2 U/kg/min reduced the platelet or fibrin deposition on the graft in a dose-dependent fashion.[130]

Ischemia is another potential setting in which protein C may play a role in preventing injury. Model studies in dogs and pigs demonstrate the rapid activation of protein C in ischemic areas in the heart following ligation of the coronary artery. Blocking this activation impairs recovery of coronary function.[131] Taken together, studies on protein C in the coronary circulation suggest that it might be important in preventing or limiting platelet deposition at sites of arterial injury and, if occlusion should occur, it could play important functions in keeping the coronary microcirculation patent.

Some of these activities are probably due to anti-inflammatory functions of activated protein C. The exact mechanisms by which the activated protein C may express anti-inflammatory activity is uncertain, but treatment of monocytes with activated protein C has been observed to dampen monocyte activation and secretion of the inflammatory cytokine, tumor necrosis factor-alpha.[132]

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THE FUNCTION OF THE THROMBIN/ THROMBOMODULIN COMPLEX IN REGULATING FIBRINOLYSIS

Recently, a plasma procarboxypeptidase B, referred to as the thrombin activatable fibrinolysis inhibitor or TAFI has been identified. Thrombin can activate this protein slowly, but thrombomodulin enhances the activation of TAFI and protein C to similar degrees. [133] Once activated, TAFI can remove carboxy terminal Arg or Lys residues. When this occurs on fibrin, it renders the fibrin resistant to lysis. Carboxy terminal Arg residues are also critical to the activity of many potent mediators like complement C5a, an anaphylotoxin. What physiologic role TAFI plays in regulation remains somewhat unclear. It is known that during inflammation infusion of soluble thrombomodulin minimizes fibrin deposition and inflammatory organ injury, [134] [135] [136] [137] [138] [139] [140] suggesting that anticoagulant and anti-inflammatory effects of thrombomodulin are dominant in vivo.

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GENETICS OF PROTEIN C

Recent advances in genetics have facilitated the analysis of the molecular basis of protein C deficiency. The protein C cDNA and genomic clones have been sequenced.[141] [142] [143] Heterozygous protein C-deficient patients usually have few clinical symptoms. In some families, however, the protein C deficiency clearly correlates with an increased risk of thrombosis. [144] [145] The genetic basis of these pedigrees has been determined and summarized in a comprehensive review. [144] More than 130 different pedigrees of families with protein C deficiency have now been characterized at the level of mutations within the gene. Of 134 apparently unrelated probands characterized, 99 had low expression levels and 18 had dysfunctional molecules with near-normal antigen levels. Single base-pair substitutions are the most common finding, with 67 different base-pair substitutions now characterized. Three mutations in the promoter have been detected. Deletions within the protein C gene appear to be relatively rare. The available mutations have not provided any striking insights into protein C function, and it is difficult to determine from the limited number of available sequences and patients whether certain mutations carry a greater risk of thrombosis than others.

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GENETICS OF PROTEIN S

The protein S gene has been sequenced and characterized, [146] [147] [148] revealing a complex gene containing 15 exons. The molecular basis of protein S-deficient patients has been determined in only a relatively few instances. This is in part because of the existence of a pseudogene for protein S that complicates the analysis. Furthermore, although protein S is clearly important for the anticoagulant pathway to function in vivo, the ability of protein S to stimulate anticoagulant activity in vitro in purified systems is modest, usually about two- to threefold. Finally, in at least some of the patients with low free protein S, the defect may lie in the C4b-BP. For these reasons, too few patients have been characterized to allow any clear picture of the molecular basis of protein S deficiency to emerge. The approaches to protein S gene analysis have been described by Schmeidel et al., [149] and a summary of all known genetic abnormalities has been published recently. [150]

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GENETICS OF THROMBOMODULIN

The multiple functions of thrombomodulin suggest that deficiencies of the protein might be associated with thrombosis. Recently, clinical studies have suggested that a C/T dimorphism at position 1,418 resulting in Ala 455-Val substitution in the 6th EGF domain in thrombomodulin may be related to risk of premature myocardial infarction. Specifically, the frequency of the C allelle was significantly higher in the Swedish population surviving premature myocardial infarction. [151] Preliminary studies from this group indicate that mutations in the thrombomodulin gene may be present in about 5% of the patients with venous thrombosis. [151] The mutations were observed at many sites throughout the thrombomodulin gene. Analysis of the thrombomodulin gene mutations is simplified to some extent by the fact that the gene lacks introns.[62]

A recent study has examined mutations in the promoter region of thrombomodulin. Mutations in the 5 regulatory region of the thrombomodulin gene were observed in 5 of 100 patients with myocardial infarctions, compared to 1 in 100 in the control group. [152] This small study suggests that mutations in thrombomodulin expression are likely to contribute to arterial thrombosis.

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GENETICS OF THE ENDOTHELIAL CELL PROTEIN C RECEPTOR

No information is known about abnormalities in this gene or the relationship between these possible abnormalities and human disease. It is known that like thrombomodulin, plasma levels of soluble EPCR rise dramatically in disease states associated with vascular injury such as lupus erythematosus. [153]

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GENETICS OF FACTOR V AS THEY APPLY TO NATURAL ANTICOAGULANT MECHANISMS

Factor Va is a major target of activated protein C anticoagulant activity. To understand the genetics, one must appreciate some of the complications in factor Va inactivation. Factor Va consists of two subunits formed by activation of single-chain factor V. Factor Va is inactivated by proteolytic cleavage at Arg residues 306 and 506.[154] Both of these sites are located on the larger subunit, the heavy chain. Another cleavage at Arg 679 occurs but its physiologic significance is uncertain. Cleavage of Arg 506 is the most rapid and results in loss of most of the factor V activity in plasma assays. In purified systems, this form of factor Va retains some activity. Complete inactivation requires cleavage at Arg 306, and this is potently facilitated by membrane surfaces.

A common dimorphism exists in Caucasians in which Arg 506 is replaced by Gln because of a single base change (G to A) at position 1,691. This renders factor Va insensitive to cleavage at residue 506 and as a result, factor Va activity is about 10 times more stable in the presence of activated protein C. [155] This trait is usually referred to as factor V Leiden or APC resistance. Estimates suggest that the variant may be found in approximately 5% of the Caucasian population and is present with much higher frequency in patients with venous thrombosis, with estimates ranging from 2060% of these patients. [155]

The factor V Leiden trait is usually associated with venous thrombosis, but recent data suggest that the Leiden trait is a risk factor for myocardial infarction in patients who smoke.[156]

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SUMMARY

Current information indicates that protein C, protein S, and thrombomodulin are potent inhibitors of the blood coagulation process. The system is down-regulated by inflammatory mediators. Total deficiencies of protein C and S are associated with severe thrombotic complications. Presumably, the down-regulation of the protein C pathway by inflammatory agents decreases the capacity of the system to redirect thrombin from a procoagulant enzyme into the initiator of a potent anticoagulant.

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Chapter 105 - Clinical Evaluation of Hemorrhagic Disorders: The Bleeding History and Differential Diagnosis of Purpura

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INTRODUCTION

The initial evaluation of patients with hemorrhagic problems involves obtaining a detailed history of bleeding symptoms and analyzing any current hemorrhagic lesions, which are most often on the skin. This chapter focuses on the bleeding history and the differential diagnosis of purpuric skin lesions that may reflect an underlying hemorrhagic or nonhemorrhagic disorder.

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THE BLEEDING HISTORY

The bleeding history forms the basis of the diagnosis and therapy of hemorrhagic disorders. In order to maximize its value, several basic principles of eliciting and interpreting this information deserve emphasis:

1. Many normal, healthy people consider their bleeding and bruising excessive. For example, using standardized, self-administered questionnaires, Miller et al. [1] found that 23% of the normal population had positive bleeding histories, and Wahlberg et al. [2] reported that a remarkable 65% of healthy women and 35% of healthy men answered Yes to the question: Do you suffer from a bleeding disorder? (Table 105-1). [2]
2. Patients with profound coagulation disorders invariably have dramatically abnormal bleeding histories, although surprisingly they may not volunteer the information unless specifically questioned.

3. Patients with mild to moderate abnormalities may not admit to having excessive bleeding symptoms or may not recognize subtle symptoms as abnormal, even though they are at risk of developing excessive bleeding if exposed to severe hemostatic challenges. In a study by Miller et al., [1] 35% of patients with heterozygous von Willebrand disease had negative bleeding histories, and in a study by Wahlberg et al., [2] approximately 54% of the men and 38% of the women with either von Willebrand disease or a platelet function defect failed to identify themselves as suffering from a bleeding disorder (Table 105-1). Identification of the group of patients with milder defects, and distinguishing them from the normal population, requires considerable skill and experience. In fact, given the low frequency of hemorrhagic disorders in the population and the high false-positive rate in the normal population, the task is truly formidable. For example, if one uses the values of 65% for the sensitivity of the bleeding history and 77% for its specificity derived from the study of Miller et al., [1] and assumes a prevalence of 10 patients with von Willebrand disease in a population of 100,000, [3] the predictive accuracy of a positive history is a dismal 0.03%. Thus, in a population of 100,000 people, there would be seven patients with von Willebrand disease with positive histories (65% of 10) to be differentiated from the 22,998 normal individuals with positive histories (23% of 99,990).
4. A search for objective confirmation of subjective symptoms provides important information in assessing the severity of the patients disorder. This is especially important when obtaining a bleeding history because some patients are extremely sensitive to even minor hemorrhagic problems, whereas others ignore major ones. Specific objective indicators are discussed later with each group of symptoms, but overall indicators include: (1) visits to other physicians for bleeding problems, along with any laboratory data obtained; (2) previous need for transfusion of whole blood, packed red blood cells, plasma, platelets, or coagulation factor concentrates; and (3) a history of documented anemia and/or physician-prescribed iron therapy.
5. A medication history is incomplete without specific questions concerning aspirin and other medications available without prescription that may affect platelet function, because patients may not recognize these agents as medications. Similarly, it is important to inquire about vitamin tablets that may contain vitamin K in patients taking oral anticoagulants.
6. Although self-administered questionnaires with Yes or No answers facilitate data collection and statistical analysis, obtaining the maximal amount of useful information requires a dialogue between patient and physician. This allows the physician to assess whether the patient truly understands what is being asked, to refine the questions in response to the initial answers, and to follow up on potentially important data that are revealed only as the discussion proceeds. In short, good history taking is not a passive process in which boxes are checked off in response to bland and ambiguous questions, but rather an extremely active process consisting of initial data gathering, hypothesis development, construction of questions to test the hypothesis, additional data gathering, and new hypothesis development. Moreover, it is an exhilarating endeavor in which the physician must meld his or her knowledge of science, medicine, and human behavior into a series of pointed questions that the patient can understand and respond to.
7. A constellation of hemorrhagic symptoms, rather than any single symptom, is most helpful in suggesting the etiology of the disorder. Thus, spontaneous hemarthroses and muscle hemorrhages are highly suggestive of severe hemophilia, whereas epistaxis, gingival bleeding, and menorrhagia are more commonly found in patients with thrombocytopenia, platelet disorders, or von Willebrand disease.
8. Excessive bruising and bleeding may be a manifestation of diseases of the blood vessel rather than diseases of coagulation or platelets. In patients with impressive bleeding histories, but no abnormalities in coagulation or platelets, the clinician must have a high index of suspicion for hereditary hemorrhagic telangiectasias, Cushing disease, scurvy, Ehlers-Danlos syndrome, and other systemic disorders. In patients whose hemorrhagic symptoms are confined to the skin, consideration must be given to a wide range of dermatologic disorders.
9. The diagnostic value of any single hemorrhagic symptom

varies according to the disorder. In studies of hemophilia carriers by Wahlberg et al. [4] and of patients with von Willebrand disease or platelet function abnormalities,[2] the individual questions were ranked on the basis of their ability to discriminate between patients and normals. Results from the hemophilia carrier study are shown in Table 105-2 . Because the patterns of hemorrhage differ in different hemorrhagic disorders, the diagnostic value of any given question will differ as well. For example, in the study on patients with von Willebrand disease and platelet disorders, [2] long bleeding after tooth extractions (Table 105-1) was a more powerful discriminator than it was in the study on hemophilia carriers [4] (Table 105-2).

10. A dietary history is important in patients taking oral anticoagulants because the patients intake of vitamin K will affect the response to the medication. In order to maintain a constant level of anticoagulation, the dietary intake of vitamin K must remain constant. [5] The absolute amount of vitamin K intake is less important, so it is reasonable to permit the patient free choice of diet with the proviso that, once selected, it remain reasonably constant. [6] If the patient wishes to change diets, it is important to monitor the level of anticoagulation more carefully. Patients should also be instructed that food additives, such as fish oils, may contain vitamin K. Patients who are not taking food by mouth are at particularly high risk of developing vitamin K deficiency, especially if they are also taking broad-spectrum antibiotics, since the latter decrease the vitamin K contribution from bacteria in the gastrointestinal tract. [7] [8] This combination of decreased oral intake and antibiotic therapy is quite common in patients with bowel disorders during the preoperative and postoperative periods. It is vital to recognize this scenario because vitamin K is required on a daily basis despite its fat solubility, and significant depression of coagulation factors can occur within only a few days.
11. Potentially confounding pharmacological and medical influences should be considered in evaluating the history. For example, because pregnancy and birth control pills can increase von Willebrand factor in some patients with the mild to moderate forms of the disease, this should be considered in assessing the bleeding history. The decrease in von Willebrand factor soon after delivery often leads to prolonged postpartum hemorrhage. Similarly, because the stress of surgery or pregnancy can lead to thrombocytopenia in type 2B von Willebrand diseases, bleeding may be especially severe at these times. [9] [10]
12. Assessing excessive bleeding in the newborn is especially difficult. For example, some neonates with Glanzmann thrombasthenia have only minimal symptoms at birth, and the symptoms of von Willebrand disease may be masked by an increase in von Willebrand factor as a result of the stress involved in the delivery. Similarly, a significant number of hemophilic neonates do not have hemorrhagic symptoms in the first weeks of life. Large cephalhematomas may be a result of birth trauma, but if they continue to progress after delivery, hemophilia or a vitamin K-dependent factor deficiency should be considered. Prolonged bleeding from the umbilical cord stump should raise the possibility of factor XIII deficiency or a fibrinogen abnormality (quantitative or qualitative).

TABLE 105-1 -- Self-administered Questionnaire for the Diagnosis of Hemorrhagic Disorders

Questions	Males		Females	
	Normals (N = 23)	Patients[a] (N = 24)	Normals (N = 20)	Patientsa[a] (N = 21)
Do you suffer from a bleeding disorder?	35[b]	46	65	62
Bleeding from the gums?	52	67	50	67
Long bleeding after tooth extraction?	4	21	10	24
Skin bleeding?	0	13	15	10
Long bleeding from small wounds?	13	25	10	10
Tendency to bruises?	22	25	55	62
Spontaneous bruises?	0	4	40	48
Nose bleeding?	57	63	85	57
Blood coughed up or vomited?	9	13	5	10
Metrorrhagia?			55	42
Muscle bleeding?	4	13	15	0
Blood in the urine?	0	8	10	10
Joint bleeding?	0	0	0	10
Blood in the stool?	13	8	5	10
Treated with vitamin K?	0	4	10	19
Treated with plasma or blood transfusion?	0	4	0	0

Adapted from Wahlberg et al.,[2] with permission.

[a] The patient group consisted of 16 individuals with von Willebrand disease, 27 individuals with a variety of qualitative platelet abnormalities (including aspirin-like defects (N = 9), isolated abnormal collagen-induced aggregation (N = 12), and isolated abnormal arachidonic acid-induced aggregation (N = 6), and 2 patients with antithrombin III deficiency.
[b] Percentage of normal patients compared with patients with von Willebrand disease, platelet abnormalities, or antithrombin III deficiency answering yes to indicated questons. Subjects were given a choice of yes, no, and dont know. The percentage answering no and dont know varied considerably from question to question.

TABLE 105-2 -- Discriminant Capacity of Questions in Distinguishing Between Normal Persons and Hemophilia Carriers

Questions	Sensitivity[a]	Specificity[b]	Difference Between Carriers and Noncarriers (*P*)
Tendency to bruises?	50	77	< 0.025
Metrorrhagia	44	81	< 0.025
Abnormal bleeding at delivery	38	97	< 0.005
Abnormal bleeding at operation	35	92	< 0.005
Tendency to nosebleeding?	32	87	< 0.05
Long bleeding after tooth extraction?	15	98	n.s.
Blood in the urine?	11	98	n.s.
Long bleeding from small wounds	8	96	n.s.
Blood in the stool?	3	100	n.s.

N.s., not significant.

From Wahlberg T, Blomback M: Improved detection of haemophilia A carriers by factor VIII related antigen (radioimmunoassay) and bleeding symptoms. Thromb Res 21:339, 1981. © 1981 with permission from Elsevier Science.

[a] Percentage of positive responses among carriers.
[b] Percentage of negative responses among normals.

Documenting the History

Table 105-3 shows a bleeding history form based on the one in use at the State University of New York at Stony Brook and Mount Sinai Medical Center, New York. It is not self-administered by the patient, but rather is filled out by the physician during discussions with the patient. The use of the form ensures that the major symptoms are elicited and recorded in a standardized manner. The form itself is then made a part of the patients permanent record.

Epistaxis

Bleeding from the nose is one of the most common manifestations of platelet disorders and von Willebrand disease; it is also the most common symptom of hereditary hemorrhagic telangiectasias. However, it is important to note that a large fraction of the normal population has experienced one or more nosebleeds. Thus, when a group of normal subjects was presented with the question, Nose bleeding? on a self-administered questionnaire, 57% of the men and 85% of the women answered Yes (see Table 105-1). To obtain more meaningful data, it is important to inquire about the frequency of nosebleeds and whether the nose bleeding occurs spontaneously or only with trauma. The latter may not always be appreciated because some individuals habitually and subconsciously traumatize their mucous membranes when manually removing crusted secretions from the nose. If the bleeding is confined to a single nostril, it is more likely the result of a localized vascular abnormality than a systemic coagulopathy. In northern locales, nosebleeding from nonhematologic etiologies is more likely to occur in the dry winter months, especially if forced air heating systems that dry out the mucous membranes are used. It is also important to ascertain the effects of aging on epistaxis because many individuals with no discernible abnormalities will have childhood epistaxis that disappears after puberty, whereas patients with hereditary hemorrhagic telangiectasias usually suffer increasingly severe epistaxis with the onset of adulthood, middle age, and old age. The length of time for the bleeding to stop will give valuable insight into the severity of the episodes. Objective information can be obtained by inquiring whether the epistaxis was severe enough to require evaluation by a physician, and if so, whether packing, cautery, or transfusions were necessary to control the bleeding.

Gingival Hemorrhage

Bleeding from the gums is another very common symptom of platelet disorders and von Willebrand disease. In addition, it is often the first sign of hemostatic compromise in patients who

TABLE 105-3 -- Bleeding History

1.Epistaxis
- A.Age when affected
- B. Frequency
- C. Spontaneous
- D. Left, right, or both nostrils
- E. Seasonal correlation
- F. Time to stop
- G. Required
  - 1. Packing
  - 2. Cautery
  - 3. Transfusions

Comments:

2.Gingival hemorrhage
- A.Frequency
- B. Spontaneous or with tooth brushing

Comments:

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3. Skin hemorrhage
- A. Petechiae
- B. Bruises
  - 1. Frequency
  - 2. Relationship to menses
  - 3. Spontaneous
  - 4. Exposed sites
    - a.Arms
    - b.Legs
  - 5.Unexposed sites
    - a. Trunk
    - b. Back
  - 6. Size
  - 7. Knots in center
  - 8. Painful
  - 9. Color
  - 10. Time to resolution
  - 11. Number currently

Comments:

---

4. Tooth extractionsages at extractions
- A. Deciduous
- B. Permanent

  Molar

  Other
- C. Duration of bleeding
  - 1. Packing
  - 2. Resuturing
  - 3. Transfusion
- D. Estimated blood loss

Comments:

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5. Bleeding from minor cuts
- A. Blade or electric razor
- B. Approximate time to stop
- C. Requires
  - 1. Direct pressure
  - 2. Tissue paper

Comments:

---

6. Bleeding from major trauma
- A. Knife wound
- B. Motor vehicle accident
- C. Need for
  - 1. Sutures
  - 2. Transfusions

Comments:

7.Hemoptysis
- A. Spontaneous
- B. Associated with respiratory infection

Comments:

---

8. Hematemesis
- A. Spontaneous
- B. Known increase in portal pressure
- C. Associated with vomiting
  - 1. Beginning of episode
  - 2. End of episode

Comments:

---

9. Hematuria
- A. Frequency
- B. Gross or microscopic
- C. Related to urinary infection
- D. Duration
- E. Required
  - 1. Cystoscopy
  - 2. Transfusion

Comments:

10. Hematochezia
    A. Frequency
    B. Duration
    C. Known hemorrhoids
    Comments:

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11. Melena
    A. Frequency
    B. Duration
    C. Known ulcer disease
    D. Required
        1. Transfusion
        2. Surgery
    E. Documented by tests for occult blood
    Comments:

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12. Central nervous system bleeding
    A. Hemorrhagic stroke
    B. Documentation
        1. Computed tomography scan
        2. Magnetic resonance imaging
    Comments:

---

13. Venipuncture site bleeding *Comments:*

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14. Ophthalmic bleeding
    A. Subconjunctival hemorrhage
    B. Retinal hemorrhage
    C. Retrobulbar hemorrhage
    Comments:

---

15. Menses
    A. Frequency
    B. Duration (in days)
        1. Heavy flow
        2. Total flow
    C. Comparison to sisters or friends
    D. Required
        1. Transfusion
        2. Iron therapy
        3. Birth control pills
        4. Dilation and curettage
        5. Hysterectomy
    E. Known fibroid tumor
    Comments:

---

16. Childbirth
    A. Pregnancies
    B. Spontaneous abortions (indicate month of gestation)
    C. Induced abortions
    D. Estimated blood loss
    E. Anemia documented
    F. Required
        1. Transfusion
        2. Dilation and curettage
        3. Hysterectomy
        4. Iron therapy
    Comments:

---

17. Hemarthroses
    A. Joints
        1. Elbow
        2. Knee
        3. Ankle
        4. Wrist
        5. Shoulder
        6. Other
    B. Frequency
    C. Required
        1. Transfusion
        2. Aspiration
    Comments:

---

18. Previous surgical procedures
    A. Procedure
    B. Date
    C. Excess bleeding
    D. Required
        1. Whole blood
        2. Plasma
        3. Platelets
        4. Coagulation-factor concentrate
    E. Reoperation
    F. Wound healing
    Comments:

---

19. Bleeding at circumcision
Comments:

---

20. Telangiectasias
    A. Mucous membranes
    B. Skin
    C. Gastrointestinal tract
    Comments:

---

21. Connective tissue
A. Double jointedness
B. Skin hyperextensibility
C. Fat distribution changes
Comments:

22. Wound healing *Comments:*

23. Medications
A. Iron
B. Birth control pills
C. Aspirin or other antiplatelet medications
Comments:

24. Family history of bleeding

Based on Coller and Hultin, unpublished data.

develop thrombocytopenia after chemotherapy. Interestingly, patients with long-standing disorders may not recognize that their gingival bleeding is excessive because they assume that everyone has gum bleeding every day. In fact, occasional, but not perennial, gum bleeding is very common among the normal population, with 52% of normal males and 50% of normal females answering Yes to the question, Bleeding from the gums? It is therefore important to establish how frequently the patients gum bleeding occurs and whether it is spontaneous, since the vast majority of normal persons will only experience gum bleeding after the trauma of tooth brushing. If spontaneous gum bleeding occurs during the night, patients may notice blood-tinged stains on their pillow cases. Daily gum bleeding with tooth brushing may or may not be abnormal, depending on whether the patient has gingival disease and whether a hard bristle or soft bristle tooth brush is used. Because routine tooth scaling performed by dental workers to remove plaque is a significant hemostatic challenge, it is useful to inquire whether the patient was told that he or she bled excessively after this procedure. In addition, some patients may have oral mucous membrane bleeding in the form of blood blisters as another manifestation of a hemorrhagic diathesis, most commonly due to severe thrombocytopenia; such blisters have a predilection to form at sites of tooth irregularities that may traumatize the inner surface of the cheek.

Skin Hemorrhage

Petechial lesions characteristically appear as crops or showers of lesions in dependent portions of the vasculature. However, the integrity of the microvasculature is dependent on a variety of vascular and extravascular factors, so it is not surprising that patients with similar platelet or coagulation disorders show considerable variability in the appearance of petechial lesions. When infants cry, they increase the pressure in the veins draining the face (as discussed later in this chapter) and this may be sufficient provocation to bring out petechial lesions in patients with platelet disorders such as Glanzmann thrombasthenia.

Bruising is one of the most difficult symptoms to evaluate because patients vary greatly in their recognition and response to the symptom. Thus, patients who always bruise excessively, even without trauma, may assume this is normal because they have experienced it all their lives, whereas normal individuals who bruise only rarely may grow inordinately concerned about a single large bruise associated with trauma. Bruising is more common in women than men. For example, 22% of normal men and 55% of normal women responded affirmatively to the question, Tendency to bruises? and none of the normal men but 40% of the normal women indicated that they had spontaneous bruises. It is necessary, therefore, to try to more clearly define the nature of the bruising in order to determine whether it is likely to be part of a hemorrhagic diathesis.

The dermatologic literature recognizes the female easy bruising syndrome (purpura simplex, discussed later in this chapter) as one in which women have excessive bruising in relationship to their menstrual cycle, although the point in the cycle when the bruising is excessive may vary from woman to woman. This diagnosis should be considered only after excluding other etiologies. If there are no underlying hematologic or nonhematologic causes, it is appropriate to reassure the patient that she is unlikely to be at risk of excessive hemorrhage, even with more severe hemostatic challenges such as surgery. It is inappropriate, however, to tell the patient that there is nothing wrong with her, because this can be misinterpreted to mean that the physician thinks the patient inappropriately sought medical attention for a trivial matter. These patients clearly do have an abnormality that can be quite frightening, even though at present the defect cannot be identified biochemically.

It is important to try to establish whether the patients hemorrhagic response is excessive when measured against the inciting trauma. Thus, spontaneous bruising is most likely to be pathologic, and the patient should be asked specifically whether bruises appear even without any recognized antecedent trauma. Unfortunately, patients vary significantly in appreciating when they have been traumatized. For example, mothers of children who are old enough to be physically active, but still young enough to be held in the mothers arms, often focus on other matters while holding the child and do not realize that they are being repeatedly kicked. Similarly, normal toddlers and youngsters who are physically active commonly have bruises on their legs and arms.

The location of a bruise may offer indirect evidence of its relationship to trauma. The vast majority of traumatic events occur on exposed sites on the arms and legs. Therefore, if the patient suffers repeated bruises on unexposed sites on the trunk or back, these are more likely to be either spontaneous or in response to minimal trauma. The size of the bruise may also give some indication of the extent of bruising. It is best to provide patients with size standards that they can understand, such as dime-sized, silver-dollar-sized, or as large as your palm. When assessing bruise size, it is important to remember that bruises often spread during the resolution phase. With severe bruises there may be hemorrhage into the bruise, resulting in very dark discoloration and the appearance of a raised knot in the center of the bruise; such bruises tend to be particularly painful.

The color of the bruise may also be quite significant. Fresh bruises associated with hemorrhagic phenomena tend to be dark purple, black-and-blue marks that evolve into shades of yellow-green as they resolve. By contrast, patients with senile purpura or Cushing syndrome commonly have bruises that are much redder in appearance. Because easy bruising may be the presenting symptom of Cushing syndrome, it is important to have a high index of suspicion for this disorder in younger patients whose bruises simulate the appearance of senile purpura.

Another color variant worth distinguishing is the jet black central area and violaceous-erythematous surrounding area characteristic of coumadin-induced skin necrosis (discussed later in this chapter). This thrombotic disorder, associated in some cases with protein C deficiency, tends to be most apparent in fatty tissues such as the breasts; it can be mistaken for a hemorrhagic abnormality because of the bruiselike quality of the lesion and the association with oral anticoagulant use.

Bruises usually take 10 days to 2 weeks to resolve, depending on the extent of the bruise. When patients indicate that their bruises take months to resolve or that they have required casting of a limb in order for the bruising to stop, consideration should be given to the poorly understood entity of psychogenic purpura (discussed later in this chapter).

Tooth Extractions

Bleeding in response to tooth extraction can provide extremely important information. The hemostatic challenge varies with the type of tooth removed; molar extractions are usually the severest tests of hemostasis. Objective information can be obtained about the duration and extent of bleeding by asking about the need to reconsult the dentist for packing, suturing, or even transfusion of blood products. A deep injection given to achieve anesthesia of the lower jaw by nerve block constitutes another important hemostatic challenge; hemorrhage from such an injection that extends down into the neck is especially important to note, since it may compromise the airway.

Bleeding from Minor Cuts

In our society, shaving nicks are the most common minor cuts suffered, and patients with platelet disorders or von Willebrand

disease usually bleed excessively from them. If a patient uses an electric razor or a depilatory instead of a razor blade, it is worthwhile asking whether a razor blade was ever used and, if so, why the switch was made. Although it may be difficult to obtain objective information about bleeding after razor nicks, it may be helpful to ask whether the patient delays leaving home in the morning because of persistent oozing from these wounds or whether the patient leaves home with small pieces of tissue paper still attached to the bleeding wounds. It is also useful to instruct patients that direct pressure for 5 minutes is usually much more effective than tissue paper, since rebleeding is very common when the paper is removed. Patients with purpura secondary to amyloidosis (discussed later in this chapter) may paradoxically choose to switch from an electric razor to a blade razor because the pressure of the electric razor causes more purpura than razor nicks.

Hemoptysis

Hemoptysis is virtually never the presenting symptom of a bleeding disorder and is rare even with serious bleeding disorders. Thus, a comprehensive search for an anatomic abnormality or an underlying infectious or neoplastic disease is required, even if the patient has a systemic coagulopathy. Patients with bleeding diatheses may, however, have blood-tinged sputum in association with acute respiratory tract infections. Occasionally, a patient with an upper respiratory tract infection associated with a postnasal drip may also complain of hemoptysis, even though the true source of blood is in the upper airway.

Bleeding from Major Trauma

In the absence of previous surgery, the extent of bleeding in response to major trauma furnishes the most reliable information about future hemostatic risk. In order to assess the appropriateness of the bleeding, the details of the injury must be determined. The need for sutures and/or transfusions to stop the bleeding provides objective information. Excessive bleeding due to thrombocytopenia or platelet dysfunction tends to occur immediately, whereas excessive bleeding due to coagulation abnormalities may be delayed. It is especially important to know whether aspirin or other antiplatelet agents were taken at the time of injury to alleviate pain.

Hematemesis

Like hemoptysis, hematemesis is virtually never the presenting symptom of a hemostatic disorder, and thus a search for an anatomic basis is mandatory. Hemostatic defects may, however, contribute significantly to the problem, as in patients with liver disease and esophageal varices, or patients with gastritis secondary to aspirin ingestion.

Hematuria

Urinary tract bleeding is also virtually never the first symptom of a hemostatic disorder and thus a full investigation to define an anatomic defect is required. Hemostatic defects may, however, exacerbate hematuria caused by other disorders; thus, patients with urinary tract infections that might ordinarily produce microscopic hematuria, may have gross hematuria. Normal subjects may also develop gross hematuria with urinary tract infections. Because women are much more likely to contract such infections, it may explain why none of the normal men but 10% of the normal women complained of blood in their urine in the study by Wahlberg et al. (see Table 105-1).[2] Even among patients with platelet disorders and von Willebrand disease, however, only 8% of the men and 10% of the women complained of hematuria (see Table 105-1).[2]

Hematochezia

Hemorrhoids are the most common cause of hematochezia, but more serious, less common, causes need to be excluded. In Wahlberg et al.s study, 13% of normal men and 5% of normal women complained of blood in the stool (see Table 105-1). [2] Although a systemic coagulopathy may exacerbate hematochezia, one should not ascribe the bleeding to the coagulopathy itself without extensive evaluation. von Willebrand disease, platelet abnormalities, and both inherited and acquired forms of angiodysplasia may all be associated with severe recurrent episodes of hematochezia and often the search for discrete bleeding sites is frustrating and inconclusive. Associations between von Willebrand disease and both angiodysplasia and hereditary hemorrhagic telangiectasias have been reported, [11] but it is possible that having von Willebrand disease merely makes it more likely that the vascular disorder will be diagnosed because the hemorrhage is more severe.

Melena

It is important to make certain that the patient understands precisely what is meant by the term melena, because many patients will answer Yes to a question about black stools when, on further questioning, it is clear that their stools are really dark brown. The black rubber tubing of a stethoscope is a good visual prompt for making this clear. Objective evidence of gastrointestinal hemorrhage can be obtained by explicitly asking whether the patients stool ever tested positive for occult blood and whether the patient ever underwent endoscopy. Like other sources of gastrointestinal bleeding, melena is virtually never the presenting symptom of an inherited hemostatic defect. Recurrent episodes of melena, like hematochezia, do occur, however, in patients with serious hemorrhagic abnormalities or angiodysplasia and on occasion may even be lethal. Objective data on previous hospitalizations, the results of endoscopic studies, and the need for blood replacement should also be obtained.

Central Nervous System Bleeding

Severe thrombocytopenia (<5,000 platelets/l) is associated with central nervous system hemorrhage, with both diffuse petechial lesions and gross hemorrhagic strokes. Hemophilia is also associated with spontaneous central nervous system hemorrhage, and it greatly increases the risk of serious hemorrhagic stroke with even minimal head trauma. It is of interest that spontaneous central nervous system hemorrhage is exceedingly rare in Glanzmann thrombasthenia. [12]

Venipuncture Site Bleeding

Patients with diffuse intravascular coagulation, hyperfibrinolysis, thrombocytopenia, or qualitative platelet disorders characteristically bleed for a long time after venipunctures, whereas patients with coagulation disorders do not. Delayed bleeding, however, may occur in the latter group.

Ophthalmologic Bleeding

Subconjunctival hemorrhages are associated with both platelet and coagulation abnormalities, especially in children when crying leads to increased venous pressure. Severe thrombocytopenia may lead to retinal hemorrhage if not effectively treated for several days to weeks. Orbital hemorrhage is more commonly associated with hemophilia than with platelet disorders.

Menses

Menorrhagia has been defined as loss of more than 80 ml of blood per cycle. [13] [14] Assessing the severity of menstrual flow

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based on the patients subjective estimation or the number of sanitary napkins or tampons used is generally unreliable because women vary greatly in their perception of excessive bleeding and their hygienic practices. [15] It is usually more helpful to establish the number of days of heavy flow and the total number of days for an average menstrual period; if the former is greater than 3 and/or the latter is greater than 6 or 7, it is likely that the menstrual bleeding is excessive. It may also help to ascertain whether the bleeding is heavy enough to require especially large sanitary napkins or to require curtailment of ordinary activities. Objective data would include whether a physician (1) prescribed birth control pills to control the bleeding, (2) told the patient she was anemic, (3) prescribed iron, (4) performed a dilation and curettage (D&C) to assess the bleeding, (5) was forced by circumstances to perform an emergency hysterectomy to secure hemostasis, and/or (6) performed elective hysterectomy or radiation sterilization of the uterus as preventive measures. When menorrhagia is confirmed by direct measurement of menstrual blood loss, it is a valuable predictor of hemostatic disorders; in one study, six of 30 women with confirmed menorrhagia were thought to have mild von Willebrand disease, one

had a low value of factor X, two had elevated D-dimer levels, and five others had prolonged bleeding times thought to reflect platelet disorders. [13]

Childbirth

A detailed history of bleeding with each pregnancy should be obtained, including objective data regarding the need for transfusions, D& C, iron therapy, and/or hysterectomy. It is useful to ask specifically whether the patients doctor commented on the bleeding being excessive at delivery, even if none of the objective criteria were met. A history of recurrent spontaneous abortion may be part of the antiphospholipid antibody syndrome (discussed later in this chapter), which may include a lupus-like anticoagulant, anticardiolipin antibodies, and/or a false-positive serologic test for syphilis. The suspicion of this syndrome should be even greater if the recurrent spontaneous abortions occur after the first trimester. Recurrent spontaneous abortions have also been reported in association with abnormalities of fibrinogen,[16] presumably due to abnormal stability of placental attachment.

Hemarthroses

Joint bleeding is the hallmark of the hemophilias and is extremely rare in all other hemostatic defects except severe von Willebrand disease. Because joint bleeding is usually not associated with discoloration, patients may not understand that their symptoms are caused by hemorrhage. It is important, therefore, to inquire specifically about pain, swelling, and limitation of motion rather than merely asking about bleeding into the joints.

Operations

The details of each surgical procedure should be recorded, including any statements made by the surgeon about the extent of bleeding. In general, excessive bleeding resulting from coagulation abnormalities may be delayed in time for hours to a day or so, whereas excessive bleeding due to platelet disorders or thrombocytopenia usually occurs immediately. Emphasis should be placed on ascertaining whether any blood products were administered. Specific questioning about tonsillectomy and appendectomy may be required because some patients forget about these operations, especially if they were performed many years before. The hospital records should be secured because they may contain important clinical and laboratory data that the patient never knew or forgot. When hospital records are unavailable, it may help to ask how long the patient was hospitalized, since delayed discharge may have been due to excessive bleeding.

Circumcision

Congenital bleeding disorders, in particular the hemophilias, may cause excessive bleeding at circumcision as their first manifestation. Delayed bleeding from the umbilical cord stump or after circumcision is said to be particularly suggestive of factor XIII deficiency, but factor VIII or factor IX deficiency can also produce these symptoms.

Telangiectasias

Patients may manifest a wide range of telangiectatic lesions, ranging from pinpoint erythematous dots that blanch when compressed, to classic cherry angiomata ranging in size to up to several centimeters (see Differential Diagnosis). The vast majority of the otherwise normal population will demonstrate an increase in skin telangiectasias with aging associated with the development of papular cherry angiomata. The latter may have a distinctive blue appearance if present in the deeper layers of the skin. Patients with hereditary hemorrhagic telangiectasias usually have progressively more severe disease as they age. In fact, it is not at all clear what constitutes the minimal criteria for hereditary hemorrhagic telangiectasias; some otherwise normal persons with no clinical manifestations in their early years may develop easy bruising in association with skin telangiectasias in their later years. The classic hallmarks of hereditary hemorrhagic telangiectasias include epistaxis and tongue telangiectasias, but lesions may be present in virtually every organ, manifesting as space-occupying lesions, sources of bleeding, or sources of arteriovenous shunting. On physical examination, the lesions may be much more subtle than the florid examples found in most textbooks; a very careful search of the integument is necessary, focusing on the face, chest, shoulders, legs, and under the nails. Lesions are also commonly found on the vermilion border of the lips and under the tongue, even when the tip of the tongue is not involved. It is important to distinguish the lesions of hereditary hemorrhagic telangiectasias from the spider telangiectasias associated with liver disease. The latter have a more splotchy appearance, are concentrated on the shoulders, chest, and face, and have a more serpiginous quality.

Connective Tissue

If Ehlers-Danlos syndrome is considered in the differential diagnosis, it is useful to inquire specifically as to whether the patient was double-jointed as a child or had unusually distensible skin. More obvious abnormalities such as lens dislocations should be apparent. Questions regarding common skin manifestations of Cushing syndrome should also be posed, including rounded faces, purple striae, truncal obesity, or fat deposition in the back of the neck. Old photographs of the patient may be extremely helpful in deciding whether facial changes are new. The full differential diagnosis of disorders affecting the integrity of the blood vessel and supporting tissues is discussed in the following section.

Wound Healing

Although abnormal wound healing is not a common problem in hemostatic disorders, defects have been reported in association with factor XIII deficiency and fibrinogen abnormalities. Patients with Ehlers-Danlos syndrome and Cushing disease also have abnormal wound healing.

Medications

The dosage of each prescription and nonprescription drug taken by the patient should be recorded. Specific questions should cover aspirin and other antiplatelet agents, birth control pills, vitamins, and food supplements, because these may not be viewed as medications by the patient. Iron therapy in the past should also be noted since it may provide information on previous episodes of anemia due to blood loss.

Family History

A pedigree going back at least one or two generations should be recorded, with emphasis on hemorrhagic and/or thrombotic manifestations for each member. A specific question regarding parental consanguinity should be included since patients may not realize that this is important information. Details about the cause of death for each deceased individual should also be obtained.

Summary

Obtaining the details of the bleeding history requires insight into the mechanisms and manifestations of the different hemorrhagic disorders as well as an understanding of the patients perceptions of symptoms. Whenever possible, objective data should be obtained to provide a more comprehensive and credible picture. Although obtaining a complete bleeding history may seem tedious, it is well worth the effort, since the patients previous responses to hemostatic challenges are much better predictors of the patients likelihood of bleeding excessively in the future than are the patients routine laboratory values. In fact, in one study, routine screening assays of coagulation and platelets failed to detect any clinically significant abnormalities in more than 100 preoperative patients with normal bleeding histories. [9] Although the clinical value of routine laboratory tests for screening for coagulation abnormalities is still in dispute, this study certainly highlights the central role of the bleeding history in evaluating hemostatic risk.

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DIFFERENTIAL DIAGNOSIS OF HEMORRHAGIC DERMATOLOGIC LESIONS

Skin hemorrhage is defined as the indiscriminate extravasation of blood cells out of the vasculature and into the skin, subcutaneous tissue, or both. The amount of blood leaking from the vessel determines the size of the lesion, with minute amounts producing pinpoint red lesions <2 mm in size (petechiae), and larger amounts producing purpuric lesions (2 mm-1 cm) or frank ecchymoses (>1 cm).[10] Despite these precise definitions, conventional usage often groups purpuric lesions and ecchymoses under the term *purpura*, and this general group of disorders, including petechiae, is often referred to as the purpuras. All these lesions can be readily differentiated from simple erythema and telangiectasias, in which the blood remains confined within the vasculature, because these latter lesions will blanch if direct pressure is applied to them. This can be easily demonstrated using a glass slide or the tip of a ballpoint pen with the penpoint in the retracted position. True purpura may demonstrate partial blanching with these maneuvers, but a nonblanchable component will remain. The color of the lesion depends on the size and location of the hemorrhage, as well as the time since the extravasation occurred. Initially, superficial lesions are bright red or deep red, and deeper lesions have a more purple appearance. With time, the lesions evolve into deep purple, brown, orange, or blue-green discolorations.

The general mechanisms by which extravasation of blood from the vasculature can occur are depicted schematically in Figure 105-1 . The integrity of the blood vessel depends on (1) the competence of the hemostatic mechanism to combat the basal level of ongoing vascular trauma, (2) the strength of the blood vessel itself and its surrounding tissues, and (3) the transmural pressure gradient tending to drive blood out of the vessel. Even if all these systems are functioning normally, however, serious trauma of diverse etiologies may be sufficient to cause hemorrhagic extravasation.

A classification of disorders producing skin hemorrhage is given in Table 105-4 .[16] [17] It is organized primarily according to etiology, but a division is made between lesions that are palpable and those that are not palpable because palpability can be readily determined at the bedside and thus has important practical significance in developing a differential diagnosis. Although the specific mechanism(s) producing palpability is still poorly understood, one hypothesis is that a lesion becomes palpable when a generalized increase in vascular permeability secondary to an inflammatory process results in marked extravasation of plasma proteins with the development of extravascular coagulation leading to fibrin deposition. Support for such a mechanism comes from studies showing that the palpable induration accompanying delayed hypersensitivity reactions can be diminished by administration of oral anticoagulants.[18] Alternatively, palpability may be secondary to extensive cellular infiltration as in certain inflammatory or malignant disorders.

Purpuric lesions secondary to hemostatic defects were described in the previous section. Among the nonhemostatic defects, we will first consider those producing nonpalpable lesions.

Nonpalpable Purpura

Increased Transmural Pressure

Acute

The clinical picture of minute petechiae of the face, especially the eyelids (Plate 105-1), neck, and upper chest may be seen after prolonged Valsalva maneuvers, coughing, vomiting, childbirth, or weight lifting.[19] [20] A similar syndrome can be found in newborns with umbilical cord strangulation. Lesions on the lower extremities, especially in the elderly, may be caused by acute venous stasis due to tight clothing or stockings. Occasionally, such dependent purpura may be palpable (Plate 105-2), even in the absence of microscopic inflammation.

Chronic

Chronic venous stasis of the lower extremity, resulting from either venous valvular incompetence or chronic use of tight-fitting garments, can convert subclinical insults of diverse etiologies into frank purpura. Thus, it is common, but not universal, for the first signs of petechiae resulting from hemostatic defects to appear at the ankles. Moreover, other common cutaneous disorders, such as drug rashes, contact dermatitis,[21] and sunburn often progress to become petechial and purpuric over the lower extremities after the first 24 hours. Chronic venous stasis accompanied by recurrent episodes of extravasation of red blood cells leads to the development of purpuric and yellow brown macules, with the latter resulting from the persistent presence of hemosiderin.

High Altitude

An increase in cutaneous petechiae has been noted in mountain climbers ascending higher than 3,800 m above sea level.[22] In this case, the mechanism for purpura may be an increase in transmural pressure due to reduced extravascular pressure rather than increased intravascular pressure.

Figure 105-1 Mechanism of nonpalpable purpura.

Decreased Mechanical Integrity of Microcirculation and Supporting Tissue

Senile Purpura

Chronic solar damage and decreased collagen, elastin, and ground substance resulting from aging may result in characteristic red to purple purpuric patches on the exterior surfaces of the forearms and hands (Plate 105-3). The skin accompanying solar purpura is particularly thin and lacks elasticity, making it highly susceptible to tears induced by shearing forces.[23] Owing to the decreased healing capacity that accompanies aging, the purpuric changes may take months to resolve. Interestingly, the syndromes of premature aging such as progeria, Werner syndrome, and acrogeria may all give rise to acral purpuric changes identical to those of senile purpura.[24]

Corticosteroid Excess

The patches of purpura in Cushing syndrome are classically described as appearing on the extensor surfaces of the forearms, but they may appear on both the flexor

and extensor aspects of both the upper and lower extremities. As with senile purpura, the lesions have a very characteristic bright red appearance, and the skin is fragile.[10] Shear stress is often the immediate cause of the purpura, and the patches may last for weeks to months. The use of potent fluorinated topical corticosteroids, either alone or with occlusive dressings, may result in cutaneous atrophy and purpura. Microscopically, there is a loss of dermal connective tissue with thinning of the epidermis.

Vitamin C Deficiency (Scurvy)

Follicular keratosis and perifollicular purpura with entrapped corkscrew hairs are the characteristic findings of vitamin C deficiency (Plate 105-4). Larger ecchymoses on the legs and mucous membranes are seen in more severe cases and may be produced by mild or inapparent trauma. Petechial lesions and hemorrhagic gingivitis or stomatitis may also occur.

Ehlers-Danlos Syndrome

Easy bruising is one of the most prominent features of Ehlers-Danlos syndrome types IV and V, but bruising may also be seen in the more common types I to III. Milder forms of Ehlers-Danlos syndrome that do not meet the diagnostic criteria for the more classic forms have been described, and mild to moderate bruising may be seen in these patients.[25] In evaluating patients for this heterogenous group of connective tissue disorders, it is important to assess the elasticity of the skin, the extensibility of the joints, and the presence of associated abnormalities such as high-arched palate and pectus excavatum.

Amyloid Infiltration of Blood Vessels

Cutaneous manifestations may be prominent in primary systemic amyloidosis associated with either mild plasmacytosis or multiple myeloma. Histologic examination demonstrates extensive infiltration of blood vessel walls with amyloid, resulting in increased vascular fragility. As a result, minimal trauma can produce hemorrhagic lesions ("pinch purpura"), and petechiae occur readily when there is an increase in transmural pressure

TABLE 105-4 -- Differential Diagnosis of Purpura

True purpura
I. Hemostatic defects A. Platelet abnormalities 1. Quantitative 2. Qualitative B. Coagulation abnormalities
II. Nonhemostatic defects
A. Nonpalpable purpura
1. Increased transmural pressure gradient a. Acute (e.g., Valsalva, coughing, vomiting, childbirth, weight lifting) b. Chronicvenous stasis c. High altitude
2. Decreased mechanical integrity of microcirculation and supporting tissue
a. Age-relatedsenile purpura b. Corticosteroid excessCushing syndrome, topical corticosteroids c. Vitamin C deficiencyscurvy d. Abnormal connective tissueEhlers-Danlos syndrome e. Amyloid infiltration of blood vesels[a] f. Hormonalfemale easy bruising syndrome (purpura simplex) g. MELAS syndrome
3. Trauma to blood vessels
a. Physical 1) Injuries 2) Child abuse 3) Factitial purpura b. Ultraviolet radiation 1) Purpuric sunburn 2) Solar purpura c. Infectious 1) Bacterial 2) Rickettsial 3) Fungal 4) Viral 5) Parasitic d. Embolic 1) Infectious organisms[a] 2) Atheroemboli (cholesterol crystal emboli) 3) Fat emboli 4) Calciphylaxis e. Allergic and/or inflammatory 1) Serum sickness 2) Contact dermatitis 3) Pigmented purpuric eruptions f. Pyoderma gangrenosum g. Neoplastic[a] h. Drug-related i. Thrombotic 1) Disseminated intravascular coagulation 2) Coumarin necrosis 3) Heparin necrosis 4) Protein C or protein S deficiency 5) Purpura fulminans 6) Paroxysmal nocturnal hemoglobinuria[a] 7) Antiphospholipid syndrome j.Drug-related
4. Unknown causepsychogenic purpura

B.Palpable purpura
1. Dysproteinemias
a. Cryoglobulinemia
b. Cryofibrinogenemia
c. Hyperglobulinemic purpura of Waldenström
2. Cutaneous vasculitis
a. Systemic vasculitides[b]
b. Paraneoplastic vasculitis
c. Henoch-Schönlein purpura
d. Acute hemorrhagic edema of infancy
3. Primary cutaneous diseases

Nonpurpuric disorders simulating purpura
I. Disorders with telangiectasias
A. Cherry angiomata
B. Hereditary hemorrhagic telangiectasia
C. CREST syndrome
D. Chronic actinic telangiectasia
E. Chronic liver disease
F. Pregnancy-related
G. Ataxia-telangiectasia
H. Other

II. Kaposi sarcoma and other vascular sarcomas[c]
III. Fabry disease
IV. Neonatal extramedullary hematopoiesis
V. Angioma serpiginosum

CREST, calcinosis, Raynaud (phenomenon), esophageal (dysfunction), sclerodactyly, telangiectasia.

[a]May also have a papable purpuric component.
[b]May also have a nonpalpable purpuric component.
[c]May also have a purpuric component, either nonpalpable or palpable.

(e.g., after a Valsalva maneuver or after proctoscopy), especially when the amyloidosis involves the eyelids and face. Many different cutaneous lesions can occur, including brown- to tan-colored translucent papules, plaques, nodules, and bullae; these may all become hemorrhagic, either spontaneously or with minimal trauma. An enlarged tongue with peripheral indentations secondary to pressure from the adjacent teeth (scalloped tongue) is also frequently seen in patients demonstrating cutaneous amyloid lesions, whereas alopecia and nail changes are less common manifestations.

Colloid Milium

Colloid milium is a cutaneous disorder characterized clinically by translucent yellow papules and plaques, and histologically by the deposition of upper dermal amorphous eosinophilic material (similar in appearance to amyloid). Lesions of colloid milium may also become purpuric after minimal trauma. [26]

Female Easy Bruising Syndrome

The female predominance and the frequent association with phases of the menstrual cycle suggest that the female easy bruising syndrome (purpura simplex) is caused by hormonal effects on the blood vessel and/or its surrounding tissues. [10] Concomitant use of nonsteroidal anti-inflammatory drugs (NSAIDs) may inhibit platelet function and contribute to the severity of the symptoms. Patients complain of frequent purpuric and ecchymotic lesions with minimal trauma. As discussed in the section on history taking, patients with this entity do not appear to be at increased risk of hemorrhage from more severe hemostatic challenges such as surgery.

Administration of Lorenzos Oil

Purpura from a defect in vessel wall function has been seen in a patient receiving Lorenzos oil (glycerol trioleate and glycerol trierucate). [27]

Trauma to Blood Vessels

Physical

Any form of injury, if severe enough, can damage blood vessels sufficiently to cause skin hemorrhage. It is thus important to

know in detail the extent of the injury before deciding whether the skin hemorrhage is consistent with the magnitude of the trauma. [19] Traumatic lesions usually have well-defined margins. Depending on the etiology (e.g., occupational, accidental, recreational), the pattern may be annular or circumferential (e.g., baseball injury), linear or loop-shaped (e.g., child beating), or subungal (e.g., running shoe injury) (Fig. 105-2). The lesions associated with child abuse often include both cutaneous purpura and petechiae of the bulbar and palpebral conjunctivae, the latter reflecting strangulation, smothering, or both. [28] [29] Patients with factitial, or self-inflicted, purpura usually have medium- to large-sized lesions on the lower extremities, but other sites may be involved as well. They characteristically express indifference to the bruises. Purpura also occurs after use of the 585 nm pulsed dye laser. [30]

Ultraviolet Radiation

Acute, severe sunburn can have a petechial component if the damage is severe enough. Patients have been described who developed petechial eruptions on the legs and trunk after only brief exposures to natural sunlight (solar purpura). It was hypothesized that their skin is particularly sensitive to long-wave ultraviolet light. [31] [32]

Infections

Bacterial, viral, rickettsial, protozoal, and parasitic infections may all produce purpura as a primary clinical manifestation. [33] [34] The pathogenesis of infectious purpura is often complex, and may include direct vascular invasion by the organism, diffuse intravascular coagulation, immune complex vasculitis, septic emboli, and/or direct toxic effects on the vasculature. Although characteristic patterns of purpura have been described for the different agents, overlap between the patterns is quite common.

One particular pattern is papular-purpuric gloves and socks syndrome. This disorder may be seen in association with parvovirus B19, measles virus, [35] cytomegalovirus, or Coxsackie B6 infections. [36]

Gram-negative sepsis with *Pseudomonas* sp., *Klebsiella* sp., or *Escherichia coli* produces characteristic lesions of ecthyma gangrenosum, which begin as plaquelike areas of edema and erythema with subsequent nodule formation surmounted by irregular purpura (Plate 105-5). The central area of purpura is often bullous, and is surrounded by concentric areas of normal skin and a thin band of erythema. Erosions and ulceration may occur. Lesions may be single or multiple, and the palms and

soles may occasionally be involved.

Meningococcemia initially produces erythematous papules, but these soon evolve into stellate purple to slate-gray purpuric lesions (Plate 105-6). The combination of purulent meningitis and petechiae strongly suggests that *Neisseria meningitides* is the etiologic agent. [37] Acrocyanosis and symmetric peripheral gangrene

Figure 105-2 "Tennis toe" subungual hemorrhage.

may ensue and are thought to be due to disseminated intravascular coagulation (DIC). The purpura of meningococcemia may be due to direct vascular invasion by the organism or an endotoxin-induced Shwartzman reaction. In chronic intermittent meningococcemia an immune complex dermatitis may develop, characterized by hemorrhagic papulovesicles over the joints.

The classic rash of scarlet fever characteristically features linear purpuric lines in the skin folds (Pastias lines). Streptococcal pharyngitis has been reported to produce perioral, neck, and truncal petechiae in 2% of patients.

Bacterial sepsis, including acute and subacute bacterial endocarditis due to gram-positive or gram-negative organisms, may cause purpuric macules, papules, hemorrhagic bullae, erosions, and/or ulcers (Plate 105-7). The first manifestations of pneumococcal sepsis in asplenic patients may be facial petechiae and purpura, accompanied by acral cyanosis or livedo reticularis. [38] [39] Although splinter hemorrhages of the nails occur in subacute bacterial endocarditis, they may also be seen in normal individuals following trauma, or in patients with trichinosis, peptic ulceration, hypertension, malignancies, severe rheumatoid arthritis, and a number of dermatologic conditions; thus, the specificity of splinter hemorrhages as a sign of endocarditis is quite limited. [40] [41]

Rickettsial infections, including Rocky Mountain spotted fever (Plate 105-8) and epidemic typhus, typically produce an array of cutaneous changes ranging from urticarial macules to petechiae, ecchymoses, and areas of hemorrhagic necrosis. Although the characteristic lesion of Lyme borreliosis is a non-purpuric annular expanding plaque (erythema migrans), the central aspect of this lesion may contain a purpuric macule, papule, or a hemorrhagic bulla.

Patients with either disseminated fungal infections (e.g., cryptococcoses, zygomycosis, candidemia, alternariosis, histoplasmosis) [42] [43] or locally invasive fungal diseases (e.g., mucomycosis) may have necrosis, purpura, and/or petechiae early in the course of the illness. [44] Viral infections may have primary purpuric eruptions as their presenting manifestations. Human parvovirus B19 infection has been described as demonstrating a petechial or confluent purpuric rash on the buttocks, axilla, and/or chest. [45]

Purpura may be the initial manifestation of parasitic infections, especially in the immunocompromised host. Migration of filariform larvae of *Strongyloides stercoralis* typically produces rapidly progressive abdominal thumbprint linear and reticulated purpura on a background of petechiae; the periumbilical region is usually most severely affected. [3] [46] Disseminated *Pneumoncystis carinii* infections in patients with AIDS may demonstrate purpuric papules and nodules that resemble the lesions of Kaposi sarcoma (discussed later in this chapter). [47]

Embolic

Atheroemboli with prominent cholesterol crystals, usually originating from atherosclerotic lesions in the aorta, produce a constellation of cutaneous findings, including acral petechiae and purpura, livedo reticularis, nodules, unilateral peripheral ulcers, and bilateral cyanosis and gangrene. [48] Distal pulses are present and occasionally the emboli can actually be seen in the retinal circulation as refractile interruptions in the column of arterial blood. The syndrome is seen most frequently in older men on anticoagulation or after vascular repair procedures. A high predilection for the pancreas makes elevations of serum amylase a common accompanying laboratory finding. Since this syndrome is often undiagnosed, it is important to have a high index of suspicion, especially in older men with livedo reticularis.

Fat embolism may occur 2 to 3 days after severe trauma. The initial findings include upper extremity, thoracic, and/or conjunctival

petechiae. The full syndrome consists of hyperthermia, respiratory distress, retinal fat emboli, neurologic symptoms, and pulmonary infiltrates. [49] [50]

Allergic and/or Inflammatory

In serum sickness, morbilliform or urticarial eruptions are the most common manifestations. Linear or serpiginous bands of erythema along the sides of the hands and feet may be seen at the margins of the palmar or plantar surfaces. [45] If the patients are thrombocytopenic, purpura usually appears within these linear bands. The eruption often heralds the onset of the syndrome. Immunologic evaluation of biopsy material often demonstrates immunoglobulin and complement deposits.

The pigmented purpuric eruptions, [47] [51] [52] [53] including Schamberg disease, Majocchi disease, and others, are a poorly understood group of disorders characterized by petechiae and purpura on a background of light to dark brown or orange hyperpigmentation (Plates 105-9 , 105-10 , and 105-11); telangiectasias may or may not be present. Occasionally scaling, lichenification, and atrophy are seen. These eruptions characteristically involve the lower extremities, but they may also be seen on either the arms or trunk, or both; on occasion, they may even be found on the palms and soles. [54] They are not associated with any systemic manifestations. There is a great deal of clinical overlap between these disorders, and an individual patient may demonstrate features of more than one pigmented purpuric eruption. Histologically, extravasation of red blood cells, hemosiderin deposits within macrophages, and a perivascular lymphohistiocytic infiltrate with endothelial cell swelling are seen. The pathogenesis of these disorders is not established, but suggested mechanisms include increased capillary fragility with rupture of capillaries in the papillary dermis, aneurysmal dilation of the microvasculature, and abnormal cellular immune responses to an unknown antigen. [55] Recently, cutaneous T-cell lymphoma was reported to produce lesions simulating those found with the pigmented purpuric eruptions [56] and the differential diagnosis of these disorders should include this possibility.

Pyoderma Gangrenosum

Pyoderma gangrenosum is a destructive, necrotizing ulceration of the skin presenting as a nodule, pustule, or hemorrhagic bulla. The lesions occur on the calves, thighs, buttocks, and face. Pyoderma gangrenosum occurs in association with inflammatory bowel disease, rheumatoid arthritis, gammopathies, blood dyscrasias, leukemia, and lymphoma. A superficial hemorrhagic bullous form occurs with acute leukemia or other myeloproliferative disorders.

Contact Dermatitis

Allergic or irritant contact dermatitis to clothing, rubber, woolen garments, elastic, benzoyl peroxide, dyes, balsam of Peru, [57] Emla[58] or detergent whiteners may result in purpuric eruptions that may simulate the pigmented purpuric eruptions. [59]

Neoplastic

Infiltration of the skin in the histiocytosis X group of disorders can result in the development of a papular and crusted dermatitis of the scalp and intertriginous areas (Plates 10512 and 10513). These lesions can have both petechial and purpuric features. As indicated previously, cutaneous T-cell lymphoma may produce cutaneous lesions similar to those seen in the pigmented purpuric eruptions. Similarly, skin infiltrations in patients with leukemias, lymphomas, and plasma cell disorders can produce red to purple papules that may simulate purpura. [60]

Drug-Related

Petechial and purpuric reactions can be observed after administration of a variety of drugs, including aspirin, alclofenac, allopurinol, atropine, belladonna, bismuth, carbamazepine, carbimazol, carbromal, chloral hydrate, chlordiazepoxide, cimetidine, desipramine, disopytramide, doxepin, fenbufen, gold salts, indomethacin, iodides, isoniazide, meclofenamate sodium, mefenamic acid, menthol, mercury, morphine, naproxen, nitrofurantoin, penicillamine, penicillin, phenacetin, phenytoin, piperazine, piroxicam, pyrazolon derivatives, quinine, quinidine, sulfonamides, sulindac, thiouracils, and tolmetin. [61] The mechanism for at least some of these reactions is presumed to be allergic hypersensitivity.

Metabolic

Relapsing diffuse petechiae on the trunk and extremities were observed in four infants with ethylmalonic aciduria, a metabolic disorder associated with acral cyanosis, chronic diarrhea, and progressive central nervous system dysfunction. [62]

Thrombotic

Diffuse (disseminated) intravascular coagulation (DIC) may result from a variety of different insults, many of which have the common denominator of producing hypotension (see Chap. 117). Because endothelial cells are active metabolically, hypotension can produce widespread ischemia and endothelial cell damage. This damage can expose subendothelial surfaces, which in turn can initiate an uncontrolled thrombotic response that ultimately selectively depletes coagulation factors and platelets. Alternative mechanisms for initiating the process involve the effects of endotoxin and other mediators such as interleukin 1 (IL-1) and tumor necrosis factor (TNF) on increasing endothelial cell and monocyte procoagulant activity. This is achieved, at least in part, by an induction of tissue factor expression, an increase in tissue plasminogen activator inhibitor-1 (PAI-1) synthesis, and a decrease in the production of thrombomodulin and tissue plasminogen activator. [63] [64] Because there is a potential for both thrombotic and hemorrhagic manifestations, it is not surprising that the skin manifestations are diverse. The most common skin findings of diffuse intravascular coagulation are acral cyanosis (Plate 105-14) with variably associated petechial, purpuric, and ecchymotic lesions (Fig. 105-3). The competence of the fibrinolytic system to digest deposited fibrin determines the extent of tissue compromise. In the most severe cases, hemorrhagic gangrene of fingers and toes can occur. [33] The presence of peripheral gangrene may be an important consideration in the often difficult decision as to whether heparin is indicated in treating the syndrome.

Coumarin Necrosis

This process affects 0.010.1% of all patients receiving coumarin and appears from day 2 to day 14 (usually days 36) of coumarin therapy. [65] Patients who are deficient in protein C appear to be at high risk because the disorder is thought to result from a temporary imbalance in the reduction of the procoagulant vitamin K factors and the anticoagulant vitamin K factors. [66] [67] [68] [69] Coumarin necrosis begins suddenly as painful erythematous patches (Plate 105-15) that become edematous and rapidly progress to irregularly hemorrhagic and necrotic plaques, nodules, and bullae; eventually large tumid indurations and infarcts occur [70] with eschar formation and sloughing (Plate 105-16). [71] This syndrome appears to be more common in women than men and the lesions often develop in the skin overlying fatty areas, such as the buttocks, thighs, and breasts. Men can also be affected, and more acral lesions can occur, as witnessed by the reports of penile involvement. [65] Lesions may be symmetric and widely distributed; on occasion, they may be severe enough to require surgical intervention. [72] Histologically, fibrin and platelet thrombi are observed in the dermal and subcutaneous vasculature. In one study, TNF was identified in the lesions and endothelial cell adhesion molecules were upregulated. [73] The lesions

Figure 105-3 Disseminated intravascular coagulation.

of coumarin necrosis can be differentiated from hemorrhagic lesions caused by excessive coumarin administration by the presence of the nearly black eschar in the center of the necrotic zone and by histologic examination. Ancillary differentiating points in favor of coumarin necrosis include the female sex predilection, the absence of an excessively prolonged prothrombin time, and the relationship to the onset of therapy. The management of this disorder and its prevention are discussed in Chapter 121 .

Protein C or Protein S Deficiency

Congenital, homozygous protein C deficiency has been reported to produce diffuse purpuric and ecchymotic skin lesions suggestive of chronic DIC and purpura fulminans. Widespread venous thrombosis usually accompanies the skin lesions. Fortunately, both the skin lesions and venous thrombosis respond rapidly to therapy with plasma. Long-term therapy may require the careful introduction of oral anticoagulants. [74] Replacement therapy with protein C concentrate has been successful. [75] Cutaneous necrosis similar to coumarin necrosis has also been reported in a patient with acquired protein C deficiency due to the development of an immunoglobulin inhibitor. [76] Post-infectious purpura fulminans has also been reported in association with production of anti-protein S IgG and IgM antibodies. [77] [78]

Purpura Fulminans

Purpura fulminans defines a syndrome of DIC manifested by massive widespread ecchymoses, especially on the upper and lower extremities, abdomen, thighs, and buttocks (Plate 105-17). [79] [80] It may be triggered by any of the infectious agents discussed previously [33] [81] or may occur without antecedent infections. Homozygous protein C deficiency can produce essentially the same pattern in the neonate. [69] [82] In addition, resistance to activated protein C caused by a single point mutation of factor V (factor V Leiden) has been shown to result in neonatal purpura fulminans. [83] Clinically, the syndrome of purpura fulminans is most often characterized by symmetric, hemorrhagic necrosis and bulla formation. The lesions enlarge with time and progress to gangrene, often with autoamputation of the digits. Associated symptoms and signs include prostration, fever, and edema of the extremities; death is not uncommon. Histopathology reveals hemorrhagic necrosis of the dermis with thromboses of the capillaries and small blood vessels. Fibrinoid necrosis of vessel walls and perivascular granulocytic infiltrates have been described.

Paroxysmal Nocturnal Hemoglobinuria

Occasional patients with paroxysmal noctural hemoglobinuria develop erythematous patches with dusky centers that may enlarge to form painful plaques of erythema with central necrosis. [84] Hemorrhagic bullae, ulcerations, petechiae, ecchymoses, palpable purpura, and eschar formation may also develop. Histologically, intravascular thrombi are seen in the absence of vasculitis.

Phospholipid Antibody Syndrome

The phospholipid antibody syndrome is characterized by the variable presence of antibodies to phospholipid-protein complexes [including cardiolipin-containing complexes and those used in coagulation assays (lupus anticoagulant)], in association with a tendency to thrombosis, thrombocytopenia, and fetal wastage (see Chaps. 120 and 129). Cutaneous manifestations are present in only a minority of patients, but they may include widespread cutaneous necrosis with thrombi within the microvasculature (similar to that seen in purpura fulminans), livedo reticularis, leg ulcers, painful skin nodules, subungual splinter hemorrhages, and acral red-to-purple macules. [85]

Unknown Origin

Psychogenic Purpura

Psychogenic purpura is the term given to a poorly understood disorder of severe, recurrent ecchymoses affecting women almost exclusively (Plate 105-18). [86] [87] [88] [89] Patients usually experience pain before or at the time the bruise appears, and the bruising is often so extensive that the patient loses the use of the limb during the healing process. In the most severe cases, the patient can become bedridden. In desperation, physicians sometimes cast the affected limb(s), and this usually succeeds in facilitating healing, but at the price of muscle atrophy. Attempts to identify the etiology have been unsuccessful. Early studies implicated allergic reactions

to blood cells or DNA, but these hypotheses are no longer fully accepted. Localized neurogenic release of fibrinolytic activity in the skin has been proposed as a mechanism to link the psychological and dermal components of the syndrome.[90] Psychological profiles of patients with this disorder have demonstrated widespread abnormalities,[87] [91] raising the possibility of self-inflicted trauma, a view supported by the healing that usually accompanies casting. It is far from certain,

however, that this is the cause in all cases, so it is best to continue to classify the etiology as unknown.

Palpable Purpura

Dysproteinemias

Cryoglobulinemia

Cryoglobulins represent cold-precipitable proteins found in plasma or serum. Single-component cryoglobulins may be IgG, IgM, or IgA. [92] These cryoproteins may be idiopathic in origin or may occur with Waldenström macroglobulinemia, myeloma, lymphoma, or benign paraproteinemias. Mixed cryoglobulins are usually composed of a rheumatoid factor IgM complexed with a monoclonal or polyclonal IgG; less frequently, IgG or IgA antibodies may have anti-IgG activity. Mixed cryoglobulins may be seen as an idiopathic phenomenon or in association with a wide variety of subacute and chronic disorders, most particularly hepatitis C. The immune complex is composed of hepatitis C virus (HCV), anti-HCV, and IgA-type rheumatoid factor.[93] Clinical lesions of cryoglobulinemia include the intermittent appearance of acral hemorrhagic necrosis (Fig. 105-4), palpable purpura, livedo reticularis, subungual hemorrhage (Plate 105-19), urticaria, leg ulcerations, Raynaud phenomenon, and erythema multiforme-like lesions.[94] [95] [96] Weakness and arthralgias are prominent noncutaneous symptoms. Monoclonal cryoglobulins may crystallize and result in livedo reticularis with purpuric necrosis, destructive arthropathy, and malignant hypertension (cryocrystalglobulinemia). [97] [98]

Cryofibrinogenemia indicates the presence in the blood of an abnormal cold-precipitable protein indistinguishable from fibrinogen or fibrin. Cutaneous manifestations include sensitivity to cold, purpura, livedo reticularis, cyanosis, ulcerations, erythema, hematoma, urticaria, gangrene, acral blisters, and Raynaud phenomenon. [99] Essential (primary) cryofibrinogenemia occurs in association with DIC. Secondary cryofibrinogenemia may occur with neoplastic, thromboembolic, or infectious disorders.

Hyperglobulinemic Purpura of Waldenström

Macular and papular, discrete or confluent purpuric lesions with hemosiderin staining are the classic cutaneous findings of benign hyperglobulinemic purpura (Plate 105-20), a syndrome that most often occurs on the legs of women between 20 and 40 years of age.[53] [100] Precipitating factors include increased hydrostatic pressure, hyperviscosity, and low temperatures.[101] [102] The polyclonal increase in globulins (mostly IgG 1) can be associated with Sjögren syndrome, systemic lupus erythematosus

Figure 105-4 Cryoglobulins.

(SLE), polymyositis, rheumatoid arthritis, myeloma, thymoma, sarcoid, or multiple sclerosis. Lesions may be recurrent and the eruption must be differentiated from the pigmented purpuric eruptions. Histologically, acute inflammatory cells, red blood cells, and arteriolar necrosis predominate. There is also a decrease in IgG_2.[103] Many patients have antibodies to Ro/SSA.[104] Circulating complexes containing IgG anti-IgG and/or IgA anti-IgG have been detected. [94]

Cutaneous Vasculitis

Patients with collagen vascular diseases as well as those with systemic large- and small-vessel vasculitis (including hypocomplementemic vasculitis, polyarteritis nodosa, microscopic polyarteritis, Wegener granulomatosis, Behçet disease, Churg-Strauss angiitis, rheumatoid vasculitis, or relapsing polychondritis) may have an array of vasculitic purpuric lesions, including petechiae, papules, ecchymoses, hemorrhagic bullae, splinter hemorrhages, and periungual hemorrhages [94] [105] [106] [107] [108] [109] [110] (Plates 105-21 and 105-22). Nonpurpuric cutaneous lesions may also be present, including ulcers, subcutaneous nodules, livedo reticularis, erythema, erythematous plaques, and telangiectasias.

Drugs and infections are common causes of small vessel vasculitis. Bacterial infections with staphylococci, streptococci, mycobacterium leprae, and others are often associated with vasculitic lesions. Viruses causing vasculitis include HIV, hepatitis B, and hepatitis C. The tetrad of necrotizing vasculitis, chronic hepatitis C infection, hypo-complementemia, and cryoglobulinemia is a late manifestation of hepatitis C infection. [111]

Alpha-1 antitrypsin deficiency has been described in association with manifestations of systemic vasculitis, including cutaneous involvement resembling that oberved in microscopic polyarteritis, Wegener granulomatosis, and Henoch-Schönlein purpura. [112]

Paraneoplastic vasculitis[113] [114] [115] describes a syndrome of petechiae, palpable purpura, urticaria, maculopapular lesions, leg ulcers, and/or erythema multiforme seen in association with hairy cell leukemia and other lympho- and myeloproliferative disorders. Intense pruritus or dysesthesias are prominent symptoms. [116] In some cases, the cutaneous lesions may precede the diagnosis of the malignancy or signal the recurrence of the malignancy. Carcinomas of the breast, lung, colon, cervix, prostate, and kidney have all been reported in association with cutaneous vasculitis. [115] [117]

Henoch-Schönlein purpura is a leukocytoclastic vasculitis that usually affects children. [99] [118] [119] Palpable purpuric lesions, abdominal pain, and arthritis of the knees and ankles are common findings, but other organs may be involved as well. Acute and chronic renal disease is an important, associated finding. Renal involvement in adults is more common but the prognosis in children and adults is the same.[120] There is no sex predilection, but there is a seasonal increase during the winter months. Several precipitating factors have been implicated, including infections, environmental chemicals, toxins, insect bites, physical trauma, complement component C2 deficiency, familial Mediterranean fever[121] and malignancies. The genetic predisposition for development of Henoch-Schönlein purpura may be an increased frequency of DQA1* 0301 as well as complement 4 (C4) gene deletion.[122] Clinical presentations include the explosive onset over the legs and buttocks of urticarial papules and plaques, with or without purpura (Plate 105-23), palpable purpura, or hemorrhagic vesicles or bullae; larger stellate, reticulate, and necrotic lesions may also occur (Plate 105-24). Occasionally, ecchymotic lesions resembling child abuse may be present, but unlike the latter, the lesions of Henoch-Schönlein purpura are usually strikingly symmetrical. Henoch-Schönlein purpura is thought to be an immune complex disease, with 50% of patients producing IgA rheumatoid factor.[123] IgA-containing immune

complexes have been detected, especially during the early phases of the syndrome. [123] [124] [125] Increased levels of von Willebrand factor, an indicator of endothelial cell damage, decreased levels of factor XIII, and increased excretion of TNF were found in a significant number of patients with Henoch-Schönlein purpura. [126]

Acute hemorrhagic edema of infancy occurs in children between the ages of 4 months and 2 years who demonstrate fever, tender iris-like or medallion-like large purpuric cutaneous lesions, and edema.[98] The lesions occur on the cheeks, eyelids, ears, extremities, and genitalia, and must be differentiated from the cutaneous findings of child abuse, Sweet syndrome, Kawasaki disease, septic emboli, drug eruption, meningococcemia, and hemorrhagic erythema multiforme.

Approximately 10% of adults with leukocytoclastic vasculitis have been reported to demonstrate livedoid superficial plaques, with multifocal areas of hemorrhage or necrosis, and reticulate margins connecting adjacent lesions. [127] These lesions contain IgA and C3 deposits in the blood vessel walls. Smooth-margined purpuric papules with uniform hemorrhage may also show IgA, but more frequently demonstrate IgG or IgM vascular deposits.

Long-distance walkers may also develop purpuric lesions of leukocytoclastic vasculitis on the lower legs. [128]

Primary Cutaneous Diseases

Insect bites (especially blackfly bites), dermatitis herpetiformis, pityriasis rosea, and other primary cutaneous disorders may present with purpuric papules and vesicles mimicking septic and vasculitic lesions.

Nonpurpuric Disorders Simulating Purpura

Disorders with Telangiectasias

Cherry angiomas are the common papular, brightly erythematous lesions seen on the trunk and extremities of middle-aged and older men and women. The lesions are progressive with age and may produce easy bruising because they tend to bleed excessively with trauma. Pinpoint or smaller cherry angiomas may be present in large numbers and may mimic a petechial eruption. Differentiating this very common disorder from hereditary hemorrhagic telangiectasias may be difficult, and these lesions may, in fact, be part of the continuum of that disorder.

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder with an estimated frequency of 1 in 50,000 manifested by widespread dermal, mucosal, and visceral telangiectasias. [129] One form of the disorder, characterized by a high frequency of pulmonary arteriovenous malformations, has been identified as being due to abnormalities in the endothelial protein endoglin on chromosome 9, which appears to mediate the response of endothelial cells to members of the transforming growth factor (TGF-) family. A number of the mutations involve the production of truncated forms of the receptor, opening up the possibility of a dominant negative mechanism to explain the autosomal dominant inheritance pattern. [130] Another form of the disease has been linked to a region of chromosome 3 containing the TGF- II receptor and a third form has been linked to the activin receptor-like kinase 1 gene on chromosome 12, which is a cell surface receptor for the TGF- superfamily of ligands. [131] [132] Clinically, venous lakes and papular, punctate, mat-like, and linear telangiectasias appear on all areas of the skin and mucous membranes, with a predominance of lesions under the tongue and on the face, lips (Plate 105-25), perioral region, tongue, nasal mucosa, fingertips, toes, and trunk. [133] Epistaxis is a nearly universal finding in this disorder, with symptoms almost always becoming worse with age. Thus the severity of the disorder can often be gauged by the age at which the nosebleeds begin, with the most severely affected patients developing recurrent epistaxis during childhood. Cutaneous changes usually begin at puberty and progress throughout life. Bleeding can occur in virtually every organ, with gastrointestinal, oral, and urogenital sites most common. In the gastrointestinal tract, the stomach and duodenum are more common sites of bleeding than the colon. Hepatic and splenic arteriovenous fistulae, as well as intracranial, aortic, and splenic aneurysms may occur; pulmonary arteriovenous fistulae are associated with oxygen desaturation, hemoptysis, hemothorax, brain abscess, and cerebral ischemia resulting from paradoxical emboli. The vessels of hereditary hemorrhagic telangiectasia show a discontinuous endothelium and an incomplete smooth muscle cell layer. The surrounding stroma lacks elastin. Thus, the bleeding tendencies are thought to be due to mechanical fragility of the abnormal vessels.

Therapy for HHT remains problematic with laser treatment for cutaneous lesions; split-thickness skin grafting, embolization, or hormonal therapy (estrogen or estrogens + progesterone) for epistaxis; pulmonary resection or embolization for pulmonary arteriovenous malformations; and hormonal therapy and laser coagulation for gastrointestinal lesions. [129] The antifibrinolytic agent epsilon amino caproic acid has been reported to be beneficial in controlling hemorrhage [134] but negative results have also been reported. [135]

Telangiectasias from chronic actinic damage (Plate 105-26), and telangiectatic mats seen in scleroderma (CREST syndrome) (Plate 105-27) may be easily confused with the lesions of hereditary hemorrhagic telangiectasia. Angiokeratoma corporis diffusum, ataxia telangiectasia, and spider telangiectasias from chronic liver disease must also be differentiated. Spider telangiectasias have a central, prominent, easily blanchable feeding vessel with several smaller telangiectasias emanating from this central vessel. Spider telangiectasias seen in patients with chronic liver disease are distributed from the head to the nipple line, and correlate with the risk of bleeding from esophageal varices. [136]

Kaposi Sarcoma and Other Vascular Sarcomas

The epidemic form of Kaposi sarcoma found among patients infected with the HIV virus is easily confused with purpuric (Plate 105-28) and ecchymotic (Plate 105-29) lesions, as well as some of the pigmented purpuric eruptions [137] (Plate 105-30). Oral lesions of epidemic Kaposi sarcoma may also mimic petechiae and purpura (Plate 105-31). Similarly, angiosarcoma may present as a purple to brown plaque resembling purpura (Plate 105-32).

Fabry Disease

Fabry disease (angiokeratoma corporis diffusum) is an X-linked inherited disorder of glycolipid metabolism with deficiency of the enzyme ceramide trihexosidase. [138] Accumulation of glycolipid throughout the body leads to cutaneous, renal, ophthalmologic, cardiac, and central nervous system manifestations. Angiokeratoma corporis diffusum lesions are pinpoint-sized to 4-mm-deep red, blue, or black macules or papules (Plate 105-33). These nonblanchable lesions are distributed over the trunk, extremities, and genitalia. In mild cases, lesions are localized to the thighs, scrotum, or periumbilical region. Grouping of lesions may occur. Superficial corneal dystrophy and varicosities of the bulbar conjunctivum are commonly seen (Plate 105-34).

Neonatal Extramedullary Hematopoiesis

Infants with congenital toxoplasmosis, rubella, or cytomegalovirus infections may have these dark red, blue, or blue-gray

macules and/or papules at birth or within the first 48 hours of life. [139] The lesions are most commonly found on the scalp, neck, and trunk but may be widely distributed. These lesions fade into tan- or copper-colored macules by 8 weeks of age.

Angioma Serpiginosum

Angioma serpiginosum is a vascular nevoid lesion showing pinpoint vascular ectasias on a background of erythema. [10] The lesion is partially blanchable and is not petechial. Capillary microscopy demonstrates punctate dilated capillaries. This lesion is usually seen on the legs and buttocks of women, but it may occur anywhere and may expand in childhood and regress with age.

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Chapter 106 - Laboratory Evaluation of Hemostatic Disorders

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INTRODUCTION

Once the presence of a bleeding disorder has been established or a high level of suspicion is generated by either the history or physical examination, or both (see Chap. 105), laboratory tests are used to establish the diagnosis. Familiarity with, and rational application of, a few laboratory procedures will enable one to place the defect in one of several broad categories. More specialized tests are subsequently necessary to establish a definitive diagnosis.

For diagnostic purposes, the hemostatic system may be somewhat simplistically divided into two parts: the plasma coagulation factors and platelets. An abnormality in either portion may give rise to a bleeding disorder. Although the history and physical examination may provide clues as to which portion of the hemostatic system is defective, the information is not definitive. Therefore, when faced with a bleeding disorder, the first task is to establish whether the disorder is due to either impairment of the reactions leading to thrombin generation and fibrin clot formation or to inadequate numbers or function of platelets. This is accomplished by performing a prothrombin time (PT), an activated partial thromboplastin time (PTT), a platelet count, and a bleeding time.

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SCREENING TESTS OF PLASMA COAGULATION FACTORS

The PT and PTT are used to assess the function of the plasma coagulation factors, with the notable exception of factor XIII. For diagnostic purposes, it is useful to divide the plasma coagulation reactions into the extrinsic, intrinsic, and common pathways, although recent data from several laboratories have indicated the existence of several interconnections between these pathways. These pathways are diagrammed schematically in Figure 1061 . The extrinsic system includes the reactions of tissue factor and factor VII that lead to the conversion of factor X to factor Xa. The intrinsic system is composed of factors VIII, IX, XI, and XII, prekallikrein, and high-molecular-weight kininogen. The common pathway includes factors V and X, prothrombin, fibrinogen, and factor XIII.

Prothrombin Time

A PT is performed by adding a crude preparation of tissue factor (commonly an extract of brain) or recombinant tissue factor reconstituted with phospholipids to citrate-anticoagulated plasma, recalcifying the plasma, and measuring the clotting

Figure 106-1 Reactions of coagulation. HMWK, high-molecular-weight kininogen; TF, tissue factor; T, thrombin; PT, prothrombin; FG, fibrinogen; F, fibrin clot (not cross-linked).

time.[1] It is standard practice to add both thromboplastin (tissue factor preparation) and Ca^{2+} in a single step. It should be apparent from Figure 1061 that abnormalities of factors VII, V, and X, prothrombin, or fibrinogen may result in prolongation of the PT. The test may be prolonged due to either a deficiency of one or more of the involved factors or to the presence of an inhibitor. The PT is also the most frequently used method to monitor the anticoagulant effect of warfarin, which causes a decrease in the activity of vitamin K-dependent factors VII, IX, and X and prothrombin. Warfarin dosage is typically adjusted to achieve a desired patient/mean normal PT ratio expressed as the International Normalized Ratio (INR) (see Chap. 121).

Commercially available thromboplastins vary in the animal source and method of preparation, resulting in differing sensitivities to factor deficiencies. [2] Although differences in instrumentation, methodology, and control plasmas also contribute to inter- and intralaboratory variability, the lack of a standard thromboplastin is thought to be the most important factor.[3] The World Health Organization has established a reference thromboplastin derived from human brain that has been used to calibrate secondary standards, available to manufacturers and laboratories for the evaluation of thromboplastin reagents. [2] The sensitivity of a thromboplastin is described by the International Sensitivity Index (ISI). Most commercial thromboplastins derived from animal sources are less sensitive to factor deficiencies than is the reference standard (ISI = 1.0) and have ISIs of 1.22.8.[4] Some authorities and panels have advocated routine reporting of PTs, especially for anticoagulant monitoring, as an INR.[5] [6] [7] The INR converts the PT patient/PT mean normal ratio to the value expected if the test had been performed with the reference thromboplastin and is given by $INR = (PT\ patient/PT\ mean\ normal)^{ISI}$. Advocates argue that use of the INR would reduce interlaboratory variation in reported PT ratios and permit the adoption of more uniform and consistent monitoring of oral anticoagulation based on INR target ranges. The use of recombinant human tissue factor is a potential alternative to the many animal thromboplastins currently in use. [8] [9] When compared with the World Health Organization standard thromboplastin, the ISI of recombinant human tissue factor was 0.961.12, with a comparable degree of precision.[9] Although the ISI is derived from analysis of plasmas from patients on chronic, stable oral anticoagulation therapy, the INR may also be superior to the PT ratio for monitoring the initiation phase of warfarin therapy. [10] When compared to the PT ratio, less variation was encountered with the INR when different thromboplastins were used to monitor the induction phase of warfarin therapy. Enthusiasm for the INR approach has been somewhat tempered by recognition that the apparent ISI of a thromboplastin used on an automated coagulation analyzer may differ from that obtained by the manual tilt-tube method used for calibration. [3] [11] [12] Instrument-specific ISI values for thromboplastins may be required to overcome this difficulty.

The use of sensitive thromboplastins or of the INR, or both, is not without drawbacks. Mild deficiencies of extrinsic or common pathway coagulation factors that represent a negligible bleeding risk do not prolong the PT when an insensitive (ISI >2) thromboplastin is used. If, however, the PT ratio is converted to the INR, or if a sensitive thromboplastin is used, more patients, especially those with acquired vitamin K deficiency or liver disease, will be identified with an abnormal coagulation test and will be at risk of receiving unnecessary plasma infusions or for having invasive procedures delayed or canceled. Thus, clinical judgment must be brought to bear in interpreting the INR within a particular clinical context.

Activated Partial Thromboplastin Time

The PTT is performed by the addition of a surface activating agent, such as kaolin, silica, or ellagic acid, and phospholipid to citrate-anticoagulated plasma. [1] After incubation for a period sufficient to provide for the optimal activation of the contact factors, the plasma is recalcified and the clotting time measured. The name of the test emanates from the phospholipid reagents being originally derived from a lipid-enriched extract of complete thromboplastin, hence the term partial thromboplastin. The PTT is dependent on factors of both the intrinsic and common pathways (Fig. 1061). The PTT may be prolonged due to a deficiency of one or more of these factors or to the presence of inhibitors that affect their function. Although it is commonly stated that decreases in factor levels to approximately 30% of normal are required to prolong the PTT, in practice the variability is considerable in the sensitivity of different commercially available PTT reagents to the various factors. In our experience, the levels vary from 2540%. Because detection of a fibrin clot does not require covalent cross-linking of the fibrin, neither the PT nor the PTT will detect even severe deficiencies of factor XIII.

Recently developed portable instruments offer the potential for rapid determination of the PT and PTT at the bedside or in an outpatient setting. [13] [14] [15] [16] [17] [18] [19] Blood from a finger stick is applied to an opening on a disposable card. The blood flows by capillary action through an enclosed channel that contains either lyophilized thromboplastin (PT) or phospholipid and surface activator (PTT), where coagulation begins. The card is inserted into a portable laser photometer, which measures the time until blood flow stops due to clot formation. The instruments convert the clotting time to a comparable PT or PTT result. Early studies indicate that these methods correlate well with traditional laboratory methods, exhibit acceptable precision, and are easily performed by health care workers and patients. One recent randomized prospective study has suggested that the use of these instruments in the cardiothoracic operating room facilitated rapid correct diagnosis in bleeding patients and resulted in a significant reduction in blood product use. [20]

Platelet Count

The platelet count is performed to detect thrombocytopenia, which is usually defined as a platelet count of $<150 \times 10^9$ /L. The procedure is now almost always performed as part of an automated blood cell profile. Currently used instruments are quite reliable, even down to platelet counts approaching 25×10^9 /L, and are

much less susceptible to the artifacts that plagued earlier generations of platelet counters. [21] [22] Nevertheless, the finding of thrombocytopenia, especially unexpected thrombocytopenia, should be confirmed by a review of the peripheral blood smear and platelet size histogram obtained from automated blood cell counters. Abnormalities of platelet size, the presence of red blood cell fragments, or evidence of pseudothrombocytopenia [23] [24] [25] [26] [27] may provide clues with which to direct the further evaluation of the patient.

Bleeding Time

The bleeding time is the screening test for platelet function. [28] [29] [30] The test is not, as its name might imply, a global test of the hemostatic system. The test is not significantly prolonged in most disorders that result in prolongation of the PT or PTT (e.g., deficiencies of factors VIII, IX, or VII). The bleeding time is prolonged not only due to functional defects of platelets, but also to deficiencies or functional abnormalities of plasma proteins such as von Willebrand factor (vWF), which are required for normal interactions of platelets with the vessel wall. The bleeding time may also be prolonged in severe hypofibrinogenemia.

The procedure is usually performed with a disposable template device that produces one or two standardized incisions on the volar aspect of the forearm. A sphygmomanometer around the arm is inflated to 40 mmHg to standardize venous pressure. The time required for bleeding to cease is determined by carefully blotting the blood emerging from the wounds with filter paper at 30-second intervals.

The test is influenced by a number of factors, including the

depth, location, and direction of the incisions; skin thickness; and skill of the technologist performing the test, in addition to platelet function. [31] [32] The bleeding time may be prolonged by some of the many medications known to inhibit platelet function (see Chap. 131). Knowledge of the platelet count is essential for accurate interpretation of the bleeding time. The test is independent of platelet count down to approximately 100×10^9 /L and is prolonged as the platelet count falls from 100×10^9 /L to 10×10^9 /L.[28] [33] Because of this, a bleeding time is generally indicated only when the platelet count is $>100 \times 10^9$ /L. In the presence of thrombocytopenia, a prolonged bleeding time must be interpreted with caution.

Analysis of the performance of the bleeding time indicates that the test functions poorly as a screening test in the general population and is a poor predictor of surgical bleeding and of the response to hemostatic therapy. [34] [35] [36] The sensitivity, specificity, and predictive value of the test have not been established. Nevertheless, we concur with Tripletts conclusion that the bleeding time can be a valuable laboratory test that can yield important diagnostic information when used in a selected patient population with suggestive or positive bleeding histories. [29]

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FURTHER EVALUATION OF ABNORMAL SCREENING TESTS

The initial battery of four tests establishes whether the patient has a quantitative disorder of platelets, a defect in platelet function, or an abnormality within either the intrinsic or extrinsic pathways of coagulation, or both.

Evaluation of Prolonged PT and/or PTT

Several tests can be performed to ensure that the prolongation of the PTT is not due to the presence of heparin in the specimen. The thrombin time is a useful test for this purpose because it is very sensitive to the presence of heparin (see following discussion). Once it is clearly established that the PT or PTT, or both, is prolonged, the task is to determine whether the prolongation is caused by factor deficiency or by the presence of an inhibitor. [37] This is usually accomplished by repeating the determinations after mixing equal volumes of the patients plasma with plasma derived from a pool of normal donors. Because factor levels of 50% of normal are sufficient to produce a normal PT and PTT, this procedure will result in correction of the abnormality if it is caused by the deficiency of one or more factors. If an inhibitory antibody is present, the mixing procedure will result in little or no correction of the abnormality. Two caveats must be borne in mind. First, relatively low-titer, low-avidity inhibitors producing only modest prolongations of the PT or PTT, or both, may be difficult to detect with the 1:1 mixing procedure. Some authorities have advocated performing inhibitor assays on other than 1:1 mixtures, in an effort to detect weaker inhibitors. In our experience, this procedure has not proved satisfactory and, as often as not, has produced ambiguous results.

INITIAL LABORATORY EVALUATION OF A BLEEDING PATIENT

Second, some classes of inhibitors seen in patients with severe factor VIII deficiency react slowly with factor VIII in the normal plasma on mixing and may not be detected if the mixture is assayed immediately. [38] The latter situation is addressed by assaying the mixture after an incubation of 0.52 hours, as well as immediately after mixing.

A series of 100 consecutive patients referred for consultation because of a prolonged PTT demonstrated that in 50% of patients the problem was indicative of a hemostatic defect. [39] Of the hemostatically compromised patients, 81% gave an abnormal hemostatic history. A laboratory approach to evaluating acquired and inherited prolongations of the PT or PTT, or both, is presented in algorithm form. This approach is discussed in greater detail in the following sections.

Inhibitors

Factor VIII inhibitors and lupus anticoagulants are the most frequently encountered inhibitors of coagulation. Factor IX inhibitors are encountered much less often; other inhibitors are seen only rarely. A detailed discussion of circulating anticoagulants is presented in Chapter 116 . One of the several approaches described following should be used to establish the presence of the lupus anticoagulant. Assays of specific coagulation factors are used to document the specificity of inhibitors against individual factors.

Quantitation of the level of factor VIII inhibitors may have therapeutic implications. [38] Two common approaches have evolved. In the Bethesda assay, patient plasma or dilutions of patient plasma and normal plasma are incubated for 2 hours at 37°C, after which the residual factor VIII activity is measured. [40] [41] One Bethesda unit/ml is the concentration that will neutralize 50% of the added factor VIII during the 2-hour incubation. In the new Oxford assay, the preliminary incubation requires 4 hours. One new Oxford unit/ml is defined as the concentration of inhibitor that will neutralize 50% of the added factor VIII (from a lyophilized concentrate) in 4 hours. [41] [42]

Lupus Anticoagulants

The so-called lupus-type anticoagulant is by far the inhibitor most likely to be encountered [43] (see Chap. 116 for a more extensive discussion). Only about one-third of these anticoagulants occur in patients with lupus erythematosus. A review of the literature indicates that this anticoagulant is present in approximately 25% of patients with systemic lupus erythematosus (SLE), if the analysis is restricted to series composed of consecutive or otherwise unselected patients. The lupus anticoagulant is paradoxically associated with an increased occurrence of thrombosis, recurrent fetal loss, and thrombocytopenia. [44] When these complications are encountered in a lupus anticoagulant-positive patient without SLE, the term primary antiphospholipid syndrome has been applied. [45]

The lupus anticoagulants react with anionic phospholipids and may therefore give rise to either a prolonged PTT (common) or prolonged PT (less common), or both. Several different laboratory procedures have been proposed as tests for the lupus anticoagulant. These have included tests based on the PTT, the PT performed with dilute thromboplastin (the tissue thromboplastin inhibition test), the dilute Russells viper venom time (activation of factor X by venom and subsequent thrombin generation) performed with limiting concentrations of venom and phospholipid, the kaolin clotting time, the plasma clotting time, and platelet or phospholipid neutralization procedures. [46] [47] No single test for the detection of lupus anticoagulants has proved entirely satisfactory or gained widespread acceptance. The sensitivity and specificity have not been adequately established for any of these procedures. [48] Problems include patient-to-patient variability due to the heterogeneity of these antibodies, as well as variation in the sensitivity of reagents to inhibitory activity. [49] [50] Minimal guidelines have been proposed. [51] These include the demonstration that (1) an abnormality of phospholipid-dependent coagulation reactions is present; (2) the abnormality is due to the presence of an inhibitor, rather than to factor deficiency; and (3) the inhibitor is directed at phospholipid rather than specific coagulation factors.

At our laboratory we use the following approach, which is consistent with the above criteria. A dilute Russells viper venom time is performed. If the initial screening test is prolonged, the test is repeated after mixing the patients plasma with an equal volume of normal pooled plasma to exclude factor deficiency as a cause of the prolongation and to demonstrate the presence of an inhibitor. Finally, the lipid dependence is established by the ability of hexagonal phase phosphatidylethanolamine to neutralize the effect of the anticoagulant.

Another test used to detect antiphospholipid immunoglobulins is the anticardiolipin antibody (ACA) assay. This test entails incubating patient serum with solid-phase

cardiolipin. Following incubation and washing, bound immunoglobulin is detected using appropriate antisera (e.g., antihuman IgG, IgM, and IgA). Progress has been made toward standardization of the ACA through the use of similar methodology and the availability of antiphospholipid immunoglobulin standards. [52] It must be borne in mind that not all ACAs exhibit lupus anticoagulant activity, nor do all lupus anticoagulants give positive results in ACA assays.

Factor Deficiency

If the prolonged PT or PTT normalizes after mixing the patients plasma with normal plasma, a deficiency of one or more of the involved factors is indicated. [37] [53] Clinical considerations, such as the presence of liver disease, may give clues to the existence of multiple factor deficiencies. If only the PT is prolonged, a deficiency of factor VII is present. If only the PTT is prolonged, a deficiency of factors VIII, IX, XI, and XII, high-molecular-weight kininogen, and/or prekallikrein should be suspected. The history and physical examination may again provide valuable clues. Deficiencies of factors VIII and IX are associated with significant bleeding disorders that conform to classic sex distribution and inheritance patterns (see Chap. 109). Factor XI deficiency is variably associated with a bleeding diathesis (see Chap. 111). Even severe deficiencies of factor XII, prekallikrein, or high-molecular-weight kininogen are asymptomatic despite the fact that the PTT may be markedly prolonged.

If both the PT and the PTT are prolonged, the patient has either a deficiency of multiple factors affecting both the intrinsic and extrinsic pathways and/or the common pathway or has a selective deficiency of factor V or X, prothrombin, or fibrinogen. In this setting, a concentration of fibrinogen adequate for clot formation should be ensured before other specific factor assays are performed. Fibrinogen levels must fall to 6080 mg/dl before hypofibrinogenemia can be accepted as the sole explanation for the prolonged PT and PTT. If hypofibrinogenemia is not the cause of the abnormal PT and PTT, specific assays for factors V and X and prothrombin should be performed.

Factors V, VII, and X and prothrombin are usually assayed by determining the ability of dilutions of the patient plasma to correct the PT of a plasma congenitally deficient in the factor to be assayed. The degree of correction is compared to that produced by equivalent dilutions of normal pooled plasma. Factors VIII, IX, XI, and XII are determined in a similar manner, except that the specific factor assays are based on the PTT.

Fibrinogen Determinations

A number of procedures for the determination of fibrinogen have been devised. These include chemical determinations of

thrombin-clottable protein, salt precipitation techniques, and immunochemical approaches such as radial immunodiffusion and nephelometry. More common, however, are assays based on the original method described by Clauss, [54] which is the basis for several assays available in kit form for routine use in clinical laboratories. These procedures involve an initial (typically 10-fold) dilution of the plasma sample to ensure that fibrinogen is rate limiting for clotting and for the subsequent measurement of a clotting time initiated by the addition of a large excess of thrombin to the sample. The length of the clotting time is inversely related to the fibrinogen concentration. Because this type of assay measures the time to formation of a detectable clot, inhibitors of fibrin polymerization, such as fibrinogen/fibrin degradation products (FDPs) produced by plasmin, which are present in patients with disseminated intravascular coagulation (DIC) or in those undergoing fibrinolytic therapy, may result in an underestimation of the actual fibrinogen concentration. Immunologic assays of fibrinogen may measure both fibrinogen and FDPs. A dysfibrinogenemia will typically result in a substantially lower value for fibrinogen concentration when determined by the Clauss technique than by immunologic assays. However, it should be noted that antigenic and functional determinations of fibrinogen tend to be somewhat discordant even in normal subjects, with the ratio of antigenic/functional fibrinogen ranging from 1.121.65. [55] [56] Congenital hypofibrinogenemia will result in low concentrations of fibrinogen by all techniques; congenital afibrinogenemia will result in virtually undetectable levels by all techniques (see Chap. 112). In contrast to the thrombin time on which it is based, determination of fibrinogen by the Clauss technique is not susceptible to interference by commonly encountered concentrations of heparin.

Thrombin Time

The thrombin clotting time has also been used to establish the presence of adequate levels of fibrinogen. We recommend, however, that one of the simple fibrinogen assays that yields quantitative information about the fibrinogen concentration be used instead for this purpose. The thrombin time is performed by measuring the clotting time after the addition of excess thrombin to undiluted plasma. [1] The test may be prolonged as a result of hypofibrinogenemia or dysfibrinogenemia; the presence of FDPs, heparin, or an antibody to thrombin; and in amyloidosis. [57] [58] Prolongation of the thrombin time due to hyperfibrinogenemia has also been described. [59] The relative sensitivity of the thrombin time to these factors depends on the precise configuration of the assay. [53] Correction of a prolonged thrombin time on the addition of protamine sulfate or of a commercially available ion-exchange resin (Heparsorb) is a presumptive test for the presence of heparin or a heparin-like anticoagulant.

Evaluation of Thrombocytopenia

Thrombocytopenia is commonly defined as a platelet count of $<150 \times 10^9$ /L. Thrombocytopenia is a laboratory finding indicative of an underlying disease process, but does not constitute the diagnosis. A number of different conditions may give rise to the finding of thrombocytopenia. The evaluation of thrombocytopenia is treated in detail in Chapters 126 , 128 , and 129 .

von Willebrand Disease

von Willebrand disease (vWD) is the most likely cause of an inherited prolongation of the bleeding time. It is now believed by some to be the most common of the inherited disorders of hemostasis. [60] [61] [62] The prevalence of this disorder was recently estimated at 0.8% in a population of children in northern Italy. [62] The disorder arises as a consequence of quantitative (types 1 and 3) or qualitative (type 2) abnormalities of the vWF protein. A recently revised classification scheme [63] is presented in Table 1061 . vWD is considered in detail elsewhere (see Chaps. 113 and 114). Although a prolonged bleeding time is the hallmark of vWD, this laboratory finding is not specific for vWD. Thus, the diagnosis is definitively established by means of additional laboratory tests. [64] These usually include measurements of the amount of vWF protein present in plasma, the functional activity of the vWF, and the procoagulant activity of the associated factor VIII molecule. Analysis of the multimeric structure and factor VIII binding capacity of vWF is useful in some settings, as described in the following sections.

The vWF protein is quantitated by immunochemical procedures. Rocket immunoelectrophoresis (electroimmunoassay) remains the most commonly used procedure, although other approaches, including enzyme-linked immunosorbent assays, are increasingly used. [1] [65] In most patients with vWD, the antigenic level of the von Willebrand protein will be reduced. However, in patients with type 2 disorders, antigenic levels of the protein may be close to, or within, the reference range.

Pregnancy, ingestion of oral contraceptives, or liver disease may elevate the plasma level of vWF and factor VIII coagulant activity, complicating the diagnosis. A recent study demonstrated that levels of vWF are a function of the ABO blood group. [66] Persons with blood group O had significantly lower levels of vWF (75 U/dl) than were found in group A (106 Udl), group B (117 U/dl), or group AB (123 U/dl) subjects.

The functional activity of vWF is assessed by measuring the ristocetin cofactor activity of the patients plasma. This assay determines the ability of vWF in the plasma to agglutinate a standardized suspension of fixed normal platelets in the presence of ristocetin from either the rate or extent of platelet agglutination. [1] [67] [68] Agglutination is usually measured in a platelet aggregometer, although other approaches have been devised. This activity is usually decreased in plasma from patients with vWD, even in those with type 2B or platelet-type pseudo-vWD in which enhanced platelet agglutination/aggregation is observed when low concentrations of ristocetin are added to the patients own platelet-rich plasma. Even in type 1 disorders, determination of ristocetin cofactor activity by the semiquantitative procedure described above appears to have a greater diagnostic sensitivity than the more qualitative determination of ristocetin-induced platelet aggregation in platelet-rich plasma. [69] This is attributable to the lower concentrations of vWF actually present in the ristocetin cofactor assays.

This functional assay tests only the ristocetin-dependent interaction of vWF with the platelet membrane GPIb/IX complex. Clinically useful tests of the interactions of vWF with the platelet membrane IIb/IIIa complex or with components of the extracellular matrix have not been devised. Defects in these interactions may, at least theoretically, also contribute to bleeding disorders.

TABLE 106-1 -- **Revised Classification of von Willebrand Disease**

Type 1Partial quantitative deficiency of vWF with normal structure and function
Type 2Qualitative abnormalities of vWF
Type 2ADecreased platelet-dependent function with loss of high-molecular-weight multimers
Type 2BIncreased affinity of vWF for platelet membrane glycoprotein IbIX complex
Type 2MDecreased platelet-dependent function but with retention of high-molecular-weight multimers
Type 2NMarkedly decreased affinity of vWF for factor VIII resulting in an autosomal mimic of hemophilia A
Type 3Essentially complete deficiency of vWF

Factor VIII procoagulant activity is also typically, but not universally, decreased in vWD. This activity is commonly measured in an assay based on the ability of various dilutions of patient plasma to correct the clotting time of authentic factor VIII-deficient plasma in a PTT-based assay. Although factor VIII levels are commonly reduced in vWD, the level may not be sufficiently low so as to prolong the PTT. Thus, a PTT within the normal range does not permit exclusion of the diagnosis of vWD.

In contrast to the type 1 forms of vWD, in which a generalized decrease in all multimeric forms of vWF occurs, the type 2A and 2B forms of the disease manifest a selective deficiency of the higher molecular-weight forms. The deficiency may have a number of causes, including impaired biosynthesis, enhanced proteolysis, or selective adsorption of the high-molecular-weight forms onto platelets. [61] Because the higher molecular-weight forms account for most of the ristocetin cofactor activity in plasma, a discrepancy between the amount of ristocetin cofactor activity present and the level of vWF antigen should lead to the suspicion of the presence of a qualitative type 2 disorder. Two approaches have been used to confirm this suspicion.

In crossed immunoelectrophoresis, the plasma sample is electrophoresed through agarose in the first dimension to separate the vWF multimers by size and then electrophoresed in the second dimension into agarose containing antibody to vWF. An immunoprecipitate forms, the shape of which is indicative of the distribution of vWF multimers. [70] Today this approach has largely been replaced by sodium dodecyl sulfate agarose gel electrophoresis.

Sodium dodecyl sulfate agarose gel electrophoresis provides a higher resolution analysis of the multimer distribution and is usually available in clinical coagulation reference laboratories. [71] Following electrophoretic separation in agarose in the presence of sodium dodecyl sulfate, the vWF multimers are detected with either ^{125}I-labeled antibodies or enzyme-linked antibodies to vWF. The discrimination of several types of vWD by this technique is illustrated in Figure 1062 .

Figure 106-2 Sodium dodecyl sulfate agarose gel electrophoretic analysis of the multimeric composition of plasma vWF from a normal subject (NP) and patients with types 1, 2A, 2B, and 3 vWD. Note the absence of high-molecular-weight multimers in the 2A and 2B disorders and the absence of all forms of vWF in the type 3 disorder. (Photo and analysis courtesy of Marlies R. Ledford, University of Miami Coagulation Reference Laboratory.)

Establishing the existence of a type 2 disorder is no longer an academic exercise. Because deamino- D-arginine vasopressin (DDAVP) is now considered front-line therapy for type 1 vWD but appears to be less effective in some of the type 2 disorders and can induce thrombocytopenia in some of the type 2B patients, this distinction has taken on important therapeutic implications. [60] [61]

Although ristocetin-induced platelet aggregation of the patients own platelet-rich plasma is not recommended as a front-line test for the diagnosis of vWD, it is useful in sorting out type 2 forms of the disease. Platelets in type 2B disease exhibit enhanced platelet aggregation to low concentrations of ristocetin when platelet-rich plasma is examined, even though the ristocetin cofactor activity of the patients plasma may be diminished. [72] This is attributed to an exceptionally high avidity of the abnormal vWF for the platelet, resulting in its adsorption from plasma. A similar phenomenon is observed in platelet-type pseudo-vWD, except that the defect resides in the patients platelets rather than in the vWF molecule. [73] [74] [75]

Type 2N is a qualitative form of vWD with a recessive mode of inheritance. [76] Mutations have been identified in the amino-terminal factor VIII binding domain of vWF that decrease affinity for factor VIII and consequently lead to lower plasma factor VIII activity. [77] vWF antigen, ristocetin cofactor activity, and multimeric patterns are normal in vWD 2N. Inheritance is homozygous or compound heterozygous, producing a laboratory profile similar to that observed in hemophilia A. Distinguishing type 2N vWD from hemophilia A has implications for both genetic counseling and patient management. However, many reference laboratories do not offer a factor VIII binding assay of vWF, most likely due to the lack of commercially available diagnostic reagents. As a result, vWD 2N is probably underdiagnosed. [78] [79]

In trying to establish the diagnosis of vWD, one must be aware that all the laboratory parameters may fluctuate significantly over time. [80] On some occasions, all four major parametersbleeding time, vWF antigen, vWF activity, and factor VIII activitymay be within the normal range. In the presence of a strong clinical suspicion, repeated testing is warranted to establish the diagnosis.

Platelet Function Testing

A prolonged bleeding time in the presence of a relatively normal platelet count may be due to an inherited defect in platelet function, but less frequently than to vWD. The inherited defects of platelet function include abnormalities or deficiencies of membrane glycoproteins; deficiencies of secretory granules or their contents, or both; and defects in the enzymatic machinery required for secretion. Hereditary disorders of platelet function are considered in Chapter 130 . Acquired defects due to associated disease states, such as uremia, multiple myeloma, myeloproliferative disorders, various inhibitory antibodies, or the presence of medications must also be considered. Acquired disorders of platelet function are discussed in Chapter 131 .

Review of the peripheral blood smear may provide a clue to the nature of the platelet function defect. In gray platelet syndrome (-granule deficiency), the characteristic purplish staining of platelets is absent, leaving them with a gray, washed-out appearance. Giant platelets are present in Bernard-Soulier syndrome and in a variety of other inherited disorders associated with thrombocytopenia. If the smear is prepared from fresh blood in the absence of anticoagulant, platelet aggregates are readily apparent in normal individuals. The aggregates are not detectable in Glanzmanns thrombasthenia. None of these observations can be considered definitive. Additional studies are required to establish the diagnosis.

Platelet aggregation studies are the mainstay of platelet function

Figure 106-3 (Figure Not Available) Platelet aggregation patterns in platelet disorders. Representative aggregation patterns in response to ADP, epinephrine (EPI), collagen (COLL), arachidonic acid (ARACH), and ristocetin (RISTO) are shown for a normal subject and for patients with a storage pool defect (SPD), Glanzmanns thrombasthenia (GT), and Bernard-Soulier syndrome (BSS). The characteristic abnormality in patients with release defects is an absent second wave of aggregation with ADP and epinephrine, an absent or greatly diminished aggregation with collagen, and a normal or variably reduced arachidonate aggregation. In Glanzmanns thrombasthenia, the characteristic abnormality is absent aggregation with all agents, except ristocetin. In Bernard-Soulier syndrome, the characteristic abnormality is absent aggregation with ristocetin and a reduced response to collagen, but aggregation with ADP, epinephrine, and arachidonate is normal. *(From White et al., [103] with permission.)*

testing in the laboratory. [81] [82] [83] Platelet aggregation is monitored by the increase in light transmission through a suspension of platelet-rich plasma as aggregation occurs. Collagen, adenosine diphosphate (ADP), epinephrine, arachidonic acid, and ristocetin are commonly used agonists in the clinical laboratory. Studies of platelet aggregation are usually performed in concert with studies of platelet secretion. The secretion of granule contents is usually monitored by following the agonist-induced release of ^{14}C-serotonin from platelet-dense granules or of adenosine triphosphate release by a chemiluminescence procedure. These rather sophisticated evaluations are obviously beyond the scope of most clinical laboratories but are usually available in referral centers.

Platelet aggregation patterns observed in some of the more common, classical inherited abnormalities are shown in Figure 1063 (Figure Not Available) . As more has been learned about platelet function defects and more disorders described, it has become clear that simple examination of the results of the usual set of platelet function studies in many cases does not permit a diagnosis at the molecular level. Although some disorders, such as Glanzmanns thrombasthenia (defect in GPIIb/IIIa) or Bernard-Soulier syndrome (defect in GPIb), give rise to unambiguous findings, defects such as storage pool deficiency, cyclo-oxygenase deficiency, or thromboxane synthase deficiency, may give rise to virtually identical patterns of aggregation with the commonly used agonists. More detailed studies, including analysis of platelet membrane proteins, electron microscopic examination of the platelets, analysis of granule contents, or the analysis of arachidonic acid metabolites, may be required for a definitive diagnosis.

Although ristocetin-induced platelet aggregation may be ab-normal both in vWD and in Bernard-Soulier syndrome, plasma ristocetin cofactor activity is normal in the latter disorder but is usually decreased in vWD. Furthermore, the defect in ristocetin-induced platelet aggregation observed in vWD can be corrected by the addition of normal plasma to the patients platelet-rich plasma. The defect in Bernard-Soulier syndrome is not corrected by normal plasma because it is due to a deficiency of the platelet membrane GPIb/IX complex. Careful attention to detail is required to obtain reproducible platelet function studies. Results are influenced by choice of anticoagulant, pH, time of storage, rate of stirring, and platelet count, as well as numerous drugs. [82] [83]

Several other tests, more common in years past, are less often used today. These include assays of platelet factor 3 activity, clot retraction, and glass bead retention. These procedures lack the diagnostic specificity of the approach to platelet function testing outlined above and are not commonly performed in most diagnostic coagulation laboratories.

The development and characterization of monoclonal antibodies reactive with platelet surface-adhesive receptors have resulted in new approaches to the investigation of platelet function disorders. Assays for the diagnosis of Bernard-Soulier syndrome (GPIb deficiency) and Glanzmanns thrombasthenia (GPIIb/IIIa complex deficiency) have been developed using antibodies directed against GPIb and IIb/IIIa, respectively. [84] [85] The availability of a panel of antibodies that can recognize the resting, activated, and ligand-occupied forms of the GPIIb/IIIa complex has been exploited in a flow cytometric approach to assess not only the presence, but also the functional activity, of the complex. [86] Although not yet in widespread use, it is likely that such assays will play an increasing role in the evaluation of platelet function in the near future.

New instruments for the rapid, in vitro assessment of platelet function using specimens of anticoagulated whole blood may soon be available for clinical use. [87] Although capable of discriminating between hemostatically normal individuals and those with well-characterized, congenital or acquired disorders of primary hemostasis, it would be inappropriate to advocate the use of these devices for screening to predict surgical bleeding risk until the application can be supported by the results of rigorous clinical trials.

Factor XIII Deficiency

When no abnormalities of the four screening tests are found despite suspicion of an underlying bleeding disorder, a deficiency of factor XIII (fibrin stabilizing factor), must be considered. Plasma factor XIII is composed of two -chains and two -chains; platelet factor XIII consists of two -chains. In the presence of Ca^{2+} , cleavage by thrombin of factor XIII -chains produces an active transglutaminase. Factor XIIIa covalently cross-links fibrin molecules, leading to clot stabilization. [88] Clinical bleeding due to factor XIII deficiency occurs at levels <2% of normal and is a rare congenital (homozygous recessive inheritance) or acquired deficiency. [88] [89] [90]

Most laboratories use a simple qualitative assay to detect a deficiency of fibrin-stabilizing activity based on the ability of 5 M urea or 1% monochloracetic acid to dissolve fibrin clots that have not been cross-linked. Normally cross-linked clots are resistant to solubilization by these agents. This is an insensitive test with a threshold for clot lysis of <2% factor XIII activity. It is below this level, however, that clinical manifestations of factor XIII deficiency occur.

More quantitative assays of factor XIII transglutaminase activity have been devised. [91] [92] These tests are more sensitive and rapid than the clot lysis assay; they can also be automated. However, they are not in widespread use because screening for factor XIII deficiency is rarely clinically indicated. Factor XIII - and subunits can be quantitated by immunologic methods using commercial polyclonal antisera. The determinations may be helpful in characterizing an inherited deficiency. [90]

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation results from the poorly regulated activation of coagulation and fibrinolysis systems in response to an underlying illness or injury that, in most cases, is clinically apparent. The generation of thrombin and plasmin predisposes the patient to both thrombotic and hemorrhagic complications. DIC syndrome is considered in detail in Chapter 117 .

Detection of diffuse fibrin deposition in the microvasculature would be definitive for the diagnosis of DIC, but obtaining such direct evidence is impractical. Red blood cell fragmentation on a peripheral blood smear signifying microangiopathic hemolysis is indirect morphologic evidence of DIC. However, this finding lacks sufficient sensitivity and specificity to be considered a reliable screening procedure for DIC.

A common approach to the laboratory investigation of DIC is to use a battery of relatively simple, rapid procedures to document the activation of both coagulation and fibrinolytic systems.[53] [93] [94] [95] [96] Interpretation of the test results can be difficult, however, because none of these tests is specific for DIC. In general, screening tests are more sensitive in the setting of acute fulminant DIC than in clinical conditions associated with chronic compensated DIC.

Thrombocytopenia occurs as a result of thrombin generation, platelet activation, and microvascular thrombus formation. Thus, the platelet count is commonly depressed in DIC and is a component of most laboratory DIC profiles. Unfortunately, thrombocytopenia is associated with several of the predisposing conditions for DIC (i.e., liver disease, leukemia, sepsis), even in the absence of DIC. Acquired platelet function defects can also be a complication of DIC, adding to the hemorrhagic risk.

The PTT and PT are frequently prolonged in DIC [94] [95] [96] due to the consumption of fibrinogen and other coagulation factors, as well as to the presence of FDPs that interfere with fibrin polymerization. Fibrinogen concentration is frequently decreased but may remain within normal limits in the chronic or compensated low-grade forms of DIC. Because fibrinogen may be markedly elevated initially in patients with sepsis, neoplasia, or other inflammatory disorders, the concentration of fibrinogen may not fall below the reference range, at least early during the course of DIC, despite substantial decreases. Because of the presence of FDPs, the concentration of fibrinogen as determined by assays based on the thrombin clotting time of diluted plasma samples (Clauss-type assays) will be lower than those determined by immunologic techniques. The thrombin clotting time may also be prolonged due both to the decrease in fibrinogen and to the presence of FDPs. Frequently, the results of the first DIC panel are normal or inconclusive. Repetition of the test battery may disclose changes over time (e.g., decreased platelets or fibrinogen) consistent with DIC.

When these screening tests are positive in an appropriate clinical setting, the diagnosis of DIC is usually confirmed by measurement of the concentration of FDPs. This determination is most commonly performed on a plasma sample collected in the presence of plasmin inhibitors to prevent in vitro fibrinogenolysis. The specimen is subsequently clotted in the laboratory with thrombin. The resulting serum sample is assayed for the presence of fibrinogen fragments D and E and sometimes other fibrinogen-related antigens by a latex agglutination procedure using particles coated with antibodies directed against the fibrinogen-related antigens. Obviously, the assay detects degradation products originating from either fibrinogen or fibrin because the antigens are expressed on both. It should also be apparent that samples that clot poorly, for example, due to the presence of a high concentration of heparin or low concentration of fibrinogen, may result in artifactually elevated levels of FDPs.

Several monoclonal antibodies directed against the fibrin-specific degradation product, D-dimer, have been used in latex agglutination assays similar to those described above. Comparisons of the D-dimer and FDP assays have led to different conclusions. Carr et al. [97] believe that the D-dimer assay is more specific but less sensitive than the FDP assay, and they recommend using the D-dimer to confirm a positive FDP result. However, Wilde et al. [98] and Boisclair et al. [99] concluded that both assays were 100% sensitive in patients fulfilling their clinical criteria for DIC. Finally, Greenberg et al. [100] reported considerable discordance between the results of the two tests in patients suspected of having DIC. Critical evaluation of these studies is hampered by variability in patient selection and by the inconsistent use of

immunoblotting as the gold standard for detection of FDP. Neither the D-dimer nor the FDP assay distinguishes between pathologic and physiologic fibrinolysis and may be elevated due to recent trauma, surgery, and thrombotic events. One advantage of the D-dimer assay is the convenience of performing the test on plasma, instead of having to perform the extra step of preparing serum, because fibrinogen and fibrin do not cross-react with the D-dimer monoclonal antibody. In addition to the semiquantitative latex agglutination methods, quantitative and automated assays have been introduced for both the D-dimer and FDP tests.[101] [102] No compelling data have been found to support the use of both tests in the laboratory evaluation of a patient with suspected DIC. Cost, convenience, and test volume are appropriate factors to consider when deciding which test and method to implement.

Bleeding due to hyperfibrinogenolysis without thrombin generation is a rare complication of various acute and chronic conditions. Typical laboratory findings are normal platelet count, decreased fibrinogen and plasminogen levels, and shortened euglobulin lysis time. Because fibrinogen degradation is increased, FDP levels are elevated. However, because fibrin degradation is not increased, D-dimer levels are not increased.

A number of more sophisticated laboratory determinations have been useful in examining and defining the pathophysiology of DIC. These include decreased plasma concentrations of antithrombin III, $_2$ -antiplasmin, factors V and VIII, and protein C. However, these determinations are not commonly used clinically

to establish the diagnosis of DIC.[53] [93] Concentrations of fibrinopeptides A and B increase in DIC, directly reflecting the action of thrombin on fibrinogen. Elevated levels of the fibrinopeptides are not, however, specific for DIC. The role of this determination in the clinical evaluation of suspected DIC has not been established. The unavailability of the results of most of the above tests in a sufficiently short time interval represents a major impediment to their use in the evaluation of DIC.

Paracoagulation tests such as the protamine sulfate precipitation test or the ethanol gelatin test, which detect qualitatively the presence of circulating complexes of fibrinogen, fibrin monomer, and the higher molecular-weight degradation products, lack sufficient sensitivity and specificity and are not recommended for routine use in the evaluation of suspected DIC.

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Chapter 107 - Structure, Biology, and Genetics of Factor VIII

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INTRODUCTION

Hemophilia A, a bleeding disorder resulting from a deficiency in factor VIII, was documented more than 1,700 years ago in the Talmud. [1] The genetics of hemophilia A was described during the early 1800s [2] and transfusion of whole blood was shown to treat a hemophilia-associated bleeding episode successfully by 1840. [3] The presence of factor VIII in plasma was demonstrated in 1911 [4] and in 1937 Patek and Taylor described its role in hemostasis. [5] In the early 1980s factor VIII was purified from human plasma [6] and subsequently the factor VIII gene was cloned. [7] [8] These developments have dramatically increased our understanding of the structure and function of factor VIII in blood coagulation and have provided a recombinant-derived source of factor VIII that is free of human plasma-borne viruses. [9] [10]

The relationship between hemophilia A and von Willebrand disease (vWD) was confused for many years because the autosomally inherited vWD is associated with factor VIII deficiency, although the hereditary factor VIII deficiency of hemophilia A is

an X-linked disease. Early preparations of antihemophilic factor were demonstrated not only to correct the clotting time of hemophilic plasma, but also to restore platelet adhesion and aggregation defects in the plasma from patients with vWD. It is now known that factor VIII and von Willebrand factor (vWF) are two separate proteins that exist as a complex in plasma and that are under separate genetic control. These proteins have distinct biochemical and immunologic properties and have unique and essential physiologic functions. Factor VIII is an X-linked gene product that accelerates the activation of factor X by factor IXa in the presence of calcium and phospholipid. vWF is an autosomal gene product that is essential for platelet adhesion to subendothelium and for ristocetin-induced platelet agglutination. Because vWF and factor VIII are found in the plasma as a complex and vWF stabilizes factor VIII and regulates its activity, the activities of these two proteins are intimately intertwined. Based on the greater understanding of factor VIII and vWF, in 1985 the Subcommittee on Factor VIII and vWF of the International Committee on Thrombosis and Haemostasis formulated nomenclature guidelines. [11] Factor VIII protein is designated VIII; factor VIII antigen is designated VIII:Ag; factor VIII procoagulant activity is designated VIII:C; von Willebrand factor is designated vWF; and von Willebrand factor antigen is designated vWF:Ag.

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FACTOR VIII FUNCTION

The physiologic response to blood vessel injury is the sequential activation of plasma proteases within the blood coagulation cascade, leading to the localized generation of thrombin and the conversion of fibrinogen to fibrin. Thrombin generation requires the interaction of proteases, protein cofactors, and substrate zymogens that assemble on a phospholipid surface or the cell surface. Factor VIII is proteolytically activated by factor Xa or thrombin to yield factor VIIIa. Factor VIIIa serves as a cofactor for factor IXa-mediated activation of factor X. Subsequently factor Xa acts in the presence of activated factor V, negatively charged phospholipids, and calcium to convert prothrombin to its active enzymatic form thrombin.

The mechanism by which factor VIIIa functions in the factor Xa-generating complex is poorly understood. Factor VIII has no enzymatic activity of its own but acts as a cofactor to increase the V_{max} of factor X activation by factor IXa by 10,000-fold in the presence of negatively charged phospholipids and calcium. [12] The mechanism by which the homologous cofactor, factor V,[13] [14] functions in the prothrombin-generating complex has been more extensively studied. Prothrombinase assembly on the surface of phospholipid vesicles requires initial protein-phospholipid rather than protein-protein interactions. The rate-limiting step is the binding of factor Xa and factor Va to separate sites on the phospholipid surface. The proteins then react rapidly by restricted diffusion on the two-dimensional surface of the phospholipid vesicle to form the prothrombinase complex.[15] [16] The association of factor Va with factor Xa on a phospholipid surface induces an allosteric conformational change in the active site of factor Xa to position the active site at a proper distance above the membrane for optimal prothrombinase activity. [15] The mechanism by which factor VIIIa accelerates the proteolysis of factor X by factor IXa also involves the interaction of factor VIIIa and factor IXa on a phospholipid surface to facilitate a conformational change in the enzyme that favors catalysis.[17]

The reported specific activity of pure factor VIII ranges from 2,300 U/mg [6] to 8,000 U/mg.[18] The definition of factor VIII activity is complicated because thrombin converts the cofactor into a much more active form. However, for standardization, 1 U of factor VIII is defined as that amount of activity in 1 ml of normal pooled human plasma measured in a factor VIII assay using factor VIII-deficient plasma.[19] For greater convenience and precision, factor VIII activity can be measured by its ability to promote activation of factor X in the presence of factor IXa, phospholipid, and calcium ions. Factor Xa is measured directly by monitoring cleavage of a synthetic chromogenic substrate.[20] Factor VIII antigen can be measured using factor VIII-specific antibodies in specific immunoassays. [21]

Characterization of factor VIII was hampered by its low concentration in plasma, its heterogeneity in size, and its exquisite sensitivity to degradation. Significant advances in our understanding of factor VIII resulted from the use of immunoaffinity chromatography for the successful purification of factor VIII from porcine [22] and human[6] [23] plasma, and from the cloning of the human factor VIII gene and elucidation of the primary structure of factor VIII. [7] [8]

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FACTOR VIII PROTEIN STRUCTURE

The deduced primary amino acid sequence of human factor VIII determined from the cloned cDNA demonstrated that factor VIII is encoded by a precursor protein of 2,351 amino acid residues from which a 19-amino acid signal peptide is cleaved on translocation into the endoplasmic reticulum (ER). Plasma factor VIII is a heterodimer processed from a larger precursor polypeptide. It consists of a COOH-terminal-derived light chain of 80,000 molecular weight (MW) in a metal ion-dependent association with an NH_2 -terminal-derived heavy chain fragment of 90,000 to 200,000 MW. [7] [24] In the plasma this complex is stabilized by association through hydrophilic and hydrophobic interactions with a 50-fold excess of vWF. The amino acid sequence revealed an organization of three structural domains that occur in the order A1:A2:B:A3:C1:C2, as shown in Fig. 107-1 .[7] [24] The A1 (amino acid residues 1329) and A2 (380711) domains of factor VIII occur in the heavy chain and the A3 (16492019) domain occurs in the light chain. The A domains have 30% identity to each other and to the triplicated A domains of ceruloplasmin and factor V and to the single A domain of ferroxidase. [7] [25] [26] The residues implicated for copper binding in ceruloplasmin are conserved in the first and third A domains of factor VIII, suggesting the A domains of factor VIII may be involved in metal ion binding. Copper ions were detected in purified preparations of factor V [27] and factor VIII,[28] and most data support that a single molecule of reduced copper (CuI) resides within a type-1 copper ion-binding site (composed of ligands His265Cys310His315Met320) in the factor VIII A1 domain.[29] Mutagenesis of the single cysteine at residue 310 to a serine, a mutation that would destroy copper ion binding to the type-1 site in the A1 domain, destroyed factor VIII activity and yielded secreted heavy and light chains that were not associated. [29] The C1 (residues 20202172) and C2 (residues 21732332) domains occur in the COOH-terminus of the factor VIII light chain and exhibit homology to milk fat globule protein and to A, C, and D chains of discoidin 1, all proteins that are capable of binding glycoconjugates and negatively charged phospholipids. [30] [31] The B domain is encoded by a single large exon of 3,100 nucleotides, has no known homology to other proteins, and contains 18 of the 25 potential asparagine (N)-linked glycosylation sites within factor VIII. Comparison to the cloned factor V cDNA and gene revealed a high degree of amino acid conservation between the A and C domains with no detectable homology within the B domains,[25] although both B domains are encoded by large single exons. The domain organization and homologies between factors V and VIII suggests that these genes evolved from a primordial ferroxidase gene by triplication of the A domain, insertion of the B domain, and addition of the two C domains. After duplication of the primordial cofactor gene, the factor V and factor VIII genes likely evolved by extensive divergence of amino acid residues within the B domain while amino acid residues within the A and C domains were conserved.

Figure 107-1 Domain structure and processing of factor VIII. The structural domains of factor VIII are depicted in the top line: (1) a triplicated A domain of 330 amino acids; (2) a unique B domain of 980 amino acids; and (3) a duplicated C domain of 150 amino acids. The hatched areas represent highly acidic regions. The location of free cysteine residues and disulfide bonds (brackets below line), potential asparagine-linked glycosylation sites, and sites of tyrosine sulfation are shown. Within the Golgi compartment in the cell, factor VIII is cleaved within the B domain to generate a 200-kDa peptide and the 80-kDa light chain polypeptide. The thrombin (IIa), activated protein C (APC), and factor Xa (Xa) cleavage sites are shown. Factor Xa also cleaves at all the thrombin cleavage sites. The two cleavages required for thrombin activation (**) and the sites of proteolytic inactivation by APC (*) are also shown.

In addition to the A, B, and C domains, there are three acidic amino acid-rich regions in the factor VIII molecule at the junction of the A1/A2 (residues 331372), A2/B (residues 700 740), and B/A3 (residues 16491689) domains and are juxtaposed to sites of thrombin cleavage (Fig. 107-1). All these acidic regions also contain the post-translationally modified amino acid, tyrosine sulfate, at residues 364, 718, 719, 721, 1664, and 1680. [32] The murine factor VIII cDNA is 8493% identical to the A and C domains of human factor VIII, whereas the acidic regions and B domain are only 4270% identical. [33] However, all thrombin cleavage sites and sulfated tyrosine residues are conserved. The murine factor VIII B domain has conserved 19 potential N-linked glycosylation sites, although in different positions than in the human sequence, suggesting that glycosylation in the B domain is important for factor VIII expression and function.

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BIOSYNTHESIS AND METABOLISM OF FACTOR VIII

The natural cell type that produces factor VIII has not been definitively identified. However, evidence obtained from liver transplantation in factor VIII-deficient dogs [34] [35] [36] and several hemophiliac patients[37] [38] strongly implicates that the liver and the reticuloendothelial system are primary sites of factor VIII synthesis. In addition, immunochemical localization by light microscopic[39] or electron microscopic[40] examination detected the factor VIII antigen in hepatocytes. However, RNA hybridization analysis has detected factor VIII mRNA in hepatocytes and in many other cells and tissues, with the spleen being the most abundant source. [41] To date there are no known established cell lines that express factor VIII. Thus, it has not been possible to study the biosynthesis of factor VIII in its natural host cell. However, the expression of factor VIII in mammalian cells transfected with the factor VIII gene allowed analysis of the biosynthesis and processing of this glycoprotein.[42]

Factor VIII Secretion

Analysis of the expression of factor VIII in Chinese hamster ovary cells provided insights into a proposed biosynthetic pathway (Fig. 107-2). On synthesis, factor VIII is translocated into the lumen of the ER where the signal peptide is cleaved. In the ER, addition of high mannose-containing core oligosaccharides to multiple asparagine residues within the factor VIII molecule occurs. A significant portion of the factor VIII in the ER is bound to resident proteins of the ER that include the glucose-regulated protein of 78,000 MW (GRP78), also known as immunoglobulin-binding protein, or BiP, [43] [44] and two lectin-binding proteins calnexin and calreticulin.[45] [46] Factor VIII undergoes cycles of binding and release from these protein chaperones and binding correlates with retention within the secretory pathway (Fig. 107-2). BiP expression is induced by glucose deprivation, inhibition of N-linked glycosylation, or the presence of malfolded protein within the ER. [47] [48] In addition, high-level factor VIII expression can also induce transcription of the BiP gene. [49] The level of BiP in the cell inversely correlates with the efficiency of factor VIII secretion.[50] [51] BiP exhibits a peptide-dependent adenosine triphosphatase activity. [52] Factor VIII dissociation from BiP and secretion requires an unusually high level of intracellular adenosine triphosphate. [53] Because factor VIII interacts with BiP, and factor V does not, a primary BiP-binding site was mapped by analysis of the secretion of factor VIII and factor V chimeric proteins. This study identified a hydrophobic pocket within the factor VIII A1 domain that occurs within the type-1 copper ion binding site. [54] Hydrophobic residues are preferred sites of BiP binding. [55] Mutation of a single phenylalanine at residue 309 to serine within the hydrophobic pocket, the residue adjacent to cysteine 310 that is part of the copper ion binding site, increased the secretion efficiency of factor VIII several fold and this correlated with a reduced requirement for adenosine triphosphate for secretion. [54] These studies suggest that BiP may provide a role in copper ion binding to factor VIII.

A significant portion of factor VIII within the ER never transits to the Golgi compartment, but rather is degraded within the cell (Fig. 107-2). Proteolysis of intracellular factor VIII is inhibited by lactacystin, a microbiotic that specifically modifies the active site of the proteasome, although secretion of factor VIII is not improved. [45] These results indicate that factor VIII within the ER is directed to the cytosolic 26S proteasomal machinery for degradation. The portion of factor VIII that is secretion competent transits to the Golgi apparatus, where the majority of factor VIII is processed at two sites within the B domain after residues 1313 and 1648 to generate the heavy chains (90,000200,000 MW) and the light chain (80,000 MW). Also within the Golgi apparatus, factor VIII is further processed by (1) modification of the asparagine-linked high mannose-containing oligosaccharides to complex types, (2) addition of carbohydrate to multiple serine and threonine residues within the B domain, and (3) addition of sulfate to six tyrosine residues within the heavy and the light chains. [56]

Stabilization of Factor VIII by vWF

Numerous in vivo observations support that the level of factor VIII and vWF in plasma is maintained at a fairly constant

Figure 107-2 Synthesis, processing, and secretion of factor VIII in mammalian cells. The factor VIII primary translation product is translocated into the lumen of the ER where asparagine-linked glycosylation occurs. A fraction of factor VIII binds tightly to the glucose-regulated protein of 78 kDa (GRP78 or BiP) and is probably destined for degradation. A proportion of the molecules transit to the Golgi apparatus where complex modification of carbohydrate on asparagine-linked sites, addition of carbohydrate to serine and threonine residues, sulfation of tyrosine residues, and cleavage of the protein to the mature heavy and light chains occur. The presence of vWF in the medium promotes stable secretion of factor VIII. In the absence of vWF, the individual chains are secreted and degraded. The A (crosshatched), B (solid), and C (dotted) domains are indicated. The acidic regions where tyrosine sulfation occurs adjacent to the A domains are shown (solid). *(From Kaufman et al.,[42] with permission.)*

ratio of 1:50. For example, patients with type 1 vWD who have a 50% reduction in vWF have a corresponding 50% reduction in factor VIII. Autosomally inherited factor VIII deficiency results from missense mutations in the first 91 amino acids of mature vWF, called vWD type Normandy or type 2N, that reduces its ability to bind and stabilize factor VIII in plasma.[57] [58] Infusion of vWF into vWF-deficient patients elicits an immediate rise in circulating levels of factor VIII. In addition, increase in the plasma concentration of vWF following infusion with 1-desamino-8-D-arginine vasopressin (DDAVP) elicits an increase in the plasma level of factor VIII. [59] It is established that vWF stabilizes factor VIII in plasma. Factor VIII and vWF are cleared with a half-life of 12 hours on infusion of the factor VIII1/vWF complex into patients with hemophilia.[60] [61] [62] Infusion of factor VIII into factor VIII-deficient patients also exhibits clearance kinetics similar to that of factor VIII/vWF complex, presumably due to rapid binding of the factor VIII to plasma vWF. [63] By contrast, infusion of factor VIII into patients with severe vWD results in rapid clearance with a half-life of 2.4 hours.[60] [64] Therefore, preparations of factor VIII that contain minimal amounts of vWF are ineffective in the treatment of patients with severe vWD. [65] These studies establish the stabilizing influence of vWF for factor VIII in the circulation. However, it is not known whether vWF also regulates factor VIII synthesis or secretion.

In vitro studies demonstrate that vWF can alter intracellular transport and secretion of factor VIII from the cell. vWF promotes the association of the light and heavy chains of factor VIII and results in stable accumulation of factor VIII activity in the conditioned medium of factor VIII-producing cultured mammalian cells. [42] [66] In the absence of vWF in the conditioned medium, the factor VIII heavy and light chains are secreted into the medium as dissociated chains that are subsequently degraded. In vitro reconstitution experiments demonstrated that vWF can directly promote reassembly of isolated heavy and light chains of factor VIII, [66] [67] suggesting a possible role of vWF in facilitating factor VIII assembly. The effect of vWF in promoting factor VIII heavy and light chain assembly and stable secretion in cell culture systems may reflect the role of vWF in regulating levels of factor VIII activity in vivo. [60] [61] [62] [63] [64] The immediate rise in vWF and factor VIII observed after administration of DDAVP suggests the existence of a pool of factor VIII/vWF complex that is DDAVP releasable. [68] Recently, DNA transfection experiments demonstrated that vWF co-expressed with factor VIII in endothelial cells can direct factor VIII into intracellular storage granules (Weibel-Palade bodies) that are released on stimulation,[69] demonstrating the potential for a factor VIII/vWF complex to be released from the cell on stimulation. However, at present there are no known natural cell types that express both factor VIII and vWF.

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INTERACTIONS OF FACTOR VIII WITH COMPONENTS OF HEMOSTASIS

Binding to vWF

In addition to increasing factor VIII survival, vWF plays a critical role in directly regulating factor VIII activity. [70] vWF prevents factor VIII binding to phospholipids [3] [71] [72] and activated platelets.[73] vWF inhibits factor VIII from activation by factor Xa, [74] from inactivation by activated protein C(APC) [74A] [74B] but does not interfere with activation by thrombin.[75] [76] It is likely that vWF mediates its inhibitory effects by preventing factor VIII interaction with phospholipids, a reaction required for both factor Xa and APC-mediated cleavage of factor VIII. It is proposed that vWF binding to platelet receptor GP1b brings factor VIII to the vicinity of platelets adhering to damaged endothelium.[73]

The affinity and stoichiometry of the factor VIII-vWF interaction has been extensively studied. [70] Binding assays that immobilize vWF onto plastic have yielded stoichiometries of one molecule of factor VIII:50100 monomers of vWF. [77] [78] In contrast, gel filtration, ultracentrifugation, and immobilization of vWF on colloidal gold or agarose microspheres yield stoichiometries close to 1:1. [78] [79] [80] [81] It appears that immobilization of vWF onto plastic alters the conformation to prevent most of the sites from being occupied. To date, these in vitro studies have not identified the mechanism by which the stoichiometry in plasma is maintained at 1:50. [63] In contrast, the dissociation constant for the vWF-factor VIII interaction is similar for all the methods described (range, 0.20.4 nM). Binding occurs within seconds. [70] [82]

The interaction of factor VIII with vWF is mediated by a major factor VIII binding site that resides within the first 272 amino acids of the mature vWF molecule [83] [84] [85] with residues 7896 and residues around 53 providing crucial roles. [85] [86] Type N vWD results from specific missense mutations within this region of vWF that cause a deficiency in factor VIII due to decreased factor VIII binding. [58] The vWF propeptide serves an essential role in multimerization of vWF, [87] a process that leads to expression of a high affinity factor VIII binding site. [78] Expression of propeptide-deleted vWF does not produce multimeric vWF, but rather produces dimeric vWF through COOH-terminal disulfide bonds. This dimeric form of vWF binds factor VIII with a sixfold reduced affinity. [78] Co-expression of the vWF propeptide promotes generation of a high affinity factor VIII binding site. However, within native pro-vWF, the propeptide needs to be cleaved to expose the factor VIII binding site. [78]

The vWF binding site on factor VIII requires both the NH_2-terminal and COOH-terminal ends of the factor VIII light chain. A vWF binding site on factor VIII was localized by monoclonal antibody inhibition to residues 16731684. [88] [89] This region is composed of a high density of acidic amino acids located at the NH_2-terminus of the factor VIII light chain and is removed by thrombin cleavage at residue 1689 (Fig. 107-1). Deletion of the acidic region in the NH_2-terminus of the light chain by site-directed mutagenesis yielded a molecule that bound vWF with a 10-fold reduced affinity, although the mutant protein had a specific activity similar to wild-type factor VIII.[73] [90] These results demonstrate that the acidic region between residues 1649 and 1689 of the light chain is critical for appropriate interaction with vWF but is not required for cofactor function of factor VIII. Recently, it was demonstrated that antibodies that interact with residues 22482312 within the factor VIII C2 domain can inhibit factor VIII binding to vWF and to phosphatidylserine. [91] This observation is consistent with the presence of a phospholipid-binding region between residues 2303 and 2332 within the C2 domain[123] and that phospholipid and vWF compete for binding to factor VIII. [71] [72] [81] It is proposed that a high affinity vWF binding site requires interactions at both the NH_2-terminal and COOH-terminal region of the factor VIII light chain. [93] [94] However, an anti-factor light chain antibody that interacts with a C2-domain epitope between residues 2248 and 2285 was able to increase vWF binding to a factor VIII molecule that had deleted the acidic region at the NH_2-terminus of the factor VIII light chain.[90] This indicates that the acidic region may not directly contact vWF, but may be required to induce a conformation at the COOH-terminus of the light chain that is capable of high affinity vWF interaction.

Factor VIII is post-translationally modified by sulfation on tyrosine residues 346, 718, 719, 721, 1664, and 1680. [32] Inhibition of tyrosine sulfation by treatment of factor VIII-expressing cells with sodium chlorate did not affect factor VIII secretion but reduced the specific activity of the factor VIII by fivefold, indicating that this modification is required for full cofactor activity. [32] The importance of this post-translational modification was also studied by the conservative mutation of tyrosine residues to phenylalanine residues to block sulfation. Tyrosine to phenylalanine mutations at residues 346 and 1664 reduced the rate of thrombin cleavage and activation.[95] Tyrosine to phenylalanine mutation at residue 1680 reduced interaction with vWF by fivefold. [95] [96] In addition, a patient with the 1680 tyrosine to phenylalanine mutation had a fivefold reduction in factor VIII antigen and activity, likely due to a defect in vWF binding. [97] These experiments show that post-translational sulfation of tyrosine residues affects factor VIII procoagulant activity and interaction with vWF.

Factor VIII Molecular Interactions in the Factor Xase Complex

Due to the lability of thrombin-activated human factor VIIIa, most binding studies have been performed with intact factor VIII. Because it is known that factor VIII activation influences the activity of factor IXa, [17] it must be considered that changes occur in binding affinities or sites of interactions on factor VIII activation. The interaction of factor VIIIa on the influence of factor IXa conformation was studied using fluorescently labeled factor IXa. These studies demonstrated that the A2 domain of factor VIIIa is required to induce a maximal conformational change in factor IXa. [17] [98] Peptide inhibition studies showed that a factor IXa binding site is localized to factor VIII residues 558 to 565 within the A2 domain. [99] [100] Monoclonal antibody inhibition experiments indicate that factor IXa binding site also exists between residues 1770 and 1840 of the factor VIII light chain, [101] and this site was further localized to residues 18111818 using synthetic peptides. [102] It is suggested that the higher affinity binding site on the factor VIII light chain interacts with the first epidermal growth factor (EGF)-like domain in factor IXa, and the weaker factor IXa interaction site within the A2 domain might induce a conformational change within the factor IXa active site. [100] [103]

The binding of substrate factor X to factor VIII has been studied by fluorescence anisotropy, solid-phase binding assays, cross-linking, and functional assays. [9] [104] The findings demonstrate that a factor X interactive site is contained within the acidic region at the COOH-terminus of the A1 subunit. The activation of factor VIII does not appear to be required for exposure of the factor X binding site. The interaction is sensitive to high ionic strength, is calcium dependent, and has a dissociation constant that is approximately several micromolars.[104]

Factor VIII Interaction with Phospholipids

Phospholipids interact with substrates, enzymes, and cofactors to play a critical role in the assembly and functional activity of the coagulation protease complexes. Negatively charged phospholipids are required for factor VIIIa-mediated enhancement of the activation of factor X. [12] [105] [106] In vivo the negatively charged phospholipids are likely provided by activated platelets and damaged endothelial cells. Factor VIII and factor V bind phosphatidylserine

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by both hydrophobic and electrostatic interactions. [107] [108] [109] [110] [111] [112] [113] However, factor V does not efficiently compete with the binding of factor VIII to phospholipid vesicles composed of 15% phosphatidyl-L-serine. [105] Under equilibrium conditions, factor VIII can bind phospholipid vesicles containing 1525% phosphatidylserine with an apparent Kd of 24 nM. [81] [105] [114] Saturation occurs at 170385 moles of phospholipid per mole of factor VIII, [105] although the process

involves both rapid and slow interactions. [115] Factor VIII binding to phospholipid involves stereoselective recognition of the O-phospho- L-serine moiety of phosphatidylserine. [81] Factor V displays a similar affinity to phospholipids but has a lower requirement for phosphatidylserine. Because the composition of phosphatidylserine exposed on the platelet membrane surface can increase from 2% to 13% after stimulation, [116] the increase in phosphatidylserine content could account for the ability for factor VIII to specifically bind the surface of thrombin-activated platelets.

Addition of negatively charged phospholipids to the factor VIII/vWF complex dissociates factor VIII from vWF. [71] [72] Thrombin-treated factor VIII does not bind vWF with high affinity, [117] [118] [119] but it does retain phospholipid-binding properties. [72] The phospholipid-binding domain within factor VIII is localized to the light chain, [120] [121] and antibody inhibition studies suggest that the phospholipid binding site likely occurs in the C2 domain. [122] Peptides corresponding to the COOH-terminus of factor VIII (residues 23032332) inhibit the interaction of factor VIII with phospholipid. [123] Deletion analysis also suggests a phospholipid-binding domain resides in the factor V C2 domain. [124] In contrast, a proteolytic fragment of the factor V A3 domain inhibits factor V binding to phospholipid, [125] suggesting a phospholipid-binding site resides in the NH_2 -terminal end of the factor Va light chain. Thus, at present it is unclear whether the phospholipid-binding sites of factors V and VIII occur at the same positions within the proteins.

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REGULATION OF FACTOR VIII ACTIVITY

Activation of Factor VIII

On treatment of intact factor VIII with thrombin or factor Xa, there is a rapid 30-fold increase and subsequent first-order decay of procoagulant activity. However, the maximal activation by thrombin is greater than that observed with factor Xa.[126] The activation coincides with proteolysis of both the heavy and light chains of factor VIII as depicted in Figure 107-1 . Cleavage within the heavy chain after arginine residue 740 generates a 90,000-MW polypeptide that is subsequently cleaved after arginine residue 372 to generate polypeptides of 50,000 MW and 43,000 MW. [127] Concomitantly, the 80,000-MW light chain is cleaved after arginine residue 1689 to generate a 73,000-MW polypeptide.[127] Each of these cleavage sites is bordered by a region rich in acidic amino acids that also contain the post-translationally modified amino acid tyrosine-sulfate.[32] The tyrosine-sulfate residues enhance thrombin cleavage at adjacent sites but do not affect factor Xa cleavage. [95] Factor V also contains tyrosine sulfate adjacent to thrombin cleavage sites and this modification also facilitates thrombin activation without affecting factor Xa activation. [128] These observations suggest that thrombin selectively utilizes tyrosine sulfate residues to facilitate interaction or cleavage. Most data support that thrombin anion-binding exosites I and II are involved in recognition of factor VIII and factor V. For example, thrombin activation of factor VIII is inhibited by either heparin, a molecule that interacts with anion-binding exosite II, or hirugen, a peptide that contains tyrosine sulfate and binds the anion-binding exosite I of thrombin. [129] [130]

Numerous studies correlated the appearance of 90,000-, 50,000-, 43,000-, and 73,000-MW polypeptides with peak factor VIII activity. [127] [131] [132] Mutagenesis studies showed that cleavages after residues 740 and 1648 were not required for cofactor activity. [133] In contrast, mutation at either arginine 372 or arginine 1689 yielded molecules that were not cleaved by thrombin at the mutated site and were not susceptible to thrombin activation. [133] [134] The importance of cleavage at residues 372 and 1689 for activation of factor VIII was also elucidated by the identification of hemophilia A patients having missense mutations that prevent cleavage at either residue 372 or residue 1689.[135] [136] These findings indicate that activation requires cleavage at both residues 372 and 1689, but does not appear to require a specific sequential order for cleavage at these sites. Cleavage at 1689 releases factor VIII from the inhibitory influence of vWF. [137] However, cleavage 1689 appears to additionally increase the activity of factor VIII in the absence of vWF. [138]

The B domain, delimited by amino acid residues 740 and 1648, is cleaved from factor VIII during activation. Comparison of the deduced amino acid sequence of porcine, murine, and human factor VIII showed a striking divergence within the B domains, whereas the bordering A2 and A3 domains exhibit 8085% homology. [24] [33] Factor VIII molecules constructed to lack most or all of the B domain have specific activities, thrombin cleavage products (except for the B domain), and thrombin activation coefficients similar to the wild-type molecule. [139] [140] The B-domain deletion molecules exhibit no detectable difference in vWF binding, survival in plasma, and ability to normalize the cuticle bleeding time after infusion into a factor VIII-deficient dog compared to the wild-type factor VIII. [76] By these analyses, removal of the B domain did not affect in vitro or in vivo procoagulant activity. In addition, B domain-deleted factor VIII has recently been used in clinical studies to demonstrate correction of bleeding episodes associated with hemophilia A. [141] One notable difference between wild-type and B domain-deleted factor VIII is that the B domain-deleted molecule is expressed at 510-fold higher levels, exhibits a reduced association with BiP in the ER, and is secreted more efficiently. [44] [76] This suggests that the B domain may regulate factor VIII biosynthesis. It is also possible that the B domain may have procoagulant, anticoagulant, or vasoactive properties heretofore unknown. For example, a portion of the B domains of factor V and factor VIII can serve as a substrate for transglutaminase activity of factor XIII. [142] [143]

Inactivation of Factor VIII

The activity of thrombin-activated factor VIII requires all polypeptides of the heterotrimer composed of the 50,000-, 43,000-, and 73,000-MW species. [144] [145] [146] The 50,000-MW A1 domain is in a Cu(I) ion-dependent association with the 73,000-MW light chain. The 43,000-MW A2 domain polypeptide is associated with the A domain by electrostatic interactions that likely involve residues 336 to 372 at the COOH-terminus of the 50,000-MW fragment. [147] [148] A detailed characterization of thrombin-activated factor VIIIa was hampered due to its marked instability that results from reversible dissociation of the 43,000-MW A2 domain polypeptide that occurs at physiologic pH.[147] [149] [150] Once activated by thrombin, the factor VIIIa heterotrimer can be stabilized by the addition of factor X and phospholipid. [151] The specific activity of porcine factor VIII is approximately fivefold greater than human factor VIIIa, and this correlates with a lower dissociation rate constant of the A2 domain polypeptide from the thrombin-activated heterotrimer.[150] [152] Covalent attachment of the A2 domain to the light chain yielded a factor VIII molecule that was stable after thrombin activation.[90] These results demonstrate the significance of A2 domain dissociation in the inactivation of factor VIII.

Factor VIII and activated factor VIII are also inactivated by proteolytic cleavage. Cleavage after arginine 336 is mediated by factor Xa, [127] factor IXa,[98] [153] APC,[127] [154] and also thrombin[32] in the presence of anionic phospholipids and correlates with inactivation of factor VIII. Additionally, APC cleaves the heavy chain

after residue 562,[155] and this cleavage at least partially inactivates factor VIII. [156] Finally, factor IXa and factor Xa cleave the light chain after residue 1719 and 1721, respectively,[127] [153] and at least cleavage at 1721 does not appear to significantly reduce factor VIII activity. [157] Factor X binding can prevent factor IXa-and APC-mediated cleavage at Arg 336,[104] and it is proposed that this may provide a mechanism to sustain maximal factor Xase activity until substrate becomes limiting.

Recently, APC resistance was shown to be one of the major causes of hereditary thrombophilia as a consequence of an arginine to glutamine mutation in the APC cleavage site in factor V at residue 506. [159] [160] [161] The APC-resistant phenotype was first reported as a poor anticoagulant response to APC in an activated partial thromboplastin time assay (called the APC resistance ratio). [159] It is possible that APC-resistant mutations in factor VIII may also lead to hereditary thrombophilia. Analysis of mutant factor VIII protein demonstrated that cleavage at either arginine 336 or arginine 562 only partially inactivated factor VIII and that resistance to cleavage at both arginine 336 or arginine 562 was necessary to detect a poor anticoagulant response in an activated partial thromboplastin time assay. [156] These results show that a single mutation in either factor APC cleavage site to yield cleavage resistance would not be detected as a poor anticoagulant response in vitro. In addition, a search did not identify any mutations at arginine 336 and 562 in factor VIII in 125 patients with venous thrombosis. [162] The sum of these studies indicates that it is unlikely mutations in factor VIII APC cleavage sites result in thrombophilia.

Modulation of Factor VIII Activity by Biologic Membranes

Procoagulant activity is profoundly affected by the presence of cellular surfaces. On platelet activation by thrombin, the platelet surface exhibits procoagulant activity. This activity results from the exposure of negatively charged phospholipids and possibly also of specific receptors for the coagulation factors. Negatively charged phospholipids are usually confined to the inner leaflet of cellular membranes [163] [164] and are exposed on cellular lysis at sites of injury. Unactivated platelets exhibit binding sites for factor Va and Xa.[165] [166] [167] Platelet activation is associated with exposure of factor VIII and factor IXa binding sites. [168] [169] [170] [171] There are approximately 400 factor VIII sites per activated platelet and factor V cannot compete with factor VIII binding. [168] The specificity in binding of factor VIII suggests that a specific protein receptor for factor VIII is involved. In addition, the kinetics of thrombin generation mediated by the prothrombinase complex on pure phospholipid surfaces compared to activated platelets suggests that a specific saturable receptor exists for prothrombinase and probably also for the factor Xa-generating enzyme

complex.[172] Platelet binding is mediated by the factor VIII light chain and is inhibited by the presence of vWF. A factor VIII mutant protein that cannot bind vWF with high affinity retains its ability to bind platelets. [73] Thus the vWF binding site and the platelet binding site appear to be distinct within the factor VIII molecule.

The present understanding of the mechanism by which factor VIII functions is presented in Figure 107-3 . The two chains of factor VIII are associated in a monovalent Cu(I)-dependent manner. vWF interacts with the NH_2 -terminus and the COOH-terminus of the light chain. The mechanism by which initial activation of factor VIII occurs is unknown, but evidence suggests that the extrinsic pathway may be the most significant physiologic initiator of factor VIIIa generation. [173] Patients deficient in factor VII have low levels of factor Xa compared with patients with deficiencies in factor VIII. [174] In addition, infusion of VIIa into a chimpanzee increases the level of circulating factor Xa.[175] These results indicate that the primary mechanism of factor Xa generation in vivo is via the extrinsic pathway. Because patients deficient in factor VIII or factor IX do have a bleeding diathesis, there is a need for the intrinsic pathway for hemostasis in vivo (Chap. 102). Two possible mechanisms could explain the need for factors VIII and IX in vivo. First, because factor Xa generation via the tissue factor pathway can bind tissue factor pathway inhibitor and subsequently inhibit the factor VIIa tissue factor complex, only small amounts of factor Xa can be generated before further extrinsic factor X activation is inhibited. [173] [176] Because tissue factor pathway inhibitor does not inhibit the intrinsic formation of factor IXa or its activity, the intrinsic route is required to amplify the response. Alternatively, because the extrinsic pathway can activate factor IX in vitro, [177] this pathway may primarily activate factor IX as opposed to factor X in vivo. Either mechanism could explain the need for factors VIII and IX for effective hemostasis.

Molecular Modeling of Factor VIII

Our knowledge of the structural requirements for factor VIII activity have dramatically increased since the original isolation of factor VIII protein from human plasma and identification of the factor VIII gene. The three-dimensional x-ray structure is now available for factor IXa [178] (Chap. 108). In addition, a molecular model of the A domains of factor VIII, based on the crystal structure of the homologous plasma protein ceruloplasmin, [179] was recently proposed.[180] Although this model comprises only the A domains and lacks the acidic regions and the B and C domains and does not consider what changes occur on activation of factor VIII to factor VIIIa, the three-dimensional model

Figure 107-3 Model for thrombin activation and inactivation of factor VIII. Factor VIII is depicted as two chains in a copper ion (CuI)-dependent association. vWF is illustrated to promote association of the light and heavy chains of factor VIII. Thrombin cleavage at residues 336 and 1689 releases the activated species from the vWF complex. Inactivation may occur through a dissociation of the A2 subunit or by a specific proteolytic event.

is a good basis for further functional studies. The model indicates that the factor IXa-binding sites in the A2 and A3 domains are in close proximity and are exposed at the same side of the molecule. With information obtained from analysis of gene defects in hemophilia A, mutagenesis studies to identify critical regions for factor VIII function, and from biochemical analyses to identify interacting regions between factor VIII and factor IXa and factor X, it should be possible to refine this model to elucidate the basis of the mechanism of action of factor VIII.

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HEMOPHILIA A

The gene for factor VIII is on the human X chromosome and therefore hemophilia A is a classic example of X-linked recessive inheritance. It occurs almost exclusively in males for they have only one X chromosome; females with one abnormal copy of the factor VIII gene are carriers because the other X chromosome contains a normal copy of the gene. The frequency of the disorder is 1/5,00010,000 male births and there is no particular ethnic group that has unusually lower or higher incidence of the disease. The severity and frequency of bleeding in the patients correlates with the factor VIII activity in plasma. [181] [182]

About 5060% of patients have severe hemophilia A with factor VIII activity <2% of normal; these patients have frequent spontaneous bleeding into joints, muscles, and internal organs. Moderately severe hemophilia A occurs in about 2530% of patients; the factor VIII activity is 25% of normal and there is bleeding after minor trauma. Mild hemophilia, which occurs in 1520% of patients, is associated with factor VIII activity of 530% and there is bleeding only after significant trauma or surgery. Individuals with factor VIII activity >30% do not have symptoms of hemophilia A. Of particular interest for the understanding of function of factor VIII is a category of patients who have considerable amount of factor VIII protein in their plasma (at least 30% of normal) but the protein is nonfunctional. Approximately 5% of patients belong to this category, termed cross-reacting material (CRM) positive. [183] CRM reduced is another category in which the factor VIII antigen and activity are reduced to approximately the same level.

Before the introduction of modern treatment, severe hemophilia A was a genetically lethal disease in which affected males produced few offspring. Therefore, nearly one-third of the mutant alleles would be lost in each generation. In 1935, Haldane predicted that to maintain a constant frequency in the population about one-third of cases would be the result of novel mutations. The prediction was proven to be correct because a large number of different mutations have been found in the factor VIII gene and many patients have been identified who carry a de novo mutation not present in the X chromosome of their mothers. One notable exception to the Haldane prediction, however, is the case of the common inversion of factor VIII.

FACTOR VIII GENE STRUCTURE AND LOCATION

The factor VIII gene is 186 kb long (approximately 0.1% of the DNA of the X chromosome) and contains 26 exons. The nucleotide sequences of the exons, intron-exon boundaries, and 5 and 3 untranslated regions have been determined. [8] [24] [184] The exon length varies from 69 to 262 nucleotides except for exon 14, which is 3,106 nucleotides, and the last exon 26, which has 1,958 nucleotides (Fig. 107-4). There are some large intervening

Figure 107-4 Schematic representation of chromosomal localization and structure of the factor VIII gene. The gene is located about 1,000 kb from the Xqter (panel B). It is 186 kb long and contains 26 exons (panel C). In the large intron 22 there are two nested genes, the intronless F8A and F8B, which utilize the exon 23 of factor VIII gene as its second exon (panel D). There are three copies of the F8A-F8B sequences on Xq28 as shown by the gray boxes of panel B. See text for further discussion of the gene structure.

sequences such as IVS22, which is 32 kb, and IVS1, IVS6, IVS13, IVS14, and IVS25, which are 1423 kb long.

The normal factor VIII mRNA is approximately 9 kb, of which the coding sequence is 7,053 nucleotides. There is a CpG island within IVS22, which is associated with two additional transcripts. One transcript of 1.8 kb is produced abundantly in a wide variety of cells. The orientation of this transcript is opposite to that of factor VIII and contains no intervening sequence.[185] This 1,739-nt long cDNA has been termed factor VIII-associated gene A (F8A) and is conserved in the mouse. [186] The second transcript of 2.5 kb is transcribed in the same direction as factor VIII and after a short exon that may encode for eight amino acids, it utilizes exons 2326 of the factor VIII gene.[187] This gene has been termed factor VIII-associated gene B (F8B). The two transcripts F8A and F8B originate within 122 bases from each other. The sequences of F8A and F8B along with few kilobases of surrounding DNA are also present in two other areas of the X chromosome approximately 400 kb telomeric to factor VIII gene.[185] [188] The function of these transcripts and their potential protein products are unknown.

The promoter (5 flanking) region of factor VIII has been studied by transient transfection studies in liver-derived cell lines, DNase I footprinting, electrophoretic mobility shift assays with nuclear extracts and purified transcription factors, and mutation analysis. DNase footprinting showed the presence of more than 12 protein-binding sites distributed up to 1 kb upstream of the transcription start site. A region up to 300 nucleotides contains all the necessary elements for maximal promoter activity.[189] [190] Mutation analysis suggested that the putative TATA box is not essential for factor VIII promoter activity. Liver-enriched transcription factors such as HNF1, NFkB, C/EBPa, and C/EBPb interact with the factor VIII promoter region.[189]

The factor VIII gene maps on the long arm of the X chromosome, in the most distal band Xq28. Haldane and Smith[191] reported linkage of hemophilia A with color blindness and Boyer and Graham[192] demonstrated close linkage of hemophilia A with polymorphisms at the glucose-6-phosphate dehydrogenase (G6PD) locus. Additional studies confirmed the close linkage of factor VIII with G6PD. [193] Patterson et al.[194] showed that G6PD and factor VIII genes lie within 500 kb of each other. Pulsed field gel electrophoresis and physical mapping of Xq28 using yeast artificial chromosomes suggested that the factor VIII gene mapped distal to G6PD. [188] [195] The order of these loci and the direction of transcription is Xcen-G6PD-3F8-5F8-Xqter. [188] [196] The distance from factor VIII gene to the Xq telomere is approximately 1 Mb.

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FACTOR VIII GENE DEFECTS

Since the cloning of the factor VIII gene, the DNA of >2,500 patients with hemophilia A has been examined for molecular defects. Initially, restriction endonuclease analysis and Southern blot, cloning, and sequencing were the methods used. The introduction of polymerase chain reaction (PCR) amplification from genomic DNA or from RNA (reverse transcriptase-PCR, RT-PCR) had revolutionized the mutation detection protocols. Several screening methods for recognition of mutations have been used, namely, denaturing gradient gel electrophoresis, single-stranded conformational analysis, RNA cleavage analysis, and subsequently direct sequencing of PCR product. A database of mutations in the factor VIII gene has been published [197] [198] and is constantly updated. A Web site of factor VIII mutations has been created (http://146.179.66.63/usr/www/WebPages/main.dir/main.htm and reference [199]). Figure 107-5 depicts the different kinds of mutations found in patients with hemophilia A from the studies of references [200] [201] [202] as updated after the identification of the common inversion by Lakich et al. [203] These studies have been selected because almost all mutations have been identified in a given sample.

Figure 107-5 Factor VIII gene defects in two samples of patients with severe and mild to moderate hemophilia.

Gross DNA Rearrangements

Common Partial Inversion of Factor VIII

The efforts to characterize all mutations in the factor VIII gene in a defined sample of patients with hemophilia A revealed a surprising and unexpected finding. After scanning all the exons of factor VIII gene using denaturing gradient gel electrophoresis, Higuchi and coworkers found that the causative mutation in about 90% of patients with mild to moderate hemophilia A.[200] However, when severely affected patients were similarly extensively studied, the causative mutation was only found in about 50%.[201] The cause of the remaining 50% of severe hemophilia A remained elusive. Subsequently Naylor et al., using RT-PCR of illegitimate transcription of the factor VIII gene, found that in about 40% of severely affected patients no RT-PCR amplification was possible between exons 22 and 23 of the gene. [204] Lakich et al. [203] and Naylor et al.[205] found that these patients have a partial inversion of the factor VIII gene (the gene up to and including exon 22 is inverted). The inversion is due to homologous recombination between the region that includes the F8A gene in intron 22 and one of the two other homologous regions located more than 400 kb 5 (telomeric) to the factor VIII gene[203] (Fig. 107-6). The regions of homology are each 9.5 kb long and there is >98% nucleotide sequence identity among them. [206] Depending on which extragenic copy of gene A is involved in the crossing-over event, two main types of inversions are recognized. Type 1 inversion affects the distal copy, whereas type 2 inversion affects the proximal copy.[203] In addition, a rare Southern blot pattern, type 3, is thought to result from an inversion occuring in individuals carrying more than two extragenic copies of gene A. The inversions originate almost exclusively in male meiosis. [207] Because the mutation occurs very rarely in female germ cells, nearly all mothers of patients with inversions are carriers of factor VIII deficiency. A recent consortium analysis of 2,093 patients with severe hemophilia A revealed an inversion of factor VIII in 890 or 43%. [208] Inversion type 1 accounts for 83% and type 2 for 16% of the inversion cases; the type 3 patterns were observed in only 1% of the cases. The discovery of this mutation is of considerable clinical significance because it accounts for about 25% of all patients with hemophilia A and >40% of those with severe disease.

Large Deletions

In 5% of the patients with hemophilia A there are large (>100 nucleotides) deletions in the factor VIII gene. [209] The mutation database contains 89 different deletions (see reference [210] and http://146.179.66.63/usr/www/WebPages/main.dir/main.htm).

Figure 107-6 Factor VIII gene inversions in hemophilia A. Due to intrachromosomal crossing-over between the homologous regions A1, A2, and A3, there is an inversion of factor VIII sequences encompassing exons 122. The various types of inversion (types 1, 2, 3A, and 3B) are shown. These inversions are easily recognizable (bottom of the figure) after Southern blot analysis using part of the homologous regions as a probe.

In the majority of these defects the deletion breakpoints have not been precisely characterized. Southern blot analysis indicated that there were not many patients with the same breakpoints suggesting that factor VIII gene does not contain sequences that are prone to become deletion breakpoints. Deletions almost always produce severe hemophilia A; however, a deletion of exon 22 and another deletion of exons 2324 were associated with moderate disease probably because of in-frame joining (after mRNA splicing) of exons 21 and 23 in one case and exons 22 and 25 in the other, to produce proteins lacking 52 and 98 amino acids, respectively.[210] [211] Few deletion breakpoints have been characterized at the nucleotide sequence level. The majority of the breakpoints examined do not occur in repetitive elements such as Alu sequences. There is usually two to four nucleotide homology at the junction point and the deletion mechanism is probably via nonhomologous recombination.[212] A total of 27 of the 73 patients (37%) with deletions for which data were available have developed factor VIII inhibitors after treatment.

Insertion of Retrotransposons

De novo insertion of LINE repetitive elements in the human genome was first reported in the factor VIII gene in two patients with severe de novo hemophilia A. [213] In one patient a 3.8-kb portion of a LINE element was inserted in exon 14 of factor VIII. The inserted DNA had a poly (A) tail, produced a target site duplication, and was inserted in a relatively adenine-rich sequence of exon 14 (Fig. 107-7). The de novo insertion in the second case was a 2.1-kb portion of a LINE element. It occurred in a different site of exon 14 and had all the characteristics of retrotransposition. LINE elements compose about 5% of the human genome and there are approximately 10^5 copies.[214] The full length of the element is 6.5 kb and most of the copies in the human genome are partial and defective. The consensus sequence of LINE element contains two open reading frames, the second of which predicts a protein with amino acid homology to viral reverse transcriptase. About 3,000 LINE copies are full length and are potential transposable elements. Only a few of these, perhaps those with open reading frames, can produce a new insertion through an RNA intermediate. They are probably transcribed into DNA and then reinserted as double-stranded DNA into a new genomic site. [215] The full length active LINE element responsible for the insertion in the first hemophilia A patient was cloned and characterized. It maps on chromosome 22 and probably encodes for a peptide that has reverse transcriptase activity. [216] [217] A third LINE element insertion in intron 10 has also been observed but since it did not co-segregate with the hemophilia A phenotype it represents a recently established private polymorphism. [218] Insertions of LINE or other retrotransposons are not common because there are only two such examples in more than 1,000 patients studied. There are about a dozen additional reports for LINE and Alu insertions in other genes. [215]

Duplications

Duplications of parts of human genes are very rare causes of mutations. There are two such lesions described in the factor VIII gene. In one there was a duplication of 23 kb of IVS22 inserted between exons 23 and 25. [219] This rearrangement found

Figure 107-7 Schematic representation of insertions of LINE retrotrans-posons in exon 14 of factor VIII gene. Color boxes represent the LINE elements and the arrows within the boxes point toward the 3 end of the sequence. The numbers on the LINE boxes represent the corresponding nucleotides of the consensus LINE sequence. Numbers in the sequences flanking the inserted LINE elements denote the corresponding nucleotides of the factor VIII cDNA. The target site duplication is bracketed. *(From Kazazian et al.,[213] with permission.)*

in two female siblings was apparently unstable and led to deletion of exons 2325 in the male offspring of one of the females. In the second case there was an in-frame duplication of exon 13 in a patient with mild hemophilia A. [220]

Chromosomal Rearrangement Breakpoints Involving the Factor VIII Gene

A complex de novo translocation of chromosomes X and 17 [46,X,t(X;17)] has been reported in a female patient with severe hemophilia A. [196] The normal X, always late replicating, contained a normal factor VIII gene. The der(17) contained a deleted factor VIII gene that lacked exons 115; exons 1626 were present in another autosome. One of the breakpoints of the complex rearrangement was therefore between exons 15 and 16 of the factor VIII gene.

Point Mutations and Small (<100 bp) Deletion/Insertions

Nucleotide substitutions and insertion/deletions of up to 100 nucleotides are discussed under point mutations.

Small Deletion/Insertions

Small deletions or insertions in the coding region of factor VIII gene that result in frameshifts and cause severe hemophilia A have been reported in 86 unrelated patients[197] [198] [199] (http://146.179.66.63/usr/www/WebPages/main.dir/main.htm). The database contains 63 small deletions (186 nucleotides) among 572 independent point mutations recorded (11%). There are 23 small insertions (110 nucleotides) (4% of the total point mutations). More than one-third of the small deletions (24 of 63) and half of the insertions (13 of 23) were found as expected in the large exon 14. The majority of the small deletions/insertions occur in DNA regions of short direct repeats. Almost all of the mutations that result in translation frameshifts cause severe hemophilia A. An exception was observed in a family with moderately severe hemophilia A and a deletion of a single nucleotide T within an A_8TA_2 sequence of exon 14. The severity of the clinical phenotype did not correspond to that expected of a frameshift mutation. A small amount of functional factor VIII protein was detected in the patients plasma. Analysis of DNA and RNA molecules from normal and affected individuals and in vitro transcription/translation suggested a partial correction of the molecular defect, because of: (1) DNA replication/RNA transcription errors resulting in restoration of the reading frame or (2) ribosomal frameshifting resulting in the production of normal factor VIII polypeptide and, thus, in a milder than expected hemophilia A. All of these mechanisms probably were promoted by the longer run of adenines, A_{10} instead of A_8TA_2 , after the delT. [221]

Nonsense Mutations

There are 115 independent nonsense mutations in 48 different codons that are included in the point mutation database comprising 20.1% of the total number of point mutations (references [197] [198] [199] and http://146.179.66.63/usr/www/WebPages/ main.dir/main.htm). This percentage is perhaps biased because many investigators have used restriction digestion analysis with Taq I that recognizes CG to TG mutations and in particular CGA to TGA (Arg to Stop) substitutions. [222] In two samples of 52 severe hemophilia A patients[200] [202] in which all point mutations have been characterized, the number of nonsense codons were 7, that is 13.5% of the total severe mutations or 29% of the severe point mutations.

CpG Dinucleotide Hypermutability

The study of point mutations in factor VIII uncovered two general lessons concerning human mutations. The first was the discovery of a mutation hotspot at CpG dinucleotides in which there is a common substitution CG to TG if the mutation occurs in the sense strand or CG to CA if the mutation occurs in the antisense strand.[222] The mutations probably occur because, in mammalian DNA, the majority of CpG dinucleotides are methylated by methyltransferase; the subsequent spontaneous deamination

of the 5-methylcytosine produces a TpG dinucleotide. The mutation usually occurs in tissues in which the gene is not expressed. [223] There are 276 independent mutations in 46 sites that conform to the CG to TG rule in the point mutation database (48%). This high proportion of CpG mutations is probably due to the deliberate screening by restriction analysis or oligonucleotide hybridization. In 35 sites recurrent mutation at CpG dinucleotides appears to have occurred. An unbiased estimate of the frequency of CG to TG mutation may be obtained from studies in which all point mutations have been characterized in a given sample of patients. [200] [201] [202] [224] In these selected studies a total of 84 point mutations have been characterized and 32 were of the CG to TG rule (38%). It has been estimated that in the factor VIII gene CG to TG or CA mutations are 1020 times more frequent than mutations of CG to any other dinucleotide. [225] The mutation hotspot has subsequently been observed in a wide variety of other human genes related to disease phenotypes.

Exon Skipping Due to Nonsense Mutations

An important observation concerning the pathophysiology of nonsense mutations has been made in the factor VIII gene and independently in the fibrillin and OAT genes using RT-PCR for mutation detection. [202] [226] In some cases a nonsense codon mutation can lead to abnormal RNA processing where the exon containing the mutation is skipped. In one case of Glu 1987 to Stop mutation in exon 19, all detectable mRNA lacked the sequences of this exon. In the second case of Arg 2116 to Stop mutation of exon 22 there was about 50% of mRNA without the sequences of exon 22 while the remaining 50% of mRNA was of normal size. The junctions of exons 1820 and 2123 do not result in translational frameshift. The mechanism, significance, and frequency of the exon skipping due to nonsense mutations are presently unknown.

Missense Mutations

The study of missense mutations is important for understanding the function of the protein and the pathophysiology of the disease. A total of 191 mutations in 457 unrelated patients leading to amino acid substitutions have been included in the database and the majority of them are schematically presented in Figure 107-8 (references [197] [198] [199] and http://146.179.66.63/usr/www/WebPages/main.dir/main.htm). These mutations are spread throughout the different domains of the gene except for exon 14 that encodes for domain B, which is devoid of amino acid substitutions that cause hemophilia A. Despite the wealth

Figure 107-8 Missense mutations in the factor VIII protein. The structural domains of the protein are shown and the amino acid substitutions are depicted using the one letter code for amino acids. For example E11V indicates a Glu to Val substitution at amino acid residue 11; similarly R282H/L/C indicates that Arg 282 is substituted by His, Leu, or Cys in different patients (bold).

of amino acid substitutions found, the mode of action of the majority of these mutations in producing reduced factor VIII activity in plasma is unknown. However, several mutations have been identified that alter thrombin cleavage sites, the vWF binding site, or otherwise introduce or destroy N-linked glycosylation sites.

Mutations at Thrombin Cleavage Sites

There are natural mutations in patients with CRM-positive hemophilia A that affect the thrombin cleavage needed for activation of the molecule. Mutations arginine 372 histidine and arginine 372 cysteine have been shown in vitro to abolish the normal cleavage by thrombin in the heavy chain. [135] [227] [228] The Serine 373 leucine mutation has also been shown to abolish normal cleavage by thrombin. [229] By inference it seems highly likely that Serine 373 proline would have the same effect, although this has not yet been tested directly. Mutations arginine 1689 cysteine and arginine 1689 histidine abolish thrombin cleavage at the light chain. [200] [204] These mutations also lead to CRM-positive hemophilia A in which there is normal amount of nonfunctional factor VIII in plasma.

Mutations at the vWF Binding Site

There are two sulfated tyrosine residues tyrosine 1664 and tyrosine 1680 in the region of factor VIII between amino acids lysine 1673 and glutamic acid 1684 in which a vWF binding site has been localized. A natural mutation tyrosine 1680 phenylalanine has been observed in patients with moderate, CRM-reduced hemophilia A. [97] Site-directed mutagenesis of tyrosine 1680 phenylalanine results in a molecule that has lost high affinity binding to vWF presumably because the phenylalanine residue cannot be sulfated. [95] [96]

Mutations at N-Glycosylation Sites

Two other CRM-positive mutations produce severe hemophilia A by creating new N-glycosylation sites in the protein. [230] The first isoleucine 566 threonine creates a new such site at asparagine 564 (Asn-Gln-Ile to Asn-Gln-Thr) in the A2 domain of the heavy chain. The second new site is in the A3 domain of the light chain; the mutation is methionine 1772 threonine, changing Asn-Ile-Met to Asn-Ile-Thr. In both cases factor VIII is present at normal levels in plasma, but it is completely inactive. When the plasma of either patient is deglycosylated, factor VIII activity is restored to a significant degree. Inhibition assays using synthetic peptides corresponding to factor VIII residues 558565 previously shown to be a factor IXa interaction site provided support that the defect in the isoleucine 566 threonine mutant factor VIII molecule is caused by steric hindrance for the interaction with factor IXa. [231] Furthermore, the isoleucine 566 threonine mutant in transiently transfected COS1 cells had a reduced mobility probably because of the additional N-glycosylation site. [231] The significance of a serine 577 proline mutation that in theory eliminates a potential N-glycosylation site at asparagine 575 is unknown.

Study of Mutations in CRM-Positive and CRM-Reduced Patients

The elucidation of mutations in this group of patients is very instructive in understanding the importance of specific amino acid residues. A small number of such mutations have been described. [198] [199] [232] Many of the CRM-positive mutations occur in the A2 domain, which consists of 228 amino acids or about 10% of the coding region of factor VIII; this region must then be important in procoagulant activity. A few of the CRM-positive mutations have been biochemically studied. The defect in the serine 558 phenylalanine factor VIII molecule has been shown to result by steric hindrance for interaction with factor IXa. [231] The majority of mutations, however, are CRM negative and probably affect the folding or stability of the protein. Because these mutations result in absence of secreted factor VIII and the in vitro functional studies depend on the analysis of the protein produced in eukaryotic cells after transfection with factor VIII cDNA, the mechanisms of action of these mutants will be difficult to elucidate. In the case of the deletion of phenylalanine 652, the secretion of the mutant factor VIII was defective and the protein was degraded within the transiently transfected COS1 cells. [231] Cases with two different mutations in the same amino acid are also of interest (Fig. 107-8). The mutations arginine 2307 leucine and arginine 2307 glutamine exhibit defective intracellular transport and secretion of apparently biologically active factor VIII molecule. [233]

Splicing Errors

Only 25 potential splicing errors have been found (references [197] [198] [199] and http:146.179.66.63/usr/www/WebPages/main.dir/main.htm). Despite the presence of 50 splice junctions in the factor VIII gene, splicing mutants do not account for a sizeable fraction of hemophilia A patients. There are 5 mutations in the invariant AG of the acceptor splice site in introns 1, 4, 5, and 6 and 14 associated as expected with severe disease. There are 4 mutations in the donor splice site consensus (nt-1 of IVS 14, nt +3 of IVS 6, nt +5 of IVS 12 and nt +3 of IVS19) and two in cryptic splice sites. No formal proof that the mutations cause abnormal splicing has been obtained.

Promoter Mutations

No examples of mutations in the 5 untranslated region of factor VIII gene have been reported to date. If such mutations do occur they are probably infrequent because two laboratories failed to find any nucleotide substitutions in 530 nucleotides of the 5 flanking region of factor VIII in 227 patients with hemophilia A. [97]

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FACTOR VIII INHIBITORS

One of the major limitations with present factor VIII replacement therapy is the development of inhibitory antibodies to factor VIII (Chap. 110) in 1020% of patients with severe hemophilia A. [234] [235] The etiology of inhibitor development is unknown and host related factors are poorly understood. [236] Studies attempting to relate inhibitor development with HLA haplotypes have had little success. The antifactor VIII antibody response recruits all subclasses of antifactor VIII antibodies, but the IgG4 isotype is somewhat overrepresented.[237] [238] Epitope mapping has shown specificities against the heavy or light chain or both in different patients. [237] [239] Studies established that the most common factor VIII epitopes that induce inhibitory antibodies are localized to the A2 domain (residues 373740), the C2 domain (residues 21732332), and possibly the A3 domain of factor VIII.[238] [240] [241] [242] [243] Because a limited number of antigenic regions have been identified, it may be possible to engineer factor VIII to be less immunogenic through recombinant DNA technology. For example, Lollar and coworkers prepared human and porcine factor VIII chimeric molecules that had residues between 484 and 508 in human factor VIII replaced by the respective porcine residues. The resultant factor VIII displayed less reactivity to antifactor VIII inhibitory antibodies. [244]

The analysis of many factor VIII mutations and their association with inhibitor development may uncover some rules, if such exist, concerning the contribution of the nature of the mutations to inhibitor formation. In the recent consortium analysis of >900 patients with factor VIII inversions, 130 of 642 patients studied had developed inhibitors (20%); in the control population of severe hemophilia A patients without inversions, 131 of 821 patients had developed inhibitors (16%). [208] These results are

not statistically different, suggesting that factor VIII inversions are not a predisposing factor for inhibitor development.

The majority of the reported inhibitor cases have nonsense mutations, deletions, or inversions in their factor VIII gene. There are, however, 10 missense mutations reported in the database that are associated with low levels of inhibitors. These may create local structural modifications with new immunogenic epitopes. Among the nonsense mutations, arginine 1941 is associated with inhibitors in 8 of 11 cases; arginine 2147 in 3 of 8, arginine 2209 in 6 of 16, lysine 1827 in 2 of 2, and arginine 1696 in 2 of 2 cases. Other nonsense mutations, however (e.g., 8 cases with arginine 336 or 9 cases with arginine 2116), are never associated with inhibitors. In these cases, exon skipping may provide a protein merely lacking 2050 amino acids (exon skipping has been detected for mutation of arginine 2116, [202] in which inhibitors have never been observed). Gross deletions of the factor VIII gene result in two- to threefold increased incidence of inhibitors than for patients without detectable deletions.[197] [245] However, no clear picture has emerged as to the correlation between the size or the breakpoints of the deletions and the development of inhibitors.

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CARRIER AND ANTENATAL DIAGNOSIS

The cloning of factor VIII gene, the discovery of DNA polymorphic markers within and closely linked to the gene, and the elucidation of molecular defects in many patients with hemophilia A have changed dramatically the practice of diagnosis of carriers and affected fetuses. The discovery of the common partial inversion of the factor VIII gene[203] provided a means of diagnosis using Southern blot analysis. This defect accounts for approximately 45% of severe hemophilia A. The diagnosis of the exact molecular defect in the remainder of the families is still not practical even in sophisticated laboratories. Because of the enormous variety of the remaining mutations, DNA diagnosis is almost always limited to indirect detection using linked DNA polymorphisms. Figure 107-9 shows the location and informativeness of DNA polymorphisms within factor VIII gene. Families requesting carrier or prenatal diagnosis (after the initial screening for the detection of the inversion) are usually asked to supply blood samples on a number of family members for linkage analysis. The affected factor VIII gene is marked within the family using polymorphic markers both within[245] [246] [247] [248] and outside[249] [250] the gene (Fig. 107-10). Short sequence repeats have been found in two introns.[251] [252] Nearly all families are informative, but 2030% for extragenic polymorphisms only. In those families the chance of error is 25% depending on the polymorphism

Figure 107-9 DNA polymorphisms within the factor VIII gene. Arrows denote the location of the polymorphisms. Percent numbers represent the observed heterozygosity of each polymorphic system in female individuals. The Msp I site in intron 22 is only polymorphic in Japanese (J). There are two polymorphic (CA)n repeats identified in introns 13 and 22.

Figure 107-10 Example of DNA diagnosis in hemophilia A. The DNA analysis is shown below the pedigree. Direct analysis shows an abnormal DNA fragment in affected individual III-2 (this fragment is due to amplification of a small rearrangement). This analysis shows that individual II-2 and her daughters III-3 and III-4 are carriers, individual III-1 is not a carrier, and the parents of II-2 do not have the abnormal fragment and, therefore, the mutation occurred de novo in one X chromosome of their germ cells. The fetus IV-1 does not have the abnormal factor VIII allele and therefore he is not affected. Indirect analysis was performed using a highly informative intragenic polymorphism that shows five different alleles in the DNA of the members of the pedigree. Affected individual III-2 has allele 1 that marks his abnormal factor VIII gene. This allele 1 was inherited from his grandfather I-1 and, therefore, the de novo mutation should have occurred in his germ cells. Note that without the results of the direct DNA analysis in this pedigree, the DNA polymorphisms were not sufficient in identifying carriers.

used. When an intragenic polymorphism is used, the chance of error is negligible (<1%).

The indirect detection of mutant genes is not feasible when only one male offspring is available and the carrier status of the mother is unknown. In these cases direct detection of molecular defect should be used. However, the large number of different molecular defects, the considerable size of the gene, and the sophistication of the mutation detection methodology make direct diagnosis not available to all families. The situation has

dramatically changed with the simple test to recognize the common partial inversion of factor VIII, which accounts for about 45% of cases of severe hemophilia A. Few laboratories will then deal with the remaining molecular defects. It seems that the method of choice is the RT-PCR amplification of illegitimate transcripts and their analysis using chemical cleavage or another mutation screening method. Further technical improvements are needed to make the analysis faster and less costly. The availability of high-density oligonucleotide arrays. [253] for the factor VIII gene will permit the rapid detection of the molecular defects in the majority of patients.

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COMBINED DEFICIENCY OF FACTORS V AND VIII

Combined factors V and VIII deficiency is an autosomal recessive disease that was described in 1954 by Oeri et al. [254] To date, a total of 89 patients in 58 families have been reported, where 24 of the families are from the Mediterranean region. Patients with this disorder exhibit factor V and factor VIII antigen levels in the range of 530% that correlates with the activity measurements. Bleeding is similar to that observed in other coagulation factor deficiencies. The levels of all other plasma proteins that have been measured appear normal in these patients, including those of the homologous copper ion binding protein ceruloplasmin. Although there have been several reports of individuals who simultaneously inherited mutations in both the factor VIII and factor V gene, [255] this is rare (approximately $1/10^{10}$).

It was originally proposed that combined deficiency of factors V and VIII resulted from a loss of protein C inhibitor. [256] However, subsequent studies failed to confirm deficiency of protein C inhibitor in these patients. [257] [258] [259] Recently, homozygosity mapping, a method originally described by Lander and Botstein [260] and positional cloning approaches were used to identify the defective gene. [261] [262] Analysis from 14 affected individuals from nine unrelated families identified that the factor V and VIII deficiency gene mapped to the long arm of chromosome 18. Positional cloning approaches identified that the gene responsible for the defect is ERGIC-53. [263] Haplotype analysis among these families suggested that two independent founders gave rise to two mutations in all of the original nine unrelated families studied. Subsequent studies have identified five different mutations in ERGIC-53 in 17 of 25 families analyzed with combined factor V and VIII deficiency. At present the genetic defect has not been identified in the remaining families.

ERGIC-53 encodes a 53,000-MW transmembrane protein of the ER-Golgi intermediate compartment (hence, ERGIC-53) that has mannose-binding capability and that cycles between the ER and the Golgi compartment.[264] [265] Analysis of lymphocytes from patients with factors V and VIII deficiency demonstrated a complete lack of ERGIC-53 protein.[263] Although ERGIC-53 has the properties expected for a protein that may facilitate protein transport from the ER to the Golgi compartment, the elucidation of its absence in combined factor V and VIII deficiency suggests the selective loss of factors V and VIII in the plasma is due to defective intracellular transport and secretion unique to these two coagulation factors. Both of these proteins contain an unusually heavily glycosylated B domain, suggesting that ERGIC-53 may interact with the B domain to facilitate their intracellular transport and secretion.

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THE FUTURE FOR HEMOPHILIA A THERAPY

Animal Models for Hemophilia A

To evaluate improved therapeutic regimens for hemophilia A and to study the role of factor VIII in vivo, animal models of hemophilia A have been studied. Dogs have been diagnosed with hemophilia A, and several colonies have been established. Bleeding tests have been established in these dogs, the most frequently used being the cuticle bleeding test.[266] A German shepherd dog model among others has been used for several studies[267] [268] that includes testing of recombinant-derived factor VIII and B domain-deleted factor VIII.[76] [269]

More recently, a mouse with severe factor VIII deficiency was made by targeted disruption of the murine factor VIII gene.[270] Exons 16 or 17 of the cloned murine factor VIII genomic DNA were interrupted by the insertion of a neomycin-resistant gene cassette and mouse embryonic stem cell lines containing the neomycin gene insertions after homologous recombination were produced. These ES cell lines were used to produce male mice with targeted disruption of the factor VIII gene. The two strains of resulting affected mice have <1% factor VIII activity and bleeding after minor trauma, a phenotype similar to that observed in severe hemophilia A in humans.[270] Affected mice do not, however, bleed spontaneously. Heterozygous females show factor VIII levels that are 50% of normal. Affected females were also produced; when bred with affected males, these animals survived pregnancy and delivery. RT-PCR of liver mRNA indicated that the factor VIII deficiency in the exon 16 knockout mice is due to truncated protein, whereas in exon 17 knockout mice it is due to either truncated or partially deleted protein.[271] Assay of cryoprecipitate from the plasma of affected mice using two antimouse factor VIII monoclonal antibodies failed to detect any factor VIII. The mice with hemophilia A provide an excellent animal model for gene therapy experiments and the study of factor VIII inhibitors.

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Chapter 108 - Biochemistry of Factor IX and Molecular Biology of Hemophilia B

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INTRODUCTION

Factor IX is a plasma protein with a molecular weight of 56,000 whose synthesis by the liver requires vitamin K. Factor IX participates in an intermediate phase of the blood coagulation pathway (see Chap. 102). It can be activated by factor XIa or factor VIIa complexed with tissue factor. In complex with factor VIIIa on membrane surfaces, factor IXa then activates factor X. The critical importance of factor IX is emphasized by the phenotype of hemophilia B, a hereditary disease characterized by factor IX deficiency.

In 1952 factor IX was discovered, distinct from factor VIII, in that after mixing of plasmas from two unrelated patients with hemophilia, the clotting time of the mixed plasma was corrected.[1] [2] Hemophilia B, or Christmas disease, is an X-linked disorder defined by the hereditary decrease in factor IX activity. The molecular defect causing factor IX deficiency in the index patient has been shown to be a point mutation causing a change from Cys 206 to serine in the catalytic domain. [3] Hemophilia B accounts for about 12% of the total cases of hemophilia. The disease is variable in phenotype, and the degree of bleeding severity usually correlates with the level of factor IX activity present in the patients plasma. As anticipated from the variable phenotypes of hemophilia B and the similarity of phenotypes within each particular family, hemophilia B is caused by a variety of genetic defects. It is estimated that one-third of cases of hemophilia B arise from spontaneous mutations.

GENE STRUCTURE AND REGULATION

The factor IX gene is located on the tip of the long arm of the X chromosome at Xq27.1,[4] close to the factor VIII gene. The gene is 34 kb in size and contains seven introns that range in size from 188 nucleotides to almost 10 kb. The mRNA is composed of a 205-bp 5 untranslated region (UTR), a 1,383-bp preprofactor IX region that encodes for a 461-amino acid precursor protein, and a 1,392-bp 3 UTR.[5] [6] [7] The protein is encoded by all eight exons (Fig. 108-1). The factor IX gene was derived ancestrally from a precursor of the prothrombin gene.[8] The genes for factor VII, factor X, and protein C share common splice junctions with the gene for factor IX, demonstrating the close relationship of the vitamin K-dependent proteins (see Fig. 102-5).

The regulation of the factor IX gene is mediated by elements in the 5 UTR, intron sequences, and the 3 UTR. The 5 proximal region extending to 270 contains the minimal promoter sequence required for maximal factor IX expression.[9] [10] Multiple promoters exist within this region, including a CAAT box at 238 and a TATA box at 181.[11] [12] A transcription start site has been defined at approximately 176.[9] Multiple negative regulatory elements (silencers) that suppress gene expression exist upstream of 416. One such silencer consists of a promoter (766 to 417) oriented in the reverse direction of the factor IX promoter activity.[12] Experiments using transgenic mice indicate that the introns of factor IX are required for its expression in vivo.[13] Furthermore, expression vectors that include the first intron of the factor IX gene produce 7- to 9-fold higher levels of mRNA than vectors lacking this sequence.[14] The 3 UTR contains polyadenylation signal sequences that are important in normal gene expression. A 653-bp deletion of this region has been found in a mild form of hemophilia B.[15]

Factor IX Leyden is a form of hemophilia B characterized by a severe bleeding disorder during childhood; factor IX activity and antigen levels are <1%.[16] With the onset of puberty or with the administration of androgen therapy,[17] the factor IX levels rise to 3060% of normal, and the hemophilia phenotype disappears (Fig. 108-2). Analysis of the factor IX genes from patients with various pedigrees with the factor IX Leyden phenotype demonstrated 11 unique point mutations clustered in an area from 40 to +20.[11] This clustering of mutations has led to the identification of binding sites for various transcription factors within the 5 regulatory region.[11] [18] Included in this region are binding sites for (1) the CCAAT/enhancer-binding protein (C/EBP) between +4 and +19,[19] (2) hepatic nuclear factor-4 (HNF-4) between 19 and 27,[20] [21] and (3) unidentified proteins between 16 to 3, 15 to +3, and +15 to +36.[9] [22] C/EBP is thought to interact with D-binding protein, a transcription factor whose expression is increased in adulthood.[23] Binding sites immediately upstream of the cluster of factor IX Leyden mutations have been found for nuclear factor-1 between 77 and 99,[11] for Ets factor GA-binding protein (GABP/) between 195 to 220,[24] and for an unidentified protein that binds

Figure 108-1 Structure of the factor IX gene and encoded factor IX domains. The factor IX gene includes eight exons and seven introns. Exons 18 are represented as solid bars and designated with roman numerals IVIII.

Figure 108-2 Factor IX Leyden. Changes in the plasma factor IX level as a function of age. *(From Briet et al.,[17] with permission.)*

between 67 and 49.[11] A transgenic mouse with a mutation at +13 in the factor IX promoter shows age-dependent, male-specific expression of factor IX.[25] In normal individuals, factor IX levels increase modestly after puberty.[26]

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SYNTHESIS OF FACTOR IX

Factor IX is synthesized in the hepatocyte as a precursor protein; it then undergoes a series of intracellular post-translational modifications and is secreted as a zymogen (Fig. 108-3). The coding sequence of the factor IX gene encodes two peptides that are removed prior to secretion of the mature factor IX into the blood: a 28-residue signal peptide that directs the nascent peptide chain to the endoplasmic reticulum and an 18-residue propeptide between the signal peptide and the mature N-terminus of factor IX.[7] The signal peptide is cleaved cotranslationally by the signal peptidase. The propeptide contains the -carboxylation recognition site that directs -carboxylation of the adjacent glutamic acid residues in the -carboxyglutamic acid (Gla) domain of mature factor IX. [27] This recognition element is defined by amino acids 18, 17, 16, 14, and 10 toward the N-terminus of this region. [28] The vitamin K-dependent carboxylase binds to the -carboxylation recognition site within the propeptide. [27] [28] [29] [30] The carboxylation reaction, catalyzed by the carboxylase, requires a reduced form of vitamin K (vitamin KH_2), molecular oxygen, carbon dioxide, and the factor IX precursor, uncarboxylated profactor IX, containing glutamic acid residues. [31] The carboxylase is an enzyme with a molecular weight of 94,000 that is sufficient to carboxylate a precursor substrate in the presence of the necessary cofactors. [32] [33] [34] [35] The carboxylase is a membrane protein, and its catalytic activity resides on the luminal side of the rough endoplasmic reticulum. [36] [37] Multiple glutamic acids are carboxylated within a single precursor upon interaction with the carboxylase.[38] The carboxylglutamic acid residues that are formed play a critical role in calcium binding.

In addition, aspartic acid 64 undergoes -hydroxylation in the rough endoplasmic reticulum. From the cDNA sequences of factor IX, [6] [7] factor X,[39] protein C,[40] [41] protein S,[42] and factor VII,[43] it is apparent that specific aspartic acid residues in precursor forms of these proteins undergo -hydroxylation post-translationally. Stenflo et al.[44] have suggested that a consensus sequence consisting of Cys-X-Asp/Asn-X-X-X-Phe/Tyr-X-Cys-X-Cys signals this event. Factor IX contains about 0.4 mol of -hydroxyaspartic acid per mol of factor IX.[45] [46] This modified amino acid is formed from aspartic acid by a post-translational process catalyzed by an -ketoglutarate-dependent dioxygenase, [47] aspartyl -hydroxylase, which has been purified [48] and cloned.[49] Mutants lacking Asp 64 have decreased calcium binding, which suggests that -hydroxyaspartic acid might be involved in calcium binding. However, detailed studies using nuclear magnetic resonance spectroscopy failed to show a significant difference in calcium binding between -hydroxylated and non--hydroxylated factor IX epidermal growth factor (EGF)-like domains. [50] Furthermore, recombinant factor IX expressed in the presence of dipyridyl, o-phenanthroline, or pyridine 2,4-dicarboxylate, which are inhibitors of 2-ketoglutarate-dependent dioxygenases, retains full activity. [51] These data are consistent with the hypothesis that Asp 64 is required for full functional activity, but -hydroxylation is not.

Bovine and human factor IX are glycoproteins that contain about 17% carbohydrate by weight. The first EGF-like domain is glycosylated at two O-linked glycosylation sites. Xyl(1 3) Glu1 O-Ser and Xyl(1 3) Xyl(1 3) Glc1 O-Ser

Figure 108-3 Biosynthesis of factor IX. Factor IX is synthesized in the liver in a precursor form. The signal peptide is responsible for translocation of the nascent peptide chain to the endoplasmic reticulum. Profactor IX undergoes post-translational processing, including -carboxylation, glycosylation, -hydroxylation, disulfide bond formation, and finally propeptide cleavage. -Carboxylation requires molecular oxygen, reduced vitamin K, and carbon dioxide in the presence of the vitamin K-dependent -carboxylase.

saccharides are glycosidically linked to Ser 53. [52] A tetrasaccharride NeuAc(2 6) Gal(1 4) GluNAc(1 3) Fuc1 O-Ser is linked to Ser 61. [53] [54] [55] A second heavily glycosylated portion of factor IX is the activation peptide (Ala 146-Arg 180). It is glycosylated at two N-linked and two O-linked glycosylation sites. [56] Asn 157 and Asn 167 are fully glycosylated. Thr 159 and Thr 169 are partially glycosylated.

Profactor IX, including the propeptide and factor IX sequence, undergoes carboxylation in the endoplasmic reticulum. Profactor IX, even in its fully carboxylated form, and in contradistinction to factor IX, is not capable of binding to membrane surfaces. [57] Presumably, this property prevents factor IX from being hung up inside the cell, where high levels of calcium ions within the endoplasmic reticulum could support factor IX-membrane interaction. Profactor IX undergoes additional post-translational processing, including disulfide bond formation and glycosylation, characteristic of other secreted plasma proteins. The propeptide is cleaved as a late processing event.[7] [58] [59] Furin or a furin-like converting enzyme cleaves peptide bonds at a site adjacent to the sequence RXRR, where X can be any amino acid and R is arginine.[60] Bristol and colleagues[61] used site-directed mutagenesis to define a hierarchy of the efficiency of cleavage given different paired amino acids at the P1 and P2 positions: Lys-Arg > Arg-Arg Thr-Arg > Arg-Lys > Lys-Lys >> Lys-Thr. -Carboxylation precedes propeptide cleavage. [62]

The carboxy-terminus of factor IX may contribute to its intracellular trafficking. Mutant factor IX with substitutions in amino acids 403415 are degraded and secreted only at low levels when expressed in a hepatoma cell line. [63] Addition of proteosome inhibitors to this system leads to recovery of factor IX secretion. [63] Consistent with these observations, naturally occurring mutations in the carboxy-terminus of factor IX lead to hemophilia B with severely depressed antigen levels in the plasma.[64]

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STRUCTURE OF FACTOR IX

Human factor IX is a single-chain glycoprotein composed of 415 amino acids (Fig. 108-4). [6] [7] [65] It has a molecular weight of 56,000.[66] [67] The structure of porcine factor IX has been determined by x-ray crystallography. [58] The protein resembles a tulip with the N-terminal Gla domain composing the bulb, the two EGF domains the stalk, and the catalytic domain the flower (Fig. 108-5A).[68]

Propeptide

The propeptide directs -carboxylation of profactor IX and then is cleaved to yield factor IX. The propeptide includes an amphipathic -helix with a hydrophobic face and hydrophilic face.[69] The carboxylation recognition site is located proximal to this helix. This recognition site binds the N-terminal third of the carboxylase. [70] [71] Mutations in the propeptide have been documented as the cause of some forms of hemophilia B. Diuguid et al. [58] determined the size of the propeptide of factor IX by analysis of a mutant factor IX, factor IX Cambridge. This mutant has an 18-residue N-terminal extension due to the mutation of Arg 1 to a serine, precluding propeptide cleavage by a propeptidase with trypsin-like specificity. Concurrently, Bentley et al. [59] evaluated factor IX Oxford 3, a mutant factor IX in which Arg 4 is mutated to glutamine; this mutation also prevents propeptide cleavage.[59] Similar mutations have been described, each characterized by the complete absence of factor IX activity and the presence of the 18-residue propeptide extension. [72] [73] [74] [75] Of the several proteins studied, [58] [72] [76] [77] there is a partial defect in -carboxylation of factor IX. Because of the failure of propeptide cleavage, these profactor IX mutants cannot bind to phospholipid vesicles, nor can they be activated by factor XIa. In fact, the propeptide prevents binding of profactor IX to membranes even if the profactor IX is fully carboxylated. [57] These data and the marked sequence homology of this domain in Gla-containing proteins led to the proposal of a role for the propeptide in designating protein precursors containing this specific propeptide for subsequent -carboxylation. [78] Using site-specific mutagenesis and an heterologous mammalian expression system, Jorgensen et al.[27] and Rabiet et al. [46] demonstrated that factor IX lacking the 18-residue propeptide was not carboxylated in vivo. Similarly, point mutations at 16 (Phe Ala) or 10 (Ala Glu), both positions highly conserved in the propeptides of the vitamin K-dependent proteins, inhibited -carboxylation. These results demonstrated that the propeptide contains a recognition element, designated the -carboxylation recognition site, that signals for the -carboxylation of the vitamin K-dependent proteins during hepatic biosynthesis. This region is sufficient to support -carboxylation of an adjacent but unrelated glutamic acid-rich substrates. [79]

-Carboxyglutamic Acid Domain

The NH_2 -terminal Gla domain of human factor IX includes 12 Gla residues, located at positions 7, 8, 15, 17, 20, 21, 26, 27, 30, 33, 36, and 40. [80] [81] The Gla domain defines some of the critical calcium-binding sites of the protein and is required for the interaction of factor IX with membrane surfaces in the presence of calcium ions.[82] Naturally occurring point mutations of glutamic acid residues destined to be -carboxylated include Glu 7, Glu 8, Glu 17, Glu 21, Glu 27, Glu 30, and Glu 33; patients with these mutations have moderate to severe hemophilia, an observation emphasizing the functional importance of these residues. [64] In contrast, factor IX that lacks Glas 36 and 40, Gla residues that are not found in other vitamin K-dependent blood coagulation proteins, maintains full function. [83] In the absence of calcium, the Gla domain consists of three well-defined structural elements linked by a flexible polypeptide backbone. [84] The three structural elements are a short amino terminal loop (residues 69), a hexapeptide loop formed by disulfide binding of cysteines at residues 18 and 23, and a carboxyl-terminal -helix (residues 3746). [85] The hexapeptide loop is essential for factor IX function, as evidenced by the fact that factor IX Zutphen (Cys 18 Arg) cannot form the Ca $^{2+}$ -dependent conformation and results in severe hemophilia.[85] When bound with calcium, the Gla domain folds in a manner that exposes an amino-terminal region, which may represent the phospholipid-binding site in factor IX (Fig. 108-5B).[86] This amino-terminal region is well structured in the Ca $^{2+}$ -bound form of factor IX (which binds membranes) but is unstructured in the Mg^{2+} -bound form of factor IX (which does not bind membranes). [87]

Epidermal Growth Factor-like Domains

The Gla domain is linked to two adjacent EGF domains by a short domain rich in aromatic amino acids. The EGF domains, each characterized by a segment of 53 amino acids and a pattern of disulfide-bonded cysteine residues, have distinct functions. The structure of the first EGF domain has been determined by two-dimensional NMR spectroscopy and x-ray crystallography. [88] [89] It consists primarily of a two-stranded beta sheet. Astermark et al., using both inhibitory peptides[90] and cross-linking experiments,[91] have suggested that the first EGF domain participates in the interaction between factor X and factor IXa in the tenase complex. A naturally occurring mutant, factor IX London-6 (Asp 64 Gly), results in mild hemophilia B. [92] Experiments using mutant factor IX in which Asp 64 has been mutated suggest that the binding of calcium to this domain enhances the enzymatic activity of factor IXa and its assembly into

Figure 108-4 Amino acid sequence of factor IX. The preprofactor IX sequence includes the signal peptide, propeptide, -carboxyglutamic acid domain, aromatic amino acid stack domain, two EGF domains, and the serine protease domain. Amino acids are designated by the one-letter code. , -carboxyglutamic acid; , -hydroxyaspartic acid.

Figure 108-5 (A) Ribbon model of the three-dimensional structure of factor IXa as determined by x-ray crystallography. Gla, glutamic acid-containing region; EGF 1, first epidermal growth factor-like region; EGF 2, second epidermal growth factor-like region; SP, serine protease-containing region. **(B)**Ribbon model of glutamic acid-containing region as determined by NMR. The cysteines of the hexapeptide disulfide loop, residues 18 and 23, are labeled by residue number and the disulfide bond is indicated. The side chains of the conserved aromatic residues of the aromatic amino acid stack domain are displayed and labeled by residue number.

the factor IXa-VIII complex.[93] In addition, deletion and chimeric mutants of the first EGF-like domain have been constructed that lead to defective activation by factor VIIa/tissue factor with normal activation by factor XIa.[94] Factor IX Alabama, which results from a point mutation in the first EGF domain (Asp 47 Gly), [95] binds normally to phospholipid vesicles[96] because the Gla domain is intact. However, the activation of factor X by factor IXa Alabama is not enhanced by factor VIIIa, [97] and its affinity for calcium is reduced.[98] Other naturally occurring mutations within this domain, including Gln 50, Cys 51, Cys 56, and Gly 60, lead to a severe hemophilia phenotype, but the basis for this defective factor IX function is not known.

The second EGF domain is required for the interaction between factor IXa and factor VIIIa in the tenase complex. [99] It appears that this second EGF domain contains contact residues that interact with factor VIIIa, but not directly with the substrate factor X. Some point mutations within this domain have only a moderate effect on

function. However, moderate to severe hemophilia is associated with mutations of Asn 92, Gly 93, Arg 94, Cys 95, Cys 99, Cys 111, Gly 114, Asn 120, Cys 124, and Ala 127.[64]

Serine Protease Domain

The C-terminal portion of factor IX demonstrates marked sequence homology with zymogens of serine proteases, such as trypsin and chymotrypsin. This region is almost 250 amino acids long and contains, in latent form, the enzyme active site of factor IXa. The mechanism of enzyme activation involves limited proteolysis. The cleavage of two peptide bonds in factor IX leads to the generation of the enzyme factor IXa. The enzyme active site is common to all serine protease and contains the enzymatic machinery for the hydrolysis of peptide bonds. Superimposed on this structure is an extended substrate-binding site, surrounding the active site, that defines the high substrate specificity of factor IXa for protein substrates. [10C]

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CALCIUM-BINDING PROPERTIES

Factor IX binds to metal ions. As with the other vitamin K-dependent proteins, there are two classes of metal-binding sites: high affinity and lower affinity. [100] Occupation of these two classes of metal-binding sites leads to two sequential conformational changes in these proteins. [102] [103] The first conformational change can be induced by calcium, magnesium, and many other divalent cations; the second conformational change is induced only by calcium ions. Fab fragments of conformation-specific antibodies, directed against factor IX after it has undergone the conformational transition that is calcium ion selective, block the binding of factor IX to phospholipid vesicles and the activation of factor IX by factor XIa. The binding of other conformation-dependent probes and the rate of conversion by factor XIa are increased in the presence of magnesium but remain dependent on calcium. These finding suggests that the conformer achieved only in the presence of calcium is necessary for the expression of a phospholipid-binding site and for the binding of factor IX by factor XIa (Fig. 108-6).[104]

The binding of calcium to the Gla-rich domain involves formation of an internal calcium-binding pocket formed by the amino-terminal nine Gla residues. The binding results in the formation of a calcium-carboxylate network with an exposed amino-terminal hydrophobic loop that is the phospholipid-binding site. [86] Refinement of this structure and incorporation of calcium ions into the Gla-rich domain emphasize the importance of Gla to the formation of the calcium-carboxylate network and its role in structure.[105] The binding of calcium to the first EGF-like domain is mediated by a consensus sequence including Asp 47, Asp 49, Gln 50, Asp 64, and Tyr 69. Asp 47, Gln 50, and Asp 64 interact directly with calcium. [90] [106] [107] Astermark et al.[108] have demonstrated that whereas the Gla and EGF domains can bind calcium ions independently, the intact Gla-EGF domains bind calcium ions with higher affinity. A high-affinity calcium-binding site has also been described in the heavy chain of factor IX involving residues 235, 237, 240, and 245. [109]

Figure 108-6 Model of the interaction of factor IX and phospholipid membrane. The hydrophobic residues (black) that form the hydrophobic patch in the phospholipid-binding site of factor IX are buried in the phospholipid bilayer. The phosphatidylserine binds specifically through electrostatic interactions with amino acid side chains of the Gla domain.

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MEMBRANE-BINDING PROPERTIES

The interaction of factor IX and factor IXa with phospholipid membranes has been studied using phospholipid vesicles. [97] Factor IX affinity for phosphatidylcholine-phosphatidylserine membranes is independent of the phosphatidylserine concentration at phosphatidylserine compositions of >2030% [110] a K_d of about 12 M has been measured. Factor IX binds to vesicles in the presence of Ca^{2+} but not in the presence of Mg^{2+} or EDTA. Furthermore, des--carboxy factor IX does not interact with phospholipid vesicles even in the presence of Ca^{2+} . These results emphasize the importance of the Gla-rich region in defining phospholipid-binding properties. Cross-linking studies using a photoactivatable amino acid at Leu 6 and Phe 9 suggest that these residues proximate or are buried in the lipid bilayer. [87]

Factors IX and IXa bind to bovine aortic endothelial cells and compete for the same site. [111] [112] Factor IX has been shown to bind to collagen IV with a K_d of 6.8 nM, which correlates with the K_d of 7.4 nM for the binding of factor IX to endothelial cells. [113] Factor IX binds to an endothelial cell extracellular matrix in the absence of intact cells. The interaction of factor IX with endothelial cells is inhibited by a Gla-containing peptide corresponding to residues 142 of factor IX; [114] the K_i for this interaction is about 0.05 M. A chimeric protein in which the Gla domain (147) of factor IX is substituted for the same domain in protein C is able to bind endothelial cells. This result suggests that the endothelial-binding site resides within this domain. [115] Cheung et al.[116] have utilized site-directed mutagenesis of the Gla domain of factor IX to show that residues 5 and 10 are required for the binding of factor IX to endothelial cells. The coagulant activity of the mutant proteins remained intact, suggesting distinct interactions between factor IX with endothelial cells and phospholipids. Although a specific receptor recognition element resides within residues 114, Gla-containing peptides of 114 and 124 do not compete with intact factor IX for binding to endothelial cells. [117] Furthermore, purified natural variants of factor IX with mutations with the hydrophobic stack domain or the first EGF-like domain did not compete effectively with wild-type factor IX for binding to endothelial cells. These results suggest that the functional integrity of the Gla domain in the interaction with endothelial cells depends on the hydrophobic stack and the first EGF-like domain.[118]

Activated platelets, but not resting platelets, bind factors IX and IXa. [119] [120] Factor IX and factor IXa share some common binding sites on activated platelets. There are 250300 sites per platelet for factor IX and 500600 sites per platelet for factor IXa. The interaction of factor IXa with platelets is at least in part mediated by the Gla domain.[121] Studies using a chimera in which the second EGF domain of factor X was substituted for that of factor IX exhibited decreased binding to platelets, suggesting that the second EGF domain may play a role in platelet binding. [122] In the presence of saturating concentrations of factors VIIIa and X, the K_d of factor IXa (K_d = 0.5 nM) binding is five times lower than that of factor IX. [123] [124]

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FORMATION OF THE TENASE COMPLEX

The assembly of factor IXa and factor VIIIa on phospholipid membranes constitutes the tenase complex (Fig. 108-7). Factor IXa and factor VIIIa interact on phospholipid membrane surfaces with a K_d of 2 nM with 1:1 molar stoichiometry.[125] The affinity of factor IXa for factor VIIIa is increased 10-fold in the presence of phospholipid. The rate of bond cleavage (k_{cat}) leading to the formation of factor Xa is increased 1,500-fold. [126] Activation of factor IX to factor IXa leads to the expression of a factor VIII-binding site and active enzyme site with full coagulant activity. [127] The factor IXa active site is distant from the membrane surface, but its structure is perturbed on interaction with factor VIIIa during the formation of the tenase complex. [128] Reciprocally, activation of factor VIII by thrombin yields factor VIIIa, which binds more tightly than factor VIII to factor IXa. [125]

The domains of factor IX involved in the tenase complex have been explored with a naturally occurring mutation and with chimeric proteins. The affinity of a naturally occurring mutation (Gly 12 Arg) for factor VIIIa is 172-fold less than that for factor IX, suggesting a role for the Gla domain in the formation of the tenase complex. [129] A chimera in which the first EGF domain of factor IX has been substituted by the first EGF domain of factor X has full or nearly full biologic activity, indicating that the first EGF domain is not involved in factor VIIIa binding. [100]

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ACTIVATION OF FACTOR IX BY THE INTRINSIC PATHWAY

Factor IX is a proenzyme with no catalytic activity. It is activated to factor IXa by factor XIa in the presence of calcium, or by factor VIIa and tissue factor in a reaction that occurs on a membrane surface and in the presence of calcium (Fig. 108-8). The kinetics of factor IX activation by factor VIIa and tissue factor or factor XIa are comparable. In contrast to the other vitamin K-dependent blood clotting proteins, membrane surfaces, including phospholipid vesicles or cell surfaces, do not enhance factor IX activation by factor XIa. The activation of factor IX New London (Pro 50 Glu) by factor XIa is abnormally slow. [130] This implicates the EGF domain in factor IX activation. When factor IX is activated by factor XIa, two peptide bonds are cleaved: one bond is located at Arg 145-Ala 146, and the other

Figure 108-7 A model of the structure of the tenase complex. The tenase complex (the enzyme complex capable of activating factor X to factor Xa) includes factor IXa as enzyme and factor VIIIa as cofactor bound to the membrane surface. The substrate factor X interacts with factor IXa, factor VIIIa, and the membrane.

bond is at Arg 180-Val 181 (Fig. 108-9).[131] With the release of the internal carbohydrate-rich activation fragment (M_r 11,000) from residues 146180, the factor IXa light chain (M_r 17,000) and heavy chain (M_r 28,000) remain bound by a single disulfide bond. The enzyme active site is located on the heavy chain. Cleavage of factor IX appears to occur via a processive mechanism in which both cleavages are made by factor XIa before factor IXa is released. [132]

Factor IX Chapel Hill (Arg 145 His), the first factor IX mutation to be defined at the molecular level, is the prototype of a genetic defect in which activation of a zymogen is impaired due to the mutation of an arginine preceding a sessile bond. [133] It has minimal coagulant activity.[97] Factors IX Chicago-2 (Arg 145 His)[134] and factor IX Albuquerque (Arg 145 Cys)[135] have mutations at the same site. In each of these mutations, the loss of the arginine at position 145 precludes the cleavage of the sessile bond separating the factor IX light chain from the activation peptide. Failure to cleave this bond results in a molecule that cleaves factor X very slowly. The active site of factor IXa is formed, however, as demonstrated by its ability to cleave synthetic substrates normally. [136] The function of the cleavage of the Arg 145-Ala 146 bond may serve to expose a binding site for factor VIIIa since factor IX binds factor VIII but is incapable

Figure 108-8 Pathways of factor IX activation. Factor IX is activated independently by either factor XIa or factor VIIa/tissue factor. Factor XIa can activate factor IX in the absence of membrane surfaces, but calcium ions are required. The complex of factor VIIa and tissue factor on membrane surfaces in the presence of calcium ions also converts factor IX to its active enzyme form.

of generating factor Xa.[137] Factor IX mutants at position 180 include factor IX Hilo (Arg 180 Gln), factor IX Milano (Arg 180 Gln), factor IX Deventer (Arg 180 Trp), and factor IX Nagoya (Arg 180 Trp), all resulting in hemophilia B_M , a type of hemophilia in which the patients mutant factor IX causes a marked prolongation of the ox brain thromboplastin time due to increased inhibition of the in vitro activation of factor VII. [138] [139] Factor IX Novara (Val 181 Phe)[139] and factor IX Tokyo (Val 182 Ala)[140] result in the hemophilia B_M phenotype, while factor IX Kashihara (Val 182 Phe) results in hemophilia B. [141] [142]

The activation of factor IX by factor XIa is highly metal selective, with optimal activation only in the presence of Ca^{2+} and suboptimal activation in the presence of Sr^{2+} .[94] [143] The K_m for the activation of factor IX by factor XIa without calcium is 18 M, and the k_{cat} is 2.4 min^{1} . In the presence of calcium, the K_m is decreased to 2 M, and the k_{cat} is increased to 10.4 min^{1} .[144] [145] Although the proteolytic activity of factor XIa is present on the light chain, the heavy chain of factor XIa may be necessary for the Ca^{2+} -dependent acceleration of factor IX activation. [145] [146] Experiments using anti-factor IX/Ca^{2+} -specific antibodies suggest that there may be a surface of factor XIa that interacts with factor IX in the presence of Ca^{2+} . An antibody directed against the heavy chain of factor XIa neutralized the enzyme competitively, but an antibody directed against the light chain neutralized the enzyme noncompetitively. [145] The region of the factor XIa heavy chain that interacts with factor IX has been localized to residues 134172 in the heavy chain of factor XI. [147] Furthermore, the presence or absence of calcium did not change the rate of activation of factor IX by factor XIa light chain.[145] On the basis of these studies and antibody inhibition studies using antibodies to factor IX, the heavy chain of factor XIa has been implicated as specifically interacting with the phospholipid-binding site of factor IX in the presence of Ca^{2+} .

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ACTIVATION OF FACTOR IX BY THE EXTRINSIC PATHWAY

Factor IX is also activated by factor VIIa and tissue factor (Fig. 108-8). [126] [148] This reaction, dependent on calcium ions, is characterized by a K_m of 0.3 M.[149] [150] The k_{cat} for this reaction is between 13 and 68 min^{-1} . [149] [150] The activation of factor IX by factor VIIa and tissue factor is accelerated by the activation of factor IX to factor IXa by factor XIa.[151]

The relative importance of factor IX activation by factors XIa or VIIa and tissue factor has been evaluated by measuring the

Figure 108-9 Activation of factor IX by limited proteolysis. The zymogen factor IX has no enzymatic activity. On cleavage of the peptide bonds adjacent to Arg 145 and Arg 180, enzymatically active factor IXa is generated. Cleavage of these peptide bonds is associated with expression of enzyme activity and a factor VIIIa-binding site.

factor IX activation peptide in patients with factors VII and XI deficiency. [152] There was a significant reduction in the baseline levels of the factor IX activation peptide in patients with factor VII deficiency but not in patients with factor XI deficiency when compared with normal controls. Furthermore, the administration of recombinant factor VIIa to chimpanzees resulted in a significant increase in both the factor IX and factor X activation peptides at the same time point. [152] Infusion of recombinant factor VIIa into patients with factor VII deficiency causes an increase in the abnormally low levels of the factors IX and X activation peptides and prothrombin fragment F1.2.[153] These studies suggest that the activation of factor IX by factor VIIa and tissue factor is important in vivo.

Almus et al.[154] have evaluated the activation of factors IX and X by factor VIIa and tissue factor in an umbilical vein model. In this system the activation of factors IX and X occurs at the same rate. These results further substantiate the importance of both of these reactions in the initiation of the extrinsic pathway of blood coagulation.

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ENZYMATIC ACTIVITY OF FACTOR IXa

Factor IXa is a serine protease that expresses its activity via the catalytic domain on the heavy chain. This activity is generated by the classic catalytic triad that in factor IXa is located at His 41, Asp 89, and Ser 185 in the heavy chain.

Once generated, factor IXa forms a complex with factor VIIIa on membrane surfaces in an interaction that is calcium dependent. In contrast to the solution-phase activation of factor IX by factor XIa, factor X is converted to its enzyme form, factor Xa, by factor IXa in complex with factor VIIIa on membrane surfaces: phospholipid vesicles, activated platelets, or endothelial cells. The activation of factor X by factor IXa has been evaluated under multiple experimental conditions. Calcium by itself accelerates factor IXa activation of factor X, but its more important role is to allow for the formation of the tenase complex on membrane surfaces. The presence of both factor VIIIa and membrane surfaces greatly accelerates the activation of factor X, while the presence of either of these cofactors alone has a minimal effect. [155] [156] The k_{cat} for factor X activation by factor IXa in the presence of factor VIIIa but without phospholipid vesicles is 0.058 min^{1} ; the same reaction without factor VIIIa but with phospholipid vesicles (25 M) has a k_{cat} of 0.095 min^{1} , whereas in the presence of factor VIIIa and phospholipid vesicles the reaction is greatly accelerated, with a k_{cat} of 1,740 min^{1} . [119] The activation of factor X by factor IXa is also supported by activated platelets. The k_{cat} for this reaction is 1,240 min^{1} , which is very close to the k_{cat} for factor X activation in the presence of phospholipid vesicles. These results suggest a physiologic role for platelets in this process. Stern et al. [157] [158] have demonstrated equal binding of factors IX and IXa to bovine aortic endothelial cells. There is an equivalent increase in binding of both factor IX and factor IXa in the presence of activated or unactivated factor VIII. [159] However, when factor X is added to the reaction mixture, a high-affinity binding site with relative specificity for factor IXa is expressed, and the interaction of factor IXa with endothelium is increased 10- to 40-fold. Factor X does not enhance factor IXa binding without the presence of factor VIII.

Physiologic inhibitors of factor IXa circulate in plasma. Factor IXa is inhibited by antithrombin III, which forms a 1:1 stoichiometric complex with factor IXa as well as other activated coagulation factors. This reaction is accelerated 1,000-fold by the addition of heparin. [160] [161] [162] [163] Protease nexin-2, an amyloid -protein precursor containing a Kunitz-type protease inhibitor domain, is an even more potent inhibitor of factor IXa than antithrombin III. [164] [165] However, it is present in plasma in only picomolar concentrations. Nonetheless, protease nexin-2 is an -granule component of platelets and may achieve physiologically significant concentrations in areas of platelet degranulation. [166]

Factor IX Eagle Rock and factor IX Bergamo are both characterized by the substitution of valine for Gly 363 and are functionally normal except for their inability to bind antithrombin III and their inability to activate factor X. [139] [167]

Defects Affecting Enzymatic Activity

Mutations within the catalytic domain of factor IX may decrease enzymatic activity. A mutation of IIe 397 Thr has been recognized in many hemophilia B families, including factor IX Vancouver, [168] factor IX Long Beach, [169] and factor IX Los Angeles. [167] This defect is near, but not within, the active site of factor IXa; it may alter the extended substrate-binding site for factor X. Factor IX Angers (Gly 396 Arg) has a mutation within the substrate-binding pocket of factor IXa that disrupts enzymatic activity. [170] Factor IX $B_{m\ Lake\ Elsinore}$ (Ala 390 Val) [171] also possesses a mutation within the substrate-binding pocket that interferes with enzymatic activity. Factor IX Eagle Rock (Gly 363 Val) [167] and factor IX Bergamo [122] both have mutations adjacent to Ser 365, the active site serine, that ablate enzymatic activity. A mutation of Arg 333 also leads to a defect in the ability to catalyze factor X cleavage.

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MOLECULAR BASIS OF HEMOPHILIA B

Defects in Gene Structure That Cause Hemophilia B

More than 800 different patterns of molecular anomalies have been described in patients with hemophilia B. [64] One-third of these are caused by de novo mutations. Gross deletions, microdeletions, insertions, and missense and nonsense point mutations have all been described, making the factor IX molecule one of the best models for studying natural mutations in humans.

Gross deletions of the factor IX gene causes severe, antigen-negative hemophilia B (Fig. 108-10).[172] [173] They account for 6% of independent factor IX mutations. A subset of large deletions is thought to result from alternating purine and pyrimidine segments in which large hairpin structures form. [174] Gross deletions have been associated with anti-factor IX antibodies in response to replacement therapy with factor IX. [172] In a series of 62 families with hemophilia B, a family member developed an inhibitor in approximately one-third of families exhibiting deletions or nonsense mutations. [175] Family members carrying missense mutations did not develop inhibitors. Thus, while antibody production can be associated with gross gene deletions, not all such patients have demonstrated such a response. [176] [177] In addition, some patients with anti-factor IX antibodies do not possess gross gene deletions, [74] [178] and in at least one case antibodies developed in an antigen-positive patient.[178] Thus, the lack of circulating factor IX protein is not the sole factor controlling the

Figure 108-10 Factor IX gene deletions as a cause of hemophilia B. Deleted portions of factor IX gene are indicated by pink rectangles; an uncertain extent of deletion is indicated by red lines. The factor IX gene (exons 18) is shown in black.

development of an immunologic response to factor IX replacement therapy.

Smaller deletions of the factor IX gene also cause hemophilia B. Although most reported microdeletions are unique, at least two deletion hotspots have been found in areas where perfect hairpin loops are generated from imperfect loops.[179] Many other patterns of small deletions have been described. Factor IX Chicago-1 possesses a complex deletion of exons 5, 7, and 8, totaling approximately 25 kb.[180] Factor IX Seattle-1 contains an intragenic deletion of exons 5 and 6 of the factor IX gene, [181] resulting in the production of a truncated factor IX protein of 36,000 molecular weight that is excreted in the urine. [182] Factor IX Yemen[178] and factor IX Tubingen[178] each possess deletions of exons 13 of the factor IX gene, with variable amounts of the 5-flanking sequence also deleted. Factor IX Hanover has a deletion of exons 4 and 5, totaling 8 kb.[178] Factor IX Strasbourg contains a 2.8-kb intragenic deletion encompassing exon 4, the exon encoding the first EGF-like domain. [183] Despite possessing 30% of normal factor IX antigen levels, this family displays a severe hemophilic phenotype. Factor IX Ratingen shows a loss of exon 7, with a deletion of 1.5 kb.[178] Factor IX London-10 contains a deletion of the codon for Arg 37, [74] resulting in a severe hemophilic phenotype despite the presence of 12% of normal factor IX antigen levels. Factor IX Seattle-2 contains a deletion of a single adenine nucleotide at position 17,699 in exon 4 of the factor IX gene, corresponding to residue 85 of the factor IX molecule.[184] This results in a frameshift mutation, creating a stop codon at codon 86. Factor IX London-11 (deletion of bases 31,05931,060), factor IX London-12 (deletion of nucleotide 6,392), and factor IX Malmo-1 (deletion of nucleotides 30,95030,957) also result in frameshifts leading to premature stop codons. [74] It is unclear whether these mRNAs are processed normally, resulting in a truncated plasma protein secreted in the urine as above, or whether these frameshift mutations result in an unstable mRNA.

In other cases, point mutations can lead to the production of little to no factor IX antigen. Rees et al. [185] have described a point mutation within the obligatory donor splice junction of exon 6. This mutation results in the production of an improperly spliced mRNA, causing a defect in transcription that results in no factor IX antigen being produced by these patients. Factor IX Bordeaux (Lys 411 Stop), [170] factor IX Portland (Arg 252 Stop), [186] factor IX Malmo-3 (Arg 248 Stop),[74] Malmo-4 (Arg 29 Stop),[74] and Malmo-5 (Trp 194 Stop),[74] factor IX New York (Arg 338 Stop),[187] factor IX Bonn-1 (Arg 338 Stop),[178] an unnamed mutant (Arg 252 Stop),[188] and other mutations all possess nonsense mutations resulting in premature stop codons. In each case, the premature stop signal yields an antigen-negative phenotype.

Finally, several missense mutations, gene insertions, and combinations of gene insertion plus gene deletion have been described that result in markedly reduced levels of factor IX antigen (Fig. 108-11). Factor IX London-8 (Cys 336 Arg) results in the loss of a cysteine conserved in serine proteases. [74] This loss disrupts the tertiary structure of the catalytic domain. Factor IX London-9 (Asn 120 Tyr) also leads to a severe,

Figure 108-11 Missense and nonsense mutations in the factor IX amino acid DNA sequence as a cause of hemophilia B. Missense mutations are designated by red shading and nonsense mutations are designated by a red circle. Amino acids are designated by the one-letter code. , -carboxyglutamic acid; , -hydroxyaspartic acid.

antigen-negative phenotype.[74] Microinsertions appear to result from polymerase slippage events as evidenced by the presence of direct repeats in most cases. [174] Hemophilia B El Salvador contains a 6.1-kb insertion near exon 4 of the factor IX gene, [189] resulting in moderate hemophilia.

Antigen-Positive Hemophilia B

Approximately one-third of cases of hemophilia B fall within the group termed cross-reactive material positive (CRM+). Such patients have normal levels of factor IX antigen and variable levels of factor IX activity, owing to the presence of a dysfunctional factor IX molecule. Mutations have been described that affect post-translational protein processing, -carboxylation, lipid binding, EGF domain function, zymogen activation, and substrate recognition and/or enzymatic activity. In addition, a series of patients have been reported to display the hemophilia B $_{M}$ phenotype. This has proved to be a heterogeneous set of mutations, as noted in the following discussion.

A developing theme in understanding the molecular basis of hemophilia B is the discovery of duplicate mutations in apparently unrelated patients. To date, 597 unique point mutations (both missense and nonsense) have been reported. [64] Several groups have postulated that the CpG dinucleotide sequence is a mutational hotspot.[190] [191] [192] The CpG sequence is a component of four of the six arginine codons; as a mutational hotspot, it may represent an important cause for the development of spontaneous point mutations. Furthermore, using a mathematical model, the 20 CpG dinucleotides in the factor IX coding sequence showed an observed mutation rate 150 times the rate that would be expected for the predicted transition rate. [193] Of 51 single-base-pair substitutions found in one study, 27 were at CpG dinucleotides, which represents a 38-fold excess for mutations at these sites. [194] In another study, point mutations at CpG dinucleotides were estimated to be increased by 77-fold over the expected rate. [195]

In patients with mutant plasma proteins, the mutation of arginine to another amino acid also appears to be a developing theme. Insofar as internal point mutations are likely to destabilize protein structure and lead to markedly diminished circulating protein levels, patients preselected for circulating mutant protein antigen are likely to have amino acid substitutions on the protein surface. Arginines, located on the protein surface, are used by proteases of the trypsin family to hydrolyze adjacent bonds during protein processing and zymogen activation. It would thus appear, given the functional importance of arginines, that their mutation leads to phenotypically obvious defects in protein function.

Molecular Diagnosis

Hemophilia B is diagnosed by the finding of isolated, hereditary factor IX deficiency. Factor IX deficiency may be observed by prolongation of the activated partial thromboplastin time and by failure of plasma from these patients to correct the clotting time of known factor IX-deficient plasma. These findings, combined with a lifelong history of bleeding or a family history of bleeding disorders, secure the diagnosis. The level of factor IX antigen present in the plasma of each patient permits the hemophilia to be characterized as antigen negative or antigen positive. Within a particular cohort, the factor IX activity level is usually constant, reflecting that each family displays the same genetic defect and that each defect is associated with a particular phenotype.

The bleeding disorder associated with moderate or severe hemophilia B and the sex-linked nature of the disorder make genetic counseling for affected persons and potential carriers an important issue. Carrier analysis has been done by differentiating between factor IX activity and factor IX antigen levels. However, this method has been at best only 80% effective, partially because of the preponderance of mutations yielding markedly diminished factor IX antigen and partially because of lyonization, which causes differential levels of inactivation of the X chromosome carrying the mutant gene. [196]

With knowledge of the sequence of the factor IX gene, it is possible to probe for the precise genetic defect among potential carriers. If the genetic defect for a particular cohort is known, the DNA of potential carriers can be screened using stringent hybridization to oligonucleotide probes to detect whether they carry that genetic defect. This method is handicapped, because as many as one-third of hemophilia cases in any generation arise from new mutations. It would likely be difficult to detect these new mutations using probes for specific mutations.

Of greater value has been the detection of several restriction fragment length polymorphisms (RFLPs) within the factor IX gene that can be used for carrier detection (Fig. 108-10). A number of polymorphisms have been described within and adjacent to the factor IX gene. [197] These polymorphisms, when informative, give a >99% probability of a correct diagnosis. Using the ten polymorphisms available, assays of >90% of families are informative. In some studies, >80% are informative when the 5 MseI RFLP, the 3 HhaI RFLP, and the DdeI polymorphism are used.[198] It should be emphasized that these figures apply only to the white population, in whom the most extensive study of RFLPs has taken place. It is clear that the frequency of each polymorphism varies with the ethnic group being studied (Fig. 108-12), such that the polymorphisms currently employed for diagnosis within the white population are inadequate for determining carrier status in either the Japanese [199] or black population. [200]

To determine whether a person is a hemophilia B carrier, genomic DNA is isolated from the potential carrier, the patient with hemophilia B, and both parents of the potential carrier. That DNA is digested with the appropriate restriction enzymes, and the digested DNA is run on a gel matrix. The DNA is transferred to nitrocellulose, and a Southern blot is performed using labeled factor IX cDNA as the probe. The restriction maps are then compared with the goal of looking for any polymorphisms found in the patient with hemophilia but not in the normal parent. The same procedure can be used on DNA obtained by chorionic villous sampling to determine whether the fetus of a hemophilia B carrier will be a normal or an affected male, and whether a female fetus will be normal or will carry the hemophilia gene.

Even in the white population, some patients cannot be diagnosed with techniques that use polymorphisms. The application of polymerase chain reaction (PCR) technology to the detection of hemophilia carriers has changed the evaluation of patients

Figure 108-12 Restriction fragment length polymorphisms in the factor IX gene.

with hemophilia B. With PCR, specific exons are amplified and sequenced to determine the abnormality that led to hemophilia B. With this information, it is possible to determine whether that mutation is present in the genome of either potential carriers or the fetuses (or both) of those carriers and patients. This obviates the problem of genetic recombination that clouds the use of closely linked restriction polymorphisms to determine the presence or absence of a mutant gene. Moreover, with the use of PCR, it becomes possible to perform antenatal testing either on cells obtained by amniocentesis or on chorionic villous samples without having to wait the several weeks required to obtain enough fetal cells to do restriction digests.

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Chapter 109 - Clinical Aspects and Therapy of Hemophilia

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INTRODUCTION

. . . if the first son of a woman is circumcised and he dies and the second son is circumcised and he dies, you must not circumcise the third son. . . . Additionally, the sons of the womans sister should not be circumcised but the sons of her brother can be circumcised.

The Talmud (Yebamot 64b)

The existence of a sex-linked hereditary coagulopathy (probably hemophilia) was first recognized and described in the Talmud in the fifth century and eventually labeled as Haemorrhaphilia, or love of bleeding, in 1828 in the German medical literature. [1] With the development of a crude laboratory assay in 1893 to determine the condition of blood coagulability in clinical and experimental situations, the clotting times of plasma from individuals with hemophilia were noted to be greatly prolonged compared to nonbleeders;[2] however, mixing two sources of plasma corrected the abnormality in vitro.[3] By 1947, the defect in hemophilia had been attributed to a single deficient plasma protein, which was required for platelet utilization and thromboplastin generation. [4] [5] Shortly thereafter, the recognition by Pavlovsky[6] that the plasmas of some hemophiliacs did not always correct the in vitro or in vivo defects of other clinically identical hemophiliacs led to the appreciation that multiple types of hemophilia existed. The clinical entity related to the protein defect described by Brinkhous and Quick was termed hemophilia A and was shown to be related to deficiency of factor VIII (antihemophilic factor or antihemophilic globulin), whereas the other type was termed hemophilia B, and was due to factor IX deficiency (antihemophilic factor B, plasma thromboplastin component, or Christmas factor, named after an individual with the factor deficiency). [7] [8] These two types of hemophilia have an indistinguishable hereditary pattern (sex-linked recessive) and are similar clinically.

The pathophysiology of hemophilia A and B is based on the insufficient generation of thrombin by the factor IXa/factor VIIIa complex through the intrinsic pathway of the coagulation cascade. This is somewhat perplexing, however, because the factor VIIa/tissue factor complex produces enough factor Xa through the extrinsic pathway to prevent the bleeding manifestations of hemophilia despite the intrinsic pathway defect. Recent information indicates that formation of the tissue factor/factor VIIa complex is the initiating event in mammalian hemostasis and is triggered immediately following vascular injury when factor VII in plasma interacts with cellular membranes extrinsic to the vasculature, which express tissue factor on their surfaces. Furthermore, factor IX in the intrinsic pathway, not factor X, is the preferred substrate for the tissue factor/factor VIIa complex because the extrinsic pathway activation of factor X is inhibited by tissue factor pathway inhibitor after a small quantity of factor Xa has been formed[9] [10] [11] (Chap. 102). In this model, a trace amount of factor Xa generates the inactive intermediate product factor IX, which is converted to factor IXa by the tissue factor/factor VIIa complex. The factor IXa/VIIIa tenase complex subsequently accelerates factor Xa generation and thrombin production more than 50-fold through the

intrinsic pathway. A deficiency in either factor VIII or IX can thereby lead to ineffective hemostasis by inadequate thrombin generation.

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EPIDEMIOLOGY

Accurate estimates of the incidence and prevalence of hemophilia depend on the reliable diagnosis and differentiation of hemophilia A and B from von Willebrand disease and other less common congenital bleeding disorders. Hemophilia A and B are clinically identical, [12] and therefore must be distinguished from von Willebrand disease, which is due to defective von Willebrand factor. [13] Hemophilia A is four to six times more common than hemophilia B, [14] [15] [16] [17] [18] [19] [20] [21] [22] [23] [24] [25] [26] [27] [28] and mutations that cause hemophilia occur throughout the genes for factor VIII [29] and factor IX. [30] Based on surveys of patients with hemophilia in North America and Europe, the annual incidence of hemophilia A has been estimated at approximately 1 in 5,000 male births (Table 109-1). The incidence of hemophilia B is estimated at about 1 in 30,000 male births. [28]

The prevalence of hemophilia indicates the number of patients alive with the disease at a given point in time. Hemophilia A and B are observed in all ethnic and racial groups. Some studies indicate a lower prevalence of hemophilia in the Chinese population, and others suggest that hemophilia occurs disproportionately in Caucasians, [31] [32] but the experience of most hemophilia clinicians in the United States is that the demographics of hemophilia mirror the general population (Table 109-2). [33]

The prevalence of hemophilia varies with age. The peak prevalence occurs in the second or third decade, [14] [15] [16] [17] [18] [19] [20] [21] [22] [23] [24] [25] [26] and it seems likely that the lower prevalence observed in younger men is due to underdiagnosis of mild hemophilia, whereas the lower prevalence in older men is likely due to excess mortality especially in those with severe disease. As the overall fitness of patients with hemophilia improves due to advances in therapy, and as the diagnosis of mild cases improves, there will probably be an overall increase in both the true and observed prevalence of hemophilia. [34] The epidemic of acquired immunodeficiency syndrome (AIDS), though devastating to infected individuals, has been predicted to have a transitory and relatively modest effect on the future prevalence of hemophilia. [32] Carrier testing and prenatal diagnosis (see box, Carrier Detection and Prenatal Diagnosis in Hemophilia) have not had a great impact on the incidence of hemophilia, because a substantial minority of cases are the result of unanticipated new mutations and not all known female carriers undergo prenatal diagnostic testing. Further,

TABLE 109-2 -- Prevalence of Hemophilia in Various Racial Groups in the United States

Combined Prevalence of Hemophilia A and B in Various Racial Groups	
Racial Group	**Prevalence (cases/100,000 males)**
Caucasian	13.4
African American	12.3
Hispanic American	11.4

Racial Distribution of Patients with Hemophilia A and B		
Group	**Hemophilia A**	**Hemophilia B**
Caucasian	71.7%	69.6%
African American	12.6%	17.2%
Hispanic American	7.8%	4.7%
Other/Unknown	7.9%	8.4%

Data from the Centers for Disease Control Hemophilia Surveillance System Project [33] show the prevalence and distribution of hemophilia A and B among different racial groups. The prevalence for each group is derived by dividing the number of cases of hemophilia A and B by the total number of males in a six-state region (Colorado, Georgia, Louisiana, Massachusetts, New York, and Oklahoma) in 1993-1994. The distribution of hemophilia A and B in various groups is derived from the number of cases of hemophilia A or B in each group divided by the total number of cases of hemophilia A and B.

pregnant carriers diagnosed with a hemophilic fetus do not always opt for pregnancy termination. [20] [21] [26] [35]

The hemophilias are more common than autosomal recessive bleeding disorders because disease is manifest in the first male to inherit a defective factor VIII or factor IX allele, unlike recessive diseases, which require inheritance of two defective alleles for disease to occur. Consanguinity is not a usual feature of families in which hemophilia occurs, in contrast to autosomal recessive bleeding disorders. Hemophilic females are exceedingly rare because the 50% factor levels typical of carriers protect against bleeding, and therefore most carriers are asymptomatic. Rarely, females may be affected due to unequal inactivation (lyonization) of factor VIII or factor IX alleles, hemizygosity for all or part of the X chromosome, or other mechanisms (see later discussion).

Three degrees of clinical severity correspond to the level of plasma coagulant factor activity. Those with factor VIII or factor IX levels <1% of normal have severe hemophilia characterized by frequent spontaneous bleeding into joints and soft tissues as

TABLE 109-1 -- Prevalence, Incidence, and Severity of Hemophilia in Various Countries

Population (year)	**Hemophilia A**			**Hemophilia B**		
	Prevalence (per 100,000 males)	**Annual Incidence (per 10,000 males)**	**Severe Disease (% of cases)**	**Prevalence (per 100,000 males)**	**Annual Incidence (per 10,000 males)**	**Severe Disease (% of cases)**
United States [14]	20.6		60%	5.3		44%
Sweden [15] [16]	10.711.1	1.4	3040%	2.63.2	0.3	2832%
Greece [17] [18]	10.712.0	1.9	3858%	1.62.0	0.4	5260%
Spain [19]	5.7			0.9		
United Kingdom [20] [21]	9.515.7		4461% [a]	1.02.8	0.4	5260% [a]
Finland [22] [23]	5.4	0.7	63%	3.2		19%
Netherlands [24]	14.5		(40%) [b]	2.6		(40%) [b]

Italy[25]	8.2		65%[a]	1.5		70%[a]
Canada[26]	11.6		34%	2.7		24%

Studies are listed in chronological order based on date of publication.

[a]Severe disease is defined as coagulant factor activity levels of <1% of normal except in the United Kingdom and Italy where severe disease includes coagulant factor activity levels of <2% of normal.
[b]Percent severe disease reported for the Netherlands is the overall average for hemophilia A and B combined.

CARRIER DETECTION AND PRENATAL DIAGNOSIS IN HEMOPHILIA

Carrier testing and prenatal diagnosis can be offered to women who are interested in childbearing and have a family history of hemophilia. Carrier testing is particularly valuable to women who are related to obligate carrier females or males with hemophilia. It may also be important to other members of the family to ascertain the mechanism of mutation of the factor VIII or factor IX genes. For instance, germ line mutation in a maternal grandfather has different implications for maternal aunts of a patient with hemophilia than does germ line mutation in the mother of the patient. This analysis is especially pertinent in de novo cases of hemophilia. The generation in which the mutation has occurred may be ascertained through carrier testing of the patients mother.

Prenatal diagnosis may be useful even when pregnancy termination is not acceptable to the family on moral or ethical grounds. For instance, a prenatal diagnosis of hemophilia may be indication for cesarean section and perinatal factor replacement and may also allow more time for psychological preparation of the parents prior to delivery. Conversely, ruling out hemophilia as soon as possible is certainly advantageous to parents of a normal child.

For many years determination of the hemophilia phenotypic by factor VIII or factor IX activity assay was the only avenue for carrier testing and prenatal diagnosis. An accuracy of >90% for carrier detection and 95% for prenatal diagnosis has been achieved in laboratories with special expertise in blood coagulation testing using assays for factor VIII, factor IX, and von Willebrand factor. Certain limitations apply, however. Sampling of fetal blood for factor activity cannot be performed before about 16 weeks gestation, and procoagulant proteins in amniotic fluid can give spurious coagulant activity on coagulation factor assays. Furthermore, maternal factor IX (but not factor VIII) is found in amniotic fluid, which may contaminate fetal blood samples and make prenatal diagnosis of hemophilia B more difficult than hemophilia A. Also, a greater proportion of hemophilia B is associated with a CRM+ phenotype than is hemophilia A, which can limit prenatal diagnosis of hemophilia B by immunoassay. Phenotypic diagnosis of carriers can be misleading in females with extreme inactivation of the X chromosome with the abnormal factor VIII or factor IX allele. The analysis of factor VIII levels in potential carriers needs to take into account the age of the woman as well as ABO blood type, which affects the normal range of factor VIII and von Willebrand factor levels. Despite these limitations, phenotypic analysis still is useful as an adjunct to genotypic analysis, and may be the only option when key family members are not available for genetic analysis.

Genotypic analysis requires either that the specific gene defect is known or that the carrier be heterozygous for at least one DNA polymorphism within, or closely linked to, the gene in question. Factor VIII and factor IX polymorphisms are detected by observing different sizes of DNA fragments obtained after digestion with specific enzymes (restriction fragment length polymorphisms, or RFLPs). DNA from the affected male must be available for analysis if the genetic defect is not known in advance. Prenatal diagnosis by genotypic analysis has certain advantages over phenotypic analysis. Results are not affected by X inactivation, ABO blood type, or von Willebrand factor levels, as are factor activity assays used for phenotype analysis. Because less material is needed for genetic analysis, the test can be done during the first trimester (e.g., chorionic villus sampling at 12 weeks gestation).

When a carrier is heterozygous for an RFLP and the hemophilic males DNA is available for analysis, it is possible to determine which RFLP is associated with the gene defect. Fetal DNA can be characterized for the presence of the RFLP, and it can be determined whether an allele associated with the defective gene has been inherited or not. The analysis of fetal DNA for inheritance of the defective allele is a probabilistic venture by virtue of the fact that there is a (small) chance of recombination occurring between the marker site and the actual gene defect. Accordingly, the best markers for analysis are within the gene (less likely to be subject to recombination with the defect) or very close to the gene of interest. When the specific gene defect is known, the analysis becomes deterministic rather than probabilistic.

A number of intragenic polymorphisms for the factor VIII and factor IX genes have been characterized as have a number of extragenic polymorphisms closely linked to the factor VIII or IX genes. Intragenic sites are preferable because the frequency of recombination approaches zero as the distance between the polymorphism and the gene defect decreases. Where the specific gene defect is not known, RFLPs are sought for which the carrier female is heterozygous. The most useful of these are polymorphisms with two alleles with 50% frequency or better yet many different alleles with more or less equal distribution of frequency of alleles to maximize the likelihood that the female carrier is heterozygous for a polymorphic site. The latter is represented by the TaqI polymorphisms at St14, an extragenic site near the factor VIII gene. By analyzing multiple polymorphisms the likelihood of finding an informative site increases. Typically the ability to find an informative site increases to nearly 100% through the analysis of three or more different sites.

Polymerase chain reaction amplification of DNA and denaturing gradient gel electrophoresis analysis may facilitate rapid screening of mutations in the known coding sequence for factor VIII. However, this analysis does not account for intervening sequences, which comprise the vast majority of the gene. If the known male with hemophilia A is not available for analysis or if informative polymorphisms are not observed in the carrier female, it may be useful to analyze fetal DNA for the common intron 22 inversions responsible for almost half of all severe cases of hemophilia A.

well as prolonged bleeding with trauma or surgery. Those with 15% of normal levels have a moderate course characterized by occasional spontaneous bleeding and excessive bleeding with surgery or trauma, and levels >5% of normal seem to protect against spontaneous bleeding, although excessive bleeding with surgery or trauma still occurs.

Patients with severe or moderate disease are readily diagnosed due to spontaneous bleeding and joint disease, but mild hemophilia probably is substantially underdiagnosed. The proportion of hemophilia A patients with severe disease differs among series, ranging from as little as 30% to as much as 80% of cases (Table 109-1). The largest of these series seem to suggest a figure of 60%. Differing amounts of mild disease reported probably account for the discrepancy between reports. Furthermore,

in some reports severe disease is defined by factor VIII levels of <2%, whereas in other series it is diagnosed on the basis of levels <1% of normal (Table 109-1). The proportion of hemophilia A that is severe is greater than that for hemophilia B in which severe disease is found in 2045% of cases. [14] [15] [16] [17] [18] [19] [20] [21] [22] [23] [24] [25] [26]

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GENETIC ASPECTS

Hemophilia A demonstrates sex-linked patterns of inheritance by virtue of the location of the factor VIII gene on the long arm of the X chromosome (Xq28). [36] The factor IX gene is also located on the long arm of the X chromosome about 1.5 megabases away, at Xq27. [37] Accordingly, males bearing either defective allele on their single X chromosome are affected with hemophilia, the severity of which is determined by the particular mutation. Hemophilic males do not transmit the gene to their sons except under extraordinary circumstances, [38] but all of their daughters are obligate carriers for hemophilia. Although female carriers are usually not affected there are a number of mechanisms by which they can be symptomatic with hemophilia, however. [39] Carriers with a defective allele on one X chromosome and a normal allele on the other X chromosome may undergo X chromosome inactivation (described by Mary Lyon), which results in unequal inactivation of alleles so that the defective (hemophilic) allele is expressed in preference to the normal allele. The lyonization process may be the result of random inactivation [40] [41] or may be nonrandom in some cases (i.e., a distinct tendency for inactivation of the normal X allele is seen in all females from the same kindred). [42] Females who have hemophilia by this process are said to be lyonized carriers. The phenotype in the lyonized carrier is no more severe than in the hemophilic male in such families because any expression from the normal allele in such carriers will ameliorate the hemophilia phenotype. Accordingly, symptomatic lyonized carriers are seen only in hemophilic kindred where the male phenotype is severe.

Other mechanisms for female hemophilia include homozygosity for the defective factor VIII allele or hemizygosity for the defective gene. Homozygosity may occur as the result of mating between a hemophilic male and a female carrier (related or not). This mechanism may be inferred if multiple hemophilic sons are born to a hemophilic woman, which has been confirmed in one case through detailed genetic analysis of the kindred. [43] Hemizygosity for the X chromosome can occur in females who have the 45, XO (Turner) karyotype limited to cells that synthesize factor VIII or IX (somatic mosaicism) [39] [44] or in all cells as a consequence of a germ line event (Turners syndrome). [45]

One report documents a female carrier who inherited an affected factor VIII allele from her mother (an unaffected carrier) and an X isochromosome from her nonhemophilic father; in this instance the expression of factor VIII from the isochromosome was defective and the daughter displayed severe hemophilia A. [46] In another case a structurally abnormal X chromosome deleted at the tip (Xq27) distal to a normal factor IX gene resulted in the inactivation of that chromosome, and expression from the other X chromosome revealed a defective factor IX gene for which she was a carrier. As a consequence she demonstrated severe hemophilia B. [47]

The evaluation of hemophilia in a female should begin with testing for von Willebrand disease as well as autosomal recessive coagulation factor deficiencies associated with a bleeding phenotype. In hemophilic females it is essential that a karyotype be done not only to rule out Turners syndrome but also to rule out a male (46, XY) karyotype associated with testicular feminization, which has been described in hemophilia A. [48] [49]

There is no single mutation responsible for hemophilia, in contrast to cystic fibrosis, sickle cell anemia, or Duchennes muscular dystrophy, which are caused by one or a limited number of mutations. Hundreds of point mutations, deletions, and inversions that cause hemophilia have been described throughout the factor VIII and factor IX genes (Chaps. 107 and 108). [28] [50]

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ETIOLOGY AND PATHOGENESIS

The hallmark of hemophilia is bleeding into joints (hemarthrosis), which is quite painful and leads to chronic inflammation and deterioration not unlike that seen in rheumatoid arthritis. Abnormal bleeding originates from the subsynovial venous plexus underlying the joint capsule, where a lack of thromboplastic activity has been demonstrated.[51] The high degree of factor Xa inhibitory activity conveyed by the tissue factor pathway inhibitor synthesized by human synovial cells may also predispose hemophilic joints to bleeding [52] and may explain the impressive hemostatic response after intravenous administration of recombinant factor VIIa for acute hemarthroses in factor VIII inhibitor patients. [53] [54] Untreated, inadequately treated, or recurrent joint bleeds result in chronic synovial and intra-articular pathology produced by the toxic effects of hemosiderin and release of proteolytic enzymes (e.g., elastase, collagenase, etc.) from the cellular blood components. Synovial hypertrophy, hemosiderin deposition, fibrosis, and damage to cartilage progress until subchondral bone cyst formation becomes apparent on x-ray. Abnormal mechanical forces of weight bearing on these joints further exacerbate the damage and ultimately result in permanent deformities with subluxation, misalignment loss of mobility, occasional ankylosis, and unequal extremity lengths. Multiple joint involvement may be the result of quantitative loading through the joint [55] so that once hemophilic arthropathy compromises the function of one joint, destruction may be precipitated in another joint because of unusual and increased stresses placed on it to compensate for the dysfunction of the damaged joint. For example, an individual with a gross deformity of the knee from recurrent hemarthrosis may be unable to assume a standing position after sitting without shifting an inordinate amount of weight onto his elbows, wrists, and hands in an attempt to project his body forward and upward. Once standing, the flexion contracture of the knee will force the individual to assume a plantar flexion of the ipsilateral foot to minimize the length discrepancy between the two legs. This results in an unnatural weight-bearing position of the ankle, leading to increased bleeding, bone destruction, pain, and eventual valgus deformity.

Radiologically and clinically, hemophilic arthropathy evolves through five stages (Fig. 109-1). Stage 1 consists of intra- and periarticular swelling due to acute bleeding. The joint structure appears normal. Stage 2 is associated with loss of bone density around the joint and epiphyseal hypertrophy secondary to chronic synovial inflammation. Progression of the epiphyseal hypertrophy with widening of the intercondylar notch characterizes the changes in stage 3. The articular cartilage surfaces are not affected until stage 4, when secondary joint space narrowing also becomes apparent. Stage 5 is characterized by advanced erosion of the cartilage with loss of joint space, the presence of joint fusion, and development of fibrosis of the joint capsule.

Because x-ray and clinical examination frequently underestimate the extent of joint disease, new approaches of evaluation are being introduced. [56] Ultrasonography is particularly helpful in the evaluation of joints affected by acute or chronic effusions. The progression of synovial hypertrophy and the resolution of acute hemarthroses following treatment can be monitored by serial sonograms; however, the technique is insensitive to changes in bone and cartilage. Although computed tomography (CT) scanning has been disappointing for evaluating hemophilia joint disease, magnetic resonance imaging (MRI) permits improved discrimination of changes in the cartilage, synovium, and joint space; furthermore, signal characteristics vary with duration of disease. [57]

Figure 109-1 Radiographic changes associated with hemophilic arthropathy. **(A)** Multiple subchondral cysts in the head of the humerus are an early finding of hemophilic arthropathy in this radiograph of the shoulder. The glenohumeral joint space is still fairly well preserved and the range of motion is normal. **(B)** Widening of the intercon-dylar notch and near fusion of the femur and the medial tibial con-dyle are seen in this knee joint affected by hemophlic arthropathy. **(C)** Narrowing/fusion of the tibiotalar joint in the ankle.

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CLINICAL PRESENTATION

The clinical presentations of patients with hemophilia A and B are indistinguishable. Hemophilia, especially severe disease with <1% of normal factor activity, is readily diagnosed, especially in patients with prior family history of hemophilia. The diagnosis of hemophilia is usually made on the basis of unusual bleeding symptoms early in life, and the age of first bleeding varies with the severity of the disease. Infants with severe disease typically present with their first bleeding between 12 and 18 months (despite undetectable factor VIII or factor IX activity); the first manifestations of disease in mild hemophilia are seen at 25 years of age. [12] [58] Severe hemophilia most often presents as easy bruising; mild to moderate disease is more often associated with bleeding after trauma or dental procedures. [12] Although all patients with severe disease have spontaneous hemarthroses, these are altogether rare before 9 months of age, presumably because there is little weight bearing until walking begins. [58]

It is remarkable that the trauma of childbirth does not cause intracranial hemorrhage any more often than it does, although some infants may be born with hemophilia A as a result of de novo mutation and die from intracranial hemorrhage at birth undiagnosed. A study of the modes of delivery and prenatal complications in 117 moderately or severely affected infants (hemophilia A and B) shows the risk of serious bleeding in conjunction with normal vaginal delivery to be small. [59] The use of vacuum extraction is a major risk factor and was associated with 10 cases of subgaleal/cephalic hematoma in this series. Cesarean section, even for those known to be affected in utero, does not eliminate the risk of intracranial hemorrhage. In addition, forceps deliveries and prolonged labor increase the risk of intracranial bleeds.[60] No bleeding complications have been reported with the use of fetal scalp electrodes and fetal blood sampling for intrapartum monitoring of affected neonates.[61]

It is often assumed that excessive bleeding with circumcision is usual for hemophilic infants. In fact, this occurs in less than half of severely affected infants after circumcision.[58] Notably, those who bleed are those circumcised several days later than those who do not. Thus, failure to bleed excessively with circumcision does not rule out hemophilia.

The individual with severe hemophilia can bleed from any anatomic site after negligible or unnoticed trauma. Although intra-articular and intramuscular bleeds are the most common and characteristic clinical symptoms of severe and moderate hemophilia, they are rare before the child enters his ambulatory years. In fact, the most common *presenting* manifestations of hemophilia A and B are soft tissue bleeds (41%), bleeding associated with intramuscular injections and surgery (16%), and oral bleeding from tongue or lip biting (11%). [62] Fourteen percent of the bleeding events were severe enough to require transfusion of packed red blood cells.

Soft tissue hemorrhage advances along tissue planes, and blood loss can be extensive because it is not confined to a closed space (e.g., the joint capsule) as in hemarthrosis. Because of this, soft tissue hemorrhage can be life-threatening. Intracranial hemorrhage was the leading cause of death prior to the AIDS epidemic and still remains the most common hemorrhagic cause of death. Hematuria and bleeding of the gastrointestinal tract are the most common problems, though less serious than intracranial hemorrhage. Retroperitoneal hematomas (Fig. 109-2) may occur spontaneously and extend downward along the psoas muscle through the inguinal canal into the thigh, or dissect upward along the vertebral muscles even as far as the neck.

Isolated hemorrhage into closed spaces such as the forearm can lead to a compartment syndrome characterized by compromise of nerve function and blood flow. If untreated by factor replacement and surgical decompression, hemorrhage may lead to permanent neuropathy or tissue necrosis. Untreated cases may result in loss of the limb. Compartment syndromes of the forearm can even be initiated by venipuncture at the antecubital fossa, and patients should be alert for its occurrence after phlebotomy.

The extreme examples of soft tissue hemorrhage, which are common in severe hemophilia A, are less common in moderate or mild disease. Moderate or mild disease may not be detected until adulthood (e.g., during dental extraction) when unexplained bleeding prompts evaluation. Routine preoperative laboratory tests may reveal a prolonged activated partial thromboplastin time (PTT) that triggers further testing and leads to a diagnosis of hemophilia. Some patients with mild disease serve

Figure 109-2 Retroperitoneal hemorrhage involving the iliopsoas muscle of the left iliac fossa in a patient with hemophilia A and a high-titer inhibitor.

in the military or engage in other hazardous pursuits, only to have hemophilia diagnosed later in life.

Intra-articular bleeding becomes problematic, usually between 2 and 3 years of age, and occurs most prominently in the knees, followed by the elbows and ankles. Bleeding into the shoulders, hips, and wrists is less common although trauma can precipitate bleeding in any joint. Bilateral involvement is not uncommon. [63]

In acute hemarthroses a tingling or burning sensation often precedes the onset of intense pain and swelling; joint mobility is compromised by pain and stiffness and the joint is maintained in a flexed position. Replacement of the deficient clotting factor reverses the pain in most cases and, once the bloody joint effusion resorbs, joint range of motion and function are regained.

Chronic hemophilic arthropathy is often painful with weight bearing and normal use, but frequently, the pain is related to the arthritic changes in the joint. Pain may disappear or subside once the joint becomes ankylosed. Muscle atrophy around the joints may lead to further instability, loss of use, valgus deformities, and increased bleeding due to loss of cushioning around the joint and uncoordinated function among muscle groups. Individuals with factor VIII and factor IX levels >20% of normal rarely develop hemophilic arthropathy even if they had experienced previous bleeds; however, one series describes an incidence of up to one-third in individuals with levels between 6% and 20% of normal.[64]

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LABORATORY EVALUATION

The hemophilias occur with various degrees of severity determined by the plasma concentration of factor VIII or factor IX. Typically, the same degree of severity exists within families, and there is little fluctuation of clotting factor levels within the individual over time. The exceptions to this include the so-called Heckathorns syndrome[65] in which factor VIII levels vary within affected family members; individuals with moderate or mild hemophilia A whose factor VIII levels vary as acute phase reactants with exercise, inflammation, and so forth; and the unusual variant of hemophilia B called factor IX Leyden [66] in which the reduced factor IX levels progressively rise after puberty, eventually approaching the normal range (34% increase per year through adolescence). This has been attributed to the effects of androgenic steroids on the mutated promoter region of the factor IX gene in these individuals. [67] [68]

Factor VIII and factor IX levels are assayed against a normal pooled plasma standard, which is designated as 100% activity or equivalent to 1.0 U/ml of factor VIII or factor IX. The normal range of factor VIII and factor IX levels varies between 50% and 150% although increases are associated in each with pregnancy and aging. The use of estrogen-containing contraceptives and replacement therapies produce substantially higher rises in factor VIII than factor IX. In the normal healthy premature and term neonate, factor IX (but not factor VIII) levels will typically be reduced to 2050% of normal and will rise to nearly normal adult levels within 6 months. This observation is attributed to hepatic immaturity at birth. In congenital factor VIII or factor IX deficiencies, individuals with undetectable activity (<0.01 U/ml) will experience the most severe and frequent spontaneous bleeding complications, whereas higher levels will manifest moderate (25% of normal activity) or mild (>5% of normal activity) symptomatology.

The whole blood clotting time, prothrombin consumption test, and the activated PTT are abnormal in hemophilia A and B because of intrinsic pathway coagulation protein deficiencies. Various other laboratory techniques are available for the specific determination of factor VIII and factor IX activities. The one-stage assay, based on the PTT, is most commonly used because of its reproducibility, simplicity, and cost. The two-stage

assay is more sensitive for the detection of low levels of factor VIII and factor IX. In Scandanavia, a chromogenic assay is used for the determination of factor VIII levels. There appear to be consistent differences in factor VIII assay results performed by chromogenic assay and by PTT in patients receiving recombinant factor VIII concentrates, with those determined by the chromogenic substrate method being approximately twice as high as those determined by the one-stage PTT method. [69]

Hemophilia A can usually be differentiated from von Willebrand disease in the laboratory by the presence of normal or increased von Willebrand factor antigen and ristocetin cofactor activity. [70] However, the rare von Willebrand 2N variant, in which the von Willebrand factor protein does not bind factor VIII, may be phenotypically identical to severe hemophilia A except for its autosomally transmitted inheritance pattern. [71] [72] Specialized assays to measure the factor VIII/von Willebrand factor protein-binding characteristics and genetic testing for the specific point mutations involved are necessary to diagnose this entity. Prolonged bleeding times, which are important for the diagnosis of von Willebrand disease, are normal in individuals with hemophilia. However, prolonged bleeding times have been documented in patients with hemophilia A and B in the absence of abnormal platelet aggregation assays or the surreptitious use of aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs) in several series of patients. [73] [74] Although there is no obvious explanation for this phenomenon, bleeding times tend to be more prolonged when the Simplate® device is used and correlate with severity of bleeding events, platelet IgG levels, circulating immune complexes, and vascular injury. The effects of concurrent hepatitis or human immunodeficiency virus (HIV) have not been evaluated.

Most individuals with severe deficiencies in factor VIII or factor IX (<1% activity) usually have undetectable levels of the corresponding factor antigen when assayed against a specific antibody and are designated cross-reactive material negative (CRM); moderately and mildly affected patients may have normal or slightly reduced levels of antigen (CRM+). [75] The clinical significance of CRM versus CRM+ status remains unclear; however, it has been conjectured that CRM individuals may be more predisposed than CRM+ individuals to the development of alloantibodies directed against their specific clotting factor protein defect. [76] A special subset of CRM+ hemophilia B, hemophilia B_M , is associated with an abnormal factor IX protein that prolongs the prothrombin time when an ox or bovine (rather than human) brain source of thromboplastin is used. This product of point mutations in the factor IX gene possesses decreased coagulation function and acts as a competitive inhibitor of factor VIIa. [77] [78]

Although an inhibitor to factor VIII or factor IX is clinically suspected when a bleeding episode fails to respond to an adequate dose of factor concentrate, laboratory confirmation is critical to the diagnosis and subsequent treatment strategy. For allo- (associated with a congenital defect) or auto- (associated with previously normal coagulation) antibody inhibitors, the patients prolonged PTT should be repeated after incubating patient plasma with normal plasma at 37°C for 12 hours. If the prolonged PTT does not correct, the presence of an inhibitor should be evaluated via specific clotting factor assays performed in multiple dilutions and subsequently quantitated according to the Bethesda method. The clotting factor against which the inhibitor is directed will remain persistently and equally deficient at all dilutions, whereas the apparent activity of other factors will increase as the inhibitor is diluted out. [79] [80] Dilutions of the patient plasma are incubated with an equal volume of normal plasma (standardized to contain 1.0 U/ml) for 2 hours at 37°C until a residual factor activity level falls within 2575% of the control mixture of normal plasma and buffer. A standard curve is established, which defines 1 Bethesda unit (BU, inhibitor potency that results in 50% neutralization of the factor VIII or factor IX activity in the pooled normal plasma). [81] Recently, the Nijmegen modification of the Bethesda method was described to increase the specificity of the assay in the very low range of factor VIII antibody inhibitor titers. This assay, which eliminates the pH-dependent variation of factor VIII activity in the incubation mixtures of the Bethesda assay and uses immunodepleted factor VIII-deficient plasma rather than a pooled normal plasma as the source of factor VIII, is usually reserved for research protocols because the clinically relevant inhibitors usually are >510 BU. [82] By convention, >0.6 BU is considered positive for the presence of an inhibitor; titers <5 BU are termed low-titer inhibitors and usually are not associated with anamnestic responses when sources of the specific clotting factor protein are administered. Inhibitors >10 BU are defined as high titer and are often associated with anamnestic responses. The most sensitive means of detecting an inhibitor antibody (which may be transitory or too low in concentration to measure in vitro), is to measure a decrease in the half-life of the factor VIII in vivo.

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DIFFERENTIAL DIAGNOSIS

The differential diagnosis of hemophilia includes congenital bleeding disorders such as von Willebrand disease, platelet disorders (e.g., Glanzmanns thrombasthenia), and deficiency of various other coagulation factors such as factors V, VII, X, XI, or fibrinogen. In addition there are autoimmune syndromes in which antibodies to factor VIII may spontaneously develop, leading to acquired hemophilia. The diagnosis of hemophilia is made on the basis of family history, bleeding symptoms, physical examination, and laboratory evaluation. The hallmarks of severe hemophilia include prolonged bleeding with surgical procedures, trauma, or dental extraction, as well as hemarthroses and spontaneous bleeding into soft tissues. The last of these distinguishes severe hemophilia A from von Willebrand disease and platelet disorders, in which soft tissue hemorrhage and hemarthopathy are uncommon (except in severe, type 3 von Willebrand disease). In addition, a family history indicating autosomal dominant transmission is characteristic of von Willebrand disease rather than hemophilia, which is sex-linked recessive in its pattern of inheritance. Family history may be unrevealing in de novo presentations of hemophilia. Severe hemophilia A is not clinically distinguishable from severe hemophilia B either by historical features (family history, bleeding symptoms) or physical examination, and the two must be distinguished by specific factor assays for factor VIII and factor IX. The accurate diagnosis of hemophilia A and hemophilia B is critical for appropriate clinical management. Autosomally inherited disorders of coagulation factors V, VII, X, XI, and fibrinogen also must be diagnosed by specific coagulation factor assays.

Mild hemophilia A may be difficult to distinguish from von Willebrand disease because factor VIII levels tend to be depressed in the latter, and prolongation of the bleeding time (characteristic of von Willebrand disease but not hemophilia) may not be reliably present. Specific assays for von Willebrand factor and analysis of the von Willebrand factor multimeric pattern by gel electrophoresis may distinguish the two disorders. Repeat testing is sometimes required to make a definitive diagnosis in the case of von Willebrand disease, especially mild (type 1) forms of the disease.

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TREATMENT

Factor Replacement Therapy

The key to treatment of bleeding in hemophilia is replacement of clotting factor to achieve hemostasis. The dose and choice of product are influenced by the severity of disease, the site and

TABLE 109-3 -- Guidelines for Replacement Dosing with Factor VIII and Factor IX

Type of Lesion	Desired Plasma Factor Level (% of normal)		Initial Dose (IU/kg body wt)		Duration of Therapy (days)
	VIII	IX	VIII	IX	
Mild (often spontaneous) hemarthrosis	30%	2030%	15	2030	q 812 h over 12 d for factor VIII; q 1224 h over 12 d for factor IX (Often only 1 dose required.)
Superficial hematoma					
Oropharyngeal or dental					
Severe epistaxis					
Persistent hematuria					
Major CNS trauma or hemorrhage; lumbar puncture or epidural anesthesia	80100%	80100%	>50	50	q 812 h over at least 1014 d for factor VIII; q 12 h over at least 1014 d for factor IX (High-purity products are recommended to avoid thrombotic complications.)
Surgery	(Levels should be maintained at least 72 h and then >50% for the duration of therapy.)				
Retroperitoneal hemorrhage					
Dental extraction with nerve block					
Severe GI bleeding					Continuous infusion with high purity products are recommended to avoid peak and trough level fluctuations and to save on overall factor concentrate utilization.

Abbreviations: CNS, central nervous system; GI, gastrointestinal.

severity of the bleeding, and the clinical scenario. The patients weight, inhibitor antibody status, and previous treatment history (especially with respect to exposure to blood-borne pathogens) also are important. The annual cost of factor replacement therapy for an adult treated weekly may range from $20,000 to $100,000, and the cost for inhibitor patients may be greater, particularly for those undergoing immune tolerance induction protocols. Therefore, patients are best managed in a comprehensive hemophilia center, where multispecialty expertise and ancillary support services exist for diagnosis, therapy, and management. The comprehensive hemophilia center also serves as a resource for patients with related bleeding disorders such as von Willebrand disease. Replacement therapy involves the education of the patient and his family in self-infusion techniques, the cornerstone of home-based treatment.

Guidelines for replacement therapy have been determined empirically over many years; however, the minimal doses of clotting factor replacement for adequate hemostasis have not been established (Tables 109-3 , 109-4 , and 109-5 and boxes, Choice of Concentrate and Treatment of Factor VIII Inhibitors). Typically, hemostasis for minor bleeding can be achieved at plasma factor levels of 2530% of normal (0.250.30 U/ml), whereas severe bleeding requires at least 50% of normal activity. Surgical procedures and life-threatening bleeding require

TABLE 109-4 -- Factor VIII Concentrates Available in the United States

Virucidal Method	Type/Name of Product	Manufacturer	Specific Activity (IU/mg nonalbumin protein)
Ultrapure Recombinant Factor VIII			
Immunoaffinity chromatography	Recombinate	Baxter	> 3,000
	Kogenate	Bayer	> 3,000
	Bioclate	Baxter[a]	>3,000
	Helixate	Bayer[a]	>3,000
Ultrapure Plasma-Derived Factor VIII			
Immunoaffinity chromatography and pasteurization (60°C, 10h)	Monoclate P	Centeon	> 3,000
Immunoaffinity chromatography, solvent detergent (TNBP/Triton X-100), heat treatment (25 ° C, >10 h)	Hemophil M	Baxter	> 3,000
	Monarc M	Baxter[b]	>3,000
Intermediate- and High-Purity Plasma-Derived Factor VIII			

Affinity chromatography, solvent detergent (TNBP and polysorbate 80), and terminal heating 80° C, 72h)	Alphanate SD	Alpha Therapeutics	830 (>400 when corrected for von Willebrand factor protein content)
Solvent detergent (TNBP/polysorbate 80)	Koate-HP	Bayer	9 22
Pasteurization (60° C, 10 h)	Humate-P	Centeon, Pharma	12
None	Hyate-C (porcine plasma-derived factor VIII concentrate used in treatment of inhibitor patients)	Speywood[a]	50

[a]Distributed by Centeon.
[b]Prepared from volunteer donor plasma provided by the American Red Cross.

TABLE 109-5 -- Factor IX Concentrates Available in the United States

Virucidal Method	Type/Name of Product	Manufacturer	Specific Activity (IU/mg nonalbumin protein)
Ultrapure Recombinant Human Factor IX			
Affinity chromatography and ultrafiltration (Chinese hamster ovary cell lines maintained in serum-free medium)	BeneFIX	Genetics Institute	> 200 (albumin free) 20% lower recovery compared to plasma-derived factor IX; increase dose by 20%
Highly Purified Plasma-Derived Factor IX			
Dual affinity chromatography, solvent detergent (TNBP/Polysorbate 80), and nanofiltration	AlphaNine SD	Alpha Therapeutics	> 200
Immunoaffinity chromatography, solvent detergent (sodium thiocyanate), and ultrafiltration	Mononine	Centeon	> 190
Low-Purity, Plasma-Derived Factor IX			
Dry heat (80° C, 72 h)	Konyne 80	Bayer	1.25
Dry heat (68 °C, 144 h)	Proplex T	Baxter	1.25
Immunoaffinity chromatography, solvent detergent (sodium thiocyanate), and ultrafiltration	Mononine	Centeon	> 190

targets of 75100% of normal activity. For factor VIII replacement one may assume that each unit of factor VIII per kg body weight raises the plasma level by 2% (0.02 U/ml). Thus, injection of 1,750 U of factor VIII will provide for an incremental rise of 50% (0.5 U/ml) of normal (1,750 U/70 kg × 0.02 U/ml),

CHOICE OF CONCENTRATE: HIGH OR LOW PURITY; RECOMBINANT OR PLASMA DERIVED

In our hemophilia treatment center, the choice of product is approached as follows. Decisions are based on the fact that there is no difference in efficacy among the concentrates. Recombinant factor VIII or factor IX concentrates are recommended for all previously untreated patients, those who have been treated but remain HCV and HIV seronegative, and for mild and moderate severity disease when desmopressin is not sufficient (e.g., surgery or trauma). Plasma-derived products are recommended for HCV- and HIV-seropositive individuals. Because of the possibility that increased purity may preserve CD4 lymphocyte levels, a higher purity concentrate is preferred; however, if the CD4 count is significantly decreased to <200/mm^3 , then a lower purity concentrate may be prescribed. The use of anti-retroviral agents is more critical to the patients survival and overall quality of life than the choice of replacement product. For surgical procedures, high-purity products are preferred both to avoid the thrombogenic side effects of the less pure factor IX products in hemophilia B, but also to facilitate continuous infusion, which reduces the overall cost of replacement therapy. High-purity products, but not necessarily recombinant products, are preferred in immune tolerance induction and prophylaxis regimens. The use of plasma-derived products in these instances can reduce the expense dramatically.

All replacement therapy options should be discussed with the patient or his family. Patient preferences always should be considered seriously, particularly because extensive scientific data are not always available to determine the best treatment and because of the wide variation in cost among the clotting factor concentrates.

which then declines according to the pharmacokinetics associated with factor VIII. This calculation estimates the peak level, and it is common practice in critical bleeding situations to double the initial dose to ensure that the trough level after one half-life exceeds the desired value. Because factor VIII has a half-life of 812 hours, repeat dosing at least two or three times per day is required to maintain the desired factor VIII level. One-half of the initial dose is given every half-life (i.e., every 812 h) to maintain the desired factor VIII level.

Similar calculations can be made for dosing factor IX concentrates. Factor IX demonstrates a volume of distribution approximately twice that of factor VIII due to equilibration with the extravascular extracellular fluid compartment (in which factor VIII does not exchange), and a half-life of about 24 hours. One unit of factor IX administered per kg body weight results in a 1% (0.01 U/ml) increment in the circulating factor IX level in plasma. Thus 3,500 U of factor IX provides an incremental rise of 50% of normal (0.5 U/ml) (3,500 U/70 kg × 0.01 U/ml). For low-purity factor IX concentrates it is best to aim for a maximum level of 50% of normal to avoid thrombotic complications that have occurred with these preparations. This is not a problem with the highly purified factor IX concentrates now available. In fact, the lower recovery of recombinant factor IX that has been observed suggests increasing the dose of recombinant factor IX by 20% over the calculated dose. [76] Subsequent maintenance doses of factor IX are given every 1824 hours. For replacement therapy of hemophilia A or B, subsequent dosing is

TREATMENT OF FACTOR VIII INHIBITORS

For high-titer factor VIII inhibitor patients, standard factor IX complex concentrates are used as first-line therapies, particularly for episodic spontaneous bleeding at home. If the patient does not improve or experiences increased frequency or severity of bleeding, activated factor IX complex concentrates are prescribed. Porcine factor VIII concentrate is reserved for surgery if the Bethesda inhibitor titer is <50 BU. Elective major surgical procedures should be approached with much caution and should only be performed in a major hemophilia treatment center. Arrangements to obtain recombinant human factor VIIa should precede the surgery.

adjusted based on measured factor levels. The duration of treatment is determined by the rapidity of pain relief and recovery of range of motion of an affected joint, or resolution of a hematoma. For major surgery, replacement therapy is usually continued for at least 1014 days to allow for efficient wound healing and scar formation.

Intermittent bolus infusions of factor concentrates have been used successfully for many years. However, pharmacokinetics may vary between products and patients, and the wide fluctuations in factor levels during therapy can make management difficult at times. Continuous infusion protocols have been developed, which reduce factor utilization, facilitate laboratory monitoring of factor levels (since laboratory values reflect a steady state rather than a peak or a trough), and may decrease the overall cost of therapy. This approach has been associated with excellent hemostasis and safety and has been used with factor VIII, factor IX, porcine factor VIII, recombinant human factor VIIa, and activated prothombin complex concentrates for therapy of inhibitor patients. Some studies note a 3075% decrease in the use of concentrate with continuous infusions administered in the surgical setting, and also report a progressive decrease in the plasma clearance of coagulation factors. [83] [84] [85] Most high-purity concentrates maintain more than 80% of initial activity after 37 days without evidence of infectious complications. [86] A typical continuous infusion protocol begins with a bolus designed to achieve 100% of normal levels followed by an infusion of 2 U/kg/h. The results of factor VIII or factor IX levels obtained thereafter are used to guide changes in the infusion regimen. Typically, if the measured factor level is low, the infusion rate can be increased or a small bolus given to bring the levels up to the desired value. Addition of small amounts of heparin (15 U/ml of infusion) can reduce the incidence of local thrombophlebitis at the venous access site.

Clotting Factor Concentrates: Benefits and Risks

Blood-Borne Pathogens

Until the availability of recombinant factor VIII products in the late 1980s and recombinant factor IX in the late 1990s, patients with hemophilia were exposed to thousands of donors via plasma-derived concentrates. Patients treated before viral inactivation procedures were introduced in 1985 were therefore infected by various transfusion-transmitted infectious agents, including HIV, hepatitis A, B, C, and G, as well as parvovirus B19. Virtually all who were treated with plasma-derived concentrates before 1985 have been infected with hepatitis C virus (HCV). The HCV genotype in hemophiliacs reflects the origin of the donors from whom concentrates were derived so that recipients of concentrates derived from United States donors have predominantly the type 1 HCV genotype (>60%). The HCV genotype 1 has a poorer response to interferon- therapy and more frequently progresses to hepatic failure. [87] HCV infection usually produces asymptomatic hepatic transaminasemia, which can either be intermittent or persistent. Liver biopsy studies indicate that chronic active hepatitis or cirrhosis can occur with either presentation. Progression to liver failure occurs in approximately 1015% of seropositive patients within 20 years of exposure. [88] Coinfection with HIV accelerates the deteroration and increases the overall risk of developing liver failure over 20-fold. HCV RNA levels are significantly higher in HIV coinfected individuals, in contrast to the apparent inhibition of HCV replication observed in those coinfected with HCV and hepatitis B virus (HBV). Ethanol consumption, acetaminophen, and hepatotoxic drugs used in AIDS treatment can also exacerbate liver disease. Chronic HCV infection in hemophilia should be considered a significant problem in its own right because there is a 30-fold increase in the incidence of hepatocellular carcinoma compared to normal populations. Interferon- therapy may prevent or delay the onset of this complication. [89] [90]

Treatment of HCV with interferon- has produced mixed results. However, this is the only agent demonstrating consistent benefits in chronic HCV. Development of spontaneous, low-titer alloantibody inhibitors to factor VIII has been reported in hemophilia A patients undergoing long-term interferon- therapy. As for nonhemophiliacs, there is a 50% response rate with the initial course of interferon- defined by a normalization of serum transaminase levels and improvement of liver histology on biopsy after a 6-month course of therapy. HCV RNA typically disappears early in the course of treatment, only to return after termination of therapy. Only 1015% of patients experience a sustained remission. Patients coinfected with mixed HCV genotypes may demonstrate a change in predominant genotype during interferon therapy, which appears to predict a favorable clinical response. [91] Combining interferon with the purine nucleoside ribavirin has resulted in an increased proportion of initial responders and an increase in the number of long-term remissions.

Liver biopsy for hemophilia patients with liver enzyme abnormalities remains controversial, especially with the improvements in polymerase chain reaction assays for HCV RNA and genotyping. Liver biopsy may be useful to predict potential efficacy of interferon because liver enzymes and HCV RNA may not always correlate with histology in the HIV-infected patient. Percutaneous liver biopsies are associated with up to a 12.5% incidence of significant intraperitoneal hemorrhage, despite adequate clotting factor concentrate replacement. [92] The transjugular approach may circumvent the significant and frequently unacceptable risks associated with the percutaneous technique and also offers a noninvasive opportunity to relieve the symptoms of portal hypertension by establishing an intrahepatic portasystemic shunt. [93]

The risks of horizontal transmission of HCV from the infected hemophiliac to his sexual partner or subsequent vertical transmission of HCV to their unborn fetus appear to be minimal (<3%) unless the patient and the pregnant female are coinfected with HIV. Vertical HIV transmission (16% incidence) is more likely to occur in these situations. [94]

Hepatitis B should not be a major problem for patients with hemophilia due to the testing of plasma donors for hepatitis B surface antigen. Furthermore, the recombinant hepatitis B vaccine should be administered shortly after birth. HBV titers may disappear with the progression of AIDS, and in these patients vaccination should be repeated. Most HBV-seropositive patients were exposed to the virus in concentrates administered before 1985; approximately 5% are chronic carriers of hepatitis B surface antigen. These individuals are more likely to develop progressive hepatic failure or hepatocellular carcinoma and are susceptible to coinfection with the hepatitis D virus (or deltavirus), which accelerates liver failure.

Hepatitis A virus (HAV) transmission was not considered an important threat to the hemophilia community due to the fecal-oral route of transmission characteristic of the virus. However, HAV seroconversions were detected in several hemophiliacs treated with solvent-detergent extracted high-purity concentrates. [95] [96] Apparently, the virus entered the plasma pool from transiently viremic donors and escaped viral inactivation, because HAV is a nonenveloped virus. This example further demonstrates the vulnerability of the blood supply to nonenveloped viruses. To minimize the risk of HAV (as well as HIV-1 and HIV-2, HBV, and HCV) some manufacturers are subjecting their plasma-derived concentrates to polymerase chain reaction testing for viral nucleic acids in addition to the serologic screening of the donors for various viral pathogens. The HAV vaccine is recommended for all seronegative hemophilic individuals over the age of 2 years. The immune response to the vaccine in HIV-seropositive patients should be checked because additional booster doses may be needed.

The transmission of hepatitis E through factor concentrates

has not been recognized, and the incidence in hemophilia patients is no different from that in the nontransfused population. This is in contrast to hepatitis G virus (HGV), the newly discovered human flavivirus pathogen, which is distantly related to HCV. Approximately 1525% of hemophiliacs who have received nonviral attenuated concentrates are seropositive for HGV, compared to 3% of healthy blood donors. [97] HGV appears to be susceptible to the same viral inactivation

processes used for HCV and other lipid-enveloped viruses so that the risk of HGV transmission from currently available plasma-derived products should be minimal.

Human parvovirus B19, a single-stranded DNA virus, is associated with fifth disease (erythema infectiosum) in children, transient aplastic anemia in patients with chronic hemolytic diseases, and chronic bone marrow aplasia in patients with AIDS. Through transplacental transmission, B19 parvovirus can induce hydrops fetalis and fetal loss. Because infection is ubiquitous and frequently asymptomatic in healthy adults, viremia is often undetected. The prevalence of B19 viremia in normal blood donor populations ranges up to 1/1,000, [98] ensuring that most large plasma pools used to make factor concentrates are contaminated with virus. The seroprevalence of hemophiliacs treated repeatedly with plasma-derived concentrates approaches 80% even in those who have only received viral-attenuated products deemed safe from HCV and HIV.[99] [100] This is considerably higher than observed in age-matched control groups (approximately 40% seroprevalence). Previous exposure to B19 parvovirus was not affected by age, type or severity of hemophilia, HIV status, CD4 lymphocyte counts, or purity of the plasma-derived concentrate.[101] Only a few anecdotes describe acute hypoplastic anemia or pancytopenia in hemophiliacs. However, the long-term consequences of parvovirus infection in hemophiliacs has not been established. To date, parvovirus B19, a nonlipid-enveloped virus, has been resistant to inactivation by all available heating and solvent-detergent techniques, used alone or in combination, in the manufacture of concentrates. Adjunctive nanofiltration steps are currently being evaluated. Of concern to many physicians who care for patients with hemophilia is the fact that parvovirus B19 is representative of other nonlipid-enveloped viral pathogens (known or unknown) that might contaminate plasma-derived concentrates. These compelling considerations have served as the rationale for administering only recombinant factor VIII or factor IX concentrates to parvovirus B19-seronegative patients, previously untreated hemophiliacs, or pregnant carriers.

Porcine parvovirus has also inadvertently contaminated multiple lots of porcine factor VIII concentrate, used exclusively in patients with factor VIII inhibitors. Retrospective evaluations of recipients of this product have demonstrated no clinical infectious sequelae or immunologic evidence of seroconversions, suggesting a lack of cross-species infectivity.

The causative agent of the progressive dementia and spongiform encephalopathy characteristic of Creutzfeldt-Jakob disease (CJD) has been considered infectious since the mid 1960s; however, its transmissibility through the transfusion of blood or blood components remains controversial. This particle is devoid of nucleic acid and composed exclusively of a modified cellular prion protein with high -sheet content. Because it cannot be detected in the asymptomatic donor, is resistant to chemical or physical inactivation, and does not induce antibodies in the carrier, which could be used as a marker of exposure, it has become a theoretical threat to the hemophilia community. Epidemiologic studies have never determined that CJD can be transmitted to humans by blood components and have never identified any CJD-related deaths in the hemophilia or other heavily transfused populations. [102] The possibility of blood-borne transmission has not been completely excluded because of its decades-long incubation period prior to symptomatic disease. Animal data suggest that CJD can be transmitted experimentally by inoculating buffy coat cells from the peripheral blood of CJD patients or the Cohn fractions of pooled plasma from infected mice into the brains of other rodents. Furthermore, a variant form of CJD has been observed to occur in individuals <40 years old in the United Kingdom. Its neurologic presentation and its histopathologic similarity with bovine spongiform encephalopathy, a prion disease of cattle first recognized in 1996, have prompted speculation that it might represent the transmission of bovine spongiform encephalopathy to humans. These observations and concerns have led to the precautionary withdrawal of factor concentrates and albumin (used to stabilize the ultrahigh-purity monoclonal antibody purified factor VIII from plasma and the recombinant factor VIII products) derived from plasma pools containing material from suspected or confirmed donors with CJD. These uncertainties have spurred the development of recombinant clotting factor concentrates, which are synthesized in cell lines maintained without animal-derived nutrients and free of human albumin in their final formulations.

Noninfectious Complications of Replacement Therapy

Besides infectious complications, clotting factor concentrates produce remarkably few clinically significant adverse reactions. Transfusion-associated anaphylaxis is rarely observed although there are recent reports of anaphylaxis to factor IX concentrates in individuals with severe hemophilia B in close association with the development of a factor IX inhibitor.[103] Genetic analysis reveals complete deletion or rearrangements of the factor IX gene in most patients. Induction of immune tolerance to factor IX has not been generally successful, and these patients may be predisposed to developing nephrotic syndrome. [104] Any plasma-derived or recombinant factor IX concentrate is capable of producing such a complication; treatment of bleeding events with recombinant human factor VIIa is the only effective alternative. Urticarial reactions and low-grade fevers can be treated with antihistamines and acetaminophen. Older, intermediate- and low-purity concentrates were contaminated by measurable anti-A or anti-B isoaggulutinins that occasionally induced significant hemolysis in patients with blood types A or B. The low- and intermediate-purity products were also associated with the rare development of primary pulmonary hypertension.

Immune Effects of Replacement Therapy

Purity and enhanced specific activity are important advantages of the monoclonal antibody purified and recombinant clotting factor concentrates; however, their benefits to the immune system are less clear. Lymphocyte proliferation and cytokine production are significantly reduced by intermediate-purity factor VIII concentrates in in vitro lymphocyte culture systems compared to the ultrahigh-purity preparations, which are essentially inert. This has been correlated with the amount of transforming growth factor- contained in the various concentrates. [105] Prospective randomized controlled studies in patients with hemophilia A have demonstrated that immune suppression with chronic use of intermediate-purity products, as indicated by declines in the absolute CD4 lymphocyte counts, are significantly greater in HIV-seropositive individuals. CD4 lymphocyte counts are more stable in HIV-seropositive patients treated with ultrahigh-purity monoclonal antibody purified concentrate. Less skin test anergy was also appreciated with these products. [106] [107] Currently available intermediate-purity concentrates with higher specific activities (50200 IU/mg) also appear to be less effective in slowing the CD4 count decline than ultrahigh-purity products. The clinical significance of this observation is difficult to assess. Although the decreases in absolute CD4 counts associated with the less pure concentrates might be considered harbingers of hastened clinical deterioration

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and progression to frank AIDS and death, no data currently support this possibility; neither does evidence suggest that the ultra-high-purity concentrates retard the development of AIDS.[108] These phenomena are attributed to the effects of contaminating plasma proteins in the intermediate-purity products, which may stun the immune system with repeated infusions. A direct correlation exists between cumulative use of these products and the decline of immune parameters.

Thrombotic Complications of Replacement Therapy

Thrombotic complications have not been commonly reported with the use of factor VIII concentrates. However, low-purity factor IX complex has been associated with occurrence of thrombotic complications when infused repeatedly in large amounts. Similar complications have been reported with administration of the activated factor IX complex concentrates used in inhibitor patients. Disseminated intravascular coagulation, deep venous thrombosis, pulmonary embolism, and fatal or life-threatening acute myocardial infarction in young men and boys have been described. The introduction of high-purity plasma-derived and recombinant factor IX preparations has virtually eliminated this thrombogenicity, and in vivo studies show little or no rise in the surrogate markers of coagulation activation (e.g., fibrinopeptide A, prothrombin fragment 1.2, etc.).

Adjunctive Therapeutic Measures

In most individuals with mild or moderate hemophilia A the expense of replacement therapy can be reduced and exposure to blood-borne pathogens can be eliminated through the administration of desmopressin (DDAVP: desamino-8-arginine vasopressin), a pharmacologic analog of the hormone 8-arginine vasopressin. DDAVP is usually administered intravenously or intranasally as a spray with subsequent rises of factor VIII coagulant activity adequate to achieve hemostasis for surgical procedures or symptomatic bleeding. Peak activity is observed within 30 minutes after intravenous infusion (DDAVP 0.3 g/kg body weight) and within 60 minutes after 300 g metered spray intranasally; peak factor VIII activity of two to five times baseline levels is typical. Because of the variability in individual response, potential candidates for this form of treatment should be tested in advance. Dosing can be repeated at 812-hour intervals; however, a progressive tachyphylaxis frequently occurs, making DDAVP less useful for long-term hemostatic support in the postoperative period. Furthermore, the antidiuretic effects of the drug may produce water intoxication and severe hyponatremia, particularly if free water intake is not restricted. Seizure activity, although uncommon, has been reported, predominantly in small children and the elderly, but may occur at any age. Monitoring of fluids and serum sodium levels should minimize these risks. Virtually all patients experience transient facial flushing; mild headaches and minimally decreased blood pressure are occasionally noted. DDAVP should be used cautiously in elderly men with risk factors for coronary or cerebral arterial thrombosis because there appears to be an increased risk of angina pectoris, myocardial infarction, or stroke following administration. This may be due to DDAVP-mediated release of supranormal von Willebrand factor protein multimers from endothelial cells, which can aggregate platelets in conditions of high shear stress. DDAVP also stimulates the release of tissue plasminogen activator from endothelial cells, producing potential fibrinolysis at the site of hemostasis. Therefore, for treatment or prevention of mucosal surface bleeding, such as dental, gastrointestinal, or vaginal (in carriers),

antifibrinolytic agents should be added immediately prior to DDAVP and continued for 57 days thereafter. Similarly, the adjunctive administration of antifibrinolytic agents such as epsilon-aminocaproic acid or tranexamic acid in combination with clotting factor concentrates enhances the control of bleeding during and after dental surgery in hemophilia A. Their use in hemophilia B should be reserved for patients receiving the high-purity factor IX concentrates because any hypercoagulable tendency induced by low-purity materials may be exacerbated by inhibiting the fibrinolytic system.

There is growing experience with the use of fibrin sealants in patients with hemophilia. Fibrin sealant can function both as a hemostatic agent and as a promoter of wound healing and has reduced blood loss and requirements for factor replacement therapy in major and minor surgical procedures, including circumcisions and dental procedures.[109] Fibrin sealants have also been used successfully to limit hemorrhage from the cavities remaining after the percutaneous evacuation of hemophilic pseudotumors (see box, Pseudotumors in Hemophilia).

Orthopedic and Musculoskeletal Bleeding

Occasionally, repetitive bleeding can occur in a single joint despite intensive prophylactic factor replacement therapy and physiotherapy. The synovitis and pain in this target joint warrant definitive treatment to prevent progressive degeneration. Open surgical or arthroscopic synovectomy can reduce the frequency and severity of hemarthoses and pain by removing inflamed tissue and most of the blood vessels in the joint. Synovectomy should not be performed with the sole intent of improving joint mobility because loss of motion in the affected joint often occurs after surgery. Synovectomy is not advisable for individuals in whom adequate hemostasis is precluded by high-titer inhibitors. Nonoperative synovectomy (synoviorthesis) using intra-articular injection of radioisotopes has been explored as a method to ablate inflamed synovial tissue. Synoviorthesis can be accomplished in an outpatient setting with only 1 or 2 days of replacement therapy and can be performed safely in inhibitor patients in whom control of bleeding is often problematic. Colloidal ^{32}P chromic phosphate has been the preferred agent for hemophilic arthropathy and results in better preservation of range of motion than operative synovectomy.[110] Synoviorthesis may not eliminate hemarthoses as effectively as operative synovectomy, however. Chromosomal breakages have been observed in 10% of a cohort of hemophiliacs (all but one seropositive for HIV) who underwent synoviorthesis since 1977,[111] and one individual has been reported to have developed acute leukemia after undergoing synoviorthesis (M. Manco-Johnson, personal communication). The risk of malignancy remains unclear because patients with rheumatoid arthritis who have received this treatment since the early 1970s have demonstrated no increase in cancer rates.

Orthotics may play an important role in management of joint disease and can serve as an interim measure before definitive reconstructive surgery. The goal of orthototic intervention is to transform a subluxed, flexed joint into a functional stiff limb rather than a functional articulation. Ankle guards and arch supports are useful in individuals with ankle arthropathy and can assist in maintaining and improving gait and weight bearing. Shoe lifts can equalize the length of the lower extremities. Otherwise, orthotics are applied most commonly to the knees and occasionally to the elbows. Dynamic braces equipped with adjustable hinges to correct extension deformities of joints with successive, painless manipulations have supplanted traditional plaster casting of the joint with serial extension wedging, which requires anesthesia, is painful, and does not permit active muscle contraction.[112] These methods do not address subluxation deformities; however, promising results have been reported with the Ilizarov apparatus, which applies significant skeletal-mediated forces simultaneously in both the subluxation and the extension planes.[112] [113]

Pain and deformity that limit mobility, quality of life, and performance status are indications for joint reconstruction. Arthroplasty with prosthetic joint replacement has been performed to regain functional knees, hips, elbows, and shoulders.

PSEUDOTUMORS IN HEMOPHILIA

Hemophilic pseudotumors are large encapsulated hematomata that represent progressive cystic swelling from persistent bleeding and incomplete resorption. This serious complication is evident in approximately 12% of severely affected patients, usually in adults, and is composed of clot and necrotic tissue. Three types of pseudotumors predominate in hemophilia. The most common type arises from repeated hemorrhage and inadequate clot resorption and is usually confined within fascial and muscle planes. Radiologically they appear as simple cysts and infrequently expand to involve adjacent structures or erode through the skin layers. The second type involves large muscle groups, such as the gluteus maximus and iliopsoas. These are especially problematic because they may gradually enlarge, develop a fibrous capsule, and eventually destroy adjacent underlying structures by pressure necrosis. Skeletal fractures and bony deformities produced by cortical erosion may result. The third and rarest type of pseudotumor arises from within bone itself, often secondary to subperiosteal bleeding. This lesion typically is observed in the long bones of the lower extremities and pelvis, but has been reported to occur within the calcaneus, cranium, and mandible (Fig. 109-3). As it expands, the periosteum is stripped and raised, leading to displacement and erosion of adjacent muscle and bone. Most pseudotumors arise in adults and occur in proximal skeletal structures; distal lesions occur more frequently in children before skeletal maturity and are associated with a better prognosis.

Pseudotumors are best diagnosed by CT scanning, magnetic resonance imaging, ultrasonography, and vascular injection studies. Although the presence of one or more progressively enlarging pelvic or skeletal masses in a hemophiliac is consistent with the diagnosis of a pseudotumor, the lesions may resemble osteomyelitis, metastatic neoplasms, Ewings sarcoma, primary osteosarcomas, tuberculosis abscesses, aneurysmal bone cysts, giant cell tumors, or plasmacytomas. Chondrosarcomas and liposarcomas have occurred in such patients. Preoperative biopsy is contraindicated because catastrophic complications including life-threatening bleeding infection are common. Distal pseudotumors respond well to conservative treatment consisting

Figure 109-3 Massive pseudotumor resulting from repeated intracranial hemorrhage in a patient with hemophilia.

of aggressive clotting factor replacement and cast immobilization. Because conservative treatment has not been nearly as successful for lesions of the proximal musculoskeleton, and complete regression is very rare,[187] this approach has been reserved primarily for individuals with high-titer inhibitors. High- and low-intensity radiotherapy regimens have been successful in eradicating pseudotumors in the long bones and may offer an alternative conservative approach.[188] Surgical extirpation is the most effective treatment for pseudotumors and is the treatment of choice when it can be carried out in major hemophilia centers. Nevertheless, operative mortality approaches 20%.[189] Depending on the size and location of the pseudotumor, the percutaneous evacuation of the cavity and subsequent introduction of fibrin sealant or cancellous bone can be considered.

[114] The improved durability and design of joint prostheses allows their use in younger patients in whom it is not unusual for knee prostheses to remain intact for over 15 years. Prosthetic loosening is observed in hip replacements within 610 years after surgery and is due to increased stresses on the joint probably due to weight bearing and abnormal torque produced by the ipsilateral or contralateral knee(s).[115] Staged surgical procedures or multiple joint procedures in a single operative session in individuals with hemophilia should be considered due to considerably lower costs of rehabilitation and clotting factor replacement using this approach.[116] An evaluation of orthopedic surgery in HIV-infected hemophiliacs with absolute CD4 lymphocyte counts of 200/mm ^{3} or less suggests that arthroplasty increases the risk of infection 10-fold, particularly *Staphylococcus* species prosthetic joint infection, chronic osteomyelitis, and sepsis.[117] This is not the universal experience,[118] but it indicates that surgical intervention should be based on the overall balance of risk and potential benefits.

Arthrodesis (surgical fixation) is reserved for painful joints with greatly compromised mobility. Fixation is most commonly performed on the ankle by interposition of autologous iliac crest bone, which fuses the juxtaposed bone surfaces. Fixation of bones into a single, well-aligned unit can be facilitated with intramedullary nails or external fixators; however, pin site infections may occur in up to 10% of patients. Recently, arthroscopic ankle arthrodesis has been developed using demineralized bone matrixbone marrow slurry as a graft substitute. Although this has not yet been widely applied for hemophilic arthropathy, it has the potential to obviate open surgery and reduce the need for factor replacement.[119] Ankle fusion allows patients good mobility in normal shoes because the joints of the feet and toes are less commonly affected by bleeds, and the pain and frequency of hemarthroses are markedly diminished. Supramalleolar varus osteotomy may be a useful alternative hemophilic arthropathy with secondary valgus deformity.[120]

Intramuscular Hematoma

Approximately 30% of bleeding episodes in individuals with hemophilia occur in muscles. Although most intramuscular hematomata are trivial and resolve spontaneously, many produce significant morbidity and, depending on their location and size, can result in mortality. Intramuscular bleeds occur less commonly than hemarthoses and usually are not life-threatening. The most serious hematomata occur in the iliopsoas muscle or retroperitoneal space, where they can bleed profusely and

mimic hip hemarthoses and appendicitis. Large hematomata can produce fever, neutrophilia, ecchymoses, hyperbilirubinemia, and elevations in lactic dehydrogenase levels. They may be painful and may impair the sensory and motor function of adjacent nerves, which they entrap and compress. Occasionally, flexion contractures involving muscles innervated by these nerves develop (i.e., the hip). Muscle bleeds can be precipitated by incidental trauma in severe hemophilia and by more significant degrees of trauma in moderate or mild disease (i.e., contact sport injuries). Iatrogenic hematomata may form in association with intramuscular injections of medications such as vaccines and can be prevented by administering replacement clotting factor beforehand. All significant muscle bleeds should be treated aggressively with clotting factor to raise the factor VIII or factor IX levels above 50% of normal.

Mucous Membrane Bleeding

Although fatal gastrointestinal tract bleeding was a greater problem prior to introduction of factor concentrates, significant hemorrhage has continued to occur in 13% of adult patients with hemophilia.[121] Gastric mucosal bleeding has become more common in recent years with the increased use of NSAIDs for treatment of the pain of chronic hemophilic hemarthropathy (see box, Pain Management in Patients with Hemophilia). NSAIDs have supplanted aspirin as the major cause of this complication. Aspirin in any form should be avoided altogether and NSAIDs prescribed judiciously for limited time periods under strict medical supervision in patients with hemophilia.

Hemorrhagic complications of portal hypertension in long-term survivors of viral hepatitis have appeared more commonly as hemophilia patients live longer. Massive, life-threatening melena and hematemesis can result from esophageal varices in patients with hemophilia complicated by portal hypertension. Sclerotherapy at the site of bleeding varices has been successful for therapy of gastrointestinal bleeding, as has the transjugular intravenous portal-systemic shunt procedure for decompression of varices, and is probably safer than aggressive open surgical procedures. In certain individuals orthotopic liver transplantation offers an additional option for the treatment of symptomatic portal hypertension complicating terminal liver failure. This approach has the additional advantage that it provides a cure of the underlying hemophilia by restoring normal coagulant factor synthesis. [122] [123] [124] Gastrointestinal blood loss in a patient with hemophilia demands an exhaustive evaluation for anatomic lesions, as in any other patient.

PAIN MANAGEMENT IN PATIENTS WITH HEMOPHILIA

Acute hemarthroses or soft tissue hemorrhages are associated with intense pain, which requires immediate analgesic relief. Chronic pain frequently accompanies hemophilic arthropathy, particularly while some joint mobility remains. Narcotic agents may be required; however, many individuals show addiction over time with frequent use. The reversible antiplatelet effects of the NSAIDs other than aspirin may be tolerated quite well in some patients for the acute and chronic pain of hemarthroses. However, aspirin is to be avoided because of its irreversible antiplatelet effects. [194] [195] Alternative agents for pain relief include a nonacetylated choline magnesium trisalicylate, paracetemol (not available in the United States), and acetaminophen combined with small amounts of codeine or synthetic codeine derivatives. These medications lack the anti-inflammatory effects of the NSAIDs.

Techniques of progressive muscle relaxation, guided imagery, and thermal biofeedback assessment of the arthritic joint may provide adjunctive means to reduce the need for narcotic analgesia,[196] but behavioral approaches to pain management in hemophilic arthropathy have had limited success when used alone. Acupuncture, transdermal nerve stimulation, and hypnosis may achieve similar results but may also mask pain such that the affected joint is not properly immobilized or attended to in a timely manner with clotting factor replacement.[197]

Bleeding from the mucous membranes of the nose and mouth is very common in hemophilia, and post-traumatic tongue bleeding must be treated aggressively to prevent progressive airway obstruction, which can develop rapidly. In infants the frenulum is a frequent site of bleeding, and as the hemophilic child grows older the loss of deciduous teeth and the eruption of secondary dentition can also be complicated by prolonged bleeding. Fortunately, these episodes usually respond well to antifibrinolytic agents (e.g., epsilon-aminocaproic acid, tranexamic acid) or fibrin sealants without factor replacement. For more difficult cases, factor replacement may be required. For major dental surgery, particularly those requiring nerve blocks, factor replacement to achieve 1525% of normal activity should be used to prevent bleeding into the tissue planes of the neck, which can lead to respiratory embarassment. In mild hemophilia, intranasal or subcutaneous/intravenous DDAVP is an excellent choice with or without antifibrinolytics or fibrin sealants. Spontaneous epistaxis is relatively uncommon without an anatomic lesion or trauma. Nevertheless, it may require factor replacement, packing, cauterization, and fibrinolytic therapy when it does occur. Urologic bleeding in hemophilia is discussed further in the box.

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DEVELOPMENT OF ALLOANTIBODY INHIBITORS

One of the most problematic complications of hemophilia treatment is the development of alloantibody inhibitors, which neutralize the coagulant effects of replacement therapy. These are typically IgG antibodies, predominantly of the IgG4 subclass. Factor VIII inhibitors do not fix complement and thus do not produce end-organ damage seen with circulating immune complexes. In contrast, factor IX inhibitors can produce anaphylaxis and nephrotic syndrome in individuals with complete gene deletions.[76] [103] [104] So-called type I alloantibody inhibitors completely inactivate factor VII or IX activity with second-order kinetics. Autoantibody inhibitors in acquired hemophilia, on the other hand, function through type II kinetics and often do not completely inactivate factor VIII activity so that these patients appear always to have some residual measurable factor VIII activity. Finally, factor VIII alloantibody inhibitors manifest varying degrees of species specificity, typically cross-reacting with heterologous factor VIII protein (e.g., porcine plasma-derived factor VIII) with less avidity than with human factor VIII. Thus, porcine factor VIII concentrates can be used for therapy of high-titer inhibitors in which human factor VIII produces no discernable increment of activity.

Factor VIII and IX inhibitors, quantified in Bethesda units, are separated into high titer or high responder and low titer or low responder. The former is defined by levels of >10 BU or the development of an anamnestic response following any exposure to the clotting factor protein antigen while the latter usually has <5 BU and manifests no anamnestic response.

Studies of inhibitor epidemiology have been complicated by differing methods for ascertaining the rate of inhibitor formation and the retrospective nature of most previous studies. Formerly, inhibitors were only identified or sought when the patient became refractory to his usual clotting factor regimen. Retrospective surveys thus reported an inhibitor prevalence of 1520% in severe hemophilia A patients. Recent studies designed

UROLOGIC BLEEDING IN HEMOPHILIA

From 6690% of hemophiliacs will have experienced at least one episode of spontaneous hematuria during their lifetimes, typically first noted after the age of 12 years.[190] [191] The bleeding is usually episodic and painless unless intraureteral clots produce renal colic. It is unusual for bleeding to produce anemia.

The pathophysiology of hematuria in hemophilia is unclear. One study suggested that microscopic renal damage may result from the abnormal clearance of immune complexes in these individuals; however, impaired renal anatomy and function are not apparent in most cases unless there are repeated bouts of hematuria and obstruction.[192] Currently, the most frequent cause of hematuria in hemophiliacs on anti-HIV therapy is the nephrolithiasis associated with the HIV protease inhibitor indinavir (Crixavan), which forms characteristic crystals of the intact drug in the urine. [193] The crystalluria may produce intrarenal sludging, renal colic, and microscopic or gross hematuria. In addition, nephrotic syndrome has recently been recognized as a complication of immune tolerance in individuals with hemophilia B and alloantibodies to factor IX, which may also be immune complex mediated.[104]

Initial treatment of hematuria should consist of conservative measures such as increased fluid intake and administration of clotting factor concentrates (520 U/kg daily for 24 days if bleeding continues). Antifibrinolytic agents such as epsilon-aminocaproic acid or tranexamic acid can cause obstruction or anuria, particularly when the bleeding originates from the upper urinary tract, and they are not recommended unless bleeding is protracted and severe. Other etiologies of hematuria must be excluded, particularly nephrolithiasis, infection, and neoplasms. Extracorporeal shock wave lithotripsy of renal calculi can be performed uneventfully in hemophiliacs (even with inhibitors) under cover of adequate replacement therapy.

specifically to assess inhibitor development associated with newly introduced factor VIII concentrates have prospectively assessed inhibitor titers every 3 months, and in these studies 2452% of individuals with severe hemophilia A develop antibodies. Most occur early after a median of 912 days of exposure to factor VIII, and one-third will disappear despite continued administration of the same factor product. Half of these inhibitors are of persistently high titer unless immune tolerance induction is initiated. [125] Inhibitors may also arise in patients with mild or moderate hemophilia but are uncommon, with an estimated incidence of 313%. Little evidence exists to suggest that higher purity concentrates produce a higher incidence of alloantibody inhibitor formation. Rather, numerous host and genetic factors appear to be more relevant.

Because of the higher than expected incidence of alloantibody inhibitors that occur in hemophilic sibs, a number of studies have attempted to identify genetic markers for predisposition to inhibitor formation. The association of certain HLA haplotypes with inhibitor formation has never been firmly established despite much study. There is no specific molecular defect in the factor VIII gene, which is responsible for development of inhibitors in hemophilia A. However, individuals with large, multidomain gene defects (74%) and nonsense point mutations in the A3 coding domain (73%) are more prone to inhibitors. Fewer inhibitors occur with missense point mutations (3%), heavy chain mutations (8%), or the intron-22 inversions (20%). [126] [127] This would suggest that inhibitors develop primarily in patients who are lacking circulating factor VIII antigen and react to factor VIII concentrates as foreign protein. Recent data indicate a significantly higher incidence of inhibitors in African-Americans and Hispanic-Americans (50%), the genetic basis for which remains unknown. [128] Other risk factors must exist to explain why inhibitors develop in mild and moderate hemophilia or why many hemophilic relatives of inhibitor patients do not themselves form inhibitors.

The prevalence of factor IX alloantibody inhibitors in hemophilia B is very uncommon in comparison to hemophilia A, ranging from 1.53% in some series. These inhibitors seem to be correlated with the presence of large gene deletions and nonsense mutations. [76] [103] [129]

The majority (85%) of factor VIII alloantibody inhibitors are directed against the A2 or C2 domains of the factor VIII molecule, whereas a much smaller proportion are directed against the amino terminal region of the A3 domain. Anti-C2 and possible anti-A3 antibodies may interfere with binding of factor VIII to von Willebrand factor or to phospholipid. This results in rapid inactivation of factor VIII activity and prevents factor VIII from participating in its tenase complex functions. Antibodies in the A2 and A3 domains are adjacent to factor IXa-binding sites and may interfere with binding of factor VIII to factor IX. The bulk of anti-factor VIII antibodies in hemophilics are non-neutralizing and are directed against other parts of the factor VIII molecule. [130] Recently it has been suggested that these antibodies may accelerate factor VIII clearance from the circulation by the reticuloendothelial system. [131]

The treatment of alloantibody inhibitors is predicated first of all on their titer. The approach to low-responder inhibitors is straightforward with the administration of large enough doses of human factor VIII or IX concentrates to saturate the inhibitor and to provide adequate clotting factor activity levels. Generally this is readily

achieved, but a low responder may convert to a high responder without warning. Conversely, high-titer inhibitors may convert to low titers in the late stages of AIDS.

For the individual with high-titer factor VIII inhibitors, several treatment options are available, but none is universally applicable to all patients. Factor IX complex concentrates (formerly prothrombin complex concentrates) of the standard or activated varieties have become the first-line therapy for home self-administration regimens and for uncomplicated bleeding events. Dosing is empirical, usually 75 U/kg, repeated every 812 hours. These products are effective in 4864% of bleeding episodes, but their use is limited by their potential for inducing thrombotic complications and the inability to predict hemostatic response on the basis of laboratory testing.[132] The prothrombin time, activated PTT, and coagulation factor assays are not useful monitors because these products contain bypassing factors that artifactually shorten in vitro clotting assays in a manner that does not correlate to clinical hemostasis. Their repeated and prolonged use for surgical coverage is problematic due to the possibility of disseminated intravascular coagulation, especially in those with liver dysfunction.

An alternative agent for high-titer inhibitor therapy is porcine factor VIII. This treatment is efficacious in about 80% of patients. [133] Furthermore, this product permits measurement of factor VIII activity in vitro to predict the clinical response in vivo. Assays for cross-reactivity to human factor VIII should be performed prior to use to rule out a high anti-porcine factor VIII titer, which will negate the effect of the porcine factor VIII. Porcine factor VIII dosing is empirical, starting typically with a bolus of 100 U/kg followed by subsequent titration of dosing as guided by the factor VIII assays. Typically, factor VIII activity response to porcine factor VIII improves with repeated dosing, suggesting in vivo saturation of the circulating inhibitor by the porcine factor VIII. Unfortunately anamnestic responses to porcine factor VIII occur in 35% of patients, thus limiting the utility

of this product. Other side effects include fever, chills, and, rarely, thrombocytopenia, which may be due to activation of platelets by porcine von Willebrand factor. Porcine factor VIII has not been associated with the transmission of blood-borne viral pathogens. Its high price and limited availability have limited its popularity as a first-line therapy for inhibitor patients. For elective surgery, porcine factor VIII should be considered the first-line therapeutic agent unless contraindicated by inhibitor cross-reactivity against porcine factor VIII. During intensive therapy for surgery, 31% of patients will make high-titer antiporcine antibodies (>10 BU). [134]

An emerging therapeutic agent for high-titer inhibitor patients is recombinant human factor VIIa. Effective hemostasis with minimal adverse side effects has been achieved for therapy of hemarthrosis and soft tissue hemorrhage and for surgical coverage, administered at 90 g/kg as an intravenous bolus followed by repeat dosing every 23 hours as necessary.[135] Most importantly, this product has provided adequate hemostasis in very high-titer inhibitor patients who otherwise would not be candidates for surgical procedures due to previous failures with other bypassing agents. Recombinant human factor VIIa is also of considerable benefit to factor IX inhibitor patients.

For hemorrhagic emergencies that do not respond to the above approaches it may be necessary to reduce the circulating inhibitor titer with extracorporeal immunoabsoption using a *Staphylococcus* protein A column, which can adsorb the inhibitor antibody, thereby permitting replacement therapy with human factor VIII. This may also be performed to lower inhibitor titers at the start of immune tolerance induction protocols.

Immune tolerance induction is advantageous to inhibitor treatment because if the antibody can be permanently suppressed, future therapy is greatly simplified. Immune tolerance induction consists of prolonged desensitization during which factor concentrates are administered daily until the alloantibody inhibitor disappears. Results from an international registry indicate that immune tolerance induction is completely successful in up to 68% of patients and partially successful in another 8%.[136] The probability of success is greatest for those receiving high-dose protocols (>100 U/kg/d), those whose protocol was initiated early in the course of inhibitor development, and those with the lowest initial titers. Concomitant immunosuppression corticosteroids and cytotoxic agents are controversial and probably best avoided, especially in children. The best predictor of success is a 50% reduction in the titer within 6 months and total disappearance of the inhibitor within 1218 months. Complete elimination is defined as a final inhibitor titer of <0.6 BU, >60% of predicted recovery of infused factor within 30 minutes of administration, and a normal half-life of infused factor VIII or factor IX. Low-dose (<50 U/kg/d) protocols have been less successful.

Immune tolerance induction is an expensive option, and is often reserved for patients who require a major surgical procedure and are highly compliant. An indwelling intravenous device is often used.

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PROGNOSIS

Before the widespread use of replacement therapy, the prognosis for patients with severe hemophilia was poor and the quality of life greatly diminished by hemophilic arthropathy. The outlook improved dramatically in the modern era of hemophilia treatment that began in the 1960s with the development of cryoprecipitate [139] and factor VIII concentrates[138] (for hemophilia A) and prothrombin complex concentrates[139] (for hemophilia B). It then became possible to achieve high levels of factor VIII or factor IX without volume overload, which limits plasma therapy. This permitted effective therapy of life-threatening soft tissue bleeding, which requires high doses of factor VIII or factor IX for prolonged periods. Furthermore, the convenience of factor concentrates made home therapy of hemarthroses practical. Use of lyophilized factor concentrates administered at home saved many patients from the delays, expense, and inconvenience of hospital- or clinic-based treatment with cryopreciptate or plasma. Factor concentrates facilitated prophylactic therapy, which has now been shown to virtually eliminate bleeding episodes and actually prevent joint deterioration, especially when started early in life (i.e., 12 years of age). [140] Many large studies of patients with hemophilia document the dramatic gains in life expectancy during the era of replacement therapy. The life expectancy of patients with severe hemophilia, which had remained more or less constant from the 19th century to the 1960s, rose from 11 years (or less) for severely affected patients to >50 or 60 years by the early 1980s in reported series. [14] [16] [20] [21] [22] [24] [141] [142] [143] Significant progress was evident even in patients with mild or moderate disease, whose life expectancies were closer to the normal population in the pretreatment era (Fig. 109-4).

Along with the benefits of replacement therapy came viral complications, the most devastating of which has been infection with HIV. HIV infection probably occurred in the late 1970s in heavily treated patients, and the first AIDS deaths in patients with hemophilia A were observed in the early 1980s. [144] Although the greatest rate of HIV seroconversion (>75%) occurred in severely affected hemophilia A patients who were heavily treated with factor VIII concentrates, 46% of patients with moderate disease and 25% with mild disease who were treated only once or infrequently also seroconverted for HIV. [145] The rate of HIV infection in severe hemophilia B (46%) was significantly lower than that of severe hemophilia A patients, as were the rates for those with moderate hemophilia B (27%) and mild hemophilia B (8%). This may reflect differences in concentrate preparation methods. [145] Overall, more than half of patients

INTRACRANIAL HEMORRHAGE

Intracranial hemorrhage is the second most frequent cause of death in individuals with hemophilia, behind AIDS. Ten percent of patients with severe hemophilia develop intracranial hemorrhage with a mortality rate of >30% and a significant incidence of psychoneurologic sequelae in the survivors. These include mental retardation, seizure disorders, and motor dysfunction. Because even minimal trauma to the head may precipitate severe hemorrhage and serious intracranial pathology, aggressive treatment with clotting factor concentrates should be initiated immediately to achieve 100% levels of factor VIII or IX, *even if there is only a clinical suspicion of bleeding*. Replacement therapy should precede any diagnostic procedures because <5% of acute head injuries in hemophiliacs are accompanied by early abnormalities on CT scan. [198] When intracranial injury is demonstrated on CT scans, neurologic deficits, vomiting, or altered mental status are apparent on physical examination. Neurosurgical intervention may be necessary but carries high risks despite maintaining adequate hemostasis with clotting factor concentrates.

Intracranial hemorrhage in the neonate is invariably preceded by mild to moderate head injury. The risk of serious bleeding associated with vaginal delivery is small, but vacuum extraction should be avoided when delivering offspring of known hemophilia carriers. In hemophilic adults, a recent increase in the incidence of intracranial bleeds has been noted and attributed to the compounded effects of end-stage hepatic failure (hepatitis C), the immune thrombocytopenia accompanying AIDS, or the bleeding complications associated with protease inhibitors for HIV treatment on the underlying congenital coagulopathy.

Figure 109-4 Effect of therapy with plasma and plasma-derived factor concentrates on the prognosis of hemophilia. Steady improvements in life expectancy are seen, particularly for patients with severe disease as factor concentrates have become widely available for treatment. Unfortunately, this trend has reversed in recent years as the viral complications of factor concentrate therapy have taken their toll. [14] [22] [141] [142] [143] [146]

with hemophilia were infected with HIV by 1983. Ironically, some patients who received certain heat-treated products early in the epidemic due to inhibitor antibodies were spared infection with HIV. The death rate for patients with hemophilia A more than tripled from a nadir of 0.4 deaths/1,000,000 population in 1979-1981 to 1.2 deaths/1,000,000 population in 1987-1989 in the United States, largely due to HIV, which caused 55% of all hemophilia deaths in the latter era. [146]

The natural history of hemophilia A depends largely on the severity of disease and the presence of concomitant viral illness. The leading cause of death has shifted in the era of replacement therapy from intracranial hemorrhage and other bleeding to viral complications of therapy, chiefly AIDS and cirrhosis from hepatitis. The overall mortality rate among all patients with hemophilia is approximately twice that of the normal male population [24] [32] [146] ; the mortality rate among severely affected patients is four to six times that of normal males. [142] [147] When viral complications of therapy are taken into account, the life expectancy of patients with no HIV or hepatitis is virtually the same as the male population for mild hemophilia A, and the life expectancy of patients with severe disease is only slightly less than the male population in general and the relative mortality is 1.2 times that of the normal male population. [147]

Bleeding events that threaten the life of patients with hemophilia A include intracranial hemorrhage (see box, Intracranial Hemorrhage) and soft tissue hemorrhage in

or around vital areas such as the airway or internal organs. The lifetime risk of intracranial hemorrhage has been estimated at 28% and accounts for one-third of all hemophilic deaths from hemorrhage despite advances in replacement therapy. [143] [148] [149] It is notable that there is only about 20% of the expected number of deaths from cardiovascular disease, suggesting a protective effect of hemophilia A. [34] [147]

Whether patients with inhibitor antibodies have a different life expectancy is not entirely clear. Although they do not bleed any more frequently than other patients with (severe) hemophilia, their bleeding episodes are more difficult to treat than those in severe hemophilia patients who can be treated with factor VIII. Where mortality and life expectancy have been compared in the two groups, the inhibitor patients life expectancy differs only slightly from that of the noninhibitor group. [147] Although inhibitor patients severe bleeding episodes are more likely to be fatal due to less than adequate therapy, fewer inhibitor patients have seroconverted for HIV. [142] [148]

Hemophilia as a chronic disease has been recognized to affect the physical and cognitive growth of individuals independent of HIV status. As primary prophylaxis emerges as a more acceptable approach to treatment, these problems may become less common; however, recent data from the United States indicate that a significant proportion (25%) of hemophilic children

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ages 618 exhibit below-normal performance in motor skills, lower than expected academic performance (measured by IQ scores), and more behavioral and emotional problems than expected, as reported by parents. [150] The presence of HIV and the absolute CD4 counts were not related to neuropsychological performance at baseline. Causality has not been established, but the potential contributions of minimal head trauma with clinically inapparent bleeding and the effects of hemophilia on the emotional well-being of the family cannot be overlooked.

A significant impairment of physical stature and sexual maturation has been observed in HIV-infected boys with hemophilia as compared to uninfected boys or population norms. Differences were exaggerated in those with absolute CD4 lymphocyte counts <200/mm^3 . The discrepancy between total serum testosterone levels (compared to age-adjusted levels in HIV-uninfected boys) and observed physical changes is hypothesized to be related to decreased free testosterone resulting from increased levels of sex hormone-binding globulin. [151]

The combined morbidity and mortality associated with hemophilia and its treatment are a powerful argument for prophylactic treatment, safer concentrates, elimination of inhibitor antibodies where they occur, and gene therapy, which currently is the only prospect (short of liver transplantation) for the cure of hemophilia.

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GENE THERAPY

In recent years considerable effort has been devoted to gene therapy as a cure for hemophilia. Gene therapy may be defined as the attempt to express a missing or defective gene to replace its missing product or function. Presently only gene transfer into differentiated cells or tissues of patients (somatic cell gene therapy) is under investigation as a treatment for hemophilia. Somatic gene therapy could be performed by removing tissue from a patient, transducing cells in vitro, and returning modified cells to the individual (ex vivo gene therapy), or by gene transfer done in the patient (in vivo gene therapy). [152] The former requires substantial tissue culture of each individual patients cells and would amount to a custom application in each case, whereas in the latter approach a single vector preparation might be administered to a large number of patients, analogous to clotting factor concentrate therapy.

Hemophilia is well suited for gene therapy because relatively low levels of coagulant activity prevent severe disease. Clinical experience with factor replacement therapy indicates the benefit of therapy, which is prophylactic rather than reactive. Maintaining factor VIII or factor IX levels of >12% of normal starting early in life (from 12 years of age) prevents virtually all spontaneous bleeding and maintains normal joint structure and function. [140] [153] Furthermore, as the total dose of prophylactic factor replacement increases and the interval between doses decreases, the outcome improves, underscoring the benefits of continuous delivery of coagulant factor.[154] Continuous delivery of factor VIII or factor IX at a steady state of 1% of normal or more would provide clinically significant benefit and should be the initial therapeutic goal of gene therapy (see box, Prophylactic Factor Replacement Therapy).

The recent development of factor VIII and factor IX knockout mice[155] [156] [157] and the previously established hemophilia A and B canine colonies and cloning of the factor VIII and factor IX genes have facilitated research on hemophilia and gene therapy. Gene transfer has been attempted using vectors targeting liver, muscle, skin, bone marrow, and endothelial cells in normal and hemophilic animals (e.g., mice and canines). Factor VIII or factor IX gene transfer to liver, muscle, skin, vascular, or bone marrow cells in vivo with retroviral vectors has typically resulted in long-term gene expression at low levels. [158] [159] [160] [161] [162] [163] [164] [165] [166] [167] [168] [169]

Adenovirus-mediated gene transfer of genes for factor VIII or factor IX into liver has resulted in high levels of gene expression

PROPHYLACTIC FACTOR REPLACEMENT THERAPY

The Swedish experience demonstrates a decreasing need for orthopedic surgery in hemophiliacs who are treated from the age of 12 years (before arthopathy has begun) with a prophylactic regimen of clotting factor concentrate replacements.[140] [153] These series and others indicate that administering factor VIII or factor IX concentrates at 2540 U/kg three times weekly for hemophilia A and twice weekly for hemophilia B beginning in toddlers results in a significant reduction in spontaneous bleeds and arthropathy. The doses and frequency of replacement therapy are adjusted to prevent the factor VIII or factor IX levels from falling below 12% of normal, thereby converting severe hemophilia into mild or moderate disease. Primary prophylaxis results in short-term and sustained improvements in objective measures of the function and radiologic appearance of joints. Joint scores have remained at zero (i.e., no affected joints) over at least 5 years of observation in 79% of individuals whose regimens were initiated between the ages of 1 and 4.5 years. Outcome is directly related to the intensity of treatment and the level of compliance,[199] [200] and translates into decreased absence from school or work, fewer bleeds and days spent in the hospital, increased personal and professional productivity, improved overall performance status, and a healthier self-image.[199] Higher than usual doses of clotting factor concentrate in an attempt to minimize damage associated with episodes of joint bleeding (on demand regimen) do not produce as good orthopedic outcomes as primary prophylaxis.[199] Factor concentrate expense is substantially greater in primary prophylaxis, and the central access devices used to facilitate vascular access are associated with a significant incidence of sepsis and thrombosis. [201] [202] [203] Cost reductions can be realized if the factor is administered via continuous infusion and dosing based on measured pharmacokinetics. [202] [203] [204] There is no evidence that prophylaxis begun early in childhood increases the incidence of inhibitor antibody formation. Although primary prophylaxis may not be optimal for all individuals with severe hemophilia (or their families), the substantial benefits demonstrated in prospective studies cannot be overlooked but must be balanced against the costs, inconvenience, and acceptance by the patient and his family, as well as the viral safety of the concentrate chosen for the regimen.[205] The initiation of a prophylaxis regimen after years of on-demand replacement therapy for recurrent hemarthroses is designated secondary prophylaxis and has caused stabilization of articular function and joint scores over time; however, joint damage is not reversed and radiographic deterioration of previously damaged joints may continue to progress.[206] This alternate strategy may be useful for those in whom there is initial reluctance to begin primary prophylaxis.

of limited duration due to humoral and cell-mediated immunity. [170] [171] [172] [173] Investigators have removed more and more of the adenovirus genes from vectors, [174] [175] administered virus in the neonatal period to induce tolerance, [176] or administered immunosuppressives to circumvent the immune response.[177] [178] These methods may not be applicable to humans with hemophilia, however, especially those with acquired immunodeficiency states. More recently, adeno-associated virus (AAV) vectors have been tested for gene therapy of hemophilia. [179] [180] [181] [182] The most

promising results are now being obtained with recombinant AAV vectors expressing factor IX. Sustained, therapeutic levels of factor IX (20% of normal) have been observed in normal mice after AAV-factor IX vectors were delivered to the liver via portal vein injection, [181] and sustained levels of 47% of normal have been demonstrated in immunodeficient mice following intramuscular injection of AAV-factor IX vectors. [182]

Sustained expression of the factor VIII gene at therapeutic levels in vivo has proven to be problematic with retroviral and adenoviral vectors, [161] [162] [163] [164] [165] [166] [167] [168] [169] [170] [171] [172] [173] [183] and the large size of the factor VIII cDNA may rule out the use of AAV vectors, which cannot normally accomodate large transgenes. Furthermore, certain sequences within the factor VIII gene appear to interfere with efficient transcription and expression of factor VIII. [184] [185] [186]

It can be anticipated that gene therapy of hemophilia will be practical when the limitations of gene transfer vectors are eliminated. Vector safety, inhibitor antibody formation, and the optimal levels of factor VIII or factor IX transgene expression will emerge as issues for the hemophilia clinician to manage in the gene therapy era.

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Chapter 110 - Inhibitors in Hemophilia

Donald I. Feinstein

INHIBITORS IN HEMOPHILIA A

Factor VIII inhibitors are antibodies that develop in patients with hemophilia A in response to factor VIII contained in various blood products. Most of these antibodies neutralize factor VIII coagulant activity. Although they do not increase the frequency of bleeding episodes, they make treatment of bleeding much more difficult. Some antibodies are directed toward nonfunctional determinants on the factor VIII molecule, but the clinical relevance of these antibodies is not clear. [1] They could result in increased clearance of transfused factor VIII and could explain why the half-life of factor VIII is reduced in some patients with undetectable levels of inhibitors affecting the function of factor VIII in vitro. The incidence of factor VIII inhibitors complicating hemophilia A is approximately 510% of all patients with hemophilia A and approximately 20% of those with severe hemophilia A.[2] [3] [4] In a meta-analysis of eight retrospective studies using crude or intermediate-purity products in high-responding, severe hemophiliac patients, the cumulative incidence of inhibitors in 451 patients was 20% after 16 years of treatment. [5]

Patients with low-titer inhibitors (35 Bethesda units) that do not rise after further exposure to factor VIII are known as low responders; this characterizes approximately 25% of hemophiliacs with inhibitors. Patients with inhibitors that rise markedly with further exposure to factor VIII (anamnestic response) are known as high responders; this characterizes approximately 75% of hemophiliacs with inhibitors. [6] Inhibitor titers usually begin to rise 23 days after exposure to factor VIII, reach a maximum within 721 days, and then decrease slowly (Fig. 110-1). Once formed, high-titer inhibitors tend to persist for long, although variable, periods, and detectable levels of inhibitor may be present 12 years later without re-exposure to factor VIII. By contrast, low-titer inhibitors in low responders occasionally disappear and may not reappear with exposure to factor VIII.

In studies of inhibitor formation in previously untreated patients receiving highly purified plasma-derived factor VIII, the incidence of inhibitors was 1835%, [7] [8] [9] and with intermediate-purity factor VIII concentrates, the incidence was 2552%. [10] [11] [12] [13] [14] In prospective studies using recombinant factor VIII in previously untreated patients, the incidence was 2429%.[11] [12] [15] In the latter studies, the inhibitors were detected early, with the median number of exposure days before inhibitor formation being 10 (range, 516 days). [11] [12] [15] Thus, most patients with severe hemophilia who are destined to develop an inhibitor do so early after exposure to factor VIII.[11] [12] [15] [16] However, the 2429% seen in the recombinant factor VIII trials may be an underestimate in that the duration of factor VIII treatment was limited in both reports. Using Kaplan-Meier analysis, the risk of inhibitor formation was 38% after 25 exposure days in the Recombinate trial and 36% after 18 exposure days in the Kogenate trial.[11] [15] [17] However,

Figure 110-1 Inducible and noninducible factor VIII inhibitors. Solid circles, high responder; open circles, low responder; arrows, factor VIII concentrate.

30% of the inhibitors were low titer and transient, and these patients were treated successfully with standard amounts of factor VIII for bleeding events. [11] [15] Low-titer, low-response inhibitors are also successfully continued on treatment. [11] [15] Thus, studies with highly purified and recombinant factor VIII have demonstrated no significant increase in the incidence of high-titer inhibitors. In addition, inhibitor development varies in the different studies because of the retrospective nature of many studies, the tendency to report mostly high-titer inhibitors, the use of more than one factor VIII product in individual patients, the variable time of patient follow-up, and the variable frequency of testing for inhibitors. The incidence of inhibitor development varies with the factor VIII product used. The most dramatic examples were the low incidence of inhibitor development reported from the Leuven Hemophilia Center when using lyophilized cryoprecipitate, [18] and the low incidence of inhibitors when using the Biotransfusion product from France, [19] contrasted to the high incidence associated with the use of a Dutch concentrate. [20] [21] [22] [23]

Although most inhibitors develop in severe or moderate forms of hemophilia A, well documented cases include patients with mild hemophilia A. [3] [24] [25] [26] [27] [28] [29] [30] In general, these patients develop only low-titer inhibitors after exposure to factor VIII. After abstinence from products containing factor VIII, the inhibitors disappear after 412 weeks, and the inhibitors do not necessarily reappear on re-exposure.

Pathophysiology

The reasons for the development of inhibitors in a minority of patients with hemophilia A is not understood. Brother pairs have a higher-than-expected incidence or absence of inhibitors. [16] [31] Another factor suggesting genetic susceptibility is the finding of a lesser incidence of human leukocyte antigen (HLA)-A1 and a higher incidence of certain complement components located close to HLA-DR in hemophiliacs with inhibitors. [32] However, recent studies correlating the molecular defect causing hemophilia A with the development of an inhibitor have shed some light on this subject. [4] [33] [34] [35] [36] The frequency of inhibitor development in patients with large multidomain deletions and in patients with nonsense mutations affecting the A3 domain of the molecule is approximately 7075%. [33] Nonsense mutations in the C1 or C2 domains, however, result in inhibitor formation in 27%, the intron 22 inversion (the most common genetic defect) in 20%, and a small deletion in 15%. [33] In contrast, factor VIII heavy chain nonsense mutations result in inhibitor formation in only 8% and a nonsense point mutation in 3%. [34] However, other factors must also be operative because hemophiliac relatives of inhibitor patients may not develop inhibitors. These other factors include immune responsiveness, product heterogeneity, and intensity of treatment. Shapiro and Hultin [24] suggested that immune tolerance might be involved to explain the development of inhibitors in patients with hemophilia A. Because factor VIII does not cross the placenta, tolerant hemophiliacs could be exposed to factor VIII in utero only as a result of maternofetal hemorrhage. Thus, according to this hypothesis, only nontolerant hemophiliacs would develop inhibitors after exposure to factor VIII. Induction of tolerance in hemophiliacs with inhibitors results in the disappearance of the inhibitor in a significant number of patients. [37] [38] [39] [40] [41] [42] [43] [44]

Characterization and Properties of Inhibitors

Factor VIII inhibitors in hemophilia A are IgG. Although light chain and heavy chain subtyping has usually demonstrated restricted heterogeneity, [45] [46] [47] [48] [49] [50] [51] [52] heavy chain subtyping of these antibodies has shown a significant predominance of the IgG_4 subtype.[47] [50] [53] IgG_4 does not fix complement, which might explain why immune complex disease does not develop in hemophiliacs. More recent studies have shown that hemophilia alloantibodies do not always have a predominance

of IgG_4 and may also contain IgG_1 and IgG_2 .[1] [54] Factor VIII inhibitors show species specificity, both in vitro and in vivo, in that human factor VIII is usually neutralized to a greater extent than is bovine or porcine factor VIII, and infusion of porcine factor VIII into a patient with an inhibitor often raises the factor VIII level.

The reaction between factor VIII and inhibitors is time- and temperature-dependent. Two different patterns of antigen antibody reaction have been described. In the type I pattern, characteristic of most alloantibodies, as seen in hemophiliacs, factor VIII is completely inactivated in the presence of excess inhibitor, [55] [56] [57] whereas in the type II pattern characteristic of most autoantibodies, the inhibitor frequently does not completely inactivate factor VIII in vitro in the presence of excess inhibitor.[45] [58] [59] [60] In the latter cases, despite the demonstration of a significant amount of residual factor VIII activity in vitro, the patient bleeds as if there were no coagulant function in vivo.[61] [62] The reason for the difference between type I and type II inhibitors is not understood. However, it has been found that most type II inhibitor plasmas have type I characteristics when tested with factor VIII that has been separated from von Willebrand factor.

The antigenic regions on the factor VIII molecule to which neutralizing factor VIII inhibitors bind have been identified. [4] [35] [54] [63] [64] [65] [66] [67] [68] [69] The epitopes to which alloantibodies or autoantibodies are directed are limited to certain areas of the factor VIII light or heavy chains [4] [35] [54] [63] [64] [65] [66] [67] [68] [69] (Fig. 110-2). The factor VIII molecule consists of two series of repeated homologous domains and a single B domain arranged in the following order: A1-A2-B-A3-C1-C2. Small regions rich in acidic amino acids are located between A1 and A2 (called AR1), A2 and B (AR2), and B and A3 (AR3).[35] The heavy chain consists of A1-AR1-A2-AR2-B, and the light chain consists of AR3-A3-C1-C2.[35] The A and C domains are required for functional activity, whereas the B domain is not. A variety of immunologic techniques have been used to characterize the parts of the factor VIII molecule to which antibodies bind. These include immunoblot analysis after sodium dodecyl sulfate-polyacrilamide gel electrophoresis of purified factor VIII, immunoprecipitation assays using recombinant polypeptide fragments of factor VIII, enzyme-linked immunosorbent assay techniques that measure antibody binding to intact recombinant or plasma-derived factor VIII, and the use of monoclonal anti-factor VIII antibodies derived from the plasma of inhibitor patients.[35] [66] [67] [68] [69] Anti-A2 and -C2 antibodies are present in approximately 68% of plasmas tested by immunoprecipitation assays using recombinant polypeptide fragments of factor VIII.[69] [70] [71] In addition, 46% of antibodies contain antibodies that bind to AR3.[69] [72] There are also less common AR1, A3, and C1 antibodies and antibodies to a light chain epitope outside of C2.[35] [54] [69] [73] [74] The antibody or combinations of antibodies that inhibit factor VIII activity usually consist of those with A2, C2, and AR3-A3-C1 epitopes because of the

Figure 110-2 Localization of factor VIII inhibitor antigens. **(A)** Thrombin cleavage fragments, **(B)** factor VIII domains, **(C)** inhibitor epitopes (hatched bars). Most antibodies are directed toward epitopes on the C2 domain, the A2 domain, or the amino terminal of the A3 domain.

ability of polypeptides containing those epitopes completely to neutralize inhibitor activity.[35] In addition to the C2 epitope, there probably exists one other distinct light chain epitope[35] and an epitope in AR1.[35]

Antibody patterns in hemophilic patients differ from those in autoantibody patients.[35] In a recent study, C2 antibodies were the only antibodies found in 48% of autoantibody patients, whereas this pattern occurred in only one of 34 hemophiliacs.[35] Moreover, 62% of autoantibody patterns were directed to a single epitope, in contrast to only 17% of hemophiliac patterns. Fewer autoantibody patients contained anti-A2 and anti-AR3-A3-C1 antibodies, and the occurrence of A2 plus C2 antibodies was also less common. Hemophiliacs treated with either plasma-derived factor VIII or recombinant factor VIII have equally complex patterns, but they differed in epitope specificity from one another.[35] The relative amounts of antibody to the A2 and C2 domains can vary over time, and neither level is always correlated with the inhibitor level.[35] [66]

Anti-C2 antibodies interfere with factor VIII function by blocking the binding of factor VIII to phospholipid, [35] [66] [75] whereas anti-A2 antibodies inhibit activated factor VIII function by blocking the conversion of factor X by the factor IXafactor VIII complex.[67] [69] Antibodies can interfere with von Willebrand factor binding, [18] [35] [69] [75] [76] and some antibodies reduce the rate at which thrombin-activated factor VIII is released from von Willebrand factor.[61] In addition, the characteristics of inhibitors produced by a given patient may change over the course of time.[1] [18] [35] [54] [63] [65] [77]

Laboratory Evaluation and Quantitation of Inhibitor Titer

The presence of an inhibitor to factor VIII should be suspected in a hemophiliac if transfused factor VIII appears either to have a short half-life or is not efficacious in achieving hemostasis, or both. This can be suspected in the laboratory whenever the partial thromboplastin time of a mixture of patient plasma and normal plasma, after incubation for 2 hours at 37°C, is longer than that of a mixture of patient plasma and hemophiliac plasma known not to contain an inhibitor. To confirm that an inhibitor is directed at factor VIII, a dilution of the patients plasma must be incubated with normal plasma, and factor VIII levels measured over 120 minutes. If the inhibitor is specific for factor VIII, factor VIII decreases over time. Although increased assay specificity can be obtained by performing parallel assays for factors XI, IX, and XII, a high-titer factor VIII inhibitor may inactivate factor VIII in the normal plasma substrate and result in a prolonged partial thromboplastin time while assaying other proteins in the intrinsic pathway. Whenever an inhibitor is suspected or when multiple factor assay results are abnormal, serial dilutions should be done. A true factor deficiency result is independent of the plasma dilution, whereas the effect of an inhibitor is dilution-dependent.

The Bethesda assay is used for quantitation of factor VIII inhibitors.[78] Factor VIII assays are done on 2-hr incubation mixtures of various dilutions of patient plasma with normal plasma. A test sample producing a residual factor VIII activity of 50% of the normal value is considered to contain 1 Bethesda unit of inhibitor per milliliter, and the inhibitor titer equals the reciprocal of the dilution of inhibitor plasma that neutralizes 50% of normal factor VIII. This assay can detect as little as 0.6 Bethesda unit. In the United Kingdom, the new Oxford method is used to quantitate factor VIII inhibitors, where factor VIII concentrate is used as the source of factor VIII along with a 4-hr incubation time.[79] One Bethesda unit equals 1.21 × 1 Oxford unit.[79] An inhibitor unit does not imply that any specific number of factor VIII units infused into the patient will neutralize any specific number of inhibitor units.

FACTORS TO BE CONSIDERED IN SELECTING A BLOOD PRODUCT FOR A BLEEDING EPISODE

1. If a hemorrhage is clinically significant, an attempt should be made to raise the plasma factor VIII level into the hemostatic range of 3050 U/ml.
2. Patients known to be high responders should not receive blood products containing factor VIII to treat minor hemorrhages, to avoid an anamnestic response (unless they are undergoing induction of immune tolerance). Conservative measures combined with the administration of factor IX complex concentrate are frequently adequate.
3. In those patients with a serious hemorrhage who are low responders with a low inhibitor level (<5 Bethesda units), high-purity human factor VIII can be given initially in an initial large bolus of 50150 U (or two to three times the patients usual dose), followed by a continuous infusion of 10 U/kg/hr. Alternatively, porcine factor VIII 50 U/kg can be used. Both low and high responders with low inhibitor levels of <5 Bethesda units with a minor hemorrhage can be most easily managed with factor IX complex concentrate in doses of 5075 U/kg repeated once or twice at 8- to 12-hour intervals as necessary.
4. If the level of inhibitor against human factor VIII is between 5 and 10 Bethesda units, larger doses of human factor VIII or porcine factor VIII, 75100 U/kg can be used.
5. If the level of inhibitor against human factor VIII is >10 Bethesda units but the level of inhibitor against porcine factor VIII is <10 Bethesda units, a larger dose of porcine factor VIII should be tried (100 U/kg).
6. If a patient has a moderately high inhibitor level of 1030 Bethesda units to both porcine and human factor VIII, the inhibitor level may be lowered by approximately 5066% by a 1.5-volume plasmapheresis or extracorporeal immunoadsorption, after which a large infusion of human or porcine factor VIII concentrate may achieve hemostatic levels. Alternatively, patients with moderately high inhibitor levels can be treated with factor IX complex or with the porcine factor VIII concentrate (particularly if the level of inhibitor against porcine factor VIII is <10 Bethesda units).
7. If a patient has a very high inhibitor level (>30 Bethesda units), the only alternatives are factor IX complex concentrates, the porcine concentrate (100150 U/kg), or recombinant factor VIIa.

Therapy

General Considerations

In hemophilia, the presence and titer of an inhibitor and whether the patient is a low or high responder are important determinants of immediate and future therapy. Minor hemorrhage may respond to conservative measures, such as immobilization and compression. For a hemorrhage requiring blood product therapy, two possible choices are available: (1) infusion of human or porcine factor VIII and, if the inhibitor titer is moderately high, preceding this therapy by maneuvers to lower the titer; or (2) infusion of an agent that bypasses the need for factor VIII, such as activated factor IX complex concentrates or factor VIIa.

High-Purity Human Factor VIII Concentrate

High-purity human factor VIII may be used successfully in the treatment of critical hemorrhages in either low or high responders with low inhibitor levels (<5 Bethesda units) and in patients with moderate inhibitor levels (1030 Bethesda units) after reduction of the inhibitor level by plasmapheresis or immunoadsorption. [80] [81] [82] [83] To neutralize the inhibitor and achieve a hemostatic level of 3050 U/ml, an adult patient is given an initial bolus of factor VIII of 50100 units/kg followed by a continuous infusion of 10 units/kg/hr. Alternatively, a large amount of factor VIII can be given every 14 hours (20 U of factor VIII for each Bethesda unit plus an additional 40 U/kg).[83] Because the dose of factor VIII concentrate needed to neutralize inhibitors and provide a hemostatic level of factor VIII cannot be predicted by the inhibitor level, factor VIII levels need to be assayed frequently to monitor the plasma factor VIII level achieved in vivo. However, although blood drawn for assay even a few minutes later may not have a measurable factor VIII level by the time the blood is processed and the assay completed, the infused factor VIII may achieve in vivo hemostasis before it is inactivated.

In patients who are high responders and who sustain a critical hemorrhage, hemostasis should be achieved early, before the anamnestic response occurs. As the factor VIII inhibitor level rises after several days of factor VIII therapy, factor VIII should be continued at a frequent interval or continuouslyhemostasis can be maintained because of the slow neutralization rate of factor VIII by the inhibitor. [55]

In a patient with low-level inhibitor (<5 Bethesda units), it may be possible to raise the level of factor VIII by infusing sufficient human factor VIII to neutralize the antibody and provide free factor VIII to achieve hemostasis. Patients with low inhibitor titers who are low responders can be treated effectively with two to three times the dose of factor VIII used in noninhibitor patients.

Porcine Factor VIII Concentrate

The suitability of a patient for porcine factor VIII depends partially on the degree of cross-reactivity of the patients inhibitor with porcine factor VIII. [84] [85] [86] [87] [88] [85] The level of inhibitor to porcine factor VIII can be determined in the Bethesda assay or the Oxford test by substituting porcine factor VIII for human factor VIII by diluting porcine factor VIII concentrate in human hemophilic plasma until the concentration of porcine factor VIII is 100 U/dl, as measured in a standard factor VIII assay. Although the degree of cross-reactivity can vary widely, according to an international survey of the use of porcine factor VIII in 154 inhibitor patients, it averaged 15%[88] (i.e., approximately 1 unit of antiporcine factor VIII equaled 6.5 units of antihuman factor VIII), with a range of 0200%. After treatment with porcine factor VIII, the degree of cross-reactivity increased. Thus, measurements of cross-reactivity are useful in predicting efficacy before the use of the porcine concentrate. In the international survey of 154 hemophilic inhibitor patients receiving porcine factor VIII, 80% of 2,472 bleeding episodes (mostly hemarthroses) had a good to excellent result, 13% had a fair result, and 7% had no response. [88] The median pretreatment cross-reactivity was 15%; 27% of patients had no cross-reactivity, and 2% of patients had 100% cross-reactivity. Most of the treatment failures were in patients with a high inhibitor titer, whereas inadequate porcine factor VIII dosage accounted for the failures in the remainder. The incidence of reactions after unselected inpatient infusions was 2.3% and usually included fever, chills, or rash. Most were dose-related and occurred only with larger doses. Only occasional patients had repeated allergic reactions unrelated to dose. A decrease in platelet count was seen in 60% of inpatient infusions but was usually insignificant, transient, and correlated

MANAGEMENT OF CRITICAL, LIFE-THREATENING HEMORRHAGES OR EMERGENCY SURGERY IN PATIENTS WITH INHIBITORS

In an emergent situation with a critical hemorrhage or with impending life-saving surgery, both a high responder and low responder should be initially treated with a bolus infusion of the porcine factor VIII concentrate 100 U/kg. Ten minutes after the infusion, blood should be drawn for factor VIII assay to determine the adequacy of the dose; if inadequate, the dose should be increased by 50 U/kg, until the patient has a postinfusion factor VIII level >3050%. Simultaneously, the preinfusion antibody titer to both porcine and human factor VIII can be determined. If the titer to porcine factor VIII is >10 Bethesda units and clinical efficacy has not been established, plasmapheresis can be performed, followed by a massive dose of porcine factor VIII. Once efficacy is established, the patient should be treated at frequent intervals or by continuous infusion at a dose of 1,000 U/hr or 10 U/kg/hr. Factor VIII levels should be monitored frequently. As long as hemostatic levels are achieved, the porcine concentrate should be continued for several days. If the inhibitor titer begins to rise and hemostatic levels can no longer be achieved, anti-inhibitor coagulant complex concentrate or factor IX complex concentrate should be used for several days.

If the patient does not respond initially to porcine factor VIII, anti-inhibitor complex concentrate may be administered, starting at a dose of 75 U/kg. Factor VIIa is currently available on a compassionate basis on research protocol by the manufacturer, but it clearly appears to be effective in these emergent situations.

with dose. In 65 evaluable patients, 31% of patients had a marked anamnestic response, 40% had an intermediate response, and 29% had no response. Some patients were continued on effective home therapy for long periods of time without developing antibodies to porcine factor VIII. However, approximately 30% of patients become resistant because of the development of high-titer porcine factor VIII antibodies. [90] Another retrospective survey of the use of porcine factor VIII in 45 patients with hemophilia A with inhibitors undergoing a variety of surgical procedures reported similar responses to those with acute bleeding episodes. [89]

Although dosage is relatively arbitrary, in patients with human inhibitor titers of <5 Bethesda units, 50 U of porcine factor VIII/kg should be given initially; in patients with 550 Bethesda units, 75100 U/kg is given initially. In patients with >50 Bethesda units, 100150 U/kg is administered. [89] The level of factor VIII achieved in vivo after the infusion is monitored to determine the adequacy of the dose administered. If the initial dose is inadequate, the dose is increased.

Factor IX Complex Concentrate

If human factor VIII cannot be used because the inhibitor level is too high, an attempt may be made to bypass the need for factor VIII by giving a factor IX complex concentrate.[91] [92] [93] [94] [95] [96] [97] [98] [99] These concentrates contain phospholipids, prothrombin, and factors VII, IX, and X, but the substance(s) responsible for factor VIII bypassing activity remain(s) unknown;[100] evidence suggests that it is activated factor IX.[101] [102] [103] These concentrates are of two types: regular unactivated intermediate-purity factor IX

complex concentrates (Konyne 80 [Miles-Cutter], profilnine HT, and Bebulin [Immuno]); and activated concentrates, so-called anti-inhibitor coagulant complex. Both types of concentrates contain various amounts of activated factors VII, IX, and X and phospholipids, and should be distinguished from the highly purified factor IX concentrates used in the treatment of hemophilia B. The activated concentrates (Autoplex [Baxter-Hyland]; FEIBA [Immuno]) contain a greater amount of the factor VIII bypassing material and are specifically designed for patients with factor VIII inhibitors. Both types of concentrates have proved efficacious in controlled clinical trials,[96] [97] and no significant differences between them have been demonstrated. However, some patients have successfully used anti-inhibitor coagulant complex in life-threatening situations in which the unactivated concentrates were ineffective. In a recent French multicenter retrospective survey of the use of FEIBA in 60 patients with factor VIII or IX inhibitors with 433 bleeding episodes or surgical procedures, efficacy was good to excellent in 81.3% of the episodes, whereas it was poor in the remainder.[104] Adverse effects were seen in only 5 of 433 episodes (12%), but an anamnestic response occurred in 17 of 54 evaluable patients (31.5%).

In addition to their unpredictable efficacy, these concentrates have other limitations. Dosage is arbitrary (5075 U/kg, three to four times per day at 8- to 12-hr intervals), and no reliable in vitro method is available that reflects in vivo efficacy. These concentrates also induce a hypercoagulable state and may be associated with thromboembolic complications when used in high dose. These concentrates contain a small amount of factor VIII antigen and can induce an anamnestic response after their use.[105] [106] If selected for use in critical situations, these concentrates should be used early in the course of the bleeding episode. [105] They are also the least expensive concentrate used in the treatment of inhibitor patients.

Human Factor VIIa Concentrate

Purified components of the prothrombin complex have been used to treat hemorrhages in patients with inhibitors. A highly purified concentrate containing factor VIIa achieved good hemostasis[107] and, subsequently, a recombinant preparation of human factor VIIa has shown significant efficacy in patients with inhibitors. [108] [109] In a survey of more than 240 patients, many of whom consisted of hemophilia A or B patients with inhibitors, the recombinant factor VIIa concentrate was used successfully for critical bleeds or for surgery. [110] Dosage and interval of dosing is still being evaluated, but usually it is initiated with doses of 90 g/kg and the same dose is repeated every 23 hours (half-life is 2.9 hours) for one to two times for spontaneous bleeds and every 23 hours for 48 hours for surgical procedures followed by tapering doses over several days. Efficacy has been reported in approximately 90% of patients with spontaneous bleeds and in approximately 80% of patients undergoing surgical procedures.[110] Treatment is not influenced by the inhibitor titer and is equally effective in hemophilia A or hemophilia B. Antibodies to factor VII do not occur except in factor VII-deficient patients.[111] No specific assay has been established that reflects clinical efficacy. The safety profile has been excellent and thrombotic events are rare.

Suppression of Inhibitors

Long-term goals in the management of these patients should be aimed at the prevention of anamnesis and suppression of further antibody production. Immunosuppressive therapy has been attempted in many patients, with variable and unpredictable results. [24] [112] [113] [114] [115] [116] [117] In general, with few exceptions, corticosteroid therapy alone is not efficacious in patients with hemophilia A and inhibitors. [117] By contrast, Dormandy and Sultan[118] collected the experience of many investigators using cyclophosphamide and factor VIII. Some degree of success was achieved on 40 occasions in 18 of 45 patients. The data reported by Hultin et al[117] are in agreement with these observations. Although total elimination of antibody occurs very rarely, immunosuppressive therapy may be helpful in patients requiring factor VIII infusion, particularly low responders with low inhibitor titers. [117] Rarely, treatment of inhibitors soon after their appearance and before reexposure to factor VIII may be an important determinant of successful outcome.[117] The use of immunosuppressive drugs has not gained wide acceptance.

The induction of immune tolerance by the frequent regular infusions of factor VIII can be effective, but the financial implications of this therapy are great. [37] [40] [41] [42] [43] [44] [85] A patient who is a high responder with a high-titer inhibitor is given daily or alternate-day infusions of human factor VIII. [37] [40] [41] [42] [44] [85] After chronic administration of factor VIII, the inhibitor titer first increases and then progressively decreases, with most patients achieving either a low or undetectable titer. Moreover, the patient no longer has anamnesis with continued exposure to factor VIII. Similar results have been achieved at some centers, either with less amounts of factor VIII[38] [43] [85] [119] [120] [121] or by giving factor VIII repeatedly but at irregular intervals. [122] Immune tolerance usually must be maintained by giving low doses of factor VIII every few days.

Recently published data from an international registry of patients treated with an immune tolerance protocol provide a concise summary of the effectiveness of these

regimens.[40] The report includes the data from 158 patients from 40 centers who were on therapy long enough to evaluate efficacy. Tolerance was attained in 107 patients (68%), 12 (8%) had a partial response (high responders converted to low responders), and 39 patients were unresponsive. Multivariate regression analysis revealed that the highest probability of success was independently associated with the dosage used (>100 IU/kg/day) and with the level of inhibitor at enrollment (<10 Bethesda units). Thus, the best results were obtained using high doses of factor VIII and starting with a low inhibitor titer (even though 82% of these patients were high responders). Therefore, it is probably important to initiate immune tolerance therapy long after the anamnestic response has dissipated. Although the best results were obtained with high doses of factor VIII, low-dose protocols can also be effective, but predominantly in those with low or intermediate inhibitor titers or in those started soon after discovery of the inhibitor. [40] [43] Mean time to the development of tolerance was 37 months, and once tolerance was achieved, only 1 of 107 patients relapsed after an average follow-up period of 5 years. Younger patients have a better chance of success in a shorter time than adults. A recent report of treatment in 21 children described elimination of the inhibitor in 19 in a median time of 4 months. [41] A successful outcome and the length of time needed to induce immune tolerance were indirectly related to the number of factor VIII exposure days or when the tolerance regimen was interrupted. There was no correlation with the length of the interval between the initial inhibitor detection and the onset of immune tolerance therapy. Thus, immune tolerance regimens should be initiated as soon as possible, after detection, preferably when the inhibitor titer is <10 Bethesda units.

The mechanism by which frequent infusions of factor VIII reduce levels of anti-factor VIII antibodies is not understood. However, patients treated successfully produce anti-idiotypic antibodies. [4] [123]

Successful regimens using factor VIII infusions combined with immunosuppressive drugs appear to shorten the induction of immune tolerance significantly, attaining remission at a much lower cost.[42] [124] High-dose intravenous immunoglobulin given together with high-dose factor VIII and cyclophosphamide sometimes preceded by extracorporeal immunoadsorption appears to have a synergistic effect in achieving a state of immune tolerance. [42] [125] [126] Intravenous IgG may have both a short- and long-term immunosuppressive effect.[127] [128] [129] [130] Anti-idiotypic

antibodies are present in the IgG preparations; [130] [131] [132] [133] the emergence of anti-idiotypic antibodies may be the explanation for the occurrence of spontaneous remission and some instances of remission induced by immunosuppressive agents. [131] [134] [135]

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INHIBITORS IN HEMOPHILIA B

Inhibitors to factor IX occur in hemophilia B secondary to alloimmunization after transfusion, similar to hemophilia A. Although the overall incidence of inhibitors complicating hemophilia B is only approximately 3%, [24] [136] the incidence in patients with severe hemophilia B is approximately 12%. [24] Patients with major gene deletions or nonsense mutations who do not have demonstrable factor IX protein antigen in their plasma may be especially susceptible to the development of factor IX antibodies.[137] [138] In contrast, in patients with missense mutations, the incidence of inhibitors is very low. [138] Approximately 80% of patients have a significant anamnestic response after transfusion with plasma products containing factor IX.

Like factor VIII inhibitors, factor IX inhibitors are IgG and some are predominantly IgG_4.[24] [139] [140] [141] Some have restricted light chain and heavy chain heterogeneity,[140] [142] whereas others are polyclonal.[141] [143] The kinetic behavior of factor IX inhibitors differs from that of factor VIII inhibitors in that factor IX inhibitors produce an immediate loss of factor IX activity, with no progressive loss on further incubation. [11] [92]

Laboratory Evaluation and Quantitation

Factor IX inhibitors should be suspected in a hemophiliac if transfused factor IX either appears to have a short half-life or is not efficacious in achieving hemostasis, or both. This can be suspected in the laboratory whenever a partial thromboplastin time of a mixture of the patients plasma and normal plasma is longer than that of a mixture of the patients plasma and factor IX-deficient plasma known not to contain an inhibitor. Specificity can be determined by doing factor IX assays of an incubation mixture of the patients plasma and normal plasma over time. A modification of the Bethesda assay is used to quantitate the inhibitor level. [78]

Therapy

In patients with hemophilia B who have inhibitors, the clinical course is not different from that seen in those with hemophilia A with inhibitors. Many of these cases can be managed with factor IX complex concentrates or the anti-inhibitor/coagulant complex. Whether the efficacy of these concentrates is due to neutralization of the inhibitor or to bypassing activity similar to that observed in hemophilia A, or both, is unknown.

Alternatively, these patients can be treated with two to three times the usual dose of highly purified factor IX concentrate in an attempt to neutralize the inhibitor and achieve a hemostatic level of factor IX. Patients who are high responders with low inhibitor titers can be treated similarly, with the caveat that anamnesis will occur within a few days. In low or high responders with moderate inhibitor titers, plasmapheresis can be used adjunctively before infusion of any of the aforementioned concentrates. In patients with high-titer inhibitors, the only alternatives are prothrombin complex concentrates, anti-inhibitor/coagulant complex concentrates, or the recombinant factor VIIa concentrate.[108] [144] The latter has been used effectively in patients with factor IX inhibitors, using dosage regimens similar to the ones discussed previously for the treatment of factor VIII inhibitors. [108]

In addition, regimens for induction of immunotolerance have been achieved in hemophilia B patients with and without the use of high doses of intravenous IgG and cyclophosphamide, sometimes preceded by extracorporeal immunoadsorption. [42] [145]

There have been several reports of life-threatening anaphylactic or anaphylactoid reactions in young children with hemophilia and inhibitors on exposure to factor IX concentrates.[146] [147] The factor IX inhibitors are detected in most simultaneously with the occurrence of the allergic reaction. Most of the patients have factor IX gene deletions or major derangements.[147] Immune tolerance regimens have resulted in very poor response in these patients, but bleeding episodes have been managed successfully with recombinant factor VIIa concentrate.

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Chapter 111 - Other Clotting Factor Deficiencies

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INTRODUCTION

This chapter describes the biology and clinical manifestations of deficiencies of prothrombin, factor V, factor VII, factors XXIII, and familial multiple clotting factor deficiencies. Table 1111 lists these clotting factor deficiencies and some of their characteristics.

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PROTHROMBIN DEFICIENCY (HYPOPROTHROMBINEMIA AND DYSPROTHROMBINEMIA)

History and Epidemiology

At the turn of the century, Morawitz (1905) formulated a basic theory of coagulation in which fibrinogen was converted to fibrin by a substance he called *fibrin ferment*, or thrombin.[1] In his theory, a precursor molecule, prothrombin, was activated by a hypothetical enzyme (thrombokinase) to yield thrombin. Morawitzs cascade was much more simple than that which is known to exist today, but it was essentially correct. True congenital hypoprothrombinemia or dysprothrombinemia is exceedingly rare; fewer than 30 kindred have been well-studied. [2] Hypoprothrombinemia or dysprothrombinemia has no known predilection for any racial or ethnic group.

Genetics and Molecular Biology

The prothrombin gene consists of 14 exons and occupies approximately 24 kilobases of DNA near the centromere of chromosome 11. [3] The gene codes for a signal peptide; a propeptide region; a gamma-carboxy glutamic acid domain, which is dependent on vitamin K for post-translational modification of ten glutamic acid residues; two kringle regions; and a catalytic domain. [4] Prothrombin is a single-chain glycoprotein with a molecular weight of 72,000. It is synthesized in the liver and is normally present in the plasma at a concentration of 100 g/ml, making it the second most abundant coagulation protein after fibrinogen. Prothrombin undergoes cleavage at several sites to

TABLE 111-1 -- Clotting Factor Deficiencies: General Information

Factor Human Deficiency	Gene Location	Normal Circulating Half-life	Estimated Incidence	Bleeding Severity
II	11p11q12	3 days	Very rare	Mildmoderate
V	1q21q25	36 hours	1:1 million	Moderate
VII	13q34	36 hours	1:500,000	Mildsevere
VIII	Xq28	12 hours	1:10,000	Mildsevere
IX	Xq27	24 hours	1:30,000	Mildsevere
X	13q34	40 hours	1:500,000	Mildsevere
XI	4q32q35	80 hours	Rare[a]	Mildmoderate
XII	5q33qter	5070 hours	?	No bleeding
XIII	*A* subunit: 6p24p25	9 days	<1:1 million	Moderatesevere
	B subunit: 1q31q32			

[a] *Rare except in those of Ashkenazi Jewish descent.*

yield the active serine protease thrombin, which consists of an A chain linked to a B chain by a disulfide bridge. The proteolytic conversion of prothrombin to thrombin requires factor Xa, calcium, and phospholipids and is accelerated 300-fold by the presence of activated factor V. [5] Thrombin performs a multitude of functions. Its procoagulant actions include: proteolysis of fibrinopeptides A and B from fibrinogen; activation of factors V, VIII, and XIII, and induction of platelet aggregation. These functions are balanced by thrombin-mediated activation of protein C. Thrombin functions as an anticoagulant when complexed with thrombomodulin, a component of the endothelial cell surface. The complex activates protein C, which in turn inhibits coagulation by degrading factors Va and VIIIa. Thrombin also has cytokine and growth factor-like activities.[6] For example, it is chemotactic and mitogenic for mononuclear phagocytes, fibroblasts, smooth muscle cells, and endothelial cells.

Pathogenesis

A variety of mutations in the prothrombin gene have been discovered. These mutations cause either decreased production of prothrombin or production of a dysfunctional molecule with reduced activity. The subsequent decrease in thrombin activity results in absent or defective clot formation, defective platelet aggregation, and defects in other pathways normally activated by thrombin. The clinical severity of bleeding depends on the particular defect. Table 1112 depicts some of the known variants of prothrombin, which are inherited in an autosomal fashion. [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18] [19] [20] [21] [22] [23] [24] [25] [26] [27] [28] [29] [30] [31] [32]

Clinical Manifestations

Bleeding associated with prothrombin deficiency typically consists of easy bruising, epistaxis, soft tissue hemorrhage, postoperative bleeding, and, in females, menorrhagia and/or metrorrhagia. Hemarthroses have also been described, but are not common. [33] The correlation between prothrombin levels and severity of bleeding is poor. For example, there are asymptomatic patients with prothrombin activities of 15% of normal (e.g., prothrombin Salatka), whereas patients with prothrombin San Juan are symptomatic with prothrombin activities in the 25% range. [34] Because all known cases of congenital hypo- or dysprothrombinemia have detectable prothrombin levels, it is possible that complete absence of prothrombin activity is incompatible with life.

Recently, a mutation in the 3 untranslated region of the prothrombin gene has been described that is associated with increased levels of circulating prothrombin. [35] It is not clear how this mutation leads to higher prothrombin levels. However, the increased level of prothrombin in these individuals is associated with an increased risk of thrombosis.

Laboratory Evaluation

Congenital prothrombin deficiency is marked by prolongation of the prothrombin time and the partial thromboplastin time (PTT). The bleeding time is normal. The prolonged prothrombin

TABLE 111-2 -- Prothrombin Variants

Variant	Mutation	Activity (%)	Antigen (%)	Dysfunction
Homozygous				
Barcelona/Madrid	Arg^{271} Cys	5	100	Activation by Factor Xa
Dharan	Arg^{271} His	5	95	
Denver		<1	13	
Frankfurt/Salatka	Glu^{466} Ala	15	100	Substrate binding
Obihiro	Arg^{271} Cys	18	100	
Poissy		2	50	Defective activation
Perija		2	70	
Segovia		720	100	
Heterozygous				
Brussels		2035	71	
Cardeza		40	100	Activation by Factor Xa; no bleeding
Padua	Arg^{271} His	52	100	Defective activation
Clamart		50	100	Activation by Factor Xa; no bleeding
Magdeburg		45	100	
Compound heterozygous				
Quick I	Arg^{382} Cys	<2	34	Fibrinogen binding; platelet activation
Quick II	Gly^{558} Val	<1		Substrate binding
San Juan I and II		725	25	
Mexico City		10	<10	
Himi I	Met^{337} Thr			No bleeding
Himi II	Arg^{388} His			Fibrinogen binding
Habana		110	50	
Metz		10	50	Thrombin domain
Molise		10	45	Thrombin domain
Tokushima	Arg^{418} Tyr and frameshift			Fibrinogen binding Platelet activation
Uncharacterized genetics				
Gainesville		25	72	
Houston		5	52	
Corpus Christi	Arg^{382} Cys	2	25	Little bleeding

In this and subsequent tables, amino acids are designated by a three-letter code. Patients exhibited a bleeding tendency unless otherwise noted.

time and PTT are corrected if the patients plasma is mixed with equal amounts of normal plasma. In true hypoprothrombinemia, both the functional and antigenic assays for prothrombin reveal decreased levels. Dysprothrombinemia results in decreased prothrombin activity, but the prothrombin antigen level may be normal or only slightly decreased. Specific diagnosis of prothrombin defects rests upon functional and antigenic assays.

Differential Diagnosis

Patients with a bleeding tendency and prolongation of the prothrombin time and PTT may be suspected of having a deficiency of prothrombin, but deficiencies of factor V, factor X, and multiple factor deficiencies should also be considered. A definitive diagnosis of hereditary prothrombin deficiency requires a specific assay for prothrombin and demonstration that levels of other clotting factors are normal.

Acquired prothrombin deficiency due to liver disease, vitamin K deficiency, or warfarin can be distinguished by demonstrating low levels of all of the vitamin K-dependent factors.

The term lupus anticoagulant is commonly used to describe an autoantibody that results in a prolonged PTT in vitro. These antibodies were once thought to be directed against phospholipids. However, the majority are directed against phospholipid-binding proteins, especially $_2$ -glycoprotein I.[36] Such antibodies are generally associated with an increased risk of thrombosis, and are rarely associated with a bleeding tendency. However, a small subset of these antibodies are directed against prothrombin. These antibodies may inhibit prothrombin activity directly,[37] or they may accelerate clearance of prothrombin, resulting in a true prothrombin deficiency.[38] The acquired hypoprothrombinemia may be associated with bleeding, particularly soft tissue hemorrhage, which may be so severe as to be life-threatening.[39] [40] In these cases, the PT is prolonged.

Therapy

Because prothrombin has a long half-life (about 3 days), most patients can be treated with plasma in doses of 1520 ml/kg body weight as a loading dose, followed by 3 ml/kg body weight every 1224 hours. Plasma levels 30% of normal are usually sufficient to treat bleeding episodes. Plasma exchange may be employed to achieve higher levels before surgery. Prothrombin complex concentrates, generally used to treat hemophilia B, also contain significant levels of prothrombin. These can be used to treat prothrombin deficiency, but care should be excercised, since their use has been associated with thromboembolic complications. Highly purified factor IX concentrates do not contain significant amounts of prothrombin and should not be used to treat prothrombin deficiency. Table 1113 lists some of the prothrombin complex concentrates and the amount of prothrombin contained in each. Highly purified prothrombin concentrates are not presently available for clinical use.

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FACTOR V DEFICIENCY

History and Epidemiology

As early as 1939, it was known that clotting factors other than prothrombin influenced the prothrombin time. In 1943, Quick found that the prothrombin time of aged plasma was prolonged. He postulated that there was a labile factor present in plasma that was different from prothrombin but was required for a normal prothrombin time.[41] At about the same time

TABLE 111-3 -- Composition of Selected Factor IX Concentrates

Product (manufacturer)	Relative Amount of Factor[a]			
	Prothrombin	VII	IX	X
Prothrombin complex concentrates				
Bebulin VH (Immuno)	120	13	100	139
Proplex T (Baxter/Hyland)	50	400	100	50
Profilnine HT (Alpha)	148	11	100	64
Konyne 80 (Bayer/Miles)	100	20	100	140
Highly purified factor IX concentrates				
Alphanine (Alpha)	<5	<5	100	<20
Mononine (Armour)	0	0	100	0

Product (manufacturer)	Amount of Factor (in nm/L)				
	Thrombin	VIIa	IXa	Xa	XIa
Activated prothrombin complex concentrates					
Autoplex T (Baxter/Hyland)	5	69	178	47	6
FEIBA (Immuno)	?				

[a] Data expressed in units of activity normalized to the amount of factor IX present.

Owren studied a woman with a lifelong history of bleeding. He concluded that she was deficient in a factor present in plasma which, in contrast to prothrombin, was not adsorbed to aluminum hydroxide. Owrens studies were carried out during the Nazi occupation of Norway, and he was unable to report his studies until 1947. [42] Eventually it was found that Owrens patient was deficient in labile factor, which we now know as factor V.

Deficiency of factor V is a rare condition with an incidence of approximately one per million. [43] About 150 cases have been reported. The deficiency shows no known predilection for members of any racial or ethnic group. Consanguinity has frequently been observed in the families of affected patients.

Genetics and Molecular Biology

Factor V deficiency is inherited as an autosomal recessive trait, with a gene frequency of $5^{10} \times 10^{4}$.[44] The factor V gene is located at 1q2125, near genes coding for the selectin leukocyte adhesion molecules.[45] Factor V is composed of several domains or modules, and is closely related to factor VIII. The two proteins are about 40% homologous in primary structure and their genes are arranged in a nearly identical fashion. [46] Both proteins have three A domains, one B domain, and two C domains.[45] The A domains of factor V bear 30% homology with the copper-binding protein, ceruloplasmin. Copper has been detected in human factor V, but its function is unclear. The tandem C domains are homologous to duplicated domains in human and murine milk fat globule proteins. The B domain of factor V is not required for procoagulant activity. It is removed from the factor V molecule during its activation by thrombin. Unlike the A and C domains, the B domain is not homologous to factor VIII. Neither is the B domain well conserved between different mammalian species.

The factor V glycoprotein (330,000 molecular weight, 13% carbohydrate) is synthesized in the liver (plasma factor V) and in the megakaryocytes (platelet factor V), although other tissues may synthesize small amounts (e.g., vascular endothelium, monocytes). Plasma factor V circulates at a concentration of about 7 g/ml. [47] Platelet factor V is found within the -granules and is released upon platelet activation and degranulation. [48] It is identical to plasma factor V and accounts for 20% of the total factor V in blood. For full activity, the factor V must be activated by thrombin or factor Xa. Thrombin cleaves arginyl residues in human factor V at positions 709, 1,018, and 1,545. The active molecule, containing a light and heavy chain linked by calcium ions, serves as an essential cofactor for factor Xa conversion of prothrombin to thrombin. The phospholipid and cell-surface binding sites of factor Va are located on the light chain, whereas the heavy chain is thought to bind prothrombin.[46]

Factor Va is inactivated when it is cleaved at two additional sites by activated protein C (APC), a reaction that requires protein S as a cofactor. This inactivation process is clearly of physiological significance, since a mutation in factor V (Arg ^{506} Gln) that abolishes one of the APC cleavage sites (factor V Leiden) is associated with an increased risk of thrombosis.[49]

Pathogenesis

The hemorrhagic tendency in factor V deficiency is a direct consequence of the lack of its activity both in plasma and platelets. Some deficient patients have normal levels of factor V antigen but low levels of functional activity. [43] [50] [51] [52] Patients severely deficient in plasma factor V are generally deficient in platelet factor V as

well. One variant, factor V Quebec, exists in which a brother and sister with abnormal bleeding had moderately reduced plasma levels (40% of normal), but severely reduced platelet factor V (24% of normal).[51] The bleeding defect in this family suggests that platelet factor V is important for hemostasis. However, it appears that the factor V Quebec disorder is associated with a defect that leads to degradation of most proteins of the -granules. [53] Thus, other abnormalities may contribute to the bleeding tendency in these patients.

Clinical Manifestations

Factor V deficiency has a wide range of clinical manifestations. Even though severe hemorrhage may occur in patients with <1% factor V, bleeding is not as frequent as that seen in severe hemophilia A patients. Mild and moderate forms of factor V deficiency most likely reflect genetic heterogeneity of the disorder. Severe factor V deficiency commonly presents with abnormal bruising, epistaxis, soft tissue hemorrhages, and occasional hemarthroses. Crippling hemophilic arthropathy is not common, but hemorrhage involving the central nervous system has been observed. Bleeding from the umbilical stump is frequent. Women commonly experience abnormal menstrual bleeding and postpartum hemorrhage. About one-half of patients are diagnosed in adulthood. One family has also been reported with congenital factor V deficiency and recurrent thromboembolic events. [54]

Laboratory Evaluation

Laboratory features of severe deficiency are shown in Table 1114 . Both the prothrombin time and PTT are prolonged, whereas the thrombin time is normal. The bleeding time may be prolonged in severe factor V deficiency since platelet factor

TABLE 111-4 -- Typical Laboratory Values for Factor V-Deficient Patients

Assay	Moderate	Severe	Normal
PT (sec)	15	55	12
aPTT (sec)	40	60	26
Thrombin time (sec)	11	11	11
Bleeding time (min)	7	7>15	39
Factor V (% normal)	27	<1	100

V is virtually undetectable in these patients. Conclusive diagnosis of the deficiency requires a specific factor V assay.

Differential Diagnosis

Hereditary factor V deficiency must be distinguished from a combined deficiency of factors V and VIII, which is also inherited. In the latter condition, both factor V and factor VIII levels are about 1520% of normal. Acquired factor V deficiency occurs in patients with liver disease. It is also reduced in patients with disseminated intravascular coagulation. Inhibitors to factor V occur in some patients with congenital factor V deficiency and rarely in otherwise normal patients who develop these inhibitors spontaneously. Factor V inhibitors have been documented in association with the use of topical bovine thrombin preparations (which contain bovine factor V) in cardiac surgery,[55] and in patients treated with antibiotics such as streptomycin. Inhibitors to factor V can be detected by showing that the patients plasma inhibits factor V activity in normal plasma.

Therapy

Fresh-frozen plasma is the mainstay of treatment for bleeding episodes in deficient patients. No commercial factor V concentrates are available, and cryoprecipitate is not rich in this protein. Although platelets contain factor V and can correct the bleeding defect in deficient patients, platelet transfusions are not the treatment of choice because of the possible induction of antiplatelet alloantibodies. However, transfusions of normal platelets may be useful in patients with circulating inhibitors to factor V, since platelet factor V may be available in a milieu protected from inhibitors. [56]

Levels of 25% of normal are usually sufficient for many mild or moderate bleeding episodes. [57] This level can be achieved by the use of plasma at an initial dose of 1520 ml/kg of body weight, followed by 36 ml/kg of body weight daily for 7 days post-surgery. Care must be taken to monitor for evidence of volume overload when large amounts of plasma are infused. Daily plasma infusions for surgery seem reasonable, since the half-life of factor V is about 36 hours. [57] In the case of severe bleeding, or in preparation for surgery, higher levels may be obtained by plasma exchange. [58]

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FACTOR VII DEFICIENCY

History and Epidemiology

The first reported case of factor VII deficiency was described by Alexander in 1951. [59] In the index case, plasma from a child with congenital bleeding displayed a prolonged prothrombin time that could be corrected by aged serum, but not by barium sulfate-adsorbed plasma. Factor VII deficiency is inherited as an autosomal recessive trait and affects males and females equally, with an incidence estimated to be about one in 500,000.

Genetics and Molecular Biology

The gene for factor VII is found on chromosome 13, adjacent to the gene for factor X. [60] Evidence exists for a regulatory element on chromosome 8 that controls factor VII expression, which occurs in the liver. [61] As a vitamin K-dependent factor, factor VII contains -carboxyglutamyl (Gla) residues that are necessary for calcium-dependent lipid binding, as well as two epidermal growth factor-like regions (EGF domains) and a catalytic domain with typical serine protease features. [62] [63]

Factor VII circulates in the plasma at a concentration of 0.5 g/ml. Factor VII is rapidly activated upon binding to its cofactor, tissue factor. Activated factor VII (factor VIIa) bound to tissue factor can catalyze activation of additional factor VII. However, it is not clear what protease is responsible for activation of the first factor VII molecules during hemostasis. Tissue factor is a membrane-associated glycoprotein that is not normally expressed by cells in contact with blood. When blood escapes from the vasculature at a site of injury, the binding of factor VII to extravascular tissue factor is the primary physiologic trigger of the coagulation cascade. [64] [65] [66] The factor VIIa/tissue factor complex then activates both factor X and factor IX. [67] Factor VIIa/tissue factor has been thought of as the first component of the extrinsic pathway, in which it activates factor X, which combines with factor Va to activate prothrombin to thrombin. However, it is now clear that discrete "instrinsic" and "extrinsic" pathways do not exist in vivo. Activation of factor X by factor VIIa/tissue factor is insufficient to support hemostasis. Factor IX activated by factor VIIa/tissue factor combines with its cofactor factor VIIIa to produce additional factor Xa on the platelet surface, [68] [69] which then activates enough prothrombin for successful clot formation.

Increased levels of plasma factor VII are a risk factor for cardiovascular disease, whereas decreased levels of factor VII may be associated with a decreased risk.

Pathogenesis

Because the factor VIIa/tissue factor complex is the key initiator of coagulation in vivo, a deficiency of factor VII can result in significant clinical manifestations. Individuals with severe deficiency (<1% activity) can have clinical bleeding as serious as those with severe classical hemophilia. However, about half of the factor VII deficient patients are asymptomatic, including at least three who apparently have <1% factor VII activity. [70] Generally, individuals who are homozygous or doubly heterozygous for a factor VII mutation are more likely than heterozygotes to express a clinical bleeding tendency. There is not, however, a very good correlation between factor VII coagulant activities measured in the laboratory and bleeding manifestations. This is partly due to the fact that different factor VII mutants express different levels of coagulant activity when assayed in the presence of tissue factor from different species. The genetic defects responsible for many of the dysfunctional factor VII molecules have been determined and catalogued. [71] A detailed database of the mutations is maintained on the World Wide Web at: http://europium.mrc.rpms.ac.uk/usr/WWW/WebPages/FVII/database.dir/titlepage.htm. Selected variants are listed in Table 1115 . In addition, three polymorphisms in the factor VII gene have been found, which affect the plasma level of the factor VII protein. An Arg Gln substitution at residue 353 is associated with a 2025% reduction in plasma factor VII activity. [72] A decanucleotide insertion at nucleotide 323 and a polymorphism in intron 7 are associated with reduced factor VII antigen and activity. [73] [74]

Diminished availability or defective function of tissue factor has never been documented as a cause of decreased factor VII activity. The phenotype of homozygous tissue factor knockout mice is that of embryonic death at about 8.5 days of gestation. [75]

Clinical Manifestations

Factor VII deficiency commonly presents as easy bruising, soft tissue hemorrhage, epistaxis, and, in women, menorrhagia and metrorrhagia. Hemarthroses and crippling hemophilic arthropathy occur in severely affected patients (<1% activity) and may be as severe as that seen in hemophilia A and B. [76] Bleeding in factor VII deficiency is usually easier to control than that seen in hemophilia A and B. Intracranial hemorrhage has been reported, especially in neonates after vaginal delivery. One series cites a 16% incidence of intracranial bleeding in 75 patients with factor VII deficiency, a complication frequently associated with a fatal outcome. [77] Postpartum hemorrhage is seen in patients

TABLE 111-5 -- Selected Dysfunctional Factor VII Variants

Variant	Antigen (%)	Activity (%)	Defect	(Domain)	Bleeding
Unnamed	30	<1	del Phe^{24}	(Gla)	Severe
Shinjo	76	20	Arg^{79} Gln	(EGF-1)	Asymptomatic
Charlotte[a]	100	<1	Arg^{79} Gln Arg^{152} Gln	(EGF-1) (cleavage site)	Severe
Kansas	60	4	Gln^{100} Arg	(EGF-2)	Asymptomatic
Mie	26	26	Arg^{247} His	(catalytic)	Asymptomatic
Unnamed	47	8	Ala^{294} Val	(catalytic)	Moderate
Unnamed	85	6	Met^{298} Ile	(catalytic)	Mild
Unnamed dbl heterozygote	50	<1	Leu^{300} Pro Cys^{310} Phe	(catalytic)	Moderate

Nagoya	87	16	Arg^{304} Trp	(catalytic)	Asymptomatic
Padua	104	9	Arg^{304} Gln	(catalytic)	Mild
Multiple[b]	100	115	Arg^{304} Gln	(catalytic)	Variable[c]
Unnamed	90	5	Cys^{310} Phe	(catalytic)	Mild
Central	38	<1	Phe^{328} Ser	(catalytic)	Severe
Toyama		1	Thr^{359} Met	(catalytic)	Severe
Polish AH dbl homozygote	2	2	Frameshift 403/4 Ala^{294} Val	(Cterminal)	Moderate/severe

[a] *Double homozygote.*
[b]Found in Richmond, Padua, Detroit, Verona (possible double heterozygote), Little Rock (possible double heterozygote), and Kansas (double heterozygote with Gln^{100} Arg).
[c] *Clinical phenotype ranges from asymptomatic to moderate bleeding.*

with factor VII levels below 1020% of normal. Postoperative bleeding is more likely in severely affected patients, although exceptions have been reported.

Laboratory Evaluation

Factor VII deficiency is the only hereditary clotting factor deficiency that causes an isolated prolongation of the prothrombin time in the presence of a normal PTT. Occasionally patients with certain mutations in factor VII have a prolonged PTT, but this is very rare. Bleeding time is usually normal. A specific factor VII assay is required to confirm the diagnosis. Typical laboratory features of factor VII deficiency are presented in Table 111-6 . The activity can vary dramatically, depending on the source of tissue factor used in the in vitro assay. Variants of factor VII deficiency can be distinguished on the basis of their reactivity toward tissue factors of different animal species.[78] [79] [80] [81] [82] [83] [84]

Differential Diagnosis

Hereditary factor VII deficiency may be suspected in any male or female patient with a lifelong history of excessive bleeding, whether spontaneous or traumatic, especially in those with isolated prolongation of the prothrombin time. Factor X deficiency and some variants of factor IX deficiency (hemophilia B_M), may also exhibit prolonged prothrombin times. In contrast to factor VII deficiency, the PTT is also prolonged in the latter two conditions.

Liver disease, warfarin ingestion, or vitamin K deficiency may mimic factor VII deficiency. These disorders may be distinguished from hereditary deficiency by measuring other vitamin K-dependent factors. There is an inherited combined deficiency

TABLE 111-6 -- Typical Laboratory Values for Severe Factor VII Deficiency

Assay	Severe	Normal
Prothrombin time (sec)	36	13
aPTT (sec)	28	26
Thrombin time (sec)	11	11
Factor VII (%)	<1	100

of prothrombin, factors VII, IX, and X (familial multiple factor deficiency, type III). In some reported cases of this syndrome, factor VII levels are lower than those of the other vitamin K-dependent factors. In addition, combined deficiency of factors VII and X has been reported (see Table 11111).

Inhibitors to factor VII have been reported to develop in some patients with hereditary factor VII deficiency following replacement therapy. [85]

Therapy

In the United States, factor VII deficiency is treated with fresh-frozen plasma or prothrombin complex concentrates that contain factor VII (Table 1113). Purified factor VII concentrates from both plasma and recombinant sources are available in Europe, but are not yet approved in the United States. Because the half-life of factor VII is about 3.5 hours, it is difficult to maintain normal levels with plasma replacement alone. Fortunately, it is not usually necessary to raise factor VII levels to 100% to achieve adequate hemostasis. Although the minimum hemostatic level is not known, an increase to 1525% of normal generally results in normal hemostasis, even as prophylaxis for surgery. Replacement therapy given every 612 hours is satisfactory for most hemorrhagic episodes despite the short in vivo half life of factor VII.

For spontaneous hemorrhage or mild trauma, therapeutic factor VII levels of 510% are sufficient to stop bleeding. This can be achieved by administering plasma at a dose of 510 ml/kg body weight and repeating the dose every 812 hours for 12 days. For major hemorrhage or surgery, plasma may be given as a loading dose of 1520 ml/kg body weight and followed by 36 ml/kg body weight every 812 hours until healing of surgical wounds occurs. This may require 57 days of treatment.

The use of plasma carries with it the risk of transmission of viral diseases, particularly hepatitis C. Other disadvantages of plasma are the potential for volume overload and the inability to achieve high levels of factor VII. For these reasons, prothrombin complex concentrates containing factor VII may be useful. Their successful use at a dose of 50 U/kg body weight every 8 hours for 24 hours, followed by plasma infusions thereafter, has been reported for major orthopaedic surgery.[86]

Highly purified factor VII concentrates are useful in treating

patients with severe bleeding or as prophylaxis for surgery or childbirth. [87] [88] Recombinant factor VIIa has proven effective in clinical trials as a treatment for bleeding in hemophilia A and B patients with inhibitors, and in limited numbers of factor VII-deficient patients. The amount of factor VII replacement required for surgery is unclear.

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FACTOR X DEFICIENCY

History and Epidemiology

During the 1950s, two groups of researchers independently discovered factor X. The index patient, named Stuart, was originally thought to have factor VII deficiency, but his plasma was later shown to correct the defect in the plasma from the original factor VII deficient patient. [89] A British group simultaneously studied a subject named Prower, leading to the original designation of factor X as Stuart-Prower factor. Factor X deficiency has no predilection for any particular racial or ethnic group and is found worldwide. The incidence is estimated at one per 500,000. Consanguinity has been shown in a number of families with factor X deficiency, including that of the index patient, whose mother and father were aunt and nephew.

Genetics and Molecular Biology

Factor X deficiency is transmitted as an autosomal recessive trait. [90] The gene encoding factor X has been localized to chromosome 13 at postition 13q34, less than three kilobases from the factor VII gene. It consists of eight exons arranged in a fashion similar to the genes of factor IX and factor VII. [91] [92] Factor X mRNA is expressed primarily, but not exclusively, in liver. [93] Mature factor X circulates as a two-chain molecule. The light chain has a molecular weight of 17,000 and the heavy chain has a weight of 40,000. Like the other vitamin K-dependent factors, the protein contains a Gla domain, two EGF-like domains, an activation peptide region, and a catalytic domain typical of serine proteases. In addition to its 11 -carboxyglutamic acid residues, factor X also has a -hydroxy-aspartic acid residue at position 63. Cleavage between Arg^{194} and Ile^{195} releases the 52 amino acid activation peptide and converts factor X to its active form. Activation is carried out physiologically by the factor VIIa/TF complex (as reflected in the prothrombin time test) or the factor IXa/VIIIa complex (as reflected in the PTT). Russell viper venom (RVV) will cleave factor X at the same site, and has been used in laboratory tests of factor X activity. Factor Xa, in the presence of its cofactors (factor Va, calcium, and phospholipids), rapidly converts prothrombin to thrombin.

Pathogenesis

The molecular defect has been identified in a number of patients with factor X deficiency. The defect in the original patient is the substitution of Val^{298} to Met. Other factor X variants have been described in varying depth (Table 1117). [94] [95] [96] [97] [98] [99] The known factor X mutations have been detailed by Cooper et al. [97]

The various missense mutations that have been described have provided information about structure-function relationships in factor X. For example, mutations in glutamic acids that are post-translationally modified by -carboxylation have shown that these residues are important in calcium-mediated binding to lipid membranes. In addition, they are critical in maintaining the protein in the correct calcium-dependent conformation for activation, cofactor binding and proteolytic activity. Roma is a variant in which a Thr^{318} Met mutation on the surface of the molecule affects activation by the factor IXa/factor VIIIa complex to a greater degree than activation by the factor VIIa/TF complex. In contrast, the Vorarlberg variant resulting from a Gla [14] Lys mutation is very poorly activated by factor VIIa/TF, but normally by factor IXa/VIIIa.

Clinical Manifestations

Hemarthroses, retroperitoneal hematomas and other soft tissue hemorrhages, hematuria, pseudotumors, and menorrhagia are observed in patients with severe factor X deficiency. Severe bleeding episodes comparable to those seen in classic hemophilia may occur, including crippling hemophilic arthropathy. Excessive bleeding is uncommon in mild disease. Patients with 15% or greater levels of factor X usually have very few hemorrhagic episodes, although bleeding may occur with major surgery or trauma.

Laboratory Evaluation

Typical laboratory findings in factor X deficiency are depicted in Table 1118 . In screening tests of coagulation, the prothrombin

TABLE 111-7 -- Selected Dysfunctional Factor X Variants

Variant	Factor X Antigen	Activity (%)			Defect[a]	(Domain)	Bleeding
		PT	aPTT	Russell Viper Venom Time			
Santo Domingo	5	1	1.5	1	Gly^{20} Arg	(propeptide)	Severe
St. Louis II	100	<1	3		Glu^{7} Gly	(Gla)	?
Vorarlberg	20	<1	32	15	Glu^{14} Lys	(Gla)	Asymptomatic
Malmo 4	100	35	43		Glu^{26} Asp	(Gla)	Mild
Unnamed[b]	72	20	45	54	Glu^{102} Lys	(EGF-2)	Asymptomatic
Prower[c] and Stockton[d]	85	8		7	Asp^{282} Asn	(active site)	Moderate
Stuart	<1	<1	<1	<1	Val^{298} Met	(catalytic)	Severe
Roma	80	3050	3	100	Thr^{318} Met	(catalytic)	Moderate
San Antonio[e]	36	14	100		Arg^{326} Cys	(catalytic)	Postoperative
Marseille and others[f]	100	21	21	26	Ser^{334} Pro	(catalytic)	Asymptomatic
Friuli	100	4	6	80	Pro^{343} Ser	(catalytic)	Moderate
Vienna	5	1	1		Gly^{395} Glu	(catalytic)	Severe
Melbourne	120	100	9	105	Unknown		
Padua	100	2530	90	88	Unknown		

[a] Numbering beginning at amino terminus of the mature factor X protein.
[b] *Found in several unrelated families.*
[c] *Double heterozygote with* Arg^{287} *Trp.*

[d] *Heterozygote.*
[e] *Double heterozygote with frameshift leading to termination at 232.*
[f] *Same mutation was found in several unrelated families in hetero- and homozygous forms.*

TABLE 111-8 -- Typical Laboratory Values in Severe Factor X Deficiency

Assay	Factor X	
	Deficiency	Normal
PT (sec)	>30	12
aPTT (sec)	60	29
Thrombin time (sec)	11	11
Bleeding time (min)	6	10
Factor X (%)	<1	100

time and the PTT are prolonged, whereas the thrombin time is normal. The Russells viper venom time, which measures the direct activation of factor X, is also prolonged. Specific factor X assays are required for the diagnosis. The bleeding time is normal even in severely affected patients.

Differential Diagnosis

Acquired factor X deficiency occurs in liver disease and as a result of warfarin ingestion or vitamin K deficiency. In these instances other vitamin K-dependent factors are also reduced. Acquired factor X deficiency may accompany amyloidosis, [100] and rarely as a consequence of an acquired inhibitor. [101] [102] In the setting of amyloidosis, there is essentially no recovery of transfused factor X, as if a circulating inhibitor to factor X were present. However, in vitro mixing assays show no inhibitor in the plasma. Factor X deficiency in amyloidosis is a consequence of adsorption of factor X by extracellular amyloid. Replacement therapy is futile and improvement is most often seen during therapy for the underlying cause of amyloidosis. [103] Splenectomy may be of value in correcting the factor X deficiency of amyloidosis, presumably via the resultant debulking of splenic amyloid. [104] Hereditary factor X deficiency must be distinguished from various hereditary combined deficiencies of clotting factors.

Therapy

Transfusion with fresh-frozen plasma may be used to treat patients with congenital factor X deficiency. Plasma is given as an initial dose of 1520 ml/kg of body weight, followed by 36 ml/kg body weight every 24 hours. The biologic half-life of factor X is about 40 hours. Thus, administration of plasma every 12 hours results in a progressive increase in factor X concentration. About 80% of administered factor X remains intravascularly shortly after infusion. Prothrombin complex concentrates, rich in factor X, have been used to prepare patients for surgery or to treat serious bleeding from trauma or surgery. Table 1113 displays the proportion of factor X present in selected concentrates.

Factor X levels of about 1015% provide adequate hemostasis for hemarthroses and uncomplicated soft tissue hemorrhage. Because of the risk of thromboembolism and DIC with high doses or prolonged administration of prothrombin complex concentrates containing factor X, it is not recommended that factor X levels of >50% of normal be exceeded unless absolutely necessary. Pure factor X concentrates have been prepared by affinity chromatographic procedures using monoclonal antibodies, but these concentrates are not yet available commercially.

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FACTOR XI DEFICIENCY

History and Epidemiology

Factor XI deficiency was first recognized as an inherited blood-clotting factor defect by Rosenthal and colleagues in 1953. [105] These workers described three related patients, one male and two females, who had a history of bleeding after dental extraction. The observation that plasma from the affected patients corrected the clotting defects of plasma from patients known to be deficient in other clotting factors implied the absence of a previously undescribed protein, later designated factor XI.

Factor XI deficiency occurs most often in Ashkenazi Jews, but also occurs in non-Jewish populations. [106] The calculated gene frequency for factor XI deficiency among Ashkenazi Jews is 4.3%, so appreciable numbers of homozygous patients may be expected. [107]

Genetics and Molecular Biology

Factor XI deficiency is inherited as an autosomal recessive trait. The gene contains fifteen exons and is located on chromosome 4q32q35. [108] After secretion as a homodimer from the liver, factor XI circulates in plasma at a concentration of approximately five g/ml. It is composed of two identical disulfide-linked monomers of 80,000 molecular weight which exhibit significant homology with prekallikrein. [109] Factor XI is activated by cleavage at the Arg^{369} -Ile^{370} bond in each monomer. This cleavage yields two amino-terminal heavy chains with a molecular weight of 50,000, and two carboxy-terminal light chains with a molecular weight of 30,000. Each heavy chain is composed of four tandem repeats called apple domains. Apple domain-1 binds high-molecular-weight kininogen; apple domain-2 binds factor IX, and apple domain-4 has sites that bind factor XIIa. [110] [111] [112] The light chains are homologous with other serine proteases, and each contains a catalytic site for activation of factor IX.

Factor XI is readily activated by factor XIIa. However, factor XII deficiency is not associated with a bleeding tendency, suggesting that another means of activating factor XI operates in vivo. Thrombin can activate factor XI in the presence of nonphysiologic cofactors, such as sulfatides. [113] [114] Platelets can support activation of sufficient factor XI by thrombin to accelerate coagulation in an in vitro model system. [115] Thus, it is likely that thrombin may serve as a physiological feedback activator of factor XI during normal coagulation.

Pathogenesis

Presently, three different point mutations account for most analyzed cases of factor XI deficiency. [116] [117] Type I mutations occur at the splice junction boundary of the last intron, resulting in either disruption of mRNA splicing or premature translation termination. A mutation in exon 5 resulting in a premature stop codon (nonsense mutation) has been designated as a type II mutation. The type III mutation in the ninth exon causes substitution of leucine for phenylalanine at postion 238 (mis-sense mutation). In small population studies, the type II and type III mutations are the most common, and compound heterozygosity (type II/III) is found frequently in affected patients. Although genotype appears to correlate with the factor XI levels observed (see Table 1119), a clear correlation of genotype with bleeding tendency has not been established.

Very low factor XI levels are not always associated with a bleeding tendency, an observation that has not been adequately explained. Evidence indicates that factor XI-like activity exists on platelet membranes and it has been suggested that such activity may compensate for deficiency of plasma factor XI in some cases. [118] Platelet factor XI has a lower molecular weight and a different electrophoretic migration than that of plasma factor XI, although the two activities cannot be distinguished immunologically.

Clinical Manifestations

Affected patients can be divided into those with major deficiency (20% of normal), and those with minor deficiency. [119]

TABLE 111-9 -- Factor XI Levels in Homozygous or Compound Heterozygous Factor XI Deficiency[a]

Mutation Type		Factor XI Level (%)	aPTT (sec)
Allele 1	**Allele 2**		
II	II	1.2	108
III	III	9.7	85
II	III	3.3	67
II		52	39
III		67	36
Normal	Normal	>100	32

[a] Adapted from Seligsohn,[107] with permission.

Patients with major deficiency may experience excessive bleeding and are usually homozygotes or compound heterozygoyes. Patients with minor deficiencies have little or no bleeding and are usually heterozygous for the disorder. Although hemorrhagic symptoms do not always strictly correlate with the factor XI level, [120] patients with a family history of bleeding in affected relatives likely will have similar bleeding tendencies. Similarly, patients without a history of bleeding usually do not experience hemorrhage even after trauma.

Those patients who do bleed usually do so only after trauma or surgery. Even patients with very low factor XI levels do not have hemorrhagic episodes as frequently or prominently as those seen in hemophilia A and B. Epistaxis, soft tissue hemorrhage, and bleeding after dental extractions may occur. Hemarthroses are very

uncommon and chronic disabling joint disease is not observed. Affected women may experience menorrhagia. Postoperative bleeding may be seen in severely affected patients. The risk of bleeding may be significantly enhanced in patients taking aspirin. [121]

Laboratory Evaluation

The PTT is prolonged in factor XI deficiency, whereas the prothrombin time and thrombin time are normal. A specific assay of factor XI activity is necessary to confirm the diagnosis. Assays of other clotting factors may be necessary to exclude a combined hereditary deficiency of factor XI and other factors. Plasma for factor XI assays should be drawn into nonwettable tubes and rapidly assayed. The clotting defect in factor XI deficiency cannot always be detected when plasma is exposed to glass or if it is assayed after the plasma is frozen and thawed.

Differential Diagnosis

Factor XI deficiency should be suspected in any patient with a prolonged PTT, especially if the family history suggests a mild to moderate life-long bleeding disorder that affects both males and females. Factor XI deficiency has been described in a familial multiple factor deficiency syndrome (FMFD type V) in which factors VIII, IX, and XI are decreased. This syndrome has been described for five patients, more than predicted on the basis of coincidence alone. A combined factor XI-factor IX deficiency has also been described as part of type VI familial multiple factor deficiency. [122]

Acquired factor XI deficiency occurs in patients who develop inhibitors to this protein. Anti-factor XI antibodies occur in patients with systemic lupus erythematosus and other immunologic diseases. Acquired deficiency can be distinguished from the hereditary form on the basis of a history of recent onset and by showing inhibiting activity in the patients plasma. Factor XI deficiency has been described as a common finding in patients with Noonans syndrome, which is characterized by congenital cardiac abnormalities, short stature, and mental retardation. [123] [124]

Therapy

Soft tissue bleeding may not require treatment. When therapy is required, fresh-frozen plasma or the supernatant from cryoprecipitate-poor plasma is used. Commercial factor XI concentrates are of limited availability. [125] Plasma exchange may be required if the hemorrhage cannot be controlled with plasma alone. The plasma half-life of factor XI is approximately 80 hours. Plasma can be administered as a loading dose of 1520 ml/kg body weight, followed by 36 ml/kg body weight every 12 hours until hemostasis is achieved. Factor XI levels of 3040% have been reported with plasma replacement therapy. The risk of hepatitis is proportional to the number of units of plasma infused.

Surgical procedures performed on factor XI deficient patients vary from cataract surgery to open heart surgery. [126] [127] These have been safely carried out with plasma replacement therapy. Antifibrinolytics, such as epsilon-aminocaproic acid or tranexamic acid, may be useful adjuncts in controlling bleeding. However, such agents should not be used in patients with hematuria, since clots refractory to lysis may be difficult to evacuate from the genitourinary tract. Bleeding following prostatectomy can be difficult to control even with aggressive therapy. Such patients may require plasma exchange to achieve higher levels of factor XI. The administration of antifibrinolytic agents alone is often sufficient to prevent hemorrhage after dental extractions.

Despite reports that some factor XI deficient patients may not bleed during surgery, the physician may have no way to ascertain which patients will not bleed. A history of past significant surgery without therapy and uncomplicated by bleeding strongly suggests but does not guarantee satisfactory hemostasis with subsequent surgery. When in doubt, factor XI deficient patients should be treated with plasma prior to surgery.

Alloantibodies to factor XI have been described and are most likely to occur in severely affected patients. These antibodies inhibit factor XI activity and represent a major complication of therapy. One patient with a factor XI inhibitor and severe bleeding failed to respond to maximal plasma therapy but responded dramatically to infusions of an activated prothrombin complex concentrate. [128]

A commercial factor XI concentrate is available in Europe, but is not licensed in the United States.

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DEFICIENCY OF THE CONTACT FACTORS

Factor XII, prekallikrein (PK), and high-molecular-weight kininogen (HK), in addition to factor XI, are the major factors involved in the so-called contact phase of coagulation. Subjects with a deficiency of factor XII, PK, or HK have no bleeding tendency, but do exhibit a prolonged PTT. These factors are thought to be important as mediators of various host-defense mechanisms including inflammation, fibrinolysis, and control of blood pressure. [129] The role of the contact factors in coagulation is not clear. They may not play a significant role in normal hemostasis, since their deficiencies are not associated with clinical bleeding, even in patients undergoing major surgery. Although deficiencies of factor XII, PK, and HK are not associated with bleeding, it is important to distinguish them from deficiencies of factors VIII, IX, and XI, which are accompanied by defective hemostasis.

Factor XII Deficiency

Factor XII (Hageman factor) deficiency was first described by Ratnoff and Colopy in 1955. [130] The original patient, Mr. John Hageman, had no history of excessive bleeding; his in vitro clotting abnormality was discovered during a preoperative evaluation. Several hundred cases of factor XII deficiency have since been described. Factor XII levels are lower in patients of Asian descent than in other ethnic groups.

TABLE 111-10 -- Selected Dysfunctional Factor XII Variants

Variant	Antigen (%)	Activity (%)	Proteolysis by Kallikrein	Defect
Washington, DC	80	<1	Normal	Cys^{571} Ser
Toronto	39	<1	ND	ND
Bern	11	<1	Normal	ND
Bari	3552	1.5	Delayed	ND
Locarno	46	<1	Delayed	Arg^{353} Pro
Valencia	4170	<1	ND	ND

ND, not determined.

The factor XII protein is synthesized in the liver and is present in normal plasma at about 30 g/ml. [131] The mature factor XII protein has a molecular weight of about 80,000, and consists of a single polypeptide chain. The protein is activated when cleaved at a single site (Arg^{353}-Val^{354}) by plasma kallikrein. The domain organization of the heavy chain of factor XII is homologous to tissue plasminogen activator and urokinase. The gene for factor XII has been mapped to 5q33qter. Several dysfunctional factor XII molecules have been described (see Table 11110). There have been no convincing reports of excessive bleeding associated with factor XII deficiency, even with major surgical procedures. Decreased plasminogen activator activity described in factor XII-deficient patients may be associated with defective fibrinolysis and an increased risk of thromboembolism. [132] In a group of patients with recurrent venous thrombosis, about 10% were found to be heterozygous for factor XII deficiency. [133] Activated factor XII is inhibited by antithrombin III, -2 antiplasmin, and C1-esterase inhibitor.

The hallmark of severe factor XII deficiency is a markedly prolonged PTT, often to greater than 100 seconds, in a patient with no history of bleeding. The prothrombin time and thrombin times are normal. In most patients the bleeding time is normal although exceptions have been noted. A specific factor XII assay is required to confirm the diagnosis.

Prekallikrein Deficiency

Prekallikrein (Fletcher factor) deficiency was first described in 1965 by Hathaway, who observed patients with a markedly prolonged PTT who had no history of abnormal bleeding. [134] The disorder is inherited in an autosomal recessive pattern. Plasma from affected patients corrected the clotting defect of plasma deficient in all other known clotting factors including factors XI and XII. In addition, the prolonged PTT of prekallikrein-deficient plasma was corrected by exposure to glass. The hallmark of prekallikrein deficiency is a markedly prolonged PTT, corrected by exposure to glass, in patients with no bleeding tendencies. [134] A specific assay is required for diagnosis.

High-Molecular-Weight Kininogen Deficiency

Patients with high-molecular-weight kininogen (also referred to as Williams, Fleaujeac, or Fitzgerald factor) deficiency also exhibit a prolonged PTT, but have no bleeding abnormality. The disorder is inherited as an autosomal recessive characteristic. Some patients are also deficient in low-molecular-weight kininogen. Both factor XI and prekallekrein circulate in a complex with high molecular weight kininogen, a necessary cofactor for activation of factor XII as well as PK and factor XI.

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FACTOR XIII DEFICIENCY

History and Epidemiology

The existence of factor XIII (fibrin-stabilizing factor) was first postulated by Robbins in 1944 when he showed that fibrin formed from purified components was soluble in weak acids, whereas fibrin formed in the presence of serum or plasma resulted in formation of an insoluble clot. [135] In 1960 Duckert described the first recognized case of a clinical bleeding diathesis due to congenital deficiency of this protein. [136] Fibrin stabilizing factor was given the designation factor XIII in 1963. Since its first description, more than 100 cases of factor XIII deficiency have been reported. No racial or ethnic group is disproportionately affected, and the incidence is estimated at one in several million.

Genetics and Molecular Biology

Factor XIII deficiency is inherited as an autosomal recessive trait. Heterozygotes are not affected with bleeding, although heterozygous females may have a higher than normal incidence of spontaneous abortions than do normal women. The gene for the *A* subunit is at 6p2425, near the HLA locus; the gene for the *B* subunit is located at 1q3132.1. [137] [138] [139] [140]

Factor XIII circulates in the plasma at a concentration of 1020 g/ml. [141] Factor XIII is not homologous to any of the other coagulation factors, but is one of a family of transglutaminases. The protein exists in plasma as a tetramer consisting of two *A* subunits and two *B* subunits. Each subunit has a molecular weight of about 85,000, and the complete protein has a molecular weight of approximately 330,000. Factor XIII catalyzes cross-linking between -glutamyl and epsilon-lysyl groups of different fibrin strands. This cross-linking renders the fibrin meshwork rigid and insoluble, even in mild organic acids. The catalytic activity of factor XIII resides in the *A* subunits. Although plasma factor XIII is synthesized in the liver, a dimer of the two *A* subunits (lacking the *B* unit but retaining activity) is produced in megakaryocytes and placental tissue. The amino acid sequence of the *A* subunit and the nucleotide sequence of its cDNA have been determined. [137] [142] A reactive thiol group is unmasked by thrombin cleavage at the amino terminus of the *A* subunit. Further thrombin cleavage at the carboxy terminus inactivates the enzyme by disrupting a calmodulin-like calcium binding site. The protein sequence and cDNA sequence of the *B* subunit, which has no enzymatic activity, reveals ten repeating homologous domains that resemble those of $_2$ -glycoprotein I. [143] The *B* subunits act primarily as carriers for the *A* subunits.

Pathogenesis

Three forms of factor XIII deficiency have been described. In type I deficiency, both subunits *A* and *B* are lacking; in type II deficiency the *A* subunit is lacking but subunit *B* is present; in type III the *B* subunit is lacking. [144] [145] Specific mutations have been reported in the A chain, including a dinucleotide deletion leading to premature termination, and a point mutation at the exon/intron junction after exon 14. A mutation in the B chain has been described at the acceptor splice junction of intron A. The majority of patients with factor XIII deficiency have an absent or defective *A* subunit. Factor XIII deficiency has been reported as the result of acquired inhibitory antibodies; these have been associated with isoniazid use. [146]

Clinical Manifestations

The hallmarks of severe factor XIII deficiency are umbilical stump bleeding in the neonatal period; intracranial hemorrhage with little or no trauma; recurrent soft tissue hemorrhage with tendency to form hemorrhagic cysts (pseudotumors); and, in females, recurrent spontaneous abortion. Males may also show oligospermia and infertility. The bleeding associated with factor XIII deficiency is usually associated with trauma, except in the case of intracranial hemorrhage which may occur in the absence of known trauma. Intracranial hemorrhage is reported in up to 25% of patients in some series. [147] Surprisingly, bleeding at the

time of surgery is not excessive, although delayed bleeding can occur. Poor wound healing and abnormal scar formation are characteristic of this disease. Unlike hemophilia A or B, hemarthroses are not usually observed.

Laboratory Evaluation

Factor XIII deficiency is marked by normal coagulation screening tests (prothrombin time, PTT, thrombin time, and bleeding time) despite a convincing history of bleeding. The most useful assays for factor XIII activity exploit the solubility of non-cross-linked fibrin clots in 5 M urea or weak organic acids. Clots formed in factor XIII-deficient plasma are soluble within minutes in such solutions, whereas normal clots remain insoluble for at least 24 hours. The test of solubility in 1% monochloroacetic acid is said to be more sensitive than solubility in 5 M urea, although the latter is more specific for factor XIII deficiency. Other tests for factor XIII activity use measurements of incorporation of fluorescent amino acids into casein, or ammonia production by the amidase activity of factor XIII. [148]

Differential Diagnosis

The clinical presentation of factor XIII deficiency may be confused with abnormalities of fibrinogen that may also present with umbilical stump bleeding, soft tissue hemorrhage, and recurrent abortions. These two conditions may be readily distinguished on the basis of screening tests of coagulation, which are all abnormal with fibrinogen defects but normal with factor XIII deficiency. Specific factor XIII assays confirm the diagnosis.

Acquired factor XIII deficiency has been reported in association with exacerbations of Henoch-Schonlein purpura. The deficiency of factor XIII seems to correlate with abdominal pain and bloody diarrhea. Infusions of factor XIII not only restore normal factor XIII levels, but also decrease the severity and duration of these symptoms during exacerbations. [149]

Therapy

Treatment of factor XIII deficiency is simplified by the fact that factor XIII has a half-life of 9 days. Minimal factor XIII activity, perhaps as little as 5%, may be sufficient to prevent bleeding complications. [150] Prophylactic therapy is practical for these reasons and is advisable due to the significant risk of intracranial hemorrhage. Prophylaxis can be accomplished by the use of fresh-frozen plasma, given as 1 or 2 units every 46 weeks. [125] Cryoprecipitate also contains factor XIII, and can be given in doses of 1 bag per 1020 kg body weight every 34 weeks. Factor XIII concentrates (Fibrogammin, Hoechst) are available in Europe, and have been used successfully in the prevention of hemorrhagic complications. [125] In one report, soft tissue hemorrhage occurred when the interval between prophylactic transfusions

exceeded 6 weeks.[150] A factor XIII-deficient patient suffered repeated spontaneous abortions until started on prophylactic plasma transfusions every 14 days or infusions of a commercial factor XIII concentrate every 21 days. The two pregnancies that followed were uncomplicated. [151]

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FAMILIAL MULTIPLE FACTOR DEFICIENCIES

A number of familial multiple factor deficiencies have been described as shown in Table 11111 . Two of these syndromes, combined factor V-factor VIII deficiency, and combined deficiency of factors II, VII, IX, and X, have been well characterized. [122] [152]

Combined Factor VFactor VIII Deficiency (Type I FMFD)

In this syndrome factor V and VIII levels range between 515% of normal. Analysis of affected members of a number of different

TABLE 111-11 -- Familial Multiple Coagulation Factor Deficiencies

Type	Deficient Factors
I	V, VIII
II	VIII, IX
III	II, VII, IX, X
IV	VII, VIII
V	VIII, IX, XI
VI	IX, XI
VII	VII, X

kindred suggested that the combined defect is due to a single genetic defect in a small region of chromosome 18. [153] The defect is due to a mutation of a protein of the endoplasmic reticulum-intermediate golgi compartment, previously named ERGIC-53. [154] The function of this protein is not known. This protein might serve as a molecular chaparone for factors V and VIII during their post-translational modification and transit through the Golgi apparatus.

Bleeding in patients with FMFD type I usually occurs only after trauma, and may be severe during and after surgery unless replacement therapy is given. Plasma is the only source of factor V, although safe and effective factor VIII concentrates are available. In preparation for surgery, plasma exchange should be performed to raise the factor V levels to near normal. After plasma exchange, factor levels can be maintained with fresh frozen plasma and factor VIII concentrates.

Deficiency of Factors II, VII, IX, and X (Type III FMFD)

A number of reports describe patients with a combined congenital deficiency of factors II, VII, IX, and X, as well as protein C and protein S. One of the first patients reported had a severe bleeding tendency requiring treatment with high doses of vitamin K. [155] Malabsorption and other causes of vitamin K deficiency or surreptitious warfarin ingestion were excluded. Parents of affected children have not been clinically affected but, interestingly, parents of one affected child had markedly decreased urinary excretion of -carboxyglutamic acid, suggesting a defect in vitamin K-dependent carboxylation of glutamic acid residues in the vitamin K-dependent factors. The clotting defect in some patients has been partially corrected by vitamin K administration; in other patients vitamin K has had no effect. The vitamin K carboxylase gene has been studied in five patients with the disorder, but only one exhibited a mutation. [156] Two of the patients appeared to have a defect in the vitamin K reductase gene. A defect in either the carboxylase or reductase could lead to impaired carboxylation of glutamic acid residues in the vitamin K-dependent clotting factors.

Bleeding in these patients may be severe. In some patients, high doses of oral vitamin K may be of benefit. When excessive bleeding occurs, patients can be treated with plasma or, if necessary, with prothrombin complex concentrates that contain all of the vitamin K-dependent factors. Care must be taken when using such concentrates to avoid thrombotic complications by using the lowest effective doses.

Other Combined Deficiencies

The other combined factor deficiencies listed in Table 11111 are very rare. Recently, a combined deficiency of factors VII and X has been reported. [157] Abnormal bleeding in these patients does occur, and requires replacement therapy with plasma or, where possible, specific clotting factor concentrates.

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PREFERRED APPROACH

The preferred treatment for any clotting factor deficiency or defect is specific replacement therapy using a purified concentrate. Purified preparations of factors VII, XI, and X are available in certain countries but these products are not yet available in the United States. However, for prothrombin, factor VII, or factor X deficiency prothrombin complex concentrates (PCCs) are widely available and represent more purified clotting factor concentrates than plasma. Several different PCC preparations are shown in Table 1113 , which lists the relative concentrations of each factor compared to 100 units of factor IX. Initially the greatest use of PCCs was for the treatment of hemophilia B. However, more highly purified preparations of factor IX are now available both from plasma and recombinant sources and these are products of choice for treatment of hemophilia B. At present, the greatest use of PCCs is for the treatment of patients with inhibitors to factor VIII or IX, since PCCs contain varying amounts of activated forms of factors VII, IX, and X and possess so-called inhibitor bypassing activity. Although PCCs are used mainly to treat patients with inhibitors, they are also quite useful for the treatment of deficiencies of the vitamin K-dependent factors. PCCs should be used with care and for the shortest time possible, however, since they have been associated with thromboembolic events including myocardial infarction, deep venous thrombosis, and pulmonary embolism.

In many instances, plasma infusions can be given to treat individual clotting factor deficiencies. For example, plasma is the main source for replacement of factor V and XI deficiency. The main drawback in the use of plasma is the danger of volume overload. Elderly patients or patients with significant cardiovascular disease may experience congestive heart failure when using large doses of plasma. In these instances, PCCs should be used if the patient is deficient in prothrombin or factors VII or X. In the case of factor XI or factor V deficiency, plasma exchange transfusion should be considered. The other danger of plasma, in addition to volume overload, is the risk of transmissible disease which although rare, still remains a possibility that is compounded when large amounts of plasma are administered. The relative risks of transmissing viral diseases following plasma transfusion are: HIV, 1 in 493,000; HCV, 1 in 103,000; HBV, 1 in 63,000; and HTLV, 1 in 641,000.

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Chapter 112 - Quantitative and Qualitative Disorders of Fibrinogen

José Martinez

INTRODUCTION

Human fibrinogen is a 340-kDa glycoprotein that circulates in plasma at a concentration of approximately 300 mg/dl. The fibrinogen molecule is a dimer consisting of two identical halves, each of which is composed of three nonidentical polypeptides termed the A-, B-, and -chains (Fig. 112-1). The halves of the molecule are connected at the amino-terminal central domain (N-terminal) by hydrophobic interactions and by interchain disulfide bonds, one linking the A-chains, two linking the -chains, and one the A-chain to the B-chain.[1] [2] [3] [4] The two -chains are linked in an antiparallel manner and the three polypeptides of each half of the fibrinogen molecule are also

Figure 112-1 Model of human fibrinogen and fibrin. Each half of the molecule is composed of three chains, A, B, and . The amino-terminal regions of the six chains are linked in the central domain (E domain) by disulfide bonds that form the dimer. In this region, fibrinopeptides A and B are cleaved from the A- and B-chains, respectively, by thrombin, which converts fibrinogen into fibrin monomer. The two nodular regions at the C-terminal (D domain) contain the complementary binding sites for the central determinants exposed on release of the fibrinopeptides. Depicted in the figure are also segments of the fibrinogen molecule that bind tissue plasminogen activator (tPA), $_2$ -antiplasmin, factor XIII, and of thrombin (IIa) to fibrin. The C-terminal -peptide or the RGD peptide of the C-terminal of the A-chain intervene in the binding of fibrinogen with platelets and other cells. *(Adapted from Mosseson,[5] with permission.)*

connected by a series of disulfide bridges.[1] [2] Elucidation of the amino acid sequence of each chain has revealed the A-chain to consist of 610, the B-chain of 461, and the -chain of 411 amino acids.[1] [2] Attached to the B- and -chains are four carbohydrate side chains, linked through *N*-acetylglucosamine to asparagine 52 of each -chain and asparagine 364 of each B-chain; the A-chain does not contain carbohydrates.[5] The molecular masses of the A-, B-, and -chains (including amino acid and carbohydrate components) are 66.5, 54.3, and 48.5 kDa, respectively.[2] Electron microscopy has revealed a trinodular structure with a central nodule linked through two thin rods to two peripheral nodules (Fig. 112-1).[6] This structure is consistent with results obtained by proteolytic cleavage of fibrinogen with cyanogen bromide or plasmin,[7] [8] a process that yields a central dimeric fragment (fragment E) corresponding to the central nodule and two peripheral monomeric fragments (fragments D) corresponding to the peripheral nodules visualized by electron microscopy (Fig. 112-1).

Plasma fibrinogen is synthesized exclusively by the hepatocyte,[9] [10] and the synthesis of the three chains is under the coordinated control of three separate genes localized on chromosome 4.[11] [12] [13] Subsequent to assembly of the constituent polypeptide chains and the addition of carbohydrate side chains, the mature molecule is secreted into the circulation where it manifests a half-life of 4 days and a fractional catabolic rate of 25%/day.[14] [15]

Fibrinogen plays a central role in three major functional processes: (1) The soluble fibrinogen molecule is converted into soluble fibrin during the process of blood coagulation. (2) The polymerized fibrin serves as a template for the localized assembly and activation of the fibrinolytic system, which modulates fibrin deposition and clot dissolution.[3] Fibrinogen binds to vascular cells such as platelets, where it supports platelet aggregation, and to endothelial cells, where it participates in tissue repair. The conversion of fibrinogen into insoluble fibrin can be divided into three distinct phases: (1) enzymatic cleavage of fibrinopeptides by thrombin, (2) fibrin polymerization, and (3) fibrin stabilization via covalent cross-linking by factor XIIIa. In the first phase, thrombin cleaves the Arg16-Gly17 bond of the A-chain and the Arg14-Gly15 bond of the B-chain, resulting in the release of two molecules of fibrinopeptide A (FPA) and two of fibrinopeptide B (FPB) per molecule of fibrinogen.[3] [16] [17] Fibrinopeptide release from the constituent A- and B-chains of fibrinogen results in the formation of fibrin monomer, the constituent chains of which are now referred to as the -, -, and -chains. Although the proteolytic cleavage of FPA and FPB by thrombin appears to be simultaneous, the cleavage of FPA actually occurs prior to and more rapidly than that of FPB.[16] [17] Moreover, the cleavage of FPA is sufficient to induce clot formation, whereas the exclusive cleavage of FPB does not lead to fibrin formation under physiologic conditions.[18] The association of thrombin with fibrinogen is, in part, mediated through its catalytic site as shown by nuclear magnetic resonance and x-ray crystallographic studies of thrombin bound to a discrete segment of the N-terminal of the A-chain. However, thrombin also binds to fibrin(ogen) through a noncatalytic site called the fibrinogen recognition site, which binds to a locus formed by the N-terminus of the -chain (2750) and the N-terminus of the -chain (1542 and Ala 68).[3] [19] Recent studies also identified a high-affinity binding site of thrombin on fibrin(ogen) on the C-terminal region of the chain variant.[20]

In intact fibrinogen, the negatively charged fibrinopeptides play a role in maintaining the dispersion of individual fibrinogen molecules, as evidenced by the fact that subsequent to their cleavage by thrombin the resulting fibrin monomers spontaneously polymerize. The polymerization process involves the reciprocal non-covalent interaction of molecular determinants in

the fragment-E region of the molecule that are exposed by the removal of FPA with complementary binding sites located in the fragment-D region of an adjacent fibrin monomer. The resulting dimer, which is arranged in a half-staggered overlap (Fig. 112-2), continues to grow in length by the staggered addition of fibrin monomers, resulting in the formation of a two-stranded, half-staggered polymer referred to as a protofibril, the basic structural unit of the fibrin clot.[3] [21] [22] The half-staggered polymerization process brings two D domains of longitudinally aligned fibrin molecules of each row of the protofibril into close contact with one another, forming DD contacts as shown by recent x-ray crystallographic analysis of fibrin fragment D.[23] These interactions further stabilize the non-covalently associated fibrin protofibril (Fig. 112-2). Polymerization continues with the formation of long, double-stranded protofibrils that ultimately associate laterally to form thick fibrin bundles.[3] [21] [22] The cleavage of FPB occurs mainly during the initial phase of fibrin polymerization and exposes determinants that are complementary with binding sites located in the C-terminal region of the molecule.[3] [16] This interaction seems to increase the rate of formation of thin fibrils as well as the lateral aggregation to form thick fibrin fibers.[3] The three-dimensional fibrin matrix is completed by branching of the fibers at specific contact points located in the fiber bundles.[3] Several of the polymerization sites within fibrin have been identified. The N-terminus of the fibrin -chain (1720; GPRV) participates in the interaction of the A determinant with the complementary binding site located in fragment D.[1] [3] [24] Crystal structural studies of the C-terminal region of the -chain showed that amino acids Gln329, Asp330, His340, and Asp364 and Arg375 form a pocket for the complementary binding of A GPRP,[24] and mutations of these amino acids cause polymerization defects as seen in several dysfibrinogenemias. In addition, the N-terminus of the fibrin -chain also participates in the assembly of fibrin by binding to a complementary site

located in the C-terminus, but the localization of this binding site remains controversial.

The final stage of fibrin formation is characterized by the factor XIIIa-mediated formation of covalent amide bonds between the epsilon-amino groups of specific lysine residues and -$CONH_2$ groups of certain glutamine residues.[25] These covalent bonds are first formed between opposing -chains of end-to-end aligned D domains (DD contact sites) of two fibrin molecules. The dimerization of the -chain formed by bridges between lysine 406 of one -chain and glutamine 398 of the opposing -chain is then followed by progressive covalent cross-linking of multiple -chains. [3] [26] As a result of this extensive covalent stabilization, the fibrin clot is rendered more compact and resistant to both mechanical disruption and dissolution by plasmin. [27]

In addition to plasma fibrinogen, the circulating blood contains a very small pool of fibrinogen, which is present within the platelet -granules. Although some studies have shown that fibrinogen is synthesized by the megakaryocyte,[28] more recent

Figure 112-2 Model of fibrin assembly showing two sets of complementary binding sites. The A fibrinopeptides are represented by semicircles and the B fibrinopeptides by triangles. After cleavage of the fibrinopeptides, the binding sites are exposed, the A site is represented by a circular hole and the B by a triangular. Complementary to these sites in the E domain are those located in the D domain, which are represented by knobs; -sites are circular knobs, and -sites are triangular. After cleavage of FPA, the A site of the E domain of one molecule interacts with the a site of the D domain of another molecule forming a half-staggered overlap dimer. Additional fibrin monomer units associate through the DD contacts. The protofibril is formed by two rows linked by DD contacts, where the monomers of one row are arranged in a half-staggered overlap with respect to the monomers of the other row. Removal of FPB exposes the B-sites, and this interaction promotes the lateral association of protofibrils into fibrin fibers. *(Adapted from Weisel,[22] with permission.)*

investigations have demonstrated that both megakaryocytes and platelets are capable of internalizing fibrinogen from plasma via a process mediated by GPIIb/IIIa. [29] Plasma fibrinogen exhibits -chain heterogeneity due to differential splicing of the hepatic mRNA. [3] This heterogeneity is manifested by the presence of a minor component, termed , in which the last four amino acids of the -chain are replaced by an extended carboxyl-terminal sequence. [3] The -chain is not present in platelet fibrinogen[3] and does not support platelet aggregation, probably due to the lack of interaction of with GPIIb/IIIa of the platelet. [3] [30] [31] Platelet fibrinogen is secreted after stimulation and plays a role in supporting hemostasis. [3]

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CLASSIFICATION OF FIBRINOGEN ABNORMALITIES

Fibrinogen abnormalities can be classified as being either congenital or acquired, with both groups manifesting quantitative defects (i.e., afibrinogenemia, hypofibrinogenemia, or hyperfibrinogenemia) or qualitative alterations of the fibrinogen molecule (dysfibrinogenemia). In a few instances, both quantitative and qualitative abnormalities can be present in the same patient (e.g., hypodysfibrinogenemia). The congenital disorders of fibrinogen are reviewed first, after which, the acquired disorders will be considered.

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CONGENITAL DISORDERS OF FIBRINOGEN

Afibrinogenemia and Hypofibrinogenemia

Congenital afibrinogenemia has been described in approximately 150 families, [32] but low levels of fibrinogen may have been undetected owing to the insensitivity of the methods used to quantitate the fibrinogen concentration. Less frequently, families with subnormal amounts of fibrinogen have been reported, and it is possible that in some of these cases the low fibrinogen concentration may have been associated with the presence of an abnormal molecule (i.e., hypodysfibrinogenemia).

Clinical manifestations in patients with fibrinogen disorders are related to the plasma fibrinogen concentration. Patients with afibrinogenemia may exhibit bleeding symptoms of variable severity. Umbilical cord bleeding can be the first hemorrhagic manifestation of this disorder and can be the cause of death in the newborn. [32] [33] [34] Later in life, gum bleeding, epistaxis, menorrhagia, [35] gastrointestinal hemorrhage, and hemarthrosis can present with varying intensity; but intracranial bleeding is the leading cause of death in this group of patients. [33] [34] [35] [36] Spontaneous abortions and abruptio placentae have also been described, even in cases with only moderate hypofibrinogenemia. [37] [38] [39] [40] Bleeding in hypofibrinogenemia usually occurs in patients with fibrinogen concentrations below 50 mg/dl, [32] and the hemorrhagic manifestations are usually associated with trauma or surgical procedures. Oddly, some patients with hypofibrinogenemia die as a result of thrombotic episodes and pulmonary embolization, although some of these episodes may have been precipitated by fibrinogen infusions. [41] [42] [43] Occasionally, the newly synthesized fibrinogen is not secreted and accumulates in the cisternae of the rough endoplasmic reticulum of the hepatocyte, resulting in a storage disease with mild elevations of serum liver enzymes. [44] [45]

Pathogenesis and Inheritance

Congenital afibrinogenemia appears to be caused by a biosynthetic defect because autologous fibrinogen injected into afibrinogenemic patients shows a normal plasma survival. [46] [47] However, it is also possible that the low plasma fibrinogen level is caused by the biosynthesis of a defective molecule that is not secreted into the circulation. [44] [45] In most cases, the fibrinolytic system is normal as are the levels of the other coagulation factors, which supports the concept of decreased biosynthesis as opposed to increased utilization as the cause of the hypofibrinogenemia.

Afibrinogenemia is inherited as an autosomal recessive disorder, and consanguinity is common in these families. [32] [33] [34] The inheritance pattern of hypofibrinogenemia is more complicated. In some families, the abnormality is only present in one parent and inheritance seems to be dominant. In other families, low levels of fibrinogen in both parents suggest a recessive mode of transmission. [32] [33] Analysis of the A-, B-, and -chain genes in two families with afibrinogenemia failed to reveal any gross abnormalities such as deletions or gross rearrangements of these genes. [48]

Laboratory Findings

All tests based on the appearance of a fibrin clot are abnormal in patients with afibrinogenemia and severe hypofibrinogenemia. These include the whole blood clotting time, the plasma recalcification time, the partial thromboplastin time (PTT), the prothrombin time (PT), and especially the thrombin and reptilase times. These abnormalities can be corrected by addition of either normal plasma or purified normal fibrinogen. Definitive diagnosis is made by immunologic measurements of the fibrinogen concentration, which are sufficiently sensitive to detect nanogram quantities of fibrinogen per milliliter of plasma. [49] [50] Measurements of fibrinogen as clottable protein are unreliable because some abnormal fibrinogens do not convert into fibrin. Other coagulation abnormalities in these patients include a prolonged bleeding time and abnormal platelet aggregation, especially with epinephrine and suboptimal concentrations of adenosine diphosphate. [50] [51] [52] The prolongation of the bleeding time and the defect in platelet aggregation can be corrected by the infusion of plasma or fibrinogen. [50] [51] [52]

Prognosis

Patients with afibrinogenemia and severe hypofibrinogenemia have a high incidence of hemorrhagic manifestations, especially in the neonatal period; and bleeding is the cause of death in approximately one-third of such patients. [33] The availability of plasma and fibrinogen concentrates in the form of cryoprecipitate and the prompt treatment of bleeding complications should improve the survival of these patients; however, some of these patients unfortunately can develop antibodies to fibrinogen, which render the treatment ineffective.

Treatment

Patients with congenital afibrinogenemia or severe hypofibrinogenemia may require replacement therapy to control episodes of active bleeding or as preparation for surgical procedures. Most patients receive fibrinogen replacement in the form of cryoprecipitate, a plasma-derived concentrate that contains approximately 300 mg fibrinogen per unit (i.e., a 3050 ml bag). An adult with afibrinogenemia and a plasma volume of 3 L will require approximately 12 U cryoprecipitate to increase the plasma fibrinogen level to about 100 mg/dl, a concentration that provides normal hemostasis for surgical procedures. [53] [54] This calculation takes into consideration an extravascular distribution of fibrinogen of 30%. Because fibrinogen is catabolized at a rate of 25%/day, the patient should subsequently receive one-third of the starting dose daily for as long as treatment is necessary. Cryoprecipitate is also used during pregnancy to prevent spontaneous abortion and to assist these patients in carrying their pregnancies to term. [55] Major complications of therapy include hepatitis and allergic reactions. Rarely, treatment with cryoprecipitate is followed by allergic reaction and

development of antifibrinogen antibodies. [33] [56] Some patients undergoing therapy have experienced thrombosis. [41] [42] [43] In cases of mild bleeding or bleeding during minor surgical procedures, treatment can be instituted by replacing one-third of the patient's estimated plasma volume with normal plasma.

Congenital Dysfibrinogenemia

Congenital dysfibrinogenemia is characterized by the biosynthesis of a structurally abnormal fibrinogen molecule that exhibits altered functional properties and commonly exhibits an abnormal thrombin-mediated conversion to fibrin. The various mutants described carry the name of the city of origin of the patient initially affected with a particular dysfibrinogenemia. [57] Since the description of the first family with dysfibrinogenemia, fibrinogen Paris, [58] in 1964, more than 250 families with abnormal fibrinogens have been reported. [59] [60] Roughly 60% of all cases exhibit no clinical manifestations, whereas hemorrhage occurs in 28%, thrombosis in 20%, and hemorrhage and thrombosis in 2% of the cases. The abnormal fibrinogen has been rarely associated with impaired wound healing or spontaneous abortion. [59]

In more than 83 of these fibrinogen mutants, the molecular structural defect has been identified. [59] [60] Approximately 55 mutations involve FPA and FPB and areas surrounding their cleavage sites, and approximately 20 variants involve the polymerization region of the -chain. In another 5 mutations, other regions of the fibrinogen molecule are involved.[59] [60] One of the aims of the study of dysfibrinogenemias is to correlate structural abnormalities of the fibrinogen molecule with functional alterations that may or may not exhibit clinical symptoms. However, this type of correlation is often hampered by inadequate knowledge concerning the molecular aberration of fibrinogen. Furthermore, clinical manifestations such as mild post-traumatic bleeding or single episodes of thrombosis may be unrelated to the presence of the abnormal fibrinogen.

Functional defects are usually reflected as abnormalities in one or more phases of the fibrinogen-to-fibrin conversion. These include: (1) impaired release of fibrinopeptides, (2) defects in fibrin polymerization, or (3) failure of the polymerized fibrin to undergo normal covalent stabilization by factor XIIIa. In addition, abnormal fibrinogens that do not support proper assembly of the fibrinolytic system or that interact abnormally with platelets, endothelial cells, or calcium have also been described (see later discussion).

In the following discussion, congenital fibrinogen disorders are classified on the basis of the most prominent functional abnormalities of the mutant molecules. Only those abnormal fibrinogens with known structural defects and very distinct functional or clinical properties are discussed. For a more complete listing of the many dysfibrinogenemias described, the reader is referred to recent reviews. [59]

Biochemical Abnormalities and Clinical Manifestations

Congenital Dysfibrinogenemia with Abnormal Fibrinopeptide Release

The proteolytic phase of fibrinogen conversion to fibrin can be studied by a detailed analysis of the rate of release of FPA and FPB by thrombin or by snake venom enzymes that specifically cleave either A or B fibrinopeptides. The rates of cleavage can be followed by radioimmunoassays specific for each fibrinopeptide or by high-performance liquid chromatography. In some of the abnormal fibrinogens, the fibrinopeptides are not released by thrombin or are released at a slow rate; in others the cleaved fibrinopeptide is structurally abnormal. [60] DNA sequencing also plays an important role in the identification of mutations of the three genes of fibrinogen. In addition to abnormal fibrinopeptide release, some congenital dysfibrinogenemias exhibit secondary delays in fibrin polymerization.

Fibrinogen Bethesda was the first mutant fibrinogen described as showing impaired release of fibrinopeptides, which was associated with a mild bleeding disorder. [61] Fibrinogen Detroit was the first abnormal fibrinogen in which a specific amino acid substitution was identified. [62] The most common mutation site in the fibrinogen molecule is Arg16 of the A-chain (Table 112-1). Because thrombin cleaves the Arg16-Gly17 bond, the release of FPA from these mutant molecules is impaired. Two different substitutions have thus far been reported to occur at this position. Most commonly, Arg16 is replaced by His, forming a bond that is not cleaved by reptilase. Almost as frequent, Arg16 is substituted by Cys, and this bond is resistant to thrombin cleavage (Table 112-1). The replacement of Arg by Cys changes the structure of the N-terminus of the mutant by forming an additional intramolecular A Cys16-A Cys16 disulfide bond. [63]

Most patients with Arg16 substitutions are heterozygous. However, homozygous cases involving the two different substitutions at the Arg thrombin cleavage site manifest hemorrhagic disorders,[64] as exemplified by fibrinogens Metz, [75] [76] Giessen I,[69] [70] and Bicêtre.[68] Some heterozygous individuals also have had bleeding symptoms due, for example, to fibrinogens Birmingham I, but this mutant was associated with abnormal von Willebrand factor. [72] Heterozygous individuals with approximately 50% normal fibrinogen should not have bleeding manifestations, but most of these mutants inhibit the conversion of the normal fibrinogen to fibrin, and this inhibition may play a role in the bleeding tendency.

Fibrinogens Detroit [16] [62] and Munich[59] [79] involve substitutions at A Arg19 by Ser and Asn, respectively; in another mutantfibrinogen Mannheim [80] A Arg19 is substituted by Gly. Substitutions at A Arg19 are usually associated with hemorrhagic tendency. Because this area of the -chain is involved in fibrin polymerization subsequent to FPA release, these mutants show a marked delay in the rate of fibrin polymerization. The delayed release of FPB reported for fibrinogen Detroit probably occurs secondary to defective polymerization related to the Arg19 substitution. [16] Three mutants with substitutions within fibrinopeptide A (Table 112-1)fibrinogens Lille, Rouen, and Mitaka IImanifest defective cleavage of FPA, [65] [66] [67] which is probably due to impaired binding of thrombin to this region of the A-chain.[81]

Abnormal cleavage of fibrinopeptides has also been observed in fibrinogen mutations involving the N-terminal region of the B-chain. A unique abnormality is present in fibrinogen New York, characterized by the slow release of FPA and a 50% decrease in total release of FPB, associated with delayed polymerization. The biochemical defect is a deletion of amino acids 9 through 72 of the B-chain, a segment that includes part of FPB (amino acids 114) and of a plasmin-derived peptide (amino acids 142). Transcriptionally, the deleted segment of the polypeptide corresponds to exon 2 of the B-chain gene. [83] The clinical expression of this abnormal fibrinogen is a tendency to thrombosis [82] (see following). Fibrinogen Ijmuiden[59] [84] has a substitution at the thrombin cleavage site Arg14-Gly15 and is replaced by Cys of the B-chain, resulting in a 50% decrease in total FPB release, thereby indicating a heterozygous condition. The additional cysteines present in the mutant either form disulfide bridges between fibrinogen and albumin or remain as free sulfhydryl groups. [84] These mutants show delayed fibrin polymerization, which confirms a role for the exposure of a polymerization site in the B-chain subsequent to the cleavage of FPB. [17] The examples described above illustrate the relationship between cleavage of fibrinopeptides and fibrin polymerization. Several members of fibrinogen Naples (same family as Milano II) were affected by venous and arterial thromboses. Characterization of the abnormal fibrinogen by DNA analysis shows a mutation GCT to ACT, which results in the substitution of B Ala68 by Thr. [86] [87] [88] The possible correlation between defective

TABLE 112-1 -- Dysfibrinogenemias with Fibrinopeptide Release Abnormalities and Known Structural Defect

	Structural Defect	Clinical Data	References		Structural Data	Clinical Data	References
A-Chain Defects							
Lille	A 7 Asp-Asn	Negative	[65]	Metz[a]	A 16 Arg-Cys	Bleeding	[75] [76]
Mitaka II	A 11 Glu-Gly	Negative	[66]	Ledyard	A 16 Arg-Cys	Bleeding	[77]
Rouen	A 12 Gly-Val	Negative	[67]	Stony Brook I	A 16 Arg-Cys	Negative	[78]
Bicêtre[a]	A 16 Arg-His	Bleeding	[68]	Kawaguchi	A 16 Arg-Cys	Negative	[63]
Giessen I[a]	A 16 Arg-His	Bleeding	[69] [70]	Detroit[a]	A 19 Arg-Ser	Bleeding	[16] [62]
Louisville	A 16 Arg-His	Bleeding	[71]	Munich I	A 19 Arg-Asn	Bleeding	[59] [79]
Birmingham	A 16 Arg-His	Bleeding	[72]	Mannheim I	A 19 Arg-Gly	Bleeding	[80]
Barcelona II	A 16 Arg-His	Bleeding	[73]				
Stony Brook II	A 16 Arg-His	Bleeding	[74]				
B-Chain Defects							
New York I	Deletion B 972	Thrombosis	[82] [83]	Ise I	B 15 Gly-Cys	Negative	[85]
Ijmuiden I	B 14 Arg-Cys	Thrombosis	[59] [84]	Naples[a]	B 68 Ala-Thr	Thrombosis	[86] [87] [88]

[a]Homozygous.

thrombin binding and thrombosis is discussed in the following sections.

Congenital Dysfibrinogenemias with Polymerization Defects

In this group, the abnormal fibrinogens exhibit a primary delay in fibrin polymerization without an obvious abnormality in fibrinopeptide release. Although the patients show an increased tendency to hemorrhage, some present with thrombosis, spontaneous abortion, or rarely, wound dehiscence. The structural aberrations usually occur in the N- or C-terminal regions of the A- and B-chains or toward the C-terminal regions of the -chains (Table 112-2). The first group is exemplified by fibrinogen Kyoto II, in which the A Pro18 is replaced by Leu.[89] Mutations toward the C-terminus affect fibrin polymerization as shown in fibrinogen Lima, a homozygous A Arg141-Ser mutant associated with a mild bleeding disorder.[90] Fibrinogen Lima and Caracas II[91] represent two of several dysfibrinogenemias where an amino acid substitution results in the formation of a new consensus sequence for the attachment of an additional N-linked oligosaccharide (Table 112-2). In the case of fibrinogen Lima, the polymerization defect appears to be due to an increased content of sialic acid because the removal of this monosaccharide markedly improved the rate of fibrin polymerization. Increased content of sialic acid, although without additional N-linked oligosaccharides, has been previously demonstrated to be responsible for the functional abnormality of the dysfibrinogenemia of liver disease (see Acquired Abnormalities of Dysfibrinogenemias). In fibrinogen Dusart (Paris V) and Chapel Hill III, the A Arg 554 is replaced by Cys, resulting in the formation of disulfide bonds that link fibrinogen to albumin. [92] [93] [94] [95] [96] [97] The linkage of albumin to fibrinogen is possibly responsible for inhibition of fibrin polymerization, decrease in plasminogen binding, and results in defective t-PA-induced activation of bound plasminogen. It is likely that the defect in the assembly and activation of the fibrinolytic system on the mutant fibrinogen is responsible for the severe thrombotic tendency of the affected individuals.[92] [93] [94] [95] [96] [97] Fibrinogen Milano III is characterized by a mutation after A 451 resulting in a frame shift that codes for insertion of two new residues, Trp452 and Ser453, which terminates the chain.[98] The propositus is a homozygous for this mutation and presented with venous thrombosis.

Fibrinogens Nijmegen and Pontoise involve mutations of the B-chain. In fibrinogen Nijmegen, B Arg64 is replaced by Cys, resulting in complexation with albumin and other proteins. A portion of mutant molecules contain free sulfhydryl groups.[84] [99] The formation of fibrinogen-protein complexes along with the free sulfhydryl groups could account for the observed alterations in fibrin polymerization and t-PA-induced activation of plasminogen bound to the mutant. [99] In fibrinogen Pontoise, B Ala335 is replaced by Thr,[100] resulting in the expression of a new N-linked glycosylation site because Asn is present at position 333.

Mutations in the -chain are more common than mutations of the B-chain. These include an Arg275 substitution for His, or by Cys, which occurs in several abnormal fibrinogens, and these substitutions at Arg275 may interfere with the longitudinal DD contacts formed during fibrin polymerization [23] (Table 112-2). Substitution of Arg275 for His is associated with bleeding or thrombosis or is clinically silent. Substitution of Arg for Cys does not lead to clinical manifestations. Several substitutions of the -chain involve the polymerization pocket,[24] as seen in fibrinogen Nagoya, Milano I, Kyoto III, Matsumoto I, and Osaka V (Table 112-2). Mutation at Met310 with Thr in fibrinogen Asahi I introduces a new consensus for an additional N-linked glycosylation site at Asn308. [111] [112] The bleeding disorder may be due to the additional oligosaccharide that could be responsible for inhibiting fibrin polymerization and cross-linking. Certain dysfibrinogenemias are structurally characterized by deletions or elongation of the -chain. The prototype of this group is fibrinogen Paris I, a mutant in which the elongated -chain is responsible both for delayed polymerization and for abnormal factor XIIIa-mediated cross-linking. [117] [118] Fibrinogen Vlissingen is structurally characterized by a six-base deletion encoding amino acids Asn319 and Asp320.[113] This mutant exhibits defects in fibrin polymerization and calcium binding. Clinically, it appears to be associated with thrombotic tendency.[113]

Congenital Dysfibrinogenemia with Alterations of Cross-linking

Although there are no abnormal fibrinogens characterized exclusively by defective factor XIIIa-mediated cross-linking,

TABLE 112-2 -- Dysfibrinogenemias with Polymerization Abnormalities and Known Structural Defects

	Structural Defect	Clinical Data	References		Structural Data	Clinical Data	References
A-Chain Defects[a]							
Fibrin Polymerization							
Kyoto II	A 18 Pro-Leu	Bleeding?	[89]	Caracas II	A 434 Ser-Asn Additional N-linkage of oligosaccharide	Negative	[91]
Lima[b]	A 141 Arg-Ser Additional N-linkage of oligosaccharide	Bleeding	[90]	Dusart (Paris V)	A 554 Arg-Cys	Thrombosis	[92] [93] [94] [95]
				Chapel Hill III	A 554 Arg-Cys	Thrombosis	[96] [97]
				Milano III[a]	Ser 453-Stop	Thrombosis	[98]
B-Chain Defects							
Nijmegen	B 64 Arg-Cys	Thrombosis	[84] [99]	Pontoise	B 335 Ala-Thr Additional N-linkage of oligosaccharide	Negative	[100]
-Chain Defects							
Kurashiki I[a]	268 Gly-Glu	Negative	[101]	Milano I	330 Asp-Val	Negative	[109]
Haifa	275 Arg-His	Bleeding, thrombosis	[59] [102]	Kyoto III	330 Asp-Tyr	Negative	[110]
Tokyo II	275 Arg-Cys	Negative	[103] [104]	Asahi I	310 Met-Thr	Bleeding	[111] [112]
					Additional N-linkage of oligosaccharide		
Baltimore I	292 Gly-Val	Bleeding, thrombosis	[57] [105]	Vlissingen I	Deletion 319320	Thrombosis	[113]
Baltimore III	308 Asn-Ile	Negative	[59] [106]	Milano VII	358 Ser-Cys	Negative	[114]
Kyoto I	308 Asn-Lys	Negative	[107]	Matsumoto I	364 Asp-His	Negative	[115]
Nagoya	329 Gln-Arg	Negative	[108]	Osaka V	375 Arg-Gly	Negative	[116]
				Paris I	-Chain elongation	Wound dehiscence	[117] [118]

[a] Includes abnormal fibrinogen A 526 Val-Glu, which demonstrates normal clotting and is clinically manifested by hereditary renal amyloidosis. [183]
[b] Homozygous.

several mutants exhibit defective cross-linking in addition to abnormalities of fibrinopeptide release or fibrin polymerization. A noted example is fibrinogen Paris I, which exhibits defective -chain cross-linking along with delayed polymerization. Both of these functional abnormalities are the result of the extended -chain. [117] [118] Fibrinogen Asahi I contains additional N-linked carbohydrate in the C-terminus of the -chain and also shows a delay in the formation of cross-linking. [111] [112] Fibrinogen Marburg also exhibits defective cross-linking of the -chains due to truncation of the C-terminal region of the A-chain (see following discussion). In contrast,

fibrinogen Dusart shows an increase rate of -chain cross-linking, an abnormality that may contribute to the thrombotic tendency. [119]

Congenital Hypodysfibrinogenemias

Congenital hypodysfibrinogenemia includes those cases in which structurally abnormal fibrinogens with altered functional activity are present with total plasma fibrinogen concentrations arbitrarily defined as below 150 mg/dl as measured immunologically or by other physicochemical methods. Measurements of clottable protein are not reliable because the abnormal molecules do not always incorporate into the clot. About 15 hypodysfibrinogenemias have been fairly well characterized, and the functional properties and clinical expression of some of these mutants are presented in Table 112-3 . In the first clear case of hypodysfibrinogenemia, involving fibrinogen Parma, [120] the patient had a severe bleeding disorder that was corrected by the infusion of normal fibrinogen, which seemed to exhibit a normal plasma survival. On the other hand, fibrinogens Philadelphia [15] and Bethesda III[121] have almost identical properties including normal fibrinopeptide release, defective fibrin polymerization, and increased catabolic rate of the autologous protein but normal survival of the homologous protein. These findings indicate that an intrinsic molecular defect of the mutant fibrinogen is responsible for the observed hypercatabolism leading to the hypofibrinogenemia.

In contrast, patients with fibrinogen Giessen II show decreased survival of the homologous (normal) protein, indicating an extrinsic (plasmin-like) factor is responsible for mediating

TABLE 112-3 -- Congenital Hypodysfibrinogenemias

	Plasma Fibrinogen Concentration (mg/dl)	Fibrinogen Half-life (h)		Functional Defect(s)	Clinical Data	References
		Homologous	Autologous			
Parma	6075	Probably normal		Unknown	Bleeding	[120]
Philadelphia I	4578	72	38	Polymerization	Bleeding	[15]
Bethesda III	100120	80	34	Polymerization	Bleeding, abortions	[121]
Giessen II	50	56		FPA release	Bleeding	[122]
				Polymerization		
Chapel Hill I	115131	65	72	Polymerization	Bleeding	[123]
Baltimore II	6296	90	81	FPB release	Negative	[124]
Marburg[a] [b]	60			Polymerization	Thrombosis	[125]
Otago[a] [c]	6	96		Polymerization	Abortions	[126]
Normal	190400	7290	7290			[14] [15]

[a]Homozygous.
[b]Deletion A 461-610.
[c]Deletion A 271.

the hypercatabolism of fibrinogen.[122] Fibrinogens Chapel Hill I [123] and Baltimore II[124] also show normal survival of both the homologous and autologous proteins, indicating that a low biosynthetic rate or impaired secretion is responsible for the hypofibrinogenemia. Fibrinogen Marburg is structurally characterized by deletion after amino acid 461-Lys of the A-chain as the result of a single base substitution A to T, forming TAA stop codon. [125] The mutant fibrinogen exhibits delayed fibrin polymerization and appears to be disulfide linked to albumin. The abnormal protein did not support the attachment of endothelial cells, suggesting that the RGD peptide of the C-terminus of the normal A-chain is important in this process. [125] Fibrinogen Otago exhibits a truncated A-chain at residue 271, caused by a cytosine insertion at A 268 producing a frame shift that translates into three new amino acids 268270 and stop codon at 271. Functionally, this mutant is characterized by severe hypofibrinogenemia and abnormal fibrin polymerization. Clinically, the major manifestations were abortions and impaired wound healing, [126] abnormalities that are also expressed by patients with severe hypofibrinogenemia.

The functional defect in hypodysfibrinogenemias is heterogenous; delayed fibrin polymerization is the predominant defect, but abnormal release of fibrinopeptides is also seen (Table 112-3). The major clinical expression in hypodysfibrinogenemia is bleeding, but repeated spontaneous abortions and thrombosis have also been reported (Table 112-3).

Pathogenesis of Thrombosis and Delayed Wound Healing in Dysfibrinogenemias

Approximately 45 dysfibrinogenemias that predispose to arterial or venous thrombosis have been described. Although some of the affected individuals experienced isolated thrombotic episodes, these may have been unrelated to the abnormal fibrinogen. [59] [64] In other cases, frequent episodes of arterial or venous thrombosis in a particular patient or in several of the affected family members suggest a causal relationship. Although the mechanisms by which the abnormal fibrinogens promote thrombus formation are largely unknown, analysis of the biochemical properties of the mutant molecules may offer some clues. Several mutant fibrinogens have an impaired ability to bind thrombin, and the excess free thrombin is available to interact with normal fibrinogen molecules or to induce platelet activation, leading to the formation of platelet aggregates. Fibrinogens New York, [82] [83] Naples,[86] [87] [88] and Pamplona II,[127] which also exhibits impaired release of FPB, belong to this group. In other cases, increased thrombosis may be the result of defective fibrinolysis as a result of impaired plasminogen activation or increased resistance of the mutant molecule to cleavage by plasmin. Indeed, fibrinogens Dusart and Chapel Hill III, [92] [93] [94] [95] [96] [97] Nijmegen,[84] [99] Ijmuiden,[84] and Pamplona II[127] exhibit decrease plasminogen binding and defective t-PA activation of the bound plasminogen. Although these mutants also show abnormal conversion of fibrinogen to fibrin, their thrombotic tendency may be related to the defective assembly or activation of the fibrinolytic system.

Other clinical manifestations of dysfibrinogenemias include delayed wound healing and wound dehiscence, as reported for fibrinogens Paris I, [117] [118] Cleveland I,[128] Caracas I,[129] and Otago.[126] The abnormal factor XIIIa-mediated cross-linking of these mutants and the resulting decreased strength of the clots may be responsible for the healing abnormalities. Patients with factor XIII deficiency also manifest defects of the healing process. Recurrent spontaneous abortions (mainly in the first trimester) associated with bleeding or thrombosis have also been reported in several dysfibrinogenemias (Tables 112-1 , 112-2 , and 112-3).

Laboratory Tests

Abnormalities in routine coagulation assays, particularly the PT or PTT, usually provide the first evidence for the presence of an abnormal fibrinogen. The PT is more sensitive than the PTT, but an even more sensitive and specific test is the thrombin time, which in some patients is so prolonged that a visible clot never forms. The reptilase and ancrod times are also useful diagnostic tests when dysfibrinogenemia is suspected. [59] [64] Measurement of the plasma fibrinogen concentration in patients with dysfibrinogenemia can give variable results depending on the type of analytic method used. Frequently, the fibrinogen concentration appears low when measured as clottable protein, especially when methods based on the rate of fibrin formation are utilized. [59] [64] This may be due to the fact that several of the abnormal fibrinogens have an inhibitory effect on the clotting of normal plasma. Furthermore, because the abnormal fibrinogen may not be incorporated into the clot, the soluble molecules that remain in serum can be mistaken for fibrin degradation

products, which thereby leads to the misdiagnosis of an active fibrinolytic system, as in disseminated intravascular coagulation (DIC). In most cases of dysfibrinogenemia, the plasma fibrinogen concentration is actually normal when measured immunologically, and the discrepancy between clottable protein and

immunologically measured fibrinogen is a characteristic feature of dysfibrinogenemia. In some instances, however, the abnormal fibrinogen is in fact present in low amounts, as in the hypodysfibrinogenemias.

Inheritance

Although the vast majority of dysfibrinogenemias are heterozygous, the homozygous condition with no normal fibrinogen has been documented in 11 families. In the case of fibrinogen Metz, the parents were consanguineous and the condition in the propositus was homozygous. [75] [76] Similar findings have been reported for fibrinogen Detroit[16] [62] among others. Some mutants release only 50% of either FPA or FPB.[59] [60] In other instances, the amino acid substitution is also present in half of the molecules.[59] [60] Using immunologic, electrophoretic, and chromatographic methods, two distinct populations of fibrinogen have been identified in several mutants.[60]

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ACQUIRED ABNORMALITIES OF FIBRINOGEN

Acquired abnormalities of fibrinogen may be classified into three groups: (1) hyperfibrinogenemia, (2) hypofibrinogenemia, and (3) dysfibrinogenemia.

Hyperfibrinogenemia

Plasma fibrinogen levels increase significantly with age, with lifestyle habits such as cigarette smoking, and in certain pathologic conditions such as hypertension, obesity, and diabetes mellitus.[130] [131] [132] [133] However, even among normal individuals, the plasma fibrinogen concentration varies, and although the exact regulatory mechanisms are not elucidated, recent studies indicate that in part fibrinogen levels may be genetically controlled. For example, high plasma fibrinogen concentrations have been observed in normal individuals exhibiting a specific nucleotide sequence polymorphism at the 5 untranslated region of the B gene. [134] [135] However, this polymorphic site is only present in a minority of the population. [134] [135] In contrast, fibrinogen behaves as an acute-phase reactant protein; therefore, its concentration is sensitive to inflammatory responses.[136] In this process, the release of interleukin-6 by macrophages leads to an increase in transcription of the B-gene, resulting in elevated levels of fibrinogen. [137] [138]

The concentration of plasma fibrinogen has important clinical implications, as indicated by several studies demonstrating that hyperfibrinogenemia is an independent risk factor in stroke and in ischemic heart disease. [139] [140] [141] [142] For example, plasma fibrinogen concentration in the upper third of the population increased the risk of ischemic heart disease threefold when compared with those in the lower third of the population. [139] Even small increments in the level of fibrinogen such as one standard deviation (0.6 mg/ml) above the mean markedly increase the risk of ischemic heart disease. [139] When other risk factors for ischemic heart disease and stroke such as hypertension, serum cholesterol, age, diabetes, and smoking were considered, high levels of fibrinogen were still evident as an important risk factor. [139] [140] [141] [142] The pathophysiologic mechanisms by which elevated fibrinogen causes vascular damage is unknown, [143] [144] and it is possible that the high concentration of plasma fibrinogen may be due to cytokines released from areas of vascular damage. In this case, hyperfibrinogenemia can be considered to be a marker of the disease and may not play a role as a causative agent in the development of vascular damage. [143] [144] The high levels of plasma fibrinogen can be part of the inflammatory response, and an increase in fibrinogen levels may be similar to the observed increase in C-reactive protein, as recently reported to occur in patients with coronary vascular disease.[145] Other studies also showed that individuals with the genetic polymorphism of the B-chain, which may be associated with high levels of plasma fibrinogen, do not show an increased incidence of coronary artery disease, [146] [147] suggesting that hyperfibrinogenemia is not the primary cause of cardiovascular diseases.

Hypofibrinogenemia

Low levels of plasma fibrinogen can be caused by a relative decrease in biosynthesis of this protein by the hepatocyte or by an increased rate of its catabolism. In some clinical disorders, both mechanisms may be involved in the development of hypofibrinogenemia. Normal or increased levels of plasma fibrinogen are common in liver disease, but moderate hypofibrinogenemia (i.e., a fibrinogen level <100 mg/dl) may be present in fulminant hepatic failure or in decompensated liver cirrhosis. [148] The low levels of fibrinogen correlate with mucosal hemorrhage that is unrelated to esophageal varices, which may be present in these patients. [149] In addition, severe hypofibrinogenemia usually carries a poor prognosis.[148] The origin of hypofibrinogenemia in this group of patients is multifactorial. In addition to impaired hepatic fibrinogen synthesis,[150] increased catabolism has been observed.[151] An active fibrinolytic system does not seem to play a crucial role in the development of hypofibrinogenemia because the treatment of these patients with fibrinolytic inhibitors does not significantly change the rate of fibrinogen catabolism. [151] These findings, along with an increase of FPA level and fibrinogen-fibrin degradation products [152] indicate that DIC is a contributing factor in the hypofibrinogenemia of some patients with liver disease. Hypofibrinogenemia is also present in patients with acute or chronic DIC of different etiologies, and a low fibrinogen level should alert the physician about this possibility (Chap. 117).

Other causes of low plasma fibrinogen concentration include the administration of certain drugs; for example, L-asparaginase can induce a severe hypofibrinogenemia, which infrequently can cause bleeding manifestations. [153] [154] [155] [156] Although the treatment with L-asparaginase leads to a decrease in the concentration of several plasma proteins, such as plasminogen and antithrombin III, [157] the levels of fibrinogen are markedly decreased, frequently below 20 mg/dl. [156] In contrast, the concentration of other coagulation factors is normal or slightly decreased. [155] [156] Some clinical studies and experimental work in rabbits indicate that impaired synthesis rather than increased catabolism is the cause of the hypofibrinogenemia in this group of patients. [158] [159] Mild decrease of plasma fibrinogen concentration has also been reported in patients treated with valproic acid. [160]

Dysfibrinogenemias

Diverse diseases affecting the liver parenchyma not only produce quantitative alterations of plasma fibrinogen but may also induce dysfibrinogenemia (Chap. 147). The alteration of functional properties of fibrinogen is the most common cause of dysfibrinogenemia. In severe liver diseases of varied etiology, including liver cirrhosis, viral and toxic hepatitis, approximately 50% of the patients exhibit the characteristic dysfibrinogenemia of liver disease. [161] [162] [163] [164] Patients with hepatoma exhibit the same abnormality.[165] [166] The abnormal fibrinogen is characterized functionally by impaired polymerization of fibrin, which explains the prolongation of the thrombin time. Both the cleavage of fibrinopeptides and the cross-linking of fibrin are normal. [161] [162] [163] [164] [165] [166] The structural defect of this abnormal fibrinogen is an increased

content of carbohydrate and in particular of sialic acid. [167] An increase in galactose and *N*-acetylglucosamine with normal mannose is consistent with increased branching of the oligosaccharides rather than the presence of new carbohydrate linkages. [168] Normalization of the thrombin time and of fibrin monomer polymerization is observed after enzymatic removal of the sialic acid from the abnormal fibrinogen. [167]

It is interesting that normal fetal fibrinogen exhibits biochemical and functional properties similar to those described in the abnormal fibrinogen of liver disease, [169] which suggests the possibility that activation of dormant carbohydrate enzymes in the diseased liver is a factor involved in the increase in carbohydrate content. [170] An abnormal fibrinogen with biochemical and functional properties similar to those found in patients with liver disease has been described in hypernephroma as part of the paraneoplastic syndrome; this abnormality abated after removal of the tumor but appeared again with the presence of metastasis. [171]

Functional abnormalities of fibrinogen can be secondary to fibrinogen autoantibodies. An inhibition of the release of fibrinopeptides by thrombin was described in a young female with a mild bleeding disorder. The isolated inhibitor was identified as an antibody of the IgG class that specifically interfered with the interaction of thrombin with fibrinogen but did not interfere with other functional activities of this enzyme. [172] Antibodies of the IgG class able to impair the polymerization of fibrin monomers have been described in association with systemic lupus erythematosus and also in a patient with ulcerative colitis and postnecrotic liver cirrhosis or without underlying disorder. [173] [174] Although some of these patients exhibit bleeding manifestations, [172] [174] in others the presence of the antibody was associated with

thrombosis,[173] but the thrombosis could be related to some other coagulation abnormality, such as lupus anticoagulant, rather than to the fibrinogen antibody. [173] Similarly, thrombotic episodes have been observed in several patients with systemic lupus erythematosus and prolonged thrombin time. [64] A third type of an IgG antibody with specificity against the cross-linking sites of fibrinogen and normal factor XIII activity was described in a patient with sarcoidosis treated with isoniazid. This antibody seemed to be responsible for the patient's mild bleeding disorder. [175]

The monoclonal immunoglobulin of multiple myeloma can prolong the thrombin time by inhibiting fibrin monomer polymerization (Chap. 118).[64] [176] [177] Although this effect is mediated by the $F(ab)_2$ fragment of the monoclonal protein, a specific antibody-antigen reaction between the monoclonal and fibrinogen has not been demonstrated. Thus, in addition to acting as specific antibodies to fibrinogen, as discussed above, [175] immunoglobulins may also act by some other as yet undefined mechanism.[64] [176] [177] [178] Some of these interactions may lead to clinical manifestations, but most frequently they are clinically silent.

In patients with active bleeding due to paraproteinemias, extensive plasma exchange seems to improve the hemorrhagic tendency. [178] Abnormal thrombin- or reptilase-induced ability of fibrinogen to clot has been noted in about 40% of patients with systemic light chain amyloidosis. [179] The isolated fibrinogen molecule demonstrated normal ability to clot, and an unidentified factor unrelated to the paraproteinemia was responsible for inhibiting the conversion of fibrinogen to fibrin. [179] Defective formation of fibrin by thrombin is also present in patients who have circulating heparin or heparin-like substance. However, in these cases, the addition of protamine to the patient's plasma shortens the thrombin time; in addition, reptilase-induced clotting ability is close to normal. [180] [181] [182] The identification of plasma factors that interfere with thrombin clotting ability of fibrinogen leading to dysfibrinogenemia can be clearly made by examining the functional properties of purified patient fibrinogen.

Cryofibrinogenemia

Cryofibrinogenemia refers to the presence of plasma, of fibrinogen that precipitates at low temperatures, usually at 4°C. The amount of cryofibrinogen present in the precipitate can be variable from traces to 100 mg percent. [184] The cold temperature-induced precipitation of proteins from plasma but not from the serum distinguishes cryofibrinogenemia from cryoglobulinemia, a condition in which proteins, mainly immunoglobulins, precipitate from serum. Cryofibrinogenemia is frequently associated with autoimmune diseases, neoplasms, thrombotic disorders, and infectious processes.[184] [185] This condition can also be present as a primary disorder [186] and can be detected in about 3% of hospitalized patients. [184] [185] Although cryofibrinogenemia is clinically silent in the majority of cases, in some patients it can cause cold hypersensitivity, cutaneous ulcerations, peripheral gangrene, and arterial or venous thrombosis. [184] [185] The cause of ulcerations and peripheral gangrene seems to be secondary to intravascular deposition of fibrin(ogen) in small arteries and arterioles, without evidence of vasculitis. [187] Several studies have demonstrated that the plasma cryoprecipitate is composed of fibrinogen-fibrin and fibronectin, and it appears that all three components are necessary for in vitro cryoprecipitation. [188] Although the natural history of this disorder is not known, it seems that some patients have spontaneous remission, whereas in others the disease progresses, requiring therapeutic intervention. Anticoagulation with heparin or coumadin appears to be effective in some cases. [186] Steroids and immunosuppressive drugs have also been used in patients who do not respond to other forms of therapy. [187] In patients with severe manifestations, such as peripheral gangrene, plasmapheresis induced a temporary improvement.[189] A marked resolution of the cutaneous manifestations of cryofibrinogenemia was observed in seven patients after treatment with stanozolol,[190] an agent that increases fibrinolytic activity. Cryofibrinogenemia should be suspected in patients with cold sensitivity and peripheral ischemia, especially with skin ulcerations, and in patients who do not have other causes for vascular occlusion.

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PROGNOSIS AND TREATMENT

The majority of patients with congenital dysfibrinogenemia are asymptomatic and do not require treatment. Active bleeding can be corrected with replacement therapy; and in those patients with a history of bleeding, replacement therapy can be instituted before surgery or procedures that involve a high risk of hemorrhage. Replacement therapy using plasma or cryoprecipitate can be prescribed according to the guidelines outlined in the afibrinogenemia section. It appears that thrombotic complications have led to the death of several patients. In patients who present with thrombosis, anticoagulant therapy should be recommended on the basis of criteria similar to those of other hypercoagulable states. Recurrent spontaneous abortions have been reported in several families, but continuous replacement of fibrinogen in the form of cryoprecipitate may permit full-term pregnancy. [126]

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Chapter 113 - Structure, Biology, and Genetics of von Willebrand Factor

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INTRODUCTION

The adhesive glycoprotein von Willebrand factor (vWF) was named after Dr. Erich von Willebrand, who described in 1926 a new bleeding disorder [1] distinct from hemophilia. von Willebrand disease (vWD) was later recognized to be caused by qualitative or quantitative defects in vWF. [2] vWF circulates in plasma at concentrations of 510 g/ml. Some of the molecules are complexed with factor VIII, apparently protecting factor VIII against degradation. [3] vWF is synthesized by only two cell types: endothelial cells and megakaryocytes. [4] [5] [6] Besides plasma, vWF is found in platelets, endothelial cells, and the basement membrane of blood vessels. [7] All these pools of vWF contribute to the proteins main function: to promote attachment of platelets to areas of vessel injury. To optimize the availability of vWF at the site of injury, a highly active form of the protein is stored in secretory granules of platelets and endothelial cells. [8] When these cells sense tissue injury (e.g., by contact with thrombin), they instantly mobilize the stored protein. The released vWF binds to glycoprotein Ib (GPIb) on the platelet surface [9] [10] [11] [12] and to components of the basement membrane, [13] forming a bridge that can withstand the high sheer stress of blood flow. vWF is necessary for this initial attachment step of platelets to the injured area. [14] Together with other adhesive proteins, such as fibrinogen, fibronectin, and thrombospondin, vWF interacts with the GPIIb/IIIa on activated platelets and contributes to platelet spreading and aggregation. [15] [16] vWF is a large protein composed of many subunits held together covalently by disulfide bonds. [17] In this way, the protein acquires multiple binding sites for platelets and the basement membrane, which may strengthen its interactions during hemostasis.

The vWF Gene

von Willebrand factor cDNA was cloned in 1985. [18] [19] [20] [21] The full-length cDNA sequence predicts a protein of 309,000 molecular weight (2,813 amino acids). In addition, the location of N- and O-linked glycosylation sites within the mature vWF subunit have been determined by direct protein analysis. [22] Comparison of the cDNA and primary amino acid sequences identified a large propeptide (741 amino acids) preceding the mature vWF subunit sequence. This propeptide is identical to a previously observed immunologic activity in plasma termed vW antigen II. This propeptide plays an important role in vWF multimer assembly and processing, but has no known function after secretion. The vWF gene is located on the short arm of human chromosome 12 [18] [20] and a partial, nonfunctional duplication (pseudogene) is on human chromosome 22. [23] The structures of the unusually large vWF gene and pseudogene are shown schematically in Figure 1131 . [24] [25] The gene is composed of 52 exons spanning a total of approximately 180 kb of the human genome. It is similar in size to the factor VIII gene (Chap. 107) and >100 times larger than the gene for -globin (Chap. 29). The vWF gene accounts for approximately 0.1% of human chromosome 12. The vWF pseudogene duplicates the middle portion of the gene from exon 23 to 34 including the intervening sequences. The pseudogene is approximately 97% homologous to the authentic gene, indicating that it has arisen fairly recently in evolution. [25]

Analysis of the vWF amino acid sequence identifies a pattern of homologous repeated segments, designated by the letters AD in Figure 1131 . This pattern suggests that the vWF gene arose by a complex series of partial gene duplications. The expression of the vWF gene is tightly regulated and restricted exclusively to endothelial cells and megakaryocytes. For this reason, vWF is frequently used as a primary histochemical marker to identify cells of endothelial cell origin. A 734-base pair DNA fragment extending from approximately 500 base pairs upstream of the transcription start site into the first intron confers endothelial cell-specific vWF gene expression in vitro, [26] and directs gene expression to a subpopulation of endothelial cells in transgenic mice. [27] However, as in the case of -globin, another highly tissue-specific regulated gene (Chap. 29), critical transcription regulatory sequences located a great distance from the 5 end of the vWF gene may be required for the high-level expression observed in a wide range of endothelial cells in vivo.

Comparison of the vWF DNA sequence to that of other known genes identifies potential relationship to a number of other adhesive proteins. A superfamily of proteins sharing sequence similarity with vWF A domains contains a number of proteins associated with the extracellular matrix, hemostasis or cell adhesion. [28] The crystal structure of the vWF A3 domain has recently been solved [29] [30] and is remarkably similar to the structure for the homologous I domain of the integrin 2/1, [31] although the metal ion-dependent structure common to at least several I domains is not required for the collagen binding function of the vWF A3 domain. [29] A possible relationship between the C repeats of vWF and segments of thrombospondin and procollagen has also been noted, [32] as well as homology of the D domains to a sperm membrane protein that binds to the extracellular matrix of the egg. [33] [34] [35]

Exons of vWF range in size from 40 base pairs to 1.4 kb for exon 28, one of the larger known single exons. This latter exon encompasses most of the A1 and A2 homologous repeats, a region containing several important vWF functional domains, as well as a large number of human mutations associated with type 2A and 2B vWD.

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DOMAIN STRUCTURE

von Willebrand factor exists as a series of multimers varying in molecular weight between 0.5 (dimer) and 20 million (multimer). Electron microscopic observations of the vWF molecules revealed that they are filamentous and contain subunits arranged in a head-to-head and tail-to-tail configuration. [36] [37] The building block of multimers is a dimer, held together by disulfide bond(s) located near the C-terminal end of each subunit. The dimers are joined to each other by disulfide bonds located near the N-terminal end of the mature subunit. [38] [39] The mature subunit has a mass of about 270 kd and contains 18.7% carbohydrate. [22] N- and O-linked carbohydrates are found clustered at both ends of the subunit. All 169 cysteine residues (8.2% of the amino acid content) are involved in interchain or intrachain

Figure 113-1 The structure of the vWF gene and protein. The structures of the vWF gene and pseudogene are indicated schematically at the top of the figure. The corresponding protein is also depicted, including the homologous repeat domain structure. The localization within vWF of point mutations associated with vWD variants are also indicated. *(Adapted from Ginsburg and Bowie, [126] with permission.)*

disulfide bonds. [40] [41] Two Arg-Gly-Asp (RGD) sequences are found in pro-vWF (Fig. 1131). The one on vWF is a part of the binding site for the platelet integrin receptor, GPIIb/IIIa. [16] The significance of the other RGD sequence located on the propeptide is not known (Fig. 1131).

Several functional domains in vWF have been identified on the vWF subunit [42] (Fig. 1131). Two collagen-binding sites have been mapped to the mature vWF subunit [43] [44] and potentially a third to the propeptide. [45] However, studies of recombinant vWF suggest that the major physiologically active binding site for fibrillar collagen lies within the A3 domain. [46] [47] vWF also has at least two binding sites for heparin [48] [49] and may interact with heparin-like molecules in the basement membrane. The factor VIII-binding domain is located in the N-terminal portion of the vWF subunit [50] and interacts with the N-terminal portion of the factor VIII light chain. [51] [52] vWF also has affinity for two platelet receptors. Its binding to GPIb plays a pivotal role in the early events of hemostasis, leading to the attachment of platelets to the area of injury. [10] [14] Several potential, short binding sequences for GPIb on vWF have been identified, all localized to a large disulfide loop contained within the vWF A1 repeat [53] [54] (Fig. 1131). This site appears to interact with the N-terminal portion of the -chain of GPIb. [55] The binding region of vWF to GPIIb/IIIa on activated platelets is located in the C-terminal portion of the mature subunit. This recognition site can be specifically inhibited by the Arg-Gly-Asp sequence contained within peptides [56] and is therefore likely to include the Arg-Gly-Asp-Ser sequence. [16] [57] vWF (with other adhesive proteins such as fibrinogen and fibronectin) may compete for binding to this glycoprotein.

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BIOSYNTHESIS

The processing steps leading to the formation of mature vWF multimers have been studied chiefly in endothelial cells, [58] but evaluation of megakaryocyte biosynthesis indicates an identical pattern of synthesis [6] (Fig. 1132). vWF is first synthesized as a pro-vWF monomer. [59] [60] [61] Co-translationally, high-mannose carbohydrate is added to the polypeptide chain. There are 13 potential N-linked glycosylation sites on mature vWF and 4 on

Figure 113-2 Map of processing steps in the biosynthesis of vWF. Interchain disulfide bonds are formed in two steps (dimerization and multimerization, pink). The propeptide is cleaved late in vWF processing (prosequence cleavage, red). *(Adapted from Handin and Wagner,[7] with permission.)*

the propeptide. While residing within the endoplasmic reticulum, the C-termini of the pro-vWF subunits are linked together by an unknown number of disulfide bonds.[62] If initial glycosylation is inhibited by the drug tunicamycin, the protein remains monomeric. The monomeric protein synthesized in the presence of tunicamycin is internally degraded and is not secreted from the cells. [63] Only dimeric molecules are transported to the Golgi apparatus, where their processing continues.

In the Golgi apparatus, common modifications, including the addition of O-linked carbohydrate, high-mannose carbohydrate processing, and sulfation, take place (Fig. 1132). In addition, a unique processing event includes the multimerization of dimers by disulfide bond formation. [7] vWF is the only protein known that has disulfide bonds formed late in protein synthesis, as disulfide bonds are usually formed in the endoplasmic reticulum. The latter process is catalyzed by the enzyme disulfide isomerase.[64] The contents of the last compartment of the Golgi apparatus (trans-Golgi) and the post-Golgi secretory vesicles are slightly acidic. [65] It is likely that multimerization occurs in these acidic compartments because this process can be completely inhibited by culturing cells in the presence of a weak base [63] that increases the pH of acidic cellular compartments. The vWF propeptide plays an active role in the interdimer disulfide bond formation. When prepro-vWF cDNA is expressed in COS (a monkey kidney cell line) or other cells that normally do not synthesize vWF, these cells are capable of supporting vWF multimerization. By contrast, if pre-vWF cDNA (with the cDNA coding for the propeptide deleted) is expressed in these cells, only dimeric molecules are synthesized and secreted. [66] [67] In in vitro multimerization experiments, pro-vWF dimers spontaneously form larger multimers, whereas vWF mature dimers remain dimeric. The in vitro multimerization is promoted by a slightly acidic environment (optimal pH is 5.8) and will not occur at a neutral or basic pH. [68] Under these acidic pH conditions the propeptide may catalyze disulfide bond formation among the mature vWF subunits. The propeptide contains sequences similar to those found at the active site of protein disulfide isomerase. These are cysteines separated by two amino acids that readily interchange with disulfide bonds in the substrate. When an additional amino acid was inserted between the cysteines of the propeptide by site specific mutagenesis, the vWF expressed did not form multimers. [69] During the biosynthesis of vWF multimers, the propeptide does self-associate, but it is not known whether this association is linked to noncovalent multimer assembly that likely precedes the interchain disulfide bond formation. [70]

The propeptide cleavage is one of the final processing steps in the biosynthesis of vWF. Similar to other prosequences, the vWF propeptide is cleaved adjacent to two basic amino acids, Lys-Arg at residues 2 and 1. An Arg at 4 position from the cleavage site is also part of the cleavage recognition sequence, providing evidence that an enzyme with furin/PACE-like specificity is responsible for the intracellular prosequence cleavage. [71] Multimerization and propeptide cleavage are separate events. Multimers secreted by endothelial cells in culture contain both pro-vWF and mature vWF subunits. [61] [72] Also, the culturing of endothelial cells in the presence of weak bases inhibits multimerization completely, whereas propeptide cleavage is only slightly affected. [63] Finally, site-specific mutagenesis of the cleavage site inhibits propeptide cleavage, but the expressed protein has a normal multimeric composition. [73] Propeptide cleavage and multimerization occur after sulfation in the trans-Golgi network, but prior to formation of the Weibel-Palade body. [74]

Although vWF processing is chiefly intracellular, some modifications to the protein likely occur after secretion. Cleavage of the propeptide, which begins in the trans-Golgi compartment, may continue extracellularly. This hypothesis is based on the observation that vWF in normal plasma is composed of only mature subunits, whereas vWF secreted constitutively from endothelial cells in culture contains both pro-vWF and mature subunits. [61] [72] The largest vWF multimers found in plasma are smaller than those found in endothelial cells. [75] A plasma protease has recently been defined that appears to specifically cleave vWF in the circulation, resulting in reduction of unusually large multimers to smaller species. [76] [77] The major proteolytic cleavage site for vWF in plasma maps to the peptide bond between Tyr 842 and Met 843 in the vWF A2 domain,[78] and recombinant vWF deleted in the A2 domain is resistant to proteolysis. [79] Mutant vWF in group II type 2A vWD exhibits increased susceptibility to cleavage by this protease, [80] the proposed mechanism for loss of large vWF multimers in this disorder. In addition, a deficiency of this protease activity has been proposed to play a role in the pathogenesis of chronic relapsing thrombotic thrombocytopenic purpura. [81]

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VON WILLEBRAND FACTOR STORAGE AND SECRETION

von Willebrand factor is the only known adhesive protein that is stored and undergoes regulated secretion from cells other than platelets. [58] Both the platelet pool and the endothelial pool of vWF are important physiologically, as demonstrated by crossed bone marrow transplantation experiments between normal and vWD pigs. [82] [83] In platelets, vWF is present in the -granules with many other stored proteins. In the Weibel-Palade bodies of endothelial cells, only vWF and its propeptide are found. [84] [85] [86] In both organelles, the stored vWF molecules appear to form tubular structures, 150 A in diameter. [87] [88] These tubules are absent in the -granules of pigs with vWD. [89] A transmembrane glycoprotein of the -granule, P-selectin, [90] [91] is a component of the Weibel-Palade body membrane. [92] Like vWF, the biosynthesis of this protein is restricted to endothelial cells and to megakaryocytes. When expressed on the plasma membrane, P-selectin promotes the adhesion of monocytes, neutrophils, and some other subsets of leukocytes. [93] [94] The only other recognized component of Weibel-Palade bodies is CD63, a highly glycosylated protein found in lysosomes, which is also expressed on activated platelets. [95] [96] Weibel-Palade bodies originate from the Golgi apparatus. They are up to 4 m long and 0.1 m thick (Fig. 1133) and are found in endothelial cells of virtually all blood vessels types. There is, however, variation in the number of Weibel-Palade bodies per cell in endothelium of different origin. [8]

Fully processed vWF is secreted from cultured endothelial cells by one of two pathways. [97] The constitutive pathway is directly coupled to vWF synthesis and occurs without stimulation. The regulated pathway, involving vWF stored in Weibel-Palade bodies, is initiated by the action of secretagogues. The two pathways differ in that the regulated pathway depends on the microtubular cytoskeleton, while constitutive secretion continues even from cells with depolymerized microtubules. [98] Constitutive secretion occurs evenly in an apical and basolateral direction. By contrast, release from Weibel-Palade bodies is highly polarized in the basolateral direction. Formation of thrombin and fibrin during vascular injury disrupts the endothelial monolayer, which may lead to the release of stored vWF into the lumen. [99] Another important difference between the two pathways is the biologic activity of the vWF secreted. Although mostly small multimers are secreted constitutively, only the largest, biologically most potent multimers are stored in Weibel-Palade bodies [86] [100] (Fig. 1134). It is not known whether the large multimers are selected for storage or whether the conditions found in the Weibel-Palade body promote multimer assembly. The vWF stored in platelet -granules is also enriched in large multimers. [101] All the N-terminal D domains (D1D3) appear to be necessary for vWF storage. Deletions of these individual domains lead to constitutive secretion of the expressed protein. [102] [103] Although the propolypeptide remains in the

Weibel-Palade bodies noncovalently associated with the mature subunit, [74] cleavage of the prosequence has to occur for efficient formation of the storage granules. [104] The biologic function of the two secretory pathways may be different. The small multimers secreted constitutively may be used as carrier proteins for factor VIII; the largest multimers released from Weibel-Palade bodies at the time of vascular injury may act to attach platelets and the injured endothelium to the vessel wall. Some of the released large vWF molecules remain associated with the endothelial membrane, [100] likely interacting with integrin-type receptors. [105] The functional significance of this transient membrane pool of vWF is unknown.

Several secretagogues for vWF that may be of physiologic importance include thrombin, [106] fibrin, [107] histamine, [108] and the terminal complement proteins C5b-9. [109] The release of Weibel-Palade bodies appears to be coupled to Ca^{2} + influx. Thrombin activity as a secretagogue is related to its proteolytic activity and the cleavage of a specific receptor. [97] [106] [110] Interaction with a cellular receptor is a likely mechanism for fibrin release of vWF. A specific N-terminal fragment of the fibrin -chain appears

Figure 113-3 Weibel-Palade bodies of endothelial cells. **(A)** Immunofluorescence staining of a human umbilical vein endothelial cell with anti-vWF antiserum. vWF is present in the perinuclear region, where it is synthesized and in the Weibel-Palade bodies (arrowhead) throughout the cytoplasm. Bar = 10 m. **(B)** Electron micrograph of Weibel-Palade bodies of the same origin. Bar = 0.5 m.

Figure 113-4 Multimeric composition of vWF secreted by endothelial cells in culture. Human umbilical vein endothelial cells were metabolically labeled with ^{35}S-methionine and the constitutively secreted protein () and that released during a 10-minute treatment with the secretagogue A23187(+) were purified and analyzed nonreduced on an agarose gel. The autoradiograph of the gel is shown. The protein secreted constitutively is composed predominantly of small multimers, while the vWF secreted from Weibel-Palade bodies is only of high molecular weight (HMW multimers). *(Adapted from Sporn et al., [100] with permission.)*

to interact with the endothelium. [111] Histamine-induced release can be blocked by histamine H_1 -receptor antagonists. [108] None of the vasoactive agents that cause the rapid rise in levels of vWF in plasma, including 1-deamino-8- D-arginine, vasopressin (DDAVP), causes vWF release from endothelial cells in vitro. [112] It is likely that these agents act through an as yet unknown intermediate. This hypothesis is supported by the observation that plasma from DDAVP-treated normal subjects stimulates release of vWF from cultured endothelial cells. [113] vWF is an unusual molecule in that it interacts specifically with two types of transmembrane receptors: the GPIb receptor of platelets and the integrin-type receptors, such as the GPIIb/IIIa complex on platelets and the vitronectin receptor on endothelial cells. Binding to these receptors is enhanced by the presence of large numbers of binding sites on the vWF multimers. The adhesive activity of the very large multimers is so great that they are kept sequestered in storage organelles and are rapidly released locally at the site of vascular injury.

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VON WILLEBRAND DISEASE

Molecular Genetics

With the availability of vWF cDNA and genomic sequences, a molecular genetic approach to the characterization of vWD became possible. Southern blot analysis identified large gene deletions as the molecular basis for vWD in a small subset of patients with severe, or type 3, disease. [23] [114] [115] [116] With the advent of the polymerase chain reaction, extensive direct DNA sequence analysis has been conducted on material from patients with a variety of vWD variants, using platelets as a source for vWF mRNA, or by direct analysis of genomic DNA. Rapid progress has been made over the past few years, and a large number of human mutations have been cataloged.[2] [117] A database of known vWF mutations and polymorphisms is maintained by a consortium of workers in the field and can be accessed through the Internet at http://mmg2.im.med.umich.edu/VWF (Fig. 1135 (Figure Not Available)). Molecular genetic analysis has shed important light on the molecular pathogenesis of vWD and promises to improve diagnosis. For some vWD variants, mutations have been identified in the majority of patients studied, and precise diagnosis and classification by DNA screening may soon become practical.

von Willebrand disease can be divided into two general categories: those variants due to a pure quantitative deficiency and

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Figure 113-5 (Figure Not Available) von Willebrand disease mutations. The location of all point mutations and small, in frame insertions/deletions reported to the vWD database (http://mmg2.im.med.umich.edu/vwf) as of 1997 are shown schematically here within the vWF domain structure. The relative positions of each of the 52 vWF exons are shown below. *(From Nichols and Ginsburg,[2] with permission.)*

those due to a qualitative structural or functional abnormality within the vWF protein. This is similar to the distinction in the hemoglobinopathies between quantitative abnormalities of globin synthesis (thalassemia) and qualitative structural or functional abnormalities within the protein subunit, such as in the other hemoglobinopathies. Total disruption of gene function by deletion of a segment of the gene or a nonsense mutation (abrupt termination of the protein by insertion of a stop codon into the coding sequence) results in quantitative vWF abnormalities. Conversely, the vast majority of qualitative variants are due to mutations resulting in single amino acid substitutions that interfere with protein structure or function. Based on this improved understanding of molecular pathogenesis, a revised classification scheme for vWD has been developed. [118]

Types 1 and 3

Quantitative vWF abnormalities can be either mild or pronounced. Patients with type 3 (severe vWD) suffer from a profound bleeding disorder (Chap. 114) with very little or no detectable plasma or platelet vWF. Type 3 vWD is a relatively rare disease, with an incidence of approximately 1/1 million. [119] By contrast, type 1 is the most common form of vWD, accounting for 7080% of cases; it is associated with a mild to moderate quantitative decrease in vWF (levels in the range of 2050% of normal). Type 3 vWD appears to result from the inheritance of a dysfunctional vWF gene from both parents. Gene deletions varying from 2.3 kb to >180 kb have been identified in several patients and may be associated with an increased risk of developing vWF inhibitor antibodies. [23] [114] [115] [116] Several other defects resulting in complete loss of vWF function have been identified in patients with type 3 vWD, including nondeletion defects resulting in loss of mRNA expression, [120] as well as specific nonsense mutations or frameshift mutations resulting in disruption of the vWF protein coding sequence [2] [121] (Fig. 1135 (Figure Not Available)).

Only a limited number of point mutations responsible for type 1 and 3 vWD have been identified, compared to the large number of mutations reported for the less common qualitative vWD variants described in the following section (Fig. 1135 (Figure Not Available)). This is due, at least in part, to the necessary complexity of screening all 52 exons of the vWF gene in a patient with type 1 or 3 vWD. In contrast, for many of the qualitative variants, the observed clustering of mutations focuses the search to a limited region of vWF. Several mutations resulting in truncation or disruption of the vWF protein coding sequence have been identified in patients with type 3 vWD. A particularly common frameshift mutation in exon 18, due to deletion of a single cytosine in sequence CCCCCC, accounts for approximately 50% of type 3 vWD alleles in Sweden and has also been shown to be the molecular defect in the original patient reported by von Willebrand. [122] The resulting frameshift produces a stable mRNA encoding a truncated protein that is rapidly degraded in the cell. [123]

This mutation is also common in the German population,[124] but not in the United States.[123]

In the most straightforward model, type 1 vWD would simply represent the heterozygous form of type 3 vWD. However, there is some controversy on this point. [121] Type 3 vWD has classically been defined as recessive in inheritance and parents, who are obligate carriers for the defect, are often completely asymptomatic, with normal laboratory numbers.[23] [114] [115] However, in other cases, parents are affected with classic type 1 vWD. Few mutations have been identified in pure type 1 families because most analyses have concentrated on type 3 vWD. It has been suggested that type 1 vWD mutations may more often produce a mutant vWF subunit that interferes in a dominant negative way with the normal allele, accounting for the autosomal dominant inheritance and resulting in particularly reduced vWF levels in these patients.[121] Mutations at cysteine residues in the D3 domain of vWF were recently identified in several patients with moderately severe vWD. Expression of recombinant vWF carrying one of these mutations (Cys 386 Arg) resulted in a mutant protein that was retained within the endoplasmic reticulum, potentially acted in a dominant negative manner when co-transfected with wild-type vWF. [125]

The reported prevalence of type 1 vWD (1% of the population [126] [127]) would predict a much higher frequency of type 3 vWD (1/40,000) than is observed (1/1 million). Thus, the possibility of locus heterogeneity (i.e., defects in other genes giving rise to a disorder clinically indistinguishable from vWD) has been proposed. [128] Consistent with this idea, studies of an animal model of type 1 vWD in the mouse have identified a single gene on mouse chromosome 11 distinct from the murine vWF gene that results in reductions of plasma vWF levels of up to 20-fold. [129] Although comparable effects for non-vWF genes in human vWD have not yet been demonstrated, other genetic factors are known to significantly modify human vWF levels, including ABO blood group (Chap. 114). The effects of such modifying genes are likely to determine the penetrance of symptomatic type 1 vWD in type 3 heterozygotes. Although the combined effects of several modifier genes could conceivably account for type 1 vWD in a subset of patients with two normal vWF gene alleles, all genetic analysis of human vWD to date has been consistent with linkage to the vWF gene.[2] [121]

Type 2A vWD

Type 2A vWD is the most common qualitative abnormality of vWF and is associated with selective loss of large and intermediate-sized vWF multimers (Chap. 114). Direct sequence analysis from platelet mRNA and genomic DNA has identified a panel of mutations within vWF exon 28 accounting for a majority of patients with type 2A vWD.[2] [117] Nearly all of these mutations are clustered within the vWF A2 repeat (Figs. 1131 and 1135 (Figure Not Available)). One particular mutation (Arg 834 Trp) accounts for approximately one-third of type 2A vWD cases around the world. In vitro analysis of recombinant vWF containing type 2A vWD mutations suggests that two distinct mechanisms contribute to the pathogenesis of this disorder [130] (Fig. 1136). In the first group, the single amino acid substitution results in a defect in vWF intracellular processing, with retention of the abnormal vWF in the endoplasmic reticulum. In the second group, vWF synthesis and processing in vitro is normal and the loss of multimers appears to occur via increased sensitivity to proteolysis in plasma [131] at a site within the type 2A vWD mutation cluster in the vWF A2 domain.[78] The protease most likely responsible for this cleavage has recently been characterized. [76] [77] Recombinant vWF carrying type 2A mutations shows increased sensitivity [80] and recombinant vWF deleted for the A2 domain is resistant to cleavage by this protease. [79] Infusion of recombinant

Figure 113-6 Two mechanisms for the molecular pathogenesis of type 2A vWD. Each vWF monomer is indicated as a gray arrow with the normal sequence indicated by white boxes and mutant sequence by black boxes. Within the cell, mixed dimers are formed, as well as homodimers of mutant and wild-type monomers. In group II, full-size multimers are assembled and proteolyzed after secretion. In group I, mutant subunits are held up in the endoplasmic reticulum; only homomultimers of normal subunits are secreted, predominantly of small size.

vWF carrying a type 2A mutation into mice suggests that cleavage also occurs in vivo. [132]

Type 2B vWD

Type 2B vWD is also characterized by the loss of large vWF multimers but through a unique mechanism, distinct from those described above for type 2A vWD. A panel of mutations has been identified in type 2B vWD patients, all located within a short stretch of the vWF A1 domain [117] [133] [134] (Fig. 1135 (Figure Not Available)). Four of these mutations, clustered within a 35 amino acid segment of the 2,813 amino acid vWF coding sequence, account for >90% of type 2B vWD patients studied.[117] Thus, screening for this panel of mutations could provide precise and rapid DNA diagnosis for this disorder.

These observations also identify a critical region of vWF involved in binding to the platelet GpIb receptor. Each of these single amino acid substitutions is thought to result in a unique gain of function, leading to spontaneous binding of vWF to platelets. [135] [136] Under normal conditions, plasma vWF is inert in its interaction toward platelets until it encounters an exposed subendothelial surface. Binding of vWF to collagen or other ligands within the vessel wall at sites of vascular injury presumably results in a secondary conformational change, which then facilitates binding to the GpIb platelet receptor. In type 2B vWD, the mutant vWF is capable of spontaneously binding to GpIb in the absence of subendothelial contact. Analysis of recombinant vWF demonstrates very similar increases in vWF binding to platelets for each of six distinct type 2B vWD mutations, supporting the hypothesis that the vWF GpIb binding domain can adopt either a discrete on or off conformation.[137] The large multimers appear to have the highest affinity for GpIb (presumably because of multivalency) and are rapidly cleared from plasma, along with the bound platelets, resulting in the characteristic loss of large multimers as well as thrombocytopenia. Recent studies also suggest that binding of type 2B vWF to circulating platelets may block platelet adhesion to collagen, contributing to the bleeding diathesis. [138]

Type 2N vWD

Type 2N is a unique variant of vWD that has important implications for the differential diagnosis of hemophilia and provides an instructive example of genetic locus heterogeneity. Rare cases of autosomal hemophilia have been reported in the past, in which deficiencies in factor VIII activity were inherited in an apparently autosomal manner.[139] [140] Biochemical analysis in several families identified a defect residing within the patients plasma vWF, interfering with its ability to bind and stabilize factor VIII.[141] [142] This variant is termed type 2N under the revised classification of vWD, [118] although sometimes referred to as vWD Normandy after the province of origin of one of the first patients. [143]

The DNA sequence analysis in type 2N vWD has identified several mutations, all clustered in a region at the N-terminus of vWF previously shown to be important for factor VIII binding[117] (Figs. 1131 and 1135 (Figure Not Available)). One of these mutations, Arg 91 Gln, appears to be particularly common. [144] Although usually silent in the heterozygote, homozygosity or compound heterozygosity for a type 2N vWF allele can result in clinically significant factor VIII deficiency. Thus, type 2N vWD should be considered in the differential diagnosis of hemophilia A, particularly in a female patient or in the context of other features suggesting an autosomal pattern of inheritance.[143] Most type 2N vWD mutations result in only mild to moderate reductions in plasma factor VIII. However, at least one mutation (Gln 24 Lys) is associated with factor VIII levels as low as 1%, when coinherited with a type 3 vWD allele. [145] This latter observation suggests that a diagnosis of type 2N vWD should also be considered in patients with more profound reductions in factor VIII level.

Prenatal Diagnosis

With the advent of molecular tools for the analysis of vWD, prenatal diagnosis has become possible and has been applied in a select number of cases. [146] [147] [148] Given the generally mild clinical manifestations of most vWD variants, prenatal diagnosis is generally not indicated. However, in the case of type 3 or severe vWD, the indications for prenatal diagnosis are similar to those of severe hemophilia. For those vWD variants for which the mutation is known, direct analysis of chorionic villus samples or amniotic fluid can be performed rapidly by polymerase chain reaction and can be expected to provide a highly accurate, specific and rapid diagnosis. For vWD families in which the specific mutation is unknown, genetic linkage analysis can be performed using a large panel of highly informative restriction fragment length polymorphisms.[149] One particularly useful polymorphism is a highly variable TCTA repeat in intron 40, which has been reported to have over 100 distinct alleles.[149] Although all cases of vWD analyzed to date appear to be linked to defects within the vWF gene, the possibility of locus heterogeneity (i.e., involvement of other genes outside of vWF) should be considered when performing this kind of analysis.

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Chapter 114 - Clinical Aspects of and Therapy for von Willebrand Disease

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von Willebrand disease (vWD) is a genetically and clinically heterogeneous inherited hemorrhagic disorder caused by a deficiency or dysfunction of von Willebrand factor (vWF), a large adhesive glycoprotein in plasma, platelets, and endothelial cells. As a result, the interaction of blood platelets with the vessel wall is defective and primary hemostasis is impaired. vWD is perhaps the most common hereditary bleeding disorder, and is more common than either hemophilia A or hemophilia B.

von Willebrand disease was first reported in 1926 by Erik von Willebrand, [1] who described a 5-year-old Finnish girl from the Aoland Islands with a bleeding disorder that could be distinguished from classic hemophilia by an autosomal pattern of inheritance, mucocutaneous hemorrhage rather than joint and soft tissue hemorrhage, a prolonged bleeding time, and normal clot retraction. Of 66 family members, 23 had a similar hemorrhagic diathesis. Two years later, George Minot [2] reported a similar bleeding disorder in five patients and described the intermittent nature of the bleeding disorder. von Willebrand originally attributed the bleeding defect in his patients to either a platelet defect or abnormal capillaries. [3] [4] [5] In 1953, three independent groups reported deficiency of factor VIII in vWD, [6] [7] [8] and in 1957, Nilsson and co-workers[9] showed that the infusion of fraction I-0 from normal human plasma led to a greater-than-expected increase in factor VIII levels in patients with vWD, suggesting that the defect might be found in the plasma. More important, the infusion of fraction I-0 from hemophilic plasma also led to a greater-than-expected rise in factor VIII, indicating that the plasma component was something other than factor VIII. [10]

The abnormal interaction between platelets and the vessel wall in vWD was first demonstrated in 1960 by Borchgrevink [11] and was confirmed by numerous workers.[12] [13] [14] [15] The next major discovery was the observation by Zimmerman et al[16] that an antigen associated with factor VIII was decreased in patients with vWD. This antigen is now known as vWF and is a protein distinct from factor VIII. The in vitro measurement of vWF activity was further facilitated by the observation by Howard and Firkin[17] that ristocetin, an antibiotic isolated from the actinomycete species *Nocardia lurida*, induced agglutination of platelets in the presence of vWF. The primary structure of vWF was determined in 1985 when the gene for vWF was independently cloned and sequenced by four groups. [18] [19] [20] [21]

PREVALENCE

Population studies indicate that the prevalence of vWD in the general population is 0.821.6%. [22] However, this is probably an underestimate of the true prevalence because of difficulties in the laboratory diagnosis of the disease. Severe, type 3 vWD is much rarer, with a prevalence of approximately 1 in 1 million.

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GENETICS

von Willebrand disease is inherited in an autosomal manner. Males and females are affected approximately equally, and each child of an individual heterozygous for a vWF abnormality has a 50% chance of inheriting the abnormal gene (Fig. 1141). In classic autosomal recessive disorders, heterozygous offspring are phenotypically normal, whereas in dominant disorders, heterozygous offspring are phenotypically affected. Most patients with vWD show an autosomal dominant pattern of inheritance, although the variability of the laboratory findings and the variability of the clinical expression of the disease implies that penetrance is often incomplete. Although most cases of vWD occur as autosomal dominants, including most type 1 and 2 cases, recent studies suggest that autosomal recessive vWD may be more common than previously recognized. The two reported families with type 2C vWD appear to fit this pattern, [23] [24] and reports of recessive inheritance in type 2A[25] and type 1[26] disease have appeared. This recessive inheritance may account for some of the laboratory variability in vWD described previously. Some patients with severe type 3 disease also show recessive genetics, as evidenced by asymptomatic consanguineous parents. However,

Figure 114-1 Autosomal pattern of inheritance typical of von Willebrand disease (vWD). In this hypothetical pedigree, two families with different defects in the von Willebrand factor gene are illustrated. The pattern of inheritance is a typical autosomal dominant pattern in which people inheriting an abnormal gene from either parent express the defect. A double heterozygote, who would have severe, type 3 vWD is illustrated. White symbols indicate normal people; pink symbols indicate a heterozygote for defect I; red symbols indicate a heterozygote for defect II; and black symbols indicate a double heterozygote.

other patients with type 3 disease appear to be homozygotes or compound heterozygotes for type 1 genetic defects, inheriting a different abnormal symptomatic gene from each parent. The severity of the disease in the latter is related to the inheritance of a double gene defect and not to a recessive defect.

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LABORATORY DIAGNOSIS

The standard laboratory work-up for vWD consists of five tests: vWF activity, vWF antigen, factor VIII activity, vWF multimeric analysis, and bleeding time (Table 1141). Analysis of the multimeric composition of vWF is required for accurate classification of vWD subtypes (see Chap. 106).

vWF Activity

Ristocetin co-factor activity (vWF:RCoF) is a functional assay for vWF that measures the vWF-supported agglutination of human platelets by ristocetin. Several methods of quantifying vWF activity have been described. [27] [28] [29] In most, ristocetin is added to a suspension of washed formalin- or paraformaldehyde-fixed platelets in the presence of patient plasma as a source of vWF, and agglutination is assessed macroscopically or in an aggregometer. The initial rate of agglutination is proportional to the concentration of plasma vWF. The vWF activity is compared with the activity of normal plasma (assigned an arbitrary activity of 1 U/ml) and expressed in units per deciliter or as a percentage of the activity in normal plasma. Normal plasma levels of vWF are 50200 U/dl. The vWF activity may be decreased artifactually in people with ristocetin-binding proteins in plasma (e.g., vancomycin). [30]

In some laboratories, vWF activity is measured by using platelet-rich plasma from the patient and performing ristocetin-induced platelet aggregation. Because this assay uses the patients own platelets, it gives abnormal results in conditions in which interaction of vWF with platelet membrane glycoprotein (GP) Ib (its receptor on the platelet surface) is impaired. Thus, in patients with Bernard-Soulier syndrome, a disorder in which there is a congenital absence of GP Ib, ristocetin-induced platelet aggregation is absent. [31] Ristocetin co-factor assay with fixed normal platelets is normal in the plasma of patients with Bernard-Soulier syndrome.

Another method for measuring vWF activity is by collagen binding assay. Patient plasma is added to pepsin-digested type III collagen immobilized on microtiter wells. Bound and therefore active vWF is detected using a polyclonal rabbit anti-human vWFhorseradish peroxidase conjugate. The vWF bound is compared with the binding from normal plasma (assigned an arbitrary activity of 1 U/ml) and expressed in units per deciliter or as a percentage of the activity in normal plasma.

TABLE 114-1 -- Laboratory Tests in von Willebrand Disease

Function	Test[a]
vWF activity	Ristocetin-induced platelet agglutination (vWF:RCoF) Ristocetin-induced platelet aggregation Botrocetin-induced platelet agglutination Glass bead retention
vWF antigen	Laurell immunoassay Enzyme-linked immunosorbent assay
Factor VIII	Coagulation assay
Plateletvessel wall	Bleeding time

RCoF, ristocetin co-factor; vWF, von Willebrand factor.

[a] Ristocetin is a glycopeptide derived from the actinomycete *Nocardia lurida.* Botrocetin is derived from the venom of *Bothrops jararaca* and other *Bothrops* spp.

vWF Antigen

The assay for vWF antigen (vWF:Ag) is usually performed with use of a rabbit antibody to vWF by either quantitative immunoassay (Laurell technique) or enzyme-linked immunosorbent assay.[32] [33] Because vWF is a very large glycoprotein, Laurell quantitative immunoelectrophoresis must be performed slowly (1218 hours) or the assay will be too sensitive to the smallest multimers of vWF, which migrate faster. The Laurell technique may overestimate the smallermolecular-weight multimers, resulting in a false elevation of vWF:Ag in type 2A vWD. The levels of vWF:Ag are compared with a normal human plasma pool and expressed as units per deciliter. Normal values are 50200 U/dl. Blood type significantly affects the normal level of vWF protein, type AB plasma having up to twice the vWF:Ag level as type O plasma. Some laboratories have developed different normal ranges for each blood type. [34] Because vWF is an acute-phase reactant, levels are increased during stress, pregnancy, and in the newborn after vaginal delivery. Reduced plasma vWF may be found in patients who are hypothyroid, but their levels return to normal when they become euthyroid.[35]

Factor VIII Activity

Factor VIII coagulant activity is usually measured by a one-stage assay using human factor VIII-deficient plasma as substrate. [36] Substrate plasmas that have been immunodepleted of factor VIII have also been used, but may give abnormal results because vWF is also removed during the immunodepletion. Normal factor VIII levels are 60150%. Factor VIII levels may also be reduced in patients with hemophilia A, factor VIII inhibitors, and diffuse intravascular coagulation.

Bleeding Time

The bleeding time is a general test of plateletvessel wall interactions [37] (see Chap. 131). In most places, the bleeding time is performed using a modification of the technique described by Ivy et al.[38] [39] [40] Standardized incisions are made on the volar aspect of the forearm, keeping venous pressure at 40 mm Hg with a blood pressure cuff. Blood is blotted to filter paper every 30 seconds and the bleeding time is taken as the time to cessation of bleeding. Although the bleeding time provides an indication of primary hemostatic function, it is difficult to standardize and dependent on the experience of the person performing the test.

Quick[41] observed that the bleeding time in vWD was sensitive to aspirin and that 650 mg of aspirin would prolong the bleeding more in a patient with vWD than in normal people. He proposed the aspirin tolerance test as a diagnostic test for vWD, although it is also prolonged in a variety of platelet function defects.

vWF Multimers

In normal human plasma, vWF is present as a series of repeating subunits, which represent multimers of the approximately 300-kd vWF protein. These multimers

range in size from a dimer of 600 kd to over 20,000 kd in increments of 600 kd (Fig. 1142). The highestmolecular-weight multimers mediate platelet adhesion. [42] Separation of vWF into its multimeric components is accomplished using sodium dodecyl sulfate (SDS)-agarose electrophoresis. [43] On SDS-agarose gels with higher amounts of agarose, fewer multimers are visible, but the resolution is enhanced so satellite bands are visualized, whereas on gels of lower agarose content, all the multimers may be visualized but the resolution is reduced. Thus, the highmolecular-weight multimers that mediate platelet adhesion are best characterized using 0.65% agarose.[44] On gels containing 23% agarose, satellite banding is enhanced such that the multimeric subunits appear in three or more discrete bands. [45]

Figure 114-2 von Willebrand factor (vWF) multimers. vWF multimers can be studied in low (0.65%) and high (23%) concentrations of agarose. The lower concentration is preferable to evaluate highmolecular-weight multimers, whereas the separation of complex multimers requires the high concentrations of agarose. **(A)** Illustration of 0.65% agarose separation of vWF multimers. Samples include plasma from patients with thrombotic thrombocytopenic purpura (lane A), diffuse intravascular coagulation (lane B), ventricular septal defects (lane C), normal plasma (lane D), severe, type 3 vWD (lane E), type 1 vWD (lane F), type 2A vWD (lane G), and type 2B vWD (lane H). **(B)** Complex multimer separation. Each multimer seen in 0.65% agarose can be resolved into three to five bands using the higher concentrations of agarose. Plasma from patients with types 2A and 2B vWD has an increase in the more rapidly moving satellite band (satellite band 5). *(Courtesy of R. R. Montgomery.)*

Other Tests

Platelet aggregation or agglutination with ristocetin or with botrocetin, a protein isolated from the venom of *Bothrops jararaca*, [46] is also useful for classification. Both compounds aggregate platelets in a vWF-dependent manner, although they interact with different sequences of the vWF molecule. [47] At 0.30.5 mg/ml, ristocetin causes aggregation of platelet-rich plasma from patients with type 2B and platelet-type, pseudo-vWD, but does not cause aggregation of platelet-rich plasma from normal patients or from patients with type 1 vWD.

Platelet adherence to columns containing glass beads is reduced in vWD, [48] but is not specific and is rarely used today. Another useful diagnostic test is the response to plasma infusion of normal or hemophilic plasma. In a patient with vWD, this results in a sustained rise in factor VIII activity, which contrasts with the shorter biologic half-life that is observed when normal plasma is transfused in patients with hemophilia. [10] This phenomenon, called de novo synthesis, may also have therapeutic benefits but is no longer used as a diagnostic test because of concerns about the viral safety of blood products.

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DIAGNOSIS

The diagnosis of vWD is established by finding reduced plasma levels of vWF activity, vWF:Ag, or factor VIII, or a prolonged bleeding time. In the classic case, all four laboratory tests are abnormal and the diagnosis is not difficult. However, on some occasions all four tests are normal, whereas on others, only one of the four is abnormal. When only the factor VIII is abnormal, vWD can be confused with hemophilia A, and when only the bleeding time is abnormal, it can be confused with a primary platelet disorder.

Repeated testing may be necessary to establish the diagnosis of vWD. The difficulty in diagnosing vWD has been emphasized by Goldin et al, [49] Miller et al,[50] and Abildgaard et al.[51] In the studies by Goldin et al, and Miller et al of two large kindreds with vWD, vWF activity, vWF:Ag, factor VIII, and bleeding time were determined and correlated with bleeding symptoms. The results indicated two important facts. First, within each kindred whose affected members all possessed the same genetic defect, there was remarkable diversity in the phenotype, with 8 different combinations of abnormal test results out of a possible 16 in one family and 10 in the other. Second, of 26 people who were genetically affected as determined by pedigree analysis, 11 had a normal panel of tests. Thus, normal values do not exclude a diagnosis of vWD. This conclusion is supported by the studies of Abildgaard et al, who found a normal panel of tests in 29 of 202 tests performed on 50 patients with confirmed vWD. In addition, they confirmed the phenotypic diversity observed by Goldin et al and Miller et al, with all 16 possible combinations of abnormal findings in their population of patients. Finally, they showed that when patients with vWD were followed with serial tests, the pattern of abnormality could change, sometimes within a matter of weeks, and that a person with vWD may demonstrate five or six different patterns [51] (Fig. 1143).

A recent study by Eikenboom and co-workers[26] may provide an explanation for some of the variability in vWD. They found that within a given kindred, the variability in laboratory findings could be roughly correlated with the gene defect, and that differences between people with the same gene defect within the kindred roughly correlated with blood group type. In addition, allelic interplay may account for some of the variability in vWD phenotype. For example, in one of Eikenbooms kindred, the phenotypic expression of the Normandy defect was affected by a second abnormal vWF allele. This suggests

Figure 114-3 Pattern of abnormalities in von Willebrand disease (vWD). From 25 families with vWD, 50 affected people were tested on a total of 202 occasions. The pattern of results in each person is shown by the dots in the column below that person. For example, in family 7, individual 1 was studied twice and showed an abnormal bleeding time and normal VIII, vWF, and vWF:Ag on both occasions. In family 5, individual 1 was studied on seven occasions and showed six different patterns of results, including all normal, isolated vWF abnormality, isolated bleeding time abnormality, bleeding time and vWF abnormality, bleeding time and vWF:Ag abnormality, and bleeding time, vWF, and vWF:Ag abnormality. BT, bleeding time; VIII, factor VIII activity; vWF, von Willebrand factor activity. *(From Abildgaard CF et al,[51] with permission.)*

that the underlying gene defect in vWD, along with other factors such as allelic interplay, blood type, pregnancy, and stress, play an important role in the phenotypic expression in the disease.

Because the factor VIII level is variably reduced in vWD, the activated partial thromboplastin time, which is typically prolonged only when the factor VIII level is <30%, is often normal in vWD. The prothrombin time and thrombin clotting time are normal, and the platelet count is normal in most patients but may be reduced in patients with type 2B or platelet-type vWD.

von Willebrand disease can usually be distinguished from classic hemophilia (Table 1142). The vWF activity, vWF:Ag, and bleeding time are normal in hemophilia. However, because some patients with vWD may present with an isolated factor VIII deficiency, it is important to take a careful family history to exclude an X-linked pattern of inheritance. Repeated testing may also be required to distinguish vWD from hemophilia. Qualitative platelet defects (i.e., defects due to aspirin ingestion) should also be considered in the differential diagnosis of vWD. A careful drug history may help to distinguish patients with drug-related platelet defects.

CLASSIFICATION

Historical studies using crossed immunoelectrophoresis showed that some patients had an electrophoretically abnormal protein, so it became possible to classify patients based on the presence or absence of such an abnormal molecule.[52] [53] [54] [55] By this technique, approximately 1015% of patients were shown to have an electrophoretically abnormal vWF protein. A subsequent classification of vWD was based on the findings of vWF multimer analysis.[56] The current classification of vWD is based more on molecular mechanisms. Three general types of vWD are

DIAGNOSTIC DIFFICULTIES IN VON WILLEBRAND DISEASE

Test	1	2	3	4	5	6
Factor VIII	89%	94%	67%	71%	35%	81%
vWF	101%	88%	81%	62%	55%	78%
vWF:Ag	95%	92%	75%	65%	52%	75%
Bleeding time	6.5	8	8	9	11.5	8

This case illustrates the difficulties that may be encountered in the diagnosis of vWD. The patient, a 34-year-old woman with a mild bleeding disorder characterized by excessive bleeding after wisdom tooth extraction, but no bleeding after an automobile accident complicated by a fractured femur, was tested on six occasions over a 2-year period. Her blood type was A, and she was not on oral contraceptives at any time during the testing period. The initial testing was negative, and multimeric analysis showed a normal pattern. Subsequent testing 6 months later was again within normal limits. Testing on a third occasion 3 months later showed a borderline factor VIII level and bleeding time, but was otherwise negative. Repeat tests 1 month later were negative, although the vWF and vWF:Ag were borderline. Testing performed 8 months later before dental work was positive and consistent with a diagnosis of vWD. Her last tests, 1 year after dental work, were again negative.

TABLE 114-2 -- Laboratory Test Results in von Willebrand Disease

	von Willebrand Disease	Hemophilia	Platelet Defect
Factor VIII	or nl		nl
vWF	or nl	nl	nl
vWF:Ag	or nl	nl	nl
Bleeding time		nl	

Ag, antigen; vWF, von Willebrand factor; nl, normal test results; , decreased test result; , increased test result.

recognized: type 1 vWD, which is a partial quantitative deficiency of vWF; type 2 vWD, which is a qualitative deficiency of vWF; and type 3 vWD, which is severe vWD (Table 1143).[57]

Type 1

In type 1 vWD, there is a partial quantitative deficiency of vWF. This is the most commonly diagnosed type of vWD, accounting for approximately 7080% of cases. The characteristic finding in type 1 vWD is the demonstration on multimer analysis that all multimers in plasma are present and in the same relative proportion as in normal plasma, but are reduced. The reduction in multimers is variable among patients and may be variable on different occasions in any given patient. Plasma levels of factor VIII, vWF, and vWF:Ag may be reduced, usually concordantly, but also may be normal, and the bleeding time may be either prolonged or normal.

Patients previously reported as IA, [58] I-1, I-2, and I-3,[59] and I-platelet normal and I-platelet low[60] are included in this category (Table 1144).

Several forms of type 1 vWD have been reported based on platelet vWF content. Most patients with type 1 vWD have a parallel reduction in plasma and platelet vWF. Patients with decreased plasma vWF multimers but normal platelet vWF multimers have been termed type I-2 [59] or type I-platelet normal[60] to distinguish them from the more common type 1 vWD, termed type I-1 or type I-platelet low. It has been suggested that platelet-low vWD is caused by defective production of

TABLE 114-4 -- Correspondence of Current Cassification of von Willebrand Disease with Previous Classification

Type	Previous Type	References
1	IA	[58]
	I-1, I-2, I-3	[59]
	I-platelet normal	[60]
	I-platelet low	[60]

2A	IIA	
	IIA-1, IIA-2, IIA-3	[59]
	I platelet discordant	[60]
	IB	[69]
	IIC	[70]
	IID	[71]
	IIE	[72]
	IIF	[73]
	IIG	[74]
	IIH	[75]
	III	[76]
2B	IIB	
	I New York	[81]
	Malmö	[82]
2M	B	[90]
	Vicenza	[91]
	IC	[92]
	ID	[93]
2N	Normandy	[95] [96]
3	III	

From Sadler, [57] with permission.

vWF, whereas platelet-normal vWD results from impaired release of vWF from cellular stores. An additional variant in which plasma vWF multimers were normal and platelet vWF multimers were reduced has been termed type I-3. [59] Interestingly, the bleeding time in patients with type I-platelet normal is normal or nearly normal, suggesting that platelet vWF may correlate more strongly with bleeding tendency. [60] [61] Furthermore, patients with normal platelet vWF may have a better response to 1-desamino-8-D-arginine vasopressin (DDAVP).

TABLE 114-3 -- Classification of von Willebrand Disease

Type	vWF	vWF:Ag	Factor VIII	Multimers	RIPA[a]	Cryo[b]
1 (partial quantitative deficiency of vWF)				Reduced multimer bands; normal multimer distribution		
2 (qualitative deficiency of vWF)						
2A (qualitative variants with decreased platelet-dependent function associated with absent highmolecular-weight multimers)				Large and middle multimers decreased		
2B (qualitative variants with increased affinity for platelet glycoprotein Ib)				Large multimers usually decreased	+	
2M (qualitative variants with decreased platelet-dependent function that is not caused by the absence of highmolecular-weight multimers)				Normal multimers, satellite banding may be abnormal		
2N (qualitative variants with markedly decreased affinity for factor VIII)	nl	nl		Normal multimers		
3 (nearly complete deficiency of vWF)				All multimers absent		
Platelet-type (qualitative defect in platelet glycoprotein Ib with increased affinity for vWF)				Large multimers decreased	+	+

Ag, antigen; nl, normal; RIPA, ristocetin-induced platelet aggregation; vWF, von Willebrand factor.

[a] Ristocetin-induced aggregation of the patients platelets in patient plasma with low concentrations (0.5 mg/ml) of ristocetin.

[b] *Cryo* refers to platelet aggregation in patient platelet-rich plasma after addition of normal human cryoprecipitate.

It is generally assumed that promoter mutations, nonsense mutations, frameshift mutations, and large deletions account for most type 1 defects. In some patients with type 1 disease, silent alleles that produce undetectable or greatly reduced amounts of mRNA for vWF have been demonstrated. [62] [63] [64] [65] Point mutations producing premature truncation of the vWF protein and missense mutations within functional domains of vWF have also been demonstrated. [26] [65]

Type 2

In type 2 vWD, there is a qualitative abnormality of vWF. Patients may have normal levels of vWF protein, but the protein is dysfunctional. These variants account for 1530% of cases.

Type 2A vWD refers to a qualitative variant of vWD in which there is defective plateletvWF interactions associated with the absence of the high and middlemolecular-weight vWF multimers in plasma. Satellite multimer bands may or may not be normal. The hemostatic abnormality in type 2A vWD is due to the absence of the hemostatically effective large vWF multimers. Platelet aggregation in response to ristocetin is reduced in proportion to the deficiency of highmolecular-weight vWF multimers. At least two distinct mechanisms account for the usual type 2A phenotype: (1) abnormal cellular assembly of vWF multimers, and (2) increased susceptibility of vWF to proteolysis in vivo. [66] [67] [68] Platelet vWF in patients with abnormal cellular assembly of vWF is similar to that in plasma and is normal in patients with increased susceptibility to proteolysis.

Patients previously classified as II-1, II-2, and II-3, [59] I-platelet discordant, [60] IIA, IB, [69] IIC, [70] IID, [71] IIE, [72] IIF, [73] IIG, [74] IIH, [75] and II-I [76] are included in this category (see Table 1144).

Evaluation of platelet mRNA from patients with type 2A vWD [67] [77] [78] [79] [80] has demonstrated missense mutations in the A2 domain (Fig. 1144). Expression of the cDNA for these defects in COS cells resulted in the suspected abnormal vWF multimers in some patients but normal multimers in others. [67]

Type 2B vWD is caused by a qualitative abnormality of vWF in which there is increased plateletvWF interaction due to an increased affinity of vWF for its platelet receptor, GP Ib. Patients previously classified as IIB, I-New York, [81] and Malmö [82] are included in this category (see Table 1144). In the usual form of type 2B vWD, there is an absence from plasma of only the

Figure 114-4 Molecular defects in types 2A and 2B von Willebrand disease. Schematic drawing of the A1 and A2 domains of von Willebrand factor (vWF) that lie within exon 28 and form the GP Ib-binding domain of vWF. Type 2A mutations are indicated by open circles. Type 2B mutations are indicated by closed circles. The mutations shown are those that have been established by in vitro expression.

highestmolecular-weight multimers. The middle and lowmolecular-weight multimers are present in normal amounts and in the same relative proportions as in normal plasma. The hallmark of type 2B vWD is an enhanced aggregation of the patients platelets in the presence of reduced concentrations of ristocetin. At a concentration of 0.30.5 mg/ml, ristocetin does not usually induce aggregation of normal platelets, but in type 2B disease, 0.30.5 mg/ml of ristocetin stimulates a full aggregation response. The highmolecular-weight multimers missing in plasma are present in platelets, suggesting that all of the vWF multimers are synthesized and that the absence of the highmolecular-weight forms is due to increased turnover of the highmolecular-weight multimers [83] because of the increased affinity of vWF for GP Ib. [84] Patients with type 2B vWD often have mild thrombocytopenia.

Most of the known mutations reported in type 2B vWD are within or very near a disulfide loop (C509C695) of vWF [85] [86] [87] [88] [89] that has been shown to interact with GP Ib (see Fig. 1144).

Type 2M vWD refers to qualitative variants in which there is decreased plateletvWF interaction that is not caused by the absence of highmolecular-weight multimers. Previous vWD types B,[90] Vicenza, [91] IC,[92] and ID[93] are grouped in this category (see Table 1144). The vWF multimer distribution is typically normal, and there may even be larger-than-normal multimers. The satellite bands may be abnormal. In vWD type B and type 2M $^{\text{Milwaukee-1}}$, the defect was shown to cause decreased binding of vWF to platelet GP Ib. [90] [94]

Type 2N vWD, also termed Normandy type vWD, is a qualitative abnormality of vWF in which there is defective interaction with factor VIII. This form of vWD masquerades as an autosomal form of hemophilia A.[95] [96] Patients with this variant have reduced levels of factor VIII, typically 515%, with normal levels of vWF and vWF:Ag. The bleeding time is normal. The molecular defect in vWD Normandy is in the region of vWF involved in binding factor VIII and results in impaired formation of the vWFfactor VIII complex. [97] [98] [99] [100] As a result, the half-life of factor VIII in the circulation is markedly shortened and factor VIII levels are reduced. The phenotypic expression of the Normandy variant occurs only if the person is homozygous for the defect or if the other allele is a second vWD allele with reduced or absent levels of normal vWF, such that only the abnormal factor VIII binding allele is predominantly expressed.

Type 3 (Severe Type)

Type 3 is a severe form of vWD, with nearly complete deficiency of vWF. Usually, vWF activity and vWF:Ag are undetectable, and factor VIII levels are typically markedly reduced (to <10%). The bleeding time is prolonged, usually to >20 minutes. Multimeric analysis of plasma from patients with type 3 disease shows essentially no multimers because of the marked reduction in vWF. This severe form of vWD may be the result of a homozygous defect or of compound heterozygosity.[101]

Four families with severe type 3 vWD have been found to have deletions of the vWF gene. [102] [103] Three families in Italy, one the product of a consanguineous marriage, were shown to have a homozygous deletion of the entire vWF gene, whereas a family in Wales was found to be homozygous for a 2.3-kb frameshift deletion that includes exon 42. In all four families, severe vWD was associated with the development of alloantibody inhibitors to vWF. Another family was identified in whom there was a complete heterozygous deletion of the vWF gene in the proband and one asymptomatic parent, suggesting that a different genetic abnormality was inherited from the second parent and that the proband was a compound heterozygote for vWF defects. An additional 20 patients with severe type 3 vWD were studied and had no evidence of a deletion.

Platelet-Type, Pseudo-vWD

Platelet-type, pseudo-vWD, first described by Miller and Castella [104] and by Weiss and co-workers,[105] is a primary platelet disorder involving the platelet receptor for vWF. Phenotypically, patients with platelet-type, pseudo-vWD are similar to patients with type 2B disease: multimeric analysis reveals absence of the highestmolecular-weight multimers; factor VIII, vWF activity, and vWF:Ag are variably reduced; and the bleeding time is prolonged. Aggregation is enhanced in response to low concentrations of ristocetin (0.30.5 mg/ml), and mild thrombocytopenia is commonly present. However, in contrast to type 2B vWD, in which the abnormality is in the vWF itself, the defect in platelet-type, pseudo-vWD is in the platelet receptor for vWF, which has an increased affinity for normal vWF. The deficiency of the highmolecular-weight multimers is thought to occur as a result of increased utilization secondary to the increased affinity of platelets for vWF. Platelet-type, pseudo-vWD can be distinguished from type 2B disease by addition of normal human cryoprecipitate to the patients platelet-rich plasma. [106] In platelet-type disease, the cryoprecipitate induces platelet aggregation, whereas in type 2B disease it does not. Platelet-type, pseudo-vWD can also be distinguished from type 2B disease by the differential binding of the patients plasma vWF to formalin-fixed platelets. Unlike the response to cryoprecipitate, this method can be performed with previously frozen plasma.[107]

The molecular causes of this disorder have yet to be clearly defined. A putative mutation at position 233 of the GP Ib -chain, resulting in a substitution of a valine for a glycine, has been reported.[108]

Acquired vWD

von Willebrand disease may be acquired in people who were previously normal. In most cases, this is caused by antibodies to vWF that neutralize vWF activity. Patients present with new-onset bruising and bleeding. vWF activity, vWF:Ag, and factor VIII are markedly reduced, and the bleeding time is prolonged. Inhibitors may occur in otherwise healthy people, but often are associated with an underlying disorder such as lymphoproliferative disorders, benign monoclonal gammopathies, and other diseases characterized by immunologic abnormalities. [109] [110] [111] [112] [113] [114] [115] [116] [117] [118]

Acquired vWD may also occur in the absence of antibodies when there is increased utilization of vWF. For example, people with cardiac disease, particularly ventricular septal defect or valvular heart disease, may acquire a deficiency of the highmolecular-weight vWF multimers and present with a pattern consistent with type 2B vWD, with decreased factor VIII, decreased vWF, decreased vWF:Ag, and an absence of high molecular-weight multimers. Repair of the cardiac defect may result in normalization of vWF.

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CLINICAL MANIFESTATIONS

The hemorrhagic tendency in vWD is highly variable and depends on the type and severity of disease. Patients with types 1 and 2 disease may have mild bleeding symptoms characterized by hemorrhage from delicate mucocutaneous tissues. Epistaxis, easy bruising, and gastrointestinal bleeding are common. Delayed hematoma formation and hemarthroses, characteristic of hemophilia A, are not a prominent feature in these patients. In women, the most frequent symptom is menorrhagia, which may be severe and out of proportion to other hemorrhagic symptoms. vWD should be prominently considered in the differential diagnosis of menorrhagia. Untreated, menorrhagia may lead to iron deficiency and anemia. Menorrhagia probably accounts for the disproportionate number of women who are

referred for evaluation of vWD. Post-traumatic, postsurgical, and dental bleeding may be severe and can be the presenting manifestation. Interestingly, pregnancy in women with vWD is usually tolerated well because plasma vWF and factor VIII levels increase during pregnancy, reaching a peak in the third trimester and falling rapidly postpartum. In patients with type 1 vWD, this rise is associated with a reduction in hemorrhagic symptoms. However, in patients with variants such as type 2B or platelet-type, pseudo-vWD, the elevated levels of abnormal vWF may increase the spontaneous binding to platelets and result in more marked thrombocytopenia. Similarly, levels of vWF and factor VIII are elevated in the newborn infant, particularly with vaginal delivery. This increase probably reduces the likelihood of bleeding but makes the diagnosis of vWD difficult in the newborn.

Although characteristic bleeding symptoms occur in patients with vWD, the absence of bleeding symptoms does not rule out this diagnosis. In the studies of Goldin et al,[49] Miller et al,[50] and Abildgaard et al,[51] approximately one half of patients with documented type 1 or 2 vWD had no history of bleeding despite reduced factor VIII or vWF levels on at least some occasions.

Patients with severe, or type 3, vWD have a severe hemorrhagic tendency. Spontaneous hemorrhage from mucous membranes and the gastrointestinal tract can be frequent and may be life threatening. Usually, hemorrhage after dental and other surgical procedures can be controlled only by replacement therapy. Perhaps because of the low factor VIII levels, deep hematoma and joint hemorrhages similar to those in hemophilia may occur. In women, menorrhagia is severe and, before hormonal therapy was available, often required extreme treatment such as ovarian ablation by radiation, a practice not currently recommended.

Angiodysplasia refers to small telangiectases in the wall of the small intestine and colon that occur in some patients with vWD, [119] [120] [121] more commonly in those >50 years of age and typically in those with type 3 disease. Although it is not clear that these are linked disorders, the presence of angiodysplasia in vWD can be a serious and disabling complication. Bleeding from angiodysplasia may present as melena or occult gastrointestinal bleeding, but typically presents with recurrent episodes of acute blood loss. Cycles of hematochezia resulting in hypotension and cessation of bleeding, followed days to weeks later by hematochezia, hypotension, and cessation of bleeding, may occur. Colonoscopy may reveal a bleeding site, but more often shows multiple telangiectases without a defined source of bleeding. Rarely, bleeding can be brisk enough to be visible by angiography. Operative intervention is not recommended unless a source of recurrent bleeding has been definitively identified. Estrogen therapy may be effective. [122]

Mitral valve prolapse has been reported to occur with increased frequency in vWD, perhaps as a linked mesenchymal disorder. [123] [124] However, a subsequent report describes a normal frequency of mitral valve prolapse in patients with vWD. [125]

Inhibitors to vWF in patients with vWD are alloantibodies, usually IgG, which inhibit the hemostatic function of vWF. They are uncommon and occur only in patients with severe type 3 disease,[126] among whom the prevalence of inhibitors has been estimated to be 78%. There is almost always a prior history of exposure to exogenous vWF, as either plasma, cryoprecipitate, or factor VIII concentrates. Patients with deletion of the vWF gene as the cause of their disease may be at higher risk for the development of inhibitors. [102] [103] A familial tendency to inhibitor formation has also been noted. [127] The presence of an inhibitor to vWF in a patient with vWD is suggested by the failure to respond clinically to replacement therapy, by decreased recovery of infused vWF, and by lack of correction of the bleeding time. Confirmation of the presence of an antibody requires the demonstration of an inhibitor of ristocetin-induced platelet aggregation

SURGERY IN VON WILLEBRAND DISEASE

Surgical and dental procedures, including major surgical procedures, may be safely undertaken in patients with most types of vWD. In patients with type 1 disease, treatment with DDAVP starting 1 hour before surgery and once a day thereafter for 23 days usually suffices for minor procedures such as dental extraction. For more extensive surgery, DDAVP may also be used, but Humate-P or Alphanate should be available should DDAVP fail to control hemostasis. Baseline factor VIII and bleeding time should be determined within 1 week of surgery. Approximately 12 hours before surgery, DDAVP is given at a dose of 0.3 g/kg body weight. If the baseline factor VIII or bleeding time is abnormal, these should be repeated 3060 minutes after DDAVP and shown to be normal before proceeding with surgery. After surgery, DDAVP may be given once a day until wound healing is complete. Factor VIII and vWF activity should be determined 3060 minutes after each dose of DDAVP.

In patients with type 2 vWD, the approach to surgery is similar to that in type 1 disease, except that replacement therapy with blood products usually is required. Because it is subjected to virucidal treatment, Humate-P or Alphanate is preferred by most physicians. Baseline factor VIII and bleeding time are determined within 1 week of surgery. Replacement therapy is started 12 hours before surgery at a dose calculated to correct the factor VIII level, and the factor VIII and bleeding time determinations are repeated to ensure correction before surgery. After surgery, treatment is given every 12 hours until wound healing is complete. Factor VIII and vWF activity are determined daily.

In patients with type 3 (severe) vWD, replacement therapy with Humate-P or Alphanate with or without DDAVP aimed at correction of the bleeding time and the factor VIII level is required for minor and major procedures. For minor procedures, treatment should be continued for 23 days; for major surgery, it should be continued for 510 days.

in the patients plasma. In most cases, antibodies to vWF also appear to inhibit factor VIII activity. [109] However, some antibodies do not inhibit function but promote accelerated clearance of transfused vWF. Those antibodies must be demonstrated by mixing the patients antibody with normal vWF and demonstrating antigenantibody binding. [126]

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THERAPY

The aim of therapy in vWD is correction of both the bleeding time and coagulation abnormalities. This is generally accomplished by raising plasma levels of vWF and factor VIII to normal. Because the bleeding time is sensitive only to the highestmolecular-weight multimeric forms, the source of the vWF and its multimeric composition are important. For example, intermediate-purity factor VIII concentrates contain large amounts of vWF, but most of this consists of low and middlemolecular-weight multimers.[128] As a result, vWF levels increase after administration of these concentrates, but the bleeding time is not corrected. [129] Several agents have been shown to provide effective therapy for patients with vWD; the choice of agent depends on the type of disease and the severity of bleeding.

DDAVP

After observations that epinephrine and stress increase levels of factor VIII and vWF in normal individuals, Ruggeri et al [127] and Mannucci et al[130] began studies to evaluate the effect of vasopressin and the synthetic vasopressin analog, DDAVP, on factor VIII levels in patients with factor VIII deficiency, including vWD. They showed that DDAVP had the same effect on factor VIII and vWF activity as vasopressin, but had <1% the pressor effect of the parent compound. Because of the rapid rise in factor VIII and vWF after administration of DDAVP, it has been suggested that the drug has a post-translational effect, perhaps through the release of vWF from intracellular storage sites in endothelial cells. One study has demonstrated the loss of vWF staining of capillary endothelial cells after DDAVP administration. [131]

DDAVP (desmopressin acetate; Rorer) is available in intranasal or intravenous forms. The intravenous preparation (4 mg/ml) can be administered intravenously or subcutaneously with essentially equivalent results. [132] [133] [134] In patients with type 1 vWD, a reproducible two- or threefold increase in vWF activity (and factor VIII) and shortening of the bleeding time occur within 1530 minutes of DDAVP administration. The dose of DDAVP is 0.20.3 g/kg body weight intravenously in a volume of 50100 ml over 30 minutes. For subcutaneous administration, the intravenous DDAVP preparation is used at the same dose with injection of <1.5 ml/site (a single treatment may require three or four subcutaneous injections). Two intranasal preparations are available, one of which, a dilute solution (100 g/ml) used for patients with diabetes insipidus, does not consistently raise plasma levels of vWF or factor VIII and should not be used in vWD. A more concentrated (1.5 mg/ml) intranasal preparation of DDAVP is effective, and at a dose of 150 mg (75 g/nostril) produces results equivalent to 0.2 g/kg of intravenous DDAVP. [135] [136] Because of the risk of hyponatremia, DDAVP should be administered no more than once a day and a serum sodium should be measured daily if DDAVP is administered on consecutive days. DDAVP should be used with extreme caution in infants because of the risk of seizures from fluid retention.

Because it stimulates a reproducible increase in vWF activity and is a synthetic product with none of the viral risks of plasma-derived products, DDAVP has become the treatment of choice in most patients with vWD. It is effective for most patients with type 1 disease, who have reduced amounts of a functionally normal vWF molecule. The effective response to DDAVP should preferably be demonstrated by carrying out a therapeutic trial infusion before the time when emergency or surgical use is required. Because it increases plasma levels of vWF by releasing endogenous stores, it usually is not effective in patients with types 2 or 3 disease, who either synthesize an abnormal protein or who do not produce functional vWF. However, reports of response to DDAVP in some patients with type 2A vWD suggest that these patients should have a trial of DDAVP to determine if it is effective. Treatment with DDAVP is ineffective in the treatment of type 3 vWD. However, DDAVP may enhance the response to cryoprecipitate in type 3 vWD, and may be useful when full correction of the bleeding time is required. [137] In type 2B and platelet-type, pseudo-vWD, the administration of DDAVP may result in increased thrombocytopenia. [138]

Plasma Products

Humate-P, a pasteurized, intermediate-purity factor VIII concentrate, and Alphanate, a solventdetergent-extracted, intermediate-purity factor VIII concentrate, have been reported to contain some of the highmolecular-weight vWF multimers and have been used to treat patients with vWD successfully, with correction of factor VIII, vWF, and bleeding time.[139] Other intermediate-purity factor VIII concentrates contain appreciable amounts of vWF, but only the middle and lowmolecular-weight multimers are present (Fig. 1145). Treatment with these concentrates can raise factor VIII and vWF:Ag levels, but the bleeding time may remain uncorrected. High-purity plasma factor VIII concentrates and recombinant factor VIII concentrates contain little or no vWF.

Cryoprecipitate is a plasma fraction that contains factor VIII, vWF, fibrinogen, and fibronectin, which is obtained by harvesting the precipitate that forms when frozen plasma is warmed to 4°C. The yield of factor VIII and vWF in cryoprecipitate can be increased two- to fivefold by administration of DDAVP to the blood donor before plasmapheresis. The full range of plasma vWF multimers is present in cryoprecipitate. Cryoprecipitate is now prepared from donors screened for antibody to human immunodeficiency virus type I (HIV-1), so that the risk of HIV transmission with cryoprecipitate is low. Nevertheless, cryoprecipitate has been reported to transmit both hepatitis B and hepatitis C, and because there is no virus inactivation step, there is a small but finite risk of HIV-1 transmission. The risk of viral transmission can be further reduced by using cryoprecipitate

Figure 114-5 von Willebrand factor multimers in commercial factor VIII concentrates. Multimer gel is run with 0.65% agarose. The lanes contain normal plasma (NP) and various commercial factor VIII concentrates: Koate-HP, Koate-HS, Humate-P, Kryobulin, Profilate-SD, and AHF-SD. The highmolecular-weight multimers are variably deficient in all of the concentrates. *(Courtesy of R. R. Montgomery.)*

TABLE 114-5 -- Therapy for von Willebrand Disease

Type	Treatment
1	DDAVP
2	
2A	Humate-P or Alphanate
2B	Humate-P or Alphanate
2M	Humate-P or Alphanate

2N	Humate-P or Alphanate
3	Humate-P or Alphanate plus DDAVP
Platelet-type	Platelet concentrates

DDAVP, 1-desamino-8-D-arginine vasopressin.

from parents or family members. [140] Each bag of cryoprecipitate contains approximately 100 U of factor VIII and 100 U of vWF activity. Because vWF levels are reduced in people with type O blood, plasma from donors with type A, B, and AB blood is preferred as the source of vWF. [141] Like DDAVP, cryoprecipitate may induce thrombocytopenia in patients with platelet-type, pseudo-vWD.

Although some patients with type 2A vWD have been reported to respond to DDAVP, the response is often transient. For this reason, plasma products are the treatment of choice in most patients with types 2 and 3 vWD (Table 1145).

Recombinant vWF

A recombinant vWF has been synthesized in Chinese hamster ovary cells, co-expressed with furin. [142] Compared with plasma vWF, the recombinant protein is fully glycosylated and undergoes proper protein processing, with dimer formation in the endoplasmic reticulum and N-terminal disulfide bonding to form multimers. Nevertheless, although recombinant vWF is highly multimered, the satellite multimer bands characteristic of plasma vWF, which result from cleavage of the Tyr842-Met843 bond by depolymerase, [143] are not present in the recombinant vWF. Thus, recombinant vWF is more like platelet vWF. Functionally, the recombinant protein binds to collagen like plasma vWF and has the same ristocetin co-factor activity as plasma vWF. The specific activity of the recombinant protein is 60 U/mg, and is comparable to plasma vWF. In preliminary studies in pigs with vWD, recombinant vWF corrects the hemostatic defect. [144]

Hormonal Therapy

Oral progestational agents increase factor VIII and vWF by unclear mechanisms. They may be useful in women, especially when menstrual bleeding constitutes the major clinical symptoms.

Other Treatments

Local hemostatic agents such as fibrinolytic inhibitors (Amicar, tranexamic acid), fibrillar collagen preparations (Avitene), and fibrin glue may be useful in external bleeding such as that due to dental procedures.

Complications

DDAVP may cause thrombocytopenia in patients with type 2B and platelet-type, pseudo-vWD. [138] [145] Reports of thrombosis associated with administration of DDAVP are rare. [146] [147] Water intoxication with extreme hyponatremia may also occur with DDAVP, and intravenous fluids must be administered with caution in patients receiving this drug. [148] Because of these effects on water balance, DDAVP is not recommended for children <1 year of age. Cryoprecipitate may transmit hepatitis viruses and, rarely, HIV-1. The risk of hepatitis must always be considered, and patients should be appropriately immunized when indicated. Humate-P and Alphanate appear to be safe from viral transmission. Inhibitors to vWF develop only in the most severely affected patients undergoing replacement therapy.

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PROGNOSIS

The prognosis in mild forms of vWD is excellent. Bleeding is typically mild, and life-threatening episodes of bleeding are rare. The risk of severe hemorrhage is greatly decreased by accurate diagnosis of the disease and education of both the patient and physician with respect to treatment. Patients with mild forms of vWD can expect a normal life span, but the prognosis in severe, type 3 vWD is more guarded; in the latter situation, life-threatening hemorrhage may occur despite the safe and effective forms of treatment available. The appearance of inhibitors in patients with severe vWD causes additional complications, often preventing adequate treatment.

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Chapter 115 - Vitamin K: Metabolism and Disorders

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INTRODUCTION

Vitamin K plays a critical role in the post-translational modification of the blood coagulation proteins, factors VII, IX, and X, and prothrombin, and of the plasma regulatory proteins, proteins C and S. Vitamin K serves as a cofactor for an enzymatic reaction that converts glutamic acid residues in the NH_2 -termini of these proteins into -carboxyglutamic acid residues (Fig. 115-1).[1] [2] There are three well-defined classes of -carboxyglutamic acid-containing proteins, although more are likely to be discovered: (1) the mammalian vitamin K-dependent blood clotting and regulatory proteins, (2) -carboxyglutamic acid-containing proteins of vertebrate mineralized tissue, and (3) conotoxins of the marine snail, *Conus*. The -carboxyglutamic acid residues confer essential metal-binding properties on the vitamin K-dependent proteins.[3] If -carboxylation is impaired either as a result of vitamin K deficiency or through pharmacologic intervention, the newly synthesized vitamin K-dependent proteins are secreted into the blood in their des--carboxy or "abnormal" forms. The des--carboxylated proteins cannot bind Ca^{2+}, and the physiologically important metal ion-mediated binding to phospholipid vesicles or cell membranes does not occur. [4] Since many critical enzymatic reactions of blood coagulation proceed on membrane surfaces, coagulation and its regulation are impaired. Therefore, vitamin K metabolism is essential to normal hemostatic and antithrombotic mechanisms. Indeed, inhibition of vitamin K action is the primary mechanism of oral anticoagulant therapy.

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MECHANISM OF ACTION OF VITAMIN K

In its role as a cofactor for the vitamin K-dependent carboxylase, vitamin K participates in a series of linked enzyme reactions that result in the generation of -carboxyglutamic acid and the regeneration of active cofactor. This series of reactions is known as the vitamin K cycle (Fig. 115-2). In addition to the enzyme, the reduced form of vitamin K and a glutamic acid-containing polypeptide substrate, the reactions require carbon dioxide and molecular oxygen. In the first reaction in the cycle (Fig. 115-2), an H^+ is abstracted from the -carbon of the glutamyl residue and a carboxyl group is added. [5] Concomitantly, the cofactor, vitamin K, is converted from the reduced to the epoxide form.[6] The enzyme that catalyzes these reactions is the vitamin K-dependent carboxylase, which catalyzes both carboxylation and epoxide formation. These two activities are associated with a

Figure 115-1 Reactants in the vitamin K-dependent carboxylation pathway.

Figure 115-2 The vitamin K cycle. Reduced vitamin K, the hydroquinone form, is a cofactor for the conversion of glutamate to -carboxyglutamate by the vitamin K-dependent carboxylase, also known as the vitamin K epoxidase. Concomitant with conversion of glutamate to -carboxyglutamate, the hydroquinone is converted to 2,3-vitamin K epoxide. The epoxide can be converted to vitamin K quinone by the vitamin K epoxide reductase. Vitamin K quinone can be reduced by two pathways to the vitamin K hydroquinone, completing the cycle.

single enzyme.[7] The vitamin K-dependent carboxylase is a single-chain protein with a molecular weight of 94,000 composed of a single polypeptide chain of 758 amino acids.[8] [9] [10] This enzyme is an integral membrane protein that binds directly to the -carboxylation recognition site on the precursor forms of the vitamin K-dependent proteins.[11] The vitamin K-dependent carboxylase is located in both the endoplasmic reticulum and the Golgi apparatus. [12] [13] However, carboxylation is an efficient process such that carboxylation of protein substrates is completed in the endoplasmic reticulum. [14] The presence of carboxylase in the Golgi apparatus is likely due to the recycling of the membrane-bound enzyme between the endoplasmic reticulum and the Golgi apparatus.

The active site responsible for glutamate binding, identified using the affinity label bromo-acetyl-FLEELY, is located in the N-terminal one-third of the protein. [15] The complementary site on the carboxylase that binds the propeptide carboxylation recognition site also resides in part in the N-terminal one-third of the protein. [16] Regions around residues 234, 406, and 513 define in part the propeptide-binding site while regions around residue 359 are involved in catalysis. [17] The vitamin K epoxidase activity of the protein requires a C-terminal region of this enzyme for this activity. [18] In the absence of propeptide and glutamate-containing substrate, the carboxylase lacks vitamin K epoxidase activity. When these substrates are added to the enzyme, vitamin K epoxidase activity is up-regulated. [19]

As epoxidation occurs concomitant with carboxylation and because vitamin K either from dietary sources or gut flora is in the quinone form, tissues in which -carboxylation takes place must have a mechanism for generating the reduced form of vitamin K. Several enzymes have been identified that perform this function. [20] Vitamin K epoxide reductase, a dithiol requiring enzyme, reduces vitamin K epoxide to vitamin K quinone (Fig. 115-2). Two enzymatic activities, including the epoxide reductase, have been identified that convert vitamin K quinone to the biologically active hydroquinone (Fig. 115-2). The epoxide reductase activity results from an enzyme complex that resides in the endoplasmic reticulum membrane and includes an epoxide hydrolase and a member(s) of the glutathione S-transferase super gene family.[21]

The mechanism by which vitamin K functions as a cofactor with the -carboxylase remains uncertain. The most attractive hypothesis is that an active oxygenated species of vitamin K abstracts a hydrogen from the -carbon of glutamic acid, with subsequent collapse of the activated vitamin K species to vitamin K epoxide and the addition of carbon dioxide to the -carbon of glutamic acid. [22] Oxygen and vitamin KH_2 -dependent exchange of tritium from tritiated water to the -carbon of a glutamic acid in a carboxylase substrate at low carbon dioxide concentrations have been taken as evidence of a carbanion mechanism. [23] Based on a nonenzymatic model, a "base strength amplification mechanism" has been proposed to explain the conversion of vitamin KH_2 into an oxygenated intermediate of sufficient basicity to abstract a hydrogen from the -carbon of a glutamyl residue in a carboxylase substrate. [24] [25] [26] This reaction scheme suggests that epoxidation of vitamin KH_2 by the vitamin K-dependent carboxylase can occur in the absence of formation of -carboxyglutamic acid but that carboxylation cannot occur in the absence of epoxidation.

NATURE OF THE SUBSTRATE

Although many tissues contain the enzymes to carry out -carboxylation and the reactions of the vitamin K cycle, the primary site of synthesis of the vitamin K-dependent proteins of the blood is the liver. These proteins are synthesized with both a signal sequence and a propeptide. The canonical leader sequences provide the signal that indicates that the protein is to be secreted and thus allows the growing polypeptide chain to be translocated to the rough endoplasmic reticulum. All sequences of the propeptides of the vitamin K-dependent proteins are highly homologous and unique to this class of proteins. [27] These propeptides serve as recognition signals that designate these proteins as substrates for the vitamin K-dependent

carboxylase.[28] The -carboxylation recognition site resides on the N-terminus of the propeptide. It is extended by a 10-residue amphipathic -helix. [29] The propeptide of the precursor forms of the vitamin K-dependent proteins serves three functions: (1) to direct -carboxylation by the -carboxylase, [28] (2) to inhibit binding of the carboxylated precursor proteins to membranes,[30] and (3) to up-regulate vitamin K epoxidase activity of the carboxylase. [19]

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VITAMIN K

Forms and Distribution

The vitamin K family of chemical compounds has many members. Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) is the major form of the vitamin found in plants. Animal tissue and bacteria produce menaquinones, a series of vitamin K forms similar in structure to vitamin K_1 but with varying lengths of unsaturated polyprenyl groups at the 3 position.

Nutritional Sources

Vitamin K is an essential fat-soluble vitamin. The diet is the primary source of vitamin K in humans. Leafy green vegetables, in particular, are a good source of vitamin K_1 ,[31] although vitamin K_1 is widely distributed in the normal human diet. A contribution to adequate vitamin K intake in humans may be provided by the vitamin K_2 synthesized by intestinal bacteria.[32] The daily dietary requirement for the vitamin has been estimated to be 100200 g/day. [33]

Physiology

Vitamin K is absorbed in the ileum. The presence of bile salts and normal fat absorption are required for effective uptake. The storage pool of vitamin K is modest. In the absence of a dietary source of the vitamin, this storage pool can be exhausted within 1 week in an otherwise normal person. Such a deficiency does not generally lead to clinical manifestations, as the vitamin K synthesized by gut flora is available to provide suboptimal but adequate synthesis of vitamin K-dependent proteins.

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VITAMIN K DISORDERS

Hemorrhagic Disease of the Newborn

Hemorrhagic disease of the newborn, due to vitamin K deficiency, develops during the first week of life, usually between days 2 and 7 (see Chap. 102).[31] [32] [33] [34] [35] [36] Clinical manifestations include bleeding in the skin or from mucosal surfaces, circumcision, or venopuncture sites. [37] Rarely, internal bleeding, including retroperitoneal or intracranial hemorrhage, is the primary manifestation of hemorrhagic disease of the newborn. These ominous complications are the rationale for the use of vitamin K prophylaxis in neonates.

Almost all neonates are vitamin K deficient, presumably as a result of deficient vitamin K nutriture in the pregnant mother during the third trimester and because of the lack of colonization of the colon by bacteria that produce vitamin K in the neonate. [38] However, this deficiency is further aggravated in some patients by inadequate dietary intake of vitamin K. This disorder is more prevalent in breast-fed babies, since human milk, in contrast to cow's milk, contains only 15 g/L of vitamin K. [37] [39]

Neonates with hemorrhagic disease of the newborn have a prolonged prothrombin time (PT) and partial thromboplastin time (PTT). However, it is critical to distinguish whether the prolongation of these times is a manifestation of the deficiency of the vitamin K-dependent proteins due to vitamin K deficiency or to decreased synthetic capacity of the liver in newborns. Elevation of the abnormal (des--carboxy) prothrombin (PIVKA-II) antigen level is indicative of vitamin K deficiency, since this form of prothrombin appears only when post-translational -carboxylation is impaired, but not when protein synthesis is impaired. [38] Administration of vitamin K (100 g) corrects the deficiency state and usually does not need to be repeated in the otherwise healthy infant.

Prophylactic vitamin K has been in use for in-hospital births for the past 30 years. Vitamin K (100 g to 1 mg) is administered intramuscularly to the newborn immediately after birth. At these doses, vitamin K administration carries little morbidity and can prevent hemorrhagic disease of the newborn. Some of these vitamin K protocols are under revision. [40]

Acquired Vitamin K Deficiency

Dietary Deficiency States and Antibiotics

The requirement for vitamin K is sufficiently low relative to the vitamin K content of a normal diet that clinically significant vitamin K deficiency does not occur as a result of inadequate dietary intake. Although sensitive markers of vitamin K deficiency, such as abnormal (des--carboxy) prothrombin antigen, [41] indicate that diet truly depleted of vitamin K can lead to mild vitamin K deficiency, no evidence shows that an inadequate diet alone can have clinical manifestations. Bacteria in the large intestine produce functional forms of vitamin K. In the absence of dietary vitamin K, small amounts of vitamin K in the large intestine are absorbed passively and prevent severe vitamin K deficiency. In patients medicated with antibiotics that destroy the intestinal flora, this vitamin K source is eliminated. Thus, a common setting of vitamin K deficiency is the case of inadequate or minimal dietary intake treated simultaneously with antibiotics (Fig. 115-3). This form of vitamin K deficiency occurs within 13 weeks, after depletion of body stores of vitamin K. [42]

Malabsorption syndromes are commonly associated with vitamin K deficiency. Defects in the enterohepatic circulation due to biliary disease interfere with absorption of fat-soluble vitamins in the ileum. Primary biliary cirrhosis, [43] cholestatic hepatitis, and other causes of cholestasis may lead to impaired absorption of vitamin K. Furthermore, intestinal malabsorption, as in sprue or regional enteritis, impairs vitamin K utilization. Older

REVERSAL OF LONG-ACTING VITAMIN K ANTAGONISTS ("SUPERWARFARINS")

The long-acting vitamin K antagonists employed as rodenticides lead to pronounced bleeding syndromes following factitious or accidental ingestion of these compounds. After diagnosis and confirmation of the diagnosis, treatment can remain challenging because the inhibition of the complete synthesis of the vitamin K-dependent proteins may continue for months after initial exposure, even in the absence of re-exposure. Fresh frozen plasma is used routinely to treat major bleeding complications, but this therapy is associated with a risk of blood-borne infection. Although it is desirable to correct or paritally correct the PT, the use of prophylactic fresh frozen plasma chronically carries significant risks and expense. Because of the potency of the second-generation rodenticides and their fat solubility, vitamin K_1 at normal doses of administration is ineffective. However, daily doses of 100150 mg of vitamin K_1 administered orally have been effective in normalizing the PT. Over time, the dose of vitamin K_1 needed to correct the PT can be adjusted downward, so that only the required amounts of vitamin K are employed.

Figure 115-3 Abnormal (des-carboxy) prothrombin antigen as a marker for vitamin K deficiency. Normal subjects have no detectable abnormal prothrombin. Vitamin K deficiency arises as a result of disorders of absorption, including mild abnormalities in otherwise normal elderly subjects, or because of impaired intake.

adults also have evidence of mild vitamin K deficiency, presumably because of intestinal malabsorption. [44]

Vitamin K Antagonists

Drugs that inhibit the reutilization of vitamin K lead to a buildup of vitamin K epoxide at the expense of vitamin K hydroquinone. Warfarin and related vitamin K antagonists, whether ingested accidentally, factitiously, or as an overdose of oral anticoagulant therapy, lead to a deficiency of the vitamin K-dependent proteins, prolongation of the PT and PTT, and clinical bleeding manifestations. [45] [46] [47] Although such patients are not vitamin K deficient, the clinical and laboratory manifestations due to vitamin K antagonists are identical to those of vitamin K deficiency. Factitious warfarin ingestion may be seen as a component of a major psychiatric disturbance. Despite repeated denials of use of such medications by suspect patients, this diagnosis must be considered in all patients with *acquired* deficiency of all the vitamin K-dependent proteins. Measurement of serum warfarin level can confirm such suspicions. With the introduction of second-generation rodenticides that inhibit vitamin K action, including brodifacoum, cases of factitious ingestion and accidental poisonings have been increasingly reported. [48] [49] [50] [51] [52]

[53] [54] [55] These long-acting poisons, now widely available (e.g., D-Con), can lead to prolongation of the PT for over 1 year after a single dose; fatalities have been reported.[56] Chemically distinct from warfarin, these compounds are not detected in serum warfarin assays. Specific serum assays have been developed. [57] [58] [59]

Other drugs cause a vitamin K deficiency-like state as well and respond to vitamin K therapy. Excessive doses of aspirin cause hypoprothrombinemia, [60] presumably due to oxidation of vitamin K. Although the mechanism is not completely understood, certain antibiotics, such as moxalactam and cefamandole, cause hypoprothrombinemia.[61] [62] [63] [64] Malnourished patients, even those with an initially normal PT, often develop vitamin K deficiency after intravenous antibiotic therapy.[65]

Hereditary Defects of Vitamin K Utilization

Hereditary combined deficiencies of the vitamin K-dependent proteins, including prothrombin, factor IX, factor X, and factor VII, have been documented in six pedigrees.[66] [67] [68] [69] [70] Although the molecular bases for this phenotype are mostly unknown, defects in the absorption and transport of vitamin K might be anticipated to manifest with this clinical presentation. In addition, abnormalities of the vitamin K-dependent carboxylase or other enzymes within the vitamin K cycle would lead to a deficiency of these proteins. In one case, a point mutation at residue 394 of the carboxylase appears to be the cause of combined deficiency of the vitamin K-dependent proteins in one family.[71] Some cases have been responsive to high daily doses of vitamin K.[66] Other cases have been resistant to vitamin K therapy, which emphasizes the heterogeneity of this disorder.

Therapy for Vitamin K Deficiency

Vitamin K deficiency is treated by the administration of vitamin K_1. The preferred route of administration depends on the urgency for correcting the bleeding tendency and on the risk of inducing local hematoma formation. If the bleeding is severe or life-threatening, fresh frozen plasma should be administered. Because of the risk of transmission of viral infection, the use of

THERAPY FOR VITAMIN K DEFICIENCY

The approach to the treatment of vitamin K deficiency depends on the clinical setting and the severity of bleeding. Except in the face of serious internal bleeding, reversal of the vitamin K deficiency by the administration of vitamin K is generally adequate. If the PT is significantly prolonged to indicate that a bleeding complication may be induced by intramuscular injection, that route of administration of vitamin K_1 (Aquamephyton) should be avoided. Since the delivery of vitamin K by the subcutaneous route is variable, intravenous vitamin K_1 (Aquamephyton, 1015 mg) is the recommended approach, as it ensures rapid delivery. However, intravenous vitamin K_1 does require monitoring, because of early reports of severe allergic reactions with the intravenous route of administration; care must be given to initiate rapid reversal of an untoward reaction. With vitamin K, the PT should return toward the normal range within 12 hours and should have corrected within 2448 hours. Serious bleeding complications attributed to vitamin K deficiency, such as intracranial bleeding, must be reversed immediately. Despite the rapid action of vitamin K, administration of vitamin K should be preceded by the infusion of fresh frozen plasma. This blood component contains all the vitamin K-dependent blood-clotting proteins. In sufficient quantities, fresh frozen plasma can correct, or nearly correct, the PT, as well as the bleeding tendency.

Patients with vitamin K deficiency without bleeding manifestations can be treated with oral vitamin K or, as in patients with chronic vitamin K deficiency secondary to malabsorption syndromes, with subcutaneous vitamin K.

blood products must be weighed carefully. There is no role for currently available concentrates of the vitamin K-dependent proteins because of the risk of transmission of viral disease.

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Chapter 116 - Inhibitors of Blood Coagulation

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Acquired inhibitors of blood coagulation, also known as circulating anticoagulants, are pathologic macromolecules in blood that directly inhibit blood clotting proteins or reactions involving blood clotting. Most have been characterized as antibodies. These inhibitors arise secondary to transfusion of plasma proteins in patients with hereditary bleeding disorders, or may arise de novo in patients with previously normal hemostatic mechanisms.

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ACQUIRED INHIBITORS OF FACTOR VIII

Clinical Setting

Virtually all inhibitors of factor VIII are recognized because of their interference with or neutralization of factor VIII activity. Most occur in four clinical situations: alloantibody inhibitors in hemophilia A, postpartum, in association with various immunologic disorders, and in older patients without any associated disorder. The last three occur with an estimated incidence of 0.21.0/1 × 10 persons/year. [1]

In 1981, Green and Lechner[2] surveyed 215 patients with factor VIII inhibitors in an effort to gather demographic information regarding spontaneously arising inhibitors to factor VIII. The gender incidence was approximately equal, and almost 60% of the patients were >60 years of age. No associated disease was found in 50% of patients, but rheumatoid arthritis, systemic lupus erythematosus (SLE), drug reactions, dermatologic disorders, and different types of malignancies (solid tumors and lymphoproliferative) were each seen in 815% of the total group. In 13.5% of cases, the disorder occurred during the postpartum period. These associations have been corroborated by other reports. [3] Overall mortality was 22%. Approximately 38% showed eventual spontaneous disappearance of the inhibitor. The spontaneous remission rate was confirmed by Lottenberg et al, [1] who reported that among 16 patients receiving no immunosuppressive therapy, 5 had spontaneous remissions.

Factor VIII Inhibitors Occurring in Patients with Immunologic Disorders and in Patients Without Underlying Disease

Immune disorders associated with factor VIII inhibitors include rheumatoid arthritis, [4] [5] SLE and other systemic autoimmune diseases,[4] [5] [6] penicillin or other drug reactions,[7] bronchial asthma, [4] [5] inflammatory bowel disease,[5] erythema multiforme,[5] dermatitis herpetiformis,[5] graft-versus-host disease,[8] and interferon-therapy.[9] [10] Occasionally, factor VIII inhibitors are associated with monoclonal gammopathies. Most patients have no underlying disease.

Factor VIII Inhibitors Occurring Postpartum

Factor VIII inhibitors can occur during the postpartum period, [4] [5] [6] [11] [12] [13] and rarely during pregnancy.[14] Most often, the inhibitor occurs after the birth of the first or second child. Although a bleeding tendency may become evident immediately or after a prolonged interval, there is usually a 2- to 5-month delay before the diagnosis is made. The course in these patients is variable, but the inhibitor disappears spontaneously in most patients after 1218 months. [11] In 51 patients with postpartum inhibitors, the survival rate was 97% at 2 years, and Kaplan-Meier analysis revealed a probability of complete remission of almost 100%. [13] The median time to complete remission was 11 months, an interval shortened with immunosuppressive drugs but not with steroids alone.

In patients who became pregnant again after the occurrence of a postpartum factor VIII inhibitor, [12] none of those in remission have had a recurrence. The cause of these postpartum inhibitors is unknown.

Properties of Spontaneously Acquired Factor VIII Inhibitors

With rare exceptions, factor VIII inhibitors are IgG antibodies. These inhibitors have restricted heterogeneity because many possess either or light chains. [5] [7] [15] [16] [17] [18] [19] [20] [21] These inhibitors frequently contain mixtures of heavy chain subclasses. Hultin et al [19] found that both hemophilic and nonhemophilic inhibitors contain mixtures of IgG_1 and IgG_4 subclasses. The high incidence of factor VIII inhibitors containing a subpopulation of IgG_4 is significant because this subclass constitutes <5% of plasma IgG. The inhibitors are specific for factor VIII activity, and most do not interfere with the activities of von Willebrand factor (vWF) within the factor VIIIvWF complex. vWF antigen and function are normal in patients with such inhibitors. Factor VIII inhibitors do not participate in complement fixation; [5] [6] they show species specificity both in vitro and in vivo in that human factor VIII is generally neutralized to a greater extent than bovine or porcine factor VIII. Infusion of bovine or porcine factor VIII into a patient with an inhibitor often raises the factor VIII level. [22]

The kinetics of the reaction between factor VIII and most hemophilic inhibitors is first order with respect to both factor VIII

and to inhibitor (type I).[23] [24] The inhibitor can be completely neutralized by excess factor VIII.[23] [25] A time dependence of the neutralization of factor VIII sometimes requires 12 hours to reach equilibrium at low inhibitor concentrations. [23] [24] Neutralization occasionally takes place in an initial rapid phase, followed by a very slow second phase.[26] [27] [28] [29] By contrast, inhibitors arising in most nonhemophiliac patients frequently differ in their reaction kinetics from inhibitors arising in hemophiliac patients.[27] [30] [31] A linear relationship between the inhibitor concentration and the amount of factor VIII inactivated is observed with hemophilia inhibitors (type I), but not with most spontaneous inhibitors (type II). Thus, most spontaneous inhibitors do not totally inactivate factor VIII in vitro. Although most factor VIII inhibitor patients have no factor VIII in the plasma, the occasional patient exhibits some plasma factor VIII activity. [6] [28] [31] [32]

Epitope Specificity of Factor VIII Antibodies

The antigenic regions on the factor VIII molecule to which neutralizing factor VIII inhibitors bind have been identified. [33] [34] [35] [36] [37] [38] [39] [40] [41] [42] [43] The epitopes to which alloantibodies or autoantibodies are directed are limited to certain areas of the factor VIII light or heavy chains. [33] [34] [35] [36] [37] [38] [39] [40] [41] [42] [43] The factor VIII molecule consists of two series of repeated homologous domains and a single B domain arranged in the following order: A1-A2-B-A3-C1-C2 (see Chap. 107). Small regions rich in acidic amino acids are located between A1 and A2 (called AR1), A2 and B (AR2), and B and A3 (AR3). [39] The heavy chain consists of A1-AR1-A2-AR2-B and the light chain consists of AR3-A3-C1-C2. [39] The A and C domains are required for functional activity, whereas the B domain is not. Anti-A2 and -C2 antibodies are present in approximately 68% of plasmas tested by immunoprecipitation assays using recombinant polypeptide fragments of factor VIII. [43] [44] [45] In addition, 46% of antibodies contain antibodies that bind to AR3. [43] [46] There are also less common AR1, A3, and C1 antibodies and antibodies to a light chain epitope outside of C2.[43] [47] [48] The antibody or combinations of antibodies that inhibit factor VIII activity usually consist of those with A2, C2, and AR3-A3-C1 epitopes because of the ability of polypeptides containing those epitopes completely to neutralize inhibitor activity. [39] In addition to the C2 epitope, there probably exists one other distinct light chain epitope and an epitope in AR1. [39]

Antibody patterns in hemophiliac patients differ from those in autoantibody patients. [39] Forty-eight percent of autoantibody patients have C2 as their sole antibody, whereas this pattern occurred in only 1 of 34 hemophiliacs. [39] Moreover, 62% of autoantibody patterns are directed to a single epitope, in contrast to only 17% of hemophilic patterns. Fewer autoantibody patients contain anti-A2 and anti-AR3-A3-C1 antibodies, and the occurrence of A2 plus C2 antibodies is also less common. Hemophiliacs treated with either plasma-derived factor VIII or recombinant factor VIII have equally complex patterns, but differ in epitope specificity from one

another.[39] The relative amounts of antibody to the A2 and C2 domains can vary, and neither level is always correlated with the inhibitor level. [39] [42]

Anti-C2 antibodies interfere with factor VIII function by blocking the binding of factor VIII to phospholipid, [39] [40] whereas anti-A2 antibodies inhibit activated factor VIII function by blocking the formation of the complex of factor IXa, factor VIII, and factor X. [41] [43] Some antibodies can interfere with vWF binding, [39] [43] [49] [50] [51] and some antibodies reduce the rate at which thrombin-activated factor VIII is released from vWF. [52] The inhibitor produced by a given patient may change over the course of time and a few non-neutralizing antibodies may occur. [1] [33] [34] [35] [36] [37]

Recognition, Identification, and Quantitation of Factor VIII Inhibitors

The presence of a factor VIII inhibitor should be suspected whenever a patient with no prior bleeding history presents with spontaneous massive bruising or with large, unexplained hematomas. The partial thromboplastin time (PTT) is prolonged, whereas the prothrombin time (PT) and thrombin time are normal. The PTT of a mixture of the patients plasma and normal plasma is also prolonged, although inactivation of factor VIII may require preincubation for 12 hours. [26] [27] [53] [54] Confirmation that an inhibitor acts specifically with factor VIII requires incubating the patients plasma diluted with an equal volume of normal plasma and performing assays of factors VIII, IX, XI, and XII at 0, 60, and 120 minutes. If the inhibitor is specific for factor VIII, only factor VIII decreases over time. A technique that uses the PTT rather than a factor VIII assay detects weak inhibitors of <0.4 Bethesda units. [53] [54]

Quantitative inhibitor measurements are important in the emergency management of hemorrhage and for evaluation of long-term therapy. A standard unit of measurement, termed the Bethesda unit,[55] is best suited for the measurement of inhibitors arising in hemophiliacs, but the complexity of reaction kinetics and variations in antibody affinities for factor VIII, particularly in nonhemophilic inhibitors with high residual factor VIII, limit standardization. Because of the latter, it may be difficult accurately to determine the inhibitor titer in nonhemophiliac patients with spontaneously acquired inhibitors. The results of the Bethesda assay underestimate the actual in vivo level of human factor VIII autoantibodies. Thus, assay results are less valuable in guiding therapy than they are in hemophiliac patients with inhibitors. Moreover, there is little correlation between the factor VIII level, titer of inhibitor, and degree of prolongation of the partial thromboplastin time with bleeding complications. In England, the New Oxford method is used to quantitate factor VIII inhibitors, [56] with 1 Bethesda unit equaling approximately 1.21 times 1 New Oxford unit. The Bethesda assay does not ensure pH control during the incubation period and this may result in nonantibody-mediated inactivation of factor VIII. A modified assay has been proposed that replaces the imidazole buffer with immunodepleted factor VIII-deficient plasma. [57]

Therapy of Patients with Factor VIII Inhibitors

The major objectives in the treatment of these patients are twofold: (1) the treatment of acute bleeding episodes, and (2) the elimination of the autoantibody to factor VIII. When a patient with an inhibitor hemorrhages, conservative measures, including immobilization, compression, and possibly epsilon-aminocaproic acid, should be considered. The use of intramuscular injections or aspirin-containing compounds is contraindicated.

The indication for the use of factor VIII concentrates or factor IX complex concentrate is serious active bleeding. If the patient is not actively bleeding, transfusion is not indicated. Unlike inhibitors associated with hemophilia A, most spontaneously acquired inhibitors are not inducible when the patient is exposed to factor VIII. When the patient is bleeding, porcine factor VIII, [3] highly purified human factor VIII, or recombinant factor VIII is indicated. Human autoantibodies usually have less affinity for porcine factor VIII than for human factor VIII and cross-reactivity with autoantibodies is <5%, compared with 2025% with alloantibodies. [3] Consequently, less concentrate is necessary to achieve effective hemostasis. [3] [22] [58] A report from the International Acquired Hemophilia Study Group provided data on 65 patients with factor VIII autoantibodies treated with porcine factor VIII. [59] The treatment schedule varied from a simple infusion to three to four infusions/day for several days. The average number of infusions per patient was 11, with the average length of treatment being 8.5 days. A wide range of doses was used, but the mean initial dose was 85 IU/kg. An excellent or good response was reported in 78% of the patients, a fair response in 12%, and poor response in 10%. The pretreatment median level of the anti-human factor VIII inhibitor was 38 Bethesda units, with a range of 1.21,024, whereas the average

MANAGEMENT OF SPONTANEOUSLY ACQUIRED FACTOR VIII INHIBITORS

CONSERVATIVE MEASURES

When a patient with an inhibitor has a hemorrhage, conservative hemostatic measures should be used to complement whatever other therapy is used. These measures include immobilization, compression, local application of hemostatic agents, and, on occasion, administration of epsilon-aminocaproic acid. The patient should not receive intramuscular injections, unnecessary venipunctures, aspirincontaining compounds, or nonsteroidal anti-inflammatory drugs. If the patient has a low inhibitor level (<3 Bethesda units) then desmopressin (DDAVP) 0.3 g/kg can be given immediately.

SELECTION OF A BLOOD PRODUCT

For patients who are actively bleeding, therapeutic options include treatment with porcine factor VIII concentrate (Hyate:C), human plasma-derived or recombinant factor VIII concentrates, factor IX complex concentrates, and recombinant factor VIIa. If the patient is not actively bleeding, transfusion is unnecessary. Unlike inhibitors associated with hemophilia A, most spontaneously acquired inhibitors do not increase in titer after exposure to factor VIII. Thus, this consideration should not impede transfusion therapy. Because human autoantibodies usually have less affinity for porcine factor VIII than for human factor VIII, porcine factor VIII concentrate is preferable to achieve effective hemostasis.

The titer of the inhibitor and its reactivity to porcine factor VIII determines the amount of factor VIII to be used. If the titer is low (<5 Bethesda units), a large dose of factor VIII sufficient to neutralize all of the circulating inhibitor and provide enough excess factor VIII to achieve a plasma factor VIII of 0.3 U/ml (30%) can be given. This can be usually achieved by giving a rapid infusion of porcine factor VIII of approximately 75 U/kg, or alternatively by giving 150 U/kg of human factor VIII concentrate followed by a continuous infusion of 10 U/kg/hr. Alternatively, a large amount of either porcine or human factor VIII can be given every 14 hours, giving 20 units of factor VIII/kg for each Bethesda unit plus an additional 40 U/kg. If a patient has a moderately high inhibitor level of 530 Bethesda units, the inhibitor level may be lowered by approximately 5065% by a 1.5-volume plasmapheresis, or alternatively by using one of several methods of extracorporeal immunoadsorption. After apheresis or immunoadsorption, the patient is initially given either porcine factor VIII (75100 U/kg) or human factor VIII (150 U/kg), followed by a continuous infusion in the same dose noted previously. If plasmapheresis cannot be done promptly or if the patient has a very high titer inhibitor (>30 Bethesda units), it is impossible to achieve hemostatic levels of factor VIII except by using porcine factor VIII (100150 U/kg) or by bypassing the site of inhibitor action with factor IX complex concentrate with a dose of 75 U/kg or recombinant factor VIIa at a dose of 90 g/kg. The inhibitor plasma should be tested against porcine factor VIII in vitro before its use but, if it is not immediately available, then assume that it is approximately <5 Bethesda units and infuse porcine factor VIII as noted previously. If neither human nor porcine factor VIII inhibitor titers are readily available, then freeze plasma to be subsequently sent to a reference laboratory and infuse porcine factor VIII. Continuous use of repeated infusions over an extended period may result in the development of antibodies to the porcine factor VIII, resulting in a lack of efficacy. In a rare patient, anaphylaxis complicates therapy with the porcine concentrate.

IMMUNOSUPPRESSION OF THE INHIBITOR

The long-term goal in the management of these patients should be aimed at immunosuppression of the autoantibody production. Although many immunosuppressive regimens have proved efficacious, we initiate therapy with intravenous IgG (1 g/kg) for 2 days. Immunosuppressive therapy with prednisone 80 mg/day PO and cyclophosphamide (2 mg/kg/day PO) is initiated concurrently with IVIG. Inhibitor titers are determined every 12 weeks. This regimen is continued for 4 weeks. If a significant decrease in titer occurs (>50% reduction), the regimen is continued until there is no further decrease in titer. If there is no significant decrease in titer, then the regimen is discontinued. Alternatively, a similar immunosuppressive regimen can be used, preceded by an infusion of factor VIII. Human factor VIII (50100 U/kg) is followed by prednisone (100 mg/day for 5 days), cyclophosphamide (500 mg/m^2 IV on day 1), and vincristine (2 mg IV on day 1). This regimen is repeated at 34 weeks. If the neutrophil nadir is >2,500/l, then the cyclophosphamide dose is increased to 750 mg/m^2. If the inhibitor titer is not decreased after three cycles, the regimen is discontinued.

If the preceding regimen is unsuccessful, the patient is placed on azathioprine (2 mg/kg) and inhibitor titers are performed. If no significant response occurs in 8 weeks, the regimen is discontinued and cyclosporine or interferon- therapy is tried.

level of antiporcine factor VIII was 2 Bethesda units, with a range of 015. The clinical response was not related to the titer of antibody to either human or porcine factor VIII, the dose of porcine factor VIII infused, or the factor VIII level after the initial infusion. The concentrate was well tolerated by 90% of patients, with only one anaphylactic reaction. No episodes of significant thrombocytopenia occurred. Ten patients (15%) had an increase in antiporcine factor VIII inhibitor titers. An important parameter in determining the type and amount of factor VIII to be infused is the titer of the inhibitor and its reactivity to porcine factor VIII. If the titer is low (<5 Bethesda units), a large dose of factor VIII sufficient to neutralize all the circulating inhibitor and provide enough excess factor VIII to achieve a plasma factor VIII of 0.3 U/ml can be given. This can usually be achieved with the rapid infusion of porcine factor VIII (5075 U/kg) or human factor VIII (150 U/kg), followed by a continuous infusion at 10 units kg/hr. Alternatively, a large amount of either porcine or human factor VIII can be given every 14 hours, giving 20 units of factor VIII/kg for each Bethesda unit plus an additional 40 units/kg. [60] If a patient has a moderately high inhibitor level of 530 Bethesda units, the inhibitor level may be lowered by approximately 5090% by plasmapheresis or, alternatively, by one of several methods of extracorporeal immunoadsorption. [61] [62] After apheresis or immunoadsorption, the patient is given a bolus of either porcine factor VIII (75100 U/kg) or human factor VIII (150 U/kg), followed by a continuous infusion of factor VIII. If the patient has a high-titer inhibitor of >30 Bethesda units, it is impossible to achieve hemostasis, except with porcine factor VIII (100150 U/kg), factor IX complex concentrate (5075 U/kg), or recombinant factor VIIa (90 g/kg). Inhibitor plasma should be tested against porcine factor VIII in vitro before its use; continuous use or repeated

infusions over an extended time may result in the development of antibodies to the porcine or human factor VIII. [3] [59] In an occasional patient, allergic reactions or thrombocytopenia complicates therapy with porcine factor VIII. [3] [59] [63]

Factor VIII levels must be assayed frequently to monitor the plasma factor VIII level achieved in vivo, particularly after the infusion of a factor VIII concentrate. The infused factor VIII may be efficacious in vivo before it is inactivated.

Factor IX complex concentrates can also be used to control bleeding in patients with factor VIII inhibitors, particularly when the inhibitor titer is high. [64] [65] [66] [67] [68] These concentrates exhibit a procoagulant activity that bypasses the factor VIII-dependent clotting reactions. Two types of factor IX complex concentrate have been used in the treatment of patients with factor VIII inhibitors: regular unactivated intermediate-purity factor IX complex concentrates (Konyne 80, profilnine HT, and Bebulin); and activated concentrates, so-called anti-inhibitor coagulant complex. Both types of concentrates contain various amounts of activated factors VII, IX, and X and phospholipids, and should be distinguished from the highly purified factor IX concentrates used in the treatment of hemophilia B. The activated concentrates (Autoplex; FEIBA) contain a greater amount of the factor VIII bypassing material and are specifically designed for patients with factor VIII inhibitors. Both types of concentrates have proved efficacious in hemophiliacs with inhibitors in controlled clinical trials. [67] [69] However, some investigators have used anti-inhibitor coagulant complex in life-threatening situations in which the unactivated concentrates were ineffective. Factor IX complex concentrates are also effective in achieving hemostasis in patients with high-titer spontaneously acquired inhibitors. In a recent French multicenter retrospective survey of the use of FEIBA in 60 patients with factor VIII or IX inhibitors (6 of whom had spontaneously acquired inhibitors) with 433 bleeding episodes or surgical procedures, efficacy was good to excellent in 81.3% of the episodes, whereas it was poor in the remainder. [70] Adverse effects were seen in only 5 of 433 episodes (12%), but an anamnestic response occurred in 17 of 54 evaluable patients (31.5%).

Purified components of the prothrombin complex have been used to treat hemorrhages in patients with inhibitors. A highly purified concentrate containing factor VIIa achieved good hemostasis[71] and, subsequently, a recombinant preparation of human factor VIIa has shown significant efficacy in patients with inhibitors. [72] [73] In a survey of more than 240 patients, many of whom consisted of patients with spontaneously acquired factor VIII inhibitors, the recombinant factor VIIa concentrate was used successfully for critical bleeds or for surgery. [74] Dosage and interval of dosing is still being evaluated, but factor VIIa therapy is initiated with doses of 90 g/kg, and this dose is repeated every 23 hours (half-life is 2.9 hours) for one to two times for spontaneous bleeds and every 23 hours for 48 hours for surgical procedures, followed by tapering doses over several days. Efficacy has been reported in approximately 90% of patients with spontaneous bleeds and in approximately 80% of patients undergoing surgical procedures. [74] Treatment is not influenced by the inhibitor titer, and no specific assay reflects clinical efficacy. Antibodies to factor VII do not occur except in factor VII deficient patients. [75] The safety profile has been excellent and thrombotic events are rare.

Long-term goals in the management of these patients should be aimed at immunosuppression of the autoantibody production. Assessment of immunosuppressive therapy is difficult because inhibitors occasionally disappear spontaneously, particularly in patients with postpartum inhibitors and patients with drug-induced inhibitors.[2] Many patients respond to corticosteroids alone; in one report of 16 patients receiving only steroids, [76] 7 were complete responders, 4 partial responders, and 5 nonresponders. The time to response averaged approximately 16 days. In their 1981 survey, Green and Lechner [2] found that inhibitors disappeared in 22 of 41 steroid-treated patients. In a prospective, multicenter, controlled clinical trial, antibody disappeared in 10 of 30 patients treated with prednisone alone (1 mg/kg) in the first three weeks, and in 3 of 4 other patients randomized to continue prednisone after the first 3 weeks of prednisone therapy. [77] In patients resistant to steroids, the use of cytotoxic agents, particularly cyclophosphamide either alone or in combination with corticosteroids, can be efficacious. In the controlled trial noted previously, antibody disappeared in three of six patients randomized to oral cyclophosphamide and prednisone after the patients were resistant to prednisone alone in the first 3 weeks. Eight patients did not respond. In this trial, the titer of antibody was significantly lower in responders than in nonresponders, and most responses occurred within 6 weeks of starting therapy.

The use of factor VIII concentrate infusion before immunosuppressive therapy was first proposed by Green. [78] The hypothesis proposed that the factor VIII concentrate infusion stimulates the abnormal clone of immune cells responsible for autoantibody synthesis and results in a greater cytotoxic effect by the immunosuppressive therapy. A combination of cyclophosphamide, vincristine, and prednisone every 3 weeks, preceded by an infusion of factor VIII concentrate (50100 U/kg) resulted in complete disappearance of the inhibitor in 11 of 12 patients. [79] The responses occurred rapidly, usually within only two or three courses, and remissions in all patients were durable over 25 years.

The initial inhibitor titer may be an important prognostic factor in the treatment of these patients. [80] Patients with inhibitor titers of <510 Bethesda units respond rapidly, whereas those with higher titers respond more slowly or fail to respond.

In a recent report, nine consecutive patients with a spontaneously acquired factor VIII autoantibody were treated with oral cyclophosphamide (100200 mg/day) and prednisone (5080 mg/day) until the inhibitor titer reached zero, followed by slowly decreasing doses. [81] All patients achieved a complete remission, with a median time of 3 weeks to resolution of all bleeding symptoms and a median time to undetectable factor VIII inhibitor of 12 weeks (range, 337 weeks). Two patients had a relapse as the immunosuppressive drugs were tapered, but the patients reattained a complete response with reinstitution of therapy. Initial titers of factor VIII inhibitor levels ranged from 2.5 to 1,040 units (mean, 174), and the patients with higher inhibitor titers required a longer duration of therapy. The results of this study, along with the results of the prospective clinical trial reported by Green et al, [77] strongly suggest that the initial immunosuppressive treatment of choice is oral prednisone and cyclophosphamide; a preliminary dose of human factor VIII is unnecessary. Immunosuppressive therapy should be continued as long as the inhibitor titer is decreasing because patients with high inhibitor titers respond more slowly than patients with low inhibitor titers. Patients with inhibitors who have high residual factor VIII levels in vitro (0.040.34 U/ml) respond well. [32]

Azathioprine in doses of 100200 mg/day (or 2 mg/kg/day) has also proved effective; Green and Lechner [2] found 19 of 28 patients to respond completely. In addition, Söhngen et al[82] reported five patients who responded to a combination of corticosteroids and azathioprine within 6 weeks of initiating therapy. There have also been cases reported in which cyclosporine [83] or interferon- [84] resulted in a complete response.

Any response to immunosuppressive therapy generally occurs within 612 weeks. If the titer has not decreased significantly in that period, therapy should be discontinued. Although most patients who obtain a complete response do not recur, an occasional patient relapses early at the time of tapering or withdrawal of immunosuppressive therapy, and an occasional patient relapses several months later.

Spontaneously acquired factor VIII inhibitors respond to

high-dose intravenous IgG (IVIG). [85] In a prospective, multicenter study of high-dose IVIG, 19 patients received either 1 g/kg × 2 days or 400 mg/kg × 5 days, followed by periodic maintenance doses. [86] A rapid decline in inhibitor titer occurred in 34 days in two patients and a gradual decline occurred in four other patients, with nadirs occurring several weeks to many months after starting therapy. Concurrent therapy with prednisone could have contributed to the response in two patients. Thus, the response rate was estimated to be between 25% and 37.5%. The mechanism by which patients with inhibitors respond to intravenous IgG is unknown. However, IgG may have both a short- and long-term immunosuppressive effect. [87] [88] [89] Anti-idiotypic antibodies are present in the IgG preparations; [90] [91] [92] [93] the emergence of anti-idiotypic antibodies may be the explanation for the occasional spontaneous remission and some instances of remission induced by immunosuppressive agents. [91] [94] [95]

The administration of 1-deamino-8-*D*-arginine vasopressin (DDAVP) can briefly raise the factor VIII level in patients who have very low inhibitor titers (<3 Bethesda units) and whose antibodies have type II reaction kinetics. [96]

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ACQUIRED INHIBITORS OF VON WILLEBRAND FACTOR

Patients with von Willebrand disease may acquire inhibitors after plasma transfusion. Moreover, an acquired syndrome similar to hereditary von Willebrand disease with onset later in life has been described. [97] [98] [99] [100] [101] [102] [103] [104] [105] [106] [107] [108] This acquired syndrome may occur in previously healthy people, or it may be associated with SLE, lymphoproliferative disorders, myeloproliferative disorders, hypothyroidism, and Wilms tumor. The presence of an inhibitor is frequently associated with a monoclonal immunoglobulin, but whether the serum M component has inhibitory activity has not been determined unequivocally. [103] [104] [106] [107] The clinical course in these patients has been variable, from mild bruising and mucous membrane bleeding to severe and life-threatening bleeding.

Laboratory studies usually show a reduction of ristocetin co-factor activity, vWF antigen, and factor VIII activity, and a prolonged bleeding time. In some cases, the patients plasma impairs ristocetin co-factor activity of normal plasma [99] [104] [105] [106] or results in a decreased level of vWF in normal plasma. Recently, in two cases of acquired von Willebrand disease, IgG antibodies could be demonstrated by a competitive enzyme-linked immunosorbent assay (ELISA) procedure. [109] In contrast, inhibitory activity cannot usually be demonstrated in the patients plasma when incubating the patients plasma and normal plasma, and measuring vWF activity, vWF antigen, or factor VIII. [104] [105] [106] [107] In one patient,[101] correction of the abnormality followed radiation therapy for lymphoma; in another patient, who had Waldenström macroglobulinemia,[103] vWF was detected on the monoclonal lymphocyte surface. These findings would suggest that in some patients with this syndrome, vWF can bind to cellular surfaces. With few exceptions, [100] none of the inhibitors had inhibitory activity in vitro against factor VIII clotting activity.

Multimer analysis of plasma from patients with acquired von Willebrand disease has yielded variable results. In 7 patients, [104] multimer analysis showed no alteration of multimeric structure (similar to type I von Willebrand disease), whereas 10 other patients [104] [105] [106] lacked highmolecular-weight multimers (similar to type II von Willebrand disease). These findings would suggest that these antibodies are heterogeneous and are directed against different epitopes of the vWF molecule.

Thus, acquired von Willebrand disease may result from several different pathophysiologic mechanisms. In one type, the inhibitor inactivates the biologic activity of vWF. In a second type, the inhibitor binds to a site on the vWF molecule lacking biologic activity, thus causing rapid clearance of vWF from the circulation because of the formation of immune complexes. In a third type, vWF binds to cellular surfaces. In the second and third types, no inhibitor activity against vWF can be demonstrated in vitro.

Infusion of plasma or cryoprecipitate in patients with acquired von Willebrand disease does not result in the expected increase in vWF activity. The secondary rise in factor VIII activity, characteristically seen in transfused patients with hereditary von Willebrand disease, does not occur. Despite these findings, transfusion of cryoprecipitate or factor VIII concentrate containing vWF activity (Humate-P, alphanate, or koate HP) or administration of DDAVP in a bleeding patient may result in a transient and blunted rise in vWF activity, vWF antigen, and factor VIII, such that effective hemostasis may be achieved. Several cases of acquired von Willebrand disease have been reported that responded rapidly to high-dose intravenous IgG (400 mg/kg/day × 5 or 1 g/kg × 2 days). [110] [111] [112] [113] Although the responses may be transient, in a few patients the response may last for 23 weeks. Moreover, after recurrence repeat administration of IVIG may result in repeated responses.

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ACQUIRED INHIBITORS OF FACTOR V

Rarely, factor V inhibitors occur in patients with hereditary factor V deficiency after transfusion. [114] However, most factor V inhibitors occur spontaneously in older, previously healthy patients in the absence of a common underlying disease. [114] [115] [116] [117] [118] [119] [120] [121] [122] [123] [124] [125] The most common association seen in approximately two-thirds of the cases is a surgical procedure preceding the development of the inhibitor. [126] Other significant associations include blood transfusion, -lactam antibiotics, and malignant diseases. [126] Many of the cases that followed surgery were associated with the use of topical bovine or fibrin glue. Both of these substances contain bovine thrombin and small amounts of bovine factor V that can result in the production of anti-bovine thrombin antibodies or anti-bovine factor V antibodies that cross-react with human thrombin and factor V. [127] Despite the various associations with factor V antibodies, in approximately 20% of cases no associations can be identified, [126] and in these patients, true autoantibodies to factor V presumably are present.

The degree of clinical bleeding in these patients varies, in contrast to the uniformly severe bleeding in patients with factor VIII inhibitors. This extreme variabilityfrom no clinical sequelae to fatal hemorrhagecan possibly be explained by the accessibility of platelet factor V to the antibody. [124] [125] Studies by Nesheim et al [124] on a patient with a factor V inhibitor suggest that platelet factor V is relatively protected from an anti-factor V antibody in whole blood, even though plasma factor V is completely neutralized. Thus, platelet factor V may play a key role in maintaining hemostatic competency in the presence of a factor V inhibitor. This hypothesis is supported by the efficacy of platelet concentrates in a factor V inhibitor patient. [119] Fresh frozen plasma failed to effect hemostasis, but on four separate occasions, effective hemostasis was achieved with platelet transfusions. Two other patients responded to platelet transfusions, but others were not responsive. The course of the factor V inhibitor is short lived in most patients, disappearing within 10 weeks. Removal of the antibody by plasmapheresis or immunoadsorption resulted in decreased bleeding in some patients. [126] Whether steroids or other immunosuppressive agents influence the natural history of the inhibitors is unclear. The inhibitor may disappear with immunosuppressive therapy, [119] [122] [123] or it may disappear spontaneously without specific therapy. [114] [118]

The laboratory identification of a factor V inhibitor is based on coagulation assays. Both the PTT and PT are prolonged, and normal plasma fails to correct these assays. These findings in the presence of a normal thrombin time should strongly suggest the presence of a factor V inhibitor. [114] The demonstration that a mixture of the patients plasma and normal plasma specifically

lacks factor V clotting activity is required for a definitive diagnosis. Most of the factor V inhibitors are polyclonal IgG, but one was identified as both IgM and IgG, and another as IgA and IgG. [118]

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ACQUIRED INHIBITORS OF FACTORS VII, IX, AND X AND PROTHROMBIN

Except for the prothrombin antibody associated with the lupus anticoagulant, spontaneously acquired inhibitors to factors VII, IX, and X or prothrombin are rare. Bajaj et al[128] described a patient in whom hypoprothrombinemia developed because of an antibody that bound to a prothrombin epitope without inhibition of coagulant activity. Unlike the hypoprothrombinemia associated with the lupus anticoagulant, this patients plasma did not contain antibody activity against anionic phospholipids, and the antibody bound to the NH_2 -terminal end of the prothrombin molecule.

Hawiger et al[129] described a patient with SLE and severe thrombocytopenia, in whom spontaneous bleeding from multiple sites developed, whose plasma contained an antibody that reacted with bovine thrombin. Scully et al [130] reported a patient who bled excessively after dental extraction and from the gastrointestinal tract. This patient had a prolonged PTT, PT, and thrombin time and a normal Reptilase time, and the immunoglobulin fraction of her plasma bound to both thrombin and prothrombin. Barthels and Heimburger[131] described a patient with cirrhosis and bleeding who had a prolonged PTT, PT, and thrombin time. The patients IgG prolonged the thrombin time but did not affect fibrin polymerization. In addition, in three other patients with an autoimmune disorder, thrombin inhibitors associated with bleeding developed,[132] [133] [134] and an occasional patient has been described with a thrombin inhibitor without any underlying disease. [135] In contrast, a rare patient may acquire a thrombin inhibitor affecting the anticoagulant function of thrombin causing thrombosis rather than bleeding. [136] [137] Stricker et al[138] described three postsurgical patients with prosthetic cardiac valves, in whom antibodies to human and bovine thrombin developed. All three patients had prolonged PTT and thrombin time. The thrombin time did not correct on mixing with normal plasma or protamine, but the Reptilase time was normal. Further studies of these patients have shown that these antibodies develop in patients exposed to topical thrombin or fibrin glue preparations during surgical procedures. [139] [140] [141] [142] In fact, in 3350% of patients exposed to such preparations, antibodies to bovine thrombin or bovine factor Va [127] [139] [140] [141] [142] develop that can cross-react with their human counterparts, resulting in prolonged clotting tests, particularly the thrombin time. [141] [142] Fortunately, most of these patients do not bleed, but some have excessive bleeding that can be fatal.

Two patients in whom IgG autoantibodies to factor VII developed had a prolonged PT but a normal PTT. [143] [144] In addition, Weisdorf et al[145] described a patient with severe aplastic anemia who had acquired factor VII deficiency. A factor VII-binding immunoglobulin in the patients plasma bound to factor VII, resulting in rapid clearance of the immune complex. There also have been several patients with hereditary factor VII deficiency who have become alloimmunized after being treated with recombinant factor VIIa concentrate.[75]

Spontaneously acquired factor IX inhibitors are extremely rare. [146] [147] [148] [149] [150] The clinical setting in which these inhibitors occur is similar to that associated with the development of factor VIII inhibitors. Three were associated with SLE and two with the postpartum state. Most seem to disappear within 17 months of onset, but whether immunosuppressive therapy alters the natural history is unknown.

Three documented cases of a factor X inhibitor have been reported. [151] [152] These patients were elderly without underlying disease who had the sudden onset of a bleeding diathesis. In two of three cases, the inhibitor was characterized as IgG antibodies and disappeared spontaneously without specific therapy.

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ACQUIRED INHIBITORS OF FACTORS XI AND XII

Inhibitors directed against factors XI or XII have been reported. [153] [154] [155] [156] [157] [158] [159] Some were detected in patients with hereditary factor XI deficiency after transfusions, whereas most occurred spontaneously in patients with SLE. Many of these patients also had low factor XII levels. Zucker et al [156] studied several patients on long-term chlorpromazine therapy in whom asymptomatic IgM inhibitors of the intrinsic phase of blood coagulation developed. The inhibitor resulted in decreased measurements of all the plasma clotting factors in the intrinsic pathway and was shown to interfere with the coagulant activity of contact product. These inhibitors may be similar to those described by Canoso et al [160] in patients on long-term, high-dose phenothiazine therapy. Except for those with hereditary factor XI deficiency, none of the patients in whom inhibitors to the contact system developed has had excessive bleeding.

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ACQUIRED INHIBITORS THAT AFFECT FIBRIN STABILIZATION, FIBRINOGEN, OR FIBRIN POLYMERIZATION

The terminal phase of blood coagulation is characterized by the cleavage of fibrinopeptides A and B from fibrinogen, polymerization of fibrin monomers, and the covalent cross-linking of the - and -chains of fibrin by factor XIIIa. Spontaneously acquired inhibitors of each of these reactions have been described.

Inhibitors of fibrinogen have been reported in patients with hereditary afibrinogenemia after transfusion. [161] In addition, Marciniak and Greenwood [162] reported a patient in whom a polyclonal IgG antibody developed that caused a delay in the release of fibrinopeptide A from normal fibrinogen reacting with thrombin, which retarded the onset of clot formation. This patient had a prolonged PTT, PT, thrombin time, and Reptilase time. The isolated inhibitor prolonged the thrombin time of normal plasma.

A high incidence of prolonged plasma thrombin time has been observed in patients with SLE. Galanakis et al [163] found that two such patients had monoclonal antibodies that reacted with different parts of fibrin and fibrinogen, delaying fibrin polymerization. In addition, autoantibodies that inhibit fibrin monomer polymerization have developed in some patients. [164] [165]

Although a variety of abnormalities of hemostasis are associated with monoclonal gammopathies, [166] isolated M proteins can interfere with the conversion of fibrinogen to fibrin monomer or with the aggregation of fibrin monomers into polymers. [166] [167] [168] Coleman et al[168] provided indirect evidence that at least some M proteins act as antibodies because Fab fragments of IgG monoclonal proteins were more inhibiting than intact protein, the isolated chains, or the Fc fragments.

Spontaneous inhibitors of fibrin stabilization can lead to severe hemorrhagic complications, some fatal. [5] [7] [169] [170] [171] [172] [173] [174] [175] [176] [177] [178] Of the 14 reported patients, 6 showed the development of an inhibitor while taking isoniazid.

Because noncross-linked fibrin clots could result from neutralization of factor XIII by (1) inhibition of activation to XIIIa, (2) inhibition of factor XIIIa, or (3) interference with the cross-linking sites on fibrinogen, the inhibitors may have more than one site of action. A patient who had been taking isoniazid for 8 years, described by Rosenberg et al,[176] was shown to have an antibody directed against the cross-linking sites on fibrinogen, but factor XIII levels were normal. By contrast, an inhibitor prevented the activation of factor XIII by thrombin but did not bind factor XIIIa. [172] [175] In other cases, the inhibitor interfered directly with factor XIIIa activity.

A classification scheme for the three types of factor XIII inhibitors is based on the step of the reaction sequence that is inhibited. Type I inhibitors are directed against the activation of the factor XIII zymogen but do not interfere with transamidase activity. Type II inhibitors impair the transamidase activity of factor XIIIa. Type III inhibitors complex with the fibrin substrate and prevent its binding by factor XIIIa. A fourth type of inhibitor was recently reported [179] where the antibody inhibited factor XIII activity by binding to the fibrin binding site on factor XIII. This inhibitor was characterized as an IgG antibody, and disappeared with immunosuppressive therapy.

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HEPARIN-LIKE ANTICOAGULANTS

The development of spontaneously acquired heparin-like anticoagulants has been reported in patients with neoplasms or those undergoing suramin therapy for the treatment of adrenocortical carcinoma.[180] [181] [182] [183] [184] [185] [186] [187] Reports of heparin-like anticoagulants associated with neoplastic disorders [180] [181] [182] [184] [185] [186] include cases of plasma cell malignancies and a severe hemorrhagic disorder.[179] [180] [183] Coagulation studies are characterized by a prolonged thrombin time that can be corrected by the addition of protamine sulfate, toluidine blue, or heparinase to the patients plasma. All these inhibitors possess biochemical and physicochemical properties of glycosaminoglycans. One patient treated intravenously with protamine sulfate showed an improvement in his laboratory study results, as well as decreased bleeding.[184]

A complex coagulopathy was reported in three women receiving suramin for adrenocortical carcinoma.[183] The severe hepatocellular dysfunction that developed accounted for some of the coagulopathy. In addition, each patient acquired a potent inhibitor to the thrombin time, which increased markedly during exacerbations of hepatic injury. Anticoagulant activity was eliminated in vitro by a combination of heparitinase and chondroitinase ABC, suggesting that the activity was mediated by heparan sulfate and dermatan sulfate. Because suramin inhibits enzymes that normally degrade glycosaminoglycans, hepatic injury was hypothesized to cause the release of glycosaminoglycans, which then accumulate because of failure of degradation.

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LUPUS ANTICOAGULANT

General Considerations

The lupus anticoagulant is an antibody that prolongs phospholipid-dependent coagulation tests in vitro. It was given this name in 1972 because clear proof of its site of action was lacking and because the anticoagulant had been recognized in patients with SLE. [7] It is a misnomer because the lupus anticoagulant is more frequently encountered in patients without lupus, [7] [188] and is associated with thrombosis rather than with bleeding. [7] [188] Immunoglobulins reacting with other hemostatic factors, such as vWF, [189] factors VIII, [190] IX, and XI, and inhibitors of thrombin and fibrin polymerization, have also been described in patients with SLE, but they are rare compared with the lupus anticoagulant.

Patients with the lupus anticoagulant who do not have established SLE fall into several different categories: (1) patients with lupus-like chronic autoimmune disorders but without findings that fit the criteria for the diagnosis of SLE; [191] [192] [193] (2) patients with other chronic systemic autoimmune disorders; (3) patients presenting with a venous or arterial thrombotic event for which no underlying cause may be apparent; [194] [195] [196] [197] [198] [199] (4) patients receiving certain drugs, including procainamide [200] and phenothiazines [16] [201] (a high prevalence of the lupus anticoagulant and a positive antinuclear antibody test are observed in psychotic patients receiving long-term chlorpromazine therapy [201]); other drugs or biologics that may induce the lupus anticoagulant include hydralazine, quinidine, and possibly -interferon; (5) patients with a recent acute viral infection, [202] [203] in whom the antibody is usually transient; (6) patients with human immunodeficiency virus infection; [204] [205] [206] [207] (7) women with recurrent fetal wastage; [192] [208] [209] [210] [211] [212] [213] [214] [215] and (8) patients seeking medical attention for a variety of disorders in whom the lupus anticoagulant is discovered as an incidental finding (frequently, such cases are discovered because of a prolonged PTT performed as a routine preoperative evaluation).

The antiphospholipid antibody syndrome can be defined by the presence of the lupus anticoagulant, the presence of elevated levels of anti-cardiolipin antibody plus one or more of the following: (1) venous thromboembolism, (2) arterial thromboembolism, (3) increased fetal loss, or (4) thrombocytopenia. The syndrome may be primary when not associated with SLE and secondary when it occurs in association with SLE. [216] [217]

The prevalence of lupus anticoagulant in patients with SLE in which the PTT test was used for its detection is approximately 10%. [7] [218] However, a higher prevalence of approximately 50% is found when a modified PTT with a reduced concentration of phospholipid is used to detect the anticoagulant. [192] Moreover, using the kaolin clotting timea test with increased sensitivity for detecting low-titer lupus anticoagulants because it contains no added exogenous phospholipidevidence of inhibitor activity can be demonstrated in the plasma of 3050% of randomly selected patients with SLE. [219] The percentage approximates the 4244% incidence of the frequency of anti-cardiolipin antibodies in patients with SLE. [220]

The prevalence of positive tests for lupus anticoagulant and anti-cardiolipin antibody in a normal population has been reported in several studies. [221] [222] [223] Because of the non-Gaussian distribution of anti-cardiolipin antibody levels in normal subjects, the cut-off points between normal and abnormal results is difficult to determine. [216] [223] The prevalences of elevated levels of IgG and IgM anti-cardiolipin antibody in a nonpregnant population were 57% and 59%, respectively, whereas the prevalence of lupus anticoagulant was 4%. The prevalences of elevated levels of IgG and IgM anti-cardiolipin antibody in healthy pregnant women were 23% and 4%, respectively. [224] [225] [226] Most of these were low titer; only 0.2% were high titer.

Properties, Epitope Specificity, Mechanism of Action, and Relationship to Anti-cardiolipin Antibodies

Both lupus anticoagulants and anti-cardiolipin antibodies are immunoglobulins that were originally thought to react only with phospholipid. Further studies of antibody specificity revealed that anti-cardiolipin antibodies were directed against epitopes on $_2$ -GPI [227] [228] and lupus anticoagulants were directed against epitopes on prothrombin. [229] [230] These studies initially postulated that these proteins, when bound to phospholipid, formed neoepitopes to which antibody developed. However, recent studies now suggest that these antibodies react directly with epitopes on $_2$ -GPI and prothrombin, and then the antigenantibody complex subsequently binds to anionic phospholipid. [229] Anti-cardiolipin antibodies are low-affinity monovalent antibodies to $_2$ -GPI when in solution, and the monovalent complexes bind weakly to anionic phospholipids. However, when the antigen density is high, bivalent complexes are formed that have a high affinity for phospholipid surfaces. [231] [232]

The lupus anticoagulant is either an IgG or IgM immunoglobulin. In some patientsfor example, those in whom it arises secondary to long-term chlorpromazine therapyit is IgM; [201] in other patients, it is IgG, [188] and in still others, it may be both IgG and IgM. [188] Transplacental transfer of the inhibitor has been reported, [233] which would be compatible with an IgG inhibitor.

The immunoglobulins responsible for the lupus anticoagulant effect react to in vitro assay systems with anionic phospholipids. Reducing the phospholipid component of clotting

DIAGNOSTIC APPROACH TO INHIBITORS

The approach to patients with abnormal coagulation tests should initially include a complete history regarding excessive bleeding, particularly as it relates to hemostatic challenges and thrombotic problems, either venous or arterial. Initial laboratory studies should include a repeat of the abnormal coagulation test on a mixture of the patients plasma and normal plasma. Correction of the abnormal test indicates a deficiency state, whereas poor correction indicates the presence of an inhibitor.

APPROACH TO INHIBITORS ASSOCIATED WITH BLEEDING

Patients who present with an acquired bleeding disorder and in whom an inhibitor is demonstrated most often have antibodies to a specific clotting factor. Subsequent laboratory evaluation should attempt to demonstrate the specific coagulation factor to which the antibody is directed and to determine the specificity of the antibody. The former is done using a specific assay for the coagulation factor suspected of being affected, and the latter is done using a specific assay for the coagulation factor of a mixture of various dilutions of patients plasma (antibody) and normal plasma (antigen) over time. If the inhibitor is specific for a single clotting factor, procoagulant activity of that coagulation factor is neutralized over time, whereas other clotting factor assays are unaffected. For example, if an acquired inhibitor of factor VIII is suspected and the factor VIII assay is low, a factor VIII assay of a mixture of the patients plasma and normal plasma should demonstrate a progressive decrease in factor VIII activity from immediately after the mixture was made to 12 hours later. By contrast, if factor IX and XI assays were done on the same mixture, factors IX and XI would be unaffected.

Rarely, a spontaneously acquired autoantibody reacts with a noncoagulant epitope of a specific hemostatic factor (e.g., prothrombin in the lupus anticoagulant-hypoprothrombinemia syndrome) or a specific clotting factor is adsorbed to cellular surfaces (e.g., vWF) or to amyloid (e.g., factor X). In these patients, the addition of normal plasma to the patients plasma results in correction of the abnormality. In these patients, the in vivo half-life of the affected hemostatic factor is shortened significantly because of rapid clearance of the antigenantibody complex or by adsorption of the clotting factor to a pathologic surface.

Spontaneously acquired heparin-like anticoagulants differ from the aforementioned inhibitors in that they are not immunoglobulins, and they do not specifically affect one specific clotting factor. These anticoagulants are easily recognized because their major effect is on the thrombin time, but the Reptilase time is normal.

APPROACH TO INHIBITORS NOT ASSOCIATED WITH EXCESSIVE BLEEDING

If the patient has a history of thrombotic disease or repeated episodes of fetal loss, he or she should be evaluated for the presence of a lupus anticoagulant or elevated levels of anticardiolipin antibody. Most commonly, patients with the former have a prolonged PTT and a normal or slightly prolonged PT. However, on occasion, the PT may be more prolonged than usual, even in the absence of an associated prothrombin deficiency. If the patient is suspected of having a lupus anticoagulant, several relatively simple tests can be used to confirm the diagnosis. For sensitivity, the kaolin clotting time as modified by Exner, the dRVVT, or the Staclot lupus anticoagulant test are excellent tests with very good sensitivity. Some believe that more than one test should be done if a lupus anticoagulant is suspected. Correction of a prolonged clotting time by the addition of phospholipid as described by Rosove or Triplett is specific for the lupus anticoagulant. In the presence of a lupus anticoagulant with a prolonged PTT, specific intrinsic clotting factor assays are frequently low but increase with increasing dilution of test plasma. For example, assays for factor IX would be 5%, factor XI 8%, factor XI 9%, and factor XII 10%, and all would progressively increase with increasing dilution of the test plasma. In some cases, the factor XI assay is more affected than the other assays. Thus, the lupus anticoagulant may affect all the specific assays, in contrast to an antibody to a specific clotting factor, for which a single assay shows irreversible inactivation of that clotting factor. In the latter case, dilution would not result in an increase in the level of that clotting factor.

mixtures amplifies the effect of the inhibitor. Thus, the most sensitive tests for detecting the inhibitor contain only limited amounts of procoagulant phospholipid: the recalcification time of platelet-free plasma, [234] the kaolin clotting time of platelet-poor plasma, [219] [235] [236] the dilute Russell viper venom time (dRVVT), [237] and the PT performed with very dilute tissue factor (the tissue thromboplastin inhibition [TTI] test). [191] [236] Adding liposomes containing phosphatidylserine, [238] rabbit brain phospholipid, [239] freeze-thawed platelets [240] [241] or hexagonal phospholipids [242] [243] markedly shortens the prolonged PTT of plasma containing the lupus anticoagulant and is used as a confirmatory test to increase specificity for presence of a lupus anticoagulant when there is a prolonged clotting test.

Patients with the lupus anticoagulant also frequently have evidence of plasma immunoglobulin reacting with the phospholipid, cardiolipin. This was first recognized as a high prevalence of an associated chronic biologic false-positive test for syphilis, [188] [234] [244] a test in which cardiolipin is the antigen. Anti-cardiolipin antibody assays are measured by an ELISA using microtiter plates coated with cardiolipin. [245] Bovine serum is added to block nonspecific antibody binding and to provide a source of $_2$ -GPI. Assay results are usually reported as either MPL units for IgM anti-cardiolipin antibody or GPL units for IgG anti-cardiolipin antibody, which are derived from a standard curve. Because of assay variability and a non-Gaussian distribution in normal people, the distinction between normal and abnormal is not clear. With the use of this sensitive assay for anti-cardiolipin antibodies, most patients with lupus anticoagulant have elevated levels of anti-cardiolipin antibodies in their plasma.

Although most patients with elevated levels of anti-cardiolipin antibodies also test positive for lupus anticoagulant activity, considerable data support lupus anticoagulant activity and anti-cardiolipin antibodies as distinct subgroups of antibodies that can be separated by affinity or physicochemical methods and have different antigenic specificities. [246] [247] Moreover, antibodies to $_2$ -GPI can be further separated into those that have anticoagulant activity and those that do not. [248] [249] In addition, lupus anticoagulants and anti-cardiolipin antibodies occur concurrently in only approximately 5075% of patients, and thus a patient may have no anticoagulant activity simultaneously with a markedly elevated anti-cardiolipin antibody titer by ELISA, and vice versa. Thus, patients who are being evaluated for a possible antiphospholipid antibody syndrome must have

tests for lupus anticoagulant activity in a clotting assay and anti-cardiolipin antibody activity by ELISA.

The mechanism by which the different antiphospholipid antibodies interfere with coagulation in vitro is not clear. However, phospholipid participates in coagulation at several known steps: as a component of the prothrombinase complex, as a co-factor for factor VIIIa and factor IXa in the tenase complex, and as a co-factor for activation of factor X by factor VIIa. The lupus anticoagulant may impede each of these reactions of blood coagulation, and it can inhibit the binding of both factor X and prothrombin to negatively charged surfaces. [219] [250] [251] Because $_2$ -GPI also binds tightly to phospholipid, it is possible that antibodies directed to epitopes on $_2$ -GPI could also interfere with phospholipid-dependent clotting reactions, particularly the tenase and prothrombinase complexes, accounting for the anticoagulant effect in vitro.

Mechanism of Hypoprothrombinemia in the Hypoprothrombinemia-Lupus Anticoagulant Syndrome

A subset of patients with the lupus anticoagulant also have a selective deficiency of prothrombin. The plasma of such patients does not contain an antibody that neutralizes prothrombin activity, [252] and the plasma prothrombin antigen is decreased to the same extent as prothrombin activity. [253] [254] Bajaj et al [255] demonstrated that the plasma from patients with the hypoprothrombinemia-lupus anticoagulant syndrome contains antibodies that bind prothrombin without neutralizing its in vitro coagulant activity. The decreased concentration of plasma prothrombin in patients with this syndrome results from the rapid removal of the prothrombinprothrombin antibody complexes. In addition, altered mobility of prothrombin antigen on crossed immunoelectrophoresis, indicative of the presence of plasma prothrombin antigenantibody complexes, has been demonstrated in 6675% of patients with the lupus anticoagulant in whom plasma prothrombin activity was not substantially decreased. [256] It is probable that these antibodies are the same antibodies to which the lupus anticoagulant is directed, and that prothrombin deficiency stems from an excess of antibody causing very rapid clearance of antigen.

Laboratory Evaluation

The results of coagulation tests usually found in patients with the lupus anticoagulant include a prolonged PTT, which is prolonged when the patients plasma is mixed with equal parts of normal plasma. The PT is minimally to moderately prolonged (0.53 seconds), and occasionally it is normal. However, the PT is prolonged more than normal plasma when diluted tissue factor is used, as in the TTI test. [191] [257] The thrombin time is normal. The sensitivity of the PTT for lupus anticoagulant detection varies with different commercial reagents used for the test, [258] [259] [260] probably reflecting, at least in part, variation in the phospholipid composition of these

reagents.[260] Moreover, some commercial PTT reagents have been created to be very sensitive to the presence of lupus anticoagulants. The prevention of platelet activation in plasma samples is crucial because procoagulant phospholipid, either in the patient plasma or in the normal plasma used for mixing studies, may neutralize weak lupus coagulant activity. [261] [262] It is therefore recommended that test plasmas and normal plasma be initially centrifuged at 10,00015,000 × g for 1015 minutes or filtered through 0.22-m screens to remove platelets before freezing. [262]

Confirmation of the Diagnosis

The pattern of abnormality of the lupus anticoagulant is often indistinguishable from that of an anticoagulant directed against any one of the several clotting factors that influence the result of the PTT, but not the PT. Therefore, further tests are needed to confirm that the prolonged PTT of a mixture of the patients plasma and normal plasma results from the lupus anticoagulant. It is particularly important to rule out a factor VIII anticoagulant, which, in contrast to the lupus anticoagulant, frequently causes serious bleeding.

Various tests have been devised to increase the sensitivity to lupus anticoagulants, including examination of clotting time in systems containing reduced phospholipid such as the kaolin clotting time,[219] the dRVVT,[237] and the dilute phospholipid time (dilute PTT). [263] [264] Some believe that the kaolin clotting time is the most sensitive, whereas the dRVVT is the most specific. By contrast, the TTI test is less sensitive and specific, [235] [236] [239] [257] but recent modifications using recombinant tissue factor have significantly improved its sensitivity. [265] However, there is evidence that to maximize the number of patients with lupus anticoagulant identified, multiple tests should be done.[209] [217] [266] Mixing studies are important in distinguishing factor deficiencies from inhibitors (deficiencies caused by warfarin are corrected by the addition of normal plasma, whereas plasma from patients taking oral anticoagulants and containing lupus anticoagulants will not be completely corrected). A recent study provides evidence that $_2$ -GPIdependent antibodies (i.e., anti-cardiolipin antibodies) prolong the dRVVT more than the kaolin clotting time, and the prothrombin-dependent antibodies prolong the kaolin clotting time to a greater extent than the dRVVT. [267] [268]

Tests based on the observation that excess phospholipid substantially shortens the prolonged PTT of lupus anticoagulant plasma are important as a means of differentiating the lupus anticoagulant from other inhibitors. The excess phospholipid is added as either freeze-thawed platelets, [241] liposomes containing phosphatidylserine,[238] platelet-derived microvesicles, [269] rabbit brain phospholipid,[239] or hexagonal phase phospholipids. [243] Correction of prolonged clotting times by excess phospholipid significantly increases specificity, and false-positive results are encountered only for heparinized patients or extremely high-titer inhibitors to other clotting factors. However, no positive results are obtained from patients with low-titer clotting factor inhibitors, congenital factor deficiencies, hepatic insufficiency, or in patients receiving warfarin therapy.

Another novel test for the detection of lupus anticoagulants uses two different snake venoms, one of which depends on the presence of phospholipid and one of which does not.[270]

One diagnostic approach that is helpful in making the diagnosis is to perform several of the specific one-stage clotting

METHODS FOR ESTABLISHING THE PRESENCE OF A LUPUS ANTICOAGULANT

1. Platelets and platelet debris must be removed from both the patients plasma and the normal plasma used for mixed studies before any testing is carried out.
2. If sensitivity is important, a screening test with very high sensitivity (e.g., kaolin clotting time, dilute phospholipid PTT, or dRVVT) should be used.
3. The clotting time of a mixture of test and normal plasma should be significantly longer than that of the normal plasma mixed with various non-lupus anticoagulant plasmas.
4. To increase specificity there should be a relative correction of the defect by the addition of lysed washed platelets, phospholipid liposomes containing phosphatidylserine, or hexagonal-phase phospholipids.
5. Testing must be repeated 24 months apart to establish the presence of a persistent abnormality.[268]

factor assays based on the PTT technique. The following pattern of results is frequently found: (1) low values for several clotting factors (artifactually decreased values reflecting the ability of the lupus anticoagulant in the test sample to impair the reactivity of the phospholipid reagent common to each assay system); and (2) increasing values for each clotting factor with increasing dilution of the test plasma in the assay system (reflecting a decreased carryover of the lupus anticoagulant into the assay system with a higher dilution of the patients plasma). Thus, inhibitors affecting more than one clotting factor using the PTT technique are usually lupus anticoagulants. In addition, for reasons that are not clear, the assay for factor XI is frequently affected more than other intrinsic assays. A comprehensive review of laboratory testing for lupus anticoagulants has been published. [271]

Laboratory Recognition of the Hypoprothrombinemia-Lupus Anticoagulant Syndrome

Although minimal to moderate prolongation of the PT, up to approximately 3 seconds beyond a control value, can be accounted for by the lupus anticoagulant, the finding of a substantially prolonged PT (e.g., 1820 seconds) represents presumptive evidence of an associated specific prothrombin deficiency. Unlike the specific clotting factors assayed in modified PTT test systems, the specific clotting factors affecting the prothrombin time test, factor VII, factor X, and factor V are assayed in clotting systems that are affected only by rare, high-titer lupus anticoagulants. Therefore, the finding of a low value in a specific prothrombin assay may be taken as convincing evidence of an associated prothrombin deficiency. If, however, further evidence is desired, three additional findings may be demonstrated: (1) a mixture of equal parts of the patients plasma and normal plasma gives the expected value calculated from the mean of the levels in individual plasmas; (2) prothrombin activity and prothrombin antigen are concordantly decreased; or (3) prothrombin has abnormal mobility on crossed immunoelectrophoresis. [272] [273]

Clinical Relationships of Lupus Anticoagulant and Anti-cardiolipin Antibodies

Both lupus anticoagulant and anti-cardiolipin antibodies are associated with thrombosis, fetal wastage, or thrombocytopenia with or without autoimmune disorders. This clinical entity is known as the antiphospholipid syndrome. [274] [275] The primary antiphospholipid syndrome is defined as venous or arterial thrombotic disease, or both, or as recurrent fetal wastage associated with elevated levels of antiphospholipid antibodies in the absence of any definite autoimmune disease. [275] These patients do not demonstrate any other clinical or serologic evidence of autoimmune disease, except for occasional low-titer (<1:160) anti-nuclear antibodies. In contrast, the secondary antiphospholipid antibody syndrome is associated with SLE and related disorders. Some patients have some evidence of a systemic autoimmune disorder but insufficient criteria to make the diagnosis of SLE (overlap syndrome). An occasional patient with high-titer anti-cardiolipin antibodies can present with fulminant disease with multiorgan system involvement (e.g., lung, kidney, brain), hypertension, and microvascular and macrovascular thrombosis. [276] [277] [278] Because these patients have a high mortality rate, they require aggressive treatment.

Most patients who test positive for the lupus anticoagulant have elevated levels of anti-cardiolipin antibody. However, although the correlation between the two is significant, many patients showing positivity for lupus anticoagulant activity do not have elevated levels of anti-cardiolipin antibody or other antiphospholipid antibodies, and vice versa.[279] [280] [281] [282]

Because anti-cardiolipin antibody levels can fluctuate significantly, [283] a negative test for both the lupus anticoagulant and anti-cardiolipin antibody does not completely rule out the presence of phospholipid antibodies. This is particularly true during an acute thrombotic episode, when antibody titers may transiently decline

to normal.[284]

Several studies[209] [266] suggest that when tests for the lupus anticoagulant and anti-cardiolipin antibodies are performed at two separate time intervals (816 weeks), a statistically significant association can be shown between persistently positive tests and prior thromboembolic events and fetal loss. The strength of the association is much reduced when transiently positive patients are included. These studies also showed that a combination of tests for detecting lupus anticoagulant activity is superior to a single test. However, the number of patients who tested positive for lupus anticoagulant activity was small, and tests were considered negative when they were <2 SD above normal.[208]

A false-positive Venereal Disease Research Laboratory (VDRL) test in low-titer (1:41:8) can be demonstrated in 30% of patients with the lupus anticoagulant or anti-cardiolipin antibodies. These cross-reacting antibodies differ from reagin, the antibody responsible for the Wasserman reaction in patients with syphilis, and do not require the presence of $_2$ -GPI for their detection. The detergent, Tween 20, can be used in the anti-cardiolipin antibody assay to distinguish $_2$ -GPIdependent and independent antibodies.[285]

Relationship of Lupus Anticoagulant and Anti-cardiolipin Antibodies to Other Antibodies

In some subjects with lupus anticoagulant and anti-cardiolipin antibody, antibodies that react to other anionic phospholipids such as phosphatidylserine, phosphatidylinositol, and phosphotidic acid have been demonstrated. [250] [282] Recently, in some patients with lupus anticoagulant or anti-cardiolipin antibody, antibodies to neutral phospholipids (phosphatidylethanolamine or phosphatidylcholine) have been demonstrated. [286] Sugi et al[287] have demonstrated that autoantibodies to phosphatidylethanolamine recognize a kininogenphosphatidylethanolamine complex. In addition, Smimov et al [288] have shown that inhibition of activated protein C is antibody and phosphatidyl-ethanolamine dependent.

Correlation Between Clinical Findings and Antiphospholipid Antibodies

Bleeding

Most patients with the lupus anticoagulant do not have a bleeding phenotype. Patients with the lupus anticoagulant have undergone needle biopsy of the kidney and the severe hemostatic challenges of major surgery,[188] [194] [289] including prostatectomy[188] and open heart surgery, [188] without excessive postoperative bleeding.

Nevertheless, patients with the lupus inhibitor may have clinically significant bleeding. In all but a few, the bleeding can be attributed to some abnormality other than the lupus inhibitor,[6] [234] such as depressed prothrombin activity. [233] [254] [290] The combination of the lupus anticoagulant and a prothrombin coagulant activity below approximately 20% of normal has resulted in severe and even fatal bleeding. In other patients, thrombocytopenia, alone or in combination with moderate prothrombin deficiency, accounted for the bleeding tendency. The hemorrhagic manifestations may be related to severe uremia. [291]

Evaluation of the risk of abnormal bleeding in patients with the lupus anticoagulant includes establishing the presence or absence of prothrombin deficiency, thrombocytopenia, or uremia. Occasionally a patient may have only a prolonged bleeding

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time associated with excessive bleeding.[292] In the absence of these coexisting risk factors, abnormal bleeding is minimal even after trauma or surgery. [194] [216] [293] [294] In summary, most patients with the isolated finding of lupus anticoagulant do not experience abnormal bleeding. Nevertheless, excessive postoperative bleeding has been attributed to the lupus anticoagulant on rare occasions. [295] [296]

Thrombosis

Since the initial observation of Bowie et al [291] in 1963, it has become clear that thrombosis is a major concern in patients with the lupus anticoagulant. The incidence has varied from 17 to 71%, averaging approximately 3040%. [192] [194] [220] [294] [297] In addition, in patients with an elevated level of anti-cardiolipin antibody, a high incidence (40%) of thrombotic disease has been reported in such patients. [220] [274] [298] Thrombosis (and fetal loss and thrombocytopenia) are more frequent as the level of anti-cardiolipin antibody increases, and very high titers (>7 SD above the normal mean) are more frequently associated with thrombotic events. In addition, although some investigators believe that elevated levels of IgG or IgA isotypes are more common than IgM in patients with thrombotic complications, [299] [300] this has not been clearly established. Moreover, the lupus anticoagulant or increased levels of anti-cardiolipin antibody must be persistently present on more than one occasion because the incidence of thrombotic complications is almost the same in patients with transiently positive tests as in patients with negative tests at two different time intervals.[208] [209] [266] In addition, the persistent presence of elevated levels of anti-cardiolipin antibody has been shown to be associated with indices of in vivo coagulation activation. In a study of patients with SLE [301] who were either persistently anti-cardiolipin antibody positive versus patients who were transiently positive or persistently negative, anti-cardiolipin antibody-positive patients had a higher mean level of F1·2 and fibrinopeptide A than patients who were transiently positive, persistently negative, or on warfarin therapy. The differences remained significant even if patients with prior thromboembolism were excluded from the analysis. These results suggest that the presence of persistently elevated levels of anti-cardiolipin antibody in SLE patients is associated with an ongoing prothrombotic state. Most recent studies suggest that the presence of lupus anticoagulants was more strongly associated with both venous and arterial thrombosis than the presence of anti-cardiolipin antibody. [302] [303] [304] Moreover, Galli et al[268] have suggestive evidence that $_2$ -GPI antibodies that prolong the dRVVT more than the kaolin clotting time (dRVVT phenotype) are associated with a higher incidence of thrombosis than the prothrombin-dependent antibodies, which prolong the kaolin clotting time more than the dRVVT (kaolin clotting time phenotype). However, either antibody may be associated with the antiphospholipid antibody syndrome.

Thrombosis may be either venous or arterial,[274] with events occurring nearly equally in the arterial and venous circulation, [305] [306] although in some series the incidence of venous thrombosis is greater (70%) than the incidence of arterial thrombosis. [303] [307] Venous thrombosis is usually manifested as deep venous thrombosis of the lower extremities with or without pulmonary emboli. In addition, unusual sites of venous thrombosis, such as hepatic veins, inferior vena cava, mesenteric veins, renal veins, cerebral venous sinuses, retinal veins, and axillary veins, have occasionally been reported. [274] Arterial thrombosis is manifested as stroke[216] [274] [308] or transient ischemic attacks.[309] The association of antiphospholipid antibodies and coronary artery disease is controversial. Hamsten et al [310] reported that young survivors of myocardial infarction had a high incidence of anti-cardiolipin antibody, and those patients with a persistently elevated anti-cardiolipin antibody had a higher incidence of recurrent events. Similar findings were reported in another prospective study of 133 patients who had a significant number of subsequent events compared with matched control subjects.[311] In contrast, other studies did not find either elevated anti-cardiolipin antibody or subsequent events in patients with an acute myocardial infarction.[312] [313] Patients admitted for peripheral vascular surgical procedures also have a high incidence of phospholipid antibodies. [314] Unusual presentations such as gangrene of the extremities and digits, multi-infarct dementia, and bowel infarction have also been reported. [315] Thromboembolic pulmonary hypertension with or without SLE can occur in association with the lupus anticoagulant or elevated levels of anti-cardiolipin antibody, or both. [274] [316] [317]

Nonbacterial mitral and aortic endocardial valve lesions, with or without SLE, accompanied by thromboembolic transient ischemic attacks and strokes associated with lupus anticoagulant activity or elevated levels of anti-cardiolipin antibody have also been reported. [318] Livedo reticularis and cerebrovascular disease (Sneddon syndrome)[275] and giant cell arteritis[319] have also been associated with the presence of these antibodies.

Although thrombosis is supposedly not associated with the lupus anticoagulant or anti-cardiolipin antibody induced by infection or drugs, there have been several reports of human immunodeficiency virus- or drug-associated thrombosis.[194] [320] In a report of the treatment of patients with melanoma with immunotherapy, 5 of 12 patients receiving interferon- had lupus anticoagulants and anti-cardiolipin antibodies within 428 days, and deep vein thrombosis developed in 4 of these 5 patients.[321] In addition, in patients with infections, anti-cardiolipin antibodies bind cardiolipin directly and do not require the presence of $_2$ -GPI. [216] [321]

Patients who are persistently positive for the lupus anticoagulant or who have persistently elevated levels of anti-cardiolipin antibody and suffer a thromboembolic event have a recurrence rate of approximately 50% within 2 years. [305] [306] [322] [323] Although one study suggested that the recurrence rate of venous thromboembolism in patients with antiphospholipid antibodies is not greater than in patients without such antibodies, [324] the average follow-up of these patients was short (8.5 months). In addition, recurrences tend to occur (90%) on the same side of the circulation as the initial eventvenous recurrences after an initial venous event and arterial recurrences after an initial arterial event. [305]

Fetal Loss, Thrombocytopenia, and Other Associations

Another major clinical manifestation associated with the lupus anticoagulant or anti-cardiolipin antibody is fetal loss. [192] [208] [209] [211] [212] [213] [214] [215] [274] [294] [300] [325] Fetal wastage may occur at any time during pregnancy.[213] [214] [274] Any patient with a history of recurrent first-trimester abortion, second- or third-trimester intrauterine death, or intrauterine growth retardation should be tested for the lupus anticoagulant and anti-cardiolipin antibody. This syndrome occurs in patients with or without SLE; thrombosis of placental vessels and placental infarction are thought to be the mechanisms by which fetal loss occurs. [212] [215] [274] The high incidence of thrombotic disease in these patients lends further support to this hypothesis.

Thrombocytopenia is a frequent finding in patients with the lupus anticoagulant or anti-cardiolipin antibody. Patients with SLE or related autoimmune disorders show an increased incidence of thrombocytopenia associated with these antiphospholipid antibodies. [326] Moreover, the incidence of these antibodies is increased (3040%) in patients with idiopathic immune thrombocytopenic purpura.[325] [327]

Coombs-positive hemolytic anemia has also been associated with lupus anticoagulants or anti-cardiolipin antibodies in patients with or without SLE. [328] Other occasional associations with antiphospholipid antibodies include migraine, chorea, transverse myelopathy, Guillain-Barré syndrome, [274] and epilepsy.[329]

Pathophysiologic Mechanism(s) of Thrombosis

The nature of the association between thrombosis and antiphospholipid antibodies such as the lupus anticoagulant and anti-cardiolipin antibody is uncertain. Because many people with antiphospholipid antibodies never experience thrombosis, it is not clear whether antiphospholipid antibodies are direct causative factors for thrombosis, or rather represent secondary consequences of thrombosis with no direct pathophysiologic role. Because we know that many of these antibodies perturb hemostasis in vitro, we have assumed to a certain degree that they also directly affect the hemostatic system in vivo so that the prothromboticantithrombotic equilibrium is perturbed enough that it becomes imbalanced, thus favoring thrombosis or placental ischemia. There is evidence substantiating this in patients with SLE and elevated anti-cardiolipin antibodies, in whom elevated levels of fibrinopeptide A [330] and the prothrombin fragment F1·2 have been demonstrated.[330] [331] In the study by Ferro et al,[331] F1·2 was elevated in patients with SLE who were lupus anticoagulant positive, but patients who were positive only for anti-cardiolipin antibody did not have elevated levels of F1·2. In other studies of patients who were lupus anticoagulant positive, the mean level of thrombinantithrombin III complexes was higher than in control subjects.[332] [333]

Antiphospholipid Antibodies as Direct Causative Factors for Thrombosis

There are a few experimental animal models that suggest that antiphospholipid antibodies directly cause thrombosis. The antiphospholipid syndrome has been produced in normal mice by passive transfer of human immunoglobulin with anti-cardiolipin activity [334] or by active immunization with a human monoclonal anti-cardiolipin antibody [335] or with $_2$ -GPI.[336] [337] In addition, pregnant mice passively [334] or actively immunized with anti-cardiolipin antibody IgG have increased fetal loss.[334] [338] In another experimental model,[339] mice injected with purified anti-cardiolipin antibody from patients with antiphospholipid syndrome followed by a pinch injury to their femoral vein had significantly larger thrombi that persisted for a longer period of time than in appropriate control animals. All of these experimental models strongly suggest that antiphospholipid antibodies have a direct pathophysiologic role in thrombosis.

Mechanism of Thrombosis

Antiphospholipid antibodies could directly contribute to thrombosis by altering platelet activity, procoagulant or anticoagulant pathways, or vascular endothelial function. Several considerations support a possible role for platelet activation in the pathogenesis of antiphospholipid antibody-associated thrombosis. First, many subjects with antiphospholipid antibody-associated thrombosis have some degree of thrombocytopenia, [194] [196] [293] [294] presumably immune in origin. Second, there is an association between antiphospholipid antibodies and antiplatelet antibodies. [325] [326] Indeed, antiphospholipid antibodies may directly cross-react with platelets. [340] [341] The third consideration is the occurrence of arterial as well as venous thrombotic episodes. Platelet activation is thought to play a role in the pathogenesis of arterial thrombotic disease, whereas other causes of increased thrombotic risk, such as deficiencies of antithrombin III, protein C, protein S, or factor V Leiden predispose primarily to increased venous thrombotic risk. Increased levels of a urinary metabolite of thromboxane A $_2$ have been demonstrated in patients with antiphospholipid antibodies.[342] However, in contrast, another study showed that although antiphospholipid antibodies could bind to circulating platelets, no evidence of measurable platelet activation was found.[340] Fourth, serum or purified IgG from subjects with antiphospholipid antibodies inhibits the release of the platelet inhibitor, prostacyclin, from vascular segments or cultured vascular endothelial cells. [343] [344] [345] [346] This finding suggests that in some subjects with antiphospholipid antibodies, impaired prostacyclin release might lead to excessive platelet activity, resulting in thrombosis. Platelets from subjects with antiphospholipid antibody-associated thrombosis are more resistant to in vitro inhibition by the inhibitory prostaglandin, PGE $_1$, than normal platelets. However, except for one study,[347] the correlation between in vitro inhibition of prostacyclin release and thrombosis in subjects with antiphospholipid antibodies has been poor. [344] [346] In addition, Shi et al[348] showed that purified IgG from patients with lupus anticoagulants and anti-cardiolipin antibodies bound to thrombin-activated but not resting platelets, and antibody binding did not produce any evidence of platelet activation. Thus, there is little evidence that lupus anticoagulants or anti-cardiolipin antibodies directly cause platelet activation. However, Arvieux et al [349] showed that murine monoclonal antibodies to $_2$ -GPI with lupus anticoagulant activity could activate platelets when there were subthreshold concentrations of epinephrine or adenosine diphosphate in the presence of $_2$ -GPI. Subsequent platelet activation was shown to depend on binding to the platelet Fc receptor, similar to the pathophysiologic process occurring in heparin-induced thrombocytopenia.

Several abnormalities of natural anticoagulant pathways have also been reported in patients with antiphospholipid antibody-associated thrombosis. In one study, thrombomodulin antibodies were found in 30% of patients with lupus anticoagulant, in 10% of patients with unexplained thromboembolism, and in 10% of control subjects.[350] However, in another study, inhibition of thrombomodulin activity was found in only 2 of 46 patients with lupus anticoagulants. [351] In that same study, plasma from 24 of 33 patients with thrombosis showed significant inhibition of activated protein C, whereas plasma from 13 patients with thrombosis showed no inhibition. In other studies, [352] [353] [354] serum and purified IgG impaired activation of protein C by thrombin complexed to the endothelial co-factor, thrombomodulin, [355] and impaired the phospholipid-dependent anticoagulant action of activated protein C and its co-factor, protein S. [352] In addition, two studies of patients with antiphospholipid antibodies have shown a higher incidence of factor V Leiden than in patients without antiphospholipid antibodies. [356] [357] Reduced plasma levels of free protein S have also been observed in subjects with antiphospholipid antibody-associated thrombosis. [320] [343] [358] In another study, purified IgG from seven patients with antiphospholipid antibodies reacted with the disaccharide present in the heparin-heparan pentasaccharide that binds antithrombin III, and these antibodies inhibited formation of thrombinantithrombin complexes.[359] Thus, it appears that plasma from some patients with antiphospholipid antibodies inhibits several natural anticoagulant pathways, and whether this inhibition stems from other antibodies has not yet been established. However, although several abnormalities of the natural anticoagulant pathway have been demonstrated in patients with antiphospholipid antibodies, a close correlation between abnormalities of these pathways and thrombosis in patients with antiphospholipid antibodies has not been consistently demonstrated. Moreover, a defect in natural anticoagulant pathways could not by itself explain the presence of arterial thromboembolism.

Because anti-cardiolipin antibodies are antibodies directed against $_2$ -GPI, [246] [247] it suggests that possibly the antihemostatic effect is due to inhibition of $_2$ -GPI function. Although patients with severe $_2$ -GPI deficiency do not have thromboembolic disease, [360] the presence of anti-$_2$ -GPI antibodies is associated with lupus anticoagulant activity, anti-cardiolipin antibodies, and thromboembolic complications in patients with SLE. [361]

Impaired fibrinolysis may contribute to thrombosis in patients with antiphospholipid antibodies. [362] [363] However, active SLE[363] [364] is independently associated with impaired fibrinolysis.[365]

More recent studies of patients with antiphospholipid antibody-associated thrombosis without SLE have failed to document either impaired fibrinolysis in vivo [366] or any effect of plasma containing antiphospholipid antibodies on in vitro endothelial secretion of either tissue plasminogen activator or plasminogen activator inhibitor-1.[367]

Antibodies against endothelial cells have been reported in some patients with antiphospholipid antibody-associated thrombosis. [368] These antibodies might cause thrombosis by directly damaging endothelial cells; by impairing endothelial, antiplatelet, or anticoagulant activities; or by bringing about procoagulant changes in endothelial function such as expression of tissue factor. [369] [370] Antiphospholipid antibody-containing sera from patients with SLE and thrombosis induced a small but significant increase of endothelial cell tissue factor activity. When added in combination with a low dose of tumor necrosis factor, a synergistic enhancement of tissue factor activity was found.[371] In addition, these antiphospholipid antibody-containing sera led to enhanced thrombosis formation in an in vitro thrombosis model. In contrast, nonantiphospholipid antibody-containing sera from patients with SLE did not generate tissue factor activity, nor did the sera produce enhanced thrombus formation.[370]

Recently, Simantov et al[371] showed that purified IgG from SLE patients with elevated anti-cardiolipin antibody and the antiphospholipid antibody syndrome causes increased monocyte adhesion when reacted with endothelial cells in vitro. Moreover, monocyte adhesion depended on the presence of $_2$ -GPI. These data strongly suggest that anti-cardiolipin antibody is capable of binding to and activating vascular endothelial cells.

Horkko et al[372] found that apolipoprotein E-deficient mice who had active atherogenesis developed high titers of autoantibodies to epitopes of oxidized low-density lipoproteins (LDL) and to cardiolipin, and sera or anti-cardiolipin antibody-IgG from patients with antiphospholipid syndrome bind to cardiolipin as it is oxidized but do not bind to reduced cardiolipin. Thus, antiphospholipid antibodies are directed at neoepitopes of oxidized lipoproteins. Moreover, it had been previously shown by Vaarla et al[373] that binding of anti-cardiolipin antibody to solid-phase cardiolipin was inhibited by oxidized LDL but not by native LDL, suggesting cross-reactivity between antiphospholipid antibodies and antibodies to oxidized LDL. These data suggest that antiphospholipid antibodies may be pathogenic in the formation of atherosclerotic lesions.

A recent study of the pathogenic mechanism of recurrent fetal loss in patients with antiphospholipid antibodies implicated annexin V, a potent anticoagulant protein found on placental trophoblast cells.[374] IgG fractions from three patients with the antiphospholipid antibody syndrome incubated with a trophoblast cell line and endothelial cells resulted in reduced levels of annexin V. Plasma overlayed on these cells had faster clotting times than controls. Thus, the results of this study suggest that antiphospholipid antibody inhibits the anticoagulant effect of annexin V, causing a prothrombotic surface.

Therapy for Patients with Lupus Anticoagulant

The lupus anticoagulant usually persists in the untreated adult patient. However, it frequently disappears spontaneously when it occurs in children in whom the anticoagulant develops after a viral infection, and may be transient in adult patients without SLE, thromboembolic disorders, or fetal loss.

When either the lupus anticoagulant or anti-cardiolipin antibody, or both, is found in patients with underlying autoimmune disease, treatment of the underlying disease with immunosuppressive therapy frequently results in reduction or disappearance of the antibody. [244] [252] [290] [375] When the anticoagulant is found in association with severe prothrombin deficiency or severe thrombocytopenia (<20,000/l), treatment with corticosteroids is indicated. There has been one case report of a patient with a lupus anticoagulant and hypoprothrombinemia who was resistant to corticosteroids but responsive to IVIG. [376] However, when the lupus anticoagulant or anti-cardiolipin antibody is discovered as an isolated finding not associated with thrombosis or fetal loss, treatment is not indicated. Nevertheless, it is very important to inform patients so that they can be educated about the symptoms of, and prophylaxis for, venous thrombosis, about the symptoms of stroke, and about the possibility of fetal loss.

Therapy for thrombosis associated with the lupus anticoagulant or anti-cardiolipin antibody should be guided by the knowledge that recurrence is common. [305] [306] [323] In one study, patients who had discontinued oral anticoagulation had a 50% probability of recurrence in two years and a 78% recurrence in eight years. [377] Similar results have been published by others.[305] [306] [323] Venous recurrence usually occurs in patients who have an initial venous event, whereas arterial recurrences occur in patients who sustain an initial arterial event. [305] However, treatment with warfarin with a target international normalized ratio (INR) of 3.0 has been associated with a significant decrease in recurrences. [305] [306] Although aspirin added to warfarin may be helpful, aspirin alone is not efficacious in preventing thrombotic events. [305] Thus, it is recommended that patients with venous or arterial thrombosis receive life-long anticoagulation with oral anticoagulants. Because of the efficacy of warfarin therapy in preventing recurrences, the use of corticosteroids and other immunosuppressive agents to suppress antibody production in the absence of autoimmune disease is not recommended.

Monitoring anticoagulant therapy may be difficult in patients with lupus anticoagulants and a prolonged PTT. Thus, it is mandatory when using heparin to monitor therapy using a specific heparin assay such as the one dependent on factor Xa inhibition (therapeutic range, 0.30.7). When using warfarin, the optimal INR for patients with lupus anticoagulants is controversial [305] [306] [324] [378] [379] because patients with lupus anticoagulants may have a variably prolonged prothrombin time [378] [380] [381] [382] and various thromboplastins have a different sensitivity in the presence of a lupus anticoagulant. [378] Therefore, it is possible that in various studies of therapy in patients with lupus anticoagulants that the degree of anticoagulation is overestimated, and the target INR of 3.0 noted earlier might be an overestimate because of the presence of the lupus anticoagulant. This hypothesis is substantiated by the report by Moll and Ortel, [378] in which the prothrombin time done by the prothrombin-proconvertin test and a chromogenic factor X level correlated well with established therapeutic ranges. Thus, if either of the latter two assays are available, they would be preferable in monitoring oral anticoagulant therapy in patients with lupus anticoagulants.

A pregnant patient with or without SLE with the lupus anticoagulant or anti-cardiolipin antibody associated with a history of recurrent fetal wastage should be treated. However, the precise regimen to be followed has not been clearly established. Lubbe et al [383] described a successful pregnancy outcome in a patient with the lupus anticoagulant and fetal wastage who was treated with prednisone and low-dose aspirin. Subsequently, the same regimen led to successful pregnancy outcomes in 10 of 16 pregnancies in 12 patients, some of whom had SLE.[384] Other investigators, using a similar regimen in patients without SLE, also reported a decrease in fetal wastage in a significant number of patients. [341] [375] However, long-term, high-dose corticosteroids during pregnancy may be associated with significant side effects, including hypertension, gestational diabetes, and premature labor and delivery. In a study by Silveira et al, [375] however, prednisone and low-dose aspirin given to 11 patients with recurrent fetal wastage (32 previous fetal losses and 5 live-born infants) resulted in 100% live-born infants (12 pregnancies and 12 live-born infants), and no significant adverse effects to either

THERAPY FOR PATIENTS WITH THE LUPUS ANTICOAGULANT OR AN INCREASED LEVEL OF ANTI-CARDIOLIPIN ANTIBODIES

The lupus anticoagulant persists in most untreated adults. Often it disappears spontaneously when it occurs in children in whom the anticoagulant is acquired after a viral infection, and it may be transient in adult patients without SLE, thromboembolic disorders, or fetal loss.

When the lupus anticoagulant or increased level of anti-cardiolipin antibodies is found in patients with underlying autoimmune disease, treatment of the underlying disease with immunosuppressive therapy frequently results in reduction or disappearance of the antibody. When the lupus anticoagulant is found in association with severe prothrombin deficiency or severe thrombocytopenia (<20,000/l), treatment with adrenal corticosteroids is indicated, beginning with a dose of 6080 mg of prednisone. However, when the lupus anticoagulant or increased level of anti-cardiolipin antibodies is discovered as an isolated finding and not associated with thrombosis or fetal loss, treatment is not indicated.

Therapy for thrombosis associated with the lupus anticoagulant or anti-cardiolipin antibodies should be guided by the knowledge that recurrence is common. The site of the first event (arterial or venous) tends to predict the site of subsequent events. It is recommended that patients with venous thrombosis receive intravenous unfractionated heparin in the usual therapeutic doses to achieve a plasma heparin level of 0.30.7 units using a factor Xa inhibition assay. Long-term anticoagulation with warfarin can be started simultaneously with a dose to maintain the INR between 2.53.5. Long-term oral anticoagulation at the same INR is also efficacious in preventing arterial thrombosis. If arterial events recur at a therapeutic INR, then low-dose aspirin (80 mg) can be added. Because of the high recurrence rate, oral anticoagulants should be given for life except during pregnancy (see later). Because of the efficacy of warfarin therapy in preventing recurrence, the use of corticosteroids and other immunosuppressive agents to suppress antibody production in the absence of autoimmune disease is not recommended.

A pregnant patient with or without SLE with the lupus anticoagulant or anti-cardiolipin antibodies associated with a history of recurrent fetal wastage should be treated either with heparin (5,000 units bid) plus low-dose aspirin (80 mg) or intermediate-dose heparin (enough to prolong to PTT by 1.21.5 times control) plus low-dose aspirin. If fetal loss recurs on this regimen, then full therapeutic doses of unfractionated heparin or, alternatively, a therapeutic dose of lowmolecular-weight heparin should be tried.

For the patient who becomes pregnant for the first time and who has not had prior thromboembolic events, no treatment is indicated. However, the patient should be educated regarding the possibility of fetal loss, the potential side effects of therapy, and the lack of controlled trials in such patients. If the patient desires therapy, it should be given. If the patient has suffered only one fetal loss, particularly in the second and third trimester, with no evidence of placental infarction, the patient should be treated with prophylactic doses of heparin and low-dose aspirin as noted previously. If the patient has the persistent presence of anti-cardiolipin antibody or the lupus anticoagulant, or both, but has not had any clinical problems and requires a major surgical procedure, prophylactic heparin and intermittent pneumatic compression should be used.

For the patient who presents with the catastrophic antiphospholipid syndrome, the favored regimen consists of aggressive therapy with daily plasmapheresis, heparin anticoagulation, pulsed high-dose methylprednisolone 2 g/day IV × 3 days, and cyclophosphamide 750 mg/m ^{2} on day 1.

mother or infants. The prednisone regimen consisted of a starting dose of 40 mg/day for 4 weeks, with the dose tapered by 10 mg/day every 4 weeks to a final maintenance dose of 5 mg/day. The levels of anti-cardiolipin antibody decreased in most patients. However, in a recent, large, randomized trial in 202 patients with autoantibodies (either lupus anticoagulant, anti-cardiolipin antibody, anti-nuclear antibody, anti-DNA, or antilymphocytic IgM) and unexplained loss of at least two fetuses, corticosteroids plus low-dose aspirin resulted in 65% viable infants, compared with 57% in the placebo group. [385]

Wallenberg and Rotmans[386] used low-dose aspirin and dipyridamole in 37 patients with obstetric histories similar to those with antiphospholipid antibodies, with a 93% success rate. Unfortunately, these patients were not systematically examined for the presence of antiphospholipid antibodies. Similarly, Elder et al [387] reported similar success using low-dose aspirin alone in 42 patients, of whom 16 had SLE (13 with antiphospholipid antibodies). However, Lockshin et al [388] have cast significant doubt regarding the efficacy of aspirin with or without corticosteroids in high-risk patients with high-titer anti-cardiolipin antibodies. In 11 pregnancies receiving corticosteroids and low-dose aspirin, there were 9 fetal losses, whereas in 10 pregnancies receiving aspirin alone or no therapy, there were 5 fetal losses.

In a recent, small, randomized trial,[389] 50 patients with at least three consecutive episodes of spontaneous pregnancy loss and positive antiphospholipid antibody were assigned either low-dose aspirin alone or low-dose aspirin plus heparin with increasing doses throughout pregnancy to achieve a PTT between 1.21.5. Viable infants were delivered by 11 of 25 (44%) women receiving aspirin alone, whereas 20 of 25 women (80%) receiving aspirin plus heparin delivered viable fetuses (r < 0.05).

In another recent randomized trial[390] in patients with persistently positive antiphospholipid antibodies and three or more fetal losses, low-dose aspirin (75 mg) plus unfractionated heparin (5,000 units bid) resulted in live births in 32 of 45 (71%) pregnancies, compared with 19 of 45 (42%) live births in patients receiving low-dose aspirin alone.

Rosove et al[391] reported 14 successful outcomes in 15 pregnancies in 14 patients (5 with lupus) using adjusted full-dose heparin therapy throughout pregnancy. None of the patients was treated with aspirin, and only one patient received a short course of corticosteroids for a lupus flare.

Thus, different therapeutic regimens in uncontrolled and controlled clinical trials have resulted in a decrease in fetal wastage. The most efficacious regimens appear to be either low- or intermediate-dose heparin plus low-dose aspirin, or adjusted-dose heparin without aspirin. Although most of the experience has been with unfractionated heparin, there is sufficient experience with lowmolecular-weight heparin to be confident that it is safe and efficacious in pregnancy. Thus, it is likely that lowmolecular-weight heparin will replace unfractionated heparin in the future. Whether it should be used in prophylactic or therapeutic

doses remains to be determined in clinical trials. Regimens containing corticosteroids appear in most studies to cause significant adverse events (hypertension, diabetes, and preterm labor and delivery).

Whether asymptomatic patients with antiphospholipid antibodies who desire pregnancy should be treated prophylactically is not clear. It is known that low-titer anti-cardiolipin antibody occurs in up to 24% of healthy young women and high-titer antibody in approximately 0.2%. [226] Thus, many young women with antiphospholipid antibody who wish to become pregnant may be referred for consultation. If the patient does have a high titer of IgG anti-cardiolipin antibody, the risk of fetal loss approximates 28%.[392] [393] If the patient has a positive lupus anticoagulant without an elevated anti-cardiolipin antibody, the risk has not been estimated. Thus, the patient should be educated regarding these data, the possibility of fetal loss, the potential side effects of therapy, and the lack of controlled trials in such patients.

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Chapter 117 - Disseminated Intravascular Coagulation

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Disseminated intravascular coagulation, frequently designated by its acronym DIC, is a pathologic syndrome arising from a heterogeneous group of medical disorders (Table 1171). It is characterized by laboratory evidence of consumption and proteolytic degradation of hemostatic components. The clinical expression varies and may be manifested by laboratory abnormalities alone or in combination with hemorrhagic and thrombotic complications. Because of the variable clinical manifestations and heterogeneity of primary disorders associated with the development of DIC, the syndrome has been characterized

TABLE 117-1 -- Underlying Conditions Associated with Disseminated Intravascular Coagulation

Acute and Subacute
Infection
Gram-negative bacteria
Encapsulated gram-positive bacteria
Viruses (e.g., varicella)
Obstetric complications
Abruptio placentae
Amniotic fluid embolism
Sepsis
Saline-induced abortion
Malignancies
Leukemia, lymphoma
Tissue injury
Burns
Heat stroke
Chronic
Malignancies
Solid tumors
Obstetric complications
Dead fetus syndrome
Localized intravascular coagulation
Aortic aneurysm
Hemangiomas (Kasabach-Merritt syndrome)
Advanced liver disease
LeVeen shunt
Fatty liver of pregnancy

under a number of designations, including *defibrination syndromes*, *consumptive coagulopathies*, or *consumptive thrombohemorrhagic disorders*.[1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] Significant controversies exist in regard to the pathophysiology, diagnosis, and treatment of DIC. However, certain generally accepted clinical and laboratory observations regarding this syndrome can provide a model to assist the clinician in the diagnosis and therapy of these patients.

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GENERAL PATHOGENESIS

Central to the pathogenesis of DIC is the unregulated and excessive generation of thrombin. This overexpression of thrombin results in the consumption of coagulation factors that are the natural substrates of this protease, such as fibrinogen, factor V, and factor VIII. Thrombin, acting as a ligand, binds to thrombin receptors on platelets and endothelial cells, [16] [17] and is a potent agonist inducing platelet activation and aggregation. [18] Thrombin also induces the endothelial release of tissue plasminogen activator (t-PA). [19] In the presence of the newly formed fibrin monomer,[20] plasmin is proteolytically formed from plasminogen. This results in an aggressive secondary fibrinolysis. Therefore, the clinical and laboratory manifestations of DIC result from generation of these two proteases, thrombin and plasmin. The overexpression of thrombin with relatively reduced expression of plasmin may result in either large-vessel thrombosis or microvascular fibrin deposition leading to organ dysfunction and ischemic necrosis. Excessive thrombin generation with a vigorous secondary fibrinolysis may result in increased consumption of hemostatic components and bleeding. Both the bleeding and thrombotic manifestations of DIC are well documented in autopsy studies. [11] [21] [22] [23] Although this model is overly simplistic, it can provide a hypothetical basis by which the clinician can approach the diagnosis and treatment of DIC.

INITIATION OF PHYSIOLOGIC AND PATHOLOGIC THROMBIN GENERATION

The physiologic formation of the hemostatic plug requires the localized and limited generation of thrombin at the site of vessel injury. DIC results from a failure of the mechanisms that limit

the regulation of blood clotting and thrombin generation. This site-specific generation of thrombin is accomplished by a combination of regulatory mechanisms (Fig. 1171). Tissue factor is exposed or expressed at the site of vessel injury. This is followed by the sequential proteolytic conversion of proenzymes (zymogens) to enzymes by a mechanism that involves the formation of macromolecular complexes of enzymes and co-factors on specific receptors present on the surface of endothelial cells and platelets. [24] [25] [26] [27] [28]

The excessive generation of thrombin is limited by several natural inhibitory mechanisms. Tissue factor pathway inhibitor modulates the local effects of tissue factorfactor VIIa expression.[29] [30] The protease inhibitors antithrombin III and heparin co-factor II are bound to endothelial- and subendothelial-associated proteoglycans.[31] [32] When bound to these endothelial-associated, heparin-like molecules, they become potent inhibitors of factors IXa and Xa and thrombin. Thrombomodulin, a thrombin-specific receptor present on the surface of endothelial cells, binds thrombin and modifies its substrate specificity. Thrombin bound to thrombomodulin can no longer act as a procoagulant enzyme[33] [34] and becomes the primary protease for the activation of protein C. [35] The resulting activated protein C, along with its co-factor protein S, degrades factors Va and VIIIa, destroying their coagulant activity. [36] Finally, the fibrinplatelet hemostatic plug is limited by a highly regulated fibrinolytic system. Plasmin, the mediator of fibrinolysis, is produced by the proteolysis of plasminogen by t-PA. Plasmin rapidly degrades newly formed fibrin, reducing the size of the clot and initiating events that lead to vascular repair. DIC arises from an overwhelming of these regulatory mechanisms that leads to excessive generation of thrombin and a failure of the normal inhibitory pathways to prevent the systemic effects of the enzyme.

Although a wide variety of disorders are associated with the development of DIC, initiation most often involves a common pathway (Fig. 1171). Initiation of DIC usually involves one or both of two mechanisms: mechanical tissue injury and endothelial activation and injury. Primary to both initiating pathways is the exposure of blood to excessive amounts of tissue factor. Mechanical injury of organs rich in tissue factor, such as brain or placenta, may result in fulminant consumption, as observed in gunshot wounds to the brain[37] or abruptio placentae. [38] A variation of tissue injury is observed in patients with malignancy-associated DIC. Malignant tissues disrupt the normal vascular hemostatic regulatory mechanisms and expose blood to tumor-associated procoagulant activity. Malignant tissues have been shown to express tissue factor-like activity,[39] and some tumors have been shown to produce a cysteine protease capable of directly activating factor X. [40] [41] An extreme example of malignancy-associated DIC is observed in patients with acute promyelocytic leukemia. The coagulopathy persists while the tissue factor-rich leukemic blasts are present in blood and bone marrow. [42] [43] The down-regulation of tissue factor expression in the leukemic blasts by all-trans retinoic acid, resulting in the correction of the coagulopathy, is an excellent demonstration of the importance of tissue factor in the initiation of DIC in this disease. [44] [45] Studies using sepsis or endotoxin models of DIC have clearly demonstrated the primary role of tissue factor. [46] [47] [48] In addition, these studies have shown that the contact-phase proteins of the classic intrinsic pathway are not directly linked to the initiation of DIC and have only an ancillary role in the coagulopathy of sepsis. [48]

Resting endothelium does not express tissue factor. [49] Endothelial cells activated by cytokines, such as interleukin-1 and tumor necrosis factor, or injured by endotoxin express tissue factor activity. [50] [51] Viruses and other pathogens capable of infecting

Figure 117-1 Regulatory pathways of hemostasis and pathophysiology of DIC. APC, activated protein C; APL, acute promyelocytic leukemia; ATIII, antithrombin III; HCII, heparin co-factor II; prothrombinase complex, membrane complex of factors Xa and Va and prothrombin; PS, protein S; tenase complex, membrane complex of factors IXa, VIIIa, and X; TF, tissue factor; TF·FVIIa, tissue factorfactor VIIa complex; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; tPA, tissue plasminogen activator.

endothelium are also capable of inducing increased tissue factor expression. [52] In conjunction with this enhanced endothelial procoagulant activity, there is a suppression of endothelial anticoagulant properties as reflected in decreased thrombomodulin expression. [47] [52] [53] In addition, there is increased plasminogen activator inhibitor synthesis and secretion, resulting in suppression of t-PAmediated activation of fibrinolysis. [54] [55] In these circumstances, even without mechanical injury or cell death, blood is exposed to a potent procoagulant surface.

Monocytes and neutrophils play an important ancillary role in the initiation of DIC. Monocytes activated by endotoxin or antibodyantigen complexes can express tissue factor activity and assemble the prothrombinase complex on their membranes.[56] [57] Activated monocytes also secrete the inflammatory cytokines, interleukin-1 and tumor necrosis factor,[58] [59] which modulate endothelial procoagulant activity. [50] [51] Platelet-activating factor, another monocyte-derived cytokine, induces platelet activation and aggregation[60] and increases vascular permeability. [61] Activated neutrophils release a number of cellular enzymes that can injure endothelium and degrade subendothelial matrix.[62] Neutrophil elastase has been shown to degrade and inactivate a number of coagulation proteins and inhibitors, including factor V, factor VIII, antithrombin III, tissue factor pathway inhibitor, and plasminogen activator inhibitor. [63] [64] [65] [66] [67]

The coagulation inhibitors antithrombin III, heparin co-factor II, and thrombomodulin, although highly effective in regulating the localized generation of thrombin, are overwhelmed in DIC by the more potent systemic generation of thrombin and its precursor enzymes. Significant acquired deficiencies of antithrombin III are rarely observed except in cases of overwhelming acute DIC or cases complicated by significant hepatic dysfunction. [12] [68] [69] [70] Interleukin-1 and tumor necrosis factor, which are elevated in sepsis, have been shown to decrease thrombomodulin expression on endothelium. [47] [52] [53] Levels of protein C also appear to decrease significantly in patients with sepsis-induced DIC. [71] [72] [73] In addition, inherited deficiencies of both protein C or protein S may be associated with microvascular thrombosis and intravascular consumption, as observed in purpura fulminans of the newborn or Coumadin necrosis in deficient adults. [74] [75] [76] [77]

The variable clinical expressions of DIC result from this complex interaction of thrombin, plasmin, proteolytic inhibitors, platelets, endothelium, monocytes, and neutrophils. The underlying disorders that initiate DIC, such as sepsis, malignancy, or mechanical tissue injury, may trigger different hemostatic and cellular pathways for thrombin and plasmin generation, resulting in a clinical pathologic process that favors either thrombosis or consumption with bleeding. Furthermore, the intensity and duration of hemostatic activation can result in a fulminant (acute), subacute, or chronic presentation. Fulminant DIC is usually characterized by coagulation factor consumption and bleeding. The chronic forms of DIC are more frequently characterized by thrombotic manifestations. However, in a significant number of cases, there are few or no clinical manifestations of DIC exclusive of the abnormal laboratory findings. Morbidity and mortality in these patients predominantly result from the

underlying disease, and no specific treatment for the DIC is indicated.

DIAGNOSIS OF DIC: CLINICAL AND LABORATORY FINDINGS

Clinical Features

The clinical manifestations of DIC are frequently obscured by the clinical features associated with the primary initiating illness (see Table 1171). There is considerable variation in the frequency of specific clinical findings described in different case series, which probably reflects differences in the underlying primary disorders reported in each study.[9] [11] [12] [78] [79] Many of the clinical findings ascribed to DIC in early case series, such as the adult respiratory distress syndrome (ARDS) or acute tubular necrosis, are probably a direct result of the primary illness, shock, and hypoperfusion. Their onset frequently has no direct relationship to the initiation of DIC, and may precede its appearance. However, the microvascular thrombosis associated with DIC may aggravate multiorgan dysfunction, and less frequent pathologic features such as pulmonary hemorrhage and renal cortical necrosis may be directly linked to the development of DIC. [9] [11] [12] [21] [23] [78]

Bleeding is the predominant clinical manifestation observed in DIC and is reported in 7090% of patients. [9] [11] [12] [78] [79] Cutaneous, gastrointestinal, genitourinary, and pulmonary bleeding, along with bleeding at surgical sites, are the most frequently reported. However, in earlier case series, the definition of DIC was based primarily on laboratory studies that clearly defined a patient population with severe consumption of hemostatic components, including fibrinogen and platelets. Studies that use laboratory criteria of DIC based primarily on markers of thrombin generation, find many more cases of DIC without clinical evidence of bleeding then previously reported.[80] [81]

Overt thromboembolic manifestations have been less frequently observed in DIC and have been reported in 1040% of patients. [9] [11] [12] [78] [79] In patients with cancer, laboratory evidence of thrombin generation is frequently present without evidence of severe coagulation factor or platelet consumption. [82] [83] [84] [85] However, activation of coagulation pathways in the patient with cancer usually results in thromboembolic manifestations. [86] [87] [88]

Laboratory Features

Laboratory studies used in the diagnosis and evaluation of patients with DIC can be characterized as tests of thrombin and plasmin generation and screening tests for hemostatic function that delineate the severity of coagulation factor consumption (Table 1172). Although abnormalities in the screening assays such as the prothrombin time (PT), partial thromboplastin time (PTT), or platelet count may provide important corroborative evidence of hemostatic component consumption, the diagnosis of DIC should not be made without at least one positive test indicative of excessive thrombin generation. The two most readily available studies that document the excess activity of thrombin are immunoassays for D-dimers and the protamine paracoagulation assay for fibrin monomer. [80] [89] [90] [91] D-dimers are

TABLE 117-2 -- Laboratory Diagnostic Studies for Disseminated Intravascular Coagulation

Laboratory markers of thrombin generation
D-dimer
Protamine paracoagulation assay for fibrin monomer
Ethanol gel assay for fibrin monomer
Fibrinopeptide A
Prothrombin fragment 1.2
Thrombinantithrombin complex
Screening assays for factor and platelet consumption
Prothrombin time
Partial thromboplastin time
Thrombin time
Quantitative fibrinogen
Platelet count
Ancillary tests
Fibrin/fibrinogen degradation products
Euglobulin or dilute whole-blood clot lysis
Antithrombin-III level
$_2$ -Antiplasmin level
Factor V level

the proteolytic by-products of plasmin degradation of cross-linked fibrin monomers. In contrast to the older assays for fibrin/fibrinogen degradation products (FDPs), which do not distinguish between the plasmin degradation by-product of either fibrin or fibrinogen, the formation of D-dimers requires prior formation of fibrin monomer, which is cross-linked by factor XIIIa. Both the formation of fibrin monomer and the generation of factor XIIIa from its zymogen require thrombin. When using the D-dimer assay as the only laboratory marker of thrombin generation, caution must be exercised in interpreting abnormal results in patients who have had recent surgery, bleeding into tissues, cirrhotic liver disease, or renal failure. In each of these clinical circumstances, D-dimers can be moderately elevated without DIC. Therefore, patients with D-dimer levels <2,000 ng/ml should not be diagnosed as having DIC unless there is other corroborating evidence. [80] [89] [90] [92]

The protamine paracoagulation test detects the presence of excess soluble fibrin monomer, which is associated with fibrinogen in plasma. The presence of fibrin monomer documents prior action of thrombin on fibrinogen. This assay can be particularly helpful in clinical circumstances when there may be other disorders that could contribute to elevations in D-dimers. However, the assay is difficult and is most reliable when the laboratory has extensive experience with the assay. Additional

assays of thrombin generation that have been used in the evaluation of patients with DIC include the ethanol gel assay for fibrin monomer, [93] immunoassays for fibrinopeptide A, [94] thrombinantithrombin complexes,[95] and prothrombin fragment 1·2. [96] However, the routine clinical use of these assays is limited by their cost and complexity for simple and rapid laboratory screening. In addition, the latter three immunoassays have an excessive degree of sensitivity to thrombin generation, and positive results may not reflect clinically significant DIC.

Laboratory studies that delineate the severity of hemostatic component consumption in the patient with DIC include quantitative platelet counts and screening assays for plasma coagulation function. The screening tests include the PT, PTT, and thrombin time. Prolongations of the PT, PTT, and thrombin time assays result from the combined primary and secondary consumption of coagulation factors. Plasmin generated by secondary fibrinolysis results in the proteolytic degradation of factors, including fibrinogen and factors V, VIII, and XII. The intermediate plasmin degradation products of fibrin inhibit fibrin polymerization, which further contributes to the prolongations observed in these assays. [97] The results from these screening assays provide the information necessary for appropriate decisions regarding replacement treatment. These screening tests direct the clinician toward a diagnosis of acute DIC.

In five case studies of acute DIC, combining over 900 patients, the most frequent laboratory abnormalities reported, in decreasing order of frequency, were thrombocytopenia, elevated FDPs, prolonged PT, prolonged thrombin time, prolonged PTT, and a low fibrinogen. [9] [11] [12] [78] [79] Although deficiencies of nearly every coagulation factor have been reported, acquired deficiencies of factor V and fibrinogen are the most frequently observed in patients with acute DIC. A fibrinogen <100 mg/dl, in the absence of severe liver disease, is invariably associated with acute DIC, usually associated with bleeding manifestations, and denotes a poor prognosis. [9] [11] [12]

In chronic DIC syndromes, the degree of factor consumption may be limited and can be balanced by enhanced hepatic synthesis of coagulation factors, resulting in only minor abnormalities in the screening assays. Platelet counts may be at low normal levels and fibrinogen levels can be normal because hepatic synthesis of fibrinogen can be significantly up-regulated by inflammatory cytokines. [98] In these circumstances, the presence of high levels of the markers of thrombin generation, in the appropriate clinical settings, may be sufficient to make the diagnosis of DIC.

Despite the presence of a significant secondary fibrinolysis, screening assays for increased fibrinolysis, such as the dilute whole-blood clot lysis test or euglobulin clot lysis test, usually give normal results in patients with DIC. [9] [12] However, the use of these screening assays should be considered in clinical circumstances such as DIC in association with severe liver disease, [99] in acute promyelocytic leukemia, [100] [101] heat stroke, [102] amniotic fluid embolism, [103] or metastatic prostate carcinoma. [104] The clinician should be aware that there can be false-positive studies if these screening tests are performed in patients with fibrinogens <100 mg/dl.

The presence of thrombocytopenia, the most frequent laboratory abnormality observed in patients with DIC, should always direct the clinician to a review of the peripheral blood film. Fragmented red blood cells, although reported in patients with DIC, rarely constitute >10% of the red cells. [12] However, in some cases of chronic DIC with elevated D-dimers but normal coagulation screening assay results, the presence of fragmented red cells can provide confirmatory evidence. In contrast, disorders such as acute leukemia, sepsis, and thrombotic thrombocytopenic purpura can result in significant thrombocytopenia and a markedly abnormal blood film without laboratory evidence of DIC.

Several systems combining clinical and laboratory data have been devised to assist in the diagnosis and determination of the prognosis of patients with DIC. When these scoring systems were proposed, they were highly predictive of patient mortality. [9] [105] However, it is uncertain whether they are as predictive of patient outcome, in view of the respiratory, cardiovascular, and hemostatic support systems available today. A diagnosis of DIC should never be made based on laboratory abnormalities, but must include consideration for the underlying primary initiating illness and the patients clinical presentation.

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DIFFERENTIAL DIAGNOSIS OF DIC

A number of clinical disorders not associated with DIC can result in an acquired bleeding diathesis and significant laboratory hemostatic laboratory abnormalities. Thrombocytopenia can result from primary bone marrow failure, an infiltrative marrow process such as leukemia, endothelial-mediated platelet activation such as in vasculitis or thrombotic thrombocytopenic purpura, or immunologic destruction. A difficult clinical problem occurs in the patient with sepsis. [104] Sepsis without DIC can be associated with a variable degree of thrombocytopenia; however, sepsis with platelet counts <50,000 are most often associated with DIC. [106]

A rare syndrome of primary fibrinolysis occurs with the independent generation of plasmin without concomitant thrombin generation. [107] Such patients can have low fibrinogen, prolonged screening assays, and elevated FDPs. In addition, patients with liver disease and primary fibrinolysis with portal hypertension may have thrombocytopenia secondary to splenic sequestration. Therefore, the differential diagnosis in these patients may prove difficult. However, in these patients, the dilute whole-blood clot lysis and euglobulin clot lysis tests are significantly shortened, and the protamine paracoagulation assay is negative for fibrin monomer. Also, despite significantly elevated FDPs, the D-dimer assay results are normal or only minimally elevated. [108]

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CLINICAL PRESENTATIONS

Acute and Subacute DIC

Infection

Bacterial sepsis is a common cause of acute DIC. Thrombin generation results from bacterial or endotoxin-associated endothelial cell perturbation and monocyte activation, leading to

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enhanced tissue factor activity and inflammatory cytokine secretion. [25] [46] [47] [50] [51] [53] Gram-negative organisms are more commonly associated with sepsis and DIC, although cases are associated with gram-positive sepsis. [109] Infection with viruses such as varicella, rubella, rubeola, and influenza, along with intracellular pathogens such as *Mycobacterium tuberculosis*, can also lead to DIC through enhanced endothelial and monocyte tissue factor activity. [110] [111] [112] [113] [114] Both fungal infections and malaria have similarly been complicated by DIC. [115] [116] [117]

There is a wide range of clinical manifestations of sepsis-associated DIC. Many cases are characterized by laboratory changes without bleeding or clinical evidence of microthrombus formation. Examples of clinical entities at the other end of the spectrum include Waterhouse-Friderichsen syndrome and purpura fulminans. The Waterhouse-Friderichsen syndrome is rare and most often seen in association with fulminant meningococcal sepsis. [118] [119] It is characterized by widespread intravascular fibrin deposition and platelet thrombi that lead to microvascular obstruction of vital organs along with hemorrhagic necrosis of the adrenal glands. The latter leads to significant, often irreversible shock associated with a high mortality rate.

Virtually any gram-negative organism can cause DIC. The gram-positive bacterial infections associated with DIC often involve encapsulated organisms such as pneumococci. The overwhelming infection observed in those with diminished circulatory clearance due to functional or anatomic asplenia is frequently implicated. [120] Clostridial species are also associated with severe DIC due to septic abortion, which carries a very high mortality rate. [121] [122]

Purpura Fulminans

Purpura fulminans is a rare, severe skin disorder associated with DIC that primarily affects children and infants. Extensive areas of skin develop blue-black hemorrhagic necrosis, and biopsy typically reveals small-vessel microthrombi and vasculitis. Distribution of necrotic areas typically depends on the clinical setting. Patients are acutely ill with fever and hemorrhage from multiple sites, are hypotensive, and manifest typical laboratory signs of DIC. [123] [124] The pathogenesis of purpura fulminans is unknown, but histologic findings have been likened to the animal model of consumptive coagulopathy, the local Swartzman reaction. [125] It has also been suggested that the development of purpura fulminans in meningococcemia may result from acquired defects in the protein C pathway because similar lesions are seen in two other protein C deficiency states, namely, neonatal purpura fulminans and Coumadin-induced skin necrosis. [126]

Purpura fulminans occurs in three clinical settings. [127] In acute sepsis-associated or secondary purpura fulminans, the initial presentation is one of overwhelming infection that is most commonly meningococcemia, but has been reported with many other gram-positive and gram-negative organisms. [127] [128] [129] [130] [131] Patients are hypotensive with reduced peripheral perfusion contributing to skin necrosis on distal extremities; alternatively, skin necrosis can occur in a patchy distribution. [132] [133] Idiopathic purpura fulminans can present within 10 days of an antecedent illness, most commonly scarlet fever and varicella in children. Most skin lesions are localized to the breasts or lower half of the body. Third, purpura fulminans can be seen in association with homozygous protein C deficiency in affected neonates, in whom massive intravascular thrombosis and abdominal wall gangrene can also develop. [75] [76] [134] Acquired inhibitors of the protein C pathway can also be associated with this presentation. [135] Patients with Coumadin-induced skin necrosis may present with a similar but usually less severe form of purpura fulminans, and thus warrant consideration of protein C or protein S deficiency. [76] [136] [137]

The mortality rate has recently been significantly reduced in purpura fulminans, largely because of more widespread use of therapeutic heparinization in these patients and aggressive red cell and clotting factor replacement. [125] [127] [138]

Obstetric Complications

Pregnancy predisposes patients to DIC through the generation of a hypercoagulable state manifested by increased levels of most coagulation factors along with reduced fibrinolytic activity due to decreased t-PA production and increased plasmin activator inhibitor levels. [139] Overt clinical DIC manifestations occur when these baseline conditions are complemented by the occurrence of any of a number of acute obstetric complications that lead to the release of tissue factor or endothelial cell perturbation, followed by consumption of clotting factors and enhanced fibrinolysis.

The manifestations of DIC associated with obstetric complications are varied, with different grades of severity noted depending on the degree of hypofibrinogenemia and depletion of other clotting factors seen in each case. Because of this variation and baseline pregnancy-associated coagulation abnormalities, it is prudent to consider serial assessment of coagulation parameters when DIC is suspected to detect progressive alterations over time. Similarly, the coagulopathy may be localized to the uterus or disseminated through the blood.

Abruptio placentae, or placental detachment, has a wide range of clinical presentations, from incidental detection on pathologic examination of the placenta, to vaginal bleeding, to pronounced DIC with accompanying shock, bleeding, severe abdominal pain due to uterine contractions, and fetal death. [139] Both the exact causes of placental abruption, outside of trauma, and the subsequent processes leading to DIC remain unclear. Placenta is rich in tissue factor activity, which likely comprises an important component of the DIC pathophysiologic process. [140] [141] Hypofibrinogenemia has been documented in 38% of abruptio placentae cases, which implies that potentially serious and life-threatening DIC is present in a significant number of patients. [38]

Amniotic fluid embolism is a rare, usually fatal catastrophe occurring in 1 in 8,000 to 1 in 80,000 deliveries. Amniotic fluid is introduced into the circulation of multiparous, postmature women with large-for-dates fetuses and in those undergoing long and vigorous labor after induction. [142] [143] DIC is triggered by amniotic fluid tissue factor and an activator of factor X. [144] [145] [146] The extensive occlusion of the pulmonary arteries that causes sudden dyspnea, cor pulmonale, shock, and seizures is due to blockage with fetal debris and meconium. This in turn enhances local plateletfibrin thrombus formation. There can be secondary fibrinolysis, further contributing to defibrination and bleeding. This is followed after a latent period of up to 3 hours by severe bleeding in 37% of cases. [145] The mortality rate associated with this condition, primarily due to respiratory failure, is >80%. [140] As with abruptio placentae, the pathogenetic mechanism for the clinical manifestations is unclear.

Because of the rapid course of this complication and its infrequency, specific treatment modalities such as heparin and fibrinolysis inhibitors have not been adequately assessed, but may be considered in individual cases. Blood and coagulation factor replacement therapy warrants consideration in patients with active bleeding, along with pulmonary and hemodynamic support.

Sepsis during pregnancy is often caused by gram-negative bacteria and *Clostridium* sp., with the patient being particularly susceptible to DIC under these circumstances. Septic abortions are often associated with severe DIC accompanied by endotoxin-mediated shock and a high mortality rate. [140] [147] Virtually any organism can be associated with septic abortion. In addition, saline-induced abortion during the second trimester without sepsis also has a clear association with hypofibrinogenemia, thrombocytopenia, and increased FDPs. [148] [149]

Malignancies

Cancer can be associated with both acute or chronic DIC, depending primarily on the type of cancer. Malignancy-associated chronic DIC associated with solid tumors is reviewed later in the chapter, whereas acute DIC associated with leukemia is discussed here. DIC has been associated with acute myelocytic and lymphocytic leukemia, chronic myeloid and myelomonocytic leukemia, hairy cell leukemia, and angioimmunoblastic lymphadenopathy. [150] [151] [152] [153] [154] [155] [156] [157] [158] [159] Among these diagnostic entities, acute promyelocytic leukemia is most commonly associated with life-threatening hemorrhage secondary to DIC and other hemostatic abnormalities.

The pathogenesis of DIC associated with acute promyelocytic leukemia has remained controversial, and consequently the optimal therapeutic approach associated with this condition remains unclear. Leukemic cells from patients with acute promyelocytic leukemia have been found to harbor tissue factor, a factor X-activating protease, and produce interleukin-1. [160] [161] [162] [163] Increased levels of prothrombin fragment 1·2 and thrombinantithrombin III complexes have also been described in these patients, and document the excessive generation of thrombin in this leukemia. [164]

Investigators have also provided evidence for primary fibrino(geno)lysis as a contributing factor associated with serious bleeding seen in patients with acute promyelocytic leukemia. In this respect, urokinase-type plasminogen activator and t-PA have been found in acute promyelocytic leukemia cells along with reduced plasma activity of plasminogen activator inhibitor and reduced $_2$ -antiplasmin plasma levels. [101] [165] [166] [167] [168] [169] [170] [171] [172] In reality, it is likely both DIC and primary fibrino(geno)lysis contribute to bleeding in the patient with acute promyelocytic leukemia.

Although bleeding predominates in acute promyelocytic leukemia, thromboembolic complications have been noted in up to 5% of patients and 1525% of autopsy results. [173] [174] One study suggested acute promyelocytic leukemia cells from a patient with coagulopathy did not in fact have procoagulant activity, but rather were capable of elaborating interleukin-1 that induced endothelial tissue factor activity when mixed with endothelial cells. [169]

Acute promyelocytic leukemia-associated bleeding due to DIC is further exacerbated by thrombocytopenia due to marrow suppression. Furthermore, once treatment is initiated, subclinical DIC often becomes clinically apparent because of chemotherapy-induced acute promyelocytic leukemia cell lysis. [175] The incidence of coagulopathy- and hemorrhage-related mortality associated with acute promyelocytic leukemia has significantly decreased since the recent introduction of the differentiating agent all-trans retinoic acid into induction regimens, with bleeding frequently abating within 48 hours of treatment initiation. [44] [176]

Tissue Injury, Burns, and Heat Stroke

Extensive trauma and burns are often associated with both localized and disseminated intravascular coagulation. This is particularly true if there is a component of head injury associated with multiple trauma because brain parenchyma is rich in tissue factor. In this respect, laboratory-based DIC scores have been shown to have prognostic value in patients with head injury. [177] [178] [179] Studies have demonstrated a high mortality rate when head injuries are accompanied by bleeding and laboratory evidence of DIC, along with a correlation between time to treatment after trauma and the development and extent of DIC. [179] [180]

Extensive exposure of circulating blood to tissue factor is probably the most prevalent causative factor in trauma-associated DIC. Other possible contributions to this clinical picture may relate to dilutional coagulopathy due to blood loss or replacement with crystalloid solutions and packed red cells alone; ischemic or trauma-associated hepatic dysfunction with reduced clotting factor and natural inhibitor synthesis; and ARDS.

Massive trauma is often accompanied by ARDS, to which hypotension followed by aggressive fluid replacement contribute. Obstruction of the pulmonary circulation secondary to multiple thrombi may also contribute to ARDS. [181] One study noted that among 30 patients with ARDS, 7 had evidence of additional thrombotic microangiopathy in the skin and kidneys, whereas an additional 12 had thrombocytopenia and 4 had pulmonary vessel fibrin deposition on postmortem examination. [182]

Serious trauma cases, usually but not always involving multiple fractures, can lead to fat embolism within the first 48 hours. [183] [184] This is associated with respiratory distress and neurologic impairment secondary to marrow fat emboli from associated fractures. If the pulmonary picture is complicated by ARDS, laboratory evidence of DIC may also be present because of endothelial perturbation, tissue factor expression, and inhibition of fibrinolysis. [184]

Burns can be associated with DIC through release into the circulation of tissue factor from damaged tissues along with associated hypovolemic shock and infection, which occur later in the clinical course. In addition to DIC, significant local consumption of clotting factors has also been associated with burns. [185]

Both DIC and primary fibrinogenolysis have been demonstrated in association with heat stroke, a syndrome defined as a body temperature >42°C due to collapse of thermoregulatory mechanisms. [186] [187] Pathophysiologic mechanisms associated with the disorder include tissue factor expression from damaged tissues and endothelial cell perturbation. [188] Extensive fibrin deposition with subsequent increased release of plasminogen activators and secondary fibrinolysis has been associated with the generalized bleeding and multiorgan failure accompanying fatal cases of heat stroke. [189]

Chronic DIC

Malignancies

The association between solid tumors and thrombotic and bleeding symptoms was initially described by Trousseau in 1865. *Trousseau syndrome* refers to either spontaneous recurrent or migratory venous thrombosis, or arterial emboli due to nonbacterial thrombotic endocarditis, or both, occurring in the setting of malignancy. [190] There is a spectrum of thrombohemorrhagic diagnostic entities seen in association with solid tumors, including low-grade DIC, venous thromboembolic disease, primary fibrino(geno)lysis, microangiopathic hemolytic anemia, and nonthrombotic valvular endocarditis. [191] These different disorders can present singly or multiply at any location and with any degree of acuteness or chronicity.

Disseminated intravascular coagulation associated with solid tumors demonstrates pathophysiologic mechanisms similar to those of the DIC seen with acute promyelocytic leukemia. These include tissue factor secreted by tumor cells and activated monocytes. [192] [193] A factor X-activating cysteine protease has been described in human carcinoma tissue. [39] [40] [41] Tumor cells or media conditioned by these cells induce platelet aggregation both in vitro and in vivo. [194] [195] [196]

Chronic DIC is classically associated with thrombosis. Among the many studies done in the area of thrombosis and bleeding in association with solid tumors, one group documented deep venous thrombosis in 113 of 182 (62%) patients with cancer. Ninety-six of the 182 (53%) manifested migratory thrombophlebitis, 41 (23%) demonstrated nonbacterial thrombotic endocarditis, a disorder classically associated with mucin-producing carcinomas, whereas 75 (41%) had bleeding manifestations. [197]

Classic laboratory findings associated with chronic DIC and

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solid tumors include thrombocytopenia and circulating FDPs. Hypofibrinogenemia also occurs, but is less common. Microangiopathic hemolytic anemia may occur in the absence of other DIC laboratory abnormalities, usually in association with disseminated mucin-secreting adenocarcinoma. Laboratory manifestations of increased thrombin generation are seen in patients with solid tumors and may not be accompanied by clinical thrombosis or bleeding.

Primary fibrino(geno)lysis is primarily seen in patients with prostate cancer and acute promyelocytic leukemia. These tumors are capable of producing activators of plasminogen and thrombin.[104] [198] Biopsy of prostate tumors is sometimes accompanied by prolonged postoperative bleeding as a result of this phenomenon, and hemorrhagic manifestations can dominate the clinical picture. [199]

Dead Fetus Syndrome

The dead fetus syndrome occurs several weeks after intrauterine fetal death and can be accompanied by DIC. [200] [201] The clinical syndrome is usually one of insidious-onset DIC mediated by tissue factor from the placenta or dead fetus entering the maternal circulation over the ensuing days, eventually causing bleeding. Serial laboratory monitoring of the mother after diagnosis of intrauterine fetal death is warranted to detect possible progressive coagulopathy; in this event immediate evacuation of uterine contents is indicated.

Localized Intravascular Coagulation

Aortic Aneurysm

Extensive aortic aneurysms have been associated with significant laboratory abnormalities and bleeding secondary to platelet and fibrinogen consumption. [202] These complications are characteristically associated with extensions and enlargements of the dissecting aneurysm, which in turn are often accompanied by sudden onset of severe pain. In one series of patients with aortic aneurysms, 40% had chronically elevated levels of fibrin split products, with 4% showing evidence of significant bleeding and laboratory findings of DIC. [203] Management of DIC in association with aortic aneurysms is outlined in the section on treatment (see later).

Hemangiomas

Kasabach and Merritt were the first to report localized intravascular coagulation in association with hemangiomas. [204] These are usually benign tumors seen mostly in infants and children that typically enlarge over time. They are associated with consumption of platelets and fibrinogen as demonstrated by radiolabeling studies showing intravascular clotting and excessive fibrinogenolysis (the latter due to t-PA release from abnormal endothelium of tumor vessels). [205] [206] [207] Microangiopathic hemolytic anemia has also been observed in this setting along with laboratory evidence of DIC. [208] [209] Clinical DIC and emboli to other organs, however, usually are not seen in association with hemangiomas. Severe bleeding is normally noted only after surgery or other invasive procedures associated with the hemangioma. [209]

Liver Disease and DIC

Patients with advanced liver disease are prone to a plethora of hemostatic and thrombotic derangements that are often very difficult to classify and hence successfully manage on a pathophysiologic basis. Alterations in hepatic synthesis and both hepatic and renal clearance of activated coagulation, fibrinolytic, and natural inhibitor proteins, along with the thrombocytopenia and the deranged hemodynamics typically seen in this patient population, collectively contribute to the difficulties encountered.

In those with advanced or acute, severe liver disease, the clinician may expect to find decreased hepatic synthesis of most coagulation proteins, protein C, protein S, antithrombin III, and plasminogen, along with $_2$ -antiplasmin. The liver also demonstrates defective clearance of activated clotting proteins under these circumstances.[210] [211] Portal hypertension leads to splenomegaly with enhanced platelet sequestration and thrombocytopenia.

Given these circumstances, the issue of whether DIC is directly associated with liver disease, as opposed to the observation that these patients may simply be more sensitive to DIC, is an unsettled one. Experimental observations exist either suggesting the existence of an association [212] [213] [214] [215] [216] [217] [218] or supporting the hypothesis these hemostatic derangements may stem from disorders other than DIC.[219] [220] [221] [222]

As is the case with the pregnancy-associated disorders, the diagnosis of DIC in patients with liver disease and generalized bleeding not due to a local source is most readily appreciated by following serial hemostatic parameters and noting a significant reduction in these values over a short period of time. If available, tests reflecting thrombin generation, such as the protamine paracoagulation assay, the prothrombin fragment 1·2 or thrombinantithrombin III complex levels, along with D-dimer levels, can also contribute to establishing the diagnosis.

Disseminated intravascular coagulation is also seen after the placement of peritoneovenous (LeVeen) shunts for complications associated with massive ascites. The incidence is variously quoted in different studies as ranging from 3 to 91%, and may depend on whether the primary disease is peritoneal or hepatic in origin. [223] [224] [225] [226] Ascitic fluid possesses both tissue factor activity in association with monocytes and thrombin activity, which likely contribute to the DIC. [227] Clamping the shunt is the only effective treatment in severe cases because heparin and antithrombin-III concentrates have not proven efficacious. [228] [229]

Fatty liver of pregnancy has also been associated with DIC on the basis of labor-induced clotting factor and platelet consumption in the presence of decreased synthesis of the clotting factors and natural inhibitors. [230] The clinical and laboratory features have been attributed to low plasma antithrombin-III levels, and in this respect patients have responded to antithrombin-III concentrate. [231]

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TREATMENT OF DIC

General Measures and Replacement Therapy

Given the heterogeneity of the underlying disorders with which DIC is associated, the difficulties often encountered with determining the extent of consumption of clotting factors and fibrino(geno)lysis in each case, and the relative lack of well controlled studies of treatment of homogeneous subgroups with DIC, it is not surprising that many aspects of treatment of DIC are controversial. Part of the problem resides with the fact that studies are very difficult to perform in view of the variety of pathophysiologic triggers and clinical presentations associated with DIC. Thus, DIC is one disorder in which management has to be individualized, with careful attention to variables such as the initiating illness and the presence of bleeding or thrombosis.

The cornerstones of management of DIC include aggressive basic support measures, paying close attention to circulatory volume status, gas exchange, and electrolyte balance, along with prompt, vigorous treatment of the underlying cause of the DIC. This includes, for example, intravenous antibiotics for gram-negative septicemia, prompt uterine evacuation for abruptio placentae, and restoration of hemodynamic stability for hypovolemic shock. The other management components of the disorder include determining whether replacement of

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REPLACEMENT THERAPY FOR DIC

The symptoms and abnormal laboratory findings associated with DIC are most readily addressed through the prompt recognition and treatment of the underlying disorder. Until the underlying cause can be definitively managed, blood product replacement therapy warrants consideration in the patient with significant bleeding or a planned procedure and coagulation parameters compatible with DIC. Replacement folate and vitamin K therapy also warrant consideration in any patient with prolonged DIC.

Platelet transfusions should be considered to maintain the count >2030 × 10^9 /L in a bleeding patient, while aiming for higher values if concurrent functional impairment is present, a procedure is planned, or blood loss is significant. DIC-associated thrombocytopenia does not usually reach levels of 20 × 10^9 /L, however, without concurrent contributing factors such as sepsis. One unit of donor platelets is expected to contain at least 5.5 × 10^9 platelets and raises the count 68 × 10^9 /L, assuming normal splenic pooling. The platelet count should be repeated 1060 minutes after the first transfusion to confirm the expected increment, and every 6 hours after that. Poor postplatelet transfusion increments are often seen in consumptive thrombocytopenia, making transfusion support difficult under these circumstances.

Cryoprecipitate administration is considered in a symptomatic patient to maintain plasma fibrinogen over 100 mg/dl. The fibrinogen content of each cryoprecipitate unit varies from 100 to 250 mg, depending on variable storage volumes and pooling methods. Thus, from 1 to 4 units/10 kg can be given, with the postinfusion increment checked 3060 minutes later and followed by repeat determinations every 6 hours.

Fresh frozen plasma is given if significant DIC-associated bleeding is accompanied by a prolonged PT and PTT. The aim is to reduce the PT, with the target in each case dependent on the nature and extent of bleeding, the presence and extent of co-existing hemostatic defects, and whether a procedure is planned, and its attendant bleeding risk. This is accomplished through administration of 1015 ml/kg of plasma.[265] Each milliliter of plasma contains 0.71 unit of activity of each clotting factor and 12 mg of fibrinogen, so an alternative formula to 1015 ml/kg incorporates estimated plasma volume, desired factor level increment, and the half-life of the factor being replaced into the equation. The PT and PTT should be checked every 6 hours and plasma given as needed based on the patients clinical response. Depending on clinical circumstances, bleeding patients with DIC given plasma transfusions can exhibit poor clinical and laboratory responses, particularly if advanced liver disease with co-existent diminished clotting factor synthesis and altered hemodynamics complicates the picture.

depleted platelets or clotting factors is necessary and whether attempted abrogation of the DIC with heparin or natural inhibitors should be considered.

The idea that blood replacement therapy may be fueling the fire of DIC has never been proven. Transfusion of platelets, cryoprecipitate, and fresh frozen plasma should be reserved for those with both clear laboratory evidence of DIC and either bleeding or a pending invasive procedure. It is typically most effective in those with very low platelet and fibrinogen levels in whom DIC is self-limited or concluded.

Patients with head injury-associated brain tissue destruction are particularly prone to acute, limited defibrination (hypofibrinogenemia, elevated levels of serum fibrinogen-related or fibrin-related split products, and low levels of factors V and VIII or platelets), in which laboratory abnormalities often return toward normal within hours.[37] These patients benefit from rapid, optimal correction of hemostatic deficiencies to minimize the neurologic consequences of intracranial bleeding, whereas heparin therapy to prevent ongoing intravascular coagulation is not helpful because of its short-lived course and associated intracranial bleeding risk.

Platelets should ideally be maintained at over 2030 × 10^9 /L in a bleeding patient, whereas those undergoing procedures or who have concurrent functional impairment or significant blood loss should have higher counts. Cryoprecipitate should be given to maintain plasma fibrinogen >100 mg/dl, whereas fresh frozen plasma is indicated to correct a prolonged PT and PTT in appropriate clinical circumstances. To determine the efficacy of transfusion, platelet and fibrinogen levels should be checked 3060 minutes after the transfusion and every 6 hours after that. Replacement therapy may be required every 8 hours while the underlying cause of the DIC is being addressed. Transfusions can be discontinued when the coagulation parameters are almost or fully normalized and symptoms have abated. If laboratory parameters fail to improve with transfusions because of DIC-associated bleeding, a concurrent heparin infusion may warrant consideration.

The Role of Heparin in DIC Management

Heparin should be considered in the management of DIC if (1) a patient has strong clinical and laboratory evidence of DIC and thromboembolic manifestations predominate (e.g., purpura fulminans, dead fetus syndrome before induction, aortic aneurysm prior to surgery); or (2) intensive blood replacement therapy fails to alleviate excessive bleeding and increase the level of clotting factors. These circumstances notwithstanding, it is important to consider that heparin is less likely to lead to clinical improvement in DIC once it is established and ongoing, which is usually the case by the time it is diagnosed. Heparin is also capable of exacerbating DIC-associated bleeding in these populations and should be used very cautiously. For these reasons, heparin usually has a limited role, if any, in acute DIC.

In chronic DIC associated with solid tumors, the dead fetus syndrome, or hemangiomas, continuous heparin infusion without a loading bolus can be given with blood products if the clinical picture warrants treatment (e.g., deteriorating coagulation parameters in dead fetus syndrome, thrombosis, before chemotherapy). Once symptoms have resolved, patients with solid tumors can be given long-term, adjusted-dose subcutaneous heparin or lowmolecular-weight heparin rather than oral anticoagulants, which are frequently ineffective under these circumstances. [232] Because the baseline PTT is often prolonged in these patients, the antithrombotic effect of heparin may be monitored by serial heparin or anti-factor Xa levels.

The role of heparin in treatment of acute promyelocytic leukemia remains controversial, with evidence supporting [160] [161] [162] [163] [164] [233] [234] [235] and refuting[236] [237] [238] its use in the complex coagulopathy associated with this disorder. Any role it plays may decline in the future in light of preliminary data suggesting the incidence of acute promyelocytic leukemia-associated coagulopathy is diminished when all-trans retinoic acid is included in induction regimens. [44] [45] [176] Similarly, the use of heparin in DIC associated with advanced liver disease is controversial. [239] [240] [241] [242] It is contraindicated in abruptio placentae because of bleeding complications associated with delivery and hysterectomy, if

HEPARIN IN THE MANAGEMENT OF DIC

The circumstances under which a trial of heparin should be considered in the management of a patient with clinical and laboratory evidence of DIC are outlined in the text. In general, if the clinical setting is more compatible with chronic DIC (e.g., secondary to solid tumor), the picture is more likely to be complicated by thromboembolic phenomena rather than bleeding, which in turn necessitates consideration of heparin.

If an initial heparin bolus is indicated, one nomogram suggests 80 U/kg intravenously (IV) can be given, followed by an 18 U/kg/hr infusion. [266] Heparin has variable sensitivity to different thromboplastin reagents incorporated in individual laboratory PTT assays. Therefore, any laboratory nomogram used in heparin dosing needs to have its dosing recommendations based on the therapeutic range for the local PTT reagent tailored to corresponding heparin levels of 0.20.4 U/ml by protamine titration or anti-factor Xa levels of 0.30.7 U/ml. If no bolus is indicated, patients can be started on an 18 U/kg/hr infusion. The PTT, heparin level, or anti-factor Xa level should be checked 6 hours after the start of therapy, and dosage adjustments made accordingly.[266]

For long-term outpatient therapy (e.g., for solid tumor-associated chronic DIC), it is also possible to achieve therapeutic heparin levels through subcutaneous injection, with its associated anticoagulant effect occurring 1 hour later and peak levels after 3 hours. Typically, 35,000 U are given daily, divided into two doses 12 hours apart, with a PTT drawn 6 hours after a dose and subsequent dosage adjustments made based on the result. Serial heparin or anti-factor Xa levels may be preferable for monitoring if the baseline PTT is prolonged. If an initial rapid effect is desired, a 5,000-U IV heparin bolus can be given before the initial subcutaneous dose. Efficacy of heparin treatment can be monitored by changes in D-dimer, fibrinogen, and platelet levels.

Early evidence suggests a potential role for lowmolecular-weight heparin in the prevention and management of DIC in association with acute promyelocytic leukemia and Trousseau syndrome.[245] [246] In this case, enoxaparin may be administered subcutaneously prophylactically at 30 mg every 12 hours, or therapeutically at 1 mg/kg every 12 hours, whereas dalteparin doses are 2,500 U daily or 100 U/kg every 12 hours, respectively.

these are carried out, and controlled trials have not been done in acute DIC associated with septic abortion.

The role of heparin in management of aortic aneurysms depends on whether the patient is a surgical candidate. Thus, although a nonsurgical patient with only laboratory evidence of DIC does not need heparin, it is prudent to obtain a DIC screen on any patient with an aortic aneurysm in whom surgery is contemplated to avoid serious intraoperative and postoperative bleeding. Preoperative correction of any hemostatic defects with replacement therapy and continuous heparin infusion is in order.[243] These maneuvers should also be considered in patients with bleeding aortic aneurysms. Those with evidence of an aneurysmal leak associated with factor consumption require transfusion of platelets and cryoprecipitate and emergency surgery, however, and should not receive heparin.

The use of lowmolecular-weight heparins in prophylaxis and treatment of DIC has recently been described in several case reports. [244] [245] [246] Although no control trials have yet been reported, these early reports suggest that lowmolecular-weight heparins may be as efficacious as adjusted-dose heparin, providing reproducible anticoagulation with less frequent dosing. In one study, six patients with acute promyelocytic leukemia received enoxaparin from the day of admission until presenting skin and mucous membrane bleeding resolved and the coagulation values returned to normal (1123 days). Three had laboratory evidence of DIC on presentation, and

significant bleeding complications developed in two patients while on lowmolecular-weight heparin, and in a third 3 days after its discontinuation. [246]

Antithrombin-III and Protein C Concentrates

Antithrombin-III concentrate has been used in the management of subsets of patients with DIC on the premise that systemic consumptive coagulopathies often lead to a relative plasma deficiency of natural inhibitors like antithrombin-III that in turn contributes to a procoagulant state. Antithrombin-III deficiency would also be expected to decrease the effect of any therapeutic heparin used to treat DIC, and so antithrombin-III concentrates warrant consideration in any heparinized patient with DIC, particularly if heparin resistance is encountered.

Exogenous antithrombin-III neutralizes thrombin generated in DIC and potentially ameliorates thrombosis, particularly if the process is associated with hepatic insufficiency with its associated decrease in antithrombin-III synthesis. In this respect, antithrombin-III shortens the duration of DIC in some patients with fatty liver of pregnancy, and should be considered in this setting. [241] Except for hepatic insufficiency, other studies have shown improved hemostatic function in most patients with DIC given antithrombin-III, but no effect on mortality. [247] [248] [249] Thus, more experience is needed with this agent in these other settings.

Activated protein C has been given to animals in concert with infusion of *Escherichia coli* with the subsequent prevention of DIC and gram-negative septicemia. [250] Conversely, when these infusions were supplemented with anti-protein C antibody, inhibition of protein C led to enhanced *E. coli*-associated lethality. Additional studies suggest activated protein C concentrates ameliorate DIC in a rabbit model, with less bleeding than was associated with heparin. [251] [252] Human studies of protein C concentrate replacement in infants with purpura fulminans due to severe protein C deficiency and in infants, children, and adults with meningococcemia appear promising. [253] [254] [255] [256] Further studies are needed to determine if protein C and activated protein C concentrates have any role in treatment of human DIC. A case series of pediatric patients with meningococcemia and DIC managed with either plasma or whole-blood exchange led to survival of seven of eight patients, with no associated bleeding or cardiovascular complications during the procedure. [257]

Fibrinolysis Inhibitors

Under most circumstances, patients with DIC should not be treated with fibrinolytic inhibitors. These agents act by blocking the secondary fibrino(geno)lysis that accompanies DIC to varying extents, and may prevent tissue perfusion. [258] [259]

The DIC setting in which fibrinolytic inhibitors may warrant consideration includes (1) when intense primary fibrino(geno)lysis can be demonstrated in association with disease states, such as acute promyelocytic leukemia, [101] Kasabach-Merritt syndrome, [260] [261] and prostate cancer; and (2) when the patient is bleeding profusely and is not responding to replacement therapy. In the latter case, FDPs may be contributing to bleeding, and the use of fibrinolytic agents should be considered only after the continuously bleeding patient has received both replacement

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therapy for depleted hemostatic factors and intravenous heparin infusions. [262] [263] epsilon-Aminocaproic acid or tranexamic acid may be useful in these cases in concert with heparin for the co-existing DIC. [264]

An approach to treatment of Kasabach-Merritt syndrome entails administering a fibrinolytic inhibitor either alone or in combination with cryoprecipitate. [101] [261] The result has been tumor shrinkage and correction of hemostatic defects through induction of thrombosis within the tumor.

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Chapter 118 - Hemostatic Defects Associated with Dysproteinemias

Howard A. Liebman

INTRODUCTION

Acquired coagulation abnormalities are frequent manifestations of dysproteinemias. [1] [2] Most of these patients do not manifest bleeding, and the hemostatic abnormalities are usually detected only on laboratory evaluation. However, 10% of patients have clinical evidence of bleeding. [3] [4] In most cases bleeding is mild, consisting of purpura, epistaxis, or hematuria. However, occasionally severe bleeding and even fatal gastrointestinal hemorrhages have been observed. The reported hemostatic abnormalities are varied and involve both coagulation and platelet dysfunction.

The pathophysiologic mechanism responsible for these coagulation defects is an abnormal interaction between a hemostatic component and a plasma paraprotein (Fig. 118-1). Paraproteins are monoclonal immunoglobulins produced by plasma cell dyscrasias and lymphoid neoplasms, such as multiple myeloma, plasma cell leukemia, Waldenström's macroglobulinemia, lymphoma, and primary (AL) amyloidosis. The circulating paraprotein may consist of the complete immunoglobulin, the immunoglobulin light chain, or other immunoglobulin fragments. The abnormal immunoglobulin does not have to be present in high concentrations in the circulation but can be expressed on the surface of the malignant lymphocyte or be deposited in the extracellular matrix. The serendipitous synthesis of an immunoglobulin by a malignant lymphocyte that possesses a structure that binds with a component of the platelet surface or coagulation proteins can result in inhibition of function or accelerated clearance of the platelet or coagulation protein.

The hemostatic defects associated with dysproteinemias include (1) coagulation abnormalities that result from plasma paraproteins that bind hemostatic components and inhibit their function; (2) abnormalities secondary to the increased clearance of coagulation proteins by forming complexes with circulating paraproteins, binding to cell-surface immunoglobulins or binding to immunoglobulin fragments deposited in the extracellular matrix; and (3) hemostatic abnormalities observed in association with dysproteinemias for which no clear pathophysiologic mechanism linking the abnormal paraprotein and the hemostatic defect can be found.

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INHIBITORS OF HEMOSTASIS

A prolonged bleeding time with a normal or a moderately decreased platelet count is a common hemostatic defect in dysproteinemias. [5] [6] [7] [8] [9] In a prospective study of coagulation abnormalities associated with dysproteinemias, 45% of patients had an abnormal bleeding time. [2] In patients who hemorrhaged, a prolonged bleeding time was observed in 67%. An abnormal bleeding time was also observed in one-third of patients who do not exhibit bleeding.

The prolonged bleeding time in these patients is associated with abnormalities of in vitro platelet function. Abnormalities include decreased platelet adhesiveness, [2] [10] [11] impaired platelet aggregation, [7] [9] [10] and reduced platelet factor 3 availability. [9] [10] [11] Abnormal platelet function studies are usually more frequent with higher concentrations of the paraprotein and with increased plasma viscosity. A paraprotein concentration above 5 g/dl is frequently associated with defective platelet function.

Several investigators have studied the effect of purified paraproteins on platelet function. [9] [11] Depressed platelet function was observed after the addition of the paraprotein to normal platelet-rich plasma. The resulting defects have been attributed to the nonspecific adsorption of the paraprotein to the platelet surface, resulting in defective platelet surface function.

A severe hemorrhagic disorder due to the development of a thrombasthenic-like platelet defect has also been reported. [12] In the patient described, there was high-affinity binding of the monoclonal paraprotein to glycoprotein IIIa on the platelet surface. The immunoglobulin completely inhibited platelet aggregation by preventing fibrinogen binding to the platelet surface.

Since markedly elevated plasma concentrations of paraproteins are more commonly associated with defective platelet function, plasmapheresis has been used in the treatment of hemorrhage.[2] [7] [9] [10] [13] There are no clinical signs or laboratory assays that predict response to plasmapheresis. However, resolution of bleeding has been reported in some patients.

Most patients have other coagulation assay abnormalities in addition to an abnormal bleeding time. [2] Approximately 50% of patients with multiple myeloma and macroglobulinemia have a prolonged thrombin time (TT). [2] This is associated with the appearance of an abnormal fibrin clot. [5] [10] [14] [15] [16] [17] [18] [19] [20] Fibrin clots formed in the presence of the paraprotein are transparent and gelatin-like and display poor retraction. Electron microscopic studies of fibrin clots from these patients show a structure that is poorly branched with narrowed fibrin strands. [10] [20]

The effect of paraproteins on fibrinogen cleavage and fibrin clot formation has been studied extensively. Early investigators suggested that the prolonged TT resulted from paraprotein inhibition of thrombin cleavage of fibrinogen. [14] [15] [16] [17] These paraproteins were termed antithrombin V. [15] [16] However, subsequent studies have failed to demonstrate antithrombin activity. [10] [18] [20]

Inhibition of fibrin polymerization by the paraprotein accounts for the prolonged TT and abnormal clot formation. [18] [19] [20] [21] [22] [23] Studies using purified monoclonal immunoglobulins have shown that these proteins inhibit polymerization of fibrin monomer. Paraproteins of all immunoglobulin subtypes can inhibit polymerization. [21] Using immunoglobulin fragments, the inhibitory activity is found to reside on the $F(ab)_2$ or Fab portions of the molecule. [19] [21] [23] This finding suggests that the antigen-binding domain of the monoclonal immunoglobulin may be responsible for fibrin/fibrinogen binding. However, direct binding of the inhibitory immunoglobulins to fibrin or fibrinogen has not been demonstrated under the conditions employed. [10] [22] [23] Therefore, the interactions between the inhibitory immunoglobulins and fibrin may be of low affinity and not a typical antigen-antibody interaction.

Despite the abnormal TT, bleeding is rarely observed in patients with inhibitors of fibrin polymerization. In patients who hemorrhage, other associated hemostatic defects are usually observed. Abnormalities of platelet function are frequently present. Large-volume plasmapheresis with or without plasma infusions has been effective in controlling hemorrhage in selected

Figure 118-1 Schematic representation of the pathophysiologic mechanisms responsible for hemostatic abnormalities in patients with dysproteinemias. **(A)** Paraproteins bind to platelets or to fibrinogen/fibrin and inhibit their function. **(B)** Circulating paraproteins bind to von Willebrand protein (vWF), resulting in increased clearance by the reticulendothelial system (RES). Factor X is rapidly cleared from plasma by binding to paraproteins deposited in the subendothelium as amyloid.

patients. [6] [9] [10] [13] Fibrinogen given as fresh frozen plasma or cryoprecipitate is not helpful. In fact, most patients have elevated plasma concentrations of fibrinogen. [24]

Circulating paraproteins that inhibit the function of von Willebrand factor (vWF) [25] [26] and factor VIII [27] [28] [29] [30] [31] [32] have been described. Unlike the inhibitors that interfere with fibrin polymerization, these inhibitors are associated with clinical bleeding syndromes. Two patients with IgG multiple myeloma have been described with circulating paraproteins that inhibited ristocetin-induced platelet agglutination and decreased platelet adhesiveness. [25] [26] These paraproteins bound vWF when studied by crossed immunoelectropheresis. Unlike patients with acquired von Willebrand disease, both patients had increased plasma von Willebrand antigen and normal factor VIII activity. A patient with acquired von Willebrand disease and an inhibitor of vWF adherence to collagen has been described. [33] The patient had a low-grade lymphoma and an IgG paraprotein. However, immunoprecipitation studies demonstrated that the inhibitor was an IgM antibody and not the IgG paraprotein.

Six patients with paraproteins that inhibit factor VIII coagulant activity have been described. The patients had IgA multiple myeloma, [27] light chain myeloma, [28] [29] Waldenström's macroglobulinemia, [30] chronic lymphocytic leukemia with an IgM paraprotein, [31] and amyloidosis with an IgA paraprotein. [32] The inhibitory immunoglobulins were characterized by their ability to inhibit factor VIII activity in normal plasma. The paraproteins resemble the type II antibody inhibitors in hemophiliacs. [34] The inhibitory paraproteins were slow-reacting antibodies of low affinity for factor VIII. There was significant residual factor VIII activity (710%) in plasma of three of these patients despite high plasma concentrations of the paraproteins, [27] [30] [32] a characteristic of type II antibodies. One patient with kappa light chain myeloma had less than 1% factor VIII activity and 5 Bethesda units of inhibitory activity in his plasma. The kappa light chains

were isolated from the patient's urine and shown to have factor VIII inhibitory activity. [29]

Large-volume plasmapheresis, combined with the infusion of cryoprecipitate or factor VIII concentrates, is only transiently effective in the treatment of hemorrhage in patients with factor VIII inhibitors. One patient with multiple myeloma experienced a clinical remission with improvement in his bleeding symptoms after treatment with alkylating agents. [26] A patient with light chain myeloma with factor VIII inhibitory activity responded rapidly to treatment with the VAD regimen, and response was closely associated with the disappearance of free light chains in the patient's urine. [29] High-dose intravenous -globulin has been reported effective in combination with infusions of factor VIII concentrate in patients with spontaneous inhibitors to factor VIII, but its clinical value in this condition is unknown. [35] Porcine factor VIII appears to be highly effective in the treatment of acquired factor VIII inhibitors [36] and may be the treatment of choice in this disorder, if the inhibitory paraprotein does not cross-react with the porcine factor VIII. Recombinant factor VIIa has also been shown to be effective in patients with acquired factor VIII inhibitors and may provide an effective alternative to porcine factor VIII. [37]

Paraproteins with a specificity for phospholipids and the characteristics of a lupus anticoagulant have been reported. [38] [39] The in vitro inhibition of coagulation protein binding to phospholipid by the patients' paraproteins was not associated with bleeding or thrombosis.

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ACCELERATED CLEARANCE OF COAGULATION PROTEINS

Decreased plasma concentrations of a number of coagulation proteins, including factor V, factor VII, factor VIII, factor X, prothrombin, protein C, and fibrinogen, have been reported with dysproteinemias.[2] [24] [40] [41] [42] Perkins et al.[2] reported severe deficiencies of coagulation proteins in eight (12%) of 62 patients. However, bleeding was observed in only two of the eight patients. Low levels of coagulant activity in these patients are not due to inhibition by the paraprotein. The deficiencies are usually corrected by transfusion of the missing plasma protein. Therefore, a circulating anticoagulant is not present. The proposed mechanism for these deficiencies is an accelerated clearance of coagulation protein and paraprotein complexes, [40] [41] [42] although many investigators have failed to find evidence of these complexes in plasma.[2] [11]

Acquired von Willebrand disease is often reported in association with dysproteinemias and lymphoproliferative disorders. [43] [44] [45] [46] [47] [48] [49] This disorder is characterized by a prolonged bleeding time, decreased platelet adhesiveness, reduced ristocetin-induced agglutination, decreased factor VIII coagulant activity, and plasma vWF. Mixing studies usually fail to detect inhibition of vWF function. The multimer pattern of vWF is normal, although the concentration of all multimers is decreased.[44]

Patients treated with infusions of cryoprecipitate have an appropriate rise in plasma von Willebrand antigen and factor VIII activity. [44] [45] [47] There is no secondary postinfusion rise in factor VIII activity, as seen in congenital von Willebrand disease. [44] [47] A rapid clearance of the von Willebrand protein, necessary for stabilization of plasma factor VIII, accounts for this loss of the secondary rise in factor VIII coagulant activity. Patients treated with DDAVP have an appropriate rise in vWF antigen and activity, followed by an accelerated disappearance of the protein from plasma. [48] [49]

Brody et al. [46] reported on a patient with macroglobulinemia and acquired von Willebrand disease who had a remission in his bleeding disorder after splenectomy. Immunofluorescence of the patient's malignant lymphocytes demonstrated surface-bound vWF. Brody and co-workers proposed that vWF preferentially

EVALUATION AND TREATMENT OF BLEEDING IN DYSPROTEINEMIAS

Clinically significant bleeding occurs in 10% of patients with dysproteinemias. A systematic approach to the evaluation and treatment of bleeding in these patients must be based on an understanding of underlying pathophysiologic mechanisms. The most common hemostatic abnormalities are due to paraprotein inhibitors of platelet function and fibrin polymerization. Patients who are bleeding and have both a prolonged bleeding time and a long TT should be treated with large-volume plasmapheresis. Plasmapheresis is particularly helpful in patients with Waldenström's macroglobulinemia. Cytotoxic chemotherapy or radiation therapy may be employed to reduce the tumor burden and decrease the production of the inhibitory paraprotein, although such therapy is rarely successful.

Patients with dysproteinemias who have inhibitors of factor VIII or vWF or patients with amyloid who have accelerated fibrinolysis or factor X deficiency are at a high risk of bleeding. Inhibitors of factor VIII are resistant to most therapies. Porcine factor VIII or recombinant factor VIIa, either therapy given alone or combined with large-volume plasmapharesis, can be employed during acute bleeding. When possible, cytotoxic chemotherapy to reduce the production of the inhibitory immunoglobulin should be employed. Treatment of acquired von Willebrand disease should be directed toward treatment of the underlying lymphoproliferative disorder. Acute bleeding episodes can be treated with DDAVP, infusions of cyroprecipitate, or factor VIII concentrates enriched in vWF.

Patients with systemic amyloidosis and factor X deficiency may have spontaneous bleeding, which is only transiently controlled by infusions of intermediate purity factor IX concentrates. Large-volume plasma exchange has proved effective in one patient. Stable patients may benefit from splenectomy. Tests for fibrinolysis should also be performed in patients who are bleeding. Patients with increased fibrinolysis should be treated with aminocaproic acid.

bound the monomeric IgM on the surface of the malignant cell.

More than 40 patients with systemic amyloidosis have been described who had an acquired deficiency in factor X. [50] [51] [52] [53] [54] [55] [56] This disorder is characterized by variable deficiencies in plasma factor X (250%) and no evidence of a factor X inhibitor in plasma. Spontaneous bleeding can occur in patients with severe factor X deficiency (<10%), and occasionally precedes the diagnosis of amyloidosis. The clinical course and survival of these patients are variable. Although death usually results from progressive amyloidosis, fatal hemorrhages do occur.

Infusion of large volumes of plasma or partial purity factor IX concentrates results in only transient elevations in factor X. Howell [51] proposed that the factor X deficiency results from factor X binding to amyloid deposits. Furie et al. [56] showed that infused ^{131}I-labeled factor X was rapidly cleared from the circulation of a patient with this disorder. The disappearance half-time was less than 30 seconds (by comparison, the half-time is 1.53 hours in healthy subjects). Total body scans of the patient showed diffuse uptake of radioactivity, but the highest concentrations were seen over the involved liver and spleen. In fact, remissions in factor X deficiency were observed in two patients after splenectomy.[57] [58] Extensive amyloid involvement of the spleen is observed in only 9% of patients with primary amyloidosis

but is reported in 40% of patients with acquired factor X deficiency. [54] Therefore, an enlarged spleen infiltrated with amyloid may be a major site of factor X clearance.[57] [58] [59] Although chemotherapy is only marginally effective in the treatment of systemic amyloidosis, [60] a remission of acquired factor X deficiency was reported in one patient after treatment with melphalan and prednisone. [61] In one patient requiring emergency surgical intervention, large-volume plasma exchange was effective in raising the factor X levels and stopping postoperative bleeding. [62]

With the use of a method of quantitative affinity chromatography, it has been shown that factor X binds to amyloid fibrils. [63] There was no difference in factor X binding to fibrils taken from patients with or without factor X deficiency. Factor IX and prothrombin bound the fibrils to a lesser extent. Combined deficiencies in other vitamin K-dependent proteins along with factor X have been reported with amyloidosis. [59] [60] [64] Also, patients may have decreased functional activity of the residual plasma factor X.[61] [65] The cause of the decreased function is unclear.

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HEMOSTATIC DEFECTS UNRELATED TO PARAPROTEINEMIA

Patients with multiple myeloma and plasma cell dyscrasias have been described with a bleeding disorder secondary to a heparin-like anticoagulant. [66] [67] [68] [69] These patients have a markedly prolonged TT that can be corrected with the addition of protamine sulfate or platelet factor 4. The anticoagulant is an effective cofactor for the inhibition of thrombin in assays using purified antithrombin III and is destroyed by treatment with heparinase. In one patient, continuous infusion of protamine sulfate successfully controlled bleeding. [69]

A prolonged TT is the most frequent hemostatic assay abnormality observed in patients with AL-type amyloidosis. [70] The presence of a plasma inhibitor of both TT and reptilase assays has been described in a number of patients with AL amyloid. [71] However, the presence of this inhibitor does not appear to be associated with an increased risk of bleeding. [70] [71]

A syndrome of pathologic and excessive fibrinolysis has been reported in 11 patients with systemic amyloidosis. [72] [73] [74] [75] [76] [77] [78] All patients had a bleeding diathesis characterized by increased fibrinolytic activity, as determined by a shortened time on the euglobulin clot lysis test, whole blood lysis time, or positive fibrin plate assay. Fibrinogen levels were moderately reduced, levels of fibrin/fibrinogen degradation products were elevated, and no evidence of intravascular coagulation was found. The pathophysiology of this disorder remains unclear. Patients have decreased plasma concentration of $_2$ -antiplasmin inhibitor,[75] but this is secondary to complex formation with plasmin.[76] Abnormalities have been reported with increased tissue plasminogen activator activity [76] and decreased plasminogen activator inhibitor activity. Elevated plasma urokinase-type activity has been reported in two patients with this disorder. [77] [78] The increased fibrinolytic activity in one patient was associated with elevated plasma levels of single-chain urokinase plasminogen activator. [78] Treatment with -aminocaproic acid effectively controlled bleeding in 10 patients.

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Chapter 119 - Disorders of Coagulation and Platelets in the Neonate

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INTRODUCTION

The hemostatic system of the fetus and neonate is unique. During fetal and early neonatal development, coagulation proteins vary in synthetic rate, post-translational processing, and metabolism, processes that are all undergoing continuous developmental change. Consequently, activities of various coagulation proteins differ not only from adult normal values, but also in proportionate relationship to other neonatal coagulation factors. [1] [2] [3] [4] [5] [6] [7] [8] Tables 1191 , 1192 , and 1193 display the means and lower limits for procoagulant, anticoagulant, and fibrinolytic activities in normal infants at various gestational ages. Many of the proteins, such as the vitamin K-dependent proteins, those of the contact factor system, antithrombin III (AT III), plasminogen, and factor XIII, show a distinct gestational dependency and often do not approximate normal adult levels until several months after birth. Other plasma proteins are only slightly lower (fibrinogen, factor V, $_2$ -antiplasmin) or normal to even increased (factor VIII, von Willebrand factor [vWF]). Most of these changes are quantitative, and in only a few instances is there convincing evidence of a distinct fetal protein with altered function. Such examples include fetal fibrinogen, [9] altered vWF, [10] plasminogen, [11] [12] and possibly protein C. [13] Vitamin K-dependent carboxylation of proteins may be incomplete. [14] Over the last several years, new data have been obtained regarding components of coagulation regulation and fibrinolysis in the newborn infant. [2] [3] [4] [5] [6] [7] [8] [11] Still missing are data regarding critical

TABLE 119-1 -- Procoagulant Coagulation Factor Concentrations for the Fetus and Newborn Infant [a]

Subjects	Fibrinogen mg/dl	Prothrombin	Factor V	Factor VII	Factor VIII	vWF (antigen)	Factor IX	Factor X	Factor XI	Factor XII	PK	HMWK	Factor XIII
Fetus (20 wk)	96	0.16	0.70	0.21	0.50	0.65	0.10	0.19	0.13				
	(40)	(0.10)	(0.40)	(0.12)	(0.23)	(0.40)	(0.05)	(0.15)	(0.08)				
Preterm newborn	250	0.32	0.80	0.37	0.75	1.50	0.22	0.38	0.12	0.22	0.26	0.28	1140
(2532 wk)	(100)	(0.18)	(0.43)	(0.24)	(0.40)	(0.90)	(0.17)	(0.20)	(0.06)	(0.09)	(0.14)	(0.20)	
Preterm newborn	300	0.45	0.82	0.59	0.93	1.66	0.41	0.44	0.15	0.25	0.33	0.40	
(3336 wk)	(120)	(0.26)	(0.48)	(0.34)	(0.54)	(1.35)	(0.20)	(0.21)	(0.06)	(0.09)	(0.23)	(0.10)	
Term newborn	240	0.52	1.00	0.57	1.50	1.60	0.35	0.45	0.37	0.44	0.35	0.64	0.61
(3741 wk)	(150)	(0.25)	(0.54)	(0.35)	(0.55)	(0.84)	(0.15)	(0.30)	(0.13)	(0.16)	(0.16)	(0.50)	(0.36)
Older infant (age and level when adult value is approximated)	340 (21 d)	0.97 (4560 d)	1.00 (1 d)	0.90 (21 d)	0.93 (12 d)	1.13 (1 wk)	0.70 (6 wk)	0.55 (6 wk)	0.52 (6 wk)	1.00 (14 d)	0.86 (6 m)	0.82 (1 wk)	1.0 36 m

Adapted from Hathaway and Bonnar, [1] *with permission.*

[a] Values (functional activity unless otherwise indicated) are expressed as compared with normal adult subject reference plasma (100% = 1 U/ml); the mean and a lower limit of range (2 SD) are shown.

TABLE 119-2 -- Anticoagulant Coagulation Factor Concentrations for the Fetus and Newborn Infant [a]

Subjects	AT-III	Hep Cof II	Protein C (antigen)	Protein S		C4b-bp	TFPI ng/ml
				Total	Free		
Fetus (20 wk)	0.23		0.10	0.15	0.22	ND	21.0
	(0.12)		(0.06)	(0.11)	(0.13)	ND	(16.0)
Preterm newborn	0.35		0.29	0.17	0.28	0.08	20.6
(2532 wk)	(0.20)		(0.21)	(0.14)	(0.19)	ND	(13.4)
Preterm newborn	0.40	0.29	0.38	0.21	0.27	0.09	20.7
(3336 wk)	(0.25)	(0.07)	(0.23)	(0.15)	(0.18)	ND	(10.4)
Term newborn	0.56	0.49	0.50	0.38	0.49	0.19	38.1
(3741 wk)	(0.32)	(0.36)	(0.30)	(0.22)	(0.33)	ND	(22.7)
Older infant (age and level when adult values are approximated)	0.82 (36 mo)	0.96 (6 mo)	0.82 (24 mo)	0.85 (3 mo)			

Abbreviations: AT-III, antithrombin III; C4b-bp, C4b-binding protein; Hep Cof II, heparin cofactor II; ND, not detectable; TFPI, tissue factor pathway inhibitor.

Data from Hathaway and Bonnar;[1] Reverdiau-Moalic et al.; [2] Moalic et al.;[3] and Andrew et al.[5]

[a] Values (functional activity unless otherwise indicated) are expressed as compared with normal adult subject reference plasma (100% = 1 U/ml); the mean and a lower limit of range (2 SD) are shown.

endothelial cell functions in coagulation protein synthesis, release, and function.

The plasma-clotting activities resulting from the fetal hemostatic system produce notable changes in the screening tests used in clinical situations. Mostly due to the low contact factors, the partial thromboplastin time (PTT) is prolonged by 2 to 3 seconds in the term infant and may be greater than twice the adult value in the small preterm infant; prolongation of the prothrombin time (PT) may be only a second in the term and up to 3 seconds in the preterm infant; a prolonged thrombin time (if performed without calcium) reflects fetal fibrinogen. These values, found in healthy infants [5] [15] and varying slightly according to technique, instruments, and reagents, are not associated with any bleeding tendency.

Because of the unique interrelationships within the fetal and neonatal coagulation system, investigations of individual components may yield misleading results. Thus, although plasma from healthy neonates shows decreased thrombin generation similar to that of warfarinized adults, [16] [17] neonates who are not vitamin K deficient almost never develop ecchymoses or gastrointestinal or intracranial hemorrhages (ICH) typical of low thrombin-generation states. Likewise, although plasminogen and tissue plasminogen activator (t-PA) are decreased in neonatal plasma, and histidine-rich glycoprotein and $_2$ -antiplasmin are relatively increased, the euglobulin clot lysis time is short and spontaneous thromboses are rare. [18] [19]

Platelet number and function, with the exception of decreased aggregation to epinephrine, low concentrations of adenosine diphosphate (ADP), and collagen, is unchanged from the adult normal values. Platelet adrenergic receptors are decreased. [20] [21] Well term and preterm infants have normal platelet counts and bleeding times.[22]

Characteristics of neonatal red blood cells including increased size and red cell mass relative to adult red cells contribute to accelerated whole blood coagulation activation at birth. Enhanced release of adenosine diphosphate, greater externalization of membrane phosphatidylserine, and increased susceptibility to lipid peroxidation may all contribute procoagulant effects to newborn blood. [23] [24]

HEREDITARY COAGULATION DISORDERS

The hereditary coagulation disorders may present during the neonatal period. These include hemophilia A and B, severe deficiencies of prothrombin or factor V, factor VII, factor X, afibrinogenemia, factor XIII, or $_2$ -antiplasmin deficiencies.[1] Affected infants present most typically with ecchymoses, cephalohematomas, umbilical stump oozing, gastrointestinal bleeding, ICH, and persistent oozing after circumcision or heel stick punctures for routine blood screening. Persistent umbilical cord stump oozing is particularly characteristic of homozygous factor XIII deficiency. ICH in a term infant should always raise suspicion of a familial bleeding diathesis including hemophilia, another hereditary coagulation deficiency, hereditary thrombocytopenia, Glanzmans thrombesthenia, or a connective tissue disorder such as Erhlers-Danlos.[25] [26] Only 50% of infants with severe hemophilia exhibit protracted oozing following circumcision. Failure to bleed excessively following circumcision does not exclude a diagnosis of hemophilia.

When hemophilia is suspected before delivery, efforts

TABLE 119-3 -- Coagulation Regulation in the Neonate

Component	Neonatal Functions	Effect on Coagulation
Reticuloendothelial system	Functionally hyposplenic	Delayed clearance of activated coagulation products leading to further activation
Native coagulation inhibitors	AT III Protein C	Diminished capacity to inhibit activated coagulation proteins
	Protein S	May promote thrombus formation
Fibrinolysis	Plasminogen	Decreased response to pharmacologic fibrinolysis
	t-PA, normal plasminogen activator inhibitor, slower plasmin generation rates	Diminished native fibronolysis in sick infants
Endothelial cells	Largely unknown	Unknown
Production of platelets and synthesis of coagulation proteins	Capacity for compensatory increase in production rate during consumption	Early depletion of platelets and coagulation proteins leading to bleeding complications

should be made to ascertain the diagnosis in the infant as soon as is practical. Prenatal diagnosis may be made by intrauterine chorionic villus sampling, amniocentesis, or periumbilical blood sampling. There is controversy regarding the optimal mode of delivery for babies with severe hemophilia. Occasional case reports and small series describe severe perinatal ICH.[25] There are no prospective data, but this complication probably does not exceed 10% of births involving affected infants. Current recommendations include a delivery as atraumatic to the infant as possible with avoidance of use of vacuum extractors or forceps. [27] Recombinant factor VIII or factor IX should be available in the event of hemorrhage. It should be noted that even though maternal levels of factor VIII and vWF increase during pregnancy, they may fall within hours of delivery and carrier mothers are at increased risk for postpartum hemorrhage. [28]

Plans should be made to obtain an umbilical cord blood sample carefully collected to avoid contamination with maternal blood or amniotic fluid for confirmation of diagnosis at the time of delivery. Laboratory investigations should include specific factor assay. Screening tests including the PTT and the PT are not adequate to confirm or exclude a diagnosis of congenital bleeding disorder in a neonate. Less severe congenital factor deficiencies including type 1 or 2 von Willebrands disease or mild deficiencies of factor IX or factor XI may not be accurately diagnosed at the time of delivery because of the overlap with normal infant values; studies at a later date (3 months of age) will usually allow diagnosis.

Detailed treatment guidelines for the hemophilias are discussed elsewhere (Chap. 109). It is important to use the safest transfusion products available. Recombinant proteins are preferred. Single donor plasma that has been appropriately screened for human immunodeficiency virus, hepatitis B, and hepatitis C and highly purified, viral inactivated plasma-derived products are acceptable if safer products are not available. All affected infants should be immunized for hepatitis A and B.

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VITAMIN K DEFICIENCY BLEEDING (VKDB)

The neonatal bleeding syndromes due to vitamin K deficiency generally affect otherwise well infants and constitute the most important preventable cause of bleeding morbidity and mortality in infants worldwide. Cord blood of normal neonates born to well-nourished healthy mothers demonstrates a marginal vitamin K status at the time of birth.[29] [30] Mean plasma concentrations of vitamin K_1 have been reported at 0.009 ± 0.02 nmol/L at birth compared with a normal adult reference range of 0.32.6 nmol/L.[31] Trace amounts of des--carboxy protein C and prothrombin, decreased vitamin K_1 , and increased vitamin K_1 epoxide were found in 27% of these infants; the data suggested decreased vitamin K stores and inefficient hepatic reductase cycling. The marked maternal/fetal gradient in vitamin K level suggests a potential population advantage of relative vitamin K deficiency in the fetus and neonate. Evidence suggests that high concentrations of vitamin K are mutagenic. [32]

After birth with the institution of oral feedings, vitamin K levels rise quickly. Formulas based on cows milk contain approximately 10 times the concentration of vitamin K as human breast milk. However, breast milk has a higher bioavailability provided the infant is fed exclusively breast milk. The incidence of vitamin K deficiency has been estimated at 0.011.5% worldwide and varies widely relating to nutritional and other factors.

The International Society for Thrombosis and Haemostasis has recently published criteria for the diagnosis, prevention, and treatment of this syndrome. [33] Those recommendations form the basis of this summary. It is proposed that the historical name, hemorrhagic disease of the newborn, be abandoned in favor of the more descriptive term, "vitamin K deficiency bleeding" (VKDB).

Vitamin K deficiency bleeding can be classified based on etiology or time of onset. The etiology may be idiopathic, which is generally found in exclusively breast-fed infants or secondary to decreased intake or absorption of vitamin K or vitamin K antagonism by drugs. Time of onset is used to divide cases into onset within the first 24 hours of life (early VKDB), from 24 hours through 7 days (classic VKDB) or up to 8 and rarely 12 weeks (late VKDB). The early form is due almost exclusively to placental transfer of maternal drugs that inhibit vitamin K activity in the baby including anticonvulsants (carbamazepine, phenytoin, and barbituates but not valproic acid),[34] antibiotics (cephalosporins, rifampicin, isoniazid), [35] and oral anticoagulants.[36] ICH or intractable oozing from skin punctures are common in this form. The incidence of bleeding in infants of mothers taking these drugs without supplementation is 612%. [33] The classic form of VKDB occurs in infants with poor oral intake or exclusive breast-feeding. Classic VKDB presents with bleeding from multiple sites, with oozing from skin punctures, the umbilical stump, the gastrointestinal tract, surgical sites, and occasionally ICH. Late VKDB usually manifests with ICH. Most cases are idiopathic but occasionally malabsorption, liver disease, or cholestasis is found. Affected infants should be evaluated for biliary cirrhosis, $_1$ -antitrypsin deficiency, cystic fibrosis, and Wilsons disease.

The diagnosis of VKDB is suggested by a clearly prolonged PT in the presence of a normal fibrinogen and platelet concentration. The PTT is usually prolonged. Severe genetic factor VII deficiency causes an isolated prolongation of the PT and should be confirmed by factor VII assay in infants in whom the PTT is normal. In VKDB, vitamin K-dependent proteins, prothrombin, factor VII, factor IX, factor X; protein C, and protein S are all reduced. Des--carboxy forms of these proteins can be detected circulating in plasma. Des--carboxy prothrombin has been used to diagnose vitamin K deficiency retrospectively. [30]

Therapeutic response to 1 mg intravenous vitamin K with correction of the PT and cessation of bleeding is prompt, thus confirming the diagnosis. Infants with life-threatening hemorrhage may benefit from immediate infusion of fresh frozen plasma in addition.

Vitamin K deficiency bleeding can be prevented with vitamin K prophylaxis. [33] Early VKDB is prevented by giving vitamin K prophylaxis to the mother during pregnancy.[37] [38] Classic vitamin K deficiency can be prevented by 1 mg vitamin K, given either intramuscularly or orally. Intramuscular vitamin K provides stores for 46 weeks, which is adequate in most cases to cover healthy infants until adequate dietary intake is established. Oral vitamin K_1 given as three doses of 1 mg has not completely eliminated VKDB. The arguments against the single intramuscular prophylaxis were potential muscle damage inflicted by the needle and potential increases in childhood cancer risk conferred by temporary unphysiologically high plasma levels of vitamin K after intramuscular injection; the latter has not been substantiated.[39] [40] The arguments against repeated oral prophylaxis chiefly involve difficulties in ensuring compliance and the devastating consequences of ICH that have occurred in treatment failures.

LIVER DISEASE

The coagulopathy of severe liver disease is complex and includes consumption of clotting factors and platelets (due to tissue necrosis, endothelial damage, disseminated intravascular coagulation [DIC], increased fibrinolysis, hypersplenism), as well as failure to synthesize procoagulant and anticoagulant proteins (Chap. 117). Clinical examples of severe neonatal hepatic disease are viral hepatitis, fetal hydrops due to erythroblastosis fetalis, cirrhosis due to $_1$-antiplasmin deficiency,

cholestatic jaundice, hepatopathy of shock (shock liver), and hereditary metabolic defects such as fructose intolerance, tyrosinemia, galactosemia, and neonatal hemachromatosis. The bleeding may be severe, especially if the infant has had surgery. Laboratory tests assessing the severity of the process and the mechanisms that are involved include platelet count, fibrinogen, PT, thrombin time, fibrin degradation (split) products, and D dimer. Specific factor assays that may guide therapy include factor V (a good marker of steady state hepatic synthetic capacity), factor VII (a marker of current liver synthesis, especially after plasma transfusion owing to its short half-life), and AT III (replacement with concentrate may control consumptive coagulopathy).

Treatment consists of replacement therapy with fresh frozen plasma, platelet concentrates, cryoprecipitates, and AT III concentrate, if necessary. In severe cases, all these agents may be needed to obtain minimal hemostatic levels. Alternatively, exchange transfusion followed by platelet transfusion can be used to obtain hemostasis. Advances in liver transplantation for the small infant have provided definitive therapy for many previously fatal infantile hepatopathies; successful interim support of coagulation is an integral part of this therapy.

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THROMBOCYTOPENIA

Thrombocytopenia, defined by a platelet count of <150,000/l, is the most common hemostatic disorder diagnosed in the neonatal period. [41] Severe thrombocytopenia (<50,000/l) has been reported in 1% of all term neonates [42] and thrombocytopenia has been determined in 22% of all neonates admitted to an intensive care unit. [43] Although many etiologies have been determined for neonatal thrombocytopenia (Table 1194), the vast majority of infants have thrombocytopenia due to conditions associated with increased destruction of circulating platelets. It is clinically useful to first categorize infants as well or with other medical conditions. In sick infants, thrombocytopenia is most frequently caused by infection, DIC and other intravascular consumptive coagulopathies such as necrotizing enterocolitis, placental chorangioma, and large vessel thrombosis (see later), and vascular disorders such as hemangiomas, heart lesions with abnormal flow mechanics or polycythemia, and pulmonary syndromes (respiratory distress syndrome, aspiration, and pulmonary hypertension). These disorders are relatively easily recognized; often many contributing conditions coexist and treatment is related to management of the underlying disease. The platelet count should be maintained >50,000 using platelet transfusion as necessary for infants who are undergoing mechanical ventilation, extracorporeal membrane oxygenation, surgery, have indwelling arterial lines or drainage tubes, or are acutely ill. Stable infants with none of these risk factors can usually tolerate a platelet count of 20,000 without spontaneous bleeding.

Thrombocytopenia that occurs in an otherwise well infant is frequently immune mediated. Since the classic paper of Pearson et al., [44] neonatal alloimmune thrombocytopenia (NAIT) has been recognized with increasing frequency and clinical concern. NAIT is caused by fetomaternal incompatibility for platelet antigens of paternal origin. The platelet-specific antigen implicated most frequently in Caucasians is PI^{Al} (present in 98% of the population) and next Br^{a} . Pen/Yuk polymorphism is the most common cause of NAIT in Asians. Studies suggest the incidence to be 1/1,0002,000 newborn babies; [45] the first pregnancy is affected in about 50% of cases. NAIT is a serious disorder. Because of the early onset and often severe thrombocytopenia, the risk of ICH is 1030% with half of the cases occurring intrauterine.

The diagnosis of NAIT begins with clinical suspicion, which should be high in any well infant, term or preterm, with unexplained thrombocytopenia, or any ill infant with the severity

TABLE 119-4 -- Causes of Neonatal Thrombocytopenia

Infection
Bacterial: sepsis, congenital syphilis
Viral: cytomegalovirus, herpes simplex, rubella, enterovirus, human immunodeficiency virus (HIV), parvo-B19
Other: toxoplasmosis
Immune Disorders
Alloimmunization
Maternal antibody induced: idiopathic thrombocytopenic purpura, systemic lupus erythematosus
Bone marrow abnormality
Congenital magakaryocytic hypoplasia
Absent radii (TAR) syndrome
Phocomelia syndrome
Fanconis pancytopenia
Aplastic anemia
Trisomy syndromes
Osteopetrosis
Congenital leukemia
Maternal drugs
Tolbutamide, hydralazine, hydantoin, azothiaprine
Infant drugs
Intralipid, tolazoline
Intravascular coagulation syndromes
Disseminated intravascular coagulation
Major vessel thrombosis: renal vein, aorta
Necrotizing enterocolitis
Placental chorangioma
Chorionic vessel thrombosis
Excessive peripheral utilization
Giant hemangioma
Hyperviscosity syndrome
Erythroblastosis fetalis
Congenital heart disease
Hereditary thrombocytopenia
Sex-linked: Wiskott-Aldrich syndrome and variants

Autosomal recessive: associated with renal diseases and deafness
Autosomal dominant: May Hegglin anomaly, Bernard Soulier
Other causes
Postexchange transfusion
Maternal hyperthyroidism
Maternal hypertension (?)
Metabolic disorders: hyperglycinemia, cirrhosis, mucolipidosis
Thrombotic thrombocytopenia purpura
Postmature and small for gestational age infants
Neonatal neuroblastoma
Neonatal cold injury
Perinatal pulmonary syndromes

Adapted from Hathaway and Bonnar,[1] with permission.

of thrombocytopenia out of proportion to the clinical setting. Maternal platelet count should be normal; mild maternal thrombocytopenia may be secondary to unrelated causes such as benign gestational thrombocytopenia. Diagnosis includes determination of parental platelet antigen incompatibility by platelet typing, which can be performed using DNA or serologic techniques. Confirmation by demonstration of maternal antibodies directed against paternal antigens is helpful when present, but a negative result does not exclude the diagnosis. Treatment of neonates with intravenous gammaglobulin, 1 g/kg/day for 23 days or intravenous methylprednisolone 1 mg/kg every 8 hours for three doses is usually successful.[41] [46] The disorder generally resolves within 2 weeks. Treatment of sick infants, infants with severe thrombocytopenia, or infants with active bleeding is by transfusion of either washed maternal or typed maternal-compatible cytomegalovirus-negative platelets. Random donor platelets should be given to an infant with life-threatening

hemorrhage, especially ICH, while specific therapy is being prepared. Although survival of untyped platelets is likely to be short, there may be a transient hemostatic effect.

When alloimmune thrombocytopenia is suspected prenatally based on a previous pregnancy or positive family history, platelet typing and antibody studies should be performed immediately. Fetal blood sampling can be performed beginning at 20 weeks to assess the infants clinical involvement. Maternal treatment with intravenous immunoglobulin/g/kg/week or steroids is generally effective.[47] Because of the increased risk of fetal hemorrhage, washed, concentrated maternal platelets should be infused into the fetus at the end of the intrauterine blood sampling procedure. Alternatively, the fetus may be treated with repeated compatible platelet transfusion commencing at 2630 weeks gestation.[48] [49] Delivery should be as atraumatic as possible and documentation of an adequate fetal platelet count before delivery is recommended.

Thrombocytopenia secondary to maternal autoantibodies generally causes less severe disease in the infant. Passively transferred antibodies from mothers with immune thrombocytopenia purpura or systemic lupus erythematosus result in varying degrees of neonatal thrombocytopenia. Mildly affected infants typically need no specific therapy. Infants with platelet counts of <50,000/l, petechiae, or oozing from skin punctures may respond to a 12-week course of prednisone, 2 mg/kg/day. Infants with severe thrombocytopenia (<10,000/l) or significant bleeding usually respond promptly to intravenous immunoglobulin. Platelet transfusions are rarely helpful unless antibody is removed by exchange transfusion.

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NEONATAL DISSEMINATED INTRAVASCULAR COAGULATION

Disseminated intravascular coagulation is the second most frequent coagulopathy in the neonate after thrombocytopenia (Chap. 117). Uncontrolled intravascular thrombin generation results when coagulation activation triggered by acidosis, poor perfusion, or endotoxin overwhelms the native regulatory mechanisms. [1] Several components of the neonates developmentally immature hemostatic system may contribute to the neonates susceptibility to DIC and have been recently reviewed. [49] Neonatal DIC is associated with a wide variety of conditions (Table 1195).

Clinically, an infant with DIC manifests poor perfusion, oozing from puncture sites, ICH, pulmonary and gastrointestinal hemorrhage, and occasionally, large vessel thrombosis. Characteristic laboratory findings include decreased platelet count; diminished levels of fibrinogen, factor V, factor VIII, protein C, and AT III; increased fibrin degradation products; decreased levels of the vitamin K-dependent factors; and a microangiopathic hemolytic anemia. A simple but helpful screening panel includes the platelet count, fibrinogen level, D-dimer assay of cross-linked fibrin, PTT, and PT. With a slightly larger sample volume, AT III and plasminogen can also be assayed. These tests can all be performed on capillary blood specimens and give an estimate both of ongoing activation as well as bleeding potential. AT III and plasminogen are often decreased to a critical level (<20% of normal adult values) and convey a poor prognosis. [50] [51] [52] [53] Other specific factor assays provide confirmatory information but neither increase diagnostic sensitivity nor aid in clinical management.

Therapy for neonatal DIC is primarily directed at reversing the etiologic trigger. Appropriate management with ventilation, intravascular volume support, and antibiotics generally results in normalization of coagulation within 2448 hours.

Infants with DIC who are actively bleeding should be supported with transfusions to maintain a platelet count of >50,000/l and a fibrinogen level of 100 mg/dl (Table 1196). In infants suffering life-threatening hemorrhage, hemostasis

TABLE 119-5 -- Conditions Associated with Neonatal Disseminated Intravascular Coagulation

Obstetric complications
Abruptio placenta
Placenta accreta
Chorioamnionitis
Fetal demise of one twin
Pre-eclampsia
Acute fatty liver of pregnancy
Neonatal infections
Rubella
Herpes
Cytomegalovirus
Enterovirus
Systemic candidiasis
Bacteria, esp. gram negative
Respiratory distress syndrome
Cardiovascular disorders
Congestive heart failure
Shock
Lactic acidosis
Massive thrombosis
Placental chorangiomas
Kasabach-Merritt (giant hemangioma)
RH isoimmunization
Nonimmune hydrops
Severe liver disease
Severe cold stress

may be achieved more emergently with a two-volume exchange transfusion. AT III concentrate in doses of 100200 U/kg may be given every 2448 hours to infants with depleted regulatory capacity. Plasminogen and protein C may be replaced using fresh frozen plasma in doses of 1020 ml/kg. Occasionally an infant with DIC manifests large vessel thrombosis or circulatory impairment caused by diffuse microthrombi, for which low-dose heparin therapy is appropriate.

Transfusion therapy in babies without bleeding signs may be reserved for a platelet count of <20,000 and a fibrinogen level of <50 mg/dl.

Intracranial hemorrhage is one of the most devastating bleeding complications in neonates. This lesion is associated with prematurity and assisted ventilation. The onset of neonatal ICH is primarily in the first 72 hours with the largest number of cases diagnosed within the first 24 hours. [54] The hemorrhages begin in the germinal matrix of the lateral ventricles (grade I ICH or germinal matrix hemorrhage). More advanced hemorrhages extend into the ventricular cavities (intraventricular hemorrhage: grade II with absence or grade III with presence of ventricular dilatation) or into the parencyma of the brain (grade IV ICH). Neurologic outcome is related to the extent of

TABLE 119-6 -- Treatment of Acquired Hemostatic Defects in the Newborn

Indication	Product	Dosage Guideline
Thrombocytopenia	Platelet concentrate	10 mg/kg will raise platelet count to 75100 × 10^3 /l
Coagulopathy (DIC, liver disease)	Fresh frozen plasma	1015 ml/kg will raise factor level by 0.2 U/ml
Severe fibrinogen depletion	Cryoprecipitate	1 U/3 kg will raise fibrinogen level to >100 mg/dl

hemorrhage with residual motor and cognitive deficits in the majority of infants with grade III or grade IV ICH. Prospective investigations of preterm infants with respiratory distress syndrome determined coagulopathies with thrombocytopenia, hypofibrinogenemia, and low plasma concentration of AT III and protein C to precede the onset of ICH. Autopsy studies demonstrate fibrin deposition in germinal matrix venules suggesting that the clinical syndrome may result from post-thrombotic hemorrhage from these poorly supported vessels. A randomized, controlled study of platelet transfusion in premature infants failed to show a decreased incidence in ICH in infants who were prophylactically supported with platelet transfusion. [55] This study would suggest that the thrombocytopenia is a marker for a process (e.g., germinal matrix infarction), which resulted in ICH rather than being causative. Therapy is supportive although there is evidence that the ICH is more likely to progress in babies with thrombocytopenia and hypofibrinogenemia. [51] Concomitant with improved neonatal intensive support, the incidence and severity of neonatal ICH has decreased.

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NEONATAL HYPERCOAGULABILITY AND THROMBOSIS

Thrombosis in the neonate is characterized by an increased incidence during the perinatal period, a predilection for major vessels, and frequent involvement of the arterial circulation. Most neonatal thromboses occur on indwelling arterial and venous catheters where the pathophysiology includes endothelial damage, mechanical obstruction to flow, the composition and design of the catheter itself, an immature regulatory system, and triggers of coagulation activation. Predisposing medical conditions in these sick infants include asphyxia, poor perfusion, infection, respiratory distress syndrome, congenital heart disease, maternal diabetes, polycythemia, and intrauterine growth retardation.

Spontaneous thromboses do occur and most frequently involve the inferior vena cava and renal veins; the aorta, renal and femoral arteries; as well as the middle cerebral arteries. Neonatal hypercoagulability has become a topic of active research and clinical interest over the past few years. Investigations of neonates with thromboses have found genetic deficiencies in the concentration or function of one or more coagulation proteins including AT III, [56] [57] [58] [59] protein C,[60] [61] [62] protein S,[63] and the factor V Leiden mutation.[64] [65] [66] [67] [68] These thromboses most frequently involve the renal veins, inferior vena cava, and central nervous system (CNS) sinuses, although the aorta may also be involved. Most infants did not have a history of antecedent trigger beyond the stress of labor and delivery. Because AT III, protein C, and protein S are easily consumed during thrombosis in an infant without a genetic deficiency, definitive diagnosis requires documentation of the same defect in a parent or persistence of the deficiency for at least 6 months after the acute event. Affected infants have responded to conventional anticoagulant and fibrinolytic therapy, although some were treated with specific protein replacement. The risk of recurrence during infancy appears to be low in all infants except those with the most severe deficiencies. All term infants with idiopathic thrombosis should be evaluated for genetic thrombophilia between 6 and 12 months of age. Preterm and sick term infants with thrombosis should be considered for a thrombophilia evaluation if the thrombus trigger was trivial or the thrombosis was unusually extensive or progressive.

Thrombophilic families with infants who manifest neonatal thromboses are at risk for recurrence in subsequent pregnancies, especially when the genetic deficiencies are severe or involve multiple gene defects. In these cases intrauterine diagnosis has been performed to plan perinatal management or provide information regarding decisions to terminate the pregnancy. In the case of an isolated factor V Leiden mutation, which affects approximately 5% of Caucasian infants, [68] [69] it is possible that reported cases of neonatal thrombosis or purpura fulminans may have involved other unidentified cofactors. [70] Because most affected individuals suffer no neonatal morbidity, caution should be exercised in advising parents on aggressive diagnostic or interventive fetal procedures based on parental carriage of the factor V Leiden gene alone.

Hypercoagulability is a common acquired condition in sick infants. Although the coagulation system of the well term and preterm infant shows low levels of many procoagulant, anticoagulant, and fibrinolytic proteins, hemostasis is functionally balanced and thromboses and hemorrhages are equally rare. The sick infant, on the other hand, is susceptible to severe acquired deficiencies of multiple anticoagulant and fibrinolytic proteins. Thromboses are common in sick neonates, especially in infants with indwelling arterial and venous catheters. Many neonatal conditions have been associated with acquired hypercoagulability and an increased risk of thrombosis including respiratory distress syndrome, [51] [71] [72] asphyxia, [73] congenital heart disease, [53] polycythemia, sepsis,[73] pulmonary hypertension, and maternal cocaine use.[74] It was first noted over 20 years ago that infants with severe respiratory distress syndrome manifest severe decreases in plasminogen and AT III and that the severity of these acquired deficiencies was correlated with a poor prognosis for survival. [71] This disorder presents along a spectrum with DIC and bleeding in a subset of infants with diffuse coagulation consumption at one end and hypercoagulability and thrombosis in infants with more limited consumption.

Acquired coagulopathies caused by transplacental transfer of the lupus anticoagulant have been reported rarely. [75] [76] [77] [78] [79] The resulting thrombotic disorder has mimicked severe genetic deficiencies of the protein affected by the maternal antibody.

The diagnosis of neonatal thrombosis is made by demonstration of the clot with an imaging technique. High-resolution real-time ultrasound produces excellent visualization of thrombi in the aorta, iliac arteries, and inferior vena cava. Color flow Doppler has extended noninvasive imaging to the intrarenal arteries and veins as well as the portal, splenic, hepatic, and cerebral arteries and veins of the neonate. Magnetic resonance imaging produces elegant intravascular images, especially of the intracranial vessels of infants who are sufficiently stable for transport to the radiology department. Contrast angiography is now rarely used in infants because of risks of increased osmotic load and fluid shifts, even using nonionic contrast; contrast studies are contraindicated in infants with renal failure or gut ischemia.

When vascular perfusion is significantly impaired by an indwelling catheter, diagnostic studies should be performed expeditiously. The catheter should be promptly removed after determining whether it can be used for local instillation of fibrinolytic therapy. Nonspecific measures, including warming of the involved or contralateral limb are sometimes helpful. Surgical thrombectomy must be guided by accessibility and local expertise. When evidence indicates recent or progressive thrombosis or organ dysfunction, either anticoagulant or thrombolytic therapy should be carefully considered.

Unfractionated heparin was the initial standard therapy for neonatal thrombosis. [80] [81] [82] [83] Heparin clearance is accelerated in the newborn partly due to neonatal differences in plasma volume and liver metabolism and partly due to the lower level of AT III in the neonate. [80] [84] Unfractionated heparin should be given to achieve and maintain a plasma anti-factor Xa activity of 0.40.7 U/mL. Typical doses required for this effect are shown in the box on Heparin Infusion Dosage. AT III concentrate can be given to overcome heparin resistance in the neonate and should be considered especially when doses of heparin in excess of 40 U/kg/hr are being infused. Confirmation of the subtherapeutic heparin level during aggressive heparin infusion therapy is more useful in gauging the potential value for AT III replacement

HEPARIN INFUSION DOSAGE

Age	Bolus (U/kg)	Maintenance (U/kg/hr)
Preterm (28 weeks)	25	15
Preterm (2836 weeks)	50	20
Full term	100	2540

than plasma concentration of AT III per se. The PTT should not be used to monitor heparin effect in preterm infants because low levels of contact factors will lead to an overestimation of heparin effect.

Low-molecular-weight (LMW) heparins are used successfully to treat neonatal thromboses. [85] [86] Because the effect of LMW heparin is limited to its action on factor Xa, the therapeutic range is somewhat higher than that for unfractionated heparin (0.51.0 U/ml). [86] LMW heparin has the advantage of being given subcutaneously and does not require frequent monitoring. In adults, LMW heparin therapy has been associated with less heparin resistance and a more predictable dose response as compared with unfractionated heparin. Although there are no prospective studies at this time, early clinical reports indicate that results of LMW heparin therapy in neonates may be similar to that in adults.

Many small series and individual case reports document the use of thrombolytic therapy in newborn infants. [81] [87] [88] In vitro and clinical experience suggests that the neonatal fibrinolytic system is less susceptible to activation resulting in requirements of up to 10 times the adult dosage for urokinase of 4,400 U/kg/hr or t-PA of 0.05 mg/kg/hr. A review of the published literature estimates the incidence of ICH in neonates receiving thrombolytic therapy at 1.2% for term infants, 13% of all preterm infants, and 25% of preterm infants treated during the first week of life. [89]

Investigations of neonatal renal vein thrombosis have reported uniform loss of renal parenchymal tissue, reduced renal function or hypertension following standard therapy with either supportive care or heparin anticoagulation. [82] Normal renal outcome has been reported in a few cases using combined therapy with heparin and thrombolytic agents. [90] [91] [92] [93] Infants with renal vein thrombosis are often near term and otherwise stable. Babies with renal vein thrombosis should be carefully evaluated for contraindications to thrombolytic therapy and should be considered for more aggressive treatment.

With both anticoagulant and fibrinolytic therapy, the bleeding risk is greater in infants with thrombocytopenia or hypofibrinogenemia. In these cases, transfusion support during therapy to maintain platelets >50,000/l and fibrinogen >100 mg/dl is appropriate. Thrombolytic therapy is contraindicated in babies with active bleeding, recent surgery (within 10 days), chest tubes, or other invasive procedures. Areas of recent infarct, especially CNS infarcts are prone to hemorrhage during thrombolytic therapy. All infants in whom thrombolytic therapy is being considered must be evaluated for risk of recent CNS ischemia or infarction. A cranial ultrasound should be taken at the initiation and conclusion of therapy to exclude ICH.

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PURPURA FULMINANS

A unique clinical syndrome of neonatal DIC and purpura fulminans has been recognized and is related to the absence of protein C or protein S in the plasma. [94] [95] [96] [97] [98] [99] [100] [101] [102] [103] [104] Babies born with homozygous or double heterozygous deficiencies of either protein C or protein S present within a few hours to days of birth with rapidly progressive skin lesions and diffuse oozing from skin puncture sites. The skin lesions often begin at the site of heel or venous puncture for routine blood sampling. These lesions, which are initially red and flat, quickly become indurated and necrotic, form an eschar, and result in gangrene. Venous thromboses of the CNS, kidneys, and primary vitrea of the eyes, often with onset in utero, are common. Coagulation screening tests, which may be normal initially, will subsequently reveal typical evidence of DIC. Family studies document heterozygous deficiencies of protein C or protein S in both parents while the infant has undetectable protein. DIC and purpura fulminans associated with homozygous deficiency of protein C respond quickly and dramatically to fresh frozen plasma, 10 mL/kg infused every 812 hours. There are now several reports of successful treatment using a viral inactivated human plasma-derived protein C concentrate. [105] [106] [107] The plasma half-disappearance time of protein C given either as fresh frozen plasma or the concentrate is approximately 8 hours. [106] [108] If administered before the development of tissue necrosis, the lesions will begin to regress within a few hours. After establishment of the diagnosis and resolution of purpuric lesions, infants are maintained on oral anticoagulation with warfarin or long-term replacement therapy with protein C concentrate. [98] [95]

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Chapter 120 - Hypercoagulable States

Kenneth A. Bauer

INTRODUCTION

Major advances have been made in our understanding of the natural anticoagulant and fibrinolytic systems that regulate the activity of the hemostatic mechanism. However, it has been only during the last few years that this information has translated into being able to diagnose clinically relevant risk factors in substantial numbers of patients presenting with thrombosis. The impact of these clinical diagnostic advances has been most dramatic in younger patients with idiopathic venous thrombosis, in >50% of whom hereditary defects can be identified. Important strides have also been made in delineating the contribution of thrombotic risk factors to arterial thrombosis, but identification of the common risk factors for atherogenesis (e.g., smoking, hyperlipidemia, diabetes mellitus) retains primacy in the evaluation of such patients.

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CLINICAL APPROACH

Differential Diagnosis

When approaching patients suspected of having an active thrombotic process or a thrombotic diathesis, it is useful to place them in one of two major categories. The first group has characteristics that suggest the presence of an inherited thrombotic disorder or a primary hypercoagulable state (Table 1201). These disorders result from mutations in single genes encoding a plasma protein component of one of the major natural anticoagulant mechanisms. The anticoagulant systems most frequently involved in the inherited hypercoagulable states include antithrombin III (AT III) in the heparan sulfate-AT III mechanism and protein C, protein S, and factor V (substitution of the amino acid Gln for Arg at amino acid 506, [1] termed factor V Leiden) in the protein C anticoagulant pathway (Fig. 1201). Elevations in plasma prothrombin levels in association with a G to A transversion at position 20210 in the 3-untranslated region of the prothrombin gene is a newly identified genetic risk

TABLE 120-1 -- Differential Diagnosis of the Patient Presenting with Thrombosis or a Thrombotic Diathesis

Inherited (Primary) Hypercoagulable States
Activated protein C resistance due to factor V Leiden mutation
Antithrombin III deficiency
Protein C deficiency
Protein S deficiency
Prothrombin gene mutation (G to A transversion at position 20210 in the 3-untranslated region)
Dysfibrinogenemia (rare)
Acquired (Secondary) Hypercoagulable States
In association with physiologic or thrombogenic stimuli
Pregnancy (especially the postpartum period)
Immobilization
Trauma
Postoperative state
Advancing age
Estrogen use
Antiphospholipid syndrome or lupus anticoagulant
In association with other clinical disorders (Table 1202)
Hyperhomocysteinemia

Figure 120-1 A schematic diagram of the pathways that generate factor Xa and thrombin, and the natural anticoagulant mechanisms that regulate the activity of these enzymes.
Factor X can be activated by factor VIIa-tissue factor (TF) complex or the factor IXa-VIIIa-activated cell surface complex. Factor Xa binds to the factor Va on activated platelets and mediates the conversion of prothrombin to thrombin under physiologic conditions. During this pro-cess, the inactive F_{1+2} fragment is released from the amino terminus of prothrombin. Thrombin is then able to act on fibrinogen to form a fibrin clot; the initial step in this conversion results in the liberation of fibrinopeptide A.
Thrombin and factor Xa are inactivated by AT III bound to heparan sulfate molecules associated with the vascular endothelium, resulting in the formation of factor Xa/AT III and thrombin/AT III complexes. Protein C is activated by thrombin bound to the endothelial cell receptor thrombomodulin. Once activated, protein C functions as a potent anticoagulant by inactivating factors VIIIa and Va. Protein S enhances the binding of activated protein C to phospholipid-containing membranes and is able to accelerate the inactivation of factors VIIIa and Va by this enzyme. The complement component, C4b-binding protein (C4b-BP), forms complexes with protein S, which neutralizes its ability to serve as a cofactor for activated protein C in inactivating factors VIIIa and Va.

factor for venous thrombosis.[2] The second category, the acquired or secondary hypercoagulable states, consists of a heterogeneous group of disorders in which there appears to be an increased risk for developing thrombotic complications as compared to the general population (Table 1202). The pathophysiologic basis for the hypercoagulable state in most of these situations is complex and multifactorial. Hyperhomocysteinemia is a common laboratory abnormality that results in an increased risk in venous as well as arterial thrombosis. Plasma homocysteine levels are determined by genetic as well as environmental factors, the latter including primarily dietary intake of folic acid and vitamins B_{12} and B_6 . It is listed separately from the inherited thrombotic disorders because it may result from defects in several genes encoding different enzymes involved in the metabolism of the amino acid.

The inherited thrombotic disorders (Table 1201) have been almost exclusively associated with *venous thrombosis*. Although hereditary deficiencies of AT III, protein C, or protein S will be found in <10% of unselected patients presenting with venous thromboembolism (Table 1203), the likelihood of identifying these defects is increased several-fold by screening patients with initial thrombosis occurring prior to age 50, a family history of venous thromboembolism and recurrent venous thrombosis.[3] Resistance to activated protein C (due to factor V Leiden), the prothrombin gene mutation, and hyperhomocysteinemia are more prevalent defects, and therefore can also be found in significant numbers of patients with first episodes of idiopathic venous thrombosis after age 50 in the absence of a positive family history.[4] [5] [6] [7] [8] Testing for the presence of factor V Leiden, the prothrombin gene mutation, and hyperhomocysteinemia should therefore be considered in such patients.

Women with thrombosis in association with oral contraceptive use or pregnancy should also undergo thorough evaluation because a significant percentage will have an inherited disorder, especially the factor V Leiden mutation. [9] [10] [11] [12] [13] Patients with venous thrombosis in uncommon sites such as the portal, hepatic, mesenteric, and cerebral veins should also undergo a complete laboratory evaluation. Interestingly, patients with axillary vein thrombosis appear to have a low prevalence of hereditary defects, even in the absence of triggering risk factors. [14] The presence of indwelling venous catheters is the most common risk factor today

for upper extremity venous thrombosis, and patients with this complication generally do not warrant evaluation for an underlying hypercoagulable state.

Although the aforementioned classification of patients into hereditary or acquired categories is useful in directing the laboratory evaluation for hypercoagulability, it is simplistic in the sense that thrombosis frequently results from an interplay of

TABLE 120-2 -- Acquired Conditions and Disorders Associated with Hypercoagulable States

Pregnancy and the postpartum period	
Immobilization	
Trauma	
Postoperative state	
Advancing age	
Estrogen administration	Associated with oral contraceptives, postmenopausal estrogens, treatment of prostate cancer with diethylstilbestrol
Antiphospholipid syndrome	With or without overt systemic lupus erythematosis
Malignancy	
Disease-related	Includes migratory superficial thrombophlebitis (Trousseaus syndrome), nonbacterial thrombotic endocarditis, thrombosis associated with chronic disseminated intravascular coagulation, thrombotic microangiopathy
Treatment-related	Associated with the administration of various chemotherapeutic agents (L-asparaginase, mitomycin, adjuvant programs for breast cancer)
Infusion of prothrombin complex concentrates	
Nephrotic syndrome	
Heparin-induced thrombocytopenia	
Thrombotic thrombocytopenic purpura	
Myeloproliferative disorders	
Paroxysmal nocturnal hemoglo-binuria	
Hyperlipidemia	
Diabetes mellitus	
Hyperviscosity	
Congestive heart failure	

genetic and acquired factors. Patients with hereditary defects are at lifelong risk of developing thrombosis, and stimuli such as pregnancy, estrogen use, or surgery trigger thrombotic episodes in perhaps 50% of individuals. Also, defects in more than one coagulation protein are now being found in venous thrombosis patients due to the high background frequency in the general population of abnormalities such as factor V Leiden (6% in US Caucasians) [15] and the prothrombin gene mutation (2% in the

TABLE 120-3 -- Prevalence of Defects in Patients with Venous Thrombosis

Activated protein C resistance (factor V Leiden)	1240%
Deficiencies of antithrombin III, protein C, protein S	515%
Prothrombin gene mutation (G to A transversion at position 20210 in the 3-untranslated region)	618%
Hyperhomocysteinemia	1020%
Unknown	1566%

Figure 120-2 Venous thrombosis can be viewed as a multigene disorder in which susceptible patients will have one or more genetic mutations. Clinical events often occur when they are exposed to exogenous prothrombotic stimuli. *(Modified from Schafer,[22] with permission. © 1994 by the Lancet, Ltd.)*

Netherlands).[2] Such individuals have a more severe thrombotic diathesis than those with a single identifiable mutation. [8] [16] [17] [18] [19] [20] [21] Thus, thrombosis can be viewed as a multigene disorder in which susceptible patients will have one or more genetic mutations, with clinical events occurring when they are exposed to exogenous prothrombotic stimuli[22] (Fig. 1202). In many cases, however, the inciting precipitant to thrombosis is not reported by the patient and is therefore subclinical.

History and Physical Examination

A complete history is mandatory in evaluating patients with a recent or remote history of thrombosis. Details should be obtained regarding the age of onset, location of prior thromboses, and results of objective diagnostic studies documenting thrombotic episodes. The latter is critical because the clinical diagnosis of deep vein thrombosis, in particular, is notoriously inaccurate (Chap. 124). The patient should be carefully questioned about circumstances that were present proximate to the time of thrombosis that might have precipitated the event. These include surgical procedures, trauma, pregnancy, immobility, or estrogen administration. Women should be carefully questioned regarding prior use of oral contraceptives or hormone replacement therapy and obstetric history. An increased risk of recurrent fetal loss is associated with the presence of lupus anticoagulants or antiphospholipid antibodies as well as the hereditary thrombotic disorders. [23] [24] [25] [26] A family history is particularly important because a well documented history of venous thrombosis in one or more first-degree relatives strongly suggests the presence of a hereditary defect. The initial manifestation of a malignancy can be a thrombotic event, so inquiry should be made regarding the presence of constitutional symptoms (diminished appetite, weight loss, fatigue), pain, hematochezia, hemoptysis, or hematuria. It should be ascertained whether the patient has an underlying disease, such as cancer, collagen-vascular disease, a myeloproliferative disorder, atherosclerotic disease, or nephrotic syndrome, or takes synthetic or natural estrogens or drugs that can induce lupus anticoagulants such as hydralazine, procainamide, and phenothiazines. Recurrent thrombosis despite therapeutic anticoagulation with oral anticoagulants is common in patients with an occult neoplasm or recurrent cancer.

In the general physical examination, special attention should be directed to the vascular system, extremities, chest, heart, abdominal organs, and skin. Because venous thromboembolism may be the first manifestation of an underlying malignancy, rectal examination and stool testing for occult blood should be done and women should undergo a pelvic examination.

The hereditary thrombotic disorders include deficiencies of AT III, protein C or protein S, resistance to activated protein C due to factor V Leiden, the prothrombin gene mutation, and rare dysfibrinogenemias. The most common sites of thrombosis

in such patients are the deep leg veins, iliac veins, and pulmonary arteries. Thrombosis in mesenteric, portal, and cerebral veins as well as superficial thrombophlebitis has been described in some series of patients with these disorders. Typical precipitating factors for thrombosis in such patients include the postoperative state, immobilization, trauma, pregnancy, oral contraceptive use, and advancing age.

Patients with thrombosis should be questioned regarding their ethnic background. Factor V Leiden is rarely if ever found in aboriginal African, Native American, or Asian populations,[27] and a recent study determined that this mutation arose only 30,000 years ago in Causcasians in Europe.[28] This information should be considered in determining whether patients should be tested for this abnormality.

Laboratory Evaluation

Routine Laboratory Tests

The initial laboratory evaluation for patients with thrombosis should include a complete blood count, review of the peripheral blood smear, and serum chemistries including liver and renal function tests, and urinalysis.

Elevations in hematocrit or platelet count should lead to consideration of polycythemia vera and essential thrombocythemia in the differential diagnosis. These myeloproliferative disorders predispose patients to venous as well as arterial thrombotic events, particularly when the hematocrit or platelet count is not controlled by therapy. In addition, secondary polycythemia or secondary thrombocytosis may provide a clue to an underlying occult neoplasm that may itself be the predisposing factor to thrombosis. Leukopenia and thrombocytopenia are often found in paroxysmal nocturnal hemoglobinuria, a rare hematologic disorder characterized by chronic intravascular hemolysis with episodes of gross hematuria and a unique constellation of thrombotic complications. These occur almost exclusively in the abdominal venous network, including the mesenteric, hepatic, portal, splenic, and renal veins, and the cerebral venous circulation. The development of thrombosis and thrombocytopenia concurrent with heparin administration should always prompt consideration of the diagnosis of heparin-induced thrombocytopenia.

If the blood smear shows evidence of schistocytes, the differential diagnosis includes disseminated intravascular coagulation (DIC) and the thrombotic microangiopathies (thrombotic thrombocytopenic purpura/hemolytic-uremic syndrome). Although bleeding is the most common coagulation problem in DIC, patients with malignancy can have a low-grade consumptive coagulopathy and can develop venous or arterial thrombosis. The latter can result from emboli arising from fibrin vegetations on the mitral or aortic valves (nonbacterial thrombotic endocarditis [NBTE]). A leukoerythroblastic picture with nucleated red blood cells or immature white blood cells on the peripheral smear should suggest the possibility of bone marrow involvement by tumor.

Patients with Budd-Chiari syndrome, which occurs when there is obstruction of the hepatic venous circulation, have ascites and hepatomegaly along with abnormal liver function tests. The nephrotic syndrome is characterized by proteinuria, hypoalbuminemia, and hyperlipidemia. Renal vein thrombosis can complicate the course of up to one-third of such patients, but patients with nephrotic syndrome are prone as well to venous thrombosis in distant, common sites.

Patients with thrombosis should routinely undergo baseline coagulation tests, including prothrombin time and activated partial thromboplastin time (PTT). If the reagent used for the latter test is sensitive to the presence of lupus anticoagulants, this will provide an initial screen for this acquired abnormality. If the PTT is normal, the use of additional specialized clotting assays for the lupus anticoagulant may facilitate making this diagnosis in patients clinically suspected of having a lupus anticoagulant (Chap. 116). Some, but not all, patients with lupus anticoagulants have elevated titers of anticardiolipin antibodies. Patients with abnormal test results should be re-evaluated because lupus anticoagulants or elevated anticardiolipin antibody levels are generally considered to be risk factors for thrombosis only if the abnormalities persist over several months.

Tests for Specific Biologic Risk Factors

Clinical judgment must be exercised with regard to the scope of the evaluation. The decision as to which tests should be ordered is determined partly by the type of thrombotic event (Table 1204). Hereditary deficiencies of AT III, protein C, and protein S and activated protein C (APC) resistance due to the factor V Leiden mutation predispose principally to venous thrombosis. Although anecdotal reports suggest an association between arterial thrombosis and the inherited thrombotic disorders (particularly protein S deficiency), it is generally not recommended to test for these defects in such patients. The presence of a lupus anticoagulant/elevated antiphospholipid antibody titer or hyperhomocysteinemia, however, is a risk factor for venous as well as arterial thrombosis. Therefore, testing for these two defects is warranted in the latter group of patients, particularly when other factors predisposing to arterial thrombosis are absent.

Additional factors will guide the extent of the laboratory evaluation. Clinical features that increase the likelihood of identifying patients with deficiencies of AT III, protein C, and protein S include an initial venous thrombotic event before age 50, recurrent thrombosis, or positive family history. These features also increase the chances of identifying patients with factor V Leiden and the prothrombin gene mutation, although these genetic defects can also frequently be identified in older patients with an initial episode of deep venous thrombosis who do not have acquired risk factors or a positive family history. It is also warranted to screen for all of these defects, hyperhomocysteinemia, and antiphospholipid syndrome/lupus anticoagulant in patients with thrombosis in unusual vascular beds (i.e., portal, hepatic, or mesenteric vein thrombosis, cerebral venous thrombosis).

Some clinical features have been associated with individual hereditary thrombotic disorders. Approximately one-third of patients who sustain the rare complication of warfarin-induced skin necrosis will prove to have underlying protein C deficiency; however, this syndrome has also been reported in patients with protein S deficiency[29] and factor V Leiden.[30] In newborn infants, the development of skin necrosis and visceral thrombosis (neonatal purpura fulminans) indicates a likely diagnosis of severe hereditary protein C deficiency, although there have been cases reported in association with homozygous protein S deficiency as well.[31] [32] Both conditions require emergent therapy if major morbidity or mortality in neonatal purpura fulminans is to be averted. Although resistance to heparins

TABLE 120-4 -- Sites of Thrombosis According to Coagulation Defect

Abnormality	Arterial	Venous
Factor V Leiden		+
Antithrombin III deficiency		+
Protein C deficiency		+
Protein S deficiency		+
Prothrombin gene mutation	?	+
Hyperhomocysteinemia	+	+
Lupus anticoagulant/antiphospholipid syndrome	+	+

TABLE 120-5 -- Screening Laboratory Evaluation for Patients Suspected of Having a Biologic Defect Predisposing to Thrombosis

Test for resistance to activated protein C

Clotting assay[a]
Genetic test for Factor V-Arg 506 Gln (factor V Leiden)
Measurement of total plasma homocysteine
Genetic test for prothrombin gene mutation (G to A transversion at position 20210 in the 3-untranslated region)
Functional assay of antithrombin III (heparin-cofactor assay)[a]
Functional assays of protein C[a]
Functional assay of protein S/immunologic assays of total and free protein S[a]
Screen for dysfibrinogenemias (Immunologic and functional assays of fibrinogen, thrombin time)[a]
Clotting assay for lupus anticoagulant[a] /serologic tests for antiphospholipid antibodies

[a] Coagulation assays are performed on platelet-poor plasma obtained from blood samples drawn into a solution containing 3.8% (wt/vol) sodium citrate. The ratio of anticoagulant to blood is 0.1:0.9 (vol/vol). In the absence of an accompanying clinical history for the patient, the performance of a prothrombin time will help to exclude the ingestion of warfarin, which will affect the measurements of protein C, protein S, and first-generation PTT-based assays for activated protein C resistance. Performance of a thrombin time as well as a PTT will help to exclude the administration of heparin.

anticoagulant effect, as measured by the PTT, often leads to consideration of AT III deficiency, congenital AT III deficiency is infrequently diagnosed in such patients.

Table 1205 gives a list of tests useful in screening patients suspected of having a biologic defect predisposing to thrombosis. Coagulation assays with high sensitivity and specificity for the factor V Leiden mutation are now widely available and are based on the resistance of the mutant factor Va molecule to inactivation by APC. Testing for the factor V Leiden mutation can also be done by analyzing DNA obtained from peripheral blood mononuclear cells. Testing for hyperhomocysteinemia should be done after an overnight fast when the patient is on a normal diet. Measuring increments in total plasma homocysteine 48 hours after an oral methionine load as well as fasting levels facilitates the identification of 10% more hyperhomocysteinemic patients and improved discrimination of affected subjects from controls. The homocysteine measurements are performed using ion-exchange chromatography, gas chromatography-mass spectrometry, or high-performance liquid chromatography with electrochemical or fluorescent detection.

The best screening tests for deficiencies of AT III, protein C, and protein S are functional assays that detect both quantitative and qualitative defects. Immunologic (antigenic) assays detect only quantitative deficiencies of these proteins. For plasma AT III, convenient functional assays are available that measure the heparin cofactor activity of the molecule. Among protein C assays, coagulation assays provide a more complete evaluation of the functional activity of the molecule than amidolytic assays. However, coagulation assays for protein C as well as protein S can give falsely low values if the factor V Leiden mutation is present, and reliable application of these assays therefore must initially assess whether this mutation is present. [33] Because factor V Leiden is much more commonly encountered than deficiencies of protein C or protein S, clinical laboratories often resort to measuring protein C activity by amidolytic assay and only perform immunoassays for total and free protein S. It is important to measure free protein S because some patients with hereditary protein S deficiency have low free levels with normal or borderline total protein S levels. To screen for a dysfibrinogenemia, the thrombin time is recommended along with measurements of plasma fibrinogen by clotting and immunologic assay.

Timing of Laboratory Testing

An important consideration in the laboratory evaluation of patients with a suspected deficiency of AT III, protein C, or protein S is the timing of testing. Erroneous diagnoses can be made due to the influence of acute thrombosis, comorbid illness, or anticoagulant therapy on the levels of these plasma proteins. Table 1206 provides a list of some of the common causes of acquired deficiencies of AT III, protein C, or protein S.

Acute thrombosis by itself can result in transiently reduced levels of AT III and occasionally protein C and protein S. Heparin therapy can be associated with up to a 30% decline in plasma AT III levels over several days, while warfarin produces a marked drop in the functional activity of protein C and protein S and a lesser decline in immunologic levels. Warfarin has also been shown to rarely elevate AT III levels significantly, sometimes into the normal range, in patients with a hereditary deficiency of this inhibitor. For these reasons, it is optimal to test for these deficiency states at least 2 weeks after completing the initial 36-month course of oral anticoagulant therapy following a thrombotic event. If, however, levels of AT III, protein C, or protein S are obtained on acute presentation that are well within the normal range, these will effectively exclude the diagnosis of their deficiency states from consideration. However, the finding of a low level during this period will need to be confirmed by repeat testing after anticoagulation is discontinued. The investigation of first-degree family members is useful to document the hereditary nature of the deficiency. Confirmation of a deficiency state in first-degree family members is particularly helpful diagnostically in patients in whom the risks of recurrent thrombosis are too great to temporarily discontinue anticoagulation. In such patients, a diagnosis of protein C or protein S can be confirmed by carrying out testing after warfarin has been discontinued for 2 weeks under the cover of intravenous or subcutaneous heparin at therapeutic doses.

The original PTT-based activated protein C-resistance tests were sensitive to the choice of reagents, instrument used for clot detection, and preanalytical conditions. Based on the choice of methodology, certain acquired conditions (e.g., elevated factor VIII levels, pregnancy, oral contraceptive use) could result in the phenotype of APC resistance, although it is not known whether this confers an increased risk of thrombosis. The original tests also could not be used to test patients with

TABLE 120-6 -- Causes of Acquired Deficiencies in Antithrombin III, Protein C, and Protein S

Antithrombin III	Protein C	Protein S
Neonatal period	Neonatal period	Neonatal period
Pregnancy		Pregnancy
Liver disease	Liver disease	Liver disease
DIC[a]	DIC	DIC
Nephrotic syndrome	Chemotherapy(CMF)[a]	
Major surgery		Inflammatory states
Acute thrombosis	Acute thrombosis	Acute thrombosis
Treatment with		
Heparin	Warfarin	Warfarin
L-asparaginase	L-asparaginase	L-asparaginase
Estrogens		Estrogens

[a] DIC, disseminated intravascular coagulation; CMF, cyclophosphamide, methotrexate, 5-fluorouracil.

lupus anticoagulants or those receiving heparin or oral anticoagulants because of the increased sensitivity of such plasmas to APC. The discovery that the APC-resistance phenotype was frequently due to the factor V Leiden mutation led to modifications in the original PTT-based activated protein resistance tests and prediluted patients plasma in factor V-deficient plasma. Such second-generation clotting assays have high sensitivity and specificity for the factor V Leiden mutation and can be used reliably in patients taking anticoagulants as well as most of the acquired conditions enumerated previously.

Other Coagulation Abnormalities

There has been renewed interest in elevated plasma factor VIII coagulant activity (VIII:C) as an independent marker of increased thrombotic risk. In a population-based case control study performed in the Netherlands, 25% of patients had levels of factor VIII coagulant activity (VIII:C) that were >150% of normal and they had an adjusted odds ratio of 4.8 for a first episode of venous thrombosis event as compared to individuals with levels under 100%. [34] Elevated VIII:C levels were also found in a similar proportion of English patients referred for evaluation of unexplained thrombosis. [35] This laboratory abnormality could not be attributed to overt inflammation because <10% of patients with VIII:C levels >150% had elevations in acute-phase reactants such as C-reactive protein, fibrinogen, and erythrocyte sedimentation rate. The levels of VIII:C were in reasonable concordance with factor VIII antigen concentrations, and it was postulated that the elevations result from a constitutive increase in synthesis or an exaggerated response to minimal inflammation. [35] Additional studies are required to determine the molecular basis underlying increased VIII:C concentrations and the clinical utility of measuring this parameter in patients with thrombosis.

There have been reports of thrombosis in association with abnormalities in other coagulation or fibrinolytic system proteins. These include heparin cofactor II deficiency,[36] [37] plasminogen deficiency (either hypoor dysplasminogenemias), factor XII deficiency, thrombomodulin mutations, and elevations in histidine-rich glycoprotein or plasminogen activator inhibitor-1 (PAI-1). However, causal associations between these abnormalities and an increased risk of thrombosis have not been clearly defined.[38] Thus, it is not currently recommended to test for these components.

Thrombosis in Association with Malignancy

Cancer is one of the most common acquired risk factors for venous or arterial thrombosis (Table 1202). The clinical manifestations that are attributable to the underlying neoplasm include deep venous thrombosis/pulmonary embolism, migratory superficial thrombophlebitis (Trousseaus syndrome), NBTE with arterial emboli, or thrombosis in the presence of chronic DIC. Thrombosis can also be associated with the administration of various chemotherapeutic agents. The occurrence of a clinical picture of a thrombotic microangiopathy can be due to the cancer itself or antineoplastic treatment, especially mitomycin-C.

Although thrombosis is most commonly encountered in the setting of advanced disease, deep venous thrombosis or pulmonary embolism can be the first sign of malignancy. In previously healthy patients presenting with a first episode of venous thrombosis, one should have a high index of suspicion for cancer because 1020% of such individuals will have cancer diagnosed at presentation or within the ensuing 24 months. [39] [40] [41] The most common sites of origin of cancer are the gastrointestinal tract, genitourinary organs, and lung. A complete medical history, physical examination including digital rectal examination and testing for fecal occult blood, pelvic examination in women, routine laboratory testing, and chest radiograph should be performed in all patients presenting with thrombosis. The presence of constitutional symptoms, unusual pain or bleeding, change in cough or hoarseness, or alterations in bowel or bladder habits warrants further diagnostic testing. It is controversial as to whether patients without any of the aforementioned symptoms or signs of neoplasia should undergo more detailed investigation including computed tomography or endoscopic evaluation. Although the literature contains studies indicating that up to 10% of patients with initial venous thrombotic episodes will have early stage cancers that were not detected by routine studies, no current data show that more aggressive diagnostic testing leads to improved survival or is cost effective. The development of recurrent idiopathic thrombosis following an adequate initial course of anticoagulant therapy is even more worrisome for an underlying cancer and warrants a thorough diagnostic evaluation. [40]

Although the treatment of acute thrombosis with heparin is usually effective in cancer patients, some will develop recurrences when they are switched over to oral anticoagulants despite a therapeutic International Normalized Ratio (INR). This scenario is particularly characteristic of cancer patients with metastatic or locally advanced disease that is refractory to antineoplastic therapy. Long-term anticoagulation with subcutaneous heparin at therapeutic doses is recommended to reduce the morbidity and mortality of thrombotic complications in such individuals.

Patients with Recurrent Thrombosis but Normal Laboratory Tests

Despite recent advances, there remains a substantial fraction of patients in whom we cannot uncover hereditary or acquired risk factors predisposing to thrombosis even after detailed laboratory evaluation (Table 1203). It is sometimes helpful for the clinician caring for such individuals to refer the patient to an appropriate specialist for review of the laboratory evaluation and consideration of additional work-up. Additional prothrombotic mutations will certainly be discovered in the future and specialists in the field will be knowledgeable as to the availability and relevance of new diagnostic developments prior to commercialization and wide clinical availability. In addition, assays are available that measure the extent of coagulation enzyme generation in the blood in vivo (e.g., prothrombin activation fragment F_{1+2}, thrombin-AT III complex, Fig. 1201), although these assays do not give information regarding the specific type of hypercoagulable state that is present. [42]

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INHERITED HYPERCOAGULABLE STATES

Patients with a tendency to thrombosis are defined as having thrombophilia, and the term inherited thrombophilia is applied to individuals with predisposing genetic defects.[43] [44] Prior to 1993, the diagnosis of a hereditary disorder could be established in only about 15% of patients under age 50 presenting with venous thromboembolism. The major disorders known at that time were deficiencies of AT III, protein C, or protein S. With the discovery that many patients with unexplained venous thrombosis have APC resistance due to the factor V Leiden mutation and more recently a prothrombin gene mutation, it is currently possible to define genetic risk factors in >50% of younger patients with idiopathic venous thrombosis. Hyperhomocysteinemia, which is determined by genetic as well as dietary factors, is a common risk factor for venous and arterial thrombosis.

AT III Deficiency

In 1965, Egeberg[45] described a Norwegian family in which certain individuals who had a history of thrombosis had plasma concentrations of AT III that were 4050% of normal. Subsequently,

other investigators described additional families with a similar constellation of clinical and laboratory abnormalities. [46] [47] [48] [49] [50] [51] [52] [53] [54]

Antithrombin III deficiency is usually inherited in an autosomal dominant fashion, and thus, affects both sexes equally. Two major types of inherited AT III deficiency have been delineated (Table 1207). The type I deficiency state is a result of reduced synthesis of biologically normal protease inhibitor molecules. [55] In these cases, the antigenic and functional activity of AT III in the blood are reduced in parallel. The molecular basis of this disorder is either a deletion of a major segment of the AT III gene or, more commonly, the occurrence of small deletions/insertions (<22 base pairs), or single base substitutions. These mutations will introduce a frameshift, a direct termination codon, a change in mRNA processing, or unstable translation products. The most recent update of the AT III mutation database includes 80 distinct mutations in patients with a type I deficiency.[56] The second type of AT III deficiency is produced by a discrete molecular defect within the protease inhibitor (type II). The plasma levels of AT III are greatly reduced as judged by functional activity, whereas AT III immunologic activity is essentially normal.

Using only an immunoassay for measuring AT III levels in the blood, initial estimates of the deficiency state in the general population were 1/2,0005,000. [57] However, recent studies using functional assays that measure AT III-heparin cofactor activity have found that the prevalence of AT III deficiency in the general population is 1/250500.[58] [59] The majority of the AT III-deficient patients identified in these studies did not have a personal or familial history of thrombosis and had a type II defect with mutations at the heparin-binding site (Table 1207).[60] On rare occasions, some of these molecular defects came to clinical attention when homozygous children of consanguinous parents who were asymptomatic carriers of such a defect developed severe venous or arterial thrombosis in association with plasma AT III-heparin cofactor levels of <10%.[61] [62]

Reviews of published cases of familial AT III deficiency indicate that 55% of affected patients experience venous thrombotic episodes. [63] [64] The initial clinical manifestations occur apparently spontaneously in about 42% of subjects, but are related to pregnancy, parturition, oral contraceptive ingestion, surgery, or trauma in the remaining 58% of patients.[63] The most common sites of disease are the deep veins of the leg and the mesenteric veins. Approximately 60% of individuals develop recurrent thrombotic episodes, and clinical signs of pulmonary embolism are evident in 40%. [63] Although cases have been reported in which AT III-deficient infants sustain cerebral venous thrombosis[54] [65] [66] affected children rarely develop thrombotic episodes before puberty. At this time, thrombotic events start to occur with some frequency and the risk of thrombosis increases substantially with advancing age. [63] The first family with a functional deficiency of AT III due to the presence of an abnormal protein (type II) was reported by Sas in 1974. [67] Many families with this type of deficiency state have now been reported and they have been further subcategorized on the basis of two different functional assays of AT III

TABLE 120-7 -- Assay Measurements in Heterozygous Antithrombin III (AT III) Deficiency

Types	Antigen	Activity	
		Heparin Cofactor	Progressive AT III
I	Low	Low	Low
II			
Active site defect	Normal	Low	Low
Heparin-binding site defect	Normal	Low	Normal

activity. The first is the AT III-heparin cofactor assay, which measures the ability of heparin to bind to lysyl residues on the inhibitor and catalyze the neutralization of coagulation enzymes such as thrombin and factor Xa. The second test is the progressive AT III activity assay, which quantifies the capacity of this inhibitor to neutralize the enzymatic activity of thrombin in the absence of heparin.

Heparin cofactor II is another protein in human plasma that exhibits heparin cofactor activity. [68] [69] [70] In contrast to AT III, this inhibitor does not inhibit factor Xa or other serine proteases and requires concentrations of heparin of at least 1 U/ml in the reaction mixture to function as an efficient inhibitor of thrombin; it therefore probably plays a minimal role when heparin is used clinically as an anticoagulant. [69] Because many functional AT III assays use heparin concentrations >1 U/ml, an assay based on factor Xa inhibition is likely to be more specific than one based on thrombin inhibition to identify patients with a congenital deficiency of AT III. [71]

The AT III-heparin cofactor and progressive AT III activity assays have identified AT III-deficient patients with reductions in heparin cofactor activity with or without concordant decrements in progressive AT III activity. Several abnormal AT III molecules have been identified with isolated reductions in heparin cofactor activity that have defects at a heparin-binding site (Table 1208). These variants generally have mutations at the amino terminal end of the molecule.[56] Variants with decreased activity in both AT III functional assays generally have mutations near the thrombin-binding site at the carboxy terminal end of the molecule (Table 1208).[56] All AT III variants cannot be neatly characterized by this schema because single amino acid substitutions can affect both functional domains of the molecule. This is perhaps best illustrated by a mutation in which arginine is replaced by histidine at residue 393. This mutation markedly decreases the ability of the protein to inhibit thrombin, but also leads to increased heparin affinity.

Another type of mutation has been described at the carboxy terminal end of the AT III molecule between amino acids 402429 (Table 1208). These type II variants are termed pleiotropic because they exhibit multiple functional defects.[56] In a large Utah family, trace amounts of an electrophoretically and functionally abnormal

inhibitor molecule have been identified. [72] Leucine is substituted for proline at position 407 in the AT III molecules of the affected members of this kindred. [73] [74] Small amounts of an electrophoretically abnormal inhibitor species have also been observed in the plasma of AT III-deficient members of the Oslo kindred first reported by Egeberg.[45] [75] In this case, threonine replaces alanine at position 404 in the abnormal protein. [76] Mutations at positions 402, 405, 406, and 407 apparently also lead to a similar type of defect.[56] The AT III-deficient subjects in the Utah and Oslo pedigrees appear, however, to have a type I deficiency state as determined by routine laboratory testing. The similar characteristics of these mutations suggest that the region of residues 402407 is important for the maintenance of normal plasma levels of AT III antigen.[74] The mutant inhibitor molecules synthesized by the liver of these deficient individuals may be susceptible to increased intracellular degradation, decreased extracellular export, or increased clearance on entry into the circulation. Finally, the mutation in the first reported AT III variant, Budapest, [67] is replacement of a proline by leucine at residue 429, leading to a molecular defect that affects both the heparin and thrombin binding sites. [77]

The prevalence of thrombosis appears to be different in heterozygous patients with type II defects at the heparin-binding site as compared to the thrombin-binding site.[78] Individuals with plasma AT III-heparin cofactor activity measurements of 50% and normal progressive AT III activity (heparin-binding site defects), have infrequent thrombotic episodes. In contrast to these observations, heterozygous type II patients with both diminished progressive AT III activity and AT III-heparin

TABLE 120-8 -- Point Mutations in Antithrombin III Deficiency (Type II)

City or Region of Propositus	Substitution	Effect of Mutation
Rouen 3	Ile 7 Asn	Defective heparin binding, new carbohydrate attachment site
Rouen 4	Arg 24 Cys	Defective heparin binding
Basel, Clichy, Dublin II, Franconville	Pro 41 Leu	Defective heparin binding
Toyama, Tours, Alger, Paris 1, Paris 2, Barcelona 2, Kumamoto, Padua 2, Amiens	Arg 47 Cys	Defective heparin binding
Rouen 1, Padua 1, Bligny	Arg 47 His	Defective heparin binding
Rouen 2	Arg 47 Ser	Defective heparin binding
Budapest 3	Leu 99 Phe	Defective heparin binding
Southport	Leu 99 Val	Defective heparin binding
Nagasaki	Ser 116 Pro	Defective heparin binding
Vienna	Gln 118 Pro	Defective heparin binding
Geneva	Arg 129 Gln	Defective heparin binding
Rouen VI	Asn 187 Asp	Defective serine protease inhibition
Glasgow III	Asn 187 Lys	Defective serine protease inhibition
Truro	Glu 237 Lys	Defective heparin binding
Unnamed	Met 251 Ile	Defective serine protease inhibition
Haslar	Ile 284 Asn	Defective serine protease inhibition
Unnamed	Glu 302 Lys	Defective serine protease inhibition
Hamilton, Glasgow-II	Ala 382 Thr	Defective serine protease inhibition
Charleville, Cambridge 1, Vicenza, Sudbury	Ala 384 Pro	Defective serine protease inhibition
Cambridge 2	Ala 384 Ser	Defective serine protease inhibition
Stockholm	Gly 392 Asp	Defective serine protease inhibition
Glasgow, Sheffield, Chicago, Waikato, Avranches	Arg 393 His	Defective serine protease inhibition
Northwick Park, Milano 1, Frankfurt 1	Arg 393 Cys	Defective serine protease inhibition
Pescara	Arg 393 Pro	Defective serine protease inhibition
Denver, Milano 2	Ser 394 Leu	Defective serine protease inhibition
Rosny	Phe 402 Cys	These mutations result in the presence of trace amounts of the abnormal antithrombin III in patients plasma.
Torino	Phe 402 Ser	
Maisons, Lafitte	Phe 402 Leu	
Oslo	Ala 404 Thr	
La Rochelle	Asn 405 Lys	
Kyoto	Arg 406 Met	
Unnamed	Arg 406 Gly	
Utah	Pro 407 Leu	
Budapest 5	Pro 407 Thr	
Unnamed	Arg 425 Thr	Abnormal heparin binding and reduced thrombin inhibitory activity.
Budapest	Pro 429 Leu	

cofactor activity (thrombin-binding site defects) sustain venous thromboembolism as often as type I patients.

The mean concentration of AT III in normal plasma is 140 g/ml. In plasmas from normal individuals, the range of AT III concentrations as determined by immunologic or functional tests is reasonably narrow. Most laboratories report a normal range between 80% and 120% for AT III-heparin cofactor determinations and a somewhat wider range for immunoassay results. The AT III-heparin cofactor assay will detect all the different subtypes of the familial deficiency state currently recognized and is therefore the best single laboratory screening test for the disorder.

Healthy newborns have about half the normal adult concentration of AT III [79] [80] and gradually reach the adult level by 6 months of age. [81] The levels may be considerably lower in infants born after 3036 weeks of gestation [81] and are even further reduced in infants with respiratory distress, necrotizing enterocolitis, sepsis, or DIC.[82] Thromboembolic events are rare in children with hereditary AT III deficiency. In the absence of heparin, AT III contributes 80% of the thrombin-neutralizing capacity of normal adult plasma.[83] [84] The levels of a second thrombin inhibitor, $_2$ -macroglobulin, are higher during the first two decades of life than in adults, and this may lessen the risk of thromboembolic complications in AT III-deficient patients during childhood. [85]

A variety of pathophysiologic conditions can reduce the concentration of AT III in the blood (Table 1206). Acute thrombosis infrequently lowers AT III levels substantially, but DIC usually reduces the level of this inhibitor. Lowered AT III concentrations occur in patients with liver disease due to decreased protein synthesis. Decreased AT III levels are also observed in individuals with the nephrotic syndrome as a consequence of urinary excretion. [86] Furthermore, modest reductions in plasma AT III concentration are found in users of oral contraceptives [87] [88] as well as in individuals receiving estrogen for other purposes. [63] [89] The levels of AT III do

not change substantially during normal pregnancies, [87] [88] but may decrease significantly in women with pregnancy-induced hypertension, preeclampsia, or eclampsia.[90] Infusions of L-asparaginase, a chemotherapeutic agent used in the treatment of acute lymphocytic leukemia, can substantially lower the plasma concentration of this inhibitor. [91] In addition, the administration of heparin decreases plasma AT III levels, [92] presumably on the basis of accelerated in vivo clearance. Evaluation of plasma samples from patients during a period of heparinization can therefore potentially lead to an erroneous diagnosis of AT III deficiency.

Due to the number of clinical disorders that can be associated with reductions in the plasma concentration of AT III, definitive diagnosis of the hereditary deficiency state is often difficult. Although

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an AT III level in the normal range is usually sufficient to exclude the disorder, low levels should be confirmed by obtaining another sample at a subsequent time. This determination is ideally performed when the patient is no longer receiving an oral anticoagulant because this medication has rarely been reported to raise plasma AT III concentrations into the normal range in individuals with the hereditary deficiency state. [47] Confirmation of the hereditary nature of the disorder requires the investigation of other family members. Diagnosis of other affected family members also allows for appropriate counseling regarding the need for prophylaxis against venous thrombosis.

Anabolic steroids such as stanozolol and danazol raise plasma AT III levels in individuals with normal [93] as well as with reduced levels of this inhibitor. [94] Despite this effect, these drugs have not been shown to prevent thrombosis in patients with hereditary AT III deficiency. [94]

Protein C Deficiency

In 1981, Griffin et al.[95] described the first kindred in which several individuals had plasma levels of protein C antigen of 50% of normal and a history of recurrent thrombotic events. Subsequently, other investigators[96] [97] [98] [99] [100] have reported numerous other families with heterozygous protein C deficiency.

Heterozygous protein C deficiency is inherited in an autosomal dominant fashion; a more severe form of protein C deficiency is an autosomal recessive disorder. The phenotype of patients with heterozygous protein C deficiency is similar to hereditary AT III deficiency. In severely affected families, 75% of protein C-deficient individuals experienced one or more thrombotic events. The initial episode occurs apparently spontaneously in 70%. The remaining 30% have the usual associated risk factors (pregnancy, parturition, contraceptive pill use, surgery, or trauma) at the time they develop acute thrombotic events. However, patients are infrequently symptomatic until their early twenties, with increasing numbers of individuals experiencing thrombotic events as they reach the age of 50. The most common sites of disease are the deep veins of the legs, the iliofemoral veins, and the mesenteric veins. Approximately 63% of affected patients develop recurrent venous thrombosis and 40% exhibit signs of pulmonary embolism.[101] There is a high frequency of superficial thrombophlebitis of the leg veins [97] as well as several cases of cerebral venous thrombosis in protein C-deficient patients. [102] There have also been reports of nonhemorrhagic arterial stroke in young adults with hereditary protein C deficiency.[103] [104] [105]

Miletich et al.[106] determined that the frequency of heterozygous protein C deficiency is 1/200300 in healthy adult blood donors, whereas Tait et al. [107] reported a prevalence of 1/500 in a healthy general population. None of the affected individuals in this population had histories of thrombosis prior to being tested. Although the inclusion criteria used for these studies do not permit estimation of the relative thrombotic risk conferred by protein C deficiency, they demonstrated that protein C deficiency can have markedly different clinical phenotypes depending on how patients are selected. The data also suggested that other factors modulate the phenotypic expression of heterozygous protein C deficiency, [108] which has become clear with the identification of other frequent prothrombotic mutations such as factor V Leiden. Data from the Leiden Thrombophilia Study indicate that individuals from the general population with heterozygous protein C deficiency have about a sevenfold increased risk of an initial episode of deep vein thrombosis as compared to normal subjects. [109]

Two major subtypes of heterozygous protein C deficiency have been delineated using immunologic and functional assays (Table 1209). The type I deficiency state is the most common form and is characterized by a reduction in both the immunologic and biologic activity of plasma protein C to 50% of normal. [101]

TABLE 120-9 -- Assay Measurements in Heterozygous Protein C Deficiency

Types	Antigen	Activity	
		Amidolytic	Coagulant
I	Low	Low	Low
II	Normal	Low	Low
	Normal	Normal	Low

Studies of the genetic defects in patients with protein C deficiency have led to the identification of more than 160 different mutations. [110] In individuals with type I deficiency, missense and nonsense mutations are most common.[110] [111] [112] In several Dutch families, the linkage between a rare polymorphism and a particular mutation with the deficiency state has indicated the presence of a founder effect. [113] Other types of mutations resulting in type I protein C deficiency include promoter mutations, splice site abnormalities, in-frame deletions, frameshift deletions, in-frame insertions, and frameshift insertions. [110] In families with a type II deficiency state, affected individuals have normal protein C levels on immunologic examination, yet possess lowered functional levels of the zymogen. The point mutations that have been identified in patients with type II protein C deficiency are shown in Table 12010 .[110] [114]

TABLE 120-10 -- Point Mutations in Protein C Deficiency (Type II)

City or Country of Propositus	Substitution	Domain of Mutation
Paris	Arg-5 Try	
Malakoff	Arg-1 His	Propeptide cleavage site; N-terminus extended with mutant His; normal -carboxylation; altered conformation of Gla domain
Osaka 10	Arg-1 Ser	Propeptide cleavage site; N-terminus extended with mutant Ser; normal -carboxylation; altered conformation of Gla domain
Paris	Arg-1 Cys	Complexation with other plasma proteins through free SH group
Spain	Arg 9 Cys	Gla domain
Netherlands; Paris	Arg 15 Try	Gla domain
Yonago	Arg 15 Gly	Gla domain
Vermont I	Gln 20 Ala	Gla domain
Mie	Glu 26 Lys	Gla domain
Vermont I	Val 34 Met	Gla domain
La Jolla I	His 66 Asn	
La Jolla III	Arg 147 Trp	
Japan; London I	Arg 169 Try	Thrombin-thrombomodulin cleavage site
Austria	Arg 169 Gln	
Spain	Arg 178 Gln	
Austria	His 211 Gln	Serine protease domain

Spain	Arg 229 Try	Serine protease domain
Marseille	Arg 229 Gln	Serine protease domain
Paris	Ser 252 Asn	Serine protease domain
Austria	Met 343 Ileu	Serine protease domain
Osaka 9	Gly 350 Arg	Serine protease domain
Netherlands	Arg 352 Try	Serine protease domain
Netherlands; La Jolla II	Asp 359 Asn	Serine protease domain
Italy	Gly 381 Ser	Serine protease domain
Cadiz	Gly 383 Cys	Serine protease domain
Osaka 3	Gly 385 Arg	Serine protease domain
Purmerend	Gly 391 Ser	Serine protease domain

Warfarin-induced skin necrosis has been associated with the presence of heterozygous protein C deficiency. [115] [116] [117] This syndrome typically occurs during the first several days of warfarin therapy, often in association with the administration of large loading doses of the medication (Chap. 121). The skin lesions occur on the extremities, breasts, trunk, and the penis and marginate over a period of hours from an initial central erythematous macule. If a product containing protein C is not rapidly administered, the affected cutaneous areas become edematous, develop central purpuric zones, and ultimately become necrotic. Biopsies demonstrate fibrin thrombi within cutaneous vessels with interstitial hemorrhage. The dermal manifestations of warfarin-induced skin necrosis are clinically and pathologically similar to those seen in infants with purpura fulminans due to severe protein C deficiency.

The pathogenesis of warfarin-induced skin necrosis is attributable to the emergence of a transient hypercoagulable state. The initiation of the drug at standard doses leads to a decrease in protein C anticoagulant activity levels to 50% of normal within 1 day. [118] Although factor VII activity measurements follow a pattern similar to that of protein C, the levels of the other vitamin K-dependent factors decline at slower rates, consistent with their longer half-lives (Fig. 1203). Increased thrombin generation has been documented in patients during this early phase of warfarin therapy using a sensitive immunochemical assay for fragment F_{1+2}, an index of the in vivo activation of prothrombin mediated by factor Xa. [119] During this period, it therefore appears that the drugs suppressive effect on protein C has a greater influence on the hemostatic mechanism than its reduction in factor VII. These effects are likely to be augmented when >10 mg warfarin daily is administered to initiate oral anticoagulation or the patient has an underlying hereditary deficiency of protein C. Only approximately one-third of patients with warfarin-induced skin necrosis, however, have an underlying inherited deficiency of protein C, [120] and this complication is only infrequently reported in individuals with the heterozygous deficiency state. Case reports have also described this syndrome in association with an acquired functional deficiency of protein C, [121] heterozygous protein S deficiency, [29] and factor V Leiden. [30]

A clinical disorder exists in which newborns develop a syndrome described as purpura fulminans and laboratory evidence of DIC in association with protein C antigen levels <1% of normal. [122] [123] [124] [125] [126] In some instances, there was a history of consanguinity in the family, making it highly likely that the affected infants were homozygous for the deficiency state. [123] [124] [126] These newborns can also be double heterozygotes, as was demonstrated in a Chinese patient [125] who had a five-nucleotide deletion in one protein C allele and a missense mutation in the other. [127] The heterozygous parents of these infants infrequently had thromboses, in contrast to patients with thrombotic histories and a hereditary partial deficiency of protein C. There have also been a number of reported cases of older patients with homozygous or doubly heterozygous protein C deficiency who do not present with lesions resembling purpura fulminans. These individuals generally had protein C levels <20% of normal in the absence of oral anticoagulant therapy, and their clinical presentation was similar to that of severely affected subjects from thrombophilic kindreds with the heterozygous deficiency state. [128] [129] Genotyping of such homozygous individuals identified several missense mutations in the protein C gene; [130] [131] [132] [133] the variant protein C molecules produced by these individuals are either synthesized at a decreased rate or are rapidly cleared from the circulation. The parents of these subjects and infants with the so-called syndrome of purpura fulminans have a type I deficiency state.

In patients with heterozygous type I protein C deficiency, it has been of interest to determine whether the genetic basis for the disorder is different between symptomatic and asymptomatic individuals. Common mutations have been found among symptomatic and asymptomatic persons, [112] [133] [134] thereby implying that the nature of the protein C gene defect alone does not explain the marked phenotypic variability among type I patients.

A variety of immunologic and functional techniques have been developed to measure protein C levels in plasma samples. The most common procedures for antigen determination are electroimmunoassay, enzyme-linked immunosorbent assay, and radioimmunoassay. Functional assays have used either thrombin [135] or the thrombin/thrombomodulin complex [118] [136] [137] to activate protein C. Enzyme activity is then assessed using either a chromogenic substrate [135] [136] [137] or by measuring its anticoagulant activity in a factor Xa one-stage clotting assay. [118] The development of simpler functional assays has been facilitated by the observation that the venom of the Southern copperhead snake (*Agkistrodon contortrix*) is able to activate protein C in plasma. After activation of protein C by this venom, the enzymes amidolytic activity can be measured using a suitable chromogenic substrate, or its anticoagulant activity can be measured in a clotting assay.

Functional assays using amidolytic and clotting end points may give useful information regarding the nature of the molecular defect in patients with type II protein C deficiency. Several individuals have been described with normal protein C antigen measurements who have substantial reductions in protein C anticoagulant

Figure 120-3 Mean levels of protein C (PC) anticoagulant activity, factor VII activity, factor X activity, protein C amidolytic activity, and protein C antigen following the initiation of warfarin therapy in patients with deep venous thrombosis. Patients were maintained on heparin infusions and 10 mg warfarin was administered for the first 3 days. Dosages were subsequently adjusted based on the prothrombin time. Measurements are expressed as percentages of prewarfarin levels. *(From DAngelo et al., [116] with permission.)*

activity, but normal or near normal amidolytic activity. [116] These defects may potentially reflect a reduced ability of APC to interact with platelet membranes or its substrates such as factor V and factor VIII. The molecular abnormality in one of these families [138] is characterized by two -carboxyglutamic acid (Gla) domain mutations (Glu 20 to Ala and Val 34 to Met). On the other hand, abnormal protein C molecules that are normally activated by the thrombin/thrombomodulin complex, but fail to exhibit proteolytic activity as measured by amidolytic or anticoagulant assays have been reported. [139] [140] This suggests that the mutations reside near the active site of the protein.

Protein C circulates in human plasma at a concentration of 4 g/ml. The logarithms of the values for protein C antigen in healthy adults are normally distributed with 95% of the values ranging from 70140%. [106] There is no significant gender dependence, but mean protein C concentrations increase by 4% per decade. The relatively wide normal range of protein C measurements in the general population occasionally makes it difficult to identify a given individual as having heterozygous protein C deficiency. [141] If medical and pharmacologic causes of low levels are excluded, patients with a protein C value of <55% are very likely to have the genetic abnormality, whereas levels of 5565% are consistent with either a deficiency state or the lower end of the normal distribution. [106] To document the presence of protein C deficiency with confidence, it is therefore useful to obtain repeat laboratory determinations as well as to perform family studies to identify an autosomal dominant inheritance pattern.

Protein C levels in newborns are 2040% of normal adult levels. [142] [143] Preterm infants have even lower levels, [144] and babies with significant perinatal thrombosis can have levels suggestive of the homozygous deficiency state. [145] Acquired protein C deficiency (Table 1206) occurs in liver disease, severe infection and septic shock, DIC, adult respiratory distress syndrome, the postoperative state, breast cancer patients receiving cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) and in association with L-asparaginase therapy. A particularly severe form of acquired protein C deficiency has been reported in association with purpura fulminans and DIC in patients with acute viral or bacterial infections. [146] [147] [148] In contrast to AT III, the antigenic concentrations of vitamin K-dependent plasma proteins, including

protein C, are often elevated in patients with the nephrotic syndrome. Most uremic patients have low levels of protein C anticoagulant activity, but normal levels of protein C amidolytic activity and antigen. [149] This is attributable to a dialyzable moiety in uremic plasma that interferes with most clotting assays for protein C activity.[149]

Warfarin therapy reduces functional [118] [135] [136] and, to a lesser extent, immunologic measurements of protein C, [95] [96] making it difficult to diagnose individuals with heterozygous protein C deficiency in this setting. Several research laboratories have used a reduced ratio of protein C antigen to prothrombin or factor X antigen to identify patients with a type I deficiency state. [95] [96] This approach, however, can only be used in subjects in a stable phase of oral anticoagulation, and the diagnostic criteria for the disorder vary with the intensity of warfarin therapy. [96] Other groups have successfully used protein C activity assays in conjunction with functional measurements of factor VII, a vitamin K-dependent zymogen with a similar plasma half-life. [150] [151] In practice, it is preferable to investigate patients suspected of having the deficiency state after oral anticoagulation has been discontinued for at least a 1-week period and to perform family studies. If it is not possible to discontinue warfarin due to the severity of the thrombotic diathesis, such individuals can be studied while receiving heparin therapy, which does not alter plasma protein C levels.

An acquired inhibitor of protein C has been documented in an Australian patient. [152] This individual had a bleeding diathesis for several years and developed purpura fulminans before his death. An autopsy showed arterial and venous thrombi in many organs. Laboratory evaluation demonstrated the presence of chronic DIC. The IgG fraction of the subjects plasma completely inhibited the functional anticoagulant activity of APC.

The acute management of thromboembolic events in heterozygous protein C-deficient patients is similar to that of subjects without this disorder. It is advisable to keep the subject fully anticoagulated with heparin during the initiation of oral anticoagulation and large loading doses of the warfarin should be avoided. Oral anticoagulants are effective in managing individuals with protein C deficiency, and the recommendations for its use in such patients who have either sustained recurrent venous thrombosis or are asymptomatic are similar to those in patients with other inherited thrombotic disorders.

Stanozolol and danazol raise protein C levels substantially in heterozygous patients with type I protein C deficiency. [153] [154] However, these drugs do not prevent thrombosis in patients with this disorder, [154] and an increase in fragment F_{1+2} levels, a measure of the in vivo activity of factor Xa on prothrombin, has been observed in conjunction with the rise in protein C levels in two protein C-deficient patients who received stanozolol for 4 weeks. [153]

Protein S Deficiency

In 1984, members from several kindreds who exhibited reduced levels of protein S were described who had a striking history of recurrent venous thrombotic disease. [155] [156] Subsequently, many additional families with this disorder have been reported. [157]

Heterozygous protein S deficiency is inherited in an autosomal dominant fashion; a more severe form of protein S deficiency is an autosomal recessive disorder. The clinical presentation of patients with heterozygous protein S deficiency is similar to that outlined for AT III or protein C deficiency. Among 71 protein S-deficient members from 12 Dutch pedigrees, [158] 74%, 72%, and 38% of the individuals sustained deep venous thrombosis, superficial thrombophlebitis, or pulmonary emboli, respectively. The mean age of the first thrombotic event was 28 years with a range between 15 and 68; 56% of the episodes were apparently spontaneous and the remainder were precipitated by an identifiable factor. Thrombosis has also been reported in the axillary, mesenteric, and cerebral veins. Warfarin-induced skin necrosis has been described in a patient with heterozygous protein S deficiency. [29]

Several case reports have described young patients with arterial thrombosis and hereditary protein S deficiency. [159] [160] [161] In a cohort of 37 consecutive young adults presenting with arterial occlusive disease, 3 had hereditary protein S deficiency. [162] However, the occurrence of arterial thromboembolic events was not increased in protein S-deficient relatives of these people as compared to their biochemically unaffected family members.

Under normal conditions, 60% of the total protein S antigen in plasma is complexed to C4b-binding protein. Only the free 40% is functionally active as a cofactor in mediating the anticoagulant effects of activated protein C. [163] This observation has led to the development of methods for measuring total [156] [164] and free protein S antigen. [165] The most reliable measurements of total protein S antigen are by radioimmunoassay [164] and enzyme-linked immunosorbent assay techniques, which involve dilution of plasma samples and thereby favor dissociation of the protein S C4b-binding protein complexes. After removing protein S C4b-binding protein complexes from plasma by polyethylene glycol precipitation, [165] free protein S may be quantified by immunoassay of the supernatant fractions. It is also now possible to measure free protein S specifically using a monoclonal antibody-based immunoenzymatic assay that uses antibodies specific for the free form. [166] Functional assay methods are based

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on the ability of protein S to serve as a cofactor for the anticoagulant effect of APC. [167] [168] Some of these assays, however, are not specific for protein S and are sensitive to the defect characterized by resistance to activated protein C, and their use can lead to an erroneous diagnosis of functional protein S deficiency. [33]

The classic type of protein S deficiency is associated with 50% of the normal total S antigen level, [156] [157] and more marked reductions in free protein S antigen and protein S functional activity [165] (Table 12011). The molecular genetic analysis of mutations in patients with protein S deficiency was initially complicated by the presence of a protein S pseudogene, [169] [170] but mutations can now be identified in 70% of thrombophilic families with protein S deficiency using mutation screening strategies. [171] Molecular analysis has identified only three cases of large deletions of the protein S gene. [172] [173] [174] Among 69 candidate causal mutations identified in patients with the classic type of protein S deficiency, 51% are missense mutations, 25% are microinsertions or deletions of base pairs, 14% are premature stop codons, and 9% affect a splice site. [171] Mutations in the protein S promoter have yet to be identified.

Another type of hereditary deficiency state has been described in which total protein S antigen measurements are in the normal range, but levels of free protein S and protein S functional activity are disproportionately reduced to <40% of normal (Table 12011). Although the mutations in the classic type of protein S deficiency are distributed throughout the coding sequence, it has only been possible to identify protein S gene mutations in 44% of patients with this phenotype raising the possibility that some cases represent acquired abnormalities. [171] Among French patients with the low free protein S phenotype in which mutations could be identified, 82% had a single mutation, Ser 460 to Pro, in the sex hormone-binding globulin domain of protein S. [175] The low free plasma protein S may result from increased binding of this abnormal protein S molecule to C4b-binding protein. [165] [176] It is not, however, clear that the Ser 460 to Pro mutation (also termed protein S Heerlen) is associated with an increase in thrombotic risk because it has been identified with similar frequency in Dutch patients with thrombosis (0.7%) and the general population (0.5%). [177] The situation is further complicated by a report of 14 Swedish thrombophilic families in whom laboratory evaluation demonstrated the coexistence of the classic deficiency state along with the low free protein S phenotype; this data led the authors to propose that the two types of protein S deficiency are phenotypic variants of the same genotype. [178]

A third type of protein S deficiency is characterized by normal total and free protein S levels, but diminished protein S functional activity (Table 12011). This phenotype has been identified infrequently, which suggests that current functional assays may not screen for all such defects. All five mutations found in such patients are located in the amino terminal end of the protein S molecule, which includes the domains that interact with APC. Two of the mutations are located in the propeptide at positions 2 (Arg to Leu) and 1 (Arg to His). [179] A substitution of Lys 9 by Glu has been identified, which may alter the conformation of protein S or interfere with carboxylation of the molecules -carboxyglutamic acid domain. [180] The other two mutations

TABLE 120-11 -- Assay Measurements in Heterozygous Protein S Deficiency

	Protein S Antigen		Protein S Activity
	Total	**Free**	
Classic	Low	Low	Low
	Normal	Low	Low
	Normal	Normal	Low

result in substitution of Thr 103 by Asn and Lys 155 by Glu in the first and second epidermal growth factor domains of protein S, respectively. [179] [181] [182]

Patients with recurrent venous thromboembolic disease in association with doubly heterozygous or homozygous protein S deficiency have been identified. [163] The parents of these patients were asymptomatic and had laboratory studies consistent with the heterozygous protein S-deficient state. The syndrome of neonatal purpura fulminans has also been described in association with homozygous protein S deficiency. [31] [32]

The average concentration of total protein S antigen in normal adults is 23 g/ml. [183] Levels increase with advancing age and are significantly lower and more variable in women than men.[184] [185] These factors have confounded the reliable estimation of the prevalence of heterozygous protein S deficiency in the normal population. Thus, it is difficult to make the diagnosis of heterozygous protein S deficiency by performing only a single assay. Repeat sampling as well as family studies are therefore usually required to establish the diagnosis firmly.

Acquired protein S deficiency (Table 1206) occurs during pregnancy[186] and in association with the use of oral contraceptives. [184] [187] Reduced protein S levels have also been noted in patients with DIC[188] [189] and acute thromboembolic disease.[189] C4b-binding protein is an acute-phase protein and the decline in protein S activity in the latter two conditions as well as in other inflammatory disorders is attributable to a shift of the protein to the complexed, inactive form. [189] The levels of total and especially free protein S are significantly reduced in men with human immunodeficiency virus infection. [190] Total protein S antigen measurements are generally increased in patients with the nephrotic syndrome, [191] [192] although functional assays give reduced values. This is, in part, due to the loss of free protein S in the urine and elevations in C4b-binding protein levels. Total and free protein S antigen concentration are moderately decreased in liver disease [164] [189] and in association with L-asparaginase chemotherapy. [193] An acquired severe deficiency of protein S has also been reported in association with cutaneous necrosis. [194] In a patient with thromboembolic disease recovering from chickenpox, a transient isolated deficiency of protein S has been reported due to the presence of an autoantibody. [195]

Interpretation of protein S measurements in individuals taking oral anticoagulants is complicated inasmuch as the antigenic and functional levels of the protein drop substantially. A few groups have used reductions in the ratio of protein S antigen to prothrombin antigen to infer a diagnosis of the classic type of protein S deficient state; this is accomplished by using a strategy similar to that described for protein C-deficient subjects. [156] [157]

Total protein S antigen values in healthy newborns at term are 1530% of normal, whereas C4b-binding protein is markedly reduced to <20%. Thus, the free form of the protein predominates in this setting and functional levels are only slightly reduced as compared to those in normal adults. [196]

The recommendations for the treatment of protein S-deficient patients with anticoagulants and thrombolytic agents are similar to those in individuals without this disorder. Heparin therapy is generally effective for the acute treatment of thrombotic episodes and standard warfarin schedules appear to be effective in preventing recurrent venous thromboembolism. Protein S concentrates have not yet been developed for clinical use. Anabolic steroids such as danazol or stanozolol have not been shown to have a role in the treatment of protein S-deficient patients.

APC Resistance

In 1993, Dahlbäck et al.[197] identified a novel mechanism for familial thrombophilia. They identified individuals with unexplained personal and familial histories of venous thromboembolism

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whose plasmas exhibited a poor response to APC in a PTT assay. Recognized mechanisms for APC resistance were excluded, such as functional protein S deficiency or inhibitors to APC. Other clinically affected relatives of the probands demonstrated APC resistance in the PTT test, suggesting that the abnormality was hereditary. Poor anticoagulant responses to APC were also demonstrable in the probands in factor IXa- and factor Xa-based assays. Based on these observations, Dahlbäck hypothesized that these patients were deficient in an unrecognized plasma cofactor that functions in concert with protein S to support the anticoagulant activity of APC.

The observations of Dahlbäck facilitated the development of a PTT-based assay to screen for APC resistance. Svensson and Dahlbäck [198] screened 104 consecutive Swedish patients referred for evaluation of venous thrombosis and found that the plasmas of 33% of the subjects showed APC resistance (i.e., defined as an anticoagulant response in the PTT-based assay below the fifth percentile of controls). Precipitating factors for thrombosis, such as pregnancy and the use of oral contraceptives, were identified in 60% of their patients. Family studies revealed that relatives with APC resistance had a significantly higher frequency of thrombosis than relatives without the defect. In a US referral population of patients under age 50 with unexplained venous thromboembolic disease, Griffin et al. [199] found that 50% had APC resistance. Other groups in Italy and Austria confirmed that APC resistance was a frequent laboratory abnormality in patients with unexplained venous thrombosis.[33] [200]

A major contribution to our understanding of the molecular basis and clinical relevance of APC resistance was made by Dutch investigators from Leiden. In 1987, they initiated the Leiden Thrombophilia Study, a large case control study to investigate risk factors for first episodes of venous thrombosis in the general population of the Netherlands.[201] This effort grew from an attempt to define the true risk of venous thrombosis associated with protein C deficiency. There was a seeming paradox in that referred patients from families with protein C deficiency had a high frequency of venous thrombosis, but the deficiency state is present in 1/200500 healthy blood donors. The criteria for accruing subjects into these studies were felt to be a major factor underlying this difference.

Following the initial observations of Dahlbäck, the group in Leiden screened 301 unselected consecutive patients for APC resistance who had been entered into the Leiden Thrombophilia Study following the completion of anticoagulant therapy. [4] Entry criteria were a first episode of deep venous thrombosis in outpatients under age 70 with the diagnosis having been confirmed by objective testing. Patients with malignancy were excluded. Each thrombosis patient was matched with a healthy age- and sex-matched control. APC resistance was found in 21% of thrombosis patients and 5% of controls. Patients with APC resistance were calculated to have a sevenfold increased risk of venous thrombosis as compared to controls (Table 12012). The lower frequency of resistance to APC in this study as compared to other studies of referral populations cited earlier is primarily attributable to different selection criteria for the thrombosis cohorts.

A defect in factor V involving the mutation of arginine 506 to glutamine 506 (Arg 506 Gln or factor V Leiden) is most often the cause of APC resistance. [202] This is the site at which APC initially cleaves factor Va, and this sequence alteration makes the mutant factor Va molecule resistant to inactivation by the enzyme. [202] The Arg 506 Gln substitution was found to be the cause of APC resistance in about 90% of Dutch patients with APC resistance in the PTT assay, [202] and the mutation was found in 24% of healthy Dutch controls. Most patients with APC resistance are heterozygous for the factor V Leiden mutation, but a number of homozygous patients with heightened APC resistance in PTT assays have been identified. [202] [203] [204] Homozygotes are at higher thrombotic risk than heterozygotes (Table 12012)[204] [205]

TABLE 120-12 -- The Leiden Thrombophilia Study Data: Relative and Absolute Risks of an Initial Episode of Deep Venous Thrombosis in the General Population Due to Common Risk Factors

	Risk	Incidence/100 Person Years	Reference
Normal		0.008	
Hyperhomocysteinemia	2.5 ×	0.02	[7]
Prothrombin gene mutation	2.8 ×	0.022	[2]
Oral contraceptives	4 ×	0.03	[9]
Factor V Leiden heterozygotes	7 ×	0.057	[4]
Oral contraceptives + factor V Leiden	35 ×	0.285	[9]
Factor V Leiden homozygotes	80 ×	0.51	[205]

as are patients with heterozygous resistance to APC combined with mutations in the genes for protein C, AT III, and probably protein S. [16] [17] [18] [19] [20] The genes for factor V and AT III are both located on the long arm of chromosome 1, thereby allowing for coinheritance of the factor V Leiden mutation and an AT III mutation within all affected members of a family. This situation is expected to lead to an even more severe thrombotic diathesis. [20]

Mutations at the arginine 306 residue in factor V, the second APC site cleavage in the activated cofactor, have recently been reported. A 49-year-old male patient with proximal venous thrombosis and APC resistance in the absence of the factor V Leiden mutation was identified. [206] Sequencing demonstrated an Arg 306 Thr mutation in the patient and his mother, who had not suffered a thrombotic event. Mutations at the Arg 306 residue appear to be quite rare because this was the only mutation at this site among a selected cohort of 17 selected patients from an initial cohort of 602 individuals with venous thromboembolism. Two Chinese patients with prior histories of venous thrombosis have been identified with an Arg 306 Gly mutation in factor V. [207] However, this abnormality was not associated with APC resistance and was identified in 1 of 40 controls, thereby lending uncertainty as to its clinical importance. Thus far, no genetic abnormalities in the factor VIII gene have been identified to cause APC resistance in humans.

Although the factor V Leiden mutation is the dominant mechanism underlying APC resistance, data exist to support the original hypothesis of Dahlbäck that a second plasma cofactor supports the anticoagulant activity of APC. [197] [208] Factor V itself, but not purified factor Va, is able to enhance the inactivation of factor VIIIa by APC. [209] [210] Factor V Leiden is significantly less effective than normal factor V in mediating this effect. [211]

The US Physicians Health Study has also provided valuable data regarding factor V Leiden as a risk factor for venous thrombosis. In a retrospective case control study of 14,916 healthy men over age 40 with a mean follow-up of 8.6 years, heterozygosity for the factor V Leiden mutation was identified in 12% of subjects (14 of 121) with a first episode of deep venous thrombosis or pulmonary embolism and 6% of controls. [15] The relative risk of venous thromboembolism was increased 3.5-fold in those individuals with no other concomitant risk factors, but surprisingly was reduced to 1.7-fold in those with pre-existent cancer or recent surgery. This study also showed that elderly patients with venous thrombosis frequently have the mutation. [5] [15] Among men over age 60 with initial episodes of venous thrombosis and no identifiable triggering factors, 26% (8 of 31) were heterozygotes for factor V Leiden. [15]

Factor V Leiden-associated thrombotic risk appears to be quite dependent on the underlying clinical setting. Although oral contraceptives and pregnancy significantly increase the risk for venous thrombosis in women with the mutation, the

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Physicians Health Study showed a 1.7-fold increased risk for an initial thrombotic event in men with pre-existent cancer or recent surgery, which was not statistically significant. [15] A retrospective study of 825 patients undergoing hip or knee replacement surgery, which is associated with a 1525% incidence of venous thrombosis despite antithrombotic prophylaxis, was unable to demonstrate an increased risk of venographically documented events postoperatively among patients with the factor V Leiden mutation. [212] Several groups have also noted that the prevalence of the mutation is lower among patients with isolated pulmonary embolism than venous thrombosis of the legs. This seeming paradox may be explained by the fact that patients with pulmonary emboli more frequently have pre-existing medical illness or have undergone recent surgery where the factor V Leiden mutation does not add much to the underlying thrombotic risk.

There has been considerable interest in determining whether a prevalent prothrombotic risk factor such as factor V Leiden leads to an increased risk for arterial events. There are no convincing data that other thrombophilic states such as deficiencies of AT III, protein C, and protein S confer an increased risk of arterial thrombosis, but evaluation of this association is complicated by the relative infrequency of these defects. In a cohort of men over age 40 in which there was a low prevalence of smoking, the Physicians Health Study did not find an association between the factor V Leiden mutation and myocardial infarction or stroke. In a younger cohort of Italian patients with myocardial infarction before age 45, an increased incidence of the factor V Leiden mutation also was not found vis à vis controls. [213] Furthermore, a report of 36 French homozygous patients with the mutation did not identify a tendency for the development of arterial thrombosis. [214]

However, recent data from a case control study suggest that heterozygosity for factor V Leiden is a risk factor for a myocardial infarction in young women (1844 years old) but only in the presence of other cardiac risk factors. [215] The presence of the factor V Leiden mutation was associated with a 2.4-fold increased risk of myocardial infarction after adjustment for age (8 of 79 or 9.5% of patients with myocardial infarction as compared to 4.1% in controls). The risk of myocardial infarction associated with factor V Leiden, however, was observed only among current cigarette smokers in whom the mutation conferred a threefold increased risk. In comparison to women who did not smoke and did not carry factor V Leiden, women who smoked and carried the mutation had a 30-fold increased risk of myocardial infarction. Interestingly, while other cardiac risk factors such as older age, obesity, hypercholesterolemia, hypertension, diabetes, family history of ischemic heart disease, and postmenopausal status (surgically induced) were associated with cardiac events, the use of low-dose oral contraceptives was not. Data from this cohort indicate that the prothrombin gene mutation is also a risk factor for myocardial infarction, but again only in current cigarette smokers. [216]

The prevalence of heterozygosity for the factor V Leiden mutation in Caucasians, including European, Jewish, Israeli Arab, and Indian populations, ranges from 1% to 8.5%. The mutation is apparently not present in African blacks, Chinese, Japanese, or Native American populations. [27] Using dimorphic sites in the factor V gene to do haplotype analysis, Zivelin et al. [28] provided data for the existence of a founder effect as the basis for the mutation in Caucasians of differing ethnic background. It was estimated that the mutation originated approximately 30,000 years ago, which came after the evolutionary divergence of Caucasian, African, and Oriental populations. [28]

There have been reports of patients in whom there is cosegregation of heterozygous APC resistance due to the factor V Leiden mutation and type I factor V deficiency. [217] [218] [219] [220] The plasma of these individuals manifests severe APC resistance in PTT assays similar to homozygous factor V Leiden patients (i.e., they are pseudohomozygous). These patients were seemingly more prone to thrombosis than heterozygous relatives with factor V Leiden alone, suggesting that their clinical phenotype is similar to homozygous factor V Leiden patients.

Several polymorphisms are present in the factor V gene. [221] [222] Two amino acid substitutions, Leu 1257 IIe and His 1299 Arg, and a codon dimorphism for Ser 1240 are present in exon 13, which codes for the proteins B domain and is removed on activation. [221] In an Italian population, the frequency of the allele encoding Arg 1299 (R2) was 30% in patients with partial factor V deficiency and 7.5% in control subjects. [221] An extended factor V gene haplotype (HR2) containing the R2 polymorphism, distinct from that encoding Arg 506 Gln, contributes to a mild APC resistant phenotype; [222] it also contains nucleotide transversions resulting in the amino acid substitutions, Arg 830 Lys and Arg 837 His. Despite its association with mild APC resistance and its increased frequency in factor V Leiden-positive patients with the lowest APC resistance ratios, the HR2 haplotype has not yet been shown to confer an increased risk of venous thrombosis.

The initial observations of Dahlbäck [197] facilitated the development of a PTT-based assay that serves as a screening test for APC resistance. The PTT is performed in the presence or absence of a standardized amount of APC, and the two clotting times are converted to an APC ratio. Results can be interpreted by comparing the ratio to the normal range, or by normalizing it to the APC resistance ratio obtained using normal pooled plasma. Although this first-generation APC resistance assay was conceptually simple and easy to perform in a coagulation laboratory, it required careful standardization and determination of the normal range in at least 50 controls. The level of APC, the PTT reagent, and the instrumentation used for clot detection affected the performance characteristics of the assay. Some assays using this format, therefore, had inadequate sensitivity and specificity for the factor V Leiden mutation. Also, patients receiving anticoagulants or with an abnormal PTT due to other coagulation defects could not be investigated with this assay, and the test was not validated in patients with acute thrombotic events or pregnant women.

The discovery that factor V Leiden apparently underlies nearly all cases of APC resistance facilitated second-generation coagulation tests that with proper standardization can give nearly 100% sensitivity and 100% specificity for the mutation. This was achieved by diluting patient plasma in a sufficient volume of factor V-deficient plasma and then performing either a PTT-based assay or a tissue factor-dependent factor V assay. [223] This modification also permits the evaluation of plasmas of patients receiving anticoagulants or with abnormal PTT results due to coagulation factor deficiencies other than factor V.

The fact that the dominant mutation underlying APC resistance is factor V Leiden also makes it attractive to diagnose this defect by analyzing genomic DNA in peripheral blood mononuclear cells. This can be readily accomplished by amplifying a DNA fragment containing the factor V mutation site by polymerase chain reaction (PCR) and analyzing the cleavage products on ethidium bromide-stained agarose gels after restriction enzyme digestion with MnlI. [202] The substitution of an A for a G at nucleotide 1691 in the factor V cDNA (CGA to CAA) results in the Arg 506 Gln mutation and the loss of a MnlI cleavage site. Other diagnostic approaches include hybridization with allele-specific oligonucleotide probes.

At present, the most cost-effective approach to diagnosing patients with factor V Leiden is to test for APC resistance using a second-generation coagulation assay. Patients with low APC resistance ratios should then be genotyped for the mutation although it can be argued that such confirmatory testing is unnecessary in labs that have perfect concordance between the results of their APC resistance assays and factor V Leiden genotype.

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Leiden mutation can be identified. The clinical implications of this type of APC resistance are uncertain. The use of such assays to identify such individuals is therefore best restricted to thrombosis research centers. Two groups, however, have reported individuals with cerebrovascular disease in association with APC resistance that is not due to the factor V Leiden mutation.[224] [225] In one of the studies, the investigators divided patients into five categories of responsiveness to APC as opposed to the usual practice of using a cut-off value for optimal separation of carriers and noncarriers of the mutation. [225] Statistical analysis showed that a low response to APC was associated with an increased risk of cerebrovascular disease independent of the factor V Leiden mutation.

The acute management of thrombosis in patients with APC resistance is no different than other patients. Due to the prevalence of this disorder, data have emerged regarding the recurrence risk following an initial venous thrombotic episode in association with the factor V Leiden mutation. This information, along with its implications with respect to long-term anticoagulation in such individuals, is presented in the following sections.

Prothrombin Gene Mutation

In 1996, investigators from Leiden reported that a G to A substitution at nucleotide 20210 in the 3-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increased risk of venous thrombosis.[2] This mutation, which was discovered by directly sequencing the prothrombin gene of selected patients with venous thrombosis, is located at the last position of the 3-untranslated region at or near the cleavage site for polyadenylation of prothrombin mRNA. There is not yet experimental evidence whether this mutation results in increased prothrombin biosynthesis by the liver or whether it is linked to another sequence variation in the prothrombin gene that was not detected by sequence analysis. In any case, it is likely that the increased thrombotic risk is attributable to the elevated plasma prothrombin concentration.

Investigation of a referral population with a personal and family history of venous thrombosis demonstrated that 18% had the mutation in the 3-untranslated region of the prothrombin gene while it was present in only 1 of 100 healthy controls.[2] Among these thrombosis patients, 40% also carried the factor V Leiden mutation, again emphasizing the current view of venous thrombosis as a multigene disorder.

In the Leiden Thrombophilia Study, 6.2% of venous thrombosis patients and 2.3% of healthy matched controls had the prothrombin gene mutation.[2] This mutation independently confers a 2.8-fold increased risk of venous thrombosis (Table 12012) and the effect is operative in both sexes and all age groups. Among heterozygotes with the prothrombin gene mutation, 87% of thrombosis patients and controls in the study had prothrombin activity levels that were >1.15 U/ml, whereas only 23% of those with a normal prothrombin genotype were elevated to this degree. Additional studies have confirmed that the prothrombin 20210 mutation is a common risk factor for venous thrombosis in unselected outpatients as well as patients with other inherited thrombotic disorders.[226] [227] [228] [229] The allele frequency of 1.2% for the prothrombin gene mutation in the Dutch population is approximately half that of factor V Leiden, making it the second most common genetic risk factor for venous thrombosis.

Dysfibrinogenemias

Qualitative abnormalities of fibrinogen are usually inherited in an autosomal dominant manner. The dysfibrinogenemias are a heterogeneous group of disorders that may present with either no clinical symptoms, a bleeding diathesis, or a history of recurrent venous or arterial thromboembolism.[230] Fewer than 20 cases of variant fibrinogens have been reported to be associated with thrombotic complications. These defects can be detected with thrombin and reptilase times, which are often prolonged. In one instance, the thrombin time has been substantially shortened.[231] [232] Functional fibrinogen measurements are usually substantially lower than antigenic measurements in the plasmas of these individuals. An occasional individual with a dysfibrinogemia may have a prolonged prothrombin time or PTT, and the inability of some abnormal fibrinogens to clot completely in vitro can result in false-positive results in fibrin(ogen) degradation product tests.

The functional and biochemical defects of a number of abnormal fibrinogens associated with thromboembolic disease have been characterized.[230] The conversion of fibrinogen to fibrin by thrombin results in the proteolytic cleavage of fibrinopeptides A and B from the molecule. Defects in the release of these two peptides [233] [234] [235] [236] [237] or abnormalities in fibrin polymerization [238] [239] [240] have been reported. Such functional defects do not, however, offer a ready explanation for the thrombotic diathesis seen in these subjects. Abnormalities in the binding of thrombin to fibrin have also been found in some dysfibrinogenemias. [241] [242] [243] In one of these kindreds, three homozygous siblings with a B chain substitution of Ala by Thr at position 68 had a severe clinical phenotype sustaining both arterial and venous thrombosis at a young age.[243] [244] It has been suggested that decreased binding of thrombin by this mutant fibrinogen may lead to the presence of excessive thrombin in the circulation and the occurrence of thrombosis.[244] Other fibrinogen mutants have been shown to cause abnormal fibrin polymerization.[238] [239] [240] [242] Some of the abnormal fibrinogens have been evaluated for their ability to resist or promote fibrinolysis on incorporation into a fibrin clot. The fibrin formed from fibrinogen Chapel Hill III has been demonstrated to be abnormally resistant to lysis by plasmin.[238] Plasminogen activation is decreased in the presence of the fibrin formed from fibrinogen Dusart, despite normal tissue-type plasminogen activator binding to the substrate.[245] [246] [247] [248] These abnormalities clearly have the potential for decreasing fibrinolytic activity in vivo, which results in a familial thrombotic diathesis in biochemically affected persons.

Inherited Abnormalities of Fibrinolysis

Although investigators have identified a few individuals with inherited abnormalities of the fibrinolytic mechanism and recurrent venous thromboembolism, the clinical association is considerably less striking than that in many kindreds with deficiencies of AT III, protein C, or protein S, or with APC resistance due to the factor V Leiden mutation and the prothrombin gene mutation. Dysplasminogenemia or hypoplasminogenemia has been reported in approximately 20 individuals with thromboembolic disease. The first case of an abnormal plasminogen was identified in Japan by Aoki et al.[249] The propositus had a history of recurrent thrombosis and family studies demonstrated that the biochemical abnormality followed an autosomal dominant inheritance pattern. Despite the hereditary nature of the defect, none of the other biochemically affected members of the kindred had thrombotic events. Other Japanese pedigrees without thrombosis have since been described with the same biochemical defect,[250] [251] and the gene frequency of this abnormality in Japan is 0.018.[252] Population studies in the United States have not uncovered any cases of this dysplasminogenemia. A study of two unrelated Japanese families with reduced functional and antigenic levels of plasminogen was unable to demonstrate a significant correlation between the deficiency state and thrombosis.[253] The non-Japanese cases of dysplasminogenemias and hypoplasminogenemias have also been remarkable for the lack of thrombotic episodes in biochemically affected family members other than the propositi.

A few reports have documented the existence of thrombophilic families with inherited abnormalities of fibrinolysis.[254] [255] [256]

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Individuals from these kindreds were initially observed to have reduced fibrinolytic potential after venous occlusion, [254] and subsequently they were noted to have high levels of PAI. Re-evaluation of two of these families [254] [255] [256] has recently demonstrated the presence of hereditary protein S deficiency and no association between PAI-1 activity and a history of thrombosis.[257] [258] These data provide further evidence that the association between defective fibrinolysis and familial thrombosis has not been established.[259] [260]

Immunochemical methods for the measurement of tissue-type plasminogen activator and functional assays for its inhibitors have been applied to the study of patients with documented venous thromboembolism. These studies have suggested that defective synthesis or release of tissue-type plasminogen activator, as well as increased levels of PAI-1 may be important pathogenetic factors in as many as one-third of these individuals.[261] [262] [263] [264] Reduced fibrinolytic activity due to increased plasma levels of a rapid inhibitor of tissue-type plasminogen activator has been found in young survivors of myocardial infarction. [265] [266] The measurements of this inhibitor correlated strongly with serum concentrations of triglycerides.

Factor XII Deficiency

Factor XII is the zymogen of a serine protease that initiates the contact activation reactions and intrinsic blood coagulation in vitro. The first patient with a deficiency of

factor XII, or Hageman factor, was reported by Ratnoff and Colopy in 1955. [267] Subjects with severe factor XII deficiency (factor XII activity <1% of normal) have markedly prolonged PTTs, but do not exhibit a bleeding diathesis. [268] The absence of a hemostatic defect in patients with a severe deficiency of factor XII, prekallikrein, or high-molecular-weight kininogen indicates that these factors are not required for in vivo hemostasis. However, there have been a number of cases of venous thromboembolism or myocardial infarction in factor XII-deficient patients, [269] including the initial patient described with the abnormality. [270] This thrombophilic tendency has been attributed to reduced plasma fibrinolytic activity. [271] A literature review of data on 121 patients with factor XII deficiency found an 8% incidence of thromboembolism, including several myocardial infarctions in relatively young individuals. [269] Interpretation of such data is difficult because such complications are likely to be reported in the literature as compared to the asymptomatic patient with factor XII deficiency. This has led some groups to perform cross-sectional analyses of thromboembolic events in larger numbers of unselected families with factor XII deficiency. [272] [273] In Swiss families with factor XII deficiency, 2 of 18 homozygous or doubly heterozygous patients had sustained deep venous thrombosis, although each occurred at a time that other predisposing thrombotic risk factors were present. [272] Among heterozygotes with factor XII deficiency, only 1 of 45 heterozygotes had a possible history of venous thrombosis. These investigators concluded that heterozygous factor XII deficiency does not constitute a major thrombotic risk factor, whereas a severe deficiency may predispose some affected persons to venous thrombosis. Other groups have found a 1020% incidence of thrombotic episodes in heterozygotes. [273] [274] Thus, it remains uncertain as to whether an increased thrombotic risk is associated with factor XII deficiency.

Thrombomodulin Mutations

The thrombomodulin gene is a potential candidate for prothrombotic mutations. A patient with a single episode of pulmonary embolism and his asymptomatic son were found to have a point mutation in the thrombomodulin gene resulting in Asp 468 Tyr. [275] Other mutations identified in the thrombomodulin gene of individual thrombophilic patients predict for Pro 483 Leu, Gly 61 Ala, and Asp 468 Tyr amino acid substitutions. [276] Additional studies are required to evaluate whether these mutations alter the structure of thrombomodulin in such a way as to either reduce its cell surface expression or functional activity as well as to determine whether thrombomodulin mutations increase the risk of thrombosis.

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HYPERHOMOCYSTEINEMIA

Although premature atherosclerosis and arterial thrombosis were known to be associated with severe hyperhomocysteinemia almost 30 years ago, [277] it has only been in the last several years that mild or moderate hyperhomocysteinemia has been established as an independent risk factor for atherosclerosis as well as thrombosis.[278] [279]

Homocysteine is an amino acid that is formed from methionine (Fig. 1204). It is converted back to methionine by one of two remethylation pathways or undergoes transulfuration to cysteine. In remethylation catalyzed by the enzyme methionine synthase, homocysteine acquires a methyl group from 5-methyltetrahydrofolate and vitamin B_{12} (cobalamin) acts as a cofactor. In a secondary pathway, betaine is the methyl donor in a reaction catalyzed by betaine-homocysteine methyltransferase. In transulfuration, homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine--synthase followed by hydrolysis by the enzyme -cystathionase to cysteine and -ketobutyrate. Vitamin B_6 (pyridoxine) is a cofactor in both of these reactions.

The normal plasma homocysteine concentration is 516 M. As a result of impaired intracellular metabolism of homocysteine, increased amounts of the amino acid accumulate in the blood, leading to hyperhomocysteinemia. Homocystinuria refers to a group of rare inborn errors of metabolism resulting in severe hyperhomocysteinemia (>100 M) and the excretion of high levels of homocysteine in the urine. The most frequent cause is homozygous cystathionine--synthase deficiency, which has a frequency in the general population of approximately 1/250,000. [280] Affected individuals exhibit premature atherosclerosis and venous thromboembolism along with mental retardation, ectopic lenses, and skeletal abnormalities. A small number of cases are due to homozygous defects encoding

Figure 120-4 Intracellular metabolism of homocysteine occurs through remethylation to methionine or transulfuration to cysteine. Numbered circles indicate the enzymes involved: 1, methionine synthase; 2, methylenetetrahydrofolate reductase (MTHFR); 3, betaine-homocysteine methyltransferase; 4, cystathionine beta-synthase. *(From De Stefano et al.,[276] with permission.)*

methylenetetrahydrofolate reductase (MTHFR), and these individuals are similarly afflicted with premature vascular disease and thrombosis along with neurological problems.

Mild (1624 M) or moderate (25100 M) hyperhomocysteinemia results from genetic and acquired abnormalities. Although heterozygous cystathionine--synthase deficiency is found in only 0.3% of the general population, a MTHFR variant with an alanine to valine substitution at amino acid 677 is more common [281] and can be present in 1.415% of the population depending on their origin. [279] This mutation causes thermolability of MTHFR and a 50% reduction in its specific activity. The most common causes of acquired hyperhomocysteinemia are deficiencies of vitamin B_{12} , folate, or vitamin B_6 , which are cofactors in homocysteine metabolism. A strong inverse correlation exists between hyperhomocysteinemia and folate levels and to a lesser extent with vitamin B_{12} and B_6 concentrations.[6] Elderly patients frequently have elevated plasma homocysteine concentrations even in the absence of vitamin deficiencies as do patients with renal failure. Cigarette smoking is also associated with acquired hyperhomocysteinemia.

The mechanisms by which hyperhomocysteinemia acts as an atherogenic and thrombogenic risk factor have only been partially elucidated, but the major effect is on the vessel wall. In vitro, homocysteine is able to promote vascular smooth muscle cell proliferation [282] and inhibit endothelial cell growth. [283] In primates, it was shown that the infusion of homocysteine causes endothelial cell desquamation, smooth muscle cell proliferation, and intimal thickening. [284] Recent data indicate that moderate hyperhomocysteinemia induced by dietary manipulation leads to vascular dysfunction with impaired vasomotor responses as well as decreased thrombomodulin activity in the aorta.[285] The thrombogenic effects of hyperhomocysteinemia that have been demonstrated in vitro include induction of tissue factor expression,[286] inhibition of heparan sulfate expression, [287] inhibition of nitric oxide and prostacyclin release, [288] [289] inhibition of tissue-type plasminogen activator binding,[290] and inhibition of thrombomodulin-dependent protein C activation. [291] [292] However, most of these studies were carried out at homocysteine concentrations that were considerably higher than are encountered in patients with severe hyperhomocysteinemia, thereby raising questions about their relevance to the prothrombotic diathesis associated with this defect.

Hyperhomocysteinemia is usually diagnosed by measuring plasma levels of homocysteine by high-pressure liquid chromatography. Although patients with homozygous homocystinuria due to cystathionine--synthase deficiency have levels >100 M, individuals with heterozygous defects in this gene or inadequate vitamin B_6 levels may have normal or only slightly elevated levels of fasting homocysteine. The discrimination of such patients from normal individuals can be improved by demonstrating an abnormal increase in plasma homocysteine 4 hours after an oral methionine load. Defects in the remethylation pathway due to MTHFR gene defects or inadequate folate or vitamin B_{12} levels tend to cause elevated homocysteine levels under fasting conditions. The prevalence of hyperhomocysteinemia is almost twice as high when based on homocysteine measurements performed after methionine loading as when based on fasting levels. It is also possible to test for the common Ala 677 Val mutation in MTHFR genetically, but this is not recommended in the initial evaluation of patients suspected of having hyperhomocysteinemia.

Mild or moderate hyperhomocysteinemia due to inherited enzyme defects or acquired vitamin deficiencies is an independent risk factor for myocardial infarction, stroke, and carotid arterial disease. [293] [294] [295] Case control studies have found that up to 40% of patients with atherosclerotic coronary or cerebrovascular disease have mild to moderate elevations in plasma homocysteine. Prospective data have shown that elevated plasma homocysteine concentrations predict for coronary events and especially mortality among patients with established coronary artery disease. [296] An association between fasting plasma homocysteine levels and arterial thrombotic events has also been shown prospectively in patients with systemic lupus erythematosus (SLE). [297]

A relationship between hyperhomocysteinemia and venous thrombosis is also now appreciated. Studies of patients with an initial episode of venous thrombosis prior to age 45 demonstrate that 1318% have moderate hyphomocysteinemia based on homocysteine measurements done fasting and following an oral methionine load.[298] [299] Many of these patients had a history of familial thrombosis in association with hyperhomocysteinemia. A Dutch study showed that 25% of patients with recurrent venous thrombosis had homocysteine levels above the 90th percentile of the control distribution, and the presence of this abnormality conferred a twofold increased risk of recurrence. [300]

Case control studies have provided strong evidence that hyperhomocysteinemia is also an independent risk factor for initial episodes of venous thrombosis in the general population. These studies did not perform homocysteine levels after methionine loading and in some instances did not even obtain homocysteine levels in the fasting state, thereby underestimating the disorders prevalence. In the Leiden Thrombophilia Study, an elevated homocysteine level exceeding the 95th percentile of the control group was found in 10% of patients and conferred a 2.5-fold increased risk of thrombosis (Table 12012). [7] However, only women over age 50 showed a statistically significant association, although a trend was present in both sexes and all age groups. In an older male population, the Physicians Health Study

demonstrated a weak association between total plasma homocysteine level and venous thromboembolism but this was confined to those with so-called idiopathic events.[8] The risk of thrombosis was increased 20-fold in patients with idiopathic thrombosis who were both hyperhomocysteinemic and had the factor V Leiden mutation. This markedly increased thrombotic risk is consistent with a report of individuals with hereditary homocystinuria in whom only those who carried factor V Leiden had sustained venous thrombosis.[301]

The fact that lower plasma levels of folate, and to a lesser extent, vitamin B_6 or vitamin B_{12}, are associated with hyperhomocysteinemia makes it attractive to prescribe vitamin supplements to slow atherogenesis as well as to reduce the risk of arterial and venous thrombosis.[6] Indeed appropriate vitamin supplements can normalize plasma homocysteine levels, but the clinical efficacy of this approach has not been demonstrated in controlled clinical trials. However, because vitamins are relatively inexpensive and have almost no side effects, it is at present reasonable to treat symptomatic hyperhomocysteinemic patients with appropriate doses of folate, vitamin B_6, or vitamin B_{12} to lower their levels of homocysteine.

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ACQUIRED HYPERCOAGULABLE STATES

See Table 1202 for a list of acquired conditions associated with hypercoagulable states.

Pregnancy and Oral Contraceptives

Pregnancy is associated with about a sixfold increased risk of venous thromboembolism.[302] Although the incidence of deep vein thrombosis and pulmonary embolism has been estimated to be as high as 1%, the true incidence is unknown due to difficulties in performing screening radiologic studies in pregnant women. Although relatively rare, pulmonary embolism causes 12 deaths per million pregnancies and is estimated to account for 12% of fatalities during pregnancy.[303]

The puerperium, defined as the 6-week period following delivery, is associated with a higher rate of thrombosis than pregnancy itself. Risk factors for thrombosis in pregnancy include

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increasing age, cesarean delivery, prolonged immobilization, obesity, and prior thromboembolism.[302] Coexistent thrombophilia represents a major risk factor, particularly during pregnancy.[304] In Sweden, 4659% of women with venous thromboembolism had APC resistance and 6575% of the thrombotic events occurred during pregnancy.[11] [13] Among a referral population of Italian women with APC resistance managed without anticoagulant prophylaxis, however, 28% sustained venous thrombosis and 62% of the events occurred postpartum.[12]

Thrombosis during pregnancy and the puerperium is attributable to pregnancy-induced alterations in hemostasis as well as venous stasis in the lower extremities caused by the gravid uterus. Trauma to the pelvic veins during vaginal delivery or tissue injury during cesarean section also contribute to the hypercoagulable state. Compression of the left iliac vein by the crossing right iliac artery is a local, mechanical factor that is believed to underlie the threefold higher incidence of deep vein thrombosis in the left leg as compared to the right.[305]

Marked changes in the coagulation mechanism occur during pregnancy.[306] Increased levels of markers of coagulation system activation can be detected in the systemic circulation by the end of the first trimester. The elevations in thrombin generation, however, likely arise from the uteroplacental circulation because the placenta is rich in tissue factor. The vasculature of the placenta is unique from other tissues in that it is lined by chorionic villi that are covered by trophoblast tissue, rather than endothelium, which is in direct contact with maternal blood. The levels of most coagulation proteins increase during pregnancy, with fibrinogen rising markedly from a normal level of 250400 mg/dl to 600 mg/dl in pregnancy. Among the natural anticoagulant proteins, there is a significant decline in the level of free and total protein S in the second trimester.[186] [307] At the same time, fibrinolytic system activity declines progressively during pregnancy, in large part due to the generation of plasminogen activator-2 by the placenta.[308] Platelet activation and increased platelet turnover also probably occur during pregnancy; mild thrombocytopenia, possibly due to increased consumption, occurs in 8.3% of healthy women at term.[309] The net effect of these hemostatic changes is to promote blood coagulation so as to provide adequate hemostasis at the time of placental separation. The altered levels of hemostatic proteins return to normal within 4 weeks after delivery; fibrinolytic activity rapidly returns to normal levels within hours after placental separation.[310]

Oral contraceptive use is associated with an increased risk of venous and arterial thrombosis. The risk of venous thrombosis is related to the estrogen dose[311] and most oral contraceptives prescribed in the United States contain <50 g ethinylestradiol in combination with a progestational agent (combined oral contraceptives). The use of low-dose estrogen preparations containing older progestational agents (levonorgestrel, lynoestrenol, and norethisterone), however, still confers about a fourfold increased risk of venous thromboembolism as compared to nonusers (Table 12012).[312] The risk of myocardial infarction and stroke is less,[313] [314] [315] which is in part related to estrogens effect in lowering low-density lipoprotein cholesterol and raising high-density lipoprotein cholesterol levels. Unexpectedly, the introduction of newer progestagens (desogestrol, gestodene, and norgestimate) with more beneficial effects on lipid profiles carries a higher risk of venous thromboembolism than the previous generation of combined oral contraceptives.[316] [317] [318] [319] This risk is particularly high among carriers of the factor V Leiden mutation[9] [10] and women with a family history of thrombosis. It is not yet known whether oral contraceptives containing the newer progestagens are associated with any increase in risk of myocardial infarction, which is an extremely rare event in young women.[313]

The mechanisms by which oral contraceptives induce a prothrombotic state are incompletely understood. Oral contraceptive use is associated with changes in the levels of many coagulation proteins.[320] It has recently been suggested that oral contraceptives induce a state of acquired APC resistance.[321]

The Postoperative State and Trauma

Deep vein thrombosis and pulmonary embolism occur with increased frequency in postoperative patients; the thrombotic risk, however, depends on the type of surgery performed. Deep vein thrombosis in this population is usually asymptomatic, requiring the use of reliable noninvasive tests or lower limb venography to establish the diagnosis. Based on patient characteristics and the type of surgery, the postoperative thrombotic risk can be estimated and an assessment made of the need for prophylactic anticoagulation.[322] Risk factors associated with higher rates of thrombosis include older age, previous venous thromboembolism, the coexistence of malignancy or medical illness (e.g., cardiac disease), thrombophilia, and longer surgical and immobilization times. In patients over age 40, the incidence of deep vein thrombosis following general or gynecologic surgical procedures is 2025% and clinically significant pulmonary embolism occurs in 12%. For urologic surgery, the incidence of deep vein thrombosis ranges from 10% for transurethral procedures and up to 40% for radical prostatectomies. Orthopedic procedures on the hip and lower extremities are among the most thrombogenic surgical procedures. In the absence of prophylaxis, the risk of deep vein thrombosis following total knee replacement is 4570% and fatal pulmonary embolism has been reported to occur in 13% of patients undergoing hip surgery. It is now recognized that the increased thrombotic risk is not confined to the immediate postoperative period and continues for several weeks.[323]

Deep vein thrombosis and pulmonary embolism are also commonly encountered after major trauma. A study of patients admitted to a Canadian regional trauma unit demonstrated that 58% of patients had lower extremity deep vein thrombosis, which was usually asymptomatic.[324] Risk factors for thrombosis in this setting were older age, the need for surgery or blood transfusions, and the presence of lower extremity fractures or spinal cord injury. Pulmonary embolism occurs in 222% of trauma patients and is the third most common cause of death in those who survive the first 24 hours.[325]

The mechanism of activation of the coagulation system following surgery or trauma has not been elucidated, but the exposure of tissue factor from injured tissue or activated monocytes is the most likely pathway for increased thrombin generation in these settings. In postoperative patients, the occurrence of thrombosis is determined by the balance between coagulation and fibrinolytic system function.[326] In a study of neurosurgical patients undergoing craniotomy, Owen et al.[327] found that individuals developing venous thrombosis exhibit increased levels of fibrinopeptide A, a marker of thrombin-mediated fibrinogen proteolysis, relative to those of B 142, a marker of plasmin action on noncross-linked fibrin, for several days prior to thrombus visualization as compared to controls. Minor alterations in a number of

hemostatic parameters have been reported in postoperative patients including elevated levels of fibrinogen and von Willebrand factor and decreased levels of AT III and protein C. Decreased venous blood flow in the lower extremities also contributes to postoperative hypercoagulability. [328] In hip replacement surgery, the femoral vein in the operated leg may kink, thereby stimulating proximal venous thrombosis in the absence of pre-existent calf vein thrombosis. [329]

Obesity

Although obesity is an independent risk factor for atherosclerosis and cardiovascular disease, the evidence regarding its role in venous thrombosis is equivocal. An early study by Kakkar et al.[330] suggested that overweight patients had an increased incidence

of venous thrombosis. More recent studies have shown that this association disappears when account is taken of other risk factors such as age, history of venous thrombosis, and the type of surgery.[331] Obesity may however be a risk factor for chronic venous insufficiency. [332]

Malignancy

The incidence of clinical thromboembolic disease in the cancer population has been estimated to be as high as 11%. [333] Autopsy series have described even higher rates of thrombosis for certain tumor types. One autopsy study found a 30% incidence of thrombosis in patients who died of pancreatic cancer and >50% with tumors in the body or tail of the pancreas.[334] Other tumor types commonly associated thromboembolic complications are carcinomas of the gastrointestinal tract, ovary, prostate, and lung. By virtue of its prevalence, lung cancer accounts for the largest number of thromboembolic events. [335]

Patients with malignancy also have a higher rate of postoperative venous thrombotic complications than the general population, with an incidence of 40%. [336] Careful attention should therefore be paid to prophylaxis of deep vein thrombosis in such patients undergoing major abdominal or pelvic surgical procedures.

In some instances, thrombosis manifests itself prior to the diagnosis of an underlying malignancy and may predate the diagnosis by several years. In Trousseaus syndrome, which is classically characterized by migratory superficial phlebitis involving the upper or lower extremities (phlegmasia alba dolens), [336] it is not unusual for a premortem diagnostic evaluation for an underlying malignancy to be unsuccessful. For years it was debated whether the presence of a venous thrombotic event identifies patients at significantly increased risk of developing cancer. Several studies demonstrated an excess risk of cancer in patients who present with pulmonary embolism or deep vein thrombosis,[337] [338] but other investigators were unable to document an increased risk of developing cancer after an idiopathic thrombotic episode. These observations, however, were obtained from small or retrospective clinical trials.

A significant and clinically important association between idiopathic venous thrombosis and the subsequent development of clinical cancer was established in 1992. Prandoni et al.[40] investigated 250 consecutive patients with symptomatic deep vein thrombosis that was objectively documented by venogram. Cancer was identified at the time of diagnosis of the thrombotic event in 3.3% (5 of 153) of patients with idiopathic venous thrombosis, but no cases were found in patients with secondary venous thrombosis due to prolonged immobilization, leg trauma, or hereditary thrombotic disorder. During a 2-year follow-up period, cancer was diagnosed in 7.6% (11 of 145) of the patients with idiopathic venous thrombosis and in 1.9% (2 of 105) of patients with secondary venous thrombosis. The incidence of cancer was considerably higher in patients with recurrent idiopathic venous thrombosis. The probability of detecting an occult neoplasm in patients with a documented thrombotic event was also evaluated by Monreal et al., [39] [339] who found a malignant lesion in 7 of 31 (22.6%) patients with idiopathic deep vein thrombosis in contrast to 5 of 82 (6.1%) patients with secondary thrombosis and 9 of 78 (11.5%) patients with pulmonary embolism. The higher incidence of cancer reported by this group is most likely due to a more aggressive diagnostic approach, which included chest radiography, upper gastrointestinal endoscopy, abdominal ultrasound, and computed tomography scan.

In patients who present with idiopathic venous thromboembolism, recommendations have varied as to the extent of evaluation for malignancy that is appropriate. [340] Decisions regarding diagnostic testing should be based on the status of the individual patient; however, a complete physical examination including digital rectal examination and testing for fecal occult blood, pelvic examination in women, and routine laboratory testing should be performed in all patients presenting with thrombosis. Prospective studies are required to assess if an aggressive diagnostic approach will result in improved survival.

Nonbacterial thrombotic endocarditis, sterile vegetations composed of platelets and fibrin on heart valves, is highly associated with malignant disorders. In autopsy series, cancer was found in as many as 75% of cases of NBTE.[341] The majority of cases of NBTE are in patients with adenocarcinomas, and its incidence among lung cancer patients may run as high as 7%.[342] Laboratory evidence for DIC is often present. The aortic and mitral valves are most commonly affected. The diagnosis of NBTE can be difficult to make antemortem because <50% of patients have audible cardiac murmurs, and small lesions may not be identified by echocardiography. The major clinical manifestations are due to systemic emboli from the vegetations on the heart valve(s) rather than from valvular dysfunction itself. Common sites of embolization include the spleen, kidney, and extremities, with the most significant morbidity arising from emboli to the central nervous system and coronary arteries. NBTE is not an uncommon disorder and should be considered in all cancer patients who develop an acute stroke syndrome as well as in others who have cerebral embolism with an unknown etiology.[343]

Disseminated intravascular coagulation is the cardinal coagulopathy associated with malignancy, and results from generalized activation of the coagulation system (Chap. 117). Malignancy is the third most common cause of DIC after infection and trauma, and accounts for 7% of cases. DIC is a common complication in cancer patients and has been reported in as many as 15% of patients with malignancy. DIC occurs in virtually all patients with acute promyelocytic leukemia. The leukemic promyelocytes contain procoagulants that can trigger DIC, as well as fibrinolytic activators. The cell lysis that results from conventional chemotherapy releases these enzymes and can exacerbate the coagulopathy. Other disease entities, particularly sepsis, which may be responsible or may contribute to the development of DIC in cancer patients, should be ruled out.

Various patterns of DIC are seen in association with malignancy. Acute forms of DIC are rare and usually present with minor bleeding from mucosal or cutaneous surfaces or extensive life-threatening hemorrhage involving visceral sites. Laboratory evaluation demonstrates prolongation of the prothrombin time, PTT, thrombin time, and reptilase time; decreased fibrinogen concentration; thrombocytopenia; and elevated levels of fibrin degradation products. More common in cancer patients are chronic forms of DIC. Many such patients are asymptomatic, and laboratory data often show modest reductions in fibrinogen and platelet count, elevated fibrin degradation products, and minimal changes in the prothrombin time or PTT. Others may have more obvious evidence of platelet, fibrinogen, and coagulation factor consumption. These patients often are hypercoagulable and can manifest deep vein thrombosis, Trousseaus syndrome, or NBTE.

Thrombotic microangiopathy is the descriptive term of a syndrome characterized by hemolytic anemia, thrombocytopenia, pathognomonic microvascular thrombotic lesions, and the involvement of various specific organs (Chap. 128). Depending on the differences in organ involvement, thrombotic microangiopathy is commonly described as thrombotic thrombocytopenic purpura or as hemolytic uremic syndrome. Severe thrombotic microangiopathy in association with thrombocytopenia may occur in as many as 5.7% of patients with metastatic carcinomas, most frequently with gastric, breast, or lung primary sites. Despite the classical manifestations of a Coomb-negative hemolytic anemia and severe thrombocytopenia, patients usually present with neurologic abnormalities, including headache, confusion or paresis, and occasionally signs of DIC.

Renal failure is an uncommon feature of carcinoma-associated thrombotic microangiopathy. The mainstay in the treatment of malignancy-related thrombotic microangiopathy is to decrease the tumor burden because other treatment modalities, such as corticosteroids, plasma exchange, or plasma infusion, only result in a moderate and transient improvement of the clinical symptoms.

Likely contributing to the thrombotic tendency in cancer patients are clinical factors such as vascular stasis due to obstruction of blood flow by the tumor or patient immobility, hepatic involvement and dysfunction, sepsis, advanced age, other comorbid conditions, and certain antineoplastic agents. Thus, the exact details of the mechanisms underlying coagulation activation in malignancy remain uncertain and may be a complex interaction of several of the above mechanisms as well as others yet to be identified.

Nephrotic Syndrome

The nephrotic syndrome results from a variety of pathologic processes that cause excessive kidney glomerular leakage of plasma proteins into the urine. It is clinically characterized by edema, proteinuria, hypoalbuminemia, and hyperlipidemia. Thrombosis is a major cause of morbidity in patients with the nephrotic syndrome, with the renal veins being the most commonly affected site. Renal vein thrombosis occurs in approximately 35% of cases, [344] but it varies considerably based on the underlying renal pathology and the severity and duration of the proteinuria. The incidence of thrombosis in other sites is 20% [344] [345] in adults, deep vein thrombosis and pulmonary embolism are the most common thrombotic complications. Arterial thrombosis is seemingly more common in children with nephrotic syndrome, resulting in stroke, mesenteric infarction, or limb ischemia.

Alterations in many blood coagulation parameters have been reported in patients with the nephrotic syndrome, although some abnormalities are inconsistently reported, of relatively minor magnitude, and poorly correlated with thrombotic events. The variability in findings is attributable to the complex nature of the nephrotic syndrome itself and the resultant marked stimulation of hepatic protein biosynthesis.

Among the natural anticoagulant proteins, plasma AT III levels are often decreased due, at least in part, to urinary excretion. [86] [346] The antigenic levels of protein C and protein S are generally increased. [191] [192] [347] Plasma levels of several components of the coagulation cascade, factors XII, XI, and X, are decreased due to urinary excretion, whereas factors XIII, X, VIII, VII, and V are characteristically elevated in these patients. Data indicate that platelet hyperreactivity [348] or increased whole blood viscosity[349] may contribute to the thrombotic diathesis in the nephrotic syndrome.

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired clonal disorder of bone marrow stem cells. Patients generally manifest chronic intravascular hemolysis with episodes of gross hemoglobinuria accompanied by leukopenia and thrombocytopenia. Many have a prior history of aplastic anemia. Despite the presence of thrombocytopenia, thrombosis is more commonly encountered than bleeding. Indeed thrombosis can be a presenting feature of PNH as well as an important cause of morbidity and mortality in this disorder. A diagnosis of PNH should be suspected in patients with a negative family history of thrombosis with evidence of pancytopenia, an elevated reticulocyte count, or iron studies consistent with iron deficiency.

A unique feature of PNH is the predilection for thrombosis to occur in the intra-abdominal venous network (mesenteric, hepatic, portal, splenic, and renal veins) and cerebral vessels as opposed to deep vein thrombosis or pulmonary embolism. Arterial events such as myocardial infarction and stroke have rarely been reported. Acute thrombotic episodes should be treated with heparin or low-molecular-weight heparin. Fibrinolytic agents have been used successfully to treat acute intra-abdominal venous thrombosis. Long-term oral anticoagulation should be considered in patients with thrombosis in association with PNH.

The abnormality of PNH erythrocytes that leads to hemolysis is their increased sensitivity to complement-mediated lysis. Deficiencies of a number of membrane proteins have been observed in PNH, including acetylcholinesterase, leukocyte alkaline phosphatase, decay accelerating factor, CD59 (membrane inhibitor of reactive lysis) antigen, homologous restriction factor, CD58, 5-ectonucleotidase, CD16, urokinase-type plasminogen activator, and CD14 antigen. These proteins are all attached to the cells via a glycosylphosphatidylinositol anchor, and PNH appears to be the consequence of somatic mutations in the X-linked phosphatidylinositol glycan class A gene that participates in an early step in glycosylphosphatidylinositol anchor synthesis. [350] Several of the membrane abnormalities involve proteins that modulate complement function and the absence of CD59 antigen on erythrocytes appears to be the most critical defect. [351] Granulocytes and platelets, like red blood cells, show increased sensitivity to complement-mediated lysis. Although it has been suggested that the membrane abnormalities in PNH may lead to increased platelet activation by complement, there is not currently an adequate explanation for the thrombotic tendency in this disease. Indeed, there is no apparent association between hemolytic and thrombotic episodes nor does the onset of thrombosis correlate with the duration or degree of hemolysis.

Hyperviscosity

Thrombosis can be a manifestation of diseases associated with hyperviscosity. Hyperviscosity of the blood may be due to increased plasma viscosity, an increased number of red or white blood cells, or decreased deformability of cells.

Increased plasma viscosity can result from hypergammaglobulinemia or hyperfibrinogenemia. Hypergammaglobulinemia associated with the hyperviscosity syndrome is most commonly encountered in patients with Waldenströms macroglobulinemia or multiple myeloma. Presenting symptoms of the hyperviscosity syndrome include bleeding due to platelet dysfunction, visual disturbances, and neurologic defects. Thrombosis in hypergammaglobulinemic states is attributable to abnormal rheology.

Hyperviscosity plays an important role in the pathogenesis of thrombosis in polycythemia vera, which is a major complication of this disorder. Patients with elevated hematocrits have increased whole blood viscosity, and these have been inversely correlated with cerebral blood flow. Acquired qualitative platelet defects have also been implicated in the pathogenesis of the hemostatic defects in polycythemia vera. Common thrombotic complications in this disorder include cerebrovascular accidents, myocardial infarction, peripheral arterial occlusion, deep venous thrombosis, pulmonary embolism, and portal and hepatic vein thrombosis (Budd-Chiari syndrome).

In the myeloid and monocytic leukemias, the presence of very elevated white blood cell counts (generally >100,000mm [3]) can increase the viscosity in the microcirculation, which can play a role in the pathogenesis of thrombosis. Small vessels in the lungs, brain, and less commonly in other organs may be obstructed by high levels of immature leukocytes.

In sickle cell disease, the increase in blood viscosity secondary to sickled erythrocytes may contribute to the occlusion of small blood vessels. Enhanced adhesion of sickle erythrocytes

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to vascular endothelium along with increased coagulation and platelet activation promote vascular occlusion. [352]

Drug-Induced Thrombosis

The contribution of cancer chemotherapy to the development of thrombotic events is difficult to evaluate because the neoplasm itself and other risk factors predispose cancer patients to thromboembolic complications.

Thrombotic events have been reported with induction chemotherapy regimens for acute lymphoblastic leukemia (ALL) that include L-asparaginase. Intracranial thrombosis with hemorrhage is observed most frequently, but deep venous thrombosis and pulmonary embolism can also occur. [353] [354] [355] In one large series of children receiving L-asparaginase as part of induction chemotherapy for ALL, the incidence of thrombotic complications was 1.2%. Generalized bleeding episodes have only rarely been observed.

The inhibition of protein synthesis by L-asparaginase causes deficiencies of numerous plasma proteins, including albumin, thyroxine-binding globulin, and various coagulation proteins. Decreased levels of prothrombin; factors V, VII, VIII, IX, X, and XI; fibrinogen; AT III; protein C; protein S; and plasminogen have all been described secondary to L-asparaginase chemotherapy. [91] [356] This results in prolongation of the prothrombin time, PTT, and thrombin time, as well as hypofibrinogenemia with levels often <100 mg/dl. These coagulation abnormalities resolve within 12 weeks after cessation of the drug.

It is difficult to assess the role of the substantial reductions in the levels of natural anticoagulant proteins such as AT III, protein C, and protein S in the pathogenesis of the thrombotic events. Both procoagulant and anticoagulant protein sythesis in the liver are decreased by L-asparaginase, leading to uncertainty as to whether there is an alteration in the balance of the opposing forces of the hemostatic mechanism. In one study of children with ALL receiving L-asparaginase, prednisone, and vincristine, no correlation was found between protein C, protein S, or AT III levels and the presence or absence of thrombosis. [193] Other clinical factors may contribute to the pathogenesis of thrombotic complications that are observed in these patients including immobility or the concurrent presence of sepsis.

Women with breast cancer receiving certain chemotherapy regimens are at increased risk for developing thrombosis. The availability of large numbers of patients receiving standard adjuvant chemotherapy programs as well as appropriate matched, untreated control populations has facilitated studies of this association. A 1981 study found a 5% incidence of symptomatic venous thrombosis among patients with stage II breast cancer treated with chemotherapy regimens that included CMF. [357] All of the thrombotic events occurred while the patients were receiving chemotherapy. In the post-treatment follow-up period, no clinically evident thromboses were observed. Goodnough et al.[358] reported a 17.6% incidence of thrombosis in a series of patients with metastatic breast cancer receiving chemotherapy, the majority of the events being pulmonary embolism or deep vein thrombosis. Levine et al. [359] randomized 205 patients receiving adjuvant chemotherapy for stage II breast cancer to either a 12-or 36-week course of chemotherapy after primary surgical treatment. During the first 12 weeks of chemotherapy there were similar numbers of thrombotic events; however, during the following 24 weeks there were five additional thrombotic events in the 36-week treatment group, while no further thromboses were seen in the 12-week treatment group that had already completed therapy. Thus, 6.8% of the patients had a thromboembolic event while on chemotherapy, but no further thrombotic events were detected during the >2,400 patient-months of follow-up postchemotherapy. No relationships were found in this study between the development of thrombosis and estrogen or progesterone receptor status, age, number of involved lymph nodes, or subsequent tumor recurrence.

The association of arterial thrombosis and cancer therapy was shown in studies by Wall et al. [360] and Saphner et al. [361] The Cancer and Acute Leukemia Group B (CALGB) study[360] found a 1.3% incidence of arterial thrombosis, either peripheral arterial or cerebrovascular, in 1,014 patients during treatment for stage II or III breast cancer on two separate chemotherapy protocols. All except one of the thrombotic events occurred while patients were receiving chemotherapy. In the study by Saphner et al.[361] including 2,352 patients, a significant increase in arterial thrombosis was found in premenopausal women who received combined chemohormonal therapy, whereas no higher risk for arterial thrombosis was seen in postmenopausal women either receiving tamoxifen alone or in combination with chemotherapy. In both studies, arterial thrombotic events tended to occur early in the course of treatment.

The pathophysiologic basis for the thrombogenicity of chemotherapy in patients with breast cancer is not well understood. In a prospective study of patients receiving adjuvant chemotherapy for breast cancer no alterations in antithrombin III levels were detected. Rogers et al. [362] found a statistically significant decrease in protein C and protein S levels during CMF chemotherapy. Some of these patients had decrements in protein C and S levels that fell below the range of values seen in hereditary thrombotic disorders. Decreases in factor VII and fibrinogen levels were also reported. None of the patients in this small study, however, had clinically evident thrombosis. Other investigators have observed significant declines in protein C concentration during CMF chemotherapy, which returned to baseline values after the completion of therapy.[363] Possible explanations for these chemotherapy-induced abnormalities include impairment of vitamin K metabolism, inhibition of DNA/RNA synthesis leading to a decrease in protein synthesis by the liver, and initiation of intravascular coagulation.

Hormonal therapies, such as estrogen therapy for prostate carcinoma, have clearly been linked to an increased incidence of thromboembolic disease. In the Veterans Administration Cooperative Urologic Research Groups studies of estrogen therapy in patients with prostate cancer, higher death rates from cardiovascular events were reported in those taking 5 mg diethylstilbestrol daily as compared to those on a 1-mg daily dose. [364] The antitumor effect of these two dosages was similar.

Lupus Anticoagulants and the Antiphospholipid Syndrome

Lupus anticoagulants are antibodies that prolong phospholipid-dependent clotting assays in vitro (Chap. 116). These immunoglobulins do not directly inhibit the activity of specific plasma coagulation factors.

The term lupus anticoagulant is a misnomer because this laboratory abnormality frequently occurs in patients without SLE. It has been observed in other autoimmune diseases, following exposure to drugs or various infectious agents, in malignancy, and in some individuals without any apparent underlying disease. [365] [366] Furthermore, subjects with lupus anticoagulants do not suffer from a bleeding diathesis unless other hemostatic defects are present. Paradoxically, the presence of lupus anticoagulants increases the apparent risk of both arterial and venous thromboembolism, [367] [368] and there is conflicting evidence as to whether recurrent events occur in the same arterial or venous distribution as the initial episode. [369] [370] Compilation of published reports suggests that about one-third of patients with such inhibitors will have thrombotic events. [366]

In coagulation tests, lupus anticoagulants are generally detected by finding a prolonged PTT. Other coagulation assays that have been used to screen patients for the presence of lupus

anticoagulants are the dilute Russell viper venom time, [371] the kaolin clotting time,[372] and the tissue thromboplastin inhibition test. [373] It is currently popular to confirm abnormal screening tests using a hexagonal phospholipid assay. If a lupus anticoagulant is present, the antibody will be neutralized by the addition of hexagonal phase phospholipid and the clotting time will normalize.

The presence of lupus anticoagulants has also been associated with the presence of biologic false-positive tests for syphilis because the Venereal Disease Research Laboratory assay depends on the presence of cardiolipin. [374] Some of these antibodies react with anionic phospholipids, [375] [376] but the binding of most antiphospholipid antibodies to cardiolipin requires the presence of a plasma cofactor, $_2$ -glycoprotein I (apolipoprotein H). [377] [378] [379] The relationship between the presence of lupus anticoagulants, antiphospholipid antibodies, and a thrombotic predisposition are complex and there are conflicting data as to which test result is associated with increased thrombotic risk. A study by Triplett et al. [380] demonstrated that antiphospholipid antibodies are not present in all patients with lupus anticoagulants and the presence of such immunoglobulins does not necessarily confer an increased thrombotic risk on these individuals. However, in patients with SLE, persistently elevated levels of IgG anticardiolipin antibodies appear to correlate more strongly with thrombosis and fetal loss than do abnormalities in clotting assays for lupus anticoagulants. [381] [382] [383] The opposite was shown in another study with respect to venous or arterial thrombosis. [384] There are conflicting data with respect to the the risk of arterial and venous thrombosis in such patients. Additional evidence that the presence of IgG anticardiolipin antibodies constitutes a thrombotic risk factor comes from a case control study of healthy adult men participating in the US Physicians Health Study. [385] An antibody titer above the 95th percentile was a significant risk factor for venous thromboembolism but not for ischemic stroke. This study, however, did not test for the presence of lupus anticoagulants. A prospective Canadian study showed that the presence of a lupus anticoagulant conferred a 9.4-fold increased risk of venous thrombosis, but the anticardiolipin antibody titer was not a significant risk factor. [386]

The management of acute venous thromboembolism in patients with lupus anticoagulants is similar to that of other individuals without this laboratory abnormality. However, the initial treatment of such subjects is complicated by the fact that the PTT cannot reliably be used to monitor unfractionated heparin dosage unless proper in vitro calibration studies are done by adding known amounts of heparin into plasma samples and measuring the response of the PTT. Thus, it is preferable to monitor anticoagulant therapy in these individuals by performing an activated whole blood clotting time or plasma heparin measurements using either factor Xa or thrombin and a suitable chromogenic substrate of these enzymes. These problems are obviated by the use of low-molecular-weight heparin, which does not require monitoring. The presence of lupus anticoagulants can also interfere with heparin monitoring during cardiac surgery. [387] Warfarin is effective in preventing recurrent thrombosis, but requires that a relatively high INR be maintained. [369] [370]

The clinical heterogeneity of the patient populations that develop lupus anticoagulants makes it difficult to generalize regarding the long-term antithrombotic management of such individuals. Although the relationship between thrombosis and the lupus anticoagulant appears to be strong in patients with SLE, [382] the significance of the association in patients without this disorder is more ambiguous. [388] Indeed, patients who develop transient lupus anticoagulants in association with infections do not usually sustain thromboembolic episodes and it is unclear whether drug-associated lupus anticoagulants are associated with thrombosis. Thus, the presence of a persistent lupus anticoagulant, a high-titer antiphospholipid antibody, or both in an asymptomatic subject with no prior thrombotic history is not an indication for anticoagulant or antiplatelet medications. However, inasmuch as it is not currently possible to determine reliably the risk of thrombosis in an asymptomatic patient with these laboratory abnormalities, all such individuals should receive appropriate prophylaxis in conjunction with major surgical procedures, [389] or prolonged immobilization, unless there is a strong contraindication to such treatment. Corticosteroids can normalize clotting assay times or reduce antiphospholipid antibody titers in patients with lupus anticoagulants. However, these and other immunosuppressive medications may not prevent recurrent thrombosis.

The presence of lupus anticoagulants, anticardiolipin antibodies, or both has also been reported in women with obstetric complications such as habitual abortions, intrauterine death, and fetal growth retardation. [390] [391] [392] [393] [394] In some cases, pathologic examination of the placenta demonstrates infarction due to decidual vessel thrombosis.[397] In a cross-sectional study of unselected women with SLE, Ginsberg et al. [383] could establish a significant association between persistently positive tests for lupus anticoagulants and anticardiolipin antibodies and pregnancy loss. However, in women without SLE, a prospective study did not demonstrate a

relationship between the presence of a lupus anticoagulant and anticardiolipin antibodies and an initial episode of spontaneous abortion or fetal death. [395]

MANAGEMENT

Approach to Management and Treatment of Acute Thrombosis

The acute management of venous thrombosis or pulmonary embolism in patients with biologic risk factors for thrombosis is generally not different from that of other patients. Thrombolytic therapy should be seriously considered in patients with massive acute venous thrombosis or pulmonary embolism. Unfractionated heparin should be initiated with an intravenous bolus of 5,000 U followed by an infusion of 1,400 Uh, [396] or, if a weight-adjusted regimen is used, a bolus of 80 Ukg body weight followed by an infusion of 18 U/kg/h.[397] The PTT should be performed approximately 6 hours after therapy is initiated, and at least daily thereafter to maintain the PTT in the therapeutic range. For many commercial PTT reagents, this corresponds to a PTT that is 1.83.0 times the mean of the normal range, or an antifactor Xa heparin level of 0.30.7 Uml.[398] For less sensitive PTT reagents, the therapeutic PTT ratio is 1.52.0 times normal. [399] Alternatively low-molecular-weight heparin in therapeutic doses can be administered, which obviates the need for monitoring the PTT. Warfarin can be started within the first 24 hours. Heparin or low-molecular-weight heparin is continued for at least 5 days [400] or until the prothrombin time is in the therapeutic range, namely, an INR of 2.03.0. The principles of anticoagulation are described in further detail in Chapters 121 and 122 .

Patients with AT III deficiency can usually be treated successfully with intravenous heparin [401] although in some situations higher than usual doses of the drug are required to achieve adequate anticoagulation. In AT III-deficient patients receiving heparin for the treatment of acute thrombosis, the adjunctive role of AT III concentrate purified from human plasma is not clearly defined because controlled trials have not been performed. [401] [402] This product should probably be administered when difficulty is encountered in achieving adequate heparinization or recurrent thrombosis is observed despite adequate anticoagulation. It is also reasonable to treat AT III-deficient subjects with concentrate before major surgeries or in obstetric situations where the risks of bleeding from anticoagulation are unacceptable.

The infusion of 50 U AT III concentrate per kilogram of body weight (1 U is defined as the amount of AT III in 1 ml of pooled

normal human plasma) will usually raise the plasma AT III level to 120% in a congenitally deficient individual with a baseline level of 50%. [403] [404] [405] [406] [407] Plasma levels should be monitored to ensure that they remain >80% the administration of 60% of the initial dose at 24-hour intervals is recommended to maintain inhibitor levels in the normal range.[407]

The infrequent occurrence of warfarin-induced skin necrosis, the relatively high frequency of asymptomatic hereditary protein C deficiency, and the diagnostic difficulty in making a rapid and definitive laboratory diagnosis of the deficiency state are arguments against the routine measurement of plasma protein C levels in all individuals with thrombosis before the initiation of oral anticoagulants. If, however, one is starting oral anticoagulants in a patient who is already known or likely to be protein C deficient, it is prudent to start the drug under the cover of full heparinization and also to increase the dose of warfarin gradually, starting from a relatively low level (e.g., 2 mg for the first 3 days and then in increasing amounts of 23 mg until therapeutic anticoagulation is achieved). Patients with heterozygous protein C deficiency and a history of warfarin-induced skin necrosis have been successfully retreated with oral anticoagulants. Protein C administration either in the form of fresh frozen plasma or protein C concentrate provides protection against the development of recurrent skin necrosis until a stable level of anticoagulation is achieved.[116] [408]

Long-term Management

After an initial episode of venous thrombosis or pulmonary embolism, patients are usually continued on oral anticoagulants for 36 months at an INR of 2.03.0 because clinical trials have established the efficacy of this regimen for preventing early recurrences. Recent data indicate that the risk of recurrence is greater in patients with permanent as opposed to temporary risk factors for thrombosis.[409] [410] In patients with inherited thrombophilia, it is therefore reasonable to continue warfarin for at least 6 months in individuals without an identifiable exogenous triggering insult (e.g., surgery, pregnancy) for thrombosis.

After 6 months of anticoagulant treatment for an acute thrombotic event, an assessment must be made as to the relative benefit conferred by long-term anticoagulant therapy in preventing future thromboembolic complications versus the potential side effects, cost, and inconvenience for the patient. Unfortunately, there is a paucity of reliable data regarding the true magnitude of the thrombotic risk in unselected patients with deficiencies of AT III, protein C, or protein S because these are relatively uncommon disorders.

Cross-sectional studies of selected kindreds with thrombosis in association with heterozygous deficiencies of AT III, protein C, and protein S have given estimates of the cumulative risk of thrombosis as a function of age. The risk is extremely low until about age 15, and then occurs at a rate of 24% per year. Cross-sectional studies of family members of unselected patients with these defects indicate that the rate of spontaneous and induced venous thrombosis are lower at 0.41% and 0.52% per year, respectively.[411] Many clinicians with experience in managing patients with these disorders feel that patients with congenital AT III deficiency (particularly type I defects) are at higher thrombotic risk than those with other defects.

Due to the relatively high frequency of the factor V Leiden mutation in patients with a first episode of venous thrombosis, data have recently become available regarding their recurrence risk. Two studies have found a significant increase. Among 77 men in the Physicians Health Study with a first episode of idiopathic venous thromboembolism followed for an average of 68 months, the rate of recurrent venous thromboembolism in factor V Leiden heterozygotes was four- to fivefold higher than in genetically unaffected men (29%, incidence rate of 7.5%/year

TABLE 120-13 -- Management of Biologic Defects Predisposing to Thrombosis

Risk Classification	Management
High risk	Indefinite anticoagulation
2 or more spontaneous thromboses	
1 spontaneous life-threatening thrombosis	
1 spontaneous thrombosis at an unusual site (mesenteric or cerebral venous)	
1 spontaneous thrombosis in the presence of more than a single biologic defect	

Moderate risk	Vigorous prophylaxis during high-risk situations
1 thrombosis with a prothrombotic stimulus	
Asymptomatic	

vs. 11%, incidence rate of 1.82%/year, respectively). [412] In a second study of 251 Italian patients with a first episode of deep vein thrombosis proven by venography, the cumulative incidence of recurrent venous thromboembolism after 8 years of follow-up was over twofold higher among 41 heterozygotes with factor V Leiden than in those without the mutation (39.7% vs. 18.3%).[413] Rintelen et al.[414] and Eichinger et al. [415] have not found an increased recurrence risk in heterozygotes with factor V Leiden, but the former study was retrospective and the latter included patients who were followed up for shorter time periods.

Although recurrent venous thromboembolism may occur more frequently in patients with factor V Leiden and an initial idiopathic event, this does not necessarily imply that oral anticoagulant treatment should be prolonged beyond 36 months; its benefits may be counterbalanced by its hemorrhagic risk. Indeed, a recent trial of long-term warfarin therapy (INR 22.85) in patients with a second venous thrombotic episode found that the drug was highly effective in preventing recurrences as compared to 6 months of therapy (2.6% vs. 21% over 4 years), but there were hemorrhagic complications (8.6% vs. 2.3%, respectively). [416]

Prospective data are not currently available regarding the utility of extending oral anticoagulation beyond 36 months in patients with biologic risk factors for thrombosis. Therefore, only general guidelines for managing and treating such patients can be offered (Table 12013).

When a heterozygous patient with one of the hereditary thrombotic disorders is identified, family studies should be conducted because approximately half of the first-degree relatives will be affected. Affected asymptomatic individuals should receive counseling regarding the implications of the diagnosis and advice regarding symptoms that require immediate medical attention. In women of child-bearing age, oral contraceptives are contraindicated in view of the increased thrombotic risk associated with the use of these medications. Recent data indicate that estrogen replacement therapy can increase the risk of venous thrombosis by two- to fourfold in perimenopausal and postmenopausal women,[417] [418] [419] but data are not yet available for women with specific hereditary thrombotic disorders. Thus, in making a decision as to whether to prescribe hormone replacement therapy to women with thrombotic histories, the potential for recurrence must be weighed against the benefits of the medication.

Management of Pregnancy

The management of pregnancies in women with thrombotic histories or hereditary thrombotic disorders poses special

MANAGEMENT DECISIONS IN PATIENTS WITH AN INHERITED THROMBOTIC DISORDER

All individuals with biologic risk factors for thrombosis should be carefully evaluated prior to surgical, medical, or obstetric procedures. They should then receive appropriate prophylactic anticoagulation regimens. If specific concentrates are available for the patients deficiency state, these can also be administered to raise the plasma levels of the protein to the normal range during the perioperative period.

In patients with or without an inherited thrombotic disorder, the occurrence of two or more spontaneously occurring thromboembolic episodes may lead to the continuation of oral anticoagulants for life. Chronic warfarin therapy is generally not recommended until an individual has had at least one documented thrombotic episode.

With regard to the issue of long-term warfarin therapy in other patients with thrombosis, recommendations must be individualized at the present and clinical features that should be considered along with the presence of biologic risk factors include:

1. The severity and site of thrombosis; e.g., a patient who previously sustained a massive pulmonary embolus is more likely to receive long-term warfarin than one who developed deep venous thrombosis in a calf vein unless there are symptoms or signs of a significant postphlebitic syndrome; patients with mesenteric, portal, or cerebral venous thrombosis are frequently anticoagulated indefinitely after diagnosis due to the potential morbidity or mortality of a recurrence at these sites.
2. Whether the thrombotic episodes were spontaneous or whether triggering factors were present; e.g., if a precipitant such as a major abdominal operation was present, it would be reasonable to manage the patient without long-term oral anticoagulation after the acute episode was adequately treated.
3. Resolution of thrombosis; e.g., if ultrasound was used to diagnose deep venous thrombosis, a follow-up study after 36 months of anticoagulant therapy can indicate whether the initial thrombus has completely resolved. The presence of persistent abnormalities suggests incomplete fibrinolysis of the clot and may prompt the physician to consider long-term anticoagulation if the patient is symptomatic from post-phlebitic syndrome. A baseline study after therapy is also useful in case the patient returns with symptoms of phlebitis in the same leg and ultrasound is to be used to document the recurrence.
4. In patients with hereditary defects, a history of thromboembolism in other biochemically affected members of the family; e.g., while marked intra- and interfamilial heterogeneity has been observed in the phenotypic expression of the inherited thrombotic disorders, it is not unreasonable to place asymptomatic, biochemically affected patients from severely affected kindreds on oral anticoagulants starting at puberty.
5. The sex and lifestyle of the individual; e.g., situations where these factors may influence the decision-making process include: women of child-bearing age planning to conceive, occupations that entail prolonged periods of immobilization and thereby might be associated with an increased risk of thromboembolism, jobs with higher than average chance of trauma that might lead to thrombotic or bleeding complications.

problems.[420] The incidence of thrombotic complications during pregnancy and the postpartum period appears to be greater in women with AT III deficiency than in those with deficiencies of protein C or protein S.[421] The factor V Leiden mutation is frequently identified in Caucasian women with venous thromboembolism during pregnancy and the puerperium,[11] [12] [13] but the thrombotic risk in asymptomatic women from the general population has not been defined. Pregnant women with factor V Leiden and a personal or familial history of thrombosis should, therefore, be considered for anticoagulant prophylaxis. Routine screening for the mutation in women of child-bearing age is not recommended because the potential risks of anticoagulation may equal or exceed its benefits. [422] During pregnancy, adjusted-dose unfractionated heparin administered by the subcutaneous route is the anticoagulant of choice because its efficacy and safety for the fetus are established. [423] Low-molecular-weight heparin is an attractive alternative to unfractionated heparin in these patients due to its better bioavailability and longer half-life. Patients with a previous history of thrombotic episodes should definitely receive treatment throughout pregnancy; affected women with AT III deficiency who have not yet experienced such events should probably likewise receive treatment. Treatment of asymptomatic women with other hereditary thrombotic disorders should be considered on an individual basis.

The dose and duration of heparin therapy in pregnancy is uncertain because appropriately designed clinical trials have not yet been performed in these patient populations. Patients considered to be at high risk should receive LMW heparin at thrombosis doses or full-dose unfractionated heparin by subcutaneous injection every 12 hours for the duration of pregnancy. The dose of unfractionated heparin should be adjusted to maintain the 6-hour postinjection PTT at 1.5 times the control value. In women considered to be at intermediate risk, lower doses of heparin can be used (5,00010,000 U subcutaneously every 12 hours) and therapy can be started during the second or third trimester and continued for approximately 6 weeks into the postpartum period. Low-risk patients can be observed closely throughout the pregnancy with duplex ultrasound imaging of the leg veins at regular intervals.

Management of Warfarin-Induced Skin Necrosis and Neonatal Purpura Fulminans

An association has been established between the rare complication of warfarin-induced skin necrosis and hereditary protein C deficiency. [115] [116] [117] [424] [425] About one-third of patients with warfarin-induced skin necrosis will prove to have hereditary protein C deficiency. [120] This complication has also been described in patients with heterozygous protein S deficiency[29] and factor V Leiden. [30]

Because warfarin-induced skin necrosis is a rare complication, therapy has been guided primarily by knowledge regarding its pathogenesis. The diagnosis should be suspected in patients with painful, red skin lesions developing within a few days after the initiation of the drug and immediate intervention is required to prevent rapid progression and to minimize complications. Therapy should consist of immediate discontinuation of warfarin, administration of vitamin K, and infusion of heparin at therapeutic doses. Lesions, however, have been reported to progress despite adequate anticoagulation with heparin. In patients with hereditary protein C deficiency, the administration of a source of protein C should be seriously considered, and it may also be appropriate in other patients with warfarin-induced skin necrosis as they invariably have reduced plasma levels of functional protein C when the skin lesions first appear. Fresh frozen plasma has been used, but improved results can be expected with the administration of a highly purified protein

C concentrate, which facilitates the rapid and complete normalization of plasma protein C levels. [426]

The management of neonatal purpura fulminans in association with homozygous or doubly heterozygous protein C deficiency is more complicated, and heparin threrapy as well as antiplatelet agents have not been shown to be effective. [122] [124] [125] [427] [428] [429] The administration of a soucre of protein C appears to be critical in the initial treatment of these patients. Fresh frozen plasma has been used with success to treat these infants. However, the half-life of protein C in the circulation is only 616 hours,[118] [430] and the administration of plasma on a frequent basis is limited by the development of hyperproteinemia, hypertension, loss of venous access, and the potential for exposure to infectious viral agents. A highly purified concentrate of protein C has been developed and is efficacious in treating neonatal purpura fulminans.[431] Warfarin has been administered to these infants without the redevelopment of skin necrosis during the phased withdrawal of fresh frozen plasma infusions,[122] [125] [126] [432] [433] and this medication has been used chronically to control the thrombotic diathesis. Successful liver transplantation has been undertaken in a child with liver failure and homozygous protein C deficiency, which resulted in normalization of the plasma protein C level and resolution of the thrombotic diathesis.[434]

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Chapter 121 - Oral Anticoagulant Therapy

Bruce Furie

STRUCTURE OF ORAL ANTICOAGULANTS

The vitamin K antagonists were discovered as the result of an intensive investigation into the etiology of a hemorrhagic disease of cattle that plagued farmers in the Great Plains during the 1920s. This disorder, characterized by hypoprothrombinemia, was associated with the ingestion of spoiled sweet clover hay contaminated with specific toxins.[1] [2] Campbell and Link[3] purified 3,3-methylene-bis-4-hydroxycoumarin from bacterial contaminants in the spoiled hay and discovered that this compound caused a syndrome similar to vitamin K deficiency. Bishydroxycoumarin, a vitamin K antagonist, was introduced into clinical practice as an anticoagulant during the 1940s[4] and is prescribed as dicumarol. Other structurally related vitamin K antagonists with varying pharmacologic properties were developed (Fig. 1211). The most important vitamin K antagonist used in the United States is warfarin sodium.

Figure 121-1 Structure of the oral anticoagulants and their relationship to vitamin K. Chemical structures include **(A)** warfarin, **(B)** phenprocoumon, **(C)** dicoumarol, and **(D)** vitamin K. Vitamin K and the coumarin anticoagulants share a common ring structure, shown in red.

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MECHANISM OF ACTION OF WARFARIN

As a vitamin K antagonist, warfarin inhibits the complete synthesis of the vitamin K-dependent blood-clotting proteins, including prothrombin and factors VII, IX, and X required for normal blood coagulation.[5] In addition, the vitamin K-dependent regulatory proteins, proteins C and S, are also affected due to a decrease in vitamin K-dependent post-translational modifications. The anticoagulant effect of warfarin depends on the reduction in the synthesis of biologically active vitamin K-dependent blood-clotting proteins and the normal clearance from the circulation of fully active vitamin K-dependent blood-clotting proteins synthesized before the introduction of the vitamin K antagonist. Importantly, the administration of warfarin does not lead to instantaneous anticoagulation. Rather, warfarin must be administered for 45 days before the anticoagulant effect is therapeutic, even when the prothrombin time (PT) is in the therapeutic range.

During the synthesis of the vitamin K-dependent proteins, the post-translational modification of glutamic acid to -carboxyglutamic acid is catalyzed by the vitamin K-dependent carboxylase.[6] -Carboxyglutamic acid is required in these proteins to permit calcium-dependent protein/membrane interaction and for the expression of full biologic activity.[7] [8] [9] The carboxylase is an integral membrane protein [10] requiring carbon dioxide, molecular oxygen, and the hydroquinone form of vitamin K to convert glutamic acid to -carboxyglutamic acid.[6] During this reaction, a -hydrogen on glutamic acid residues on the precursor protein is extracted, and carbon dioxide reacted to form the new carboxyl group. The vitamin K hydroquinone is converted to vitamin K epoxide in a reaction that is closely linked to the carboxylase activity. Although some vitamin K antagonists inhibit the carboxylase directly, warfarin does not inhibit the vitamin K-dependent carboxylase, except at very high concentration,[11] and is thus not a direct competitor of the action of vitamin K.

Inhibition of -carboxylation of the vitamin K-dependent proteins by warfarin yields proteins in the plasma that are partially carboxylated. [12] [13] The elimination of even a single -carboxyglutamic acid usually disables the function of these proteins; some -carboxyglutamic acid residues are so critical that their absence abolishes biologic activity of the protein.[14] Therefore, the induction of acarboxy- and des--carboxyprothrombin, for example, in the plasma of patients treated with warfarin, yields primarily functionally inert vitamin K-dependent blood-clotting proteins. The coagulant activity correlates closely with the quantity of fully carboxylated proteins that remain in the plasma of patients treated with warfarin.[15]

The vitamin K cycle represents a metabolic pathway for the utilization and recovery of vitamin K after its participation in vitamin K-dependent carboxylation reactions (Fig. 1212) (Chap. 115). Warfarin inhibits some of the enzymes involved in this cycle, blocking the availability of the proper form of vitamin K for participation as a cofactor in the reaction catalyzed by the carboxylase.[16] [17] [18] Vitamin K is reduced to the vitamin K hydroquinone by a vitamin K reductase. This enzyme is inhibited by warfarin. The vitamin K hydroquinone is the cofactor for the

Figure 121-2 Vitamin K cycle and its inhibition by warfarin. Vitamin K is reduced to the vitamin K hydroquinone by a vitamin K reductase inhibited by warfarin. The vitamin K hydroquinone is a cofactor for the vitamin K-dependent carboxylase. In this reaction, the hydroquinone is oxidized to the vitamin K epoxide and glutamic acid is converted to -carboxyglutamic acid. The carboxylase is not inhibited by warfarin. The vitamin K epoxide is salvaged by a vitamin K epoxide reductase, an enzyme sensitive to warfarin inhibition, and is recycled back to vitamin K.

carboxylase. During carboxylation of glutamic acid residues by the carboxylase, the vitamin K hydroquinone is oxidized to the vitamin K epoxide. Warfarin has no effect on this reaction, except at very high concentration. The vitamin K epoxide is recycled to vitamin K via a pathway that requires the vitamin K epoxide reductase. This reaction is inhibited by warfarin,[16] leading to increased plasma levels of vitamin K_1 epoxide.

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PHARMACOLOGY

Warfarin sodium is almost always administered by the oral route, hence its role in a class of compounds known as oral anticoagulants. Warfarin is nearly completely absorbed, although considerable variation remains in the individual absorption patterns. Peak warfarin levels are observed at 0.54 hours after oral administration. [19] Warfarin circulates in the blood bound to albumin. With 97% of warfarin bound to albumin, only small amounts of warfarin circulate free in plasma. [20] However, it is this free warfarin that is biologically active. The mean half-life of warfarin in the blood is 44 hours, but this varies widely in different subjects from 15 to 48 hours. [21] This plasma half-life depends on the rate of warfarin absorption and on the rate of warfarin degradation. The individual variation in the half-life of this drug is dominated by the variability of the degradation rate. Renal clearance of warfarin is low because most of the warfarin is bound to albumin. Warfarin is metabolized in the liver to yield a series of inactive metabolites that are recovered in

Figure 121-3 The reduction of the activities of the vitamin K-dependent blood clotting proteins by warfarin. Warfarin (10 mg) was administered to a normal subject on 4 consecutive days. The activities of factors VII, X, and IX and prothrombin are presented as a function of time after the initiation of the medication. Factor VII activity disappears most rapidly, while prothrombin activity decreases more slowly. Factor IX and factor X activities decrease at an intermediate rate. Because the PT is so sensitive to the factor VII concentration, with the initiation of warfarin therapy the PT can be disproportionately prolonged before the levels of factors IX and X and prothrombin are reduced sufficiently to promote an antithrombotic effect.

the urine. [22] Oxidation of warfarin is associated with the hepatic cytochrome P-450 complex.

The biologic effect of warfarin, the decrease in the plasma activity of the vitamin K-dependent proteins, lags behind the peak plasma warfarin concentration. The coagulant activity of each of these proteins in plasma represents a balance between the synthesis of the protein in a completely active -carboxylated form and its utilization and metabolism. The administration of warfarin impairs the complete synthesis of the vitamin K-dependent proteins by inhibiting -carboxylation but does not affect the plasma half-life of these proteins. Thus, the disappearance of biologically active, fully -carboxylated factors VII, X, and IX and prothrombin coagulant activities from the plasma is related to the half-life of each of these proteins. Factor VII activity disappears most rapidly with a half-life of about 6 hours (Fig. 1213). Factors X and IX have half-lives of about 24 hours; these activities decrease less rapidly from the plasma than that of factor VII. Prothrombin has a half-life of about 72 hours, disappearing at the slowest rate of the vitamin K-dependent blood-clotting proteins.

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INDICATIONS FOR THERAPY

Warfarin and related oral anticoagulants (see box on Initiation of Warfarin Therapy) are used for the prevention of thromboembolic disorders in patients at risk. [23] [24] Because of the 56% incidence of systemic embolization associated with atrial fibrillation and valvular heart disease, chronic warfarin therapy is routinely used in this clinical setting. Recently, oral anticoagulants have been advocated in all patients with atrial fibrillation. [25] [26] [27] Patients with prosthetic heart valves, particularly of the mechanical type, require chronic warfarin administration to prevent embolism of formed thrombi (see box on Management of Chronic Warfarin Therapy). Other patients, such as the family members of patients with serious thromboembolic disorders secondary to defects in the proteins that regulate blood coagulation, may be placed on oral anticoagulant therapy if they also demonstrate a deficiency in these activities and are considered

INITIATION OF WARFARIN THERAPY

When warfarin therapy is planned for chronic anticoagulation after pulmonary embolism or deep venous thrombosis, patients are routinely treated with heparin during the acute phase of anticoagulation. Heparin is usually administered by continuous intravenous infusion for a period of 714 days. Because warfarin will not prolong the PT for 1 or 2 days after the initiation of therapy and because the anticoagulant action of warfarin is adequate only after 4 or 5 days of therapy, warfarin and heparin are given simultaneously for 4 or 5 days. A large loading dose of warfarin is unnecessary. Warfarin therapy (5 mg/day) can be instituted at the initiation of heparin therapy. Subsequently, the warfarin dose can be adjusted to optimize the prolongation of the PT. When adequate control has been established, heparin therapy may be discontinued.

Warfarin is also used for the initiation of anticoagulant therapy without prior overlapping heparin therapy. A typical example is a patient with valvular heart disease with atrial fibrillation who requires prophylaxis with antithrombotic agents. Although warfarin-induced necrosis is a rare disorder, the prevalence of heterozygous protein C deficiency has been estimated to be 1/300 in the United States. To lower the risk of warfarin-induced necrosis, consideration may be given to either the measurement of the functional protein C level in the plasma of a patient to be treated for the first time with warfarin or to the coadministration of subcutaneous heparin therapy for the first 4 or 5 days of warfarin therapy.

at risk of thromboembolic disease. Warfarin is effective in decreasing the incidence of venous thrombosis after hip surgery [28] [29] and has also been used at low fixed doses in patients with indwelling catheters. [30]

Warfarin is also used to prevent the recurrence of thromboembolic disease. After the acute treatment of thromboembolic diseases, such as pulmonary embolism and deep venous thrombosis, with heparin or fibrinolytic agents, patients are routinely maintained on warfarin for 36 months. The decision to extend treatment for longer periods depends on an analysis of benefit versus risk. Risk factors for recurrent thromboembolic disease include specific abnormalities of coagulation or regulatory proteins,

MANAGEMENT OF CHRONIC WARFARIN THERAPY IN PATIENTS REQUIRING MINOR SURGERY

In patients requiring chronic anticoagulation for such conditions as heart valve prostheses or protein C deficiency, warfarin therapy may need to be discontinued briefly to permit the safe performance of surgical procedures. Warfarin therapy may be stopped 1 week prior to surgery and anticoagulation begun using subcutaneous heparin. With a normal diet, the PT will return to the normal range within the week. Heparin can be discontinued just before surgery. Either heparin and warfarin, or warfarin alone, can be reinstituted after the successful completion of surgery. The period between surgery and the reinitiation of anticoagulants depends on the balance between the risk of bleeding and the risk of thromboembolic disease.

a history of recurrent thromboembolism, and a family history positive for thromboembolism (Chap. 120). Other uses of warfarin therapy are more controversial. Despite the widespread use of warfarin for more than four decades for the prevention of second myocardial infarction as well as pooled data showing a statistically significant, albeit modest, effect, definitive clinical evidence that warfarin alters the incidence of reinfarction is lacking. [31] [32] This question has been revisited in that high-intensity warfarin therapy caused a reduction of recurrent infarction, stroke, and mortality. [33] Despite these results, warfarin has not found a major application in patients with myocardial infarction. Nevertheless, anticoagulant therapy, including heparin and warfarin, does play a role in preventing the arterial and venous thromboembolic complications that may occur during the immediate postinfarction period. It is currently recommended that patients with an anterior myocardial infarction, a subset at high risk of thromboembolism, be treated with prophylactic anticoagulants. [34] [35] The role of warfarin in the treatment of stroke is unclear.

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CONTRAINDICATIONS

Warfarin is contraindicated in patients who fail to comply with the routine administration of the drug and in patients who are unable to obtain regular measurements of the PT. Furthermore, oral anticoagulants should not be used or should be used with extreme caution in patients with either hereditary or acquired bleeding disorders or in patients with anatomic lesions that predispose them to serious bleeding (e.g., active duodenal ulcer). Warfarin crosses the placenta in pregnant women. Because it is teratogenic and has been associated with a fetal warfarin syndrome, [3€] warfarin use is contraindicated during pregnancy. Other anticoagulants, such as subcutaneous heparin or enoxaparin, are used preferentially instead of warfarin to prevent thromboembolism in pregnant women at risk of thromboembolism.

REGULATION OF THERAPY

The PT is routinely used for monitoring therapy with the oral anticoagulants, including warfarin. The PT is a biologic test in which recalcified, citrated patient plasma is clotted by the addition of a crude preparation of tissue factor and lipid, also known as thromboplastin, or highly purified recombinant tissue factor. Thromboplastin is an extract of either brain or lung tissue, or a mixture of both. Tissue factor initiates the extrinsic pathway of blood coagulation, including factor VII, factor X, factor V, prothrombin, and fibrinogen. Decreases in the activities of three vitamin K-dependent proteins, factors VII and X and prothrombin, prolong the PT. The patients PT is usually reported as the clotting time, in seconds, and is compared with the PT of a normal plasma sample. Warfarin is administered to prolong the PT from the normal level (12 seconds) into the therapeutic range. The use of the International Normalized Ratio (INR) is also reported to correct for the variation in potency of different thromboplastins (see following discussion). The warfarin dose is adjusted to obtain the recommended therapeutic INR for a given indication. Because factor VII is at the beginning of the extrinsic pathway and factor VII has the shortest half-life of the vitamin K-dependent blood coagulation proteins, the PT is very sensitive to the factor VII levels; furthermore, the factor VII level is suppressed more quickly than the other vitamin K-dependent proteins. The optimal therapeutic range varies with the indication for warfarin anticoagulation [37] (Chaps. 124 and 125). Standard-intensity warfarin therapy includes a therapeutic PT prolongation of 1.52.0 times the control PT value using the thromboplastin preparations currently used in the United States. When the anticoagulant effect is aimed at this level, bleeding complications occur in about 1020% of patients, with

INTENSITY OF ANTICOAGULANT THERAPY: RECOMMENDED INR VALUES	
Prevention of	
Deep vein thrombosis	2.03.0
Subclavian vein thrombosis	2.03.0
Pulmonary embolism	2.03.0
Prophylaxis against systemic thromboembolic disease	
Atrial fibrillation	2.03.0
Cardiac valve replacement	
Mechanical valves	2.53.5
Tissue valves	2.03.0

about half of these complications occurring when the PT is within the therapeutic range. Low-intensity therapy, with a PT of 1.31.8 times control, has been associated with a significantly lower incidence of bleeding. [38] [39] For these reasons, lower-intensity therapy, with a PT of 1.31.5 times the control PT, appears reasonable in the clinical setting of deep venous thrombosis. However, whether low-intensity therapy provides adequate prophylaxis of thromboembolic disease comparable to standard-dose warfarin therapy in other clinical situations has not been definitely demonstrated. Clinicians should use the INR value in making therapeutic decisions regarding the appropriate dose of warfarin (see box on Recommended INR Values). Furthermore, the INR has been shown to have utility during the induction phase of warfarin therapy.[40]

International Normalized Ratio

Thromboplastins used for PT measurements are derived from a variety of tissues, including brain and lung, and various species, including rabbit, bovine, and human. To standardize the potency of these different thromboplastin reagents, these reagents were calibrated using the standardized human brain thromboplastin reagent, the Manchester comparative reagent, as an international reference preparation. More recently, recombinant tissue factor has been used. A calibration system, based on the linear relationship of the logarithm of the PT of the patient compared to the logarithm of the PT of control plasma, yields the INR:

where the ratio of the patient PT to the control PT is the observed ratio, and c represents the International Sensitivity Index (ISI). Because each thromboplastin yields a unique c value, PTs obtained using different thromboplastins can be compared, hence the INR. Currently, all commercially available thromboplastins are calibrated with regard to the ISI, and individual laboratories report both the PT and the INR for a given sample. The ISI varies, at 1.01.2 in thromboplastins used in Europe to 1.82.8 for the North American thromboplastins. [41] [42]

An independent method of monitoring warfarin anticoagulation has been undergoing clinical evaluation. [15] [43] This method does not rely on the PT or the use of thromboplastins. By monitoring the level of fully -carboxylated prothrombin, or native prothrombin, by immunoassay, it has been possible to identify a therapeutic range of 1224 g/ml in which bleeding complications are minimized and at which the warfarin dosage is adequate to prevent thromboembolic disease. [44] [45] This method has been further evaluated in patients undergoing hip replacement. [46]

Low-dose or minidose warfarin therapy in fixed-dose regimens has been advocated for general prophylaxis of thromboembolic disease following surgery as well as to prevent thrombosis in central venous catheters. [47] [48] Because of the highly individual rates of absorption, utilization, and degradation of warfarin, fixed warfarin doses have variable potencies in different patients. However, the PT is insensitive to low-intensity warfarin therapy. The native prothrombin assay may offer an alternative method to monitor low-intensity therapy because it is sensitive to small amounts of warfarin, permitting adjusted low-dose therapy.

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COMPLIANCE

Because of the low toxic/therapeutic ratio, the risk of bleeding associated with warfarin therapy is highest in patients who are not carefully monitored. Therefore, only patients who are willing to subject themselves to the regular inconvenience of the PT measurement should be treated with warfarin. During the initiation of therapy, PT measurements may be required two or three times a week, then once weekly, until an adequate dosage is determined. Patients treated chronically with warfarin who have had stable PT measurements require a PT every 3 or 4 weeks. Furthermore, only reliable patients who will take their medication regularly should be considered for warfarin therapy.

DIET AND DRUG INTERACTION

Both diet and the coadministration of certain drugs can have a marked effect on the magnitude of warfarin action [49] [50] (Table 1211). Certain drugs diminish the pharmacologic response to warfarin. Barbiturates induce hepatic microsomal enzymes, enhancing the degradation of warfarin. Vitamin K antagonizes the inhibitory effect of warfarin on the synthesis of vitamin

TABLE 121-1 -- Effect of Drugs on Warfarin Response

Medications That Potentiate the Effect of Warfarin	
Acetaminophen	Isoniazid
Acetohexamide	Mefenamic acid
Allopurinol	Methimazole
Androgenic and anabolic steroids	Methotrexate
-Methyldopa	Methylphenidate
Antibiotics that disrupt intestinal flora (tetracyclines, streptomycin, erythromycin, kanamycin, nalidixic acid, neomycine)	Nalidixic acid
	Nortriptyline
	Oxyphenbutazone*p*-Aminosalicyclic acid
	Paromomycin
Cephaloridine	Phenylbutazone
Chloramphenicol	Phenytoin
Chlorpromazine	Phenyramidol
Chlorpropamide	Propylthiouracil
Chloral hydrate	Quinidine
Cimetidine	Salicylate
Clofibrate	Sulfinpyrazone
Diazoxide	Sulfonamides
Disulfiram	Thyroid hormone
Ethacrynic acid	Tolbutamide
Glucagon	
Guanethidine indomethacin	
Medications That Depress the Effect of Warfarin	
Antipyrine	Glutethimide
Barbiturates	Griseofulvin
Carbamazepine	Haloperidol
Chlorthalidone	Oral contraceptives
Cholestyramine	Phenobarbital
Digitalis	Prednisone
Ethanol	All vitamin preparations containing vitamin K
Ethchlorvynol	

K-dependent proteins. Whether vitamin K is administered parenterally or orally, or in excess because of dietary intake, the effect is a blunting of the anticoagulant effect of warfarin. A common mechanism for the enhancement of warfarin action is the displacement of warfarin bound to albumin by other drugs that compete for this albumin-binding site. Albumin-binding drugs displace warfarin, thereby increasing the amount of free warfarin in the plasma. It is the free form of warfarin that is pharmacologically active, enhancing warfarin inhibition of vitamin K action. Because the clearance of warfarin from the blood is also a function of the free warfarin level, enhancement of the biologic action of warfarin is followed several days later by a reduction in warfarin action. Numerous drugs displace warfarin from albumin, but model examples include phenylbutazone and chloral hydrate. Chloramphenicol inhibits liver microsomal enzyme synthesis, reducing the normal rate of degradation of warfarin and leading to the enhancement of the effect of a particular warfarin dose.

It is good practice to stabilize the PT on a given dose of warfarin while avoiding changes in medications or diet, including alcohol consumption. In addition to the drugs listed in Table 1211 , a large number of drugs perturb the PT of patients treated with warfarin. Some drug interactions have not been clearly defined. For these reasons, the introduction of any new medication in a patient on stable warfarin therapy requires careful monitoring of the PT during the transition period so as to permit adjustments of the warfarin dosage to accommodate the effects of the new medication. Other anticoagulants or antiplatelet drugs should be avoided during warfarin administration. For example, aspirin increases bleeding complications during warfarin therapy.

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WARFARIN RESISTANCE

Warfarin resistance is an occasional problem in some patients who have previously taken warfarin but are now refractory to normal doses and in other patients who are refractory to warfarin from the onset of therapy. The consideration of warfarin resistance is usually reserved for patients who require in excess of 1520 mg/day of warfarin to maintain the PT within the therapeutic range.

The molecular basis of warfarin resistance is unknown in most patients. Patients unknowingly supplemented with vitamin K, such as those undergoing parenteral nutrition or feedings with enteral nutrition products, are resistant to the action of vitamin K antagonists administered at customary doses. [51] Patients with unusual dietary intake, including large amounts of cruciferous vegetables (e.g., spinach, cabbage, or broccoli) may also be resistant to warfarin because of the large quantities of vitamin K in these foods.[52] [53] Once excess vitamin K intake has been ruled out, the mechanism of warfarin resistance remains speculative. There are no proven examples of antibodies arising to warfarin. A rare condition, hereditary warfarin resistance, is characterized by the absence of warfarin effect on the synthesis of the vitamin K-dependent blood-clotting proteins despite the achievement of very high plasma warfarin levels. [54] [55] Based on the study of the warfarin-resistant rat, it appears that the vitamin K epoxide reductase from these rats is less sensitive to warfarin than is the normal enzyme. [55] [56] [57]

Patients resistant to the effects of warfarin can still be treated with warfarin, but higher doses are necessary. With appropriate care, the warfarin dose can be gradually increased until the PT prolongs into the therapeutic range. In these patients, the warfarin dosage threshold at which the PT becomes unacceptably long is higher than in normal persons, but these patients are just as brittle. Small increases in the warfarin dose can prolong the PT significantly beyond the therapeutic range. Treatment with large doses of warfarin (e.g., >50 mg/day) may have toxicities not observed at conventional doses, so the treating physician may elect to use alternative therapies, such as subcutaneous heparin.

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COMPLICATIONS

Bleeding complications are observed in 1020% of patients treated with warfarin therapy. [38] [39] [44] [58] [59] [60] [61] [62] About one-half of these complications occur when the therapeutic range of the PT has been exceeded, but one-half occur despite a PT within the therapeutic range. These complications may be mild, such as epistaxis, purpura, or hematuria, or they may be more serious, such as retroperitoneal bleeding, the formation of large hematomas, or significant gastrointestinal bleeding (see box on Therapy for Prolonged PT). Life-threatening bleeding, such as intracranial bleeding, does occur, and patients should be warned of the symptom complex of such an event so that they can seek medical attention immediately. The most common agents or disorders associated with excessive anticoagulation (INR >6.0) were acetaminophen, advanced malignancy, recent diarrheal disease, decreased oral intake, and warfarin use in excess of that prescribed. [63]

Patients with missense mutations in the propeptide of factor IX are extremely sensitive to the action of warfarin. Although these patients have a normal partial thromboplastin time, normal factor IX activity, and no bleeding phenotype, administration of warfarin leads to a marked decrease in factor IX activity and a marked increase in bleeding risk. [64] [65]

Warfarin-induced skin necrosis is a rare complication. During the onset of warfarin therapy, usually between days 2 and 7, the patient develops a bluish purple lesion on the thigh, breast,

THERAPY FOR PROLONGED PROTHROMBIN TIMES AND BLEEDING COMPLICATIONS

Bleeding complications occur frequently in association with warfarin therapy. Severe complications, such as intracranial bleeding or massive gastrointestinal bleeding, must be treated rapidly and effectively. The PT should be normalized as quickly as possible by the infusion of 23 U of fresh frozen plasma to replenish the activities of the vitamin K-dependent proteins. Current preparations of the vitamin K-dependent proteins labeled as factor IX concentrations (e.g., Konyne, Proplex) have no role in the treatment of warfarin overdosage because these agents carry an unacceptable risk of hepatitis transmission. Vitamin K can be administered to reverse the effects of warfarin, but it must be realized that vitamin K will not be effective until 1236 hours later. Patients given vitamin K may be refractory to the action of warfarin for a period of time after anticoagulant therapy is reinstituted.

Moderate complications, such as hematomas, hemarthrosis, or mild gastrointestinal bleeding, may be treated by the discontinuation of warfarin therapy and the administration of vitamin K. Vitamin K_1 (Aquamephyton) can be administered intravenously (with appropriate caution and monitoring of severe allergic reactions) at a dose of 10 mg. The PT will return toward the normal range within 24 hours.

Mild complications, such as purpura, ecchymoses, or epistaxis, can be treated by withholding or reducing warfarin therapy until the PT returns into the therapeutic range. In this situation, fresh frozen plasma carries an unnecessary risk of viral infection and vitamin K administration greatly complicates reinstitution of anticoagulant therapy with warfarin.

buttock, or toes. This lesion is characterized by a clear line of demarcation between the affected area and surrounding tissue. Over several days, this lesion becomes increasingly necrotic, while the surrounding area becomes erythematous and inflamed. The histology of the lesion reveals thrombi in the microvasculature. This syndrome is often due to the exaggeration of protein C deficiency by warfarin in a patient who has hereditary heterozygous protein C deficiency. [66] Protein C, a regulatory plasma protein that serves as a natural anticoagulant, requires vitamin K for its complete synthesis (Chap. 104). Because protein C has a relatively short plasma half-life (Fig. 1213), warfarin induces a relative protein C deficiency before it reduces the activity of the vitamin K-dependent blood-clotting proteins to an anticoagulant level. Because patients with heterozygous protein C deficiency are especially susceptible to this complication, they should only be treated with warfarin when heparin is used simultaneously during the first 4 or 5 days of therapy. Warfarin-induced skin necrosis has also been reported in rare cases of hereditary protein S deficiency.

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REVERSAL OF WARFARIN EFFECT

Overdosage of warfarin is a common problem that occasionally leads to serious bleeding manifestations. Although generally observed in patients being treated for thromboembolism, warfarin overdosage may also be seen after accidental or surreptitious ingestion. Proper therapy depends on the level of bleeding complications. In patients with prolongation of the PT without signs of bleeding, warfarin can be withheld until the PT approaches the therapeutic range. Patients with life-threatening bleeding disorders, including intra-abdominal or intracranial hemorrhage, require urgent, immediate correction of the PT. These patients must be treated with fresh frozen plasma (23 U) as a source of the vitamin K-dependent blood coagulation proteins. Because fresh frozen plasma carries the risk of infection, the severity of the bleeding episode must be balanced against the risk of hepatitis. Factor IX concentrates include the vitamin K-dependent blood-clotting proteins in a partially purified form. However, because current products essentially ensure the transmission of hepatitis to patients who lack immunity to hepatitis, they are contraindicated. Improvements in the large-scale preparation of purified vitamin K-dependent proteins may offer new agents that will prove useful. [67] [68] [69] Vitamin K can be administered to reverse the effects of warfarin. However, the response to vitamin K is delayed for 1224 hours due to the requirement for de novo synthesis of the vitamin K-dependent blood-clotting proteins. Furthermore, vitamin K administration usually results in normalization of the PT and will interfere with re-anticoagulation with vitamin K antagonists. Furthermore, patients treated with vitamin K may be resistant to warfarin action once warfarin therapy is reinitiated. For these reasons, vitamin K therapy is best reserved for patients with serious bleeding complications initially treated with fresh frozen plasma and whose anticoagulant management can be approached with alternative methods (e.g., heparin). Because the absorption of oral vitamin K, vitamin K_1 (Aquamephyton), is variable, the treatment of warfarin overdosage best involves administration of intravenous vitamin K_1 .

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Chapter 122 - Heparin

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INTRODUCTION

In 1923, Howell[1] used the term heparin to describe an aqueous extract of canine liver that inhibited coagulation of blood in vitro. During the next decade, similar extracts were shown to consist of mixtures of sulfated polysaccharides containing uronic acid and glucosamine. [2] In 1939, investigators in Canada and Sweden reported the successful use of heparin as a treatment for recurrent thrombosis and pulmonary embolism. [2] [3] The indications for heparin were soon expanded to include vascular surgical procedures, extracorporeal circulation, and prophylaxis of thromboembolism.

Heparin is a sulfated glycosaminoglycan that, when administered intravenously, binds to antithrombin, leading to rapid inhibition of proteases of the coagulation pathways. This interaction produces a potent anticoagulant effect. Endogenous heparin-like molecules appear to be involved in the inhibition of coagulation within normal blood vessels and may have a variety of other biologic functions. Although heparin is an effective agent for the treatment and prevention of venous thromboembolic disease, it can produce bleeding and can trigger thrombocytopenia, which is associated with both venous and arterial thrombosis.

Low-molecular-weight (LMW) heparins are produced by enzymatic or chemical depolymerization of standard heparin. LMW heparins produce the majority of their anticoagulant effect by catalyzing the interaction between antithrombin and factor Xa. In clinical practice, the LMW heparins offer substantial benefits over standard heparin; their principle advantages are a predictable pharmacokinetic profile (allowing weight-adjusted subcutaneous treatment without monitoring), a reduced risk of heparin-induced thrombocytopenia, and a potential reduction in the risks of bleeding and osteoporosis.

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STRUCTURE AND BIOSYNTHESIS

Heparin

Heparin is found in the secretory granules of mast cells. It is synthesized from uridine diphosphate-sugar precursors as a highly sulfated polymer of alternating uronic acid and D-glucosamine.[4] The glycosaminoglycan chains are built on a core structure consisting of one xylose and two galactose residues covalently attached to serine in a polypeptide backbone. About 1015 glycosaminoglycan chains, each containing 200300 monosaccharide

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units, are attached to a single core protein to yield a proteoglycan with a molecular weight of 750,0001,000,000.

Related Glycosaminoglycans

Two other glycosaminoglycans, heparan sulfate and dermatan sulfate, also have anticoagulant activity. [5] Heparan sulfate is closely related to heparin and is found on the surface of most eukaryotic cells and in the extracellular matrix. Heparan sulfate proteoglycans vary considerably in structure. In general, they are smaller than the heparin proteoglycan and contain fewer glycosaminoglycan chains linked to a larger and more complex core protein. In some cases, the core protein has a hydrophobic domain that anchors the proteoglycan to the cell membrane. Dermatan sulfate is a repeating polymer of L-iduronic acid and *N*-acetyl-D-galactosamine. [6] Like heparan sulfate, dermatan sulfate is a component of proteoglycans on the cell surface and in the extracellular matrix.

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ANTICOAGULANT ACTIVITY

Antithrombin

Studies by Brinkhous et al.[7] in 1939 indicated that the anticoagulant effect of heparin is mediated by an endogenous plasma component, termed heparin cofactor. Thirty years later, antithrombin was purified from plasma and shown to have heparin cofactor activity, that is, the ability to inhibit thrombin rapidly only in the presence of heparin.[8] [9] Antithrombin is a M_r 58,000 single-chain glycoprotein that is homologous to members of the $_1$ -antitrypsin family of serine protease inhibitors (serpins).[10] [11] [12] It is synthesized in the liver and circulates in plasma at a concentration of 3 M. [13] Antithrombin inhibits activated coagulation factors of the intrinsic and common pathways, including thrombin, factor Xa, and factor IXa, but has relatively little activity against factor VIIa. [14] Antithrombin acts as a suicide substrate for its target proteases. Inhibition occurs when a protease attacks the reactive site Arg 393-Ser 394 peptide bond in antithrombin and becomes trapped as a stable 1:1 covalent complex.[15] [16]

The concentration of antithrombin in plasma exceeds that of any of the target proteases that are generated during coagulation. Under these conditions, protease inhibition in plasma can be described by pseudo-first-order kinetics. In the absence of heparin, thrombin and factor Xa are inhibited by antithrombin with a half-life of about 0.51.5 minutes, whereas factor IXa is inhibited about 10 times more slowly. [17] These rates of inhibition are too slow to affect coagulation in vitro. Heparin increases the rate of inhibition of thrombin, factor Xa, and factor IXa by antithrombin approximately 1,000-fold. [17] As a result, inhibition of these proteases in heparinized plasma is essentially instantaneous.

Factor Xa bound to platelets in the prothrombinase complex[18] and thrombin bound to fibrin[19] [20] are both protected from inhibition by antithrombin in the presence of heparin. Thus, heparin may promote inhibition of factor Xa and thrombin in solution only after they have diffused away from these binding sites. By contrast, factor VIIa bound to tissue factor is inhibited by antithrombin in the presence of heparin with a half-life of about 2 minutes, whereas the rate of inhibition of free factor VIIa is negligible.[21] [22] Whether the rate of inhibition of factor VIIa/tissue factor by antithrombin is sufficient to affect coagulation in vivo is unknown. The major anticoagulant effect of heparin is apparently to blunt the positive feedback reactions of thrombin on activation of factors V and VIII and thus to decrease the rate of thrombin generation.[23] [24] [25]

When heparin is added to plasma at a therapeutic concentration (0.11.0 U/ml), factors IXa and Xa and thrombin are inhibited almost exclusively by antithrombin. In the presence of higher concentrations of heparin or dermatan sulfate, thrombin is inhibited primarily by heparin cofactor II. [26] [27] Heparin also stimulates inhibition of thrombin by plasminogen activator inhibitor 1,[28] protein C inhibitor,[29] and protease nexin-1 (glia-derived nexin)[30] and inhibition of factor Xa by tissue factor pathway inhibitor (TFPI).[31] [32] The latter four inhibitors are present in plasma at > 1/100 the concentration of antithrombin. Intravenous infusion of heparin increases the level of circulating TFPI severalfold, presumably by causing release of TFPI from binding sites on the endothelium. [33]

Heparin accelerates plasminogen activation by tissue plasminogen activator (t-PA) or urokinase but decreases the stimulatory effect of fibrin on t-PA activity. [34] Inhibition of t-PA or urokinase by plasminogen activator inhibitor 1 is unaffected by heparin. [35] The net effect of an intravenous infusion of heparin may be to enhance fibrinolysis mediated by endogenous t-PA.[36] However, heparin does not appear to enhance the thrombolytic effect of exogenously administered t-PA. [37]

Binding of Heparin to Antithrombin

Binding of heparin to antithrombin is essential to accelerate formation of antithrombin/protease complexes. When heparin is fractionated according to its ability to bind to antithrombin, the high-affinity molecules possess virtually all of the anticoagulant activity of the starting material, whereas the low-affinity molecules are inactive. [38] [39] [40] Heparin binds non-covalently to antithrombin with a dissociation constant (K_d) of 2×10^{-8} M.[41]

The high-affinity binding site for antithrombin within a heparin chain is a specific pentasaccharide structure. [42] [43] [44] This structure contains a 3-O-sulfate group that appears to be unique to the high-affinity binding site for antithrombin. Several of the sulfate groups within the pentasaccharide are essential for binding to antithrombin, whereas others do not appear to be required. In commercial heparin preparations, approximately 30% of the molecules contain this structure and bind to antithrombin with high affinity.[38] [39] [40] An identical structure is thought to arise during the biosynthesis of heparan sulfate chains, although at a much lower frequency. Heparan sulfate chains that contain this structure bind to antithrombin and stimulate protease inhibition. Other glycosaminoglycans that lack the specific pentasaccharide structure (e.g., dermatan sulfate, chondroitin-4-sulfate, or chondroitin-6-sulfate) do not bind to antithrombin with high affinity. [27]

Heparin binding induces a conformational change in antithrombin that appears to lock the glycosaminoglycan into place on the surface of the inhibitor. [41] The heparin/antithrombin complex then reacts rapidly with a target protease. Interaction with a protease reduces the affinity of antithrombin for heparin, allowing the antithrombin/protease complex to dissociate from the glycosaminoglycan chain.[45] Thus, a single heparin molecule can catalyze the formation of many antithrombin/protease complexes.

Catalytic Mechanism

Two models have been proposed to explain the catalysis of antithrombin/protease reactions by heparin. In the first model, heparin binding induces a conformational change in the reactive site of antithrombin that allows a target protease to interact more efficiently with this site. [9] [17] In the second model, the heparin chain functions as a template that binds antithrombin and the target protease simultaneously to form a ternary complex, and catalysis occurs mainly by an approximation effect. [46] Current evidence suggests that both mechanisms are valid but differ in their relative importance depending on the target protease.

The balance between the two mechanisms may explain differences in the rate enhancement for inhibition of thrombin and

factor Xa produced by heparin chains of varying length. For example, the synthetic pentasaccharide that contains only the antithrombin binding site of heparin increases the rate of inhibition of factor Xa about 270-fold but has relatively little effect on the rate of inhibition of thrombin. [47] Because an oligosaccharide of this size is unlikely to function as a template, induction of a conformational change in antithrombin may be sufficient to catalyze inhibition of factor Xa. Longer heparin chains produce an additional twofold increase in the rate of factor Xa inhibition, which may represent the contribution of the template mechanism. Stimulation of the thrombin/antithrombin reaction requires heparin molecules that contain at least 18 sugar residues (molecular weight 5,400), which is the smallest chain able to form a ternary complex with antithrombin and thrombin.[48] The factor IXa/antithrombin reaction has a similar requirement for longer heparin chains. Therefore, inhibition of thrombin and factor IXa may depend primarily on the template mechanism.

Thrombin binds to heparin with a K_d of 610 × 10^6 M under physiologic conditions.[49] Chemical modifications of thrombin that decrease its affinity for heparin greatly reduce the ability of heparin to stimulate the thrombin/antithrombin reaction. [50] Factor Xa also binds to heparin, but with a much lower affinity in comparison with thrombin. Inhibition of factor Xa by antithrombin in the presence or absence of heparin is unaffected by changes in ionic strength or by chemical modification of factor Xa to reduce its affinity for heparin,[47] [51] suggesting that catalysis of the reaction between antithrombin and thrombin, but not factor Xa, requires binding of heparin both to antithrombin and to the protease.

Heparin Cofactor II

Heparin cofactor II (M_r 66,000) is a single-chain glycoprotein that is about 30% identical in amino acid sequence to antithrombin. [52] [53] [54] It is present in plasma at a concentration of 1 M.[52] Heparin cofactor II inhibits only thrombin; it does not inhibit the other proteases generated during coagulation or fibrinolysis. [55] In contrast to antithrombin, heparin cofactor II also inhibits chymotrypsin-like proteases, albeit slowly. [55] [56]

The affinity of heparin cofactor II for heparin is lower than that of antithrombin. Therefore, an approximately 10-fold higher concentration of heparin is necessary to accelerate thrombin inhibition by heparin cofactor II in plasma. [27] By contrast, the activity of heparin cofactor II is stimulated approximately 1,000-fold by dermatan sulfate, which has no effect on the activity of antithrombin. [27] Heparin cofactor II binds to a high-affinity site in dermatan sulfate. [57]

Addition of dermatan sulfate to plasma causes prolongation of the thrombin time and activated partial thromboplastin time (PTT), [5] and intravenous infusion of dermatan sulfate produces an antithrombotic effect in experimental animals[58] [59] [60] [61] [62] and humans.[63] [64] [65] These effects appear to be mediated primarily by heparin cofactor II.[27] [66] [67] Several other natural or synthetic polyanions, including pentosan polysulfate, [68] fucoidan, [69] and chondroitin sulfate E,[70] have been reported to stimulate inhibition of thrombin by heparin cofactor II; these compounds also appear to have some activity with antithrombin.

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PHYSIOLOGIC FUNCTIONS

Vascular Heparan Sulfate

Because of the dramatic effect of heparin on the activity of antithrombin in vitro, it has been assumed that an endogenous heparin-like substance must stimulate antithrombin in vivo. Current evidence suggests that heparan sulfate proteoglycans anchored in the vessel wall interact with circulating antithrombin to produce an antithrombotic effect.

Glycosaminoglycans extracted from cloned endothelial cells have anticoagulant activity, and de novo biosynthesis of heparan sulfate proteoglycans has been demonstrated in cultured endothelial cells. [71] [72] Approximately 110% of the labeled heparan sulfate from endothelial cells binds to immobilized antithrombin with high affinity, suggesting that only this proportion contains the antithrombin-binding pentasaccharide of heparin.

Antithrombin binds to cultured bovine aortic endothelial cells (about 60,000 sites per cell) with a K_d of 12×10^{9} M, and binding is diminished by pretreatment of the cells with heparinase. [72] Electron microscopic autoradiography of ^{125}I-labeled antithrombin bound to endothelial cells in culture or after perfusion of segments of rat aorta ex vivo indicates that >90% of the antithrombin is associated with the extracellular matrix located in the subendothelium. [73] Binding of antithrombin to the subendothelial matrix of the aorta is greatly increased after crush injury, which causes detachment of most of the endothelial cells. Interaction of coagulation proteases with antithrombin bound to subendothelial heparan sulfate proteoglycans may inhibit thrombosis. [73] Interleukin-1 and tumor necrosis factor decrease heparan sulfate biosynthesis by cultured endothelial cells and reduce the amount of antithrombin that can be bound per cell by about 50%. [74] This mechanism may contribute to the increased thrombogenicity of the endothelium induced by cytokines.

When a trace amount of thrombin is injected into the circulation, the thrombin appears to become bound initially to thrombomodulin on the endothelial cell surface. [75] In comparison with free thrombin, thrombin bound to thrombomodulin reacts less rapidly with fibrinogen and heparin cofactor II, more rapidly with protein C, and at about the same rate with antithrombin. [76] The net effect of these changes in substrate specificity may be a small increase (about threefold) in the rate of the thrombin/antithrombin reaction because of diminished competition from other substrates. After the thrombomodulin has become saturated with thrombin, the excess thrombin may be inhibited rapidly by antithrombin bound to heparan sulfate proteoglycans. [77] Platelet factor 4, released by the platelet during platelet aggregation, competitively inhibits binding of antithrombin to heparan sulfate and may promote local clot formation at the site of hemostasis. [78]

Mast Cell Heparin

Heparin binds cationic molecules such as histamine and neutral proteases to form crystalline arrays within the secretory granules of mast cells, [79] but its physiologic function is otherwise unknown. Under normal circumstances, heparin is not released from mast cells into the circulation and cannot be detected in plasma. However, a small amount of heparin may appear in the circulation of patients with systemic mastocytosis and produce mild prolongation of the PTT. [80]

Tumor-Derived Heparan Sulfate

Circulating heparan sulfate, apparently released from tumor cells, has been reported to cause marked prolongation of the thrombin time and PTT and to cause bleeding in a few severely ill patients with malignancies. [81] [82] [83] [84] Protamine neutralizes the heparan sulfate anticoagulant in vitro and may be an effective treatment for patients with this type of anticoagulant. [84] [85] A coagulopathy associated with circulating heparan sulfate and dermatan sulfate has also been observed in a few patients who were treated with high-dose suramin for metastatic adrenocortical carcinoma. [86]

Inhibition of Cell Proliferation

Heparin inhibits growth of a variety of cultured cells, including endothelial cells, vascular smooth muscle cells, and renal mesangial

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cells. In addition, heparin prevents the extraordinary proliferation of vascular smooth muscle cells that follows damage to the endothelium in vivo, as demonstrated in the rat carotid artery. [87] These effects are independent of the anticoagulant activity of heparin. [88] Heparan sulfate proteoglycans synthesized by postconfluent vascular smooth muscle cells and endothelial cells also have antiproliferative activity and can be removed from the cells by a platelet heparinase. [89] This phenomenon suggests that smooth muscle cell proliferation in a damaged blood vessel might be triggered by heparinase in combination with growth factors released from the platelets. The mechanism by which heparin and heparan sulfate suppress cell growth appears to involve inhibition of a protein kinase C-dependent signaling pathway early in the cell cycle. [90]

Angiogenesis

Acidic and basic fibroblast growth factors (FGF) bind to heparin with very high affinity. [91] These growth factors are mitogens for endothelial cells, smooth muscle cells, and other mesenchymal cells, and they induce angiogenesis. Although heparin inhibits growth of capillary endothelial cells in vitro, it potentiates the growth-promoting effect of acidic FGF on these cells. [92] This effect depends on the size and degree of sulfation of the heparin molecule but not on its anticoagulant activity. Heparan sulfate proteoglycans in the extracellular matrix bind and stabilize basic FGF and may serve as a reservoir from which the growth factor can be released by an excess of heparin or digestion with heparitinase. [93] Heparan sulfate provides a low-affinity binding site for basic FGF on the surface of target mesenchymal cells. Furthermore, cell surface heparan sulfate or exogenous heparin promotes the binding of basic FGF to its high-affinity receptor (a transmembrane protein with tyrosine kinase activity) and is required for the biologic activity of basic FGF. [94] [95] Highly sulfated oligosaccharides that bind basic FGF have been isolated from heparin or heparan sulfate. [96] [97]

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CLINICAL APPLICATIONS

Pharmacologic Effects

Heparin produces an immediate anticoagulant effect when administered intravenously. At a plasma concentration of 0.11.0 U/ml, heparin causes thrombin and factor Xa to be inhibited very rapidly (half-lives <0.1 second) by antithrombin. Higher concentrations of heparin (>5 U/ml), which may be present transiently after a bolus intravenous infusion, may also stimulate thrombin inhibition by heparin cofactor II. [26] These effects result in prolongation of the PTT and the thrombin time. Although the prothrombin time may also be prolonged, it is affected to a lesser degree by heparin. LMW heparin and dermatan sulfate have substantial antithrombotic activity at doses that do not predictably affect the PTT. [58] [98]

Heparin clears lipemic plasma in vivo by causing the release of lipoprotein lipase from the endothelium into the circulation. Lipoprotein lipase hydrolyzes triglycerides present in chylomicrons and very-low-density lipoproteins to glycerol and free fatty acids. [99] The clearing of lipemic plasma may occur at a dose of heparin below that which produces an anticoagulant effect, and rebound hyperlipemia may occur after heparin is discontinued. The effects of heparin on lipid metabolism are striking but have no obvious clinical importance.

Preparations

Heparin for therapeutic use is commonly extracted from porcine intestinal mucosa or bovine lung. During the isolation of heparin from these sources, the core protein is removed, and the glycosaminoglycan chains become degraded slightly to yield a heterogeneous mixture of fragments with a mean molecular weight of 12,000 (range 5,00030,000). These preparations may also contain small amounts of other glycosaminoglycans. [100] Despite the heterogeneity in source and composition among different commercial heparin preparations, their biologic activities are similar (150 USP U/mg).

The USP unit is defined as the quantity of heparin that will prevent 1.0 ml of citrate-anticoagulated sheep plasma from clotting for 1 hour after the addition of 0.2 ml of 1% calcium chloride. Standard heparin extracted from porcine intestinal mucosa or bovine lung is available as the sodium salt in aqueous solutions of 10,00040,000 USP U/ml. The calcium salt of heparin from porcine intestinal mucosa has been associated with a lower frequency of local hematoma when injected subcutaneously and is used commonly in Europe in a low-dose regimen for prevention of thromboembolism. [101]

The LMW heparins (mean molecular weight 4,0006,500) are isolated from standard heparin by gel filtration chromatography, precipitation with ethanol, partial depolymerization with nitrous acid, or other chemical techniques. [102] LMW heparin preparations accelerate factor Xa inhibition at a dosage similar to that of standard heparin but have greatly reduced thrombin inhibitory activities. The ratio of antithrombin to anti-factor Xa activity of a given preparation depends largely on the percentage of molecules that are of sufficient length to accelerate the thrombin/antithrombin reaction. [103] The LMW heparins are being used with increasing frequency in patients because of their increased convenience, and in some cases efficacy, when compared to standard heparin.

The heparinoid danaparoid sodium is a mixture of several types of nonheparin glycosaminoglycans (84% heparan sulfate, 12% dermatan sulfate, 4% chondroitin sulfate) extracted from porcine intestinal mucosa. Danaparoid sodium is an effective anticoagulant that has been used for the treatment of acute deep vein thrombosis,[104] prevention of deep vein thrombosis in patients at high risk, [105] [106] [107] [108] and the treatment of patients with heparin-associated thrombocytopenia. [109] [110]

Absorption and Pharmacokinetics

Heparin is not absorbed through the gastrointestinal mucosa and, therefore, must be given parenterally. It is usually administered by continuous intravenous infusion or subcutaneous injection. Heparin has an immediate onset of action when given intravenously. The onset of action of subcutaneous heparin generally occurs within 2060 minutes. The pharmacokinetic profiles of standard heparin vary widely between individuals, as a result of protein binding of the heparin within the plasma. [111] [112] [113] [114] The LMW heparins demonstrate much less protein binding. Thus they have a predictable dose-response profile when administered by subcutaneous or intravenous injection. [102] [111]

Following a bolus intravenous injection, elimination of heparin activity from the blood can be described by first-order kinetics. However, the half-life of standard heparin varies with the dose administered. [115] The half-life of the anticoagulant activity in humans is approximately 1, 1.5, and 2.5 hours at doses of 100, 200, and 400 U/kg, respectively. Heparin appears to be cleared and degraded primarily by the reticuloendothelial system. A small amount of undergraded heparin is excreted in the urine. The half-life of heparin may be shortened in patients with pulmonary embolism and prolonged in patients with hepatic cirrhosis or end-stage renal disease. [116]

The LMW heparins have longer biologic half-lives than standard heparin preparations. Although it is thought that LMW heparins are eliminated in large part by renal excretion,[117] the half-life of reviparine (an LMW heparin) was only slightly prolonged in dialysis-dependent patients when compared with healthy controls. In addition, the half-life of reviparine was near normal while the patients were undergoing dialysis. [118] Of note,

each LMW heparin preparation is produced using a unique process; as a result, the products are not interchangeable, having different pharmacokinetic profiles. [119]

Administration and Dosage

Continuous intravenous infusion is the usual method of administration of full-dose heparin because the incidence of bleeding complications is lower in comparison with intermittent infusions. [120] Heparin therapy can be initiated with a bolus injection of 5,000 U followed by 1,0001,300 U/hr delivered by an infusion pump. Adjusted-dose subcutaneous administration can be used for the long-term management of patients in whom warfarin is contraindicated (e.g., during pregnancy). A daily dose of 25,00035,000 U given as divided doses every 12 hours is usually sufficient to achieve a therapeutic effect similar to that of intravenous heparin. If therapeutic doses of subcutaneous heparin are used, a PTT should be performed 6 hours after the injection, and the dose titrated to achieve a PTT in the therapeutic range. Low-dose heparin (5,000 U subcutaneously every 8 or 12 hours) decreases the incidence of venous thromboembolism in certain high-risk patients; however, it is associated with a slight increase in the risk of perioperative hemorrhage. [121]

The LMW heparins are usually administered using a weight-adjusted dose subcutaneously once or twice daily, but they can be administered intravenously. Dosing of LMW heparins varies among products (Table 1221). The LMW heparins may be preferable to standard heparin for long-term subcutaneous administration because

they do not require monitoring, have a lower risk of producing heparin-induced thrombocytopenia, [122] and may be associated with a reduced risk of osteoporosis. [123] [124]

Laboratory Monitoring

Therapy with standard heparin is usually monitored with a global test of coagulation, such as the whole blood clotting time, whole blood recalcification time, or PTT. Because of its convenience and rapid turn-around time, the PTT is the most widely used test. The optimal PTT therapeutic range for standard heparin is that which produces an equivalent anti-Xa heparin level of 0.30.7 U/ml. The PTT value that corresponds to this range can vary widely between PTT reagents and testing equipment. In most centers, the optimal range will be 6590 seconds. Major bleeding complications can occur even though the PTT is within the therapeutic range. [125] [126] [127]

Because of the fear of bleeding from overdosage, intuitive adjustments in the heparin infusion rate often result in subtherapeutic PTT results during the first 2448 hours of treatment.[127] Recent prescription guidelines for continuous intravenous heparin have reduced the incidence of subtherapeutic PTT results to <2% of patients during the first 24 hours. Supratherapeutic PTT occurred in about two-thirds of patients who received warfarin simultaneously, but the risk of bleeding was not increased in these patients.[127] Although laboratory monitoring of low-dose subcutaneous heparin is generally unnecessary, care should be taken in patients with low body mass, who may become therapeutically anticoagulated at a standard prophylactic dose.

The LMW heparins do not produce a predictable prolongation of the PTT at therapeutic doses. Fortunately, the LMW heparins have predictable pharmacokinetics and can be administered using a weight-based algorithm without laboratory monitoring. [128] However, if monitoring is required, an anti-Xa heparin level using an LMW heparin standard should be performed. The target anti-Xa heparin range for the treatment of acute venous thromboembolism is 0.51.5 anti-Xa U/ml; for the prophylaxis of venous thromboembolism, a level of 0.20.5 U/ml is adequate.

As with the LMW heparins, monitoring of danaparoid sodium cannot be performed using the PTT. Rather an anti-Xa heparin level, using a standard curve of known danaparoid sodium concentrations, is used. The optimal anti-Xa range for danaparoid sodium anticoagulation (either therapeutic or prophylactic) has not been established. Therefore, it is clinical practice to target a range similar to that used for the LMW heparins.

TABLE 122-1 -- Indications and Doses of Low-Molecular-Weight Heparins

Indication	Drug	Dose	Frequency
Prophylaxis			
General surgery			
Low risk	Dalteparin	2,500 U	12 hr preoperatively then once daily after surgery
	Enoxaparin	2,000 U	12 hr preoperatively then once daily after surgery
	Nadroparin	3,100 U	2 hr preoperatively then once daily after surgery
High risk	Dalteparin	5,000 U	1012 hr preoperatively then once daily after surgery
	Enoxaparin	4,000 U	1012 hr preoperatively then once daily after surgery
Orthopedic surgery	Ardeparin	50 U/kg	Twice daily after surgery
	Dalteparin	5,000 U	812 hr preoperatively and once daily after surgery
	Enoxaparin	3,000 U	Twice daily after surgery
	Enoxaparin	4,000 U	1012 hr preoperatively then once daily after surgery
	Nadroparin	40 U/kg	2 hr preoperatively then once daily after surgery for 3 days, then increase dose to 60 U/kg
	Tinzaparin	50 U/kg	2 hr preoperatively then once daily after surgery
	Tinzaparin	75 U/kg	Once daily beginning 1224 hr after surgery
Acute spinal injury	Enoxaparin	3,000 U	Twice daily
Multiple trauma	Enoxaparin	3,000 U	Twice daily
Medical conditions	Dalteparin	2,500 U	Once daily
	Enoxaparin	2,000 U	Once daily
Treatment			
Venous thrombosis	Dalteparin	100 U/kg	Twice daily
	Enoxaparin	100 U/kg	Twice daily
	Nadroparin	90 U/kg	Twice daily
	Tinzaparin	175 U/kg	Once daily
Unstable angina	Dalteparin	100 U/kg	Twice daily
	Enoxaparin	100 U/kg	Twice daily

Heparin Resistance

The dose of heparin required to produce a therapeutic PTT varies from patient to patient. The variability is due to differences in the plasma concentrations of heparin-binding proteins such as histidine-rich glycoprotein, vitronectin, and platelet factor 4, which competitively inhibit binding of heparin to antithrombin. [78] [129] [130] In patients with venous thromboembolism, the therapeutic dose is usually <35,000 U/day. Occasionally, the PTT will fail to become prolonged unless a very high dose (>50,000 U/day) of heparin is administered. The level of heparin in these patients may be therapeutic at the usual dose when measured by other tests (e.g., anti-factor Xa activity or protamine sulfate titration). The majority of these "heparin-resistant" patients have high levels of factor VIII as well as heparin-binding proteins (which are acute phase reactants) in their plasma. Patients with inherited antithrombin deficiency ordinarily have 4060% of the normal plasma concentration of this inhibitor, and the PTT responds normally to heparin in most of these patients. [131] [132] However, acquired antithrombin levels of <25% of normal may occur in patients with hepatic cirrhosis,[133] nephrotic syndrome,[134] or disseminated intravascular coagulation (DIC).[135] In these instances, large doses of heparin may not prolong the PTT. Heparin itself can cause a modest (15%) decrease in the circulating antithrombin concentration, but this effect is unlikely to have clinical significance. [136] [137]

Indications for Therapy

Venous Thromboembolism

Standard or LMW heparins are used in the initial treatment of venous thromboembolic disease because of their rapid onset of action. In patients with deep vein thrombosis, initial treatment with a vitamin K antagonist alone is inferior to the combination of heparin plus a vitamin K antagonist in preventing further thromboembolic complications.[138] Under most circumstances, oral vitamin K antagonists are preferred to heparin or LMW heparin for long-term treatment of thromboembolism because of their convenience and lower cost. However, it is likely that long-term treatment with subcutaneous LMW heparin or standard heparin is at least as safe and

effective as treatment with oral anticoagulants.

Patients with acute venous thromboembolism can be treated safely and effectively with subcutaneous LMW heparin administered out of hospital. Three large, well-designed studies have demonstrated that outpatient treatment of acute deep vein thrombosis using unmonitored, weight-adjusted LMW heparin is as safe and effective as inpatient treatment with adjusted dose intravenous standard heparin. [139] [140] [141] In addition, one of these trials and a separate study [142] have demonstrated the safety and efficacy of treatment of acute pulmonary embolism with LMW heparin. Subsequent clinical experience has demonstrated that the majority (>80%) of unselected patients presenting with acute venous thromboembolism can be safely and effectively treated as outpatients. [143]

Oral anticoagulants can be initiated on the day of presentation with acute venous thromboembolism. However, heparin therapy should be continued for a minimum of 4 days, irrespective of the International Normalized Ratio value. The 4-day overlap of heparin and warfarin is required to ensure adequate anticoagulation during the initial days of warfarin, when an acute warfarin-induced fall in the levels of protein C produces a potential hypercoagulable state, during which acute extension of thrombosis could occur.

Occasional patients will suffer recurrent thromboembolism despite therapeutic anticoagulation with warfarin. These patients can be effectively treated with long-term adjusted-dose standard heparin, or long-term weight-adjusted unmonitored LMW heparin.

Acute Coronary Syndromes

When used alone, heparin is effective in the short-term treatment of unstable angina, but a rebound is seen when the drug is stopped. [144] Aspirin appears to prevent the cluster of ischemic events that occurs when heparin is discontinued. Evidence suggests that the addition of heparin to aspirin improves short-term outcome in patients with unstable angina, but it is uncertain whether this effect is sustained.

Several large studies have recently suggested that LMW heparin in combination with aspirin is at least as effective as standard heparin in patients with unstable angina. [145] [146] [147] These studies, two of which used dalteparin and one of which used enoxaparin, compared the use of subcutaneous LMW heparin with intravenous standard heparin. Once again, the major advantage of LMW heparin in this patient population is its predictable pharmacokinetics that allow weight-based dosing without monitoring.

In patients with acute myocardial infarction, heparin has been reported to reduce reinfarction and death. Moderate doses of heparin (12,500 U subcutaneously 12 hourly) reduce the incidence of mural thrombosis detected by two-dimensional echocardiography, which is a particular problem in patients with anterior wall myocardial infarction. [148]

Heparin prevents early reocclusion of the infarct-related artery after successful thrombolysis with t-PA. However, a recent meta-analysis [149] has suggested that routine administration of therapeutic doses of heparin to patients with acute myocardial infarction is not indicated. This analysis demonstrated that heparin therapy reduced mortality by 6% (95% confidence intervals 010%), and the rate of reinfarction by 1.3%; however, heparin was associated with a clinically important increase in the risk of major hemorrhage that outweighed its observed benefit. Patients with acute myocardial infarction should continue to receive prophylactic doses of heparin (5,000 U every 8 or 12 hours) to reduce the risk of venous thromboembolism.

Cerebrovascular Thromboembolism

The appropriate use of heparin or LMW heparin in patients with ischemic stroke remains poorly defined. Nonambulatory patients may benefit from low doses of heparin to prevent venous thromboembolism. Heparin and LMW heparin do not appear to improve the outcome of patients with partial stable or complete stroke. [150] Although an initial study [151] suggested the administration of fraxiparine to patients with acute cerebral ischemic improved long-term outcome, this finding has not been replicated in several subsequent larger studies. The use of heparin to treat patients with acute embolic stroke of cardiac origin, usually in the presence of nonrheumatic atrial fibrillation, remains controversial because the risk of central nervous system bleeding appears to be high in this situation. [152] Heparin therapy in the management of transient ischemic attacks has not been shown to offer improved survival or a reduced incidence of subsequent strokes. [150]

Other Indications

Disseminated Intravascular Coagulation

Acute DIC is frequently treated with low-dose heparin. DIC is characterized by uncontrolled activation of coagulation, which results in microvascular thrombosis and bleeding due to clotting factor depletion. It is felt that heparin, by catalyzing the inactivation of thrombin, will reduce uncontrolled activation of thrombin and thus lessen the rate at which coagulation factors are being depleted. There is no clinical evidence that this treatment strategy is valid. Furthermore, the potential to exacerbate hemorrhage is substantial. As a result, anticoagulant therapy in patients with acute DIC should be reserved for those patients with objectively confirmed thrombosis.

Patients with acute promyelocytic leukemia frequently have bleeding associated with laboratory evidence of DIC. In the past, low-dose heparin by continuous infusion has been recommended for these patients. The DIC associated with acute promyelocytic leukemia frequently resolves within 48 days after the initiation of all-trans retinoic acid, although this therapy might not reduce the risk of thrombosis in these patients. [153] In the absence of acute thromboembolism, treatment with heparin is not recommended in this setting.

Trousseaus syndrome is a complication of malignancy (most often adenocarcinoma) characterized by venous thromboembolism, arterial embolic disease, and nonbacterial endocarditis. Laboratory findings are consistent with chronic DIC, including mild or moderately severe thrombocytopenia. Many patients with Trousseaus syndrome will suffer recurrent thromboembolism despite adequate anticoagulation with warfarin. In such patients long-term treatment with subcutaneous standard heparin or LMW heparin is recommended. [154] [155] The role of heparin in DIC of other etiologies has yet to be established.

Heparin (100 U/kg/day) has been used in the prevention of hepatic veno-occlusive disease after bone marrow transplantation. A prospective, randomized study found this regimen to be highly effective in the prevention of veno-occlusive disease, but other studies have been less successful. [156] [157] Treatment of veno-occlusive disease with heparin after organ dysfunction has developed is generally unsuccessful. [158]

Toxicities

Bleeding

Bleeding is the principal toxicity of heparin. Major bleeding has been reported to occur in 133% of patients receiving various forms of heparin therapy, and fatal bleeding occurred in 3 of 647 patients treated with heparin in one series. [159] Often an underlying cause for bleeding is present, such as recent surgery, trauma, peptic ulcer disease, or platelet dysfunction. Heparin can interfere with platelet function and prolong the bleeding time, but it is unclear to what extent the antiplatelet effect contributes to the hemorrhagic complications of heparin therapy. [160] There is no evidence that the PTT response achieved during intravenous standard heparin therapy correlates with the risk of hemorrhage. Furthermore, there is only weak evidence of a dose-dependent relationship in this bleeding risk. [161]

Four randomized studies have compared the incidence of bleeding during intermittent and continuous intravenous infusion of heparin. Two of the studies demonstrated a significantly lower incidence of bleeding in the patients receiving continuous intravenous infusions (01% versus 933%), [126] [162] and a third study reported a similar trend. [163] However, the total dose of heparin administered by intermittent infusion was higher than that administered by continuous infusion. When the same doses were used in one study, the incidence of bleeding was identical, implying that bleeding was related to the dosage and not to the method of administration. [164] In a study in which patients considered to be at high risk of bleeding were initially treated with a lower dose of heparin (30,000 U/day) than those at low risk for bleeding (40,000 U/day), the incidence of major bleeding was 11% in the high-risk group compared with 1% in the low-risk group. [165] Taken together, these

studies suggest that the incidence of bleeding depends both on the total daily dose of heparin and on the presence of underlying risk factors.

Several randomized studies have compared a continuous intravenous infusion of standard heparin with a fixed dose of subcutaneous LMW heparin in treatment of venous thrombosis.[166] [167] [168] The incidence of major bleeding appears to be lower during therapy with LMW heparin, although this finding was not confirmed in larger recent studies.[139] [140] [141]

Heparin-Induced Thrombocytopenia (HIT)

Heparin-induced thrombocytopenia (as defined by a fall in the platelet count to <150 × 10^9 /L or a decrease by 50% in patients with pre-existing thrombocytopenia) occurs in 2.7% of patients receiving intravenous standard heparin for the treatment of acute venous thrombosis. It occurs less frequently in patients receiving therapeutic doses of LMW heparin.[122] HIT is due to an autoantibody that is directed against heparin in association with platelet factor 4.[169] This antibody binds to and activates the Fc receptor on the platelet surface, leading to platelet activation. A diagnosis of HIT should be considered in any patients receiving heparin (either intravenously or subcutaneously) who have an unexplained 50% or greater reduction in platelet count that begins 5 or more days after starting heparin, provided that the patient has not previously received heparin. HIT can develop within 48 hours in patients with previous heparin exposure. Heparin should be discontinued immediately in patients with suspected HIT. In patients with evidence of arterial or venous thrombosis, an alternate anticoagulant should be administered. LMW heparin should *NOT* be used in this situation because of a high risk of cross-reactivity of the heparin-dependent antibody with the LMW heparin. In this situation another anticoagulant such as danaparoid sodium or a direct thrombin inhibitor (such as hirudin or argatroban) should be given.[170] Ancrod, a defibrinogenating snake venom, has also been used in this setting but may not be as effective as other agents, because it does not block thrombin generation and subsequent thrombin-mediated platelet activation. Warfarin should not be started until adequate anticoagulation with these agents has been achieved and the platelet count has returned to the normal range, because it can induce skin necrosis or worsen venous or arterial thromboembolism in patients with HIT.[171] Patients with HIT who have not had thrombosis are likely at high risk for the development of thrombosis. Whether such patients should receive anticoagulants is controversial. Such patients can receive prophylactic or therapeutic doses of danaparoid, although the most appropriate treatment for these patients is currently under investigation.

Osteoporosis

Radiographic evidence of osteopenia has been reported in 17% of women treated with heparin throughout pregnancy.[172] In most cases, the osteopenia resolves within 1 year after delivery. Spontaneous osteoporotic vertebral fractures occur in 23% of pregnant women receiving heparin.[172] [173] High-dose heparin and prolonged duration of treatment appear to enhance the risk of osteoporosis. Several pregnant patients with thromboembolism have been treated successfully with LMW heparin, but the relative risk of osteoporosis in comparison with standard heparin is unknown.[102] [174] The risk of osteoporosis in nonpregnant patients receiving long-term heparin has not been established.

Other Toxicities

Abnormalities in liver function tests occur frequently in patients receiving intravenous or subcutaneous heparin. Mild elevations of the aspartate transaminase and alanine transaminase occur without a concomitant increase in serum bilirubin or alkaline phosphatase.[175] Cutaneous allergic reactions to subcutaneous heparin occur rarely. In many cases, these reactions do not occur when LMW heparin is substituted for standard heparin. In addition, if a cutaneous allergic reaction occurs with one form of LMW heparin, clinical experience suggests it may not occur if another form is substituted.

Use in Pregnancy

In contrast to warfarin, heparin does not cross the placenta and has not been associated with fetal malformations. Therefore, despite

the risk of osteoporosis, heparin is the drug of choice for the prophylaxis and treatment of venous thromboembolism during pregnancy.[176] Heparin may also prevent thromboembolic complications associated with mechanical heart valves.[176] Heparin can be administered either by intermittent adjusted-dose subcutaneous injections or by continuous intravenous infusion. Ambulatory management may be facilitated by the use of implantable intravenous ports and indwelling subcutaneous catheters. It has been recommended that pregnant patients with venous thromboembolism be anticoagulated throughout the course of pregnancy and for approximately 68 weeks after delivery.[176]

There are no methodologically adequate clinical studies examining the efficacy of LMW heparin in pregnant patients. LMW heparin does not appear to cross the placenta.[177] [178] Their reduced requirement for monitoring and potentially lower risk of osteoporosis make the LMW heparins attractive anticoagulants in pregnancy.

An elevated PTT may be associated with increased bleeding at the time of delivery. In the nonpregnant patient, a single dose of subcutaneous heparin is usually cleared within 12 hours, but during the third trimester of pregnancy the PTT may remain prolonged as long as 28 hours after a subcutaneous dose of heparin.[179] It has been recommended that heparin be discontinued 24 hours before induction of delivery or immediately at the onset of labor.

Antagonist

The anticoagulant effect of heparin disappears within hours after discontinuation of the drug, and mild bleeding due to heparin can usually be controlled without administration of an antagonist. If life-threatening hemorrhage occurs, the effect of heparin can be reversed quickly by intravenous infusion of the sulfate salt of protamine, a mixture of basic polypeptides isolated from salmon sperm. Protamine binds tightly to heparin in vitro and thereby neutralizes its anticoagulant effect. The typical dose of protamine sulfate is 1 mg/100 U heparin. Neither the route (intravenous or arterial) nor the rate of administration of protamine has been found to be of clinical importance.[180]

Protamine is used routinely to reverse the anticoagulant effect of heparin following cardiac surgery and other vascular procedures. An antibody-mediated anaphylactic reaction may occur within minutes after receiving the antagonist. This complication occurs in approximately 1% of patients with diabetes mellitus who have received protamine-containing insulin (neutral protein Hagedorn insulin or protamine zinc insulin) but is not limited to this group.[181] This reaction can be fatal.[182] A less common nonimmunologic reaction consisting of pulmonary vasoconstriction, right ventricular dysfunction, and systemic hypotension associated with transient neutropenia may also occur after administration of protamine.[183] [184]

The effectiveness of protamine in patients who have received LMW heparin is unknown. However, as there is no other effective antidote, administration of protamine to a bleeding patient who has received a dose of LMW heparin seems appropriate.

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Chapter 123 - Thrombolytic Therapy

Victor J. Marder

Thrombolytic therapy represents a conjoint approach to vascular reperfusion, based primarily on the use of fibrinolytic agents delivered systemically or directly into the offending thrombus and complemented by anticoagulation, antiplatelet, and even mechanical strategies. In theory, any major thrombosed vessel that is the cause of clinically significant disease is amenable to this therapeutic approach, and the decision to use thrombolytic therapy requires an informed choice between the potential for clinical benefit and the risk for serious complication. In actuality, thrombolytic therapy represents an acute phase of a prolonged antithrombotic management plan, and the decision regarding the benefit:risk ratio relates to this usually brief but more aggressive approach to vascular occlusion. This review and others [1] [2] [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] describe the properties of thrombolytic agents (the plasminogen activators), the regimens that include adjunctive antithrombotics, the physiopathology of successful thrombolysis and bleeding complications, effects of treatment on coagulation and fibrinolytic parameters, and clinical results in the major venous and arterial thrombotic disorders. The clinical trials that have been systematically pursued for deep vein thrombosis, pulmonary embolism, acute myocardial infarction, peripheral arterial occlusion, and ischemic stroke (cerebrovascular accident [CVA]) are presented.

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FIBRINOLYTIC AGENTS

Mode of Action

All of the plasminogen activators share the potential of inducing plasmin action on fibrin contained within a thrombus, with an associated greater or lesser degree of plasma fibrinogenolysis (lytic state). Degradation of fibrin produces the beneficial effect of reducing thrombus size (thrombolysis), but at the same time, the activators may lyse hemostatic plugs or induce matrix degradation at sites of vascular injury or abnormality (Table 1231). Rethrombosis of the vessel may follow initial reperfusion, usually as a result of a persistent local vascular lesion and a permissive or predisposing state of plasma coagulation. The relationships of these biologic actions of thrombolysisloss of vascular integrity, rethrombosis, and the plasma lytic statedetermine

TABLE 123-1 -- Principal Biologic Effects of Thrombolytic Therapy

Clinical Result	Process	Cellular and Biochemical Events
Benefit	Thrombolysis	Degradation of fibrin and disruption of platelets in the thrombus
Side effect	Systemic lytic state	Plasma fibrinogenolysis and derangement of platelet function
Complications	Bleeding	Degradation of fibrin in hemostatic plugs and of matrix in abnormal vessels, plus blood hypocoagulability
	Rethrombosis	Persistent local vascular lesions plus permissive status of blood coagulation

Figure 123-1 (Figure Not Available) Molecular interactions of physiologic and therapeutic thrombolysis. In the top panel, therapeutic administration of plasminogen activator (PAI-1) accelerates thrombolysis. Free plasmin in the blood exceeds the capacity of antiplasmin to neutralize the protease activity, resulting in fibrinogen degradation and the so-called plasma proteolytic state. In the bottom panel, tissue plasminogen activator is released from endothelial cells in response to a variety of stimuli, mimicking therapeutic administration of plasminogen activator. Free plasmin degrades fibrinogen and soluble fibrin, producing both fibrinogen and fibrin degradation products. *(Reproduced with permission from Francis and Marder.[15])*

the effectiveness and safety of thrombolytic treatment.

Figure 1231 (Figure Not Available) illustrates the molecular interactions by which such treatment is locally augmented at sites of thrombi. [15] [16] Plasminogen and plasminogen activators bind to or react in contiguity with fibrin in the thrombus. Fibrin facilitates the conversion of plasminogen to plasmin, because plasminogen activator inhibitor-1 (PAI-1) and $_2$ -plasmin inhibitor are not efficient for inhibiting thrombus-bound plasminogen activator and plasmin. Plasmin in turn degrades fibrin to fibrin degradation products. Under physiologic conditions, systemic fibrinogenolysis does not occur because tissue plasminogen activator (t-PA) and plasmin are efficiently inhibited by PAI-1 and $_2$ -plasmin inhibitor in the blood. However, with administration of therapeutic dosages of plasminogen activator, virtually all of the plasma plasminogen is converted to plasmin, overwhelming the neutralizing capacity of antiplasmin and leading to some degree of fibrinogenolysis. At the same time, the administered plasminogen activator maximally converts thrombus-bound plasminogen to plasmin, resulting in accelerated thrombolysis. The relationship of the lytic state to hemorrhagic complications and to vascular reperfusion and rethrombosis is an ongoing question of study, with significant implications for the application of laboratory results in predicting clinical outcome.

Available Plasminogen Activators

Five plasminogen activators, streptokinase, [17] urokinase,[18] [19] alteplase (t-PA),[20] [21] anistreplase,[22] [23] and reteplase[24] have been approved by the Food and Drug Administration (FDA) for use in the major thrombotic diseases (Table 1232). These and other plasminogen activators have also been applied in virtually all potential clinical indications, not always with proof of efficacy. However, their clear benefit in reducing short- and long-term mortality after acute myocardial infarction has spawned a plethora of new plasminogen activator variants, principally by

TABLE 123-2 -- Comparison of Plasminogen Activators

Agent	FDA Approval	Source	Relative Cost	Antigenic	Half-life (min)	Regimen	Lytic State	Bleeding
Streptokinase	Yes	*Streptococcus*	1	Yes	20	Infusion	4+	4+
Urokinase	Yes	Cell culture	6	No	15	Infusion	4+	4+
Alteplase (t-PA)	Yes	Recombinant	8	No	5	Infusion	2+	4+
Anistreplase	Yes	*Streptococcus* + plasma product	5	No	70	Bolus	4+	4+
Reteplase	Yes	Recombinant		No	15	Double bolus	3+	4+
Saruplase (scu-PA)	No	Recombinant		No	5	Infusion	13+	4+
Staphylokinase	No	Recombinant		Yes		Infusion	1+	4+
TNK-t-PA[a]	No	Recombinant		No	15	Bolus	1+	4+
BAT-PA[b]	No	Recombinant		Minimal		Infusion	1+	Probable

t-PA, tissue plasminogen activator.

[a] TNK-t-PA is the abbreviation for a t-PA mutant that has been altered at six sites: threonine (T) at position 103, asparagine (N) at position 117, and four residues starting with lysine (K) at positions 296299.

[b] BAT-PA is derived from the salivary gland of a bat species by recombinant technology.

recombinant DNA technology, in attempts to attain improved properties, such as greater activity, prolonged half-life, or a lessened lytic state. For example, the newest approved plasminogen activator, reteplase, consists of the second kringle and the protease domain of alteplase, and the prolonged half-life (from 5 to 15 minutes), allows it to be administered as a double-bolus regimen. [25] [26] A second alteplase variant, TNK-PA,[27] is altered at three specific amino acid sites and is effective as a single-bolus injection.[28] Recombinant forms of urokinase, [29] saruplase (pro-urokinase, scu-PA),[30] [31] staphylokinase,[32] and BAT-PA[33] (from the salivary gland of *Desmodus rotundus*) are at various stages of testing, and chimerics of t-PA and pro-urokinase [34] and bifunctional agents composed of anti-fibrin or anti-platelet antibodies complexed to plasminogen activator[35] show encouraging results in animal models.

The major distinctions between the agents relate to their antigenicity, half-life, potential for inducing a lytic state, and hemorrhagic potential (Table 1232). Those plasminogen activators that are derived from a human protein (urokinase, alteplase, reteplase) are essentially nonantigenic, whereas those from a bacterial species, whether purified from streptococci as streptokinase complexed with human plasminogen (anistreplase), or prepared by recombinant technology from staphylococci as staphylokinase,[36] can induce antibody formation and present a potential for allergic response that could preclude prolonged or follow-up administration. The experience with BAT-PA in humans is limited, but data suggest that treatment is tolerated and that antibody induction may not preclude a full course of therapy.

The half-life of each plasminogen activator determines whether it can be administered as a bolus injection or short infusion, or if a continuous infusion is needed. Among the approved agents, the most suitable for bolus injection is anistreplase (half-life of æ40 minutes) [37] and the least suitable is alteplase (half-life of 5 minutes),[38] which requires continuous infusion for therapy. The rate of infusion of streptokinase may be limited by side effects of tachycardia, fever, and muscle aches even though its half-life is 20 minutes. [37] Results in patients suggest that some of the newer agents have less of a plasma lytic state, especially TNK-t-PA. [39] Although it is an attractive hypothesis that minimizing fibrinogen degradation will result in less clinical bleeding, [40] this potential has not been borne out in clinical trial (see later). To date, all of the approved plasminogen activators and those with extensive clinical trial data are associated with a significant bleeding risk, roughly equivalent for all agents, except for the higher rate of intracranial hemorrhage with t-PA. [41] The anticipation of greater safety with a new plasminogen activator, based on biochemical studies, must be treated with caution, and no agent has been shown to be free of hemorrhagic risk.

Adjunctive Antithrombotic Agents

Inadequate response to plasminogen activator therapy occurs if the thrombosed vessel does not manifest full reperfusion, or if reocclusion quickly follows initial success (Table 1233). In

TABLE 123-3 -- Potential Avenues for Improving Patency Results of Thrombolysis Using New Antithrombotics

Agent	Vascular Response	Need for Better Adjunctive Agent	Example
Streptokinase	Slow-progressive reperfusion Low reocclusion	Potent antithrombin to accelerate reperfusion	Hirudin, hirulog small molecule inhibitors
Tissue plasminogen activator	Rapid early reperfusion Later reocclusion (58%)	Improved antiplatelet agent to passify the vessel wall	Abciximab, other antiglycoprotein IIb/IIIa agents

general, t-PA and recombinant derivatives have potent thrombolytic activity and achieve early vascular patency with relatively mild effects on blood coagulation, but are vulnerable to early reocclusion. Agents such as streptokinase elicit a more potent effect on coagulation and reperfuse vessels more gradually, but tend to maintain vascular patency. Variations in plasminogen activator use have attempted to circumvent one or the other limitation, such as by combined therapy with the two types of plasminogen activator. Unfortunately, the combination of t-PA plus streptokinase does not produce a superior clinical result [42] for patients with acute myocardial infarction. Acknowledging that early and persistent patency is a principal objective of treatment, considerable effort has been directed to adjunctive management with antithrombin and antiplatelet agents.

The first definitive demonstration of antiplatelet efficacy in the treatment of an acute thrombotic event was with the use of a simple aspirin regimen of 160 mg/day for 30 days to decrease mortality after acute myocardial infarction (Second International Study of Infarct Survival [ISIS-2]). [43] In patients treated within 6 hours, aspirin reduced the 35-day mortality rate by 23%, equal to the reduction achieved by streptokinase alone, and both agents together had an additive effect amounting to a 39% relative risk reduction compared with patients who received neither thrombolytic nor aspirin. Whether the addition of heparin to a regimen of plasminogen activator plus aspirin would further improve the clinical outcome in patients with acute myocardial infarction is resolved for streptokinase use but still controversial for t-PA. The Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) study [42] definitively illustrated that early intravenous heparin is no more effective than delayed subcutaneous heparin in patient survival at 30 days after acute myocardial infarction. Coronary artery patency studies show that intravenous heparin in addition to streptokinase or anistreplase (plus adequate aspirin) does not increase the incidence of vascular patency, [44] [45] but does increase bleeding complications.[45] Although the use of delayed subcutaneous heparin in addition to aspirin is relatively safe, Collins et al [10] conclude that there is little evidence of any additional clinical advantage with subcutaneous or with intravenous heparin added to plasminogen activator therapy. There still is a difference of opinion as to the value of heparin added to a regimen of t-PA plus aspirin. When the dose of aspirin is 160 mg/day or more, heparin produces either no increase in patency [46] [47] or a marginal improvement.[48] With 80 mg/day or less aspirin, heparin exerts a marked effect on vascular patency. [49] [50] Thus, although there is general agreement that heparin does not contribute significantly to a regimen of streptokinase plus aspirin, [10] [51] the practice of physicians perseveres in using intravenous heparin in concert with t-PA and aspirin.[51] [52]

In patients with nonlife-threatening thrombotic disease, such as deep vein thrombosis or even many cases of peripheral arterial occlusion, where prolonged plasminogen activator therapy increases the at-risk interval for bleeding complications, concomitant anticoagulant therapy with heparin is usually avoided.

Among the multitude of potential improved adjunctive agents, two groups have received the most attention, the hirudin/hirulog [53] [54] or small molecule inhibitors of thrombin [55] and inhibitors of the glycoprotein (GP) IIb/IIIa platelet fibrinogen receptor by small peptides or chemicals or by monoclonal antibody. [56] [57]

Pilot studies in patients with acute myocardial infarction showed encouraging increases in 90-minute patency rates with hirudin rather than heparin. [58] [59] [60] Unfortunately, these initial studies used relatively high dosage schedules of anticoagulant, and unacceptable rates of intracranial hemorrhage resulted. [61] [62] [63] With a scaled-back dosage regimen of anticoagulant in patients receiving t-PA or streptokinase therapy along with aspirin, a large trial of 3,002 patients (Thrombolysis and Thrombin Inhibition

in Myocardial Infarction [TIMI] 9B) showed no significant difference in clinical outcome using heparin or hirudin. [64] Because most of the patients in this study received t-PA, it remained to be determined whether a newer antithrombin might be beneficial in patients receiving streptokinase. Based on promising results of hirulog in patients with unstable angina,[65] a patency study of 412 patients showed significantly greater patency (46% vs. 35%) and less bleeding using hirulog rather than heparin.[66] It is possible that the relatively high 90-minute patency rate attained with t-PA plus aspirin and heparin is close to the maximal response, whereas the relatively low initial patency rate with streptokinase leaves room for improvement with better antithrombin agents.

Effective GP IIb/IIIa inhibition can be achieved by the monoclonal antibody 7E3 (Abciximab) and by small molecule inhibitors, which effectively block fibrinogen binding to platelets and prevent the cascade of biochemical events leading to aggregation. These agents are effective in preventing adverse outcome after unstable angina and angioplasty,[67] [68] and appropriate dosaging of concomitant anticoagulant therapy prevents excessive bleeding associated with these procedures. [69] Considering that small molecule inhibitors of the fibrinogen receptor can accelerate reperfusion of coronary arteries in patients with acute myocardial infarction, [70] it is not unreasonable to anticipate that inhibitors will ultimately find a role as adjunctive therapy to plasminogen activators. The rationale for this approach would be that passivation of the arterial wall by a long-acting or bound GP IIb/IIIa inhibitor could prevent vascular reocclusion, especially that which follows t-PA therapy. [71]

Other Adjunctive Approaches to Thrombolysis

Because fibrinolysis depends on conversion of plasminogen to plasmin, the rate is determined at least in part by the plasminogen concentration incorporated into a

clot,[72] as well as by the presence of soluble plasminogen in the clot interstices. [73] A decrease in plasma plasminogen is part of the lytic state [74] (see later), which is less striking with fibrinogen-sparing agents such alteplase, saruplase, reteplase, and BAT-PA. [14] Thus, enrichment of plasminogen enhances the thrombolytic rate in vitro[75] and in animal models,[76] especially with agents such as streptokinase, urokinase, and anistreplase. Clinical proof of efficacy of plasminogen supplementation is not yet available, although preliminary observations in patients with deep vein thrombosis, peripheral arterial occlusion, acute myocardial infarction, and acute pulmonary embolism have been reported.[77] [78] [79] [80] Most studies have used plasminogen derived from human plasma, but a recombinant form of human plasminogen has been prepared and studied in animals, [81] and is undergoing clinical trials.

Ultrasound has been applied directly into the thrombus by thin wire, [82] and appropriate conditions of frequency and energy can mechanically disrupt and disintegrate clots and atherosclerotic plaque. Percutaneous, transvascular ultrasonic disruption of a femoral artery thrombus has been reported in patients, [83] and a similar application has been used to enhance t-PAmediated fibrinolysis in animal models. [84] The mechanism whereby ultrasound may produce its accentuation of plasminogen activator-mediated lysis is not known, but potentially such therapy could be achieved locally at the same time that systemic hemorrhagic complications may be avoided. More recent experiments with this approach suggest that transcutaneous ultrasound may induce thrombolysis when combined with systemic infusion of microbubbles, even in the absence of plasminogen activator administration. [85] The role of ultrasound as isolated therapy or as adjunct to plasminogen activator administration remains to be established, but the greatest potential would be in patients with thrombosis that may be amenable to the percutaneous approach, such as peripheral arterial occlusion, deep vein thrombosis, or thrombosed shunts and catheters.

Catheter-Directed (Regional) Infusion

A recurrent issue concerns the administration of plasminogen activator directly through a catheter onto or into a thrombus. The rationale for such regional therapy is compelling for arterial occlusions because the offending thrombus is usually localized to a single vessel downstream from the catheter tip, and the catheter also provides angiographic documentation of progress. It is the hope of the practitioner to confine treatment to the thrombus only, thereby maximizing thrombolysis by minimizing distant bleeding. Local therapy of venous thromboembolic disease has a less compelling rationale, but an extensive experience in which patients are treated by catheter delivery is receiving attention.

Regional treatment of arterial thrombi does achieve better thrombolysis than systemic therapy, but activity of the plasminogen activator is not restricted to the region in question. During the period of infusion, the lytic state may be comparable to that following systemic administration, [86] and bleeding, even intracranial hemorrhage, may complicate therapy. Whether a totally fibrin-specific agent could in fact avoid bleeding complications that accompany regional infusions remains to be determined.

The main decision regarding regional treatment is the clinical exigency of the patient. For example, even though regional infusion into the coronary artery may dissolve clot more effectively, systemic administration is more efficient for first-line treatment of the at-risk population with myocardial infarction. Still, with maximization of effort, such as has been applied to early angioplasty for acute myocardial infarction, some centers have demonstrated the feasibility of local revascularization therapy. Local therapy may also be useful as follow-up for failed systemic treatment of acute myocardial infarction. Regional treatment for slower-progressing illnesses such as peripheral arterial occlusion or deep vein thrombosis have the luxury of time required for this approach, and it is most applicable for dissolving thrombus in occluded catheters. To date, local therapy has proven to be least effective in patients with pulmonary embolism. For the treatment of thrombotic stroke, most studies have used systemic treatment to save time, but results of a trial using a local approach are still to be reported.

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EFFECTS OF THROMBOLYTIC TREATMENT ON THE BLOOD

Lytic State

The *lytic state* is a general description of the effects of plasmin activity on the circulation [74] (Table 1234). The shortening of the euglobulin lysis time measures free activator in the circulation. Laboratory parameters reflect the biochemical events that culminate in the degradation of plasma fibrinogen and the hypocoagulable state, manifested principally by prolongation of the thrombin time. Because hemostatic plug dissolution and fibrinolytic hemorrhage can occur in the presence of a minimal lytic state, the simplest and perhaps most physiologic definition of the lytic state is a demonstration of a decrease in plasma fibrinogen.

Free plasmin also affects platelet function by decreasing aggregation induced by adenosine diphosphate or other agonists. [87] Plasmin also cleaves the platelet membrane glycoprotein receptor for von Willebrand factor and reduces ristocetin-induced platelet aggregation. [88] However, the effect of plasminogen activators on platelets and blood coagulation factors is not a direct decrease in functional integrity and activity. For example, platelet hyperactivity and hyperaggregability may result from initial exposure of activator to platelets, [89] [90] and exposure of fresh thrombus surface or underlying vascular lesions, as well as the direct effect of activators on fibrinogen, [91] [92] may actually induce increased local coagulation. Thus, a patient may experience sequential hypercoagulability and hypocoagulability or

TABLE 123-4 -- Development of the Plasma Proteolytic (Lytic) State

Biochemical Step	Laboratory Parameter
Circulating plasminogen activator	Short euglobulin lysis time, variable platelet hyperreactivity
Plasminogen converted to plasmin	Decreased plasminogen
Antiplasmin complexes with and inhibits plasmin	Plasmin-antiplasmin complexes, decreased antiplasmin
Free plasmin	
Direct action	Increased plasma fibrin lysis and chromogenic activity
Degradation of fibrinogen	Decreased clottable protein, increased degradation products
Degradation of other plasma proteins	Decreased factors V and VIII
Action on platelets	Glycoprotein cleavage, decreased aggregation
Hypocoagulable state	Prolonged coagulation tests (thrombin time) and bleeding time

even the simultaneous occurrence of these opposite influences, depending on the phase of lytic state. [93]

Predictive Value of Laboratory Changes

Laboratory markers of hematologic events that occur after plasminogen activator administration have potential relevance for reflecting or predicting vascular events of clinical importance (Table 1235). According to the concept that the actions of plasminogen activators in the blood and on the thrombus are parallel but separate events (see Table 1231 , Fig. 1231 (Figure Not Available)), successful dissolution of the pathologic thrombus through fibrin degradation would not necessarily correlate with the extent of fibrinogenolysis

TABLE 123-5 -- Relationship of Vascular and Hematologic Parameters of Thrombolysis and the Potential for Laboratory Markers of Related Events

Vascular	Hematologic	Potential Marker
Reperfusion	Thrombolysis	Hypofibrinogenemia, elevated D-dimer, fibrin degradation products
Bleeding	Hemostatic plug or matrix disruption	Long bleeding time, hypofibrinogenemia
No reperfusion or reocclusion	Hypercoagulable state, thrombus and vessel thrombogenecity	Increased fibronopeptide A, prothrombin 1.2, thrombinantithrombin complexes, soluble fibrin, persistent high fibrinogen level
Late bleeding (with new injury)	Hypocoagulable state	Long bleeding time, persistent lytic state
Lasting patency	Hypocoagulable state, vascular passivation	Hypofibrinogenemia, long bleeding time, abnormal platelet aggregation/adhesion

(lytic state). Likewise, bleeding that would result from hemostatic plug disruption or vascular matrix degradation could occur independently of changes in hemostatic or platelet parameters in the blood. [3] [4] The controversy of this approach could be stated as follows: does the degree of hypocoagulable state contribute to hemorrhagic events over what might be expected by direct activity of the plasminogen activator on a hemostatic plug or the vessel wall? Proponents of this thesis would state that a plasminogen activator with fibrinogen-sparing properties would cause less bleeding because the induced hypocoagulability is minimized. Similar questions have been asked with regard to the effect of blood changes on vascular reperfusion and reocclusion.

The potential markers of bleeding could include any change in the lytic state cascade (Table 1236), including a decrease in plasminogen, hypofibrinogenemia, increased fibrinogen degradation products, prolonged bleeding time, and deranged platelet aggregation. Markers of vascular reperfusion as a surrogate of clinical recovery could potentially use lytic state markers, including changes in platelet function or direct reflections of thrombolytic degradation of fibrin such as increased D-dimer levels. Resistance to vascular reperfusion or late reocclusion (and clinical deterioration) might be predictable by hypercoagulable assays such as fibrinopeptide A, prothrombin intermediate fragment 1.2, thrombinantithrombin complexes, and soluble fibrin. [94] Retrospective studies have assessed laboratory values with the goal of finding correlations that could be of predictive value in individual patients, but the data show either no correlation with clinical events or only general trends that are of limited value for individual patients.

Bleeding Events

Although patients who receive thrombolytic agents are at risk of bleeding, such complications are at best only weakly correlated with laboratory parameters of the lytic state. For example, urokinase or streptokinase treatment of pulmonary embolism or deep vein thrombosis regularly induces the lytic state, but the laboratory values, including fibrinogen concentration, plasminogen concentration, and euglobulin lysis time, are not significantly different in patients with or without a hemorrhagic complication.[95] [96] In patients with acute myocardial infarction, streptokinase produced a similar fibrinogen decrease in patients with or without bleeding complications.[97] The direct comparison of t-PA and streptokinase in the TIMI trial (phase I) showed significantly lower fibrinogen values with streptokinase than with t-PA, but the incidence of major and minor bleeding complications was the same with both treatments.[98] A subsequent analysis of patients treated with t-PA[99] showed a small and nonsignificant trend toward a lower nadir fibrinogen concentration (100 vs. 130 mg/dl) and higher peak fibrin degradation products concentration (300 vs. 280 g/ml) in patients with major bleeding episodes. Furthermore, patients treated with t-PA who had ischemic (thrombotic) or hemorrhagic CVA had the same nadir concentration of fibrinogen.[100] The only predictive blood

TABLE 123-6 -- Absolute Contraindications to Fibrinolytic Therapy

Risk	Condition
Intracranial bleeding	Hemorrhagic cerebrovascular accident, intracranial neoplasm, recent cranial surgery or trauma (10 days), uncontrolled severe hypertension
Massive hemorrhage	Major surgery of thorax or abdomen (10 days), prolonged cardiopulmonary resuscitation, current severe bleeding (e.g., gastrointestinal)

value for hemorrhagic complication is the plasma t-PA antigen concentration (3.4 g/ml in bleeders vs. 2.2 g/ml in nonbleeders).[99] The higher value in those who bled suggests that t-PA has a direct effect on susceptible hemostatic plugs or vascular sites, greater with higher local concentrations, that could cause bleeding independent of the changes in blood coagulation parameters. This effect might also help to explain the higher rate of intracranial hemorrhage with high therapeutic dosages of t-PA.[101] These data support the concept that bleeding occurs independent of the plasminogen activator effect on blood coagulation, and is probably the result of action at sites of vascular injury or malformation (see Table 1231).

The template bleeding time is prolonged during thrombolytic therapy, either as a direct result of the plasminogen activator or of concomitant aspirin therapy. One report correlates the prolonged bleeding time with spontaneous bleeding,[102] but a subsequent analysis of the bleeding time showed no distinction in patients with or without hemorrhagic complication.[103]

The occurrence of intracranial hemorrhage with plasminogen activator treatment is infrequent but of great clinical import in that more than 50% of patients have lethal outcomes or are left with significant neurologic deficit. Although major hemorrhagic complications other than intracranial hemorrhage are induced with approximately equal frequency by all of the plasminogen activators, the more potent agents such as t-PA have a consistently higher risk of inducing intracranial hemorrhage. The reason is not clear; it is not likely to reflect the presence of a fresh hemostatic plug that is dissolved, but may be related to a vascular abnormality associated with amyloid deposition.[104] As with other bleeding complications, the degree of lytic state does not explain the event because the fibrinogen is the same in patients with intracranial hemorrhage or with a thrombotic CVA.[100] Subcutaneous heparin added to aspirin during plasminogen activator infusion increases the risk slightly (0.6% vs. 0.4%),[8] but the use of t-PA is a most important predictor,[105] along with age greater than 65 years, weight less than 70 kg, hypertension on admission, and a prior history of transient ischemic attack (TIA) or CVA.[101] A retrospective comparison of streptokinase (41,000 patients), anistreplase (21,000 patients), and t-PA (41,000 patients) showed the incidence of intracranial hemorrhage to be 0.2%, 0.6%, and 0.6%, respectively.[14] Recent observations of accelerated regimen t-PA versus streptokinase, reteplase, or double-bolus t-PA show an overall risk of approximately 0.8% for accelerated t-PA, with the risk in elderly patients (>75 years of age) being significantly higher (2.1%)[42] [106] [107] (Table 1237).

Vascular Patency and Reocclusion

Laboratory results also do not predict vascular reperfusion or degree of patency. In the Urokinase Pulmonary Embolism Trial (UPET) study, in which a large systemic dose of urokinase was administered, there was no correlation of laboratory results with decrease of embolus size,[95] and deep vein thrombosis trials also showed a lack of correlation of venographic change after plasminogen activator therapy with degrees of alteration in fibrinogen, thrombin time, plasminogen, or fibrin degradation products.[96] [108] Even intracoronary administration of streptokinase for treatment of acute myocardial infarction showed no difference in nadir plasma fibrinogen in patients who did or did not reperfuse,[109] and similar measurements in patients with acute myocardial infarction receiving t-PA also failed to show a correlation with post-treatment coronary artery patency.[99] Although there is not universal agreement, the data do suggest that changes in blood coagulation or fibrinolytic assays after plasminogen activator administration do not directly influence the degree of thrombus dissolution.

A reasonable hypothesis for noninvasive measurement of successful thrombolysis would be to measure plasma levels of cross-linked fibrin degradation products, but the data thus far are not conclusive, with some showing high predictability for successful vascular reperfusion[110] [111] and others failing to document such a correlation.[112] [113] Prediction of coronary artery reocclusion in an individual patient is problematic, but trials using t-PA provide instructive information.[99] Among 55 patients with reocclusion, the decrease in fibrinogen was less (120 vs. 180 mg/dl), as was the increase in fibrin degradation products (200 vs. 310 g/ml) than in patients whose vessels stayed patent. The data suggest that a more profound lytic state protects against reocclusion and may partially explain the lower tendency for reocclusion with streptokinase, urokinase, and anistreplase than with t-PA.[42] [114] [115] [116] Markers of hypercoagulability such as fibrinopeptide A, thrombinantithrombin complexes, and prothrombin fragment 1.2 tend to be higher in patients with increased mortality rates and lower TIMI flow grades, but application for predicting clinical outcomes in individual patients remains to be proven.

Laboratory Monitoring

Unlike treatment regimens with anti-thrombin agents such as heparin, the plasminogen activator dosage regimens are standardized, either on a total dosage or weight-adjusted basis, and only the duration of infusion leaves room for variation. Thus, the major role for monitoring thrombolytic therapy with laboratory studies would be to document that a given dosage of agent has indeed achieved a lytic state. Furthermore, time constraints in patients with acute myocardial infarction allow little chance to react to a laboratory result during the acute phase of the illness. Once a lytic state has been attained, there is no need to regulate the dose of plasminogen activator because alterations based on laboratory markers would neither improve the chance of thrombolysis nor decrease the risk of hemorrhagic complication. On the other hand, in patients who are anticipated to receive more than bolus injections or short infusions, the absence of a lytic state might reasonably be cause for discontinuation

TABLE 123-7 -- **Rates of Intracranial Hemorrhage in Three Large Studies of Accelerated-Regimen Tissue Plasminogen Activator**

	GUSTO I (19901993)		GUSTO III (19951997)		COBALT	
	SK (17,804)[a]	t-PA (9,039)	Reteplase (10,138)	t-PA (4,921)	Bolus t-PA (3,585)	t-PA (3,585)
Age <75 y	0.4	0.5	0.7	0.8	0.7	0.6
Age >75 y	1.2	2.5	2.5	1.7	3.6	2.6
Overall	0.5	0.7	0.9	0.9	1.1	0.8

SK, streptokinase; t-PA, tissue plasminogen activator; COBALT, Continuous Infusion Versus Double-Bolus Administration of Alteplase; GUSTO, Global Use of Strategies to Open Occluded Coronary Arteries.

[a] Numbers of patients.

of therapy and initiation of standard anticoagulation. Another recourse is to administer plasminogen in purified form or as whole plasma, but no evidence for converting a nonresponder to a responder exists, except perhaps by regional plasminogen activator delivery.

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MANAGEMENT OF BLEEDING COMPLICATIONS

Prevention of Bleeding

The absolute contraindications to plasminogen activator therapy are those that may result in intracranial hemorrhage or massive, life-threatening hemorrhage (see Table 1236). The risks of minor bleeding or even of the need for blood transfusion are not absolute contraindications. The decision regarding plasminogen activator use must weigh the expected severity of the complication with the potential benefit associated with accelerated thrombolysis. Thus, some patients with obvious life-threatening thrombotic diseasefor example, massive pulmonary embolism with profound shock or a large anterior wall acute myocardial infarctionmay warrant potentially life-saving thrombolytic therapy even in the face of serious contraindications. On the other hand, patients with relatively less threatening thrombotic disease, such as deep vein thrombosis, would reasonably not receive plasminogen activator therapy if there is a relevant history that significantly increases the risk of intracranial hemorrhage (e.g., TIA, prior thrombotic stroke, or current hypertension). The risk of major bleeding after recent surgery of the head, thorax, or abdomen is obvious, but the risk of serious bleeding after brief and relatively nontraumatic cardiopulmonary resuscitation is probably less than feared. Some clinical situations are associated with an increased risk of symptomatic but not serious bleeding, including patients with recent superficial wounds, arteriotomy or venotomy, a remote history of gastrointestinal or genitourinary bleeding, active menses, and minor surgical or biopsy procedures.

The elderly have a higher risk of bleeding, especially intracranial hemorrhage, but they also have a higher risk of death from acute myocardial infarction than do younger patients. Therefore, the survival benefit in patients older than age 70 years is relatively more than in patients younger than 60 years of age (80 vs. 25 lives saved per 1,000 treated).[43] [117] It is important not to withhold plasminogen activator therapy from the elderly, but the clinician must take into account age-related risk factors that may preclude therapy.

Management of Acute Bleeding and Post-treatment Surgery

In patients who have life-threatening bleeding or who have sudden emergencies that require surgical intervention, normal hemostasis can quickly be re-established by discontinuing plasminogen activator infusion, replenishing plasma fibrinogen with either whole plasma or cryoprecipitate, and providing fresh platelets to correct a prolonged bleeding time (Table 1238). If the plasminogen activator is not yet cleared from the circulation (see Table 1231), fibrinolytic activity can be neutralized by infusion of fibrinolytic inhibitors such as epsilon-aminocaproic acid or aprotinin.

Reports of post-thrombolytic surgical intervention exist, but controlled studies of management have not been performed. The reported cases of coronary artery bypass graft surgery after streptokinase usage generally indicate that the operation can be performed immediately, [118] [119] but that such therapy may result in bleeding complications and the need for blood transfusion. [120] Such results are relevant for invasive procedures after t-PA treatment as well, even if performed after a delay of 12 and sometimes 24 hours.[121] In the TIMI-IIA study,[99] angioplasty performed immediately after stopping t-PA resulted in a significantly

TABLE 123-8 -- Management of Hypocoagulable State Induced by Plasminogen Activators

	During Treatment	Immediately After Treatment	636 Hours Later
Plasma fibrinogen	Low	Nadir	Progressive recovery
Need for cryoprecipitate	Yes	Yes	Yes
Bleeding time	Long	Variable	Normal[a]
Need for platelets	Yes	Yes	No
Circulating activator	Present	Variable	Absent
Need for antifibrinolytic agent	Yes	Yes	Yes

[a] Unless aspirin also is administered, in which case the bleeding time and potential for bleeding may persist.

MANAGEMENT OF FIBRINOLYTIC BLEEDING

Most bleeding complications can be anticipated by careful clinical evaluation. Recent and unhealed vascular trauma sites are susceptible to bleeding for approximately 10 days. Depending on its location, the bleeding complication can be unimportant (ecchymoses, hematuria), bothersome and requiring transfusion (duodenal ulcer, accessible surgical site), or life threatening (thoracic or cranial surgery, recent or even remote stroke).

Once a patient is to be treated with a plasminogen activator, avoid unnecessary trauma to the patient. Medication should be administered by mouth or intravenously, but not intramuscularly. Phlebotomy sites should be compressed for 510 minutes, unnecessary arteriotomy should be postponed or avoided, and manual manipulation of the patient should be minimized. Treatment of some thrombotic conditions requires concomitant antiplatelet or antithrombotic treatment, especially certain arterial occlusions, but for deep vein thrombosis and pulmonary embolism, such therapies usually increase the risk of bleeding without adding to the clinical result. The use of intravenous heparin or even subcutaneous heparin in addition to aspirin and streptokinase is probably not reasonable, but heparin use with alteplase is still the rule of thumb, even though definitive evidence of its requirement is not available.

Bleeding that occurs during plasminogen activator infusion should be managed appropriately with blood replacement and, if necessary and possible, by discontinuing the plasminogen activator treatment. If bleeding is to be controlled immediately, the plasminogen activator must be neutralized by fibrinolytic inhibitors, such as epsilon-aminocaproic acid or aprotinin, and cryoprecipitate and platelets should be transfused.

If surgical intervention is required for up to 24 hours after plasminogen activator administration, the hemostatic mechanisms of the patient should be evaluated and maximized to avoid operative bleeding. Depending on the half-life of the activator, fibrinolytic inhibitor may or may not be required, but fresh fibrinogen replacement and often platelet transfusion are often required to ensure hemostasis, because plasma fibrinogen concentration does not recover spontaneously until 2436 hours after discontinuing plasminogen activator treatment. This pertains for both the long- and short-acting agents and also for those that induce a potent or a mild fibrinolytic state.

greater need for blood transfusion than if performed at 1848 hours later. The reason for bleeding coincident with interventional procedures after t-PA, which is cleared quickly from the circulation, is that the nadir fibrinogen remains low for 24 hours. [99] Normal fibrinogen concentrations are not achieved until full repletion by hepatic synthesis of fibrinogen, a process that requires 2436 hours regardless of the plasminogen activator that was administered.

In anticipation of invasive surgery immediately after plasminogen activator treatment, it is reasonable to replenish fibrinogen with cryoprecipitate, because essentially no normal fibrinogen is present during streptokinase [122] or t-PA infusion,[123] and to restore platelet function with fresh platelet transfusion for up to 24 hours after plasminogen activator administration. In addition, antifibrinolytic therapy should be administered prophylactically if activator is still circulating [124] (see Tables 1231 and 1236).

Other Side Effects and Complications

Termination of treatment may be necessitated by allergic or pyrogenic side effects. These complications can be managed with corticosteroids, antihistamines, or adrenergic agents for anaphylaxis and antipyretics for fever. Allergic reactions are three to five times more frequent using bacterially derived products than t-PAtype derivatives, but patients who receive t-PA rarely manifest allergic reactions such as bronchospasm or angioneurotic edema. [125] [126] A second side effect is hypotension, especially in patients with acute myocardial infarction, occurring in approximately 12% of patients exposed to streptokinase or anistreplase and 7% of those exposed to t-PA, compared with only 1.5% of patients not exposed to a plasminogen activator. [127] Whether cardiac rupture occurs more frequently in patients with acute myocardial infarction who receive plasminogen activator than in those who do not is in dispute. [128] [129]

Embolic phenomena of clinical importance are distinctly unusual in patients with venous thromboembolic disease who received plasminogen activator treatment. In the UPET study,[95] recurrent pulmonary embolism occurred in 15% of patients who received either urokinase or only heparin. A venous thrombus breaking off to become a fatal pulmonary embolus is distinctly unusual and may have alternative explanations other than lysis of a vulnerable, free-floating thrombus. On the other hand, distal systemic embolization from a cardiac source during plasminogen activator treatment, although unusual, is clinically apparent, and up to 20% of patients with peripheral arterial occlusion experience sudden, new ischemia of the distal extremity. [130] In this circumstance, the best treatment mode is continued local instillation of plasminogen activator, a treatment that usually succeeds in dissolving the new embolus. Rarely, systemic treatment with plasminogen activator results in cholesterol crystal embolization in the setting of extensive pre-existing atherosclerotic disease. [131]

RESULTS OF CLINICAL TRIALS OF MAJOR VASCULAR OCCLUSIONS

Table 1239 summarizes the FDA-approved agents in the major categories of vascular thrombotic disease, namely myocardial infarction, peripheral arterial occlusion, deep vein thrombosis, pulmonary embolism, acute ischemic stroke (CVA), and catheter occlusion. These approvals are based on pivotal clinical trials, but actual practice may not reflect the specifics of dosage schedules or mode of administration. For example, there has been an evolution of dosage change in the management of patients with pulmonary embolism, mostly resulting from comparative trials of t-PA with urokinase. Thus, a new and short (2-hour) protocol for t-PA proves to be superior to the FDA-approved 24-hour infusion of urokinase, and a similar condensation of urokinase dosage over 2 hours showed results equivalent to those obtained with the 2-hour infusion of t-PA. Given prior evidence showing streptokinase to be equivalent to urokinase, it is reasonable to anticipate a similar improved outcome for high-dose streptokinase over 2 hours. An evolution of the

TABLE 123-9 -- Commentary on FDA Approval[a] and Common Usage of Plasminogen Activators

Agent	Clinical Indication	FDA-Approved Regimen	Common Usage
Urokinase (Abbokinase)	PE	12-h infusion	2-h infusion suffices
	Acute MI	Intracoronary only	Infrequent, for reocclusion or with percutaneous transluminal coronary angioplasty
	Intravenous catheter	5,000 U in 1 ml	Popular therapy
	PAO, DVT	Not approved	Widespread use off-label, catheter delivery
Streptokinase (Streptase)	Acute MI	IV or IC	Intravenous use only
	PE	Duration 24 h for PE,	Therapy tailored for each patient
	DVT	72 h for DVT,	
	PAO	2472 h for PAO	
	Arteriovenous cannulae	Local installation	
Reteplase (Retavase)	Acute MI	Two bolus injections, 30 min apart	Just approved
Alteplase (Activase)	Acute MI	3 h or 90 min	Accelerated dosage used
	Acute ischemic stroke	90 mg/1 h, within 3 h of symptoms	Limited usage
	PE	100 mg/2 h	Urokinase over 2 h has equal efficacy
	PAO, catheter	Not approved	Used off-label
Anistreplase (Eminase)	Acute MI	30 U over 25 min	Infrequently used

DVT, deep vein thrombosis; FDA, Food and Drug Administration; MI, myocardial infarction; PAO, peripheral arterial occlusion; PE, pulmonary embolism.

[a] Based on *Physicians Desk Reference,* 1998.

dosage schedule for t-PA in acute myocardial infarction is reflected in FDA recommendations, with the current approved methodology calling for an accelerated regimen over 90 minutes instead of the prior front-loaded regimen over 180 minutes. Agents under development and clinical trial according to research protocols significantly influence clinical practice, such as in the use of regional (catheter-delivered) urokinase for deep vein thrombosis and of urokinase or t-PA for peripheral arterial occlusion, even though these are not yet approved modalities.

Deep Vein Thrombosis

Save for the unusual patient who has a marked proximal extension of thrombus and unfortunate massive embolization despite apparently effective anticoagulant treatment, the typical patient with deep vein thrombosis is not at risk of a lethal outcome and manifests an apparently benign course of recovery. Thus, most reviews of this clinical situation conclude that the use of thrombolytic treatment in such patients is not a straightforward decision, and a report of the American Heart Association[12] concludes that thrombolytic treatment may be indicated only in selected patients with extensive proximal deep vein thrombosis.

The most discouraging and disabling outcome of proximal deep vein thrombosis and a most compelling rationale for plasminogen activator use is to prevent the postphlebitic syndrome, a condition that usually develops over some years in up to 25% of patients. [132] There is no doubt that a venous thrombus can be lysed more rapidly and in more patients using plasminogen activator in addition to heparin: approximately 50% versus only 10% with heparin alone. [96] [133] Furthermore, a small but important subgroup of patients with more acute symptoms treated only with heparin actually have extension of thrombus, [96] apparently the result of inefficient neutralization of thrombus-bound thrombin by antithrombin III. The time of maximal benefit of plasminogen activator infusion is approximately 7 days after onset of clinical symptoms, after which significant lysis is less likely compared with heparin alone.

The evidence supports the conclusion that effective thrombolysis does reduce the incidence of symptomatic cases of postphlebitic syndrome, from approximately 40% to 10%.[134] [135] Presumably, the desired clinical result depends on rapid clot lysis and maintenance of venous valve leaflet function. This is a desirable outcome considering the debilitating nature of chronic edema and the risk of recurrent infected ulcerations that require hospitalization. Although not life threatening, this complication of deep vein thrombosis significantly affects the patients quality of life. Thus, thrombolytic treatment for proximal deep vein thrombosis induces more rapid clot lysis than does heparin, may dissolve coincidental pulmonary emboli that accompany the process, and reduces the occurrence of severe or symptomatic

postphlebitic syndrome.

Why then is plasminogen activator therapy used so infrequently for deep vein thrombosis? The explanation is the reasonable emphasis by the clinician on clinical outcome rather than a radiologic or physiologic result (Table 12310). Although most patients with significant deep vein thrombosis may have venous duplex evidence of obstruction or reflux,[136] severe postphlebitic syndrome develops only in a minority. Furthermore, even though very few patients actually dissolve a significant amount of thrombus with heparin anticoagulation alone, only the rare patient has a clinically important pulmonary embolism despite heparin treatment.

Because a lethal outcome is rare, careful attention must be paid to the risk of hemorrhage, which is greater than that with heparin treatment alone. With systemic (intravenous) administration over an interval of 27 days, the period of hemorrhagic risk is prolonged and the incidence of intracranial hemorrhage approaches 1%. [137] Thus, the relatively benign course of deep vein thrombosis treated with heparin usually convinces the clinician to reserve plasminogen activator only for the patient with proximal thrombus and without risk factors for serious bleeding.

Since the mid-1990s, a more aggressive approach to plasminogen activator delivery has used regional perfusion by catheter directly into the venous thrombus. Impressive recanalization rates have been attained, with up to 90% complete and partial lysis, and in anecdotal cases, substantial benefit has been noted even in relatively old thrombi, in patients with symptoms longer than 4 weeks.[138] [139] [140] [141] It is likely that the clinical impression that old thrombi are resistant to thrombolysis may in fact reflect

TABLE 123-10 -- Natural History and Anticipated Clinical Benefit of Thrombolytic Treatment for Acute Thrombosis

Thrombotic Disorder	Natural History (with Heparin)	Potential Benefit of Thrombolytics	Clinical Decision
Deep vein thrombosis	Slow resolution, subclinical PE frequent, post-phlebitic syndrome in 2550% with proximal thrombi.	Treatment within 7 days, complete lysis in half the cases; decreased incidence of postphlebitic syndrome.	Treat large proximal thrombi, especially with coexistent PE. Catheter delivery (urokinase, tissue plasminogen activator) under study.
PE	Slow (1 wk) reduction of pulmonary hypertension. Overall mortality 7%, up to 30% with massive PE and hypotension.	Earlier treatment (within 48 h) induces rapid thrombolysis and accelerates reversal of pulmonary hypertension. Possible increased survival in patients with clinical shock.	Thrombolytics for massive PA with shock, coexistent cardiopulmonary disease or submassive PE if no contraindications.
Peripheral arterial occlusion	Major surgical intervention in 95%, cardiopulmonary complications in 50%, loss of limb in 20%, 1-y mortality up to 40%.	Striking thrombolysis in 70%, surgery avoided in 35%, no change in limb salvage but potential for decrease in 1-y mortality.	Catheter delivery of PA superior to systemic infusion. Thrombolytic preferred over surgery for initial. Treatment for the ischemic but salvageable limb.
MI	Overall mortality 11.5%, 8% for inferior MI, 17% for anterior MI.	Overall mortality reduced by 2050%, most striking in patients with anterior MI treated within 4 h after symptom onset.	Indicated for most patients within 6 h of symptoms; angioplasty available in some centers.
Cardiovascular accident	Irreversible deficits and risk of death depending on location; hemorrhagic transformation relatively infrequent (3%).	Good results only if treatment initiated within 3 h, reversal of neurologic deficit at a cost of increased parenchymal hematoma.	Clear-cut functional benefit without increased mortality risk if treatment before 3 h, but with increased risk of hemorrhagic transformation.

MI, myocardial infarction; PA, plasminogen activator; PE, pulmonary embolism.

TABLE 123-11 -- Comparison of Outcome Using Different Plasminogen Activators

Thrombotic Disorder	Comparison of Agent
Deep vein thrombosis	No direct comparison, but equivalent results in separate trials.
Pulmonary embolism	SK (12 h) equal to UK (12 h or 24 h), UK (2 h) equal to t-PA (2 h), no comparison of SK with t-PA in equivalent regimens.
Peripheral arterial occlusion	t-PA equal to UK, historically better results for UK and t-PA than SK, but no direct comparisons.
Myocardial infarction	Comparable results with SK, t-PA, and APSAC in two megatrials, marginal survival advantage (0.9%) of t-PA over SK in a third, reteplase equivalent to t-PA and SK. Intracranial hemorrhage less common with SK than t-PA, reteplase, or APSAC.
Cerebrovascular accident	Only t-PA approved, but no comparative trials and other agents had longer delays (>3 h) before treatment.

APSAC, anistreplase; SK, streptokinase; t-PA, tissue plasminogen activator; UK, urokinase.

a problem of inadequate intravenous delivery of plasminogen activator to a fully occluded vessel, a situation that is overcome by direct catheter delivery of plasminogen activator to an actually susceptible thrombus. This approach often combines stent placement, thereby providing a second revascularization approach in the single invasive vascular procedure. Long-term follow-up studies and comparative trials against systemic therapy [96] [142] [143] [144] [145] [146] are needed to determine their relative values for safety against bleeding and for prevention of the postphlebitic syndrome and recurrence of thrombotic disease. It is likely that all of the plasminogen activators can accelerate thrombolysis relative to heparin treatment alone, but direct comparative trials have not been performed to determine if one agent or regimen is superior to another. Whatever agent is chosen (only streptokinase is thus far approved by the FDA), the use of thrombolytic agents continues to be highly individualized (Table 12311).[147]

Pulmonary Embolism

The rationale for the use of plasminogen activator in patients with acute pulmonary embolism is to reduce acute morbidity, reperfuse the pulmonary vascular bed, and prevent late consequences such as persistent perfusion defects, reduced pulmonary capillary blood volume, and chronic pulmonary hypertension. The anticipated clinical course of patients with pulmonary embolism varies according to the size of the embolus and the cardiopulmonary reserve of the patient. [95] [148] [149] With routine heparin anticoagulant therapy, the overall mortality risk is 79%, with the greatest risk being in patients with a massive embolus and concomitant shock secondary to acute cor pulmonale.[95] [150] In this situation, acute changes associated with the embolus induce right ventricular dilatation and right-sided failure with secondary effects of decreased left ventricular filling and decreased cardiac output, followed inexorably by hypotension, decreased coronary blood flow, worsening right ventricular failure, and, ultimately, circulatory arrest. Other than general measures for support of respiration, blood pressure, and cardiac function, therapy is directed at the thrombotic process using antithrombin agents, plasminogen activators, surgical embolectomy, intrapulmonary artery catheter retrieval of thrombus, and placement of inferior vena caval umbrella filters.

Short-term improvement using urokinase, streptokinase, or t-PA[95] [151] [152] plus heparin, compared with heparin alone, is reflected by rapid thrombolysis and reperfusion of the pulmonary artery, more rapid recovery of abnormal perfusion lung scans, a decrease in pulmonary artery pressure, and improved right ventricular wall motion. These benefits in physiologic response are most evident in patients with relatively recent onset of symptoms (within 48 hours), [95] but some acceleration of response can be seen in patients treated for up to 2 weeks after symptom onset.[153] Studies comparing urokinase and streptokinase document equal efficacy with 12- or 24-hour infusions and for both agents.[151] When administered over 2 hours, t-PA is clearly superior to heparin therapy [152] and achieves more rapid thrombolysis than a 24-hour infusion of urokinase.[154] [155] However, large doses of t-PA and urokinase administered in an equivalent manner over 2 hours produce equal angiographic improvement.[156] Whether a similarly modified streptokinase regimen has results equivalent to those of the 2-hour urokinase or t-PA infusion remains to be established, but prior experience suggests that such would be the case. Short courses of t-PA therapy (50100 mg over 10 or 15 minutes) have proved to be no better than the 2-hour regimen.[157]

Most patients with pulmonary embolism recover with heparin therapy alone. In a population of patients with massive, submassive, or small pulmonary embolism, controlled trials of plasminogen activator versus heparin showed no difference in mortality. [95] [151] This result would tend to obscure any potential benefit in subgroups of patients at higher risk, such as those with massive embolization and clinical shock who have the most dramatic thrombolytic responses and early reversal of elevated pulmonary artery pressures. In uncontrolled studies, clinical experience indicates that patients with massive pulmonary embolism and clinical shock have better outcomes with plasminogen activator therapy than with an invasive surgical procedure using thoracotomy and pulmonary embolectomy. [158] Although one analysis[159] suggests an improved 30-day survival in a review of 719 consecutive patients with major pulmonary embolism (5% vs. 11%), results in any individual trial fail to show a survival benefit with plasminogen activator therapy.

Considering that the risk of serious bleeding complication is doubled (4% vs. 9%) and that the risk of intracranial hemorrhage remains at least 1% with all of the plasminogen activators, some clinicians are inclined to use heparin rather than fibrinolytic agents in patients with favorable-prognosis pulmonary embolism. The time window for maximal therapeutic response likewise limits the exercise of more aggressive therapy using plasminogen activators because natural resolution of the thrombus is not very different using heparin or plasminogen activator in patients with a remote history of symptoms (>48 hours). [95]

Selection of the patient who merits thrombolytic therapy therefore depends on a complex of clinical circumstances that emphasizes minimum delay before initiating treatment, selection of the most seriously ill patient for maximal short-term benefit, and avoidance of patients with absolute contraindications, especially those who are predisposed to intracranial hemorrhage. [160] The American Heart Association guidelines suggest plasminogen activator therapy when it has life-saving potential for patients with massive pulmonary embolism, especially in those who have experienced syncope, hypotension, severe hypoxemia or heart failure, or patients with submassive embolism in the presence of underlying cardiac or respiratory disease. [12] In the circumstance of life-threatening pulmonary embolism, the clinician may opt for plasminogen activator use even in the presence of a relative contraindicationfor example, in patients who are postoperative, during pregnancy, after cardiopulmonary resuscitation, and even within 1 week after neurosurgery. [161] [162] [163] [164] Administration of plasminogen activator directly into the pulmonary artery does not provide results superior to those obtained with systemic therapy. [165]

Insertion of inferior vena caval umbrella filters has been a

popular intervention in patients with pulmonary embolism, and filters are often introduced into patients with deep vein thrombosis who have no evidence but only a risk of pulmonary embolism. This approach was originally developed for management of patients with contraindications to anticoagulant or fibrinolytic therapy, but few studies of efficacy in comparison with medical management have been performed. A recent report followed patients with deep vein thrombosis who were randomized to filter placement or anticoagulation alone, and the final conclusion was that an initial benefit of the filter for preventing pulmonary embolism was counterbalanced by an excess of recurrent deep vein thrombosis without any difference in mortality. [166] Problems exist with inferior vena caval filters, including thrombosis of the filter with proximal propagation of thrombus, caval perforation, and the potential for postphlebitic symptoms secondary to a greater deep vein thrombosis recurrence rate, thus arguing against indiscriminate use of filters.

There is considerable disagreement in the application of thrombolytics for patients with pulmonary embolism, and in general the pulmonologist is reluctant to administer thrombolytic agents.[167] Most clinicians agree, however, that thrombolytic therapy can quickly reverse the pathophysiology of cor pulmonale and that survival of the most severely affected patient is more likely using plasminogen activator therapy, although this is not proven.

Peripheral Arterial Occlusion

More than with any of the other major vessel occlusions, peripheral arterial occlusion illustrates the advantages of regional, catheter-delivered plasminogen activator over systemic (intravenous) administration (Fig. 1232). The benefit is primarily due to more effective thrombolysis than to greater safety from hemorrhage, but the data indicate that such a combined radiologic and medical approach is comparable with and in some ways superior to surgery as initial therapy of the acutely threatened, but not irreversibly ischemic limb. The presence of generalized atherosclerosis, hypertension, and other manifestations of arterial disease in many of these patients makes thrombolytic therapy more problematic than in patients with venous thromboembolic disease. For example, partial dissolution of a mural thrombus may result in systemic embolization during treatment of a peripheral arterial embolus, whereas pulmonary embolization from a treated deep vein thrombosis is seldom of clinical importance.

Chronic arterial occlusions are not amenable to systemic plasminogen activator administration, not only for the lack of clinical benefit but for the absence of vascular reperfusion.[168] [169] Acute arterial occlusions proved to be more amenable to systemic plasminogen activator treatment, especially if initiated within hours of onset of symptoms and if such symptoms were

Figure 123-2 (A) Angiogram of the abdominal aorta showing complete occlusion of the right iliac artery (*arrow*). **(B)** Catheter tip is located within the thrombus proper for effective infusion of plasminogen activator (*arrow*).**(C)** Repeat angiography shows reconstitution of blood flow in the right iliac artery (*arrow*). Extensive atheromatous plaque is visible distal to the original thrombus. **(D)**

due to embolic phenomena rather than secondary to in situ atherothrombosis. [170] [171] Occlusions of small, distal arteries that are less amenable to a surgical approach may be managed by plasminogen activator infusion. The first demonstration of therapeutic efficacy for regional (intra-arterial) administration in peripheral arterial occlusion was reported by Dotter et al[172] in 1974, which showed vascular reperfusion. However, this approach did not prevent a lytic state or avoid bleeding complications. Subsequent studies have verified that reperfusion can be attained in 5080% of patients, but that in a significant proportion of patients, an underlying stenotic lesion requires angioplasty or bypass surgery to protect against reocclusion. [173] [174] [175] [176] [177] [178] [179] [180]

Despite an extensive clinical experience, a prospective, randomized comparison of regional therapy versus bypass surgery as initial management of patients with ischemic limbs secondary to arterial occlusion was not reported until 1994. [181] In a 114-patient study, catheter-directed urokinase infusion into the thrombus reperfused the affected vessels in 70% of cases, and allowed simple angioplasty in the other third. Although overall limb salvage and in-hospital mortality were the same for patients receiving intra-arterial urokinase and surgical repair as initial therapy, the 1-year mortality rate was lower in the thrombolytic group (16% vs. 42%), arguably resulting from a lower incidence of cardiopulmonary complications. This result has been confirmed in a similar study of recombinant urokinase, which achieved patency in 70% and reduced the need for major surgical procedures by 40%. [182] In this follow-up study, however, there was no survival benefit, possibly because the sickest patients were not entered into the study, but rather treated off-protocol with regional plasminogen activator infusion. Systemic activation of fibrinolysis (lytic state) occurred in many patients receiving plasminogen activator and serious bleeding occurred in 13% (vs. 6% with surgery), including intracranial hemorrhage in 1.5%.[182] In a similar trial of t-PA and urokinase, there also was no clinical advantage for plasminogen activator administration over surgery. [183]

It is generally agreed that lysis of occluded arterial grafts can be accomplished equally as well as for thrombosed native arteries. [181] [182] [184] [185] Retrospective analyses[185] [186] [187] show that results with t-PA or urokinase are superior to those with streptokinase for both thrombolytic revascularization (80% vs. 64%) and hemorrhagic complications (5% vs. 25%). One study that compared t-PA with urokinase[183] showed no difference in clinical outcome, but as yet there have been no prospective comparisons of streptokinase with either t-PA or urokinase in peripheral arterial occlusion. The total experience does not represent a strong foundation for definitive conclusions on the relative benefit of t-PA, urokinase, or streptokinase. A recent report suggests that recombinant staphylokinase also is effective in recanalization of peripheral arterial occlusion, albeit also with attendant major hemorrhagic complications, including fatal intracranial hemorrhage. [188] Although only one study has shown a 12-month

survival advantage over surgery (84% vs. 58%),[181] the similar efficacy of different plasminogen activators in comparative trials of pulmonary embolism and acute myocardial infarction suggest but do not provide convincing evidence [189] that all of the plasminogen activators will have equivalent advantages relative to surgery in the management of the ischemic but salvageable limb.

Myocardial Infarction

The potential of thrombolytic therapy for acute myocardial infarction [190] was not recognized until coronary angiography documented thrombus as the cause [191] and the ability of plasminogen activators to lyse such thrombi. [192] In retrospect, evidence of benefit existed in the early 1980s, [193] [194] but definitive data awaited the trials that compared streptokinase, alteplase, and anistreplase with placebo. [43] [117] [195] [196] These studies document that prompt treatment of the coronary artery thrombus by systemic administration of plasminogen activator reduces mortality by more than 40% when administered within 12 hours, and by 2025% at 46 hours after symptoms.[8] Tangible benefit can still be effected at up to 12 hours after symptom onset, but thereafter, plasminogen activator treatment is not better than antithrombotics alone. Aspirin administration virtually doubles the benefit. Even without thrombolytic therapy, mortality is significantly reduced when aspirin is administered within 1 hour or 46 hours after symptom onset. [43] The impact of plasminogen activator therapy on the clinical outcome of patients with acute myocardial infarction has been dramatic, and the survival advantage persists for years after therapy. [197] [198] [199] Although advanced age (>75 years) translates to a higher mortality rate after acute myocardial infarction, thrombolytic therapy saves more lives per 1,000 elderly patients who are so treated than in younger patients. [8] [200] Patients with anterior infarcts show relatively greater benefit than those with inferior infarcts, probably because of the inherent higher mortality risk with the former, and treatment is effective regardless of concurrent hypotension or history of diabetes or prior myocardial infarction, and in both men and women. [43] However, patients with ST segment depression or a normal electrocardiogram do not show the benefit in survival shown by those with ST elevation or bundle branch block. [43]

Because of the prevalence of and high mortality rate associated with acute myocardial infarction, this illness has been the most intensively studied thrombotic disorder to determine the relative value of different plasminogen activators or of regimens of plasminogen activators with anticoagulant or antiplatelet agents.

The trials of note (Table 12312) have involved well in excess of 100,000 patients treated expeditiously, most often within 6 hours of symptom onset. [8] The Gruppo Italiano per lo Studio

TABLE 123-12 -- Mortality at 30 Days in Five Comparative Trials of Plasminogen Activator in Acute Myocardial Infarction

	GISSI-2 (20,000)[a]	ISIS-3 (40,000)	GUSTO (20,000)	INJECT (6,000)	GUSTO-III (15,000)
Streptokinase	8.5%	10.6%	7.2%	9.5%	
Tissue plasminogen activator					
180 min	8.9%	10.3%			
90 min			6.3%		7.24%
Anistreplase		10.5%			
Reteplase				9.02%	7.47%

GISSI, Gruppo Italiano per lo Studio della Streptochinasi nellInfarto Miocardica; GUSTO, Global Use of Strategies to Open Occluded Coronary Arteries; INJECT, International Joint Efficacy Comparison of Thrombolytics; ISIS, International Study of Infarct Survival.

[a] Number of patients.

della Streptochinasi nellInfarto Miocardica (GISSI)-2 and ISIS-3 studies [38] [201] [202] performed in the early 1990s showed no statistical difference in short-term mortality rates between streptokinase and t-PA and between streptokinase, t-PA, or anistreplase. The studies showed a significantly lower rate of intracranial hemorrhage for streptokinase than for the other agents, approximately 0.7% versus 0.35%. There was no advantage in overall 35-day mortality rates using subcutaneous heparin beginning at 4 hours over no heparin, but the intracranial hemorrhage rate was increased (from 0.4% to 0.6%), suggesting that heparin did not improve results with plasminogen activator plus aspirin.

The issue of adjunctive intravenous heparin as a substantial contributor to results using t-PA, more so than with streptokinase, was based on patency results that in some cases failed adequately to control for the contribution of concomitant aspirin therapy. [49] [50] Nevertheless, the possibility that inadequate heparin masked a potential benefit of t-PA over streptokinase and the possibility that a more intensive regimen of t-PA is needed for maximal clinical results was the foundation for the accelerated t-PA regimen over 90 minutes.[42] Several conclusions are available from this large, prospective, open-label study. First, the overall mortality rate at 30 days was no different with streptokinase plus intravenous heparin than with streptokinase plus subcutaneous heparin, providing the necessary foundation to document that aggressive heparin use with streptokinase is unnecessary. Second, the combination of t-PA plus streptokinase was no better than streptokinase alone, despite prior patency evidence that the combination was advantageous. [203] Third, there was a small but statistically significant mortality advantage in favor of the accelerated t-PA plus intravenous heparin regimen, over the streptokinase-treated patients (6.3% vs. 7.2%), representing a difference of 9 patients per 1,000 treated. Fourth, the incidence of intracranial hemorrhage was significantly higher in the t-PAtreated group (0.7% vs. 0.5%), somewhat militating against a clear-cut advantage for t-PA in the entire population of treated patients. Subgroup analysis of patients in the GUSTO study showed no difference between streptokinase and t-PA in patients treated after 4 hours or with inferior myocardial infarction. Considering that the risk of intracranial hemorrhage is considerably higher in the elderly treated with t-PA than with streptokinase,[43] tailored therapy with one or other regimen has been proposed, rather than a standard regimen. [9] [204]

The small survival advantage of the accelerated t-PA regimen in GUSTO has been explained by the higher 90-minute coronary artery patency rates with t-PA, [205] almost a 2:1 higher TIMI-3 result in favor of t-PA (54% vs. 30%). Coronary artery patency categorically influences outcome, best exemplified by the higher mortality rate after intracoronary administration in patients without reperfusion than in those with reperfusion (14% vs. 8%). [206] [207] Furthermore, full patency (TIMI grade 3) rather than lesser degrees of patency (TIMI grade 2) or persistent occlusion of the coronary artery (TIMI grades 0 or 1) is dramatically associated with improved survival.[208]

However, the patency results do not fully explain the relatively small difference in survival noted in the GUSTO study, because the patency at 180 minutes was the same for t-PA and streptokinase (42% vs. 35%),[205] as predicted on the basis of prior information.[209] Effort has been directed toward improving early patency rates and preventing the clinical deterioration that follows early reocclusion. [210] In one retrospective analysis of 5,475 patients, [211] an estimate was made that accelerated or front-loaded t-PA induced TIMI grade 3 flow at 90 minutes in 63% or 50% of patients, respectively, compared with only 32% after streptokinase, and that reocclusion occurred in 6%, 12%, and 4%, respectively. The potential for improved early patency is greatest for streptokinase, and that for lessened early reocclusion is greatest for t-PA. One novel approach has been the development of new plasminogen activators with more potent

early thrombolytic effect. Thus, reteplase infusion results in a 90-minute TIMI grade 3 patency rate of 6063% versus only 4549% for accelerated t-PA. [212] [213] Despite favorable reviews for the reteplase data, [214] [215] a subsequent mortality trial (GUSTO-III) [107] showed that the 30-day mortality rate with reteplase was not statistically different from that with accelerated t-PA in a trial of 15,000 patients (7.47% vs. 7.24%). Similarly, reteplase showed no statistically significant difference in mortality from streptokinase (9.02% vs. 9.53%) in a trial of 6,000 patients, [216] despite a clear advantage in 90-minute TIMI grade 3 patency results. In further support of the concept that improved patency rates do not uniformly result in higher survival, a double-bolus regimen of t-PA, which had shown a TIMI grade 3 patency rate of 88%,[217] resulted in a slightly higher mortality rate (7.98% vs. 7.53%) and higher intracranial hemorrhage rate than accelerated t-PA. [106] TIMI grade 3 patency rates after staphylokinase administration or a 60-minute t-PA regimen have been reported to be 68% [218] and 81%,[219] and a 63% TIMI grade 3 patency was noted after a single bolus (40 mg) of TNK-tPA,[220] but these striking findings must be tested in mortality trials before acceptance as superior plasminogen activator agents or regimens.

It is possible that improved antiplatelet strategies, such as using antibodies, small peptides, or chemicals directed against the platelet fibrinogen receptor, [56] [57] will decrease late reocclusion after t-PA, and improved antithrombin agents such as hirudin [53] [54] rather than heparin may accelerate early reperfusion with the relatively slow-acting agents such as streptokinase. The potential benefit for improved antiplatelet therapy could be that a lower dosage of t-PA could be used, [71] which could in theory improve the safety profile of t-PA relative to intracranial hemorrhage. On the other hand, a significant increase in TIMI grade 3 patency at 90 minutes with streptokinase might translate into results that are equivalent to those achieved with accelerated t-PA, but proof of survival benefit with this regimen is not available.

A reasonable alternative approach to early coronary artery revascularization is immediate angioplasty, [221] and this approach is feasible for large centers that can expedite therapy without delay. Although there is some disagreement in the interpretation of results, [204] angioplasty would certainly have advantages for patients with relative contraindications to thrombolytic therapy, especially if there were high risk of intracranial hemorrhage. Intracoronary plasminogen activator administration probably still has a role in patients who fail to reperfuse with systemic treatment or who deteriorate secondary to early reocclusion, [222] in which case local infusion of plasminogen activator may be attempted as a prelude to bypass surgery or concomitant with angioplasty. The risk of serious hemorrhage, even intracranial hemorrhage, is tangibly less than the chance of improved survival after plasminogen activator treatment of an anterior myocardial infarction. However, in the setting of pre-existent hypertension or a prior CVA or TIA, the incidence of intracranial hemorrhage is significantly increased to more than 3%, [101] depending on the plasminogen activator that is infused. The risk of intracranial hemorrhage correlates with four risk factors [41] specifically, age >65 years, body weight <70 kg, hypertension on admission, and the administration of alteplase.

Thus, although the benefit for application of plasminogen activator therapy is striking and physicians have applied these agents for routine care, the risk of serious bleeding may significantly influence the decision in some patients. For example, patients with an inferior myocardial infarction who present more than 6 hours after onset of pain may best be managed conservatively with antithrombin or antiplatelet agents only. On the other hand, a massive anterior myocardial infarction may well warrant plasminogen activator therapy even in the face of a significant risk of serious bleeding, because the mortality rate can be reduced by as much as 25% using thrombolytic agents.

Ischemic Stroke

Because intracranial hemorrhage is the most feared complication of plasminogen activator treatment, the rationale of dissolving cerebral artery thrombi that cause ischemic stroke has presented both a therapeutic dilemma and a technical obstacle. The risk of inducing an intracranial hemorrhage or of converting a subclinical, petechial hemorrhagic stroke into a parenchymal hematomawith its attendant 60% mortality rateis high. Until the availability of accurate technology for distinguishing a hemorrhagic from a thrombotic stroke, the chance of a catastrophic outcome has been unacceptably high. The only satisfactory exception to the general aversion to plasminogen activator treatment of stroke was the application of regional agent into the vertebrobasilar arterial system in patients with severe brain stem thrombotic stroke.[223] In this situation of dire prognosis for coma and death, the results were sometimes dramatically beneficial and helped to establish the feasibility and potential usefulness of this approach.

In a spurt of activity in the mid-1990s, five randomized and blinded trials of intravenous plasminogen activator were completed for ischemic stroke, three of which used streptokinase at 1.5 million units over 1 hour [224] [225] [226] and two of which used alteplase at a maximal dose of 90 or 100 mg over 1 hour.[227] [228] Table 12313 summarizes the results, which were different in various

TABLE 123-13 -- Five Major Placebo-Controlled Thrombolytic Trials of Acute Ischemic Stroke

	MAST-I	MAST-E	ECASS	NINDS	ASK
Agent	SK	SK	rt-PA	rt-PA	SK
No. of patients	622	310	620	291,333	340
Timing to treatment	6 h	6 h	6 h	3 h	4 h
10 days	27% SK vs. 12%[a]	34% SK vs. 18%[a]			
13 mo			18% t-PA vs. 13%	17% t-PA vs. 21%	36% SK vs. 21%[b]
6 mo	36% SK vs. 24%	47% SK vs. 38%			
Intracranial hemorrhage	6% SK vs. 0.7% (5 d)	22% SK vs. 3% (5 d)	20% t-PA vs. 6.5% (13 mo)	912% t-PA vs. 25% (36 h)	13% SK vs. 3% (0.01)
Functional tests (36 months)	20% disabled SK/ASA, 34% SK vs. 40%	8.4% severe Rankin vs. 21.1%	Rankin 100 vs. 90 (<0.001)	Rankin 39 vs. 26 NIHSS 31 vs. 20 (.03)	Barthel index <60 48% SK vs. 45%

ASA, acetylsalicylic acid; ASK, Australian Streptokinase Trial; ECASS, European Cooperative Acute Stroke Study; MAST (I, E), Multicentre Acute Stroke Trial (Italian Study Group, European Study Group); NIHSS, National Institutes of Health Stroke Scale; NINDS, National Institute of Neurological Disorders and Stroke; SK, streptokinase; (r)t-PA, (recombinant human) tissue plasminogen activator.

[a] Study terminated because of excess early mortality.
[b] Increased mortality with SK (over placebo) only in patients treated at 3 h or longer after symptom onset.

details of patient characteristics, diagnosis, treatment, and follow-up evaluation. The most important distinction was the delay from symptoms until treatment, with three studies accepting patients at up to 6 hours after symptoms, one until 4 hours, and one cutting off accession at 3 hours maximum. The major end points were usually a combination of mortality plus severe disability (dependence) at 16 months, the rate of intracranial hemorrhage (parenchymal hematoma) complicating treatment, and functional assessment at 36 months. Of the five studies, three were terminated wholly or in part before completion because of an excess in short-term (10 days) mortality, [224] [225] [226] and a fourth study that was completed showed a nearly significant increase in 30-day mortality in the intention-to-treat group of patients.[227] The only study that showed no increase in mortality at 30 days was the National Institute of Neurological Disorders and Stroke (NINDS) rt-PA Stroke Study Group,[227] which assessed t-PA in patients who were started on treatment before 3 hours. The other trials entered patients at up to 4 hours [225] or 6 hours.[224] [225] [227] The Australian Streptokinase Trial showed an excess mortality for patients entered after 3 hours but not before 3 hours. [226]

Functional tests on survivors at 36 months regularly showed higher scores in the treated group compared with placebo, but this advantage [229] must be tempered by the unacceptable high early mortality rates of most trials. Uniformly, the patients who received any plasminogen activator had an increased rate of intracranial hemorrhage, especially of parenchymal hematoma, evident as early as 1.5 days or as a measured accumulation over several months. This remains an especially severe complication, associated with a mortality rate of approximately 60%, especially in patients who are screened by computed tomography scanning to rule out overt hemorrhage before treatment.

A summary of 12 trials, representing an experience in almost 3,500 patients, has been published by Wardlaw et al, [13] and draws the following conclusions:

- Thrombolytic treatment is associated with a significant increase in early deaths and total deaths, but with a reduction in combined outcome of death or dependence.
- Symptomatic intracranial hemorrhage occurs in some patients, a large percentage of whom had fatal outcome.
- For all patients randomized within 3 hours, there was a nonsignificant, slight increase in deaths and a reduction in death plus dependence.
- There was significant heterogeneity between trials, most pointedly with regard to the severity of stroke, time before treatment, dosage of plasminogen activator, and use of other antithrombotics.
- Because there has been no direct comparison of t-PA with streptokinase or urokinase, the poor outcome noted in streptokinase trials could be explained by

differing characteristics of the trials, most especially with regard to the time before treatment.

They conclude that there is substantial risk and uncertain benefit with the use of plasminogen activator therapy for acute ischemic stroke. Clearly, more testing is needed, but it is difficult to enter patients into a study within 3 hours, even when a research team is trained and on call. These technical problems are certainly at the root of the limited applicability of conditions described in the NINDS trial [228] for general practice. Of potential relevance is an ongoing trial of recombinant saruplase (pro-urokinase) administered by regional perfusion and a study of the antithrombotic, ancrod, which may prove advantageous by avoiding the complication of intracranial hemorrhage.

Other Indications

A variety of thrombotic disorders have been treated by plasminogen activator infusion, some with dramatic results. [14] Local installation of plasminogen activator into thrombosed access catheters or arteriovenous shunts can lyse thrombus and re-establish flow, but reocclusion may follow. Local administration of plasminogen activator through special catheters that contain multiple pulse-spray openings have been applied for the treatment of otherwise inaccessible thrombi, such as in the mesenteric venous or arterial system, thrombosed cerebral or basilar arteries, upper extremity deep vein thrombi (subclavian/axillary system), and for thrombosed superior vena cavae, especially in the presence of external compression by tumor. In many examples of thrombolysis, residual stenotic areas of vein or artery are identified, and vascular radiologists are increasingly inserting stents into such vessels to impede rethrombosis. A number of patients with retinal artery or vein thrombi have been treated with plasminogen activator, but results are either difficult to interpret or unimpressive, especially considering that retinal vein occlusions are often clinically undetected for days or weeks. Cerebral venous sinus thrombi should be especially amenable to plasminogen activator infusions because relief of the venous obstruction would be expected to correct the venous hypertension and cerebral edema behind the obstructive lesion.

Many unusual thrombotic conditions are potentially correctable by plasminogen activator infusion, including heparin-associated thrombocytopenia with thrombosis, mobile right heart thrombus, inferior vena cava filter thrombosis, central line thrombosis in premature infants, hepatic vein occlusion, renal allograft vein thrombosis, portal vein thrombosis after liver transplantation, and paradoxical embolus through a patent foramen ovale. In all such clinical situations, the potential for clinical improvement must be weighed against that for serious hemorrhagic complication when deciding on a course of treatment.

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Chapter 124 - Venous Thromboembolism

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INTRODUCTION

Although venous thrombosis can occur in any vein in the body, it most commonly occurs in the lower limbs. Thrombosis of the superficial veins of the legs frequently occurs in varicosities and is usually benign and self-limiting. In contrast to superficial vein thrombosis, involvement of the deep veins of the leg is a more serious condition. Thrombi localized to the deep veins of the calf are less serious than those involving the proximal veins (popliteal, femoral, or iliac veins) because they are often smaller and therefore less commonly associated with long-term disability or clinically important pulmonary embolism (PE). On the other hand, proximal vein thrombosis is frequently complicated

by PE. In addition, extensive damage to the valves in the veins often leads to disability from the postphlebitic syndrome.

Venous thrombosis and its major complication, PE, have long been known to be important causes of morbidity and mortality in hospitalized patients. Recently, it has become apparent that venous thromboembolism also occurs in otherwise well, ambulant outpatients. The diagnosis of acute thromboembolism in outpatients is now being made more frequently because of increased diagnostic suspicion and the availability of reliable, noninvasive diagnostic tests. Other possible reasons for the increase in the reported rate of venous thrombosis in outpatients include the trend toward early hospital discharge of postsurgical patients who may not have received adequate in-hospital prophylaxis or whose in-hospital prophylaxis was terminated at discharge even through the patient remained at risk for venous thrombosis.

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PATHOGENESIS OF VENOUS THROMBOEMBOLISM AND CLINICAL RISK FACTORS

Venous thrombi are composed predominantly of fibrin and red cells [1] and usually arise in the large venous sinuses in the calf, in valve cusp pockets in the deep veins of the calf, or at sites of vessel damage. Venous thrombosis occurs when activation of blood coagulation exceeds the ability of the natural anticoagulant mechanisms and the fibrinolytic system to prevent fibrin formation. [1] Activation of blood coagulation is usually initiated by tissue or vascular trauma or by inflammation, and is augmented by venous stasis. Vascular wall damage is an important factor predisposing to venous thrombosis after major hip or knee surgery. [1] In addition to activating coagulation, tissue damage can also lead to impaired fibrinolysis because inflammatory cytokines induce endothelial cell synthesis of plasminogen activator inhibitor-1 (PAI-1), the major inhibitor of fibrinolysis. [2]

Under normal circumstances, activated coagulation factors are diluted in the flowing blood and are neutralized by inhibitors on the surface of endothelial cells or by circulating antiproteinases. [3] However, activated clotting factors that escape regulation can trigger the coagulation system, thereby leading to fibrin formation. This in turn activates the fibrinolytic system by inducing the release of tissue plasminogen activator (t-PA) from endothelial cells [4] and urokinase from monocytes and leukocytes, which are attracted to the thrombus by released fibrinopeptides and platelet products.

Thrombogenic Factors

Activation of Blood Coagulation

The coagulation proteins circulate as inactive precursors or zymogens. Each zymogen is converted into an active enzyme that then activates the next zymogen in the coagulation pathway (see Chap. 102).[5] Although coagulation can be activated through either the intrinsic or the extrinsic pathway in vitro, coagulation in vivo is initiated via the extrinsic, or tissue factor, pathway. In this pathway, activated factor VII binds to tissue factor on stimulated monocytes at sites of vascular injury. The tissue factor-factor VIIa complex then activates factors IX and X. The direct activation of factor X by the tissue factor-factor VIIa complex represents the extrinsic pathway, whereas activation of factor IX links the extrinsic and intrinsic pathways. [6] The intrinsic pathway is initiated in vitro by the activation of factor XII that occurs when blood is exposed to an activator, such as kaolin or celite. Activated factor XII then activates factor XI, which in turn activates factor IX. Finally, activated factor IX activates factor X in the presence of factor VIII, phospholipid, and calcium. The rate of this reaction is markedly enhanced by the presence of phospholipid and by prior exposure of factor VIII to thrombin or activated factor X.

Activated factor X completes the coagulation cascade by converting prothrombin to thrombin in the presence of activated factor V, phospholipid, and calcium. The rate of this reaction is accelerated by the presence of phospholipid and by thrombin or factor Xa activation of factor V. Thrombin then converts fibrinogen to fibrin, activates platelets, and activates factor XIII, which, in the presence of calcium, cross-links the fibrin, thereby stabilizing the clot.

Blood coagulation is modulated by positive and negative feedback loops. Thrombin and activated factor X activate factors VIII and V, markedly accelerating the coagulation reactions involving these two cofactors. [5] Although activated factor X can feed back to activate factor VII, this reaction is down-regulated by tissue factor pathway inhibitor (TFPI), which first complexes activated factor X, thereby rendering it inactive. The TFPI-Xa complex then binds the factor VIIa-tissue factor complex and prevents further activation of factor X. [7]

A low level of activation of blood coagulation occurs continuously in normal subjects. [8] However, factor VIIa, which is responsible for the initiation of coagulation in vivo, is the only coagulation factor that normally circulates in its enzyme form. In vivo activation is reduced in patients with hemophilia and is increased above normal in some patients with hereditary deficiencies of antithrombin, protein C, and protein S, [8] in patients with activated protein C resistance, [9] and in patients at increased risk of thrombosis after elective orthopedic surgery (Table 1241).[10] Activation of blood coagulation also increases with age in patients over the age of 45.

Coagulation may be activated by contact of factor XII with collagen on exposed subendothelium of damaged vessels or by contact with prosthetic surfaces. Coagulation is further augmented by activated platelets. Coagulation may also be initiated by the exposure of blood to tissue factor made available locally as a result of vascular wall damage, [6] by activation of endothelial cells by cytokines, and by activated monocytes that migrate to areas of vascular injury. [11] Factor X can be activated directly by extracts of malignant cells that contain a cysteine protease, [12] which may be one of the mechanisms by which thrombosis is induced in patients with malignant disease. A factor elaborated by hypoxic endothelial cells can also directly activate factor X [13] and could lead to thrombosis in patients with severe venous stasis, in whom stagnant hypoxia occurs in the valve cusps.

Clinical risk factors that predispose to venous thromboembolism by activating blood coagulation include malignancy, extensive surgery, trauma, burns, myocardial infarction, and possibly local hypoxia produced by venous stasis (Table 1241).[13]

Venous Stasis

Venous return from the legs is enhanced by contraction of the calf muscles, which propels blood upward from the extremities.

TABLE 124-1 -- Thrombogenesis

Stimulation
Activation of coagulation
Vessel damage
Stasis
Inhibition
Circulating inhibitors
(antithrombin III, protein C, protein S)
Endothelial cell components
(heparan sulfate, thrombomodulin)
Fibrinolytic system
(tissue plasminogen activator, plasminogen)

Venous stasis contributes to thrombogenesis by allowing activated coagulation factors to accumulate. In addition, stagnation of the blood could lead to local hypoxia, which stimulates endothelial cell release of an activator of factor X. [13] Venous stasis is produced by immobility, venous obstruction, increased venous pressure, venous dilation, and increased blood viscosity.

Immobility

Venous thrombosis occurs in immobilized individuals because blood pools in the intramuscular sinuses of the calf, which are dilated during recumbency. Many clinical examples highlight the role of stasis in the pathogenesis of venous thrombosis. Thus, the prevalence of venous thrombosis found at autopsy is markedly increased in individuals who were confined to bed for more than a week before death. Further, preoperative immobility is associated with a higher frequency of perioperative venous thrombosis. After surgery, patients remain at risk for venous thromboembolism for as long as they are immobile. Finally, immobility contributes to the high incidence of postoperative venous thrombosis in patients who have undergone hysterectomy, transabdominal prostatectomy, hip surgery, knee surgery, or surgery for fractures of the lower limb. The effect of immobility on thrombosis is well illustrated by comparing the location of thrombosis in paraplegics with that in stroke patients. Whereas thrombosis occurs with equal frequency in both legs in paraplegics, it occurs more frequently in the paralyzed limb in stroke patients.

Venous Obstruction

Venous obstruction contributes to the risk of venous thrombosis in patients with pelvic tumors, and to recurrent venous thrombosis in patients with persistent obstruction due to proximal vein thrombosis. Raised central venous pressure produces venous stasis in the extremities, which may explain the high prevalence of venous thrombosis in patients with heart failure. A similar mechanism may underlie the propensity for thrombosis in the left leg during pregnancy, presumably from obstruction of the left common iliac vein by the right common iliac artery.

Increased Blood Viscosity and Venous Dilation

Venous stasis can be caused by increased blood viscosity or venous dilation. The blood viscosity can be increased by an elevated hematocrit (polycythemia), hypergammaglobulinemia, dysproteinemias, or an increased fibrinogen concentration. Stasis due to venous dilation could contribute to the increased risk of thrombosis in patients with varicose veins and in elderly patients, particularly if they are bedridden. The ability of estrogen to cause venous dilation may explain the increased prevalence of thrombosis during pregnancy, [14] in individuals taking estrogen-containing oral contraceptive pills, [14] and in patients taking postmenopausal estrogen replacement therapy.[15] [16] [17]

Vessel Wall Damage

Although the normal endothelium is nonthrombogenic, damage or injury to the endothelium can trigger the activation of platelets and coagulation. Thus, vascular injury leads to the expression of tissue factor, either directly by endothelial cells or by monocytes that are attracted to the site of damage. In addition, the exposure of blood to subendothelium leads to platelet adhesion and aggregation.

The vascular endothelium can be damaged by direct trauma or it can be perturbed by exposure to endotoxin, inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), thrombin, or low oxygen tension. Perturbed endothelial cells synthesize tissue factor and PAI-1 and internalize thrombomodulinchanges that promote thrombogenesis.[18]

Direct venous damage may lead to venous thrombosis in patients undergoing hip surgery, knee surgery, or varicose vein stripping and in patients with severe burns or lower limb trauma. Perturbation of vascular endothelium by exposure to thrombin or inflammatory mediators probably contributes to the thrombosis that occurs after tissue damage, such as surgery or trauma, and in patients with acute or chronic inflammatory states (Table 124-1).

Protective Mechanisms

Endothelial Protective Mechanisms

Normal vascular endothelium is nonthrombogenic to flowing blood. Endothelial cell-surface glycosaminoglycans and thrombomodulin are potent inhibitors of coagulation, whereas vessel wall generation of prostacyclin and nitric oxide and synthesis of plasminogen activators limit platelet aggregation and fibrin deposition.

Thrombomodulin and heparan sulfate present on the luminal surface of endothelial cells are important modulators of thrombin activity. Heparan sulfate, a glycosaminoglycan similar to heparin, catalyzes the inhibition of thrombin and factor Xa by antithrombin. [3] Thrombomodulin serves as a surface-bound receptor for thrombin.[18] Once complexed with thrombomodulin, thrombin undergoes a conformational change that markedly alters its substrate specificity such that thrombin is no longer capable of activating platelets, of converting fibrinogen to fibrin, or of activating factors V, VIII, and XIII. [3] Rather, complexed thrombin acquires enhanced ability to activate protein C, which in turn proteolytically inactivates factors Va and VIIIa. This reaction requires protein S as a cofactor. Thus, once bound to thrombomodulin, thrombin not only loses its procoagulant activity but, by activating protein C, triggers a potent anticoagulant pathway.

The importance of the protein C anticoagulant pathway in the regulation of physiologic hemostasis has been highlighted by the syndrome of activated protein C resistance.[19] [20] [21] Activated protein C resistance is the most frequent hereditary condition predisposing to venous thrombosis, being found in 1630% of patients with acute venous thromboembolism. In 90% of cases, patients with activated protein C resistance have an inherited abnormality in their factor V gene, known as factor V Leiden (Arg506 Gln). This mutation inhibits the inactivation of factor Va by activated protein C. As a result, activated factor V persists, resulting in continued and excessive thrombin generation, thereby predisposing to thromboembolism (Table 1242).

Generation of plasminogen activators[22] by vascular wall cells limits fibrin deposition, while platelet aggregation is inhibited by the release of prostacyclin (PGI_2) and endothelium-derived nitric oxide. [23] Endothelial cell affinity for t-PA, plasminogen, and activated protein C and protein S may also contribute to the thromboresistance of the vessel wall.[3] Plasminogen binds to the cell surface, where it can be activated to plasmin by t-PA, thereby promoting local fibrinolytic activity. Bound activated protein C and protein S have a potent anticoagulant function.

Endothelial cells also have properties that are potentially procoagulant. Intact endothelium possesses receptors for activated

TABLE 124-2 -- Odds Ratio for Thromboembolism in Patients with Congenital Hypercoagulable States

Hypercoagulable State	Odds Ratio
Antithrombin deficiency type I	13.7
Antithrombin deficiency type II	9.8
Protein C deficiency	11.9
Protein S deficiency	10.0
Dysfibrinogenemia	18.0
Activated protein C resistance	2.7[a]

[a]Relative risk derived from large prospective cohort series. [123]

factors IX, X, and V. Perturbed endothelial cells also synthesize endothelins, which cause local vasoconstriction.

Inhibitors of Blood Coagulation

Activated coagulation factors are serine proteases, and their activity is modulated by several naturally occurring plasma inhibitors. The most important inhibitors of the blood coagulation system are antithrombin, protein C, and protein S. [3] An inherited deficiency of one of these three proteins is found in about 15% of patients who present with venous thrombosis before the age of 45.[24] Some congenital dysfibrinogenemias can also predispose to thrombosis, as can congenital deficiency of plasminogen (Table 124-2). A deficiency in heparin cofactor II, a secondary inhibitor of thrombin, has been associated with thrombosis, but a clear causal relationship has yet to be established.

Antithrombin

Antithrombin primarily inhibits thrombin and factor Xa, but it also inactivates factors XIIa, XIa and IXa to a lesser degree. The rate at which antithrombin inhibits these enzymes is markedly accelerated by unfractionated heparin and by naturally occurring heparin sulfate found on the luminal surface of endothelial cells. Antithrombin levels about 50% of normal are associated with venous thrombosis.

Congenital antithrombin deficiency is inherited as an autosomal dominant trait. Affected patients have antithrombin levels 4060% of normal, and 70% of those affected experience thromboembolic events before the age of 50. Although thrombosis usually occurs in the deep veins of the legs or manifests as PE, it may also occur at unusual sites such as the mesenteric, renal, and cerebral veins. Antithrombin is decreased slightly during heparin therapy, but there is no evidence that this modest reduction is clinically important. Levels of antithrombin are also reduced in protein-losing states, which may contribute to the increased risk of thrombosis in the nephrotic syndrome. Marked reductions in antithrombin levels occur in patients with liver disease or disseminated intravascular coagulation.

Protein C

Like other vitamin K-dependent coagulation factors, protein C contains -carboxyglutamic acid residues that are required for the normal function of this inhibitor. Protein C is activated by thrombin,[3] a process greatly enhanced by the interaction of thrombin with thrombomodulin. [3] [18] Activated protein C proteolytically inactivates factors Va and VIIIa[3] on the platelet and endothelial cell surface and also stimulates fibrinolysis. [3] Protein S, another vitamin K-dependent protein, is a necessary cofactor for activated protein C.[3]

Protein C deficiency is inherited in an autosomal dominant manner and is associated with familial venous thrombosis. [24] The level of protein C in affected heterozygotes is 4060% of normal. Although the clinical manifestations are similar to those of antithrombin deficiency, there are three unique features of protein C deficiency. Homozygotes can develop a severe thrombotic tendency in infancy characterized as purpura fulminans, heterozygotes have an increased risk of developing warfarin-induced skin necrosis because of the short half-life of this protein, and heterozygotes who are identified by mass screening may have a benign form of the condition with a low risk of venous thrombosis.

Protein S

This vitamin K-dependent plasma protein serves as a cofactor for activated protein C. [3] Protein S deficiency is also inherited in an autosomal dominant manner and is at least as common as antithrombin and protein C deficiency.[24] The clinical manifestations are similar to those seen with antithrombin and protein C deficiency. Thrombosis occurs in heterozygotes whose levels of functional protein S are in the range of 1550% of normal.

Dysfibrinogenemia

The majority of patients with dysfibrinogenemia are asymptomatic, about one-third have a bleeding tendency, and a much smaller proportion are predisposed to thrombosis.[25] In this last group, however, the risk of thrombosis in affected family members is much higher than in those who are unaffected, suggesting that there is a true relationship between certain dyfibrinogenemias and thrombosis. [24] The possible mechanisms by which a dysfibrinogenemia may lead to thrombosis include defective thrombin binding to fibrin, and the production of fibrin that is resistant to natural fibrinolysis.

Heparin Cofactor II

A secondary inhibitor of thrombin, heparin cofactor II complexes and inactivates the enzyme. The rate of this reaction is accelerated by both unfractionated heparin and dermatan sulfate. Although there have been reports of thrombosis associated with heparin cofactor II deficiency, a causal relationship remains to be established because the risk of thrombosis in family members with heparin cofactor II deficiency is similar to that in unaffected family members. [24]

Fibrinolytic System

The basic reaction of the plasma fibrinolytic system is the conversion of plasminogen to the active enzyme, plasmin, by limited peptide bond cleavage produced by a variety of plasminogen activators (Chap. 103). Two endogenous plasminogen activators, t-PA and urokinase, are synthesized by and released from endothelial cells. Urokinase is also synthesized by monocytes. Plasmin hydrolyzes fibrin and various plasma coagulation proteins, including fibrinogen, and factors V and VIII. As with the coagulation system, the activity of the fibrinolytic system is modulated by inhibitors that regulate both the plasminogen activators and the proteolytic effect of plasmin.[26]

Decreased fibrinolytic activity occurs in the early postoperative period, [2] in individuals taking oral contraceptives, [27] during the last trimester of pregnancy, [27] and in obese subjects.[28] Fibrinolytic activity in the leg veins is less than that in arm veins, which may partly explain the greater tendency for venous thrombosis to occur in the lower extremities. The relative reduction in fibrinolytic activity in the leg veins is more marked in the elderly.

Impaired fibrinolysis predisposes to postoperative venous thrombosis and possibly to coronary artery thrombosis in patients with coronary artery disease. [2] The impaired fibrinolytic activity reported in patients with venous thrombosis is acquired and is caused by increased levels of plasminogen activator inhibitor. [2] However, there are occasional reports of familial abnormalities of the fibrinolytic system in association with venous thrombosis. [29] [30]

Congenital Plasminogen Deficiency

Congenital plasminogen deficiency has been reported in association with venous thromboembolism in a number of families. [31] The pattern of inheritance appears to be autosomal, with heterozygotes having plasminogen levels (both activity and antigen) decreased to about 50% of normal. Functional abnormalities of plasminogen have also been described, some of which have been associated with venous thromboembolism. [32]

Other Clinical Disorders That Predispose to Venous Thromboembolism

Recently, an association between elevated levels of prothrombin (typically >1.50 U/liter) and venous thromboembolism has

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been proposed and confirmed. In most cases, the elevated levels are due to a mutation in the regulatory sequence of the prothrombin gene. This results in elevated basal prothrombin levels, which predispose to thrombosis by an unknown mechanism.

Other conditions reported to be associated with venous thromboembolism include thrombocytosis, polycythemia vera, and antiphospholipid antibodies. The antiphospholipid antibodies are a group of autoantibodies directed against anionic phospholipids in concert with cell-surface or plasma proteins (see Chap. 116).[33] [34] [35] There are two major groups of antiphospholipid antibodies, anticardiolipin antibodies and the lupus anticoagulant. The mechanism by which these antibodies predispose to thromboembolism is unclear. The absolute risk of thrombosis in patients with the lupus anticoagulant is increased. This finding has led to recommendations that patients with antiphospholipid antibodies and a history of thrombosis receive long-term, high-intensity warfarin therapy to prevent recurrence. [33] [36] This recommendation is based on methodologically inadequate studies and requires verification prior to its widespread application.

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NATURAL HISTORY OF VENOUS THROMBOEMBOLISM

Most thrombi are asymptomatic and confined to the intramuscular veins of the calf. These calf vein thrombi often undergo spontaneous lysis and rarely, if ever, produce long-term sequelae.[37] In contrast, complete lysis of proximal vein thrombosis is uncommon even when heparin treatment is given. [38]

The symptoms and signs of venous thromboembolism are caused by obstruction to venous outflow, inflammation of the vessel wall or perivascular tissues, or embolization of thrombus into the pulmonary circulation. Asymptomatic PE is detected by perfusion lung scanning in about 50% of patients with documented proximal vein thrombosis.[39] Most clinically significant and fatal PE probably arise from thrombi in the proximal veins of the legs. Although PE may also complicate calf vein thrombosis, these emboli tend to be smaller in size and occur less commonly than in patients with proximal vein thrombosis. [39] Asymptomatic venous thrombosis is found in 70% of patients who present with confirmed PE.[40] These thrombi are usually large and involve the proximal veins.

Extensive venous thrombosis causes venous valvular damage, which leads to the postphlebitic syndrome. [41] In addition, a previous history of venous thrombosis is associated with an increased risk of further episodes, particularly when patients are exposed to high-risk situations. [42]

Prognosis of Venous Thrombosis

Untreated or inadequately treated venous thrombosis is associated with a high complication rate, which can be decreased markedly by adequate anticoagulant therapy. About 20% of untreated silent calf vein thrombi and 2030% of untreated symptomatic calf vein thrombi extend into the popliteal vein. When extension occurs, it is associated with a 4050% risk of clinically detectable PE. [37] Patients with proximal vein thrombosis who are inadequately treated[43] have a 47% frequency of recurrent venous thromboembolism over 3 months, and patients with symptomatic calf vein thrombosis who are treated with a 5-day course of intermittent intravenous (IV) unfractionated heparin without continuing oral anticoagulants have a recurrence rate greater than 20% over the next 3 months. [44]

In contrast, clinically detectable recurrence occurs in less than 2% of patients with proximal vein thrombosis during the initial period of unfractionated heparin therapy[39] if an adequate anticoagulant response is achieved, and the recurrence rate during the subsequent 3 months of treatment with oral anticoagulants or moderate doses of subcutaneous unfractionated heparin is 24%. [43] [45] [46] After 3 months of anticoagulant therapy, the recurrence rate is 510% in the subsequent year.[43] [45] [46] Patients whose first episode of venous thrombosis was idiopathic and those who have ongoing risk factors, such as prolonged immobilization or cancer, are at higher risk of recurrence.

The significance of asymptomatic calf deep vein thrombosis discovered incidentally by screening venography after orthopedic surgery is unclear; recent studies suggest that the risk of these thrombi producing clinical sequelae is low. [47] As a result, if adequate perioperative deep vein thrombosis prophylaxis is given, it is likely not necessary to perform screening tests for deep vein thrombosis in asymptomatic patients.

Postphlebitic Syndrome

The postphlebitic syndrome is caused by venous hypertension, which is usually the result of valve destruction but occasionally can be produced by large proximal vein thrombi that block overflow.[48] Valve destruction results in malfunction of the muscular pump mechanism, which leads to increased pressure in the deep calf veins during ambulation. The high pressure ultimately renders the perforating veins of the calf incompetent, so that blood flow is directed from the deep veins into the superficial venous system during muscular contraction. This leads to edema and impaired viability of subcutaneous tissues and, in its most severe form, to venous ulceration. Outflow obstruction may initially be bypassed by the development of collateral veins, but with time, the veins distal to the obstruction become dilated and their valves become incompetent.

In patients whose thrombosis extends into the iliofemoral veins, the swelling may never disappear. In contrast, the swelling may subside after initial treatment but recur months or years later in patients with less extensive proximal vein thrombosis. Other symptoms and signs of the postphlebitic syndrome may be delayed for 510 years after the initial thrombotic event. These symptoms include pain in the calf that is relieved with rest and leg elevation, pigmentation and induration around the ankle and lower third of the calf, and ulceration, which usually occurs in the region of the medial malleolus.

Patients with extensive thrombosis involving the iliofemoral vein frequently have greater disability and may even have venous claudication, which is characterized by incapacitating, bursting pain with exercise. [49] This complication rarely occurs in patients with thrombosis involving the more distal veins.

The risk of postphlebitic syndrome has recently been quantified. Prandoni et al., [50] in a prospective cohort study, demonstrated that 29.1% of patients with acute deep vein thrombosis will develop the syndrome after 8 years of follow-up. The development of ipsilateral recurrent thrombosis was associated with a large increase in risk for the development of postthrombotic syndrome.

Whether therapy for acute venous thrombosis reduces the long-term risk of postphlebitic syndrome is unclear. Practically, therapy that reduces thrombus extension or prevents the development of profound outflow obstruction from the limb is likely to reduce the long-term risk of postphlebitic syndrome. In addition, the use of graduated compression stockings may reduce the risk of postphlebitic syndrome in patients with deep vein thrombosis.

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DIAGNOSIS OF VENOUS THROMBOSIS

The approach to the diagnosis of venous thrombosis has changed radically over the past 20 years. It is now accepted that the clinical diagnosis of venous thrombosis is nonspecific[51] and that objective tests are necessary to confirm the diagnosis. This concept has led to three new developments. The first is the widespread use of venography to confirm a clinical suspicion

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of venous thrombosis, the second is an improvement in the technique and safety of venography, and the third has been the development and careful evaluation of noninvasive tests to replace venography.

In symptomatic patients, venous ultrasonography (US) is sensitive and specific for proximal deep vein thrombosis (thrombosis of the popliteal or more proximal veins); however, US is insensitive to calf vein thrombosis and to deep vein thrombosis occurring after orthopedic surgery. [52] To increase the sensitivity of US for clinically important calf thrombosis and to improve the safety of diagnostic strategies that do not include venography in patients with suspected calf vein thrombosis, US can be repeated 7 days after the initial study. This strategy will detect the 1030% of calf vein thrombi that extend proximally. If the test remains negative after 7 days, the risk of clinically important proximal extension is negligible, and it is safe to withhold treatment. [53] [54] [55] [56]

Clinical Diagnosis

Although clinical diagnosis is nonspecific, careful documentation of clinical symptoms and signs is helpful in ruling out venous thrombosis when an alternative cause is identified (e.g., sciatica, cellulitis, ruptured Bakers cyst, muscle or tendon tear, cutaneous vasculitis, arthritis). In addition, a recent study found that patients could be classified into high-, intermediate-, and low-probability groups based on their clinical manifestations and the presence or absence of risk factors such as recent immobilization, hospitalization within the past 6 months, or malignancy. [57] Patients with the classic symptoms and signs of deep vein thrombosis, such as localized pain, tenderness, swelling, and discoloration, and who had at least one risk factor had an 85% probability of venous thrombosis, while those with atypical symptoms and no risk factors had only a 5% probability of venous thrombosis. These low and high pretest probabilities can be combined with the results of objective noninvasive tests to make clinical decisions. Thus, a low pretest clinical probability and a negative result on noninvasive testing can be used to exclude a diagnosis of venous thrombosis, thereby obviating further testing. In contrast, a high pretest clinical probability and a negative noninvasive test result should prompt further investigation with venography.

About 70% of patients referred for clinically suspected venous thrombosis will not have the diagnosis confirmed by objective tests. [53] [54] Of the 30% who have venous thrombosis, about 85% have proximal vein thrombosis and the remainder have thrombosis confined to the calf. [53] [54] The conditions that most frequently simulate venous thrombosis are a ruptured Bakers cyst, cellulitis, muscle tear, muscle cramp, muscle hematoma, external venous compression, superficial thrombophlebitis, and the postphlebitic syndrome.

Objective Tests for the Diagnosis of Venous Thrombosis

Both invasive and noninvasive tests are useful for the diagnosis of venous thrombosis. Venography is the only invasive test of proven value, whereas the noninvasive studies that are useful are impedance plethysmography and venous US. Because venous US is the most sensitive and specific noninvasive test, it is considered the test of choice. Unlike venography, both of the noninvasive tests lack sensitivity for calf vein thrombosis.

Venography

Venography remains the reference standard for the diagnosis of venous thrombosis. [58] It is technically difficult to perform, and its proper execution and interpretation require considerable experience. With good technique, ascending venography outlines the entire deep venous system of the lower extremities, including the external and common iliac veins. Venography may produce superficial phlebitis and can cause deep vein thrombosis. [58]

Impedance Plethysmography

This noninvasive test uses electrical resistance (impedance) to detect blood volume changes in the calf produced by inflation and deflation of a pneumatic thigh cuff. The changes in blood volume are reduced if the popliteal or more proximal veins are obstructed.

The impedance test does not distinguish between thrombotic and nonthrombotic obstruction to venous outflow. Thus, falsely positive results may be obtained during pregnancy if a patient is positioned incorrectly or inadequately relaxed, if the vein is compressed by an extravascular mass, or if venous outflow is impaired by increased central venous pressure. Reduced arterial inflow to the limb due to severe obstructive arterial disease can also lead to reduced outflow, thereby producing a falsely positive result.

The reported sensitivities and specificities for the diagnosis of symptomatic proximal venous thrombosis range from as low as 65% to as high as 95%. [53] [59] [60] [61] [62] [63] The test may not detect large, nonocclusive proximal vein thrombi and fails to detect most thrombi within the calf veins.

In patients with proximal vein thrombosis, the result of impedance plethysmography returns to normal in about 30% of patients by 3 weeks, in 60% of patients at 6 weeks and 3 months, in 80% of patients at 6 months, and in 90% of patients at 1 year. [61] Information on the status of impedance plethysmographic findings is clinically useful if the patient returns with recurrent symptoms. Thus, in patients whose study returned to normal after an episode of venous thrombosis, a positive test result and appropriate clinical symptoms are highly suggestive of recurrent venous thrombosis.

Venous Ultrasonography

This test is performed using a high-resolution real-time scanner equipped with a 5-MHz electronically focused linear array transducer. The common femoral vein and femoral artery are first located in the groin with the patient in a supine position. The superficial femoral vein is then examined along its course. Once this has been done, the popliteal vein is located and examined down to the level of its trifurcation into the peroneal and tibial veins. At each of these locations, the vein being examined is compressed gently but firmly with the transducer probe and the results are observed on the monitor. Hard copies from freeze frame images of both stages

of the procedure are obtained and serve as a permanent record.

Venous US is very accurate for the detection of proximal vein thrombosis in symptomatic patients but relatively insensitive to calf vein thrombosis. If the field of examination is extended to the distal popliteal vein and the proximal deep calf veins, venous US detects about 50% of calf vein thrombi in symptomatic patients. [64] [65] Potential limitations of venous US include inability to visualize the iliac veins and the segment of the superficial femoral vein within the femoral canal. This is not a serious limitation because isolated thrombi within the femoral canal or the iliac vein are rare. [66] Further, the obstruction produced by iliac vein thrombi often limits the compressibility of the common femoral vein segment and hence will be detected by venous US.

Other Techniques

A number of new techniques are being evaluated for the diagnosis of acute and recurrent deep vein thrombosis. One such technique is the D-dimer assay, which uses mono- or polyspecific antibodies against D-dimer to provide quantitative or

CLINICALLY SUSPECTED VENOUS THROMBOSIS

A practical noninvasive approach for the diagnosis of clinically suspected deep vein thrombosis (DVT) using serial venous ultrasonography. [a]

[a] *If noninvasive testing is unavailable, venography should be performed.*

qualitative data on the concentration of D-dimer in whole blood or plasma. D-dimer is a product of fibrin lysis and as such is increased in patients with acute venous thromboembolism. However, the test is nonspecific, because the level of D-dimer is also increased in a variety of acute illness, including malignancy and infections. Therefore, the D-dimer assay is most useful as a tool to rule out suspected deep vein thrombosis. [67] [68]

Other techniques that may prove useful in the diagnosis of acute venous thromboembolism include contrast agentenhanced co-axial tomography (CT), spiral CT, and magnetic resonance angiography. Techniques such as thermography do not have sufficient accuracy to be useful.

Approach to the Diagnosis of Venous Thrombosis

Venous US is the noninvasive method of choice because it is the most sensitive and specific. Although it is relatively insensitive to calf vein thrombosis, this shortcoming can be overcome by repeating the test after 57 days.

A diagnostic algorithm for the noninvasive diagnosis of clinically suspected venous thrombosis is shown in the box. If venous US is not available or does not reveal evidence of thrombosis in a patient with a high pretest likelihood of deep vein thrombosis, the diagnosis should be confirmed with ascending venography. Alternatively, in patients with a moderate pretest likelihood of thrombosis who have a negative initial US study, serial US examination is a safe and effective alternative to venography.

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DIAGNOSIS OF PULMONARY EMBOLISM

The clinical diagnosis of PE is as nonspecific as the diagnosis of venous thrombosis. Therefore, objective tests are necessary. However, PE is more difficult to diagnose than venous thrombosis, and the diagnosis is confirmed in less than half of patients with clinically suspected PE. [40] [69]

The chest radiograph is not specific for PE and usually does not show any diagnostic abnormality. However, it is useful to exclude other causes for the presenting symptoms (e.g., pneumothorax) and is essential for interpreting the lung scan findings. Like the chest x-ray, the electrocardiogram (ECG) is frequently normal or shows nonspecific abnormalities. However, in the appropriate clinical setting, ECG evidence of right ventricular strain is strongly suggestive of PE.

Perfusion lung scanning is useful because a normal scan excludes the diagnosis of PE, whereas a large perfusion defect that is not matched on the ventilation scan is highly suggestive of PE. Other lung scan findings, which are seen in about 70% of patients, cannot be used to rule out or rule in PE. It is in these patients that pulmonary angiography or objective tests for venous thrombosis are needed. Tests for venous thrombosis are useful because 70% of PE are associated with venographically detected deep vein thrombosis of the legs.

Clinical Manifestations of Pulmonary Embolism

Most PE are clinically silent. Dyspnea is the most frequently reported symptom. [69] [70] Chest pain is common and is usually pleuritic in nature but may be substernal and compressing. Hemoptysis is a less frequent feature of PE. [40] [69] [70]

The physical signs of PE are nonspecific. Syncope is usually associated with massive PE and is caused by a reduction in cardiac output. This in turn results in hypotension and transient impairment of cerebral blood flow.

Although 70% of patients with PE have venographic evidence of thrombosis at presentation, less than 20% of these patients have leg symptoms. [40] Massive PE causes tachypnea, tachycardia, cyanosis, and hypotension. In these patients, cardiac examination may reveal a right ventricular heave, a loud pulmonary second sound, and a gallop rhythm. Physical examination of the chest may be normal or nonspecific abnormalities may be detected. Patients with pulmonary infarction or atelectasis may have reduced movement of the affected portion of the chest. There may be signs of pulmonary consolidation or atelectasis, a pleural friction rub, crackles, or a pleural effusion, and a low-grade fever may be present.

Differential Diagnosis

The differential diagnosis of shortness of breath includes atelectasis, pneumothorax, pneumonia, acute bronchitis, acute bronchiolitis, acute bronchial obstruction due to mucous plugging or bronchoconstriction, and acute PE. Entities in the differential diagnosis of pleuritic chest pain include pneumonia, viral pleurisy, chest wall pain from trauma or viral infection, pericarditis, and pleural inflammation caused by an immune disorder.

Diagnostic Tests for Pulmonary Embolism

The diagnostic tests for PE include pulmonary angiography and ventilation-perfusion lung scanning. Recently, spiral CT has been introduced for the diagnosis of acute PE. This test, which involves taking high-resolution, high-speed scans of the lung after a bolus injection of contrast agent, is able to demonstrate intraluminal filling defects in the pulmonary vasculature. However, spiral CT has not been compared, in methodologically rigorous studies, to currently available widely used imaging techniques for diagnosing PE. Therefore, its role in the diagnosis of PE is uncertain.

Pulmonary Angiography

This test is the reference standard for establishing the presence or absence of PE. [71] Selective angiography and magnification views improve resolution and reduce the risk of the procedure. [71] When adequately performed, a negative result on pulmonary

angiography excludes the diagnosis of PE. [71] However, unless the tertiary pulmonary arteries are visualized in a patient with a small perfusion defect, the diagnosis of PE cannot be excluded.

Arrhythmias, cardiac perforation, cardiac arrest, and hypersensitivity reactions to contrast medium occur in up to 34% of patients undergoing pulmonary angiography. [72] Patients with a history of allergy to radiopaque dye should not undergo pulmonary angiography.

Lung Scan

The lung scan consists of both a perfusion and a ventilation component. For the perfusion component, particles of isotopically labeled microaggregates of human albumin are injected IV and become trapped in the pulmonary capillary bed. Their distribution reflects lung blood flow and is recorded with an external photoscanner. A normal perfusion scan excludes PE, but an abnormal perfusion scan is nonspecific. [40] [70] [73]

Ventilation lung scanning is performed using either radioactive gases or aerosols that are inhaled and exhaled by the patient while a gamma camera records the distribution of radioactivity within the alveolar gas exchange units. The purpose of ventilation imaging is to improve the specificity of perfusion scanning for the diagnosis of PE. When ventilation scanning was initially introduced, it was assumed that ventilation would be preserved in areas of reduced perfusion caused by PE (so-called ventilation-perfusion mismatch), whereas ventilation would be abnormal when perfusion defects occurred as a consequence of primary abnormalities of ventilation (ventilation-perfusion match). This assumption has proved to be only partially correct. [40] [70] [73]

Approach to the Diagnosis of Pulmonary Embolism

Patients with large perfusion defects (one or more segments or more extensive defects) and a ventilation mismatch have a 90% probability of PE. However, the frequency of PE is not sufficiently low to rule out PE in patients with a large ventilation-perfusion match and in those with small perfusion defects. [40] [70] [73] As a result, patients with these lung scan findings require further investigation with either pulmonary angiography or objective tests for venous thrombosis. Although a positive

result on venography or compression US can be used to make the diagnosis of venous thromboembolism in a patient with an abnormal perfusion scan, a negative result cannot be used to exclude venous thrombosis because the thrombus may have completely embolized to the lungs. [40] [70]

A diagnostic algorithm for the management of clinically suspected PE is shown in the box. After a history and physical examination, ECG, and chest radiography, all patients should undergo ventilation-perfusion lung scanning. A negative perfusion lung scan rules out clinically significant PE, and anticoagulant therapy is withheld. If the perfusion scan demonstrates one or more segmental (or larger) defects and ventilation to these regions is normal, a diagnosis of PE is made. Although a ventilation-perfusion mismatch supports a diagnosis of PE, a

DIAGNOSTIC APPROACH WHEN PULMONARY EMBOLISM SUSPECTED

ventilation-perfusion match does not exclude PE, and further objective testing is required in these patients. Similarly, the diagnosis of PE cannot be excluded in patients with small perfusion defects (one or more subsegmental defects) or those with indeterminate lung scan findings (in which the perfusion defects correspond to abnormalities on the chest radiograph). In these cases, bilateral venography or venous US should be performed. If deep vein thrombosis is documented, anticoagulant therapy can be started, thereby obviating pulmonary angiography. However, if these tests are negative, pulmonary angiography is required in patients in whom the clinical suspicion of PE is high. For those with a lower pretest likelihood of PE, an alternative strategy is to withhold anticoagulants and to perform serial noninvasive tests to detect ongoing venous thrombosis. In patients with a low or low to moderate pretest likelihood of PE, a D-dimer test may prove useful; if the D-dimer assay is negative, further testing could be avoided, whereas if it is positive (increasing the likelihood of acute thrombosis), further testing is likely warranted. [74] [75]

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DIAGNOSIS OF RECURRENT VENOUS THROMBOEMBOLISM AND THE POSTPHLEBITIC SYNDROME

The majority of patients with a history of venous thrombosis who develop new-onset leg pain do not have recurrent deep vein thrombosis when investigated with the appropriate diagnostic tests. Some of these patients have the postphlebitic syndrome, while in others the pain is unrelated to venous thrombosis. [76]

Diagnosis of Acute Recurrent Venous Thrombosis

The diagnosis of acute recurrent venous thrombosis is difficult to make because the clinical manifestations of recurrence are nonspecific. In addition, all of the validated diagnostic tests for acute venous thrombosis have limitations in this setting because the venous occlusion produced by the initial episode of venous thrombosis makes it difficult to identify new abnormalities.

Our approach to the diagnosis of clinically suspected recurrent venous thrombosis is to use a combination of impedance plethysmography, compression US, and venography. Once anticoagulants are discontinued, patients undergo serial impedance plethysmography and compression US at 3- to 6-month intervals until the test results normalize. Although the impedance plethysmogram normalizes more frequently than venous US, both tests are useful because they provide results that can be used for comparison should the patient present with recurrent symptoms.

The diagnostic algorithm for patients with suspected recurrent venous thrombosis is shown in the box. Both the impedance plethysmography and compression US are performed. If either test is positive and the previous result was negative, a diagnosis of recurrence is made. Similarly, if venous US shows more extensive thrombosis than that seen on previous examinations, a diagnosis of recurrent disease can also be established. However, if both impedance plethysmography and venous US are positive and the previous tests also were positive, venography is performed. If this study shows a new intraluminal filling defect or evidence of thrombus extension when compared with the previous venogram, a diagnosis of recurrence is made. If no new defect is found, the diagnosis of recurrent thromboembolism is based on clinical features. Finally, if both impedance plethysmography and venous US are negative at presentation, the patient is followed with compression US repeated once over the next week.

Diagnosis of the Postphlebitic Syndrome

The clinical spectrum of the postphlebitic syndrome varies from a course that may mimic acute venous thrombosis to one of persistent leg pain that is worse at the end of the day and is associated

CLINICALLY SUSPECTED RECURRENT VENOUS THROMBOSIS

with dependent edema, stasis pigmentation, and, in its most severe form, skin ulceration. [77] Rarely, patients may complain of venous claudication on walking. [78] When symptoms are acute or subacute in onset, a diagnosis of postphlebitic syndrome should be considered only after recurrent venous thrombosis has been excluded by objective tests. There is no single definitive diagnostic test for the postphlebitic syndrome, but a past history of objectively documented deep vein thrombosis, appropriate clinical findings, and evidence of venous reflux or outflow obstruction on venous US constitute sufficient evidence to make this diagnosis.

Other causes of recurrent leg pain or swelling include recurrent muscle strain, internal derangement of the knee or hip, recurrent cellulitis, extrinsic compression of the vein, lumbosacral disk disease, sciatic pain, and factitious causes of leg pain and swelling. In some patients there is no explanation for the leg pain, and the possibility of thromboneurosis should be considered. These patients may complain of leg pain and tenderness that is disproportionate to the physical findings, or they may present with highly atypical symptoms. Patients with severe thromboneurosis may be incapacitated by the fear of recurrent venous thrombosis or death. Frequently, there is a history of multiple hospital admissions for treatment of recurrent venous thrombosis, and many of these patients are on long-term anticoagulant therapy, and some have had caval interruption procedures. Thromboneurosis may have an iatrogenic component, as the fear of recurrent thrombosis is reinforced each time the attending physician admits the patient to the hospital and orders treatment on the basis of clinical suspicion alone. To prevent this, it is important that a clinical suspicion of acute venous thrombosis (either the first episode or recurrent episodes) is always confirmed by appropriate objective tests. In some patients, the fear of thrombosis is relieved only after suspected recurrent episodes of venous thrombosis have been excluded by appropriate tests on multiple occasions.

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PROPHYLAXIS OF VENOUS THROMBOEMBOLISM

PE is the most common preventable cause of death in hospital.[79] Hospitalized patients can be classified as at low, moderate, or high risk for developing venous thromboembolism (Table 1243).[79] In the absence of prophylaxis, the frequency of postoperative fatal PE ranges from 0.10.8% in patients undergoing elective general surgery to 0.31.7% in patients undergoing elective hip surgery and 47% in patients undergoing emergency hip surgery.[79] Effective prophylaxis is very cost-effective and is available for most high-risk groups.[80]

Prophylaxis is directed toward either suppressing activation of blood coagulation or preventing venous stasis. These objectives can be achieved by using one of the following proven prophylactic approaches: low-dose subcutaneous unfractionated heparin,[79] [81] [82] intermittent pneumatic compression of the legs,[79] [82] IV dextran,[79] oral anticoagulants,[79] [82] [83] adjusted doses of subcutaneous unfractionated heparin,[84] graduated compression stockings,[82] [85] or low molecular weight heparins.[83] [86] Antiplatelet agents such as aspirin are relatively ineffective for preventing venous thromboembolism.[79] [82] [83]

Low-Dose Unfractionated Heparin

Low doses of unfractionated heparin prevent thrombosis by catalyzing the inhibition of thrombin and factor Xa by antithrombin. Unfractionated heparin is given subcutaneously at a dose of 5,000 U 2 hours prior to surgery and is continued postoperatively at a dose of 5,000 U every 12 hours. Low-dose unfractionated heparin prophylaxis does not require laboratory monitoring and is simple and convenient to administer. It is the method of choice for moderate-risk general surgical and medical patients, and it reduces the risk of venous thromboembolism by 5070%.[79] [81] [82] [83] When used in these doses, unfractionated heparin is safe and free of serious bleeding complications, but because of the potential for minor bleeding, it should not be used in patients undergoing cerebral, eye, or spinal surgery. Although low-dose unfractionated heparin is effective in patients undergoing elective hip surgery and reduces the incidence of venous thrombosis by about 40%, it is less effective than warfarin, adjusted-dose unfractionated heparin,[84] and low molecular weight heparin.[83] [86] Low-dose unfractionated heparin has not been shown to be effective in patients with hip fractures or those undergoing major knee surgery.

Intermittent Pneumatic Compression

Intermittent pneumatic compression of the legs enhances blood flow in the deep veins and increases blood fibrinolytic activity.[79] It is the method of choice for preventing venous thrombosis in patients undergoing neurosurgery,[85] is effective in patients undergoing major knee surgery,[87] and is as effective as low-dose unfractionated heparin in patients undergoing abdominal surgery.[82] Intermittent pneumatic leg compression is virtually free of clinically important side effects and is particularly useful in patients who have a high risk of bleeding.

Graduated Compression Stockings

Graduated compression stockings also reduce venous stasis in the legs and are effective for preventing postoperative venous thrombosis in general surgical patients[82] and in medical or surgical patients with neurologic disorders, including paralysis of the lower limbs.[85] In surgical patients, the combination of graduated compression stockings and low-dose unfractionated heparin is significantly more effective than low-dose unfractionated heparin alone.[88] [89] However, graduated compression stockings alone are inadequate prophylaxis in patients undergoing

TABLE 124-3 -- Risk Categories for Venous Thromboembolism

Risk Category	Risk of Venous Thromboembolism (%)		
	Calf Vein Thrombosis	Proximal Vein Thrombosis	Fetal Pulmonary Embolism
High Risk			
1. General surgery in patients >40 years with recent history of DVT or PE	4080	1020	15
2. Extensive pelvic or abdominal surgery for malignant disease			
3. Major orthopedic surgery on lower limbs			
Moderate Risk[a]			
1. General surgery in patients >40 years lasting 30 minutes or more	1040	210	0.10.7
2. Immobilization with major medical illness, including stroke, cardiac disease, chronic respiratory disease, bowel disease, and malignancy			

[a]Risk is increased by advancing age, malignancy, prolonged immobility, varicose veins, and cardiac failure.

surgery associated with a very high risk of thromboembolism.[90] Graduated compression stockings are inexpensive and should be considered in all high-risk surgical patients, even if other forms of prophylaxis are used.

Oral Anticoagulants

When administered in doses that prolong the prothrombin time to an International Normalized Ratio (INR) of 2.0, oral anticoagulants effectively prevent postoperative venous thromboembolism in patients in all risk categories.[79] [83] Oral anticoagulants can be given preoperatively, at the time of operation, or in the early postoperative period. When started at the time of surgery or in the early postoperative period, the anticoagulant effect is not achieved until the third or fourth postoperative day. Nevertheless, when used in this fashion, oral anticoagulants are effective in very high-risk patient groups, including patients with hip fractures.[91] However, prophylaxis with oral anticoagulants is relatively inconvenient because careful laboratory monitoring is necessary.

Dextran

Dextran 40 is usually given IV at a daily dose of 500 ml over 46 hours. Treatment is started at the time of surgery and continued for 25 days postoperatively. [79] [83] Although dextran is an effective form of prophylaxis, it is inconvenient, can cause fluid overload in patients with cardiovascular impairment, and increases the risk of postoperative bleeding. In addition, dextran is less effective than warfarin [92] and low molecular weight heparin. [93] Given these limitations, dextran is rarely used for prophylaxis.

Adjusted-Dose Subcutaneous Unfractionated Heparin

In this regimen, unfractionated heparin is given subcutaneously at a dose of 3,500 U three times daily starting 2 days before surgery. The unfractionated heparin dose is then adjusted to maintain the activated partial thromboplastin time (aPTT) at the upper limit of the normal range. Leyvarz and associates [84] and Green and associates [94] have reported that adjusted-dose unfractionated heparin regimens are more effective than fixed low-dose unfractionated heparin in patients undergoing elective hip surgery and in those with spinal cord injury. When the unfractionated heparin dose was adjusted to prolong the postoperative aPTT into the upper normal range, there was no increase in bleeding. [84] However, an unfractionated heparin dose that prolonged the aPTT to 1.5 times normal did cause excessive bleeding. [94]

Low Molecular Weight Heparins

Low molecular weight heparin is an effective and safe form of prophylaxis in the following high-risk groups [86] : patients undergoing elective hip surgery, [86] those with hip fractures, patients undergoing major general surgery or major knee surgery, those with spinal injury, and those who have sustained a stroke. Low molecular weight heparin is more effective than standard low-dose unfractionated heparin in general surgical patients, [86] patients undergoing elective hip surgery, [86] and patients with stroke [86] or spinal injury. [86] In addition, low molecular weight heparin has also been shown to be more effective than warfarin in patients undergoing hip or major knee surgery, [86] better than adjusted-dose unfractionated heparin in preventing proximal vein thrombosis after elective hip surgery, [86] and superior to dextran or aspirin after hip surgery. [86]

Choice of Prophylaxis in Different Patient Groups

The recommended prophylactic approaches for patients in the various risk categories are summarized in Table 124-4 .

TABLE 124-4 -- Recommended Prophylactic Approaches for the Alternative Risk Categories and Different Surgical Groups

Risk Category	Recommended Approaches[a]
Moderate Risk	
General surgery or major medical illness	Low-dose heparin or intermittent compression
Neurosurgery or genitourinary surgery	Intermittent compression
High Risk[a]	
Elective hip surgery or very high-risk general surgery	Low molecular weight heparin or oral anticoagulants
Fractured hip or major knee surgery	Low molecular weight heparin or oral anticoagulants

[a] In high-risk patients.

General Surgical and Medical Patients

Early ambulation should be encouraged, and graduated compression stockings should be considered for all patients. Moderate-risk patients should be given prophylaxis with low-dose unfractionated heparin. If anticoagulants are contraindicated because of an unusually high risk of bleeding, intermittent pneumatic compression should be used. Patients at very high risk, for example, those with recent venous thrombosis, should receive low molecular weight heparin, oral anticoagulants, or adjusted-dose unfractionated heparin.

Hip Surgery

Low molecular weight heparin, oral anticoagulants, or adjusted-dose unfractionated heparin is effective. In direct comparisons, fixed-dose low molecular weight heparin was more effective than warfarin in preventing thrombosis and was better than adjusted-dose unfractionated heparin in preventing proximal vein thrombosis.

Major Knee Surgery

Although intermittent pneumatic compression is effective in preventing venous thrombosis after major knee surgery, low molecular weight heparin is more convenient and is the treatment of choice.

Genitourinary Surgery, Neurosurgery, Ocular Surgery

Intermittent pneumatic compression, with or without the use of static graduated compression stockings, is effective and does not increase the risk of bleeding.

Stroke and Spinal Injury

Low molecular weight heparin is more effective than low-dose standard unfractionated heparin in these patient groups and is the prophylactic method of choice.

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TREATMENT OF VENOUS THROMBOEMBOLISM

The objectives of treating patients with venous thromboembolism are to prevent death from PE, to reduce morbidity from the acute event, to minimize postphlebitic symptoms, and to prevent thromboembolic pulmonary hypertension. All of these goals can be achieved with adequate anticoagulant therapy.

Preventing Death Due to Pulmonary Embolism

Anticoagulants are effective in reducing mortality from PE. [95] Vena caval interruption, usually with an inferior vena caval filter,

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should be considered when anticoagulant therapy is contraindicated because of the risk of previous or life-threatening hemorrhage. [95] Far less commonly, caval interruption is used when anticoagulant therapy has failed.

Thrombolytic therapy with streptokinase, urokinase, or t-PA is more effective than unfractionated heparin alone in improving the angiographic defects produced by PE,[95] and may be better than unfractionated heparin in preventing death in patients with massive PE associated with shock. [96] [97] Based on these findings, thrombolytic therapy is the treatment of choice for patients with massive PE, or in those with underlying cardiac or pulmonary disease, in whom even a small or moderate embolus may be life-threatening.

Thromboendarterectomy is effective in selected cases of chronic thromboembolic pulmonary hypertension with proximal pulmonary arterial obstruction. [98] Urgent pulmonary embolectomy is usually reserved for patients with a saddle embolism lodged in the main pulmonary artery or for those with massive embolism whose blood pressure cannot be maintained despite thrombolytic therapy and vasopressor agents. [95]

Anticoagulant Therapy to Reduce Morbidity from the Acute Event

Anticoagulants are the mainstay of treatment for most patients with venous thromboembolism. A recent study in patients with proximal vein thrombosis highlights the importance of early unfractionated heparin therapy. [99]

Three large clinical trials have recently been published that demonstrate the effectiveness and safety of outpatient treatment of acute deep vein thrombosis in otherwise well patients.[100] [101] [102] In each of these studies, fixed-dose, weight-adjusted, low molecular weight heparin was compared with adjusted-dose, inpatient treatment with IV unfractionated heparin. When the results of these three studies are combined, outpatient treatment was not associated with either an increase in the risk of recurrent thromboembolism or an increased rate of significant hemorrhage. Based on these findings, outpatient treatment of acute deep vein thrombosis should be considered in all patients who present with acute venous thromboembolism but are otherwise well.

Treatment should consist of weight-adjusted once or twice daily subcutaneous low molecular weight heparin. Monitoring of the anticoagulant effect of the low molecular weight heparin is not required. If anticoagulant-related complications develop, if the patient is very large (>100 kg) or very small (<40 kg), or if the patient has renal insufficiency, then the anticoagulant effect of the low molecular weight heparin can be assessed using anti-factor Xa heparin levels. The target anti-factor Xa heparin level is 0.51.5 U/ml. Low molecular weight heparin does not have a predictable effect on the aPTT; thus this test cannot be used for monitoring low molecular weight heparin. Warfarin should be initiated on the same day as the unfractionated heparin. The low molecular weight heparin should be continued for at least 4 days and until the INR has been between 2.0 and 3.0 on 2 consecutive days.

Patients with co-morbid conditions that preclude outpatient treatment, with symptomatic PE, or who develop thromboembolism during hospitalization should be treated with either unfractionated heparin or low molecular weight heparin in the hospital. [103] Prospective randomized trials have compared the safety and effectiveness of unfractionated heparin given by either continuous IV infusion, intermittent IV injection, or subcutaneous injection. Although continuous IV unfractionated heparin infusion[95] produces less bleeding than intermittent IV injections, unfractionated heparin delivery by intermittent subcutaneous injections appears to be as safe and efficacious as continuous IV unfractionated heparin infusion in the treatment of venous thrombosis. [95] [104] Regardless of whether unfractionated heparin is given by continuous IV infusion or by subcutaneous injection, it is important to give doses sufficient to produce an adequate antithrombotic effect (see section on Practical Recommendations).

Unfractionated heparin has a half-life that varies considerably among individuals. [105] After a single IV injection, there is an initial rapid disappearance owing to a saturable clearance mechanism, followed by a more gradual linear clearance, [106] with a mean heparin half-life of approximately 60 minutes. Whereas IV unfractionated heparin produces an immediate anticoagulant effect, peak heparin levels are not achieved until 3 hours after subcutaneous injection, but the levels will remain therapeutic for up to 12 (or more) hours, depending on the dose.

Unfractionated heparin therapy can be monitored using the activated clotting time and the aPTT, or by heparin assays that measure the ability of heparin to accelerate the inactivation of factor Xa or thrombin. It is important to give adequate initial unfractionated heparin doses, as the risk of recurrent venous thromboembolism is likely increased if insufficient unfractionated heparin is given. Accordingly, the aPTT should be maintained above a level equivalent to a heparin level of 0.3 U/ml as determined by measuring the anti-factor Xa activity. For most currently used aPTT reagents, this is equivalent to an aPTT ratio of 1.8 times the control value. Thus, the recommended therapeutic range is an aPTT ratio of 1.82.5. [107] Higher unfractionated heparin doses may be associated with an increase in the risk of bleeding.[106]

In the past, heparin was administered for a period of 710 days, and oral anticoagulants were started after 35 days and overlapped with heparin for 45 days. The period of overlap is necessary because the antithrombotic effects of oral anticoagulants are delayed. [109] [110] Two studies have demonstrated that 910 days of unfractionated heparin therapy is no better than a 4- to 5-day course of unfractionated heparin with overlapping warfarin. [108] Since neither study included many patients with major PE or extensive iliofemoral vein thrombosis, it would be prudent to use heparin for at least 7 days in these individuals.

After an initial course of heparin therapy, patients with venous thromboembolism require continuing anticoagulant therapy for weeks or months to prevent recurrence.[43] [44] Both therapeutic doses of subcutaneous heparin and oral anticoagulants are effective for this indication. Although adjusted-dose unfractionated heparin produces less bleeding than high-intensity warfarin (target INR of 3.55.0) in this setting, [45] less intense warfarin therapy (target INR of 2.03.0) is just as effective as the higher-intensity regimen but produces significantly less bleeding.

Preventing Morbidity from the Postphlebitic Syndrome

The postphlebitic syndrome is a major cause of morbidity. The risk of postphlebitic syndrome in patients with symptomatic deep vein thrombosis is 22.8% after 2 years, 28.0% after 5 years, and 29.1% after 8 years.[50] Surgical thrombectomy does not prevent the disorder because most thrombi recur after surgical removal.[95] [111] The ability of thrombolytic therapy to prevent the postphlebitic syndrome has been evaluated to a limited extent. Streptokinase treatment produces complete lysis of acute venous thrombi in 3040% of cases and causes partial lysis in an additional 30%.[95] In contrast, complete lysis of venous thrombi occurs in less than 10% of patients treated with unfractionated heparin.[95] It is estimated that thrombolysis occurs 3.7 times more often in patients treated with streptokinase than in those given unfractionated heparin, but major bleeding occurs 2.9 times more frequently with streptokinase.[95] Although five randomized studies have reported that the frequency of the postphlebitic syndrome is significantly higher in patients treated with unfractionated heparin than in those given streptokinase, [95]

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one trial[112] reported that patients treated with streptokinase and those treated with unfractionated heparin had similar manifestations of the postphlebitic state after 5 years of follow-up. Two recent studies reported that the incidence of the postphlebitic syndrome was reduced by the early use of graduated compression stockings.[95] Accordingly, patients with proximal vein thrombosis should be encouraged to wear these stockings at the first sign of leg swelling.

Prevention of Late Effects of Pulmonary Embolism

Clinically significant pulmonary hypertension is an uncommon complication of PE. In randomized trials comparing unfractionated heparin with either streptokinase or urokinase for the treatment of patients with acute PE, thrombolytic therapy produced greater improvement in lung scandetected perfusion defects than unfractionated heparin during the first week. However, there were no apparent differences in the lung scan findings at 2 weeks, 3 months, and 1 year.[113] Subsequent long-term follow-up in small subgroups of these patients indicated that pulmonary capillary blood volume and both pulmonary vascular resistance and functional status were significantly better in patients treated with fibrinolytic therapy than in those given unfractionated heparin.[95]

Moser and colleagues[98] have reported their experience with surgical pulmonary endarterectomy in carefully selected patients with chronic thromboembolic pulmonary hypertension. Most patients with obstruction of the proximal pulmonary arteries show impressive improvement after endarterectomy. The authors stress the importance of careful patient selection and expert perioperative management.

Side Effects, Cost, and Clinical Utility of Thrombolytic Therapy

Bleeding occurs more frequently with thrombolytic therapy than with unfractionated heparin.[95] [114] The risk of hemorrhage increases with the duration of thrombolytic infusion and usually occurs at a site of previous surgery or trauma. Intracranial hemorrhage occurs in about 1% of patients at risk, about twice as frequently as with unfractionated heparin treatment.

Practical Recommendations

The majority of patients with proximal vein thrombosis or calf vein thrombosis should be treated with outpatient low molecular weight heparin, while those with symptomatic PE should be treated in the hospital with low molecular weight heparin or with high-dose IV unfractionated heparin. High-dose unfractionated heparin can be given either by continuous IV infusion or by twice daily subcutaneous injection. For IV unfractionated heparin therapy, a bolus of 5,000 U should be followed by a continuous IV infusion at a dose of 1,300 U/hour. The initial dose of subcutaneous unfractionated heparin should be 35,000 U/day given in two divided doses. Because of its delayed onset of action after subcutaneous injection, a 5,000 U IV bolus of unfractionated heparin should be given together with the first subcutaneous injection in high-risk patients. Low molecular weight heparin should be weight adjusted and administered once or twice daily, depending on the manufacturers recommendations. Heparin should be administered for a total of 47 days. Warfarin should be started at the same time as the heparin, using an initial dose of 5 mg if the patient weighs less than 80 kg and 7.5 mg if the patient weighs more than 80 kg. The duration of warfarin therapy is variable. Patients with a persistent, major risk factor for recurrence, such as malignancy, should receive warfarin for as long as the risk factor persists. Those with a secondary thrombosis (such as after orthopedic surgery) who have returned to normal mobility may need warfarin for as little as 6 weeks. Patients with idiopathic deep vein thrombosis (i.e., those without a clear precipitant) and patients with a known hereditary predisposition to thrombosis should receive warfarin for an indefinite period of time. Based on currently available literature, this period should be in excess of 1 year, although the optimal duration of warfarin therapy is unknown.

The dose of unfractionated heparin should be adjusted to maintain the aPTT at 1.82.5 times control. To monitor unfractionated heparin given by continuous IV infusion, the aPTT should be measured 6 hours after the bolus dose so that it reflects the anticoagulant effects of the infusion. If twice daily subcutaneous unfractionated heparin is given, a midinterval aPTT should be measured (i.e., 6 hours after the injection).

The INR is used to monitor oral anticoagulant therapy, and the dose of warfarin should be adjusted to achieve an INR of 2.03.0. Heparin can be discontinued when the INR has been therapeutic for 2 successive days.

Thrombolytic therapy is only indicated in patients with major PE (i.e., those with hemodynamic compromise) and in young patients with very extensive proximal vein thrombosis and no contraindications to thrombolytic therapy.

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VENOUS THROMBOSIS IN UNUSUAL SITES

Subclavian or Axillary Vein Thrombosis

Although thrombosis of the subclavian or axillary veins most frequently occurs as a complication of a chronic indwelling catheter, it may also occur as a consequence of local malignancy, and is a well-documented complication of mastectomy and radiationtherapy. [115] Subclavian vein thrombosis is particularly common in children with indwelling central venous catheters; this complication develops in up to 20% of children who have catheters placed for cancer chemotherapy or parenteral nutrition. In adults, the cause of subclavian or axillary vein thrombosis is uncertain, and idiopathic subclavian or axillary vein thrombosis often occurs in young patients and may be preceded by repetitive, strenuous activity that involves the affected arm. Occasionally, subclavian or axillary vein thrombosis can occur in patients with activated protein C resistance or congenital deficiency of antithrombin, protein C, or protein S, or in patients with antiphospholipid antibodies. Finally, thrombosis of the axillary or subclavian vein or the superior vena cava is a rare complication of perivenous endocardial pacing using an implantable system. [116] [117] Upper extremity deep vein thrombosis is associated with PE, a complication seen frequently in children with catheter-related thrombosis.

Patients with subclavian or axillary thrombosis experience pain in the axilla and edema and cyanosis of the arm. If thrombosis extends into the superior vena cava, patients can develop edema and cyanosis of the face, neck, and upper extremities. Dilated superficial veins on the chest and arms appear after a number of days. Occasionally, asymptomatic thrombosis of the internal jugular vein accompanies subclavian vein thrombosis. The definitive diagnosis is made with venography, although venous US may also be useful.

Subclavian or axillary vein thrombosis is usually treated with anticoagulants. Thrombolytic therapy also is effective and should be considered because it is estimated that over two-thirds of the patients with subclavian vein thrombosis will have persistent symptoms in the affected arm. However, subclavian vein stenosis that persists after thrombolytic therapy may predispose to recurrence, thereby limiting the benefits of this treatment modality. [118]

Mesenteric Vein Thrombosis

Mesenteric vein thrombosis is uncommon. In an extensive literature review, [119] only 367 patients with mesenteric vein thrombosis

were identified. The disorder occurs most commonly in the sixth and seventh decades of life and affects segments of the small bowel (rarely the colon), leading to hemorrhagic infarction rather than gangrene. Bowel infarction often produces bloody ascites, and adhesions frequently develop between the involved bowel and the omentum.

Many of the affected patients have associated disorders, such as thrombosis at other sites, inflammatory bowel disease, recent abdominal surgery, malignant disease, and portal hypertension. Mesenteric vein thrombosis may also complicate hypercoagulable states (such as antithrombin, protein C, or protein S deficiency), polycythemia vera, or the use of oral conceptives, [120] and it has also been reported in late pregnancy. In about 20% of cases no underlying cause is found.

The clinical manifestations of mesenteric vein thrombosis include intermittent abdominal pain, abdominal distention in the later stages of the disorder, vomiting, diarrhea, and melena. Although the diagnosis is often difficult to make, blood-stained ascites fluid on abdominal paracentesis and hemorrhagic bowel infarcts at peritoneoscopy are characteristic findings.

Management includes supportive care and surgical resection followed by anticoagulant therapy. The mortality is about 20%, and recurrence occurs in up to 20% of cases.

Renal Vein Thrombosis

Renal vein thrombosis can occur as a complication of the nephrotic syndrome, possibly because antithrombin is lost in the urine, and is commonly seen in association with membranous glomerulonephritis. [121] Patients may be asymptomatic, may present with mild symptoms of abdominal or back pain, or may have severe flank pain and tenderness. PE has been described as a relatively common complication of renal vein thrombosis. With anticoagulant therapy there is a gradual improvement in renal function tests, although patients may suffer from long-standing proteinuria. Thrombolytic therapy has been used successfully in a small number of patients with renal vein thrombosis. [122]

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Chapter 125 - Arterial Thromboembolism

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INTRODUCTION

Arterial thrombosis usually occurs as a complication of atherosclerosis. Atherosclerosis is the most common underlying cause of coronary heart disease, cerebrovascular disease, and peripheral arterial disease and as such is the single most common cause of mortality and morbidity in the Western population. Arterial narrowing due to atherosclerosis limits blood flow and causes ischemic symptoms when the oxygen requirements are increased by exercise. For example, angina pectoris occurs when the coronary arteries cannot supply sufficient blood flow to meet the demands of the myocardium. Similarly, intermittent claudication reflects ischemia of the leg muscles as a result of an imbalance between arterial blood supply and the demands of the exercising muscles, whereas intestinal angina occurs after a large meal when the blood supply to the gut is insufficient to meet the requirements.

Tissue infarction occurs when the arterial supply is completely occluded for a critical period of time. Arterial occlusion usually is the result of the rupture or fissuring of an atherosclerotic plaque, which exposes thrombogenic material and triggers the formation of a platelet-fibrin thrombus.

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PATHOGENESIS OF ATHEROSCLEROSIS

The term atherosclerosis was introduced by Marchand, who recognized the association of fatty degeneration and vessel stiffening. [1] Atherosclerosis affects medium and large-sized arteries and is characterized by patchy intramural thickening of the subintima that encroaches on the arterial lumen and eventually leads to vascular obstruction. The earliest lesion of atherosclerosis is the fatty streak, which is due to an accumulation of lipid-laden foam cells in the intimal layer of the artery. With time, the fatty streak evolves into the fibrous plaque, the hallmark of established atherosclerosis.

Atherosclerotic lesions are composed of three major components. The first is a cellular component with increased numbers of intimal smooth muscle cells and an accumulation of macrophages. The second component is the connective tissue matrix, large amounts of which are produced by the proliferating smooth muscle cells, and extracellular lipid. The third component is lipid that accumulates within the smooth muscle cells and the macrophages, thereby converting them into foam cells. Thus, the development of atherosclerotic lesions involves proliferation of smooth muscle cells, synthesis of connective tissue matrix, and accumulation of macrophages and lipid.

Over the years, two main hypotheses have been proposed to explain the pathogenesis of the atherosclerotic process, the lipid hypothesis [2] [3] and the chronic endothelial injury hypothesis. [4] Experimentally, atherosclerosis can be produced by cholesterol feeding [2] or by chronic endothelial injury. [4]

The Lipid Hypothesis

The lipid hypothesis postulates that with increased levels of low-density lipoproteins (LDL), lipid accumulates in smooth muscle cells and macrophages as the LDL penetrates the arterial wall. LDL is oxidized in the presence of endothelial cells, and in its oxidized form becomes more atherogenic. Thus, oxidized LDL causes endothelial damage[5] and is chemotactic for monocyte-macrophages. The monocytes first adhere to the altered endothelium by interacting with surface adhesion molecules, and then migrate through the endothelium and basement membrane by elaborating hydrolases that degrade connective tissue matrix. As these cells accumulate, they take up lipid and are converted to foam cells. Macrophages bind intra-intimal LDL via a family of novel receptors known as scavenger receptors, which recognize LDL only after it has been oxidized. Oxidation affects both lipid and apoprotein moieties of the LDL molecule; the lecithin component of LDL phospholipid undergoes

conversion to lysolecithin, which is chemotactic for monocytes. [5] In addition, uptake of oxidized LDL renders the macrophages less mobile, thereby promoting the accumulation of these lipid-laden cells in the intima.

An experimental model of atherosclerosis has been carefully studied in monkeys fed a diet rich in cholesterol. [2] Within 2 weeks of induction of hypercholesterolemia, monocytes become attached to the surface of the arterial endothelium, migrate into the subendothelium, and accumulate lipid, which gives them the appearance of foam cells. Proliferating smooth muscle cells then emerge, and they also accumulate lipid. As the fibrous plaque enlarges, the endothelial cells retract, exposing the subendothelium to the blood and triggering the formation of platelet-fibrin thrombi. The release of platelet-derived growth factor leads to further smooth muscle proliferation, as described in the response-to-injury hypothesis. Support for the important role of oxidized LDL in atherogenesis comes from several sources. Thus, administration of antioxidants to hyperlipidemic rabbits inhibits the formation of fatty streaks. Oxidized LDL can be extracted from human lesions, and many humans have circulating antibodies that are specific for epitopes of oxidized LDL. [6]

The Chronic Endothelial Injury Hypothesis

The response-to-injury hypothesis has been reviewed by Ross. [4] It postulates that endothelial injury results in loss of endothelium, adhesion of platelets to subendothelium, aggregation of platelets, release of platelet-derived growth factors and elaboration of chemotactic factors that attract leukocytes, which in turn release other growth factors. The growth factors induce replication and migration of smooth muscle cells into the intima and result in the formation of a fibrous plaque. The smooth muscle cells synthesize and secrete connective tissue matrix containing collagen, proteoglycans, and elastic fibers, which contributes to the mass of the lesion. Plaque fissure or rupture may trigger the formation of a platelet-fibrin plug, which is then incorporated into the lesion. With repeated injury there is further intimal proliferation and progressive narrowing of the lumen.

The lipid hypothesis and the endothelial injury hypothesis are closely linked. Oxidized LDL is cytotoxic to cultured endothelial cells and may cause endothelial injury, attract monocytes, and stimulate smooth muscle growth. Modified LDL is also a potent inhibitor of macrophage mobility. Stimulated macrophages release growth factors that promote the proliferation and migration of smooth muscle cells. The smooth muscle cells in turn elaborate a monocyte chemotactic factor and synthesize connective tissue matrix.

With the development of atherosclerosis, there is impaired regulation of vascular tone. This is due in part to decreased production of endothelial-derived nitric oxide. The impaired release of nitric oxide is associated with increased vascular tone, and may be associated with increased platelet activation and intimal proliferation. [7]

Atherosclerotic lesions can develop in the absence of endothelial denudation and without the involvement of platelet-derived growth factors. [2] Thus, smooth muscle hyperplasia occurs in hypertension without associated endothelial cell loss [8] [9] [10] [11] and persists long after platelet interaction with the vessel wall has ceased. [12] [13] [14] Cells other than platelets produce growth factors, [15] including macrophages, [16] endothelial cells, [17] and arterial smooth muscle cells, [18] and these may contribute to the growth of the atherosclerotic plaque. [4] In this context, local synthesis of platelet-derived growth factor has been demonstrated in human atherosclerotic plaques removed at surgery. [19]

Growth of the Atherosclerotic Plaque

The atherosclerotic plaque grows slowly over a period of years and can produce severe stenosis or even total vascular occlusion. The mature plaque consists of a fibrous cap composed of collagen, a lipid core containing intracellular and extracellular lipid, and areas of calcification. Lipid-rich plaques are prone to spontaneous fissuring or rupture when exposed to high shear stress at sites of stenosis and arterial branching. Two forms of plaque injury are recognized, superficial and deep. Superficial injury produces areas of focal endothelial denudation that can enlarge and lead to the formation of mural or even occlusive thrombi. Plaques that are capped with superficial collagen fibers separated by large number of lipid-filled macrophages tend to predispose to superficial injury. [20] [21]

Deep intimal injury is characterized by a split or tear that extends from the luminal surface of a plaque deep down into the plaque substance. This type of injury, which

tends to occur in plaques that contain a large lipid-rich pool, exposes blood to the highly thrombogenic contents of the plaque and leads to intraluminal and extraluminal thrombosis. Intraluminal thrombi become incorporated into, and increase the size of, the atherosclerotic plaque, while extraluminal thrombi can be partly or completely occlusive.[22]

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PATHOGENESIS OF ARTERIAL THROMBOSIS

Arterial thrombi form under conditions of high flow and are composed mainly of platelet aggregates held together by fibrin strands. Thrombosis is initiated by rupture of the atherosclerotic plaque, which exposes thrombogenic material in the subendothelium to the blood. [22] [23] If the thrombus is nonocclusive and blood flow remains rapid, the thrombi may organize and become incorporated into the atherosclerotic plaque. [23] With more marked arterial narrowing, shear rates increase and promote more extensive platelet and fibrin deposition, which can result in the formation of an occlusive thrombosis.

Thrombogenic Factors

Thrombosis occurs when the balance between thrombogenic factors and protective mechanisms is perturbed. The protective mechanisms include the nonthrombogenic properties of intact endothelial cells, fluid-phase antiproteases, and the dissolution of fibrin by the fibrinolytic system. Thrombogenic stimuli include perturbation or loss of endothelial cells and activation of platelets and coagulation.

Nonthrombogenic Properties of Endothelial Cells

The fluidity of blood in vivo is enhanced by the thromboresistant properties of intact vascular endothelium and is threatened by damage to the vessel wall. [24] Endothelial cell-surface glycosaminoglycans and thrombomodulin are potent inhibitors of coagulation, while vessel wall generation of prostacyclin and nitric oxide, and plasminogen activators limit platelet aggregation and fibrin deposition, respectively. [25]

Damage to the Vessel Wall

Thrombogenesis is promoted by loss of endothelium, which may be caused by direct physical damage such as occurs with angioplasty, hemodynamic stress, use of tobacco products, high blood cholesterol levels, or enzymes released from platelets and leukocytes. [15] The shedding of endothelial cells exposes the subendothelium to platelets and blood coagulation factors. Platelets that adhere to the subendothelium undergo a shape change, aggregate, and secrete their granular contents, thereby recruiting more platelets. At physiologic shear rates, platelet adhesion to subendothelial collagen is mediated by von Willebrand factor and possibly other adhesive proteins, which bind to a glycoprotein receptor (GPIb) on the platelet surface [26] as well as to subendothelial components.

Although endothelial cell loss represents the most severe form of vascular damage, more subtle injury may also promote thrombogenesis. Thus, endothelial cells exposed to endotoxin, cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), thrombin, hypoxia, or increased shear stress synthesize tissue factor and internalize thrombomodulin, thereby promoting coagulation. In addition, these perturbed cells also produce PAI-1, which impairs fibrinolysis, and acquire receptors to which leukocytes and platelets adhere. [25] Finally, the altered endothelial cells synthesize factors that regulate local blood flow. These include vasoconstrictors known as endothelins, as well as vasodilators such as prostacyclin and nitric oxide. [7] [25]

Platelet Activation

Platelets adhering to collagen undergo a shape change, secrete their granular contents, and aggregate. In addition to collagen, a variety of other agonists, including thrombin, epinephrine, and thromboxane A_2 (TXA_2), also promote platelet aggregation. [27] Whereas all of these agents stimulate the synthesis of TXA_2, collagen, thrombin, and TXA_2 also induce the release of adenosine diphosphate (ADP) from platelet granules, which amplifies the aggregation process. In addition to these pathways, thrombin-induced platelet aggregation occurs through a third mechanism that may involve the activation of platelet calpain. [28]

Virtually all agonists that induce platelet aggregation act through a common pathway that involves the accumulation of ionized calcium within the platelet cytoplasm either by mobilization of calcium from the dense tubular system [28] or by increased transport across the membrane. This in turn results in the functional expression of the platelet glycoprotein GPIIb/IIIa, which serves as a receptor for fibrinogen and other Arg-Gly-Asp-containing adhesive proteins such as fibronectin, von Willebrand factor, and thrombospondin. [28] Binding of fibrinogen to GPIIb/IIIa is essential for platelet aggregation because the dimeric fibrinogen bridges the platelets together.

An increase in platelet cyclic adenosine monophosphate (AMP) levels reduces the calcium-mobilizing effect of all agonists. Prostacyclin produced by endothelial cells inhibits platelet aggregation by activating platelet adenylate cyclase, thereby elevating cyclic AMP levels. [29]

Activation of Coagulation

Damage to the vessel wall activates the blood coagulation pathway by exposing blood to tissue factor and by activating platelets. Stimulated platelets promote further activation of coagulation by providing a phospholipid surface on which efficient assembly of coagulation factor complexes can occur. Thrombin formed as a result of these processes then serves to convert fibrinogen to fibrin, which stabilizes the platelet aggregates.

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EPIDEMIOLOGY AND PREVENTION OF ATHEROSCLEROSIS

The most effective means of preventing arterial thrombosis is to prevent atherosclerosis. The proven risk factors for atherosclerosis are hypercholesterolemia, hypertension, cigarette smoking, obesity, physical inactivity, age, family history, diabetes and male sex. The first five of these risk factors are potentially reversible, and there is evidence that their reversal reduces the complications of atherosclerosis.

Cholesterol and Lipids

The plasma level of cholesterol is determined by genetic factors, by the type and amount of fat in the diet, and by other factors such as obesity, physical activity, and disease states. Based on the results of animal studies, epidemiologic data, and interventional studies, there is good evidence for an association between hypercholesterolemia and atherosclerosis.

Of the three major classes of lipoproteins, very-low-density lipoproteins (VLDL), LDL, and high-density lipoproteins (HDL), LDL contain 6070% of the total serum cholesterol and are the most atherogenic. In contrast, the levels of HDL are inversely correlated with the risk of coronary heart disease.

The association between serum cholesterol levels and the risk of coronary heart disease is continuous. [30] [31] Familial hypercholesterolemia, a disorder caused by an absent or defective LDL receptor, causes premature coronary heart disease. [32] [33] In the heterozygous form of this disorder, which occurs in 1 in 500 people, the total cholesterol concentration is usually in excess of 300 mg/dl. Approximately 5% of all patients who present with acute myocardial infarction (MI) before the age of 60 have heterozygous familial hypercholesterolemia. The homozygous form of familial hypercholesterolemia occurs in 1 in 10^{6} subjects. These individuals have cholesterol levels ranging from 6001,000 mg/dl and usually develop severe coronary heart disease before the age of 20.

Reduced levels of HDL cholesterol are associated with an increased risk of coronary heart disease. The main causes of reduced HDL cholesterol include cigarette smoking, obesity, physical inactivity, androgenic and related steroids (including anabolic steroids), -blocking agents, hypertriglyceridemia, and genetic factors. In contrast, weight reduction and exercise elevate HDL cholesterol levels.

Both the cholesterol level and the prevalence of coronary heart disease are influenced by environmental factors, including diet. Thus, individuals who immigrate from countries where the prevalence of coronary heart disease and the serum cholesterol levels are low to a country with a high prevalence of coronary heart disease will often sustain increases in both serum cholesterol levels and rates of coronary heart disease.

The evidence that decreasing serum cholesterol levels with cholesterol-lowering drugs or dietary modification slows or reverses the progression of coronary atherosclerosis[34] [35] and reduces coronary events[36] comes from over 20 randomized trials that included almost 40,000 subjects. [34] [36] [37] [38] Lowering the serum cholesterol level with diet or drug therapy also slows the progression of angiographically documented coronary atherosclerosis in patients with arterial bypass grafts.[34] Modifying several risk factors, such as by lowering the serum cholesterol level, the blood pressure, and the levels of LDL cholesterol and by cessation of smoking,[36] reduces the risk of ischemic heart disease. [39] [40] Individuals with several risk factors benefit most from these measures. [41]

Aggressive lowering of the serum cholesterol level in patients with recent MI results in a rapid decrease in the risk of subsequent ischemic cardiac complications, the need for surgical revascularization, and death rates. [42] [43] This effect occurs even when the total cholesterol level falls within the upper range of normal (5.55.8 mmol/l, 213310 mg/dl).

Smoking

Cigarette smoking increases the risk of coronary heart disease, peripheral arterial disease, cerebrovascular disease, and graft occlusion after reconstructive arterial surgery. It is particularly hazardous in those with a poor cardiovascular risk profile and in women taking estrogens, and there is a dose relationship between the risk of coronary heart disease and the number of cigarettes smoked daily. [44] Those who stop smoking have only half the risk of those who continue to smoke, regardless of how long they smoked. Smoking cessation also reduces mortality after coronary bypass surgery, reduces morbidity and mortality in patients with peripheral vascular disease, and decreases mortality after MI.

Although the mechanism by which smoking increases the

risk of atherosclerosis is uncertain, there are several possibilities. Cigarette smoking decreases the levels of HDL, increases LDL cholesterol, and, by raising levels of carbon dioxide, leads to hypoxia, thereby perturbing the anticoagulant properties of the endothelium. In addition, smoking increases platelet reactivity and, by elevating the plasma fibrinogen and the hematocrit, also increases blood viscosity.

Hypertension

Hypertension is a risk factor for stroke, MI, and cardiac and renal failure. [45] Treatment of hypertension reduces the incidence of stroke and lowers overall mortality, but there is less convincing evidence that it affects coronary events. [46] Thus, pooled analysis of all studies examining the effects of lowering the blood pressure shows a 10% risk reduction for mortality and a 40% risk reduction for stroke. However, there is only an 8% risk reduction for fatal and nonfatal MI, a difference that is not statistically significant.

Physical Inactivity

Individuals who exercise regularly have a lower incidence of MI and death, [47] but it is uncertain whether the association is causal or whether it merely reflects the fact that healthier individuals are more likely to exercise. In patients who have recovered from an acute MI, [48] exercise produces a 19% reduction in the risk of recurrent infarction or death. Regular exercise may exert these protective effects by increasing the levels of HDL cholesterol and lowering the blood pressure. [49] [50]

Obesity

Although some observational studies[51] have suggested that obesity is an independent risk factor for coronary heart disease, this has not been a universal finding.

Emerging Risk Factors

Evidence is increasing that a variety of additional risk factors for atherosclerotic disease exist. Congenital or acquired hyperhomocysteinemia is associated with an increase in the risk of both arterial and venous thromboembolism. In some cases, homocysteine levels may be reduced with the administration of folic acid or vitamins B_6 or B_{12} . Whether these interventions reduce the risk of atherosclerosis and its complications is unclear.

Impaired fibrinolysis has been linked to atherosclerotic vascular disease in some but not all studies. Antiphospholipid antibodies are clearly associated with premature arterial thromboembolism, and may be associated with accelerated atherosclerosis. Both thoracic radiation therapy and heart transplantation are associated with accelerated atherosclerosis and ischemic cardiac syndromes.

NONSURGICAL TREATMENT OF ARTERIAL THROMBOEMBOLISM

Treatment of atherosclerosis and its thromboembolic complications includes surgical procedures such as endarterectomy, embolectomy, arterial bypass surgery, and angioplasty, as well as medical management. The three forms of medical therapy that are of proven effectiveness in the treatment of arterial thrombosis are antiplatelet drugs, thrombolytic agents, and anticoagulants.

Antiplatelet Agents

Three antiplatelet agents, aspirin, ticlopidine, and clopidogrel, a new thienopyridine derivative similar to ticlopidine, have been shown to be effective in the prevention and treatment of arterial thrombosis.[52] [53] In addition, agents that inhibit the interaction between fibrinogen and its receptors on platelets reduce the risk of acute ischemic cardiac syndromes after coronary angioplasty.

Aspirin

The antithrombotic effects of aspirin reflect its ability to inhibit the synthesis of TXA_2, a potent inducer of platelet aggregation and vasoconstriction, by inactivating platelet cyclooxygenase. Aspirin is rapidly absorbed from the gastrointestinal tract and peak levels are reached 1520 minutes after ingestion. Despite its rapid clearance from the circulation, the inhibitory effects of aspirin persist for the life span of the platelets because the drug irreversibly acetylates cyclooxygenase. To achieve rapid and complete inhibition of TXA_2 production, for example, in a patient with an evolving MI, a 325-mg tablet of non-enteric-coated aspirin should be used. However, lower aspirin doses can be used for maintenance treatment[54] because as little as 80 mg/day is sufficient to maintain the inhibition of TXA_2 synthesis.

Aspirin reduces the incidence of MI and death in the following groups: men and asymptomatic women over the age of 50, and patients with asymptomatic myocardial ischemia, stable angina, unstable angina with or without non-Q-wave infarction, acute MI, or cerebrovascular disease. In addition, aspirin also reduces the risk of stroke in patients with cerebral ischemia and decreases the risk of acute thrombosis after either aortocoronary bypass surgery or coronary angioplasty. Finally, aspirin was shown to lower the incidence of nonfatal MI in healthy U.S. physicians.[52]

The side effects of aspirin are primarily gastrointestinal, and gastrointestinal hemorrhage can occur in some patients. These complications are dose related and are reduced if enteric-coated aspirin is used. Aspirin is contraindicated in patients with active peptic ulcer disease or aspirin-induced asthma, and it should be discontinued if gastrointestinal side effects are severe.

In most randomized studies, patients with a history of active peptic ulcer disease were excluded, and the beneficial effects of aspirin occurred without hemorrhagic complications.[55] However, in the U.S. Physicians Study the use of aspirin was associated with an increased incidence of cerebral hemorrhage, a finding not observed in the thousands of symptomatic patients treated with aspirin to prevent the complications of atherosclerosis.[55] [56] The preoperative use of aspirin in patients undergoing bypass surgery was associated with an increased incidence of operative bleeding, which probably reflects the combined antiplatelet effect of aspirin and the anticoagulant activity of high-dose heparin used during cardiac surgery.[57]

In studies in which aspirin alone was compared with the combination of aspirin and dipyridamole, no additional benefit was observed from the addition of dipyridamole.[55] However, like aspirin, dipyridamole also augments the antithrombotic effect of oral anticoagulants in patients with mechanical prosthetic valves.[58]

Ticlopidine and Clopidogrel

Ticlopidine and clopidogrel are thienopyridine derivatives that inhibit ADP-dependent platelet aggregation. They are metabolized by the liver after consumption, producing active metabolites that inhibit platelet aggregation. Because the production of intermediate active metabolites is required, the inhibitory effects of ticlopidine and clopidogrel on platelet function are delayed for 2448 hours after administration.

Ticlopidine has been shown to be more effective than aspirin in reducing stroke in patients with transient cerebral ischemia or minor stroke. Ticlopidine is also effective in (1) reducing the risk of the combined outcome of stroke, MI, or vascular death in patients with thromboembolic stroke, (2) decreasing vascular death and MI in patients with unstable angina, (3) reducing

TABLE 125-1 -- Thrombotic Disorders for Which Aspirin or Ticlopidine Has Been Shown to Be Effective

Indication	Effective	
	Aspirin (minimum effective dose)	Ticlopidine (250 mg bid)
Asymptomatic males and females >50 years old	Yes (325 mg every 2nd day)	
Silent myocardial infarction	Yes (75 mg)	
Stable angina	Yes (325 mg every 2nd day)	
Unstable angina	Yes (75 mg)	Yes
Acute myocardial infarction	Yes (160 mg)	
Aortocoronary bypass surgery	Yes (100325 mg)	Yes
Acute occlusion following coronary angioplasty	Yes (650 mg)	Yes
Peripheral vascular disease	Yes (325 mg)	Yes
Transient cerebral ischemia and incomplete stroke	Yes (30 mg)	Yes
Complete stroke	No evidence	Yes
Placental insufficiency	Yes (60150 mg)	

Atrial fibrillation	Yes (325 mg)[a]	
Prosthetic heart valves	Yes (100 mg)[b]	

From Hirsh, [88] with permission.

[a]In combination with warfarin.
[b]Not as effective as warfarin.

acute occlusion of coronary bypass grafts, and (4) improving walking distance and decreasing vascular complications in patients with peripheral vascular disease. [52]

The most common side effects of ticlopidine are diarrhea and skin rash; the most serious complication is irreversible neutropenia. Nevertheless, ticlopidine should be considered in place of aspirin in patients who are allergic to or intolerant of aspirin, or who have had a vascular event despite the use of aspirin.

Clopidogrel and ticlopidine are more effective than aspirin for the prevention of ischemic stroke, MI, or vascular death in patients at high risk of these complications. [53] Clopidogrel, unlike ticlopidine, is not associated with the development of neutropenia.

The thromboembolic disorders for which aspirin or ticlopidine are effective are listed in Table 125-1 . Ticlopidine is more effective than aspirin in patients with transient cerebral ischemia and incomplete stroke, but aspirin is safer and less expensive. The role of clopidogrel is not yet clear: although it is safer than ticlopidine and more effective than aspirin, cost constraints are likely to limit its initial use to patients who have arterial thromboembolism while receiving aspirin.

Platelet Glycoprotein IIb/IIIa Blockade

Blockade of the platelet fibrinogen receptor (GPIIB/IIIa) using antibody fragments (abiximab, c7E3 Fab) or peptides reduces the risk of clinically significant ischemic events in patients undergoing coronary angioplasty. Three studies [59] [60] [61] that enrolled a total of 4,774 evaluable patients have demonstrated that c7E3 reduces the risk of death, MI, or urgent coronary artery bypass grafting in the first 30 days after percutaneous coronary angioplasty in patients with acute or chronic coronary artery syndromes. c7E3 produces an immediate and profound inhibition of platelet activity that persists for 612 hours after termination of its infusion. Administration of c7E3 is associated with an increase in the risk of hemorrhage, although this risk can be moderated with the judicious use of heparin and careful patient selection.

Thrombolytic Therapy

Thrombolytic therapy is useful in the treatment of arterial thrombosis because rapid clot lysis and restoration of blood flow prevent permanent tissue damage. More than 80% of patients with acute MI have thrombotic occlusion of the infarct-related coronary artery, [62] and thrombolytic agents produce rapid lysis of these thrombi in 5075% of cases.[62] Tissue plasminogen activator (t-PA) is more effective than streptokinase in achieving early coronary lysis, [63] but both agents improve left ventricular function and reduce mortality. Thus, streptokinase and t-PA decrease mortality by approximately 25% [62] [64] when used without adjunctive aspirin or intravenous heparin, and reduce mortality by 4050% when either agent is combined with aspirin. [62] In the GUSTO study, an accelerated t-PA regimen combined with high-dose intravenous heparin produced a 14% greater reduction in mortality than streptokinase. This difference was most evident in patients treated within 4 hours of onset of symptoms.

Following successful thrombolysis there is a 1020% reocclusion rate, and a 34% reinfarction rate. [62] Aspirin reduces the incidence of reinfarction. [56] Reocclusion is less common with streptokinase than with t-PA because streptokinase causes extensive plasma proteolysis and the resultant fibrinogen degradation products produce a systemic anticoagulant state. There is experimental evidence that direct thrombin inhibitors, such as hirulog and hirudin, are more effective than heparin at preventing reocclusion after coronary thrombolysis. However, large-scale clinical trials have been disappointing because treatment with the direct thrombin inhibitors has been complicated by a high rate of hemorrhage. [65] [66] [67]

Anticoagulants

The role of anticoagulant therapy in the treatment of arterial thrombosis is controversial. [68] [69] When used alone, heparin is effective in the short-term treatment of unstable angina,[68] but a rebound effect is seen when the drug is stopped. Aspirin appears to prevent the cluster of ischemic events that occur when heparin is discontinued. There is suggestive evidence that the addition of heparin to aspirin improves the short-term outcome in patients with unstable angina, but it is uncertain whether this effect is sustained.

In patients with acute MI, heparin has been reported to reduce reinfarction and death. [68] Moderate doses of heparin (12,500 U subcutaneously every 12 hours) also reduce the incidence of mural thrombosis detected by two-dimensional echocardiography, which is a particular problem in patients with anterior infarction.

Heparin prevents early reocclusion of the infarct-related artery after successful thrombolysis with t-PA. However, a recent meta-analysis [70] has suggested that routine administration of heparin to patients with acute MI is not indicated. This analysis demonstrated that heparin therapy reduced mortality by 6% (95% confidence intervals: 0, 10%), and the rate of reinfarction by 1.3%. However, heparin was associated with a clinically important increase in the risk of major hemorrhage that outweighed its observed benefit.

Oral anticoagulants are effective for the treatment of arterial thrombosis. [68] Two studies have shown that oral anticoagulants are effective for long-term treatment of patients with MI.[71] [72] Both studies used high-intensity warfarin regimens (INR 2.74.5 and 2.84.8, respectively), and the risk of bleeding was increased with anticoagulants. The recently reported Coumadin Aspirin Reinfarction Study demonstrated that fixed low-dose warfarin (1 or 3 mg) combined with 80 mg of aspirin per day does not provide benefit beyond that achieved with 160 mg of aspirin/day. [73]

Direct thrombin inhibitors, such as hirudin, are currently under evaluation for the treatment of acute ischemic coronary syndromes. Two large studies have failed to show that the administration of hirudin reduces the risk of death or nonfatal reinfarction at 30 days in patients who present with coronary ischemia. [74] Evidence from three large clinical trials shows that

hirudin in high dose is associated with a clinically important increase in the risk of cerebral hemorrhage. Currently, the value of direct thrombin inhibitors in patients with arterial thromboembolic disease is unclear.

Atrial Fibrillation

Patients with nonvalvular atrial fibrillation are at increased risk of stroke, which occurs at a frequency of 5% per year. [75] [76] [77] [78] [79] The risk of stroke increases with age,[80] [81] and is increased by a number of associated cardiac disorders, including a history of MI, angina, heart failure, or thromboembolic event, and the presence of left atrial dilation, left ventricular dysfunction, mitral calcification, or hypertension. In such patients, warfarin (INR 2.03.0) produces a 6080% reduction in the risk of stroke, with only a modest increase in bleeding complications. [75] [76] [77] [78] [79] Therefore, warfarin (target INR 2.03.0) should be administered to all patients with nonvalvular atrial fibrillation who do not have a contraindication to anticoagulant therapy. This recommendation applies particularly to those patients with nonvalvular atrial fibrillation who have an additional risk factor for stroke, such as hypertension, diabetes, or previous cerebral ischemia, in whom warfarin therapy (target INR 2.03.0) is clearly more effective than either fixed low-dose warfarin or aspirin. [82] Aspirin should be used in patients with nonvalvular atrial fibrillation who are at high risk for stroke when warfarin is contraindicated. The risk of cerebral hemorrhage increases with age; as a result, warfarin therapy should be initiated in the very elderly only after careful evaluation of the bleeding risk and discussion with the

TABLE 125-2 -- Indications for Anticoagulant Therapy and Aspirin in Arterial Thromboembolism

Indication	Anti-coagulants Effective	Aspirin Effective	Drug of Choice

Acute myocardial infarction			
Prevent systemic embolism	Yes[a]	No	Anticoagulants
Prevent reinfarction	Yes[a]	Yes[a]	Either
Prevent death	Yes[a]	Yes[a]	Aspirin
Angina			
Unstable angina	Yes[a]	Yes[a]	Both
Stable angina	No	Yes[a]	Aspirin
Coronary artery bypass graft	?	Yes	Aspirin
Atrial fibrillation			
Prevent systemic embolism	Yes[a]	Yes	Anticoagulants
Treat systemic embolism (including stroke)	Yes[a]	No	Anticoagulants
Valvular heart disease			
Prosthetic	Yes[a]	(Augment effect of anticoagulants)	Anticoagulants
Rheumatic	Yes	No	Anticoagulants
Systemic embolism			
Prevention	Yes	No	Anticoagulants
Treatment	Yes	No	Anticoagulants
Peripheral arterial disease	No	?	Aspirin
Cerebrovascular disease	No	Yes[a]	Aspirin

[a] Evidence from randomized clinical trials.

patient. Low-risk patients (i.e., those with intermittent atrial fibrillation or who are less than 65 years old and without a history of cerebral ischemia, diabetes, or hypertension) should receive aspirin, 325 mg/day.

Mechanical and Bioprosthetic Valves

Patients with prosthetic heart valves are at risk of systemic embolism, which most often manifests as a stroke. The embolic risk is greater with mechanical than with bioprosthetic valves, with prosthetic mitral rather than aortic valves, and when there is associated atrial fibrillation. [83] For patients with tissue prosthetic valves who are in sinus rhythm, the risk of embolism is largely confined to the first 3 months after valve insertion, [83] whereas patients with mechanical prosthetic valves (particularly in the mitral position) have a lifelong risk of systemic embolism. [83]

Randomized trials in patients with mechanical prosthetic valves have shown that warfarin is effective in reducing the risk of systemic embolism, even when given at a lower intensity than that used in the past. [84] [85] For this reason, an INR of 2.53.5 is recommended for these patients. [83] Furthermore, the risk of systemic embolization, valve thrombosis and vascular death is reduced by the addition of aspirin (100 mg/day) to warfarin in these patients. [86]

The risk of thromboembolism is lower with uncomplicated bioprosthetic valves than with mechanical valves. In the absence of complications or atrial fibrillation, warfarin should be given for 3 months. [83] [87]

The relative effectiveness of anticoagulants and aspirin in the treatment of arterial thromboembolism is summarized in Table 125-2 .

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Chapter 126 - Immune Throbocytopenic Purpura, Neonatal Alloimmune Thrombocytopenia, and Posttransfusion Purpura

James Bussel
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INTRODUCTION

Immune thrombocytopenic purpura (ITP) is one of the most common causes of thrombocytopenia encountered in medical practice. The disorder has been estimated to affect approximately 1 in 10,000 in the general population and to account for 0.18% of hospital admissions (see Berchtold and McMillan [1] for review). ITP is caused by autoreactive antibodies that bind to platelets and shorten their life span. The clinical presentation varies widely, from the acute onset of petechiae and severe thrombocytopenia to the discovery of asymptomatic mild thrombocytopenia during the evaluation of another illness. ITP may accompany other systemic diseases having more than one potential cause of thrombocytopenia. In such cases, recognition and treatment of the autoimmune component may have an important impact on morbidity. Pregnancy in women with ITP may be complicated by neonatal thrombocytopenia.

Over the past few years several of the antigens recognized by the autoantibodies that cause ITP have been identified. This has enabled new tests to be developed that may facilitate the diagnosis of ITP and its differentiation from other causes of thrombocytopenia. Several important new treatment modalities have also been introduced into practice, each of which is effective in some patients. However, each of these also has important potential side effects and some have considerable cost. In addition, a practice guideline was developed by a committee of the American Society of Hematology that provides one approach to diagnosis and management.[2]

This chapter reviews the general clinical features of ITP, with an emphasis on an appraisal of new developments in diagnosis and management. The authors also describe how these newer treatment modalities are best utilized in the most common clinical settings they encounter. Discussion of ITP in adults and children is separated in order to emphasize the separate diagnostic and treatment issues pertinent to each age group.

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ITP IN ADULTS

Presentation

Among adults, ITP occurs most commonly in women during the second and third decades of life, [3] [4] but the disorder occurs in both sexes and at any age. Most patients come to medical attention because they develop petechiae, purpura, or ecchymoses over the course of several days. Evidence of cutaneous bleeding may occasionally be widespread and may be accompanied by bleeding from mucosal sites such as epistaxis, gingival bleeding, hematuria, menorrhagia or, less commonly, melena. Rarely, patients present with signs of intracranial hemorrhage or bleeding at other internal sites. It is not uncommon for patients to have a history of easy bruisability for several months or longer prior to an acute exacerbation. Most patients are otherwise in good health, although some complain of easy fatiguability. In contrast to children, few adults have had a systemic viral illness in the weeks preceding the onset of bleeding. A few others have evidence of a preexisting systemic disorder such as systemic lupus, antiphospholipid syndrome, chronic lymphocytic leukemia, common variable immunodeficiency, or HIV infection. The typical patient, however, is entirely well with the exception of bleeding.

Differential Diagnosis

ITP is the most common cause of severe thrombocytopenia in an otherwise healthy young adult, but remains a diagnosis of exclusion. [2] Other disorders that need to be considered can be divided into those that cause isolated thrombocytopenia and those in which there are usually additional hematologic abnormalities. The former include familial thrombocytopenia, hypersplenism, or systemic lupus, drug-induced thrombocytopenias, (see Chap. 129) and HIV infection (see Chap. 155). During pregnancy, the differential diagnosis also includes gestational thrombocytopenia and preeclampsia. Other causes of thrombocytopenia are less common and generally are evident from the initial presentation. Among older adults, the major additional consideration is myelodysplasia, in which thrombocytopenia may precede other manifestations of the disease by months to years. [5]

Initial Evaluation

The physical examination should reveal only signs of bleeding, typically petechiae and purpura. The presence of splenomegaly

or lymphadenopathy suggests another diagnosis or an underlying illness with secondary immune thrombocytopenia. The blood counts are normal except for the platelet count unless there has been significant bleeding. Thrombocytopenia determined by automated analyzer must be confirmed by a thorough examination of the peripheral blood smear to exclude the diagnosis of pseudothrombocytopenia (see the following section) and other common hematologic conditions that generally affect multiple cell lines. The mean platelet size is increased in most, but not all patients, although platelets consistently approaching the size of erythrocytes are more typical of a hereditary thrombocytopenia. Coagulation tests to exclude a diagnosis of disseminated intravascular coagulation are rarely needed other than in an occasional patient who presents during pregnancy. Bone marrow aspiration and biopsy may be performed in older individuals to exclude other causes of thrombocytopenia, [2] but are rarely revealing when the presentation is typical. Erythroid and myeloid development should be normal. Megakaryocytes are generally normal or increased in number and there should be no dysplastic features such as megakaryocyte clumping. No additional studies are required to make a diagnosis of ITP in the typical case. [2]

Pseudothrombocytopenia

The blood count of every patient should be repeated and the blood smear must be inspected visually before making a diagnosis of ITP, both to exclude another cause of thrombocytopenia and to exclude pseudothrombocytopenia. Failure to diagnose pseudothrombocytopenia may lead to serious errors in management. [6] Pseudothrombocytopenia is an in vitro artifact of automated cell counting that occurs with a frequency of between 1 in 1,000 and 1 in 10,000 blood specimens collected in EDTA. [7] [8] The phenomenon occurs when platelets are agglutinated by IgG, and less commonly when they are agglutinated by IgM or IgA autoantibodies or monoclonal proteins, [9] [10] which bind to a divalent cation-dependent conformer of the platelet glycoprotein IIb-IIIa complex [11] [12] or another cryptic antigen [10] induced by a low concentration of ionized calcium attainable only in vitro. The diagnosis should be suspected when platelet clumping is observed on a blood smear made from EDTA-anticoagulated blood. [7] In some patients, the clumping is extensive enough to cause a spurious leukocytosis. [13] The diagnosis is confirmed by demonstrating that the platelet count is substantially higher when measured in citrated or heparinized blood, or in blood obtained without an anticoagulant. The in vitro clumping occurs at body temperature, which distinguishes this phenomenon from clumping caused by cold agglutinins, [7] [14] although the two phenomena may coexist. [9] [11] [15] Less commonly, the peripheral blood smear also shows platelets adherent to [16] or within [17] [18] granulocytes or monocytes, a phenomenon known as platelet satellitism. Occasionally, pseudothrombocytopenia may occur when giant platelets are excluded from automated analysis. [19] Because pseudothrombocytopenia and ITP, or other causes of thrombocytopenia, can coexist, [17] [20] the blood smear of patients with ITP must be analyzed to confirm the automated platelet count before therapy is initiated or changed.

Pathogenesis

Prior to 1950, the pathogenesis of ITP remained a matter of debate. An immunologic cause was suspected based on the prevalence of neonatal thrombocytopenia in affected women, the frequency of coexisting immune hemolysis, and the clinical response to steroids and splenectomy. However, it was not until the studies of Harrington et al., showing that infusion of plasma from ITP patients induced transient but severe thrombocytopenia in normal recipients, that the importance of platelet autoantibodies was generally accepted. [21] This observation was followed by efforts to identify and to isolate the autoantibodies and to identify the platelet antigens recognized. The cumulative efforts of many investigators have provided definitive evidence that ITP is caused by platelet-reactive autoantibodies, but the nature of the derangement that leads to autoantibody production remains unknown.

Platelet life span is reduced in essentially all patients with ITP, [22] [23] primarily as a result of the clearance of antibody-coated platelets by tissue macrophages. [24] [25] The contribution of intravascular destruction, if any, has not been established, but the possibility has been suggested because platelet-derived microparticles [26] and antibodies to cytoplasmic components of platelet glycoproteins [27] are found in the plasma of some patients. However, the rapid response to splenectomy in many patients suggests extravascular clearance is the major cause of thrombocytopenia in most patients.

The extent of compensatory *platelet production* in ITP is a matter of contention. Initial studies were consistent with the notion that platelet production increased as much as fivefold in most patients in response to shortened platelet survival. [23] This conclusion was in keeping with the increased number of megakaryocytes in the bone marrow and the larger, presumably younger, platelets seen on peripheral smear. In addition, bleeding time studies (see discussion later in this chapter) suggested enhanced hemostatic effectiveness of the residual platelets, consistent with their presumed greater mass. Other studies suggest that platelet production is

more heterogeneous than previously understood.[28] [29] Impaired platelet production, presumably resulting from destruction of antibody-coated platelets by intramedullary macrophages[30] or a direct effect of autoantibodies on the later stages of megakaryocyte differentiation [31] may contribute to the thrombocytopenia in some refractory patients. Again, the rapid response to splenectomy remains strong supportive evidence for the importance of extramedullary platelet destruction as the primary cause of the thrombocytopenia in most patients with ITP.

Platelet function also appears to be more variable than was previously understood. Most autoantibodies identified to date are directed at platelet glycoproteins that serve important hemostatic functions. Yet, the extent of bleeding generally correlates with the severity of thrombocytopenia, with spontaneous bleeding rarely becoming a problem until the platelet count falls below 30,000/l. This clinical experience is consistent with the observation that bleeding times remain within the normal range in at least some patients with ITP at somewhat lower platelet counts than among patients with aplastic anemia. [32] However, a subset of patients with a clear history of easy bruising and only moderate thrombocytopenia has been identified, in whom acquired storage pool deficiency and impaired release reaction caused by antiplatelet antibodies have been demonstrated. [33] [34] [35] In extreme cases, platelet autoantibodies cause an acquired thrombasthenia [36] [37] or Bernard-Soulier-like disorder which can be clinically severe. [38] A few patients have been described in whom bruising has persisted despite normalization of the platelet count and who were benefited by measures to inhibit antibody production. [39] [40] Such severe qualitative platelet defects are infrequent, however, and it is sufficient in most ITP patients to monitor the platelet count to predict the risk of hemorrhage (see discussion later in this chapter).

Laboratory Diagnosis

IgG and/or IgM antibodies against platelet glycoprotein (GP) complexes, including GP IIb/IIIa, Ib/IX, Ia/IIa, V, and IV and other specific platelet glycoproteins, [41] [42] [43] [44] have been identified on the platelets or in the plasma of most ITP patients. Detection is based on the principle of antigen capture in which, for example, a monoclonal antibody to a cell-specific glycoprotein is immobilized on a solid support to which a lysate of the patients

platelets or normal platelets sensitized with the patients plasma is added. This step enables the antigen, and any associated human antibody, to be captured onto the solid support where it can then be detected with an appropriately tagged antihuman immunoglobulin by radioimmunoassay or ELISA. These assays have largely replaced antiglobulin tests, which have a high incidence of false-positives presumably as a result of the uptake of plasma immunoglobulin by megakaryocytes followed by secretion and rebinding of plasma IgG when platelets are activated in the circulation. [45] However, antiplatelet antibodies are not detected in at least 20% of typical cases of ITP even using these newly developed techniques. False-negative test results may be caused by competition between the monoclonal and human antibodies for the same epitope on the platelet and antibodies may dissociate from the antigen during test performance either due to low affinity or loss of the epitope during solubilization of the platelet. Or, serum may contain antibodies to cytoplasmic portions of these glycoproteins that are exposed in vitro but not on the surface of circulating platelets in vivo. [27] Little information is available on the incidence of false-positives, that is, specific antiplatelet glycoprotein antibodies in presumably nonimmune thrombocytopenic conditions that may be hard to distinguish from ITP clinically, including myelodysplasia, preeclampsia, chronic liver disease, sepsis, and so forth, in which such tests would provide the most benefit. Additional clues may come from the absence of markedly elevated levels of thrombopoietin that occur in hypomegakaryocytic disorders [46] [47] and elevated plasma levels of glycocalicin, [48] but neither assay is clinically available at this time. Therefore, ITP remains a clinical diagnosis and a diagnosis of exclusion, and it is premature to rely upon available platelet antibody or other tests either to confirm or to exclude this diagnosis.[2]

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INITIAL TREATMENT OF ADULTS WITH ITP

The choice of initial management depends in large part on the clinical presentation. Life-threatening hemorrhage rarely occurs at platelet counts >10,000/l, [49] [50] and therapy is generally not required at platelet counts >3050,000/l in the absence of bleeding or predisposing comorbid conditions. [2] However, because it is uncommon for ITP to remit spontaneously in adults, [2] [49] some form of treatment is generally indicated for the typical patient who presents with bruising or bleeding, especially in patients over the age of 60 and those with a previous history of bleeding. [49] [51] In the absence of hemorrhage or another medical emergency, such as concurrent trauma or surgery (see discussion later in this chapter), treatment is generally initiated with prednisone 12 mg/kg/day, although somewhat lower doses (0.250.5 mg/kg/day) may provide comparable benefit.[35] [52] The goal of initial treatment is to identify the lowest total dose of prednisone that will reverse and prevent severe thrombocytopenia for a sufficient period to permit the occasional adult who will have a durable response to be identified. [2] [53]

The response rate to prednisone varies from 6090% depending on the intensity and duration of treatment. [2] [53] [54] It is unlikely that additional benefit is achieved by continuing prednisone beyond 34 weeks, by which time considerable toxicity is generally evident. Therefore, if the patient is not in complete remission by this time, alternative forms of management should be sought, since there is no compelling evidence that prolonging treatment further will alter the natural history of the disease. In the authors experience less than 15% of adults with chronic ITP have a durable remission once prednisone is tapered, although remission rates approaching 25% have been reported by others.[2] [53] [54] Therefore, it is likely that even steroid responders will eventually require alternative forms of treatment.

The mechanism of action of prednisone in ITP remains controversial. Evidence suggests that glucocorticoids impair the clearance of antibody-coated platelets by tissue macrophages,[55] inhibit antibody production, [56] and increase platelet production, possibly by inhibiting phagocytosis of platelets by bone marrow macrophages.[30] In addition, cutaneous bleeding may resolve before an increase in the platelet count is seen, either because prednisone has a direct effect on vascular integrity or because the first platelets to circulate are recruited preferentially to sites of bleeding.

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EMERGENCY TREATMENT

Emergency treatment is indicated for internal or widespread mucocutaneous bleeding. Hospitalization should be considered even for asymptomatic patients presenting with platelet counts of <20,000/l, and certainly for those with counts <10,000/l, until a beneficial effect of treatment can be documented. General measures to reduce the risk of bleeding should be instituted including avoidance of drugs that interfere with platelet function, control of blood pressure and other medical conditions that inhibit coagulation, and measures to minimize the risk of trauma. Platelet transfusions are indicated if there is evidence of life-threatening bleeding and are often surprisingly effective in this setting, [57] [58] especially when given in conjunction with other therapies. [59] Treatment should be initiated with either intravenous immunoglobulin (1 g/kg/day for 2 days) (see discussion later in this chapter) or intravenous methylprednisolone (12 g/day for 13 days). [2] [60] [61] Emergency splenectomy should be reserved for the rare patient who fails to respond and requires additional treatment, for example, prior to emergency craniotomy for intracranial hemorrhage. Plasmapheresis has been used widely with few lasting effects but may play an adjunctive role with IVIG in emergency settings. [62] [63]

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SPLENECTOMY

Although there are no controlled studies showing that the duration of treatment with glucocorticoids alters the clinical course of responding patients, most hematologists taper prednisone over a period of 14 months. Most adults relapse during or after prednisone is discontinued, at which time splenectomy is considered. In general, splenectomy is the treatment of choice in any ITP patient who requires additional medical treatment (platelet count of <3050,000/l) and who is capable of undergoing the procedure.

Some investigators have reported a correlation between response to splenectomy and prior response to steroids, [57] [64] [65] intravenous immunoglobulin (IVIG),[66] or site of platelet clearance,[67] but there are too many exceptions to exclude a splenectomy from consideration based on any combination of clinical or laboratory parameters.[56] [68] [69] [70] Approximately two-thirds of adults will have an initial complete response to splenectomy and another 15% will have a stable partial response. [2] [53] [54] Approximately 15% of responding patients will relapse either soon after splenectomy or, less commonly, many years later. [49] Laparoscopic splenectomy may provide an alternative approach in some patients,[71] although there is some concern that operating time is longer and accessory spleens may be more likely to be overlooked. Using either approach, clinically significant bleeding is rare even when the patient is severely thrombocytopenic, and the practice of prophylactic platelet transfusions is to be discouraged.[2] Splenic irradiation has been used successfully in a few patients in whom splenectomy was judged to be medically hazardous, [72] but the procedure requires further evaluation.

The major risk of splenectomy is that of overwhelming bacterial sepsis, which occurs in less than 1% of otherwise healthy adults with uncomplicated ITP managed appropriately.[73] Patients should be immunized with polyvalent pneumoccocal vaccine at least 2 weeks prior to splenectomy, and in some settings, with *Hemophilus influenzae* type b vaccine and quadrivalent

meningococal polysaccharide vaccines.[2] It is also our practice to have patients seek medical attention and begin antibiotics immediately at the onset of a systemic febrile illness; fever 102°F should be treated in a hospitalized setting with IV antibiotics. Evidence does not suggest any benefit of prophylactic antibiotics in otherwise healthy adults after splenectomy for ITP.

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TREATMENT OF SPLENECTOMY FAILURES

Overview

Approximately 30% of adults require additional therapy after splenectomy because of recurrent or persistent thrombocytopenia; [53] [54] the response rate is lower in patients with Evan syndrome or systemic lupus erythematosus (SLE). Spontaneous remissions are uncommon[74] and a mortality rate in excess of 5% has been reported for those in whom severe thrombocytopenia recurs or persists after splenectomy. [1] However, in the authors experience, this rate can be reduced substantially with the proper use of newer forms of treatment.

The initial approach to patients with recurrent bleeding or severe thrombocytopenia after splenectomy is generally the same as for those who present with severe, acute ITP: the diagnosis must be affirmed, measures to limit bleeding must be instituted, and treatment with corticosteroids and/or IVIG must be reinstituted depending on the severity of the presentation, even if these modalities were ineffective prior to splenectomy. Once the platelet count has increased to a safe level, the process of tapering steroids is resumed. An occasional patient will prove to be manageable with alternate-day corticosteroids or sufficiently low daily doses of prednisone (510 mg) to permit long-term use, but most will require one or more of the treatments described later in this chapter. Consideration should be given to modalities to decrease the incidence of osteoporosis if more than 10 mg of prednisone per day is required. [35]

Multiple treatment options are available. [2] [53] [54] However, it is important to be clear that the goals of therapy for splenectomy failures differ from those for patients at initial presentation because the chance of inducing a durable, complete, and unmaintained remission is much lower. Treatment must be therefore individualized with the aim of achieving a platelet count that is sufficient to prevent spontaneous bleeding and, if possible, one that affords protection from injury or other emergencies (generally a platelet count >2050,000/l). This risk also depends on the patients age and bleeding history [51] [75] as well as comorbid conditions. Further, an assessment of the bleeding risk must be balanced against a similar estimate of potential complications that may result from each form of treatment. Therefore, no single treatment paradigm is applicable to all patients.

Search for an Accessory Spleen

The presence of an accessory spleen should be sought in any patient who has relapsed and is likely to require additional treatment, since antibody-coated platelets may be removed even if all Howell-Jolly bodies are not. [76] [77] Most accessory spleens can be detected using ^{99m} technetium sulfur colloid, although more sensitive radionucleotide imaging techniques or CT or MRI scanning may be required in select cases. [77] [78] Neither the size of the accessory spleen nor any other characteristic has been useful in predicting response to surgery. The reported response rates to removal of an accessory spleen vary widely. [76] [77] Less than one-quarter of patients will have a long-term remission after removal of an accessory spleen. Therefore, the decision to recommend removal of an accessory spleen depends upon its location, the severity of the thrombocytopenia, the perceived risk of surgery, and the response to alternative forms of therapy.

Agents That Impair Platelet Clearance

Most patients do not have accessory spleens and require intolerable doses of corticosteroids to maintain adequate platelet counts. The options for treatment in this setting are described. Although each is discussed in the context of being used as the sole modality of therapy, it is often possible and desirable to use these agents in combination at lower doses or at longer intervals with synergistic benefits and reduced toxicity.

Intravenous Immunoglobulin (IVIG)

A major treatment advance was made in 1981 when Imbach and coworkers observed an increase in the platelet count of two children with immunodeficiency who had been treated with IVIG for infection.[79] A single course of therapy will increase the platelet counts to >50,000/l in approximately 85% of adults and to >100,000/l in approximately 65% of adults.[80] [81] IVIG is generally given by intermittent intravenous infusion over 12 days in total doses ranging from 0.52 g/kg/course with comparable response rates.[82] The infusions are generally repeated as needed every 1021 days, consistent with the half-life of IgG in the circulation, although unexpectedly high rates of durable responses have been reported when IVIG was given on a fixed schedule. [83] [84]

IVIG impairs the clearance of IgG-coated platelets presumably by competing for binding to tissue macrophage Fc IIA receptors. [80] [84] The biologically active fraction of IVIG is unknown, that is, it is uncertain whether monomeric IgG competes directly with antibody-coated platelets for binding to macrophages, or whether IgG aggregates in the preparations or cell-associated or soluble immune complexes formed in the recipient are responsible for the drugs effectiveness. Anti-idiotypic antibodies that interfere with binding or formation of antiplatelet autoantibodies may contribute in the occasional patient who develops long-term remissions, [85] but the observation that Fc fragments are as effective and the comparable efficacy of anti-D argues that Fc IIA blockade is of prime importance. [61] There is no evidence that currently available commercial preparations differ in their efficacy or toxicity other than in the rare patient with IgA deficiency, but the possibility of transmission of hepatitis C mandates that at least one effective form of viral inactivation be included (see discussion later in this chapter). Preferable characteristics, in addition to lower cost, include solvent detergent and/or other antiviral treatment, liquid state, and lower incidence of reactions during infusion.

Toxicity is generally mild and self-limited. As many as half the patients complain of headache (most often during the initial infusion), which is responsive to oral analgesics and slowing of the rate of administration. Occasional patients experience a more severe, but generally self-limited, migraine syndrome beginning 12 days after infusion, which may be associated with a pleocytosis in the cerebrospinal fluid. [86] [87] Positive direct antiglobulin tests occur commonly but are typically short-lived,[88] whereas overt hemolytic anemia has occasionally been reported to occur from infusion of high-titer isoagglutinins, [89] as has the formation of red cell isoagglutinins resulting from transfer of blood group A substance. [90] Other rare side effects include thrombosis,[91] pulmonary or renal failure,[92] and anaphylaxis in IgA-deficient patients with IgE anti-IgA antibodies, in whom IgA-depleted preparations should be used. [93] [94] Antiviral antibodies[95] can be passively acquired after administration of IVIG, but there are no documented cases of HIV or hepatitis A or B having been acquired as a result of treatment. A single large outbreak of hepatitis C was reported in 19935 and was attributed to a single preparation affected by a change in donor screening and the absence of viral inactivation, [96] but no cases have been reported after infusion of a solvent-treated product. Lots continue to be recalled if a donor is at risk for Creutzfeld-Jacob

disease, although no cases of IV transmission have been documented.

Major limitations of IVIG treatment have been the need for repeated intravenous infusion and its high cost, which averages almost $3,000 per course in adults. Approximately one-third of patients become refractory after multiple courses of therapy. [81] Whether maintenance treatment alters the natural history of chronic ITP

remains an open question. In the absence of a controlled study the major indications for IVIG are: (1) treatment of medical emergencies; (2) preparation for splenectomy in patients intolerant or resistant to corticosteroids; (3) as a means to defer splenectomy in young children or debilitated adults; (4) during pregnancy when potentially teratogenic drugs must be avoided; (5) in chronic management of the rare patient who is refractory to other measures; and (6) while awaiting a response to slower-acting agents such as azathioprine or danazol.

Anti-D

Salama and Mueller-Eckhardt proposed that induction of a mild hemolytic anemia by infusing anti-D into Rh(D)+ individuals might inhibit macrophage Fc receptor function and clearance of antibody-coated platelets analogous to the infusion of large amounts of nonspecific IgG. [97] Comparable response rates are seen after administration of 1/100 as much IgG given as anti-D compared with the amount given in a typical course of IVIG. [98] Further, anti-D, given at a dose of 75 g/kg, induces a comparable increase in the platelet count within the first 24 hours of treatment (37,000/l). [99] Treatment requires approximately 5 minutes, is less expensive than IVIG, and the systemic side effects of IVIG are avoided. The dose-limiting toxicity of anti-D is hemolytic anemia with a mean decrease in hemoglobin of 1.0 g/dl, occasionally accompanied by chills and nausea.[98] A higher response rate is seen in those in whom the hemoglobin in reduced by 2 g/dl when the dose of anti-D is increased. Other limitations of anti-D treatment include its inapplicability to Rh(D) individuals, lower increments in the platelet count with a mean increase of 45,000/l, and minimal efficacy in splenectomized patients.[98] The indications for anti-D are essentially the same as those for IVIG. Anti-D is especially useful in HIV-positive individuals and others who require chronic treatment in whom it is desirable to avoid both long-term treatment with corticosteroids and splenectomy.

Danazol

The impeded androgen, danazol, given at a dose of 1015 mg/kg/day for 46 months, is effective in the majority of adults, [100] [101] [102] as well as in some children[103] and patients with secondary immune thrombocytopenia.[104] Danazol may impair macrophage-mediated clearance of antibody-coated platelets initially, [105] but inhibition of antibody production [100] [101] [106] may contribute to its effectiveness after prolonged use. Despite these favorable reports, the efficacy of danazol in refractory patients remains controversial.[107] [108] The variation in the observed response rates may in part be due to differences in the duration of treatment. [105] In one series, the mean duration of treatment was 2.7 months, but some individuals required as much as 6 months of treatment until a response was seen. [101] In our experience, the major indications for danazol are to defer splenectomy and as a potential corticosteroid-sparing drug. Although an occasional patient will experience an extended, unmaintained remission after a prolonged course of treatment, remissions usually require continued drug administration, albeit at much lower daily doses, [109] with relapses typically occurring weeks to months later if therapy is stopped. Danazol shares with other androgens the potential for causing hepatic injury including cholestatic hepatitis, peliosis, [110] and neoplasia.[111] Thrombocytopenia was reported as a complication of danazol in a few patients treated for disorders other than ITP.[112]

Vinca Alkaloids

Vinca alkaloids bind avidly to platelets from which they are released slowly into the plasma. [113] This may permit the drug to be delivered by antibody-coated platelets to tissue macrophages, thereby inhibiting their phagocytic capabilities. [105] [113] Vincristine (0.025 mg/kg or 11.5 mg/m^2 , not to exceed 2 mg) and vinblastine (46 mg/m^2 , not to exceed 10 mg) can be administered either by injection or by slow infusion. The latter method is reported to be somewhat more efficacious and potentially more toxic.[114] Reported response rates vary widely. Contrary to initial favorable reports, the results of more recent and larger series indicate that the response rate to either drug is closer to 1020% among patients with chronic refractory ITP, and sustained complete remissions are uncommon. [68] [115] [116] [117] Treatment must be given every 24 weeks for an indefinite period; many patients become refractory or treatment must be stopped because of neurologic or hematologic toxicity.

Immunosuppressants

Although immunosuppressive drugs have generally been reserved only for refractory patients intolerant of the treatments mentioned earlier in this chapter, their proper place in the treatment of refractory patients is being reassessed. Among patients treated with azathioprine, 2040% attain a complete remission and an additional 1020% have a partial response, [74] [117] [118] although lower response rates have been noted by some.[117] [119] Again, the variable outcomes may reflect differences in the intensity or duration of treatment. For example, the median time to response among patients in one study was 4 months, and maximal benefit was not attained in some cases until 78 months of treatment.[118] Responses are generally durable and, at least in some patients, persist after treatment is discontinued. Side effects are rare and predominantly limited to reversible elevations of serum transamidases.

Comparable or only slightly lower response rates have been reported with the use of cyclophosphamide as a single agent. [117] [119] [120] Cyclophosphamide may be given orally at a dose of 12 mg/kg/day titrated based on the neutrophil count, or 12 courses may be given intravenously at a dose of 11.5 g/m^2 every 3 weeks. The use of cyclophosphamide should be restricted, especially among younger patients, because of the risk of infertility and leukemia. The combination of cyclophosphamide and prednisone with one of a number of additional agents has been effective in a small number of refractory patients with acceptable toxicity. [121] [122] A few patients have been treated successfully with cyclosporin.[123] The high response rate reported for pulse dexamethasone has not been confirmed in subsequent studies but remains under study.[124]

Treatments Whose Mechanism of Action Is Unknown

A few patients with chronic ITP have been treated by incubating their plasma with columns coated with staphylococcal protein A. An overall response rate approaching 30%, including many sustained responses, has been reported by the manufacturer of the columns. [125] The mechanism of action is unknown. Approximately one-third of the patients developed an acute hypersensitivity-type reaction; 5% required discontinuation of treatment. [125] [126] Cases of hypotension and fatal anaphylaxis have been reported in patients treated with protein A pheresis for other disorders. [126] Dapsone[127] and colchicine[128] have been reported to be effective in relatively small numbers of patients. Their mechanism of action is not established and the response rates in otherwise refractory patients have not been studied prospectively. Occasional patients have been treated successfully with recombinant -2b interferon [129] although worsening of thrombocytopenia has been noted.[130]

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SECONDARY IMMUNE THROMBOCYTOPENIAS

Introduction

Secondary immune thrombocytopenias comprise 4050% of cases in some series.[131] [132] The reason for this increased recognition of secondary ITP is not apparent although it may be related to the recent epidemic of HIV-1 infection (see Chap. 155). The incidence of secondary immune thrombocytopenias should be considered in determining when to treat a patient with a systemic illness for presumed ITP when other causes of thrombocytopenia are more frequent or may coexist. Because ITP remains a diagnosis of exclusion, the accuracy of these decisions depends on the familiarity of the treating physician with the prevalence, implications, and proper management of secondary immune thrombocytopenia in each of its associated conditions.

Systemic Lupus Erythematosus

Presentation

Approximately 1525% of patients with systemic lupus erythematosus (SLE) develop thrombocytopenia during the course of their illness, although the problem is severe enough to require treatment in only 510% of patients. [133] [134] Thrombocytopenia may precede the diagnosis of SLE by months to years,[135] but such progression occurs in <5% of patients with classic ITP. [3] [136] [137] Therefore, serologic evaluation should be restricted to those individuals who show additional evidence of lupus. [2]

The prognostic significance of thrombocytopenia in patients with SLE depends on the clinical context. Four clinical presentations have been described. The clinical course of patients who develop immune thrombocytopenia without a worsening of other disease manifestations is similar to that of patients with ITP. [133] [138] [139] In contrast, severe thrombocytopenia that develops during a systemic exacerbation of vasculitis may have a different pathogenesis (see the following section) and carries a poorer prognosis. [133] [134] [139] [140] Thrombocytopenia also occurs in patients with antiphospholipid antibodies, who may be at a somewhat higher risk for thrombosis and recurrent spontaneous abortions. [141] [142] Amegakaryocytic thrombocytopenia has been detected in a few patients. [143]

Pathogenesis

The pathogenesis of thrombocytopenia in SLE patients may be multifactorial. First, circulating platelet-reactive antibodies with diverse specificities have been identified,[144] [145] [146] but which of these cause thrombocytopenia has not been determined. Second, plasma from most patients contains immune complexes that bind to platelets in vitro [147] and both DNA and anti-DNA antibodies have been identified on patient platelets. [148] However, since such complexes are found in many patients in the absence of thrombocytopenia, their importance in accelerating platelet clearance or altering platelet function remains uncertain. [149] [150] Further, platelet clearance may be influenced not only by the composition of these immune complexes, which are undoubtedly heterogeneous, but also by impaired macrophage IgG Fc receptor-mediated clearance resulting from the underlying disease and its treatment. [151] Finally, when severe thrombocytopenia occurs as part of a systemic relapse, platelet activation and deposition may also occur within vessels involved by vasculitis, such as the kidney. [152] Thrombocytopenia caused by vascular injury may not respond to measures that suffice to manage ITP.

Management

For several reasons, treatment of thrombocytopenic episodes in patients with SLE is more complicated than the management of patients with classic ITP. First, thrombocytopenia caused by platelet-specific antibodies must be distinguished from that caused by systemic vasculitis. In extreme cases, the distinction may be relatively straightforward. At one end of the spectrum, patients with severe thrombocytopenia in whom there is no other evidence of active SLE should be treated as if they had ITP. At the other extreme, it is generally necessary to control the systemic vasculitis before thrombocytopenia will improve. However, in many patients the extent to which vasculitis contributes to the thrombocytopenia is not apparent and may vary. Second, because patients with SLE may experience repeated episodes of thrombocytopenia, treatment should only be used when a risk of bleeding exists in order to avoid excessive use of corticosteroids or other medications in these heavily treated patients. Third, attempts to induce long-term remission with splenectomy should be tempered by the knowledge that many patients continue to require corticosteroids to control the underlying disease. Therefore, treatment decisions should be predicated on the extent to which thrombocytopenia per se is the predominant reason for treatment and the likelihood that systemic vasculitis will necessitate treatment in the future.

When thrombocytopenia occurs in the absence of a systemic flare-up, the short-term response to corticosteroids, danazol, IVIG, and immunosuppression in patients with SLE and classic ITP are comparable.[153] [154] [155] [156] The role of splenectomy remains controversial. Variability in the long-term response rates is undoubtedly influenced by differences in the severity of the underlying disease. [157] [158] [159] It is our opinion that splenectomy is indicated only when thrombocytopenia is severe and persistent, and when systemic manifestations of the SLE are otherwise well-controlled. In such cases, the procedure is as effective as in ITP. Profound thrombocytopenia that occurs during the course of a systemic exacerbation of SLE may require pulses of high-dose corticosteroids [160] or cyclophosphamide,[161] or both.

Lymphoproliferative Disorders

Chronic Lymphocytic Leukemia

Thrombocytopenia caused by marrow and splenic infiltration is a common feature of advanced chronic lymphocytic leukemia (CLL) [162] and has a mean survival time of about 19 months.[163] In contrast, immune thrombocytopenia, which occurs in approximately 2% of patients with CLL, [164] [165] does not confer a poor prognosis[166] [167] [168] and requires distinct treatment. ITP may precede the diagnosis of CLL or develop at any stage. [167] [169] The pathophysiology of autoantibody production in CLL and its relationship to clonal B-cell proliferation has been reviewed. [170]

The diagnosis of an antibody-mediated thrombocytopenia becomes progressively more difficult as the disease progresses. ITP should be suspected in patients with early stage CLL whose bone marrow contains adequate numbers of megakaryocytes or in patients at a more advanced stage in whom the severity of thrombocytopenia is disproportionate to the extent of marrow infiltration or splenomegaly. The concurrent development of autoimmune hemolytic anemia may also suggest the diagnosis.[167] The cause of thrombocytopenia may be difficult to establish in patients who have a significant tumor burden. A short course of therapy, such as IVIG, may be indicated. Although most patients with CLL and immune thrombocytopenia respond to corticosteroids or splenectomy, [166] [167] [168] immunosuppressive therapy that inhibits B-cell proliferation and antibody production may be required. [166] [167] [168] [169] IVIG prophylaxis (400 mg/kg/month) should be considered in patients postsplenectomy and for those who require immunosuppressive therapy.

Hodgkin Disease and Non-Hodgkin Lymphomas

ITP occurs in approximately 1% of patients with Hodgkin disease. The clinical courses of approximately 50 patients have

been reported. [132] [164] [171] In three-quarters of the reported cases, ITP was diagnosed an average of 52 months (1 month22 years) after Hodgkin disease was detected.[172] Hodgkin disease was discovered at the time of splenectomy for ITP in a few patients. [3] In most of the remaining cases, ITP preceded the diagnosis of Hodgkin disease, although in some instances the interval was so long as to place the significance of the association in doubt. [172] [173] [174] One persistent dilemma is whether the development of ITP in a patient with Hodgkin disease signals recurrence of the lymphoma. Although such cases have been reported, [132] [172] approximately two-thirds of the patients did not have any evidence of active Hodgkin disease. [171] [172] [175] [176] Virtually all patients in whom there was no evidence of recurrent Hodgkin disease responded to some combination of corticosteroids, [172] [176] splenectomy,[175] [177] and azathioprine.[176] Treatment of the Hodgkin disease has been required in some patients before the thrombocytopenia resolves. [167] [178] [179]

The association between immune thrombocytopenia and non-Hodgkin lymphoma appears almost coincidental, and few generalizations can be made. [132] [175] [180] [181] [182] The histologic type of lymphoma and extent of the underlying disease has varied widely. In most cases, thrombocytopenia has proved difficult to manage unless the underlying malignancy responded to chemotherapy.

Leukemia of Large Granular Lymphocytes

The term leukemia of large granular lymphocytes (LGL) is applied to two disorders that can be distinguished by the phenotype of the clonally expanded cell population.[183] Thrombocytopenia occurs in both forms of the disease, although the pathogenesis and treatment differ. In >90% of the patients there is a clonal expansion of $CD3^+$, $CD16^+$ T cells (T-LGL), and the natural history is generally one of stability or slow expansion of this cell population. In the remaining patients, the LGL cells express an NK phenotype (CD3 , $CD16^+$, $CD57^+$). NK-LGL is more typically aggressive, and severe thrombocytopenia, caused by marrow replacement and splenic infiltration, may develop over months. [183]

Most patients with T-LGL come to medical attention because of recurrent infections secondary to neutropenia. [184] Thrombocytopenia, which occurs in 520% of these patients, is generally mild and may be secondary to splenic enlargement, [185] but occasional patients present with severe thrombocytopenia and a clinical picture compatible with ITP.[184] [185] The diagnosis of LGL should be suspected in patients with thrombocytopenia accompanied by neutropenia, arthralgia, splenomegaly, and a positive test for rheumatoid factor. On occasion the number of LDL cells is increased only modestly, [183] making the disorder hard to distinguish from severe ITP in which an increase in $CD56^+$ CD53 cells has also been observed. [186] Data is not sufficient to determine whether the response to treatment of immune thrombocytopenia in patients with T-LGL differs from typical ITP.

Miscellaneous Disorders

Mild thrombocytopenia, attributed to hypersplenism, has been noted in patients with autoimmune hyperthyroidism and resolves slowly with return of the euthyroid state.[187] [188] Immune thrombocytopenia probably also occurs more commonly in these patients.[188] [189] [190] Although most of these patients respond to measures effective in ITP, others require control of the hyperthyroid state before the thrombocytopenia abates. [188] [189] In patients with sarcoidosis, immune platelet destruction may be superimposed on thrombocytopenia caused by splenic and marrow infiltration with granulomas. [191] [192] ITP has also been reported in association with myasthenia gravis[193] and in patients with a variety of benign and malignant neoplasms, most of whom responded to conventional treatment for ITP. [182] [194] [195] [196] Thrombocytopenia commonly occurs after allogeneic bone marrow transplantation in the setting of graft-versus-host disease in which platelet autoantibodies, vascular injury, viral infection, and hypersplenism all may contribute to accelerated platelet destruction. [197] [198] Both transient and persistent immune thrombocytopenias have also been reported after organ and bone marrow allografts donated by patients with presumed compensated ITP. [199] [200] ITP also occurs with increased frequency in patients with common variable immunodeficiency (CVI)**.** The average age at diagnosis was 28 years in one large series. [201] CVI should be suspected in patients with recurrent infections and allergies. Splenectomy and immunosuppressive agents should be avoided, to the extent possible. Thrombocytopenia associated with HIV infection is discussed in Chapter 155 .

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ITP IN CHILDHOOD

Introduction

Treatment of children with ITP differs substantially from that for adults with respect to the differential diagnosis that must be considered at presentation, the much higher frequency of spontaneous remission, and, therefore, the greater delay before splenectomy is considered. In addition, the potential long-term effects of treatment must be taken into consideration in a population with a >50-year life expectancy. No clear pathophysiologic explanation has emerged that explains the obvious difference in the clinical course of ITP in these two age groups.

Presentation

As in adults, children with acute ITP typically present with the sudden onset of petechiae and purpura and platelet counts <2030,000/l. The relationship between the risk of bleeding and the platelet count appears to be the same as in adults. However, intracranial hemorrhage (ICH) has been reported in approximately 0.51% of children,[202] an incidence that may be higher than that seen in adults. Both sexes are affected with equal frequency. The peak incidence occurs between the ages of 3 and 5 but the disorder may occur at any age.[203] Although many patients have a history of nonspecific viral infection in the weeks prior to developing symptoms, such symptoms are common in children and a relationship of preceding infection to the development of ITP is hard to document. Occasional patients present with ITP during or within 4 weeks after an otherwise typical systemic viral infection caused by Epstein-Barr virus, [204] varicella, [205] cytomegalovirus,[206] rubella,[207] or hepatitis A, B, or C,[208] [209] [210] parvovirus,[211] and others,[212] or after vaccination with attenuated live virus. [213] The parents and pediatrician are usually more concerned than the child who continues to feel and act completely well. As in adults, the physical examination is notable only for signs of bleeding, the absence of hepatomegaly or lymphadenopathy, and two normal radial rays. Minimal splenomegaly is detectable in less than 10% of children. The CBC should be within normal limits for age with the exception of the platelet count. This includes the white blood cell differential, which normally has more lymphocytes than neutrophils in children <45 years of age.[203]

Evaluation

To experienced pediatric hematologists, this constellation of findings (i.e., an otherwise healthy child with isolated thrombocytopenia and a physical examination notable only for bruising or petechiae) is sufficient to make a diagnosis of ITP, which is by far the most common (>95%) cause of isolated, especially severe, thrombocytopenia in childhood. In general, no additional testing, including a bone marrow biopsy, is required if the presentation is typical, irrespective of therapy. [2] [214] The outcome of cooperative childhood cancer study group trials, which

included thousands of children, demonstrated that it is extremely unlikely (<0.1%) for acute leukemia to present with isolated thrombocytopenia, an otherwise normal blood count and smear, and the absence of hepatosplenomegaly or lymphadenopathy. [202] [215] One survey showed that most pediatric hematologists no longer believe that examination of the bone marrow is necessary to make a diagnosis of ITP when the presentation is typical, but that such an examination is required if there are abnormalities in the physical examination or in nonplatelet components of the CBC or if the patient fails to respond to treatment. [214] A response to IVIG in this setting is also essentially diagnostic of ITP. [216] HIV testing should be performed if the child or mother is considered to be at risk, including expremature infants who received transfusions or when significant cervical or axillary adenopathy is detected. [217] [218]

Differential Diagnosis

Once thrombocytopenia is documented in a healthy child with an otherwise normal CBC and no abnormalities other than bleeding manifestations on physical examination, the first diagnostic consideration is to exclude familial, nonimmune thrombocytopenias. These disorders are seen in 210% of children presenting with isolated thrombocytopenia.[219] A bone marrow examination will not help to differentiate ITP from these inherited syndromes. The diagnosis of a familial disorder may not be suspected despite careful questioning, since other affected family members may be asymptomatic. On occasion, an inherited cause of thrombocytopenia may only be suspected after the child has failed to respond to treatment for ITP. Fortunately, most children with inherited thrombocytopenias have mild to moderate thrombocytopenia (40100,000/l) and do not have symptoms or require treatment other than when platelet production is further impaired during periods of infection. The presence of uniformly small platelets should raise a suspicion of Wiskott-Aldrich syndrome, which may present with thrombocytopenia alone. Bernard-Soulier syndrome should be suspected with moderate thrombocytopenia and giant platelets. The causes of inherited thrombocytopenia are considered in detail in Chapter 127 . Amegakaryocytic thrombocytopenia may be indistinguishable from ITP on clinical presentation, and the diagnosis is generally considered only when the child fails treatment. Bone marrow biopsy is the definitive diagnostic test. Too few cases have been reported in children to determine the response to measures effective in adults. However, approximately 50% of the cases progressed to marrow aplasia, some of whom have been treated successfully with bone marrow transplantation. [220]

The second diagnostic consideration is to exclude an underlying disorder associated with ITP. Only HIV, SLE, and humoral immunodeficiency states occur with sufficient frequency in childhood to merit consideration. HIV as a cause of ITP in childhood is being seen with increased frequency. In utero transmission is the most common mode of infection. Usually the diagnosis is made apparent by maternal history or during the initial examination of the child, which typically reveals axillary adenopathy, hepatosplenomegaly and/or an abnormal hemoglobin, total white blood cell count, or white cell differential. However, some affected children have no clinical evidence of HIV even after many years of infection. HIV testing is reliable for diagnosis in children over 12 months of age. SLE may develop in as many as 25% of female adolescents who present with ITP.[221] This concern has led some pediatricians to an inappropriate search for antinuclear antibodies (ANA) in every child with ITP. A positive ANA does not predict the development of SLE, its severity, its responsiveness, or the incidence of spontaneous remission in otherwise healthy children.[222] Therefore, testing for antinuclear antibodies should only be performed if additional specific symptoms or laboratory findings are present that suggest the presence of SLE. Humoral immunodeficiency is found in 12% of children with ITP, most commonly as a result of IgA deficiency. [223] ITP also occurs in 15% of children with CVI and IgG_2 deficiency.[201] This incidence may be sufficiently high to warrant measuring immunoglobulin levels and documenting response to polyvalent pneumococcal vaccine prior to splenectomy.[224] Immunoglobulin measurements may therefore also be indicated in children who are to receive IVIG, because the use of IgA-depleted preparations may prevent formation of anti-IgA antibodies and is effective when such antibodies already exist. [80]

Third, additional diagnosis must be considered if other cytopenias are present. For example, the finding of severe anemia, even when there has been significant epistaxis, should lead to an investigation of alternative diagnoses such as aplastic anemia, myelodysplasia, and disorders associated with accelerated erythrocyte and platelet destruction such as thrombotic thrombocytopenic purpura and Evans syndrome. The diagnosis of the latter may be complicated if the patient has recently been treated either with IVIG, which may contain anti-A or -B isohemagglutinins, or with anti-D, since both therapies may cause a positive direct antiglobulin test. [225]

Management at Presentation

Which children with acute ITP should be treated and which treatment should be used, if any, remains a matter of intense controversy. [2] [226] [227] Treatment is intended primarily to prevent ICH. However, the incidence of ICH is too low [228] to perform a controlled study. The risk of ICH may be higher in children with platelet counts <20,000/l,[229] children presenting with extensive wet purpura or internal bleeding, and those with SLE or coexisting coagulopathies. However, because ICH can occur in the absence of bleeding at other sites, our approach is to treat all children with platelet counts <20,000/l. It should be emphasized, however, that no trial has demonstrated a reduction in ICH and other experienced pediatric hematologists may use more restrictive criteria. A secondary goal of therapy is to prevent the development of chronic disease. However, at least 80% of affected children recover spontaneously, many within the first 2 months. [230] [231] Chronicity is more likely to develop in teenagers, in children with SLE, and in those with Evans syndrome. However, children at any age can develop chronic ITP even with an otherwise typical presentation and there is as yet no firm evidence that the natural history is altered by early treatment. [124]

It is clear through the results of several recent controlled trials that prednisone (24 mg/kg/day), [232] intravenous methylprednisolone (30 mg/kg/day for 3 days), [233] IVIG (1 g/kg/day for 2 days),[234] and anti-D given at a dose of 75 g/kg [235] all shorten the duration of severe thrombocytopenia compared with no treatment. With IVIG, the mean increase in the platelet count was 54,000/l within the first 24 hours. [216] The combination of IVIG and corticosteroids may be synergistic. [236] Platelet transfusions should be reserved for ongoing or imminent major hemorrhage.[56] We recommend that all severely thrombocytopenic children with acute ITP (platelet counts <20,000/l) be treated with a combination of IVIG (1 g/kg/day) and intravenous methylprednisolone (30 mg/kg/day), both for 13 days, until a platelet count >2030,000/l is achieved and there is no evidence of bleeding. The diagnosis of ITP should be reconsidered in the rare situation in which a child fails to demonstrate a partial response to IVIG and/or methylprednisolone.

Management of Acute ITP after the First Week

The goal of treatment is to maintain a platelet count >2030,000/l with a minimum of toxicity. Drugs that impair platelet function, such as aspirin and glycerol guaiacolate, should be

CHRONIC ITP IN CHILDREN: MANAGEMENT AFTER 6 MONTHS

Most pediatric hematologists delay recommending splenectomy for at least 1 year for two reasons. First, children who have platelet counts >2030,000/l and who are asymptomatic can be followed safely with little or no treatment. Virtually all children can be managed with IVIG or anti-D if the platelet count falls below 2030,000/l, with small doses of prednisone (<0.2 mg/kg/qod) added if necessary. Our experience and those of others is that platelet count generally improves over time. However, the platelet count may fall at puberty in previously stable patients with mennorhagia as a complicating feature. Second, the risk of postsplenectomy sepsis may be as high as 2% over 10 years in children and greater in those less than 12 years of age.[73] Indeed, there have been as many reported deaths from postsplenectomy sepsis as from bleeding in this age group. [240] [241] It remains to be determined whether this risk is reduced substantially by the use of pneumococcal and H Flu B vaccines in conjunction with prophylactic antibiotics, which is the current standard of care. How often to revaccinate and the value of prophylactic antibiotics in this setting remain unsettled. We currently recommend that Pneumovax be given every 510 years, but do not administer hepatitis vaccine unless a special risk is identified. We recommend that prophylactic antibiotics be used for the first year after splenectomy and thereafter should be given immediately for any infection; intravenous antibiotics should be initiated urgently for children with a fever >102°C. No other long-term health risks have been identified in children who have been splenectomized for ITP. Therefore, most pediatric hematologists prefer waiting until a child has reached 5 years of age before recommending splenectomy because of the frequency of infection and high fevers in this population, although there is insufficient data on the risk of sepsis after the age of 2 to make firm recommendations. In the youngest children, it may be wise to document protective titers of antibody to the most common serotypes of pneumococcus before proceeding to splenectomy. Durable clinical remission occurs in 7090% of children with typical ITP after splenectomy. [242]

Among the few children refractory to splenectomy, alternate diagnoses should be investigated. Few children have significant bleeding problems even if they remain thrombocytopenic, which must be borne in mind when recommending potentially toxic forms of treatment. The rare, symptomatic, refractory pediatric patient should be approached in the same manner as a comparable adult, although the potential side effects of various drugs may differ depending on the age of the child.

avoided. Guidelines must be set for physical activity. Competitive contact sports, such as football or basketball, lacrosse, soccer, and hockey, should be avoided by children with platelet counts <30,000/l, but other physical activities including track, swimming, bicycling, baseball, and tennis need not be restricted for most children.

Primary reliance is often placed upon prednisone because it is relatively inexpensive, can be given orally, and the toxicity is tolerable when the duration of treatment is limited. One approach is to taper the dose of prednisone over 2 weeks to 3 months, although there is no evidence that any specific duration of treatment increases the probability of sustained complete remission. An attempt should be made to administer prednisone on an alternate day regimen to avoid toxicity, including osteoporosis, which may be the most medically significant, long-term side effect of corticosteroids in children. The utility of high-dose dexamethasone remains to be defined.[124]

The authors recommend the use of IVIG[237] or IV anti-D (Winrho-SD) [235] for children who require more than 0.2 mg/kg/day of prednisone as their initial treatment or when treatment beyond 23 months is required. Both IVIG and anti-D are generally administered on an as needed basis rather than at fixed intervals. The combination of prednisone with IVIG (and probably anti-D) may be synergistic. Treatment should be continued for at least 12 months if a response is seen, since spontaneous remissions may be delayed. With this combination of drugs, few children require splenectomy. Treatment with vinca alkaloids, [238] azathioprine,[239] or danazol[103] may be chosen in a few children in order to defer splenectomy. Because the disease will remit between the first and twelfth month in >80% of children with typical ITP, splenectomy should be deferred, if at all possible, for at least 1 year from the time of diagnosis.

The advantage of IVIG in managing children includes its lack of toxicity, its high rate of efficacy, and the possibility of curative effects. [216] [227] The disadvantages are that it must be given intravenously for hours, optimal use in maintenance depends upon the platelet count (making scheduling difficult), postinfusion headaches are common, and its cost is high.[237] In contrast to adults, less than 10% of pediatric patients become refractory even with prolonged treatment. There is no evidence that any of the commercially available products differ in clinical effectiveness. Anti-D appears to be equally safe and comparably effective to IVIG for Rh+ patients who have not undergone splenectomy.[235] The cost is considerably less and infusion is complete within minutes. Whether early treatment with IVIG or anti-D will alter the

incidence of chronic ITP remains to be determined. Children have been treated with each modality for years without evidence of toxicity.

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ITP DURING PREGNANCY

Diagnosis of ITP in Pregnancy

Because ITP commonly occurs in young females, hematologists are often asked to manage this disorder during pregnancy. The incidence in referral institutions is approximately 12 per 1,000 deliveries,[243] but is likely to be lower in the general community. Proper management of ITP in pregnancy requires consideration of two patients: the mother and the fetus. Therefore, management involves not only careful attention to the maternal platelet count and risk of bleeding, but an awareness of the incidence and consequences of neonatal thrombocytopenia caused by transplacental passage of maternal autoantibodies, as well as the risks and benefits to the fetus of maternal therapy, invasive monitoring, and surgical delivery. Several comprehensive reviews offer a more in-depth discussion of the differential diagnosis and management of thrombocytopenia in pregnancy.[244] [245]

It may be difficult to diagnose ITP when a woman presents near term with no prior platelet counts. The most common condition that must be distinguished from ITP is gestational thrombocytopenia, which occurs in approximately 4% of women (see Chap. 129). Gestational thrombocytopenia is quite likely when an otherwise entirely healthy women known to have had a normal platelet count develops mild thrombocytopenia (>100,000/l) toward the end of the third trimester of an uncomplicated pregnancy.[246] The importance of making the intrapartum distinction from ITP is that severe neonatal thrombocytopenia is rare and there is no indication for diagnostic or therapeutic intervention other than following the maternal platelet count. [246] However, gestational thrombocytopenia and ITP are both diagnoses of exclusion. Although the incidence of gestational thrombocytopenia in the typical setting vastly exceeds all other causes of thrombocytopenia combined, on occasion a previously healthy woman will present for the first time at term with mild-to-moderate ITP, early preeclampsia, type IIb

THERAPY OF ITP IN PREGNANCY

Most women with ITP can be managed without great difficulty during pregnancy, although occasional patients experience severe exacerbations. The only contraindication of treatment during pregnancy involves the use of cytotoxic drugs having teratogenic potential during the first trimester. [249] Pregnancy does not alter the indications for maternal treatment, since platelet counts >30,000/l are considered safe for otherwise uncomplicated pregnancy and delivery. [250] Corticosteroids are the mainstay of treatment. Although adverse effects have been noted in some experimental and clinical studies, it is generally accepted that corticosteroids pose little risk to the human fetus even during the first trimester. [250] Refractory patients and those intolerant of prednisone can generally be managed with IVIG. [251] Splenectomy has been performed successfully during pregnancy,[252] but is rarely necessary. Experience with azathioprine during pregnancy for ITP is limited, although it has been used in other settings without clear evidence of teratogenicity. [250] Other drugs, including danazol and dapsone, should be avoided if possible, because far less is known about their potential effects on the fetus.

The major concern comes at the time of delivery. Platelet counts >50,000/l suffice for cesarean section [253] and it is probably wise to use this number as a guidepost for treatment during the third trimester, when emergent obstetrical indications occur more commonly. Some anesthesiologists require higher platelet counts to perform epidural anesthesia, although studies to support this position are lacking.

The other major goal of management is to prevent intracranial hemorrhage from occurring in a severely thrombocytopenic neonate during a difficult vaginal delivery.[254] [255] However, there is no generally accepted noninvasive means to identify neonates at risk, the benefits of cesarean section have not been proven to reduce the occurrence of ICH,[256] and the relative risks of newer interventional diagnostic and therapeutic measures may be even greater in some settings. [257] [258] Therefore, the best approach to managing delivery in women with ITP remains controversial. Initial studies suggesting that women with ITP are at high risk for severe neonatal thrombocytopenia may have been biased by the overinclusion of women first diagnosed after delivery of a clinically affected child. [259] The risk of severe neonatal thrombocytopenia, defined as <50,000/l, is now estimated to be 1015%, but the incidence of platelet count below 20,000/l is probably <5% and the risk of intracranial hemorrhage in the neonate is <1%. [243] [258] [260] Further, antenatal intracranial hemorrhage occurs rarely, [243] [253] [260] arguing against a role for maternal treatment of the fetus as has been proposed for alloimmune thrombocytopenia (see discussion later in this chapter).

Unfortunately, there is no noninvasive means by which the fetal platelet count can be predicted. No significant correlation between fetal and maternal platelet counts either before or after treatment has been found.[243] [248] [253] [256] [261] Severely thrombocytopenic neonates have been born to mothers with normal platelet counts before[262] or after splenectomy, [256] as well as to women who have responded to corticosteroids or IVIG.[248] [263] [264] The risk of severe neonatal thrombocytopenia appears higher if the mother has had a previously affected fetus.[265] It is possible to directly measure the neonatal platelet count before delivery by means of fetal scalp sampling[266] or by percutaneous umbilical blood sampling (PUBS).[248] However, neonatal platelet counts determined by fetal scalp sampling may be complicated by falsely low values resulting from platelet clumping, leading to unnecessary emergency cesarean sections unless the adequacy of the count is affirmed by direct inspection of the blood smear.[267] [268] The risk to the fetus of PUBS may exceed that of ICH even in the hands of a skilled operator [248] [253] [269] [270] and the procedure is now rarely performed for ITP in the United States. [243] [257] [258] There is also no direct evidence that cesarean section prevents ICH, although no affected children born by this route of delivery have been reported and some experts would recommend the procedure when severe neonatal thrombocytopenia has been documented.[2]

The neonatal platelet count commonly falls in the first few days postpartum, possibly related to the maturation of the fetal reticulo-endothelial cell system. [256] The fall is transient but may infrequently require treatment. Therefore, it is mandatory to obtain a radiological investigation to diagnose or to exclude ICH as soon after birth as possible in all severely thrombocytopenic neonates. Management with platelet transfusions, corticosteroids, and/or IVIG [271] should be instituted emergently pending the results of the study if there is any suspicion of bleeding, even in the absence of signs of neurologic impairment and even when the anterior fontanel is flat.

von Willebrand disease, or a previously undiagnosed congenital thrombocytopenic condition. The prevalence of these other disorders is likely to be higher among women with platelet counts <75,000/l and when thrombocytopenia is detected before the third trimester. Conventional antiglobulin tests do not distinguish ITP from gestational thrombocytopenia, although antigen capture assays may prove to be helpful. [247] Rigorous criteria for making the diagnosis have not been identified, especially at earlier time points in gestation, and the rare reported examples of neonatal thrombocytopenia occurring in women with presumed gestational thrombocytopenia may be examples of maternal ITP.[248]

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NEONATAL ALLOIMMUNE THROMBOCYTOPENIA

Clinical Presentation

Neonatal alloimmune thrombocytopenia (NAIT) is the platelet analogue of hemolytic disease of the newborn, although there are important differences in their natural history and management. NAIT occurs in 1 in 15,000 term pregnancies (approximately 8004000 cases per year in the United States), [272] [273] accounting for approximately 1020% of the total number of cases of neonatal thrombocytopenia. [272] [274] [275] The higher estimate is probably more accurate, as additional affected neonates with moderate thrombocytopenia are being identified as a result of greater awareness combined with the use of recently developed serologic techniques. In contrast to hemolytic disease of the newborn, almost half of the cases of NAIT occur during the first term pregnancy, [272] [276] although the incidence of prior miscarriage in these women is unknown.[272] Typically, the diagnosis is suspected when bleeding and severe thrombocytopenia occur in a neonate born after an otherwise uneventful pregnancy. ICH is common (1020%) and is fatal in up to 5% of cases. [272] [277] [278] The affected child may show signs and symptoms of hydrocephalus or porenchephaly as a result of ICH in utero in up to 10% of cases, [272] [278] [279] which may occasionally occur before the 30th week of gestation. [278] [280] [281] Thus, in contrast to neonatal thrombocytopenia caused by maternal ITP, NAIT is often severe and potentially life-threatening. [282] The platelet count may

continue to fall and bleeding may occur during the first several days after delivery, necessitating prompt diagnosis and management. [272] Thrombocytopenia typically persists for several days to 3 weeks.[274]

Pathogenesis

NAIT is caused by the transplacental passage of maternal alloantibodies against platelet antigens shared by the father and the fetus. Shed fetal platelet antigens can pass into the maternal circulation by the 14th week of gestation, [283] at which time the placenta has already developed the capacity to transport maternal antibodies to the fetus. Several biallelic antigen systems have been implicated. Most identified cases in whites occur in homozygous HPA-1b (PI^{A2}/PI^{A2}) mothers.[272] Most cases of NAIT due to anti-HPA-1a have been severe, as have the few cases attributable to alloantibodies against other determinants on platelet GP IIIa, i.e., anti-HPA-1b [284] and anti-HPA-4 (Yuk^a/Pen)[285] and GP IIb, i.e., anti-HPA-3a (Bak^a/Lek).[286] The clinical picture is generally milder in NAIT caused by anti-HPA-5a (Br^a), which accounts for 1520% of the cases of NAIT in Europe,[272] [287] presumably because platelets express far fewer copies of GP Ia/IIa complex on which the HPA-5 alleles are located. This difference may also contribute to the observations that more cases of NAIT caused by anti-HPA-5b (Br^a) occur for the first time in second or subsequent pregnancies than those caused by anti-HPA-1a and that as many as 60% of the neonates are asymptomatic at birth, [287] although two fatal cases have been reported.[272] [288] Too few cases of NAIT due to antibodies to other antigens [289] [290] [291] [292] [293] have been reported to estimate the risk of severe NAIT. The prevalence of ABO and HLA antibodies as a cause of NAIT remains unsettled, [294] although an association between anti-HLA antibodies and neonatal amegakaryocytic thrombocytopenia has been reported in a few patients. [274] [295] Additional novel alloantibody specificities will likely be identified in the future. These advances place additional demands on referral laboratories to acquire and maintain the appropriate roster of typed controls, reference sera, and DNA-based techniques for haplotyping. Serologic diagnosis of NAIT requires demonstrating parental platelet incompatibility and antiplatelet antibody in maternal serum of matching specificity.

Additional genetic factors modify the risk of NAIT in HPA-1b homozygous mothers. Neonatal thrombocytopenia occurs in only 16% of women who are homozygous for the HPA-1b allele and who carry an HPA-1a fetus.[272] [273] Production of anti-HPA-1a antibodies occurs predominantly in women who carry the HLA haplotype B8-DR3[296] [297] [298] and the supertypic determinant DRw52a, [299] [300] [301] in whom the incidence of NAIT exceeds 25%,[273] a relative risk 10- to 100-fold above HPA-1b/1b women with other HLA haplotypes. Genetic restriction in the development of anti-HPA-5a antibodies may exist as well. [302] These genetic linkages may have important implications in the management of relatives of affected women. Because fetal blood sampling may induce sensitization, it is our policy to test all HPA-1b/1b, Dw52a-positive women for anti-HPA-1a antibodies monthly and at 3638 weeks in cases when the childs father is HPA-1a positive. PUBS is then performed immediately if antibodies are detected.

Management of the Affected Child

The index case can be devastating and typically occurs without warning. Because ICH develops in 10% of affected neonates during the peripartum period and may extend in those with antenatal hemorrhage, immediate diagnosis and treatment are mandatory. The maternal platelet count should be documented as normal, although NAIT can occur coincidentally with ITP or other causes of thrombocytopenia. [303] [304] A neonate born with a platelet count <20,000/l should be presumed to have NAIT even if there is evidence of bacterial sepsis, disseminated intravascular coagulation, or congenital viral (herpes, CMV, varicella, rubella) or toxoplasma infections. NAIT should also be considered in any neonate with ICH, if there is a positive family history, or if thrombocytopenia is unexplained.

The urgency of treatment should be governed by the likelihood of ICH. If the ultrasound is normal and there is no clinical evidence of ICH, IVIG should be started, [272] [305] [306] [307] although responses can take 23 days. Corticosteroids provide marginal, if any, benefit and should not be used as the sole modality. [272] [288] [308] If ICH has occurred, then random donor platelets should be given for immediate benefit along with IVIG and corticosteroids while antigen negative, usually maternal, platelets are procured.[309] Although theoretically platelets should be washed to remove circulating alloantibody and resuspended in AB-positive plasma, in actual practice, maternal platelets can be administered safely after centrifugation. However, the platelets should be irradiated to lessen the risk of graft-versus-host disease. [310] A rise in the platelet count should be verified. Random donor platelets have been effective in cases shown subsequently to be due to anti-HPA-5b (Br^a), since approximately 80% of the population lack this antigen. Serologic studies should be performed on the parents to confirm the diagnosis because of the implications for subsequent pregnancies and the need for genetic counseling of other family members. Maternal antibodies that react with paternal platelets are generally demonstrable, although on occasion they may not be detected initially, presumably because of adsorption to fetal platelets. In up to 15% of typical cases, they may not be detectable at all.[272]

Management of Subsequent Pregnancies

The introduction of PUBS has permitted children at risk for severe thrombocytopenia and hemorrhage to be identified. An invasive approach is warranted, especially in cases of HPA-1a incompatibility[311] for several reasons: (1) severe thrombocytopenia occurs in >99% of subsequent pregnancies; (2) the majority of fetuses will be more severely affected than their previously affected sibling, and >50% will have initial platelet counts <20,000/l; (3) severe fetal thrombocytopenia occurs early, with 42% of patients having platelet counts <20,000/l by 24 weeks; (4) neither the presence nor the absence of alloantibody in the mother nor a change in antibody titer clearly predicts the neonatal platelet count; [273] [288] (5) up to one-half of the cases of ICH occur antenatally and, therefore, would not be prevented by elective cesarean section at term; (6) when treatment is withheld, fetal platelet counts commonly fall rapidly or remain very low during gestation; [312] and (7) effective antenatal treatment

is available.[311] [312]

Neonates at risk for NAIT during subsequent pregnancies should be identified. PUBS is probably warranted in all HPA-1a-negative women if the childs father is homozygous HPA-1a positive. Fetal genotypes can now be determined at 1018 weeks gestation using cells obtained by chorionic villus sampling or amniocentesis [313] [314] if the father is unknown, is unavailable for testing, or is heterozygous for the antigen in question. The fetal platelet count should be determined by PUBS as early as 2024 weeks gestation in women with a history of NAIT and an antigen-incompatible fetus. Maternal or antigen-negative platelets can be transfused to lessen the risk of hemorrhage from the procedure itself. The optimal approach for an HPA-1a-negative relative is less clear. Because fetal blood sampling can cause sensitization, it is our policy to obtain monthly antibody testing in women without a previously affected sibling and to restrict PUBS to those women with positive tests.

Two approaches to antenatal management of thrombocytopenic fetuses have been studied. The more aggressive approach, pursued primarily in several European centers, involves weekly transfusion of washed, irradiated, maternal antigen-matched

platelets in utero,[312] [315] starting as early as 2630 weeks gestation. [312] [316] This approach requires matched donors in addition to the mother because of transfusion frequency, although frozen maternal platelets have been used successfully. Each procedure also carries a risk of fetal exsanguination and side effects from transfusion[317] and is labor-intensive and costly. In the United States, it has become the practice to administer IVIG, 1g/kg/week, to the mother once an HPA-1a-positive fetus has been identified and thrombocytopenia has been documented. PUBS is repeated 46 weeks later to document response. [311] [318] In utero transfusion of maternal platelets is given during the procedure to guard against exsanguination of the thrombocytopenic fetus. [270] [312] [319] [320] Prednisone (1 mg/kg) is given in addition if a response is not seen. Therapy is begun at 12 weeks if there has been ICH during a prior pregnancy. IVIG has also been administered directly to the fetus in utero with limited success.[321] [322] In the authors experience, ICH has not been detected in any of the 73 fetuses managed in this way compared with an approximate 20% incidence during the index pregnancies.

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POSTTRANSFUSION PURPURA

Introduction

Posttransfusion purpura (PTP) is an uncommon, acquired thrombocytopenia that develops approximately 1 week after blood transfusion. Plasmas from affected individuals contain alloantibodies to antigens expressed on intact platelets or on platelet membranes in the transfused blood. Through a mechanism yet to be defined, alloantibody production is accompanied by destruction of the recipients platelets. The temporal association of thrombocytopenia with blood transfusion was first described by Zucker et al.[323] and by van Loghmen et al. [324] A relationship between the development of platelet-specific isoantibodies and the consumption of host platelets was first suggested by Shulman et al.[325] The clinical course of untreated PTP is typically severe and protracted and may end in fatal hemorrhage. Therapy is rapidly effective in almost all cases, making an early, accurate diagnosis of this disorder imperative.

Clinical Presentation

The prototypic clinical presentation of PTP is now well-defined. The typical patient is a multiparous middle-aged female who presents with the acute onset of bleeding resulting from severe thrombocytopenia 1 week after the administration of a blood product containing platelet material. This may include fresh or stored whole blood, packed or washed red blood cells, or fresh or frozen plasma. In a recent retrospective analysis of 104 affected individuals, 99 were women with a mean age of 58.4 years.[326] Thrombocytopenia was first noted a mean of 68 days (range 114 days) after transfusion and platelet counts were found to be <10,000/l in over 80% of cases at presentation.[326] Approximately one-third of the patients reported having had chills or fever after the transfusion. In the absence of prior pregnancies, almost all of the remaining patients reported have had a history of previous exposure to blood through prior transfusions, [276] [327] suggesting that PTP occurs as part of an anamnestic immune response. PTP is therefore exceptionally rare in males or in nulliparous females who have no history of prior transfusion. [326] [327] [328] In these patients, the onset may be delayed (24 days), consistent with a primary immune response.[329] Subclinical cases have been reported,[330] [331] but the actual incidence of asymptomatic, milder forms of PTP is unknown. PTP after bone marrow transplantation has recently been described.[332]

On rare occasions, thrombocytopenia has developed within hours after transfusion of plasma, whole blood, or packed red blood cells. [333] [334] [335] In these cases, isoantibodies reactive with recipient platelets have been identified in the transfused blood product. In each case, the donor was a multiparous female, homozygous for an allele not found on the recipients platelets.

Pathogenesis

The pathogenesis of PTP remains unclear. The enigma is why the recipients platelets are destroyed by alloantibodies directed at a determinant they seemingly do not express. Any proposed explanation for this disorder must also account for the low incidence in susceptible individuals exposed to blood products known to contain the provoking antigen, the absence to date of the PTP syndrome in women whose pregnancies are complicated by NAIT due to alloantibodies with same apparent specificity, the prolonged duration of the thrombocytopenia, the reported lack of response to transfusion of antigen-negative platelets during the acute phase, [336] and the fact that the syndrome may or may not recur after inadvertent or deliberate re-exposure to antigen-mismatched blood products.[276] [330] [337]

It is known that serum from over 90% of patients with PTP contains antibodies to the platelet antigen Pl^{A1} (HPA-1a), which is expressed on the platelets of 9798% of the Caucasian population. The remaining cases have been associated with antibodies to the Pl^{A2} (HPA-1b) allele[326] [338] and other epitopes on platelet glycoproteins IIb-IIIa,[326] [338] [339] [340] [341] [342] or, less commonly, those found on another platelet surface protein.[343] Although in a few patients only antibodies directed to HLA determinants were detected,[326] [344] it is uncertain whether these were the actual cause of the PTP syndrome.[345] Despite the ease with which these isoantibodies are demonstrated in essentially every patient with PTP, their role in the destruction of autologous platelets is unproven. Indeed, in at least one instance, reinfusion of plasma obtained during the period of severe thrombocytopenia did not reproduce the syndrome.[325] Moreover, although alloantibodies with the same apparent specificity circulate in many women with NAIT, PTP has never developed concomitantly.

Another unanswered question is why PTP is a rare disorder. Based on the frequency of HPA-1b homozygous individuals, anti-HPA-1a antibodies would be expected to develop after 13% of random donor transfusions. One factor that may contribute to the lower than expected frequency is the apparent genetic restriction on the production of anti-HPA-1a antibodies. Development of PTP and NAIT is closely linked to the HLA B8 and DRw52 loci.[273] [296] [300] [346] Yet, only a single instance of sisters with PTP[347] and PTP occurring in a woman with a history of NAIT have been reported.[348] Therefore, additional factors, including the specifics of the anamnestic response (see discussion later in this chapter) may be required to develop severe thrombocytopenia and overt bleeding.

An increased amount of IgG and/or IgM has been detected on platelets of affected individuals during the thrombocytopenic episode. However, the composition and the antigen(s) recognized by the eluted immunoglobulin has not been identified. Three theories have been advanced for which at least some supportive experimental data has been obtained.

The initial hypothesis, proposed by Shulman and coworkers, was that donor platelets are lysed by a complement-dependent interaction between the alloantibody (e.g., anti-HPA-1a) and the transfused platelets, which releases antigen-antibody complexes into the circulation.[325] These complexes may then bind to HPA-1a-negative host platelets, possibly via Fc receptors, thereby perpetuating the destructive process. Such destruction of platelets in an antigen-independent manner by immune complexes might explain why transfusion with HPA-1a-negative platelets may be ineffective in acute cases. This hypothesis derived support initially from the failure to detect platelet autoantibody in plasma obtained when, or soon after, thrombocytopenia developed. More recently, immunoglobulin with anti-HPA-1a reactivity has been found in platelet eluates from affected patients for periods as long as 100 days after the acute

episode.[349] [350] In addition, reactivity of eluted immunoglobulin with autologous and other HPA-1-negative platelets has been noted, [349] [351] as would be expected if complexes were present and played a role in vivo. However, there has been no direct evidence to date that HPA-1a-anti-HPA-1a complexes are present in PTP sera or that these immune complexes can volley for several weeks between rapidly destroyed platelets and their replacements without themselves being cleared from the circulation. Nor has it been shown directly that sera or platelet eluates from affected individuals contain anti-HPA-1a complexed with specific antigen, and alternate explanations for the serologic activity of the eluates have been proposed (see discussion later in this chapter).

A second, interrelated hypothesis is that the HPA-1a antigen, released into plasma from lysed donor platelets, binds specifically and with high avidity to antigen-negative platelets. This interaction may permit HPA-1a containing immune complexes to bind to platelets initially through a site on the antigen itself, and this binding may then be stabilized through secondary interactions with platelet Fc receptors, or the immune complexes may form in situ. Only during an anamnestic

response would sufficient alloantibody and donor platelet antigens be found coincidentally in the host to initiate the syndrome. The interaction of HPA-1a antigen with the platelet membrane may also explain why such complexes are not more rapidly cleared by tissue macrophages. In support of this hypothesis, soluble HPA-1a antigen has been detected in plasma, and antigen negative platelets incubated with this material acquire the capacity to bind anti-HPA-1a antibody. [352] [353] The biochemical basis of this interaction is unknown and it is possible that the HPA-1a antigen circulates and binds to platelets as microparticles. [354] [355] Theoretically, recycling of the antigen and/or antigen-antibody complexes from destroyed to newly synthesized platelets would prolong antigen expression on the host platelets for longer than might otherwise be expected. It has been deduced that binding of <5% of the amount of soluble HPA-1a antigen estimated to be present in a single unit of blood would permit sufficient alloantibody to bind and destroy all of the circulating platelets in vivo. [356] [357] Sufficient circulating antigen-antibody complexes would remain to perpetuate this level of sensitization of the markedly reduced number of residual platelets after the first round of destruction. [356] Although measurable amounts of HPA-1a antigen have been detected in PTP plasma during the acute phase of the syndrome, [356] [357] it remains to be proven that HPA-1a antigen is present on host platelets complexed with anti-HPA-1a antibody in vivo during the period of thrombocytopenia.

An equally compelling case can be made for a third possibility, that a subset of anti-HPA-1a antibodies cross-react with undefined determinants on HPA-1b platelets.[358] Although the HPA-1a and 1b antigens differ at a single amino acid, the epitope recognized by naturally occurring anti-HPA-1a antibodies is not composed of a linear amino acid sequence. Rather, the amino acid substitution induces a conformational change within the glycoprotein IIb-IIIa complex that generates a distinct neoepitope.[359] [360] In some patients, a variable proportion of anti-HPA-1a antibodies formed against this neoantigen may cross-react with the backbone structure of glycoprotein IIIa. The alternative is that autoantibodies directed at entirely different epitopes may be generated coincidentally. There are several pieces of evidence in support of this possibility. First, in certain experimental models, animals immunized with allogeneic or xenogenic platelet or erythrocyte antigens produce autoreactive antibodies, and occasionally develop immune destruction of the sensitized host cells. [361] [362] [363] In the best-studied example, a substantial proportion of marmosets sensitized to platelets from another species developed acute, persistent (30100 days) immune thrombocytopenia several weeks after immunization. Platelet eluates from the affected animals contained both autoreactive as well as anti-donor-specific antibodies. [364] Second, autoreactive anti-erythrocyte antibodies have been detected after transfusion [362] [363] [365] [366] or experimental sensitization to heterologous or alloantigens, [361] in some cases accompanied by overt hemolysis.[363] [365] [366] Such antibodies may persist beyond the expected life span of the transfused red cells. [363] [367] Finally, serum and platelet eluates from some individuals with PTP have been found to contain immunoglobulin reactive with autologous platelets and platelets presumably totally lacking in glycoprotein IIb-IIIa.[368] A similar finding has been reported in HPA-1a-negative women sensitized during pregnancy. [351]

The hypothesis that only a subset of alloantibodies cross-react with autologous platelets is consistent with: (1) the infrequent occurrence of overt PTP in HPA-1-negative patients exposed or re-exposed to blood products containing the antigen; (2) the absence of the PTP syndrome in HPA-1a-negative mothers with NAIT; (3) the prolonged course of thrombocytopenia in the absence of treatment; and (4) the presence of anti-HPA-1a reactivity in eluates from autologous platelets and those congenitally lacking platelet glycoprotein IIIa. However, none of these clinical and serologic observations exclude the role of HPA-1a-antibody complexes. It is also possible that the alloantibodies recognize an epitope that is only generated in vivo. To date, the inability to obtain sufficient $F(ab)_2$ fragments of immunoglobulin from in vivo eluates in the presence of profound thrombocytopenia has precluded definitive analysis of the pathologic antibody.

Diagnosis

PTP should be suspected in any patient who develops severe thrombocytopenia approximately 1 week after transfusion. Suspicion is heightened if the patients plasma is capable of lysing platelets from random normal donors, because few other conditions, other than quinidine purpura or the presence of multiple HLA antibodies, are associated with lytic antibodies. Treatment must be initiated immediately based on clinical criteria, because it generally takes several days until the results of confirmatory tests become available.

The presence of alloantibodies to platelet proteins can be demonstrated by Western blotting or an antigen-capture assay. The diagnosis is generally confirmed by demonstrating that serum from the affected patient contains antibodies to a specific alloantigen, this is, binding of antibody to platelets containing, but not to those lacking, a specific antigen. This is generally accomplished by an antiglobulin test such as immunofluorescence assay or ELISA, or by demonstrating lysis of stored, frozen platelets obtained from known antigen positive donors. Upon recovery, the patients platelets can be phenotyped using reference sera that contain antibodies of known specificity. Typically, platelets obtained from the patient upon recovery will not aggregate or lyse when incubated with plasma obtained during the acute phase. This inability to reproduce the syndrome in vitro, the sine qua non of the PTP syndrome, is the basis of the uncertainty as to the pathogenesis of this syndrome discussed in the previous section.

Treatment

The clinical course of untreated or nonresponsive patients is typically prolonged. In the series cited earlier, bleeding lasted a mean of 10.2 days. [326] It took a mean of 14.6 days until the platelet count exceeded 50,000/l (range 390), and 19.5 days until it exceeded 100,000/l (3130). Thrombocytopenia has been reported to last as long as 60120 days.[330] [369] [370] Fatality rates as high as 10% were reported before effective therapy was identified. [276] [336] [371] [372] [373] Therefore, immediate diagnosis and intervention must be made once the disorder is suspected clinically.

The treatment of choice is intravenous immunoglobulin

(IVIG) 1 g/kg/day for 2 days, although a second course may be required in some patients.[374] Over 90% of the reported patients have responded, generally beginning within 23 days of treatment.[326] [370] [374] Therefore, IVIG has replaced plasmapheresis [375] and exchange transfusion[325] [376] as the initial treatment of choice, both because of its somewhat higher response rate and its ease of administration. Although occasional patients respond to corticosteroids given as a single agent, [377] [378] [379] [380] most do not,[326] [371] and steroids should be considered to have an adjunctive role, if any, in treatment. A single response to splenectomy in a refractory patient has been reported.[381] Recurrent thrombocytopenia may occur within days of stopping treatment; however, these relapses are generally milder and more easily managed than the initial episode. [357] [370] [374]

The role of prophylactic platelet transfusions in patients with profound thrombocytopenia but non-life-threatening bleeding is unclear. Sera from affected patients commonly contains antibodies to multiple HLA determinants, making provision of antigen matched platelets problematic. Administration of random donor platelets, which is commonly associated with moderate to severe transfusion reactions including bronchospasm and hypotension, rarely succeeds in raising the platelet count, and theoretically may perpetuate the disease. [323] [324] [325] [326] [382] Even when antigen-matched platelets can be procured in time, they may[276] [336] [383] or may not[384] [385] suffer the same fate as the patients own antigen-negative cells.

Anti-HPA-1a antibodies may persist for over a year after the initial episode. [386] It has been theorized by some that persistence of high antibody titers may protect against recurrence by immediately destroying potentially sensitizing platelets, thereby precluding coincidence of circulating antigen at the time of an anamnestic response.[276] Exposure [350] or re-exposure[325] [357] to blood products containing the relevant antigen does not invariably provoke PTP, but recurrences have been reported.[330] [337] [357] [387] Therefore, most experts recommend limiting the exposure of affected individuals by transfusing only antigen-matched (including autologous) blood products on subsequent occasions.[326] Whether current techniques of washing and filtering erythrocytes or the use of frozen, thawed, and washed red cells remove sufficient platelet antigen to prevent recurrence is unknown. [387] [388] It is not known whether the risk of PTP developing in unaffected multiparous or previously transfused siblings that share HLA haplotypes is sufficient to recommend similar restrictions on the source of transfused blood products. The risk of PTP developing in women whose pregnancies were complicated by NAIT appears sufficiently low that only awareness and close monitoring of platelet counts after transfusion appears warranted.

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Chapter 127 - Thrombocytopenia Due to Decreased Platelet Production

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An estimated 15×10^6 megakaryocytes/kg body weight, averaging 12×10^3 fl in volume, produce approximately 1,0001,500 platelets each. [1] When measured as platelet turnover (calculated by dividing the platelet count by the platelet survival time and correcting for splenic pooling), most of the megakaryocyte cytoplasm is delivered to the circulation as platelets (effective platelet production). [2] In disease states, decreased platelet production is due either to a decrease in the megakaryocyte mass (virtually always a decrease in the number of megakaryocytes) or to a failure of delivery of an appropriate number of viable platelets by an adequate megakaryocyte cytoplasmic mass, a process termed *ineffective platelet production*[2] (Fig. 127-1). Table 127-1 shows typical kinetic characteristics for some of these disorders.

A decrease in numbers of megakaryocytes may be due to direct toxicity or damage to their immediate progenitors, the megakaryocyte colony-forming unit (CFU-Mk) or their precursors, the megakaryocyte burst-forming unit (BFU-Mk; see Chap. 17). Damage to more primitive stem cells (or to the regulatory mechanisms promoting their growth) would give rise to multilineage cytopenias (see Chap. 19).

Abnormalities of growth factors or growth factor pathways that promote commitment, proliferation, or differentiation of BFU-Mk, CFU-Mk, or their progeny may also be involved in the pathogenesis of platelet production abnormalities. The

Figure 127-1 Ineffective platelet production. In thrombocytopenia, the relationship between marrow megakaryocyte cytoplasmic mass and the turnover of platelet mass in the peripheral blood is usually direct. Platelet mass turnover represents the product of the mean megakaryocyte cytoplasmic volume multiplied by the total number of marrow megakaryocytes. The results in normal subjects are indicated by the arrow, and the stippled area represents 95% confidence limits in thrombocytopenic patients with effective production. Ineffective thrombocytopoiesis is identified as a disparity between available marrow substrate (megakaryocyte cytoplasmic mass) and delivery of platelet mass to the peripheral blood (platelet mass turnover). Results in patients with autosomal dominant thrombocytopenia (open circles), Wiskott-Aldrich syndrome (open triangles), megaloblastic anemia (open squares), and preleukemia (closed triangles) are characterized by ineffective platelet production. *(From Thompson A, Harker L: Quantitative platelet disorders. In Manual of Hemostasis and Thrombosis. FA Davis, Philadelphia, 1983, p. 65, with permission.)*

availability of assays for thrombopoietin (TPO) and other cytokines that may be involved in platelet production permits a better assessment of humoral involvement in production thrombocytopenia.[3] [4] For example, recent studies have suggested that the thrombocytopenia often accompanying cirrhosis may in part be due to TPO deficiency, although the data remain equivocal. [5] [6] [7]

Classically, the diagnosis of thrombocytopenia due to decreased platelet production depends on a bone marrow examination to assess the quantity and quality of the megakaryocytes. Noninvasive methods to assess platelet production have been described, although they are not in common use. The serum concentration of the glycocalicin fragment of glycoprotein (GP) Ib, released into the plasma at thrombocytopoiesis, has been shown to be significantly below the normal range in patients with production thrombocytopenia.[8] [9] Another method is theoretically based on the content of residual RNA in platelets that have recently entered into the circulation (analogous to the reticulocyte count). This may be detectable by flow cytometry using fluorescent dyes, such as thiazole orange, that bind to the RNA. Thus, fewer platelets would be expected to be positive for RNA if platelet production were decreased. [10] [11] Another approach to measure platelet production indirectly is measurement of TPO levels. TPO would be expected to be increased in patients with a platelet production deficit, and several studies have shown this to be the case.[9] [12] [13] [14] [15] Concurrent measurements of TPO concentration together with glycocalicin or thiazole orange binding may prove to be of value. [9] However, significant technical constraints remain in the reliable quantitation of thiazole orange binding to platelet RNA, and the physiology and normal range of blood TPO levels in various disease states remain to be elaborated.

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ACQUIRED PRODUCTION DISORDERS

Platelet production abnormalities may be divided arbitrarily into acquired and hereditary disorders.

Marrow Infiltration

Production disorders are commonly observed when the marrow is involved with metastatic cancer, lymphoma, or leukemia. Table 127-2 categorizes the infiltrative processes associated with thrombocytopenia. Although physical replacement of marrow is the etiology of the thrombocytopenia in many cases, it is also possible that inhibitory factors produced by the infiltrating cells are toxic to the cells of the megakaryocytic lineage or interfere with normal regulatory mechanisms. The diagnosis of infiltrative disease is made by marrow examination, although diagnostic clues are usually provided by history, physical examination, and a leukoerythroblastic blood smear. The marrow shows decreased megakaryocytes, which may be larger than normal because of a compensatory physiologic response to the thrombocytopenia. The treatment approach is specific to the infiltrative process.

Chemotherapy and Irradiation

Direct destruction of megakaryocytes, their progenitors, or both can be observed after administration of chemotherapeutic

TABLE 127-1 -- Typical Kinetic Profiles in Patients with Thrombocytopenia Due to Production Abnormalities

Category	Megakaryocytes			Platelets			
	Cell No. ($\times 10^6$ /kg)	Cytoplasmic Volume (fl)	Cytoplasmic Mass ($\times$ 1011 fl/kg)	Concentration (platelets/mm^6)	Volume (fl)	Survival (days)	Mass Turnover ($\times 10^5$ fl/mm^3 /day)
Normal	15 ± 4	12,000 ± 1,700	1.8 ± 0.4	250,000 ± 40,000	8.7 ± 0.8	9.6 ± 0.6	3.2 ± 0.5
Decreased megakaryocytopoiesisdamaged marrow	r2	14,000	0.3	22,000	9.1	5.2	0.7
Ineffective production							
Autosomal dominant	49	9,400	4.6	64,000	8.9	8.4	1.1
Wiskott-Aldrich syndrome	20	9,000	1.8	40,000	4.0	5.0	0.4
Vitamin B_{12} deficiency	51	8,900	4.5	62,000	8.5	8.4	0.8
Preleukemia	18	13,300	2.4	16,000	9.0	6.7	0.4

Adapted from Thompson A, Harker L: Quantitative platelet disorders. In Manual of Hemostasis and Thrombosis. FA Davis, Philadelphia, 1983, p. 65, with permission.

agents or irradiation. Thrombocytopenia is one of the most frequent adverse effects of total body irradiation. [16] After either allogeneic or autologous marrow transplantation, thrombocytopenia may persist despite resolution of leukopenia and anemia. The etiology of prolonged thrombocytopenia after transplantation is unclear; it may be multifactorial (e.g., graft-versus-host disease, infections), but decreased production is likely important in some cases. [17] Transplantation with peripheral blood stem cells may improve the rate of platelet recovery compared with marrow transplantation, but thrombocytopenia remains a problem. [18] Transplantation of umbilical cord stem cells is associated with delayed platelet recovery; in 39 pediatric patients receiving such transplants, the median time to recovery was 49 days (range, 15117 days).[19]

Isolated thrombocytopenia with decreased megakaryocytes has been reported after chemotherapy for acute myeloid leukemia. Cyclosporine may augment the platelet count in such patients.[20]

Agents that are more likely to induce leukopenia usually induce thrombocytopenia as well. Alkylating agents in general produce more prolonged thrombocytopenia than do antimetabolites. Although it has been claimed that some alkylating agents are sparing of the megakaryocytes (e.g., cyclophosphamide), this is a relative phenomenon; conceivably, agents such as busulfan, the nitrosoureas, or platinum cause cumulative damage in the more primitive progenitors. Other chemotherapeutic agents, such as the vinca alkaloids, may not decrease the platelet count significantly.

Reducing the intensity of the chemotherapy dose remains the most appropriate approach to management, although in the near future hematopoietic growth factors that enhance thrombocytopoiesis may prove to be of value in these patients. A TPO derivative, pegylated megakaryocyte growth and development factor (MGDF), has been administered to 38 patients with lung cancer who received the agent after treatment with carboplatin

TABLE 127-2 -- Infiltrative Marrow Disorders Associated with Thrombocytopenia

Metastatic cancer
Leukemia
Lymphoma
Myeloma
Myelofibrosis
Gauchers disease
Osteopetrosis

and paclitaxel in a randomized, double-blinded trial. In the treated patients, the nadir platelet count was 188,000/mm ^{3} , whereas it was 111,000/mm^3 in 12 control patients receiving placebo. The platelet count returned to baseline levels in the treated patients in 14 days, whereas the control patients required 21 days. [21] Another study using the same TPO derivative was performed in 41 patients with a variety of advanced cancers treated with carboplatin and cyclophosphamide. In this study, patients also received granulocyte colony-stimulating factor. The platelet nadir occurred earlier in patients given MGDF, although there was no difference in the depth of the nadir. Recovery to the baseline platelet count was observed by day 17 in the treated patients, compared with day 22 in the placebo group. Moreover, patients who had previously received MGDF recovered their platelet counts more rapidly. [22]

Other cytokines, including interleukin-1 (IL-1), [23] IL-3,[24] IL-6,[25] IL-11,[26] and IL-3/granulocyte/macrophage colony-stimulating factor (GM-CSF) fusion protein [27] have been shown to enhance the platelet count in patients receiving chemotherapy. It is possible that these broadly acting cytokines may prove to be of value in augmenting hemostasis and reconstituting other cell lineages. Nonetheless, it has not yet been shown that the capacity of TPO and other cytokines to augment the platelet count after chemotherapy will diminish bleeding in patients with marked thrombocytopenia.

It is possible that drugs other than growth factors may ameliorate chemotherapy-induced thrombocytopenia. Studies with amifostine, a precursor of a thiol compound, suggest a reduction in thrombocytopenia in some patients. [28] Additional clinical trials are required to investigate the general utility of this approach, whose mechanism of action is unclear. Beyond attempts to raise the platelet count with cytokines or other drugs, amelioration of the bleeding tendency may be achievable by augmentation of other aspects of the hemostatic system. epsilon-Aminocaproic acid may be of benefit in some patients with refractory thrombocytopenia and bleeding.[29]

Ethanol-Related Disorders

Thrombocytopenia is the most common hematologic abnormality in severe alcoholism [30] (see Chap. 148). In one analysis, alcohol was a contributing factor in 26% of patients admitted to hospitals with a platelet count of <100,000/mm ^{3} . Alcohol abuse may result in thrombocytopenia due to several mechanisms, including ineffective production related to folate deficiency (which may lead to severe thrombocytopenia) and increased splenic pooling due to portal hypertension (which usually results in a modest decline in platelets). Ingestion of alcohol inhibits the expected response to thrombocytopenia induced by plateletpheresis. [31] A mild shortening of platelet survival has

been reported.[32] However, ethanol itself can be directly toxic to the marrow. [33] [34] In vitro studies have shown that alcohol concentrations achievable in vivo inhibit megakaryocyte maturation but do not inhibit megakaryocyte colony formation. [34] [35] Megakaryocyte numbers usually are normal, but markedly decreased megakaryocytes have been observed. In one such case, labeling with platelet-specific antibodies demonstrated that numerous, small, unidentifiable cells were immature megakaryocytes.[35] Rarely, marrow panhypoplasia has been observed in association with alcohol ingestion. [36] Anemia and macrocytosis accompanied by megaloblastic changes and ringed sideroblasts in the erythroid marrow are commonly noted in association with ethanol abuse. However, the severity of the anemia shows no correlation with the thrombocytopenia.[30] Treatment consists of withdrawal of ethanol and administration of a normal diet. Recovery of the platelet count, often with a rebound thrombocytosis, usually occurs within 2 weeks.

Viruses

Mild thrombocytopenia is frequently associated with viral infection. [37] Although the pathophysiologic mechanisms have not been systematically sought, a production deficit is probably important in the etiology of many of the cases. Megakaryocytes are capable of harboring a variety of viruses, although viral entry into megakaryocytes is not well understood. Infected marrow may exhibit dysmorphic megakaryocytes containing inclusion bodies, vacuoles, or degenerating nuclei, or show naked nuclei. Thrombocytopenia has been reported in mumps, rubella, measles, varicella, cytomegalovirus, infectious mononucleosis, chickenpox, dengue and other hemorrhagic fevers, hepatitis, and parvovirus infections. [38] [39] [40] [41] Live measles virus vaccination can also induce thrombocytopenia due to decreased production.[42]

Human Immunodeficiency Virus (HIV)

Thrombocytopenia after human immunodeficiency virus (HIV) infection is a common complication (see Chap. 155). Almost one half of adults and one fourth of children with hemophilia exhibit a decreased platelet count within 10 years after seroconversion. [43] The thrombocytopenia may be due both to immune mechanisms and to a decrease in platelet production (see Chap. 129). Production thrombocytopenia may be related directly to HIV infection, adverse effects of drug therapy, or to secondary malignancy or myelodysplasia. Platelet kinetic studies have shown that patients infected with HIV have a moderate reduction in platelet survival, but all have decreased platelet production regardless of the degree of thrombocytopenia. [44] A retrospective study of 85 patients with HIV and thrombocytopenia suggested that in early stages of infection, platelet destruction is predominant, whereas in patients with full-blown acquired immunodeficiency syndrome, thrombocytopenia is more often due to a production deficit. [45] Based on the finding of HIV mRNA and p24 antigen in megakaryocytic cytoplasm, [46] [47] [48] HIV has been shown to infect megakaryocytes directly, perhaps through megakaryocyte surface CD4. [49] [50] Morphologic abnormalities of megakaryocytes, including denuded nuclei, [51] together with growth anomalies of progenitor cells in culture have also been observed in patients with HIV, [52] [53] supporting a direct effect of the virus on megakaryocytopoiesis. Apoptosis of GP IIb/IIIa+ marrow cells has been suggested as an additional mechanism of impaired platelet production that may be independent of either direct megakaryocyte infection or CD4 count. [54] Isolated amegakaryocytic thrombocytopenia has been observed in a child with HIV. [55] Treatment with zidovudine has been shown to increase platelet production in some patients with HIV. [56] [57] The mechanism for this effect is controversial, with one kinetic study suggesting that the drug augments

INVESTIGATION OF DECREASED PLATELET PRODUCTION

Suspicion of thrombocytopenia due to decreased production should be provoked by a history of irradiation or chemotherapy, thrombocytopenia present at birth or in early infancy in the presence of a normal maternal platelet count, a family history of thrombocytopenia, concurrent anemia, leukopenia, or both, and alcohol abuse. A careful physical examination may suggest a production disorder if signs of alcohol-related liver disease or a hematologic malignancy are present. Although the thrombocytopenia due to splenomegaly is not related to a production abnormality, splenomegaly is often observed as a feature of portal hypertension, lymphoma, or other processes associated with marrow damage, and it may be a concurrent pathophysiologic mechanism of the thrombocytopenia.

The peripheral blood smear is critical to rule out pseudothrombocytopenia (see Chap. 126). Moreover, the blood smear provides additional clues to both the pathophysiologic mechanism of the thrombocytopenia and the diagnosis. For example, giant platelets suggest a hereditary or myelodysplastic syndrome; oval macrocytosis and hypersegmented neutrophils suggest folate or vitamin B_{12} deficiency; and a leukoerythroblastic smear points to an infiltrative process.

A marrow examination is required to evaluate the megakaryocytes. A biopsy is more reliable than an aspirate to determine whether megakaryocytes are decreased. However, an aspirate showing abundant megakaryocytes in the presence of thrombocytopenia is sufficient to suggest platelet destruction or ineffective production. Unfortunately, no clear guidelines are available to determine precisely what defines a decreased or increased number of megakaryocytes. Megakaryocyte morphology is occasionally valuable. The normal compensatory response to thrombocytopenia is enlargement of the cells with increased ploidy. Small, microlobulated, or hypolobulated megakaryocytes may be seen in myelodysplastic syndromes. Dysmorphic megakaryocytes may be also observed in viral infections, including HIV. In the future, flow cytometry may provide a more objective analysis of megakaryocytes.

Establishment of the pathologic process of ineffective production is one of exclusion. The marrow examination reveals quantitatively normal megakaryocytes, and the apparent absence of peripheral platelet destruction together with the appropriate clinical circumstances (e.g., folate or vitamin B_{12} deficiency) often points to this mechanism. The bleeding time, although not a useful measurement to predict the propensity for bleeding, may be helpful in distinguishing ineffective production from platelet destruction. In destructive processes such as immune thrombocytopenia, the bleeding time may be shorter than predicted on the basis of the platelet count alone, whereas in ineffective platelet production, the bleeding time is more often related to the platelet count. In complex cases, platelet survival studies may be necessary to show that consumption or splenic pooling are not significant contributors to the thrombocytopenia; however, survival studies are rarely required for clinical purposes. In the future, flow cytometric estimation of platelet production rate and measurement of TPO levels may permit a more facile approach to the differential diagnosis of thrombocytopenia.

platelet production,[44] and another claiming that it decreases production.[45] The specific role of other antiretroviral agents in HIV-related production thrombocytopenia has not been elucidated. Interferon- has been shown to increase the platelet count in zidovudine-resistant HIV-related thrombocytopenia.[58]

Drug-Related Disorders

A variety of drugs and toxins have been implicated in the etiology of aplastic anemia (see Chap. 19); several have been implicated in isolated thrombocytopenia believed to be due to a production deficit. Estrogen was reported to decrease platelet counts;[59] although megakaryocytes possess the estrogen receptor,[60] detailed thrombokinetic studies have not been carried out. Thrombocytopenia due to thiazide diuretics has been reported frequently.[61] Although the etiology in most cases is probably destructive, decreased megakaryocytes have been noted.[62] Chemotherapy may result in prolonged thrombocytopenia that may be isolated.[63] Interferons and IL-2 may induce thrombocytopenia, perhaps the result of inhibited megakaryocytic colony formation.[64] [65] Anagrelide, an agent used to lower the platelet count in patients with malignant thrombocytosis, appears to operate by reducing megakaryocyte size and ploidy, and by disrupting maturation.[66]

Folate and Vitamin B12 Deficiency

Varying degrees of thrombocytopenia may be observed with either folate or vitamin B_{12} deficiency; in some cases it may be severe[67] [68] (see Chap. 28). The pathophysiologic mechanism is ineffective production[1] because megakaryocyte numbers are normal or increased in the marrow, and platelet survival is normal or slightly shortened.[69] Amegakaryocytic thrombocytopenia has been reported in vitamin B_{12} deficiency.[70] Folate deficiency is frequently associated with ethanol abuse; the etiology of the thrombocytopenia may be more complex in these patients.

The blood may show macrocytosis and hypersegmented neutrophils in addition to the thrombocytopenia. Platelets are morphologically normal, but the count is variably decreased. Extremely severe thrombocytopenia may be observed. Often (but not necessarily), the marrow shows megaloblastic changes in the erythroid and myeloid lineages, as well as normal numbers of megakaryocytes, some of which may appear large. Although multiple disconnected nuclear lobulations have been described,[71] distinctive morphologic abnormalities of the megakaryocytes are not often apparent. Rapid recovery of the platelet count can be achieved with administration of the appropriate vitamin or by abstention from ethanol intake.

Iron Deficiency

Although thrombocytosis develops in most patients with iron deficiency, rare patients become thrombocytopenic.[72] In some instances, this is accompanied by decreased megakaryocytes.[73] Recovery of the platelet count has been observed with iron replacement,[73] [74] whereas induction of thrombocytopenia during iron therapy has been reported.[75] Hypotheses have been presented concerning the role of iron in platelet production,[76] but little investigation has been performed in this area.

Acquired Aplastic Anemia

Aplastic anemia involves multiple hematopoietic lineages, although isolated thrombocytopenia can be the presenting feature.[77] Many of the same pathogenetic

mechanisms seem to be operative in aplastic anemia and isolated amegakaryocytic thrombocytopenia (see later). TPO levels have been shown to be elevated in patients with aplastic anemia[12] (aplastic anemia is discussed in Chap. 19).

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal disorder resulting from mutations in the X-linked gene *PIG-A* that encodes for an enzyme required in the initial step of biosynthesis of glycosylphosphatidylinositol anchors (PNH is discussed in Chap. 20). Approximately 25% of patients with PNH have significant marrow aplasia.[78] Thrombocytopenia at diagnosis is a poor prognostic indicator. [79] Because platelet survival is usually normal in PNH, [80] the mechanism of the thrombocytopenia is decreased or ineffective production. Megakaryocyte progenitors show decreased proliferative activity and exhibit increased sensitivity to complement.[81] [82] Treatment with antithymocyte globulin or granulocyte colony-stimulating factor and cyclosporine has ameliorated the thrombocytopenia in some patients,[83] [84] whereas marrow transplantation has resulted in long-term remissions in patients with aplasia associated with PNH. [85]

Acquired Pure Amegakaryocytic Thrombocytopenic Purpura

Acquired pure amegakaryocytic thrombocytopenic purpura is a rare disorder that may be analogous to other isolated unicellular marrow aplasias. The pathognomonic finding is a normal bone marrow biopsy with markedly decreased or absent megakaryocytes. Platelet survival is normal in these patients, and the platelet counts reflect the degree of megakaryocytic hypoplasia. Amegakaryocytic thrombocytopenia may be a harbinger of aplastic anemia [86] or a presenting finding of lymphoma.[87] The pathogenetic mechanisms underlying this disorder are diverse and may be related to drugs or toxins, viruses, cytokine anomalies, and humoral or cell-mediated megakaryocyte suppression.[88] [89] [90] The Philadelphia chromosome has been described in a patient with this disorder in whom marrow aplasia subsequently developed.[91] TPO levels in pure amegakaryocytic thrombocytopenia have been reported as high. [92]

Patients present with bleeding manifestations due to severe thrombocytopenia. Macrocytosis is seen in almost all patients, presumably due to reticulocytosis, related to acute blood loss anemia, but other abnormalities of the peripheral blood are not typically seen. Except for the absence of megakaryocytes, the marrow shows normal cellularity. A decrease in megakaryocytic colony growth may be noted; it may be corrected by removal of an inhibitory cell population or antibody. [89] [90]

Because the causes of this disorder are multiple, the natural history and therapeutic approach depend on the specific etiology. Determination of the pathophysiologic mechanisms with megakaryocyte progenitor cell assays may be useful in guiding treatment because humoral or cell-mediated abnormalities may be discerned with these methods.[89] [93] In the absence of these uncommonly available laboratory studies, management is approached on an empiric basis.

If the etiology is viral, intravenous IgG or anti-HIV therapies are indicated. [88] In the rare patient with isolated amegakaryocytic thrombocytopenia due to ethanol or drugs, avoidance of the offending agent is indicated. Cytotoxic antibodies directed toward the CFU-Mk may be approached with corticosteroids, plasmapheresis, intravenous IgG, danazol, cyclosporine, or cyclophosphamide. [88] In a patient in whom an IgG antibody was found to be blocking GM-CSF action, a complete response to cyclophosphamide was observed.[93] Patients with T-cell mediated inhibition of megakaryocytopoiesis may respond to antithymocyte globulin, cyclosporine, or hematopoietic growth factors.[88] Despite the varied etiologies of this disorder, a review

of 30 patients found that sustained remissions were achieved in 8 patients with immunosuppressive agents. [94] Nonetheless, most patients do not respond to treatment, and intensive immunosuppressive therapy may not prevent progression to aplastic anemia. [86]

Refractory Thrombocytopenia Due to Myelodysplasia

A small proportion of patients (as low as 0.6%) with the myelodysplastic syndrome present with isolated thrombocytopeniadesignated *refractory thrombocytopenia*.[95] Clonal chromosomal abnormalities are required to confirm the diagnosis, with chromosomes 3, 5, 8, or 20 most commonly involved, but partial deletions of other chromosomes have been reported.[96] The usual laboratory findings include macrocytosis of platelets and red cells. Abnormal megakaryocyte morphology, typically mononuclear megakaryocytes, is often observed,[95] sometimes in combination with an increased number of megakaryocytes.[97] Dysmorphic erythroblasts or myeloid cells may also be noted. The clinical course of these patients is the progressive development of additional cytopenias and the myelodysplastic syndrome, with a significant number evolving into acute myeloid leukemia. [95] Therapy has not been shown to be beneficial. Some patients with a full-blown myelodysplastic syndrome associated with marked thrombocytopenia and <10% blasts have shown an increase in platelet count after androgen therapy. [98] Because some of these cases have been misdiagnosed and treated for idiopathic thrombocytopenic purpura (ITP), recognition of this uncommon syndrome is important. [97]

Cyclic Thrombocytopenia

Cyclic oscillations in the platelet count have been observed occasionally. Occurring predominantly in women, these fluctuations can result in thrombocytopenia sufficiently severe to result in bleeding. [99] Most frequently, the cycling occurs in association with the menstrual cycle. It is likely that a production abnormality is not the pathophysiologic mechanism for the thrombocytopenia in most patients with this disorder, and that circulating autoantibodies may be important. [100] [101] A study of 10 patients with cyclic thrombocytopenia suggested varied etiologies of the thrombocytopenia, with cyclic variations in platelet production being responsible for some cases, including the two male patients in that group.[102] The possibility that fluctuating cytokine levels may contribute to the pathogenesis of the disorder has been raised by several studies, although it is difficult to discern cause from effect. [103] [104] [105] Cyclic thrombocytopenia may rarely be a presenting manifestation of myelodysplasia. [106] Treatment has been variable; responses to low-dose contraceptives and intravenous gamma-globulin have been reported. [101] [107]

CONGENITAL OR HEREDITARY PRODUCTION DISORDERS

Thrombocytopenia due to decreased platelet production in the newborn is uncommon, accounting for <5% of neonatal thrombocytopenia. [108] Most of the thrombocytopenic disorders that are referred to as congenital have a genetic basis, although some may be acquired in the perinatal period (e.g., congenital rubella). [109]

Congenital Aplastic Anemia Syndromes

Congenital aplastic anemia, Fanconis anemia, and other syndromes associated with failure of several hematopoietic lineages are discussed in Chapters 18 , 19 , 20 . These syndromes occasionally present with thrombocytopenia, [110] but it soon becomes apparent that other lineages are involved.

Congenital Amegakaryocytic Thrombocytopenia

Congenital amegakaryocytic thrombocytopenia is a rare disorder of infancy and early childhood of unknown etiology. The inheritance pattern is mixed, with some of the cases being X-linked; others are autosomal recessive. [110] Approximately 40% of affected patients have somatic anomalies fitting no other congenital syndrome. [110] Patients present with isolated severe thrombocytopenia with normal marrow cellularity, but scant or no megakaryocytes. Platelet survival is normal. Anemia and macrocytosis are commonly observed; occasionally hemoglobin F is elevated. [111] The disorder may be an intrinsic stem cell defect because the marrow microenvironment appears to be normal. [112] In four of five cases analyzed, CFU-Mkderived colonies were very low in number, but increased in the presence of IL-3, GM-CSF, or both, although not to normal levels. [113] Studies in a single patient suggest that the response of megakaryocytic progenitors to TPO is defective (despite an elevation in serum TPO level), perhaps due to impaired expression of the TPO receptor mpl. [114] These findings suggest that therapeutic use of TPO would not be of benefit, but this remains to be tested. The natural history of the disorder is not well defined, but most patients with isolated thrombocytopenia either die of bleeding complications, progress to aplastic anemia, or acquire leukemia. [110] Projected survival is poor6 years for those who do not have associated physical anomalies and 2 years for those who do. [110]

Treatment with corticosteroids with or without androgens has been useful in some cases, but most patients relapse. [110] Splenectomy is ineffective. Bone marrow transplantation may be useful for these patients if a suitable donor is available. [115] Administration of IL-3, but not GM-CSF, was shown to augment the platelet count and decrease bleeding complications in five patients. [113]

Thrombocytopenia with Absent Radius Syndrome

Thrombocytopenia with absent radius syndrome (TAR) is an autosomal recessive disorder manifest by hypomegakaryocytic thrombocytopenia and absent radii. The general lack of consanguinity for this uncommon syndrome suggests that the gene may be more prevalent than previously suspected, or that other modes of inheritance occur. [116] [117] Chromosomal abnormalities are absent. Bone marrow culture studies of a patient with TAR showed absence of megakaryocyte colony formation accompanied by high levels of megakaryocyte colony-stimulating activity. [118] This activity declined as the patients platelet count increased, accompanied by an increase in megakaryocytic colonies. No significant abnormalities of colony formation or colony-stimulating activity were observed in the patients parents. [118] In five patients with this syndrome, TPO levels were measured and found to be elevated. [119] The TPO receptor mpl was found to be expressed normally on the platelet surface of these patients, and to be of the same molecular weight as in normal control subjects. Although platelet responsiveness to adenosine diphosphate and thrombin receptor agonist peptide was normal, addition of TPO to these agonists did not induce synergy as is normally seen. Moreover, TPO-induced tyrosine phosphorylation of platelet proteins was markedly reduced or absent. These data suggest that the TPO signal transduction pathway is abnormal in the TAR syndrome. [119]

Absence of the radii with thumbs present is pathognomonic of the syndrome (Fig. 127-2), and the diagnosis should not be considered unless these findings are present. [120] The muscles that normally attach to the radius are inserted into the carpal bones. Short stature accompanied by other skeletal malformations, including absence or malformation of the ulnar bones, and abnormalities of the humerus, shoulder joint, and lower extremities, are frequent. [110] Tetralogy of Fallot and atrial septal defects occur in one third of patients. [121] Symptomatic milk allergy has been observed frequently and may cause severe bloody diarrhea. [120] [122]

Figure 127-2 Thrombocytopenia with absent radius syndrome. **(Left)** A typical upper extremity deformity. **(Right)** Radiograph showing complete absence of the radius. *(Adapted from Hoffbrand A, Pettit J: Sandoz Atlas of Clinical Hematology. Gower Medical, London, 1988, with permission.)*

Thrombocytopenia is noted at birth, at which time it is most severe, ranging from 15 to 30,000/mm ^{3} . Intercurrent infection, surgery, gastrointestinal disturbances, and other types of stress are associated with even lower counts, and these are often accompanied by a myeloid leukemoid reaction, noted in two thirds of patients. Eosinophilia, seen in one half of the patients, is common in those with milk allergy. [120] Many patients have anemia related to bleeding and hemolysis, the latter observed in the first year of life. The bone marrow shows decreased or absent megakaryocytes, sometimes accompanied by erythroid hyperplasia. Megakaryocytes are often small, basophilic, and vacuolated. [120]

The diagnosis is suspected by the typical somatic abnormalities. Prenatal diagnosis is possible using ultrasonography, skeletal radiography, and fetal blood sampling. [123] [124] [125] TAR is distinguished from Fanconis syndrome by the presence of the thumbs and by the lack of chromosomal abnormalities. The platelet count and clinical course of these patients tend to improve with age. If patients survive the initial 12 years of life, survival appears to be normal. [120] Thrombocytopenia is best managed by platelet transfusions; orthopedic surgery should be postponed during the first few years of life. Steroids, splenectomy, and intravenous IgG treatments are usually ineffective. [121] [126] Rarely, thrombocytopenia develops when the patient is an adult; in such cases, splenectomy may be effective. [127] [128] Marrow transplantation is an option for the rare patients who continue to remain severely thrombocytopenic with bleeding symptoms throughout childhood. [129]

Other Autosomal Recessive Thrombocytopenias

The Bernard-Soulier syndrome is associated with moderate thrombocytopenia, but it occasionally may be severe. It is not clear whether a production deficit is involved in the etiology of the thrombocytopenia in most patients because platelet survival study results may be shortened or normal, [130] [131] [132] whereas megakaryocyte numbers have been reported to be increased or decreased. [130] [132] [133] Platelet functional defects are likely more important in the pathogenesis of bleeding in this disorder, as in other giant platelet syndromes (see Chap. 130). Nonetheless, it is clear that there are megakaryocytic abnormalities in the

Bernard-Soulier syndrome, including failure to express the GPIb-IX complex.[132] [134] Although in most cases the megakaryocytes are not enlarged despite the marked increase in platelet size, there is abnormal development of the membrane system. Conceivably, deficiency or mutations of the GPIb-IX complex may be important in the formation of platelet territories.[134]

A production deficit may contribute to the thrombocytopenia often noted in the gray platelet syndrome, a disorder in which megakaryocytes are unable to package endogenously synthesized proteins into developing granules [135] (see Chap. 130).

Familial Thrombocytopenia with Micromegakaryocytes

Several members of a family initially believed to have ITP exhibited thrombocytopenia with decreased megakaryocytes. Marrow examination showed a decrease in mean megakaryocyte diameter and the presence of numerous micromegakaryocytes.[136] Another family has been described that also exhibited numerous micromegakaryocytes.[137] Modest thrombocytopenia accompanied by giant granules (15% of all platelets) and a mild hemorrhagic tendency was observed. Platelet survival was normal. Despite a threefold increase in megakaryocytes, platelet production was calculated as 30% of normal. Although platelet function was normal globally, the abnormal population of platelets with giant granules failed to release their contents on stimulation. Culture of megakaryocyte progenitors showed a normal number of colonies, but micromegakaryocytes were always noted. Cell death was observed at the end of the culture period due to lysis, apoptosis, or cell activation. The major distinguishing features between this family (designated Paris-Trousseau thrombocytopenia) and the formerly described family was the presence of a deletion in the long arm of chromosome 23 in the latter (del[11]q23.3qter). That this genetic locus may be important in thrombocytopoiesis is supported by a case of severe thrombocytopenia associated with micromegakaryocytes,[138] and 35 cases of children with a similar chromosomal deletion, 47% of whom also exhibited thrombocytopenia associated with a wide variety of somatic abnormalities.[139]

Autosomal Dominant Thrombocytopenias

May-Hegglin Anomaly

May-Hegglin anomaly is an autosomal dominant disorder characterized by giant platelets, moderate thrombocytopenia (40,00080,000/mm ^{3}), and leukocyte inclusion bodies[140] (Fig. 127-3). Patients may have easy bruising or menorrhagia, but 40% are asymptomatic. No deaths due to bleeding have been

Figure 127-3 Peripheral blood in the May-Hegglin anomaly. Note the giant platelet and the typical basophilic inclusion in the neutrophil. *(Adapted from Hoffbrand A, Pettit J: Sandoz Atlas of Clinical Hematology. Gower Medical, London, 1988, with permission.)*

reported.[141] A variety of overlap syndromes manifest characteristics of Alports syndrome[142] [143] (see later).

The pathognomonic feature is the Döhle-bodylike inclusion bodies found in neutrophils and eosinophils and occasionally in monocytes. These bright blue, spindle-shaped inclusions are composed of parallel 7- to 10-nm filaments.[141] The platelets are large but exhibit no intrinsic structural abnormalities and have normal surface membrane proteins.[144] Platelet volume may exceed that of red cells, with one third at 3080 fl.[144] The mechanism by which the giant platelets are formed in the May-Hegglin anomaly and in other macrothrombocytopenias is unknown, but presumably involves abnormal megakaryocyte fragmentation.[145] Bleeding times are variable.[141] [145] Because marrow megakaryocytes appear normal and platelet survival has been shown to be normal or modestly decreased in most cases,[131] [145] [146] ineffective production is the likely etiology of the thrombocytopenia.

Owing to the modest thrombocytopenia, treatment is not usually required. Platelet transfusions have frequently been given for thrombocytopenia, but their requirement is not clear.[141] [146] [147] Pregnancy and normal vaginal delivery in patients with this disorder can be managed uneventfully.[148] Two patients with symptomatic thrombocytopenia underwent splenectomy without significant response.[149]

Alports Syndrome Variants

The syndrome of hereditary interstitial nephritis, cataracts, and high-frequency sensorineural deafness, designated Alports syndrome,[150] has been associated with thrombocytopenia and giant platelets in a number of families, referred to as Epsteins syndrome.[151] Ultrastructural abnormalities have been described in megakaryocytes.[134] This syndrome has been seen in association with leukocyte inclusions (resembling those in the May-Hegglin anomaly), and designated Fechtners syndrome.[152] [153] The Sebastian platelet syndrome is a variant of Fechtners syndrome without the other defects seen in Alports syndrome.[154] [155] A family with macrothrombocytopenia, late-onset hearing loss, and expression of glycophorin A on half of the giant platelets has been described that appears to be distinct form other variants.[156] The genetic defects in Alports syndrome are deletions or rearrangements in the collagen IV gene located on Xq22,[157] [158] and may be involved in the abnormalities in some Alports syndrome variants. However, whether the platelet and megakaryocyte abnormalities are related to these gene defects remains unknown.

In these autosomal dominant disorders, platelet counts are variable, although thrombocytopenia to levels of 20,000/mm ^{3} may be seen. The mean platelet volume ranges between 20 and 27 fl.[151] [152] [159] Platelet ultrastructure is normal, as is the bleeding time.[151] [159] [160] Platelet aggregation has been claimed to be normal in some reports and abnormal in another.[151] [152] [161] Marrow megakaryocytes are normal, but one report suggests that this may not always be the case.[151] [159] [162]

Cases are most often diagnosed in adult patients. Many were frequently misdiagnosed as having ITP, and failed to respond to treatment with corticosteroids or splenectomy.[151] [159] [162] Bleeding tends to be mild or moderate and is manifested as bleeding after dental extraction, easy bruising, postoperative bleeding and hematoma formation, and postpartum hemorrhage.[163] Fatalities have been observed in patients with Sebastian syndrome.[154] Nevertheless, renal failure, rather than thrombocytopenia, is the usual etiology of the morbidity and mortality in Alports syndrome variants.

Other Autosomal Dominant Thrombocytopenias

Thrombocytopenia without other congenital abnormalities has been reported in a number of kindreds with an autosomal dominant inheritance pattern.[164] [165] Most of these patients have modest thrombocytopenia with few clinical symptoms, normal platelet morphology, and normal numbers of megakaryocytes, but there are exceptions.[1] [164] [166] [167] Some patients exhibit abnormal megakaryocytic lobulations.[168] Families exhibiting macrothrombocytopenia have been described.[167] [168] [169] Platelet function is variable.[164] [165] [168] [169] [170] [171] Platelet survival has been reported to be normal in most families, suggesting ineffective production.[1] [164] However, in one kindred, platelet survival was short; this was demonstrated to be an intrinsic platelet abnormality.[170] In one pedigree with macrothrombocytopenia, an abnormality of GPIV (CD36) was identified.[172] Mediterranean macrothrombocytopenia has been defined as a syndrome of asymptomatic thrombocytopenia and large platelets in people of Mediterranean descent.[173] [174] Their inheritance pattern is not well defined, and little published information is available.[173] [174]

Patients with autosomal dominant thrombocytopenia may be relatively more common than previously believed. Most cases are not diagnosed until adulthood; these patients usually are asymptomatic, although exceptions have been reported.[165] Many may be misdiagnosed as having ITP and treated for that disorder.[166] [171] Family studies of 54 patients referred with the diagnosis of refractory ITP showed that these patients had autosomal dominant thrombocytopenia with large platelets, normal megakaryocytes, and normal platelet survival.[175] Treatment is usually not indicated. Corticosteroids are ineffective, although splenectomy occasionally results in a modest response.[170] [171] Conceivably, the poor responses to standard therapy in some patients with presumed ITP may reflect the possibility that some of those patients have misdiagnosed hereditary thrombocytopenia.

Wiskott-Aldrich Syndrome

The syndrome of immunodeficiency, microthrombocytopenia, and eczema defines the Wiskott-Aldrich syndrome (WAS), a rare X-linked disorder. At the level of the peripheral blood, the syndrome is characterized by depletion of the protease calpain in platelets, decreased mitogenic responsiveness of lymphocytes, decreased surface microvilli in T cells, a loss of cell surface sialoglycoproteins, and abnormal glycosyl transferases.[176] The gene involved in the pathogenesis of the syndrome

has been identified to be on the short arm of the X chromosome (Xp11.22), cloned and designated *WASP*.[177] The mechanism by which the product of this gene, a 53-kd, proline-rich protein, induces its pathologic sequelae remains speculative. One hypothesis is that WASP is important in cytoskeletal structure or cell signaling,[178] and that defects or absence of WASP lead to instability or abnormal assembly or function of the cytoskeleton in platelets and T cells.[176]

Analysis of the X-chromosome inactivation pattern in female heterozygotes (who have no abnormalities) has revealed nonrandom inactivation of the defective gene in multiple hematopoietic lineages, including CD34+ progenitors.[179] This finding suggests that WASP plays a crucial function in cell development. There are a variety of mutations involving the *WASP* gene leading to either absent or mutated protein,[180] [181] [182] [183] but the relationship between these mutations and the wide range of clinical symptomatology is yet to be explained.[184]

Platelet survival time in these patients is approximately half-normal;[185] [186] however, this moderately reduced survival time does not explain the degree of thrombocytopenia in all patients. Platelet turnover is approximately one-fourth normal and is associated with a normal megakaryocyte mass;[1] [187] thus, ineffective production appears to be an important pathophysiologic defect. Because survival time is shortened and splenectomy results in improvement of the platelet count in many patients, platelet destruction contributes substantially to the thrombocytopenia.

Platelets are abnormally small, approximately one-half normal size,[185] [188] but thrombocytopenia is usually moderate. Platelet function is variable;[186] [189] [190] an abnormality of GPIb may be observed in some patients, but not in others.[191] [192] Calpain in affected platelets is reduced relative to normal platelets, but not in affected lymphocytes.[193] The role of this deficiency in the shortened survival of WAS platelets is unclear, but may be involved in inappropriate platelet stimulation followed by clearance from the circulation.[193] Megakaryocytes are normal or increased in number.[1] [187] It has been proposed that WASP is involved in megakaryocytopoiesis and in the assembly of actin filaments.[194] [195]

Diagnostically, the X-chromosomal inactivation pattern, probe hybridization, and direct sequencing can be used for prenatal diagnosis and for detection of the carrier state.[181] [182] [196] [197] The classic triad of infections, thrombocytopenia, and eczema is seen in approximately one fourth of patients at diagnosis.[198] One third of cases have no family history. The diagnosis is often suspected on the basis of the small platelets. Patients usually present with bleeding within the first few months of life. Bloody diarrhea and epistaxis are the common manifestations, but intracranial hemorrhage may also occur. As might be expected, patients having the highest risk for bleeding are those with a platelet count of <10,000/mm^3 at diagnosis.[198] Within 6 months, eczema and infections ensue. Beyond the complications of infections and bleeding, there is a 2%/year risk of the development of a malignancy.[199] Acute myeloblastic leukemia and large cell non-Hodgkin lymphomas, often occurring in the brain and gastrointestinal tract, are the malignancies usually observed. Enlarged lymph nodes and hepatosplenomegaly resulting from lymphoid hyperplasia occur commonly and must be distinguished from the development of a lymphoid malignancy. One half of deaths associated with this syndrome are infectious, one fourth related to hemorrhage, and 5% to malignancy.[199] Life expectancy is usually <10 years.

Corticosteroids are of no benefit and may be deleterious because of the propensity for recurrent infections. Patients who exhibit significant bleeding have benefited from splenectomy, which has been shown to augment platelet number, size, and function in many cases.[188] [200] [201] Median survival in 39 untransplanted, splenectomized patients was 25 years.[201] Nevertheless, thrombocytopenia recurs in a number of patients.[202] Antibiotic prophylaxis and intravenous gamma-globulin are useful in preventing infections but generally do not improve the thrombocytopenia.[202] Should a human leukocyte antigen (HLA)-compatible sibling donor be available, allogeneic marrow transplantation is the most appropriate therapy.[201] [203] [204] Umbilical cord blood infusion from a HLA-identical sibling has been shown to be effective in WAS.[205]

Wiskott-Aldrich Syndrome Variants and Other X-Linked Recessive Thrombocytopenias

A number of kindreds with a WAS-like picture or with X-linked thrombocytopenia have been described.[196] [206] [207] Family studies of a girl with the phenotype of WAS suggested an autosomal recessive mode of inheritance.[208] One family has been described with a WAS variant that appears to be transmitted in an autosomal dominant fashion.[209] Some WAS variants have X-linked microthrombocytopenia and no associated abnormalities, whereas others have variable degrees of eczema and immunologic defects.[207] A WAS-like syndrome associated with renal disease has been described, although the kidney disease may be secondary to the underlying immune defect of the WAS, rather than a separate entity.[210] Lymphocyte mitogenic response to periodate is abnormal in WAS variants, as it is in classic WAS, and this abnormality may be a useful diagnostic test in distinguishing variant WAS from other congenital thrombocytopenias.[211] It is now apparent that X-linked isolated thrombocytopenia and WAS represent varying clinical manifestations consequent to mutations of *WASP*,[181] [212] [213] [214] and thus molecular analysis would provide the precise diagnosis if a WAS variant is considered in the differential diagnosis. The thrombocytopenia usually is mild in these families; most cases have been discovered incidentally.[214] Marrow megakaryocytes are normal or increased.[206] [215] [216] In those few patients reported in whom thrombocytopenia was severe and associated with bleeding, responses to splenectomy have been noted.[206] [215]

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Chapter 128 - Thrombotic Thrombocytopenic Purpura and Hemolytic Uremic Syndrome

Keith R. McCrae
Douglas Cines

INTRODUCTION

In 1924, Moschowitz described a 16-year-old girl who died of a previously undescribed illness characterized by microangiopathic hemolytic anemia, petechiae, hemiparesis, and fever, which he termed acute febrile pleiochromic anemia with hyaline thrombosis of terminal arterioles and capillaries. [1] Postmortem examination revealed numerous hyaline thrombi, most prevalent in the terminal arterioles and capillaries of the heart and kidneys. [1] In 1936, four similar cases were reported by Baehr et al., who proposed that the hyaline thrombi were secondary to agglutinated platelets. [2] In 1947, Symmers[3] suggested that the term thrombotic microangiopathy be used to describe this disorder, and in 1955 Gasser used the term hemolytic uremic syndrome to describe a related syndrome that consisted of Coombs negative hemolytic anemia, thrombocytopenia, and renal failure. [4]

For many years, thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) were considered separate disorders. More recently, overlap in the pathogenesis and clinical features of these syndromes has been appreciated, [5] leading some to adopt the term TTP-HUS to describe both. These disorders are also referred to as thrombotic microangiopathies (TMA), based on their characteristic microvascular thrombotic lesions. [3] In our view, TMA is best considered as a family of related disorders with overlapping clinical and pathologic features. Such considerations have led to a newly proposed classification scheme, [5] [6] [7] which we have modified for use in this chapter (Table 128-1). In this scheme, the category of idiopathic TMA includes cases commonly referred to as either TTP or sporadic HUS. Other TMA syndromes are designated either by their etiology (when known) or the clinical situation with which they are associated. The utility of this scheme is that it highlights differences in the natural history, prognosis, and response to therapy of TMA syndromes of diverse etiology.

TABLE 128-1 -- Terminology for Syndromes of Thrombotic Microangiopathy

Idiopathic TMA
Thrombotic thrombocytopenic purpura (TMA-TTP)
Sporadic hemolytic uremic syndrome (TMA-HUS)
Toxin-associated TMA
Verocytotoxin-producing *Escherichia coli*-associated TMA (VTEC-TMA)
Shigella dysenteriae-associated TMA (Shigella-TMA)
Familial TMA
Autosomal recessive
Autosomal dominant
Quinine and miscellaneous drug-associated TMA
Transplantation and cyclosporine A-associated TMA
Malignancy and chemotherapy-associated TMA
Neuraminidase-associated TMA (*Streptococcus pneumoniae*-TMA)
Human immunodeficiency virus-associated TMA
Pregnancy-associated TMA

Modified from Kaplan BS: Thrombotic microangiopathy: a proposal for a new terminology for hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. J Nephrol 8:3, 1995, with permission.

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GENETIC ASSOCIATIONS AND FAMILIAL TMA

Little information concerning the genetics of TMA is available. Recent reports indicate that HLA-B40 and the related antigens B60, 61, 41, and 47 predispose to development of verocytotoxin-producing *Escherichia coli* (VTEC) associated TMA.[8] [9] Although no specific haplotypes have been implicated in the development of idiopathic TMA, the presence of the supertypic class II antigen, DR53 may be protective, [10] possibly due to inability of the corresponding DRB4 chain to bind a relevant antigen or toxin. [10]

Two distinct patterns of familial TMA have been defined. [11] In the first, the disease begins simultaneously in family members of different ages, who often reside in areas where VTEC infection is endemic. Recovery exceeds 80%, and relapses are uncommon. [11] [12] An example is the simultaneous development of VTEC-TMA in five siblings following VTEC-induced gastroenteritis. [12] In the second, affected siblings develop the disease at similar ages but different times. [11] [13] Some of these have occurred during or soon after pregnancy, concomitant with the use of oral contraceptives, [14] or in association with consanguinity [15] or chronic hypocomplementemia. [16] Families with this pattern may be subdivided into those in which TMA follows an autosomal recessive mode of inheritance, usually developing more commonly during infancy or childhood, [11] [15] or an autosomal dominant pattern, in which the age of onset is more variable. [11] [13] [14] [17]

CLINICAL PRESENTATION

Idiopathic TMA

Idiopathic TMA-TTP

Clinical Manifestations

The clinical manifestations of TMA-TTP have been the subject of extensive review. [18] [19] This disorder occurs with an annual incidence of 3.7 cases per 100,000 residents, [20] is slightly more common in females (female/male ratio of 3:2), [18] [19] [21] [22] and has a peak incidence in the third decade. [18] [19] [22] Ten to 40% of patients complain of an upper respiratory tract infection or flulike syndrome in the 2 weeks preceding the diagnosis. [18] Occasional patients present with malaise, fatigue, fever, or other nonspecific symptoms of days to weeks duration that are unresponsive to antibiotics or symptomatic management. [18] [19] The diagnosis of TMA may be overlooked until these prodomal symptoms become unrelenting or neurologic dysfunction develops.

The classic pentad of symptoms associated with TMA-TTP include microangiopathic hemolytic anemia (MAHA), thrombocytopenia, neurologic symptoms, fever, and renal dysfunction. [18] However, only 40% of patients develop the complete pentad, [18] whereas 75% present with a triad of MAHA, neurologic symptoms, and thrombocytopenic purpura [18] [19] (Table 128-2). In data collected from patients during the last decade,

TABLE 128-2 -- Clinical Presentation of Patients with TMA

Study	Parameters (% of episodes)					
	Fever	Neurologic Dysfunction	Renal Dysfunction	Triada[a]	Pentadb[b]	Reference
Thompson et al.	60	70	54	70	30	[23]
Amorosi and Ultmann	98	60	50	NS	NS	[19]
Ridolfi and Bell	59	52	45	74	40	[18]
Kennedy et al.	14	71	58	NS	NS	[25]
Petitt	87	100	18	100	NS	[22]
Cuttner	100	95	55	95	55	[21]
Byrnes	61	89	89	89	44	[65]
Bell et al.	NS	NS	NS	NS	77	[71]
Rock et al.	24	63	52	63	NS	[70]

Abbreviation: NS, data not stated.

Modified from Thompson et al., with permission.[23]

[a] Historical triad of anemia, thrombocytopenia, and renal dysfunction.
[b] Classic pentad of anemia, thrombocytopenia, neurologic dysfunction, fever, and renal dysfunction.

the incidence of symptoms such as fever appears to have diminished, perhaps due to earlier diagnosis. [23]

Neurologic symptoms ranging from headache and confusion, to somnolence, seizures, aphasia, or coma often dominate the clinical picture in TMA-TTP, [18] [19] [23] [24] [25] and may fluctuate in severity, a characteristic attributed to the repetitive formation and dissolution of microthrombi in the cerebral microvasculature. [26] [27] Thrombocytopenia may be severe, with 25% of patients presenting with platelet counts below 20,000/l. [18] [25] Approximately 90% of patients experience bleeding complications, [18] [19] [24] which may be disproportionate to the severity of thrombocytopenia, perhaps because of concomitant damage to the microvasculature. Despite bleeding events, the prothrombin time, partial thromboplastin time, and fibrinogen levels are usually normal or only mildly perturbed. [28] However, mild elevations in the levels of fibrinogen degradation products occur in 50% of patients, [28] and the use of more sensitive assays, such as those for prothrombin fragment 1+2, thrombin-antithrombin, and plasmin-antiplasmin complexes and fibrin D dimers, demonstrates activation of coagulation and fibrinolytic pathways. [29] Evidence of renal involvement, such as hematuria, proteinuria, or mild azotemia is common, though on presentation the severity is generally mild. [19] MAHA, resulting from fragmentation of red blood cells traversing fibrin strands in the microvasculature [18] [19] or oxidant damage to the red blood cell membrane, is a sine qua non of this disorder. This results in the schistocytes or helmet cells seen on the peripheral blood smear, which are characteristic of TMA (Fig. 128-1), as well as markedly elevated levels of plasma lactate dehydrogenase (LDH). Nucleated red blood cells are present in most patients, and their number may be disproportionately increased in comparison to the degree of reticulocytosis, [18] suggesting an element of marrow injury. With rare exceptions, the direct antiglobulin test is negative. [19] Other less common symptoms include abdominal pain and respiratory distress (see box, Approach to the Patient with TMA).

Pathogenesis

The factors that initiate idiopathic TMA are unknown. However, extensive evidence suggests that endothelial cell damage resulting in accelerated platelet/vessel wall interactions is a critical intermediary in the pathogenesis of this disease.

Pathology.

The prototypic vascular lesions are characterized by platelet-rich thrombi within or beneath a damaged endothelium. [30] Involved microvascular endothelial cells are swollen and in some cases detached. The subendothelium contains fibrinoid material composed, in part, of fibrin(ogen), von Willebrand factor (vWF), and platelet

remnants.[30] [31] Involved vessel walls characteristically display a paucity of mononuclear leukocytes; fibrinoid necrosis, aneurysm formation, and other evidence of vasculitis are absent in primary cases. Medium and large-sized arteries show less extensive involvement and the venous system is typically spared. The most commonly involved organs are the brain, kidney, pancreas, heart, and adrenal glands. [18] Involvement of other sites such as the spleen, gingiva, bone marrow, and skin (Fig. 128-2) also occur.[25]

Endothelial Injury.

Plasma from patients with TTP and other forms of TMA often contain elevated concentrations of endothelial-derived proteins such as thrombomodulin, P-selectin, plasminogen activator inhibitor type 1 (PAI-1), and vWF. [27] [32] Circulating endothelial cells, as well as decreased levels of tissue factor pathway inhibitor, have also been reported.[27] [32] Antiendothelial cell antibodies, some directed at CD36, have been described, but their role has not been delineated. [33] [34] It has also been reported that plasma from patients with idiopathic and human immunodeficiency virus (HIV)-associated, but not VTEC-TMA, induce apoptosis of dermal microvascular, renal, and cerebral endothelial cells, but not those of pulmonary or hepatic origin, through a pathway involving induction of Fas (CD95). [35]

Abundant evidence indicates that the capacity of endothelial cells to inhibit platelet activation and mediate vasodilatation through the elaboration of prostacyclin may be impaired.[36] Plasma levels of the prostacyclin metabolite, 6-keto-PGF_{1}, are

Figure 128-1 This peripheral blood film was obtained from a 28-year-old woman who presented with fever, epistaxis, and altered mental status. Note the absence of platelets and the presence of a nucleated erythrocyte and schistocytes (arrows), consistent with a microangiopathic process.

Figure 128-2 This skin biopsy was obtained from a 54-year-old woman who presented with low-grade fever, MAHA, and thrombocytopenia. The biopsy was performed at the site of a petechial lesion. Note the numerous thrombotic lesions in dermal arterioles (arrows). Petechial hemorrhage is noted in the upper dermis. Inspection of the arteriolar walls reveals prominent, swollen endothelial cells with no evidence of infiltrating leukocytes. Hematoxylin and eosin, original magnification × 100. (Photograph courtesy of Dr. I. Bruce Elfenbeim, Department of Pathology, and Dr. Alexander Zweibach, Division of Hematology, Temple University School of Medicine.)

reduced during the acute phase of the disease and normalize on remission. [37] Impaired renal generation of prostacylin has been observed in children with VTEC-TMA.[38] Vein biopsies from patients with TMA-TTP are deficient in prostacyclin synthesis in vitro, a finding attributed to inhibition of cyclo-oxygenase by free radicals or enhanced lipid peroxidation.[37] Plasma from patients with idiopathic, but not VTEC-TMA, has been reported to lack a 300400 kDa cyclo-oxygenase cofactor that is present in normal plasma, and stimulates vascular prostacyclin production. [39] It is presumed this factor is replenished by plasma therapy.[39] Defects in serum prostacyclin binding and enhanced prostacyclin degradation by TMA sera have also been described. [37] [39] Treatment of patients with TMA with intravenous prostacyclin, however, has met with limited success to date.[40]

Endothelial cell injury may also promote platelet adhesion to the vasculature by disrupting the production or processing of vWF. [32] Pioneering studies by Moake et al. demonstrated the presence of unusually large multimeric forms of vWF (ULvWF) in the plasma of four patients with chronic relapsing TMA (CR-TMA) during remission.[41] These ULvWF, which are exceedingly potent in supporting shear stress-induced platelet aggregation, [42] disappeared during relapse, presumably due to proteolysis under the high shear stress conditions within the partially occluded microvasculature of patients with TMA, [43] or consumption during systemic platelet aggregation.[41] Since these reports, both increased as well as diminished levels of ULvWF have been reported in a variable percentage of patients with idiopathic, VTEC- and drug-associated TMA.[32] These inconsistent results may reflect differences in the sampling time and method of plasma collection, resulting in ex vivo proteolysis.[44]

Several mechanisms may contribute to the accumulation of ULvWF in TMA plasma. Larger multimeric forms of vWF are normally processed at high shear forces by a plasma vWF-cleaving protease.[45] It is possible that the amount of ULvWF released by damaged endothelium may exceed the processing capacity of this metalloprotease, or that the protease may be deficient in individuals with CR-TMA. [46] Deficient protease activity, due to the presence of neutralizing IgG antibodies, has been recently reported in approximately 80% of patients with idiopathic, non-familial TMA (TMA-TTP). [46A] [46B] In patients with familial TMA syndromes with clinical manifestations most closely resembling those of TTP, protease activity was not detectable, though no inhibitor was demonstrated. [46B] Protease

APPROACH TO THE PATIENT WITH TMA

The diagnosis of TMA must be made in a timely manner because delay carries with it the attendant risk of sudden death. The disorder should be considered when prodromal symptoms such as malaise, fatigue, gastrointestinal distress, or low-grade fever persist or progress in the face of symptomatic therapy. A complete blood count and an analysis of the peripheral blood smear are indicated in any patient with a protracted course. Suspicion should be heightened in women who are or were recently pregnant, in patients who are infected or are at risk for infection with human immunodeficiency virus (HIV), in allograft recipients, and in those with a history of underlying vasculitis or exposed to specific drugs described in the text. Patients with early, idiopathic TMA-TTP may present without the classic triad or pentad of signs and symptoms (Table 128-2). Thus, it is the authors opinion that a provisional diagnosis of TMA should be made, and plasma-based therapy instituted, in any patient with the above-mentioned prodromal symptoms in whom MAHA and thrombocytopenia are discovered, unless an alternative diagnosis is evident. We consider an average of one or more schistocytes per high-power field to be of concern, especially when accompanied by thrombocytopenia and nucleated red blood cells, or an increased serum LDH level. The differential diagnosis of TMA-TTP, MAHA, and acquired thrombocytopenia is extensive and can be extraordinarily difficult in some cases. Vascular damage secondary to systemic lupus erythematosus, scleroderma, septic or tumor emboli, immune complex-mediated vasculitis (e.g., infective endocarditis), malignant hypertension, cryoglobulinemia, or infection with rickettsial or, more rarely, hemorrhage-inducing viral organisms, may mimic TMA. TMA may also be superimposed on pre-existing immune vascular disorders such as systemic lupus erythematosus or the antiphospholipid syndrome; in such patients, plasma exchange should be added to existing anti-inflammatory and immunosuppressive therapy. Occasional patients with disseminated intravascular coagulation secondary to malignancy or sepsis (e.g., purpura fulminans in the setting of protein C deficiency) present with occlusive microangiopathy of sufficient severity to be confused with TMA. In the setting of renal transplantation, there are occasions in which a biopsy may be required to distinguish TMA from allograft rejection or recurrence of a pre-existing renal vascular disorder. Occasional patients with TMA present with acute pancreatitis, acute respiratory distress syndrome, or memory and personality changes or other poorly defined neurologic symptoms that themselves have a broad differential diagnosis. Again, the finding of MAHA and thrombocytopenia provides important clues to the underlying problems and the urgent need for intervention with plasma-based therapy.

activity, however, was normal in the majority of patients with either sporadic or familial variants of HUS. [46B] These intriguing observations may provide insight into the greater therapeutic efficacy of either fresh frozen plasma (FFP) or cryosupernatant, sources of the vWF-cleaving protease, [32] [46C] in TTP versus HUS (see later). Further definition of the role of the protease in TMA awaits its cloning and molecular characterization. It has also been proposed that calpain or a related protease, present in elevated levels in TMA plasma, may generate novel fragments of vWF with enhanced affinity for the platelet glycoprotein (GP) IIb/IIIa complex. [47]

Platelet Activation.

In addition to alterations in endothelial cell function, TTP plasma may promote platelet aggregation directly. [48] Platelet microparticles have been detected in essentially all patients with TMA, their number correlating inversely with the platelet count and their disappearance with clinical improvement. [49] Platelet aggregation has been attributed by one group to a 37-kDa protein that binds specifically to platelet glycoprotein IV (CD36), [50] whereas others have implicated a lysosomal, cathepsin-type cysteine protease present in the plasma of patients with TMA. [51] However, Kelton et al. have argued that this latter activity is more likely attributable to the calcium-dependent, cytosolic protease, calpain. [52] Calpain was detected on the surface of platelet microparticles isolated from patients with TMA, [53] an environment that might protect the enzyme from its plasma inhibitors. [52]

Other Mechanisms.

Plasminogen activator activity is reportedly absent from vessels at sites of microthrombi in TMA. [54] In addition, plasma levels of PAI-1 are markedly elevated, [55] [56] far exceeding those measured in patients with disseminated intravascular coagulation (DIC). Plasma concentrations of PAI-1 have prognostic significance in children with TMA-HUS, and the recovery of renal function in a cohort of children with TMA correlated with the removal of PAI-1 by peritoneal dialysis. [57] Elevated levels of the vasoconstrictor, endothelin, have been reported in a few patients with idiopathic and VTEC-TMA. [58] Increased levels of nitric oxide metabolites are present in the plasma of patients with acute TMA, [59] perhaps generated following nitrous oxide release in response to elevated shear stress. [30] Nitrous oxide, however, may interact with neutrophil-derived superoxide anions to form lipid peroxides, as well as cytotoxic peroxynitrite and hydroxyl radicals. [59] Other products of activated leukocytes, such as elastase and cathepsin G, [60] have also been detected in patients with TMA. [61]

Therapy

The mortality rate of TTP-TMA approaches 100% without therapy. Through the mid-1970s, splenectomy remained the only modality with more than an anecdotal response rate. [19] Since that time, however, there has been a dramatic reversal of this grim prognosis with the advent of empiric, plasma-based therapy, such that long-term survival now approaches 90%. [62]

Plasma Therapy.

Anecdotal reports of responses to whole blood infusion have existed since Moschowitzs initial description of the disease. [63] Yet, the remarkable benefit of plasma therapy was not appreciated until 1976, when Bukowski et al. reported a 50% survival rate in 16 patients with TMA treated by exchange transfusion. [64] The following year, Byrnes and Kharuna reported that the active component in whole blood was contained in plasma, an observation that led to the widespread use of plasma infusion. [65] Thereafter, others began to explore the efficacy of plasma exchange under the assumption that the response rate might be improved by removing a toxic substance from patient plasma. [66] Numerous confirmatory studies followed, [26] [62] based on which plasma has become the therapy of choice for TMA-TTP.

The results of a prospective trial reported in 1991 appeared to resolve the long-debated issue of the relative efficacy of plasma exchange versus infusion in favor of the former. [67] At 6 months, complete remissions were seen in 78% of patients treated with exchange versus 31% in those treated with plasma infusion alone. [67] It has been argued, however, that patients in the plasma exchange arm of this study received a threefold greater volume of plasma, obscuring the question as to whether removal of a disease-inciting agent accounts for the apparently superior response. In support of this, no significant difference in outcome was observed in a retrospective analysis of patients who received equal volumes of plasma given by exchange or infusion. [68] Plasma infusion has also been effective in well-documented cases of chronic relapsing TMA. [69] Nevertheless, from a practical perspective, large volumes of plasma may be more easily administered by plasma exchange, which is, therefore, the standard initial therapy in almost all situations. [26] Exchange should be performed daily with the goal of exchanging 11.5 plasma

volumes (4060 ml/kg) with each procedure. Neurologic improvement occurs most rapidly, often within hours to days. [23] The serum LDH level typically falls by 50% within 3 days in responders, and the platelet count begins to rise in a mean of 5 days, though normalization may take several weeks. [23] Impaired renal function is generally the last to improve.

Fresh frozen plasma remains the most commonly used replacement product. However, up to 50% of patients seemingly refractory to exchange with FFP may respond subsequently to exchange with cryosupernatant, [70] which is essentially FFP from which vWF, fibrinogen, fibronectin, and factor XIII have been depleted. [62] As with all secondary treatments, the response to a newly introduced agent, a delayed response to a previously used modality and the natural history of the disease cannot be distinguished.

Plasma exchange should be continued until neurologic symptoms have resolved and both a normal serum LDH and platelet count have been maintained for 3 days; a shorter duration of therapy risks immediate and occasionally fatal relapse. Approximately 90% of patients will show a clinical and laboratory response to plasma exchange within 3 weeks, most by 10 days. Even with optimal therapy, approximately 30% of patients will relapse, half within 6 weeks of initial treatment (see box, Relapsing TMA). The decision to switch from FFP to cryosupernatant or to introduce another form of therapy remains a matter of clinical judgment but, in general, is not considered until the patient has received a minimum of 1014 days of daily exchange with FFP, unless extraordinary circumstances intervene.

Auxiliary Treatments and Management of Refractory Patients

Corticosteroids.

The rationale for the use of corticosteroids is uncertain. Corticosteroids are of little benefit on their own, [26] [62] and retrospective studies do not suggest that they improve the response to plasma exchange. [62] However, Bell et al. recently reported a response rate of 30% in patients with mild, idiopathic TMA (minimal symptoms, no neurologic abnormalities) treated with 200 mg prednisone daily. [71] It is the authors opinion that patients with seemingly asymptomatic TMA-TTP can die suddenly, and that corticosteroids as the sole modality of therapy is neither sufficient nor appropriate until these findings can be confirmed.

Splenectomy.

Before the introduction of plasma therapy, splenectomy was considered first-line treatment for TMA-TTP, inducing remission in up to 50% of patients when used in conjunction with other agents. [62] The use of splenectomy is now confined to refractory patients, with variable response rates reported. [23] [71] [72] Some series suggest that splenectomy induces an enhanced response to plasma exchange in patients initially refractory to this modality. [23] [26] In the authors opinion, splenectomy should be considered in patients refractory to large volume plasma exchange, perhaps after a trial of cryosupernatant. Exchange should be continued after surgery until the remission

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RELAPSING TMA

More than 80% of patients presenting de novo with TMA-TTP can now be treated successfully with plasma-based therapy, with a concomitant increase in long-term survival. However, with the advent of effective therapy, it has become apparent that at least 30% of successfully treated adult patients will experience one or more relapses. Approximately half of these occur within the first 2 months after therapy has been discontinued, although some prefer to characterize these episodes as incomplete responses. The remainder can occur from a few months to many years after the initial episode. Approximately half the patients experience multiple relapses with no periodicity or obvious inciting event, with the possible exception of pregnancy in a few cases. There are also rare infants, children, and adults in whom relapses occur at regular intervals in the absence of therapy. Unusually large von Willebrand factor multimers may be found in the plasma of some of these patients, and relapses have been successfully pre-empted by regularly timed infusions of plasma. Recent reports suggest that a decrease in plasma vWF-cleaving protease activity may also portend relapse in some patients. In contrast, no serologic markers of relapse have been identified in adults with sporadic TMA-TTP, and no therapy (including antiplatelet agents, corticosteroids, or immunosuppressants), with the possible exception of splenectomy, has been shown to clearly reduce the incidence of recurrence outside the setting of pregnancy. However, patients often recognize the prodomal symptoms and bring themselves to medical attention promptly. The physician should respect these self-evaluations and investigate the possibility of recurrent TTP immediately. The prognosis of relapsed TTP is excellent if management is initiated promptly, but every episode carries the potential for irreversible tissue injury.

criteria mentioned above are attained. Splenectomy during hematologic remission may reduce the incidence or extend the duration between relapses in patients with chronic relapsing TMA. [73]

Other Modalities.

The response rate to *antiplatelet agents* such as aspirin, dipyridamole, sulfinpyrazone, or ticlopidine is 10%, [26] [62] essentially indistinguishable from the natural history of TMA. Antiplatelet agents have also not been convincingly shown to increase the response to plasma exchange, [67] [71] and may promote bleeding in the setting of severe thrombocytopenia. [26] Hence, their use as first-line therapy cannot be recommended, [26] though the role of ticlopidine in preventing TMA relapse is currently under investigation. [74] Some authors also recommend the use of aspirin to prevent thrombosis in patients who exhibit a rapid rise in the platelet count in response to plasma therapy. [26] [62] The use of *intravenous immunoglobulin* is predicated on the observation that IgG from healthy individuals inhibits the capacity of TTP plasma to agglutinate platelets in vitro, [75] a finding that others have failed to reproduce. Most reports supporting the efficacy of intravenous immunoglobulin [76] are compromised by the delay in the initiation of treatment or concomitant administration of other agents, [26] [62] and others have failed to demonstrate clinical responses to intravenous immunoglobulin after failure of plasma exchange. [26] There are anecdotal reports of favorable responses to *vincristine*, [26] [62] [77] as well as other immunosuppressive therapies such as azathioprine, cyclophosphamide, cyclosporine, and staphyloccal protein A immunoadsorption. [78]

Idiopathic TMA-HUS

Idiopathic TMA-HUS is a variant of TMA in which severe renal failure, developing in the absence of an obvious precipitating factor, is the predominant disturbance. This disorder encompasses the adult, atypical, or sporadic hemolytic uremic syndromes and may account for up to 10% of cases of pediatric HUS. In some instances, sporadic cases have occurred in more than one family member, [79] suggesting a cryptic autosomal recessive inheritance. [80] Though TMA-HUS is considered a rare disorder, up to 16% of patients in large series of idiopathic TMA (TTP and HUS) have required dialysis. [81] On the other hand, the incidence of idiopathic TMA-HUS in adults is decreasing as it becomes apparent that VTEC-TMA may account for a greater percentage of adult cases than previously appreciated. [82]

Most patients with TMA-HUS experience a prodrome suggestive of an upper respiratory tract infection, or nonspecific symptoms of malaise and fatigue, but severe

abdominal pain and bloody diarrhea, typical of VTEC-TMA, is notably absent. Hence, idiopathic TMA-HUS is sometimes also referred to as D(-)HUS. The occurrence of idiopathic TMA-HUS does not display the seasonal variability characteristic of VTEC-TMA. [83] Renal involvement is more severe than in TMA-TTP, with up to 60% of patients requiring dialysis. [84] MAHA is universal, and the reticulocyte count and LDH concentration are elevated. [85] The severity of thrombocytopenia is generally less than that seen in patients with TTP. [85] [86] Neurologic manifestations are also typically less common and severe than in TMA-TTP, and rarely dominate the clinical course, [82] though cerebral microthrombi are frequently detected at autopsy. [87]

The response to therapy of patients with TMA-HUS is ill-defined, due to its rarity, lack of clear diagnostic criteria, and the inclusion of patients with TMA-HUS in series consisting primarily of patients with TMA-TTP. This disorder is also frequently grouped with pregnancy, cancer, and chemotherapy-associated TMA, which may have different natural histories. Nevertheless, TMA-HUS is associated with a poor prognosis, with a mortality rate approaching 25%, and 50% of surviving patients developing chronic renal insufficiency. [83] Responses to plasma exchange have been documented, but their frequency is difficult to assess. Likewise, whether this disorder responds to the above-mentioned ancillary agents used in TTP-TMA has not been established. Older age and severity of renal dysfunction are associated with a poorer prognosis. [84] Nevertheless, a trial of plasma exchange is appropriate in patients able to undergo the procedure safely.

Verotoxin-Associated TMA (VTEC-TMA)

Epidemiology

This variant is commonly referred to as typical, epidemic, childhood, or D(+) HUS. Although the incidence is highest in children <5 years old, [88] cases have been reported in individuals of all ages. [88] [89] The pathogenesis of this form of HUS, and in particular its association with verotoxin-producing *E. coli*, [83] has been investigated extensively. The relationship of verotoxin-producing organisms to human disease was first recognized in 1983, based on an association of *E. coli* strain 0157:H7 with two outbreaks of hemorrhagic gastroenteritis. [90] VTEC were first implicated in the development of TMA following the detection of these organisms in the stools of 11 of 15 Canadian children with HUS. [91] Additional studies have demonstrated an association of other verotoxin-producing strains of *E. coli*, [92] of which >100 have now been identified, with epidemics of TMA. [93] VTEC are also associated with sporadic cases of TMA, [88] [93] [94] although molecular subtyping may be required when an association between apparently isolated cases is sought. [95] The disease occurs more frequently during summer months in temperate climates. [83]

The major reservoir of verotoxin-producing *E. coli* is domestic

cattle, approximately 1% of which harbor *E. coli* in their gastrointestinal tract. Outbreaks of VTEC-TMA are associated most frequently with ingestion of inadequately cooked ground beef from an identified source, [88] [94] though contamination of other food sources, such as poultry and cheese, has been reported. Other cases have been attributed to ingestion of contaminated water [96] or unpasteurized milk. [97] Person-to-person transmission may contribute to outbreaks of VTEC-TMA in settings such as day care centers, institutions, [98] and nursing homes. [99] A few instances of asymptomatic carriage of VTEC have been reported. [100]

Clinical Manifestations

This variant occurs most commonly after an episode of *E. coli* 0157:H7-induced gastroenteritis, [101] [102] although rare cases have been reported after infection of the urinary tract or skin. The disease begins with the sudden onset of abdominal pain and watery diarrhea 1 to 9 days after toxin exposure. [93] In some individuals, abdominal pain may be severe and precede the onset of diarrhea; this presentation, particularly in the absence of fever, may be difficult to differentiate from inflammatory bowel disease, appendicitis, ischemic colitis, or intussception. Bloody diarrhea ensues on the second day, accompanied in some cases by nausea and vomiting. [93] Fever is absent or mild. Colonoscopy shows an edematous colonic mucosa, with occasional ulceration and pseudomembrane formation. Thumbprinting in the distal ascending and proximal transverse colon, suggesting an ischemic colitis, may be seen on barium enema, and is caused by exotoxin-induced thrombi in the microvasculature of the bowel wall. [93]

It has been estimated that 930% of individuals with VTEC-associated hemorrhagic gastroenteritis develop HUS. [24] Young children and the elderly are at greatest risk for progression, [88] perhaps due to a slower rate of clearance of the organism from the gastrointestinal tract. Antimotility agents further delay clearance and may increase the risk of progression to TMA. [103] Antibiotics do not diminish progression and may theoretically increase the risk of progression by enhancing exotoxin production or release. [103] Patients who develop TMA present 2 days to 2 weeks after the onset of bloody diarrhea with oliguria or other evidence of renal impairment; [93] [101] 60% of patients ultimately require dialysis, at least temporarily. [101] The extent of MAHA varies considerably. Thrombocytopenia is common, but is mild or absent in 30% of cases at presentation. Thirty to 50% of patients develop neurologic manifestations, which may include irritability and somnolence, and less commonly, confusion, paresis, and seizures. [93] [104]

The disease should be suspected in a patient who presents with the characteristic clinical manifestations after an episode of bloody diarrhea, though the prototypic history of a preceding hemorrhagic gastroenteritis is absent in approximately 10% of cases. [101] [105] The diagnosis may be verified by a positive stool culture. [101] *E. coli* 0157:H7 ferment sorbitol slowly, and thus appear as colorless colonies on sorbitol-MacConkey agar; sorbitol-negative cultures may be characterized further using commercially available 0157:H7-specific antisera. [106] Methods to detect Shiga-like toxins or their structural genes [93] improve diagnostic sensitivity. Demonstration of a fourfold or greater rise in antibody titer to either Shiga-like toxins or 0157 lipopolysaccharide may be used to confirm a diagnosis of VTEC-TMA. [93] Serologic assays are of particular value when stool cultures are not performed within 7 days after the onset of diarrhea, because cultures obtained after this time are frequently negative. [107] However, methods to identify non-0157:H7 strains of verotoxin-producing *E. coli*, which account for up to 25% of cases of VTEC-TMA, are not widely available.

Pathogenesis

Pathology

Examination of renal biopsy material, though rarely required for diagnosis in endemic areas, suggests that endothelial damage is central to the pathogenesis of VTEC-TMA. Involved glomeruli show widening of the subendothelial space, which is filled with cellular debris and fibrin. Concurrent thickening of the capillary wall results in a double contour appearance. [30] In some specimens, infiltration of glomeruli by monocytes, and, to a lesser extent, neutrophils has been observed, perhaps as a result of the elevated urinary levels of monocyte chemoattractant protein-1 (MCP-1) and interleukin 8 in these patients. [108A] Mesangiolysis is present in most cases. Glomerular capillary endothelial cells are swollen and occasionally detached, leading to obliteration of the capillary lumens. [108] Thrombi in glomerular capillaries may be sparse, [39] though thrombotic occlusion of afferent arterioles occurs in some cases (Fig. 128-3A (Figure Not Available)). The presence of glomerular involvement with sparing of larger arterioles and arteries is generally associated with a good prognosis. [109] In older children and adults (who primarily develop idiopathic or familial TMA), involvement of arterioles and interlobular arteries, with relative sparing of glomeruli, is observed more commonly. These lesions are characterized by edema and myointimal proliferation, necrosis of the vascular wall, and patchy cortical necrosis. Glomeruli exhibit ischemic changes, characterized by collapse, retraction, and wrinkling of the capillary loops (Fig. 128-3B (Figure Not Available)). This pattern is associated with a poor prognosis and a high incidence of chronic renal insufficiency. [109]

Toxins

Escherichia coli verotoxin is a 70-kD bacterial exotoxin, [110] named for its cytotoxicity against African green monkey kidney (Vero) cells. Verotoxin is homologous to Shigella toxin [88] [110] and is therefore also referred to as Shiga-like toxin 1 (SLT-1). Many strains of pathogenic *E. coli* also produce a second, less closely related toxin, SLT-2, usually in conjunction with SLT-1. [111] The intact, 70-kD SLT holotoxins consist of a 32-kD A subunit and five 7.7-kD B receptor-binding subunits. [111] The toxin binds to Gala1-4Galb (galabiose) residues on globosyltriaosylceramide (Gb_3; also known as CD77, Burkitts lymphoma antigen, and the human blood group P^k antigen). [112] Receptor-bound toxin is internalized, [113] and transported to the endoplasmic reticulum, [114] where the A subunit is cleaved to a 27-kD A1 subunit, which binds and inactivates the 60s ribosomal subunit and prevents elongation factor 1-dependent binding of aminoacyl tRNA. [115] This blocks peptide elongation and leads to endothelial cell death.

The predilection of children exposed to verotoxin to develop renal disease may relate to the greater expression of Gb_3 by glomerular capillary endothelium in children compared to adults, [116] as well as the relatively greater expression of Gb_3 by glomerular endothelium versus other vascular beds. [116] [117] This pattern of expression

may account for the 1,000-fold greater cytotoxicity of SLTs toward human renal, compared with umbilical vein, endothelial cells in vitro. [116] The predilection of rabbits injected with SLT-1 to develop neurologic dysfunction with sparing of the kidneys may likewise be due to enhanced expression of Gb_3 by brain endothelial cells in this species.[118] The expression of Gb_3 and cytotoxicity of SLT-1 are enhanced by tumor necrosis factor-alpha (TNF-) and interleukin-1 (IL-1), which are released by monocytes following stimulation by *E. coli* lipopolysaccharide and SLT.[119] SLTs also induce the expression of TNF- within the kidney,[120] potentially increasing the expression of their own receptor through a positive feedback loop.

Therapy

Verotoxin-associated TMA may be the most common cause of acute renal failure in children[83] [105] Although approximately 60% of children require dialysis initially, the disorder is typically self-limited and the mortality rate has been reduced to 56% with appropriate supportive care. [83] Renal insufficiency

Figure 128-3 (Figure Not Available) **(A**)3-year-old boy who died 3 days after the onset of fulminant TMA following hemorrhagic colitis. Note thrombi in afferent arteriole and glomerular capillaries. Periodic acid-Schiff, × 400. **(B)** Transplanted kidney from a 3-year-old girl who had presented with idiopathic TMA-HUS at age 7 months. She received this cadaveric transplant at age 3 years. During the first 2.5 days post-transplant, she developed progressive thrombocytopenia and anemia followed by tonic-clonic seizures due to a diffuse cerebral infarction involving the left hemisphere. Note double contouring of the capillary wall (arrow) and collapsed, ischemic glomerulus. Periodic acid-Schiff, × 400. (Photos courtesy of Dr. Laura Finn, Department of Pathology, and Bernard S. Kaplan, M.B., B.Ch., Division of Nephrology, The Childrens Hospital of Philadelphia. From Meyers and Kaplan, [201] with permission.)

generally resolves within 23 weeks, although some patients have prolonged anuria, requiring several months before renal function returns to normal. Despite a favorable short-term outcome, it has become apparent that many patients with VTEC-TMA develop chronic renal insufficiency over time. [109] In one report of 29 patients with typical childhood TMA followed for 1528 years, 7 developed chronic renal failure and 12 developed related symptoms (hypertension, proteinuria, or mildly reduced glomerular filtration rate), whereas only 10 had no residual abnormalities. [109] Thus, complete recovery and long-term preservation of renal function approximates 5060%.[121] Presenting features associated with a poorer long-term outcome include prolonged anuria, [122] an elevated leukocyte count,[123] and the severity of the preceding gastroenteritis. There is also a relationship between the extent of histologic damage on presentation and eventual outcome; virtually all patients with cortical necrosis are left with significant chronic renal disease. [109] Recent studies demonstrate that persistent proteinuria more than 1 year after the initial episode of TMA is also associated with progressive renal disease. [105]

In randomized trials, neither plasma infusion nor exchange have been shown to benefit children with VTEC-TMA. [121] In one study of older children with TMA at high risk for poor outcome, plasma exchange did not prevent the development of renal insufficiency,

although all patients requiring long-term dialysis were in the nontreated group. [124] Likewise, treatment with heparin, urokinase, aspirin, or dipyridamole has not been beneficial. The utility of protein restriction and angiotensin-converting enzyme inhibitors to reduce the progression of renal disease in patients with persistent proteinuria after their initial episode of TMA is under investigation. [105]

Transplantation and Cyclosporine A-Associated TMA

Thrombotic microangiopathies occur most commonly in three transplant-related settings: (1) in association with the use of cyclosporine A (CyA), (2) as recurrent disease after transplantation for a TMA-related disorder, and (3) in the setting of bone marrow transplantation.

Cyclosporine A is the most common cause of drug-induced TMA.[125] TMA occurs in 35% of renal transplant recipients, [126] [127] [128] as well as in occasional heart[129] and liver [130] recipients treated with CyA. CyA-induced TMA typically develops insidiously with progressive, otherwise unexplained renal insufficiency. [126] MAHA or thrombocytopenia develops in only half the patients and is typically mild. [126] [127] The appearance of the glomerular capillary and arteriolar thrombotic lesions is similar to de novo cases of TMA.[131] The distinction of CyA-TMA from acute tubular necrosis or rejection can often be made only by biopsy, and not in every case can an unequivocal distinction be established. [132] Broad alloantibody reactivity,[126] [133] coinfection with cytomegalovirus,[128] and the use of non-heart-beating renal donors[134] may predispose to CyA-associated TMA.

Cyclosporine A is cytotoxic to cultured endothelial cells at concentrations similar to the peak plasma levels and tissue concentrations achieved in vivo. [135] CyA acts synergistically with interleukin-1 (IL-1), IL-1, and TNF- to induce endothelial cell tissue factor expression [136] and inhibit thrombomodulin activity.[137] CyA also enhances the secretion of prostacyclin and thromboxane by cultured endothelial cells [135] and inhibits the production or activity of a putative plasma prostacyclin stimulating factor in vivo.[138] Plasma levels of vWF[139] and endothelin-1[140] are elevated during episodes of CyA-induced nephrotoxicity, consistent with endothelial cell injury. CyA also enhances platelet aggregation and thromboxane release. [141] Therapy for CyA-induced TMA involves reduction of the dose or discontinuation of CyA. The prognosis is generally good, although occasional patients develop permanent renal failure. [125] In some patients, CyA can be reintroduced later at a lower dose[126] ; in others, FK 506 has been substituted successfully,[142] although the latter may also precipitate TMA on occasion. [143]

Approximately 50% of patients with idiopathic TMA-HUS develop end-stage renal insufficiency, becoming candidates for renal transplantation. TMA can recur in the transplanted kidney, even in the absence of CyA. The reported frequency of this complication varies widely. [144] [145] [146] A careful, multigenerational history must be taken to exclude the possibility of familial TMA when a living related donation is being considered. [80]

Thrombotic angiopathies also occur in at least 4% [147] of patients who undergo autologous or allogeneic bone marrow transplantation. [148] [149] [150] The use of total body irradiation as part of the pretransplant conditioning regime, [151] immunosuppression with CyA post-transplant, [147] [148] and the development of graft-versus-host disease[147] increase the incidence and severity of TMA. TMA can be particularly difficult to diagnose in this setting [148] because most marrow recipients develop thrombocytopenia, microangiopathic red cell changes, and mild renal insufficiency at some point in their course. [152] It has been proposed that bone marrow transplant recipients with TMA can be stratified into three groups for prognostic purposes, based on a number of identified risk factors that include: (1) the development of signs/symptoms within 120 days of transplant, (2) exposure to CyA or FK 506, and (3) the presence of neurologic symptoms. [150] Mortality exceeds 50% in high-risk patients (two or three risk factors), [150] for whom aggressive therapy with plasma exchange is indicated.[153] No controlled trials, however, have been performed to establish the benefit of intervention. In contrast, isolated mild renal insufficiency and microangiopathy developing insidiously months to years after transplant may result from radiation nephritis, perhaps exacerbated by chemotherapy, and often resolves or stabilizes with supportive care alone. [154]

Cancer and Chemotherapy-Associated TMA

Thrombotic angiopathies may terminate the course of some patients with disseminated malignant neoplasms and large tumor burdens, most commonly adenocarcinoma of gastrointestinal tract origin. [155] Patients present with the abrupt onset of moderate to severe MAHA and thrombocytopenia. [155] Renal insufficiency and neurologic dysfunction occur less commonly than in idiopathic or chemotherapy-induced TMA, [156] and may result from concurrent metabolic disturbances, central nervous system metastases, or hemorrhage.

The pathogenesis of cancer-associated TMA remains poorly understood. Laboratory evidence of disseminated intravascular coagulation (DIC) is found in 2580% of patients based on findings of elevated fibrin split products [155] [156] [157] or more sophisticated measurements of fibrinogen turnover.[155] However, the observation that TMA occurs in only 5% of patients with disseminated malignancy and DIC [158] suggests that additional factors, such as microvascular occlusion or intimal proliferation induced by tumor emboli within the pulmonary vasculature, [156] [159] [160] or formation of an incompletely endothelialized tumor vasculature that predisposes to platelet adhesion, must be involved in its development. The survival of patients with cancer-associated TMA is generally measured in weeks. The only effective therapy is reduction of the tumor burden, a goal that is not often attainable. A role for plasma exchange has not been established.

Specific cancer chemotherapeutic agents have also been implicated in the development of TMA (see references 159 161 for reviews). Most patients with this disorder had been receiving therapy for an adenocarcinoma when TMA developed, [162] [163] but the syndrome has been reported in individuals with other histologic types of neoplasms.[162] [164] Unlike cancer-associated TMA, most patients with chemotherapy-associated TMA do not carry a high tumor burden. [159] [161] [162] Mitomycin-C,[162] a known nephrotoxin,[165] is the most commonly reported cause. Other cases have been reported in patients treated with cis-platininum, [163] either alone or in combination

with other agents.[164] Most patients who develop TMA do so after having received a cumulative dose of mitomycin-C in excess of 60 mg,[162] but the syndrome has been reported after minimal exposure.[164] Most (77%) cases occur within 4 months of the last exposure to chemotherapy, and it is uncommon for cases to develop beyond 1 year.[162]

Patients with chemotherapy-associated TMA present with moderate to severe MAHA, thrombocytopenia, and renal insufficiency (median creatinine value of 4.2 mg/dl).[162] Approximately 1520% of patients develop neurologic dysfunction, which may include confusion, headache, and seizures. [162] [163] A unique feature is the development of noncardiogenic pulmonary edema in over 50% of patients. [163] Pulmonary function may also deteriorate rapidly after red blood cell or platelet transfusion,[162] which should be used with extreme caution in this setting.

Chemotherapy-associated TMA is presumed to result from direct toxicity to the endothelium. Mitomycin has been reported to inhibit the production of prostacyclin by umbilical vein endothelium, and the infusion of mitomycin-C into the rat kidney induces histologic changes similar to those of chemotherapy-induced

TMA.[166] Plasma levels of vWF increase after platinum-based chemotherapy, [167] and some,[168] but not all investigators,[169] have observed increased levels of ULvWF in affected patients. Chemotherapy-induced TMA confers a grave prognosis. Fewer than 20% of affected patients respond to plasma exchange and corticosteroids, [159] [162] and >50% die within 2 months. [162] More recently, however, responses to immunopheresis with staphylococcal protein A columns (ProSorba) have been reported in up to 50% of treated patients, typically those with no clinically evident tumor. [162] [170]

Miscellaneous, Drug-Associated TMA

Numerous anecdotal reports link the ingestion of specific drugs with the development of TMA. However, in only a few cases, such as quinine and possibly ticlopidine, can the association be considered confirmed. Quinine and its stereoisomer quinidine are common causes of drug-induced immune thrombocytopenia. [171] However, an association of quinine with TMA was first reported by Gottschall et al. in 1991, who described the course of three patients with a syndrome resembling idiopathic TMA-HUS.[172] Patients generally present with severe MAHA, platelet counts <50,000/l, and renal insufficiency, usually requiring dialysis. [173] Neurologic dysfunction occurs in a minority of patients, but can be severe, and occasional patients develop granulocytopenia and lymphopenia. [174] Although this disorder most commonly occurs after the ingestion of quinine tablets, cases have been reported after exposure to quinine in beverages such as tonic water. [175] Though the pathogenesis of this disorder is not well understood, quinine-dependent antibodies reactive with platelet-endothelial cell glycoproteins IIb/IIIa and Ib/IX are found in the serum of some patients,[172] [173] [174] and antibodies to neutrophil and T-lymphocyte membrane proteins occur in others. [173] [174] Antibodies reactive with an endothelial cell protein of similar size to platelet glycoprotein IIIa were reported in one study, [176] whereas in another, patient sera induced neutrophil aggregation and binding to cultured endothelial cells in a quinine-dependent manner. [174] The prognosis is favorable once quinine is withdrawn and plasma exchange instituted. [173] Symptoms may recur on re-exposure to quinine.[175]

Anecdotal evidence suggests an association between other drugs, including ticlopidine, [177] [177A] histamine H_2 -receptor antagonists,[178] and the Norplant contraceptive[179] with TMA. A thorough review of this topic has recently appeared. [161]

HIV-Associated TMA

An association between HIV infection and TMA has been recognized since 1984, [180] with a marked increase in the number of reported cases in recent years. HIV infection occurs in 1420% of patients presenting with TMA in retrospective series, [181] [182] and the association has been confirmed in a case-control study. [181] The prevalence of HIV infection in patients with TMA may be as high as 50% in some geographic regions. [183]

The presentation of HIV-associated TMA and idiopathic TMA are similar. [23] [183] [184] Initial diagnosis may be difficult because fever, anemia, [185] thrombocytopenia,[186] nephropathy, neurologic dysfunction, and elevated LDH levels secondary to lymphoma, drug reactions, or pulmonary infection with *Pneumocystis carinii* occur commonly in this population.[183]

The pathogenesis of HIV-associated TMA is not well understood. It has been speculated that the function of the microvascular endothelium is disrupted by opportunistic organisms, drugs such as fluconazole,[187] infection with HIV and alterations in plasma cytokine levels. [183] A single case of VTEC-TMA in a patient with HIV has been reported.[188]

Although HIV-TMA responds to plasma exchange,[23] [183] the role of antiplatelet agents or splenectomy is less certain. A case of TMA responding to therapy with zidovudine has been reported.[189] Most patients have advanced HIV infection on presentation and die within 1 year of their underlying disease. [23] [183]

Neuraminidase-Associated TMA

Neuraminidase-associated TMA is a rare disorder that occurs primarily in children in the setting of pneumonia and bacteremia caused by *Streptococcus pneumonia*.[190] All pathogenic pneumococci synthesize neuraminidase, which cleaves terminal sialic acid groups from carbohydrate residues expressed on the surface of erythrocytes, platelets, and glomerular cells, thereby exposing the Thomsen-Friedenreich (T-F) antigen. [24] [190] The exposed antigen is recognized by naturally occurring IgM antibodies leading to complement fixation and cellular damage. [24] [191] The renal tissue of affected individuals bind peanut agglutinin, indicative of exposed T-F antigen.[190] [191] Neuraminidase is also expressed by myxoviruses, influenza 2, *Streptococci, Bacteroides, Pseudomonas, Vibrio cholera, Clostridia*, and *Corynebacterium*. [24] Patients present with MAHA, thrombocytopenia, and acute renal failure in the setting of pneumonia. [190] Neurologic symptoms similar to those in other TMA syndromes may be present. Exposure of the T-F antigen also leads to difficulty in ABO cross-matching and may cause a positive direct antiglobulin test, providing additional clues to the diagnosis. [24] Few reports concerning treatment are available. Some cases resolved with supportive care alone. [190]

Pregnancy-Associated TMA

The differential diagnosis of MAHA in association with gestation is complex and has been the subject of recent review. [192] In some cases, TMA may be difficult to distinguish from other causes of MAHA unique to pregnancy, such as pre-eclampsia, the syndrome of hemolysis-elevated liver function tests and thrombocytopenia (HELLP), and acute fatty liver of pregnancy associated with DIC, each of which is managed differently. [192] The severity of the renal and neurologic abnormalities and the time during gestation at which the signs and symptoms of TMA first appear often provide clues needed to prevent critical delays in therapy. [192] [193]

Approximately 10% of cases of idiopathic TMA-TTP occur in association with pregnancy. [18] [194] In one series, 40 of 45 cases of TMA occurred antepartum; the mean gestational age at the onset of symptoms was 23.5 weeks.[195] In the absence of appropriate therapy, maternal and fetal mortality approach 90% and there is no evidence that uterine evacuation, by itself, leads to resolution. [196] [197] On the other hand, the prognosis has improved dramatically since the advent of plasma exchange, and there is no evidence that continuing pregnancy impairs the rate of response. [194] [195] Many patients carry to term successfully, although the overall risk of fetal loss remains appreciable.[196] Some patients with chronic, relapsing TMA develop recurrent episodes during pregnancy. [194] Anecdotal evidence suggests that prophylactic treatment with antiplatelet agents or corticosteroids may reduce the frequency and severity of these relapses. [194] Other patients have been managed with periodic transfusion of plasma or plasma fractions.[198] For the woman with a single episode of idiopathic TMA-TTP during pregnancy, the risk of recurrence appears small.

A second variant of TMA, previously termed malignant nephrosclerosis, irreversible postpartum renal failure, or postpartum intravascular coagulation, [193] also occurs in association with pregnancy. The disorder primarily affects primiparas who present with MAHA, thrombocytopenia, renal insufficiency, and hypertension beginning a minimum of 48 hours after an otherwise normal pregnancy. Neurologic symptoms are less frequent and severe. [192] In one series, the mean time to onset of symptoms was 26.6 days after delivery,[195] a finding that helps to distinguish TMA-HUS from other causes of pregnancy-associated

TRANSFUSION THERAPY IN TMA

Patients with TTP often develop symptomatic anemia because of bleeding and partially compensated hemolytic anemia. Packed red blood cells can be transfused safely in this setting. In elderly patients and in those with an impaired cardiovascular system, it may be prudent to provide some margin of safety when choosing a threshold for transfusion. In contrast, platelet transfusions are generally considered to be contraindicated in TTP. There are several published reports of patients under treatment for idiopathic TMA whose clinical situation deteriorated markedly within 124 hours of receiving allogeneic platelets; the authors are aware of similar experiences. Clinical deterioration after platelet transfusion can also occur in patients with VTEC-associated and drug-induced TMA. Postmortem examination of such patients has revealed widespread microthrombi involving the brain, heart, lung, kidney, and multiple other organs. Many of these lesions appeared fresh, in that they showed no evidence of overgrowth by proliferative endothelial cells. Sudden death has also been reported on rare occasions in patients who responded to plasma therapy with a rapid rise in the platelet count. Thus, we consider platelet transfusions to be contraindicated in patients with TMA in the absence of overt life-threatening bleeding or a comparable hemostatic challenge.

MAHA and thrombocytopenia such as pre-eclampsia, HELLP, and TMA-TTP. Occasional patients with familial TMA display clinical manifestations for the first time during pregnancy, and occasional cases associated with VTEC infection have occurred intrapartum. [199] The prognosis of pregnancy-associated TMA-HUS remains relatively poor. In older series, the mortality rate approached 50% and an additional 1015% were left with chronic renal insufficiency. [200] More recently, responses to plasma exchange or infusion have been reported, [192] though their frequency is uncertain (see box on transfusion therapy). Uterine evacuation does not lead to remission, and fetal outcome depends on successful treatment of the mother. [192]

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FUTURE DIRECTIONS

The TMA syndromes remain relatively uncommon. However, the incidence and mortality associated with idiopathic and VTEC-associated TMA are increasing, [20] despite the fact that effective therapy for these syndromes has been available for two decades. [26] [62] [64] Further, the etiology and the pathogenesis of TMA remain poorly understood. Additional insight into the biology of TMA may result from cloning and molecular characterization of the vWF-cleaving protease and a clearer definition of the causes of endothelial damage and apoptosis. Nevertheless, a disorder universally fatal 30 years ago has a far better prognosis today, though, remarkably, treatment remains largely empiric.

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Chapter 129 - Thrombocytopenia Due to Platelet Destruction and Hypersplenism

Theodore E. Warkentin
John G. Kelton

INTRODUCTION

Thrombocytopenia is defined as a platelet count below the lower limit of the normal range (150×10^9/L). Sometimes, an expanded definition of thrombocytopenia is appropriate. For example, an abrupt drop in the platelet count can signify the onset of a platelet-destructive process such as heparin-induced thrombocytopenia or bacteremia even if the platelet count remains $>150 \times 10^9$/L.

When a clinician is faced with a thrombocytopenic patient, three questions must be asked. First, could the patient have pseudothrombocytopenia? Second, what is the most likely explanation for the thrombocytopenia? And third, what are the likely risks to the patient suggested by the cause and severity of the thrombocytopenia? For example, very severe thrombocytopenia caused by drug-dependent antibodies or platelet-reactive autoantibodies is usually associated with bleeding. In contrast, mild to moderate thrombocytopenia caused by heparin-dependent antibodies or attributable to disseminated intravascular coagulation (DIC) secondary to adenocarcinoma is strongly associated with thrombosis. Often, the underlying cause of the thrombocytopenia (e.g., bacteremia, cancer, cirrhosis), rather than the thrombocytopenia itself, poses the greatest risk.

Thrombocytopenia can be caused by four general mechanisms: (1) platelet underproduction; (2) increased platelet destruction; (3) platelet sequestration; and (4) hemodilution. Platelet underproduction usually occurs with underproduction of other blood cell lines, and is therefore often accompanied by pancytopenia. Thrombocytopenia caused by increased platelet destruction develops when the rate of platelet destruction surpasses

TABLE 129-1 -- General Mechanisms of Platelet Destruction

Type of Thrombocytopenia	Specific Examples
Immune-mediated	
Autoantibody-mediated platelet destruction via reticuloendothelial system (RES)	ITP; secondary ITP (e.g., immune thrombocytopenia associated with lymphoproliferative disease, collagen vascular disease, infections such as infectious mononucleosis,[a] HIV)
Alloantibody-mediated platelet destruction via RES	Neonatal alloimmune thrombocytopenia;[a] post-transfusion purpura; passive alloimmune thrombocytopenia; alloimmune platelet transfusion refractoriness[a]
Antibodies against microbial antigens adsorbed onto platelets	Malaria-associated thrombocytopenia
Drug-dependent, antibody-mediated platelet destruction via RES	Drug-induced immune thrombocytopenic purpura (e.g., quinine, quinidine, sulfa drugs, vancomycin, etc). See Table 129-6
Platelet activation by binding of IgG Fc of drug-dependent IgG to platelet FcRIIa receptors	Heparin-induced thrombocytopenia
Non-immune mediated	
Platelet activation via thrombin or inflammatory cytokines	Disseminated intravascular coagulation (DIC);[a] septicemia and other systemic inflammatory response syndromes (e.g., adult respiratory distress syndrome, fat embolism syndrome, pancreatitis)
Platelet destruction via ingestion by macrophages (hemophagocytosis)	Infections, malignant lymphoproliferative disorders
Platelet destruction via uncertain mechanisms (e.g., possible platelet-activating proteinase)	Thrombotic thrombocytopenic purpura (TTP);[a] hemolytic-uremic syndrome (HUS)[a]
Platelet losses on artificial surfaces	Cardiopulmonary bypass surgery; use of intravascular catheters
Decreased platelet survival associated with cardiovascular disease	Congenital and acquired heart disease; cardiomyopathy; pulmonary embolism

[a] See chapters 126 and 128 for a discussion of thrombocytopenia in these disorders.

the ability of the bone marrow to produce platelets, and can be caused by immune or nonimmune mechanisms (Table 129-1). Thrombocytopenia caused by platelet sequestration involves platelet redistribution, usually resulting from an increase in the splenic platelet pool. Hemodilution is characterized by a decrease in the numbers of red cells, white cells, and platelets caused by the administration of colloids, crystalloids, or blood products.

This classification can be helpful for all thrombocytopenic patients, including those who develop thrombocytopenia in pregnancy. However, in pregnant patients, the differential diagnosis must be expanded to include causes unique to these patients (Table 129-2).

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APPROACH TO THE THROMBOCYTOPENIC PATIENT

History and Physical Examination

Certain questions should be asked, including (1) the location and severity of bleeding (if any); (2) the temporal profile of the hemostatic defect (acute, chronic or relapsing); (3) symptoms of a secondary illness, such as a neoplasm, infection, or systemic lupus erythematosus (SLE); (4) a history of recent medication use, alcohol ingestion, or a transfusion; (5) risk factors for infection with human immunodeficiency virus (HIV); and (6) family history of thrombocytopenia.

TABLE 129-2 -- Differential Diagnosis of Thrombocytopenia in Pregnancy

Incidental thrombocytopenia of pregnancy (gestational thrombocytopenia)
Pre-eclampsia/eclampsia[a]
Thrombotic microangiopathy
Thrombotic thrombocytopenic purpura (TTP)
Hemolytic-uremic syndrome (note: post-partum HUS syndrome)
Disseminated intravascular coagulation (DIC) secondary to
Endometritis
Amniotic fluid embolism
Retained fetus
Abruptio placentae
Pre-eclampsia/eclampsia

[a]Pre-eclampsia/eclampsia usually is not associated with overt DIC.

During the physical examination, evidence of hemostatic impairment should be sought. The signs of platelet bleeding include petechiae and purpura. Petechiae typically occur on the dependent regions of the body or on traumatized areas. Spontaneous mucous membrane bleeding (wet purpura), epistaxis, and gastrointestinal bleeding indicate more serious hemostatic defects. Although petechiae are common in patients whose platelet count is <1020 × 10^9 /L, most patients whose platelet count is >50 × 10^9 /L do not exhibit any signs of hemostatic impairment. The physical examination often suggests an explanation for the thrombocytopenia. For example, enlarged lymph nodes can indicate a viral infection, such as infectious mononucleosis or HIV, or a neoplastic lymphoproliferative disorder. An enlarged spleen suggests hypersplenism.

Laboratory Evaluation

Laboratory tests used for the investigation of a patient with thrombocytopenia are summarized in Table 129-3 . The blood film is examined to exclude pseudothrombocytopenia, which is characterized by in vitro platelet clumping. This phenomenon, which occurs in about 1 in 1,000 blood samples examined, is caused by naturally-occurring, platelet GPIIb/IIIa-reactive autoantibodies that produce aggregation of platelets in the presence of the calcium-chelating anticoagulant, ethylenediaminetetraacetate (EDTA).[1] [2] The platelet aggregates are not counted by the electronic particle counter, so the automated platelet count appears falsely low. The correct platelet count can usually be determined by collecting the blood into sodium citrate or heparin anticoagulants or performing a count of non-anticoagulated fingerprick samples; maintaining the blood sample at 37°C will also reduce platelet clumping in many instances. [2] EDTA-dependent pseudothrombocytopenia has no

TABLE 129-3 -- Laboratory Tests Used to Investigate a Patient with Thrombocytopenia

Test	Rationale
Common tests	
Complete blood count (CBC)	Isolated thrombocytopenia is usually caused by platelet destruction, whereas involvement of all cell lines suggests underproduction or sequestration
Examination of the blood film	Pseudothrombocytopenia
	Toxic changes and granulocyte left shift suggest septicemia
	Atypical lymphocytes suggest viral infection
	Red cell fragments suggest TTP or HUS
	Parasites (e.g., malaria)
	White cell inclusions suggest hereditary macrothrombocytopenia
Blood cultures	Bacteremia, fungemia
Antinuclear antibody	Systemic lupus erythematosus (SLE)
Thyroid stimulating hormone (TSH) level	Thyroid dysfunction can accompany ITP

Direct antiglobulin test	Exclude concomitant immune hemolysis and ITP (Evans syndrome)
Coagulation tests	
Activated partial thromboplastin time, prothrombin time (INR), thrombin time, fibrinogen, D-dimer	Disseminated intravascular coagulation (DIC)
Lupus anticoagulant assay	Lupus anticoagulant syndrome
Anticardiolipin antibody assay	Antiphospholipid antibody syndrome
Serum protein electrophoresis; quantitative immunoglobulins	ITP associated with lymphoproliferative disorders (monoclonal); hypersplenism associated with chronic hepatitis (polyclonal)
HIV serology	HIV-associated thrombocytopenia
Bone marrow aspirate, biopsy	Assess megakaryocyte numbers and morphology; exclude primary marrow disorder
Specialized tests	
Glycoprotein-specific platelet antibody assays (e.g., MAIPA)	Specific assay for primary and secondary ITP
Drug-dependent platelet-associated IgG	Specific assay for drug-induced immune thrombocytopenic purpura (DITP)
Drug-dependent platelet activation (e.g., platelet serotonin release assay)	Heparin-induced thrombocytopenia (HIT)
Heparin/platelet factor 4 ELISA	Heparin-induced thrombocytopenia (HIT)
Radionuclide platelet lifespan studies (e.g., ^{111}In platelet survival study)	Define the mechanism of thrombocytopenia; identify an accessory spleen post-splenectomy

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; HUS, hemolytic uremic syndrome; IgG, immunoglobulin G; INR, international normalized ratio; ITP, idiopathic (immune) thrombocytopenic purpura; MAIPA, monoclonal antibody immobilization of platelet antigens; TTP, thrombotic thrombocytopenic purpura.

pathologic significance, other than potentially placing a patient in jeopardy for inappropriate treatment for thrombocytopenia that does not exist. A much less common (1 in 10,000 blood samples), antibody-mediated pseudothrombocytopenic disorder is platelet satellitism, in which rosette-like clusters of platelets are seen around neutrophils.[3] This entity is produced by IgG antibodies that recognize EDTA-induced cryptic epitopes both on platelet GPIIb/IIIa complexes and neutrophil FcIII receptors.

Bone marrow examination can be helpful in assessing platelet production, particularly if megakaryocytes are reduced or abnormal in appearance. Examination of the bone marrow can be diagnostic in a few disorders (e.g., leukemia, metastatic tumor, megaloblastic anemia).

Elevated platelet-associated IgG (PAIgG) is observed in patients with either immune or non-immune thrombocytopenia; [4] therefore, these assays are not useful. There is evidence that glycoprotein-specific platelet antibody assays, such as the monoclonal antibody immobilization of platelet antigens (MAIPA) assay, [5] are relatively specific for autoimmune thrombocytopenic disorders.[6]

In patients in whom the mechanism of chronic thrombocytopenia is unclear, an ^{111}In-labeled autologous platelet survival study is performed. Three patterns can be seen: (1) a normal platelet survival and recovery (underproduction); (2) a marked reduction in the platelet life span (increased destruction); and (3) a reduced recovery, but a normal or near normal life span (sequestration). ^{111}In-labeled platelets can also be used to image accessory splenic tissue in patients with idiopathic thrombocytopenic purpura (ITP) who fail splenectomy or develop post-splenectomy relapse.

Therapy

Bleeding risk for thrombocytopenic patients can be reduced by avoiding drugs that can impair hemostasis (e.g., alcohol, antiplatelet agents, anticoagulants) and invasive procedures (intramuscular injections). If drug-induced thrombocytopenia is suspected, as many medications as possible should be stopped. Life-threatening bleeding episodes should be treated with platelet transfusions regardless of the mechanism of the thrombocytopenia.

The underlying cause and anticipated natural history of the thrombocytopenic disorder influences the decision of whether to transfuse platelets for prophylaxis against bleeding. As a general rule, patients with chronic thrombocytopenic disorders characterized by increased platelet destruction (e.g., chronic ITP) or chronic underproduction (e.g., aplastic anemia, myelodysplasia) can often tolerate long periods of severe thrombocytopenia without major bleeding. In addition, a prophylactic transfusion can trigger alloimmunization against HLA or platelet antigens and thereby jeopardize future therapeutic platelet transfusions. Therefore, prophylactic platelet transfusions are seldom indicated for these patients. However, when such patients have a transient but major hemostatic risk such as a significant head injury, trauma or following major surgery, platelet count should be maintained above 50×10^9 /L. Invasive procedures

such as thoracentesis, paracentesis, and liver biopsy are usually not associated with excess bleeding if the platelet count is >50×10^9 /L. [7] [8] Although a platelet count trigger of 20×10^9 /L has traditionally been used at many centers to guide prophylactic platelet transfusions in leukemic patients, most physicians now use a lower threshold.[9]

Prophylactic platelet transfusions should not be given to patients with heparin-induced thrombocytopenia, thrombotic thrombocytopenic purpura, or hemolytic-uremic syndrome, since platelet transfusions could exacerbate platelet-mediated thrombotic complications.

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ANATOMY AND PHYSIOLOGY

The Spleen: Anatomy and Function

The spleen is a small, well-perfused organ receiving about 5% of the cardiac output. [10] Its anatomy is uniquely suited for its function; the progressive branching of the splenic artery into the trabecular and then central arteries helps separate the plasma from the cellular elements. [10] The central arteries arise perpendicularly from the trabecular arteries and skim off the plasma layer from the cells. Soluble antigens in the plasma are delivered to the white pulp, where phagocytic cells process the antigens and initiate antibody production.

The cellular-rich hemoconcentrated fraction of the blood is delivered to the red pulp. A small percentage of this blood flows directly to the splenic veins (the closed system), but most moves into the splenic cords (the open system). Here the cellular elements percolate through a meshwork of reticulum fibers, reticuloendothelial cells, and supporting cells to reach the splenic sinuses. The cells enter the sinuses by passing through fenestrations in the basement membrane of the endothelial cells lining the sinuses. The blood exits through the splenic vein into the portal system. Because the veins in the portal system lack valves, any increase in portal pressure is transmitted to the splenic microcirculation.

The spleen has a number of important roles. [10] It is the largest lymphoid organ in the body and plays a pivotal role in host defense by clearing microorganisms and antibody-coated cells. The spleen is also important for antibody synthesis, especially against soluble antigens. The filtering function of the spleen includes (1) culling (removal of damaged or senescent cells and bacteria), (2) pitting (removal of red cell inclusions and parasites), and (3) remodeling (reticulocyte sequestration and maturation). The spleen also acts as a (4) large reservoir for platelets (about one-third of the platelet mass). [11] In contrast, the human spleen contains <2% of the total red blood cell mass, although in some animals (dogs and cats), the spleen is a much more important red cell reservoir.

Physiologic Platelet Sequestration

Radiolabeled platelet studies have shown that approximately 30% of the total platelet mass exists as a freely exchangeable pool in the spleen. [11] Since the normal platelet life span is 910 days, a platelet spends about one-third of its life, or 3 days, within the spleen. In patients with hypersplenism, as many as 90% of platelets are found in the spleen.

After the labeled platelets are injected, accumulation is apparent in both the liver and spleen. [12] [13] An initial, irreversible phase of hepatic uptake occurs. This equilibrates during the first 5 minutes and may reflect hepatic clearance of platelets damaged in the labeling procedure. Simultaneously, there is a slow rise in activity in the spleen that peaks in about 20 minutes. Splenic platelet uptake is thus dependent on input (spleen blood flow) and output (clearance).

The splenic platelet pool size can be decreased and the platelet count increased with intravenous infusions of epinephrine in normal individuals and patients with splenomegaly. [11] In contrast, isoproterenol (a $_1$ - and $_2$ -agonist) increases the pool size. [14] Splenic blood flow increases with increasing spleen size, although perfusion (flow per unit tissue volume) falls. [15] Blood flow can be increased in some inflammatory disorders (e.g., SLE) without an increase in spleen size. A marked increase or decrease in splenic perfusion alters the proportion of platelets within the spleen.

The most important determinant of the splenic platelet pool is the spleen size. [15] The measurement of spleen size can thus be helpful in predicting the degree of thrombocytopenia expected from excess platelet pooling in the spleen. For example, if the splenic platelet pool is 90% (i.e., 10% outside the spleen), the platelet count will be reduced by a factor of 7 (since normally 70% of platelets lie outside the spleen). Consequently, and as a general rule, even if the spleen is massively enlarged, very severe thrombocytopenia is virtually never seen. On the other hand, mild splenomegaly that may not be detectible on physical examination can explain mild thrombocytopenia.

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PATHOLOGIC PLATELET SEQUESTRATION: HYPERSPLENISM

Definition

Hypersplenism is a syndrome characterized by splenomegaly and any or all of the following cytopenias: anemia, leukopenia, or thrombocytopenia. Implicit in the definition is that the cytopenias will be corrected following splenectomy. Although splenomegaly is almost always present in hypersplenism, many patients with splenomegaly do not have hypersplenism. Almost always, the hypersplenism is the result of an identifiable pathologic process, but in some cases the cause of the splenomegaly remains elusive and is termed *primary*.

Pathogenesis

A list of many of the disorders producing splenomegaly and hypersplenism is presented in Table 129-4 . An increase in the size of the spleen can be caused by several mechanisms. An increase in the workload of the spleen can result from immunologic stress (infection, inflammation, or an autoimmune disorder) or from increased red cell removal (red cell membrane disorders, hemoglobinopathies). Portal hypertension will also increase the size of the spleen, and is termed congestive splenomegaly. Benign and malignant infiltrative disorders can also increase splenic size (infiltrative splenomegaly) and cause hypersplenism. Some of these disorders produce thrombocytopenia by more than just hypersplenism: e.g., marrow replacement by tumor, immune-mediated platelet clearance. Thus, the demonstration of an enlarged spleen does not necessarily mean that the cytopenias are caused by hypersplenism.

Cytopenias

The thrombocytopenia of hypersplenism is caused primarily by increased splenic platelet pooling. [11] [15] A massively enlarged spleen can hold >90% of the total platelet mass. In the absence of pathologic platelet overproduction, the total body platelet mass is usually normal and the platelet life span is near-normal. [11] Usually, the splenic transit time remains normal (approximately 10 minutes), but the absolute number of platelets retained within the enlarged spleen is increased. All these platelets remain part of the exchangeable pool. In hypersplenism, the platelet count is usually between 50150 × 10^{9} /L, and almost never <20 × 10^{9} /L. Therefore, patients with hypersplenism almost never have evidence of hemostatic impairment

TABLE 129-4 -- Differential Diagnosis of Splenomegaly and Hypersplenism

Infections
Acute
Viral (viral hepatitis, infectious mononucleosis, cytomegalovirus)
Bacterial (septicemia, salmonellosis, brucellosis, splenic abscess)
Parasite (toxoplasmosis)
Subacute and chronic
Subacute bacterial endocarditis
Tuberculosis
Malaria
Kala-azar
Fungal disease
Inflammation
Felty syndrome
Systemic lupus erythematosus
Serum sickness
Rheumatic fever
Sarcoidosis
Congestive splenomegaly
Intrahepatic
Cirrhosis
Extrahepatic
Portal vein obstruction
Splenic vein obstruction
Hepatic vein occlusion (Budd-Chiari syndrome)
Chronic passive congestion
Heart failure
Hematologic disorders
Red cell disorders: hemolytic anemias, thalassemia, sickle cell disorders

Neoplasia
Malignant
Myeloproliferative disorders
Myeloid metaplasia
Polycythemia rubra vera
Essential thrombocythemia
Chronic leukemia
Chronic myeloid leukemia
Chronic lymphocytic leukemia
Hairy cell leukemia
Lymphoma
Acute leukemia
Hairy cell leukemia
Malignant histiocytosis
Benign
Hamartoma
Hemangioma
Lymphangioma
Fibroma
Storage disease
Gaucher disease
Niemann-Pick disease
Miscellaneous
Amyloidosis
Cysts

attributable to thrombocytopenia, nor do they need specific interventions to raise the platelet count. Plasma volume expansion occurs in hypersplenism, but hemodilution plays a relatively minor role in the thrombocytopenia. Similarly, some patients have evidence of marrow suppression, but, again, its contribution to the thrombocytopenia tends to be modest.

Sequestration and hemodilution combine to produce the anemia of hypersplenism. [16] An expansion of the plasma volume accompanies hypersplenism; the degree of expansion is proportional to the size of the spleen. In the absence of persistent portal hypertension, this expansion can be improved or corrected by splenectomy.

The neutropenia of hypersplenism is caused by an increase in the marginated granulocyte pool, a portion of which is located in the spleen. [17] Neutropenia of hypersplenism is usually asymptomatic.

Diagnosis

Thrombocytopenia is likely to be caused by hypersplenism when (1) splenomegaly is present; (2) the thrombocytopenia is mild to moderate in severity; (3) moderately-reduced or low-normal neutrophil and hemoglobin levels are found; and (4) no or minimal evidence for impaired hematopoiesis is observed on bone marrow examination. Ultrasonography and radionuclide imaging are of comparable sensitivity in documenting splenomegaly, [18] and either test should be performed when suspected splenomegaly is not evident on clinical examination. The mean platelet volume is usually slightly decreased in hypersplenism, but this finding is not sufficiently specific to be diagnostically useful. An ^{111} In-labeled platelet survival study can be diagnostic of hypersplenism, demonstrating reduced platelet recovery and a normal platelet life span. Determining the cause of the splenomegaly is usually the most important issue.

Therapy

Several maneuvers can improve or correct the cytopenias attributable to hypersplenism, including total or partial [19] [20] splenectomy, partial splenic embolization, [21] [22] [23] andin patients with congestive splenomegalysurgical [24] or transjugular intrahepatic portosystemic shunting. [25] However, cytopenias secondary to hypersplenism, per se, are almost never sufficiently severe to justify treatment. Consequently, the decision to perform one of these interventions usually depends upon other considerations. For example, splenectomy should be considered for relief of pain or early satiety associated with massive splenomegaly (e.g., myelo- or lymphoproliferative disorders [26]), or for splenomegaly of unknown origin (investigation of possible splenic lymphoma [27]).

Short-term complications from splenectomy include infections, bleeding, and thromboembolism. The major long-term risk of splenectomy is overwhelming septicemia, which can be reduced by vaccination. All patients should be vaccinated against pneumococcus, meningococcus, and hemophilus, preferably at least 2 weeks prior to splenectomy. [28] Splenectomy for congestive hypersplenism in the setting of portal hypertension has a high morbidity and mortality; consequently, splenectomy is generally considered contraindicated for cytopenias in these patients. [29] Splenectomy is also associated with a high morbidity (50%) and mortality (1015%) in myeloid metaplasia, and does not alter the natural history of this disorder. [30] Thus, splenectomy is usually performed for palliation of intractable symptoms in these patients.

Splenectomy in Gaucher disease usually corrects the cytopenias, relieves abdominal discomfort, and in children, improves growth. [31] Partial rather than total splenectomy has been used in an attempt to avoid shifting the deposition of glucocerebroside from spleen to bones. Often, however, recurrence of splenomegaly and hypersplenism results after partial splenectomy, [19] and this technique should still be considered experimental. [31] Enzyme replacement therapy can reduce the morbidity from hypersplenism.

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DRUG-INDUCED THROMBOCYTOPENIC SYNDROMES

Many drugs can cause thrombocytopenia. Some drugs (e.g., anticancer chemotherapy, valproic acid [32]) cause dose-dependent thrombocytopenia, generally via myelosuppressive mechanisms. An important disorder encountered by hematologists is

unexpected thrombocytopenia caused by immunologic (idiosyncratic) mechanisms. The frequency of these reactions varies considerably among drugs, and ranges from very rare (<1/10,000) for commonly used drugs such as acetaminophen, aspirin, quinine/quinidine, and trimethoprim/sulfamethoxazole, [33] to common (13%) for other drugs such as gold[34] and unfractionated heparin. [35] [36] [37]

Immunologic drug-induced thrombocytopenia can be divided into two general groups. One group, which represents the largest number of drugs implicated, is exemplified by quinine- and quinidine-induced thrombocytopenia. These disorders are mediated by the binding of the Fab terminus of IgG to a complex comprised of drug (or drug metabolite) and a platelet membrane component (typically platelet GPIb/IX or GPIIb/IIIa) (Fig. 129-1). The Fc portions of the IgG molecules are not involved in the binding to platelets, but are available for interaction with the Fc receptors on phagocytic cells of the reticuloendothelial system. Severe thrombocytopenia (platelet count usually <20 × 10^9 /L) is often observed in these patients, and they generally present with the abrupt onset of bleeding.

The other general type of drug-induced thrombocytopenia is exemplified by heparin-induced thrombocytopenia (HIT). In this disorder, the Fab portion of the pathogenic IgG binds to platelet factor 4 (PF4), an -granule protein that is immunogenic when complexed to heparin or other sulfated polysaccharides. The Fc portions of the IgG molecules bind to the platelet FcIIa receptors, triggering platelet activation. Because the Fc portion of the IgG molecules are bound to the platelet Fc receptor, they may not be available to the Fc receptors of the reticuloendothelial system, which may explain the less-severe thrombocytopenia that characterizes HIT.

Heparin-Induced Thrombocytopenia

Heparin is the most important cause of immune-mediated drug-induced thrombocytopenia for several reasons. First, heparin is a widely used anticoagulant. Second, HIT is relatively common, occurring in 13% of patients who receive unfractionated heparin for 714 days, respectively. [35] [36] Third, HIT paradoxically can cause life- or limb-threatening venous or arterial thrombosis.[35]

Pathogenesis

Heparin binds reversibly and saturably to platelets and can weakly activate platelets in vitro. [36] In general, the direct platelet-activating effects of heparin, as well as its immunogenicity, are proportional to its size and degree of sulfation; consequently, low-molecular-weight heparin is less likely to cause HIT than is unfractionated heparin (see Fig. 129-2). [35]

HIT is caused by heparin-dependent IgG that activates platelets via their FcIIa receptors. [38] The antigen is a complex between heparin and platelet factor 4. [39] [40] [41] HIT antibodies can also recognize PF4-related chemokines, such as interleukin-8 and neutrophil-activating peptide-2, [42] but the relevance of this fact is unknown. Some patients form IgM and IgA heparin-dependent antibodies,[43] but the clinical significance of this is uncertain. A unique laboratory characteristic of HIT is that large heparin concentrations (10100 U/ml) inhibit platelet activation by the pathogenic IgG, [44] most likely by displacing the pathogenic IgG from surface-bound heparin/PF4 complex. This laboratory feature is exploited in diagnostic testing for HIT (see the section, Diagnosis).

In vivo thrombin generation contributes to the pathogenesis

Figure 129-1 Proposed binding of quinine/quinidine-induced thrombocytopenia.

Figure 129-2 Pathogenesis of heparin-induced thrombocytopenia. Heparin produces mild platelet activation, resulting in release of platelet factor 4 (PF4) from platelet granules, and the formation of immunogenic heparin/PF4 complexes. B lymphocytes generate IgG that recognize the heparin/PF4 complexes; the Fc tails of the IgG bind to platelet FcII receptors, resulting in Fc receptor clustering and consequent strong platelet activation. Platelet-derived microparticles are generated that accelerate thrombin generation. The HIT antibodies also recognize PF4 bound to endothelial heparan sulfate, leading to immunoinjury that causes endothelial activation. Tissue factor expressed on the endothelial surface further produces thrombin generation. This model of thrombin generation in HIT helps explain some of the unusual clinical manifestations of HIT (e.g., venous limb gangrene, DIC) and provides a rationale for treatment that reduces thrombin generation (e.g., danaparoid, recombinant hirudin, argatroban).

of some of the unusual sequelae of HIT,[37] which can include venous thrombosis,[35] warfarin-associated venous limb gangrene,[45] [46] and DIC.[36] Thrombin generation in HIT results from the generation of procoagulant microparticles from platelets activated by HIT antibodies, [47] as well as via expression of tissue factor by endothelial cells that have been activated by HIT antibody that recognizes PF4 bound to endothelial heparan sulfate. [41] [48]

Only a minority of patients who generate HIT antibodies develop thrombocytopenia. [35] Differing risk for HIT results from variability among platelets in their ability to be activated by HIT IgG,[49] [50] as well as varying titres of HIT antibodies. Clinical factors also appear to explain differences in risk for sequelae: e.g., postoperative orthopedic patients are at high risk for venous thrombosis, whereas postoperative cardiovascular patients are at relatively high risk for arterial thrombotic events. [35] [51]

Clinical Features

Most patients with HIT develop mild to moderate thrombocytopenia: the mean platelet count nadir in many series is approximately 50 × 10^9 /L. [36] Very rarely, the platelet count is as low as 5 × 10^9 /L. DIC and red cell fragmentation may be present in these patients with severe thrombocytopenia. Thrombosis in HIT can

complicate a relative platelet count fall of >30%, but prior to the development of thrombocytopenia as conventionally defined (<150 × 10^9 /L).[35] A high heparin requirement to maintain a therapeutic partial thromboplastin time (heparin resistance) is a common, but nonspecific, finding in patients with HIT.

The thrombocytopenia almost always begins 510 days after the initiation of heparin therapy. [35] HIT is prothrombotic: at least 3050% of patients will develop a thrombosis in association with HIT.[35] [36] [52] Both venous (deep vein thrombosis, pulmonary embolism) and arterial (especially aortic and iliofemoral arterial thrombosis, cerebrovascular accidents, myocardial infarctions) thrombi occur. Other complications include painful dermal erythema or necrosis at the sites of subcutaneous heparin injection,[53] and acute inflammatory [54] or transient memory disturbances[55] following heparin bolus injections to sensitized individuals. Unexplained hypotension or abdominal pain in a HIT patient suggests bilateral adrenal hemorrhagic infarction, which can lead to acute adrenal failure; this hemorrhagic necrosis appears to result from adrenal vein thrombosis. [56]

Diagnosis

Sensitive and specific assays for HIT antibodies have been developed based on the observation that the pathogenic IgG activates washed platelets. [44] [49] [57] These assays are more sensitive and specific than the simpler and more widely-used citrated platelet-rich plasma methods. [58] Important quality control maneuvers include the selection of platelet donors whose platelets respond well to Fc receptor stimulation, as well as the inclusion of negative

and positive HIT sera of varying reactivity to ascertain that the test platelets can identify weaker HIT sera. [49] A characteristic activation profile is produced by HIT serum: increased platelet activation at low heparin concentrations (0.050.3 U/ml), but background platelet activation at high heparin concentrations (10100 U/ml). [44] [49] [57] A gold standard assay (the ^{14}C-serotonin release assay[44] [49]) has been shown to have high sensitivity and specificity (>95%) for HIT antibodies in a blinded investigation.[35] Sometimes, heparin-independent platelet activation can be triggered by HIT serum ("indeterminate" result); for these sera, an enzyme-linked immunosorbent assay (ELISA) utilizing a heparin/platelet factor 4 target can be diagnostic. [37] Conversely, HIT antibodies that do not recognize the major antigen, heparin/PF4, require an activation assay, such as the serotonin release assay, for diagnosis. Thus, reference laboratories should be able to perform both activation and antigen assays.[37] [59]

Management

Heparin should be discontinued in patients with suspected HIT. However, there appears to be a high risk (approximately 50%) for subsequent thrombosis in patients with serologically confirmed HIT in whom heparin is simply discontinued. [52] Some physicians therefore consider continued use of anticoagulant agents even in patients with isolated thrombocytopenia attributable to HIT. [37] The recognition for an important role for thrombin generation in the pathogenesis of HIT has provided a rationale for the use of anticoagulant agents that reduce thrombin generation. Currently, three such anticoagulantsdanaparoid, recombinant hirudin, and argatrobanappear to be effective for the treatment of HIT. Differing pharmacokinetic and other considerations ensure that no single agent is ideal for all patients.

Danaparoid sodium (Orgaran), formerly known as Org 10172, is a mixture of anticoagulant glycosaminoglycans with predominant anti-factor Xa activity. In 1993, an uncontrolled report[60] of 230 HIT patients treated with danaparoid suggested that about 90% of patients respond favorably, as defined by platelet count recovery without new or progressive thrombosis during treatment. The authors have used this drug successfully in HIT patients, and have observed that a marker of in vivo thrombin generation (thrombinantithrombin complexes) falls during danaparoid treatment. [61] Anticoagulant monitoring can be performed using anti-factor Xa levels, although this is usually not necessary because of predictable anticoagulant effects with standard dosing (see Table 129-5). Danaparoid levels can accumulate in patients with renal failure, and monitoring should be performed in these patients. Anywhere from 1040% of HIT patient sera exhibit some degree of in vitro cross-reactivity with danaparoid; however, clinically-significant cross-reactivity

TABLE 129-5 -- Treatment Protocols for HIT Complicated by Thrombosis

Danaparoid
Loading dose: 2,250 U IV bolus,[a] followed by 400 U/hour × 4 hours, then 300 U/hour × 4 hours. *Maintenance*: 150200 U/hour, with subsequent dose adjustments made using anti-Xa levels (target range 0.50.8 anti-Xa U/mL), if assay available [b]
Recombinant hirudin
Loading dose: 0.4 mg/kg bolus. *Maintenance*: 0.15 mg/kg/hr infusion, with dose-adjustments to maintain APTT 1.53.0 times the median of the normal laboratory APTT range.

Modified from Warkentin, et al.,[37] with permission.

[a]Adjust bolus for body weight: <60 kg, 1,500 U; 6075 kg, 2,250 U; 7590 kg, 3,000 U; >90 kg, 3,750. The recommendations are based on 750 U ampule availability; for 1,250 U ampules, the loading dose would be 2,500 U, etc.
[b]The calibration curve for anti-Xa testing must be derived using danaparoid rather than low-molecular-weight heparin.

is uncommon (<5%). Our practice is to use danaparoid for its immediate anticoagulant effect in HIT patients without delaying treatment to perform in vitro cross-reactivity testing.[59] Danaparoid has been approved for the treatment of HIT in some countries (e.g., the Netherlands, New Zealand), although it is often used off-label in other countries (e.g., United States, Canada) where it has been approved for antithrombotic prophylaxis. [59]

The recombinant hirudin derivative, lepirudin (Refludan), is a 65-amino acid polypeptide that inactivates thrombin by forming a tight, noncovalent 1:1 complex with it. Evaluation of a standard lepirudin protocol (Table 129-5) to treat HIT complicated by thrombosis in a historically controlled study in Germany [62] led to its approval for this indication first in the European Union (March 1997), and then in the United States (March 1998). Hirudin is metabolized and excreted by the kidney, and the drug must be avoided or the dosage reduced in patients with renal failure. Anticoagulant monitoring is performed using the partial thromboplastin time (target PTT, 1.53.0 times the median of the laboratory normal range).

Argatroban is a synthetic antithrombin that has been used to treat HIT in Japan and the United States. [63] A recent, historically-controlled study suggested that this agent reduces the frequency of thrombosis in patients with HIT. Argatroban is metabolized by the liver, and may be used in patients with renal failure.

Warfarin and other oral anticoagulants should not be used to treat patients with active HIT. [37] [45] [46] HIT associated with deep vein thrombosis (DVT) is a high-risk situation; this is because DVT in a HIT patient treated with warfarin can progress to venous limb gangrene. [45] The laboratory marker of this unusual syndrome is a high INR (generally, >4.0), which corresponds to the combination of a marked reduction of protein C together with persisting thrombin generation (as shown by elevated thrombinantithrombin complex levels) during warfarin therapy. [45] [46] However, warfarin can be given safely to patients whose thrombin generation is controlled (e.g., using danaparoid or hirudin), or following resolution of the HIT.

A defibrinogenating snake venom, ancrod (Arvin), has been used in uncontrolled studies to treat HIT, [64] and is approved in Canada for this indication. The drug, given at a dose of 1 U/kg over 1224 hours, reduces the fibrinogen to low levels (<0.1 g/L), thereby reducing plasma viscosity. Ancrod does not reduce, and may even increase, thrombin generation in HIT; [61] this may explain why some patients have developed venous limb gangrene during combined treatment with ancrod and warfarin.[45] Ancrod should not be used in HIT patients with evidence of increased thrombin generation.

Using sensitive assays, all low-molecular-weight heparin preparations are as capable of activating platelets in the presence of HIT serum as unfractionated heparin (i.e., 100% in vitro cross-reactivity). Further, because many patients with HIT who are treated with low-molecular-weight heparin also develop progressive or recurrent thrombocytopenia or thrombosis (i.e., in vivo cross-reactivity), these agents are not recommended to treat HIT. [37] [59]

Although primary treatment for HIT should include an anticoagulant that reduces thrombin generation, certain treatment adjuncts [37] can benefit selected patients, including thromboembolectomy, thrombolytic therapy, high-dose intravenous gammaglobulin, aspirin, or plasmapheresis.

Prevention

Platelet monitoring in patients treated with heparin should reflect the overall risk of HIT (i.e., the preparation, the dose, and the clinical situation). The authors recommend daily platelet count monitoring in patients at relatively high risk for developing HIT (e.g., postoperative orthopedic and cardiovascular

patients receiving prophylactic-dose unfractionated heparin, and medical patients receiving therapeutic-dose unfractionated heparin). The frequency of HIT is much lower with low-molecular-weight heparin,[35] and no consensus exists regarding frequency of platelet count monitoring in patients receiving these preparations. The patient with a past history of HIT who requires future anticoagulation presents a problem, since the risk of recurrence is unknown. Danaparoid has been used successfully in these patients.[60] HIT antibodies are transient, and are usually undetectable even using a sensitive assay two months after an episode of HIT. [37] Thus, if HIT antibodies are not detectable, it may be acceptable to use heparin for a brief indication (e.g., heart surgery using cardiopulmonary bypass). Patients requiring urgent heart surgery during acute HIT have been successfully anticoagulated with alternate anticoagulants (e.g., danaparoid, [65] lepirudin[66]), but each of these approaches has certain drawbacks.

Drug-Induced Immune Thrombocytopenia

A large number of drugs can cause a syndrome that mimics acute ITP,[33] [67] [68] [69] [70] (Table 129-6 [33] [34] [69] [70] [71] [72] [73] [74] [75] [76] [77] [78] [79] [80] [81] [82] [83] [84] [85] [86] [87] [88] [89] [90] [91] [92] [93] [94] [95] [96] [97] [98] [99] [100]). Typically, these patients develop severe thrombocytopenia (platelet count usually <20 × 10^9 /L) with petechiae and purpura, usually within weeks, but occasionally much longer after starting drug therapy. Drug-induced immune thrombocytopenia is much less common than HIT. Perhaps the most common cause of this syndrome is trimethoprim/sulfamethoxazole (co-trimoxazole), even though it occurs in only approximately 1 in 25,000 patients treated with this drug combination.[33]

Pathogenesis

Drug-dependent binding of the Fab component of IgG to platelet glycoproteins leads to platelet destruction. This occurs because the IgG-sensitized platelets are recognized by the Fc receptors of reticuloendothelial macrophages. For quinine- and quinidine-induced immune thrombocytopenia, both the GPIIb/IIIa and GPIb/IX complexes have been implicated as targets for the drug-dependent IgG.[69] In contrast, for sulfa antibiotic-induced immune thrombocytopenia, the GPIIb/IIIa complex is predominantly involved.[82] Sometimes, drug metabolites rather than the parent drug form the antigen.[83] [88] A trimolecular complex is formed among IgG-Fab, the drug (or metabolite), and the platelet glycoprotein. In contrast to HIT, platelet Fc receptors are not involved. Drug-dependent IgG binding is remarkably heterogeneous with respect to binding affinity, number of binding sites per platelet, and the range of drug concentration required.

Clinical Features

Patients with drug-induced immune thrombocytopenia typically present with petechiae, purpura, and severe thrombocytopenia (platelet count often <20 × 10^9 /L). Sometimes systemic symptoms such as fever and chills occur in patients with abrupt-onset thrombocytopenia. Usually, the thrombocytopenia becomes clinically apparent 12 weeks after starting the drug, but the thrombocytopenia can begin even after a patient has been taking a drug for several years. Usually the platelet count begins to rise within a few days of discontinuing the implicated drug, but occasionally several weeks are required for recovery, possibly because of generation of drug-independent IgG (platelet autoantibodies). [70]

Rarely, distinct drug-dependent IgG destroy red and/or white cells in addition to platelets. For example, both platelet- and leukocyte-reactive quinidine-dependent IgG have been reported in a patient with quinidine-induced bicytopenia. [71] Sometimes, the immune process is directed against a pluripotent hematopoietic stem cell, resulting in pancytopenia accompanied by marrow aplasia or hypoplasia (e.g., gold-, carbamazepine-, or quinidine-induced pancytopenia). A bone marrow should be performed in patients with suspected drug-induced bicytopenia or pancytopenia, as a hypoplastic marrow can indicate the serious complication of drug-induced aplastic anemia.

Diagnosis

A high index of clinical suspicion is often required to make the diagnosis. Quinine is widely available, e.g., as an ingredient in tonic water, or as an additive to street drugs. Demonstration of drug-dependent binding of IgG to platelets in vitro is important in diagnosis. Labeled immunoglobulin-specific probes (phase II assays) or glycoprotein capture techniques (phase III assays) appear to have the highest sensitivity and specificity. Investigators have reported that a commercially available solid-phase red cell adherence system can be modified to detect drug-dependent platelet antibodies, [100] but the predictive value of these assays is uncertain. A number of detection systems including flow cytometry can be used.[68] [82]

There are certain caveats in diagnostic testing. First, metabolites rather than parent drug must sometimes be used to detect the IgG. [83] [88] Second, wash buffer in these assays must include the target drug (or metabolite). Third, even despite these maneuvers, the sensitivity of in vitro assays is relatively low, and the diagnosis of drug-induced immune thrombocytopenia often must be made on clinical grounds alone. Sometimes the diagnosis is confirmed by inadvertent or deliberate re-exposure to the suspected drug. However, deliberate drug rechallenge is not recommended because of the potential hazard.

Management

As many drugs as possible should be discontinued in patients with suspected drug-induced immune thrombocytopenia. If further drug treatment is necessary, an alternate, immunologically non-cross-reactive substitute should be prescribed. Platelet transfusions should be given to patients with life-threatening bleeding. High-dose intravenous -globulin, 1 g/kg given over 68 hours for 2 consecutive days, usually is effective. [72] [95] Corticosteroids appear to be relatively ineffective in treating drug-induced immune thrombocytopenia.[101]

Gold-Induced Thrombocytopenia

Gold-induced immune thrombocytopenia occurs in as many as 13% of treated patients.[34] A genetic predisposition is suggested by the association with HLA-DR3, which is found in approximately 85% of affected patients.[34] The thrombocytopenia typically occurs during the first 20 weeks of therapy, before 1,000 mg of gold has been given. Rarely, the thrombocytopenia begins much later, sometimes even several months after discontinuation of the gold. Although the onset of thrombocytopenia is typically abrupt, regular platelet count monitoring is important because an early diagnosis can be made in some patients. The thrombocytopenia often persists for several months despite discontinuation of the gold. It is uncertain whether this is caused by gold-induced autoimmune thrombocytopenia [74] or whether prolonged release of gold from tissue stores produces long-term drug-dependent thrombocytopenia.[73] Although most patients will eventually respond to corticosteroids, immediate albeit generally transient correction of severe thrombocytopenia can usually be achieved with high-dose intravenous -globulin. [72] Some patients with persisting thrombocytopenia benefit by splenectomy or gold-chelating agents (dimercaprol, *N*-acetylcysteine).

TABLE 129-6 -- Drug-Induced Immune Thrombocytopenia[a]

	Platelet Count x<20 × 10^9 /L	Drug Rechallenge	Drug-Dependent in Vitro Testing		Platelet Count <20 × 10^9 /L	Drug Rechallenge	Drug-Dependent in Vitro Testing
Quinine/Quinidine Group				Procainamide[91]	Y	Y	Y[I]

Quinine[69]	Y	Y	Y[I,II,III]	Quinidine (see above)			
Quinidine[65] [70] [71]	Y	Y	Y[I,II,III]	-Blockers			
				Alprenolol	Y	Y	
Gold salts				Oxprenolol	Y	Y	
Gold sodium thiomalate[34] [72]	Y	Y	Y[II]	Other antihypertensive drugs			
Auronofin (oral gold)[73]	Y	Y	Y[II]	Captopril	Y		
Aurothioglucose[74]	Y			Diazoxide[d]		Y	
Antimicrobials				-Methyldopa	Y	Y	Y[I]
Antimony-containing				Diuretics			
Stibophen	Y	Y	Y[I]	Acetazolamide[e]	Y		Y[I]
Sodium stibogluconate		Y		Chlorothiazide	Y	Y	Y[I]
Cephalosporins				Chlorthalidone			Y[I]
Cefamandole	Y		Y[II]	Furosemide	Y	Y	Y[I]
Cefotetan	Y		Y[II]	Hydrochlorothiazide	Y	Y	Y[I]
Ceftazidime[75]	Y			Spironolactone	Y		Y[I]
Cephalothin	Y	Y	Y[I]	Benzodiazepines			
Ciprofloxacin[76]		Y		Diazepam	Y	Y	Y[I,II]
Clarithromycin[77]	Y						
Fluconazole[78]	Y			Antiepileptic drugs			
Fusidic acid[79]	Y	Y	Y[II,III]	Carbamazepine [92] [93]	Y	Y	Y[I,II]
Gentamicin	Y	Y	Y[I]	Phenytoin	Y		Y[I]
Nalidixic acid	Y	Y		Valproic acid	Y	Y	
Penicillins				H_2 -antagonists			
Ampicillin	Y		Y[II]	Cimetidine	Y	Y	Y[II]
Apalcillin	Y			Ranitidine			Y[I,II]
Methicillin	Y	Y	Y[I]				
Mezlocillin[80]	Y		Y[I]	Sulfonylurea drugs			
Penicillin	Y		Y[II]	Chlorpropamide	Y		
Piperacillin	Y	Y		Glibenclamide (glyburide)	Y		
Pentamidine[81]	Y		Y [II,III]	Iodinated contrast agents			
Rifampin	Y	Y	Y[I,II]	Diatrizoate[94]	Y	Y	
Sulfa antibiotics[b]				Iocetamic acid	Y		
Sulfamethoxazole[82] [83]	Y		Y[I,II,III][c]	Iopanoic acid	Y	Y	Y[I]
Sulfamethoxypyridazine	Y			Sodium ipodate	Y	Y	Y[I]
Sulfisoxazole[82]	Y		Y[I,II,III]				
Suramin[84]	Y		Y[I]	Retinoids			
Vancomycin[85]	Y	Y	Y[II,III]	Isotretinoin	Y	Y	
				Etretinate			
Anti-inflammatory drugs							
Acetaminophen	Y	Y	Y[I][b]	Antihistamines			
Salicylates				Antazoline	Y	Y	Y[I]
Acetylsalicylic acid[33]	Y	Y	Y[I]	Chlorpheniramine	Y	Y	Y[I]
Diflunisal	Y		Y[I]	Illicit drugs			
Sodium -aminosalicylic acid	Y	Y	Y[I]	Cocaine [95]	Y	Y	
				Heroin	Y	Y	
Sulfasalazine	Y	Y		Quinine contaminant ("filler")	Y		Y[I,II]
Other nonsteroidal anti-inflammatory drugs				Antidepressants			
Diclofenac[86] [87]	Y	Y	Y[I]	Tricyclic antidepressants			
Fenoprofen	Y			Amitriptyline[96]	Y	Y	
Ibuprofen[88]	Y		Y[II][b]	Desipramine			Y[I]
Indomethacin	Y			Doxepin	Y		
Meclofenamate	Y	Y		Imipramine			Y[I]
Mefenamic acid[89]	Y		Y[I,II]	Tetracyclic antidepressants			
Naproxen[90]	Y			Mianserin	Y	Y	Y[I]
Oxyphenbutazone	Y			Antineoplastic drugs			
Phenylbutazone			Y[II]	Actinomycin D	Y	Y	

Piroxicam	Y			Aminoglutethimide	Y	Y	
Sulindac			Y[I]	Tamoxifen[97]		Y	Y [II]
Tolmetin	Y	Y		Miscellaneous drugs			
Cardiac and antihypertensive drugs				Danazol	Y	Y	
				Desferrioxamine		Y	
Positive inotropic drugs				Levamisole[98]		Y	
Digitoxin		Y	Y[I]	Lidocaine	Y	Y	Y[I]
Digoxin		Y	Y[I]	Morphine	Y		Y[I]
Antiarrhythmic drugs				Papaverine[99]	Y	Y	Y[II]
Amiodarone	Y	Y		Ticlopidine			Y[II]

[a] Key evidence implicating the various drugs is shown, including documentation of severe thrombocytopenia, drug rechallenge (deliberate or inadvertent), and in vitro testing. In vitro testing is classified as phase I (IgG-nonspecific endpoints such as platelet aggregation, platelet factor 3 generation, clot retraction inhibition, and so forth), phase II (increase in drug-dependent platelet-associated IgG by patient serum), and phase III (drug-dependent binding to specific platelet glycoproteins). A recent report [100] that did not provide detailed clinical information used a phase I assay to suggest the following additional drugs might also cause immune thrombocytopenia: acyclovir, albuterol, amlodipine, amoxicillin, amphotericin, atenolol, axid, cefazolin, ceftriaxone, cephalexin, clindamycin, diltiazem, famotidine, fluoxetime, imipenem, metronidazole, nafcillin, normodyne, prilosec, and tobramycin. Other references for this table can be found in previous editions of this textbook.

[d] Although classified here as an antihypertensive, note that diazoxide is a thiazide derivative.

[e] Although classified here as a diuretic, note that acetazolamide is a sulfonamide derivative.

[b] Several other sulfa antibiotics have been implicated in DITP.

[c] Metabolites of the drug have been implicated in the pathogenesis of DITP.

Drug-Induced Autoimmune Thrombocytopenia

Certain drugs besides gold have been reported to initiate autoimmune thrombocytopenia, e.g., procainamide. [102] However, as the pathogenic antibodies are by definition drug-independent, it is difficult to establish causation. The mumps-measles-rubella vaccine can cause a severe, but generally self-limited thrombocytopenia that is clinically and serologically indistinguishable from childhood acute ITP. [103]

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MISCELLANEOUS DRUG-INDUCED THROMBOCYTOPENIC SYNDROMES

Drug-Induced Thrombotic Microangiopathy

Several drugs can trigger a syndrome of thrombocytopenia, fragmentation hemolysis, and renal failure (drug-induced hemolytic-uremic syndrome) (see Chap. 128). This syndrome has been established for quinine: multiple quinine-dependent antibodies reactive against platelets, red cells, leukocytes, and endothelial cells have been reported.[104] Patients treated with plasmapheresis usually recover, although some also require short-term hemodialysis. [104] Although a similar syndrome may be caused by mitomycin C,[105] cyclosporine, and penicillamine, it should be noted that many patients who receive these drugs have underlying illness (gastric adenocarcinoma, bone marrow transplantation, collagen vascular disease, respectively) that are themselves associated with hemolytic uremic syndrome. Growing evidence indicates that thrombotic thrombocytopenic purpura (TTP) rarely can be caused by the antiplatelet drug, ticlopidine. [106]

Nonidiosyncratic Drug-Induced Thrombocytopenia

Most antineoplastic drugs produce a dose-dependent pancytopenia caused by injury to hematopoietic cells, including megakaryocytes and their progenitor cells. Typically, the platelet count nadir occurs at a predictable point following treatment and then quickly recovers. Unexpectedly severe or prolonged thrombocytopenia in patients receiving chemotherapy should suggest alternate explanations for the thrombocytopenia (e.g., idiosyncratic thrombocytopenia caused by another drug).

Approximately 20% of patients who receive valproic acid (an anti-epileptic agent) develop mild to moderate thrombocytopenia; bleeding symptoms are uncommon. [32] This mechanism of thrombocytopenia is unknown, but appears to be nonidiosyncratic, as the risk of thrombocytopenia correlates strongly with serum concentrations of valproic acid metabolite.[32] Amrinone is another agent that can cause mild, dose-dependent thrombocytopenia. [107]

Rapid Drug-Induced Thrombocytopenia

Some drugs produce rapid but generally mild and transient drops in the platelet count. These include heparin, [36] protamine, bleomycin, hematin, ristocetin (no longer clinically used), desmopressin (particularly in patients with type 2B von Willebrand disease [108]), and porcine factor VIII. The mechanism(s) for thrombocytopenia in these syndromes is obscure. Occasionally acute thrombocytopenia is IgG-mediated: for example, some rapid-onset thrombocytopenic reactions to heparinparticularly when accompanied by systemic inflammatory symptoms and signsare attributable to IgG-mediated HIT in patients who were recently sensitized to heparin. [54]

Abciximab (c7E3, or ReoPro) is a humanized chimeric Fab fragment of a murine monoclonal antibody (7E3) directed against GPIIb/IIIa. This agent reduces restenosis following percutaneous transluminal coronary angioplasty. About 0.5% of patients develop severe thrombocytopenia within several hours of treatment with abciximab.[109] [110] Most patients do not have associated bleeding complications, but platelet transfusions can be used if bleeding occurs. The mechanism of the thrombocytopenia is uncertain, although the abrupt onset of severe thrombocytopenia suggests an immune pathogenesis.

Drug Hypersensitivity Reactions

Mild to moderate thrombocytopenia is sometimes observed in patients with systemic drug hypersensitivity reactions. Comorbid clinical features can include generalized rashes, fever, cholestasis, and leukopenia. Allopurinol, isoniazid, sulphasalazine, and phenothiazine drugs, among others, have been implicated in these reactions.

Thrombocytopenia Secondary to Biologic Response Modifiers

Use of purified or recombinant biologic response modifiers such as interferon, [111] interleukin-2, [112] and certain colony-stimulating factors [113] has resulted severe, reversible thrombocytopenia in some patients. Anti-lymphocyte globulins can also produce severe thrombocytopenia in some patients. [114] The mechanism of causation of this thrombocytopenia is uncertain.

OTHER CAUSES OF DESTRUCTIVE THROMBOCYTOPENIA

Incidental Thrombocytopenia of Pregnancy

Maternal thrombocytopenia occurs in 57% of pregnancies.[115] [116] [117] Most of these women are healthy, have no prior history of thrombocytopenia, and are detected by routine blood testing. The cause of the mild reduction in platelet count (usually 75150 × 10^9 /L) is unknown. This condition is benign, without an increased risk for maternal bleeding or neonatal thrombocytopenia.[115] [116] Accordingly, no special treatments are indicated in these women, and the route of delivery should be determined by obstetrical indications.

Pre-eclampsia/Eclampsia

Pre-eclampsia is characterized by the onset of hypertension and proteinuria during pregnancy, especially in a primigravida near term. Pre-eclampsia complicates approximately 5% of pregnancies. Thrombocytopenia occurs in about 20% of pre-eclamptic patients, and almost half of all patients with eclampsia.[117] [118] [119] In addition, some patients have evidence for a platelet function defect, exhibiting a prolonged bleeding time despite a normal platelet count.[120] A subset of patients with pre-eclampsia have microangiopathic *h*emolysis, *e*levated *l*iver enzymes, and *l*ow *p*latelets (HELLP syndrome).[121] This condition usually indicates severe pre-eclampsia, and is associated with a higher risk of maternal and fetal complications.[121] [122] [123] Repeated clinical and laboratory assessment of these patients is important, as this syndrome can mimic other life-threatening complications of pregnancy, such as overt DIC, thrombotic thrombocytopenic purpura, septicemia, or acute fatty liver of pregnancy.[124]

Increased platelet destruction is the mechanism for the thrombocytopenia in pre-eclampsia.[125] However, activation of the coagulation system is relatively modest,[126] [127] suggesting that thrombin generation may not be a major factor in the thrombocytopenia. Endothelial dysfunction is another potential explanation for increased platelet turnover in pre-eclampsia.[128]

Pharmacologic control of hypertension and rapid delivery are the treatments of choice for pre-eclampsia, and will usually result in resolution of the thrombocytopenia within a few days. Because of the associated platelet function defects, some physicians recommend demonstrating a normal bleeding time in moderately thrombocytopenic patients before performing epidural

anesthesia in these patients.[119] However, epidural anesthesia is considered to be relatively safe even in patients with moderate thrombocytopenia.[129] In patients in whom delivery is not an option (i.e., premature fetus), treatment with bed rest and aggressive antihypertensive therapy has been reported to result in an improved platelet count. However, the clinical course is markedly variable, and some patients develop life-threatening organ failure.[122] Plasmapheresis has been used in some patients, especially if there is evidence of thrombotic microangiopathy and organ dysfunction.[130] The authors would perform plasma exchange in such a patient whose clinical picture has features that overlap with TTP.

Infection

Infection is a common cause of thrombocytopenia, occurring in approximately 5075% of patients with bacteremia or fungemia, and almost all patients with septic shock or DIC.[131] Even when caused by bacteremia, the thrombocytopenia is generally mild to moderate in severity, and usually not accompanied by significant coagulation abnormalities or bleeding. However, the likelihood of laboratory evidence for DIC increases as the platelet count falls to <50 × 10^9 /L. The mechanism(s) for thrombocytopenia in septicemia in the absence of DIC is uncertain, but could include chemokine-induced macrophage ingestion of platelets (hemophagocytosis) and direct activation of platelets by endogenous mediators of inflammation (e.g., platelet-activating factor[132]) or certain microbial products.[133] Various explanations for thrombocytopenia among different types of infection are listed in Table 129-7 .[134] [135] [136] [137] [138] [139] [140] [141] [142] [143] [144] [145] [146]

Unexplained thrombocytopenia in any hospitalized patient

TABLE 129-7 -- Mechanisms for Thrombocytopenia Complicating Infections

Mechanisms for Thrombocytopenia	Selected Examples
Increased platelet destruction	
Disseminated intravascular coagulation	Meningococcemia[134]
Hemophagocytosis	Septicemia,[135] Epstein-Barr virus[136]
Platelet-reactive autoantibodies: acute	Varicella[137]
Platelet-reactive autoantibodies: chronic	HIV[138]
Hemolytic-uremic syndrome	Verocytotoxin-producing*e. coli,*[139] HIV[140]
Antibodies against platelet-adsorbed microbial antigens	Malaria[141]
Hypersplenism	
Acute	Disseminated mycobacterium avium in HIV[142]
Chronic	Viral chronic active hepatitis, malaria
Decreased platelet production	
Replacement of marrow by granulomata	Ehrlichiosis,[143] tuberculosis
Infection of megakaryocytes	HIV [144]
Transient virus-induced aplastia	Parvovirus B19[145] (erythroblastopenia predominates)
Multiple mechanisms	

e.g., platelet destruction + hypersplenism	Recurrent malaria
e.g., increased platelet destruction, decreased platelet production, hypersplenism	Chronic HIV infection [146]

warrants studies, such as blood cultures, to exclude infection. Prompt recognition and treatment of the infection is most important, as platelet count recovery tends to parallel the resolution of the infection. Prophylactic platelet transfusions are generally not required unless the platelet count falls to <1020 × 10 ^{9} /L, or unless comorbid clinical features increase the likelihood of serious bleeding (e.g., concomitant coagulopathy, an invasive procedure, uremic platelet dysfunction). The use of heparin for patients with septic shock and DIC is controversial. However, heparin may benefit a subset of patients with clinical evidence of DIC and microvascular thrombosis (e.g., meningococcemia with acral tissue necrosis). The possibility of acquired protein C deficiency complicating acute DIC should also be considered in septic patients with limb ischemia (e.g., meningococcemia), as treatment with vitamin K, plasma, and, possibly, protein C concentrates, [134] could be beneficial.

Patients infected with HIV pose a special diagnostic problem, as many potential causes of thrombocytopenia often coexist in these patients (Chap. 155).[146] These include: immune platelet destruction, impaired platelet production secondary to HIV infection of megakaryocytes, drug-induced myelosuppression (commonly, zidovudine, ganciclovir, and trimethoprim/sulfamethoxazole), HIV-associated thrombotic microangiopathy, hypersplenism, and marrow infiltration by tumor or opportunistic infections. Platelet kinetic studies have shown a complex interaction of decreased platelet production, increased platelet destruction, and splenic platelet sequestration. Immune platelet destruction may be related to antibodies that cross-react with GPIIb/IIIa complexes. [135] Zidovudine often raises the platelet count in patients with HIV-associated thrombocytopenia. Most patients with HIV-associated thrombocytopenia respond to conventional treatments for ITP, including corticosteroids, splenectomy, and gammaglobulin.[136]

Systemic Lupus Erythematosus

Thrombocytopenia occurs in as many as 25% of patients with systemic lupus erythematosus (SLE), generally because of immune mechanisms.[147] Many different types of platelet-IgG interactions are described (anti-glycoprotein, [148] antiglycolipid,[149] antiphospholipid, platelet-reactive immune complexes) and the predominant explanation for thrombocytopenia is unknown.

Several thrombocytopenic syndromes are seen in SLE patients. For many patients, the thrombocytopenia is chronic, resembling ITP, and is the predominant clinical manifestation of the lupus.[147] Often, these patients have a prolonged bleeding time and platelet function abnormalities despite the relatively mild thrombocytopenia.[150] Some thrombocytopenic SLE patients have antiphospholipid antibodies and are at increased risk for thrombotic rather than bleeding complications.[151] Acute, severe thrombocytopenia can be a prominent feature of patients with a severe multisystem exacerbation of lupus. [147] Rarely, patients with SLE develop an illness that closely resembles TTP or HUS; these patients should be treated with plasma exchange. [152]

Treatment of the thrombocytopenia of SLE is similar to that of ITP (see Chap. 126). Corticosteroids are the first line of therapy, but many patients do not respond or require high doses. This can produce serious adverse effects such as avascular necrosis of the femoral head. Before proceeding to splenectomy, we usually try danazol (an attenuated androgen), in doses of 2001,200 mg/day. [153] Usually, several weeks are required before results are visible. The efficacy of splenectomy in SLE is controversial. Although many patients respond, the overall success rate appears to be lower than that for ITP, with a higher operative morbidity. [154] Patients with refractory thrombocytopenia can benefit with more aggressive therapies, including high-dose intravenous -globulin, [155] azathioprine, cyclophosphamide combined with plasmapheresis,[156] and cyclosporine.

Antiphospholipid Antibody Syndrome

The antiphospholipid antibody syndrome is a clinicopathologic disorder characterized by a wide spectrum of clinical events (particularly thrombosis and recurrent miscarriages) associated with antibodies that recognize a complex of negatively charged phospholipid and various protein cofactors such as $_{2}$ -glycoprotein I, prothrombin, protein C, protein S, and annexin V.[157] [158] [159] Many patients with this syndrome have thrombocytopenia, [160] which is typically mild and intermittent. The antiphospholipid antibody syndrome should be considered in patients who develop cardiac valvulitis, idiopathic lower limb or abdominal vein thrombosis, dural sinus thrombosis, non-atheromatous arterial thrombosis, or microvascular thrombosis (e.g., acrocyanosis, digital ulceration or gangrene, livedo reticularis). [161] [162] [163] [164] The mechanism of the prothrombotic tendency in patients with antiphospholipid antibody syndrome remains elusive, but interference with endothelial cell function, impaired fibrinolysis, interference with the protein C anticoagulant pathway, and antibody-mediated platelet activation have all been described. [159]

Antiphospholipid antibodies are detected using solid-phase enzyme-linked immunoassays (ELISA) with purified phospholipids (usually cardiolipin) as target antigens. Lupus anticoagulant activity is shown by demonstrating inhibition of certain phospholipid-dependent coagulation assays. Although antiphospholipid antibodies are frequently detected in patients with SLE, they can also be found in patients with other autoimmune disorders, malignancy, infections, or as a complication of certain drugs (e.g., procainamide). Often no associated condition is identified (primary antiphospholipid antibody syndrome). [165] Evidence indicates that thrombocytopenia in patients with antiphospholipid antibody syndrome is associated with platelet glycoprotein-reactive autoantibodies. [148] [166]

Specific treatment for the thrombocytopenia is not usually required. For many patients, long-term anticoagulant or antiplatelet therapy, or both, are needed to prevent recurrent thromboses. Some clinical observations suggest that corticosteroid treatment may exacerbate certain thrombotic complications, [167] although others have noted benefit from corticosteroids. Anticoagulant therapy for asymptomatic patients without a prior history of thrombosis is not currently recommended.

Malignancy

Thrombocytopenia complicating malignant disorders most frequently results from antineoplastic treatment or marrow replacement by tumor. However, certain thrombocytopenic syndromes have been associated with malignancy, including autoimmune thrombocytopenia, DIC, and thrombotic microangiopathy.

Thrombocytopenia attributable to platelet glycoprotein-reactive autoantibodies can complicate neoplastic lymphoproliferative diseases such as Hodgkin disease, non-Hodgkin lymphoma, and chronic lymphocytic leukemia.[168] [169] Sometimes the thrombocytopenia responds to treatment of the neoplasm, although in some patients (particularly those with Hodgkin disease) the thrombocytopenia is indistinguishable from ITP and is not related to the activity of the lymphoma (see Chap. 126).

DIC occurs in certain malignancies, particularly adenocarcinomas of the pancreas, stomach, lung, colon, breast, and prostate. [170] [171] Some patients present with venous or, less commonly, arterial thrombosis as the first clinical manifestation of their malignancy. In these patients the presence of thrombocytopenia is an important clue that should prompt specific investigations for DIC. The platelet count typically rises to normal or even elevated levels during heparin therapy; however, recurrent thrombocytopenia and thrombosis can occur within hours or days of discontinuing the heparin therapy. Cancer patients with DIC and DVT are also at risk for warfarin-associated venous limb gangrene (HIT complicated by DVT). Thus, cancer patients with DIC should receive heparin rather than warfarin anticoagulation.

DIC with hemorrhagic manifestations is characteristically seen in some patients with prostate adenocarcinoma, and in most patients with acute promyelocytic leukemia. It is crucial to recognize promyelocytic leukemia, since treatment with all-trans retinoic acid produces differentiation of the malignant cells, and rapidly reduces the life-threatening bleeding risks attributable to hyperfibrinolysis in this disorder (see Chaps. 53 and 54).[172]

A destructive thrombocytopenic disorder that resembles hemolytic uremic syndrome or thrombotic thrombocytopenic purpura has been described in patients with adenocarcinoma of the breast and colon.[105] This syndrome has a high prevalence in females, frequently those with a history of therapy with mitomycin C. DIC is not usually present. Some patients respond transiently to plasmapheresis or protein A immunoadsorption. [173]

Hemophagocytic Syndrome

The hemophagocytic syndrome is characterized by pancytopenia and morphologic evidence of phagocytosis of red cells, granulocytes, and platelets by reactive macrophages. Some adult patients have a fatal disease process characterized by high fever, weight loss, prominent hepatosplenomegaly, severe pancytopenia, elevated liver enzymes, and a terminal infection.[174] A T-cell lymphoma is the usual explanation for this dramatic illness. [174] [175] However, similar patients with fulminant

illness have been described following otherwise unremarkable bacterial [176] or viral infections (particularly the Epstein-Barr virus [136] [177] [178]). In some cases, the viral infection could have initiated the lymphoma;[177] in others, the infection may represent the initial manifestation of a lymphoma accompanied by a severe immunocompromised state. In children, the high mortality associated with hemophagocytic lymphohistiocytosis warrants aggressive treatment, including antineoplastic chemotherapy and bone marrow transplantation.[136]

Severe, but potentially reversible, hemophagocytosis can be seen in patients with certain unusual infections (e.g., babesiosis, [179] ehrlichiosis[180]). Hemophagocytosis has also been observed in patients with SLE,[181] Still disease, and HIV infection.[182] Treatment should be directed at the underlying illness, with blood product support as needed.

Solid Organ and Bone Marrow Transplantation

Thrombocytopenia commonly occurs during episodes of solid organ allograft rejection. [183] It is possible that platelet activation and deposition in the transplanted organ vasculature contribute to the rejection process. Anti-rejection therapies also can cause thrombocytopenia via increased platelet destruction (antilymphocyte globulin[114] [184]) or marrow suppression (azathioprine[184]). About 5% of renal transplant recipients,[185] [186] and even fewer recipients of liver or heart transplants,[186] develop post-transplant HUS. Although HUS is often attributed to cyclosporine treatment in these patients, it has been noted that cyclosporine usually can be safely resumed following graft recovery.[185]

Early, severe thrombocytopenia caused by marrow ablative therapy invariably accompanies bone marrow transplantation (BMT). Platelet count recovery to >50 × 10^9 /L is more rapid (16 vs. 35 days) in patients receiving autologous mobilized peripheral blood progenitor cells compared with autologous BMT. [187] Severe, persistent thrombocytopenia despite recovery of red and white cells is relatively common following marrow or peripheral blood transplantion; [188] autoimmune thrombocytopenia has been implicated in some patients.[189] Late-onset thrombocytopenia following BMT that responds to corticosteroids, intravenous -globulin, and splenectomy has also been attributed

to autoimmune thrombocytopenia. Rarely, transplantation-associated alloimmune thrombocytopenia can be caused by donor/recipient incompatibility involving platelet-specific alloantigens such as Pl^{A1} or Br^a . [190]

A syndrome of thrombocytopenia, red cell fragmentation, and renal impairment can occur in as many as 10% of BMT patients, usually beginning 312 months following transplantation (BMT-associated hemolytic uremic syndrome).[191] The hematologic abnormalities can be mild and remit spontaneously, although patients often have residual azotemia and hypertension. More severely affected patients can benefit from plasmapheresis.Unfortunately, the syndrome has a poor overall prognosis and many patients will die despite any interventions.

Cardiopulmonary Bypass Surgery

Excess bleeding is an important problem in patients who undergo heart surgery utilizing cardiopulmonary bypass. [192] Most of these patients receive blood transfusions, and approximately 5% require urgent reoperation for postoperative bleeding.

Thrombocytopenia and transient platelet dysfunction are observed in virtually every patient. Typically, the platelet count falls by 3350%, primarily due to hemodilution, but also as a result of bleeding and losses within the extracorporeal perfusion device. The bleeding time rises markedly during heart surgery (>30 minutes), but usually improves to <15 minutes shortly after surgery, and to normal several hours later. In contrast, the thrombocytopenia persists for 34 days, followed by recovery of the platelet count to values exceeding the preoperative baseline.

The pathogenesis and clinical significance of the hemostatic defect in these patients remain uncertain, but it is probably multifactorial. Studies have described transient, *intrinsic* defects in platelet function[192] [193] [194] (see Chap. 131). These include decreased in vitro platelet aggregation, decreased platelet surface membrane proteins,[195] selective depletion of platelet -granules, and evidence of in vivo platelet activation [196] and platelet vesiculation. [196] The platelet dysfunction in heart surgery is also attributable to an *extrinsic* platelet defect resulting from thrombin inhibition by the high doses of heparin. [197] Further, an important role for hyperfibrinolysis in the pathogenesis of bleeding is shown by elevated D-dimers levels in bleeding patients, [198] as well as the efficacy of antifibrinolytic agents [199] [200] in the prevention and treatment of heart surgery-associated bleeding. Other factors in some patients include residual heparin effect post-bypass (including heparin rebound[201]) and preoperative use of aspirin. Treatment of platelet dysfunction after cardiopulmonary bypass is discussed in Chapter 131 .

Thrombocytopenia Associated with Cardiovascular Disease

Congenital Cyanotic Heart Disease

Thrombocytopenia caused by a decrease in platelet life span occurs in some patients with severe cyanotic congenital heart disease, and is approximately related to the severity of the polycythemia. Bleeding occurs in a few patients and can be related to platelet function defects, coagulopathy, or hyperfibrinolysis. Reducing the hematocrit by phlebotomy sometimes improves the hemostatic defects.

Valvular Heart Disease

Although increased platelet turnover is common in valvular heart disease, mild thrombocytopenia occurs infrequently. The pathogenesis of the platelet consumption is not well understood, but could be related to increased plateletvon Willebrand factor interactions at high shear. [202] Indeed, high-molecular-weight multimers of von Willebrand factor are reduced in some of these patients,[203] suggesting a possible explanation for the characteristic bleeding from gastrointestinal angiodysplasia observed in some patients with aortic stenosis or hypertrophic cardiomyopathy.[204]

Pulmonary Vascular Disorders

Disorders characterized by pulmonary hypertension can be accompanied by thrombocytopenia, the pathogenesis of which is poorly defined. [205] Thrombocytopenia has also been reported to occur in association with pulmonary embolism,[206] possibly as a result of mild DIC. When evaluating such a patient, the clinician should inquire about current or recent heparin exposure, as HIT and pulmonary embolism are strongly associated. [35] [52]

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Chapter 130 - Hereditary Disorders of Platelet Function

Joel S. Bennett

INTRODUCTION

A prolonged bleeding time in a patient with a normal platelet count implies a diagnosis of von Willebrand disease or a disorder of platelet function. von Willebrand disease is discussed in Chapter 114 and acquired disorders of platelet function are discussed in Chapter 131 . Hereditary disorders of platelet function can be subclassified into disorders of platelet adhesion, aggregation, secretion, or procoagulant activity according to the predominant phase of platelet function affected.

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DISORDERS OF PLATELET ADHESION

Bernard-Soulier Syndrome (Glycoprotein Ib-IX Deficiency)

The Bernard-Soulier syndrome, described in 1948 by Bernard and Soulier, is a rare disorder of platelet function characterized by a prolonged bleeding time, very large platelets, and thrombocytopenia.[1] Individuals with the Bernard-Soulier syndrome have a bleeding diathesis because their platelets are unable adhere to von Willebrand factor (vWf) in the subendothelial matrix. [2] The basis for this abnormality is deficiency or dysfunction of the glycoprotein Ib-IX (GPIb-IX) complex. [3] [4]

Biology and Molecular Aspects

Following disruption of the endothelial lining of the vasculature, circulating platelets adhere to exposed subendothelial connective tissue. At low rates of shear in the blood, platelets adhere to subendothelial collagen, fibronectin, or laminin. However, at higher rates of shear, such as those present in arterioles and in the microcirculation, platelet adhesion requires the presence of subendothelial vWf. [5] [6] vWf is an elongated multimeric glycoprotein, each monomer of which contains two domains that interact with platelets.[7] [8] One domain binds to the glycoprotein IIb-IIIa complex (GPIIb-IIIa) on activated platelets. [9] The other binds to GPIb-IX, thereby mediating the adhesion of unactivated platelets to subendothelial vWf. [10] vWf is also present in plasma but is not found on the platelet surface. There is as yet no adequate explanation for this paradox. Exposing vWf to the antibiotic ristocetin or the snake venom protein botrocetin in vitro induces binding to GPIb-IX. [11] [12] However, the factors that induce vWf binding to GPIb-IX in vivo are uncertain. One possibility is that the conformation of vWf changes when it is present in the subendothelium, allowing it to bind to GPIb-IX. [8] [13] [14] A second possibility is that shear stress induces vWf binding by changing the conformation of GPIb-IX, vWf, or both.[15] [16] [17]

The protein GPIb-IX, affected in Bernard-Soulier platelets, is a non-covalent heterodimer, consisting of the heavily glycosylated 165,000-molecular weight GPIb and the 20,000-molecular weight GPIX.[15] [18] There are approximately 25,000 copies of GPIb-IX on the platelet surface. [15] GPIb itself is a disulfide-linked heterodimer containing a 143,000-molecular weight -subunit (GPIb) and 22,000-molecular weight -subunit (GPIb). [15] GPIb can be cleaved by trypsin and a calcium-dependent platelet protease, giving rise to a soluble 135,000-molecular weight heavily glycosylated amino terminal fragment termed glycocalicin [15] and a membrane-associated 25,000-molecular weight remnant that remains disulfide-linked to GPIb and associated with GPIX. [18] The amino terminal portion of glycocalicin contains binding sites for both vWf and thrombin[15] and its carboxyl-terminal portion contains multiple sialic acid-rich hexasaccharides O-linked to serine and threonine residues. [19] The latter consists of several tandem repeats of a mucin-like sequence and is polymorphic due to differences in the number of repeats of a 13 amino acid motif. [20] GPIb extends into the platelet cytoplasm where it is linked to the platelet cytoskeleton [21] via actin-binding protein [22] and possibly to platelet signaling pathways via the protein 14-3-3.[23] The cytoplasmic portions of GPIb and GPIX are acylated with palmitic acid through thioester linkages, probably at a free cysteine residue. [24] This modification likely stabilizes the interaction of these subunits with the membrane lipid bilayer or with other membrane proteins. In addition, the cytoplasmic portion of GPIb can be phosphorylated at Ser 166 by the cyclic adenosine monophosphate (AMP)-dependent protein kinase, [25] a reaction that inhibits collagen-induced platelet actin polymerization.[26]

The GPIb, GPIb, and GPIX each contain leucine-rich motifs (LRMs) with the consensus sequence L-LN-LLPPGLL-GL-. [19] [27] [28] Similar sequences were first noted in the plasma protein leucine-rich $_2$ glycoprotein and have now been detected in 13 other proteins. [15] There are seven LRMs in GPIb and GPIb and GPIX each contain a single LRM. The amino acids flanking the LRMs are also conserved. [15] The function of the LRMs is not known. The presence of multiple leucine residues suggests that they could act like leucine-zippers involved in protein oligmerization. [15] However, the classic leucine zipper motif consists of an amphipathic -helix, whereas the LRM appears to be an amphipathic -sheet.[29] Moreover, the motifs in GPIb, GPIb, and GPIX are not located in regions known to be involved in intermolecular associations.[15] On the other hand, they could be involved in intramolecular associations that contribute to the tertiary structure of each molecule.

A fourth protein, glycoprotein V (GPV), is also missing from Bernard-Soulier platelets. [3] [30] GPV is an 82,000-molecular weight substrate for thrombin. [31] [32] However, GPV cleavage is not required for thrombin-induced platelet activation [32] nor is it required for GPIb-IX function. [33] GPV contains 15 tandem LRMs homologous to those of GPIb, GPIb, and GPIX,[34] but they do not appear to be involved in the formation of a GPIb-IX-V complex. [35] The association of GPV with GPIb-IX is mediated by GPIb.[36] Because there are only half as many GPV molecules on the platelet surface as there are GPIb-IX complexes, [35] it is likely that GPIb-IX-V is a trimer with a stoichiometry of 2:2:2:1 for GPIb, GPIb, GPIX, and GPV, respectively. [36]

Studies of GPIb-IX biosynthesis indicate that although GPIb and GPIb can be expressed independently on the cell surface, their expression is much more efficient in the presence of GPIX.[33] Further, GPIb is the subunit that links GPIb to GPIX. [37] Thus, mutations that result in the deficient expression of any one of the three components of GPIb-IX can produce the Bernard-Soulier syndrome. On the other hand, GPV is not required for GPIb-IX expression and the basis for its absence from Bernard-Soulier platelets is not known.

Etiology and Pathogenesis

The effects of GPIb-IX deficiency are not limited to the inability of affected platelets to adhere to vWf. The majority of Bernard-Soulier platelets are large when observed on peripheral blood smears. However, there is contradictory evidence about the size of the platelets in the circulation. It has been reported that circulating Bernard-Soulier platelets are of normal size, [38] but have increased membrane, perhaps located in the surface-connected open-canalicular system and extruded when platelets spread during the preparation of a blood smear. [39] Others have found that circulating Bernard-Soulier platelets have an increased volume, [40] a more spherical shape,[40] and a more deformable membrane than normal platelets.[41] Although the serum prothrombin time has been reported to be shortened in the Bernard-Soulier syndrome[42] and collagen-induced platelet procoagulant activity and factor XI binding to be absent, [43] prothrombin consumption, measured by a two-stage assay, is normal[44] and the expression of platelet factor 3 [44] and prothrombinase activity by Bernard-Soulier platelets is increased. [45] An unexplained decrease in thrombin-, trypsin-, and thromboxane-stimulated phospholipase C activity has also been reported. [46] GPIb-IX is a target for some drug-dependent antiplatelet antibodies induced by quinidine and quinine [47] [48] [49] and as expected, these antibodies fail to bind to Bernard-Soulier platelets. [50]

Genetic Aspects

The Bernard-Soulier syndrome is an autosomal recessive disorder. [51] In most instances, GPIb-IX is not detectable on the surface of Bernard-Soulier platelets, but in a few cases, 747% residual

GPIb or GPIb-IX has been detected.[3] [30] [52] When it has been possible to study heterozygotes, most have had platelets that were larger than normal and a GPIb

content intermediate between normals and affected individuals. [30] [53] Consistent with the rarity of the syndrome, consanguinity has often been noted in affected families.

The gene for each component of the GPIb-IX-V complex has been localized and characterized. The GPIb gene is located on chromosome 17p12-ter, [54] the GPIb gene on chromosome 22q11.2,[55] [56] the GPIX gene on chromosome 3q21,[57] [58] and the GPV gene on chromosome 3q29.[58] Each gene has a compact intron-depleted structure, such that the genes for GPIb, GPIb, and GPV contain two exons and the gene for GPV contains three. [58] Moreover, the open reading frame and 3 untranslated region of each is encoded by a single exon except for the GPIb gene where its single intron is inserted into the codon for the fourth residue of its signal peptide.[55] The genes are also similar in that their 5 flanking regions contain potential binding sites for the GATA, *ets*, and Sp-1 transcription factors like other genes expressed by megakaryocytes.[58] [59] [60] [61] Although potential TATA elements are located in the GPV 5 flanking region, it is uncertain whether these are utilized. [58]

A number of mutations responsible for the Bernard-Soulier syndrome have been identified (Table 130-1). As would be expected from the studies of GPIb-IX-V biosynthesis discussed previously, these mutations involve the genes for either GPIb, GPIb, or GPIX. Most of the detected mutations have been missense mutations or frameshifts resulting in premature stop codons. However, two unique mutations were identified in the GPIb genes of a patient with the velo-cardio-facial syndrome and consisted of deletion of a portion of chromosome 22q11.2 including the GPIb locus [62] and a mutation in a GATA binding site in the promoter of the remaining GPIb allele resulting in decreased GPIb gene transcription. [61] It is noteworthy that seven mutations are located in the LRMs of either GPIb or GPIX (Table 130-1). Those involving GPIb produced dysfunctional GPIb-IX complexes, [63] [64] [65] [66] whereas those involving GPIX decreased GPIb-IX expression. [67] In addition, a Asn 45 Ser mutation in GPIX has been detected in separate kindreds from the United Kingdom, [68] Austria,[69] and Sweden,[70] a Cys 73 Tyr mutation in GPIX has been identified in two kindreds in Japan, [71] and a dinucleotide deletion involving codon 492 of GPIb has been identified in a Caucasian [72] and an African-American kindred in the United States,[73] suggesting that these sites may be hot spots for mutation in the GPIX and GPIb genes.

Clinical Presentation

The Bernard-Soulier syndrome presents in infancy or childhood with bleeding characteristic of defective platelet function: ecchymoses, epistaxis, and gingival bleeding.[51] Later manifestations include menorrhagia and postpartum, gastrointestinal, and post-traumatic hemorrhage. [81] [82] Hemarthroses and expanding hematomas are unusual. Although the severity of hemorrhage among affected individuals is variable, it may require frequent transfusions and suppression of menses. In a report of 59 cases, there were 10 deaths.[83] In some patients the severity of hemorrhage inexplicably declines over the course of the disease. [83]

Laboratory Findings

The bleeding time in patients with the Bernard-Soulier syndrome is markedly prolonged to >20 minutes. [44] Platelet counts may vary within kindreds and in a given patient. Most patients are thrombocytopenic to some degree and patients with platelet counts <20,000/l have been reported. [84] Platelets seen on stained peripheral blood smears are large, with 3080% having a mean diameter >3.5 m.[53] Occasional platelets are as large as 2030 m in diameter. The red blood cells and white blood cells are normal and distinctive abnormalities have not been observed in marrow megakaryocytes by light microscopy, [85] although abnormalities in the demarcation membrane system have been observed by electron microscopy.[86] The laboratory tests that distinguish Bernard-Soulier from normal platelets are the failure of Bernard-Soulier platelets to agglutinate in the presence of ristocetin and decreased [87] or absent agglutination[88] after exposure to botrocetin. In contrast to von Willebrand disease, these abnormalities cannot be corrected by the addition of normal plasma. [84] Absent ristocetin-induced agglutination and normal platelet aggregation can be reproduced in the whole blood aggregometer, obviating the difficulty of preparing platelet-rich plasma containing a sufficient number of Bernard-Soulier platelets for conventional aggregometry. [89] Aggregation of Bernard-Soulier platelets induced by agonists such as adenosine diphosphate (ADP), collagen, thrombin, and epinephrine is normal (Fig. 130-1).[51] Although a defect in prothrombin consumption, manifested by a short serum prothrombin time, has

TABLE 130-1 -- Mutations Resulting in the Bernard-Soulier Syndrome

Affected Protein	Mutation	Consequence	Reference
GPIb	Trp 343 stop	Truncated GPIb	[74]
GPIb	Leu 57 Phe[a]	Autosomal dominant BSS	[63]
GPIb	Ala 156 Val[a]	Dysfunctional GPIb-IX	[64]
GPIb	Deletion of T317	Frameshift truncated GPIb	[75]
GPIb	Ser 444 stop	Truncated GPIb	[76]
GPIb	Cys 209 Ser	Decreased GPIb expression	[77]
GPIb	Deletion of Leu 179[a]	Dysfunctional GPIb-IX	[65]
GPIb	Deletion of an adenine between bases 1932 1938	Frameshift truncated GPIb	[78]
GPIb	Leu 129 Pro[a]	Decreased GPIb expression and function	[66]
GPIb	Adenine deletion in codon 19	Frameshift truncated GPIb	[79]
GPIb	Dinucleotide deletion in codon 492	Frameshift soluble, truncated GPIb	[72] [73]
GPIb	Deletion of DiGeorge/velo-cardio-facial region in 22q11.2	Absence of one GPIb allele	[62]
GPIb	C G transversion in GATA bindng site	Decreased GPIb gene transcription	[61]
GPIX	Asp 21 Gly[a]	Decreased GPIb-IX expression	[68]
GPIX	Asn 45 Ser[a]	Decreased GPIb-IX expression	[68] [69] [70]
GPIX	Trp 126 Stop	No detectable GPIX	[78]
GPIX	Cys 73 Tyr	Decreased GPIb-IX expression	[71]
GPIX	Phe 55 Ser[a]	Decreased GPIb-IX expression	[80]

[a]Mutation involving a LRM or a flanking region.

Figure 130-1 Platelet aggregation in response to ADP. Following the addition of ADP to stirred aliquots of platelet-rich plasma in an aggregometer, there is a brief decrease in light transmission indicated by the solid arrow, due to platelet shape change. When normal (NRML) or Bernard-Soulier platelets (BSS) are examined, shape change is followed by either a single continuous wave of aggregation at high ADP concentrations or two waves of aggregation at lower ADP concentrations. The hatched arrow at the inflection point between the first and second waves of aggregation is the point at which platelet secretion occurs. -Storage pool deficient platelets (SPD) generally undergo only a first wave of aggregation and the aggregates may dissociate. Thrombasthenic platelets (TSA) undergo shape change but do not aggregate.

been reported,[44] expression of platelet factor 3 activity by Bernard-Soulier platelets is normal or increased. [44]

Differential Diagnosis

Conditions associated with thrombocytopenia and large platelets can be differentiated from the Bernard-Soulier syndrome because Bernard-Soulier platelets fail to agglutinate in the presence of ristocetin. Giant platelets, thrombocytopenia, and Döhle bodies in leukocytes are seen in the *May-Hegglin anomaly*.[90] The function and membrane proteins of May-Hegglin platelets are normal. [91] When hemorrhage does occur in these patients, it correlates with the extent of thrombocytopenia. [90]

Several variants of Alports syndrome have been described with large platelets, thrombocytopenia, hereditary nephritis, sensorineural deafness plus (*Fechtner syndrome*)[92] or minus leukocyte inclusions (*Epsteins syndrome*). [93] A third variant with only macrothrombocytopenia and characteristic leukocyte inclusions has been observed (*Sebastian syndrome*). [94] Platelet function in some affected individuals is normal, [92] [95] in others platelet aggregation and secretion induced by ADP, collagen, and epinephrine is defective. [93] [96] The *gray platelet syndrome* due to a deficiency of platelet -granules is associated large platelets and thrombocytopenia.[97] In contrast to Bernard-Soulier platelets, these platelets are pale gray on Wright-stained blood smears and agglutinate normally in the presence of ristocetin.[96] In the autosomal dominant *Montreal platelet syndrome* giant platelets, thrombocytopenia, and a prolonged bleeding time are associated with spontaneous platelet aggregation at pH 7.4 and normal agonist-induced platelet aggregation. [95] So-called *Mediterranean macrothrombocytopenia*[100] is not a unique entity, but refers to a putative difference in platelet count and platelet size among Europeans of northern and Mediterranean origin. There are also anecdotal reports of thrombocytopenia and giant platelets, often associated with a mild bleeding diathesis, but lacking the features of either Bernard-Soulier syndrome or the May-Hegglin anomaly.[101] [102] [103] [104] In *platelet-type von Willebrand disease*, mutations within the vWf binding domain of GPIb produce spontaneous vWf binding and the clinical picture of type 2B von Willebrand disease. [105] [106] [107]

Acquired Bernard-Soulier-like disorders can be differentiated from the congenital Bernard-Soulier syndrome by history. Antibodies to GPIb occur in some patients with idiopathic thrombocytopenic purpura,[108] [109] [110] but it has not been possible to determine if the antibodies are responsible for bleeding because of the concomitant thrombocytopenia. Two patients with myelodysplasia and an acquired Bernard-Soulier-like syndrome have been reported. [111] [112] A patient has been reported with a lymphoproliferative disorder and clinical bleeding who had a prolonged bleeding time, normal platelet count and platelet morphology, and a circulating IgG antibody that inhibited ristocetin-induced platelet agglutination but was directed against an unidentified 210,000-molecular weight platelet protein. [113]

Therapy

Treatment of hemorrhage in patients with the Bernard-Soulier syndrome usually requires platelet transfusion (Table 130-2). Hormonal control of menses has been effective in managing menorrhagia.[114] Splenectomy has been attempted to increase the platelet count, [115] but has resulted in only transient increases and has not ameliorated the platelet function defect. Corticosteroids are not beneficial in this disorder. Desmopressin acetate (DDAVP) has been reported to shorten the bleeding time in several affected individuals, [116] [117] [118] but its efficacy in patients with hemorrhage has not been reported.

Deficiency of Platelet Collagen Receptors

Collagen is a substrate for platelet adhesion and an agonist for platelet aggregation and secretion. A number of platelet proteins

TABLE 130-2 -- Treatment of Inherited Disorders of Platelet Function

Disorder	Defect in Platelet Function	Structural Defect	Treatment Options		
			Platelet Transfusion	DDAVP	Corticosteroids
Bernard-Soulier syndrome	Adhesion	GPIb-IX deficiency	Y	?	N
Thrombasthenia	Aggregation	GPIIb-IIIa deficiency	Y	N	N
Gray platelet syndrome	Secretion	-granule deficiency	Y	Y	?
Storage pool disease	Secretion	-granule deficiency	Y	Y	?
Primary secretion defects	Secretion	Defective signal transfuction	Y	Y	?
Scott syndrome	Procoagulant activity	Decreased exposure of anionic phospholipids	Y	N	N

Abbreviations: Y, yes; N, No; ?, may be effective in some cases; DDAVP, desmopressin.

that interact with collagen have been identified, including GPIIb, [119] GPIV,[120] GPVI,[121] the GPIa-IIa (21) complex,[122] a 65,000-molecular weight protein that binds to type 1 collagen[123] and a protein with a molecular weight of 85,00090,000.[124] It is unclear which of these proteins functions as a physiologic receptor for collagen. However, several clinical reports suggest that GPVI and the GPIa-IIa complex can function in this manner.

GPVI

A 62,000-molecular weight protein, GPVI, is stably associated with the Fc receptor -chain in the plasma membrane of human platelets. [125] [126] Exposure of normal platelets,[126] [127] but not GPVI-deficient platelets,[125] to collagen or collagen-like peptides results in the phosphorylation of the Fc receptor -chain on tyrosine residues, followed by the tyrosine phosphorylation of tyrosine kinase Syk and phospholipase C2. [127] Moreover, murine platelets lacking either the Fc receptor -chain or Syk are unable to respond to collagen stimulation,[128] whereas the interaction of GPVI-deficient human platelets with either immobilized [129] or soluble[130] collagen is markedly impaired. Thus, it appears that platelet function can be induced by a collagen-stimulated pathway consisting of GPVI, the Fc receptor -chain, Syk, and phospholipase C2.

The description of three patients with mild bleeding disorders whose platelets specifically lacked GPVI is additional evidence that GPVI is a functional receptor for collagen on platelets.[121] [131] [132] The platelets of each patient failed to aggregate following stimulation with collagen, despite the presence of normal amounts of GPIa-IIa, although the platelets of two retained some ability to adhere to collagen-coated surfaces. [131] [132] Further, an antibody against GPVI has been reported in a patient with idiopathic thrombocytopenic purpura that inhibits collagen-induced platelet function. [133]

GPIa-IIa

The GPIa-IIa complex is indistinguishable from the VLA-2 complex on lymphocytes and the integrin 21 present on a variety of cells. [134] [135] In many of these cells (including platelets), 21 functions as a Mg^{2+} -dependent receptor for collagen.[122] [136] [137] [138] In platelets, the collagen-stimulated phosphorylation of Syk [139] [140] and phospholipase C2[139] can be blocked by an anti-GPIa-IIa antibody, whereas cross-linking GPIa-IIa with a monoclonal antibody induces the FcRII-dependent phosphorylation of these proteins.[139] Moreover, two patients with histories of bleeding have been reported whose platelets failed to respond normally to collagen and lacked GPIa-IIa. The platelets of the first contained æ1525% of the normal amount of GPIa and failed to aggregate in response to collagen, to adhere to collagen under static conditions,[141] and to adhere and spread on collagen under conditions of flow. [142] The platelets of the second completely lacked GPIa plus the -granule protein thrombospondin, failed to aggregate in response to low concentrations of collagen and adhered to, but did not spread on, collagen-coated surfaces. [143] A third patient has also been reported with a myeloproliferative disorder, a prolonged bleeding time, and no bleeding symptoms whose platelets were unresponsive to collagen and contained GPVI but no GPIa-IIa.[144] Thus, GPIa-IIa appears to play a role in the platelet response to collagen. On the other hand, the inability of platelets lacking GPVI to respond to collagen despite the presence of GPIa-IIa, and vice versa, suggests that both proteins may be required for normal platelet responsiveness to collagen.

GPIV

A single chain 88,000-molecular weight protein, GPIV (CD36), is present on the surface of a variety of cells and functions as a receptor for red blood cells parasitized by *Plasmodium falciparum* on endothelium,[145] a binding site for thrombospondin, [146] [147] a receptor for oxidized low-density lipoprotein, [148] and a signal transduction molecule.[149] Because monoclonal anti-GPIV antibodies inhibit collagen-mediated platelet adhesion [150] and purified GPIV binds to collagen and inhibits collagen-induced platelet aggregation, [120] it has been suggested that GPIV is a platelet receptor for collagen. [120] GPIV carries the platelet antigen Nak^{a151} and platelets of Nak^{a} -negative individuals lack GPIV[152] as a result of one of several mutations. [153] [154] [155] However, GPIV-deficient individuals, usually identified because their platelets lack Nak^{a} , are asymptomatic and in most,[156] [157] [158] [159] but not all,[160] [161] studies, their platelets interact normally with collagen. [156] Thus, GPIV does not appear to be a physiologically relevant platelet receptor for collagen.

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DISORDERS OF PLATELET AGGREGATION

Glanzmann Thrombasthenia (Glycoprotein IIb-IIIa Deficiency)

Glanzmann thrombasthenia is a rare disorder characterized by a prolonged bleeding time, normal platelet count, and absent macroscopic platelet aggregation. [51] Thrombasthenia was originally described by Glanzmann in 1918 as a bleeding disorder with a normal platelet count and abnormal clot retraction. [162] Affected platelets in Glanzmann thrombasthenia are unable to aggregate. The basis for their defective function is deficiency or dysfunction of the GPIIb-IIIa complex. [163]

Biology and Molecular Aspects

Platelet aggregation follows platelet adhesion to form a hemostatic plug. [164] In contrast to adhesion, however, aggregation is an active metabolic process in which agonists such as thrombin or ADP bind to their cognate receptors to stimulate the exposure of a fibrinogen or vWf binding site on the platelet membrane GPIIb-IIIa complex.[165] [166] [167] [168] [169] Fibrinogen or vWf bound to GPIIb-IIIa then cross-links adjacent platelets into an occlusive plug. GPIIb-IIIa is already present on the surface of resting platelets. Thus, the signals induced by platelet stimulation cause a change in GPIIb-IIIa that exposes its ligand binding site. [170] Following ligand binding, the cytoplasmic tails of GPIIb-IIIa associate with submembranous actin filaments via intermediary cytoskeletal proteins. [171] This provides a mechanism for transmitting the force of cytoskeletal contraction to the fibrin clot resulting in clot retraction. [172] [173] GPIIb-IIIa can also bind to vWf and fibronectin in the subendothelial matrix, resulting in the spreading of adherent platelets. [174] [175] There are approximately 40,00080,000 GPIIb-IIIa complexes on the surface of resting platelets [176] and additional complexes, present in the membrane of the platelet -granules, can be translocated to the platelet surface by platelet stimulation. [177] [178] Glanzmann thrombasthenia results when platelets lack sufficient numbers of functional GPIIb-IIIa complexes on their surface to support platelet aggregation. [163] Because the platelets of obligate heterozygotes aggregate normally, but contain one-half the normal number of GPIIb-IIIa complexes, 50% of the normal number of GPIIb-IIIa complexes per platelet is at least sufficient to support platelet aggregation. [179]

The GPIIb-IIIa complex is a calcium-dependent heterodimer and dissociates into GPIIb and GPIIIa monomers in the presence of calcium chelators. [180] By electron microscopy, it appears as a 12 × 8 nm globular head with two 18-nm tails extending from one side. [181] When incorporated into phospholipid vesicles, the globular head extends æ20 nm above the vesicle surface with the tips of the tails inserted into the phospholipid. [182] GPIIb-IIIa is a member of the integrin superfamily of adhesion receptors that mediate a variety of cell-cell and cell-extracellular

matrix interactions.[183] Integrins are /-heterodimers with -subunits homologous to GPIIb and -subunits homologous to GPIIIa. Because each integrin -subunit interacts with a restricted number of -subunits, integrins can be classified into subfamilies. GPIIb-IIIa belongs to a small subfamily consisting of GPIIb-IIIa or IIb3 and the vitronectin receptor v3. The expression of GPIIb-IIIa is restricted to cells of the megakaryocytic lineage because the GPIIb gene is only active in these cells, [184] whereas v3 is expressed by a variety of cells including endothelium and platelets. [185]

GPIIb has a molecular weight of 136,000 and is composed of disulfide-linked heavy (GPIIb) and light (GPIIb) chains with molecular weights of 125,000 and 23,000, respectively.[180] The light chain anchors the protein in the platelet membrane [186] and the heavy chain interacts with GPIIIa.[187] GPIIb is synthesized as single chain precursor (Pro-GPIIb).[186] In association with GPIIIa, Pro-GPIIb is transported to the Golgi complex where it is cleaved into heavy and light chains. [188] A feature of GPIIb is the presence of four domains in the heavy chain that are similar to the calcium-binding domains of calmodulin [186] and likely bind calcium in the mature protein.[189] A region proximal to the second of these domains (amino acids 294314) can be cross-linked to a peptide corresponding to the carboxyl terminus of the fibrinogen -chain, suggesting that these amino acids are part of GPIIb-IIIas ligand binding site. [190] [191] [192] Studies in vitro suggest that GPIIb plays a major role in determining which protein ligands bind to GPIIb-IIIa. [193] [194]

GPIIIa is a single-chain transmembrane protein that contains 56 cysteine residues and 28 disulfide bonds. [184] [195] Accordingly, its apparent molecular weight of 90,000 on unreduced sodium dodecyl sulfate gels increases to 110,000 following disulfide bond reduction. [180] Disulfide bonds in GPIIIa are distributed in three regions in the extracellular portion of the molecule: a cysteine-rich, protease-resistant amino terminus (residues 162), a protease-sensitive central region (residues 101422), and a disulfide-rich, protease-resistant core (residues 423622). [196] In addition, there is a long-range disulfide bond between cysteine residues 5 and 435 that results in a large protease-sensitive loop in the molecule. [196] [197] [198]

The GPIIb-IIIa heterodimers are assembled in the calcium-rich milieu of the endoplasmic reticulum. [199] In the absence of heterodimer formation, GPIIb and GPIIIa are retained eventually degraded in this organelle. [199] The factors responsible for the retention of GPIIb and GPIIIa monomers in the endoplasmic reticulum are unknown. However, it is clear that formation of GPIIb-IIIa heterodimers alone is not sufficient to guarantee egress because mutations that distort the conformation of the assembled heterodimers also result in their retention. [199] [200]

Etiology and Pathogenesis

The fundamental defect of thrombasthenic platelets is their inability to aggregate. However, GPIIb-IIIa deficiency is responsible for additional abnormalities of platelet function. Thrombasthenic platelets do not spread normally on the subendothelial matrix, an aspect of platelet adhesion that is mediated by GPIIb-IIIa binding to fibronectin and vWf. [174] [201] The amount of fibrinogen in the -granules of thrombasthenic platelets is decreased to absent. [202] Megakaryocytes do not synthesize fibrinogen,[203] [204] [205] consequently, -granule fibrinogen results from the endocytosis of plasma fibrinogen. [206] [207] Kistrin and barbourin, snake venom proteins that inhibit fibrinogen binding to GPIIb-IIIa, also inhibit the endocytosis of fibrinogen by guinea pig megakaryocytes. [208] [209] Thus, the absence of functional GPIIb-IIIa on thrombasthenic platelets likely accounts for their deficit in -granule fibrinogen. [210] A number of biochemical reactions in platelets require the presence of GPIIb-IIIa or platelet aggregation and are impaired in thrombasthenic platelets. For example, phosphorylation on tyrosine of multiple platelet proteins including proteins with molecular weights of 140,000 and 50,00068,000 depends on ligand binding to GPIIb-IIIa and the tyrosine phosphorylation of proteins such as pp125 ^{FAK} as well as proteins with molecular weights of 95,00097,000 requires platelet aggregation. [211] [212] [213] [214] Calpain, a calcium-dependent thiol protease, is activated by thrombin-stimulation of stirred normal platelets, but not following similar treatment of thrombasthenic platelets. [215] The redistribution of a number of proteins, including spectrin, talin, vinculin, pp60 ^{src} and p62^{yes} , to the detergent-insoluble platelet cytoskeleton follows thrombin-stimulated platelet aggregation, but is not detected when thrombasthenic platelets are studied. [216] The kinetics of calcium exchange in unstimulated thrombasthenic platelets is decreased. [217] However, it is still unclear whether GPIIb-IIIa plays a direct role in calcium transport. [218] [219] [220] Clot retraction in blood containing thrombasthenic platelets is either absent or reduced, [221] a finding consistent with the ability of GPIIb-IIIa to link the fibrin clot to the contractile elements of the platelet cytoskeleton. [172] [173]

On the other hand, those aspects of platelet function that do not depend on GPIIb-IIIa are normal in thrombasthenia. Thrombasthenic platelets interact normally with collagen fibrils[221] and undergo secretion when stimulated by strong agonists such as thrombin. [202] Incubation of thrombasthenic platelets with ristocetin induces vWf

binding to GPIb-IX[11] and thrombasthenic platelets adhere to vWf in the subendothelium. [174] [201] Thrombasthenic platelets express normal amounts of procoagulant activity following lysis,[45] but expression may[202] or may not [222] be decreased under conditions that require platelet aggregation.

Genetic Aspects

The genes for GPIIb and GPIIIa are both located on the long arm of chromosome 17 at q21 23. [223] [224] [225] The GPIIb gene spans 18 kb and is located in a region that contains at least seven complete and three incomplete Alu repeats. [226] The gene consists of 30 exons ranging in size from 46 bp to 220 bp. [226] Like the genes for other proteins expressed in megakaryocytes, its 5 flanking region lacks TATA or CAAT boxes. Instead it contains a linear array of regulatory elements including two motifs recognized by the GATA-binding transcription factors, [227] [228] a sequence recognized by a member of the Ets family of transcription factors, [229] and a binding site for the transcription factor Sp1. [230] In addition, a repressor element located between the GATA motifs may be involved in controlling the tissue specificity of GPIIb gene expression. [231] The gene for GPIIIa spans 63 kb and contains 15 exons. [232] [233] Like the GPIIb gene, the 5 flanking region of the GPIIIa gene lacks TATA or CAAT boxes. [234] However, specific regulatory motifs in this region that might control GPIIIa gene expression have not yet been identified. Although the organization of exons in many genes reflects the distribution of functional domains in the proteins they encode, [235] this does not appear to be true for either the genes for GPIIb or GPIIIa.

Glanzmann thrombasthenia is inherited as an autosomal recessive disorder with clusters of disease in populations where consanguinity is common. [236] [237] [238] It has been classified into three types based on the amount of GPIIb-IIIa present and the presence or absence of -granule fibrinogen and clot retraction. [202] In type I, platelets contain <5% of the normal amount of GPIIb-IIIa and clot retraction and -granule fibrinogen are absent. In type II, platelets contain 1020% of the normal amount of GPIIb-IIIa, clot retraction is decreased, and -granule fibrinogen is present. [239] In variant thrombasthenia, the platelet content of GPIIb-IIIa is 50% of normal so that the GPIIb-IIIa abnormality is qualitative, rather than quantitative. [239]

Expression of GPIIb-IIIa on the cell surface requires that both GPIIb and GPIIIa are present in the endoplasmic reticulum. [240] Thus, a mutation in the gene for either protein will produce thrombasthenia. On occasion, it has been possible to determine which gene is affected by measuring the number of v3

TABLE 130-3 -- GPIIb Mutations Resulting in Glanzmann Thrombasthenia

Mutation	Type	Comments	Reference
4.5 kb deletion in GPIIb gene	I	Deletion between Alu repeats in introns 1 and 9 no detectable protein	[244]
Deletion of amino acids 106111	I	Alternative splicing due 13-base deletion including splice acceptor site of exon 4	[245]
Arg 584 stop	I	Unstable mRNA	[247] [253] [254]
Mutation of splice acceptor for exon 26	I	Alternative splicing loss of exons 2527 and synthesis of single-chain truncated protein	[247]
Mutation of splice acceptor for exon 28	I	Alternative splicing loss of exon 28, intracellular degradation of GPIIb-IIIa	[248]
Gly 273 Asp	I	Intracellular retention of malfolded GPIIb-IIIa	[200]
Gly 418 Asp	I	Intracellular retention of malfolded GPIIb-IIIa	[256]
Deletion of 6 bp and insertion of 31 bp in exon 25	I	Alternative splicing at a cryptic acceptor site in the inserted sequence	[249]
Arg 327 His	II	GPIIb-IIIa expression 710% of normal, clot retraction decreased	[242] [243]
Mutation in exon 17 disrupting a splice donor site	I	Use of cryptic donor site within exon 17 leading to the synthesis of an unstable mRNA	[250]
Mutation of splice donor site in intron 15	I	Use of cryptic donor site in exon 15 frame shift and premature stop codon	[251] [252]
Ser 870 stop	I	Intracellular retention of malfolded GPIIb-IIIa	[255]
Deletion of Val 425 and Asp 426	I	Intracellular retention of malfolded GPIIb-IIIa	[246]

complexes per platelet, because they are increased when the GPIIb gene is affected and decreased when the GPIIIa gene is affected. [241]

A variety of mutations in the genes for GPIIb and GPIIIa have been detected in patients with thrombasthenia (Tables 130-3 and 130-4). Except for one example of type II thrombasthenia,[242] [243] all reported mutations of the GPIIb gene have produced type I disease. The identified mutations have included large [244] and small deletions,[245] [246] mRNA-splicing abnormalities leading to shifts in the GPIIb reading frame and aberrant GPIIb proteins, [247] [248] [249] [250] [251] [252] nonsense mutations,[247] [253] [254] [255] and missense mutations.[200] [242] [243] [253] Six mutations[200] [242] [246] [248] [255] [256] are of particular interest because the translated product of the mutant gene is able to associate with GPIIIa in the endoplasmic reticulum, but the conformation of the resulting heterodimers is sufficiently perturbed to impair their egress from the endoplasmic reticulum to the Golgi apparatus. Four of the latter mutations [200] [242] [246] [256] are located in the putative calcium-binding region of GPIIb, suggesting that folding of this portion of GPIIb makes an important contribution to the overall conformation of GPIIb-IIIa. It is also noteworthy that examples of variant thrombasthenia due to mutations in GPIIb have not yet been identified.

In contrast, GPIIIa mutations have produced all three types of thrombasthenia. Type 1 disease has resulted from complex

TABLE 130-4 -- GPIIa Mutations Resulting in Glanzmann Thrombasthenia

Mutation	Type	Comments	Reference
Complex gene rearrangement	I	Homologous recombination among 3 Alu sequences causing a 15 kb-inversion and 1 kb-deletion	[257] [258]
Asp 119 Tyr	V[a]	Perturbed ligand binding domain in GPIIb-IIIa	[268]
Deletion of 11 bp in exon 12	I	Frameshift premature stop codon and the synthesis of a truncated protein	[245]
Arg 214 Gln	V	Decreased ligand binding to GPIIb-IIIa	[275]
Ser 752 Pro	V	Defective agonist-stimulated GPIIb-IIIa activation	[267]
Arg 214 Trp	V	Decreased ligand binding to GPIIb-IIIa, corrected by incubating platelets with dithiothreitol	[269] [273] [275]
Mutation of splice donor site in intron 1	I	Frameshift premature stop codon and synthesis of a severely truncated protein	[261]
34 kb insertion into intron 8	I	Abnormal mRNA splicing decreased mRNA stability	[260]
Arg 62 stop	I	Nonsense mutation truncated protein	[254]
G A mutation 6 bp upstream from exon 9 splice donor site	I	Alternative splicing of exon 9 frameshift, premature stop codon, and mRNA instability	[262]
Cys 374 Tyr	II	Reduced GPIIb-IIIa expression on the platelet surface	[266]
Dinucleotide deletion affecting codons 210 and 211 in exon 4		Frameshift premature stop codon and synthesis of a severely truncated protein. Patient is carrier for type I thrombasthenia.	[263]
Leu 117 Trp	I	Intracellular retention of malfolded GPIIb-IIIa	[264]
11.2-kb deletion between Alu repeat in intron 9 and exon 13	I	Mutant mRNA lacked exons 10, 11, 12, and 13 and contained a frameshift premature stop codon	[259]
Deletion of IIe 325, Pro 326, and Gly 327	I	Mutant GPIIIa unable to associate with v when expressed in CHO cells	[265]

Arg 724 stop	V	Truncation deleting 39 C-terminal amino acids of the GPIIIa cytoplasmic tail defective agonist-stimulated GPIIb-IIIa activation	[274]

[a]V, variant.

rearrangements in the GPIIIa gene;[257] [258] deletions,[259] insertions,[260] and point mutations[261] [262] affecting mRNA splice donor or splice acceptor sites; deletions or point mutations producing frameshifts and premature stop codons;[245] [263] nonsense mutations;[254] and missense mutations.[264] Type 1 disease has also resulted from the deletion of three amino acids (IIe 325, Pro 326, Gly 327), thereby impairing the ability of GPIIIa to associate with an -subunit. [265] One missense mutation (Gys 374 Tyr) has produced in type II disease. [266] Five mutations have produced variant thrombasthenia in which platelets contain nearly normal amounts of nonfunctional GPIIb-IIIa. Four of the mutations are missense mutations. In one kindred, a Ser 752 Pro substitution in the cytoplasmic domain of GPIIIa prevented GPIIb-IIIa activation by cellular agonists.[267] In each of the remaining kindreds, a charged amino acid in the extracellular domain of GPIIIa was converted to an uncharged residue. These mutations (Asp 119 Tyr;[268] Arg 214 Trp;[269] Arg 214 GIn[270]) are located in regions of GPIIIa that have been implicated in ligand binding. [271] [272] However, incubation of platelets containing the Arg 214 Trp mutant with the reducing agent dithiothreitol restored platelet aggregation, [273] suggesting that Arg 214 is necessary to maintain the conformation of the ligand binding site in GPIIIa. The fifth mutation is a nonsense mutation in which the codon for Arg 724 is converted to a stop codon.[274] This results in the synthesis of a truncated GPIIIa molecule lacking the carboxyl terminal 39 amino acids of its cytoplasmic domain and the presence of GPIIb-IIIa heterodimers that are unable to transduce either inside-out or outside-in signals.

Additional cases of thrombasthenia, have been reported in which abnormalities in GPIIb or GPIIIa structure or distribution have been detected, but the responsible mutations have yet to be identified. In one patient with variant thrombasthenia, GPIIb-IIIa was present, but dissociated abnormally in the presence of low concentrations of ethylenediamine titra-acetic acid (EDTA). [276] The GPIIb-IIIa of the second patient was also more sensitive to EDTA and failed to react with a anti-GPIIb-IIIa monoclonal antibody, suggesting that its conformation was abnormal. [277] The platelets of a third patient contained decreased amounts of both normal and an abnormal form of GPIIb.[278] Finally, the platelets of the fourth patient with a mild thrombasthenia-like illness aggregated poorly in response to ADP and other agonists and had a deficit in the surface pool, but not the internal pool, of GPIIb-IIIa. [279]

Clinical Presentation

The clinical manifestations of thrombasthenia in a large cohort have been described in detail. [239] Thrombasthenia typically presents with mucocutaneous bleeding in the neonatal period or infancy, occasionally with bleeding following circumcision. Spontaneous petechiae are uncommon and bleeding generally results from a condition that would cause bleeding in an otherwise normal individual. Next to purpura, epistaxis is the most frequent type of bleeding, especially in childhood. Gingivalbleeding, often related to poor dental hygiene, and menorrhagia are frequent. Bleeding at menarche may be severe and require transfusion. Similarly, parturition represents a severe hemorrhagic risk. Other hemorrhagic manifestations include gastrointestinal bleeding and hematuria, but hemarthroses and deep hematomas are unusual. Serious bleeding may follow trauma or surgery in patients not prepared with normal platelets. The severity of the hemorrhagic diathesis is not predictable, even within single kindreds, and does not correlate with the extent of GPIIb-IIIa deficiency. Thus, one sibling may experience life-threatening bleeding requiring frequent transfusion, whereas another will suffer only mild bruising and epistaxis. The apparent decline in the clinical severity of thrombasthenia with age is likely due to a decrease in the incidence of conditions such as epistaxis with aging.

Laboratory Findings

Platelet counts and platelet morphology on peripheral blood smears are normal. The bleeding time of affected individuals is markedly prolonged. [221] [236] [237] The diagnosis thrombasthenia is usually suspected after platelet aggregometry. In contrast to normal platelets, addition of agonists (except for epinephrine) to a stirred suspension of thrombasthenic platelets results in only platelet shape change and platelet aggregation is absent (Fig. 130-1).[51] Platelet secretion induced by strong agonists such as thrombin is normal, but secretion in response to weak agonists such ADP and epinephrine is absent. Coagulation tests, such as the prothrombin time and the partial thromboplastin time, are normal in thrombasthenia. Clot retraction in the presence of thrombasthenic platelets is either absent or reduced.

Differential Diagnosis

A history of lifelong bleeding, a prolonged bleeding time, and absent platelet aggregation are diagnostic of thrombasthenia and differentiate it from other disorders of platelet adhesion and secretion. Instances of acquired thrombasthenia have been reported and can be differentiated from congenital thrombasthenia by history. Although autoantibodies against GPIIb-IIIa have been detected frequently in patients with idiopathic thrombocytopenic purpura, [280] [281] autoantibodies that induce a thrombasthenic-like state are unusual.[282] [283] [284] A patient with multiple myeloma has also been reported whose IgG_1 paraprotein was directed against GPIIIa and inhibited GPIIb-IIIa function.[285] Congenital afibrinogenemia may be associated with a prolonged bleeding time and decreased in vitro platelet aggregation due to the absence of sufficient fibrinogen to support platelet aggregation. [286]

Therapy

Bleeding in thrombasthenia requires the transfusion of normal platelets. (Table 130-2). Because bleeding is a lifelong problem, the use of human leukocyte antigen-matched platelets should be considered to lessen the chance of refractoriness to transfusion due to platelet alloimmunization. In rare instances, thrombasthenic patients have developed antibodies against normal GPIIb and GPIIIa following transfusion. [287] [288] Such antibodies could potentially limit the effectiveness of transfused platelets. Oral contraceptives have been useful in controlling menorrhagia. Regular dental care is essential in minimizing gingival bleeding. Fibrinolytic inhibitors such as \?\-aminocaproic acid, in addition to platelet transfusions, may be useful in controlling bleeding after dental extractions. Corticosteroids are not efficacious in managing bleeding in thrombasthenic patients. [221] Although DDAVP shortened the bleeding time in a patient with thrombasthenia and had a beneficial effect during minor dental surgery, [116] this is the only report of an effect of DDAVP in thrombasthenia.[259] The thrombasthenia of two severely affected children has been corrected by bone marrow transplantation. [290] [291] Prenatal diagnosis of Glanzmann thrombosthenia is discussed in Chapter 159 .

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DISORDERS OF PLATELET SECRETION

The platelet cytoplasm contains four types of granules: dense granules containing ADP, adenosine triphosphate (ATP) calcium, serotonin, and pyrophosphate; -granules containing a variety of proteins, some derived from the plasma, others synthesized by the megakaryocyte; lysosomes containing acid hydrolases; and microperoxisomes containing a peroxidase activity.[292] Following platelet activation, the contents of these granules are extruded in a process known as platelet secretion. Inherited disorders of platelet secretion result from a deficiency

of one or more of the types of platelet granules or from abnormalities in the platelet secretory mechanism. Secretion disorders are a frequent cause of mild to moderate bleeding manifested by easy bruising, menorrhagia, and excessive postoperative and postpartum blood loss.[293] In one group of 145 patients presenting with such symptoms, 18% were found to have a deficiency of platelet granules, 19% to have an abnormality in the platelet secretory mechanism, and 36% to have von Willebrand disease.[294] No explanation for the bleeding diathesis could be found in 27% of the patients. Patients with disorders of platelet secretion usually have a prolonged bleeding time, absence of the second wave of platelet aggregation when platelets are stimulated by ADP and epinephrine, and decreased aggregation when platelets are stimulated by collagen.[293] These disorders must be differentiated from the acquired abnormalities of platelet secretion induced by drugs such as aspirin or systemic diseases such as uremia and multiple myeloma (Chap. 131) and from the various types of von Willebrand disease (Chap. 114).

Disorders of Secretion Due to Abnormalities of Platelet -Granules

The Gray Platelet Syndrome (-Granule Deficiency, -Storage Pool Disease)

The gray platelet syndrome, described by Raccuglia in 1971,[97] is a rare disorder that results from the specific absence of morphologically recognizable -granules in the platelets of affected individuals.

Biology and Molecular Aspects

Normal platelets contain æ50 spherical or elongated structures termed -granules that contain a variety of proteins, some of which are specific or relatively specific for platelets and others that are also found in plasma.[295] The former include platelet factor 4, (PF4), -thromboglobulin (TG) platelet-derived growth factor (PDGF), and thrombospondin (TSP) and the latter include fibrinogen, vWf, albumin, coagulation factor V (FV), IgG, fibronectin, and a number of protease inhibitors. [292] [295] [296] The platelet-specific proteins and several of the plasma proteins (vWf, [297] FV[298]) are synthesized by megakaryocytes. The others reach the -granules via endocytosis of circulating protein.[203] [206] [207] The -granule membrane contains many of the same proteins present in the platelet plasma membrane (GPIIb-IIIa[299] and GPIb-IX-V[300]) and others specific for -granules (P-selectin[301] [302] and osteonectin[303]). Each can be translocated to the platelet surface following platelet-activation. [177] [178] [301]

Etiology and Pathogenesis

Electron micrographs of megakaryocytes and platelets from patients with the gray platelet syndrome reveal absence of normal -granules and the presence of vacuoles and small -granule precursors containing material that stains for vWf and fibrinogen. [304] [305] The other types of platelet granules are present in normal numbers.[304] The outer membranes of the vacuoles and small -granule precursors contain P-selectin and GPIIb-IIIa, [299] [306] which can be translocated to the platelet surface after thrombin stimulation.[306] These observations indicate that -granules are present in gray platelets and suggest that the abnormality responsible for the gray platelet syndrome is an inability to target proteins to these structures. The inability to package and retain PF4, TG, and PDGF in -granules would account for the elevated plasma concentrations of PF4 and TG and the bone marrow fibrosis observed in patients with the syndrome. [307] [308] The abnormality responsible for the gray platelet syndromes appears to be restricted to megakaryocytes because electron micrographs of endothelial cells from affected patients reveal typical Weibel-Palade bodies suggesting that these cells are capable of packing vWf normally. [309]

Clinical Presentation and Laboratory Findings

Patients with the gray platelet syndrome present with a lifelong history of mild to moderate mucocutaneous bleeding, [97] [98] [309] [310] although one patient developed subgaleal and epidural hematomas following head trauma.[311] Patients also exhibit variably prolonged bleeding times, moderate thrombocytopenia, reticulin fibrosis of the bone marrow, and large platelets whose gray appearance on a Wright-stained blood smear gives the name to the disorder. [97] [309] Platelet counts vary from 25,000 to 150,000/l, but generally range between 60,000 and 100,000/l. Studies of platelet aggregation have produced variable results. Aggregation induced by ADP, epinephrine, arachidonic acid, and the calcium ionophore A23187 has generally been normal or nearly normal, [97] [307] but collagen-induced aggregation has been decreased to absent in some cases[98] [307] and normal in others.[97] [311] Responses to thrombin have also been variable, with reports of decreased aggregation and secretion in response to low thrombin concentrations, [98] [307] [310] impaired increments in intracellular calcium, [312] and decreased generation of IP_3 .[313] Gray platelets agglutinate normally when exposed to ristocetin and contents of their dense granules is normal. [98] [314] As expected, the PF4, TG, fibrinogen, vWf, FV, fibronectin, and thrombospondin content of their -granules is markedly decreased. Surprisingly, gray platelets contain substantial quantities of albumin and IgG, [306] [315] proteins normally present in -granules. The PF4 and TG concentrations in the plasma of affected individuals are normal or elevated. [96] [307]

Genetic Aspects

Although nearly 40 cases of the gray platelet syndrome have been reported,[316] the mode of inheritance of the gray platelet syndrome remains uncertain, but the presence of the disorder in males and females in the same family indicates that it is an autosomal disease.[314] A disorder resembling the gray platelet syndrome is present in the Wistar Furth strain of rat and is inherited in an autosomal recessive fashion. [317]

Therapy

Treatment of bleeding episodes, if severe, may require the transfusion of normal platelets (Table 130-2).[311] DDAVP shortened the bleeding time in one patient and was used as successful prophylaxis for a dental extraction.[318]

The Quebec Platelet Disorder (Factor V Quebec)

Two unrelated families with an autosomal dominant bleeding disorder of moderate severity have been identified in Quebec. [319] [320] Their disorder is characterized by

mild thrombocytopenia, defective epinephrine-induced platelet aggregation, a decrease in the concentration of multiple -granule proteins due to degradation, and bleeding unresponsive to platelet transfusion. The disorder of the index family was initially attributed to a decrease in platelet factor V, hence the name factor V Quebec.[321] In contrast to the gray platelet syndrome, the -granules in platelets of the Quebec platelet disorder appear to be morphologically normal. [320] However, many -granule proteins, including factor V, multimerin, fibrinogen, vWf, fibronectin, osteonectin, and TSP, are decreased in concentration and substantially degraded.[322] The pathogenesis of this disorder is unknown.

Disorders of Secretion Due to Deficiency of Platelet Granules

Dense Granule Deficiency (-Storage Pool Disease)

Biology and Molecular Aspects

Normal platelets contain three to six 200300-nm dense or -granules that serve as intracellular storage sites for ADP, ATP,

calcium, pyrophosphate, and serotonin.[323] Because of their high calcium content, -granules can be visualized in electron micrographs of unfixed, unstained platelet whole mounts.[323] They can also be visualized by electron microscopy of thin sections of fixed platelets after staining with uranyl ions (uranaffin reaction) and by fluorescence microscopy after staining the platelets with mepacrine (quinacrine). [323] The -granule membrane contains a 40,000-molecular weight protein named granulophysin that may be related to the synaptosomal membrane protein synaptophysin.[324] Granulophysin is translocated to the platelet surface following platelet activation.[325] The membrane may also contain P-selectin, GPIb, and GPIIb-IIIa.[325] [326] -Granules undergo exocytosis coincident with the second wave of platelet aggregation.[327] Although -granule ADP is thought to be involved in the propagation of the primary platelet response, [328] [329] the function of the other -granule contents is not known. The biogenesis of -granules in megakaryocytes is poorly understood. -storage pool deficiency occurs in two syndromes of tyrosinase-positive albinism in humans,[330] [331] in several pigment dilution syndromes in mice[332] and rats,[333] and as part of a Chediak-Higashi-like syndrome in cattle, [334] cats,[335] mink,[336] and foxes.[337] This suggests that the biogenesis of dense granules, melanosomes, and lysosomes is somehow related. The mechanism by which dense granules accumulate ATP and ADP is also not known. It is clear, however, that two pools of adenine nucleotides exist in normal platelets that exchange very slowly, if at all. [338] One pool is a metabolic nongranule pool in which the ratio ATP/ADP is 810:1. The second pool is present in the -granules and contains 65% of the platelet adenine nucleotides with an ATP/ADP ratio of 2:3. Thus, in normal platelets, the whole platelet ATP/ADP ratio is <2.5. On the other hand, serotonin is not synthesized by megakaryocytes, but is taken up from the plasma and stored in the -granules where it is protected from platelet monoamine oxidase. [339]

Etiology and Pathogenesis

-Storage pool disease represents a heterogeneous group of disorders with -granule abnormalities as the common feature. [323] The disorder can be subdivided into deficiency states associated with albinism and those in otherwise normal individuals. -Storage pool disease has also been observed in some patients with the Wiscott-Aldrich syndrome, [340] the syndrome of thrombocytopenia with absent radii,[341] the Ehlers-Danlos syndrome, [342] and osteogenesis imperfecta.[343] When -storage pool disease is associated with albinism, as in the Hermansky-Pudlack syndrome (oculocutaneous tyrosinase-positive albinism, platelet dense granule deficiency, and ceroid like inclusions in cells of the reticuloendothelial system) [330] and the Chediak-Higashi syndrome (partial oculocutaneous albinism, frequent pyogenic infections, and giant lysosomal granules in cells of hemotologic and nonhematologic origin), [344] there is a quantitative deficiency of -granules.[324] [345] In the platelets of nonalbinos, however, the number of uranaffin-positive and mepacrine-positive granules is normal to only slightly decreased, suggesting an inability to package the -granule contents in these patients.[323] [346] The platelet content of granulophysin also parallels the apparent number of -granules in storage pool-deficient patients, being low in patients with the Hermansky-Pudlack and Chediak-Higashi syndromes, but normal in nonalbinos. [345] [346] In some nonalbino patients, the -granule abnormality is associated with a variable deficiency of -granules (-storage pool disease). [347] [348] The nature of the -granule deficiency in these patients is also heterogeneous. For example, in one patient with severe -storage pool disease, a decrease in the platelet content of P-selectin suggested that a defect in -granule formation accompanied the deficit in -granules. In a family with less severe disease, however, the platelet content of P-selectin was normal, suggesting that the -granule abnormality might be similar to that seen in the gray platelet syndrome.[348] The content of lysosomal acid hydrolases in platelets from patients with -storage pool disease is normal,[329] but thrombin-induced acid hydrolase secretion may be impaired in severely affected individuals, an abnormality that can be corrected in vitro by the addition of ADP.[329] The ability of -storage pool-deficient platelets to form thrombi on subendothelium has been studied ex vivo under various conditions of shear.[201] It was found that thrombus dimensions were reduced in proportion to the magnitude of the dense granule deficit and were reduced even further when -storage pool disease platelets were examined. These studies suggest that the contents of the -and -granules either potentiate the growth of thrombi on the subendothelium or help to stabilize the thrombi in the presence of high shear stress.

Clinical and Laboratory Findings

Patients with -storage pool disease present with a mild to moderate bleeding diathesis characteristic of patients with platelet secretion defects. [293] Platelet counts and morphology are usually normal, but bleeding times are usually, but not always, prolonged. [294] Moreover, the volume of blood that emerges from the bleeding time wounds in these patients is increased over normal, particularly at early time points, suggesting that -granule contents are involved in the contraction that follows vascular-transection.[349] The quantity of thromboxane B_2 , a metabolite of thromboxane A_2 , in the collected blood is also decreased, especially in patients with -storage pool disease, a finding consistent with the impaired agonist-induced prostaglandin and thromboxane synthesis often detected in these platelets. [350] In the aggregometer, -storage pool deficient platelets usually lack a second wave of aggregation when stimulated by agonists such as ADP and epinephrine (Fig. 130-1).[351] Responses to low concentrations of collagen are also diminished to absent, but responses to high collagen concentrations may be normal or nearly normal. [351] [352] Although this pattern of aggregation is typical for -storage pool disease, patients have been reported with normal aggregation studies. [294] Although the steady-state serotonin content of -storage pool-deficient platelets is decreased, the rate of uptake of serotonin is normal. [338] Without storage sites, however, the serotonin either leaks out of the platelet or is metabolized by platelet monoamine oxidase. [352] [353] In -storage pool deficiency, the dense granule pool of ADP no longer contributes to the whole platelet content of ADP and the ATP:ADP ratio of -storage pool deficient platelets is 3.0. [293] This alteration in ATP:ADP ratio is extremely helpful in the diagnosis of -storage pool deficiency, especially when typical aggregation abnormalities are absent. [294] Flow cytometry of mepacrine-labeled platelets may be a rapid method to detect -storage pool-deficient platelets,[354] [355] but its sensitivity compared to measurement of ATP:ADP ratios has not been tested.

Genetic Aspects

The Hermansky-Pudlak and Chediak-Higashi syndromes are inherited as autosomal recessive disorders. [331] [345] Although studies of the inheritance of -storage pool disease in patients without albinism are limited, examination of three generations of one family with this disorder suggest that it is transmitted in autosomal dominant fashion.[356] The nature of the mutations responsible for -storage pool disease is unknown. A number of murine mutations result in the disorder, [332] [357] [358] [359] [360] indicating that multiple genetic loci are involved. Studies of patients in Puerto Rico and Switzerland have localized the gene for the Hermansky-Pudlak syndrome to human chromosome 10q23.123.3.[361] It is noteworthy that this region is syntenic to the region of murine chromosome 19 that includes the mutations *pale ear* and *ruby-eye*.[362] Although the gene for the erythrocyte protein 4.2 was initially thought to be a candidate for the murine *pallid* mutation, [363] they are now thought to be distinct loci. [364]

Therapy

Bleeding can be controlled by the transfusion of normal platelets (Table 130-2). However, this is seldom necessary because the potential risks of platelet transfusion far outweigh the benefits when bleeding is not life-threatening. Moreover, other methods to readily improve hemostasis without the risks of transfusion are available. Bleeding has been controlled by the administration of cryoprecipitate [365] or DDAVP.[366] [367] The latter avoids the transfusion of blood products and should be regarded as the initial therapy of choice. DDAVP is a vasopressin analog whose pressor effects (V_1 vasopressin receptors) are substantially less than its antidiuretic effects (V_2 vasopressin receptors). Infusion of the drug releases large multimers of vWf and tissue plasminogen activator from tissue stores. [368] The released vWf accounts for the beneficial effect of the drug on the bleeding time in some forms of von Willebrand disease. The precise mechanism of its ability to shorten the bleeding time in patients with platelet function disorders is uncertain. Nonetheless, in several series of patients with platelet disorders, shortening of the bleeding time in response to

DDAVP occurred in 43100% of patients.[366] [367] [369] This variability in response rate indicates that if DDAVP is to be used for surgical prophylaxis, a bleeding time must be obtained after the DDAVP infusion to ensure that the bleeding time has responded.

In a single study, prednisone at doses of 2050 mg for 34 days was used to improve hemostasis in patients with inherited platelet disorders. [370] Shortening of the bleeding time was seen within 3 days and persisted for up to 37 days after the prednisone was stopped. However, prednisone treatment was ineffective in shortening the bleeding time in patients with thrombasthenia [221] or in normal individuals given aspirin. [371]

Disorders of Secretion Due to an Abnormal Secretory Mechanism

Platelet function in this group of disorders resembles that of individuals who have been given platelet inhibitory drugs such as aspirin. [372] There is marked impairment of aggregation and secretion in response to weak platelet agonists such as ADP, epinephrine, and low concentrations of collagen, whereas responses to stronger agonists such as higher concentrations of collagen may be normal or near normal. The bleeding time of patients with this type of secretion defect is usually prolonged. However, when it is normal or only slightly prolonged, it may become markedly prolonged 2 hours after the ingestion of three aspirin tablets. [373]

Biology and Molecular Aspects

Stimulated platelets secrete the contents of their cytoplasmic granules by a process that involves granule convergence at the center of the platelet, fusion of the granule membranes with the membranes of the surface-connected open canalicular system, and extrusion of the granule contents into the platelets external milieu. [374] [375] This process requires coupling agonist receptors on the platelet surface to the effector systems responsible for platelet aggregation and secretion and is discussed in detail in Chapter 100 . Briefly, platelet stimulation results in the activation of the phospholipases C and A2 and the serine/threonine kinases protein kinase C, calcium-calmodulin dependent protein kinase, and myosin light chain kinase. [376] Phospholipase C activation in platelets is a G protein-mediated process [376] and generates of diacylglycerol and inositol 1,4,5-triphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate. [377] Diacylglycerol is a protein kinase C activator and IP3 is responsible for the rise in intracellular calcium that accompanies platelet activation induced by most platelet agonists. [378] Platelets contain high levels of the protein tyrosine kinase pp60$^{c\text{-}src}$, [379] the src-related kinases Fyn, Lyn, Hck, and Yes,[380] and the protein kinases p72^{syk} and CPTK71[381] and platelet activation results in the phosphorylation of a number of intracellular proteins on serine, threonine, and tyrosine residues. [382] [383] [384] Which phosphorylated proteins are directly involved in platelet secretion remains to be determined.

Etiology and Pathogenesis

These disorders constitute a heterogeneous collection of abnormalities of stimulus-response coupling in platelets. In many instances, the evidence linking observed biochemical abnormalities to defective platelet secretion is not definitive. Patients have been reported whose platelets display abnormalities in arachidonic acid metabolism including impaired coupling of the thromboxane A_2 receptor and phospholipase C[385] and deficiencies of cyclo-oxygenase[386] [387] [388] [389] [390] [391] and thromboxane synthetase.[392] [393] Diminished platelet responsiveness to endogenous and exogenous thromboxane A_2 has been attributed to abnormalities of platelet endoperoxide/thromboxane A_2 receptors.[394] [395] [396] Impaired platelet responsiveness to the ionophores A23187 [395] [397] and ionomycin[398] has been observed, as well as diminished IP3 production and calcium mobilization due to defective activation of phospholipase C-2. [399] [400] [401] Impaired GPIIb-IIIa activation and pleckstrin phosphorylation following platelet stimulation by multiple agonists has been noted. [402] A patient whose platelets were initially reported as having impaired receptor-mediated release of arachidonic acid from phospholipids and calcium mobilization [403] was subsequently found to have decreased platelet membrane GTPase activity and decreased Gq. [404] This is a notable observation in view of the report that Gq-deficient mice fail to aggregate in response to thrombin, ADP, collagen, arachidonic acid, and U46619. [405] Patients have been reported whose platelets respond poorly to ADP likely due to a decrease in the number of ADP receptors.[406] [407] Others show decreased responsiveness epinephrine [408] [409] [410] [411] and platelet aggregating factor.[410] [411] In one group of 11 patients with impaired platelet responses to low concentrations of collagen, 8 were noted to have abnormal responses to other weak-agonists, such as ADP, epinephrine, thromboxane A_2, and the prostaglandin endoperoxide analog U44069, but normal responses to strong agonists such as thrombin and high concentrations of collagen. [412] This suggests a defect in coupling stimulation by weak agonists to secretion, rather than a defect in the secretory mechanism itself. In remaining patients, responses to strong agonists were abnormal and the platelets appeared unable to synthesize thromboxane A_2. Finally, in patients with the neuropsychiatric attention deficit disorder and easy bruising, abnormal dense granule and lysosome secretion was inexplicably observed when gel-filtered platelets, but not platelets in platelet-rich plasma, were stimulated with thrombin or the ionophore A23187.[413]

Genetic Aspects

Most reports have been of individual patients so that determination of the mode of inheritance has not been possible. Where families have been studied, the mode of inheritance appeared to be autosomal dominant. [388] [390] [393] [394] [408]

Therapy

The approach to therapy in patients with these disorders is identical to that described above for patients with storage pool disease.

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DISORDERS OF PLATELET PROCOAGULANT ACTIVITY

The plasma membrane of activated platelets provides a surface for the assembly of the tenase and prothrombinase complexes that activate coagulation factor X and prothrombin, respectively (see Chap. 102 for a detailed discussion). This procoagulant activity was previously termed platelet factor 3. Although it is uncertain whether specific membrane structures

serve as receptors for this activity, it is clear that it is associated with a calcium-induced exposure of anionic phospholipids on the platelet surface. The phospholipid composition of the platelet plasma membrane, like that of other cells, is asymmetric with phosphatidylcholine and sphinomyelin localized predominantly in the outer membrane leaflet and phosphatidylserine and phosphatidylethanolamine concentrated in the inner leaflet. This asymmetry is catalyzed by an aminophospholipid translocase, an Mg^{82+} -dependent ATPase.[414] [415] Following an agonist-stimulated rise in intraplatelet Ca^{2+} , however, membrane asymmetry is lost with exposure of anionic phospholipids on the platelet surface [416] and with membrane vesiculation.[417] A similar response to increased intracellular calcium has been detected in erythrocytes[418] and lymphocytes.[419] The rapid Ca^{2+} -induced movement of phospholipids between membrane leaflets is mediated by a membrane-associated phospholipid scramblase.[420] The phospholipid scramblase, cloned from a human K562 cell cDNA library, is a proline-rich 37,000-molecular weight type II membrane protein present not only in erythrocytes and platelets, but in a variety of hematologic and nonhematologic tissues and cell lines as well. [421]

Isolated deficiency of platelet procoagulant activity is exceedingly rare. In addition to the one well-studied patient after whom the disorder was named (Scott syndrome),[422] data on three additional patients have been reported. [222] [423] The initial patient presented with bleeding after surgery and dental procedures and a spontaneous retroperitoneal hematoma.[422] Although prothrombin and partial thromboplastin times, bleeding times, and platelet function studies were normal, serum prothrombin times were short and the patients platelets were unable to express normal procoagulant activity. Moreover, although the patients platelets secreted normal quantities of factor Va activity, they expressed only 2025% of the normal number of factor Xa binding sites. [424] In addition, factor X activation by factor IXa and factor VIIIa was impaired, suggesting a concomitant deficiency of factor IXa and factor VIIIa binding sites. [425] When stimulated with the combination of thrombin and collagen, there was decreased exposure of anionic phospholipids on the platelet surface [426] and decreased membrane vesiculation.[427] Similarly, there was decreased membrane vesiculation and procoagulant activity when the patients erythrocytes were treated with the ionophore A23187. [418] However, whereas membrane-associated phospholipid scramblase activity in the patients platelets, erythrocytes, and lymphocytes was impaired, Ca^{2+} -induced function of the isolated scramblase incorporated into proteoliposomes was indistinguishable from normal, [420] suggesting that another membrane component affecting the function of the scramblase may be responsible for the syndrome.

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Chapter 131 - Acquired Disorders of Platelet Function

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INTRODUCTION

The acquired disorders of platelet function are among the most common of all hematologic abnormalities, an observation supported by the predictable effect on platelet function of the ubiquitous drug, aspirin. This chapter discusses the clinical disorders associated with acquired defects in platelet function (Table 131-1). A major goal is to attempt to provide a balanced view of the clinical importance of each acquired platelet defect. This task may not be possible in all cases. An illustration of the real difficulty in predicting the risk for hemorrhage is the clinical experience with Glanzmann thrombasthenia, a congenital platelet disease that is defined by a long bleeding time and absent platelet aggregationabnormalities similar to but typically more severe than those found in many acquired disorders. Among thrombasthenic patients who have equally severe abnormalities of platelet function the range of bleeding symptoms is great, and some patients experience no excessive bleeding throughout their lives. In most patients, severe hemorrhagic episodes are sporadic and unpredictable. [1] Therefore, since acquired platelet function defects generally produce milder symptoms than Glanzmann thrombasthenia, it should be expected that bleeding in patients with acquired defects will be even less predictable and may occur only when additional hemostatic defects are present. The preservation of normal hemostasis despite diminished platelet function is consistent with the observation that spontaneous bleeding does not usually occur in thrombocytopenic patients until the platelet count is <10,000/ml, or <5% of the normal value.[2]

Part of the problem in the clinical assessment of these disorders is the difficulty in interpreting the laboratory measures of

TABLE 131-1 -- Principal Causes of Acquired Disorders of Platelet Function

Drugs that affect platelet function
Nonsteroidal anti-inflammatory drugs
Ticlopidine
-Lactam antibiotics
Other drugs (see Table 131-2)
Foods and food additives
Systemic conditions associated with abnormal platelet function
Chronic renal disease
Cardiopulmonary bypass surgery
Antiplatelet antibodies
Hematologic diseases associated with abnormal platelet function
Chronic myeloproliferative disorders
Leukemias and myelodysplastic syndromes
Dysproteinemias

platelet function by which they are defined: platelet aggregation and the bleeding time. These diagnostic procedures are discussed more fully in Chapter 106 , but their interpretation requires emphasis here. There are several reasons why it cannot be assumed that abnormal in vitro platelet aggregation or a prolonged bleeding time predict an increased risk for bleeding. First, it is difficult to accurately express results of aggregation studies in quantitative terms, and there is no universally accepted definition of a normal range of values. Second, aggregation studies can be inconsistent and abnormal in normal subjects who have ingested no medication [3] and may vary with the age of the subject.[4] Third, aspirin ingestion is widespread in our society, and this drug causes abnormal patterns of aggregation (see below). Fourth, the secondary wave of aggregation following adenosine diphosphate (ADP) stimulation occurs in citrate-anticoagulated platelet-rich plasma, but not in the presence of physiologic concentrations of calcium, which suggests that this normal aggregation pattern occurs in vitro but may not occur in vivo. [5] Even with these limitations, platelet aggregation studies can be useful in the diagnostic evaluation of selected patients, but the ability of abnormal results to predict an increased risk for bleeding is unknown. Finally, although the bleeding time is an important aid in the diagnosis of selected congenital disorders, [6] its value in predicting a risk for hemorrhage is unproved.[7]

Despite difficulty in assessing individual risks, acquired platelet function disorders do have a clear effect on hemostasis and thrombosis in large studies. Again the case of aspirin is pertinent, as recent data demonstrate that a small but sustained dose of aspirin (325 mg every other day) may cause a reduction in the risk for myocardial infarction in a selected study group but may also increase the risk for hemorrhage. [8]

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DRUGS THAT AFFECT PLATELET FUNCTION

A vast array of drugs are known to affect platelet function (Table 131-2). [9] Some of the agents listed in Table 131-2 are used specifically for their antithrombotic activity, with diminished platelet function being the therapeutic goal. [10] These agents are also discussed in Chapter 125 . For the others, the occurrence of abnormal platelet function is an unwanted side effect. For all of these drugs, their effect on platelet function is defined by an abnormality of platelet aggregation or of the bleeding time, but whether they contribute to a risk of excessive bleeding is definitively established only for aspirin.

Aspirin

Aspirin has become a common household drug since its introduction nearly 100 years ago, and estimates of its current use in the United States are staggering: 10,00020,000 tons annually, [11] which is equivalent to almost one tablet for every person every day.

Definition of the Aspirin-Induced Platelet Defect

The mechanism of aspirin impairment of platelet function is the acetylation and irreversible inhibition of platelet cyclooxygenase. [10] [12] For platelets, which have minimal protein synthetic capacity, a single small dose of aspirin, 40100 mg, or as little as 10 mg taken daily for a week, can totally inhibit thromboxane production and therefore impair the function of a cohort of platelets for its entire circulating life span of 10 days. These observations are consistent with data suggesting a therapeutic antithrombotic effect of aspirin at doses as low as 30 mg/day. [12] Although the effect of aspirin on platelet cyclooxygenase function is clear, it may not be the only mechanism by which aspirin interferes with hemostasis. Aspirin depresses thrombin formation in clotting blood, possibly by acetylating prothrombin, and the acetylation of fibrinogen after therapeutic doses of aspirin significantly interferes with fibrin formation and allows accelerated fibrinolysis. [13]

Aspirin also acetylates cyclooxygenase in endothelial cells, blocking synthesis of the final product of endothelial cell eicosanoid metabolism, prostacyclin, which is a strong inhibitor of platelet function. However, the endothelial enzyme is less sensitive than platelet cyclooxygenase to low doses of aspirin, and, in contrast to platelets, endothelial cells can recover cyclooxygenase activity by synthesizing new enzyme. [12] [13] Therefore, a consideration for therapeutic trials with aspirin has been to use a low dose, minimizing the inhibition of endothelial cell prostacyclin synthesis, which could have a prothrombotic effect. A significant portion of aspirins effect on platelets occurs in the portal circulation; [14] half of an oral dose is deacetylated in the liver to form inactive salicylic acid. This provides an additional therapeutic advantage by allowing small oral doses of aspirin to react more effectively with circulating platelets than with endothelial cells in the systemic circulation.

Aspirin and Platelet Aggregation

A predictable abnormality after exposure of platelets to aspirin either in vivo or in vitro is impaired aggregation with epinephrine, ADP, arachidonic acid, and low concentrations of collagen and thrombin. [15] These abnormalities are a direct result of inhibition of cyclooxygenase, with the resulting deficient synthesis of the final product of platelet arachidonic acid metabolism, thromboxane A_2 . [10] Thromboxane A_2 diffuses out of the platelet, binds to specific platelet membrane receptors, and reinforces aggregation by promoting alpha-granule and dense granule secretion. With cyclooxygenase inhibition, only a primary, reversible wave of platelet aggregation without platelet secretion occurs following stimulation by epinephrine and low concentrations of ADP, thrombin, and collagen. The aspirin-induced abnormality of platelet aggregation is so characteristic that abnormal platelet aggregation patterns of any cause are often designated as aspirin-like. Stronger agonists (high concentrations of thrombin and collagen) do not require thromboxane A_2 synthesis to cause platelet secretion and irreversible aggregation. The occurrence of a complete platelet response in the presence of total inhibition of the enzyme cyclooxygenase demonstrates the presence of multiple pathways of platelet activation, and this may explain why normal subjects almost always have normal hemostasis while taking aspirin.

Aspirin and the Bleeding Time

Aspirin also prolongs the bleeding time, but in individual subjects this is less consistent than the platelet aggregation abnormality. Initial studies demonstrated that 2 hours after ingestion of two to three aspirin tablets (650975 mg), the mean template bleeding time of a group of normal subjects was significantly increased. [6] [16] Prolongation of the bleeding time can be demonstrated for as long as 4 days after a single dose of aspirin, [17] until normal platelet turnover results in the appearance of new platelets with normal function. However, aspirin actually prolongs the bleeding time to a value greater than the normal range in only about half of normal subjects. [6] [16] It is assumed, but has not been clearly demonstrated, that individual subjects have a consistent bleeding time response to aspirin on repeated challenges. [6] [17] Smaller single doses of aspirin (150160 mg) that consistently cause abnormal platelet aggregation do not cause a prolonged bleeding time; however, daily ingestion of 30 mg of aspirin (but not 10 mg) does cause an increased bleeding time. [18]

The bleeding time has a reputation for being the most direct test of platelet function, because it is performed directly on the patient and because it correlates with the platelet count in selected patients. [19] However, its limitations need to be

TABLE 131-2 -- Drugs That Inibit Platelet Functiona [a]

Nonsteroidal anti-inflammatory drugs	Nifedipine (Procardia)
Aspirin [b]	Verapamil (Calan, Isoptin)
Sulfinpyrazone (Anturane) [b]	Diltiazem (Cardizem)
Indomethacin (Indocin)	Quinidine
Ibuprofen (Advil, Motrin, Nuprin)	Psychotropic drugs
Sulindac (Clinoril)	Tricyclic antidepressants
Naproxen (Naprosyn)	Imipramine (Tofranil)
Phenylbutazone (Butazolidin)	Amitriptyline
Meclofenamic acid (Meclomen)	Nortriptyline (Pamelor)

Mefanamic acid (Ponstel)	Phenothiazines
Diflunisal (Dolobid)	Chlorpromazine (Thorazine)
Piroxican (Feldene)	Promethazine (Phenergan)
Tolmetin (Tolectin)	Trifluoperazine (Stelazine)
Zompirac (Zomax)[c]	Anesthetics
	Local
-Lactam antibiotics	Dibucaine (Lidocaine)
Penicillin G	Tetracaine (Carbocaine)
Penicillin G	Cyclaine
Carbenacillin (Geopen)	Butacaine
Ticarcillin (Ticar, Timentin)	Nepercaine
Methicillin (Staphcillin)	Procaine
Ampicillin (Polycillin, Omnipen)	Cocaine
Nafcillin (Nafcil, Unipen)	Plaquenil
Piperacillin (Pipracil)	General
Azlocillin (Axlin)	Halothane (Fluothane)
Mezlocillin (Mezlin)	Narcotic
Apalcillin	Heroin
Sulbenicillin	Oncologic drugs
Temocillin	Mithramycin
Cephalosporins	Daunorubicin
Cephalothin (Keflin, Seffin)	BCNU
Moxalactam (Moxam)	Miscellaneous drugs
Cefoxitin (Mefoxin)	Ticlopidine
Cefotaxime (Claforan)	Clofibrate (Atromid-S)
Cefazolin (Ancef, Kefzol)	Ketanserin
Other drugs	Antihistamines
Antibiotics	Diphenhydramine (Benadryl, Allerdryl)
Nitrofurantoin (Furadantin, Macrodantin)	Chlorpheniramine (Donatussin, Probahist)
Drugs that increase platelet cAMP concentration	Mepyramine
Prostacyclin	Radiographic contrast agents
Iloprost	Renografin-76
Dipyridamole (Persantin)[b]	Renovist II
Anticoagulant	Conray-60
Heparin	
Fibrinolytic agents	Foods and food additives
Plasma expanders	-3 Fatty acids (eicosapentaenoic acid)
Dextrans	Ethanol
Hydroxyethyl starch (hetastarch)	Chinese black tree fungus
Cardiovascular drugs	Onion extract
Nitroglycerin	Ajoene (garlic component)
Isosorbide dinitrate (Isordil, Diltrate)	Cumin
Propranolol (Inderal)	Turmeric
Nitroprusside (Nitropress)	Clove

[a] Of these drugs, only aspirin has been demonstrated to cause a significant increase in bleeding (Table 1313). The other drugs are described as affecting platelet aggregation or the bleeding time, and case reports have suggested an association with increased bleeding. Brand names for these agents are given in parentheses when they may be more familiar.
[b] Used as a therapeutic antithrombotic agent.
[c] Withdrawn from the U.S. market in 1983.

recognized.[7] For example, to demonstrate an effect of aspirin on the template bleeding time, certain technical details must be followed. If the bleeding time test is performed using a vertical rather than the traditional transverse skin incision and without the usual venostasis, there is no prolongation after aspirin treatment, despite the characteristic changes in platelet aggregation and secretion.[17] An older bleeding time technique, the Duke earlobe puncture, is insensitive to aspirin treatment.[16] It is usually assumed that the skin bleeding time can be used to assess the risk for bleeding from other organs. However, in one study of patients taking aspirin for rheumatic disease and who were undergoing gastric endoscopic biopsy, this assumption was shown not to be true.[20] These data emphasize that the bleeding time cannot be automatically accepted as an accurate representation of systemic hemostasis.[7]

The most dramatic demonstrations of the aspirins effect on the bleeding time have been in patients with severe hemophilia.[21] The bleeding time is already slightly prolonged in some patients with severe hemophilia A.[22] However, following the administration of two or three aspirin tablets, sustained hemorrhage from the bleeding time incision was observed in five of 11 patients with severe hemophilia A, and this ultimately required an infusion of plasma or factor VIII to arrest the bleeding.[21] Patients with mild hemophilia appeared to have less risk

from aspirin, as in none of 16 patients was the bleeding time prolonged greater than values in the normal control group.[21] Also, small doses of aspirin cause a greater than normal prolongation of the bleeding time in patients with chronic renal failure and have been associated with reports of major bleeding.[23] The combination of ethanol and aspirin has a synergistic effect on increasing the bleeding time in normal subjects.[24]

Clinical Importance of the Aspirin-Induced Defect in Platelet Function

In contrast to its antithrombotic effect, which appears not to be dose related above small threshold doses, aspirin-induced gastrointestinal (GI) mucosal toxicity is dose related.[25] The mechanism of mucosal injury appears to be separate from the effect on hemostasis. The risk for GI bleeding with aspirin, from both the upper and lower GI tract and from both discrete ulceration and more diffuse mucosal damage, is increased 1.52.0-fold, [26] even with low doses of aspirin, 100 mg/day.

The overall clinical importance of aspirin on hemostasis was demonstrated by the administration of a low dose of aspirin (325 mg every other day) or a placebo to 22,071 physicians over the course of 5 years (Table 131-3).[8] Aspirin-treated subjects had a 44% decrease in the risk for myocardial infarction. Aspirin treatment was also associated with a small but significant increase in the risk for serious hemorrhage. Some 2,979 subjects in the aspirin group and 2,248 of those taking placebo reported problems such as easy bruising, hematemesis, melena, and epistaxis (relative risk, 1.32; $P < 0.00001$), and 48 in the aspirin group, compared to 28 in the placebo group, required a blood transfusion during the 5-year period ($P = 0.02$). Therefore, in spite of the difficulty in assessing the effect of aspirin on the hemostatic function of individual normal subjects, the clinical importance of aspirin-induced impairment of hemostasis is certain. Aspirin may cause more bleeding symptoms in patients with other hemostatic defects. The risk of bleeding is increased if aspirin is given simultaneously with warfarin in high-intensity regimens (INR 3.04.5), although the occurrence of major hemorrhagic events may not be increased.[27] On the other hand, aspirin given during treatment of acute coronary syndromes in combination with fibrinolytic and other antithrombotic agents does not appear to increase the incidence of major bleeding episodes. [13] [28]

TABLE 131-3 -- Effect of Aspirin on the Risk of Bleeding in Normal Subjectsa[a]

	Aspirin	Placebo	Relative Risk	*P* Value
No. of subjects	11,037	11,034		
No. with hemorrhagic complications	2,979	2,248	1.32	<0.00001
Easy bruising	1,587	1,027	1.55	<0.0001
Epistaxis	862	640	1.35	<0.0001
Melena	364	246	1.48	<0.00001
Hematemesis	38	28	1.36	0.22
Nonspecific gastrointestinal bleeding[b]	440	422	1.04	0.55
Other bleeding[c]	724	596	1.21	0.0004
Number of requiring blood transfusion	48	28	1.71	<0.02

[a] These data are adapted from the Physicians Health Study Research Group,[g] and they represent the only definitive documentation of a hemorrhagic risk from drug-induced platelet dysfunction, other than the direct platelet GPIIb/IIIa receptor antagonists. The subjects were healthy male physicians, treated with one aspirin tablet (325 mg) every other day or a placebo, and followed for an average of 60.2 months.
[b] No further defined.
[c] Hemorrhagic complications described as other bleeding: 29% were related to shaving or brushing the teeth (32% in the aspirin group and 27% in the placebo group), and 72% were hematuria (70% in the aspirin group and 75% in the placebo group).

MANAGEMENT OF PATIENTS TAKING ASPIRIN

The therapeutic recommendations for management of a patient taking aspirin are straightforward. In a patient with a clear therapeutic indication for aspirin, whether it be a headache or unstable angina, and no apparent increased risk for bleeding, aspirin is one of the safest drugs. In patients who already have greatly increased hemorrhagic risks, such as severe hemophilia, aspirin must be avoided.[21] When a hemostatically normal patient who is taking aspirin is scheduled for an invasive procedure, aspirin should be discontinued for at least 5 days.[35] This period of time is sufficient for about half of the circulating platelets to be replaced by platelets from megakaryocytes that were not exposed to aspirin, and half of the normal number of platelets (e.g., approximately 100,000/l) is sufficient for normal hemostasis during any surgical procedure. However, since aspirin use is so common[11] and since the drug is present in hundreds of pharmaceutical products, it may not be feasible to discontinue aspirin and wait 5 days for a procedure in most patients. Then only careful observation is necessary. Significant excessive bleeding due to aspirin is rare, and any serious bleeding that does occur during an operative procedure is likely due to a surgically correctible lesion. If severe hemorrhage due to deficient platelet function is suspected, a platelet transfusion would be promptly effective, but this will be indicated only very rarely. Perhaps the recommendations for surgery in a patient who has taken aspirin should parallel the recommendations for surgery in patients taking prophylactic subcutaneous heparin. [36] [37] With both aspirin and the conventional fixed doses of subcutaneous heparin (5,000 units twice daily), the risk for increased bleeding during and after surgery is significant in large studies but is negligible in any individual patient. Therefore, except for those patients in whom the nature of the surgical procedure requires the absolute minimum risk for excessive bleedingas in some plastic surgical, neurosurgical, or ophthalmologic proceduresthe increased risk for bleeding with aspirin use does not appear to be a reason to delay necessary surgery. The use of prophylactic platelet transfusions in this clinical setting is discouraged.

Furthermore, relatively low doses of aspirin (60 mg/day) given during pregnancy did not increase maternal or neonatal bleeding complications. [29]

On balance, the hemostatic risks of aspirin treatment may be similar to those observed in patients with Glanzmann thrombasthenia, in which the hemorrhagic complications are greater when there is an additional cause for bleeding. [1]

Several studies have addressed the risk of postoperative hemorhage in patients taking aspirin. The results, as may be expected with a very mild hemostatic defect, are not consistent. Some studies report increased blood loss in patients taking aspirin who undergo cardiac surgery, but others do not. [9] Reports of increased chest tube blood loss in aspirin-treated patients are not associated with worse clinical outcomes, such as a need for reoperation or greater numbers of units of blood transfused.[30] [31]

In studies of patients undergoing elective total hip replacement by a single surgeon, aspirin treatment (1.23.6 g/day) was begun the day before surgery and continued for 7 days.[32] The blood loss was 20% greater in the treated group in one study, but this was considered clinically unimportant and required no additional care. [32] A study of 200 patients undergoing general

or gynecologic surgery also suggested that there was greater operative blood loss in patients who had taken aspirin. [33] The surgeon and the anesthesiologist observed greater than expected bleeding in 12 of 55 patients who had taken aspirin and had abnormal results on platelet aggregation studies, compared to 7 of 97 patients who had not taken aspirin and had normal aggregation studies. The other 48 patients in this study either claimed they had not taken aspirin but were found to have abnormal platelet aggregationa frequent occurrence in many studies owing to habitual and forgotten aspirin use (33 patients)or had taken aspirin but their platelet aggregation was normal (15 patients). Eight of these 48 patients were thought to have excessive bleeding at surgery. Although the results were interpreted as demonstrating increased operative bleeding following aspirin treatment, it is important to note that greater than expected bleeding also occurred in the control patients. This observation is inevitable in all subjective evaluations of bleeding, either surgical or medical. The observer always expectshopes forthe normal bleeding response to be minimal, but the range of normal bleeding is great, and patients with more bleeding are often viewed with concern.

It may be concluded from the sum of these reports that aspirin has a small, inconsistent, but sometimes important effect on surgical bleeding. Minimally invasive trauma, such as spinal or epidural anesthesia, is not associated with increased bleeding in patients taking aspirin. [29] [34] These data on aspirin represent well the clinical paradigm for acquired disorders of platelet function: (1) their occurrence is common; (2) the defect is usually mild; (3) normal individuals vary in their sensitivity to aspirin; (4) platelet aggregation and the bleeding time are subject to technical vagaries; (5) the risk for increased bleeding in the individual subject who is otherwise normal is minimal and unpredictable; (6) however, a definite risk for increased bleeding with aspirin can be demonstrated when large groups of normal subjects are analyzed or when individuals are studied who have concomitant pathologic (e.g., hemophilia) or physiologic (e.g., childbirth) conditions that can predispose to hemorrhage.

Other Nonsteroidal Anti-inflammatory Drugs

In addition to aspirin, many other drugs used for their anti-inflammatory and analgesic properties can cause decreased platelet function (Table 131-2).[9] As with aspirin, their mechanism of action appears to be the inhibition of the activity of platelet cyclooxygenase. In contrast to aspirin, each of these agents has only a temporary effect on cyclooxygenase function, causing enzyme inhibition only as long as the active drug is present in the circulation. Therefore, among these agents only drugs such as piroxicam, which has a plasma half-life of >2 days, [38] affect platelets for more than a few hours. As with aspirin, the most sensitive indication of impaired platelet function is the inhibition of in vitro aggregation and secretion. These agents prolong the bleeding time minimally and transiently or not at all, consistent with the bleeding time being a less sensitive measure of the aspirin-induced defect. [9]

Clinical reports are consistent with the observations on platelet function and suggest that these drugs cause less risk for increased bleeding than aspirin. As with aspirin, they may increase the bleeding time in patients with severe hemophilia, [35] although in two studies therapeutic doses of ibuprofen had no effect on the bleeding time in 19 of 20 patients with hemophilia. [39] Therefore, the clinical approach to patients taking any drug that can inhibit platelet cyclooxygenase should be similar, although the hemostatic risk with drugs other than aspirin usually disappears a few hours after the drug is stopped. The additional risk for bleeding with surgery should be negligible, but a prudent course would be to discontinue these drugs the day before a procedure. Analgesics such as acetaminophen and sodium or choline salicylate do not inhibit platelet function and have no adverse effect on hemostasis. [39]

Ticlopidine and Clopidogrel

Ticlopidine is a thienopyridine that is used to reduce the risk of thrombotic strokes in patients who have cerebrovascular disease, and for the prevention of myocardial infarction.[10] [40] The degree of prolongation of the bleeding time is equivalent to that seen with aspirin. Ticlopidine impairs fibrinogen binding to GPIIb/IIIa and inhibits platelet aggregation to many agonists, particularly ADP. The major site of action appears to be on the pathway providing stimulusresponse coupling between the ADP receptor and fibrinogen binding. The effects of ticlopidine on platelet function appear within 2448 hours after ingestion, are maximal in 46 days, and continue for 410 days after the drug has been discontinued.[40] Ticlopidine may be associated with a small increased risk for bruising and epistaxis, but the major complications are neutropenia, aplastic anemia, and thrombocytopenia.[40] It is recommended that ticlopidine be discontinued at least 10 days before elective surgical procedures. Clopidogrel is a congener of ticlopidine that has similar effects on platelet function and similar antithrombotic efficacy in atherosclerotic vascular diseases, although with a reduced tendency to cause cytopenias.[41]

Platelet GPIIb/IIIa Receptor Antagonists

A new class of agents has been developed for use in acute coronary syndromes; these agents block the fibrinogen receptor function of platelet GPIIb/IIIa, essentially creating a phenotype of Glanzmann thrombasthenia.[28] [42] The first agent that was approved for use in 1996, abciximab (ReoPro), is a human-murine chimeric monoclonal antibody to human platelet GPIIb/IIIa, and it also reacts with another $_3$ integrin, $_v{}_3$. Agents developed subsequently are highly selective for GPIIb/IIIa and include RGD- (or KGD)-containing peptides or small peptidomimetic molecules.[43] Abciximab and the recently approved agents, integrilin and tirofiban, are given by intravenous infusion; some of the subsequent agents will be orally administered. Initial clinical trials have demonstrated efficacy in preventing restenosis after coronary angioplasty and in the treatment of unstable angina. [28] [43] [44] Bleeding complications were significantly greater with abciximab plus heparin than with conventional treatment with heparin alone in initial trials, but this adverse effect was reduced by decreasing the heparin dose in subsequent trials. [44] An important complication of use of these agents is the occurrence of acute, profound thrombocytopenia, which occurs in 1% of patients. [42] [45] The cause of the thrombocytopenia is unknown, but the clinical course suggests that these patients have a preformed, naturally occurring antibody against epitopes on GPIIb/IIIa that is exposed by binding of the receptor antagonist molecules. Drugs of this class in general have a high affinity for circulating platelets, and their clearance when dissociated is rapid. As might be expected, therefore, platelet transfusions are effective in the management of bleeding in these patients, either in the presence or in the absence of thrombocytopenia.

-Lactam Antibiotics

Antibiotics are the other major category of therapeutic agents, along with the nonsteroidal anti-inflammatory drugs (NSAIDs), that affect platelet function (Table 131-2).[9] Like NSAIDs, antibiotics are widely used. The antibiotics that affect platelet function all share a common -lactam ring structure, a characteristic of the penicillins and cephalosporins. Some of the antibiotics listed in Table 131-2 have a predictable dose- and duration-related effect on the bleeding time. [46] [47] Because the effect on bleeding time is seen only in patients who are receiving large parenteral

doses of antibiotics, this is a potential problem only for hospitalized patients. In a study of 74 hospitalized patients with a consistently prolonged bleeding time, the likely cause was penicillin in 39 patients (30 patients were receiving penicillin G, more than 15,000,000 U/day, and 9 were receiving ampicillin, 68 gm/day) and aspirin or related drugs in 7 patients.[48]

The structural properties that cause some, but not all, of the penicillins and cephalosporins to affect platelet function are unknown. The diversity of side chain structure alters the antibacterial and pharmacologic properties of the penicillins and cephalosporins and may also determine their effect on platelet function. It is postulated that the antibiotic associates with the platelet plasma membrane by a lipophilic mechanism, causing a perturbation that blocks multiple receptoragonist interactions or stimulusresponse coupling.[49] The characteristic laboratory observation is the occurrence of a prolonged bleeding time and abnormal platelet aggregation after several days of high-dose parenteral therapy. [46] [47] These abnormalities do not subside until several days after the antibiotic is discontinued.

The frequency of clinically important bleeding in patients taking -lactam antibiotics appears to be low and is not predicted by a prolonged bleeding time, and the causal relationship to antibiotic treatment is unproved. [50] Certainly many patients who need to receive high doses of these antibiotics have risk factors for hemorrhage, such as thrombocytopenia, sepsis, malignancy, and renal failure. Typical case reports suggesting antibiotic-related hemorrhage (e.g., with the use of carbenicillin and nafcillin [51] [52]) occur in such complicated patients. In these cases, the antibiotic was assumed to have contributed to the bleeding because the diagnostic bleeding times were markedly abnormal. An alternative explanation is that the bleeding time is not related to a risk for hemorrhage [7] and that the antibiotics were innocent. For each report implicating an antibiotic as a cause of hemorrhage, many more patients receive the same antibiotics in large doses without bleeding complications.[50] In conclusion, antibiotic-induced platelet dysfunction appears to have little clinical importance, and the potential effect on platelet function should not

be considered in the decision to use any antibiotic in any clinical situation.

An exception to this conclusion may be moxalactam. The frequency of clinically important hemorrhagic complications with moxolactam appears to be greater than with other antibiotics.[50] Its effect on the bleeding time and platelet aggregation are no different from that of other antibiotics, [47] so it probably has no more profound effect on platelet function. However, unlike most other -lactam antibiotics, moxalactam contains a methylthiotetrazole-leaving group that has been implicated in the inhibition of synthesis of vitamin K-dependent proteins.[53] Therefore, moxolactam-induced bleeding may be due to the combination of deficiencies in coagulation factors II, VII, IX, and X and impaired platelet function.

Other Drugs

Antibiotics

Nitrofurantoin, an antibiotic structurally unrelated to the -lactam antibiotics, may cause a mild prolongation of the bleeding time and impair platelet aggregation when blood levels of the drug are >20 M.[54] Nitrofurantoin is not known to cause clinical bleeding.

Drugs That Increase Platelet Cyclic AMP Concentration

Elevation of the platelet concentration of cyclic adenosine monophosphate (cAMP) by any mechanism inhibits platelet function. Prostacyclin and its analogs increase cAMP synthesis by stimulation of adenyl cyclase, and they have been studied as antithrombotic substitutes for heparin in cardiopulmonary bypass surgery and hemodialysis.[10] [55] In spite of the potent inhibition of platelet aggregation by prostacyclin in vitro, the effects on the bleeding time are minimal and inconsistent. [55] Dipyridamole increases cAMP concentration by inhibition of the cAMP phosphodiesterase; it has been used extensively as an antithrombotic agent despite the lack of clearly documented efficacy.[10] There are no data to suggest that dipyridamole causes increased bleeding.

Anticoagulants

Although heparin is best known for its anticoagulant effect and its adverse effect in causing thrombocytopenia (see Chaps. 122 and 129), it has the potential to affect platelet function. Heparin can bind to the platelet surface, [56] cause platelet aggregation and secretion, [57] and impair von Willebrand factor (vWF)-dependent platelet function.[58] Heparin can also cause a prolonged bleeding time.[59] Whether these phenomena are related to the bleeding complications of heparin use is unknown. The prolonged bleeding time is probably due to heparins inhibition of thrombin generation, analogous to the slight but significant increase in bleeding time in patients with hemophilia.[22]

Fibrinolytic Agents

Bleeding during therapy with plasminogen activators is predominantly due to the effects of hypofibrinogenemia and increased fibrin(ogen) degradation products on fibrin clot formation, usually combined with a structural lesion in the blood vessel wall (see Chap. 123). Pharmacologic doses of streptokinase, urokinase, and tissue plasminogen activator (t-PA) may impair platelet function.[60] Several mechanisms may be involved. First, very high levels of fibrin(ogen) degradation products coupled with very low levels of fibrinogen may impair platelet aggregation. Second, plasminogen can bind to the platelet surface where it is converted to the proteolytic enzyme, plasmin, by plasminogen activator.[61] On the platelet surface, plasmin can degrade both GPIb (thereby impairing the interaction of the platelet with vWF [62]) and fibrinogen (thereby dispersing platelet aggregates [63]). Third, plasmin can inhibit platelet aggregation by blocking the release of arachidonic acid from platelet membranes, thereby limiting thromboxane production.[64] The clinical importance of these observations is unknown.

Plasma Expanders

Dextrans are partially hydrolyzed branched polysaccharides of glucose. The two preparations in clinical use have average molecular sizes of 70,00075,000 (often termed dextran 70) and 40,000 (termed dextran 40, or referred to as low-molecular-weight dextran). Both preparations are effective plasma expanders and both can affect platelet function, though there are some data to suggest that the high-molecular-weight molecules have a greater effect on hemostasis. [65] An infusion of a liter of 6% dextran solution over 1 hour prolonged the bleeding time in about half of 163 normal subjects, with some bleeding times prolonged to >30 minutes. [65] Dextran infusion also impairs platelet aggregation and platelet procoagulant activity and can cause a modest reduction in plasma vWF concentration. However, dextran has no effect on platelet function when added directly to platelet-rich plasma in vitro. [66] Because of these effects on platelet function, dextran has been used as an antithrombotic agent, and its efficacy in preventing fatal postoperative pulmonary emboli appears to be equivalent to that of subcutaneous heparin. [67] This therapeutic effect is achieved without any increased risk for bleeding, as demonstrated in a large double-blind trial comparing dextran and placebo during general surgery. [68] Therefore dextran infusions represent yet another example of an agent that can alter platelet function and prolong the bleeding time without an apparent increased risk for hemorrhage. Hydroxyethyl starch, known as hetastarch, is a synthetic

glucose polymer with an average molecular weight of 450,000 (range, 10,0001,000,000) that is also used for plasma expansion. Like dextran, it may prolong the bleeding time, particularly if administered in doses of >20 ml/kg of a 6% solution, and may predispose to bleeding if administered simultaneously with heparin or used in a patient with a preexisting hemostatic defect.[69]

Cardiovascular Drugs

Administration of nitroglycerin, isosorbide dinitrate, propranolol, or nitroprusside can decrease platelet aggregation and secretion, although the effects are minimal and inconsistent in some studies.[9] Some of the observed effects may also be mediated by desensitization of platelets by increased plasma concentrations of epinephrine.[70] There are also numerous reports on the effect on platelets by drugs used clinically for their ability to inhibit the influx of calcium ions across the membranes of excitable cells, known as calcium channel blockers: nifedipine, verapamil, and diltiazem. [9] These studies have demonstrated inhibition of platelet aggregation in vitro when high concentrations (micromolar) of the drug were used with washed platelets. [71] This effect is seen primarily with epinephrine as the agonist and it does not appear to be related to inhibition of calcium ion flux. The proposed mechanisms of action include inhibition of epinephrine binding to $_2$ -adrenergic receptors, inhibition of the platelet response to thromboxane A_2 , and inhibition of serotonin-induced aggregation. In therapeutic doses, the calcium channel blockers do not prolong the bleeding time. Quinidine, an anti-arrhythmic drug, can act as an antagonist at platelet $_2$ -adrenergic receptors when in high concentrations. In a single report, a patient taking 800 mg of quinidine and 650 mg of aspirin daily developed melena and generalized petechiae with a normal platelet count and a bleeding time of >35 minutes.[72] Subsequent studies on two normal volunteers showed that quinidine caused a mild increase in the bleeding time that was apparently potentiated by aspirin.

Psychotropic Agents

Platelets from patients taking tricyclic antidepressent drugs (imipramine, amitriptyline, nortriptyline) or phenothiazines (chlorpromazine, promethazine, trifluoroperazine) may have impaired in vitro aggregation and secretion responses to ADP, epinephrine, and collagen, but this effect is not associated with an increased risk for bleeding.[9]

Anesthetics

Both local and general anesthetics may impair in vitro platelet aggregation. Halothane may cause a slight prolongation of the bleeding time, but it has no adverse effect on surgical hemostasis. Cocaine may increase platelet responsiveness to ADP. [9]

Heroin

A study of ten patients admitted to a methadone program who denied intake of any other drugs for the previous 2 weeks demonstrated a long bleeding time in two and abnormal platelet aggregation in eight.[73] None of these patients had any evidence of a clinically important hemostatic defect, and the cause of the abnormalities is

unclear.

Oncologic Drugs

Administration of mithramycin to a total dose of 621 mg has been associated with decreased platelet aggregation, an increased bleeding time, and mucocutaneous bleeding. Daunorubicin and BCNU can each inhibit platelet aggregation and secretion when added to platelet-rich plasma, but there has been no suggestion of clinically important bleeding caused by defective platelet function. [9]

Miscellaneous Drugs

Clofibrate, a drug that can lower the plasma concentration of lipoproteins, diminishes platelet responsiveness to ADP, collagen, and epinephrine when given to patients with type II hyperbetalipoproteinemia, and can diminish the responsiveness of normal platelets to ADP and epinephrine in vitro. Ketanserin, which has been studied for its potential to prevent atherosclerotic complications, causes decreased platelet aggregation in response to serotonin. Antihistamines and some radiographic contrast agents can also impair platelet aggregation. The mechanism of action of these agents is not known. [9]

Foods and Food Additives

In our nutrition-conscious society, the effect of certain food components on platelet function is an increasingly important issue. A diet rich in fish and other marine oils, that contain a high concentration of -3 fatty acids (eicosapentaenoic acid, EPA, C20:5-3; and docosahexaenoic acid, DCHA, C22:6-3) may decrease the development of atherosclerosis and the occurrence of myocardial infarction. [10] One proposed mechanism of this effect is inhibition of the platelets role in the pathogenesis of atherosclerosis. Subjects ingesting a diet rich in marine oils or supplemented with -3 fatty acids have a slight prolongation of the bleeding time. These lipids act by reducing the platelet content of arachidonic acid and by competing with arachidonic acid for cyclooxygenase, thereby reducing thromboxane production. [10] Whether these subjects have any increased risk for clinically important bleeding is unknown.

Ethanol is a commonly imbibed substance that acts synergistically with aspirin to prolong the bleeding time. [24] Ethanol can itself significantly impair platelet function in vitro, and a blood level as low as 0.05% can impair platelet adhesion to vessel subendothelium in an in vitro perfusion system. [74]

Other food components or additives may affect platelet function and have been suspected of increasing the risk for minor bleeding. Easy bruising noted after eating Chinese food has been attributed to an effect on platelets by a black tree fungus. [75] A component of onion extract can inhibit platelet arachidonic acid metabolism. [76] Ajoene, a component of garlic, is an inhibitor of fibrinogen binding to platelets and platelet aggregation. [77] Extracts from frequently consumed spices, cumin, turmeric, and clove can decrease platelet thromboxane production and inhibit platelet aggregation. [78]

It can be concluded that platelets are sensitive to an enormous variety of therapeutic and dietary compounds. [9] The inhibition of platelet aggregation is a common observation in patients receiving a wide variety of agents; prolongation of the bleeding time is less common. However, an increased risk for clinically important bleeding has been demonstrated only for aspirin (Table 131-3).[8] Reports of increased bleeding with all other agents must be viewed with caution. Despite this qualification, it would be prudent for the clinician to have a thorough understanding of the antiplatelet effects of drugs prescribed, and to always consider the potential impact of drug-induced platelet dysfunction, particularly in patients with coexisting hemostatic defects.

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SYSTEMIC DISORDERS THAT AFFECT PLATELET FUNCTION

Chronic Renal Failure

Clinical Features

The clinical importance of bleeding in chronic renal failure is difficult to assess. The recognition of severe hemorrhage as a distinct complication of renal failure is generally attributed to Reismans description in 1907 of two patients with Brights disease who had severe and generalized bleeding. [79] Reisman proposed

that the cause was a toxin analogous to the hemorrhagins of snake venom. However, there could have been multiple causes of the observed bleeding problems in both of Reismans patients.[79] Others observed a tendency to hemorrhage in patients with chronic glomerulonephritis in the predialysis era, and the descriptions of epistaxis and menometrorrhagia[80] are similar to the symptoms of patients with a primary platelet abnormality.[1] Chronic nephritis was also noted to be one of the common causes of cerebral hemorrhage in young people.[80] However before dialysis, death occurred primarily from hyperkalemia and pulmonary edema. [81] After dialysis became established treatment, the major causes of death became infection and the underlying cause of the renal failure. Hemorrhage caused six of 100 deaths in one series, but additional factors could have contributed in each patient. [81] Others noted that management with dialysis, allowing better nutrition and improved general health, appeared to diminish the occurrence of hemorrhagic symptoms.[82] The current prognosis for patients with end-stage renal disease is excellent. Patients who are <46 years old have a 97% 5-year survival rate with no medical complications; for patients <61 years old, the 5-year survival rate is 84%. [83] Bleeding problems were not mentioned in this discussion,[83] and bleeding was not considered in reviews published in the past 25 years on the clinical course and management of patients with chronic renal failure. However, bleeding and thrombosis in chronic uremia continue to be discussed. [84]

More objective indication of the rarity of a significant bleeding diathesis in patients with renal failure is the extensive experience with percutaneous renal biopsy. A study of 1,000 consecutive percutaneous renal biopsies performed at the Mayo Clinic reported that 69 patients had hematuria, of whom 50 experienced clearance within 2 days and two needed blood transfusions. [85] Fourteen patients had clinically detectable perirenal hematomas, and two underwent surgical exploration and evacuation. A bleeding time was determined in 707 of these patients before biopsy; it was abnormal in 24 (3%), and one of these patients had a perirenal hematoma. The other 13 patients with perirenal hematomas had a normal bleeding time. Hypertension was a more significant factor than the bleeding time in predicting the occurrence of complications.[85] Another study of 183 consecutively performed renal biopsies reported three hemorrhagic complications that required surgical intervention; all were the result of needle lacerations of the kidney or spleen. [86] In a discussion of 5,120 renal biopsies performed at 15 institutions, there were no deaths, and the severe bleeding complications appeared to be related to anomalous vessels, heparin anticoagulation, or the presence of amyloid in the kidney. [87] The incidence of small perirenal hematomas after percutaneous renal biopsy, as diagnosed by computed tomography, is high, 85%, [88] but this may not be unexpected for the puncture of an organ that receives 20% of the cardiac output. The reports with other invasive procedures in patients with chronic renal disease are few, but they suggest that bleeding complications after abdominal surgery, liver and bone marrow biopsies, and tooth extractions may be rare. [89]

By contrast, GI tract hemorrhage is a common complication and frequent cause of death in patients with acute renal failure, and patients with chronic renal failure account for a significant proportion of patients undergoing endoscopy for upper GI bleeding. [90] Experimental data suggest that chronic uremia causes increased susceptibility of gastric mucosa to acid injury.[91] More than 90% of patients with renal failure and GI bleeding have an anatomic diagnosis at endoscopy: angiodysplasia is most common, with peptic lesions (gastric or duodenal ulcer, erosive esophagitis, gastritis, or duodenitis) also very common. [90] Rectal ulcers can also cause sudden, massive lower GI hemorrhage.

These observations suggest that serious, spontaneous hemorrhage is very uncommon in patients with chronic renal failure. However, the many individual case reports of serious, spontaneous hemorrhage support the existence of a hemostatic defect in at least some uremic patients. Patients have been described with subdural hematoma, subarachnoid hemorrhage, hemorrhagic pericardial effusion, hemorrhagic pleural effusion, subcapsular liver hematoma, retroperitoneal hematoma, mediastinal hemorrhage, and bleeding into the anterior chamber of the eye.[9] In these patients the heparin therapy used for hemodialysis could have been a factor in the bleeding because the nature of the bleeding, primarily visceral hematomas, is more consistent with a heparin-induced coagulopathy than with the typically mucocutaneous bleeding of a platelet function disorder. [1]

The primary hemostatic abnormality in uremia is thought to be a defect in platelet function. [9] Descriptions of bleeding in uremia often focused on the laboratory phenomena of abnormal platelet aggregation or a prolonged bleeding time, rather than actual hemorrhagic episodes in patients. [84] As with the drug-induced disorders of platelet function discussed above, abnormal platelet function in uremia is far more common than clinically important bleeding. Furthermore, laboratory evaluation of platelet function may not be a good predictor of the risk of hemorrhage.

Tests of Hemostasis and Platelet Function in Uremia

Platelet aggregation studies are frequently abnormal in uremic patients, but the occurrence of the abnormality does not appear to correlate with the severity of the renal failure or the occurrence of bleeding. [92] The bleeding time may also be prolonged in uremia and does appear to correlate with the severity of the renal failure [92] or the occurrence of clinically important bleeding. [7]

Anemia, which does correlate with the severity of renal failure, is an independent cause of a prolonged bleeding time. [93] [94] [95] [96] The relationship of the hematocrit to the bleeding time was first discerned by Duke, in 1910, who found that the bleeding time in thrombocytopenic patients was corrected by transfusion of fresh whole blood, but when thrombocytopenia recurred the bleeding time was less abnormal in the presence of a higher hematocrit. Bleeding times can be prolonged with severe anemia due to vitamin B_{12} or iron deficiency, and are corrected when the anemia is corrected with transfusions of washed red cells. [93] Therefore it should be expected that severely anemic patients with chronic renal failure will have a prolonged bleeding time. The relationship of the bleeding time to hematocrit in these patients has been confirmed by correction of the bleeding time with the transfusion of washed red cells and by treatment with erythropoietin. [95] [97] There was a suggestion in these reports that bleeding symptoms also improved with the correction of the anemia. In other experiments, it has been shown that the presence of red cells displaces the less dense platelets to the periphery of the column of circulating blood, thereby increasing the interaction of platelets with the vessel wall. [98] Since a prolonged bleeding time, rather than clinical bleeding, has been the focus of concern and discussion in many studies of the hemostatic defect in uremia, it is likely that this issue will receive less emphasis as patients with chronic renal failure are routinely treated with erythropoietin.

In contrast to the studies of platelet function performed to understand the possible increased risk for bleeding in patients with chronic renal failure, coagulation and fibrinolytic activities have been investigated to understand the increased risk for thrombotic complicationsa major cause of mortality. [84] Although the data are

inconsistent, markers of activated coagulation are commonly present and suggest the presence of a prothrombotic state. [84] [99]

Pathogenesis of Abnormal Platelet Function in Uremia

Even though the anemia of renal failure appears to be the major cause of the prolonged bleeding time, there is other evidence of abnormal platelet function. Platelet aggregation abnormalities

MANAGEMENT OF PATIENTS WITH CHRONIC RENAL FAILURE AND ABNORMAL PLATELET FUNCTION STUDIES

The first principle of management is to determine if an increased risk for clinically important bleeding is present. This must be determined by a careful history and examination of the patient (see Chap. 105). Since abnormal platelet aggregation and a prolonged bleeding time are common in uremic patients and do not predict an increased risk for hemorrhage, they are not an indication for transfusion or therapeutic intervention. If a patient with abnormal platelet function and a long bleeding time requires an invasive procedure, for example a kidney biopsy, lung biopsy, or laparotomy, and there is no history to suggest an increased risk for bleeding, there is probably less risk to doing the procedure without specific treatment to correct the platelet defect than to delay and attempt to normalize a laboratory value.[85] [86] [87] [88] If bleeding does complicate a procedure, a surgical complication is the most likely cause and the initial management is no different than in patients without renal failure. If a patient with chronic renal failure presents with a hemorrhagic episode, the patient should be initially evaluated for the cause of bleeding without the physician assuming that uremia is the cause. Several treatment modalities may improve the bleeding time, and anecdotal observations suggest that they may also improve hemostasis. However, these modalities have not been uniformly effective, and prospective studies have not been performed. Therefore, therapy for bleeding in a patient with chronic renal failure should take into consideration the severity of bleeding, the anticipated severity of the hemostatic risk from surgery or trauma, and the risks of the therapy.

Dialysis with correction of the renal failure is often, but not always, effective in correcting the abnormal platelet function and the long bleeding time in uremia and diminishing the risk for clinical bleeding. For example, intensive dialysis decreases the occurrence of acute GI bleeding, a major cause of morbidity and mortality in patients with acute renal failure, probably by decreasing the ulcerative intestinal complication. [104] Peritoneal dialysis and hemodialysis are equally effective.

Infusion of DDAVP (1-deamino-8-D-arginine vasopressin desmopressin) avoids the risk of blood-borne infection and has been used in the management of bleeding in uremia.[105] DDAVP has less pressor effect than natural vasopressin, and side effects are uncommon. However, the reported complications of DDAVP therapy include facial flushing, mild tachycardia, water retention, hyponatremia severe enough to cause seizures, arterial thrombosis, and memory loss. DDAVP stimulates the release of von Willebrand factor from endothelial cells, and this may be the reason for its therapeutic effect in uremia, although other mechanisms may be involved. DDAVP, administered intravenously or subcutaneously in a dose of 0.3 mg/kg over 1530 minutes, shortens the bleeding time in 5075% of uremic patients. This correction occurs in 3060 minutes and lasts for 4 hours, correlating with an increase in the plasma concentration of von Willebrand factor and an increase in the proportion of the higher-molecular-weight multimers of von Willebrand factor. There are reports of the effectiveness of DDAVP in preventing bleeding at surgery in uremic patients, but no controlled trial has been performed, and the occurrence of excessive bleeding with surgery in uremic patients who have not received specific treatment may be negligible.

An increase in the hematocrit, either by red cell transfusion or by treatment with recombinant human erythropoietin, is associated with a correction of the bleeding time and a suggestion of diminished clinical bleeding. [94] [106] It seems unusual to suggest red cell transfusion as a therapeutic measure for correction of abnormal hemostasis, but if hemorrhage occurs and red cell replacement is required, transfusion up to a hematocrit of 30% may provide some improvement in hemostasis. However, the inevitable risk of infection associated with transfusion of blood products outweighs the potential benefit unless red cell replacement is required. Similarly, the use of cyroprecipitate infusions, which have been reported to shorten the bleeding time in some studies but not others, may have greater risk than benefit for patients with chronic renal failure.

Conjugated estrogens have also been reported to shorten prolonged bleeding times in uremic patients. One report suggests that oral conjugated estrogen (Premarin) is effective at a single dose of 50 mg.[107] The mechanism of this effect and its clinical relevance are unknown.

persist when the hematocrit is normalized by red cell transfusion [94] [95] or erythropoietin treatment.[100] Washed uremic platelets suspended in normal plasma do not adhere normally to de-endothelialized rabbit vessels at a high shear rate, and normal platelets suspended in uremic plasma acquire an adhesion defect that may be related to an abnormality of the interaction of vWF with GPIIb/IIIa. This has led to the suggestion that there may be an abnormality of vWF in uremia, but measurement of vWF antigen and activity (measured as ristocetin cofactor activity) demonstrates normal or elevated values. Other studies have found that vWF function is diminished relative to the amount of immunologic vWF present; however, no abnormalities of the interaction of vWF with its constitutive platelet membrane receptor, GPIb-IX, have been reported. [9] [96]

Platelets from uremic patients frequently exhibit reduced fibrinogen binding, aggregation, and secretion in response to a wide variety of agonists. This abnormality may be retained by the platelets after their separation from uremic plasma, and in some experiments uremic plasma has induced these defects in normal platelets. Uremic platelets may also exhibit a reduction in several of the biochemical responses necessary for aggregation and secretion, including the rise in cytoplasmic free calcium levels, release of arachidonic acid from membrane phospholipids, conversion of arachidonic acid to thromboxane A_2, and dense-granule and alpha-granule secretion. In addition, a decrease in the dense granule content of ADP and serotonin and an increase in cAMP have been demonstrated. However, others have noted increased mobilization of internal calcium stores in platelets from patients with chronic renal failure. [9] [96]

The pathogenesis of the diverse platelet function defects in uremia remains undefined. They may be caused by both dialyzable and nondialyzable substances present in uremic plasma.[96] For example, platelet aggregation can be inhibited by dialyzable substances, such as guanidinosuccinic acid or phenolic acids, and by poorly characterized substances of intermediate molecular sizes at concentrations found in uremic plasma. The reduced aggregation responses may improve after the uremia is corrected by dialysis. Venous and arterial segments from uremic patients produce more of the platelet inhibitor prostacyclin than normal vessels, and this abnormality is not corrected by dialysis. In rats, uremic plasma stimulates excessive prostacyclin production by endothelial cells. Endothelial cells from uremic

patients also generate more nitric oxide (NO), possibly related to higher plasma levels of the NO precursor, L-arginine, and NO may be responsible for the higher platelet concentrations of cGMP and deficient platelet function. [100] [101] Furthermore, the response of platelets from patients on hemodialysis to NO may be impaired. [100] Some substances found in high concentrations in uremic plasma, such as urea and parathyroid hormone, appear to have no role in inhibiting platelet function. Dialysis itself may be associated with alterations in platelet function, although the clinical importance of such changes is not known. For example, hemodialysis may cause transient increases in the bleeding time and reduced responsiveness to platelet agonists in vitro, [102] and both chronic hemodialysis and peritoneal dialysis are associated with an increase in reticulated platelets, suggesting accelerated platelet turnover. [103]

Procoagulant activity describes an important property of platelets to provide a catalytic membrane surface to accelerate the generation of thrombin. Congenital deficiency of platelet procoagulant activity is not associated with an abnormality of aggregation or of the bleeding time but may be associated with increased bleeding. Based on crude tests, the serum prothrombin time, and platelet factor 3 assay, this property of platelets is consistently diminished in patients with uremia. Insofar as optimal procoagulant activity requires platelet activation, decreased procoagulant activity in uremia may reflect the abnormalities of activation described previously. [9] [96]

Platelets from uremic patients may be unusually sensitive to medicines that decrease platelet function. Aspirin has been reported to be more effective in prolonging the bleeding time in uremic patients than in control subjects, and this effect may occur by mechanisms other than the irreversible inhibition of cyclooxygenase function. Similarly, -lactam antibiotics that prolong the bleeding time may have a greater effect in uremic patients and may increase the occurrence of bleeding,

particularly if renal clearance of the antibiotic is reduced. [9] [96]

Mild thrombocytopenia has been reported in chronic renal failure, in which setting it is probably due to a combination of diminished marrow production and diminished platelet survival.[96] However, a platelet count of <100,000/l should alert the physician to the possibility of thrombocytopenia due to a systemic disease, such as multiple myeloma, systemic vasculitis, the hemolytic-uremic syndrome, pre-eclampsia, renal allograft rejection, or an adverse reaction to a medicine such as heparin.

Cardiopulmonary Bypass Surgery

Clinical and Laboratory Features

Although cardiopulmonary bypass is accompanied by decreased plasma levels of coagulation factors and increased fibrinolytic activity, abnormal platelet function is a prominent observation in these patients. A fall in the platelet count and platelet dysfunction are seen in most patients undergoing bypass surgery with either a bubble or membrane oxygenator.[108] A prolonged bleeding time (longer than expected for the degree of thrombocytopenia), decreased platelet aggregation, decreased platelet agglutination in response to ristocetin, and a decreased concentration of alpha-granule and/or dense granule contents are typically found in patients following bypass surgery.[108] Despite these abnormalities, excessive bleeding is uncommon, occurring in only 5% of patients. Bleeding, usually manifesting as excessive chest tube blood loss (defined as >100 ml/hour), is due to multiple causes: surgical complications, excessive protamine dose, and possibly also platelet function abnormalities.[109] [110] [111] [112] [113]

Pathogenesis of the Platelet Disorder

The platelet defect caused by cardiopulmonary bypass is most likely due to the effects of platelet activation and fragmentation within the extracorporeal circulation. The severity of the platelet abnormalities correlates with the duration of the bypass procedure. [114] [115] Following uncomplicated surgery, platelet function returns to normal in 1 hour,[114] though a much longer time may be required in some patients[116] and the platelet count typically does not return to normal for several days. [113] Thrombocytopenia is caused by hemodilution, by accumulation of platelets within the bypass circuit, and to a lesser extent by sequestration of damaged platelets in the liver. Platelet dysfunction following bypass may be due to reversible adhesion and aggregation of platelets to fibrinogen adsorbed from plasma onto the bypass circuit material, mechanical trauma and shear stress, cardiotomy suction, trace concentrations of circulating thrombin and ADP, complement activation, hypothermia, blood conservation devices, bypass priming solutions, and, with bubble oxygenators, exposure of platelets to the bloodair interface. [9] Cardiopulmonary bypass also consistently causes the appearance of platelet fragments, or membrane microparticles, evidence that the platelet surface membrane is subjected to severe mechanical stress during the procedure.[115] Surface membrane alterations may play a significant role in platelet dysfunction. Other data suggest that the hemostatic abnormality is not intrinsic to platelets but rather is primarily due to reduced formation of the platelet agonist thrombin during heparin administration. Furthermore, abnormalities of hemostasis measured by laboratory studies are transient and do not correlate with clinical bleeding. [113]

Management

It is controversial whether to determine the bleeding time preoperatively in patients without a bleeding history in the hope of identifying those patients more likely to have excessive postoperative bleeding. In cardiac surgery as well as in other major surgery, the bleeding time should not be relied on as a good predictor of postoperative hemorrhage.[7] In fact, if a careful history is taken, few laboratory assays are helpful in the preoperative evaluation. [117] Prophylactic platelet transfusions are not routinely indicated for cardiopulmonary bypass surgery. However, patients who have a prolonged bleeding time and excessive blood loss in the postoperative period usually will respond to platelet transfusions. [114]

Postoperative blood loss may be diminished by the use of DDAVP after cessation of bypass but most clinical trials have not demonstrated a beneficial effect of DDAVP.[105] [118] In these studies, a benefit of DDAVP seemed more apparent in patients undergoing more complex cardiac surgical procedures. Potential risks of DDAVP include hyponatremia, hypotension, and thrombosis.[105]

Attempts have been made to prevent platelet activation during bypass surgery in both man and experimental animals by infusion of prostaglandin E_1 or prostacyclin. By increasing the platelet concentration of cAMP and reducing platelet responsiveness, these agents partially prevent the occurrence of thrombocytopenia and platelet function abnormalities.[55] However, the hypotensive properties of these agents limit their usefulness. Aprotinin (Trasylol), a peptide serine protease inhibitor, has been studied for its potential to protect platelets from activation by thrombin, plasmin, and other proteases generated during cardiopulmonary bypass and may decrease blood loss.[119] Allergic reactions preclude its use in patients who have previously been treated with aprotinin.

Anti-platelet Antibodies

Immunoglobulin molecules can bind to the platelet surface in several pathologic conditions, including idiopathic thrombocytopenic purpura (ITP), systemic lupus erythematosus (SLE), and platelet alloimmunization.[120] The common pathologic result

of antibody binding, with or without complement binding, is accelerated platelet destruction and thrombocytopenia. In most cases the surviving platelets appear to function normally; indeed, bleeding times in ITP may be shorter than expected for the degree of thrombocytopenia. On the other hand, some individuals with circulating antiplatelet antibodies appear to have impaired platelet function, although the frequency of this problem, compared to the occurrence of thrombocytopenia, must be very low. Immune thrombocytopenia is a common disease; significantly impaired hemostasis caused primarily by antibody-mediated platelet dysfunction has been demonstrated in only a few reports.

Clinical and Laboratory Features

In some patients with ITP or SLE, platelet dysfunction may be suspected because mucocutaneous bleeding symptoms occur at a platelet count that is usually sufficient for normal hemostasis (>50,000/l), and the bleeding time may be longer than expected for the degree of thrombocytopenia. Patients with antiplatelet antibodies may exhibit defective platelet function in vitro, even if they do not have a prolonged bleeding time or clinical symptoms of excessive bleedinga situation similar to that described above for the effects of aspirin ingestion and renal disease. For example, in two studies, 13 of 19 patients with ITP demonstrated impaired platelet aggregation to ADP, epinephrine, or collagen. [121] [122] In two other studies, 22 of 35 patients with SLE were found to have decreased platelet aggregation in response to these agonists.[123] [124] These platelet function abnormalities appeared to be antibody-mediated because IgG purified from the plasma or eluted from the platelets of some of the patients inhibited the aggregation of normal platelets.

Several aspects of platelet function may be impaired by antiplatelet antibodies. The most frequently observed abnormality is absence of platelet aggregation in response to low concentrations of collagen, and absence of the second wave of irreversible aggregation in response to ADP or epinephrine. This pattern is identical to the abnormalities caused by aspirin, described above. In ITP and SLE, the abnormal aggregation may be related to a decreased platelet content of dense-granule and alpha-granule contents or to an activation defect manifested by diminished conversion of arachidonic acid to thromboxane A_2. In addition, experiments using an ex vivo perfusion system indicated that some antiplatelet antibodies may inhibit the adhesion of platelets to subendothelial matrix. [125]

Pathogenesis

How autoantibodies or alloantibodies may impair platelet function is unknown. Antibody binding to specific platelet membrane proteins, lipids, or glycosphingolipids could affect the participation of these membrane components in stimulusresponse coupling. For example, antibodies against $_2$-microglobulin from the sera of patients with SLE or rheumatoid arthritis inhibit ADP-induced platelet aggregation, although the normal function of $_2$-microglobulin on the surface of platelets is unknown. Some HLA antibodies inhibit the uptake of serotonin by platelets. Anti-PI^{A1} alloantibodies bind to GPIIIa, and some inhibit aggregation by interfering with fibrinogen binding to the GPIIb/IIIa complex.[126] Autoantibodies with specificity for GPIIb/IIIa may cause a functional platelet disorder indistinguishable from Glanzmann thrombasthenia,[127] and autoantibodies reacting with glycoprotein Ib-IX-V may cause a functional disorder indistinguishible from Bernard-Soulier syndrome. [128]

Some antiplatelet antibodies can induce aggregation and secretion. In vitro, antibodies may activate platelets through the binding of IgG immune complexes to platelet

Fc receptors, with the deposition onto the cell of sublytic quantities of C5b9, the membrane attack complex of the complement system. [129] If bound antibody were to cause platelet secretion in vivo and the platelets continued to circulate, they might be expected to be refractory to platelet agonists and deficient in secretory granule contents. Although such activation of circulating platelets is an attractive hypothesis for the acquired storage pool deficiency seen occasionally in ITP or SLE, alternative mechanisms need to be considered. For example, some antibodies may affect the uptake of substances into platelet granules, either during megakaryocytopoiesis or in the circulation. [130]

Management

Antibody-mediated platelet dysfunction is a syndrome of only investigative interest and not of clinical importance, except in those instances where an acquired platelet disorder was responsible for bleeding symptoms. [127] [128]

Miscellaneous Disorders

Patients with disseminated intravascular coagulation (DIC) may have reduced platelet aggregation associated with a deficiency of secretory granule contents, a phenomenon termed acquired storage pool disease or, more graphically, exhausted platelets [131] and resulting from platelet stimulation by thrombin or other agonists. It has also been suggested that the elevated fibrin(ogen) degradation products and low fibrinogen levels that occur in DIC may inhibit platelet function. Although purified fibrin(ogen) degradation products can impair platelet aggregation in vitro, this requires high concentrations that are unlikely to occur in vivo. Furthermore, hypofibrinogenemia would contribute to a defect in platelet aggregation only in extreme cases in which the plasma fibrinogen is <10 mg/dl because that concentration is sufficient to saturate platelet membrane fibrinogen receptors. Chronic liver disease and hepatic cirrhosis of various causes have been reported to prolong the bleeding time and cause other platelet function abnormalities that may be related to a decrease in GDIb. [132] However, studies demonstrating that the bleeding time and platelet aggregation abnormalities correlated with the degree of thrombocytopenia [133] suggest that no specific platelet function defect exists in liver disease. Bartters syndrome, a disorder of primary renal potassium wasting and hypokalemic alkalosis, has been reported to be associated with a prolonged bleeding time and decreased platelet aggregation and secretion in response to ADP and epinephrine. [134] There are isolated reports of a slight prolongation of the bleeding time or of platelet aggregation defects in a number of other clinical conditions: nonthrombocytopenic purpura with eosinophilia, [135] atopic asthma and hay fever, [136] acute respiratory failure, [137] and Wilms tumor. [138] The clinical importance of these observations is unknown.

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HEMATOLOGIC DISORDERS THAT AFFECT PLATELET FUNCTION

Chronic Myeloproliferative Disorders

Bleeding and thrombosis are important causes of morbidity and mortality in the chronic myeloproliferative disorders, which include essential thrombocythemia, polycythemia rubra vera, and myelofibrosis with myeloid metaplasia. Abnormal platelet function has been postulated as a contributing cause for these complications. Thrombocytosis is present by definition in thrombocythemia and may also occur in each of the other disorders. Bleeding manifestations are comparable to those seen in patients with platelet function disorders [1] but contribute to mortality in <3% of patients. [139] The incidence of thrombotic complications is 3.4% per year and is correlated with age.[139] Both arterial and venous thromboses can occur, and they may be in

unusual locations, such as the hepatic, portal, and mesenteric circulations. Patients with thrombocythemia can develop ischemia of the fingers and toes due to digital artery thrombosis, microvascular occlusion in the coronary circulation, and transient neurologic symptoms due to cerebrovascular occlusion. [140]

Pathogenesis of the Bleeding and Thrombosis

A number of intrinsic platelet function defects have been reported, but their clinical importance is uncertain. The bleeding time is prolonged in only a minority of patients and significant bleeding complications can occur in patients with a normal bleeding time. Elevation of the platelet count per se, correlates poorly with the risk for hemorrhage or thrombosis in some studies but spontaneous bleeding is rare in patients with platelet counts of <1,000,000/l. A clear risk factor for excessive bleeding, particularly after surgery, is the increased whole blood viscosity in patients with uncontrolled polycythemia vera and a high hematocrit. [140]

Platelets in these disorders may be larger or smaller than normal, abnormally shaped, and have a decreased number of secretory granules. Platelet survival may be decreased in essential thrombocythemia. The most common platelet abnormality is decreased aggregation and secretion in response to epinephrine, ADP, and collagen. These abnormalities are not due merely to the high platelet count, since patients with reactive thrombocytosis have functionally normal platelets. [141] In what may appear to be a paradox, some patients may demonstrate spontaneous in vitro platelet aggregation in platelet-rich plasma. The significance of this is unknown because it is also occasionally seen in normal individuals. Decreased platelet aggregation or secretion, and decreased procoagulant activity, may be caused by: (1) decreased agonist-induced release of arachidonic acid from membrane phospholipids; (2) decreased conversion of arachidonic acid to prostaglandin endoperoxides or lipoxygenase products; (3) decreased platelet responsiveness to thromboxane A_2; (4) decreased dense-granule or alpha-granule contents; or (5) decreased $_2$ -adrenergic receptors. Specific platelet membrane abnormalities have also been reported, including a deficiency of glycoproteins Ib and IX, causing an acquired form of the Bernard-Soulier syndrome; [142] a deficiency of receptors for the prostaglandin PGD_2; [143] a deficiency in the number of c-mpl receptors; [144] and an increased number of receptors for the Fc portion of IgG. [145] Myeloproliferative disorders are clonal in origin, and therefore the platelet abnormalities may develop from a clone of abnormal megakaryocytes. Acquired von Willebrand disease has also been reported in these patients. [146]

It is important to emphasize several features about the platelet function defects reported in myeloproliferative disorders. First, none has consistently predicted a risk for bleeding or thrombosis. Second, none is unique to a particular myeloproliferative disorder. Third, their relative frequency has varied widely in different reported series. Therefore the clinical importance of the described abnormalities of platelet function in myeloproliferative disorders is unknown.

Management

The most effective preventive treatment is to maintain the hematocrit at <45% in patients with polycythemia vera. Decreasing the platelet count with alkylating agents, such as hydroxyurea, or with anagrelide[147] is important in patients with signs of or risks for bleeding and thrombosis, [140] although there are reports documenting the lack of symptoms associated with untreated extreme thrombocytosis of long duration. Assuming a potential leukemogenic risk of alkylating agents, asymptomatic patients should probably not be treated with such drugs to decrease their platelet count, especially if they are young, except for specific indications of serious thrombotic or hemorrhagic complications. However, hydroxyurea has never been demonstrated to increase the risk for leukemic transformation in patients with chronic myeloproliferative syndromes.[148] Aspirin may be effective therapy for acute digital ischemia and for diminishing the risk for thrombosis, and in low doses (40 mg/day) it does not increase the risk for bleeding. [149]

Leukemias and Myelodysplastic Syndromes

Bleeding in these disorders is most always due to thrombocytopenia, though abnormalities of platelet function have been described. In acute myelocytic leukemia and its variants, platelets may be larger than normal, abnormally shaped, and have a marked variation in the number of granules. There may be decreased aggregation and secretion in response to ADP, epinephrine, or collagen, as well as decreased platelet procoagulant activity. Identical abnormalities can be seen in the myelodysplastic syndromes, although the platelets may be less uniformly affected, perhaps owing to the presence of normal platelets mixed with those produced by the neoplastic clone. Abnormal platelet function and acquired von Willebrand disease have been reported in hairy cell leukemia.

Dysproteinemias

Clinical Features

Platelet dysfunction is observed in approximately one-third of patients with IgA myeloma or Waldenström macroglobulinemia, in 15% of patients with IgG myeloma, and occasionally in patients with benign monoclonal gammopathy. [150] However, thrombocytopenia is much more likely to be a major cause of bleeding. Additional hemostatic problems in these patients can be due to the hyperviscosity syndrome, to a heparin-like coagulation inhibitor, [151] or to complications of amyloidosis (i.e., acquired factor X deficiency[152] or fibrinolysis[153]). A diagnostic problem in patients with myeloma can be the interpretation of laboratory assays of coagulation when the paraprotein interferes with fibrin polymerization and the function of other coagulation proteins. [150] Patients may have markedly abnormal results on laboratory tests (for example, the thrombin time) with no evidence of clinical bleeding. [150] Also, the bleeding time may be prolonged in patients with dysproteinemias in the absence of clinical bleeding.

Pathogenesis

Abnormalities of platelet function correlate with the concentration of the plasma paraprotein. Myeloma proteins can inhibit all platelet functionsaggregation, secretion,

procoagulant activity, and clot retractionand normal platelets can acquire these defects when incubated with the purified monoclonal immunoglobulin. In some cases, specific interactions of the monoclonal protein have been described. One IgA myeloma protein inhibited the ability of a suspension of aortic connective tissue to aggregate normal platelets.[154] The bleeding time and bleeding symptoms of the patient from whom this paraprotein was isolated were corrected when the IgA myeloma protein was removed by plasmapheresis. In another patient, an IgG myeloma protein bound specifically to platelet membrane GPIIIa. [155] Both the intact IgG and its $F(ab')_2$ fragment inhibited binding of fibrinogen to activated platelets, thus inducing a defect comparable to thrombasthenia. This patient also had bleeding reminiscent of classic Glanzmann thrombasthenia[1] and died of hemorrhage from a gastric ulcer. [155] There are a number of reports of acquired von Willebrand disease in patients with myeloma, benign monoclonal gammopathy, or chronic lymphocytic leukemia. [156] In some the plasma concentration of

vWF was decreased, in others the larger multimers were deficient. The myeloma protein may interact with vWF and accelerate its clearance from plasma, or interfere with its binding to platelet GPIb.

Management

A high plasma concentration of myeloma protein can cause severe hemorrhagic symptoms, in part due to hyperviscosity and in part due to platelet dysfunction. Under these conditions, bleeding should be regarded as a medical emergency and treatment initiated by means of plasmapheresis to reduce the concentration of the myeloma protein. The goal of therapy is the cessation of bleeding. Chemotherapy for the underlying plasma cell neoplasm should be begun to effect a longer-lasting reduction of the paraprotein. DDAVP infusion, with or without plasmapheresis, may be transiently effective in patients with acquired von Willebrand disease. [156]

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Chapter 132 - Human Blood Group Antigens and Antibodies

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PART 1
Carbohydrates Determinants

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INTRODUCTION

Biochemical studies demonstrated that many important blood group antigens are complex carbohydrates (i.e., glycoconjugates). These are grouped together in this section because they have some similarities in their biosynthesis, in the human immune response to these antigens, and in the outcome in vivo after transfusion of incompatible blood.

The biosynthetic pathways used in forming antigens in the ABH, Lewis, P, and I blood group systems are interrelated. These oligosaccharide antigens may exist free in solution. In addition, they may be covalently attached to lipid molecules (ceramides) to form glycosphingolipids, or to polypeptides to form mucins, integral membrane glycoproteins, or soluble glycoproteins. The formation of the relevant glycosidic linkages (i.e., the bonds between monosaccharides) are specifically catalyzed by various glycosyltransferases. Some glycosyltransferases, found in all individuals, form framework structures. The genes encoding other glycosyltransferases are allelically inherited and the resulting enzymes specify the synthesis of variable structures. Because of their variable inheritance and expression, the latter may form blood group antigens. As described below, antigens in the ABH, Lewis, P, and I blood group systems are synthesized on common precursor framework molecules. Competition between genetically inherited blood group-specific glycosyltransferases results in a rich mixture of antigenic molecules. In addition, a single oligosaccharide may contain several different blood group specificities (epitopes). The absence of particular blood group antigens in certain individuals may result in specific antibody production after stimulation by the foreign antigen.

The immune response to carbohydrate antigens, particularly when presented as repetitive epitopes, is usually thymus independent (for review, see reference [1]). . In this case, multivalent antigens directly stimulate B cells to synthesize antibodies without the aid of helper T cells. Thymus-independent immune responses classically result in the production of IgM antibodies, and most antibodies to carbohydrate blood group antigens are, in fact, of the IgM class. Surprisingly, individuals lacking a carbohydrate blood group antigen on their red blood cells (RBCs) often have naturally occurring IgM antibodies to that particular antigen in their serum, even if they have not been previously exposed to human blood products. The current understanding of this phenomenon is not that these antibodies spontaneously arise without prior antigenic stimulation, but that cross-reacting antigens present in the environment, such as on gut bacteria, stimulate specific IgM production. By contrast, high-titered IgG antibodies to carbohydrate antigens can be found in certain individuals. The latter may be stimulated by a thymus-dependent form of these oligosaccharides, perhaps as individual epitopes on glycoproteins, in which T-cell help leads to an isotype switch from IgM to IgG production. A complete understanding of this phenomenon is not yet available, however.

Because carbohydrate-specific blood group antibodies are predominantly IgM, these pentameric decavalent molecules directly agglutinate antigen-positive human RBCs without the aid of an antiglobulin reagent. These antibodies, for unclear reasons, often cause more significant agglutination in vitro at temperatures <37°C. Because most IgM molecules directly fix complement, these antibodies can cause immediate intravascular hemolysis after transfusion of incompatible, antigen-positive RBCs. In unusual cases, high-titered IgG carbohydrate-specific antibodies coat RBCs and lead to extravascular hemolysis after incompatible transfusion. Alternatively, the latter may cross the placenta resulting in hemolytic disease of the newborn.

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ABH, SECRETOR, AND LEWIS SYSTEMS

ABH Antigens

At the turn of the 20th century, Karl Landsteiner discovered the ABO blood group system, which is the most important one with respect to blood transfusion and renal transplantation. He was fortunate to discover the first human alloantigens by using a conceptually simple experiment (Table 132-1). The RBCs of each individual were found either to lack or to have one or both of the two antigens, A and B. In addition, the serum of each subject contained naturally occurring directly agglutinating antibodies that recognized the antigens absent from their own RBCs. This experiment can be explained in modern terms as follows: cross-reacting carbohydrate structures on environmental agents stimulate the thymus-independent production of IgM anti-A and/or anti-B antibodies in individuals not tolerant to these antigens. The IgM antibodies then directly agglutinate the appropriate antigen-positive RBCs, preferentially at room temperature. There are interesting variations in the frequencies of these blood types in different human populations (Table 132-2).[2] [3]

Although the ABH antigens (the H antigen is the relevant carbohydrate structure present on group O RBCs) are typically described as blood group antigens because of their presence on RBCs, they are also found on other tissues and may be more appropriately termed histo-blood group antigens. [4] In blood they exist in both a cellular form on platelets and a soluble form as blood group-active glycosphingolipids inserted into plasma lipoprotein particles. They exist as membrane antigens on such diverse cells as vascular endothelial cells and intestinal, cervical,

TABLE 132-1 -- ABO Blood Group Antigens and Antibodies

Blood Group	RBC Antigens		Serum Antibodies	
	A	B	Anti-A	Anti-B
A	+			+
B		+	+	
AB	+	+		
O			+	+

TABLE 132-2 -- Frequencies of ABO Groups in Selected Populations

Population[a] (no. tested)	Various Phenotypes (%)					
	O	A_1	A_2	B	A_1 B	A_2 B
South American Indians (539)	100	0	0	0	0	0
Vietnamese (220)	45	21	0	29	4	0
Australian aborigines (126)	44	55	0	0	0	0
Germans (100,000)	43	33	9	11	3	1
Bengalese (241)	22	22	2	38	15	1
Lapps (324)	18	36	19	15	6	6

Data from Mourant et al.[2] and Walker.[3]

[a] Figures are for selected populations and do not necessarily apply to the racial group as a whole.

urothelial, and mammary epithelial cells. Soluble forms are also found in various secretions and excretions, such as saliva, milk, urine, and feces. In some tissues, their appearance is developmentally regulated. Despite their wide distribution, genetic inheritance, developmental regulation, and importance in transfusion and transplantation, their normal physiologic function, if any, remains a mystery.

To appreciate fully the structure and antigenicity of ABH antigens and their relationship to other blood group systems, it is necessary to understand the underlying biochemistry.

Early studies indicated that anti-A, anti-B, and anti-H specifically recognize epitopes composed of the terminal trisaccharides or disaccharides illustrated in Figure 132-1 . From these results it is possible to conclude that the A, B, and H antigens are not directly encoded by the corresponding genes, but rather the genes code for particular glycosyltransferases, commonly

Figure 132-1 Biochemical structures of the immunodominant oligosaccharides of the H, A, and B antigens. The monosaccharides are abbreviated as follows: D-galactose is Gal; D-*N*-acetylgalactosamine is GalNAc; and L-fucose is Fuc.

called the A, B, and H transferases, or equivalently, the A, B, and H enzymes. The H enzyme is a fucosyltransferase that specifically adds in an (12) linkage to a terminal galactose. The A or B enzymes then add *N*-acetylgalactosamine or galactose, respectively, an (13) linkage to the same terminal galactose. However, the substrate for the A or B enzymes is a terminal H antigen sequence; these enzymes do not transfer the appropriate sugar to galactose in the absence of (12)-linked fucose. Similarly, the H enzyme does not function if this galactose is substituted with a different sugar. Thus, the biosynthetic pathway is:

where reaction 1 is catalyzed by the H enzyme and reaction 2 by the A or B enzyme.

The finding that the *A* and *B* genes code for glycosyltransferases explains some results obtained from classic genetic analysis of family pedigrees. In particular, the *A* and *B* genes are inherited in a strict mendelian fashion and are dominant compared to *O*, but the *A* and *B* genes are co-dominant with each other. That is, an individual with the genotype *AO* (or *BO*) is phenotypically A (or B), an individual of genotype *AB* is phenotypically AB, and an individual of genotype *OO* is phenotypically group O. Because the A and B enzymes both use the H antigen as substrate, even the presence of only 50% of these enzymes in an *AO* (or *BO*) heterozygote is sufficient to convert the RBCs to the corresponding A (or B) phenotype. Similarly, if both the A and B enzymes are present, they each convert 50% of the available H antigen substrate, yielding RBCs expressing both the A and B antigens.

Recent studies have begun to elucidate the biology of the ABH system at the level of the gene. The allelic genes encoding the A and B enzymes are located on chromosome 9[5] [6] (Table 132-3). They encode membrane-bound glycosyltransferases containing 354 amino acids (Fig. 132-2). Interestingly, these two enzymes exhibit a great degree of mutual homology and, at the amino acid level, differ at only four residues. [7] [8] Their substrate specificity is primarily determined by the two adjacent residues near the C-terminus of the protein, amino acids 266 and 268 [9] [10] (Fig. 132-2). The predominant allele corresponding to blood group O, O_1, contains a single nucleotide deletion near the N-terminus of the protein. This leads to a shift in reading frame and a prematurely terminated translation product lacking enzymatic activity.[8] (Fig. 132-3). Recently several other *O* alleles have been identified [11] [12] [13] [14] [15] (Fig. 132-3). For example, the O_2 allele has a missense mutation that abolishes enzyme activity.[11] [12] [13]

Not only do these findings using molecular biology confirm and extend the results obtained from carbohydrate biochemistry and enzymology, but they also suggest new ways of blood typing using molecular biology techniques. [16] [17] [18]

TABLE 132-3 -- Chromosomal Assignment of Cloned Genes in the ABH, Se, Le, I, and P Blood Group Systems

Gene	Gene Product	Location	Reference
H (*FUT1*)	(12) fucosyltransferase	19q13.3	[24]
Se (*FUT2*)	(12) fucosyltransferase	19q13.3	[24]
Le (*FUT3*)	(13/4) fucosyltransferase	19p13.3	[24] [33]
A	(13) *N*-acetylgalactosaminyl transferase	9q34.1-q34.2	[5] [6]
B	(13) galactosyltransferase	9q34.1-q34.2	[5] [6]
I	(16) *N*-acetylglucosaminyl transferase	9q21	[60]
P_1	(14) galactosyltransferase	22q12.3-q13.1	[68] [69] [70]

The *A* and *B* genes are allelic. The *H* and *Se* genes are encoded at distinct, but closely linked, loci and are not alleles of each other. The *H*, *Se*, *Le*, *A*, *B*, and *I* cDNA sequences and some genomic sequences have been determined. Neither the P_1 gene nor its cDNA have yet been cloned.

Figure 132-2 cDNA structures and amino acid sequences of the A, B, and related glycosyltransferases. The A_1 and B transferases are 354 amino acid, type II, membrane glycoproteins. All sequences in this figure are compared with the A_1 sequence. Above each bar is the relevant nucleotide sequence; uppercase letters indicate the existence of a mutation leading to a change in amino acid sequence, and lowercase letters indicate that a mutation does not lead to a change in amino acid sequence. Below each bar is the relevant amino acid sequence using the single letter code for amino acids. The hatched portions of the bar indicate that a frameshift mutation resulted in translation of a significant length of amino acid sequence that differs from the A_1 sequence. The designation del indicates that there is a one nucleotide deletion; ins indicates that there is a one nucleotide insertion.

Secretor Gene

The ABH antigens are found not only on RBCs but also in secretions, particularly saliva and plasma. The ability to secrete ABH antigens is genetically inherited: 80% of whites are secretors and 20% are nonsecretors. This trait is inherited as a single locus gene in simple mendelian fashion. The secretor gene (*Se*) is dominant; nonsecretor (*se*) is recessive. The terminal carbohydrate sequences of the ABH antigens in saliva and plasma are identical to those on RBCs. However, the backbone or framework carbohydrate structures are different. ABH antigens on glycosphingolipids and glycoproteins synthesized by RBC precursors are primarily coupled to framework type 2 chains (Gal(14)GlcNAc-R); the same antigens on plasma glycosphingolipids and salivary mucins are coupled to type 1 chains (Gal(13)GlcNAc-R) (Table 132-4). Because ABH blood group-active glycosphingolipids on plasma lipoproteins are also passively transferred onto RBCs, the RBCs of secretors have ABH antigens on both type 1 and type 2 precursor chains, but RBCs of nonsecretors only have ABH antigens on type 2 chains.

Initially, it was thought that the *H* gene was a structural gene coding for the H enzyme and the secretor locus encoded a regulatory gene that permitted expression of the *H* gene in the appropriate tissues. This hypothesis suggested that a single H enzyme transferred fucose residues onto either type 1 or type 2 precursor chains. In this model, the H enzyme is always expressed in RBC precursors, but its expression in secretory tissues (e.g., salivary epithelium) comes under the control of the secretor locus. However, this model does not explain all the available information, and recent biochemical, immunologic, and genetic studies demonstrated that an alternative model is correct.[19] [20] The latter hypothesizes that there are two different H transferases: one that adds fucose to type 1 chains (H type 1 enzyme) and one that acts on type 2 chains (H type 2 enzyme). Therefore, the H type 1 enzyme is the structural protein coded for by the secretor gene (*Se*) and is expressed in secretory tissues. In the last several years the genes encoding multiple fucosyltransferases have been cloned. These results indicate that the *Se* gene is equivalent to the *FUT2* gene and that the product of the *Se* gene, the H type 1 enzyme, is the same as the FUT2 enzyme.[21] [22] At least one copy of the *Se* gene is found in 80% of the population and leads to the expression of ABH antigens in secretions. By contrast, the traditional H locus is a structural gene for the H type 2 enzyme; the latter is equivalent to the FUT1 fucosyltransferase.[23] This gene (*H* or *FUT1*) is active in virtually all individuals (see later) and leads to the formation of ABH antigens on RBCs and other tissues. These two enzymes, FUT1 (or, equivalently, the H type 2 enzyme) and FUT2 (or, equivalently, the H type 1 enzyme) are encoded by genes at two closely linked loci on chromosome 19[24] (Table 132-3).

Following the cloning of the *Se* gene in 1995,[21] [22] multiple variants and mutations of this gene have been described. For example, many individuals, particularly of Asian descent, have a normally functioning variant, Se^1, that contains a silent mutation [25] [26] [27] [28] (Fig. 132-4). In addition, the Se^w mutant encodes a functional, but less active, enzyme, also primarily in individuals of Asian descent. [25] [27] [29] This mutation leads to the partial expression of the secretor phenotype. As described below, this results in individuals whose RBCs have the Lewis blood group phenotype of Le(a+b+). Finally, at least six nonenzymatically active variants of the *Se* gene have been described (Fig. 132-4). Most (se^1-se^5) are caused by premature stop codons that result in the expression of truncated, nonfunctional enzyme proteins; [22] [26] [27] [28] [30] one (se^6) is the result of a fusion gene between *Se* and an adjacent pseudogene.[28]

Lewis System

The two Lewis blood group antigens Le^a (Lewis a) and Le^b (Lewis b) were discovered in the 1940s (for review, see reference 31). . Virtually all individuals fall into one of three different Lewis types: Le(a+b), Le(ab+), and Le(ab) (Table 132-5). These molecules are not intrinsic RBC antigens; they are synthesized in another tissue (probably the intestinal epithelium), circulate in plasma attached to lipoproteins, and then passively transfer onto RBCs. [32] Biochemical studies demonstrated that these are carbohydrate antigens on glycosphingolipids (Fig. 132-5). They are structurally similar to the type 1 ABH antigens found on plasma glycosphingolipids; the latter also transfer onto RBCs. The Lewis gene (*Le*) resides on chromosome 19,[24] [33] and its locus is distantly linked to the H and Se loci (Table 132-3). The gene codes for an enzyme, an (14) fucosyltransferase

Figure 132-3 cDNA structures and amino acid sequences of the *O* alleles of the A and B transferases. As in Figure 132-2 , all sequences in this figure are compared with the A_1 sequence. The hatched portions of the bar indicate that a frameshift mutation resulted in translation of a significant length of amino acid sequence that differs from the A_1 sequence. When a frameshift mutation leads to a stop codon resulting in premature termination of translation, the unshaded portions of the bar indicate that this section of the nucleotide sequence is not translated.

denoted FUT3, and thus behaves in a dominant fashion. A human cDNA derived from the *Le* gene, the *FUT3* gene, has recently been cloned and encodes a 361 amino acid type II membrane-bound enzyme[34] (Fig. 132-6). Approximately 95% of whites and 75% of blacks have at least one *Le* allele.

The transfer of fucose to a type 1 precursor by the Lewis

TABLE 132-4 -- Biochemical Structures of ABH Antigens

Blood Group	Secretions (Type 1 Chain)	RBCs (Type 2 Chain)

enzyme results in the formation of the Le^a antigen; the addition of (14)-linked fucose to the H type 1 structure leads to the formation of the Le^b antigen. Thus, the latter is formed through the cooperation of two glycosyltransferases encoded by the two genes, one gene from the Lewis system (*Le* on chromosome 19) and one from the ABH system (*Se* at a different, unlinked locus on chromosome 19). The biosynthetic pathways demonstrating the connections of the ABH, secretor, and Lewis systems (encoded by genes at three distinct loci on two chromosomes, 9 and 19) are illustrated in Figure 132-7 . Interestingly, cooperation of the *Le, Se*, and *A* (or *B*) genes leads to the formation of a minor antigen, ALe^b (or BLe^b), recognized by both anti-A (or anti-B) and anti-Le^b antibodies. Because the H type 1 enzyme converts virtually all type 1 precursor chains into H type 1, whether or not the Lewis enzyme is present, Lewis-positive secretors have virtually no Le^a antigen and their RBCs type as Le(ab+). By contrast, Lewis-positive nonsecretors have Le(a+b) RBCs. This is summarized in Table 132-6 .

Individuals whose RBCs type as Le(a+b+) are rarely identified in Caucasian populations, but are reasonably common in Asian populations. In these individuals the *Le* gene is normal but the secretor gene is partially defective due to an Ile 129 Phe mutation (Se^w) [25] [27] [29] (Fig. 132-4). Because the resulting Se^w

Figure 132-4 cDNA structures and amino acid sequences of the functional and nonfunctional alleles of the secretor (*Se* or *FUT2*) gene. The H type 1 transferase (FUT2) is a 365 amino acid, type II membrane glycoprotein. All sequences in this figure are compared with the *Se* sequence. The hatched portion of the bar represents sequences derived from a homologous pseudogene. The open portion of the bar represents a length of sequence in which the pseudogene and the *Se* gene are identical; the fusion between these two genes occurred in this region.[28]

TABLE 132-5 -- The Lewis Blood Group System

Blood Group	RBC Antigens		Serum Antibodies	
	Le^a	Le^b	Anti-Le^a	Anti-Le^b
Le(a+b)	+			Very rarely
Le(ab+)		+	Very rarely	
Le(ab)			Occasionally	Occasionally

Figure 132-5 Biochemical structure of the Lewis-active glycosphingolipids. The monosaccharides are abbreviated as follows: D-glucose is Glc and D-*N*-acetylglucosamine is GlcNAc. Abbreviations are as in Figure 132-1 .

Figure 132-6 cDNA structures and amino acid sequences of the functional and nonfunctional alleles of the *Lewis* (*Le*) gene. The Le transferase is a 361 amino acid, type II membrane glycoprotein. All sequences in this figure are compared with the *Le* sequence.

Figure 132-7 Biosynthesis of blood group antigens with type 1 chains. The genes encoding the relevant glycosyltransferases are denoted in italics and the reactions catalyzed are indicated by arrows. The names of the individual blood group antigens are shown in boxes next to the relevant structures. The abbreviations for the monosaccharides are as described in Figures 132-1 and 132-5 .

enzyme has partial activity, some type 1 precursor chains are converted to H type 1 and are subsequently converted to Le^b by the normal Lewis enzyme, FUT3 (Fig. 132-7). In addition, some type 1 precursor chains avoid (12) fucosylation by the partially defective FUT2 transferase, Se^w , and are converted into Le^a by the normal Lewis FUT3 enzyme (Fig. 132-7). Thus, the RBCs of these individuals contain both the Le^a and Le^b antigens and type as Le(a+b+).

Subsequent to the cloning of the *Le* cDNA, at least six different defective alleles (le^1 -le^6) have been sequenced from individuals whose RBCs type as Le(ab) [35] [36] [37] [38] [39] [40] [41] (Fig. 132-6). Based both on family studies and expression of transfected chimeric cDNAs in vitro, the mutations at nucleotides 508, [36] [37] [39] 1067, [36] [38] [39] and 202[41] severely inhibit or abolish enzyme activity. Interestingly, the mutation at nucleotide 59, which changes an amino acid in the transmembrane domain, may affect the Golgi localization of this enzyme, but not its activity, yielding the paradoxical non-genuine Lewis-negative phenotype. [36] [37] [38] [39] The RBCs of these individuals lack the relevant blood group-active glycosphingolipids and type as Le (ab); however, the mucin glycoproteins in their saliva do contain Lewis antigens. [38]

Although hemolytic transfusion reactions and hemolytic disease of the newborn are rarely caused by antibodies to the Lewis blood group antigens, this blood group system may be important

TABLE 132-6 -- Interactions of the ABH, Lewis, and Secretor Systems

Genes	A *Le Se*	A *Le se*	A *le Se*	A *le se*
RBC antigens	A type 1 A type 2 Le^b (type 1) ALe^b (type 1)	A type 2 Le^a (type 1)	A type 1 A type 2	A type 2

Salivary blood group antigens	A type 1 Le^b (type 1) ALe^b (type 1)	Le^a (type 1)	A type 1	

in renal transplantation[42] and in infection by *Helicobacter pylori*.[43] [44]

Bombay and para-Bombay Phenotypes

The ABH system has several unusual but instructive antigen variants. The most striking is the Bombay phenotype (O_h)[45] . The RBCs of these individuals type as group O, and they are nonsecretors of ABH antigens. However, they do not express H antigens on their RBCs and their serum contains high-titered, hemolytic anti-H antibodies that lyse RBCs of individuals of any ABO group, except those from another individual of the Bombay phenotype. Based on the premise that the *H* gene codes for the H type 2 glycosyltransferase (FUT1), then the allelic *h* gene codes for a nonfunctional enzyme. In addition, because individuals of the Bombay phenotype are nonsecretors (i.e., they lack a functional H type 1 transferase [FUT2]), their genotype is *hh sese*. Interestingly some of these individuals do express active A and/or B enzymes, but the A (or B) antigen is not detected because the appropriate substrate for these enzymes (H type 1 or H type 2 chains) is not synthesized (Fig. 132-7). Nonetheless, the functional *A* or *B* genes can be transmitted to the next generation, yielding the apparently paradoxical pedigree O × O A (or B). On rare occasions, individuals are identified with ABH-deficient RBCs but with ABH antigens in their secretions; this is referred to as the para-Bombay phenotype.[46] Again, in terms of the model described above, these individuals have the *hh Sese* or *hh SeSe* genotype. That is, they are able to synthesize functional H type 1 transferase (FUT2), but not H type 2 transferase (FUT1). These variations are summarized in Table 132-7 .

Since the cloning and sequencing of the *H* gene in 1990, [23] various groups have investigated the molecular nature of the defective *h* alleles.[47] [48] [49] [50] [51] At least 20 different *h* alleles have been identified to date, variously containing missense, nonsense, and frameshift mutations. Interestingly, virtually every individual examined in these studies had a different mutation leading to

TABLE 132-7 -- Genetic Inheritance of H and Se Enzymes

	H Type 1 Transferase (Se, FUT2)	H Type 2 Transferase (H, FUT1)
Normal secretor	+	+
Normal nonsecretor		+
Bombay phenotype		
para-Bombay phenotype	+	

inactivation of FUT1 activity. In addition, one individual had a completely normal coding sequence, but defective enzyme activity, [51] suggesting a defect in transcriptional regulation rather than in protein structure. This latter result, in particular, also suggests that caution will be required if molecular approaches for blood typing based on *genotype* become widely used as a replacement for classical methods, which directly determine blood group *phenotype*.

Antigenic Variants of the ABH System

Some relatively common variations in the ABH system relate to the strength of the A antigen on group A cells. Several subgroups of A that have weak expression of the A antigen have been identified. The RBCs of most group A individuals (80% of whites) type as A_1 (Table 132-2) and correspond to the results originally described by Landsteiner. Most of the remaining group A individuals have weaker expression and are denoted A_2 . Other rarer subgroups of A (e.g., A_3 , A_x , A_m , A_{el}) have progressively weaker A antigen expression. Interestingly, many of these latter individuals produce an antibody, anti-A_1 , which does not agglutinate their own RBCs but does agglutinate A_1 RBCs. This phenomenon is explained by quantitative and qualitative differences in antigen expression. The number of A antigen sites per RBC varies from 800,000 sites for A_1 cells to 250,000 sites for A_2 cells to 700 sites for A_m cells.[52] The finding that A_2 individuals can synthesize A_1 -specific antibodies suggests that there are also qualitative differences. Recent biochemical investigations demonstrate that A antigens on A_1 RBCs differ from those on cells of the various subgroups of A.[53] In addition, the molecular biologic approach has identified mutations in allelic A transferase cDNA and genomic DNA sequences in individuals with RBCs expressing weak A activity[54] [55] [56] [57] [58] (Fig. 132-2). For example, the A transferase in A_2 individuals has a Pro156 Leu missense mutation and a frameshift mutation near the 3-end of the coding sequence.[54] In addition, A_3 individuals have an Asp 291 Arg mutation in the coding sequence.[56] These findings are summarized in Figure 132-2 . In some cases, transfection studies have been used to prove that these mutations result in weakened or variant enzyme activity of the encoded glycosyltransferases.[54]

By analogy with the subgroups of A, weak subgroups of B have also been described (e.g., B_3 , B_x , B_m , B_{el}). For example, the gene encoding the *B* allele responsible for the B_3 subgroup, has an additional Arg 352 Trp mutation in the coding sequence[56] (Fig. 132-2).

The B(A) and cis AB phenotypes are interesting and unusual variations in the ABH system. In each case, one chromosome in the affected individual apparently encodes for a gene (or genes) that leads to the synthesis of both the A and B antigens. In the B(A) phenotype, the RBCs predominantly have B antigens with small amounts of A antigens.[55] In the cis AB phenotype, the RBCs have equivalent amounts of the A and B antigens.[59] Recent molecular studies demonstrated that the coding sequence of the B(A) transferase is virtually identical to the B transferase, except that it lacks the Gly 235 Ser mutation [55] (Fig. 132-2). Thus, at three of the relevant amino acid positions the $B_{(A)}$ allele is identical to *B* and at one position it is identical to *A*; this allows the resulting glycosyltansferase to then create small amounts of A antigen in addition to large amounts of the B antigen.[55]

In the two cases of cis AB that have been studied, the *cisAB* allele has mutations resulting in two amino acid substitutions [59] (Fig. 132-2). The Pro 156 Leu mutation is identical to that found in A_2; the Gly 268 Ala mutation is a change that is important for conferring B transferase activity to the resulting enzyme. [9] [10] Thus, the *cisAB* allele seems to have arisen by point mutations of the A_1 transferase during evolution. These changes resulted in an enzyme that functions as an AB transferase chimera. Evidence for an alternative model, in which crossing-over results in one chromosome that contains both an intact A transferase and a separate, intact B transferase, is not yet available.

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Ii BLOOD GROUP SYSTEM

The Ii blood group system has been intensively studied but is still not completely understood. The Ii antigens are oligosaccharides that form the type 2 chain precursors for the ABH antigens (Fig. 132-8). The best available evidence indicates that the difference between the I and i antigens relates to branching of the oligosaccharide chain; anti-i antibodies recognize an unbranched oligosaccharide chain, and anti-I antibodies recognize a similar chain that is also branched. Fetal and cord RBCs contain mostly i antigen with small amounts of I; adult RBCs demonstrate the opposite pattern. This suggests that the glycosyltransferase necessary for synthesis of the branched structure, a (16) *N*-acetylglucosaminyltransferase, is developmentally regulated. The availability of the cloned cDNA encoding this enzyme[60] (Table 132-3) will now allow this hypothesis to be tested.[61] Rare adults have RBCs that type as i; presumably their RBC precursors lack this glycosyltransferase. Antibodies specific for the Ii antigens are clinically relevant in the setting of cold type autoimmune hemolytic anemia. The sera of these patients typically contain high titers of a monoclonal antibody, usually with anti-I specificity. In patients demonstrating hemolysis in vivo, the antibodies bind to RBCs at or near 37°C in vitro and have the ability to fix complement. Surprisingly, almost all normal individuals have low titers of presumably polyclonal anti-I. These autoantibodies have a low thermal amplitude, agglutinating RBCs only at room temperature or below, and do not cause accelerated RBC destruction in vivo. Patients with particular infectious diseases such as infectious mononucleosis and mycoplasma pneumonia often develop cold agglutinins, typically with anti-i and anti-I specificity, respectively. [62] In unusual cases, this may result in immune-mediated hemolytic anemia. At present, no completely satisfactory explanation that describes, at a molecular level, the differences between cold agglutinins that cause hemolysis in vivo, and those that do not, is available. [63] [64]

Figure 132-8 Ii blood group antigens. The monosaccharide abbreviations are as described in Figures 132-1 and 132-5 .

TABLE 132-8 -- Structures of P Blood Group and Related Glycosphingolipid Antigens

Lactosylceramide	Gal14Glc11cer
P^k	Gal14Gal14Glc11cer
P	GalNAc13Gal14Gal14Glc11cer
Lacto-*N*-neotriaosylceramide	GlcNAc13Gal14Glc11cer
Lacto-*N*-neotetraosylceramide	Gal14GlcNAc13Gal14Glc11cer
P_1	Gal14Gal14GlcNAc13Gal14Glc11cer
H type 2	Fuc12Gal14GlcNAc13Gal14Glc11cer
A type 2	GalNAc13[Fuc12]Gal14GlcNAc13Gal14Glc11cer

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P BLOOD GROUP SYSTEM

The P blood group system consists of three glycosphingolipid antigens: P^k, P, and P_1 (Table 132-8) (for review, see reference 65). . These carbohydrate chains are structurally and biosynthetically related [66] Each has a common precursor, Gal(14)Glc-ceramide (also called lactosylceramide); the P^k antigen is the biosynthetic precursor of the P antigen; both P^k and P_1 share a common disaccharide structure, Gal(14)Gal(14)-R, at the nonreducing end of the glycosphingolipid. These antigenic structures are not found on RBC membrane glycoproteins.[67] The glycosyltransferase that converts lactosylceramide to the P^k antigen is denoted as P^k synthase, that which converts P^k to P is denoted as P synthase, and that which converts paragloboside to P_1 is denoted as P_1 synthase (Table 132-8 and Fig. 132-9). Although none of the genes encoding these enzymes has yet been cloned, the gene encoding the P_1 synthase has been localized to chromosome 22[68] [69] [70] (Table 132-3).

Five different RBC phenotypes in which various combinations of these three antigens are present have been described (Table 132-9). The P_1 and P_2 phenotypes are common and account for almost all of the population. The serum of some P_2 individuals contains anti-P_1 antibodies. These antibodies are usually IgM low-titered cold agglutinins and are rarely of clinical significance. By contrast, the rare individuals with the P_1^k, P_2^k, and p phenotypes have naturally occurring high-titered IgM antibodies with specificity either for the P antigen (anti-P) or for all the antigens in the P blood group system (anti-P_1PP^k, or, equivalently, anti-Tj^a). These antibodies are clinically relevant in that they can cause severe hemolytic transfusion reactions. An unusual syndrome of recurrent spontaneous abortions has also been associated with these antibodies,[71] presumably due to the presence of P^k - and P-active glycosphingolipids on trophoblastic tissue. [72] In addition, the syndrome of paroxysmal cold hemoglobinuria, originally associated with

Figure 132-9 Biosynthetic pathway of antigens in the P blood group system. The biochemical structures of these glycosphingolipids are shown in Table 132-8 .

syphilis, is caused by Donath-Landsteiner antibodies. The latter are complement-fixing IgG antibodies with anti-P specificity that cause immune-mediated hemolysis in vivo.[73] Finally, recent studies have shown that the P blood group antigen is the receptor for parvovirus B19 on erythropoietic precursors. [74] [75] This virus causes both transient aplastic crises in patients with underlying hemolysis and anemia in immunocompromised patients. [76]

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POLYAGGLUTINATION AND T AND Tn ANTIGENS

The major glycoproteins on RBC membranes, glycophorins A, B, and C, each carry 16, 11, and 12 O-linked oligosaccharides per molecule, respectively. [77] These oligosaccharides are disialylated tetrasaccharides with the structure shown in Figure 132-10 . In certain individuals, cryptic carbohydrate antigens are exposed by removing sialic acid residues from the mature tetrasaccharide (denoted T activation) revealing the T antigen. In other individuals, cryptic antigens are exposed by preventing enzymatic attachment of (13)-linked galactose (Tn activation) resulting in expression of the Tn antigen. [78] Interestingly, the T and Tn structures are also tumor-associated antigens.[79]

Red blood cells expressing the T or Tn antigens are polyagglutinable because they are agglutinated by IgM antibodies present in all adult sera. These naturally occurring anti-T and anti-Tn antibodies presumably arise after exposure of individuals to cross-reacting structures present on bacteria.

The T antigen is transiently exposed on RBCs in vivo by the action of microbial neuraminidases (sialidases), typically in individuals with a bacterial infection caused by organisms such as *Escherichia coli*.[80] However, T activation has also been reported to occur in normal individuals. Although this phenomenon causes serologic problems in blood banks, it rarely causes hemolysis in vivo.

The Tn phenotype is an acquired condition of unknown etiology that may be transient but is usually persistent. It is a clonal disorder resulting from diminished (13) galactosyltransferase activity arising in hematopoietic stem cells. [81] [82] Thus, the Tn antigen is not only expressed on RBCs but it is also found on platelets and granulocytes. In most affected individuals, two populations of RBCs are found: one normal and one polyagglutinable. Although this disorder may occur in healthy

TABLE 132-9 -- RBC Phenotypes in the P Blood Group System

Phenotype	Frequency	RBC Antigens	Enzymes Present	Serum Antibodies
P_1	75%	P_1 , P, P^k	P_1 , P, P^k synthases	None
P_2	25%	P, P^k	P, P^k synthases	anti-P_1
$P_1{}^k$	Rare	P_1 , P^k	P_1 , P^k synthases	anti-P
$P_2{}^k$	Rare	P^k	P^k synthase	anti-P_1 , anti-P
p	Rare	None	None	anti-P_1 PP^k (anti-Tj^a)

Figure 132-10 Biochemical structures of O-linked oligosaccharides on glycophorins of normal RBCs and of T- and Tn-activated polyagglutinable RBCs. Oligosaccharides with the NeuAc(26)Gal(1O) Ser/Thr structure are also found on Tn-activated RBCs. [78] *N*-acetylneuraminic acid is abbreviated as NeuAc; serine as Ser; and threonine as Thr. Other abbreviations are as in Figure 132-1 .

individuals, patients may present with immune-mediated hemolytic anemia, thrombocytopenia, or granulocytopenia, or may subsequently develop acute leukemia. [83] [84]

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PART 2
Protein Determinants

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INTRODUCTION

Numerous blood group antigens reside on membrane proteins and comprise determinants dependent primarily on amino acid sequence. The biochemical and genetic basis of many of these antigens has been elucidated in recent years (Table 132-10), and these advances have led to the use of molecular diagnostic techniques to predict risk of hemolytic disease of the newborn and even expression of recombinant blood group antigen proteins to define antigenic epitopes. Some protein antigens typically stimulate brisk thymus-dependent immune responses, and the resulting high-titer IgG antibodies to such antigens may often result in rapid extravascular clearance of antigen-positive cells. These IgG antibodies may also cross the placenta, resulting in hemolytic disease of the newborn. For unknown reasons, other antigens, however, only rarely stimulate antibody production.

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Rh BLOOD GROUP

The Rh blood group antigens are clinically the most important of the protein antigens. They are the most frequent targets of alloantibodies produced by transfusion recipients as well as by mothers alloimmunized to antigens expressed by fetal RBCs. The Rh antigen proteins are also frequent targets of autoantibodies responsible for idiopathic and drug-induced hemolytic anemias. Antibodies to Rh antigens are usually IgG and rarely fix complement. Nevertheless, they may induce rapid extravascular clearance of antibody-sensitized cells.

The Rh blood group comprises 45 individual antigens, of which 5 are routinely identified: D, C, c, E, and e. These reside on two proteins that are the products of two extremely closely linked and homologous genes. The first Rh antigen to be defined was the Rho, or D, antigen. This antigen may be expressed or absent, giving rise to the so-called Rh-positive (D-positive) and Rh-negative (D-negative) phenotypes, respectively. No antigen antithetical to D has ever been identified; however, the symbol d has sometimes been used to denote absence of the D antigen. The other four antigens comprise two antithetical pairs: C and c, and E and e. Because of the tight linkage between the D and CcEe loci, these five antigens are inherited en bloc. Certain genotypes encoding specific combinations of D and CcEe antigens are more common than others, although gene frequencies vary from one population to another (Table 132-11).[85]

The Rh blood group system also encompasses a large number of antigens that represent epitopes determined by rare Rh protein polymorphisms or epitopes common to most Rh proteins but recognized immunologically only by those rare persons who are deficient in Rh proteins or whose cells carry only

TABLE 132-10 -- Blood Group Antigens That Reside on Identified Erythrocyte Membrane Proteins

Blood Group System	Symbols for System and Major Antigens	Molecular Characterization, Other Nomenclature
Cartwright	Yt^a /Yt^b	Acetylcholinesterase
Chido/Rodgers	Ch^a , Rg^a	C4 complement component
Colton	Co^a /Co^b	28-kd water channel protein (aquaporin-1)
Cromer	Cr^a , Tc^a , Dr^a , etc.	Decay accelerating factor (CD55)
Diego	Di^a /Di^b , Wr^a /Wr^b	Band 3, anion transport protein (AE-1)
Duffy	Fy^a /Fy^b	3546-kd promiscuous chemokine receptor (DARC)
Gerbich	Ge:2,3	Glycophorins C and D, sialoglycoproteins and
Indian	In^a /In^b	Hyaluronan receptor (CD44)
JMH	JMH	76-kd phosphatidylinositol-linked glycoprotein (CD108)
Kell	K/k, Js^a /Js^b , Kp^a /Kp^b , etc.	93-kd protein of zinc-binding neutral endopeptidase family
Kidd	Jk^a /Jk^b	Urea transporter
Knops/McCoy	Kn^a , McC^a	Complement receptor type 1 (CR1, CD35)
Kx	Kx	34 kD protein, possibly a neurotransmitter transporter
Landsteiner-Weiner	LW^a /LW^b	3747 kd-glycoprotein related to intracellular adhesion molecule subfamily
Lutheran	Lu^a /Lu^b	78- and 85-kd laminin receptor
MN	M/N	Glycophorin A, sialoglycoprotein
Ok^a	Ok^a	Neurothelin
Rh	D, E/e, C/c	3032-kd multipass integral proteins related to NH_4^+ transporters
Scianna	Sc: 1,2	60-kd glycoprotein
SsU	S/s, U	Glycophorin B, sialoglycoprotein
Xg	Xg^a	PBDX (Xg glycoprotein)

TABLE 132-11 -- Frequencies of Rh Gene Complexes in Different Populations

Gene Complex	Frequency	
	White Population	Black Population
DCe	0.42	0.17
dce	0.37	0.26
DcE	0.14	0.11
Dce	0.04	0.44
dCe	0.02	0.02
dcE	0.01	0.001
DCE	0.001	0.001
dCE	0.001	0.001

abnormal Rh protein molecules. These are the low- and high-frequency antigens of the Rh system. In addition, some Rh antigens exist only when certain antigens are encoded by the same gene. We now know that these cis antigens exist because the Cc and Ee epitopes constitute different immunogenic sites of the same protein. The most common example of a cis antigen is the f antigen, produced when the c and e antigens are encoded by the same gene. For example, f is expressed by the genotype *CDE/ce*, but not by the genotype *CDe/cDE*, even though both lead to expression of both the c and e antigens. Numerous other cis antigens have also been identified.

The null phenotype in the Rh blood group system has been extensively studied. Rh_{null} erythrocytes express no Rh antigens. Most interestingly, these cells also exhibit a wide range of abnormalities,[86] [87] [88] including stomatocytosis, reduced cation and water content, increased adenosine triphosphatase activity, abnormal membrane phospholipid distribution, reduced membrane stability, and shortened survival in vivo. Thus, individuals with this phenotype have a mild-to-moderate chronic hemolytic anemia.

Biochemistry

The proteins bearing Rh antigens were originally identified and characterized as 28,00033,000-d membrane proteins, all with common N-terminal sequences and a high degree of homology over the remaining protein domains (Fig. 132-11).[89] [90] [91] [92] These proteins are highly hydrophobic and lack any detectable glycosylation, a highly unusual trait for membrane proteins. The Rh proteins make up a large portion of the fatty acid acylated proteins of the plasma membrane [93] and may also interact with the cytoskeleton. [94] [95] Fatty acid acylation appears to be the result of a continuous on and off process of constant exchange of free palmitate for palmitate esterified onto cysteine residues within the portion of the molecule residing near the cytoplasmic border of the inner membrane leaflet.

Despite the marked abnormalities demonstrated by Rh_{null} erythrocytes, in which Rh peptides are not expressed, no function has yet been attributed to the Rh proteins. The multiple membrane spanning domains of the Rh peptides are reminiscent of transport proteins, but no transport function has been successfully demonstrated for Rh peptides. Rh peptides have been shown to be homologous to nonmammalian NH_4^+ transporters;[96] however, proteins with NH_4^+ transport function have thus far not been identified in animals.

When Rh proteins are deficient, abnormalities in the expression of other membrane proteins are also detected. These include reduced or absent expression of the U antigen, known to be at least partly dependent on expression of glycophorin B. Expression of another glycoprotein, CD47 (see later discussion), is also markedly reduced in Rh_{null} cells. Some human and murine monoclonal antibodies coprecipitate several membrane proteins, including the Rh peptides and either CD47 and/or another glycoprotein, Rh50.[97] This and additional evidence has led to the belief that the Rh peptides exist in the membrane as part of a multiprotein complex that may include the Rh peptides, the Rh50 protein, CD47, and glycophorin B. Serologic evidence suggests that expression of the Rh and Duffy proteins may also be associated.

Genetics

The two closely linked *RHD* and *RHCE* genes on chromosome 1p36.1334.3 encode the proteins bearing the D and CcEe

Figure 132-11 (A) Structure of the RhD and RhCE proteins. The RhD and RhCE proteins are highly homologous multipass integral membrane proteins that lack glycosylation. The upper figure indicates the individual amino acid positions at which the RhD and RhCE proteins differ. The lower figure indicates the polymorphisms that correspond to expression of the C/c and E/e antigen polymorphisms. **(B)** Examples of the genetic basis of variant Rh genes. A large number of complex variant *RHD* and *RHCE* genes have arisen, apparently due to the high homology between and close proximity of the *RHD* and *RHCE* genes. As shown by the variants illustrated here, many *RHD* and *RHCE* variants contain one or more exons derived from the other gene, leading to aberrant expression of Rh epitones and, in some cases, expression of rare novel antigens.

antigens, respectively.[98] [99] [100] In persons with the Rh-negative (D-negative) phenotype, the *RHD* gene is most often missing entirely, so that Southern blots of genomic DNA show absence of a set of bands corresponding to the *RHD* gene.[100] The *RHCE* gene encodes a protein that bears either E or e, along with either C or c. The difference between the coding regions of the D and CcEe cDNAs consists of scattered nucleotide differences encoding only a relatively small number of amino acid differences. Both the D and CcEe proteins have similar overall structure, including 30% hydrophobic amino acid residues and 12 predicted transmembrane domains (Fig. 132-10). The difference between the E and e proteins depends on a single amino acid polymorphism (proline versus alanine at position 226) in the fourth extracellular domain. The C and c proteins differ at four amino acids, although expression of either a serine or proline at position 103 in the second extracellular domain is probably the critical amino acid substitution on which the antigenic Cc epitopes depend. [101]

A large number of *D* and *CE* variant genes have been identified and are apparently derived from genetic events that occur because the *D* and *CE* genes are both highly homologous and physically close.[102] These genes frequently bear one or more exons deriving from the other gene (Fig. 132-11). For example, the *DVa* gene is normal except for the replacement of exon 5 of the *D* gene with exon 5 of the *CE* gene.[103] Some genes contain more extensive substitutions; one type of *DVI* gene contains three exons from *CE*,[104] and persons with the Dc-phenotype, who lack all Ee antigens, have a *CE* gene in which exons 49 are derived from the *D* gene.[105]

The cDNAs of two additional Rh-associated proteins have been cloned. The Rh-related glycoprotein Rh50 is encoded by chromosome 6, and its cDNA bears partial sequence homology to the Rh cDNAs encoded by chromosome 1.13. In addition, the predicted structure of the protein is analogous to that of the D and CcEe proteins. However, the Rh50 protein is glycosylated, unlike the Rh proteins. Thus far, it has been mutations in the *RH50* gene that have been identified as leading to the Rh_{null} phenotype, presumably because expression of Rh50 protein is necessary for normal expression of Rh proteins. [106]

The second Rh-related protein, which is more widely expressed by human tissues, has been described by several names. Identification on leukocytes via monoclonal antibodies has led to its designation as CD47, [107] while its association with integrins in certain tissues has led to its description as an integrin-associated protein. [108] Unlike the Rh50 protein, CD47 bears no significant homology to the Rh polypeptides. [109]

LW Antigens

The LW antigen, named for two pioneers in blood group antigens, Landsteiner and Weiner, is more strongly expressed by D+ than D RBCs. Indeed, LW is the Rhesus antigen originally identified by these investigators using heterologous antisera to rhesus monkey RBCs. Since its original identification, LW has been subdivided into LW^a and LW^b . Both antigens are borne by 3747-kd glycoproteins encoded by a gene on chromosome 19. [110] Structurally, the LW protein is related to the intercellular adhesion molecule subgroup of the Ig superfamily of molecules, [111] and LW has been shown capable of binding certain leukocyte integrins. [112] The molecular basis for the $LW^{a/t}$ polymorphism, as well as for the LW (ab) phenotype, have been defined. [113] [114] Although expression of LW antigens is abolished in the Rh_{null} phenotype, the full structural relationship between the LW antigens and the Rh system remains to be determined.

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MNSs BLOOD GROUP ANTIGENS

The M, N, and S/s antigens reside on two highly related integral membrane glycoproteins most often called glycophorin A (GPA) and glycophorin B (GPB). [115] GPA is one of the two most abundant erythrocyte integral membrane proteins, each of which is expressed at about 1×10^6 copies per cell. GPB is somewhat less abundant (0.83×10^5 copies per cell). Together they account for 95% of the periodic acid-Schiff-staining material in the RBC membrane and bear the great majority of the sialic acid residues that confer a negative surface charge to erythrocytes.

Antibodies to the M and N determinants of GPA may be immune or naturally occurring and may be allo- or autoimmune. These antibodies are rarely of clinical significance in transfusion, hemolytic disease of the newborn, and autoimmune hemolysis. Antibodies to the S and s antigens, however, are most often IgG alloimmune antibodies that are capable of stimulating immune clearance of transfused or fetal RBCs. In addition, individuals lacking either GPA or GPB or only expressing rare variants of these proteins often produce clinically significant antibodies to epitopes expressed by normal GPA or GPB.

Biochemistry and Genetics

The glycophorin genes are highly homologous and lie close to each other on chromosome 4q2831, apparently having arisen through gene duplication. [116] GPB is smaller than GPA, because the region of the *GPB* gene corresponding to exon 3 of GPA is not used due to mutation of the intron-exon junction in the *GPB* gene. In addition, the cytoplasmic portion of the GPB molecule is truncated (Fig. 132-12).

Glycophorin A bears an M or N antigen at its N-terminus, depending on whether the N-terminus comprises Ser-Ser-Thr-Thr-Glyor Leu-Ser-Thr-Thr-Glu, respectively (Fig. 132-13).[115]

Figure 132-12 Structure of the *GPA* and *GPB* genes and some GPA and GPB variants. GPA is encoded by seven exons; while GPB is encoded by five exons. The genetic material corresponding to exon 3 of the GPA gene does not contribute to mature mRNA due to a splice junction defect. In addition, a stop codon in the GPB exon corresponding to exon 6 of GPA prevents extension of the cytoplasmic domain of GPB. Many so-called Miltenberger (Mi) phenotypes result from gene conversion events resulting in substitution of material from one glycophorin gene into the genetic structure of the other glycophorin gene.

Figure 132-13 The MN and Ss polymorphisms of GPA and GPB. Numbers indicate amino acid position, and small dark circles indicate O-linked glycosylation sites. Small circles indicate presence of additional amino acids.

However, human anti-M and anti-N, which are usually IgM antibodies, often depend both on primary amino acid structure as well as appropriate glycosylation of the second, third, and fourth amino acids (-Ser-Thr-Thr-).

The N-terminal structure of GPB is homologous to that of the N variant of GPA through residue 26. At amino acid 29, GPB is polymorphic; the existence of a methionine corresponds to expression of the S antigen, while a threonine at this site gives rise to the s antigen (Fig. 132-13).[115]

Null and Variant Phenotypes

Red blood cells may be totally deficient in either GPA or GPB, or both, without apparent shortening of RBC half-life or functional abnormalities. Lack of GPA or GPB, or both, is denoted as the En(a), S-s-U-, and MkMk phenotypes, respectively.

The *GPA* and *GPB* genes have also given rise to a large number of genetic variants, often associated with unique antigens. The variant phenotypes created by genetic events involving these genes have been designated Miltenberger phenotypes. These phenotypes arise from a number of different mechanisms. Some represent simple point mutations. Others appear to arise from formation of variant *GPA/B* genes via unequal crossing over,[117] [118] [119] a phenomenon apparently dependent on the close proximity to one another of the highly homologous *GPA* and *GPB* genes, along with a third homologous gene, denoted glycophorin E; thus, some molecules may consist of the N-terminal portion of GPA, along with the C-terminal portion of GPB, or vice versa (Fig. 132-13). Other variant glycophorin genes appear to result from gene conversion events that result in genes that have small internal segments that correspond to the homologous gene.

Antibodies to the En^a and U antigens made by En(a), U-, and M^k M^k individuals are almost always capable of markedly accelerating clearance of antigen-positive cells. Likewise, antibodies made to antigens carried by variant glycophorin molecules are frequently capable of causing hemolytic transfusion reactions and hemolytic disease of the newborn.

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GERBICH ANTIGENS

Although antibodies to the Gerbich antigens are rarely encountered, their mention is appropriate because they reside on two molecules that also bear the name glycophorins and that are important to erythrocyte membrane integrity. Glycophorins C and D (GPC/D) are membrane glycoproteins that arise from a single gene by the use of alternate translation start sites (Fig. 132-14).[120] [121] Unlike GPA and GPB, to which they are unrelated, GPC/D are physiologically important membrane proteins that interact with the peripheral membrane protein band 4.1 to mediate attachment of the cytoskeleton to the membrane. [122] [123] [124] Complete lack of these glycoproteins, a phenotype designated Leach, results in absence of the Gerbich blood group antigens as well as a rare form of hereditary elliptocytosis. [125] [126]

A number of variant forms of GPC/D also exist and are occasional targets of clinically significant alloantibodies, but are not associated with elliptocytosis. Some of these variants arise from a deletion or duplication of one of two internal exons (Fig. 132-14), which are themselves highly homologous to one another.[120] This homology apparently leads to unequal crossing-over events. The resultant variant proteins may both lack some common Gerbich epitopes as well as present novel Gerbich antigens.

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KELL BLOOD GROUP ANTIGENS

The Kell blood group system is extremely important from the point of view of transfusion medicine because antibodies to the K antigen are among the alloantibodies most often identified. Antibodies to K, as well as to other Kell system antigens (Table 132-12), are in general IgG antibodies that are capable of rapidly clearing antigen-positive cells. The Kell blood group system includes three sets of well-defined antithetical antigens that have been mapped to chromosome 7q33. [127]

Figure 132-14 Structure of the GP*C/D* gene and some variants. The GP*C/D* gene contains four exons that encode the mature protein. GPD, however, results from translation initiation at an alternative site, located near the 5 end of exon 2. The regions encoding the Ge:2 and Ge:3 antigens are indicated.

TABLE 132-12 -- Commonly Identified Kell System Antigens

Symbol and Original Name	ISBT Alphanumeric Symbol	Frequency
K (Kell)	K1	Low
k (Cellano)	K2	High
Kp^a (Penney)	K3	Low
Kp^b (Rautenberg)	K4	High
Js^a (Sutter)	K6	Low
Js^b (Matthews)	K7	High

Abbreviation: ISBT, International Society of Blood Transfusion.

Null and Variant Kell Phenotypes

Although most Kell system-related immune problems are caused by antibodies to antigens that belong to one of the three antithetical antigen pairs listed in Table 132-12 , the unusual Kell phenotypes are quite interesting from a number of viewpoints. Rare individuals have been found who fail to express all Kell antigens, apparently due to inheritance of two nonfunctional (amorphic) Kell alleles. This phenotype, designated Ko, is associated with no morphologic or physiologic RBC abnormalities.

Another phenotype, associated with weakened Kell antigens, presents with acanthocytosis and mild chronic hemolytic anemia. This latter phenotype is associated with absence of the Kx antigen, and is often designated the McLeod phenotype. The McLeod phenotype has now been shown to be part of the syndrome of neuroacanthocytosis and is associated with late-onset neurologic symptoms. [128] [129] The Kx antigen is encoded at Xp21, near the loci for X-linked chronic granulomatous disease and Duchenne muscular dystrophy.[130] Therefore, the Kell and Kx antigens have been recognized as distinct for some time. The serologic characteristics of the McLeod phenotype, namely the weakening of Kell blood group antigen expression and the absence of Kx antigen expression, are thought to result from failure of the normal interaction of the Kell and Kx proteins.

Biochemistry and Genetics

The protein carrying the Kell antigens has been purified and its cDNA isolated and cloned. [131] This protein of 93,000 daltons belongs to the family of zinc-binding neutral endopeptidases and is highly homologous to CD10 (CALLA, common acute lymphocytic leukemia antigen). The Kell protein comprises 732 amino acids, organized with one membrane-spanning domain, so that the C-terminus is extracellular and the N-terminus is in the cytoplasm. All Kell system antigens appear to reside on a single protein, so that the various antithetical antigen pairs represent polymorphic epitopes on this protein, usually determined by single amino acid substitutions.

Immunoprecipitation studies using antibody to Kell protein, however, also demonstrate co-precipitation of a second protein. This 34,000-dalton protein has been identified as the Kx protein, which is the *XK* gene product.[132] The *XK* gene encodes a membrane protein with structural similarity, but not amino acid homology, to a family of neurotransmitter transport proteins. [133] A function for KX has not yet been proven.

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KIDD BLOOD GROUP SYSTEM

The Kidd antigens are clinically important and often problematic antigens. Antibodies to these antigens are relatively common alloimmune antibodies that often fix complement. However, antibodies to Kidd antigens often wane to undetectable levels after primary or secondary exposure, making these antibodies frequent causes of delayed hemolytic transfusion reactions.

The two common antigens in this systemJk^a and Jk^b occur at about the same frequency in the white population. A rare allele that apparently encodes no Kidd protein is denoted *Jk*. Individuals homozygous for this amorphic allele express no Kidd antigens and may, after exposure to normal RBCs, make anti-Jk3, an antibody that reacts with all cells that express Jk antigens. The Jk(ab) phenotype is of added interest because it can be shown to have a defect in urea transport; unlike normal RBCs, Jk(ab) RBCs cells will not lyse in 2 M urea. [134] [135]

Biochemistry and Genetics

The Kidd antigens reside on a urea transport protein. [136] The gene encoding this protein, *HUT11*, has been mapped to chromosome 18q11q12. The *HUT11* gene product is also expressed in the kidney, and individuals lacking expression of *HUT11* have reduced urinary concentrating ability. [137] The Jk^a and Jk^b antigens arise from a single amino acid substitution. [138]

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DUFFY BLOOD GROUP ANTIGENS

The Duffy (Fy) antigensFy^{a} and Fy^{b} occur with nearly similar frequencies in the white population, while approximately two-thirds of American and 90% of West African blacks are Fy(ab). Antibodies to Fy antigens are relatively common, most often IgG and immune in nature. Anti-Fy ^{a} is slightly more common that anti-Fy^{b} .

The Duffy antigens have been investigated with intense interest because of their relationship to malarial resistance. *Plasmodium vivax*, a cause of human malaria, and *Plasmodium knowlesi*, a causative agent of malaria in monkeys, cannot invade Fy(ab) RBCs, [139] although *Plasmodium falciparum*the malarial parasite causing the most serious human morbidity and mortalitycan both attach to and invade Fy(ab) RBCs.

Biochemistry and Genetics

The Fy antigens reside on a protein of 35,00046,000 daltons that has been shown to be part of the chemokine receptor family of proteins. [140] The RBC Duffy protein can bind a number of chemokines, including interleukin-8. Duffy protein is also expressed in other tissues, including endothelial cells. [141] Current investigations are examining whether Duffy protein may play a role in leukocyte migration across endothelium.

The Fy(ab) phenotype in people of African origin does not arise from nonfunctional *FY* alleles. Instead, it is caused by mutation of the promoter region of the *FY* gene.[142] This mutation disrupts the nucleotide sequence normally recognized by the GATA-1 transcription factor, a factor of critical importance in the expression of a large number of erythroid genes. The result of this mutation is that the *FY* gene is not transcribed in erythroid cells but is expressed in nonerythroid cells, in which other transcription factors presumably drive expression of the *FYB* gene.[143] Thus far, this promoter mutation has only been found in association with the *FYB* allele.

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LUTHERAN ANTIGENS AND RELATED PROTEIN ANTIGENS

The Lutheran blood group system is another system that comprises a large number of individual antigens (18), of which eight comprise four antithetical pairs. Only Lu^a and Lu^b , however, are frequently identified. Lu^b is expressed by > 99% of individuals, while Lu^a is expressed by about 8%. In general, antibodies to Lutheran antigens are IgG in nature, although IgA and IgM antibodies

have been reported; most are not effective in causing accelerated clearance of antigen-positive RBC.

The Lutheran blood group system is of especial interest, however, because of the unusual genetic regulation of expression of Lutheran antigens. Although some Lu(ab) persons have inherited two amorphic (nonfunctional) Lutheran alleles, most examples of the Lu(ab) phenotype are due to inheritance of a single, dominant *In(Lu)* gene. This gene causes markedly reduced expression of Lutheran antigens, so that routine typing results in identification of a Lu(ab) phenotype. However, more sensitive techniques demonstrate expression of very small amounts of apparently normal Lutheran protein and antigens by these cells. In addition, the *In(Lu)* gene also suppresses expression of unrelated proteins, including the CD44 hyaluronan receptor, [144] a protein that carries another blood group, In. [145] [146] In addition, *In(Lu)* may affect expression of other antigens, including the Knops and AnWj antigens.

A third type of Lu(ab) phenotype has been shown to be due to an X-linked gene. Like in the *In(Lu)* phenotype, Lutheran antigens in the X-linked Lu(ab) phenotype are suppressed but not totally absent, while expression of antigens outside the Lutheran blood group system, such as CD44, is unaffected. This phenotype is inherited in an X-linked recessive manner,[147] [148] whereas the *In(Lu)* phenotype is inherited as an autosomal dominant trait.

Biochemistry and Genetics

Lutheran antigens reside on two related proteins of 78,000 and 85,000 daltons that are themselves products of alternatively spliced mRNA from a single gene. [149] [150] [151] The *LU* gene is at chromosome 19q13.2, a locus linked to that of the secretor (*Se*) gene. The Lutheran protein is an Ig superfamily molecule containing two V and three C2 type domains; it has been shown capable of mediating cell adhesion to laminin. [152] Although this function is only minimally demonstrable on normal RBCs, it is present on sickle RBCs, as well as in the diverse epithelial tissues in which Lutheran protein is also expressed. [152] Lutheran protein, in its 78,000-dalton form, has also been called the basal cell adhesion molecule. [153] Thus far, several Lutheran antigens have been mapped to the various IgSF domains of Lutheran protein, and the molecular basis for the Lu^a /Lu^b phenotype has been identified as a single nucleotide substitution leading to a single amino acid change in the IgSF domain nearest the N-terminus of the protein. [154] [155]

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COLTON ANTIGENS

The Colton blood group system comprises antigens expressed by the water channel protein of erythrocytes, also known as channel-forming integral protein-1 or aquaporin-1.[156] [157] This protein accounts for >2% of the total membrane protein and facilitates rapid water transport across the erythrocyte membrane. It most likely occurs in the membrane as a tetramer, in which one of the four protein molecules is glycosylated and also carries ABH determinants on a polylactosaminoglycan moiety. The gene encoding aquaporin-1 is on chromosome 7p14, and weak expression of Colton antigens may sometimes occur in leukemias and myelodysplastic conditions in which there is monosomy 7. However, total absence of aquaporin-1, as seen in the Co(ab) phenotype, is not associated with clinically significant defects in circulating erythrocytes nor in renal function, despite the fact that aquaporin-1 is responsible for about 80% of the water reabsorption in normal kidneys. [158] [159]

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DIEGO AND WRIGHT ANTIGENS

The Diego blood group system comprises two pairs of antithetical antigens (Di^{a} /Di^{b} and Wr^{a} /Wr^{b}), as well as a number of

Figure 132-15 The anion transport protein (band 3). The anion transport protein, also called AE1 or band 3, carries both Diego and Wright antigens. Band 3 also carries ABH and Ii antigens on a single highly branched N-linked oligosaccharide, represented by the branching structure on the fourth extracellular domain. As do many transport molecules, band 3 crosses the membrane multiple times. Band 3 is thought to occur in the membrane as a homodimer and form a pore through which anions are exchanged between the extracellular environment and the cytosol.

low-incidence antigens. Alloantibodies to these antigens are only occasionally encountered and are usually IgG; they vary widely in their ability to clear transfused incompatible RBCs and to cause hemolytic disease of the newborn.

Diego and Wright antigens reside on the erythrocyte anion channel protein, [160] [161] [162] long known as band 3, a nomenclature earned when RBC membrane proteins were first examined in Coomassie blue-stained gels (Fig. 132-15). This protein, which occurs in approximately 1,000,000 copies per RBC, is also known as AE-1 (anion exchanger-1), as it is a member of a family of anion exchange proteins expressed in many tissues. [163] [164] [165] The band 3 protein also subserves an important structural role in human erythrocytes by providing a means of connection between the cytoskeleton and the plasma membrane via its interactions with ankyrin, and abnormalities of this protein account for the Southeast Asian ovalocytosis syndrome, [166] [167] [168] as well as between one-tenth and one-third of all cases of dominantly inherited hereditary spherocytosis. [169] [170] Band 3 is also a frequent target of RBC autoantibodies in warm-type autoimmune hemolytic anemia. [171]

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OTHER-BLOOD GROUP SYSTEMS

A large number of blood group systems and individual unrelated blood group antigens have been identified, and the genes for many of them have been cloned (Table 132-10). The reader should keep in mind that some proteins bearing blood group antigens are functionally important, and that somebut by no means allpolymorphisms or deficiencies of such proteins may be accompanied by physiologically significant changes in RBC morphology, function, or survival. Antibodies to blood group antigens will continue to serve as obstacles to transfusion, as well as tools for investigating the surface structure of the erythrocyte and other cell types.

LABORATORY METHODS FOR DETECTING BLOOD GROUP ANTIGENS AND ANTIBODIES

Manual methods based on agglutination of RBCs are still the most common serologic assays performed in blood banks. Automated assays based on agglutination, and manual or automated solid-phase assays adherence assays not based on agglutination, are used in large blood centers or in research environments.

The serologic behavior of RBC antibodies primarily relates to their immunoglobulin isotype. Thus most antibodies to carbohydrate antigens such as those in the ABO, Lewis II, and P blood group systems are naturally occurring and are of the IgM isotype. These multivalent antibodies optimally bind to RBCs at temperatures <37°C and directly agglutinate these cells suspended in saline. By contrast, antibodies occurring following immunization to protein antigens such as those in the Rh, Kell, and Duffy blood group systems, are of the IgG isotype. These bivalent antibodies optimally bind to RBCs at 37°C and will bind to, but not directly agglutinate, RBCs suspended in saline. The addition of an antiglobulin reagent (i.e., rabbit anti-human IgG) is required to induce agglutination.

ANTIGEN TYPING OF HUMAN RBC

Commercially available human polyclonal and mouse monoclonal IgM anti-A and anti-B antibodies are used to determine the ABO type of patient and donor RBCs. These antibodies will directly agglutinate RBCs at room temperature. To confirm a patients ABO type the presence of the corresponding serum isoagglutinins is also determined by incubating the serum at room temperature with commercially available group A and group B RBCs. In both cases, hemagglutination is macroscopically visible, and an antiglobulin reagent is not required.

Patient and donor RBCs are also routinely tested for the presence of the D antigen in the Rh system. A commonly used anti-D reagent is a chemically reduced IgG antibody that directly agglutinates D-positive RBCs suspended in saline at room temperature.

IDENTIFICATION OF SERUM ALLOANTIBODIES OR AUTOANTIBODIES DIRECTED AGAINST RBC

Most alloantibodies found in patient sera are of the IgG isotype and therefore do not directly agglutinate antigen positive RBCs. Thus,the most common assay used to detect these antibodies is the indirect antiglobulin test, formerly known as the indirect Coombs test. In this assay, patient serum is incubated at 37°C with commercially available reagent RBCs of known antigen type. Following incubation, unbound antibodies are removed from the RBCs by washing with saline, and an antiglobulin reagent containing either rabbit anti-human IgG or a mixture of rabbit anti-human IgG and anti-human complement is added. If the RBCs are agglutinated, this indicates the presence of an alloantibody or an autoantibody or both. The distinction between alloantibody and autoantibody and the determination of the specificity of the alloantibody are determined by performing this assay with a panel of 10 different reagent RBCs varying in antigen phenotype. Agglutination of all panel cells suggests the presence of an autoantibody; differential reactivity suggests the presence of an alloantibody to a specific RBC antigen.

The indirect antiglobulin test is also used to cross-match donor blood for transfusion into patients. In this case, patient serum is tested against RBCs from a donor with a compatible ABO and D antigen type. A positive reaction indicates the probable presence of an alloantibody in the recipient directed against a donor RBC antigen. The specificity of this alloantibody can be determined by the approach outlined previously.

DETECTION OF RBC COATED IN VIVO WITH IMMUNOGLOBULIN AND COMPLEMENT

The direct antiglobulin test, formerly known as the direct Coombs test, detects the presence of antibody or complement (or both) on the surface of RBCs. This technique is used in the analysis of delayed hemolytic transfusion reactions and in the evaluation of patients with suspected autoimmune hemolytic anemia. In this assay, unagglutinated patient RBCs are obtained from ethylenediaminetetraacetic acid anticoagulated blood, washed with saline, and then incubated with a commercially available antiglobulin reagent containing either rabbit anti-human IgG or a mixture of rabbit anti-human IgG and anti-human complement. The test is positive if agglutination is seen after the addition of the antiglobulin reagent. Antiglobulin reagents containing anti-IgM or anti-IgA are available in specialized centers to detect coating of RBCs in vivo by antibodies of these isotypes.

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Chapter 133 - Human Platelet Antigens

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INTRODUCTION

Platelet Membrane Glycoproteins

Among the variety of glycoproteins on the human platelet surface, there are several that contribute to the immunogenic makeup of the platelet (Table 133-1).

Integrins

The integrins are membrane glycoprotein heterodimers, each consisting of noncovalently associated and subunits. [1] [2] [3] [4] [5] [6] The specificity of an integrin is dictated in large part by the identity of its subunit, even though ligand binding per se may occur with a significant portion of the subunit. The number of possible combinations is apparently limited, however, and selective pairing in humans between 16 and 8 subunits has led to the discovery of 22 different integrins (Fig. 133-1). These ubiquitous receptors mediate a wide range of cell adhesion events that are important to every fundamental area of human biology, including embryonal development, immunocompetence, wound healing, and hemostasis. Two platelet membrane receptors that figure prominently in the antigenic profile of platelets, the cohesion receptor $_{IIb\ 3}$ and the collagen receptor $_{2\ 1}$, are integrins.[1]

The Platelet Cohesion Receptor, Integrin $_{IIb\ 3}$

The numerically predominant platelet integrin $_{IIb\ 3}$ (initially designated glycoprotein [GP] IIb/IIIa) mediates the common cohesive pathway that results from platelet activation in vivo, namely, platelet aggregation supported by the binding of adhesive proteins, such as fibrinogen and von Willebrand factor (vWF). The subunit of this integrin, $_{IIb}$, is synthesized exclusively by megakaryocytes. Consequently, $_{IIb\ 3}$ is a unique marker for platelets or cell lines with a megakaryocytic phenotype. Glanzmann thrombasthenia is an inherited disorder of platelet function characterized by an inability of platelets to bind fibrinogen and undergo agonist-induced aggregation.[7] The

TABLE 133-1 -- Functional Properties of Selected Membrane Glycoprotein (GP) Complexes

GP	Alternate Names	Receptor Function	Protein Ligands
Ib-IX-V		Adhesion	vWF
$_{IIb\ 3}$	GPIIb/IIIa, CD41/CD61	Adhesion Cohesion	Fibrinogen Fibrinogen Fibronectin Vitronectin vWF
$_{2\ 1}$	GPIa/IIa, VLA-2, CD49b/CD29	Adhesion	Collagen
$_{5\ 1}$	GPIc/IIa, VLA-5, CD49e/CD29	Adhesion	Fibronectin
$_{6\ 1}$	GPIc/IIa, VLA-6, CD49f/CD29	Adhesion	Laminin

Abbreviations: vWF, von Willebrand factor; VLA, very late activation (antigen); CD, cell differentiation (antigen).

Figure 133-1 The integrin receptor family. Integrin and subunits are paired to form functional receptors, as indicated by solid connecting lines. Those integrins that exist on human platelets are highlighted and include $_{2\ 1}$, $_{5\ 1}$, $_{6\ 1}$, $_{v\ 3}$, and $_{IIb\ 3}$.

molecular defect in this disease involves either a quantitative or a qualitative abnormality of $_{IIb\ 3}$. The $_{IIb\ 3}$ heterodimer complex is depicted schematically in Figure 133-2 .

The Platelet Collagen Receptor, Integrin $_{2\ 1}$

Another integrin that contributes significantly to platelet function is the collagen receptor $_{2\ 1}$ (GPIa/IIa).[8] [9] [10] [11] This integrin plays a fundamental role in adhesion of blood platelets to both fibrillar (types IIII and V) or nonfibrillar (types IV, VI, VII, and VIII) collagens. [8] [9] [12] [13] [14] [15] Unlike $_{IIb}$, the $_{2}$ subunit is a single-chain molecule[16] and, like several other integrin subunits, contains an additional 129-amino acid segment known as the I-domain (Fig. 133-3). Inherited platelet deficiencies of the $_{2}$ subunit have been described; affected individuals exhibit chronic mucocutaneous bleeding and prolonged bleeding times. [17] [18] The expression of $_{2\ 1}$ on platelets differs markedly between normal subjects and depends on the inheritance of at least three alleles of the $_{2}$ gene.[19] [20] Although the levels of the integrins $_{5\ 1}$ or $_{IIb\ 3}$ and the glycoprotein Ib-IX-V complex on platelets (molecules per platelet) certainly vary from one individual to the next, [19] [21] these differences never exceed a fraction of the mean population level. On the other hand, the number of $_{2\ 1}$ molecules per platelet varies by as much as an order of magnitude and correlates precisely with quantitative estimates of platelet adhesion to type I or type III collagens. [19]

Regulation of $_{2\ 1}$ expression could certainly modulate the antigenicity of this membrane receptor. It is also possible that similar factors influence expression of other

platelet integrins, including $_{IIb\ 3}$, but the extent to which that occurs remains to be determined. Two other integrins, the fibronectin receptor $_{5\ 1}$ [22] and the laminin receptor $_{6\ 1}$, [23] are expressed by platelets, but neither has yet been shown to contribute significantly to platelet immunogenicity.

Figure 133-2 The integrin $_{IIb\ 3}$, $_{IIb}$, and $_{3}$ form a noncovalently associated heterodimer. The hatched area of $_{IIb}$ represents the decapeptide recognition site. On $_{3}$, the stippled region represents the RGD recognition site; the hatched region, an alternative fibrinogen recognition site. Divalent cations (positively charged spheres) are required for both complex integrity and ligand binding. In this and subsequent figures, the locations of the polymorphisms that give rise to alloantigens are indicated by a black circle within which is the corresponding HPA designation (see Table 133-3). Regions that contain autoantigenic determinants are indicated by boxed areas and include two sequences on the cytoplasmic tail of $_{3}$ (721762), a portion of the cysteine-rich domain of $_{3}$ (479656), and the discrete epitope 2E7, located at residues 222238 of $_{IIb}$.

Figure 133-3 The $_{2}$ subunit. $_{2}$ belongs to the subfamily of subunits that are single-chain molecules with an additional I-domain (large open bar) sequence. Three putative calcium-binding repeats are indicated (small open bars), as is the site of the alloantigenic HPA-5 polymorphism. The locations of disulfide bonds (thin lines) are hypothetical.

The Receptor Complex Glycoprotein Ib-IX-V

The pattern of membrane glycoproteins existing as functional complexes is repeated in the case of the non-integrin adhesion receptor complex Ib-IX-V. [24] [25] The functional complex is actually a heptamer composed of one molecule of glycoprotein V associated with two molecules of each of three other gene products, glycoproteins Ib, Ib, and IX ((2)Ib:(2)Ib:(2)IX:(1)V) (Fig. 133-4).[26] This complex is a receptor for vWF and the initial mediator of transient platelet contact and attachment to the vessel wall under essentially all conditions of flow or shear stress. The vWF-binding site on the complex is located within the amino-terminal domain of GPIb.[25] [27] [28] Bernard-Soulier syndrome is an inherited disorder of platelet function characterized by defective platelet adhesion to subendothelium and a quantitative or qualitative defect in the Ib-IX-V complex caused by mutations in genes encoding either the GPIb or GPIX components. [29] [30] [31]

Platelet Glycolipids

Glycolipids play an important role in the structural and functional characteristics of the platelet membrane, as is true of all cell types. Less is known about platelet glycolipid structure and immunogenicity than about platelet glycoproteins, but important contributions have been made in this area, permitting the identification of a number of immunogenic glycolipid components of the membrane (Table 133-2). [32] The predominant glycolipid on human platelets is lactosyl ceramide, representing 64% of the neutral glycolipids. Other neutral glycolipids include trihexosyl ceramide, glycosyl ceramide, and globoside. The principal fatty acids associated with these neutral glycolipids are behenic acid (22:0), arachiditic acid (20:0), and lignoceric acid (24:0). Ganglioside I (identified as hematoside or GM3) represents 92% of the acidic glycolipids, and ganglioside II represents 5% of the platelet acidic glycolipid composition.

Figure 133-4 Glycoprotein Ib-IX-V complex. GPIb is composed of a heavy chain (Ib) and light chain (Ib) linked by a disulfide bond. The Ib molecule is noncovalently associated with glycoprotein IX. All three polypeptides span the membrane, are glycosylated (open diamonds or circles), and contain repetitive leucine-rich glycoprotein sequences (open areas). Two each of Ib, Ib, and IX are associated with one molecule of V. In the case of Ib, vWf binds at the amino-terminal region of the molecule, and five additional O-glycosylated repeats generate a carbohydrate-rich (macroglycopeptide) carboxy region. The positions of the Ib polymorphism that gives rise to the HPA-2 alloantigen system and the tandem 13-amino acid repeats (*) are indicated.

TABLE 133-2 -- Glycolipid Antigens of Platelets

Cardiolipin (CL)
Lactosyl ceramide
Glycosphingolipids (GSL)
Acidic
Sulfatides/gangliosides
Monogalactosyl sulfatide (16/6 idiotype)
Neutral
Globotriosyl ceramide
Globotetraosyl ceramide

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ALLOANTIGENS

The proliferation of serologically defined alloantigens on platelet glycoproteins has led to the development of a consensus nomenclature in which each of the alloantigens is prefixed by HPA-, for human platelet antigen.[33] These designations as well as the alternate (original) names are given in Table 133-3 . Unfortunately, the HPA nomenclature is not based on current knowledge of the linkage between several of these polymorphisms. As pointed out by Newman et al.,[34] [35] these polymorphisms really define a limited number of allelic variants (Table 133-4 (Table Not Available)), and it is important to understand the molecular basis for distinguishing the allelic variants of each immunogenic glycoprotein.

In the case of GPIb and integrin subunit $_{2}$, each is represented by two allelic variants. For $_{2}$, residue Glu_{505} defines the antigen HPA-5a, while Lys_{505} is defined as HPA-5b.[36] With GPIb, the Gly_{15} /Glu_{15} substitution gives rise to the antigens Iy^{b} and Iy^{a} , respectively.[37] The next highest degree of complexity is found with the integrin subunit $_{IIb}$. Three allelic variants of $_{IIb}$ have been defined that differ at either residue 837 (Val or Met) [38] or residue 843 (Ser or Ile).[39] When this kind of analysis is applied at the molecular level, it is apparent that the hypothetical variant Met_{837} , Ile_{843} does not exist. Consequently, the serologic phenotype HPA-3a, HPA-9b is not possible. For GPIb (the heavy chain), alloantigens HPA-2a and HPA-2b are distributed among five alleles that differ by the single amino acid replacement, Thr/Met, respectively, at residue 145 [40] and four polymorphic size variants, termed A, B, C, and D (ranging from largest to smallest), that are generated by differences in the number of tandem 13-amino acid repeats present in the macroglycopeptide portion of the molecule.[41] [42] The D isoform of GPIb contains one repeat, the C isoform contains two repeats, and so on. The most complex scenario occurs with the integrin subunit $_{3}$. Eight different alleles of the $_{3}$ can be distinguished that differ at six positions within the coding sequence. These dimorphic residues and their serologic designations are: Leu_{33} /Pro_{33} (HPA-1a/HPA-1b);[43] Arg_{62} /Gln_{62} (HPA-10wa/HPA-10wb);[44] Arg_{143} /Gln_{143} (HPA-4a/HPA-4b);[45] Pro_{407} /Ala_{407} (HPA-7a/HPA-7b);[46] Arg_{489} /Gln_{489} (HPA-6a/HPA-6b);[47] and Arg_{636} /Cys_{636} (HPA-8a/HPA-8b).[48] The eighth, rare HPA-1b allele characterized by the presence of Arg_{40} does not appear to be serologically distinguishable from the HPA-1b allele bearing the more common Leu_{40} .[49]

TABLE 133-3 -- Alloantigens Associated with Alloimmune Thrombocytopenia (NATP or PTP)

Alloantigen[a]	Platelet Glycoprotein	NATP	PTP	References
HPA-				
$_{IIb\ 3}$ Public				
1a	Pl^{A1} Zw^{a}	3	++	[94] [197] [198] [199] [200] [201] [202] [203] [204] [205] [206] [207]
1b	Pl^{A2} Zw^{b}	3	+	[94] [207] [208] [209] [210] [211] [212] [213] [214]
3a	Bak^{a} Lek^{a}	IIb	+	[94] [212] [214] [215] [216] [217] [218] [219]
3b	Bak^{b}	IIb	?	[207] [220] [221] [222]
4a	Pen^{a} Yuk^{b}	3	+	[206] [223] [224] [225] [226]
$_{IIb\ 3}$ Private				
4b	Pen^{b} Yuk^{a}	3	+	[227] [228]
6b	TU CA	3	+	[75] [76]
7b	Mo	3	+	[46]
8b	Sr^{a}	3	+	[229]
	Va^{a}	$_{IIb\ 3}$	+	[82]
	Gro^{a}	3	+	[80]
9b	Max^{a}	IIb	+	[38]
	Oe^{a}	3	+	[81]
10b	La^{a}	3	+	[44]
$_{2\ 1}$ Public				
5a	Br^{b} Zav^{b} Hc^{a}	2	+	? [230] [231] [232] [233] [234] [235]
5b	Br^{a} Zav^{a} Hc^{b}	2	++	+ [94] [236] [237] [238] [239] [240]
GPIb-IX-V Public				
2a	Ko^{b} Sib^{b}	Ib		[241]
2b	Ko^{a} Sib^{a}	Ib	+	? [61] [234] [241] [242] [243] [244] [245]
GPIb-IX-V Private				
	Iy^{a}	Ib-IX	+	[37]
CD109 Public				
	Gov^{a}	CD109	+	? [100] [101]
	Gov^{b}	CD109	+	[100] [101]

Abbreviations: NATP, neonatal alloimmune thrombocytopenia; PTP, post-transfusion purpura.

Adapted with permission from Warkentin and Smith.[196]

[a] Public, gene frequency > 0.02; private, gene frequency < 0.02 (Western populations).

TABLE 133-4 -- Alloantigenic Alleles of Platelet Membrane Glycoproteins

(Not Available)

Adapted with permission from Newman et al.[34] [35]

Immunochemistry of Platelet Alloantigens

HPA-1

The HPA-1 alloantigen system is defined by the Leu$_{33}$ /Pro$_{33}$ polymorphism that, according to the disulfide bonding scheme proposed by Calvete et al., [50] would be enclosed within a small 13-amino acid loop formed by the pairing of Cys$_{26}$ with Cys$_{38}$. This region of the molecule is held proximal to the distal cysteine-rich region in the middle of $_3$ by a long-range disulfide bond linking Cys$_5$ and Cys$_{435}$.[51] The importance of the three-dimensional structure imposed by the Cys$_{26}$ -Cys$_{38}$ disulfide bond constraint to expression of the HPA-1 determinants is indicated by the facts that linear peptides corresponding to the $_3$ (2638) sequence are not antigenic,[52] and the antigens are not lost following denaturation of $_3$ in ionic detergents. However, they are immediately destroyed on subsequent disulfide bond reduction. The complex structure of the $_3$ molecule and the sensitivity of the determinants to this structure are the likely explanation for the observed heterogeneity in binding properties of anti-HPA-1a alloantibodies. [53] [54] Although all alloantibodies would bind to the denatured molecule or to recombinant amino-terminal segments of the molecule expressed in *E. coli*,[55] [56] a subset of antibodies appear to require presentation of the antigenic loop within a more native environment, such as the nondenatured molecule.[53] [54]

Anti-HPA-1a antibodies inhibit clot retraction and platelet aggregationin the latter case, presumably because they block the binding of fibrinogen. [57] [58] Ryu et al.[59] have also reported that there is a dose-dependent stimulation versus inhibition of fibrinogen binding induced by anti-HPA-1a. A similar effect has been attributed to other platelet inhibitors, particularly the disintegrins, RGD peptides, and certain snake venoms. [60]

HPA-2

Two previously described polymorphisms of the Ib-IX-V complex, termed Ko and Sib, are now known to be reflections of two linked polymorphisms, one of which defines the diallelic system, HPA-2.[40] [61] [62] [63] [64] In the GPIb sequence, a Thr/Met polymorphism at residue 145 is associated with HPA-2a and HPA-2b epitopes, respectively (Fig. 133-4).

HPA-3

The HPA-3 system is associated with an Ile$_{843}$ /Ser$_{843}$ polymorphism of $_{IIb}$ (Fig. 133-5).[39] In addition, Take et al.[65] have reported that the binding of certain anti-HPA-3a antisera to $_{IIb}$ is decreased following desialation of $_{IIb}$, which raises the possibility that glycosylation of $_{IIb}$ may contribute to or influence the expression of the HPA-3 epitopes. Moreover, anti-HPA-3 alloantibodies do not bind to the precursor form of the $_{IIb}$ molecule, pro-$_{IIb}$.[39] Thus, O-glycosylation at the polymorphic Ser 843 may influence specificity or accessibility of HPA-3 determinants.

At this time, there has not yet appeared any report concerning the effect of antibodies specific for HPA-3a antigens on fibrinogen binding, platelet aggregation, or clot retraction. Because the $_{IIb}$ molecule is expressed only on platelets, megakaryocytes,

Figure 133-5 Schematic diagram of the $_{IIb}$ subunit. $_{IIb}$ is composed of two chains linked by a single interchain disulfide bond. The light chain is a transmembrane molecule. The open areas represent four putative divalent cation (calcium)-binding repeats, and the location of the binding site for the fibrinogen -chain carboxydodecapeptide is indicated by a large arrow. The positions of the polymorphisms that define the alloantigen systems HPA-3 and HPA-9 are indicated.

or cells with a megakaryocyte lineage, the HPA-3 epitopes are not found on other cell types.

HPA-4

Another diallelic human alloantigen system known as Pen (or Yuk) is found on $_3$ and is associated with an Arg$_{185}$ /Gln$_{186}$ polymorphism.[45] [58] [66] [67] Given the proximity of the Pen polymorphism to the RGD-binding domain (residues 109171) of $_3$, it is not surprising that anti-HPA-4a antibodies completely inhibit aggregation of HPA-4a homozygous platelets.[68]

Other cells that express $_3$ as the subunit of the vitronectin receptor, including endothelial cells, fibroblasts, and smooth muscle cells, also express HPA-1 and HPA-4 epitopes.[68] [69] [70] [71] This could contribute to the complexity of the clinical symptoms in alloimmune-mediated thrombocytopenia. At this time, little is known about the involvement of tissues other than platelets in these conditions.

HPA-5

The HPA-5 system is located on the integrin subunit $_2$ (Fig. 133-3).[72] [73] The detection of this system was facilitated by the development of a highly sensitive murine monoclonal antibody-based immobilization of platelet antigen (MAIPA) assay. [74] Like the preceding alloantigenic systems, the HPA-5 system is diallelic. Roughly 2002,000 copies of $_2$ are present on the surface of normal platelets, and each $_2$ molecule expresses a single HPA-5 epitope.[72] The integrin $_2$ is distributed on a wide variety of cells, but currently nothing is known about the antigenicity of the HPA-5 determinants on receptors expressed by cell types other than platelets.

HPA-6

This system, originally known as Ca or Tu, is defined by the $_3$ Arg$_{489}$ /Gln$_{489}$ polymorphism resulting from a G/A substitution at base 1564.[47] [75] [76]

HPA-7 and HPA-8

Two novel alloantigens, HPA-8b and HPA-7b, have recently been localized to $_3$.[46] [77] The alloantigen HPA-8b is classified as a private alloantigen because it appears to be inherited within a single family or family group but is not expressed by the general population. The unique feature of the HPA-8b polymorphism is that it is associated with an Arg$_{636}$ /Cys$_{636}$ substitution and thereby results in an additional unpaired cysteine residue. [77] Since its initial characterization, it has been accepted that all cysteine residues in $_3$ are involved in disulfide bridges. Despite the addition of this new sulfhydryl group, the HPA-8b-positive $_3$ subunit still associates with $_{IIb}$ and contributes to an expressed $_{IIb}$ $_3$ complex without apparent impairment of function. The alloantigen HPA-7b results from a C to G substitution at bp 1,267 of $_3$, resulting in replacement of Pro$_{407}$ by Ala$_{407}$.[46] HPA-7b is a very low-frequency alloantigen and has been detected in only one of 450 random donors outside of the birth family of the propositus.

HPA-9

The HPA-9 system, originally named Max, is the second system to be localized to the integrin $_{IIb}$ subunit. It is defined by the Val_{837} (Max^a)/Met_{837} (Max^b) polymorphism.[38]

HPA-10w

This system, initially named La, is defined by the $_3$ Arg_{62} /Gln_{62} polymorphism.[44]

Gov

The alloantigenic polymorphism Gov is uniquely expressed by a different protein receptor. Kelton et al. [78] initially described the Gov system, which is now known to be carried by the 175-kd glycosyl phosphatidylinositol-anchored glycoprotein CD109. Alloantibodies defining each of two alleles (Gov^a/Gov^b) were detected in two patients who had received multiple platelet transfusions [78] and, more recently, in three patients who had developed neonatal alloimmune thrombocytopenia. [79] The genotypic frequencies in the Canadian population are: for Gov^a, 81%; for Gov^b, 74%.

Additional Suspected Alloantigens

Five additional alloantigens have been serologically defined whose precise polymorphic residues or structures have not yet been identified (Table 133-3). Two are associated with the integrin $_3$ subunit: the private antigen Gro^a and the low-frequency antigen Oe^a. [80] [81] One additional low-frequency antigen, Va^a, is associated with the integrin $_{IIb\ 3}$, although it has not been determined which subunit bears the determinants. [82] The remaining two alloantigens are localized to the glycoprotein Ib-IX-V complex: Ly^a, found in one of 249 unrelated German donors and not yet precisely localized within the Ib-IX-V complex, [37] and PI^T, implicated in a single case of neonatal alloimmune thrombocytopenia, localized by Beardsley et al. [83] to glycoprotein V by immunoblot assay and expressed by platelets from all of 50 normal donors tested.

Neonatal Alloimmune Thrombocytopenia and Post-transfusion Purpura

Two clinically significant syndromes are the direct result of sensitization to platelet-specific alloantigens: neonatal alloimmune thrombocytopenia and post-transfusion purpura.

Neonatal Alloimmune Thrombocytopenia

Neonatal alloimmune thrombocytopenia is caused by maternal sensitization to paternal alloantigens on fetal platelets (Table 133-5). It is intriguing that only a fraction of those mothers negative for the platelet antigen in question deliver infants affected with neonatal alloimmune thrombocytopenia. For example, in the western world, responsiveness to HPA-1a is most commonly the cause of neonatal alloimmune thrombocytopenia, yet the frequency of homozygous HPA-1b mothers in the general caucasian population is 2%, and estimates of the incidence of neonatal alloimmune thrombocytopenia are no greater than 0.05%. A key to understanding this discrepancy lies in the finding that responsiveness to HPA-1a shows an HLA restriction. [84] [85] [86] HPA-1a and HPA-1b are defined by the Leu_{33} /$Pro_{33\ 3}$ polymorphism.[43] [50] [87] [88] [89] Individuals who are homozygous for Pro_{33} (homozygous HPA-1b) and responsive to the predominant HPA-1a antigen are almost exclusively HLA DRB3*0101[85] or DQB1*02. [90] [91] In the case of DRB3*0101, the calculated risk factor is 141, a risk level equivalent to that of the hallmark of HLA restriction in autoimmune disease, ankylosing spondylitis, and HLA-B27. [91] In contrast, responsiveness of homozygous HPA-1a individuals to the HPA-1b allele is not linked to HLA.[91] [92] T cells are the likely candidates for providing HLA restriction in this case, and Maslanka et al. [91] provided elegant evidence that in one case of neonatal alloimmune thrombocytopenia, T cells that share CDR3 motifs are stimulated by peptides that contain the same Leu $_{33}$ polymorphism that is recognized by anti-HPA-1a alloantibodies. In the case of another, less frequent antigen, HPA-6b, there appears to be an association between responsiveness and the MHC genes *HLA-DRB1*1501, HLA-DQA1*0102*, or *HLA-DQB1*0602*.[93]

TABLE 133-5 -- Alloimmune Thrombocytopenias

Neonatal autoimmune thrombocytopenia
Incidence: 1 per 3,000 in a retrospective study, 1 per 2,200 births in one prospective study.
Maternal antibodies produced against paternal antigens on fetal platelets.
Similar to erythroblastosis fetalis, except that 50% of cases occur during first pregnancy.
Most frequently implicated antigens are HPA-1a and HPA-5b (United States/Europe).
In the case of responsiveness to HPA-1a, there is a high-risk association with *HLA-DRB3*0101* or *HLA-DQB1*02.*
In the case of responsiveness to HPA-6b, there is an increased association with *HLA-DRB1*1501*, *HLA-DQA1*0102,* or *HLA-DQB1*0602*.
Post-transfusion purpura
Nearly all of the reported patients have been females previously sensitized by pregnancy or transfusion (<5% were males).
Thrombocytopenia usually occurs 1 week after transfusion.
Homozygous HPA-1b individuals account for a majority (>60%) of cases.
High-risk association with *HLA-DRB3*0101* or *HLA-DQB1*02.*
Enigmatically, the recipients antigen-negative platelets are destroyed by autologous antibody.

Responsiveness to HPA-1a is not the sole cause of neonatal alloimmune thrombocytopenia. In a large study of 348 cases of clinically suspected neonatal alloimmune thrombocytopenia,[94] 78% of serologically confirmed cases were due to anti-HPA-1a and 19% to anti-HPA-5b. All other specificities accounted for no more than 5% of cases. In reports from other laboratories, the association of neonatal alloimmune thrombocytopenia with other alloantigens, such as HPA-3a, HPA-3b, HPA-1b, or HPA-2b, was noted but is rare.[95] [96] [97] [98] Obviously, differences in allelic gene frequencies between different racial or ethnic populations will have an important impact on the frequency of responsiveness to a particular alloantigen. Table 133-6 summarizes some of the known variation in allelic gene frequencies between different world populations. Thus, in the Japanese population, anti-HPA-1a has never been shown to be involved in neonatal alloimmune thrombocytopenia, and antibodies specific for HPA-4b play a dominant clinical role. [99] This is probably because the gene frequency for the HPA-1b allele among the Japanese (0.02) is much lower than that found in Western populations (0.15). Conversely, the gene frequency of the HPA-4b allele in Japan (0.0083) is higher than that observed in Western populations (<0.001).

Post-transfusion Purpura

Post-transfusion purpura follows 710 days after an immunogenic blood (platelet) transfusion (Table 133-5). It most often affects previously nontransfused, multiparous women. As with neonatal alloimmune thrombocytopenia, the risk for developing post-transfusion purpura is increased among HLA-DR3-positive individuals, and HPA-1a is the antigen most often implicated (in Western populations). [84] [100]

The exact mechanism by which the recipients antigen-negative platelets are also cleared from the circulation in post-transfusion purpura is not yet fully understood.

Proposed mechanisms include the following: (1) During the first phase of post-transfusion purpura, the recipient develops antibodies that recognize framework determinants (conserved protein structures surrounding the specific polymorphic sites), and these react with each of the allelic forms of the antigen, resulting in autoantibody formation (see Chap. 11); (2) recipient antibodies form immune complexes with soluble antigens from donor platelets, and these interact with autologous platelets via an Fc receptor-dependent mechanism; and (3) soluble antigen from the transfused product is adsorbed onto recipient platelets, rendering them passively positive for the antigen in question. Platelet membrane microparticles are known to be a constituent of fresh frozen plasma and platelet concentrates.[101] It is conceivable that the $_{IIb\ 3}$ complex could become adsorbed onto neighboring platelets via this process.

HPA-1b platelets had been reported to become HPA-1a-positive when incubated with plasma from HPA-1a-positive individuals. [102] [103] [104] Although this passive transfer of soluble antigen has been proposed as a mechanism for clearance of the recipient platelets in post-transfusion purpura, Ehmann et al. [105] contend that this is an in vitro artifact. These authors provided evidence in patients with post-transfusion purpura for the presence of immune complexes composed of donor antigen and recipient antibody. We have found that centrifugation, even at forces exceeding 10,000 times g, fails to remove antigen-positive material (residual platelets and/or fragments) from plasma preparations. However, clearance of antigen-positive material is reliably accomplished by the passage of such plasma through a 0.45-m filter (D. Warejka, R.H. Aster, and T.J. Kunicki, unpublished observations). These findings support the contention of Ehmann et al. that antigen transfer using nonfiltered plasmas is an in vitro artifact.

At this time, there remains no conclusive evidence to support

TABLE 133-6 -- Gene Frequencies of the Major Alloantigenic Alleles in Various World Populations

HPA-	Western	Japan	Amerindian	Black	Korean	African American	Finnish	Thai
1a	0.85	0.998	>0.993	0.885	0.995		0.86	>0.998
1b	0.15	0.002	<0.007	0.115	0.005		0.14	<0.002
2a	0.93		0.058	0.852		0.82	0.91	0.917
2b	0.07		0.042	0.148		0.18	0.09	0.083
3a	0.61						0.59	0.37
3b	0.39						0.41	0.63
4a	>0.99	0.989						0.991
4b	<0.01	0.011						0.009
5a	0.89					0.79	0.95	0.973
5b	0.11					0.21	0.05	0.027

Data from references , [34] , [196] , [246] (Western), [247] (Japanese), [248] (Amerindian), [249] (black), [249] (Korean), [249] (African American), [250] (Finnish), and [251] (Thai).

or refute any one of the proposed mechanisms for the pathology of post-transfusion purpura. See Chapters 11 and 126 for further discussion of neonatal alloimmune thrombocytopenia and post-transfusion purpura.

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ISOANTIGENS

Isoantibodies are produced against an epitope that is expressed by all normal individuals and is not polymorphic. In the area of human platelet immunology, a classic example of isoimmunization can and does occur when a patient with an inherited deficiency of a membrane glycoprotein has received multiple platelet transfusions to correct a bleeding diathesis. Glanzmann thrombasthenia and Bernard-Soulier syndrome are examples of inherited disorders in which the individual either lacks or expresses an altered form of $_{IIb\ 3}$ (Glanzmann thrombasthenia) or Ib-IX-V (Bernard-Soulier syndrome), respectively. Isoantibodies developed by transfused patients do not distinguish any of the allelic forms of the glycoproteins (such as HPA-3 or HPA-1 alloantigens on $_{IIb\ 3}$) but react with the platelets of all normal persons tested. Because the propositus does not express the platelet glycoprotein that carries the epitope in question, these antibodies do not bind to their own platelets.

Isoantibodies in Glanzmann Thrombasthenia

Several cases have been documented in which patients with Glanzmann thrombasthenia produced antibodies specific for $_{IIb\ ,\ 3}$, or the $_{IIb\ 3}$ complex.[106] [107] [108] [109] [110] Recently, we defined an idiotype (OG) that is associated at high frequency with isoantibodies specific for integrin subunit $_{3}$ generated by patients with Glanzmann thrombasthenia.[108] [111] [112] [113] Patient O.G. suffered from persistent, often serious bleeding episodes as a result of both his Glanzmann thrombasthenia phenotype and the fact that he had generated a very high-titered, IgG isoantibody inhibitor of platelet cohesion. [108] [113] The study of the OG idiotype presented an excellent opportunity to compare and contrast disease-related, $_{IIb\ 3}$ inhibitors of human origin with other natural or synthetic inhibitors of this integrin receptor. Rabbit polyclonal anti-OG idiotype binds to IgG specific for $_{IIb\ 3}$ obtained from 11 nonrelated patients with Glanzmann thrombasthenia, including an unrelated patient, E.S., studied extensively by Coller et al., [107] who had developed isoantibodies of very similar specificity. On the other hand, anti-OG does not recognize $_{IIb\ 3}$ -specific antibodies produced by other unrelated patients with Glanzmann thrombasthenia, who had developed isoantibodies with specificities distinct from that of the OG isoantibody. Moreover, anti-OG does not recognize $_{IIb\ 3}$ -specific antibodies developed by any patients with idiopathic thrombocytopenic purpura or six representative patients with alloimmune thrombocytopenias, and anti-OG never binds to IgG from any of several nonimmunized control individuals.

Anti-OG binds to selected protein ligands of $_{IIb\ 3}$, namely, fibrinogen, vitronectin, and vWF, but not to other known protein ligands, such as fibronectin or type I collagen. The epitope(s) recognized by anti-OG on these three adhesive proteins are either very similar or identical, since each protein can inhibit the binding of anti-OG to any of the others. The epitope on fibrinogen recognized by anti-OG resides in the B chain and is likely contained within the first 42 amino acids from the amino-terminus.[114] Because OG IgG inhibits fibrinogen binding to $_{IIb\ 3}$, the specificity of the OG idiotype defines a novel binding motif for the integrin $_{IIb\ 3}$ that is shared by fibrinogen, vitronectin, and vWF but is distinct from previously described RGD-containing sites on the fibrinogen A chain or the fibrinogen -chain carboxyl-terminal decapeptide site. Moskowitz et al. [114] employed specific proteolytic forms of fibrinogen to confirm that the epitope recognized by anti-OG-2 is located within B 142. This represents an excellent example of molecular mimicry in which an antigen-selected, IgG inhibitor of $_{IIb\ 3}$ function shares a novel recognition sequence common to three physiologic protein ligands of that receptor.

Isoantibodies in Bernard-Soulier Syndrome

Because Bernard-Soulier syndrome is less frequently encountered than Glanzmann thrombasthenia, the isoantibodies produced in conjunction with this syndrome are also less frequently encountered. In the only clear case of such an isoantibody, [115] the isolated IgG impaired both normal platelet adhesion to subendothelial elements and in vitro aggregation in response to ristocetin and bovine factor VIII.

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AUTOANTIGENS

Autoimmune (or idiopathic) thrombocytopenic purpura (AITP or ITP) is the most frequently encountered form of immune thrombocytopenia. [116] [117] This disorder can be classified as acute or chronic on the basis of the duration of the thrombocytopenia, the chronic form persisting >612 months (see Chap. 126). The acute, self-limiting form occurs predominantly in children, often following a viral illness or immunization, and affects males and females with equal frequency. The chronic form is mainly an adult illness and affects twice as many females as males. Life-threatening bleeding occurs in up to 1% of patients with ITP. The reason why some patients sustain severe hemorrhagic complications and others do not remains unexplained, but because of differences seen in the clinical expression of chronic and acute ITP, it has been theorized that the mechanisms of disease for each form are probably different.

Glycoproteins as Autoantigens

The integrin $_{IIb\ 3}$ was the first platelet membrane component to be identified as a dominant antigen in chronic ITP, [118] and subsequent studies from several laboratories have confirmed the important contribution of this receptor to the autoantigenic makeup of the human platelet. [96] [119] [120] [121] [122] [123] [124]

Attempts to further localize autoepitopes on either integrin subunit have been more successful with regard to $_{3}$. Early on, Kekomaki et al. [124] defined a prominent autoantigenic region as the 33-kd chymotryptic fragment of $_{3}$ located within the cysteine-rich region of $_{3}$ (Fig. 133-6) that was bound by both plasma autoantibodies and autoantibody eluted from patients platelets. Fujisawa et al. [125] [126] [127] subsequently determined that plasma autoantibodies in five of 13 patients with chronic ITP bound to peptides representing $_{3}$ residues 721744 or 742762, namely, the carboxy-terminal region of $_{3}$ that is presumed to be located in the cytoplasm of the platelet, while autoantibodies eluted from the platelets of additional ITP patients bound to other areas of $_{3}$ within the extracellular portion of the molecule (perhaps identical to the 33-kd region defined by Kekomaki et al. [124]). Most important, they determined that certain platelet-associated autoantibodies bind preferentially to cation-dependent conformational antigens on the $_{IIb\ 3}$ complex. [127]

More recently, additional autoantigenic epitopes have been identified on the $_{3}$ subunit. Nardi et al. [128] identified the peptide $_{3}$ (4966) as a site that is bound by a majority of affinity-purified antibodies isolated from serum immune complexes of immunologic thrombocytopenic HIV-1-infected patients. This is not a peptide that is bound by serum IgG antibodies from control subjects or patients with the classic form of ITP. Using random peptide phage display libraries, Bowditch et al. [129] determined that plasma antibody eluates from one ITP patient bound to two distinct hexapeptides, and the binding to one of these could be inhibited by the sequence $_{3}$ (734739).

Figure 133-6 Schematic diagram of the $_{3}$ subunit. The $_{3}$ subunit is a single-chain transmembrane molecule. Twenty-eight intrachain disulfide bonds are present, at least one of which is thought to bring the amino-terminal domain (N) proximal to the cysteine-rich domain at the carboxy (C) third of the molecule. The approximate positions of RGD- and fibrinogen (Fbg)-binding sites are indicated. The locations of the polymorphisms that give rise to alloantigen systems HPA-1, HPA-4, HPA-6, HPA-7, HPA-8, and HPA-10w are indicated.

Autoantibodies reactive with $_{IIb}$ were identified in two patients with chronic ITP, [122] [123] and in one of these patients the antibody was subsequently shown to react with a chymotryptic, 65-kd COOH-terminal fragment of the $_{IIb}$ heavy chain (Fig. 133-5). [123] Ethylenediaminetetraacetic acid (EDTA)-dependent autoantibodies represent a special category that are adsorbed by autologous platelets when whole blood is drawn in EDTA. [130] In one such case of EDTA-dependent pseudothrombocytopenia, an IgM antibody was shown to bind to $_{IIb}$ by immunoblot assay and crossed immunoelectrophoresis. [131] As a final note, it should be remembered that, although most autoantibodies apparently induce thrombocytopenia, a minority can induce platelet dysfunction without an increase in platelet clearance (see Chap. 126). [132] [133] The proportion of the two types of autoantibodyone leading to platelet clearance, one blocking platelet functionmay be an important factor controlling the unpredictable clinical severity of ITP.

$_{IIb\ 3}$ is not the only integrin implicated as the antigen target for human autoantibodies. Serum IgG autoantibodies specific for integrin subunit $_{2}$ were identified in a unique case of autoimmune platelet dysfunction following myasthenia gravis. [134] This autoantibody inhibited aggregation of normal platelets induced by collagen or wheat germ agglutinin. This is the first case in which autoantibodies to Ia were associated with a chronic hemorrhagic disorder, and this study provides strong indirect support for a role of this integrin in hemostasis in vivo.

Autoantibodies to components of the Ib-IX-V complex are also frequently encountered in adult chronic ITP. [135] He et al. [136] have made progress in the localization of selected autoantigenic epitopes on the glycoprotein Ib molecule. Epitopes were most frequently found on a recombinant fragment of Ib corresponding to residues 240485, and next most often on a fragment representing residues 1247. In the case of those antibodies reactive with the former sequence, further epitope mapping identified the dominant determinant as the 9-amino acid sequence TKEQTTFPP (residues 333341). [136] In some cases in which autoantibody to Ib-IX was detected, the clinical presentation proved to be particularly severe and refractory to therapy. [137] One case of pseudo-Bernard-Soulier syndrome (dysfunction of the Ib-IX-V receptor complex) was reported to be caused by an autoantibody to Ib. [138] Finally, in a subset of childhood ITP, that associated with varicella zoster infection, the glycoprotein V component of this receptor was found to be the dominant target of serum autoantibodies that do not cross-react with viral antigens. [139] At the same time, in another group of children with this disease, it was found that serum antibodies specific for viral antigens can cross-react with normal platelet antigens and thus may contribute to platelet clearance. [140]

It had been hoped that the antigenic targets in acute and chronic forms of ITP might be different so that antigen identity might one day be used as an early indicator of clinical outcome. Subsequent studies have shed more light on this issue and have demonstrated that autoantibody specificity in chronic and acute ITP is quite similar, particularly in children. [141] [142] Nonetheless, a distinction between antigen specificity and an acute versus chronic course in ITP may yet be found in the early stages of the autoimmune response. Prospective studies aimed at answering this question are warranted.

How large is the autoepitope repertoire on a given glycoprotein antigen? The answer to this important question will have an impact on the feasibility of developing therapeutic and diagnostic measures based on epitope specificity. Because the autoantibodies that react with a given epitope are likely to share idiotypes, one can approach this question from two angles, analyzing the epitope repertoire, the idiotype repertoire, or both. Two studies have addressed the extent of the autoantigen repertoire on $_{IIb\ 3}$ by analyzing the competitive binding between human autoantibodies and murine monoclonal antibodies. [143] [144] However, the limited number of studies that have used this approach have generated conflicting results, and there remain insufficient data to judge the size of the autoepitope repertoire on $_{IIb\ 3}$. Additional analyses aimed at epitope localization will be necessary, and perhaps novel approaches will expedite this task. One such novel approach is the

development of human monoclonal autoantibodies specific for $_{IIb\ 3}$ and other platelet glycoproteins.

Human monoclonal antibodies are an alternative tool in the search for glycoprotein epitopes that are autoimmunogenic in humans. The first human monoclonal antibody against a platelet glycoprotein was developed by Nugent et al., [145] derived from an individual with ITP who produced an antibody against $_{3}$. This human monoclonal antibody detects a neoantigen associated with $_{3}$ that is expressed only on stored or thrombin-activated platelets. A number of human monoclonal autoantibodies specific for the heavy chain of GPIb were generated from the lymphocytes of an ITP patient with serum autoantibody specific for Ib. [146] The heavy chain variable region genes of four of these antibodies have been sequenced and found to be markedly homologous to human immunoglobulin, germ-line, heavy chain variable region genes. [146] Most recently, another human monoclonal autoantibody was produced that is specific for the heavy chain of $_{IIb}$.[147] The epitope recognized by this antibody (2E7) has been identified as a contiguous amino acid sequence with residues 231238 with an immunodominant tryptophan residue at position 235 (Fig. 133-5). [148] This is the first time that the precise epitope on $_{IIb}$ or $_{3}$ recognized by a human antibody has been identified.

From the foregoing analysis, it is clear that further studies are required to determine the extent to which the production of human autoantibodies to platelet glycoproteins is clonally restricted. Given a selected number of idiotypes (Ids) related to autoimmunity to $_{IIb\ 3}$, one could potentially use the anti-idiotype (anti-Id) to modulate immunization to $_{IIb\ 3}$. Along these lines, it has been reported that intravenous immune globulin G (IVIgG), which is routinely used to reverse acute thrombocytopenia in ITP, may contain anti-Id directed to idiotypes of autoantibody but not alloantibodies that recognize $_{IIb\ 3}$.[149] Recently, Nugent et al.[137] defined a DM idiotype that is characteristic of human autoantibodies that are specific for the Ib heavy chain. The latter study clearly suggests that the repertoire

of idiotypes expressed by human autoantibodies specific for membrane glycoproteins, such as those of the human platelet, will be narrowly defined and, thus, amenable to study.

Glycolipids as Antigens

A number of reports have implicated cardiolipin, lactosyl ceramide and other glycosphingolipids as autoantigenic targets (Table 133-2).[150] [151] [152] [153] [154] [155] [156] van Vliet et al.[154] analyzed the binding of serum IgG/IgM antibodies from 30 patients with ITP to platelet glycosphingolipids separated by high-performance thin-layer chromatography (HPTLC). Acidic glycosphingolipids, namely sulfatides and gangliosides, were identified as the major targets of serum autoantibodies. Thirteen of the 30 sera, five with anticardiolipin antibodies, had antibodies that bound to sulfatides, while four sera showed antibody binding to gangliosides. Koerner et al. [150] employed a more efficient phase partition separation of acidic glycosphingolipids from neutral glycosphingolipids and were able to demonstrate that serum antibodies specific for neutral glycosphingolipids were more characteristic of ITP. Two classes of glycosphingolipid autoantigens were defined: those associated with general autoimmunity and detected in the sera of patients with either systemic lupus erythematosus (SLE) or ITP, and those peculiar to platelet-specific autoimmunity and detected only in the sera of ITP patients. Two glycosphingolipid forms belong to the platelet-specific group, but they are present at minute levels, and further characterization awaits large-scale purification. One-half (6/12) of patients with ITP had serum IgG or IgM antibodies that bound these platelet-specific glycosphingolipids. Sera from none of ten patients with nonimmune thrombocytopenia, none of ten patients with SLE, and only one of 18 normal subjects gave positive reactions with the platelet-specific glycosphingolipid group. The general glycosphingolipid antigen group includes globotriaosyl ceramide, globotetraosyl ceramide, and a third unidentified neutral glycosphingolipid. Antigens in the general group were bound by IgG or IgM antibodies in the sera of ten of ten patients with SLE, eight of 12 patients with ITP, and none of ten patients with nonimmune thrombocytopenia or 18 control subjects. These findings provide compelling support for a role of neutral glycosphingolipids as antigenic targets in selected cases of ITP.

Animal Models of ITP

Many investigators have searched in animals for clinical correlates of human ITP. These attempts have been largely unsuccessful, but an exception may have recently emerged from concerted studies in dogs. As pointed out in a recent series of reports by Lewis and Meyers, [157] there is now substantial evidence that ITP in dogs and ITP in humans are clinically analogous syndromes. In 32 cases of canine ITP, increased platelet-bound IgG was detected in a majority of the animals (30), and Ig eluted from the platelets of 11 of 19 affected dogs bound to homologous normal canine platelets. [157] Furthermore, Ig specific for integrin subunits $_{IIb}$ and/or $_{3}$ was detected in sera of four of 17 dogs tested. Lewis and Meyers concluded that, in canine ITP, Ig bound to the surface of platelets is directed against host antigens and that the target antigen is frequently $_{IIb\ 3}$. Given the similarities between these findings and the cumulative experience with human ITP, the canine model may prove to be a valuable tool to further understand the pathogenesis of the autoimmune response to platelet antigens.

Quinine/Quinidine Purpura

Although drug-induced thrombocytopenic purpura (DITP) may be a complication of therapy with a variety of drugs, it is most frequently seen in the United States with the administration of quinine and quinidine. [158] It has been proposed that the following criteria be met before an individual can be considered to have DITP: (1) the patient is not thrombocytopenic prior to administration of the drug; (2) thrombocytopenia follows drug ingestion and begins to reverse shortly after cessation of drug; (3) thrombocytopenia does not reoccur after cessation of drug treatment; and (4) all other causes of thrombocytopenia are ruled out. [159]

The exact mechanism for platelet clearance is not yet certain. However, cumulative evidence now favors a mechanism whereby the drug induces the expression of a neoantigen on the platelet surface[160] [161] [162] [163] [164] [165] that is recognized by circulating antibodies only in the presence of the drug. The observation that platelets from patients with Bernard-Soulier syndrome (lacking Ib-IX-V) failed to lyse in the presence of drug-dependent antibody, specific drug, and complement was the first indication that a specific platelet antigen is recognized by such antibodies. [165] This finding led other laboratories to confirm that purified Ib-IX would compete for drug plus antibody and was therefore likely to contain the antigenic epitope in question. Evidence of direct binding of such antibodies to Ib-IX was first provided by Chong et al.,[163] and Berndt et al.[162] established that the complex of Ib and IX is likely required for maximum antigen expression. Whereas it has been confirmed that Ib-IX-V is a major antigen in DITP,[165] [166] [167] [168] there is now ample evidence that the integrin $_{IIb\ 3}$ is also a dominant target for many quinine- and quinidine-dependent antibodies.[169] [170] Continued study of the drug-dependent autoimmune phenomena and their relationship to platelets is warranted, with particular attention to a comparison of the clinical significance of drug-dependent autoantibodies that bind to either Ib-IX or $_{IIb\ 3}$.

In order to understand the mechanism whereby drugs like quinine or quinidine induce neoantigen formation, one must first consider the direct effects of these drugs on platelet membrane components, a subject that has not received enough attention by those interested in DITP. Deykin and Hellerstein [171] were probably the first to show that quinidine inhibits in vitro platelet aggregation induced by adenosine diphosphate (ADP), collagen, or adrenaline. Lawson et al. [172] demonstrated that quinidine administration will induce the prolongation of the bleeding time without thrombocytopenia. Connellan et al. [173] extended these findings and showed that quinine, both ex vivo and in vitro, will inhibit platelet aggregation induced by weak agonists like ADP or adrenaline. Aggregation by strong agonists, for example, collagen, thrombin, or arachidonate, was inhibited only in vitro. The combination of ddAb and quinine inhibited the binding of two monoclonal antibodies, HuPlm1, specific for $_{3}$, and FMC25, specific for GPIX. On the other hand, the binding of another antibody, AN51, specific for GPIb, was enhanced. The studies clearly point to a general platelet dysfunction that can be attributed to exposure to these drugs. That this dysfunction should be the result of perturbations of specific membrane glycoprotein receptors is not surprising. Nor should it be surprising that one effect of such perturbations might be the development of neoantigens or the exposure of cryptic antigens.

In 12 patients with DITP, Chong et al.[170] used a panel of murine monoclonal antibodies in competitive binding assays to map the domains on Ib-IX-V that are bound by drug-dependent antibodies. The combined data showed that one quinine-dependent antibody binds to an epitope on the amino-terminal portion of Ib and five other quinine-dependent antibodies recognize a complex-specific epitope proximal to the membrane-associated region of Ib-IX. Each of six quinidine-dependent antibodies contained two specificities, one for the same Ib-IX complex epitope described above, the other for IX alone. Additional observations were that antibodies reactive with Ib-IX are more predominant (12/12 patients) than those that bind $_{IIb\ 3}$ (3/12 patients) and that antibodies specific for Ib-IX-V are present in titers 8- to 32-fold higher than the corresponding antibodies

that bind to IIb 3 in the same patient samples. In each case, those antibodies that bound to Ib-IX-V were distinct from those that recognized IIb 3 . The specificity of quinine- and quinidine-dependent antibodies for a conformation-sensitive epitope(s) on the GPIX component of the complex was confirmed by Lopez et al. [174] using monoclonal antibody inhibition assays.

Regions of IIb 3 that bind to quinine- or quinidine-dependent antibodies have also been further localized by Visentin et al. [175] Of 13 patient sera containing such antibodies, ten were reactive with both Ib-IX and IIb 3 , two reacted with Ib-IX alone, and one reacted with IIb 3 alone. Again, in those sera where both specificities were identified, the anti-Ib-IX-V antibodies were distinct from those that bound to IIb 3 . Seven sera containing anti- IIb 3 antibodies were further characterized: three bound only to the IIb 3 complex, one bound to IIb alone, and three bound to 3 alone. Those that recognized 3 alone were found to bind to epitopes on the major 61-kd chymotryptic fragment of 3 that are resistant to deglycosylation with endo-H. In the case of sulfonamide-induced immune thrombocytopenia, [176] the causative antibodies are almost exclusively specific for calcium-dependent (complex-specific) epitopes on the integrin IIb 3 .

One additional intriguing aspect of certain cases of DITP is worthy of mention, because it may have an important bearing on our understanding of the autoimmune response to platelets in general. Based on anecdotal evidence, it has been suspected that some cases of chronic ITP are initiated in clinical situations that, from all appearances, could be classified as DITP. [135] The only difference is that selected autoantibodies persist long after exposure to the insulting drug. Direct evidence to support of this contention was obtained by Nieminen and Kekomaki. [177] They reported that the DITP patients with Ib-IX specific antibodies, despite a very intense and acute thrombocytopenia, recovered promptly after drug removal. On the other hand, DITP patients with more prolonged thrombocytopenia and persistently elevated platelet Ig levels >1 month after drug removal had antibodies that reacted with additional target antigens, including integrin IIb 3 . To better understand the pathogenesis of both DITP and classic ITP, in the future it may become important to distinguish the acute but readily reversible clinical situation that we accept as DITP from the more complex disease situation that may be initiated by drug exposure but evolve into a more classic form of ITP.

Heparin-Associated Thrombocytopenia

Heparin-associated immune thrombocytopenia (HAT) is a life-threatening condition that results in thrombocytopenia and can be associated with thrombosis. [178] [179] [180] [181] Unlike quinine- or quinidine-dependent antibodies, the actual binding of heparin-dependent antibodies (HDAs) to the platelet surface appears to be of very low affinity and was difficult to demonstrate in earlier studies until the report of Lynch and Howe. [178] It was also determined that HDAs differ from other forms of drug-dependent antibodies in that they can often be *activating*, causing not only thrombocytopenia but also heparin-dependent platelet aggregation, thromboxane synthesis, and granule release that can be quantitated by preloading platelets with [^{14} C]serotonin. The consequences of HAT therefore are multiplied by often serious thrombotic complications. Approximately 30% of these patients die, with an additional 20% developing vascular occlusions that result in gangrene and subsequent amputation.[182]

Although the precise mechanism of HAD binding to platelets eluded investigators for many years, it was generally accepted that the activating properties of HAD must be mediated by Fc-dependent binding to platelets. In this regard, Kelton et al. [179] showed that the platelet release reaction induced by HDA could be blocked by pretreating platelets with human or goat IgG Fc fragments, Adelman et al. showed that the Fab regions of HDA alone are not sufficient to cause platelet activation, [183] Chong et al. [184] showed that purified rabbit IgG and its Fc, but not Fab, fragments markedly inhibited platelet aggregation induced by HDAs, and a number of groups observed that the monoclonal anti-Fc receptor antibody, IV.3, could block platelet activation by HDA. [179] [185]

Subsequent findings from several study groups have led to the consensus that the dominant pathologic factor in HAT is circulating antibody specific for a complex of heparin (H) and platelet factor 4 (PF4). [186] [187] [188] [189] [190] It is now accepted that H-PF4 complexes, which are detected in 85% of patients with HAT, [191] can bind to the membrane of platelets[187] [192] [193] or endothelial cells,[187] leading directly to thrombocytopenia in the former case. In those cases where the HDAs are IgG, the Fc portion can cross-link to the platelet FcRIIA receptor, inducing platelet activation, release, aggregation, and thrombosis. [187] [189] [190] However, HAT can also be associated with antibodies of IgM and IgA isotypes, [194] in which case the Fc()R of lymphocytes or the Fc()R of monocytes and neutrophils, as well as complement activation, may play a role in the pathology of this disorder.

Thus, it is now generally accepted that one of the major stimuli for the thrombosis associated with HAT is the Fc receptor-mediated immune complex-dependent activation of platelets. In support of this theory, Warkentin et al. [181] have pointed out that this likely involves and is complicated by the propensity of these immune complexes to generate procoagulant platelet-derived microparticles.

Alternative protein targets may be involved in selected cases of HAT. For example, in nine of 15 patients with HAT who lacked detectable antibodies to H-PF4 complexes, Amiral et al.[195] found evidence of autoantibodies specific for the chemokines neutrophil-activating peptide-2 (NAP2) or interleukin-8 (IL-8). PF4 is 60% homologous to NAP2 and 40% homologous to IL-8, leading to the proposal that HAT involving these proteins could proceed along a mechanism similar to that invoked for PF4.

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Chapter 134 - HLA and Neutrophil Antigen and Antibody Systems

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INTRODUCTION

This chapter reviews human leukocyte antigen (HLA) and neutrophil-specific antigens and antibodies. The structure and function of the HLA genetic complex and some important clinical applications of HLA testing are discussed. Analysis of HLA gene products is used in four clinical settings: (1) to select compatible donor-recipient pairs for organ and bone marrow transplantation, (2) to select HLA-compatible single donor platelet products for thrombocytopenic patients who are refractory to random pooled platelets, (3) to analyze genetic factors that contribute to the expression of HLA-associated diseases, and (4) to resolve parentage disputes or forensic issues. Only HLA testing in the settings of organ transplantation and blood transfusion is discussed in this chapter. Neutrophil-specific alloantigen systems are reviewed and clinical neutropenic disorders associated with the production of specific antibodies induced through alloimmune, isoimmune, or autoimmune mechanisms are discussed.

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HLA ANTIGENS AND ANTIBODIES

The HLA genetic system is the major histocompatibility complex (MHC) in humans. MHC gene products were initially defined in mice as transplantation antigens; [1] for several years, they were of interest primarily to transplantation immunologists. In the mid-1960s, two lines of evidence suggested that MHC genes might participate more broadly in immune responses. First, Lilly et al. [2] discovered that genetic susceptibility of inbred mice to Gross virus-induced leukemia was linked to the murine MHC, H-2. This observation was the basis for a major survey of HLA and disease associations that began in 1967 and has continued to the present time. [3] Second, certain inbred mouse strains were observed to respond poorly or not at all to selected simple antigens. [4] Studies demonstrated that the capacity to respond to specific antigens was genetically determined by factors linked to the H-2 complex, [5] giving rise to the concept of immune response (Ir) genes. In 1974, Zinkernagel and Dougherty identified the critical role of MHC genes in immune responses by demonstrating that antigen-primed T cells recognized a foreign antigen only when it was presented on cells sharing the same MHC antigens as the initial antigen-presenting cell. [6] This phenomenon became known as MHC restriction. The investigators shared the 1996 Nobel prize in medicine for this important observation. Finally, MHC molecules were found to direct the educational process, occurring in the thymus, that ultimately determines the repertoire of expressed T-cell receptors. [7] MHC studies now are an integral component of immunobiology. MHC genes, together with the B-cell and T-cell receptor genes, form a major triad of gene families responsible for genetic control of immune responses.

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HLA NOMENCLATURE

HLA nomenclature in the 1950s consisted of multiple, locally formulated assignments. As the field matured and developed structure, a need for uniform nomenclature became apparent. In 1967, an HLA nomenclature committee, formed under the auspices of the World Health Organization (WHO), received responsibility to develop and update HLA nomenclature.[8] Major nomenclature revisions were made in 1967, 1975, 1984, and 1987. Currently, two nomenclature systems are used: one based on immunologically defined epitopes using either serologic or cellular techniques, and a second based on defined nucleotide sequences of allelic genes.

Immunologically Defined HLA Nomenclature

The current immunologically defined nomenclature, implemented in 1975 and modified in 1984, is based on the following principles: *HLA* followed by a hyphen designates the MHC, capital letters (e.g., *A, B, C, DR, DQ, DP*) designate segregant series, and a numeric designates a specific allele. Numerics for each locus, with the exception of the A and B loci, start with the number 1.[9] HLA-A and -B alleles are still considered as a single group for sequential numbering of new specificities. When the nomenclature was first developed, HLA-A and -B locus alleles were thought to be encoded by a single locus. Provisional allelic specificities originally were preceded by a *w*, but the letter is no longer used for that purpose. However, the *w* is used in four other circumstances. To prevent possible confusion between HLA-C locus alleles and complement components, the *w* prefix in all C locus alleles is permanently retained. DP and Dw alleles also retain a *w* to indicate that both series were originally defined by cellular techniques.[10] Finally, the public specificities, Bw4 and Bw6, retain the *w* to indicate the specificities are epitopes rather than allelic gene products.

A minor modification has been made since 1987. New serologically defined variant specificities that detect a single gene product of known nucleotide sequence receive an extended numeric that coincides with the molecular nomenclature of the allele.[11] For example, a unique serologic specificity that detects the class I gene product HLA-A*0203 is called HLA-A203.

Sequence-Defined Allelic Nomenclature

An additional nomenclature, based on nucleotide sequences, was recommended during the Tenth International Histocompatibility Workshop in 1987.[11] Refined gene mapping and nucleotide sequence data revealed that more than one locus could code for alternate protein chains in D region molecules. Further, many HLA allelic variants that are not detectable by traditional serologic techniques were discovered. This complexity necessitated development of the following nomenclature for HLA genes: (1) HLA designates the MHC, and (2) a series of capital letters, with or without a numeric, designates a specific locus (Table 134-1). Specific alleles (Table 134-2) are designated by an asterisk (*), followed by a two-digit number indicating the most closely associated serologic specificity, followed by a two-digit number defining the allele. For example, there currently are 25 officially recognized molecular variants of the serologically defined specificity HLA-A2. They are designated as HLA-A*0201 through 0225. Similarly, 24 molecular variants of HLA-DR4 are called HLA-DRB1*0401 through *0424. When a fifth digit appears in the nomenclature (e.g., *11041 and *11042), it identifies two alleles differing only by a single nucleotide that does not change the encoded amino acid. Occasionally, the capital letter N or L, with or without a preceding 3-digit number, such as

TABLE 134-1 -- Names for Class I and II Genes

Name	Previous Equivalents	Molecular Characteristics
HLA-A		Class I -chain
HLA-B		Class I -chain
HLA-C		Class I -chain
HLA-E	E, 6.2	Associated with class I 6.2-kb Hind III fragment
HLA-F	F, 5.4	Associated with class I 5.4-kb Hind III fragment
HLA-G	G, 6.0	Associated with class I 6.0-kb Hind III fragment
HLA-H	H, AR, 12.4	Class I pseudogene associated with 5.4-kb Hind III fragment
HLA-J	cda12	Class I pseudogene associated with 5.9-kb Hind III fragment
HLA-K	HLA-70	Class I pseudogene associated with 7.0-kb Hind III fragment
HLA-L	HLA-92	Class I pseudogene associated with 9.2-kb Hind III fragment
HLA-DRA	DR	DR -chain
HLA-DRB1	DRI, DR1B	DR_1 -chain determining specificities DR1, DR2, DR3, DR4, DR5, etc.
HLA-DRB2	DRII	Pseudogene with DR -like sequences
HLA-DRB3	DRIII, DR3B	DR_3 -chain determining DR52 and Dw24, Dw25, Dw26 specificities
HLA-DRB4	DRIV, DR4B	DR_4 -chain determining DR53
HLA-DRB5	DRIII	DR_5 -chain determining DR51
HLA-DRB6	DRBX, DRB	DRB pseudogene found on DR1, DR2, and DR10 haplotypes
HLA-DRB7	DRB1	DRB pseudogene found on DR4, DR7, and DR9 haplotypes
HLA-DRB8	DRB2	DRB pseudogene found on DR4, DR7, and DR9 haplotypes
HLA-DRB9	M4.2 exon	DRB pseudogene, isolated fragment
HLA-DQA1	DQ1, DQ1A	DQ -chain as expressed

HLA-DQB1	DQ1, DQ1B	DQ -chain as expressed
HLA-DQA2	DX, DQ2A	DQ -chain-related sequence, not known to be expressed
HLA-DQB2	DX, DQ2B	DQ -chain-related sequence, not known to be expressed
HLA-DQB3	DV, DQB3	DQ -chain-related sequence, not known to be expressed
HLA-DOB	DO	DO -chain
HLA-DMA	RING6	DM -chain
HLA-DMB	RING7	DM -chain
HLA-DNA	DZ, DO	DN -chain
HLA-DPA1	DP1, DP1A	DP -chain as expressed
HLA-DPB1	DP1, DP1B	DP -chain as expressed
HLA-DPA2	DP2, DP2A	DP -chain-related pseudogene
HLA-DPB2	DP2, DP2B	DP -chain-related pseudogene
TAP1	RING4, Y3, PSF1	ABC (ATP-binding cassette) transporter
TAP2	RING11, Y1, PSF2	ABC (ATP-binding cassette) transporter
LMP2	RING12	Proteasome-related sequence
LMP7	RING10	Proteasome-related sequence

101 or 102, may be added to the alleleic designation. *N* indicates a nonexpressed or null allele. *L* indicates a mutation in a noncoding region that causes significantly lower expression of the specific gene product. For example, there is a noncoding variant of A*2402. The normal allele is designated A*2402101, and the poorly expressed variant is A2402102L. If there is no normal counterpart to a null or poorly expressed gene product, it is designated with the letter *N* or *L* without the additional preceding three-digit number. [12]

To summarize, two HLA nomenclatures are in use: a nomenclature that is used to describe immunologically defined specificities (antigenic determinants) and a nomenclature that is used to define alleles, based on confirmed nucleotide sequences. Immunologically defined specificities are commonly used in organ transplantation and platelet donor selection. Molecular nomenclature is commonly used for defining HLA class II alleles in donors and recipients of unrelated hematopoietic stem cell transplants. As class I and II serology is replaced by molecular typing techniques, molecular nomenclature will increasingly replace the classical serologic nomenclature.

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STRUCTURE OF THE HLA GENETIC COMPLEX

The HLA genetic complex, located on the short arm of chromosome 6, spans a distance of approximately 4,000 kilobases (kb).[13] The complex contains more than 100 genes. Thirty-three class I and class II genes are officially recognized by the WHO nomenclature committee (Table 134-1). Within the MHC, defined genes are physically grouped into three regions (Fig. 134-1). The class I region encodes genes for heavy chains of the classical transplantation molecules, HLA-A, -B, -C, the nonclassical class I genes, HLA-E, -F, and -G, several pseudogenes, and other genes of undetermined function. The class II region encodes both - and -chains of HLA-DR, -DQ, -DP, -DM, and -DO. At least four genes in the class II region code proteins that transport peptides into the endoplasmic reticulum for loading into class I molecules (TAP1, TAP2) or have proteasome-like structures that partially digest endogenously derived proteins (LMP2, LMP7).[14] The class III region encodes several structurally and functionally diverse molecules (C4, 21-hydroxylase [21-OH], C2, factor B, tumor necrosis factor [TNF], heat shock protein Hsp 70). DRA, the most telomeric class II locus, is separated from 21-OHB by about 400 kb; the position of TNF- and - loci is approximately 200 kb centromeric to the HLA-B locus, with the gene on the HLA-B locus side.[14]

Class I and II genes are the most polymorphic genetic systems known. More than 500 class I and II allelic gene products have been sequenced, of which 136 can be detected by serologic techniques.[12] [15] The true number of gene products is still underestimated, since a significant number of variant alleles remain to be sequenced in nonwhite populations throughout the world.[16] [17] The occurrence of the class III genes within the MHC is still puzzling. It is not clear if this group of somewhat unrelated genes is located in the MHC complex by chance occurrence or if there are important functional relationships between these genes and the class I and II genes. Because this chapter focuses on the HLA class I and II genes and molecules, no further discussion of class III genes is presented.

TABLE 134-2 -- Listing of Serologically Definable HLA Specificities

A	B		C	DR	DQ	DP
A1	B5	B50(21)	Cw1	DR1	DQ1	DPw1
A2	B7	B51(5)	Cw2	DR103	DQ2	DPw2
A203	B703	B5102	Cw3	DR2	DQ3	DPw3
A210	B8	B5103	Cw4	DR3	DQ4	DPw4
A3	B12	B52(5)	Cw5	DR4	DQ5(1)	DPw5
A9	B13	B53	Cw6	DR5	DQ6(1)	DPw6
A10	B14	B54(22)	Cw7	DR6	DQ7(3)	
A11	B15	B55(22)	Cw8	DR7	DQ8(3)	
A19	B16	B56(22)	Cw9(w3)	DR8	DQ9(3)	
A23(9)	B17	B57(17)	Cw10(w3)	DR9		
A24(9)	B18	B58(17)		DR10		
A2403	B21	B59		DR11(5)		
A25(10)	B22	B60(40)		DR12(5)		
A26(10)	B27	B61(40)		DR13(6)		
A28	B35	B62(15)		DR14(6)		
A29(19)	B37	B63(15)		DR1403		
A30(19)	B38(16)	B64(14)		DR1404		
A31(19)	B39(16)	B65(14)		DR15(2)		
A32(19)	B3901	B67		DR16(2)		
A33(19)	B3902	B70		DR17(3)		
A34(10)	B40	B71(70)		DR18(3)		
A36	B4005	B72(70)				
A43	B41	B73		DR51		
A66(10)	B42	B75(15)				
A68(28)	B44(12)	B76(15)		DR52		
A69(28)	B45(12)	B77(15)				
A74(19)	B46	B78		DR53		
A80	B47	B81				
	B48					
	B49(21)	Bw4				
		Bw6				

Data from Bodmer et al.[13]

Class I and II Genes

Class I and II genes, with minor exceptions, have similar structures (Fig. 134-2). They are split genes containing six to eight exons coding sequences that correspond to functional regions of the gene product. The exons code a leader sequence, two to three extracytoplasmic domains, a transmembrane sequence, and two to three intracytoplasmic sequences.[18] [19]

Only the heavy chain of the HLA class I molecule is encoded in the MHC. Genes encoding HLA-A, -B, and -C alleles each contain three extracytoplasmic exons coding for; $_1$, $_2$, and $_3$ domains of the heavy chain, a transmembrane, and three cytoplasmic tail exons. The associated class I light chain, $_2$ -microglobulin, is encoded on chromosome 15.[20] By contrast, both and protein chains of HLA class II molecules are encoded within the MHC. -Chain genes usually contain four exons coding for a leader sequence, $_1$ and $_2$ domains, and a combined transmembrane-cytoplasmic tail. -Chain genes, in addition to leader, $_1$, and $_2$ exons, have a separate transmembrane and two distinct cytoplasmic tail exons.

Each class II molecule contains an - and a -chain. All DR molecules use DRA for -chains but can use alleles coded by DRB1 (the classical DR specificities), DRB3 (DR52 molecules), DRB4 (DR53), or DRB5 (DR51). DP molecules are the product of DPA1 and DPB1 alleles; DQ molecules are the product of DQA1 and DQB1 alleles; DM molecules are the product of DMA and DMB alleles; and DO molecules are the product of DNA and DOB alleles. Several class I and II genes, as noted in Table 134-1 , are pseudogenes. Other class II genes are potentially functional but are not known to be expressed in adults (DQA2, DQB2, DQB3). [13] (The structures of the various HLA gene products are discussed later in more detail under the Physical Structure of HLA Gene Products.) Important functional roles for two additional HLA class II gene products, HLA-DM and HLA-DO, have been identified during the past 5 years. [21] [22] DM is not expressed on the cell membrane but is found primarily in endosomal compartments. The DM molecule physically associates with classical class II molecules during their transit to the endosomal compartments. DM appears to stabilize class II molecules during the time period that the invariant chain-derived peptide has dissociated until another stable peptide is firmly bound. DO and DM have similar distributions because they associate together in the endocytoplasmic reticulum and endosomal compartments. Preliminary evidence suggests that DO may negatively regulate the function of DM.

Expression of HLA Molecules

HLA molecules are expressed constitutively on the cell membranes of many different cell types. HLA class I molecules are found on most nucleated cells. [23] They also occur on platelets, in plasma, and, to a limited extent, on reticulocytes. [24] Constitutive expression of class II molecules, by contrast, is restricted to relatively few cells, principally B cells, tissue dendritic cells, Langerhans cells, and some endothelial cells. [25]

Expression of class I and II chains can also be up-regulated in most cells by the action of cytokines or through direct cell-cell contacts. [26] The expression of class II molecules in certain physiologic and pathologic conditions contributes significantly to the pathogenesis of the allograft rejection process and in certain types of autoimmune disease. Induction is controlled by regulatory elements found in the 5-flanking regions and intron regions contiguous to exon 1. [27] [28] Major advances have been made in defining the regulatory elements that govern MHC expression. (For reviews of these elements and key trans-acting DNA-binding proteins, refer to references[29] [30] [31]) The major cytokine that initiates the sequence of nuclear events leading to increased expression of HLA molecules in differentiated cells is interferon- (IFN-).[32] [33] [34]

The existence of additional MHC class I genes in mice has

Figure 134-1 Physical map of the HLA genetic complex, illustrating the clusters of genes according to the class of encoded gene products. The symbol represents four DRB pseudogenes, designated DRB6, 7, 8, and 9. Other pseudogenes are shown in gray.

Figure 134-2 Organization of class I and II MHC genes. 5 UT and 3 UT, untranslated regions in the 5 and 3 ends of the gene; L, leader sequence; , , exons encoding extracellular domains; TM, transmembrane exon; CY, exons encoding the cytoplasmic tail. *(From Germain and Malissen. [15])*

been known for several years. These molecules are called class Ib to distinguish them from the classical class Ia genes.

More than 17 class Ib genes have been identified in humans. These sequences are found on either side of the HLA-A locus. Although a majority of the sequences are pseudogenes, at least six are potentially capable of generating expressed gene products. Orr and colleagues characterized three class I genes contained within Hind III restriction enzyme fragments encompassing the HLA-A region, HLA-E, -F, and -G. [35] The genes transcribe mRNA for class I-like heavy chains that associate with $_2$ -microglobulin. HLA-G codes for a transmembrane class I molecule of limited polymorphism that is expressed on cytotrophoblasts and is ubiquitously expressed in low levels on other cells, including lymphocytes, cells of dendritic lineage, and mononuclear phagocytes. [36] HLA-G serves as a ligand that can inhibit the cytotoxic response of many natural killer (NK) cells. [37] [38] Expression at the fetomaternal interface thus may protect the trophoblast from NK-mediated fetal rejection during pregnancy. Transfection experiments demonstrate that HLA-E and -F genes produce class I-like heavy chains that associate with $_2$ -microglobulin within the cytoplasm, but the molecules do not insert into the cell membrane. [39] The functions of these gene products are currently not clear. Four additional class I pseudogenes, HLA-H, -J, -K, and -L, flank HLA-A. [14]

To summarize, most of the human MHC has been mapped and sequenced. In addition to class I, II, and III genes of known function, there are many other potentially functional genes. Some of the novel class I molecules appear to be analogous to the murine Qa molecules. If the observed MHC-linked mating preference [40] extends to humans, some of the class I-like genes could mediate chemosensory recognition between members of the same species.

Inheritance and Linkage Disequilibrium

Because of their close physical linkage, HLA genes are normally inherited en bloc from the parents, unless a recombination event has occurred. Thus, despite the complexity of the gene clusters, there are normally only four genotypes transmitted to offspring, and the probability of genotypic HLA identity of two siblings is 25%. Recombinant HLA haplotypes (the portion of a single chromosome carrying one set of the HLA genes) are observed in 23% of offspring, a frequency that would be expected based on the length of the genetic complex. HLA genes are expressed codominantly, and while null or amorphic class I or class II alleles are observed, they are uncommon. On some chromosomes, specific functional HLA-DR loci are commonly deleted on different HLA haplotypes. The DRB1 locus, which codes for the -chain of classical DR molecules, is found on all HLA haplotypes. Some DR1 haplotypes carry only the DRB5 locus, while other DR1 haplotypes do not bear DRB3, 4, or 5 loci. DR2 haplotypes carry only DRB5. DR3, 5, and 6 haplotypes carry only DRB3. DR4, 7, and 9 haplotypes carry only DRB4. Finally, DR8 and DR10 haplotypes carry only DRB1.[13] Null alleles are common in the C4 and C2 class III genes. [41]

Certain combinations of genes occur in HLA haplotypes within the population far more frequently than expected on the basis of gene frequencies. This phenomenon, referred to as linkage disequilibrium, is quite common in the HLA system and has important biologic and clinical implications. In large, randomly mating populations, such as humans, gene frequencies rapidly achieve equilibrium within the population after one or two generations, unless some positive or negative selection pressure is acting on the gene (Hardy-Weinberg principle). When equilibrium exists, the occurrence of any two HLA genes on a particular haplotype should be an independent event governed only

Figure 134-3 Three-dimensional configuration of HLA-A2, modeled from x-ray crystallographic studies. *(From Bjorkman et al.,[45] with permission.)*

by the gene frequencies in the population. For example, if HLA-A1 and HLA-B8 gene frequencies are respectively 0.16 and 0.1 in a population, the expected occurrence of an HLA haplotype bearing both A1 and B8 should be 0.16 × 0.1 × 100 = 1.6%. In certain white populations with origins in Northern Europe, the actual occurrence of this haplotype in the population may be as high as 8%. Linkage disequilibrium effects often can be seen throughout an entire haplotype, involving class III as well as class I and II MHC encoded genes.[42] The possible causes and significance of linkage disequilibrium are discussed in more detail later in the section titled Evolution and Functions of HLA Gene Products.

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PHYSICAL STRUCTURE OF HLA GENE PRODUCTS

Class I and II molecules are heterodimeric glycoproteins. Although the peptide chains are different for each class of molecules, the overall three-dimensional configurations of the molecules are probably very similar (Fig. 134-3). The protein chains of each class of molecule, the class I heavy chain, $_2$ -microglobulin, and the class II - and -chains, belong to the immunoglobulin gene superfamily. [43] Each protein contains one of the immunoglobulin-like domain structures typical of this family, and these domains show sequence homologies with domains of other members of that family.

Class I Molecules

HLA class I molecules contain a heavy protein chain, of approximately 45 kd mass, that is noncovalently associated with $_2$ -microglobulin, a nonpolymorphic protein of 12 kd mass. The heavy chain structure correlates closely with the exonic arrangement of the gene. Beginning with the N-terminal amino acid, there are three extracytoplasmic domains ($_1$, $_2$, and $_3$), followed by a hydrophobic transmembrane stretch of amino acids and terminating in a cytoplasmic tail. $_2$ -Microglobulin, together with the heavy chain domain, constitutes the membrane-proximal portion of the molecule. The amino acid sequences of 4050 class I molecules, deduced from class I nucleotide sequences, [44] demonstrate that amino acid sequences of the $_3$ domain are highly conserved. $_2$ -Microglobulin in humans is essentially monomorphic. All class I polymorphism detected by serologic and cellular techniques resides in the $_1$ and $_2$ domains. Like immunoglobulins, most class I polymorphism occurs in specific sites referred to as hypervariable regions. Comparisons of the various class I amino acid sequences to HLA-A2 indicate that virtually all hypervariable sites occur within or in close proximity to the peptide groove. [45]

Early structural models of class I molecules indicated that both the $_1$ and $_2$ domains contained stretches of amino acid sequences in an -helical configuration. The significance of this observation was not appreciated until further landmark studies of Bjorkman and colleagues [45] [46] identified the three-dimensional structure of HLA-A2 (Fig. 134-4). The $_1$ and $_2$ domains form a platform of eight antiparallel strands overlain by the two helices (Fig. 134-5). This configuration forms a groove that is about 25 A long, 10 A wide, and 11 A deep. This site, referred to as the peptide groove, accommodates processed peptides for presentation to T-cell receptors (see the section titled Evolution and Functions of the HLA Gene Products). The ends of the class I peptide groove are relatively closed, restricting the size of

Figure 134-4 Comparative configurations of HLA-A2 (class I) and of HLA-DRI (class II). *(From Hammer and Sinigaglia,[207] with permission from Hogrefe & Huber Publishers, Seattle, Toronto, Bern, Göttingen.)*

Figure 134-5 Scheme of the peptide groove of an HLA class I molecule, formed by the $_1$, and $_2$ domains. The view is looking down on the vertically oriented molecule. *(From Bjorkman et al.,[46] with permission.)*

peptides that can be accommodated. The size range of class I peptides is 711, with a modal length of 9. Peptide grooves of both class I and II molecules contain recesses (pockets) that favor certain types of amino acid side chains. [46] These anchor residues largely determine if a particular peptide will bind with high affinity to a specific HLA molecule. Clusters of HLA molecules share similar pockets that favor the binding of like peptides. Peptides bearing the appropriate amino acid anchor residues that favor binding to a cluster of HLA molecules are referred to as a supermotif. [47] This observation has important implications for the production of peptide-based vaccines.

Class II Molecules

HLA class II molecules contain two noncovalently associated protein chains, , of approximately 33 kd mass, and , of approximately 29 kd mass. Each chain is a transmembrane protein that consists of a cytoplasmic tail, a hydrophobic transmembrane segment, and two membrane-distal domains. An HLA class II molecule has been crystallized and its structure deduced (Fig. 134-4). The peptide-binding site structure, previously projected to be similar to class I, was documented. In contrast to class I molecules, the peptide groove of class II molecules is open-ended, and a broader array of peptides, up to 18 amino acids in length, can be accommodated. Pockets that favor the binding of specific anchor residues are also characteristic of class II peptide grooves.

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ORIGIN OF MHC-ASSOCIATED PEPTIDES

Peptides presented by MHC molecules are derived from two distinct sources. Exogenously derived antigens enter cells by endocytotic processes and bind primarily to class II MHC.[48] The peptide-class II complex serves as the ligand for $CD4^+$ helper T lymphocytes. This is the classical pathway utilized by antigen-presenting cells (APCs) to capture, process, and present exogenous antigens for subsequent induction of delayed-type hypersensitivity reactions or the production of antibodies. Endogenous antigens originate in the cytosolic environment of the cell. [49] Endogenous peptides, which may include peptides from self antigens or may be derived from early viral proteins produced in a virus-infected cell, are presented primarily by class I MHC molecules and are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL). The routes through which the peptides and the class II or I molecules pass are referred to respectively as the exogenous and endogenous pathways, according to the origin of the peptides.

Exogenous Pathway

With appropriate route of administration, foreign antigen derived from the external environment is taken up by APCs via random or receptor-mediated endocytosis. The antigen undergoes limited proteolysis within membrane-bound acidic endosomal compartments, which are now referred to as MHC class II peptide-loading compartments (MIICs). Concurrently, MHC class II molecules are assembled in the endoplasmic reticulum. Early class II molecules contain a third chain, in addition to the and , called the invariant chain (Ii). The chain serves several important functions in the exogenous pathway. It occupies the peptide-binding groove and prevents most endogenously derived peptides from binding to the newly assembled class II molecules. It functions to stabilize class II molecules, and it also contains an amino acid sequence that targets the molecular complex to MIICs. Upon entry into MIICs, most of the invariant chain is degraded, leaving only the small portion that is bound in the peptide groove (called class II-associated Ii chain, or CLIP). The nonclassical class II molecule HLA-DM facilitates the process of loading specific endogenous peptide into classical HLA class II molecules. HLA-DM and Ii expression are co-regulated with the classical class II genes. DM is largely absent from the cell membrane, but accumulates in MIICs. DM molecules do not bind peptides within their own peptide grooves. The role of DM in peptide loading is to function as a chaperone for class II molecules that are empty, or loaded with low-stability peptides. Class II molecules are susceptible to denaturation during this period, and DM molecules prevent this until a high-stability peptide is loaded. The DM molecule then dissociates from the stable peptide-MHC trimer, which is then transported to the plasma membrane and is re-expressed on its surface.

Endogenous Pathway

Peptides presented by class I MHC molecules have their origins mainly from proteins found in the cytosol. Most are derived from self proteins under normal circumstances. Endogenous peptides are also derived from viral proteins when these proteins are actively synthesized by a virus-infected cell. [50] Peptides from phagocytosed microbial antigens and other endocytosed material also can gain access to newly synthesized class I molecules via leakage into the cytosol. This may constitute a minor component of the endogenous pathway. It appears that evolution provided the endogenous pathway and class I MHC molecules as a mechanism to survey the internal environment of the cell for antigens from replicating organs and from mutated self proteins that would otherwise escape recognition by antibody or other extracellular immune mechanisms.

In contrast to class II molecules, whose structure is temporarily stabilized by invariant chain, then by HLA-DM prior to peptide binding in MIIC compartments, class I molecules must bind peptides within the endoplasmic reticulum. Otherwise, the physical association of the class I heavy chain with $_2$ -microglobulin is unstable.[49] [51] [52] Until the time that peptides are loaded into their peptide grooves, class I molecules are stabilized by a complex series of cofactors that include two calcium-dependent chaperones and the protein, tapasin. [53] The newly formed HLA heavy chain first associates with calnexin. When $_2$ -microglobulin

associates with the heavy chain, calnexin dissociates and calreticulin binds. During this time period, tapasin also associates with the class I-calreticulin complex. The complex then binds to MHC-encoded transporter associated with antigen processing molecules (TAP1/TAP2 heterodimer) via the class I molecule. TAP molecules insert through the endoplasmic reticulum membrane. Endogenous peptides that will be transported through TAP molecules are derived from proteins that are degraded into a series of peptides through the action of cytosolic proteasomes. Two proteins, LMP2 and LMP7, that are involved in this process are encoded in the class II region of HLA.[54] Since proteasomes contain multiple protease enzymes, a variety of different peptides may be obtained from the same protein. The class I molecules ultimately select the appropriate peptides based on anchor residues and affinity. If no peptides meet the criteria for binding after a period of time, the class I molecules are released and ultimately degraded in the cytosol by proteasomes. This process produces class I-derived peptides that can be loaded and presented by other HLA molecules, a process that has clinical relevance in allotransplantation. Peptides transported into the endoplasmic reticulum insert into the peptide groove of the docked class I molecules. Stable trimeric complexes are transported to the plasma membrane and re-expressed on the membrane surface.

To summarize, the high degree of polymorphism found in both class I and class II molecules is restricted to specific, hypervariable regions of the membrane distal domains that form the peptide groove. The remaining portions of the molecules are relatively conserved, reflecting structural constraints imposed by the functions of the molecules in antigen presentation and cellular interactions. Indeed, studies of the types of amino acid substitutions found in the hypervariable regions compared to those in conserved regions indicate that both positive and negative selection pressures may be exerted on the molecules: positive selection in the hypervariable regions that favors conformational or charge change and negative selection that favors no conformational or charge change.

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ANTIGENIC STRUCTURE OF HLA MOLECULES

HLA molecules contain multiple alloepitopes that can induce humoral and cellular responses during alloimmunization. Serologically defined HLA alloepitopes are defined with well-characterized, operationally monospecific alloantibodies. T-cell epitopes are characterized using cloned T lymphocytes. The availability of HLA structure and amino acid sequence data, together with monoclonal antibodies and the technology of site-directed mutagenesis, now makes mapping of alloepitopes on the HLA molecules technically feasible. [55] As expected, serologically definable epitopes are located on and adjacent to the peptide groove. Epitopes recognized by T cells are distinct from the serologically defined epitopes. [56] [57] This was anticipated, since T and B cells recognize epitopes through different mechanisms (see the section titled Evolution and Functions of the HLA Gene Products).

Two general types of alloepitopes, based on their distribution pattern, have been defined serologically. [58] Certain epitopes (private epitopes) occur only on a single gene product or its allelic molecular variants, (for example, HLA-A2). Serologic reagents that recognize private epitopes have been important for HLA antigen typing. Other alloepitopes, termed public epitopes, differentially occur on more than one distinct HLA gene product. Many public epitopes are widely distributed among HLA molecules. Antibodies to public epitopes have been used to categorize HLA gene products into major cross-reactive groups (CREGs). The current significance of public epitopes, however, is their clinical relevance for patients awaiting transplants or requiring repetitive platelet transfusions. A single alloantibody directed against a high-frequency public epitope can have devastating consequences for potential transplant recipients or patients who need repetitive platelet transfusions (see the section titled HLA Testing for Transplantation and Transfusion). The HLA-Bw4 and -Bw6 specificities are prototypic examples of public epitopes. These two specificities were originally thought to define a diallelic locus closely linked to, but distinct from, HLA. All individuals studied were either Bw4, Bw6, or both. Sequential immunoprecipitation studies subsequently demonstrated that Bw4 and Bw6 are alternative specificities present on virtually all HLA-B molecules and a few HLA-A molecules. Parham and colleagues[59] identified $_1$ -domain amino acid patterns in positions 7983 that correlate with Bw4, Bw6, or the absence of Bw4 or Bw6 (most A-locus alleles). Table 134-2 lists the currently recognized HLA specificities. [13] Table 134-3 is a summary of the major public epitopes and their approximate distribution among HLA molecules.

TABLE 134-3 -- Population Frequencies of Major Cross-Reactive or Determinants Present on HLA-A and HLA-B Gene Products

Major Cross-Reactive Group	Public Epitope	Associated Private Epitopes	Approximate Epitope Frequency (%)[a]
1C	1p 10p	A1, 3, 9 (23, 24), 11, 29, 30, 31, 36, 80 A10 (25, 26, 34, 43, 66), 11, 28 (68, 69), 32, 33, 74	79
2C	28p 9p 17p	A2, 28 (68, 69), 9, 17 A2, 28 (68, 69), 9 (23, 24) A2, B17 (57, 58)	70
5C	5p 21p	B5 (51, 52), 18, 35, 53, 78 B5 (51, 52), 15 (62, 63, 75, 76, 77), 17 (57, 58) 21 (49, 50), 35, 53, 70 (71, 72), 73, 74, 78	50
7C	7p 22p 27p	B7, 8, 41, 42, 48, 81 B7, 22 (54, 55, 56), 27, 42, 46 B7, 13, 27, 40 (60, 61), 47	54
8C	8p	B8, 14 (64, 65), 16 (38, 39), 18	38
12C	12p	B12 (44, 45), 13, 21 (49, 50), 40 (60, 61), 41	44
Bw4	Bw4	B13, 27, 37, 38, 44, 47, 49, 51, 52, 53, 57, 58, 59, 63, 77; A24, 25, 32	79
Bw6	Bw6	B7, 8, 18, 35, 39, 41, 42, 45, 46, 48, 50, 54, 55, 56, 60, 61, 62, 64, 65, 67, 71, 72, 73, 75, 76, 78, 81	87

Modified from Rodey and Fuller,[56] with permission.

[a] North American white populations of European origin.

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HLA ALLOIMMUNIZATION

Alloimmunization to HLA antigens occurs through pregnancy, transfusion of blood products, or prior transplantation. HLA molecules traditionally have been considered strong immunogens because of their influence on allograft rejection and the development of graft-versus-host disease (GvHD). Surprisingly, only about one-third of individuals who are alloimmunized develop HLA alloantibodies, [60] suggesting to some that these molecules may be relatively poor immunogens. Since cell-mediated immunity is a principal mechanism of graft rejection in nonimmune recipients, it also is possible that HLA molecules preferentially induce T-cell-mediated responses. T-cell responses, however, are difficult to measure in vitro because the frequency of precursor T cells activated in vitro to an HLA molecule may be an order of magnitude greater than the frequency of precursor T cells activated to strong antigens, such as tetanus toxoid. [61] [62] Generally, there is no consistent difference in CTL or proliferative cell precursor frequency between nonimmune and alloimmunized individuals. [63]

An alternative to poor immunogenicity as an explanation for the relatively low percentage of antibody responses to alloimmunization is that the molecules are strong immunogens, but immune effector responses are modified by concurrent immune responses that down-regulate effector products such as antibodies or CTL. Evidence suggests that regulatory immune responses do occur.[64] [65] Suciu-Foca and others[66] [67] [68] have described the occurrence of autoanti-idiotypic-like antibodies (AB2) in the serum of many alloimmunized individuals who do not make HLA alloantibodies. AB2 appear to have specificity for cross-reactive idiotypes present on HLA-specific alloantibodies, as shown by the capacity of AB2 to inhibit binding of the HLA alloantibodies to relevant HLA targets. Reed et al. [69] reported that the presence of AB2 inhibiting one or more HLA antibodies to incompatible HLA antigens on donor grafts is associated with excellent graft outcome, whereas the absence of AB2 predicts early graft loss. Although there is no direct evidence that these autoantibodies are important components in early activation of immune regulation, their occurrence suggests that alloimmunization with specific HLA incompatibilities will modify subsequent immune responses to the incompatibilities, either positively or negatively.

Immune regulation of effector responses may also be mediated through differential cytokine production by T cells. Helper T cells with distinctive patterns of cytokine secretion occur in mice[70] and in humans.[71] [72] One T-cell phenotype, Th1, which secretes predominantly IL-2, TNF-, and IFN-, mediates classical delayed-type hypersensitivity reactions. The second type, Th2, secretes predominantly IL-4, IL-5, and IL-10, and is largely responsible for B-cell activation and differentiation into antibody-secreting cells.[73] Once differentiated, these two helper T-cell types are mutually regulatory: IL-4 and INF- each reciprocally inhibit synthesis of the other cytokine. Thus, preferential activation of one Th type subsequently inhibits activation of the other type. Predominant Th2 cytokine profiles in an allograft is associated with decreased acute cellular rejection because cellular immune effector mechanisms are suppressed. However, it can be associated with humoral and chronic rejection processes.[74]

Two types of HLA allorecognition, direct and indirect, are recognized based on the mode of peptide presentation. Direct allorecognition occurs only in the context of HLA alloimmunization because of the unique role MHC molecules play in antigen presentation. T cells responding to donor-derived, allogeneic APCs can be activated directly by HLA molecules present on the APCs. Besides antigenic differences in the HLA molecules themselves, they contain a large array of peptides that are potentially antigenic. The cumulative number of different antigens in this setting activates large numbers of T cells. Direct allorecognition is a vigorous and early HLA immunization event that is largely responsible for the acute cellular rejection response observed in organ transplantation, and for acute GvHD. Indirect allorecognition is the classical antigen presentation pathway, in which an exogenous antigen, in this case donor-derived HLA antigens, is taken up by endocytosis into the responders own APCs. The antigen is then processed and the peptides presented on self-HLA molecules. Indirect antigen presentation may play a prominent role in chronic rejection of solid organ allografts, and possibly in chronic GvHD. [75] [76]

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LABORATORY DETECTION OF HLA ANTIGENS AND ANTIBODIES

Historically, HLA antigens and antibodies have been defined by serologic techniques, using complement-dependent lymphocytotoxicity (CDC) techniques. Although serology remains a keystone for clinical HLA testing, newer procedures are available that supplement or may gradually replace CDC. These include DNA-based typing procedures, flow cytometry for crossmatching and HLA antibody screening, and solid phase immunoassays for HLA antibody detection. Oligonucleotide primers, probes, and direct sequencing, used in conjunction with the polymerase chain reaction (PCR) or other process that amplifies copies of the relevant gene, are emerging as the principal adjunct procedure for class II typing. Many centers no longer type class II antigens using serologic techniques because DNA typing, which provides more information, can be performed for the same charge and with comparable turnaround time. Commercial kits for antigen-level HLA class I typing are now available. Molecular genetic typing techniques will most likely replace serology as the primary form of HLA testing as probes become available and the procedures become cost- and time-effective. With the availability of allele-level HLA class II molecular typing, the unidirectional mixed leukocyte reaction (MLR), once a common clinical procedure used to identify composite HLA-D region compatibility between donor and recipient, now is rarely used for compatibility testing. [77]

Microlymphocytotoxicity Procedure

The standard procedure[78] for HLA testing is a complement-dependent microlymphocytotoxicity test (CDC). The test is miniaturized, requiring 1 l and 2,000 cells per well, on microplates containing up to 96 wells. Because of extensive polymorphism of the HLA system, multiple antisera (or cells) must be used for complete testing. It is not uncommon to employ 120140 antisera to determine the HLA phenotype of an individual or to test against 60 cells when screening patients sera for HLA antibodies. The general procedure, summarized in Figure 134-6 , involves an initial incubation of antiserum and lymphocytes, followed by the addition of rabbit serum as a source of complement. If specific binding of antibody to relevant HLA molecules has efficiently activated the complement cascade, cell membrane injury occurs and is visualized microscopically by the addition of a vital dye such as eosin Y, trypan blue, or ethidium bromide.

Class I HLA molecules are present on both T and B lymphocytes. Class II molecules, however, are normally present only on the B-cell population. To perform class II typing, relatively purified B-cell lymphocytes must be used as targets (nylon wool adherence or antibody-specific capture to a solid phase) or B cells must be tagged so that they can be distinguished from T cells (tagged with fluorochrome-labeled, B-cell-specific antibody). [79]

CDC is used for HLA typing (unknown cell tested against panels of HLA antisera), HLA serum analysis (unknown serum tested against panels of HLA typed cells), or crossmatches between

Figure 134-6 Summary of the complement-dependent lymphocytotoxicity (CDC) procedure, showing the optional antiglobulin augmentation step.

potential recipients and donor pairs (recipient serum tested against donor cells). A limitation of CDC in antibody detection is that not all HLA antibodies efficiently activate the complement system and certain HLA specificities are not detected. Thus, antibody reactivity can be greatly underestimated in some patients. Failure to detect certain antibodies with CDC is referred to as the cytotoxicity negative but adsorption positive (CYNAP) phenomenon. [80] CYNAP can be minimized by the addition of anti-human light chain-specific or $(Fab)_2$ -specific antiglobulin reagent. [81] [82] This modified test is termed the antiglobulin-augmented microlymphocytotoxicity (AHG-CDC) test. The mechanism of augmentation is that more efficient complement activation occurs with the provision of additional heavy chain complement-binding sites in proximity to bound HLA antibodies. CYNAP commonly occurs with antibodies directed against public or CREG specificities. Consequently, standard CDC defines only partial specificity of the antibody. An example of the CYNAP phenomenon is given in Table 134-4 . The serum, directed against the 2C public epitope, shows no reactivity with the HLA-A9 (A23, A24) epitopes until tested by AHG-CDC.

Indirect immunofluorescence using flow cytometry is more sensitive than AHG-CDC for the detection of HLA antibodies and is gradually replacing CDC or AHG-CDC as the final crossmatch procedure used for organ transplantation. [83] Limited HLA specificity analysis of selected sera by flow cytometry also is performed in some centers, and its use may increase as better reagents become available to increase throughput. Solid phase immunoassays that use solubilized HLA molecules as targets now are commercially available for HLA antibody screening. They are formatted either as ELISA trays or as coated microparticles for use in flow cytometry. As solid phase immunoassays that employ purified single HLA molecular species per well or particle continue to improve, they will replace CDC procedures as the standard HLA antibody screening technique.

Molecular Typing

HLA class II DR and DQ typing by molecular techniques is routinely performed in many clinical laboratories that support transplantation programs. Two general procedures are employed, depending on the level of resolution desired to discriminate between variant alleles. One procedure, referred to as sequence-specific primer typing (PCR-SSP), utilizes DNA primers that are specific for individual or similar groups of class II alleles. The primers are used in conjunction with PCR to amplify the relevant genomic DNA. The amplified products for each primer pair are subjected to gel electrophoresis and visualized by staining. [84] Amplification occurs only when the appropriate sequence is present, and a product of known base pair size is generated. This technique is especially useful for typing individual patients but loses efficiency when larger numbers of patients (>50 per week) are typed, owing to the need for larger numbers of electrophoresis units. Additional labeled probes are not necessary to routinely define the amplified DNA, and the test can be performed within 2 hours using nonradioactive techniques. The procedure is often used for antigen-level class II typing (comparable to or better than serologically defined typing), but can be used for allele-level typing by performing further amplifications with additional primers.

A second common procedure, PCR-SSO, uses locus-specific or group-specific primers to amplify the desired genomic DNA, followed by the application of a labeled oligonucleotide probe that binds to an allele-specific sequence. For dot blotting, amplified DNA is usually applied to a membrane as multiple dots, and labeled probes are added to each DNA dot for hybridization and subsequent visualization. [85] In reverse dot blotting, nonlabeled probes are attached to a membrane through linking molecules and stored. Amplified, labeled DNA is then added at the time of testing. A number of sensitive, nonradioactive labels are now available. Reverse dot blotting is often used for allele-level typing of individual patients, whereas dot blotting is used when large numbers of samples can be tested at one time.

All of the molecular typing procedures are evolving rapidly. Typing for HLA class I is more complex because the number of variant alleles is greater and two, rather than one, exons are polymorphic. Finally, the HLA complex contains a number of HLA class I pseudogenes that could be inappropriately amplified with a poorly designed primer or probe. The technology for semi-automated, radioisotope-free, direct nucleotide sequencing is also developing rapidly and could become the

principal molecular typing method in the near future. [86]

TABLE 134-4 -- The CYNAP Phenomenon in an Alloantibody Directed Against the 2C Public Epitopea[a]

Cell Phenotype	Procedure	1:1	1:2	1:4	1:8	1:16	1:32
A1, 2, Bw53, w42	CDC	8	8	8	4	1	1
	AHG-CDC	8	8	8	8	4	1
A23, w36, Bw53, 58	CDC	1	1	1	1	1	1
	AHG-CDC	8	8	6	1	1	1
A24, w34, Bw42, 45	CDC	1	1	1	1	1	1
	AHG-CDC	8	8	8	6	4	1
A3, 28, B45, 49	CDC	8	8	8	6	1	1
	AHG-CDC	8	8	8	6	4	1
A34, w36, Bw53, 42	CDC	1	1	1	1	1	1
	AHG-CDC	1	1	1	1	1	1

[a] Underlined specificities in the cell phenotypes belong to the 2C cross-reactive groups. Sera were tested at doubling dilutions (1:11:32).

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EVOLUTION AND FUNCTIONS OF HLA GENE PRODUCTS

MHC molecules are crucial to the general process by which an organism discriminates, at a molecular, cellular, and perhaps species member level, between relevant self and non-self elements. This function is most clearly demonstrated in the immune system, which functions mainly to eliminate foreign or altered self-products from the internal environment. The selection of a T-cell receptor repertoire during thymus maturation of T cells, for example, is determined by the type of MHC-peptide configuration present in the thymic cortex and medulla.[7] [87] Differentiating thymocyte receptors with T-cell receptors having any affinity for self MHC molecules are positively selected for clonal expansion. Next, there is an elimination or suppression of high-affinity self-reactive receptors that could result in lethal autoimmunity. The final repertoire of cells contains receptors that recognize alterations of self MHC caused by physical association of foreign peptides, but generally lacks high-affinity receptors that recognize autologous peptides bound to self MHC molecules.

A second major function of MHC molecules is antigen presentation to T cells. After the three-dimensional structure of HLA-A2 was determined, the molecular basis of the MHC restriction became apparent. The ligand of a T-cell receptor is a complex that consists of a peptide bound within the peptide groove of an MHC molecule. The configuration imparted by this combination determines the affinity of this complex for a set of T-cell receptors and subsequent activation of the T cell. The estimated length of peptides that can be accommodated in the MHC peptide groove is 717 amino acids, depending on the class of HLA molecule. [88] [89] The peptide groove of class II molecules, which appears to be open at both ends, can accommodate peptides 1317 amino acids in length. Class I peptide grooves are closed at one end and will only carry peptides of 711 amino acids in length. Most peptides presented to T-cell receptors by MHC molecules are derived from larger protein chains through limited proteolysis within cells. Although the MHC-peptide complex is essential for antigen-specific T-cell activation, it is not sufficient. APCs must provide additional signals, either cytokines or other expressed membrane receptors, [90] [91] to allow T-cell activation to occur. Finally, a variety of accessory molecules that enhance contact between APCs and T cells promote T-cell activation.[90] Two T-cell accessory molecules, CD8 and CD4, respectively interact directly with class I and II MHC molecules to enhance T cell-APC contact and T-cell activation. [92] [93]

Class I and II molecules have related but distinct roles in the antigen presentation process. The class I-peptide complex interacts primarily with T-cell receptors of $CD8^+$ T cells. This effector arm of the immune response is particularly important for endogenously derived peptides. [94] Thus, viral proteins produced very early during the initial replication cycle can be processed and presented before significant viral replication occurred. Similarly, presentation of peptides derived from abnormal self-proteins may permit recognition of mutant cells, such as chronic myelocytic leukemia cells. [95]

The predominant function of class II molecules is presentation of peptides from exogenously derived proteins to $CD4^+$ T cells. This heterogeneous population includes helper T-cell subsets, Th1 and Th2, [70] [71] [72] [73] that secrete distinct cytokine profiles and are associated with classical delayed-type hypersensitivity and B-cell helper activity, respectively. A subset of $CD4^+$ T cells also facilitates induction of $CD8^+$ T cells with suppressor activity.[96] [97]

HLA molecules also have a significant biologic role in innate immunity, serving as ligands for inhibitory receptors present on different subsets of NK cells. [98] [99] [100] Absence of MHC class I expression on mutant cells has been known to confer resistance to killing by NK cells. Some NK cells are inhibited by certain HLA-C locus alleles and by the class I public epitope, Bw4. Selective inhibition of NK killing is mediated through a complex series of NK cell inhibitory receptors (KIRs). Through these pathways, MHC molecules coordinate reciprocal functional activities of NK and T cells.

Biologic Basis of Alloreactivity

The estimated precursor frequency of T cells from immunized donors that will proliferate in vitro to tetanus toxoid or other strong immunogens is about 1 in 5,00020,000. By contrast, the precursor frequency of T cells from nonimmune donors to an allogeneic MHC molecule is 1 in 5002,000. [61] [62] Similar differences are observed when CTL precursor frequencies are estimated. This large discrepancy has puzzled investigators for a number of years, particularly since exposure to allogeneic molecules is not known to occur in nature except during pregnancy. Recognition of allogeneic MHC molecules and self MHC-foreign peptide complexes may not involve distinct sets of receptors, since cloned T cells that can react with both are described. This evidence suggested that MHC alloreactivity is actually a form of cross-reactivity with altered self. That is, a given allogeneic MHC molecule and its bound peptide may have a configuration that is similar to that recognized by a set of T cells that react with self MHC complexed with a foreign peptide. [101] [102] In the absence of processed foreign peptides, peptide grooves of MHC molecules are normally occupied by self peptides. Thus, exposure to an apparently homogeneous allogeneic MHC molecule by serologic testing is actually, at the T-cell level, exposure to MHC molecules already complexed with a large array of potentially foreign peptides. [103] Consequently, a proportionally broader set of T cells is activated.

Evolutionary Basis of MHC Polymorphism

Every individual who is heterozygous for each known MHC gene product has six class I molecules (two each of HLA-A, -B, and -C gene products) and eight class II molecules (two each of DR, DP, DQ, and DRw51, w52, and w53). Within the human species, however, there are at least 500 discrete class I and II gene products. Two general theories, selectionist and neutralist, are proposed to explain the level of polymorphism. [104] [105] Both theories propose that a high level of polymorphism has been established and maintained because of heterozygous advantage. However, the theories differ in proposed reason and mechanism. Selectionist theories propose that new MHC molecules are selected and maintained in the species because they confer some form of survival advantage. Neutralist theories propose that positive selection does not occur but that neutral random mutations are accumulated to favor outbreeding and heterozygosity. The conclusion that the mutations favor genetic heterozygosity is based partly on the observation that H-2 linked mating preference in mice promotes MHC-incompatible matings. [40]

Recent observations that positive selection appears to influence amino acid substitutions in the MHC hypervariable region favor a selectionist theory. [44] Such theories propose that the major evolutionary pressure for developing polymorphism was a need to select an optimal number of alternative antigen presenting molecules within the species to attain parity with constantly mutating environmental pathogens. The limited number of gene products carried by a single individual must be used to present all foreign peptides encountered by the individual. An MHC molecule can present many but not all different peptides. A single amino acid substitution in the peptide groove of an MHC can abrogate the molecules capacity to carry a specific peptide. [106] Therefore, the capacity of one MHC molecule to accommodate different foreign peptides is broad but not limitless. It is possible that some peptides cannot be effectively presented

to or recognized by T cells in a given individual. Some Ir gene phenomena in inbred animals are a reflection of these physiologic blind spots.

A final question concerns the mechanisms through which MHC polymorphism is generated. Initially, genetic hotspots of hypermutability that favored a higher than normal mutation rate in MHC molecules were thought to exist. This mechanism would tend to produce an ever-changing set of MHC molecules of relatively recent origin. Comparative amino acid sequence data in different animal strains, however, indicate that most of the observed MHC polymorphism in rodents and primates actually predates speciation and has been retained as long as 5 to 10 million years. Gene conversion, originally suggested as a major source of class I polymorphism

in inbred mice,[106] was not as apparent in highly outbred human populations. However, sequencing of HLA class I alleles in Amerindian populations with limited HLA gene pools serologically reveals highly diverse alleleic microvariants that developed through gene conversion events. Thus, gene conversion may play a prominent role in sustaining HLA heterogeneity within relatively inbred populations. [16] [17]

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HLA TESTING FOR TRANSPLANTATION AND TRANSFUSION

Three general procedures are used for clinical HLA testing: HLA typing to identify HLA specificities, serum analysis to detect HLA alloantibodies, and crossmatch procedures to determine compatibility between a specific donor-recipient pair. Transfusion of blood products and pregnancy conceptually are allografts. As such, they induce similar immunologic responses. The responses, however, are modified according to differences such as the physical structure of the graft, the manner in which it is introduced into the host, and the duration of time in the host. Transfused leukocytes, for example, persist for relatively short periods in individuals with normal immune function compared to fetal allografts or vascularized organ allografts. Immune responses differ even among solid organs. Liver allografts, which shed large amounts of HLA antigen as well as passenger leukocytes, have a large capacity to clear preformed donor-specific antibodies and evoke different types of immunity tolerance. Organs, because they express different levels of HLA molecules and have different patterns of vascularization, also are affected differently by immune effector responses. Because of these differences, it is not possible to make categorical statements concerning HLA that apply to all transplant situations. In the following sections renal transplantation data will be used for most of the discussion of transplantation principles, with a brief indication of cases in which the principles do not apply to other organ transplants.

Renal Transplantation

When an allogeneic organ is transplanted, incompatible alloantigens present in the tissues are recognized appropriately as foreign. This recognition initiates a complex sequence of immunologic events that leads to the production of immune effector as well as immune regulatory responses. In a nonimmunosuppressed recipient, effector responses usually dominate early in the course of the immune response, leading to rejection of the tissue. Although primary rejection is mediated by cellular mechanisms in the nonimmune recipient,[107] preformed donor-specific alloantibodies directed against alloantigens in the endothelium (ABH blood group antigens, HLA antigens, and possibly endothelial-specific alloantigens) can cause the rapid loss of a vascularized graft.

Three clinical strategies have been employed to increase the probability of graft acceptance: (1) use of immunosuppressive agents to blunt immune effector mechanisms, (2) histocompatibility matching to reduce graft foreignness, and (3) pretransplantation alloimmunization to initiate the early phases of donor-specific hyporesponsiveness to alloantigens present in the graft.[108]

Immunosuppressive agents are a keystone of transplantation. Without some form of immunosuppression, all but grafts from monozygotic twins will be rejected. The first successful combination of drugs for organ transplantation was azathioprine and prednisone.[109] This drug therapy has been replaced by or supplemented with more effective immunosuppressive drugs, Cyclosporin A and tacrolimus (FK506). A principal mode of action of these agents as an immunosuppressive agent is the inhibition of factors that activate IL-2 and IL-2 receptor genes of T cells. IL-2 production plays a central role in the early amplification of cellular responses to the allograft.[110] Immunosuppression in transplant recipients, however, is a two-edged sword. Sufficient immunosuppression must be given to sustain engraftment while allowing the patient to respond adequately to environmental pathogens. There are no effective in vitro assays to quantify immunosuppression, so it is difficult to define adequate immunosuppression in the organ transplant recipient. In comparison to bone marrow transplantation, in which the immune system must be ablated with lethal doses of x-irradiation or drugs, the degree of immunosuppression used in organ transplantation is modest, yet the majority of grafts survive. As noted below, the role of immunosuppressive drugs in organ transplantation may be partly indirect, by facilitating immunologically mediated donor-specific acquired tolerance, as well as directly suppressing immune effector responses. The success of Cyclosporin A and tacrolimus in improving graft survival, particularly for nonrenal organ transplants, continues to spur searches for additional immunosuppressive agents, and a number of promising drugs are currently being tested. One drug, mycophenolate mefotil, is now approved for clinical use for supplemental immunosuppression in conjunction with Cyclosporin A or tacrolimus.[111] [112]

The two major transplantation antigen systems in human organ transplantation are the ABO and HLA systems. ABH substances are present in endothelial cell membranes. Naturally occurring isohemagglutinins can bind to the endothelium and cause hyperacute rejection of kidney and heart allografts. ABO matching of organs is therefore performed according to the same principles used for red cell transfusion. The second major transplantation antigen system is HLA. In humans, the most compelling evidence that HLA is the MHC is the superior graft survival rate of organs obtained from genotypic HLA-identical siblings when compared with graft survival from any other donors.[113] [114] Patients receiving these grafts have fewer rejection episodes and require lower doses of immunosuppressive drugs. Renal graft survival rates in this group exceed 90% at 2 years.

The importance in improving graft survival rates of HLA-A, -B, and -DR matching of kidney donors derived from cadaveric sources is well documented,[115] [116] but it has been difficult to establish significant improvement with lesser degrees of HLA matching. This is especially true if graft survival rates are evaluated only during the 2-year immediate post-transplantation period. However, a statistically significant improvement in graft survival rates can be demonstrated in major registries that monitor renal transplant results submitted by multiple centers.[113] [115] [116] Terasaki and colleagues[117] presented evidence that the beneficial effect of HLA matching is most apparent when long-term graft outcome (1520 years) is considered. Because graft loss after the first year following transplantation is linear, graft biologic half-lives can be estimated for grafts in different risk categories. The half-life of a genotypic HLA-identical graft, for example, is 25 years, and it is 13 years for parental one-haplotype matched grafts. By contrast, calculated half-lives for cadaveric grafts matched or mismatched for HLA-A, -B, and -DR are 17 and 8 years, respectively. This means that after 10 years,

60% of mismatched cadaveric grafts are lost. Thus, the chances of long-term graft survival are improved if HLA-compatible matches are provided, an increasingly important consideration in an era of donor organ shortages and limited health care funds.

Prior alloimmunization, through blood transfusion, pregnancy, or prior allografts, poses an additional risk for potential recipients. The presence of donor-specific HLA antibodies, especially antibodies directed against donor HLA class I antigens, in a patients serum is associated with a high incidence of early graft failure.[118] [119] These antibodies, detected by crossmatching procedures, preclude the use of that donor. Serum of potential recipients is regularly monitored for HLA antibodies against panels of HLA typed cells. Antibody reactivity to the panel, expressed as percent reactive antibody (PRA), is an indication of the likelihood that a compatible donor will be found. Alloimmunized patients serum may react against as many as 80100% of panel cells. In such patients, defining the specificity of the HLA antibodies becomes an important factor in predicting which donors will be compatible. Interestingly, the high PRA in the highly sensitized patients is not due to multiple HLA antibodies. Rather, it is almost always due to the presence of a few HLA antibodies with specificity to the high-frequency public or CREG epitopes (see the section titled HLA Alloimmunization).

Although prior alloimmunization may be detrimental to potential transplant recipients who make HLA antibodies, it paradoxically promotes graft acceptance in crossmatch-negative transplants.[60] [120] [121] Opelz et al.[120] noted that renal cadaveric graft survival rates in crossmatch-negative recipients who received no blood transfusions prior to transplantation were 60% lower than in comparable patients who had received blood. The beneficial effect of blood transfusion was most striking in patients who had received repetitive donor-specific blood transfusions before receiving kidneys from living-related donors.[60] [121] Following these observations, most

centers established a policy of deliberate pretransplantation blood transfusion. Although the mechanisms responsible for the blood transfusion effect have not been established, evidence suggests that pretransplantation alloimmunization may promote establishment of donor-specific hyporesponsiveness (DSH). (This concept is discussed in the section titled Concept of Allograft Acceptance.) Interestingly, the beneficial effect of blood transfusion of graft survival has not been so apparent in the cyclosporine era.[122] The apparent loss of this effect is attributed to the general use of Cyclosporin A and to improved procedures for management of early rejection episodes. Most centers now do not perform deliberate blood transfusion. However, the concept of pretransplantation exposure to donor-specific antigens as a means to promote DSH has re-emerged in different formats, driven by the need to improve long-term graft survival by reducing the incidence of chronic rejection.

Other Organ Transplantation

Heart, liver, pancreas, and lung transplantation are now established forms of therapy for end-stage organ failure. A beneficial effect of HLA matching is also observed in heart, pancreas, and more recently lung transplants.[114] [123] No beneficial effect of HLA matching has been observed in patients receiving primary liver transplants.

Concept of Allograft Acceptance

During the first two decades of organ transplantation, most investigations focused on mechanisms that lead to allograft rejection. An important by-product of the observed beneficial effect of blood transfusion was raised awareness that graft acceptance might be an active immunologic process. This concept suggests that a primary role of immunosuppressive drugs in successful allograft acceptance is to facilitate the emergence of DSH. Long-term graft survival would thus depend on how effectively this process is achieved. Multiple mechanisms of active immune regulation have been identified in experimental transplant models. Donor-specific T-suppressor cells, adoptively transferred from an engrafted animal to a nonimmune animal, support acceptance of a similar graft in that animal.[124] [125] [126] Early graft acceptance also may be promoted through the induction of anti-idiotypic antibodies,[66] [69] although the relationships between the early and late regulatory events have not been formulated. Recent studies of cytokine profiles locally secreted by graft-infiltrating cells may reflect the state of immunologic balance in the allograft. Acute rejection is associated with elevations in IL-2, IFN-, and CTL-derived enzymes granzyme B and perforin. IL-2 and IFN- are secreted by the Th1 type of helper T cell. Cytokine patterns characteristic of Th2-type cells, IL-4 and IL-10, are reported more frequently during graft acceptance.[127] [128] Finally, donor graft-derived passenger leukocytes have been observed to persist in the peripheral lymphoid tissues of some animals and patients with long-term allograft acceptance.[129] [130] Persistence of mononuclear leukocyte microchimerism has been proposed, through unknown mechanisms, to induce or sustain a state of donor-specific unresponsiveness.[128] However, this theory remains controversial. Other investigators, noting that microchimerism can also be detected in patients with failed grafts, suggest that is an epiphenomenon associated with effective general immunosuppression.

Techniques and reagents for DSH induction during the pre- or peritransplantion period are actively sought. A variety of reagents, including monoclonal anti-CD4, a recombinant molecule, CTLA-Ig, and others, have been used to study DSH induction experimentally.[131] [132] Anti-CD4 and CTLA-Ig are in Phase I clinical trials. The general events that seem to favor DSH induction are the production of generalized immunosuppression in the recipient during graft or donor antigen placement, while concurrently giving a reagent that effectively prevents a primary immune response to specific donor antigens. In many models, DSH persists after the period of general immunosuppression has abated. Implementation of effective DSH induction will clearly provide a major new treatment modality for organ transplantation. There is no current evidence that the new protocols result in true acquired tolerance, but they do appear to shift the balance in favor of graft acceptance.

To summarize, alloimmunization initiates two opposing immune responses that are in dynamic balance: strong effector immune responses that cause allograft rejection, and relatively weaker regulatory immune responses that potentially suppress graft rejection responses that are waning or have been actively blunted through immunosuppression. The intensity of effector immune responses is less when allografts are well matched for histocompatibility antigens. When effector responses predominate, graft rejection occurs. When regulatory mechanisms predominate, the rejection process is down-regulated and a state of graft acceptance ensues.

HLA Alloimmunization in Platelet Transfusion

HLA class I molecules, expressed on platelets, are a major cause of immunologic refractoriness in the alloimmunized thrombocytopenic patient.[133] [134] [135] Although it would be useful to provide HLA-identical platelet products to patients who need repetitive platelet transfusions, extensive HLA polymorphism precludes this as a routine practice. Further, additional factors contribute to the refractory state, especially in patients who are treated intensively with chemotherapeutic agents that damage vascular endothelium.[136] Platelet-specific antigens, such as HPA-1a ($P1^{A1}$), are uncommon causes of refractoriness because each diallelic system contains a high-frequency allele.[137] In about one-third of transfusion events, the cause of apparent immunologic refractoriness cannot be identified. Thus, most patients receive either random pooled platelets or ABO-compatible random

single-donor platelets until signs of immunologic refractoriness develop; then efforts are made to provide HLA-compatible single-donor platelet products.

The optimal clinical approach to this problem is to prevent HLA alloimmunization. Two methods that prevent or forestall the development of the refractory state, leukocyte depletion of platelet products[138] [139] and ultraviolet (UV) irradiation of platelet products,[140] [141] can reduce HLA alloimmunization by 50%, presumably by eliminating APCs.[142] Class II molecules are important during the cognitive phase of primary alloimmunization. Experimental models of alloimmunization indicate that recognition of class I alloantigens may not occur in the absence of metabolically functional class II-bearing APCs.[143] Platelets bear only class I HLA antigens. Both clinical methods remove the class II-bearing APCs (monocytes, dendritic cells) either physically (through leukodepletion) or functionally (through UV irradiation).

Although the number of thrombocytopenic patients alloimmunized by repetitive transfusion of blood products may be dramatically reduced with these procedures, alloimmunization is not eliminated. Patients at risk for the development of refractoriness to platelet transfusions can be alloimmunized from prior transplantation, pregnancies, and transfusions in which depleted products are not used. In these cases, HLA-matched platelets may be required. At least 1,5003,000 HLA-typed apheresis donors are needed for a reasonable chance of procuring several compatible platelet products for the average patient.[144] [145] When compatible donors are not found, subsequent selection of donors having one or more mismatched antigens is often random. However, it is possible to increase the likelihood of providing mismatches to which the patient is not yet alloimmunized through an understanding of the types of HLA antibodies induced by alloimmunization.

Concepts of HLA Matching and Permissible Mismatching

Table 134-5 summarizes a hierarchical strategy used in our laboratory for the selection of HLA-typed single-donor platelet

TABLE 134-5 -- Search Strategy for Potential HLA-Typed Platelet Donors That Includes Analyses of Public Epitopes and Antibodies to Public Epitopes

Data needed for search
Recipient phenotype (based on private epitopes): A1, 3, B8, 27
Recipient phenotype (based on public epitopes); 1C, , 7C, 8C, 4C, 6C (see Table 134-3)
Recipient serum analysis: anti-2C (A2, 28, 9, B17) HLA antibodies detected
Prioritized donor search
HLA match
HLA compatible[a]
HLA public match[b]
Permissible mismatch[c]
Random crossmatching
Acceptable donor phenotype(s) for:
HLA match: A1, 3, B8, 27

HLA compatible: Any donor with one or two blanks in the phenotype (e.g., A1, , B8,)
HLA public match: Any donor with no public epitope incompatibilities
Permissible mismatch: Any donor lacking the 2C public epitope
Random crossmatching: Any crossmatch-negative donor[d]

From Rodey and Fuller,[5ε] with permission.

[a] Platelet donor has blanks in phenotype due to either homozygosity or rare HLA alleles.
[b] Donor phenotype usually contains mismatched private epitopes, previously referred to as cross-reactive mismatching.
[c] Selection requires careful analysis of recipient serum for HLA alloantibodies.
[d] No evidence of donor-specific HLA or platelet antibodies in recipient serum.

products.[5ε] HLA match (also referred to as A match) requires full HLA-A and -B phenotypes in both recipient and donor and matching of all four alleles. HLA-compatible means that no mismatches exist. This occurs when the donor phenotype contains blanks in the phenotype (fewer than four HLA-A and -B alleles identified). Incomplete phenotyping is usually due to homozygosity or, less commonly, to a rare allele for which the laboratory has no reagents. When it is due to homozygosity, the products are actually matched. HLA public match is also referred to as cross-reactive matching. This is an important partial matching strategy, because antibodies directed against the public epitopes are the most frequent cause of the highly alloimmunized state. Selection of donors matched for public epitopes thus increases the odds that the patient will not have antibodies directed against the platelet product.

Most centers that provide HLA-typed single-donor platelets already select products for refractory patients on the basis of the criteria summarized previously. When donors cannot be found in these groups, the next step traditionally has been to select donors with one or more arbitrarily mismatched antigens. The process can be greatly improved if the specificity of HLA antibodies can be ascertained in advance. When HLA antibody specifications are known, mismatched antigens to which the patient has developed antibodies can be avoided. This strategy is referred to as selective or permissible mismatching. Because the highly HLA sensitized state is caused by relatively few antibodies directed against high-frequency public specificities rather than many antibodies against low-frequency specificities, determination of antibody specificity is usually feasible, even in patients with reactivity against >75% of donor cells. [146] [147]

Finally, selection of HLA-compatible donors by crossmatching is effective if sufficiently sensitive procedures are used to detect anti-HLA antibodies. Crossmatching does not need to be random, and the probability of selecting crossmatch-negative products from available single-donor products is significantly improved by selecting products that are public epitope- or CREG-compatible and do not contain HLA specificities to which the patient has known HLA antibodies.

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SUMMARY

HLA molecules were originally defined serologically as transplantation antigens. Clinically, the most common application of histocompatibility testing is still the selection of donors for recipients who require organ and tissue transplantation or compatible single-donor platelet products. The biologic significance of HLA molecules, however, extends far beyond their applications in transplantation, and the greatest potential clinical applications of HLA in transplantation and in the treatment or prevention of other diseases have not been realized. As the crucial role of HLA molecules in immunologic reactivity is further defined and understood, additional therapeutic applications will undoubtedly emerge.

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NEUTROPHIL ANTIGENS AND IMMUNE NEUTROPENIAS

The development of antileukocyte antibodies after blood transfusion was first reported by Doan in 1926. [148] Later, the presence of leukoagglutinins was described in association with agranulocytosis. [149] [150] The demonstration of leukocyte antibodies in sera of multiparous women [151] [152] subsequently led to the discovery of the HLA antigens. Concurrent with this event, neonatal neutropenia due to fetomaternal incompatibility was described, [153] and investigation of the involved antibodies demonstrated the existence of several alloantigens expressed only on neutrophils. [154] [155] [156] Further studies of these antigens established their polymorphism and their role in autoimmune neutropenia, [157] [158] febrile and pulmonary transfusion reactions, [159] [160] [161] [162] quinine-induced

TABLE 134-6 -- Neutrophil Antigens Identified by Alloantibodies

Neutrophil-specific antigens (N series)
NA: (NA1, NA2, NC, SH, SAR, LAN)
NB: (NB1, NB2)
Antigens shared with other leukocytes
Mart, Ond
Antigens with wide tissue distribution
HLA, group 5
Antigens defined by cold-reactive antibodies
I, i

NA series are located on FcRIIIb, Ond on CD11a (LFA-1), and Mart on CD11b (C3bi) receptors.

neutropenia, [163] and in some neutropenias following bone marrow transplantation. [164]

In addition to neutrophil-specific antigens, other clinically important antigens, not restricted to neutrophils, have been identified by serologic methods. Also, the use of monoclonal antibodies has led to recognition on neutrophils of functionally important structures such as adhesion molecules and receptors. In this section, the emphasis will be on neutrophil antigens defined by alloantibodies.

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NEUTROPHIL ANTIGEN CLASSIFICATION

The classification in Table 134-6 is based on differences in distribution patterns, serology and relationships with known functional structures. Neutrophil alloantigens are divided into those expressed only on neutrophils, those shared with other leukocytes, and those with wide tissue distribution. Phenotype and gene frequencies of the antigens commonly associated with neutrophils are described in Table 134-7 .

Neutrophil-Specific Antigens (N Series)

The letter N used in the nomenclature of neutrophil-specific antigens indicates their restriction to neutrophilic leukocytes; genetic loci and individual alleles are identified alphabetically and numerically, respectively. The antigens thus far identified in this group (Table 134-6) all have been involved in the development of alloimmune neonatal neutropenia and represent epitopes on the low-affinity Fc receptor on human neutrophils (FcRIIIb, CD16). Another common property is their absence from myeloid precursors and their appearance after metamyelocyte differentiation. Biochemically, the NA antigens are sialoglycoproteins varying in molecular weight between 50 and 80 kd, depending on the degree of N-glycosylation. Anti-NA antibodies bind to a 2328 kd Mr protein backbone. Neutrophils usually express two types of low-affinity IgG receptors known as FcRII (CD32) and FcRIII (CD16). A third receptor, the high-affinity FcRI (CD64), appears only on neutrophils produced after administration of growth factors (granulocyte colony-stimulating factor, G-CSF). Characterization of the Fc receptors on human leukocytes began with their discovery by Messner and Jelinek in 1970,[165] followed by recognition of their high- and low-affinity forms[166] and further characterization of the low-affinity forms with the use of monoclonal antibodies.[167] [168] In 1985 Werner et al.[169] discovered the localization of the NA antigens on the neutrophils Fc receptor. Subsequently, the low-affinity receptors on neutrophils were found to differ from

Figure 134-7 Comparison of amino acid sequence differences in CD16 molecules as FcRIIIb expressed on neutrophils (NA1, NA2, SH) and FcRIIIa on NK cells.

those expressed on other cells (NK cells, large granular lymphocytes, T_s , and monocytes),[170] the receptor on NK cells having a 610 kd larger molecular mass with transmembrane and cytoplasmic domains. The receptor on neutrophils was found to be susceptible to dissociation by phosphatidylinositol (PI)-specific phospholipase C, and thus it was established to be anchored to the outer leaflet of the membrane lipid bilayer through a PI-anchoring mechanism (Fig. 134-7). The genes controlling these two forms of receptors (FcRIIIA on NK cells and B on neutrophils) are linked and mapped on chromosome 1q22. In the FcRIIIA gene, a single codon difference converts an in-frame UGA termination codon to CGA (Arg), thereby extending the reading frame for the transmembrane and cytoplasmic domains. The two FcRIII molecules also differ in their amino acid sequences,[171] as shown in Figure 134-7 . Whereas no polymorphism is recognized for FcRIIIA, several point mutations have been identified in FcRIIIB that relate to NA1, NA2,[172] and SH[173] alleles. The amplified cDNAs encoding FcRIII differ in NA1 and NA2 at five nucleotides, predicting four amino acid substitutions. As a result, NA1 has four and NA2 has six glycosylation sites, which accounts for higher molecular weight of NA2. Also, one point mutation, 266 C A (A1a Asp), accounts for the difference between NA2 and SH. In addition to a mutation, the SH phenotype has been recently shown to be the result of a gene duplication.[174] Similar to SH, NC1 is associated with NA2. The gene frequencies of NA1 and NA2 have been extensively studied in different populations.[175] [176] [177] NA2 has been found to be the most frequent allelomorph among whites, whereas NA1 constitutes the more frequent gene among Asians.[175] LAN[178] and SAR,[179] causing alloimmune neonatal neutropenia, are high-frequency antigens. Several cases of alloimmune neonatal neutropenia have been described in which the maternal neutrophils

TABLE 134-7 -- Phenotype and Gene Frequencies of Neutrophil Antigens

	NA1	NA2	NC1	NB1	NB2	5^a	5^b
Phenotype (%)	46	88	91	97	32	29	96
Gene frequency	0.367	0.633	0.72	0.827	0.173	0.181	0.819

did not react with either anti-NA1 or anti-NA2 antibodies.[180] [181] [182] Because neutrophils from these individuals do not react with monoclonal anti-CD16, they are considered to be NA^{null} . Foremont et al.[183] found four NA^{null} individuals among 3,377 randomly tested French donors. Several other antigens (ND1, NE1, and HGA-3) have been described that appear to belong to the NA series. Unfortunately, the unavailability of antisera has prohibited their further characterization.

Extensive studies on FcRIII biology have clarified the functions of the NA antigens. They serve as a binding site for dimeric, complex and aggregated IgG, and participate in phagocytosis of antibody-coated particles. Neutrophils with NA1 phenotypes have been reported to be more efficient than NA2 in the phagocytosis of IgG1 opsonized bacteria, and in forming rosettes with erythrocytes coated with IgG3 anti-Rh antibodies.[184] Clinically, in addition to their high immunogenicity, which leads to alloimmunization and autoimmune disorders, their PI-linked property provides a convenient tool for the diagnosis of paroxysmal nocturnal hemoglobinuria, in which a defective anchoring mechanism results in their reduced expression on the affected neutrophils. The absence of an NA gene does not seem to lead to clinically discernible neutrophil dysfunction. In our laboratory, several members of a family were identified to be NA^{null} and all have remained in perfect health over two decades of observation. It has also been shown that the neutrophil activation results in the release of the NA antigens into plasma.[185] These soluble forms have been considered to influence Ig synthesis by B cells and in HIV infection, and a progressive reduction in their plasma concentration has been suggested to have a prognostic value.[186]

NB System

These highly immunogeneic antigens share many properties with the NA group, even though they segregate independently.[155] [187] The NB antigens are neutrophil specific, appear after differentiation to metamyelocytes, are PI-linked,[188] and their antibodies cause alloimmune neonatal neutropenia and transfusion reactions. In a family with multiple members having the NA^{null} phenotype, we observed absence of NB antigens together with NA. Further clarification of the NB system awaits their study by new molecular methods.

The neutrophil antigens, defined by human antibodies, are not detected on nonprimate neutrophils. In primates, NA2 and NBI (and 5^b) were demonstrated on chimpanzee neutrophils and NBI was detected in baboons.

Antigens (Ond and Mart) Shared by Neutrophils and Other Leukocytes

Antibodies to two high-frequency antigens, Ond^a and $Mart^a$, were found in sera of a multitransfused and a multiparous donor, respectively.[189] [190] Using

antigen-immobilization and immunoprecipitation methods, Simsek et al.[191] have identified Ond^a on $_L$ (CD11a) and Mart^a on $_m$ (CD11b) subunits of $_2$ -integrins, respectively. Molecular characterization has determined that substitutions in $_L$ (Arg 766 Thr) and in $_m$ (Arg 61 His) account for the antigenic differences. It has been speculated that monoclonal antibodies with properties similar to Ond and Mart may have clinical applications in the control of GvHD.

Antigens with Wide Tissue Distribution

ABH

Early reports on the expression of ABH antigens on human neutrophils have not been confirmed. ABO incompatibility does not alter the in vivo survival of [111]In-labeled granulocytes or their ability to localize at the sites of infection.[192] Nevertheless, in granulocyte transfusion, ABO compatibility is required because of the high red cell content of the cell preparation.

HLA Antigens on Neutrophils

A general feature of HLA antigens is their reduced expression or loss concurrent with cell maturation and differentiation. In general, HLA antigens, particularly class II, cannot be detected on cells that do not proliferate. Accordingly, class II molecules are not demonstrable on neutrophils, even with the use of monoclonal antibodies. Class I antigens are detected but at a level much lower than the level at which they are found on lymphocytes.

Group 5

Antigens 5^a and 5^b , the two alleles known in group 5,[187] are expressed on all blood and solid tissue cells except erythrocytes.[193] Antibodies to 5^b cause febrile and pulmonary transfusion reactions,[161] [194] but not alloimmune neonatal neutropenia.

Leukocyte Antigens Defined by Cold-Reactive Antibodies

Anti-I and anti-i antibodies cause reversible cold agglutination of neutrophils similar to red cell hemagglutination.[195] These antigens also have been demonstrated on lymphocytes by cytotoxicity assays. Other types of cold-reactive leukocyte antigens have also been described that react with a cold leukoagglutinin present in low titers in 25% of normal donors. A high-titer cold leukoagglutinin was observed in the serum of a severely neutropenic patient,[196] presumably representing a disorder equivalent of cold agglutinin-associated hemolytic anemia.

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METHODS FOR DETECTION OF NEUTROPHIL ANTIGEN/ANTIBODY REACTIONS

The agglutination technique is the method of choice for detection of antibodies against the N series and group 5. These antibodies are not usually cytotoxic. Unlike hemagglutination, neutrophil agglutination is not mediated by the formation of immunologic bridges. Rather, the reaction is a temperature-dependent activity that requires viable cells. Neutrophils, activated by the effects of antibodies, form pseudopods and need hours of incubation to move toward each other and form aggregates. Thus, many reactions will be missed if the incubation period is inadequate. The immunofluorescence assays, [159] including flow cytometry, are also suitable methods. NB2 (9^a) and 5^b, however, do not react in the immunofluorescence tests, indicating the need to use multiple techniques for neutrophil antibody screening. Also, the immunofluorescence test employed for detection of neutrophil-bound IgG may produce misleading results because of nonspecific positive reactions found on neutrophils of patients who receive G-CSF. Subsequent studies in our and other laboratories [197] [198] revealed that G-CSF treatment induces expression on neutrophils of the high-affinity receptor for IgG (FcR1, CD64), which binds IgG from serum. A proper interpretation of a positive immunofluorescence reaction, therefore, must consider conditions likely to lead to expression of FcR1 on the test neutrophils. For monoclonal antibodies, flow cytometry remains the method of choice. Monoclonal antibody-specific immobilization of granulocyte antigen assay (MAIGA) [191] is a promising new method that allows the simultaneous detection of alloantibodies and determination of their position on any cell membrane structure to which a specific monoclonal antibody is available. In this assay, neutrophils are incubated with the test sera and with the desired monoclonal antibody. The cells are washed and then solubilized by detergents to release the alloantibody-Ag-monoclonal antibody

complex. A goat anti-mouse antibody, attached to a solid surface, is used to capture monoclonal antibody, and the presence of human Ig on the captured complex is detected by ELISA. In another method called luminoimmunoprecipitation, [191] neutrophils are biotinylated, incubated with the antibody source, washed, and solubilized, and the complexes are captured by agarose beads coated with staphylococcus protein A. The captured complexes are then subjected to SDS-PAGE, trans-blotted, and then visualized with enzyme-conjugated streptavidin and quantified. With the advent of molecular biology, neutrophil genotyping also has become practical, using well-defined primers to obtain NA1, NA2, and SH PCR products. Other methods used for detection of leukocyte antibodies are reviewed elsewhere. [192]

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CLINICAL DISORDERS RELATED TO NEUTROPHIL ANTIGENS AND ANTIBODIES

Immunologically Induced Neutropenias During Infancy

Four types of immunologically induced neutropenia during infancy are recognized: alloimmune neonatal neutropenia, transient neutropenia secondary to maternal autoimmune neutropenia, autoimmune neutropenia of infancy, and autoimmune neutropenia associated with hemolytic anemia or thrombocytopenia.

Alloimmune Neonatal Neutropenia

Alloimmune neonatal neutropenia is a disorder analogous to erythroblastosis that is caused by transplacental transfer of maternal neutrophil-specific alloantibodies into the fetal circulation. [153] [154] [155] [156]

Etiology and Pathophysiology

Maternal alloimmunization can occur any time after the first trimester of pregnancy when the hematopoietic cells are developed. The incidence of pregnancy-induced neutrophil alloimmunization has been estimated to be 0.10.2%, [199] [200] in contrast to 20% for the HLA antigens. The target for antineutrophil antibodies in the fetus and neonates is a small mass confined to mature neutrophils. This results in selective destruction of the mature cells, but the precursors as well as the monocytes and eosinophils are spared. These effects are distinct from those anticipated for the HLA-type antibodies, which must overcome the placental barrier where some cells express HLA. The transferred HLA antibodies are then likely to be neutralized by the antigens in circulation and on the surface of various tissue cells, reducing the antibody levels to the extent that adverse effects can occur only if the antibody concentration is overwhelming.

Infants affected by alloimmune neonatal neutropenia are, as a rule, neutropenic at birth. In some cases, however, neutropenia does not occur at birth but is recognized after a delay of 13 days. The immaturity of the macrophages involved in neutrophil destruction may be one explanation for this delay.

Among various neutrophil specificities, NA1 is involved in 34%, NB1 in 13%, and NA2 in 12% of cases of alloimmune neonatal neutropenia. In one-third of cases other specificities, many not yet defined, have been involved, and in 4% of cases no antibodies are detected. An unexplained high incidence of spontaneous abortion, stillbirth, prematurity, and twin pregnancy has been reported in the affected families.

Pathogenesis

Complement-dependent cell lysis does not seem to occur, because the antibodies do not fix complement. Phagocytosis of the opsonized neutrophils by macrophages has been shown in vitro and in the bone marrow of some of the patients with autoimmune neutropenia of infancy. [201] Antibody-dependent neutrophil agglutination may also prove to be a factor. Such aggregates, produced by activated C5, or experimentally produced by phorbol myristate acetate, become trapped in the lung capillaries. Entrapment of neutrophils may similarly occur in the splenic sinusoids and liver capillaries.

Clinical Presentation and Laboratory Evaluation

Alloimmune neonatal neutropenia is often asymptomatic, and the absence of neutrophils is discovered only if a routine blood examination is performed at birth. In symptomatic cases, fever, respiratory, urinary, or skin infections, mostly caused by staphylococci, -hemolytic *Streptococcus*, and *Escherichia coli*, develop within 1 or 2 weeks. Neutropenia may last from 2 weeks to 6 months. We have recorded only four deaths due to sepsis, all occurring before the disease was recognized.

The total leukocyte count is usually normal, and the diagnosis requires manual differential counts. The blood smear reveals paucity or complete absence of the mature neutrophils, often with eosinophilia and monocytosis (commonly reported as atypical lymphocytes). Band forms or more immature myeloid cells may also be seen. Red blood cells and platelets are normal unless there is an associated neonatal thrombocytopenia. In a retrospective study of sera from 33 affected families, we detected antineutrophil and platelet antibodies in 10. The bone marrow shows myeloid hyperplasia with a characteristic left shift often described as maturation arrest at either myelocyte, metamyelocyte, or band stage. Occasionally, the segmented neutrophils are found in the marrow in near-normal numbers.

Once neutropenia is recognized, the alloimmune nature of the disorder must be established by demonstrating neutrophil-specific alloantibodies in the maternal sera. Neutrophil specificity of the antibodies can be established by absorption of the maternal plasma with paternal platelets to remove non-neutrophilic antibodies.

Transient Congenital Neutropenia

Mothers with autoimmune neutropenia may give birth to infants who develop transient neutropenia. [202] In one of our patients, the mother had a persistent leukopenia, mild Crohns disease, and a palpable spleen. The fourth child, a premature infant, was born with a normal leukocyte count but on day 5 had only 1% neutrophils. The neutropenia lasted 6 weeks. Four years later the mother gave birth to her fifth child, who also developed a transient but severe neutropenia lasting 2 weeks. In the second family, the mother had had chronic neutropenia and a slightly enlarged spleen for at least 3 years when she gave birth to her first infant. On the second day of life, the infant had an absolute neutrophil count of 500, which returned to normal within 4 months. The following year, a second child was born and found to be neutropenic after 1 week, recovering within 4 weeks. The autoimmune nature of the mothers neutropenia was established by positive serologic tests.

Autoimmune Neutropenia of Infancy

Chronic benign neutropenia of infancy and early childhood has long been recognized. The autoimmune nature of this disorder was established when autoantibodies with specificity for neutrophils were demonstrated. [157] Autoimmune neutropenia of infancy is the most common form of chronic neutropenia in infants. [158] Severe neutropenia is usually recognized when the child is 47 months old. The diagnosis of neutropenia is made either by a routine blood test or during evaluation of an infection. The chronicity becomes apparent when the blood tests, repeated after recovery from infection, remain abnormal. The clinical course of autoimmune neutropenia of infancy is relatively mild, with occasional stomatitis, otitis, diarrhea, and respiratory infection. The incidence of these complications diminishes as the child grows older. Occasionally, chronic otitis results in

hearing loss, and persistent gingivitis in older children leads to tooth loss. Neutropenia is self-limited, and complete recovery within 16 years is the rule. Recurrence of

the autoimmunity has not thus far been observed on long-term follow-up. The cause of and the reason for the spontaneous loss of the autoantibodies have not been clarified.

Autoimmune Neutropenias Associated with Hemolytic Anemia and Thrombocytopenia

Autoimmune neutropenia may occur in combination with autoimmune hemolytic anemia and thrombocytopenia during the perinatal period. This disorder is distinct from autoimmune neutropenia of infancy, the clinical course often being much more severe.

Management of Immune Neutropenias in Infancy

These types of neutropenias are usually self-limited and compatible with normal growth and development. Affected infants, however, should receive protective mouth and skin care and kept from exposure to potential sources of infection. Hospitalization and vigorous antibiotic therapy are required only for severe infections. Recombinant G-CSF has temporarily corrected neutropenia in both alloimmune neonatal neutropenia and autoimmune neutropenia of infancy. The dose required varies from 1 to 5 g/kg and can be administered subcutaneously one to three times per week after a few initial daily doses. This treatment should be used only in symptomatic cases, and prophylactically when patients need surgery. Long-term treatment should be avoided. Intravenous administration of large doses of -globulin also reverses the neutropenia in autoimmune neutropenia of infancy. [203] In alloimmune neonatal neutropenia, the demonstration of neutrophil antibodies in the maternal sera indicates potential occurrence in future pregnancies. Preventive treatment with IV IgG during the last trimester, as recommended for alloimmune neonatal thrombocytopenia, is not warranted because of the benign nature of the disorder. But mothers with neutrophil alloantibodies should be warned about the possibility of febrile reactions if they receive blood transfusions. They should also be advised that transfusion of their blood, or even their packed red blood cells, into normal recipients might cause a severe and potentially fatal pulmonary transfusion reaction.

Differential Diagnosis of Neutropenias in Infants

The prognosis of neutropenias in infancy varies according to cause. Therefore, once neutropenia is recognized, every effort should be made to determine its cause. Immunologically induced neutropenias, except those associated with hemolytic anemia and thrombocytopenia, have a benign course. The nonimmune congenital forms, by contrast, last for life and may be complicated by repeated life-threatening infections. In alloimmune neonatal neutropenia, alloantibodies are demonstrated in the maternal sera. In autoimmune neutropenia of infancy, documentation of a normal neutrophil count before the apparent onset of the disease is most valuable. The diagnosis of autoimmune neutropenia of infancy is established by demonstrating antibodies in the affected children rather than in the maternal sera. A positive diagnosis of the transient congenital form is made by documenting an autoimmune neutropenia in the mother. The differential diagnosis includes disorders of neutrophil production, stem cell abnormalities, and neutropenias associated with infection.

Neutropenia Associated with Infections

The relative neutrophil reserve (the total number of neutrophils, bands, and metamyelocytes) in the bone marrow in newborn infants is small compared to adults. The bone marrow reserve in newborn rats has been estimated to be $2 \pm 0.1 \times 10^6$ /g, compared to $4.5 \pm 0.2 \times 10^6$ /g in adult rats.[204] Moreover, newborn rats, unlike adults, cannot increase the rate of stem cell proliferation in response to bacterial inoculation, presumably because their myeloid cells are already maximally stimulated. Similar limitations may contribute to the neutropenia associated with viral and bacterial infections in infants. In all these cases, other diagnoses should be suspected if the neutropenia persists after the acute inflammation has subsided.

Primary Autoimmune Neutropenias in Adolescents and Adults

The hematologic profile is that of an absence or reduction in the number of neutrophils, with or without a reduction in other granulocytes and lymphocytes. Monocytosis is commonly present. The bone marrow typically reveals an absence of mature cells with an increased number of myeloid precursors. Splenomegaly is not a consistent finding, and clinical manifestations vary. The serologic diagnosis is made by demonstrating neutrophil antibodies, and in some cases with specificity for the known antigens. In rare instances, neutropenia may be caused by cold-reactive antibodies.

Secondary Immune Neutropenias in Adults

Autoimmune neutropenias may be associated with systemic lupus erythematosus, rheumatoid arthritis, Sjögren syndrome, Grave disease, and lymphoproliferative disorders (e.g., T-cell or hairy cell leukemia). A combination with hemolytic anemia or thrombocytopenia, in a characteristic sequence, has been designated alternating autoimmune hemocytopenia. In this form, the disease is initially limited to only one of the cell types. Remissions occur with or without treatment, but relapses may involve other cell types. Alternation between neutropenia, thrombocytopenia, and hemolytic anemia continues until pancytopenia, often resistant to treatment, prevails. Neutropenia may also occur in infectious mononucleosis or *Mycoplasma* pneumonia. In these cases, cold-reactive antibodies have been considered to be a contributory factor.

Febrile Transfusion Reactions

The association between leukoagglutinins and febrile transfusion reaction has long been established. Anti-HLA, 5^b, and antibodies against neutrophil-specific antigens have been implicated. Because these antibodies do not occur naturally, the reaction occurs only in previously alloimmunized patients. More recently, nonhemolytic transfusion reactions have been attributed to the presence of cytokines such as IL-I and IL-6 in the donors plasma. [205] These cytokines are probably generated during storage of non-leuko-reduced blood products. In either case, transfusion of leuko-reduced products will minimize the reaction.

Pulmonary Transfusion Reaction

This serious complication is a noncardiogenic pulmonary edema attributed to the presence of leukocyte antibodies in the donors plasma and should be suspected in all patients who experience unexplained acute respiratory distress syndrome following transfusion of blood or blood products. The radiologic appearance of bilateral pulmonary infiltrates, without cardiomegaly or pulmonary vascular congestion, is diagnostic.

FUTURE DIRECTIONS

The development of new methods such as MAIGA and the more frequent use of techniques of molecular biology should

further facilitate characterization of the ever-increasing number of neutrophil antigens, especially those that represent polymorphism of adhesion molecules and their receptors. Significant progress has already been made in elucidating the functions of the FcRIII molecule. It is now known that collaboration between this receptor and FcRIIa is involved in the phagocytosis of opsonized bacteria, and that the binding of soluble CD16 molecules to its receptor, the CR3 on monocytes, induces production of IL-6 and IL-8. [206]

In autoimmune neutropenia, a disorder far more common than is generally appreciated, often neutrophils are the only affected cells. This model of autoimmunity, in which the target can be defined at its amino acid sequence (i.e., NA antigens), offers an opportunity for research on the pathophysiology of the immune system. Examples of autoimmune leukopenia in which neutrophils and other leukocytes are affected together may now be explained by the discovery of new target antigens that are shared by neutrophils and other leukocytes, like those representing adhesion molecules and their receptors. Moreover, understanding of the relationships between the target antigens and functional structures may reveal why some neutropenic patients remain asymptomatic whereas others, even with higher leukocyte counts, develop serious symptoms.

A promising new approach in the treatment of autoimmune neutropenia is the administration of G-CSF. Treatment with low doses of G-CSF leads to elevated neutrophil counts and elimination of symptoms. In these patients, treatment with G-CSF probably converts an uncompensated disorder to a compensated form, analogous to compensated forms of autoimmune hemolytic anemia in which the patient maintains a normal hematocrit. High doses and long-term administration of G-CSF, however, invariably result in thrombocytopenia, a complication that should be avoided but is correctable by reducing the dose. The effects of G-CSF on immunologically induced neutropenias may prove to be more profound. In this model, the autoimmune disease is controlled not by immunosuppression but by increasing the mass of the target organ, a feat not achievable in other tissues. The impact of this challenge to the immune system needs to be evaluated. The increase in the target mass may drive the affected immune system either to exhaustion, leading to a full recovery, or perhaps to further aggression.

With the introduction of G-CSF, there will be a reduction in the use of intravenous immunoglobulins. IV IgG, however, may be needed in cases of leukopenia in which cells other than neutrophils are destroyed. The future will determine whether growth factors with broader hematopoietic activities would be beneficial in these forms of leukopenia.

ACKNOWLEDGMENT

This work was supported by the Jimmy Pollock Research Foundation.

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Chapter 135 - Principles of Red Blood Cell Transfusion

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INTRODUCTION

Approximately 12 million units of blood were transfused in the United States in 1992. This figure is striking if one remembers that the era of blood transfusion began only about a century ago, with the discovery of the ABO system by Landsteiner.[1] Actually, it was not until about four decades later, with the description of the Rh system by Levine, Stetson, Landsteiner, Wiener, and Peters[2] [3] [4] and, perhaps even more critically, with the introduction of a safe and effective anticoagulant-preservative solution suggested by Loutit and Mollison,[5] that the clinical practice of transfusion as we know it began. Another important landmark at about the same time was the development of the antiglobulin test by Coombs,[6] which permitted the detection of antibodies in recipient plasma that did not produce direct agglutination in the test tube but might be capable of causing in vivo red cell destruction.

After these initial breakthroughs, the next several decades of development in transfusion practice focused on improvements in the preservation of red cells and on expansion of knowledge of red cell antigens and their clinical significance. However, it was not until the mid-1960s, with the introduction of plastic blood packs by Walter and Murphy,[7] that a new phase, the era of component therapy, began. This innovation, combined with the ability to store blood for extended periods, again revolutionized transfusion practice.

The next two decades saw major advances in transplantation and open heart surgery and in therapy for many malignancies, supported by the ready availability of blood component support. During this period, although some adverse effects of transfusion, such as transmission of hepatitis, became well established, the new-found benefits of transfusion seemed to outweigh any risks.

Since the recognition in late 1982 that the human immunodeficiency virus is transmissible by blood transfusion, the risks of transfusion are weighed more heavily against the benefits. Donor blood is subjected to an ever-growing battery of tests to detect potentially transmissible infectious agents, and the era in which ABO compatibility was the major hurdle seems eons away.

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RED BLOOD CELL PRESERVATION AND STORAGE

The first key to storage of blood for later transfusion is a stable, minimally toxic anticoagulant with preservative properties. During the early 1900s it was recognized that citrate met these criteria.[8] [9] [10] Although slightly more toxic than heparin, if given rapidly and in large amounts, citrate has preservative action that heparin lacks. Citrate has the added advantage of not causing systemic anticoagulation in the recipient.

The other factor essential for long-term storage is a mechanism to maintain cell viability and function. Fresh transfused red cells have a good survival rate in the recipients circulation, with a destruction rate approximately equal to that of the recipients own cells: 1%/day. [11] The arbitrary standard used for stored red cells requires 70% to remain in the circulation for 24 hours.

Adenosine Triphosphate Levels

Adenosine triphosphate (ATP) levels appear to be a major determinant of red cell viability. [12] [13] [14] The drop in cellular ATP levels during storage has been correlated with the change from a disk to a sphere configuration, with increased cell rigidity, and with loss of membrane lipid leading to decreased critical hemolytic volume. [15] For this reason, most efforts to extend the period of red cell storage have focused on ways to maintain intracellular ATP levels. First, dextrose was introduced into the citrate solution (citrate-phosphate-dextrose [CPD]: 21 days), to which adenine was added (CPDA-1: 35 days). Recently, two additive solutions containing additional dextrose and adenine (Nutricell, or AS-3) or dextrose and adenine plus mannitol (Adsol, or AS-1) have been licensed, extending the maximum storage time to 42 days (Table 135-1). Unfortunately, ATP is not the sole determinant of red blood cell viability. [16] It appears that when intracellular ATP is maintained at >1.5 mol/g hemoglobin (Hb), other factors also predict viability. Because these factors remain undefined, no in vitro test is available to predict the in vivo survival rate of stored red cells.

2,3-Diphosphoglycerate Levels

Stored red cells must also maintain their capacity to deliver oxygen. It was not until 1967 that the central role of 2,3-diphosphoglycerate (2,3-DPG) in releasing oxygen from oxyhemoglobin was recognized.[17] [18] Interestingly, it had already been observed that the oxygen dissociation curve of stored red cells was shifted to the left. [19] Attention was then focused on ways to maintain

TABLE 135-1 -- Biochemical Changes in Stored Red Cells

Variable	CPDA-1		Adsol
	Fresh	35 Days	35 Days
In vivo survival (at 24 hours) (%)	100	<71.0	<88.0
pH	<7.5	< 6.7	< 6.7
ATP (% initial)	100	<45.0	<76.0
2,3-DPG (% initial)	100	<10.0	<10.0
Plasma K^+ (mEq/L)	5.1	<78.5	<49.0

Data from Fenwal Laboratories, [117] Zuck et al.,[118] and Moore et al.[119]

high levels of 2,3-DPG in stored cells. The first anticoagulant introduced on a large scale, acid citrate-dextrose, was ineffective because of its low initial pH, so that the new CPD, with its higher initial pH and slower fall in pH, was superior. CPDA-1, Adsol, and Nutricell have not further improved 2,3-DPG maintenance. Although 2,3-DPG depletion of stored red cells is known to decrease their oxygen delivery, the clinical significance of these findings is unclear. 2,3-DPG levels in stored cells are rapidly regenerated in vivo, rising to 50% of normal within several hours and to normal within 24 hours. [20] Although a patient with normal cardiac status should be able to compensate by increasing cardiac output to maintain normal oxygen delivery until 2,3-DPG levels are regenerated, an improvement in 2,3-DPG preservation in stored red cells is still desirable. New additive systems containing ascorbate, ascorbate-2-phosphate, or other materials that maintain pH and 2,3-DPG levels are under study but have not yet been licensed for clinical use. [21] [22]

By-products of Red Cell Storage with Potentially Adverse Effects

Infusion of large volumes of blood with citrate anticoagulant over a short period may cause plasma citrate levels to reach the toxic range. The primary concern is the cardiovascular effects of hypocalcemia caused by chelation of calcium by citrate. The risk of citrate toxicity is exacerbated in the setting of liver dysfunction or liver immaturity, so that the situations of greatest risk appear to be exchange transfusion of premature infants and massive transfusion. Despite these theoretical considerations, there is little documented evidence of clinical citrate toxicity, and the problem can usually be prevented by slower infusion. [23] If large amounts of blood have to be infused over a very short period, administration of calcium gluconate can be considered, but whether the benefits justify the risk is controversial. [24]

Another issue with prolonged storage is the question of excess potassium in the red cell supernatant that could cause cardiac problems. At a storage temperature of 4°C the red cell sodium-potassium pump is essentially nonfunctional, and intra- and extracellular levels gradually equilibrate. In addition, the hemolysis that occurs during the storage period results in increased potassium in the supernatant. However, because the total volume of plasma in red cell concentrates is low (70 ml), the total potassium burden is only 5.5 mEq at product expiration. Practically speaking, the potassium load is rarely a clinical problem except in the setting of preexisting hyperkalemia and renal failure. In this situation, fresher units of red cells or washed red cells may be used.

Microaggregates consisting of platelets, leukocytes, and fibrin form during blood storage. Although several studies have shown that pulmonary dysfunction after massive transfusion may be significantly diminished by the use of a microaggregate filter of pore size 2040 m, [25] [26] other studies have not confirmed microaggregates as the cause of hypoxia.[27] [28] Since microaggregate filters have been found to slow the infusion rate, their use is not uniformly recommended in cases of trauma. Some authorities suggest their application in cardiopulmonary bypass and when more than 5 units of blood are to be given. [29]

Since their introduction in the 1960s, plastic blood bags used for storing red cells have been made from polyvinylchloride containing the plasticizer di(2-ethyl)phthalate (DEHP), which confers pliability. Nevertheless, questions have been raised for many years about the safety of DEHP, which is known to be lipophilic, to leach from the bag and to be present at levels of 5070 mg/L in stored red cells. [30] Numerous studies in animal models have addressed the toxicity of the substance and are summarized in a comprehensive review.[31] The DEHP effects found in animals range from a shock lung-like picture, to loss of fertility, to

hepatocarcinogenicity, to teratogenicity. Although

none of these adverse effects has been documented in humans, the question of potential toxicity has obviously been raised, initiating a search for alternative materials.[32] The situation is complicated by DEHP having been shown to have a significant stabilizing effect on the red blood cell membrane, and red cells stored in various containers lacking DEHP have shown unacceptable recovery at the end of the storage period. This decreased recovery has been shown to be due to increased hemolysis and increased osmotic fragility. [33] Recently, however, Baxter-Fenwal has introduced a new plastic, PL 2209, a polyvinylchloride plasticized with butyryl-*n*-trihexyl-citrate (BTHC) in place of DEHP. BTHC undergoes less leaching from the bag than does DEHP and has passed numerous safety tests. In addition, the 24-hour post-transfusion recovery of red cells held in these bags to the end of the permitted storage period is excellent (83%), and hemolysis is minimal. [34]

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RED BLOOD CELL COMPONENTS

Modern transfusion medicine assumes that it is preferable to give the patient the specific portion of the blood required rather than whole blood: red cells for oxygen-carrying capacity, plasma for coagulation proteins, and platelets for microvascular bleeding. The component therapy approach allows for optimal use of a limited community resource (Table 135-2). Methods for extended storage of red cells do not result in optimal preservation of other blood elements.

Today the clinician wishing to increase the patients oxygen-carrying capacity is more likely to be using a red cell concentrate than whole blood, although there may still be situations in which whole blood, if available, is appropriate. Several modifications can be made to red cell products to render them leukocyte or plasma depleted that have particular clinical applications. Red cells can also be frozen for long-term storage.

Whole Blood

A unit of whole blood is collected in CPDA-1 anticoagulant, giving it a shelf-life of 35 days and a volume of approximately

TABLE 135-2 -- Red Cell Components: Characteristics and Indications

Component	Characteristics	Indications
Whole blood	High volume; good flow	Combined red cell/volume deficit (massive hemorrhage; exchange transfusion)
Red blood cells	Lower volume	Red cell deficit
	Higher hematocrit	
	Good flow in AS-1	
Leukocyte-reduced red blood cells	$<10^8$ leukocytes	Prevention of febrile reactions
	$<10^8$ leukocytes	Reduction of alloimmunization
Washed red cells	Plasma depletion	Prevention of severe allergic reactions
	Must use within 24 hr	Prevention of anaphylaxis in IgA deficiency
Frozen red cells	Long-term storage	Rare donor unit storage
	Plasma and leukocyte depletion	Autologous storage for postponed surgery
	Must use within 24 hr of thawing	

510 ml (450 ml of blood plus 63 ml of CPDA-1). Within 24 hours of collection, the platelets in the unit, [35] as well as the granulocytes, are dysfunctional, and several plasma coagulation factors have fallen to suboptimal levels. [36]

Whole blood has the single advantage of correcting simultaneous deficits in oxygen-carrying capacity and blood volume. Therefore, whole blood is indicated in the management of trauma or in surgical cases involving extensive blood loss. In this setting, whole blood has two distinct advantages: (1) it provides colloid osmotic pressure and some coagulation factors not supplied by crystalloid solutions, and (2) it does not expose the recipient to red cells and plasma from different donors.

However, the goal of using whole blood for all cases of concomitant red cell and volume deficit is a very difficult one to achieve in practice. Not only is it difficult to balance the overall community need for production of other blood components, but it may be difficult for the hospital to predict inventory needs. Most cases requiring massive transfusion involve either trauma victims or emergency cardiovascular surgery. Although various studies of this issue maintain that 2050% of red cell transfusions should be met with whole blood, [37] [38] [39] some trauma centers manage patients exclusively with red cell concentrates. [40]

In the absence of whole blood, the simultaneous need for volume and oxygen-carrying capacity can be met by combining red cell concentrates with one of several volume expanders. If blood loss is mild to moderate, volume can generally be replaced with crystalloid (e.g., normal saline), which has the dual advantages of sterility and economy. In the setting of massive transfusion, there may be a need to replace intravascular proteins. Colloid solutions (albumin and plasma protein fraction) are expensive but are preferred to plasma because they carry no risk of disease transmission. In fact, plasma is rarely indicated, even in the setting of massive transfusion, unless there is a well-documented coagulopathy, as in the setting of liver failure. [41] [42] In addition, in some cases of trauma and cardiovascular surgery platelet transfusion may be indicated to combat microvascular bleeding from dilutional thrombocytopenia or bypass-associated platelet dysfunction. The transfusion of an average adult dose of platelets usually supplies the equivalent of several units of relatively fresh plasma, so that there is no reason for further donor exposure by the administration of frozen plasma.

Whole blood that is <5 days old is indicated for exchange transfusion in infants to provide the proper hematocrit and coagulation factors while limiting donor exposure. For elective surgery commonly associated with massive transfusion, particularly liver transplantation, in which large amounts of plasma are transfused, whole blood would certainly be advantageous if it could be made available.

Red Cell Concentrates

Red cell concentrates are obtained from whole blood after removal of most of the plasma for the production of frozen plasma or platelets, or both. At most blood centers, the red cells are then mixed with 100 ml of an additive nutrient solution that extends the storage period to 42 days [43] and results in flow properties similar to those of whole blood. [44] Alternatively, red cells can be produced from CPDA-1-anticoagulated blood and stored for 35 days.

Red cell concentrates are the product of choice for the correction of an isolated defect in oxygen-carrying capacity, as in chronic anemia. These concentrates have a particular advantage over whole blood in patients with cardiovascular compromise, who might be unable to deal with the volume of whole blood.

Another setting in which packed cells rather than whole blood must be used is the emergency transfusion of patients of unknown ABO type. Concentrated type O red cells are transfused after the plasma containing isohemagglutinins is

removed to prevent potential hemolysis of the recipients red cells.

Leukocyte-Reduced Red Cells

Leukocyte-reduced red cells (LRRCs) can be prepared by a variety of methods, resulting in differing degrees of white cell removal. The minimum standard, set by the American Association of Blood Banks (AABB), is a leukocyte number in the final component of $<5 \times 10^8$.[45] Early techniques of preparation involved centrifugation or washing with saline, whereby the buffy coat was repeatedly removed. Subsequently, a second-generation technique known as the spin-cool-filter method was introduced. This required use of 1-week-old red cells, which were centrifuged and then cooled for 4 hours to enhance microaggregate formation before passage through a microaggregate filter. Currently the most widely used method of leuko-reduction is filtration, which can be performed either in the laboratory or at the bedside. The various filters on the market result in >99% leukocyte reduction while depleting <10% of the red cells. [46] Most recently, blood bags with in-line filters, which allow prestorage leuko-reduction, have become available.

The major indication for the use of LRRCs has been the prevention of the nonhemolytic febrile transfusion reaction, which is the most common adverse effect of transfusion, particularly in multiply transfused patients or multiparous females. [47] Traditionally these reactions have been felt to be mediated by antibodies directed against leukocyte antigens,[48] but whether lymphocyte HLA antigens or granulocyte-specific antigens are the major targets has remained a subject of controversy. [49] Depletion of leukocytes to $<5 \times 10^8$ has been shown to prevent, or at least ameliorate, such reactions in most patients. [50] [51] Increasing evidence has accumulated suggesting that cytokines play a role in causing these reactions. [52] [53] Because cytokines may be released from leukocytes during storage, it has been suggested that prestorage leuko-depletion may be more helpful. At present, however, the practice is relatively limited, and it remains unclear whether this method will prove cost-effective in preventing such reactions. [53]

A second important indication for LRRCs is the prevention of alloimmunization to HLA antigens that can adversely affect post-transfusion platelet increments, such as in cancer patients undergoing chemotherapy. [54] [55] This approach will be effective only if platelets for transfusion can also be white cell depleted. According to current AABB standards,[45] the total leukocyte number must be $<5 \times 10^6$ when intended for this purpose. With the introduction of third-generation leuko-reduction filters for both red cells and platelets, this goal is achievable. However, there is little consensus on the degree of leukocyte depletion necessary to significantly diminish or eliminate alloimmunization.[56] [57] A recent multicenter study known as TRAP addressed this issue. The recently published data showed that the use of leuko-reduction filters significantly decreased the rate of alloimmunization but did not totally eliminate the problem. [58]

Leuko-reduction has also been studied for its ability to prevent transfusion-mediated immunosuppression, which is of particular concern in the postoperative period. (See further discussion under Appropriate Transfusion Practice in Various Clinical SettingsPerioperative Period.) Data collected to date suggest that this practice may be of value.[59] Indeed, even simple removal of the buffy coat from red cells (which is a routine procedure during component preparation in Europe) may provide a sufficient degree of leuko-reduction. [60] Because component preparation in the United States does not include a buffy coat removal step and because soluble white cell fragments may be capable of mediating immunosuppression, a prestorage leuko-reduction method might be required to abrogate this adverse effect in transfusion recipients in the United States. However, despite numerous studies in this area, the data remain contradictory. Therefore, at the present time, leuko-depletion for the sole purpose of preventing immunosuppression does not appear to be justified.

Washed Red Cells

Red cells are washed, using isotonic saline solutions, by either automated or manual techniques. Automated techniques are more efficient, but there is always some degree of red cell loss with each wash cycle. Because the washing is performed in an open system, the resulting product must be transfused within 24 hours.

The primary aim of washing is to remove plasma proteins, although some leukocytes and platelets are removed simultaneously. The major indication for washed red cells is the prevention of severe allergic transfusion reactions, thought to be mediated by recipient antibodies (most likely IgE) to donor plasma proteins. Washing is recommended when reactions are recurrent and severe, even in the face of antihistamine administration. In IgA-deficient patients who have preformed antibody to IgA, IgA-containing plasma can actually cause anaphylaxis (see Chap. 141). [61] Multiple cell washes may be required to remove the contaminating plasma protein. [62] [63]

Another setting in which washed cells may be indicated is paroxysmal nocturnal hemoglobinuria. [64] However, one study has suggested that these patients do not require washed cells.[65] In theory, washing removes complement components that could cause lysis of the patients complement-sensitive red cells.

Frozen Red Cells

Red cells can be frozen (with glycerol used as a cryoprotective agent) and stored in liquid nitrogen or mechanical freezers. The required concentration of glycerol depends on the rate and the temperature of freezing.[66] [67] [68] The freezing process destroys other blood constituents, except for a small percentage of immunocompetent lymphocytes.[69] Red cells are prepared for transfusion by thawing and washing away the glycerol using a series of progressively less hypertonic saline solutions, allowing glycerol to diffuse gradually from the cells to prevent hemolysis. The cells are resuspended in isotonic saline solution containing glucose. The extensive washing removes 99.9% of the plasma[70] as well as cellular debris.

Red cells can be stored in the frozen state for 10 years by regulation [45] and for considerably longer periods, with good viability. [71] After thawing and washing, storage is limited to 24 hours because of the open system. The 24-hour post-transfusion survival rate is 8590%. [68]

Frozen cells have been shown to maintain prefreezing ATP and 2,3-DPG levels. The standard is to freeze within 6 days of collection, while these factors are still present in high levels. When it is necessary to freeze older units, rejuvenation with a solution containing pyruvate, glucose, phosphate, and adenine has provided excellent results.[72]

The major indication for frozen red cells is the maintenance of a collection of rare donor units for transfusion to patients with unusual red cell phenotypes who have developed alloantibodies. Sometimes patients with such rare phenotypes can make autologous donations that can be frozen for later use. Cells from autologous donors can be frozen if more units are required than can be collected in the 42-day liquid storage period or if surgery is postponed. One other legitimate use for frozen red cells deserves mention. In patients with severe aplastic anemia awaiting bone marrow transplantation and in whom transfusion cannot be avoided, frozen cells have the least adverse effect on subsequent graft survival. [73] [74]

Because of the high cost and cumbersome nature of freeze-thaw procedures, other uses of frozen red cells are somewhat

difficult to justify. Occasionally, frozen red cells are effective in preventing severe febrile transfusion reactions when LRRCs in conjunction with medications have failed.

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APPROPRIATE TRANSFUSION PRACTICE IN VARIOUS CLINICAL SETTINGS

The response to red cell transfusion varies from patient to patient. In the absence of increased red cell destruction or sequestration, 1 unit of red cells can be expected to increase the Hb level by 1 g/dl or the hematocrit by approximately 3%. This rise is usually not measurable until 24 hours after transfusion, when the plasma volume has had time to return to normal. On the basis of a half-life of about 57.7 days for donor red cells, Mollison et al. [11] calculated that an average-sized adult requires 24 ml red cells/day to maintain a given hematocrit, assuming no red cell production. Petz and Tomasulo [75] estimated this requirement to be 2 units of red cells every 2 weeks.

Several factors can adversely affect the survival of transfused red cells. Hemolysis caused by either immune red cell damage or mechanical trauma shortens the survival of transfused cells, much as it shortens the survival of the patients own cells. Hypersplenism can lead to initial sequestration as well as increased destruction of red cells. Continued blood loss is another obvious cause of suboptimal response to transfusion. It should also be emphasized that transfusion suppresses erythropoiesis, so that the net result of transfusion may be less than expected. [76]

Chronic Anemia

As a rule, signs and symptoms attributable to anemia are unlikely to develop at an Hb level of >78 g/dl. [75] When the anemia is of gradual onset, the bodys compensatory mechanisms for maintaining oxygen delivery to the tissues come into play. Cardiac output is increased and intracellular 2,3-DPG is increased; thus, oxygen unloads at a lower oxygen saturation of Hb. When chronic anemia is due to red cell destruction, the healthy bone marrow can respond by increasing production by up to sixfold.

Red cell transfusion is always symptomatic and supportive rather than definitive therapy for anemia. Transfusion should be used only when there is no definitive treatment for the underlying cause, or when the severity of the anemia and the clinical manifestations in the patient make it impossible to wait for the effects of the treatment to be realized.

Generalizations about whether or when to transfuse red cells, and how many, are difficult to make and are usually inappropriate. The clinical impact of anemia varies, depending on its pathogenesis, rate of onset, the presence or absence of accompanying hypovolemia, and, most important, the individual patient. The Hb level at which a given individual manifests the signs and symptoms of anemia relates, in part, to underlying health status, cardiorespiratory reserve, and activity level.

Perioperative Period

Many generalizations have been made about the appropriate transfusion management of acute blood loss, often with very little hard data to support the arguments. One rule of thumb is that blood losses of 10% of total blood volume require no replacement therapy at all, losses of up to 20% can be replaced exclusively with crystalloid solutions, while losses of >25% generally require red cell transfusion to restore oxygen-carrying capacity, along with crystalloid and sometimes colloid solutions to restore intravascular volume and maintain perfusion. For years the figure of 10 g/dl of Hb has been used as the gold standard for the red cell transfusion trigger during the perioperative period. [77]

In the acquired immunodeficiency syndrome era, however, this approach is no longer justified. Each case must be evaluated individually on the basis of clinical signs and symptoms, rather than on the basis of laboratory values. If the cardiovascular system is healthy and the degree of hypoperfusion is not significant, good tissue oxygenation can be maintained at much lower Hb levels. A recent National Institutes of Health consensus conference suggested that many surgical patients do not need transfusion unless the Hb level falls to <7 g/dl. [77] Recent re-examination of these issues has laid to rest the long-held belief that these lower Hb levels interfere with wound healing[78] or automatically make general anesthesia a risk. [79]

Given that red blood cell transfusion should be tailored to individual needs, the question arises, is there any readily available, objective measurement that can be used to determine how low the Hb level can safely be allowed to fall before red cell transfusion is initiated? Several recent studies of acute normovolemic anemia in rats,[80] dogs,[81] and baboons[82] [83] have focused on the whole body oxygen extraction ratio (ER) as an indicator of when to transfuse. These studies make the seemingly valid assumption that the heart is the major organ at risk. With progressive hemodilution, healthy animals with normal coronary trees were able to maintain normal levels of oxygen consumption through a moderate increase in cardiac output, an increase in coronary blood flow, and a linear increase in the ER up to a ratio of 50%. As the hematocrit fell to <10%, however, oxygen consumption began to fall off, and the animals were no longer able to increase the ER enough to compensate for the low oxygen blood tension. An ER of 50% was found to represent the critical point at which the myocardium converted from aerobic to anaerobic metabolism, reflected in net lactate production. At this point, metabolic acidosis set in, resulting in hemodynamic instability. Most recently, experimenters compared the response to acute normovolemic anemia in healthy dogs to the response in dogs with critical coronary stenosis and found that both groups converted to anaerobic metabolism and went into congestive heart failure at an ER of >50%, but that an ER of >50% occurred at a hematocrit of 17.0% in the dogs with critical stenosis, compared to a hematocrit of 8.6% in the healthy group.[84] On the basis of these studies, it seems reasonable to extrapolate to humans and to suggest that the ER, which is a variable readily obtainable from standard hemodynamic monitoring devices, can be used to help assess transfusion need and that an ER of 50% can be used as a red cell transfusion trigger, since it appears to be a valid indicator of marginal myocardial oxygen reserve in both healthy persons and individuals with coronary artery disease.

Because the potential immunomodulatory effects of transfusion have been studied mainly in surgical patients, this topic is appropriately addressed here. The possibility of immunomodulation by allogeneic transfusions in humans was first suggested about 25 years ago when improved renal allograft survival was reported in pretransplant transfusion recipients. [85] A potential link between allogeneic transfusion and an increased rate of cancer recurrence and postoperative infection was first raised in the 1980s, [86] [87] [88] and numerous retrospective and prospective observational studies supporting these contentions have been reported in the literature since that time. These studies, as well as reports from investigations with opposing data, have been analyzed in two recent reviews on the topic. [89] [90] Studies in experimental animals have also yielded contradictory results. Research using animal models and human subjects on the specific immunomodulatory effects of transfusion has consistently pointed to depression of cell-mediated immunity, although the specific defects seen have varied. [91] [92] The mediators of these immunomodulatory effects appear to be leukocytes present in cellular blood components, including whole blood and red blood cells. [89] However, the fact that plasma transfusions have also been associated with immunosuppression suggests that soluble leukocyte fragments may also be capable of mediating the

adverse effect.[93] [94] Several well-designed, prospective, randomized controlled trials have been conducted comparing transfusion with leuko-reduced blood components or autologous blood to transfusion with unmodified allogeneic blood components. Based on previous reports and the apparent pathogenesis of the immunosuppression, it was predicted that the recipients of leuko-reduced blood or autologous blood would have a significantly lower risk of immunosuppression.

However, the results of these studies do not fully support the findings of the earlier observational studies, and controversy continues to rage. [60] [89] [90]

Red Cell Transfusion in Neonates

In neonates, as is often the case with adult patients, it is convenient to consider periodic, small-volume transfusion separately from massive transfusion situations. Not only are the trigger for transfusion and the optimal type of component very different in the two settings, but, particularly in infants, the potential adverse effects may be quite distinct.

Low-volume red cell transfusion is rarely indicated in full-term infants unless some acute blood loss has occurred at birth or an intrauterine situation has led to prenatal anemia. In contrast, premature infants are frequent recipients of these transfusions. In the intensive care setting, the premature infant is subjected to frequent blood sampling, and iatrogenic anemia may necessitate transfusion. Anemia of prematurity is also a well-recognized entity, with premature infants having a slightly lower Hb value at birth and with the postnatal decline in Hb occurring earlier and being more pronounced than in full-term infants. [95] The mechanism for this anemia of prematurity appears to involve relatively lower output of erythropoietin (EPO) in response to a given degree of anemia in premature infants. [96] [97] This may be due in part to the fact that the liver, rather than the kidney, is the major site of EPO production in these infants. [98] Although some have considered this degree of anemia to be physiologic, whether it is truly benign remains an area of controversy.

Another debate among neonatologists concerns what clinical signs and symptoms are valid reflections of poor tissue oxygenation and so should be used as triggers for red cell transfusion. Congestive heart failure and severe pulmonary disease are generally accepted indications for transfusion; however, recurrent apnea, tachypnea, tachycardia, and failure to thrive as transfusion triggers are more controversial. [95] [99] [100] Recently, the AABB has published very general guidelines for neonatal transfusion, and similar documents have been developed in Britain and Canada, but none of the three addresses the critical issue of how to define symptomatic anemia in this patient population.[101] Although this controversy has continued, the rate of transfusion and the donor exposure rate of premature infants have consistently declined. These changes, rather than being attributable to changes in the transfusion trigger, reflect improvements in patient care (e.g., microtesting methods resulting in less iatrogenic blood loss; the use of surfactant resulting in decreases in respiratory distress) as well as the use of a single unit to supply one infant over a longer period. The latter has become possible with the advent of the sterile docking technology, which preserves the full shelf-life of the unit of red cells, combined with accumulating solid evidence that fresh blood is not necessary for low-volume transfusions in neonates, because supernatant potassium and decreased pH are not of concern in this setting. [95] [100] Finally, several recent trials of EPO therapy in premature infants have been undertaken and are summarized in a recent review.[102] Although administration of relatively high-dose EPO has been shown to raise Hb levels and reticulocyte counts in healthy premature infants, the effect in sicker neonates is unclear. Although transfusion exposure was decreased, the significance of this is diminished, given the promise of the new strategies for limiting transfusions and donor exposure outlined above. Therefore, the high cost of EPO treatment may not be justified in this patient population. [103]

In the case of massive transfusion, the situation differs, with marked increases in transfusion in recent years in full-term as well as premature infants. With the advent of highly sophisticated techniques of open heart surgery in neonates and the introduction of extracorporeal membrane oxygenation (ECMO) as a means of temporarily sustaining life in neonates with severe but reversible cardiorespiratory disease, massive transfusion is no longer confined to those infants requiring exchange transfusion.

Despite the decline in Rh hemolytic disease of the newborn, this disease remains the main indication for exchange transfusion. The two triggers for exchange are (1) rapidly rising levels of unconjugated bilirubin that may lead to kernicterus and permanent central nervous system damage, and (2) congestive heart failure secondary to severe anemia. Exchange transfusion is especially beneficial in hemolytic disease of the newborn because it clears the bilirubin and the offending antibody from the circulation and removes antibody-coated red cells before lysis, while at the same time providing a source of red cells that cannot be attacked by the antibody. A two-blood-volume exchange is commonly performed by using a fresh unit of blood concentrated to a final hematocrit of about 50%. [104] In hyperbilirubinemia resulting from other causes (e.g., that associated with liver immaturity in premature infants), phototherapy is the treatment of choice because its effects are usually more sustained, and exchange transfusion is only used for marked elevations. [105] ECMO and open heart surgery are two other situations in which the neonate is exposed to large volumes of allogeneic red cells. The ECMO circuit requires a prime with 2 units of red cells, as do many of the types of extracorporeal circuits in use for cardiopulmonary bypass. Transfusion support for these two types of procedures was the subject of a very complete, recent review. [105]

Although accumulating evidence supports the safety of using red cell units of any age and with any preservative solution for low-volume transfusions in neonates, the same transfusion policies may not apply to massive transfusion. Newborn physiology is unique in several ways that may have implications for massive transfusion therapy. The newborn does not handle metabolites in a mature fashion. Renal immaturity may lead to problems in clearing potassium or acid from stored red cells, and the immature liver may not catabolize citrate efficiently. These problems are accentuated and protracted in the premature infant. To address the concern about potassium load, fresh (<5 days old) or washed red cells are often used, although the necessity of this practice is actively debated. [106] [107] Fresh blood may also be preferred because of its higher 2,3-DPG levels and better red cell integrity. [105] The citrate problem is probably best handled by using slow infusion rates, [23] since the use of bicarbonate or calcium replacement to counteract the acid load or calcium-chelating effects of citrate is controversial. [24] Finally, the use of red cells stored in the newer preservative solutions (CPDA-1, Adsol, Nutricell) is rejected by some authorities because of the risk of renal damage and renal stones due to adenine metabolites.[108] In addition, the potential toxicity of the various additives is of concern, as is a significant depletion of plasma proteins associated with transfusion of Adsol or Nutricell units.[109] [110] If CPD red cells are not available, the additive solution can be removed and the cells washed. Red cells preserved in Adsol or Nutricell also have a lower hematocrit, which must be taken into account in making calculations for exchange transfusion. [105]

The humoral and cellular immune systems of the neonate are also immature; again, the problem is accentuated in the premature infant. This has two implications for transfusion therapy. There is a small but real risk of transfusion-induced

graft-versus-host disease in premature infants receiving red cell transfusions [111] [112] and in the fetus undergoing intrauterine transfusion. [113] Irradiation of red cells is performed in these two settings. Another risk of transfusion in low birth weight (<1,500 g), premature infants is the development of clinical cytomegalovirus (CMV) infection in infants of CMV-seronegative mothers. [114] CMV-seronegative blood is now routinely used in these neonates. However, it appears that leuko-reduction by filtration may offer an alternative to CMV screening [115] [116] and is currently being used by some institutions when the supply of CMV-seronegative blood is inadequate.

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Chapter 136 - Principles of Platelet Transfusion Therapy

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INTRODUCTION

In 1910 a Johns Hopkins physician, W.W. Duke, demonstrated the relation of blood platelets to hemorrhagic disease, described a method for determining the bleeding time, and for the first time showed that hemorrhagic disease caused by thrombocytopenia could be relieved by transfusion. However, reliable methods for platelet preparation were not developed until 1950. [1] Even in 1970, platelet transfusion therapy was readily available only in specialized medical centers. Before 1960, two-thirds of patients with acute leukemia died of hemorrhage. [2] The development of potentially curable chemotherapeutic regimens, which led to severe myelosuppression, necessitated intensive research

into the biology of platelets, methods and devices for their procurement and storage, and platelet transfusion practices. This research allowed the widespread availability of platelet transfusions for the treatment of benign and malignant medical disorders, trauma cases, and complicated surgical procedures. Unlike the surgery patient to whom platelet transfusions are given during the perioperative period, medical patients frequently receive longer courses of platelet transfusions. Consequently, platelet alloimmunization and refractoriness to platelet transfusions occur. This is especially true in patients undergoing myelosuppressive therapy resulting in amegakaryocytic thrombocytopenia and in aplastic anemia. These groups of patients represent the largest recipients of platelet transfusions.

Alloimmunization is the single most important factor limiting the therapeutic effectiveness of platelet transfusion therapy. [3] The purpose of this chapter is to describe the effective use of platelet transfusions in medical patients and the immune and nonimmune reactions to platelet transfusions. Because refractoriness to platelet transfusions is a serious problem, methods to circumvent or prevent it are also described.

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PLATELET PREPARATIONS

Platelet Concentrates and Apheresis Platelets

Platelet transfusions are available as platelet concentrates or as apheresis units. [4] The former are prepared from units of whole blood by centrifugation and the latter are collected by pheresis devices. Various arguments have been proposed for the superiority of apheresis platelets, including reduced rates of alloimmunization and transfusion reactions.[5] [6] However, the only compelling argument seems to be the reduced risk of infection with apheresis platelets.

In the United States, platelet concentrates are separated from whole blood by first preparing platelet-rich plasma and then centrifuging the platelets with a second centrifugation.[4] Although the contents of the platelet concentrates are highly variable, depending on the preparation technique, the 50-ml platelet concentrate usually has at least 5.5×10^{10} platelets. In Europe the buffy coat method is the most common method of platelet preparation.[7] [8] This method involves centrifugation to prepare a buffy coat, from which platelets are separated by an additional centrifugation. The white cell contamination is approximately 10^{8} WBCs per bag when the platelet-rich plasma method of concentration is used and 10^{6} WBCs per bag for platelets prepared by the buffy coat method. The relatively lower WBC content in platelets prepared by the buffy coat method may be advantageous in reducing alloimmunization and febrile transfusion reactions.

Pheresis platelets are collected from donors by continuous centrifugation using a large intravenous catheter that allows processing of a large volume of blood and the removal of platelets by means of an automated system. Since a conventional transfusion dose for an adult patient is approximately 6 units of pooled platelets, collection parameters have been used to collect this number of platelets from a donor. Modern pheresis devices are equipped to predict the yield from the donors size, platelet count, and hematocrit. The leukocyte content of a pheresis platelet depends on the technology used, but most devices yield a WBC contamination of less than 10^{6} per bag. Platelets from single donors pose less infectious risk to the recipient than do pooled platelets simply because there are fewer donor exposures. Much has been written about the benefits of pheresis platelets in reducing the risk of alloimmunization. Data from a recently completed multicenter trial to reduce alloimmunization to platelets, however, have shown that leukocyte-reduced pheresis platelets provide no additional reduction in alloimmunization over that afforded by leukocyte-reduced platelet concentrates. [9]

Platelet blood components are licensed for a storage time of 5 days at room temperature. Clinical studies indicate that there is little loss of platelet function and viability with 5-day-old platelets. [10] Maintenance of the function and viability of liquid stored platelets is limited to a relatively short period of time because of the storage lesion that develops. There is considerable interest in improving the storage condition for platelets to reduce the functional abnormalities that occur during storage. When platelets are removed from the human circulation and exposed to the foreign conditions of even the most carefully designed collection and storage system, a variety of changes collectively referred to as platelet activation begin. [11] [12] [13] A number of variables may produce minimal changes that perpetuate the process as more platelets are recruited into the activated state. These activation changes may be reflected in platelet shape change, adhesion, or aggregation, the secretion of platelet granular contents, and the expression of activation antigens. The challenge of preparing platelets for transfusion has been to minimize the damaging effects of preparation and storage.

A major advance in improving stored platelets was the elucidation of the metabolic factors that lead to a deleterious fall in the pH. [10] Early in the development of platelet storage techniques, it was recognized that a drop in the pH to <6 was associated with a marked reduction in platelet viability. The drop in pH was caused by an accumulation of lactic acid through glycolysis and was promoted by the relatively hypoxic storage conditions found in the older generation of platelet storage bags. With improved transmission of oxygen through gas-permeable storage bags (second-generation containers), metabolism by oxidative phosphorylation was facilitated, thereby largely circumventing the fall in pH and allowing improved platelet viability.

Because of the possible deleterious effects caused by plasma factors, such as thrombin and complement, on platelets, there has been an increased interest in developing different storage solutions for platelets. A variety of crystalloid solutions are under development that allow for improved platelet metabolism while reducing the amount of plasma to low limits. If these new media ultimately are shown to improve platelet function, other benefits of plasma removal from platelet concentrates are foreseeable.[14] First, the unneeded plasma could be used in plasma fractionation. Second, transfusion practices may be improved. By removing plasma, there would be less concern about administering ABO-incompatible plasma, which would minimize the risk of hemolysis in susceptible transfusion recipients. If viral inactivation of platelets by photosensitive dyes should become feasible, the absence of plasma in platelets would be an advantage. A problem with viral photoinactivation of blood products is the competition between serum proteins and viral particles for viricidal dyes. [15]

Cryopreserved Platelets

Cryopreserved platelets have been developed for long-term platelet storage using dimethylsulfoxide (DMSO) or glycerol. The average post-transfusion recovery of cryopreserved platelets is 50%. Hemostasis seems to be maintained with DMSOpreserved platelets. Despite early promising results with glycerol-preserved platelets, this technique has not been successfully adapted for clinical practice. Cryopreserved platelets were especially developed for patients who become alloimmunized during induction chemotherapy for acute leukemia and later needed additional marrow suppressive therapy. Platelets could be collected during remission, frozen, and subsequently used when necessary. Because using frozen platelets is accompanied by some logistical considerations, they are not widely used. [16]

Lyophilized Platelets

The lyophilization of platelets is an alternative that circumvents the logistical concerns inherent to the use of frozen stored platelets. In 1995 Read and co-workers reported that lyophilized platelets may support hemostasis in an animal model. [17] They reported that they could correct the bleeding time in thrombocytopenic animals, and also that transfused reconstituted lyophilized platelets participated in carotid arterial thrombus formation in a canine model. This form of platelet is currently undergoing human clinical trials and is not yet approved for routine use. Although these studies are provocative, additional studies are needed to substantiate the usefulness and safety of lyophilized platelets and to investigate other potential forms of platelet substitutes.

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CLINICAL APPLICATIONS OF PLATELET TRANSFUSIONS

The Thrombocytopenic Patient

Platelet transfusions are given prophylactically or therapeutically to thrombocytopenic patients and to patients undergoing invasive procedures. There has been considerable interest in defining the lowest safe platelet concentration at which bleeding is unlikely, so that fewer prophylactic transfusions would be needed. This interest was initially stimulated by the hypothesis that if fewer platelets were given, alloimmunization rates would be lower. This hypothesis, however, does not appear to hold true in patients with acute leukemia. There does not appear to be a dose-response relationship between the number of platelet concentrates given and the rate of alloimmunization.[18] Certainly, by lowering the level at which one transfuses platelets, the expense of platelet transfusions may be decreased. Currently >3 million units of platelets are transfused annually in the United States. The majority of these units are transfused in tertiary care hospitals to manage hematologic malignancies. In a recent review of practices at the University of Minnesota Hospital and Clinics, 86% of platelet transfusions were used in patients with hematologic malignancies. Sixty-eight percent of transfusions were given prophylactically to prevent bleeding, and some 32% were given therapeutically during episodes of bleeding.[19]

Clinicians have been generally aware that hemorrhage is more common during the most severe stages of thrombocytopenia. In an effort to identify a hemorrhage threshold related to the degree of thrombocytopenia, Gaydos et al. reviewed hemorrhagic episodes in 92 consecutive patients treated for acute hemorrhage between 1956 and 1959 at the National Cancer Institute.[20] Platelet counts of <100,000/mm^3 were associated with an increased risk of bleeding. Patients with platelet counts of 5,000/mm^3 manifested gross hemorrhage on approximately one-third of days at risk. The authors could not, however, identify a distinct threshold. Rather, the risk of hemorrhage increased progressively as the platelet count fell. In examining patients with fatal hemorrhage, Gaydos et al. also noted that patients in blast crisis hemorrhaged despite having adequate platelet counts. When such patients are excluded, only eight of the 92 patients experienced fatal hemorrhage. Of these eight patients, only one had a platelet count of >5,000/mm^3 , and none had a platelet count of >10,000/mm^3 .

Based on the experience of Gaydos et al., several centers tested strategies of prophylactic platelet transfusion. Higby et al. compared prophylactic platelet transfusions and prophylactic transfusions with platelet-poor plasma.[21] Of note, both groups in this study experienced equivalent degrees of thrombocytopenia, which suggests that marrow ablation was not complete or that the quality of transfused platelets may not have been optimal. Nonetheless, an increased rate of bleeding episodes was noted in patients receiving platelet-rich plasma, and the study was terminated early. At approximately the same time, Roy et al. compared two doses of platelet transfusions given prophylactically with results in historical patients.[22] Overall, bleeding episodes were reduced from 56% in historical controls to 58% in patients receiving platelet transfusions. Rates of both minor and major bleeding episodes were decreased in the treated groups. However, there was no difference between the low dose or high dose of platelets.

Based on these studies, a prophylactic platelet transfusion strategy has been widely adopted by nearly all American oncologists. Undoubtedly, practical matters were a major factor in the adoption of this strategy. In the 1960s and 1970s, platelet concentrates were not available on an emergency basis. Various methods for storing platelets, some clearly less than optimal, were employed, resulting in platelet concentrates of variable efficacy. Further, there was a general feeling among clinicians that hemorrhagic episodes were catastrophic and irreversible. In this context, a prophylactic platelet transfusion strategy was generally accepted and a 20,000/mm^3 triggerd was adopted.

Since the adoption of this policy, the availability of platelets has changed dramatically. Today, platelets are easily available on an emergency basis. Nonetheless, American oncologists have persisted in using a platelet count of 20,000/mm^3 as a trigger for transfusion, despite the improved availability of platelet concentrates and considerable physiologic and clinical data that question the efficacy of this strategy.

In 1997 Heckman and co-workers, in a randomized study of prophylactic platelet transfusion thresholds during induction therapy in adult patients with acute leukemia, reported that giving prophylactic transfusions only when the platelet count dipped to <10,000/mm^3 decreased platelet utilization, with only a small adverse effect on bleeding and no effect on mortality.[23] It therefore appears that with amegakaryocytic thrombocytopenia, prophylactic transfusions should be given if the count falls to <5,000/mm^3 . At values between 5,000 and 10,000/mm^3 , the clinician may be able to abstain from transfusing if the patient is stable and if no other conditions make spontaneous bleeding likely. These conditions include blast crisis, anticoagulation with heparin for disseminated intravascular coagulation (DIC), the use of drugs that affect platelet function, uremia, and recent invasive procedures, including spinal taps or the placement of central venous catheters. This approach has recently been confirmed by other investigators, who showed that lowering the transfusion threshold to 10,000/mm^3 was not only safe but reduced the number of platelet transfusions by 22%.[24]

Platelet Transfusion Dose

The dose administered to a thrombocytopenic patient depends on the therapeutic goal. A clinician administering prophylactic platelet transfusions to a myelosuppressed patient may wish to give only enough platelets to prevent bleeding. In this situation the general practice is to administer sufficient platelets to maintain a sustained platelet count of >10,00020,000/mm^3 . To accomplish this, one must take into consideration different physiologic factors, including the presence of fever, active bleeding, or DIC. Approximately one-third of transfused platelets are sequestered in the splenic pool.[25] Because of this, approximately one-third more platelets need to be transfused to accomplish a target platelet count. Although normal platelet survival is 9 days, there exists a direct relationship between the platelet count and the survival of platelets in thrombocytopenic individuals. The survival of transfused platelets may be followed by frequent platelet counts to estimate the frequency of transfusion. Knowing the concentration, or average concentration, of platelets supplied by a blood center, one can calculate the volume of platelets to transfuse. FDA guidelines dictate that pheresis platelets must contain >3 × 10^{11} platelets (6 equivalent

units) or >5.5 × 10^{10} in platelet concentrates prepared from units of whole blood. In a normal-sized individual, 3 × 10^{11} platelets is considered an appropriate dose. If a patient is being managed as an outpatient, larger doses of platelets may extend the interval between transfusions. In calculating the administration dose, the physician should realize that in many instances a platelet count is not always available on the platelet bag, or if it is, the accuracy of the count may have a variability of 20%.[10]

Other Quantitative Platelet Disorders

Thrombocytopenia may develop on the basis of increased consumption or splenic sequestration of platelets. In addition, when a patient is massively transfused (one total blood volume replacement), thrombocytopenia may develop. This type of thrombocytopenia has been called dilutional, because stored red cell transfusions have

very few platelets.[26] Increased platelet destruction is seen in autoimmune thrombocytopenia, drug-induced thrombocytopenia, DIC, and thrombotic thrombocytopenia purpura (TTP). Thrombocytopenia in these conditions can be quite severe, and profound bleeding and purpura are not uncommon presenting symptoms in autoimmune or drug-induced thrombocytopenia; therefore, platelet transfusions may be considered necessary in patients who are having catastrophic bleeding.

Autoimmune Thrombocytopenia and Drug-Induced Thrombocytopenia

Autoimmune thrombocytopenia is due to antibodies directed toward antigenic targets located on platelet glycoproteins. In drug-induced thrombocytopenia, the drug may serve as a hapten to bind to platelet glycoproteins, inciting an immune response. Thus, transfused platelets are as likely to undergo immune destruction as are autologous platelets, and through the same mechanisms. Platelet transfusions are never the only means of treating autoimmune thrombocytopenia. A variety of approaches are used, as well as intravenous -globulin, that interfere with platelet destruction. In some clinical situations, such as intracranial hemorrhage, platelet transfusions are only one of multiple methods used to try to prevent life-threatening bleeding until other treatments have an effect. One combinational approach is to use both intravenous -globulin and platelet transfusions to block the effect of the autoantibody on the transfused platelets. [27] This approach may prove beneficial in patients who may need surgery or undergo invasive procedures. There is no evidence that preoperative platelet transfusions have any use when a patient is to undergo splenectomy as part of the treatment of chronic autoimmune thrombocytopenia.

Qualitative Platelet Disorders

There are a variety of acquired and congenital qualitative platelet disorders. The most common acquired qualitative platelet abnormalities are either drug-induced or caused by uremia. Other acquired platelet disorders are seen in myeloproliferative disorders, in the dysgammaglobulinemias, or consequent on the use of mechanical cardiac assist devices. Aspirin is probably the most common drug that affects platelet function. Platelet transfusions can improve the bleeding time in patients taking aspirin if the transfused platelets constitute 10% of the platelet population. This is especially important in cardiac patients who are taking aspirin prophylactically and need emergency platelet transfusions. Platelet transfusions are not usually considered first-line management in uremia-induced platelet dysfunction. Rather, hemodialysis, cryoprecipitate administration, or correction of severe anemia by transfusion or erythropoietin is tried first. [4] [10]

A variety of inherited qualitative platelet disorders exist that can be accompanied by severe bleeding, particularly following trauma. These disorders include Glanzmann thrombasthenia and Bernard-Soulier syndrome. Platelet transfusions are effective in these disorders. However, because the disorders are characterized by absence of the glycoproteins that are carried on transfused platelets, isoimmunization to these proteins can occur. When this happens, platelet transfusions are ineffective.[28]

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PLATELET TRANSFUSION REFRACTORINESS

Failure to achieve an expected increment in platelet count in response to a platelet transfusion is called platelet transfusion refractoriness. Refractoriness may occur on an immune or nonimmune basis.[29] Management of refractoriness to platelet transfusion requires identifying the specific cause. Different patient populations may be at greater risk for certain types of platelet refractoriness than others. Patients with acute promyelocytic leukemia may be expected to have a higher incidence of DIC-related transfusion failures. Bone marrow transplant recipients may experience a higher rate of veno-occlusive disease. Several authors have reported multivariate analyses of platelet transfusion failures. [29]

Clinically, the response to a platelet transfusion can be assessed by measuring the increment in the platelet count over time. The post-transfusion platelet response should be calculated on the basis of the patients body surface area and corrected for the number of platelets transfused. The corrected platelet count increment is calculated using the following formula:

In general, a successful corrected count increment should be >7,500 within 160 minutes of a transfusion and >4,500 if measured 1824 hours after transfusion. It has been suggested that the corrected platelet count increment 1 hour after platelet transfusion is a useful indirect measure with which to establish alloimmunization versus nonimmune causes of platelet transfusion failure. HLA antibodies are only one of many factors that influence 1-hour platelet count increments. It should also be noted that even when platelet antibodies are present, acceptable 1-hour increments may be achieved. This latter phenomenon may be seen when platelets with weak expression of HLA-B locus antigens are given. [30]

Immunologic Basis of Platelet Transfusion Refractoriness

Immunologic destruction of transfused platelets may be the result of alloantibodies, autoantibodies, drug-induced antibodies, or immune complexes. Any of these factors may contribute to immune platelet transfusion refractoriness. The platelet membrane carries class I HLA antigens and the human platelet antigens (PLA, Bak, Br, Ko, and Pen). However, antibodies to class I HLA-A and HLA-B loci antigens account for most cases of immune-mediated platelet destruction. Antibodies to the human platelet antigens are relatively infrequent causes of platelet transfusion refractoriness. [29]

HLA Alloantigens

Soon after the introduction of prophylactic platelet transfusion, it became apparent that in many patients, serial transfusion resulted in decreasing effectiveness. Through the work of Yankee and others, it is now recognized that transfusion failure results

TABLE 136-1 -- Recognized HLA Class I Specificities

HLA-A Loci Antigens	HLA-B Loci Antigens	
A1	B5	Bw49
A2	B7	Bw50
A3	B8	Bw51
A9	B12	Bw52
A10	B13	Bw53
A11	B14	Bw54
Aw19	B15	Bw55
A23	B16	Bw56
A24	B17	Bw57
A25	B18	Bw58
A26	B21	Bw59
A28	Bw22	Bw60
A30	B27	Bw61
A31	B35	Bw62
A32	B37	Bw63
Aw33	B38	Bw64
Aw34	B39	Bw65
Aw36	B40	Bw67
Aw43	Bw41	Bw71
Aw66	Bw42	Bw73
Aw68	B44	Bw75
Aw69	B45	Bw76

Aw74	Bw46	Bw77
A29	Bw47	Bw4
	Bw48	Bw6

from the induction of alloantibodies to HLA and other antigens. [3] Yankee et al. were able to show that most cases of platelet refractoriness could be reversed by use of platelet transfusions that were phenotypically matched at the HLA-A and -B loci. It was also recognized that the development of HLA antibodies as measured by lymphocytotoxic activity correlated with the development of the immune refractory state. [31] It is now well recognized that response to HLA-A and B loci antigens is the major cause of post-transfusion alloimmune transfusion failure.

Understanding the HLA system is important so that compatible platelet transfusions may be selected for alloimmunized patients (Table 136-1). Only HLA-A and HLA-B antigens have been shown to be important in causing immune-mediated platelet transfusion refractoriness. [31] [32] [33] There are two broad types of HLA antibodies made in response to platelet transfusions. The first type recognizes epitopes unique to a particular HLA allele and referred to as antibodies to private specificities. Antibodies to A2 or B12 fall into this group. The second type of HLA antibodies recognize more than one gene product. These antibodies recognize structural similarities between gene products (cross-reactive epitopes) or identical epitopes present on different gene products of different alleles and are referred to as antibodies to public epitopes (Table 136-2). Traditionally, HLA serology has placed the greatest emphasis on classifying the private antigens. More importance has recently been placed on the clinical importance of public HLA specificities. The best-known

TABLE 136-2 -- Class I Cross-Reactive HLA Groups (associated private epitopes)

A1C (1, 3, 11, 10, W19, 9, 28)
A2C (2, 28, 9, 17, 10, 33)
B5C (5, 53, 35, 18, 15, 17, 70, 49)
B7C (7, 27, 22, 42, 48, 40, 41, 13, 47)
B8C (8, 14, 16, 22, 42, 48, 40, 41, 13, 47)
B8C (8, 14, 16, 22, 52)
B12C (12, 21, 40, 41, 13)

examples of public specificities are Bw4 and Bw6. These antigens are encoded by a diallelic system and are associated with two different groups of HLA-B class I antigens. Other public antigens carried by HLA-B class I antigens have been divided into four cross-reactive groups, B5, B7, B8, and B12. The observation that the specificity of HLA antibodies in multiply transfused individuals is generally against public epitopes suggests that matching for these public antigens is important. With improved serologic approaches to identifying specificities to class I HLA antigens, selection of platelets for transfusion may be simplified by relying on a process based on public specificities. [34] [35] [36]

Human Platelet Antigens

Human platelet antigens (HPA), previously called platelet-specific antigens, arise as the result of polymorphisms of platelet membrane glycoproteins. The nomenclature for these antigens is shown in Table 136-3 . [37] [38] [39] Since the antigen systems arise because of polymorphisms of integrins, some of the antigen systems are not specific for platelets. Because these antigens are epitopes on integrins, some platelet-specific antigens may be expressed on endothelial cells, fibroblasts, and smooth muscle cells. For example, antigens associated with glycoprotein IIIa (GPIIIa) may have this diverse tissue distribution, whereas antigens associated with GPIIb are restricted to platelets.

Antibodies to the human platelet antigens HPA-1 to HPA-5 (PLA, Bak, Ko, Br, Pen) are only rarely causes of platelet transfusion refractoriness. In a large series of patients who received platelet transfusions, only 2% of patients had detectable antibodies to the human platelet antigens. Most of these antibodies were found in patients who were also alloimmunized to class I HLA antigens. In patients in whom HLA-identical platelet transfusions fail, antibodies to the human platelet antigens should be suspected. [40]

Detection of Antibodies in Platelet Transfusion Refractoriness

Specific identification of alloimmunization can be done by measuring HLA antibodies using lymphocytotoxicity testing. [3] Serial lymphocytotoxic antibody measurements are helpful in managing alloimmunized patients. [41] Some patients may have decreases or a loss of lymphocytotoxic antibodies, either permanently or transiently, and can be successfully transfused with platelet concentrates. It should be noted that some patients may have antibodies to HLA class I antigens and yet not experience platelet transfusion failure. A new development for detection of HLA antibodies has been reported by Kao and co-workers. [42] This solid phase enzyme-linked immunoassay detects the presence of IgG anti-HLA antibodies using purified HLA antigens

TABLE 136-3 -- Gene Frequencies of Platelet-Specific Antigens Calculated from Genotype Frequencies

Haplotype	Caucasians	African Americans	Korean	Dutch[a]
HPA-1a (PI^{A1})	0.89	0.92	0.995	0.846
HPA-1b (PI^{A2})	0.11	0.08	0.005	0.154
HPA-2a (Ko^{b})	0.92	0.82	0.87	0.934
HPA-2b (Ko^{a})	0.09	0.18	0.13	0.066
HPA-3a (Bak^{a})	0.67	0.63	0.67	0.555
HPA-3b (Bak^{b})	0.33	0.37	0.33	0.445
HPA-4a (Pen^{a})	1.00	1.00	1.00	1.000
HPA-4b (Pen^{b})	0.00	0.00	0.00	0.000
HPA-5a (Br^{b})	0.89	0.79	0.97	0.902
HPA-5b (Br^{a})	0.11	0.21	0.03	0.098

[a]Dutch gene frequency as reported by Simsek.[66]

prepared from platelet concentrates. The test is commercially available and is now widely used to screen for HLA antibodies in a variety of transfusion and transplantation conditions. Antibody specificity to the platelet-specific antigens can be identified using isolated platelet glycoproteins. In general, few donor centers have a donor population typed for the human platelet antigens except for those in the HPA-1 system. The recent introduction of DNA-based typing for the human platelet antigens permits accurate typing of donors and even thrombocytopenic patients because lymphocyte-derived DNA is used. [37]

Clinical Features of Alloimmune Refractoriness

The incidence of alloimmunization to class I HLA antigens in repeatedly transfused recipients may vary from 20% to 80%, depending on the population studied. [9] [40] In

general, patients with aplastic anemia have higher rates of alloimmunization than those with acute leukemia. The lower alloimmunization rates in leukemia may be due to the underlying disease or to immunosuppressive therapy. In a series of 114 patients reported by Dutcher et al., 42% of patients developed lymphocytotoxic antibody within the first 8 weeks of transfusion. [18] Approximately one-quarter of these developed it within 1 week, suggesting the presence of an amnestic response. Seventy-five percent of responders appeared to have a de novo response. Of those patients who manifested lymphocytotoxic antibody, 17 patients ultimately lost it despite further transfusion. More interestingly, of those initial patients who did not respond by developing lymphocytotoxic antibody in the first 8 weeks, 92% remained unresponsive despite continued transfusion therapy. Thus the development of an alloimmune response post transfusion seems to be an early event in transfusion therapy. Responsiveness or nonresponsiveness to HLA antigens develops in the first weeks of therapy, and this pattern is apparently maintained throughout subsequent therapy.

Anti-platelet Glycoprotein Antibodies

Besides the phenotypic differences that arise because of polymorphisms in the platelet glycoproteins, patients may form antibodies to the entire platelet glycoprotein molecule. Therefore, individuals who have the Bernard-Soulier syndrome may become immunized to GPIb, and those with Glanzmann thrombasthenia may become immunized to GPIIb/IIIa. Patients lacking these important membrane glycoproteins and needing platelet transfusions may become immunized to normal platelets. This results in transfusion refractoriness and significantly complicates the transfusion management of patients with qualitative platelet disorders. [28]

ABH Antigens

Initially it was thought that platelets could be transfused without regard to their ABH type. Studies by Duquesnoy and co-workers indicated that at most, ABH incompatibilities could account for only a 20% decrease in survival. [32] More recently Brand and co-workers described two patients who had absolute platelet refractoriness based on ABH antibodies. [43]

Nonimmune Refractoriness

Platelet transfusions may not result in an increment in the platelet count if the stored platelets are defective. This should be relatively uncommon, given the close scrutiny devoted to quality control of blood products. However, the clinician should not fail to consider freshness of a given platelet transfusion as a cause of a single platelet transfusion failure. Other causes of nonimmune-mediated platelet refractoriness are listed as follows and should be considered in the differential diagnosis of platelet transfusion refractoriness: [29]

1. *Microangiopathic platelet destruction.* Consumption of platelets in microangiopathic hemolytic anemias has been classically described in the syndromes of TTP and the hemolytic uremic syndrome. This microangiopathic process can also be observed during bone marrow transplantation. In some cases, therapy with Cyclosporin A has been incriminated as the trigger for this process. Platelet transfusions in patients with active TTP may lead to acute worsening of both renal and cerebral function.
2. *Coagulopathy.* DIC has been classically associated with nonimmune platelet refractoriness. This syndrome has been associated with bacterial sepsis, which is common in the transfused patients, as well as with acute progranulocytic leukemia. The quantitative role of DIC in the platelet refractory state has not been well characterized. Patients with active hemorrhage will have shortened platelet survival.
3. *Splenic sequestration.* Splenomegaly is a major cause of platelet transfusion failure. Approximately 30% of the platelet mass in humans is contained within the spleen. With increases in splenic size, up to 90% of circulating platelets can be sequestered in this organ. Characteristically, splenic sequestration is associated with a reduced 1-hour platelet recovery but normal survival.
4. *Fever and infection.* Studies by several groups have implicated both fever and infection as causes of decreased platelet survival. One study noted that platelet transfusion requirements were increased by 50% in febrile patients. This may be increased further in patients with major infections, particularly those with DIC.
5. *Hepatic dysfunction/VOD.* Hepatic sequestration of platelets has been reported in association with a wide spectrum of liver diseases. Recently a syndrome of veno-occlusive disease of the liver (VOD) characterized by deposition of platelets in the hepatic venules and the development of an intrahepatic thrombosis with hepatomegaly, right upper quadrant pain, and ascites has been described. This organ dysfunction has been noted after administration of a wide variety of cytotoxic agents, including total body irradiation, alkylating agents, and vinca alkaloids.
6. *Other factors.* Platelet refractoriness has been reported with a number of medications. Amphotericin in particular has been implicated in decreasing platelet recovery and survival. Similarly, vancomycin has been reported to be a major cause of platelet refractoriness, as have antithymocyte globulin, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, and the interferons. In view of the large number of drugs cancer patients characteristically receive, it would not be surprising if a number of agents were implicated in accelerated platelet destruction.

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MANAGING THE ALLOIMMUNIZED PATIENT

Transfusion Selection

Because alloimmunization to HLA antigens accounts for the majority of cases of alloimmune platelet transfusion refractoriness, the clinician should select platelets on the basis of HLA matching. Table 136-4 outlines the fundamentals of HLA matching. In early studies, it was found unnecessary to match for HLA-C and -D loci antigens.[32] [33] Based on experience with tissue matching in renal transplantation, it was recognized that using cross-reactive HLA types could circumvent the problem of finding exactly identical HLA-typed platelets. Depending on the HLA type of an individual, one may have little difficulty in locating platelets that are identical, whereas in some patients with an unusual HLA type, such as some ethnic populations, HLA matching is difficult. The practice of using either

TABLE 136-4 -- General Guidelines for HLA Matching

1. If HLA-identical platelets are unavailable, platelets from donors whose HLA types are serologically cross-reactive with the recipients may be substituted.
2. Matching for antigens of the HLA-C locus is not necessary.
3. Mismatching for some HLA-B locus antigens that are weakly expressed on platelets is acceptable for some donor-recipient pairs.
4. If cross-reactive platelets are ineffective for some patients, attention to linked HLA specificities may be important (i.e., Bw4/Bw6).
5. Although expressed on platelets, ABO matching is less critical than HLA matching.

HLA-identical platelets or HLA- matched (not identical but serologically cross-reactive, with only minor epitope differences) has given rise to a classification of HLA match grades that should be kept in mind when discussing the level of match that a patient is receiving. This classification is given in Table 136-5 .

Relying only on HLA matching may have shortcomings. In some cases it is overly restrictive, in that in some cases some HLA-B loci antigens are not located on platelets. Because of this, in addition to antigen matching, there has been a great deal of interest in adopting an approach similar to that used for testing red cell compatibility. In this paradigm, a red cell phenotype that matches the patients phenotype is selected and then a major crossmatch is done. This same approach can be used with platelets by performing a platelet crossmatch.[44] [45] [46]

Kickler and co-workers studied the effectiveness of HLA matching as the sole method of platelet selection for alloimmunized patients. Of 50 HLA-identical platelet transfusions, 20% were unsuccessful. Of the transfusions selected on the basis of cross-reactivities without regard to matching of public specificities, 41% were failures (23 of 56 transfusions). When one or two antigen-mismatched platelets were used, approximately one-third of transfusions were failures. These observations indicate that relying solely on matching platelets on the basis of HLA private antigens is frequently unreliable. Furthermore, even if patients are alloimmunized, they may receive mismatched platelets and have successful transfusions.[44] [45] [46]

For these reasons, refining the process of selecting platelets for alloimmunized patients has been the subject of much investigation. A practical approach to selecting platelets for the alloimmunized patient that takes account of the importance of HLA matching and confirming this by platelet compatibility testing is outlined in the box. Numerous investigators have evaluated the usefulness of crossmatching a recipient against potential donors. This approach can be readily accomplished, for pheresis platelets are stored for 5 days. By taking an aliquot of platelets

TABLE 136-5 -- Donor and Recipient HLA Match Grades

Grade	Definition
A	All four donor antigens are identical to the recipients
B1U	All donor antigens are identical to those of the recipient; only three antigens are detected in the donor
B2U	All donor antigens are identical to those in the recipient but only two are detected in the donor
B1X	Three donor antigens are identical to those in the recipient; the fourth is serologically cross-reactive with a recipients antigen
B2UX	Two donor antigens are identical to those in the recipient; a third is cross-reactive; only three antigens are detected in the donor
B2X	Two antigens are identical to those in the recipient; two are cross-reactive
C	One donor antigen is mismatched with antigens in the recipient
D	Two or more donor antigens are mismatched with antigens in the recipient

AUTHORS APPROACH TO SELECTION OF PLATELETS FOR ALLOIMMUNIZED PATIENTS

1. Determine HLA phenotype and ABO type.
2. Screen patients serum for lymphocytotoxic antibody.
3. Screen patients serum for antibodies to platelet-specific antigen (optional if there is a history of failing HLA-identical platelets is not available).
4. Select from the donor pool the most compatible antigens in the HLA and, if possible, ABO systems. If a platelet-specific antibody is detected, matching for this antigen is required.
5. Crossmatch the recipients serum with the selected potential donors and select the most compatible crossmatch.
6. Determine 1-hour and 18- to 24-hour post-transfusion platelet counts, not only to assess transfusion outcome but also to guide the selection of platelets for future transfusions.
7. Before deciding that platelet refractoriness exists, measure the platelet count increment on more than one occassion to ensure that a poor increment was not related to the storage time of the platelets that were transfused.

from an integrally attached tubing segment, compatible platelets can be found in the inventory of stored platelets. Compatible donors may be found even if a patient is broadly alloimmunized. A variety of techniques exist for platelet crossmatching, most involving a labeled antiglobulin technique. Many referral centers have developed their own techniques, which they have validated in their own patient populations. Recently, commercially available platelet compatibility tests have resulted in the wider use of platelet crossmatching. In general, no consensus has emerged concerning all the methodologic requirements for platelet compatibility testing. These include the importance of detecting different immunoglobulin classes, interference by immune complexes, or the effect of isoagglutinins.

Management of Platelet Transfusion Refractoriness and Bleeding

A variety of approaches have been tried when no compatible platelets can be found for a patient who is alloimmunized, is bleeding, or may be undergoing invasive procedures. Therapeutic modalities have included splenectomy, corticosteroid administration, plasmapheresis, administration of intravenous immune globulin (IVIG), and repeated platelet transfusions. Except for IVIG, there is little evidence that any of these approaches work. [47]

Kickler and co-workers performed a randomized, placebo-controlled clinical trial investigating the use of IVIG in alloimmunized thrombocytopenic patients. [47] IVIG was administered at a dose of 400 mg/kg for 5 days. An incompatible platelet transfusion from the same donor was used before and after the patient received study drug or placebo. Seven patients received IVIG and five received placebo. Although platelet recovery in 16 hours was satisfactory in five patients after IVIG treatment, 24-hour platelet survival was not improved in most of these patients. It could not be excluded that this poor 24-hour survival was unrelated to nonimmune causes of shortened platelet survival. None of the placebo group achieved satisfactory 1-hour platelet corrected count increments. By *t*-test, the post-treatment mean values 1 hour after transfusion were significantly greater than in the control group. When a regression model was used to adjust for any distributional assumptions of the study population,

the parameter estimate for IVIG treatment was positive. This indicated that IVIG may improve 1-hour platelet recovery. These studies also suggest that the kinetics of platelet survival may be affected by IVIG. It is likely that if additional platelets were administered, severe thrombocytopenia may be corrected in these patients. Furthermore, the authors suggest that this approach may permit the performance of invasive procedures.

When conventional methods fail to increase the platelet count to hemostatic levels, the only remaining alternative that has been tried is the continuous transfusion of platelets (massive transfusion). [29] [44] It has been argued that even when there is no incremental increase in the platelet count, transfused platelets still exert some effect, permitting platelet plug formation or maintenance of endothelial integrity. These arguments are based on clinical observations. In one well-established animal model of alloimmune thrombocytopenia, if the platelet count did not increase to >60,000/mm ^{3} , capillary leakage of blood still persisted. Nonetheless, given the potentially serious risk of doing nothing in a patient experiencing active bleeding, the clinician must try to support the patient while other, more definitive treatments are undertaken to stop the bleeding or ameliorate those nonimmune factors that may be contributing to platelet refractoriness. [48]

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PREVENTION OF ALLOIMMUNIZATION

Reduction of Donor Exposures

Early studies suggested a relationship between the number of platelet transfusions and the rate of alloimmunization to HLA antigens. Other studies have not documented a dose-response relationship. The reasons for these contradictory conclusions are not clear. In part, clinical differences in the study populations, including multiparity of the transfusion recipients, may be the main explanation. The definition of alloimmunization may also be important. For a primary immune response, 23 weeks may be required. If sera are not collected over a sufficiently long period of time, an antibody response may not be measured. Alternatively, some patients may lose antibodies by the time of testing, contributing to negative responses. The data are also consistent with the hypothesis that the alloimmune response may have a threshold effect. Over a low range of exposure, there may be a dose-response effect. This may explain the dose effect seen by Perkins [49] and Sintnicolaas.[6] With larger numbers of exposures, further alloimmunization may not develop if tolerance has been established. [50]

Blood Product Modification

In animal studies, depletion of contaminating leukocytes from donor blood components or ultraviolet irradiation of platelets was effective in preventing an alloantibody response to MHC antigens.[51] [52] [53] With the development of efficient methods to remove leukocytes from blood products, clinical trials were instituted to test whether leukocyte reduction would reduce alloimmunization to class I HLA antigens. Some small trials suggested that if $<10^{6}$ contaminating leukocytes remained, alloimmunization could be reduced by 3050%. Other studies were not as conclusive. Because of this, a large multicenter clinical trial was done in the United States. The Trial for the Reduction of Alloimmunization to Platelets (TRAP) tested the efficacy of transfusing platelets that were modified by leukocyte reduction or ultraviolet irradiation, or of transfusing platelets from leukocyte-depleted, single-donor platelets. The results of transfusing these modified platelet products were compared with the results of transfusing platelet concentrates. All patients received leukocyte-depleted red cells. [9]

Of 530 patients with no alloantibodies at baseline, 13% of those in the control group formed HLA antibodies and their thrombocytopenia became refractory to platelet transfusions, as compared with 3% in the group receiving filtered platelet concentrates, 5% in the group receiving ultraviolet-irradiated platelet concentrates, and 4% in the group receiving filtered apheresis platelets. Results in the treatment group were significantly different from results in the control group, but not among the treatment groups. HLA antibodies were found in 45% of the controls, compared to 1721% of the treated patients. These maneuvers were effective even though 26% of the patients had previously received nontreated blood products. Of note, none of the treatment maneuvers reduced the rate (610%) of alloimmunization to the human platelet alloantigens. Thus, this large study documents the usefulness of either leukocyte removal or inactivation by ultraviolet irradiation in the chronically transfused leukemic patient who is immunosuppressed. Interestingly, the use of random donor platelet concentrates appeared to offer no increased risk of alloimmunization over the use of single-donor apheresis concentrates.

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TRANSFUSION REACTIONS

Febrile and allergic platelet transfusions are two of the most common types of platelet transfusion reactions. [54] Because platelets are suspended in donor plasma, if group O platelets from six to eight donors are pooled and transfused, a substantial amount of anti-A and anti-B isoagglutinins is present in the approximately 300 to 400-ml bag of pooled platelets. If these platelets are given to a group A or group B individual, acute hemolysis may result from the passive administration of the isoagglutinins. For this reason, it is preferable to also consider plasma compatibility when transfusing platelets. This is especially important in children and in patients who are receiving multiple transfusions each day. Another problem to consider is volume overload. If a patient is anuric or receiving multiple transfusions of platelets and other blood products, it is sometimes necessary to concentrate platelets immediately before transfusion. This can usually be done without difficulty. A preferable approach to preventing volume overload is to use pheresis platelets, since these generally have a lower volume. Because platelets contain 12 ml of red cells, they have the potential for immunizing an Rh(D)-negative patient. This is only of concern in treating females of childbearing age. Because of this concern, one may wish to use only Rh(D)-negative platelets.

Systemic Reactions to Platelet Transfusions

Acute systemic reactions to platelets are seen in 2% of all transfusions. [9] Severe reactions occur with equal frequency after the transition of platelet concentrates or pheresis platelets, and even if leukocyte reduction is done at the time of transfusion. Acute symptoms include rash, wheezing, fever, chills, dyspnea, urticaria, and hypotension. [54] These symptoms were previously attributed to either antileukocyte antibodies or IgE-mediated reactions to transfused plasma proteins. However, medical understanding of the pathophysiology of platelet reactions has changed with a better characterization of cytokines present in blood products. Several investigators have identified cytokines as possibly important factors in certain transfusion reactions associated with the administration of platelet products.

Platelet transfusions are associated with a significantly higher incidence of patient reactions than red cell transfusions. [55] [56] It was generally thought for many years that antileukocyte antibodies accounted for most febrile reactions to platelets. However, clinical observations of patients suggested that this was not the complete answer. Reactions occur not uncommonly with the first transfusion in nearly one-third of cases and are independent of the total number of transfusions. Furthermore, the majority of first reactions occur during the first three transfusions. Although presensitization might account for some

of the occurrence of early reactions, this observation suggested that other factors may be involved. [57]

A variety of cytokines can be generated during the in vitro storage of platelets. [55] Heddle et al. reported that blood product age predicted transfusion reactions. [55] [56] This was one of the first pieces of evidence that some type of biomediator was present in platelets. Transfusion reactions were fivefold greater with platelets, and the older the platelet product and the higher the white cell count, the more likely that a reaction would occur on transfusion. Similar observations were reported by Muylle et al. [58] [59] [60] In a retrospective study, Muylle and Peetermans found an increased incidence and severity of reactions to platelet transfusion associated with the use of plastic bags allowing 5-day platelet storage. [59] These reactions included bronchospasm, wheezing, and dyspnea and were related to the length of the storage time and the white cell content of the platelet concentrate.

Cytokines in Platelet Transfusions

Because blood components all contain leukocytes, which produce cytokines, it is not surprising that these hormone-like substances accumulate in products such as platelet concentrates during storage. Muylle et al. investigated cytokine levels in stored platelet concentrates and the relationship between these levels and transfusion reactions. They found increasing amounts of such cytokines as tumor necrosis factor (TNF), interleukin 1 (IL-1), and IL-6 in the plasma of stored platelet concentrates. They speculated that the source of these cytokines could be damage to leukocytes in the platelet concentrate, leading to leakage of cytokines or the activation of monocytes in the platelet concentrates. [61]

Another group of investigators has also reported significant accumulations of IL-8, IL-1, IL-6 and TNF- in platelet concentrates during storage. Stack and Snyder found that leukocyte reduction by third-generation filters on day 1 of storage prevented the generation of IL-8 and IL-1 out to day 5 of storage. [62] Other investigators have also reported that leukocyte depletion of platelets after processing and prior to storage prevented the accumulation of the above substances. Whether or not prestorage leukocyte depletion will lead to reduced platelet transfusions is not known. [63]

Bacterial Contamination of Platelets

Because platelets are stored at room temperature, organisms that were introduced during phlebotomy or during transient donor bacteremia may proliferate. Bacterial contamination of platelet products can lead to transfusion-associated sepsis. Characteristic symptoms of transfusion-associated sepsis include chills, fever, hypotension, and hypoxia. Cultures of blood drawn from the patient experiencing such reactions are positive in less than half of episodes. Estimates of the incidence of bacterial contamination in platelet products range from a high of 10% of all platelet pools to more conservative estimates of 4 in every 1,000 pools. [64] [65]

If the bacteria contaminating the platelet product are gram negative, there is an added risk of the infusion of endotoxins, which can result in endotoxic shock. Clinical features associated with endotoxins include fever, chills, hypotension, respiratory failure, and gastrointestinal symptoms, to mention a few.

It is not clear what intervention can prevent or reduce the incidence of bacterial contamination in platelet products. Although shortening the storage shelf-life to 3 days has been considered, this may be of little real value, since bacteria can achieve log growth and relatively high concentrations by day 3 of storage. [64] [65]

Because platelet transfusions are frequently given to neutropenic patients, the morbidity and mortality of septic reactions are of serious concern. Because of this, it is recommended that the untransfused contents of an aborted transfusion should be cultured and Gram stained. If the contents are positive on Gram stain, the patient should be treated for a possible septic reaction.

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Chapter 137 - Principles of Neutrophil (Granulocyte) Transfusions

Ronald G. Strauss

INTRODUCTION

Current cytapheresis technology permits collection of highly enriched fractions of several types of blood leukocytes from either healthy donors or patients for transfusion and transplantation or further processing (e.g., ex vivo expansion, genetic manipulation, etc.) and storage. Neutrophils (PMNs) are collected routinely and issued as a standard blood component (granulocytes, pheresis). This chapter analyzes the use of granulocyte transfusions (GTX) as an adjunct to antimicrobial drugs in the treatment and possible prevention of progressive infections.

Serious and repeated infections with bacteria, yeast, and fungi continue to be a consequence of severe neutropenia (<0.5 × 10^{9}/L blood PMNs) and disorders of PMN function. In the recent multicenter NIH Trial to Reduce Alloimmunization to Platelets (TRAP) study, [1] 7% of adult patients with acute nonlymphocytic leukemia died of infection during first remission induction therapy. Previous attempts to prevent infections in severely neutropenic patients (prophylactic GTX) achieved only modest success (i.e., rates of certain infections were partially reduced, but GTX were toxic and expensive). Similarly,

the therapeutic use of GTX has diminished strikingly over the past several years, despite many reports that have shown them to offer benefit in certain experimental and clinical settings. [2] This lack of acceptance of GTX can be explained by the development of effective antimicrobial agents to prevent and treat infections and by the availability of recombinant hematopoietic growth factors and peripheral blood hematopoietic progenitor cell transfusions to hasten marrow recovery.

Many physicians hold strong negative opinions about the value of GTX that have been reinforced by knowledge that historically, PMN concentrates collected for transfusion contained woefully inadequate numbers of PMNs. It is now possible to collect very large numbers of PMNs from granulocyte colony-stimulating factor (G-CSF)-stimulated normal donors using large-volume leukapheresis. This fact, along with the historical success of GTX in treating bacterial infections, suggests that a critical reassessment of therapeutic GTX is warranted. Further, because of the relatively rapid engraftment following the use of peripheral blood progenitor cell transfusions as a replacement for either autologous or allogeneic bone marrow transplantation, it is reasonable to explore the possibility that severe neutropenia following intense myeloablative therapy might actually be eliminated by prophylactic GTX.

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THERAPEUTIC GRANULOCYTE TRANSFUSIONS IN NEUTROPENIC PATIENTS

Recently, 34 papers that reported the therapeutic use of GTX in severely neutropenic patients (<5 × 10^9 /L blood PMNs) were reviewed.[3] The data are tabulated (Table 137-1 (Table Not Available)) according to the index infection that prompted GTX therapy. Patients are counted only once (e.g., patients with septicemia were listed only in the septicemia group, even if they had another infection such as pneumonia). As an exception, all patients with invasive fungal infections are counted together because it was impossible to accurately separate sepsis, pneumonia, sinusitis, and so forth, into distinct categories. All patients given GTX for a designated type of infection are enumerated in the Treated column. Of the treated patients, those for whom the actual course and mortality of the index infection could be clearly documented are enumerated again in the Evaluable column. GTX therapy was considered successful if so stated by the author. Several of the 34 reports described uncontrolled studies of small numbers of patients with diverse underlying diseases, types of infections, antimicrobial therapies, GTX management strategies (i.e., variable dose and quality of PMNs), and varying definitions of success. Because of these confounding factors, combining data from multiple reports is of limited value in drawing conclusions, and it was done simply to document the breadth of the reported experience (Table 137-1 (Table Not Available)).

To obtain more definitive information regarding efficacy, the seven controlled studies were analyzed in more detail. [4] [5] [6] [7] [8] [9] [10] In these seven studies, the response of infected neutropenic patients

TABLE 137-1 -- Infectious Problems in Neutropenic Patients Treated with GTX in 34 Studies

(Not Available)

From Strauss,[3] with permission.

TABLE 137-2 -- Results of Seven Controlled Studies Evaluating Therapeutic Granulocyte Transfusions in Neutropenic Patients

Investigators	Success	Study Group		Control Group	
		N	Survival (%)	N	Survival (%)
Higby et al.[8]	Yes	17	76	19	26
Volger and Winton[9]	Yes	17	59	13	15
Herzig et al.[7]	Yes	13	75	14	36
Alavi et al.[4]	Partial	12	82	19	62
Graw et al.[6]	Partial	39	46	37	30
Winston et al.[10]	No	48	63	47	72
Fortuny et al.[5]	No	17	78	22	80

to treatment with GTX plus antibiotics (study group) was compared to that of comparable patients given antibiotics alone and evaluated concurrently (control group). The design, size, and results of these seven studies are presented in Tables 137-2 and 137-3 . Three of the seven studies reported a significant overall benefit for GTX.[7] [8] [9] In two additional studies,[4] [6] overall success was not demonstrated for GTX, but certain subgroups of patients were found to benefit significantly. For example, in the first controlled study,[4] many patients received an inadequate dose of GTX by current standards, and overall success was not demonstrated. However, 100% of patients who received GTX on at least four occasions and 80% of those who received GTX on at least three occasions survived, compared with only a 30% survival among controls. In the other study that found partial benefit, no advantage for GTX could be demonstrated when all patients were analyzed. However, when the subgroup of patients with persistent bone marrow failure were analyzed separately, 75% of those receiving GTX responded favorably, compared with only 20% of controls. Thus, some measure of success for GTX was evident in five of the seven controlled studies. However, this success was counterbalanced by four studies that were negative in some respect, two totally[5] [10] and two partially negative.[4] [6]

An explanation of these inconsistent results is evident on critical analysis of the adequacy of GTX support (Table 137-3). Patients in the three successful trials received relatively high doses of PMNs (generally 1.7 × 10^{10} /day).[7] [8] [9] Moreover, donors were selected to be both erythrocyte and leukocyte compatible. By contrast, the four controlled studies yielding negative results can legitimately be criticized in light of current technology. Data for three [4] [5] [6] of the four negative studies were collected before 1977, when the PMNs transfused were clearly inferior, in both quality and quantity, to those available today. Two of the four studies with negative conclusions used PMNs collected by filtration leukapheresis for at least some patients. [4] [6] It is now known that such PMNs are defective, and they are no longer transfused. Although three of the four negative studies used PMNs collected by centrifugation leukapheresis, [5] [6] [10] the dose was extremely low (0.410.56 × 10^{10} per concentrate). This daily dose is approximately one-tenth the dose that could be transfused currently, and it is not surprising that GTX were unsuccessful when given in such a grossly inadequate fashion. As another factor, investigators in two of the four negative studies [4] [10] made no provision for the possibility of leukocyte alloimmunization and selected donors solely on the basis of erythrocyte compatibility. Finally, control subjects responded reasonably well to antibiotics alone in three of the four negative studies, [4] [6] [10] suggesting that some patients have no apparent need for additional therapeutic modalities.

The preceding analysis is qualitative and suffers from the imprecision of combining data from studies that, although controlled, are not truly comparable (i.e., nonuniformity among

TABLE 137-3 -- Design of Seven Controlled Studies Evaluating Therapeutic Granulocyte Transfusions in Neutropenic Patients

Investigators	Randomized?	Characteristics of Neutrophil Concentrates				
		Collection Method	Dose (× 10^{10})	Schedule	HLA[a]	WBC[a]
Higby et al.[8]	Yes	Filtration	2.2	Daily	No	Yes
Vogler and Winton[9]	Yes	Centrifugation	2.7	Daily	Yes	Yes

Herzig et al.[7]	Yes	Filtration	1.7	Daily	No	Yes
		Centrifugation	0.4	Daily	No	Yes
Alavi et al.[4]	Yes	Filtration	5.9	Daily	No	No
Graw et al.[6]	No	Filtration	2.0	Daily	No	Yes
		Centrifugation	0.6	Daily	No	Yes
Winston et al.[10]	Yes	Centrifugation	0.5	Daily	No	No
Fortuny et al.[5]	No	Centrifugation	0.4	Daily	No	Yes

[a] Donors selected to be compatible with recipient either by HLA typing (A and B loci matched, at least in part) or by leukocyte crossmatching.

control subjects, patient clinical status, GTX dose, compatibility, etc.). Recently, data from the seven controlled GTX trials were analyzed quantitatively by formal meta-analysis,[11] and many of the impressions of the preceding qualitative analysis were confirmedspecifically, that the dose of PMNs transfused and the survival rate of the nontransfused control subjects were primarily responsible for the differing success rates of the studies. In clinical settings in which the survival rate of nontransfused control subjects was low, study subjects benefited from receiving adequate doses of GTX. The authors concluded that severely neutropenic patients with infections known to carry a high mortality should be considered for GTX given in an adequate dose. [11]

The most frequent infection for which GTX were considered previously was bacterial sepsis, and many patients treated with GTX for septicemia have been reported (Table 137-1 (Table Not Available)). Most neutropenic patients with bacterial sepsis, who experience bone marrow recovery during the early days of infections, will respond to antibiotics alone.[2] [3] Most patients with newly diagnosed acute leukemia and in whom induction chemotherapy is successful fit into this category and will not need GTX. By contrast, septic patients with persistent neutropenia due to continuing marrow failure may benefit from GTX added to antibiotic therapy. [2] Examples are patients in the later stages of leukemia undergoing investigational chemotherapy or recipients of marrow grafts, in whom hematopoietic recovery may be delayed for >23 weeks.

Regarding the other infections listed in Table 137-1 (Table Not Available) , information published to date is insufficient to determine definitively whether therapeutic GTX offer benefits over those of antibiotics alone. Currently, yeast and fungal infections pose difficult problems that deserve additional discussion. Occasional case reports, experimental studies in animals, and experience in treating patients with chronic granulomatous disease support the success of GTX in some patients with yeast and fungal infections. In addition, an uncontrolled study of 15 patients [12] found a 60% favorable response to transfusion of granulocytes collected from G-CSF-stimulated donors when the granulocytes were given to neutropenic patients with fungal infectionsa success rate higher than expected from the usual clinical experience. In contrast, a large clinical study[13] comparing infected bone marrow transplant patients given GTX ($n = 50$) to those treated without GTX ($n = 37$) found no benefit for GTX in treating fungal and yeast infections. This study was not designed to provide definitive answers. It was a retrospective review. Patients were not randomly selected for therapy; instead, the decision to use GTX or not was determined according to individual physician preference, making it impossible to exclude selection bias. Neither patient characteristics nor the types of infection being treated were evenly distributed between the GTX group and the patients not given GTX. The dose of PMNs transfused was known for only 15% of the GTX administered and likely was quite low because of the collection techniques used. [13]

To determine the optimal role for therapeutic GTX, individual physicians must survey the outcome of life-threatening infections with bacteria, yeast, and fungi in their own neutropenic patients. If infections in these patients respond promptly to antibiotics alone and survival approaches 100%, GTX are unnecessary and should not be used, as the benefits would not outweigh the potential risks. However, if significant numbers of infected neutropenic patients fail to respond to antibiotics alone, the addition of GTX should be considered, along with other modifications of therapy (e.g., selection of different antibiotics, closer monitoring of antibiotic blood levels, intravenous immunoglobulin [IVIG] therapy, G-CSF or other recombinant growth factors, and immune-modulating agents). Once the decision to use therapeutic GTX has been made, they must be given effectively, and recommendations for PMN collection are discussed later in this chapter. A daily infusion of approximately 48×10^{10} PMNs should be given to patients with persistent neutropenia ($<0.5 \times 10^9$ /L blood PMNs) and infections that have failed to respond to a reasonable course (48 hours) of combination antimicrobial agents. GTX are continued until the infection has resolved or until the blood PMN count is maintained at $>1.0 \times 10^9$ /L. This end point may be difficult to detect because transfused PMNs, when collected from G-CSF-stimulated donors and transfused at doses of 48×10^{10} PMNsmay elevate the recipients blood PMN count to 13×10^9 /L for several hours post infusion. Thus, accurately distinguishing transfused PMNs from marrow recovery is challenging and must be based on a sustained increase in blood PMN counts.

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THERAPEUTIC GRANULOCYTE TRANSFUSIONS IN SPECIAL CIRCUMSTANCES

Most patients with congenital disorders of PMN dysfunction have adequate numbers of blood PMNs, but they are susceptible to serious infections because their PMNs fail to kill pathogenic microorganisms. Patients with severe forms of PMN dysfunction are relatively rare, and few definitive studies have been reported to establish the efficacy of therapeutic GTX in their management. Thus, firm recommendations about the use of GTX to treat patients cannot be made. However, several patients with chronic granulomatous disease complicated by progressive life-threatening fungal infections have been reported to benefit. [14] [15] [16] [17] [18] [19] [20] [21] [22] Because of the possibility of alloimmunization and transfusion-transmitted infections, therapeutic GTX are recommended only for progressive infections that cannot be controlled with antimicrobial drugs. Because of lifetime problems with infections, prophylactic GTX are impractical.

Neonates (infants within the first month of life) are another

TABLE 137-4 -- Five Controlled Trials (Six Reports) of Neonatal Granulocyte Transfusions

Investigators	Randomized?	Infants Transfused		Infants Not Transfused	
		No.	Survival (%)	No.	Survival (%)
Laurenti et al.[23]	No	20	90[a]	18	28
Christensen et al.[22]	Yes	7	100[a]	9	11
	No[b]			10	100
Cairo et al.[24]	Yes	13	100[a]	10	60
Cairo et al.[25]	Yes[c]	21	95[a]	14	64
Baley et al.[26]	Yes	12	58	13	69
Wheeler et al.[27]	Yes	4	50	5	40
	No[b]			11	91

[a]Transfused infants survived significantly better than nontransfused infants.
[b]Additional nontransfused infants who were not randomized because all had adequate marrow storage pools.
[c]Expanded version of study reported earlier by Cairo et al. [24]

group of patients who may suffer life-threatening bacterial infections caused, at least in part, by PMN dysfunction and neutropenia. [23] [24] Neutropenia must be viewed differently in neonates than in older patients, in whom GTX are considered usually when the blood PMN count falls to $<0.5 \times 10^9$ /L. By contrast, absolute blood PMN counts as high as 3.0×10^9 /L might prompt consideration of GTX in neonates. [20] [21] The blood neutrophil count varies greatly during the first days of life, and a transient neutrophilia with absolute PMN counts of 1025×10^9 /L is commonly seen in healthy neonates. Although not completely specific, sepsis should be suspected in any sick neonate with an absolute PMN count of $<3.0 \times 10^9$ /L during the first week of life. The mechanism of neutropenia cannot always be identified, but in some infants a marked decrease in the marrow PMN storage pool can be demonstrated. For example, these forms (metamyelocytes and segmented PMNs) account for 2665% of all nucleated cells in normal marrow, but in some neonates with sepsis this value will be <10% of nucleated marrow cells.

Several investigators have reported using GTX to treat neonatal sepsis. Four [25] [26] [27] [28] of the six controlled studies[25] [26] [27] [28] [29] [30] demonstrated a significant benefit of GTX (Table 137-4). However, these studies can be criticized for small size, faulty design, and heterogeneity of both patients and quality of GTX. Thus, the use of GTX for neonatal sepsis remains controversial and, as discussed in the following section, alternative therapies must be considered.

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ALTERNATIVE OR ADDITIVE MEASURES TO THERAPEUTIC GRANULOCYTE TRANSFUSIONS

Neonates and older patients with severe neutropenia, particularly those undergoing chemotherapy or bone marrow transplantation, exhibit a variety of abnormalities in body defense mechanisms, not all of which can be corrected by GTX. Consequently, infections that occur in these patients do not always respond to GTX, and a number of additional therapies have been evaluated. Two that appear to hold promise are the use of recombinant myeloid growth factors and IVIG.

Recombinant myeloid growth factors such as G-CSF and granuloctye-macrophage colony-stimulating factor (GM-CSF) are glycoprotein cytokines that enhance the production, differentiation, and function of myeloid cells. [30] In neonates, studies of cytokine production have yielded conflicting results, with investigators reporting values that are higher than, equal to, or lower than adult values. [32] [33] [34] [35] [36] [37] [38] [39] Generally, G-CSF and GM-CSF levels are lower in preterm than in term neonates, and the ability of preterm leukocytes to further increase production when stimulated is diminished. Plasma G-CSF levels were reported to be markedly elevated in neonates with infection, [33] [34] but other investigators found G-CSF mRNA expression and protein production to be decreased in neonatal leukocytes, particularly in activated cells. [35] [36] In a controlled study, [40] 42 neonates with presumed bacterial sepsis were randomized to receive three doses of either G-CSF or placebo. Therapy with G-CSF significantly increased the marrow PMN storage pool, blood PMNs, and expression of PMN membrane C3bi. [40] In a similar study of GM-CSF, [41] 20 preterm neonates were randomized within 72 hours of birth to receive either GM-CSF or placebo for 7 days; GM-CSF increased marrow PMNs, blood PMNs, and C3bi receptor expression. Unfortunately, neither the G-CSF nor the GM-CSF studies reported sufficient information to assess their efficacy in the prevention or treatment of neonatal sepsis. However, two randomized clinical trials have been conducted, more recently, to assess the efficacy of G-CSF and GM-CSF. Neither has demonstrated clear clinical benefit. In the G-CSF trial, 20 infants with neutropenia and sepsis received either G-CSF (10 g/kg/day) or placebo for three days. [42] Recognizing that the number of study subjects was quite small, G-CSF did not significantly improve severity of illness, morbidity, or mortality. In a preliminary report of GM-CSF, [43] preterm infants received either GM-CSF (8 g/kg/day) or placebo for the first 28 days of life in an attempt to reduce the incidence of infections. Although GM-CSF was well tolerated and significantly increased blood leukocyte counts, it did not significantly decrease infection rates.

In older children and adult patients, both G-CSF and GM-CSF have been used to accelerate marrow recovery following chemotherapy and to successfully diminish the rate of acquiring infections and the need for prolonged hospitalization. [31] In contrast to this success in preventing neutropenic infections, the role of G-CSF and GM-CSF in treating already established infections is not as firmly documented. Regardless, these growth factors frequently are given to patients with neutropenia and severe infections, and it seems reasonable to consider adding GTX to the treatment of severe bacterial, yeast, or fungal infections that are progressing in neutropenic patients despite the use of appropriate antibiotics plus recombinant myeloid growth factors.

The use of IVIG, either to prevent or to treat neonatal sepsis, has appeal because this therapy may correct abnormalities of both humoral immunity and neutrophil function. In studies of experimental infections, IVIG proved beneficial by increasing opsonic activity and by improving PMN kinetics in animals. [44] [45] [46] Although the precise role of IVIG in the management of human infants is incompletely defined, this therapy has been given (with mixed results) both to prevent and to treat infections. Most prophylactic studies evaluating IVIG to prevent infections have found little or only modest benefit, [47] with a few studies suggesting true benefit. [48] [49] [50] In contrast, several therapeutic studies reported benefit from adding IVIG to antibiotics during the treatment of neonatal infections. [47] However, the data remain insufficient to justify the routine use of IVIG as standard therapy to prevent and/or treat infections in preterm infants. Moreover, caution is warranted before IVIG is broadly applied as treatment for neonatal sepsis. In high doses, IVIG has been demonstrated to impair body defense mechanisms and to increase susceptibility to fatal infections. [51] [52] [53] IVIG has great appeal because of the ease with which it can be prescribed, its apparent benefits to multiple body defense systems, and, finally, its safety in terms of failing to transmit donor infectious diseases. However, the genuine benefits and risks of IVIG must be carefully examined before it is prescribed with impunity.

Thus, the role of GTX, IVIG, and recombinant myeloid growth factors in the treatment of neonatal infections is unclear, and none of these approaches can be recommended as a standard of routine neonatal practice. Moreover, in many studies,

standard supportive care with antibiotics seems to provide adequate therapy. Each institution must assess its own experience with neonatal sepsis and local management. If nearly all infants survive without apparent long-term morbidity, GTX or alternative therapies are unnecessary, and attention should be focused on prompt diagnosis and optimal antibiotic therapy. If the outcome of standard supportive therapy is less than optimal, additional therapies such as GTX must be explored.

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PROPHYLACTIC GRANULOCYTE TRANSFUSIONS IN NEUTROPENIC PATIENTS

Based on existing reports, prophylactic GTX are of marginal value. In 12 reports, [54] [55] [56] [57] [58] [59] [60] [61] [62] [63] [64] [65] benefits were few, while risks and expenses were substantial. However, partial success was demonstrated when certain subgroups of patients were examined separately. Some measure of success was found in seven of 12 studies; the remaining five studies failed to show a benefit for prophylactic GTX. [61] [62] [63] [64] [65] (R.M. Cooper, personal communication). In none of these five negative studies were large numbers of PMNs obtained from matched donors and transfused daily. Thus, in a situation analogous to that for the negative therapeutic GTX trials, the failure of prophylactic GTX might be explained, at least in part, by inadequate transfusions.

A major concern raised by prophylactic GTX has been that of transfusion-related risks, a concern heightened because of the marginal benefits of prophylactic GTX. In the therapeutic setting, where GTX efficacy is more apparent, risks are more acceptable. Leukocyte alloimmunization poses a risk of special importance for GTX therapy. Although reports are controversial, it seems likely that most patients receiving multiple GTX from random donors will develop antileukocyte antibodies. Antileukocyte antibodies mediate transfusion reactions, have an adverse effect on post-transfusion increments in blood PMN counts, alter the circulating kinetics of infused PMNs, and decrease the antimicrobial effects of GTX. [66] [67] [68] [69] Many of the other risks of GTX have been greatly diminished by current practices, such as -irradiation to prevent graft-versus-host disease and the use of cytomegalovirus-seronegative units to eliminate cytomegalovirus transmission.

Clearly, prophylactic GTX cannot be recommended at this time to treat patients with acute leukemia undergoing first remission induction or consolidation therapy. However, consideration should be given to the use of prophylactic GTX in bone marrow transplant recipients. Progressive infections, particularly with yeast and fungi, occur frequently in bone marrow transplant recipients who are neutropenic, exhibit PMN dysfunction, and manifest defective cellular and humoral immunity for months following transplantation. Altered immunity is particularly profound when marrow is T-lymphocyte depleted to diminish graft-versus-host disease. Although all types of infection pose a threat, 10% of 1,186 marrow transplant patients developed a noncandidal fungal infection, with only 17% of infected patients surviving. [70] Thus, the possible role of GTX, collected by modern techniques from G-CSF-stimulated donors, needs to be reexamined as a means to approach this important clinical problem.

Autologous bone marrow transplantation has been supplanted by transfusion of peripheral blood hematopoietic/immunologic progenitor cells collected following cytokine (usually G-CSF) stimulation. This technique is being used increasingly in the allogeneic setting because it is convenient, economical, and leads to relatively rapid engraftment.[71] Recovery to a blood PMN count of 0.5×10^9 /L occurs within 614 days after transfusion of progenitor cells. In some patients, the period of severe neutropenia <0.2×10^9 /L) persists only a few days, particularly when G-CSF is given to the patients following myeloablation. Thus, the complete elimination of severe neutropenia

AUTHORS APPROACH TO THERAPEUTIC GTX

Indicated for severe bacterial, yeast, or fungal infection in a neutropenic patient (<0.5×10^9 PMN/L blood) when the infection progresses despite optimal antimicrobial therapy.
Collect PMNs (48×10^{10}) from allogeneic blood donors, as follows:

1. Stimulate neutrophilia by giving the donor 300 g of G-CSF subcutaneously 12 hours before beginning leukapheresis, plus three doses of oral prednisone, 20 mg, taken approximately 18, 12, and 4 hours before beginning leukapheresis.
2. Process 10 L of donor blood using a continuous-flow blood separator with citrated hydroxyethyl starch solution infused throughout the collection.

Transfuse one granulocyte concentrate (48×10^{10} PMNs) daily until either bone marrow recovery (blood PMNs >1.0×10^9 /L without GTX) or clinical resolution of infection.

using this transplantation approach plus prophylactic GTX is a distinct possibility that deserves careful study.

Allogeneic peripheral blood progenitor cell donors receive G-CSF daily for 47 days, with leukapheresis usually performed on days 57 to collect sufficient progenitor cells for transfusion. Donor blood PMN counts increase, often to >50×10^9 /L, with G-CSF, and extraordinarily large doses of PMNs could be collected on the days following progenitor cell leukapheresis. Obviously, systematic studies are needed to define optimal G-CSF dose and to schedule the proper timing of leukapheresis procedures, first to collect progenitor cells for transplantation and then to collect PMNs for GTX; to examine the coordination of myeloablation, progenitor cell collection, storage, and transfusion, and subsequent PMN collection and transfusion; and to identify potential risks and inconvenience to the donor of prolonged G-CSF stimulation and multiple leukapheresis procedures. Moreover, the efficacy of prophylactic GTX used as part of this progenitor cell then PMN transfusion approach versus peripheral blood progenitor cell transfusion followed by cytokine therapy of the patient without GTX must be investigated by properly designed randomized trials.

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PREPARATION AND STORAGE OF NEUTROPHIL CONCENTRATES

To ensure adequate numbers and quality of PMNs, granulocyte concentrates must be collected from stimulated donors by automated leukapheresis using an erythrocyte sedimenting agent such as hydroxyethyl starch.[72] A major limitation of GTX efficacy has been the inability to transfuse adequate numbers of perfectly functioning PMNs. Under the stress of a severe bacterial infection, the marrow of an otherwise healthy adult will produce between 10^{11} and 10^{12} PMNs in 24 hours. Granulocyte concentrates collected from healthy donors who are not stimulated with corticosteroids or G-CSF will contain between 0.2 and 0.8 × 10^{10} PMNsabout 1% of a healthy marrows output. Hence, donor stimulation is mandatory to achieve even a hope of a reasonable PMN dose per GTX. Donor stimulation with properly timed corticosteroids (4 hours before leukapheresis) will increase the yield to about 2 × 10^{10} PMNs.[73] Stimulation with G-CSF alone or in combination with corticosteroids will produce higher but variable PMN yields, depending on G-CSF dose and schedule of administration. Yields of 48 × 10^{10} PMNs

are achieved regularly, and post-transfusion blood PMN counts frequently increase to 13 × 10^{9} /L, with PMNs detected in the recipients bloodstream for several hours following GTX.[74] [75] [76] Thus, PMN donors are optimally stimulated using G-CSF plus corticosteroids, with the dose and schedule best determined by local needs. [75]

Granulocyte concentrates should be transfused as soon as possible after collection because PMN functions begin to deteriorate almost immediately. [77] [78] Some delay between collection and transfusion is inevitable, and granulocyte concentrates are usually stored briefly at 22°C, with little or no agitation. Lane et al. [78] have published a number of reports on the properties of PMNs collected for transfusion, and aberrations of nearly every function become evident within 2472 hours. It is highly desirable to transfuse granulocyte concentrates within 6 hours of collection; they should not be given if >24 hours old.

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Chapter 138 - Transfusion of Plasma Derivatives: Fresh-Frozen Plasma, Cryoprecipitate, Albumin, and Immunoglobulins

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INTRODUCTION

Plasma and its derivatives represent a valuable resource, but the risk of infectious disease transmission, although rare, and other adverse effects mandate their appropriate use. The increased use of plasma and plasma derivatives has led to a decline in the use and availability of whole blood. Plasma can be separated from packed red cells (PRBC) through centrifugation of whole blood at the time of collection, or plasma can be collected by apheresis as a single product or as a byproduct of platelet apheresis or red blood cell apheresis. Plasma is further processed into its derivatives through the cold ethanol fractionation (method of Cohn). [1] In this chapter, the features and uses of fresh-frozen plasma (FFP), cryoprecipitate, albumin, and intravenous (IVIg) and intramuscular immunoglobulins are discussed. The use of plasma-derived clotting factor concentrates for therapy of specific congenital clotting factor deficiencies is discussed in Chapter 140 .

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FRESH-FROZEN PLASMA

Plasma is the fluid compartment of blood and consists of 90% water, 7% protein and colloids, and 23% nutrients, crystalloids, hormones, and vitamins. The protein fraction contains the soluble clotting factors, the constituents for which transfusion of plasma is most often required.

Plasma frozen at 18°C or colder within 6 hours of donation is labeled FFP. This product may be stored up to 1 year before use, at which time it is thawed over 2030 minutes. The activities of the labile coagulation factors (V and VIII) decrease after thawing but remain adequate for at least 24 hours. Plasma that is not immediately frozen as FFP becomes either liquid plasma (stored at 16°C) or source plasma (stored at 18°C or colder). These products are used for the preparation of plasma derivatives: albumin, clotting factor concentrates, and immunoglobulin preparations. When FFP is thawed at 4°C, a precipitate forms that is separated by centrifugation; the

supernantant is decanted. These are termed cryoprecipitate and cryo-poor-supernatant, respectively. Their use is discussed later in this chapter.

Solvent Detergent-Treated Plasma

Recently, the Food and Drug Administration (FDA) approved the clinical use of a new plasma product, solvent detergent (SD) plasma. SD plasma is a pooled product (2,500 donor units per pool) and subjected to solvent detergent treatment, a process combining organic solvent tri(n-butyl)phosphate with nonionic detergent, Triton X-100. The SD process inactivates lipid-enveloped viruses, including human immunodeficiency virus (HIV) type 1 and 2, hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell lymphotropic virus (HTLV) type 1 and 2, hepatitis G virus (HGV), vesicular stomatitis virus, Sindbis virus, and Sendai virus, and preserves the structural and functional integrity of selected plasma proteins. It does not, however, inactivate nonenveloped viruses, particularly parvovirus B19 and hepatitis A virus (HAV), or the causative agents of various encephalopathies, or Creutzfeldt-Jakob disease. [1A] [1B] [1C] [1D] Although the transfusion-related transmission of Creutzfeldt-Jakob disease is only a theoretical one (no cases have been reported), the increased prevalence of parvovirus B19 and HAV make transmission by transfusion of pooled blood components a real concern. [1E] [1F] [1G] Unlike other factor concentrates and FFP, pooled SD plasma contains significant amounts of neutralizing antibody to both parvovirus B19 and HAV, which have protective value. [1H] [1I] Although the indications for SD plasma are similar to those for FFP, the per-unit cost of SD plasma is much greater than that of FFP.

Indications

The indications for the use of FFP, the clinical conditions for which FFP administration is not indicated, and investigational uses are listed in Table 138-1 . Audits of transfusion practices have consistently demonstrated that FFP use is inappropriately high. [2] Specifically, FFP should not be used (1) to reconstitute whole blood by coadministration with red blood cells (RBCs), (2) as a volume expander, or (3) as a source of nutrients. Appropriate uses are described in detail in the following sections.

TABLE 138-1 -- Administration of Fresh-Frozen Plasma

Indicated
Multiple acquired coagulation factor deficiency [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16]
Liver disease
Massive transfusion
Disseminated intravascular coagulation
Rapid reversal of warfarin effect
Plasma infusion or exchange for thrombotic thrombocytic purpura, hemolytic uremic syndrome, syndrome of hemolysis, elevated liver enzymes, and low platelets or Refsums disease[17] [18] [19] [20] [21]
Congenital coagulation defects (see Chap. 140)[a]
C1-esterase inhibitor deficiency[a] acute episodes and prophylaxis of angioedema [22] [23]
Investigational
Meningococcal sepsis[24] [25]
Acute renal failure in the context of multiorgan failure [26]
Not indicated
Immunodeficiency
Burns
Wound healing
Reconstitution of packed red blood cells
Volume expansion
Source of nutrients

[a]Fresh-frozen plasma is used in the absence of specific factor concentrate.

Liver Disease and Transplantations

Patients with severe liver disease may have low levels of the vitamin K-dependent clotting factors (II, VII, IX, and X) (see Chap. 115). These patients develop a prolonged prothrombin time (PT) and partial thromboplastin time (PTT). In addition, the thrombin time (TT) may be prolonged, fibrin split products may be elevated, and in later stages the fibrinogen level decreases. Hemorrhage, most often secondary to an anatomic lesion, may be complicated by the coagulopathy represented by these abnormalities.

Infusion of FFP is indicated during severe bleeding if the coagulation test results are abnormal. In the absence of anatomic lesions, bleeding does not usually occur until the PT is >1618 seconds or the PTT is >5560 seconds. FFP is not recommended prophylactically before a surgical challenge or liver biopsy unless these values are exceeded.[3] In fact, the PT and PTT are poor predictors of surgical bleeding, and mild abnormalities in these coagulation tests may be impossible to correct even with infusion of large quantities of FFP. [4] [5] Orthotopic liver transplantation complicated by pre-existing severe liver disease, lack of clotting factor synthesis during the anhepatic stage, massive PRBC transfusion, and disseminated intravascular coagulation (DIC) may require large volumes of FFP. [6] Transfusion should be guided by clinical assessment of bleeding, coagulation test results, and thromboelastographic changes. [7] [8]

Massive Transfusion

The administration of FFP to patients receiving large quantities of PRBC (>1 blood volume in 24 hours) is often recommended because of a dilutional coagulopathy arising from blood replacement with PRBC and crystalloid solutions, both of which lack coagulation factors. Studies addressing the use of FFP in massive transfusion have yielded confusing and conflicting results. [9] [10] Dilutional thrombocytopenia may also complicate the coagulopathy in these patients. [11]

In general, FFP transfusions are of little value in nonbleeding patients who have received <610 units of PRBC. In some patients, however, shock associated with DIC and dilutional coagulopathy may occur, necessitating FFP. [12] While several studies have demonstrated the ineffectiveness of routine FFP infusions in patients receiving >10 units of PRBC within 24 hours, many physicians still opt to transfuse FFP in the face of continued bleeding. It is far more effective to use FFP to replace depleted clotting factors reflected by abnormal laboratory tests than to use predetermined formulations (e.g., 1 unit of FFP for every 5 units of PRBC transfused). [13]

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation may be secondary to sepsis, liver disease, hypotension, surgery-associated hypoperfusion, trauma, obstetric complications, leukemia (usually promyelocytic), or underlying malignancy (see Chap. 117). Successful treatment of the underlying cause is paramount. Patients with DIC and bleeding should be given FFP in amounts sufficient to correct or ameliorate the hemorrhagic diathesis, although in patients with severe liver disease, bleeding, and DIC, FFP infusions in any amount often fail to normalize the PT and PTT. [14]

Rapid Reversal of Warfarin Effect

Warfarin inhibits the hepatic synthesis of vitamin K-dependent clotting factors (II, VII, IX, and X) by blocking the recovery of the form of vitamin K that is active in the carboxylation of these proteins (see Chap. 115). Thus, warfarin therapy induces functional deficiencies of these factors, which correct within 48 hours after the discontinuation of warfarin if diet and vitamin

K absorption are normal. Vitamin K administration corrects the coagulopathy in 1218 hours. [15] In patients anticoagulated with warfarin who have active bleeding, require emergency surgery, or have serious trauma, the deficient clotting factors can be immediately provided by FFP transfusion. To replenish the vitamin K-dependent coagulation factors such that adequate hemostasis (normalized PT) is established, a large volume of FFP may be required. Thus, when volume overload is a concern, prothrombin complex concentrates may be used to reverse the warfarin effect. In patients without bleeding but with significantly prolonged PTs, the potential danger of significant hemorrhage is often overemphasized and must be weighed against the risks of FFP infusion. [16]

Thrombotic Thrombocytopenic Purpura and Hemolytic Uremic Syndrome

In some patients with thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS), or the syndrome of hemolysis, elevated liver enzymes, and low platelets, FFP transfusion or exchange has induced clinical remission. [17] [18] [19] Plasmapheresis may be preferred in patients who are at risk for fluid overload, such as those with renal or cardiac impairment (see Chap. 128). Several mechanisms through which FFP exerts its beneficial effects have been suggested: FFP may restore prostaglandin I_2 (PGI_2), it may inhibit the agglutinating activity of the abnormal plasma, or it may inhibit the release of large-molecular-weight von Willebrand factor (vWF) multimers or restore their normal processing in the circulation. Some authorities have suggested the use of cryo-poor-supernatant for refractory TTP. [20] [21]

Dosage

One unit of FFP derived from a unit of whole blood contains 200280 ml. When plasma is collected by apheresis, as much as 800 ml can be obtained from one individual (jumbo plasma units). On average, there are 0.71 unit/ml of activity of each coagulation factor per ml of FFP and 12 mg/ml fibrinogen. The appropriate dose of FFP may be estimated from the plasma volume, the desired increment of factor activity, and the expected half-life of the factor being replaced. Alternatively, the FFP dosage may be estimated as 810 ml/kg, and should be ordered as the number of milliliters to be infused. [27] The frequency of administration depends on the clinical response to the infusion and normalization of laboratory parameters.

Compatibility and Side Effects

Fresh-frozen plasma is screened for unexpected RBC antibodies and should be ABO-type compatible. Tests for serologic compatibility are not performed before administration. The Rh(D) type is matched in only some parts of the United States because immunization to Rh(D) antigen has rarely been reported as a result of transfusion of Rh(D)-positive plasma to Rh(D)-negative individuals. [28] Plasma from multiparous women should not be used. Hemolytic reactions due to infusion of an undetected antibody directed toward recipient RBC antigens have been reported, but these cases are extremely rare. [29] Alloimmunization to RBC antigens may occur with FFP transfusion, but this is also rare. [30]

Fever, chills, and allergic reactions may occur and are treated symptomatically. Occasionally, severe allergic reactions with symptoms of bronchoconstriction or noncardiogenic pulmonary edema are seen. These cases are thought to be due to antibodies present in donor plasma that react with recipient leukocytes. Anaphylactic reactions may occur after infusion of plasma (containing IgA) into patients with IgA deficiency and antibodies to IgA. [31] For such patients, it is possible to obtain IgA-deficient plasma from donors in the national registry.

Transmission of infectious disease by FFP has been significantly reduced, but the risk is not yet completely eliminated. FFP is not thought to transmit the cell-associated cytomegalovirus. [32]

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CRYOPRECIPITATE

Cryoprecipitate is prepared from 1 unit of FFP thawed at 4°C. The precipitate is then refrozen in 10 to 15 ml of plasma and stored at 18°C or colder for 1 year. Cryoprecipitate contains 80100 units of factor VIII, 100250 mg of fibrinogen, and 5060 mg of fibronectin. Initially the plasma also contains 4070% of vWF. Anti-A and anti-B antibodies may also be present.

For clinical purposes, cryoprecipitate should be considered the blood product most rich in factor VIII, vWF, XIII, fibrinogen, and fibronectin. Cryoprecipitate is used predominately to treat bleeding associated with fibrinogen or factor XIII deficiency states, occasionally in vWF deficiency, and only rarely in factor VIII deficiency. Congenital deficiencies of these factors are discussed in Chapters 109 , 111 , and 114 .

Indications

The indications and clinical conditions for which the administration of cryoprecipitate is not indicated are listed in Table 138-2 . Specific indications and inappropriate uses are discussed in the following sections.

Fibrinogen Deficiency

Fibrinogen deficiency is the primary indication for cryoprecipitate transfusion. It may be in the form of congenital afibrinogenemia (rare) or dysfibrinogenemia due to severe liver disease and DIC.[41] These patients often have concomitant decreases in clotting factor levels and require the coadministration of FFP. It is important to obtain fibrinogen measurements because levels <100 mg/dl cause prolongation of both the PT and PTT despite adequate clotting factor replacement. Very low levels of fibrinogen occur during liver transplantation, where transfusion support with cryoprecipitate is vital. [6] [16]

von Willebrand Disease

von Willebrand factor, required for platelet binding to disrupted vascular endothelium, is low or dysfunctional in von Willebrand disease. DDAVP (desmopressin acetate[1-deamino, 8-D-arginine], vasopressin) remains the treatment of choice, although severe bleeding may require cryoprecipitate. Recent

TABLE 138-2 -- Administration of Cryoprecipitate

Indicated
Some patients with hemophilia A or von Willebrands disease [34]
Fibrinogen deficiency
Congenital afibrinogenemia
Dysfibrinogenemia[33]
Factor XIII deficiency[a]
Fibrin glue[35] [36] [37] [38] [39] [40] [41] [42] [43] ,[b]
Renal stone removal[48]
Investigational
Wound healing (as a source of fibronectin) [49]
Not indicated
Uremic bleeding
Sepsis (postoperative)

[a]Cryoprecipitate is used in the absence of specific factor concentrate.
[b]This product is not FDA-approved in the United States.

advances in clotting factor concentrates and viral inactivation methods may make the use of wet cryoprecipitate obsolete. [34]

Fibrin Glue

Fibrin glue results from the mixture of a fibrinogen source (either from FFP, platelet-rich plasma, or heterologous [90%] or more recently autologous cryoprecipitate) with bovine thrombin. The enhanced local hemostasis achieved by fibrin glue is through the action of thrombin on fibrinogen. As adjunct therapy, this product has been used successfullly in cardiac surgery to control multiple small blood leaks due to adhesions, [35] and to reduce blood loss and the factor concentrate requirement in patients with von Willebrands disease undergoing various surgical procedures. [36] Both autologous and heterologous fibrin clot preparations have been used in meniscal tear repair,[37] in establishing patency of anastomosis in vasovasostomy,[38] in the treatment of postoperative neonatal chylothorax,[39] and in other surgical procedures.[40] [41] [42] In otologic and neurotologic procedures, autologous fibrin glue is extensively used (e.g., ensuring a watertight seal in wound closure, securing prosthetic devices during reconstruction procedures, stabilizing fascial and dural homografts, and in nerve anastamosis [43]). The infectious risks (e.g., human immunodeficiency virus [HIV], hepatitis) associated with the use of heterologous fibrin glue are eliminated by replacement with the autologous source. Other complications related to bovine thrombin (anaphylaxis, formation of antibodies to factor V) or related to the activation of coagulation have been reported. [44]

Uremic Bleeding

Abnormal bleeding is a common complication of uremia. In 1980, a single study led to the widespread but temporary use of cryoprecipitate for the treatment of uremic

bleeding.[45] No data currently support this use.[46] Cryoprecipitate should not be used in the routine management of uremic bleeding, but its use may be considered if other therapeutic interventions have failed.[47]

Dosage

The dosage of cryoprecipitate is calculated on the basis of the amount of fibrinogen present in 1 unit of cryoprecipitate, the plasma volume, and the desired increment. The difficulty in determining the correct amount to administer is due primarily to variability in the fibrinogen content of cryoprecipitate, secondary to variable storage volume and pooling methods used. The goal of therapy should be to maintain the measured fibrinogen at >100 ml/dl. This can usually be accomplished by the transfusion of 24 units/10 kg if the fibrinogen content of the concentrate is low and by 12 units/10 kg if the fibrinogen content is high.

Compatibility and Side Effects

Because cryoprecipitate can contain anti-A and/or anti-B antibody, infused units should be ABO compatible. The risks of fevers, chills, allergic reactions, and infectious disease transmission are similar to those of FFP.

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ALBUMIN

Albumin, an important plasma protein, contributes primarily to the maintenance of plasma colloid oncotic pressure. It is clinically available in three forms: a 5% solution in saline, a 25% solution in distilled water, and purified protein fraction (PPF), which is 5% total protein (88% albumin, 12% globulins). These products are heat-treated and unable to transmit viruses. The high cost, periodic shortages, and lack of efficacy in some clinical situations have led to increased scrutiny over the appropriate use of this important plasma derivative. [50] [51] In many clinical situations, the use of albumin remains controversial and is the subject of continued debate. We believe that albumin is extensively overused. Thus, in Table 138-3 , we have listed our preferences for indications, possible indications, investigational uses, and clinical situations in which we believe albumin administration is not indicated. Under common usages, we have listed clinical situations in which albumin is frequently used.

Indications

A decrease in measured plasma albumin is found in many situations and is often not a clinical concern. Mild edema due to hypoalbuminemia does not require albumin therapy. However, inadequate synthesis, as seen in severe liver disease and severe malnutrition, or excessive loss, as seen in nephrotic syndrome and protein-losing enteropathy, can lead to significant hypoalbuminemia with intravascular volume depletion, anasarca, ascites, and pleural effusions. Historically, albumin has had a broader use (i.e., nutritional support, correction of hypoalbuminemia, volume replacement), but recent studies support its benefit in fewer situations, including nephrotic syndrome resistant to potent diuretic therapy, following large-volume paracentesis, and in ovarian hyperstimulation

TABLE 138-3 -- Administration of Albumin

Indicated
After large-volume paracentesis[62] [63] [64] [65]
Nephrotic syndrome resistant to potent diuretics[66] [67]
Ovarian hyperstimulation syndrome[69] [70] [71] [72]
Volume/fluid replacement in plasmapheresis[116]
Possibly indicated
Adult respiratory distress syndrome
Cardiopulmonary bypass pump priming[54] [55]
Fluid resuscitation in shock/sepsis/burns [56] [57] [58]
Neonatal kernicterus
To reduce enteral feeding intolerance[117]
Not indicated
Correction of measured hypoalbuminemia or hypoproteinemia [56]
Nutritional deficiency, total parenteral nutrition [118] [119]
Pre-eclampsia[120]
Red blood cell suspension
Simple volume expansion (surgery, burns)[57] [58]
Wound healing
Investigational
Cadaveric renal transplantation[121]
Cerebral ischemia[122] [123]
Stroke[124]
Common usages
Serum albumin <20 g/dl
Nephrotic syndrome, proteinuria and hypoalbuminemia
Labile pulmonary, cardiovascular status
Cardiopulmonary bypass, pump priming
Extensive burns
Plasma exchange
Hypotension
Liver disease, hypoalbuminemia, diuresis
Protein-losing enteropathy, hypoalbuminemia
Resuscitation
Intraoperative fluid requirement exceeding 56 L in adults
Premature infant undergoing major surgery

syndrome (OHSS). The indications and inappropriate uses of albumin are described in detail in the following sections.

Intravascular Volume Expansion

Albumin provides the majority (80%) of plasma colloid oncotic pressure. Infused albumin provides colloid oncotic pressure; however, 50% of the infused protein is lost to the extravascular fluid compartment within 4 hours. Crystalloid may also provide volume expansion and is more quickly redistributed into total body fluids. Studies investigating the use of albumin in various situations including volume expansion during and after surgery, [52] [53] as priming solution in cardiopulmonary bypass, [54] [55] or in maintaining colloid oncotic pressure, [56] [58] found no clinical benefit compared with controls. An apparent benefit of albumin followed by diuretic therapy may be found in postoperative patients with intrapulmonary shunting, although the studies have been criticized. [59] [60] One prospective trial of colloid and crystalloid solutions has been performed in burn patients; [61] it found a difference in cardiac index in the albumin group but failed to show a significant difference in outcome. Thus, albumin therapy cannot be recommended for simple volume expansion or for the maintenance of albumin levels or colloid oncotic pressure.

Cirrhosis

The use of albumin in cirrhotic patients dates to before 1950. In this setting, albumin is recommended for temporary improvement in hyponatremia or prevention of the complications associated with paracentesis, including volume shifts and hyponatremia. Several studies demonstrated that after large-volume paracentesis, both hyponatremia and renal insufficiency were improved with albumin infusion compared with other volume-expanding agents. [62] [63] [64] [65]

Nephrotic Syndrome

Albumin has been used to increase colloid oncotic pressure with the intention of increasing diuresis. This use is currently limited to patients in whom diuretic therapy is poorly tolerated or ineffective or those with massive ascites or anasarca. [66] [67]

Ovarian Hyperstimulation Syndrome

Ovarian hyperstimulation syndrome is characterized by ovarian enlargement and increased capillary permeability that results in ascites and renal impairment with fluid and electrolyte imbalance. This potentially lethal syndrome is associated with exogenously administered gonadotrophins for the induction of ovulation in women undergoing reproductive procedures. [68] Several vasoactive substances, including an unidentified factor released by the hyperstimulated ovary, have been implicated in the pathogenesis of OHSS. In high-risk patients, the use of prophylactic IV human albumin at the time of oocyte retrieval has been shown to decrease significantly the incidence and severity of OHSS, [69] [70] [71] although the pregnancy rate may be less than that associated with embryo cryopreservation. [72] It is postulated that albumin exerts its beneficial effect through binding and inactivating the OHSS-associated factor.

Dose

The volume and speed of administration should be determined by the patients volume status, condition, and response to the product. Albumin 5% is oncotically equivalent to normal human plasma. Albumin 25% provides less infusion volume per amount of albumin.

Compatibility and Side Effects

Albumin is a plasma derivative used widely and associated with rare adverse reactions. Still, allergic reactions, including urticaria, may be encountered. Volume overload may occur with excessive doses, and a decrease in ionized calcium is at least a theoretical possibility. Albumin is an excellent growth medium for bacteria, and bacterial contamination can lead to febrile, and more serious, reactions. Albumin lots are tested for contaminants and pyrogens before shipment. Transmission of infectious diseases is no longer a concern.

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INTRAVENOUS IMMUNOGLOBULIN

Intravenous immunoglobulin is prepared by fractionation of large pools of human plasma. There are numerous preparations available in the United States and throughout the world. Each preparation is slightly different and has theoretical advantages and disadvantages and specific licensed indications. Ideally, IVIg should contain each IgG subclass; retain Fc receptor activity; have a normal half-life; demonstrate virus neutralization, opsonization, and intracellular killing; and have antibacterial capsular polysaccharide antibody. Further, vasoactive impurities should be absent, and no transmissible infectious agents should be present. [73]

Indications

Indications, possible indications, and investigational uses of IVIg are listed in Table 138-4 .

Primary Immunodeficiency Syndromes

Primary congenital immunodeficiency syndromes have been treated with intramuscular immunoglobulin for the past 30 years. The use of intramuscular immunoglobulin has certain disadvantages: delayed absorption, delivery of inadequate amounts due to small muscle mass, and pain at the injection site. IVIg overcomes these disadvantages, and used prophylactically in patients with primary immunodeficiency [74] [75] [76] has been demonstrated to reduce the number of febrile and infectious episodes as well as improve survival rate. [77] IVIg use in IgG subclass deficiencies is also beneficial. [78] [79]

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) may be associated with hypogammaglobulinemia and complications of repeated bacterial infections (see Chap. 72). IVIg decreases the incidence and severity of bacterial infections in CLL patients with hypogammaglobulinemia and has become accepted prophylactic therapy. [80] [81]

Bone Marrow Transplantation

The use of prophylactic IVIg or cytomegalovirus (CMV)IVIg in CMV-negative bone marrow transplant recipients during the first 100 days post-transplant has been demonstrated to reduce the incidence of symptomatic CMV-associated disease, including CMV interstitial pneumonia, in some trials. [82] [83] [84] Due to the high cost of this treatment and the increasing use of prophylactic ganciclovir, IVIg is currently not indicated for the

TABLE 138-4 -- Administration of Intravenous Immunoglobulins

Indicated
Primary immunodeficiency syndromes
Common variable immunodeficiency[94]
X-linked agammaglobulinemia
Severe combined immunodeficiency
Ataxia-telangiectasia
Wiskott-Aldrich syndrome[76]
IgG subclass deficiency[78] [79]
Cytomegalovirus-interstitial pneumonia after bone marrow transplant
Chronic inflammatory demyelinating polyneuropathy [134] [135] [136]
Chronic lymphocytic leukemia[80] [81]
Guillain-Barré syndrome[139] [140] [141] [142]
Idiopathic thrombocytopenic purpura (refractory) [92] [93] [94] [95] [96] [97] [98] [99] [100]
Inflammatory myopathies (refactory, dermatomyositis and polymyositis)[148] [149]
Mucocutaneous lymph node syndrome[102] [103]
Pediatric HIV infection[90] [91]
Parvovirus infection
Possibly indicated
Antiphospholipid syndrome in pregnancy[128] [129]
Autoimmune hemolytic anemia (warm-type unresponsive to prednisone)[130] [131]
Factor VIII inhibitors (refractory)[103] [185]
Graves ophthalmopathy[154]
Immune neutropenia[146]
Multiple myeloma (stable disease, high risk for infections) [152] [153]
Myasthenia gravis (refractory, acute severe decompensation) [155]
Systemic lupus erythematosus (refractory, severe active) [179] [180]

Thrombocytopenia refractory to platelet transfusion [181] [182]
Vasculitis (refractory to standard therapy) [183] [184]
Investigational
Acquired von Willebrand disease [126]
Amyotrophic lateral sclerosis[127]
Burn patients[132] [133]
Chronic fatigue syndrome[137] [138]
Fetomaternal alloimmune thrombocytopenia[162] [163]
Graft-versus-host disease[88] [89]
Human immunodeficiency virus infection[125] [143] [144] [145]
Immune-mediated aplastic anemia[81]
Inflammatory bowel disease[147]
Intractable childhood epilepsy (West syndrome and Lennox-Gastaut syndrome) [150] [151]
Multiple sclerosis[156] [157]
Neonatal hemolytic disease[164] [165] [166]
Neonatal sepsis[158] [161]
Prevention of nosocomial postoperative infections [167] [168]
Prophylaxis in transplant recipients against cytomegalovirus infection [169] [170]
Recurrent unexplained spontaneous abortions [171] [172] [173]
Rheumatoid arthritis[174] [175]
Solid organ transplantation[176] [177] [178]

prophylaxis of CMV infections in bone marrow transplant recipients. In established CMV-interstitial pheumonia, IVIg in combination with ganciclovir has been shown to reduce the mortality rate and has become the recommended treatment modality.[85] [86] [87] Its role in preventing severe graft-versus-host disease is controversial. [88] [89]

Pediatric Human Immunodeficiency Virus Infection

The defects in humoral and cellular immunity observed in children with HIV infection predispose them to life-threatening bacterial infections. Studies have shown that the administration of IVIg to HIV-infected children can reduce the incidence and severity of bacterial infections as well as the frequency of hospitalization. [90] [91] Prevention of maternalfetal transmission of HIV infection through the use of hyperimmune HIV-IVIg is being investigated.

Idiopathic Thrombocytopenic Purpura

The use of IVIg represents a major advance in the treatment of both acute and chronic idiopathic thrombocytopenic purpura (ITP) (see Chap. 126). IVIg significantly raises the platelet count within 5 days in both adults with chronic ITP and children with acute ITP. [92] [93] The mechanism of action of IVIg in ITP is unknown, but most believe that Fc receptor blockade decreases the removal of antibody-coated platelets. Other proposed mechanisms include suppressed antiplatelet antibody synthesis, increased antiviral immunity, and blockage by anti-idiotypic antibodies. [94] In general, IVIg induces responses in most patients within 12 days. Responses are of variable duration and rarely sustained, although maintenance therapy may be of some value. [95] IVIg may be effective in chronic ITP refractory to corticosteroids or splenectomy,[96] may show greater efficacy in conjunction with corticosteroids, [93] [97] and has been demonstrated to be superior to oral corticosteroids in children with acute ITP.[98] [99] [100]

Although IVIg has shown equal efficacy with corticosteroids in pediatric acute ITP and in 75% of adults with chronic ITP, because of the transient responses and high cost, its use is justified only in clinical situations requiring rapid elevation of platelet count or if standard therapy has failed. IVIg is therefore indicated in acute bleeding episodes or before urgent surgery, including splenectomy; in patients at high risk of intracranial hemorrhage; and in those in whom corticosteroids are contraindicated or ineffective. IVIg has also been used to treat ITP during pregnancy, postinfectious thrombocytopenia, ITP associated with HIV infection, and neonatal thrombocytopenia. Intravenous anti-D immune globulin has demonstrated efficacy in Rh-positive, nonsplenectomized individuals with ITP. It has been suggested that the mechanism of action may involve a shift in the immune-mediated destruction from platelets to the antibody-coated RBCs. [101]

Mucocutaneous Lymph Node Syndrome

The mucocutaneous lymph node syndrome (Kawasaki disease) has been treated with aspirin with or without concomitant IVIg administration. Coronary artery aneurysm, a serious complication of this disease, was significantly reduced in the IVIg-treated group. [102] [103]

Dosage

Patients with primary immunodeficiency syndromes and IgG levels <200 mg% are candidates for immunoglobulin replacement. Most patients require 100200 mg/kg IV every 34 weeks to achieve adequate IgG levels (usually >800 mg%) and protection against infection. Initially, serial IgG level determination may allow the physician to individualize the dose and schedule. These are affected by the recovery, half-life, redistribution, and catabolism of IVIg, which vary from product to product and patient to patient. Patients with ITP are usually treated with 400 mg/kg daily for 5 days or 1,000 mg/kg daily for 2 days (see Chap. 123). Repeat single doses (400 mg/kg) have been used intermittently in some patients (particularly children) to maintain platelet counts >50,000/l. Kawasaki disease is also treated with 400 mg/kg daily for 35 days. The adult dose of IV anti-D is 2 mg over 30 minutes.

Compatibility and Side Effects

Infusions of IVIg should be started slowly and patients should be closely monitored. If the initial rate (0.5 ml/kg/hr) is well

tolerated, the rate can be increased gradually, but not more than eightfold. Fever, headache, nausea, vomiting, fatigue, backache, leg cramps, urticaria, flushing, elevation of blood pressure, and thrombophlebitis may be seen. IgA-deficient patients may have IgG anti-IgA antibodies, which can cause anaphylactic reactions. [104] This complication is rare and may be avoided by using products with a lower concentration of IgA. [105] Aggregated IgG may produce chills, nausea, flushing, chest tightness, and wheezing. Rarely, IVIg preparations contain IgG anti-A and/or anti-B RBC antibodies that can produce hemolysis [106] or interfere with serologic evaluations, including RBC compatibility testing. [107] Serum sickness can occur. Improved manufacturing processes currently in place render IVIg free of envelope and non-envelope viruses.[108] Nevertheless, hepatitis C virus transmission has been reported in certain patient populations. [109] [110] High-dose IVIg therapy has been associated with reversible, acute renal failure [111] and aseptic meningitis.[112]

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HYPERIMMUNE AND INTRAMUSCULAR IMMUNOGLOBULINS

Hyperimmune immunolglobulin is prepared from large pools of plasma known to contain elevated antibody titers against specific infectious agents. The intramuscular and hyperimmune immunoglobuins[73] are listed in Table 138-5 .

Antithymocyte globulin (ATG) is a purified concentrated -globulin made from hyperimmune serum of horses immunized with human T lymphocytes. ATG is used in renal transplant patients as an adjunct therapy in the treatment of graft rejection. [113] [114] It is also used in patients with aplastic anemia who are not candidates for bone marrow transplantation (see Chap. 19). [115]

Hyperimmune immunoglobulin is used to prevent the development of specific clinical disease or alter its symptomatology. CMV Ig is indicated in all kidney and liver transplant recipients who are seronegative for CMV and who receive the respective organ from a seropositive donor and in bone marrow transplant patients with CMV interstitial pneumonia. [85] [86] Hepatitis B immunoglobulin is used to provide passive immunity to hepatitis B virus associated with needlestick exposure or sexual contact with HBsAg-positive individuals.

Rh immunoglobulin is used when fetal Rh(D)-positive RBCs may have entered the maternal circulation of an Rh(D)-negative mother. This means that Rh(D) immunoglobulin is given to Rh(D)-negative mothers after abortion or amniocentesis, as well as before delivery and again postpartum if the child proves to be Rh(D) positive. [186] [187] [188] The therapeutic effect is thought to be due to antibody feedback with T-cell suppression of the B-cell clone responsible for the formation of anti-Rh antibody. Rh immunoglobulin can also be given to prevent immunization in Rh(D)-negative individuals given Rh(D)-positive components such as platelets. Transfusion of Rh(D)-positive RBCs to Rh(D)-negative recipients is occasionally necessary, and prevention of immunization is not possible unless IV Rh immunoglobulin is

TABLE 138-5 -- Available Hyperimmune and Intramuscular Immunoglobulins

Antithymocyte globulin
Cytomegalovirus immunoglobulin
Hepatitis B immunoglobulin
Rabies immunoglobulin
Rh(D) immunoglobulin
Tetanus immunoglobulin
Vaccinia immunoglobulin
Varicella-zoster immunoglobulin
Western equine encephalitis immunoglobulin

made available by the manufacturers. Rh immunoglobulin has also been used to treat ITP.

Experimentally, immunoglobulin has also been administered via oral, intrathecal, and aerosolized routes. Oral administration has been tried with some success in the prevention of rotavirus infection and necrotizing enterocolitis in premature infants, in the treatment of *Cryptosporidia*-associated diarrhea, and in the prevention of viral gastroenteritis after bone marrow transplant. Intrathecal administration has been tried in the treatment of viral encephalitis and tetanus, aerosolized administration in infections with respiratory syncytial virus.

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Chapter 139 - Preparation of Plasma-Derived and Recombinant Human Plasma Proteins

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INTRODUCTION

The development of large-scale methods for the preparation of human plasma proteins began more than 50 years ago. In 1940, after the outbreak of World War II, a meeting was called to discuss the urgent request of the United States Armed Forces for 300,000 units of human whole blood or plasma for transfusion, which at that time seemed an impossibly large amount.[1] [2] Dr. Edwin J. Cohn of the Harvard Medical School was approached to determine whether animal plasma could be made safe for human use. Cohn drew together a task force of investigators and developed a method for the fractionation of bovine plasma based on differential precipitation of various plasma proteins achieved by appropriate combinations of ethanol concentration, pH, low temperature, ionic strength, and protein concentration. Within a short time, highly purified preparations of bovine serum albumin were available for clinical trials. Although there were no immediate reactions, the frequency of severe delayed reactions made it obvious that these preparations were unsuitable for clinical use. Meanwhile, Cohn had arranged with the American Red Cross to provide his laboratory with a supply of human plasma, and the ethanol method was quickly adapted for human plasma fractionation. Although albumin was the only product distributed during the war, the remaining plasma fractions were carefully preserved, and other preparations, including fibrinogen and immunoglobulins, were soon developed.

Since World War II, major improvements have occurred in the preparation of human plasma proteins. The ethanol process has been supplemented by more selective purification techniques. A variety of methods have been developed for inactivation or removal of viruses that might be present in the starting materials, which may be pooled from the plasma of thousands of human donors.[3] [4] Advances in recombinant DNA technology have allowed recombinant human plasma proteins to be produced in tissue culture systems. This chapter describes current methods and future directions for the preparation of plasma-derived and recombinant human plasma proteins for clinical use.

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FRESH FROZEN PLASMA

Plasma that has been frozen solid within 6 hours after phlebotomy and stored at 18°C meets the criteria for labeling as fresh frozen plasma (FFP). [5] Clinical indications for the administration of FFP are described in Chapter 138 . At the present time, the viral safety of FFP depends entirely on donor selection and blood screening techniques. However, a method has recently been developed for the treatment of plasma with a solvent/detergent mixture to inactivate any lipid-enveloped viruses that might be present.[6] The coagulation factor content of solvent/detergent-treated plasma (SD-plasma) has been found to be comparable to that of untreated FFP.[7] Clinical trials have shown that SD-plasma is efficacious in the treatment of acquired or inherited coagulation factor deficiencies and thrombotic thrombocytopenic purpura (TTP).[8] [9] The product is currently available in Europe and was recently licensed in the United States.

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ALBUMIN AND PLASMA PROTEIN FRACTION

Albumin is still one of the major products of human plasma fractionation. During the 50 years since the initial use of human serum albumin in treating the casualties at Pearl Harbor, literally tons of albumin have been isolated and millions of units have been infused. [10] Cohns method 6 is the basis for most of the large-scale production of albumin today. [11] Most manufacturers use cryoprecipitation as the first step in order to obtain the factor VIII/von Willebrand factor (Factor VIII/vWF) complex, which can then be further purified by a variety of techniques. Factor IX complex and antithrombin III (AT III) may be separated from the cryosupernatant before it is processed by the ethanol method to obtain immunoglobulins and albumin. Albumin, having the highest solubility and the lowest isoelectric point of all the major plasma proteins, remains in solution as the ethanol concentration is raised in stages from zero to 40%, with an overall decrease in pH from neutrality to 5.8 and a temperature adjustment to 5°C. It is only when the pH is adjusted to 4.8, in the presence of 40% ethanol at 5°C, that the bulk of the albumin is finally precipitated in fraction V. [10] [12] After the removal of ethanol and salts, sodium acetyltryptophanate and sodium caprylate are added as stabilizers, and the albumin is sterile-filtered and bottled. The vials are then heated for 10 hours at 60°C to inactivate any remaining blood-borne viruses.

Three albumin products are manufactured in the United States: Albumin (human) 25%, Albumin (human) 5%, and plasma protein fraction (PPF). To be designated Albumin, >96% of the protein content must be albumin by cellulose acetate electrophoresis (CAE). PPF is an albumin product of lower purity, obtained by co-precipitating fraction IV-4 with fraction V. [13] The total protein in PPF must be 83% albumin by CAE, with 17% globulins, and 1% -globulin. PPF is more economical to produce than albumin, [14] but the rapid infusion of PPF has been associated with hypotensive episodes. [15] [16] Clinical indications for the use of Albumin and PPF are described in Chapter 138 . Albumin (human) 25% is also often used as a stabilizer in the formulation of other highly purified plasma products.

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IMMUNOGLOBULINS

Immuno- and Hyperimmunoglobulins

Since the early 1950s, immunoglobulins have been prepared from the fraction II + III precipitate obtained by Cohns method 6, with additional precipitation steps to remove lipoproteins, IgA, IgM, and other plasma proteins.[17] [18] [19] Immunoglobulin is prepared from the plasma of unselected normal donors, while hyperimmunoglobulins are prepared from the plasma of donors with high antibody titers against specific antigens (e.g., rho(D), hepatitis B, rabies, and tetanus). These donors may be identified during convalescent periods after infection or transfusion, or they may be specifically immunized to produce the desired antibodies. Immunoglobulin products currently available in the United States are described in Chapter 138 .

Intravenous Immunoglobulins

The original immunoglobulin concentrates, termed immune serum globulin (ISG), were administered by the intramuscular route, with the associated problems of limited injectable volume, poor bioavailability, and discomfort at the injection site. [20]

Intravenous injection of ISG caused serious clinical reactions, attributed to complement-activating aggregates in these products. [21]

To overcome these limitations, intravenous immunoglobulin (IVIg) products have been developed using a variety of methods to remove or inactivate anticomplementary aggregates. Processing and formulation techniques have been improved, and conditions have been identified, to permit these products to be prepared as either freeze-dried or liquid concentrates. [22] Today most intramuscular ISG usage is limited to hyperimmune products. Immunoglobulin products tend to be self-protecting from viral transmission because of the large pools of antibodies they contain. [23] However, postinfusion viral infections have occurred in a few instances. As a result, most manufacturers have incorporated viral inactivation or removal steps in the production of IVIg. [24]

The development of IVIg has permitted the administration of much higher doses, with a subsequent expansion in immunoglobulin therapy. In addition to providing passive immune protection, IVIg has been found to modulate the immune response to autoimmune diseases such as idiopathic thrombocytopenic purpura. Clinical indications for the use of IVIg are described in Chapter 138 .

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COAGULATION FACTOR CONCENTRATES

The distinction between hemophilia A and B was unknown when it was first shown that the transfusion of whole blood could be used to curtail bleeding in patients with hemophilia.[25] [26] By 1940, bleeding episodes were being routinely treated with plasma. However, large amounts of plasma were needed, and this method of therapy could not provide normal levels of coagulation factors without producing hypervolemia. [27] The development of coagulation factor concentrates has resulted in dramatic increases in the life expectancy and the quality of life of patients with hemophilia.

The major issues in the preparation and use of coagulation factor concentrates are viral safety and purity. With the exception of cryoprecipitate, all these concentrates are now subjected to some form of treatment for the inactivation or removal of viruses that might be present in the starting materials. The purity may be important for both immediate and long-term safety. As discussed below, the purity of factor IX (FIX) concentrates has definitely been shown to affect product safety; however, the situation for factor VIII products is less clear. Although in vitro testing has shown deleterious effects on immunocompetence with lower purity products, the few in vivo studies performed have shown no signs of immunomodulation.[28] The development of improved coagulation factor concentrates continues to be a major focus of research.

Factor VIII Concentrates

Factor VIII or antihemophilic factor (human) (AHF) concentrates for the treatment of hemophilia A have evolved from cryoprecipitates to very-high-purity immunoaffinity-purified products.[29] Four recombinant products have also been licensed for clinical use. The various AHF concentrates available in the United States are shown in Table 139-1 .

Cryoprecipitate

In 1959, Pool and Robinson[30] reported that when frozen plasma is slowly thawed, a residual cryoprecipitate results that contains

TABLE 139-1 -- Antihemophilic Factor Concentrates Marketed in the United States[a]

Antihemophilic Factor	Manufacturer/Distributor	Product Name	Specific Activity[b]	Purification Methods	Virus Inactivation/Removal Methods
Human	Alpha	Alphanate	140[c]	Cryoprecipitation, IEC, heparin affinity chromatography	S/D (TNBP/Polysorbate 80), dry heat treatment at 80°C for 72 hours, heparin chromatography, PEG precipitation
	Centeon	Monoclate-P	>3,000[c]	Cryoprecipitation, cold purification, $Al(OH)_3$ adsorption, IAC, AH-Sepharose chromatography	Pasteurization at 60°C for 10 hr
	Baxter/American Red Cross	MONARC-M	2,000[c]	Cryoprecipitation, cold precipitation, IAC, IEC	S/D (TNBP/Triton X-100), IAC, IEC
	Baxter	Hemofil M	2,000[a]	Cryoprecipitation, cold precipitation, IAC, IEC	S/D (TNBP/Triton X-100), IAC, IEC
	Centeon	Humate-P	25	Cryoprecipitation, $Al(OH)_3$ adsorption, glycine precipitation	Pasteurization at 60°C for 10 hr
	Bayer	Koate HP	922	Cryoprecipitation, SEC	S/D (TNBP/Polysorbate 80)
Recombinant	Baxter	Recombinate	>3,000[c]	IAC, IEC, IEC	Purification steps
	Bayer	Kogenate	>3,000[c]	IAC, SEC, IEC	Inactivation/purification steps
	Baxter/Centeon	Bioclate	>3,000[c]	IAC, IEC, IEC	Purification steps
	Bayer/Centeon	Helixate	>3,000[c]	IAC, SEC, IEC	Inactivation/purification steps
Porcine	Speywood	HYATE:C	>50[d]	Polyelectrolyte fractionation	Purification steps

Abbreviations: IAC, immunoaffinity chromatography; IEC, ion-exchange chromatography; SEC, size exclusion chromatography; S/D, solvent/detergent; TNBP, tri-*n*-butyl phosphate; PEG, polyethylene glycol.

[a] These concentrates were marketed in the United States in 1997. Data were obtained from manufacturers, distributors, and available literature.
[b] Factor VIII U/mg of protein.
[c] Prior to addition of human albumin.
[d] Porcine factor VIII g/mg of protein.

most of the factor VIII activity from the original plasma. By 1965, single-donor cryoprecipitate with factor VIII concentrations 520 times that of plasma became widely available for use in the treatment of hemophilia A.[31] Although single-donor cryoprecipitate now has only limited clinical use, cryoprecipitation is still the first step in most procedures for the preparation of plasma derivatives containing high concentrations of the factor VIII/vWF complex, fibrinogen, fibronectin, and/or factor XIII. [32] Cryoprecipitate contains approximately one-half of the factor VIII/vWF complex and one-third of the fibrinogen and factor XIII in whole plasma. [33]

Intermediate- and High-Purity AHF Concentrates

The development of intermediate-purity AHF concentrates was the next significant advance in the treatment of hemophilia A. Cryoprecipitate was used as the starting material, and a variety of methods were developed to remove fibrinogen, immunoglobulins, and other proteins. Fibrinogen can be removed at specific conditions of

temperature and pH[34] or by precipitation with polyethylene glycol, [35] while aluminum hydroxide may be used to adsorb and remove the vitamin K-dependent clotting factors II, VII, IX, and X.[35] However, even though these concentrates are enriched approximately 400-fold over plasma, factor VIII is still <1% of the total protein. [36] Thus, patients receiving these concentrates are exposed to large quantities of other plasma proteins. In addition, each lot is prepared from cryoprecipitate obtained from the plasma of several thousand donors. Until effective methods were developed for viral inactivation, patients receiving these concentrates were exposed to all of the blood-borne viruses in the donor population. It is not surprising that frequently treated patients uniformly became infected with hepatitis [37] [38] and, later, often HIV.[39]

Intermediate-purity AHF concentrates were the mainstay of hemophilia A treatment until the development of high-purity concentrates in the late 1980s. Various ion-exchange and gel filtration techniques have been added to the precipitation methods to produce concentrates with specific activities of 50200 IU/mg of protein, in contrast to 150 IU/mg for intermediate-purity AHF and <1 IU/mg for cryoprecipitate.[26] However, even though these concentrates are highly purified in comparison with the intermediate-purity concentrates, high molecular weight proteins such as fibrinogen, fibronectin, and vWF tend to fractionate with factor VIII and remain in these preparations.[40]

AHF Concentrates Purified by Immunoaffinity Chromatography

The next major advance in the preparation of AHF concentrates was the use of murine monoclonal antibodies (mAbs) for the purification of factor VIII by immunoaffinity chromatography. Two different methods have been developed that both start with low-purity factor VIII concentrates partially purified by conventional means.[41] [42] [43] One procedure uses an mAb that binds to the vWF portion of the factor VIII/vWF complex; the other uses an mAb that binds directly to factor VIII. In both processes, the columns are loaded with the low-purity intermediate, washed extensively to remove unwanted proteins, and then eluted, respectively, by separation of factor VIII from the vWF/mAb complex or directly from the mAb itself. Both processes use a final chromatography step to remove the harsh elution solutions as well as any mAb that might have leached off the column. These processes produce factor VIII that is essentially pure prior to the addition of albumin as a stabilizer. Both processes include several steps that inactivate or remove viruses.

Recombinant AHF Concentrates

One of the remarkable accomplishments of molecular biologists has been the elucidation of the structure of factor VIII, its molecular cloning, and the successful production of recombinant human factor VIII. Almost simultaneously, two groups cloned the entire factor VIII gene, the largest gene cloned at that time, and isolated a cDNA encoding the structural regions of the factor VIII molecule. [44] [45] [46] [47]

Because post-translational processing is essential to factor VIII functionality, both groups have expressed the recombinant factor VIII molecule in mammalian cells. One group uses Chinese hamster ovary cells that co-express recombinant factor VIII and recombinant vWF, as the recombinant vWF substantially increases the recovery of recombinant factor VIII from the culture medium.[48] The recombinant factor VIII is purified by immunoaffinity and ion-exchange chromatography. [49] The other process uses baby hamster kidney cells to express recombinant factor VIII, which is purified by immunoaffinity, size exclusion, and ion-exchange chromatography.[50] [51] Both products contain minimal or no recombinant vWF and only trace quantities of mouse immunoglobulin, hamster protein, and cellular DNA. The products are essentially pure prior to the addition of human albumin to stabilize the recombinant factor VIII. These recombinant factor VIIIs have been shown to be similar to plasma-derived factor VIII in in vitro [49] [52] [53] [54] as well as clinical [55] [56] [57] studies. These products are termed antihemophilic factor (recombinant). A fully functional recombinant factor VIII has also recently been expressed in the milk of transgenic pigs. [58] The large amounts of protein that can be produced relatively inexpensively in transgenic animals may someday permit the widespread prophylactic treatment of hemophilic patients so that joint and soft tissue bleeding can be prevented.

Factor IX Concentrates

Two types of factor IX concentrates are available today: factor IX complex, which contains significant amounts of the other vitamin K-dependent proteins, including factors II, VII, and X; and coagulation factor IX (human), a preparation substantially free of these other proteins. The factor IX concentrates available in the United States are shown in Table 139-2 .

Factor IX Complex Concentrates

The vitamin K-dependent clotting factors, because of their similar structures, tend to co-purify by most of the methods used to isolate them from plasma. Thus, the initial preparations containing factor IX for treatment of hemophilia B were in fact complex mixtures of the vitamin K-dependent proteins. Because the protein in highest concentration in these products is prothrombin, they have also been identified as prothrombin complex concentrates; however, factor IX complex is the generic name in the United States.

The first factor IX-rich concentrate used for the treatment of hemophilia B was developed in France more than 30 years ago. [59] It was produced by the adsorption of the plasma vitamin K-dependent clotting factors with tricalcium phosphate and was successfully used to treat hemophilia B for a number of years. The original production process had a number of disadvantages and was eventually discontinued. [60] However, this method led to the development of several other processes based on tricalcium phosphate adsorption. [61] Factor IX complex produced by these methods contains approximately equal amounts of factor II, VII, IX, and X activity units.

The next major development in the production of factor IX complex concentrates was the introduction of ion-exchange chromatography. [62] This represented one of the first major uses of chromatography in plasma fractionation. As shown in the box, the vitamin K-dependent clotting factors can be removed from cryoprecipitate-poor plasma by adsorption onto DEAE-cellulose or DEAE-Sephadex, and the plasma can then be further fractionated by Cohns method for the production of

TABLE 139-2 -- Factor IX Concentrates and Related Products Marketed in the United States[a]

Product Type	Manufacturer/Distributor	Product Name	Specific Activity[b]	Purification Methods	Virus Inactivation/Removal Methods
Factor IX complex	Alpha	Profilnine SD	45	DEAE cellulose adsorption	S/D (TNBP/polysorbate 80), DEAE cellulose chromatography
	Baxter	Proplex T	NA	Tricalcium phosphate adsorption	Dry heat treatment at 60°C for 144 hr
	Immuno	Bebulin VH	NA	NA	Vapor heating: 60°C for 10 hr at 190 mbar, then 80°C for 1 hr at 375 mbar
	Bayer	Konyne 80	1	IEC	Dry heat treatment at 80°C for 72 hr
Coagulation factor IX (human)	Alpha	AlphaNine SD	150	DEAE adsorption, barium citrate adsorption, dual AC	S/D (TNBP/polysorbate 80), nanofiltration, dual AC, DEAE cellulose chromatography
	Centeon	Mononine	150	IEC, IAC, AH-Sepharose chromatography	Sodium thiocyanate; IAC, ultrafiltration
Anti-inhibitor coagulant complex	Baxter/NABI	Autoplex T	NA	NA	Dry heat treatment at 60°C for 144 hr
	Immuno	FEIBA VH	NA	NA	Vapor heating: 60°C for 10 hr at 190 mbar, then 80°C for 1 hr at 375 mbar
Coagulation factor IX (recombinant)	Genetics Institute	BeneFix	200	Q Sepharose, Cellufine sulfate, ceramic hydroxyapatite, and IMAC chromatographies	Chromatography, nanofiltration

Abbreviations: NA, not available; S/D, solvent/detergent; AC, affinity chromatography; IAC, immunoaffinity chromatography; IEC, ion-exchange chromatography; IMAC, immobilized metal affinity chromatography; TNBP, tri-*n*-butyl phosphate.

[a]These concentrates were marketed in the United States in 1997. Data were obtained from manufacturers, distributors, and available literature.
[b] Factor IX U/mg of protein.

immunoglobulin and albumin. The eluate from the DEAE resin may be further purified by additional precipitation or adsorption steps. [63] [64] Such processes give 100-fold or more purification of factors II, IX, and X. Factor VII does not bind to DEAE under the conditions used, however, and is present in only very low concentrations in concentrates prepared in this way.

Coagulation Factor IX

With the widespread use of factor IX complex, it soon became apparent that serious thromboembolic episodes and acute myocardial infarction were major complications of their infusion, especially when used in large quantities for extended periods, such as for surgical procedures or in patients with liver disease. [65] [66] As discussed in Chapter 140 , the cause of the thrombogenicity has not been rigorously defined. It may be due to the presence of activated clotting factors. [67] [68] contaminating phospholipids,[69] or the result of zymogen overload.[70] Zymogen overload refers to the co-administration of large, unnecessary quantities of factors II and X in the factor IX complex preparations.[70] [71] These factors persist in the circulation (half-lives of about 4 days and 3050 hours, respectively, compared with about 20 hours for factor IX), so that they accumulate after repeated infusions of the complex, upsetting the normal balance and leading to a hypercoaguable state. [66]

On the basis of these hypotheses, methods have been developed for the preparation of second-generation factor IX concentrates that are more highly purified and essentially free of the other vitamin K-dependent clotting factors. These have been designated coagulation factor IX (human). Coagulation factor IX concentrates are manufactured from factor IX complex by one or more additional steps, such as chromatography on sulfated dextran, [71] a second DEAE column and a heparin-Sepharose column,[72] or barium citrate adsorption followed by an affinity column. [73] Coagulation factor IX concentrates are also prepared by immunoaffinity chromatography using mAbs to factor IX.[74] [75] All these methods also include viral inactivation steps. These preparations have been shown to be nonthrombogenic when compared with factor IX complex in animal models[76] and also in clinical studies. [77]

A recombinant coagulation factor IX has also been developed and was recently licensed in the United States. Recombinant factor IX is produced by cell culture in Chinese hamster ovary cells that co-express a protease needed to properly modify the factor IX molecule. [78] It is purified with several chromatography steps and treated to remove viruses.[79] The final product has been shown to be structurally and functionally similar to plasma-derived factor IX. [80] [81] However, for reasons not yet determined, it gives a lower recovery when infused into patients. [82]

Other Coagulation Factor Concentrates

As described in Chapter 140 , one of the major complications in the treatment of hemophilic patients is the development of inhibitors, antibodies directed against factor VIII or factor IX. While low-titer inhibitors can be saturated by administering large amounts of factor VIII or factor IX, this may not be feasible when the inhibitor level is >510 Bethesda U/ml plasma.[83] [84] One of several approaches for treating hemophilia A patients with high-titer inhibitors is the use of a porcine factor VIII concentrate. Porcine factor VIII is efficacious in controlling hemostasis and is less readily inactivated by some human factor VIII inhibitors. [85] [86] [87] [88] The critical factor for repeated use of porcine factor VIII, anamnesis with the development of high-titer inhibitor levels against porcine factor VIII, is seen in approximately one-third of patients. [87] Another widely used method of treatment for patients with high-titer inhibitors is administration of either factor IX complex or a specially activated factor IX complex termed anti-inhibitor coagulant complex to bypass the factor

PLASMA ALGORITHM BASED ON COHN'S METHOD 6[a]

[a] *Dotted lines indicate optional steps used by manufacturers.*

VIII step in the coagulation cascade.[89] [90] These concentrates are effective in 5060% of cases, but thromboembolic complications have occurred in some patients treated in this way.

The bypass rationale has led to the use of activated factor VII (factor VIIa) for the treatment of both hemophilia A and B patients with inhibitors. [91] Factor VII or factor VIIa may be the active substance in the anti-inhibitor coagulant complexes. [92] [93] Early work with plasma-derived factor VIIa gave good results.[94] However, since it is impossible to purify enough factor VIIa from plasma for widespread patient use, recombinant factor VIIa concentrates have been developed. [95] Recombinant factor VIIa is produced in tissue culture in baby hamster kidney cells and spontaneously activates during purification by immunoaffinity chromatography. [95] [96] Recombinant factor VIIa has been used successfully in a number of clinical trials [91] [93] [94] [97] but is not yet licensed in the United States. However, the manufacturer has made the product available on a compassionate use basis for several years. [84]

Patients with von Willebrand disease (vWD) have been treated with plasma, with cryoprecipitate, and with some AHF concentrates that contain vWF. Cryoprecipitate has a population of vWF multimers similar to that of normal plasma, while AHF concentrates are often deficient in the multimeric forms. However, during the past 10 years the ratios of ristocetin cofactor activity (vWF:Rco) to vWF antigen (vWF:ag) in many plasma-derived AHFs have increased to the point that they are now preferred over cryoprecipitate because of their viral safety. [98] The vWF:Rco/vWF:ag ratio appears to be a good indicator of vWD treatment efficacy. Current AHF products vary considerably, with vWF:Rco/vWF:ag ratios ranging from 0.25 to 1.4 and 0.5 to 5.3 units of vWF:Rco per unit of factor VIII. [98] A vWF concentrate depleted of factor VIII has also been developed in Europe, with plans to seek licensure in the United States.

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FIBRIN SEALANTS

Fibrin sealants, or fibrin glues, are topical hemostatic agents that have been used in a variety of surgical situations for their hemostatic and adhesive properties. [99] The preparations consist of two components that are mixed together immediately prior to use. The first component is derived from human plasma and contains fibrinogen, along with factor XIII, and other plasma proteins. Depending on the manufacturer, the fibrinogen may be isolated directly from Cohn fraction I or from cryoprecipitate as a by-product of AHF manufacture. [99] These fibrinogen concentrates also typically contain either an added plasmin inhibitor to delay clot lysis [99] [100] or else are purified to remove endogenous plasminogen. [100] [101] The second component contains either bovine or human plasma-derived thrombin and possibly other proteins. Although bovine thrombin has been in widespread use for many years, recent research suggests that it may be responsible for many postsurgical hemostatic problems. [100] [102]

Fibrin sealant has been advocated by many surgeons as the material that best approaches the ideal operative sealant. [103] It appears to have no tissue toxicity, forms a firm seal within seconds, is completely readsorbed within days or weeks following application, and may promote local tissue growth and repair. [99] [100] [103] Although preparations of fibrin sealant have been available in Europe since the mid-1970s, the United States Food and Drug Administration revoked all licenses for the manufacture of fibrinogen concentrates in 1978 owing to frequent viral contamination. [104] However, several manufacturers have developed improved, virally inactivated products that have been licensed or that are currently pending licensure in the United States. [100] Additional products containing fibrin sealant, such as a fibrin sealant-coated bandage, are also being developed. [100] [105]

In the absence of licensed products, a variety of methods had been used to prepare fibrin sealant for use in the treatment of individual patients. The preparations are made from single-donor cryoprecipitate or other plasma fractions plus bovine thrombin. [106] [107] However, the methods are somewhat cumbersome, the concentration of fibrinogen in these preparations is highly variable, and, most important, the products are not treated to prevent viral transmission. [99] The use of a standardized manufactured product should increase the likelihood of safe, uniform performance in the surgical field.

PLASMA PROTEINASE INHIBITORS

The proteinase inhibitors that are present in human plasma play critical roles in the regulation of the proteolytic cascades of the coagulation, fibrinolytic, complement, and kinin systems. Most of these inhibitors have similar amino acid and structural properties and are members of a superfamily of proteins designated serpins (serine proteinase inhibitors).[108] Hereditary deficiencies in $_1$ -antitrypsin, AT III, and C_1 esterase inhibitor can cause specific disease states, and inhibitor concentrates have been developed. The plasma proteinase inhibitor concentrates available in the United States are shown in Table 139-3 .

$_1$ -Proteinase Inhibitor

$_1$ -Antitrypsin, licensed as $_1$ -proteinase inhibitor (human) (API), was the first of the serpins to be isolated and characterized. [109] Although the protein was originally named for its antitrypsin activity, its primary physiologic function appears to be the inhibition of neutrophil elastase. Patients with hereditary deficiencies of this inhibitor develop pulmonary emphysema and liver disease. [110] [111]

API therapy is indicated for chronic treatment of individuals with hereditary deficiency who have clinical evidence of panacinar emphysema. Weekly intravenous infusions are recommended in order to maintain an adequate level of functional API in the epithelial lining of the lower respiratory tract. [110] [111]

TABLE 139-3 -- Plasma Proteinase Inhibitor Concentrates Marketed in the United States[a]

Product Type	Manufacturer/Distributor	Product Name	Specific Activity	Purification Methods	Virus Inactivation/Removal Methods
$_1$ -Proteinase inhibitor (human)	Bayer	Prolastin	0.35[b]	Ethanol fractionation, PEG precipitation, IEC	Pasteurization at 60°C for 10 hr
Antithrombin III (human)	Kabi/Baxter Bayer	ATnativ Thrombate III	NA 8[c]	AC, IEC Ethanol fractionation, dual AC	Pasteurization at 60°C for 10 hr Pasteurization at 60°C for 10 hr

Abbreviations: AC, affinity chromatography; IEC, ion-exchange chromatography; PEG, polyethylene glycol; NA, not available.

[a]These concentrates were marketed in the United States in 1997. Data were obtained from manufacturers, distributors, and available literature.
[b]Milligrams functional $_1$ -PI/mg protein.
[c]AT III U/mg of protein.

However, intravenous administration is an inefficient therapy; it has been estimated that only 2% of the infused API is present in the lung. Clinical studies suggest that aerosol delivery of API directly into the lungs by inhalator would be efficacious and could replace intravenous administration because of its lower cost and greater convenience.[112] API is also currently in clinical trials for the treatment of cystic fibrosis.

An API concentrate prepared from human plasma is licensed for use in the United States. The product is prepared from Cohn fraction IV-1 by fractional precipitation with polyethylene glycol followed by ion-exchange chromatography on DEAE-Sepharose. It is pasteurized by heating in solution for 10 hours at 60°C to ensure viral inactivation.[113] [114] Several new plasma-derived API concentrates are being developed, as are recombinant concentrates from both cell culture [115] [116] and transgenic animals. The large amounts of API that can be produced in the milk of transgenic animals may be important in the future. Because relatively large doses of API are needed even with aerosol delivery, the amount available from plasma will be insufficient, especially with the additional indications being studied. Using recombinant DNA techniques, it may also be possible to modify the API molecule so as to give it advantageous new properties, such as resistance to oxidation and a broader antiprotease inhibitory spectrum. [112]

Antithrombin III

Antithrombin III, as the major physiologic inhibitor of thrombin and factor Xa, plays a critical role in the regulation of hemostasis. Heterozygous hereditary deficiency of AT III has been linked to an increased tendency to thrombosis. [117] [118] Acquired deficiencies have also been reported in women taking oral contraceptives and as a consequence of pregnancy, surgery, cirrhosis, and hepatic malignancies.

The affinity of heparin for AT III, essential for the pharmacologic effect of the drug, has also been employed as a means of purifying AT III. Use of immobilized heparin as an affinity adsorbent for AT III [119] is the key step in large-scale preparation of AT III concentrates for clinical use. [120] As shown in the box, AT III may be isolated from cryosupernatant,[121] from Cohn fraction I supernatant,[122] or from Cohn fraction IV-1.[123] Although heparin-based adsorbents are not specific for AT III, other plasma proteins are bound less tightly and may be washed off with medium-ionic-strength buffers prior to the elution of AT III with a high-ionic-strength buffer. Although these eluates may contain small amounts of other proteins, additional purification steps produce concentrates that contain >95% AT III. [120] All the AT III concentrates now being manufactured are pasteurized by heating in solution for 10 hours at 60°C to inactivate blood-borne viruses. Clinical studies by a number of investigators have shown that these AT III concentrates are effective in the prophylaxis or treatment of thromboembolic disorders in patients with hereditary AT III deficiency.[117] [124]

C_1 Esterase Inhibitor

C_1 esterase inhibitor (C_1 -INH) plays an important role in the regulation of the complement system cascade. In 1963 it was established that a deficiency in C_1 -INH is the underlying biochemical defect in hereditary angioedema, an autosomal dominant disease that is characterized by episodic swelling of the subcutaneous tissues and the mucosa of the gastrointestinal and respiratory tracts.[125] The swelling can lead to acute airway obstruction, a major cause of mortality among patients with this disease.[126] FFP has been used in replacement therapy for the treatment of acute attacks as well as for short-term prophylaxis prior to surgery, but the volume of plasma needed and the time required for thawing and infusion are drawbacks to this approach. [127]

C_1 -INH concentrates are not yet licensed in the United States, although preparations have been available in Europe for several years. [128] [129] [130] [131] C_1 -INH concentrates have been used there for short-term and long-term prophylaxis, as well as for treatment of acute episodes of hereditary angioedema. The products are

treated for viral inactivation and are supplied as freeze-dried concentrates.

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FUTURE DIRECTIONS

Almost all plasma proteins licensed for human use have been cloned and expressed in a biologically active form in animal cells, and several have been developed into licensed products.[132] [133] Other plasma proteins such as fibrinogen and albumin have been cloned and expressed by a variety of laboratories, but remain untested in clinical trials. The reasons for this include the inability to produce these proteins in adequate quantities or in the inappropriate post-translational modification of the recombinant protein.[134] [135] The main advantages of recombinantly produced plasma proteins include freedom from human viruses and a potentially unlimited supply. Recombinant proteins can also be produced in modified forms that may give them advantageous new properties such as increased potency, longer half-life, or varied specificity.

Transgenic Animal Production of Human Plasma Proteins

Advances in molecular biology and embryology have led to the production of plasma proteins in the milk of genetically engineered farm animals. [136] [137] [138] This technology has overcome at least one of the apparent shortcomings of tissue culture production systems. Transgenic cows, goats, pigs, and sheep can produce large quantities of human proteins, typically 110 g/L in milk, whereas animal tissue culture systems routinely produce substantially less protein, typically 10100 mg/L. [139] In addition, transgenic animals appear to be able to perform most of the post-translational modifications required for protein activity, even when the protein is being made at relatively high levels of production.[139] Most recently, even the highly modified coagulation factor VIII protein has been made in the milk of pigs in a biologically active form.[56] Proteins produced in transgenic animals should be free of human viruses, less expensive to produce, and available in unlimited quantities. Thus, the production of human plasma proteins in transgenic animals is an attractive alternative to their isolation from human plasma or production in tissue culture systems.

Gene Therapy

Of course, the most satisfying solution to human genetic deficiencies would be the replacement of the dysfunctional gene in the affected individual so that the consequence of the gene abnormality goes unnoticed. This process, termed gene therapy, is the subject of intense research, especially for treatment of hemophilia A and B.[140] [141] [142] Although the number of hemophilia B patients is relatively small, the characteristics of both the disease and the factor IX molecule are such that it has become an excellent model system for the development of generic gene therapies.[140] There are two general approaches. Fully functional new genes may be inserted into various body cells using a modified virus or other carrier. Alternatively, cells containing functional genes may be implanted into various body tissues. The main issues with either approach are the production of sufficient protein and the persistence of the production. In addition, hemophiliacs could still develop inhibitors to these proteins. Although these problems are far from being solved, the pace of the research has been rapid and an actual cure for diseases like hemophilia may be in sight.

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Chapter 140 - Transfusion Principles for Congenital Coagulation Disorders

Joan Cox Gill

INTRODUCTION

Rapid advances in knowledge over the past several decades have led to an increased understanding of both hemorrhagic and thrombophilic plasma deficiency states and to the development of sophisticated therapeutic products to replace many of the deficient proteins. This chapter outlines the general principles of replacement transfusion therapy for clotting factor and other plasma protein deficiencies and provides recommendations for the use of transfusion and pharmacologic products for these disorders.

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GENERAL PRINCIPLES OF PLASMA PROTEIN TRANSFUSION THERAPY

Of major importance in the consideration of therapeutic options for a particular patient is the establishment of an accurate diagnosis and determination of the severity of the patients coagulopathy. Because hemostasis testing is often technically difficult and time-consuming, the clinician who relies on an inexperienced laboratory will have great difficulty in the treatment of a patient when the proper diagnosis is masked by laboratory artifact. Therefore, a high-quality and experienced coagulation laboratory should be used.

Once an accurate diagnosis has been established and the severity of the plasma protein deficiency has been determined, replacement therapy and other therapeutic maneuvers can be planned to restore balance to the hemostatic system. For a rational approach, one must first know the hemostatic level that must be achieved to treat a particular clinical manifestation. [1] For example, it has been established that patients with hemophilia A require 30-40% of normal factor VIII levels to control joint hemorrhages;[2] however, for major surgery, a 100% correction is desirable. [3] Hemostatic levels have been established for most of the coagulation factors and are summarized in Table 140-1 .

Second, the in vivo volume of distribution of the plasma protein must be considered. The volume of distribution differs for each protein because of varying molecular size, interaction with other plasma proteins, and interaction with cellular elements of blood as well as the endothelial cell. The dose needed to raise the plasma level a desired amount has been determined by recovery and half-life studies for the various coagulation factor proteins and is included in Table 140-1 . For example, infusion of 1 U factor VIII/kg results in a 2% (2 U/dl) rise in plasma factor VIII. Thus, a 70-kg man with severe hemophilia A (<1 U/dl factor VIII) would require 20 U/kg or 1,400 U factor VIII to achieve a 40% (U/dl) factor VIII level, the effective hemostatic level known to achieve hemostasis for a significant hemarthrosis.

Third, knowledge of the half-life of the infused protein is necessary if the plasma level of the coagulation factor must be maintained over time, as is necessary in perioperative management. For example, in the postoperative treatment of hemophilia A, because factor VIII has a 12-hour half-life, to maintain a plasma factor VIII level >4050% (U/dl), a 50% (25 U/kg) correction every 12 hours must be administered. The biologic half-lives of the coagulation factors are listed in Table 140-1 .

Finally, the concentration of the coagulation factor in the product must be known to calculate the amount of product to be administered. Fresh-frozen plasma, because of its content of approximately 1 IU/ml of each of the coagulation factors, can theoretically be used to treat any plasma protein deficiency, but frequently the dose needed to attain a particular in vivo hemostatic level would result in circulatory volume overload. Therefore, the coagulation factor concentrates, because of their convenience and now increased safety over most plasma and cryoprecipitate products, have become the preferred product for treatment of most disorders; the amount contained in each vial is printed on the label. In cases of life-threatening bleeding or major surgery, or if the patient is not responding clinically as expected, laboratory monitoring of factor levels is advisable. A detailed description of the preparation and properties of products containing coagulation factor proteins is found in Chapter 139 .

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THERAPY FOR PROCOAGULANT DEFICIENCIES

Hemophilia A

Once the diagnosis of hemophilia A (factor VIII deficiency) has been established, the clinician may choose from a variety of therapeutic alternatives to treat bleeding manifestations or to prepare the patient for surgery, tooth extractions, or other invasive procedures. The desired concentration of plasma factor VIII (hemostatic level) to be achieved depends on the type and location of the bleed or planned procedure. Two dose classifications are generally accepted. For tooth extractions, routine joint and muscle hemorrhages, and lacerations, one should attain a plasma factor VIII level of 3040% (U/dl) (Table 140-2). In contrast, more aggressive replacement therapy (80100% [U/dl]

TABLE 140-1 -- Characteristics of Coagulation Factors Important for Transfusion Therapy

Factor	Factor Source (concentrate)	Minimum Hemostatic Level[a]	Desired Initial Level for Major Surgery	Increase in Plasma Level with Dose of 1 U/Kg	Biologic Half-life	Initial Dose[b]
Fibrinogen (I)	Cryoprecipitate (200300 mg/bag)	75150 mg/dl	150 mg/dl	[c]	45 days	23 bags/15 kg daily
Prothrombin (II)	Plasma PCC[d]	1540 U/dl	40 U/dl	1 U/dl	3 days	1520 ml/kg 40 U/kg
V	Plasma	1025 U/dl	30 U/dl	1.5 U/dl	1236 hours	1520 ml/kg
VII	Plasma PCC[d]	510 U/dl	25 U/dl	1 U/dl	46 hours	1520 ml/kg 2050 U/kg
VIII	Factor VIII concentrate	3050 U/dl	100 U/dl	2 U/dl	1215 hours	50 U/kg
IX	Factor IX concentrate	2050 U/dl	80 U/dl	1 U/dl	1830 hours	80 U/kg
X	Plasma PCC[d]	1020 U/dl	25 U/dl	1 U/dl	1.52.5 days	1520 U/kg 25 U/kg
XI	Plasma	1030 U/dl	30 U/dl	2 U/dl	13 days	1520 ml/kg
XIII	Plasma Cryoprecipitate (50100 U/bag)	15 U/dl	5 U/dl	13 U/dl	310 days	10 ml/kg 1 bag/20 kg
von Willebrand factor	Humate-P	3050 U/dl	80 U/dl	2 U/dl	1215 hours	4050 U/kg

[a] The lower value is the minimum hemostatic level required for treatment of minor bleeding and the higher value is the maintenance nadir level required for treatment of major bleeds and surgery.
[b] For severe deficiency for surgery.
[c] 1020 ml/kg fresh-frozen plasma or 1 donor unit (bag)/5 kg cryoprecipitate will raise the plasma fibrinogen level 50100 mg/dl.
[d] Prothrombin complex concentrate; factor VII content variable.

TABLE 140-2 -- Treatment of Specific Hemorrhages in Hemophilia

Type of Bleed	Hemophilia A	Hemophilia B
Hemarthrosis[a]	20 U/kg FVIII concentrate;[b] 15 U/kg if treated early. Repeat dose the following day if severe bleed.	30 U/kg FIX concentrate;[c] 20 U/kg if treated early
Muscle or significant subcutaneous hematoma	20 U/kg FVIII concentrate; may need every-other-day treatment until well controlled	30 U/kg FIX concentrate;[c] may need treatment every 2 or 3 days until well controlled
Mouth, deciduous tooth, or tooth extraction	20 U/kg FVIII concentrate; antifibrinolytic therapy; remove loose deciduous tooth	30 U/kg FIX concentrate;[c] antifibrinolytic therapy;[d] remove loose deciduous tooth
Epistaxis	Pressure for 1520 min; pack with Vaseline gauze; antifibrinolytic therapy; 20 U/kg FVIII concentrate if above fails	Pressure for 1520 min; pack with Vaseline gauze; antifibrinolytic therapy; 30U/kg FIX concentrate if above fails (4 hrs after antifibrinolytic dose)
Major surgery, life-threatening hemorrhage (e.g., CNS, GI, airway)	50 U/kg FVIII concentrate, then 25 U/kg q 12 hrs or continuous infusion to maintain FVIII >100 U/dl for 24 hrs, then >50 U/dl for 57 days and then >30 U/dl for 57 days	80 U/kg FIX concentrate,[c] then 2040 U/kg q 1224 hrs to maintain FIX >40 U/dl for 57 days and then >30 U/dl for 57 days[c]
Iliopsoas hemorrhage	50 U/kg FVIII concentrate, then 25 U/kg q 12 hrs until asymptomatic, then 20 U/kg qod for total 1014 days[e]	80 U/kg FIX concentrate,[c] then 2040 U/kg q 1224 hrs to maintain FIX >40 U/dl until asymptomatic, then 30 U/kg qod for total 1014 days[c] [e]
Hematuria	Bed rest; 1.5 × maintenance fluids; if not controlled in 12 days, 20 U/kg FVIII concentrate; if not controlled, prednisone if HIV negative	Bed rest; 1.5 × maintenance fluids; if not controlled in 12 days, 30 U/kg FIX concentrate; if not controlled, prednisone if HIV negative

[a] For hip hemarthrosis, orthopedic evaluation for possible aspiration is advisable.
[b] For mild or moderate hemophilia, DDAVP (0.3 mcg/kg) should be used instead of FVIII concentrate if patient is known to respond with a hemostatic level of FVIII; if repeated doses are given, monitor FVIII levels for tachyphylaxis.
[c] If repeated doses of FIX concentrate are given, highly purified coagulation FIX concentrate should be used. If recombinant FIX concentrate (BeneFix) is used, multiply the dose by a factor of 1.2.

[d]Do not give antifibrinolytic therapy until 46 hrs after a dose of prothrombin complex factor IX concentrate.
[e]Repeat radiologic assessment should be performed before discontinuation of therapy.

plasma factor VIII) should be instituted for patients undergoing major surgery or with limb- or life-threatening bleeding episodes such as central nervous system bleeding, hemorrhage around the neck or retroperitoneum, and compartment syndromes from muscle or subcutaneous hemorrhage. Calculation of the dose required to achieve a desired hemostatic level is based on the following formula: [4]

Thus, for treatment of a hemarthrosis in a 70-kg man with severe hemophilia A, the dose would be 40 (U/dl) × 70 (kg) × 0.5 = 1,400 IU factor VIII.

For patients undergoing major surgery or with serious hemorrhage, the plasma factor VIII level should rapidly be corrected to 100% (U/dl) or if for surgery, immediately before the procedure, and then maintained at 75100% for 24 hours, then >4050% (U/dl) for 57 days and >2030% (U/dl) for an additional 57 days (Table 140-2). This can be accomplished by infusion of intermittent doses of factor VIII based on the half-life of 12 hours; for example, by administration of 25 U/kg (50% [U/dl] correction) every 12 hours. Alternatively, a 50% (U/dl) level can be maintained by continuous infusion of 2 IU factor VIII/kg/hr (0.4 IU/kg/hr/% [U/dl] desired factor VIII level).[5] It is advisable to administer a 50% (U/dl) correction of factor VIII concentrate immediately after major surgery to compensate for the loss of factor VIII by increased consumption and blood loss during the surgical procedure. If a continuous infusion is chosen for postoperative therapy, a dose of 4 IU factor VIII/kg/hr for the first 24 hours will maintain the factor VIII level in the 75100% range. For surgical procedures and limb- and life-threatening hemorrhages, a factor VIII assay should be monitored at least daily. Central nervous system bleeding requires a full 2 weeks of therapy, and patients are at increased risk of recurrence for approximately 6 months after an episode. Iliopsoas and other retroperitoneal hemorrhages must be treated aggressively with maintenance of a 50% (U/dl) plasma factor VIII level until symptoms have abated; it is then possible to taper the factor VIII therapy to every other day for a total of 1014 days.

For treatment of more routine bleeding, such as hemarthrosis, usually only a single dose of clotting factor replacement (plasma factor VIII level 3040% [U/dl]) is required. However, with severe joint bleeds or in the case of many muscle bleeds, additional infusions of factor VIII must be given. Generally, administration of a 40% correction every other day for 710 days, or until a muscle hemorrhage or large subcutaneous hematoma has nearly resolved, is adequate. For tooth extractions, mouth lacerations, or recurrent epistaxis, it is often helpful to use antifibrinolytic therapy as an adjunct (Table 140-2).

Prophylaxis is routinely being used in many centers now that recombinant concentrates are available. This strategy has been shown to prevent the chronic arthropathy seen when hemarthroses are treated episodically. A dose of 2040 U/kg of factor VIII concentrate is given every other day or three times weekly. The dose and frequency are adjusted to maintain a plasma nadir level of >1 U/dl. [6] [7]

Therapy Products

Recombinant Factor VIII Concentrates

The factor VIII gene has been cloned, sequenced, and successfully transfected into mammalian cell cultures to produce recombinant factor VIII. [8] [9] Two recombinant factor VIII products are currently available, Recombinate and Kogenate. Clinical trials have documented that the half-life, recovery, and efficacy of recombinant factor VIII is indistinguishable from plasma-derived factor VIII. [10] [11] The clear advantage to the use of recombinant factor VIII is safety from human viral contamination, making these products preferable to plasma-derived concentrates in most cases if cost is not an issue. The need for human albumin as a stabilizer in these preparations is a disadvantage, however, because any human blood-derived protein could theoretically become contaminated with an as-yet-unknown infectious agent; for example, the agent of Creutzfeldt-Jakob disease has recently been a source of anxiety, although there have been no known cases of transmission of the disease by any blood product. [12] [13] Two newly developed recombinant factor VIII concentrates, one a B-domain-deleted preparation, are currently in clinical trials; the absence of human albumin as a stabilizer in these products should provide an additional measure of safety from human bloodborne infectious agents. Initial concern that the incidence of inhibitor development was increased in previously untreated patients treated with highly purified factor VIII concentrates was probably due to the differences in follow-up and data collection performed in the setting of these clinical trials. [14] [15] Subsequent carefully performed retrospective studies of patients treated with intermediate-purity products suggested that there is no difference in the incidence of inhibitor development. [16]

Plasma-Derived Factor VIII Concentrates

Several commercial plasma-derived factor VIII concentrates are available. Concentrates of intermediate purity are produced from large pools of plasma by combinations of cryoprecipitation and precipitation with glycine, polyethylene glycol, or ethanol. [17] These products contain, on the average, 25 units of antihemophilic factor (AHF) (factor VIII) per mg protein. More recently, products of high purity have been developed. These products, also derived from large pools of human plasma, are produced by immunopurification of cryoprecipitate on monoclonal antibody columns using mouse monoclonal antibodies to factor VIII [18] or von Willebrand factor (vWF). [19] Extraneous proteins are washed from the column, the factor VIII is eluted, albumin is added as a stabilizer (resulting in 215 AHF U/mg protein), and the final product is lyophilized. Products produced by gel filtration chromatography are also considered to be of high purity and contain approximately 922 AHF U/mg protein, similar to the monoclonal antibody-purified factor VIII concentrates after albumin has been added during the formulation. The diversity of foreign antigens contaminating the products is higher, however, because the specific activity is 3560 AHF U/mg protein before the addition of albumin, whereas the monoclonal antibody immunopurification results in 1,5002,500 AHF U/mg protein before the addition of albumin.

With improved purity of plasma-derived products, the frequency of complications due to exposure to extraneous plasma proteins has greatly diminished. Complications such as allergic reactions, isohemagglutinin-induced hemolytic anemia, immune complex-mediated hematuria, and granulocyte antibody-induced acute lung injury are rarely seen. Use of monoclonal antibody immunopurified concentrates appears to stabilize CD4 counts in human immunodeficiency virus (HIV)-infected hemophilic patients.[20] [21]

All plasma protein concentrates now undergo a viral attenuation process in addition to purification. Dry heating in the lyophilized state from 60°C to 68°C for 3072 hours, initially developed to eliminate hepatitis from concentrates, was soon shown to be ineffective for this purpose; hepatitis C developed in most treated patients. [22] However, it was fairly effective in reduction of HIV transmission, although a few cases were reported. With the development of improved viral attenuation methods, all dry-heated factor VIII concentrates of intermediate purity were removed from the market. Newer virus attenuation methods include heating in the wet state (pasteurization),

solvent/detergent treatment with a variety of agents, vapor heating, superdry heat (80°C for 72 hours), and affinity chromatography plus dry heat or solvent/detergent treatment. These methods have been successful in eliminating HIV seroconversions and possibly hepatitis C. [23] The exceptions have included the transmission of hepatitis C in products heated in n-heptane and probable transmission of hepatitis A in products treated with a particular solvent/detergent method. [24] Parvovirus can be transmitted by all current virus-inactivated human plasma-derived concentrates. [25] The risk of hepatitis B transmission can be further diminished by scrupulous immunization at the time of diagnosis of all patients, including infants, with one of the hepatitis B vaccines. [26] If clotting factor replacement therapy is needed at the time of diagnosis, hepatitis B immunoglobulin with the first dose of vaccine may provide passive protection until vaccine-induced antibodies are formed. Family members of chronic carriers of hepatitis B antigen and those involved in the preparation and administration of clotting factor concentrates should also be immunized. Patients older than 2 years should undergo immunization with the hepatitis A vaccine as well. [27]

Factor VIII concentrates are formulated in vials that may be stored either refrigerated or, for short periods, at room temperature. These commercial products are packaged in vials with the number of units of activity of the coagulation factor printed on the label, and most packages also contain diluent and needles necessary for

reconstitution and administration of the product; thus, they are very convenient for outpatient use and home care.

Cryoprecipitate

The first widely used concentrated factor VIII preparation, cryoprecipitate, was prepared by Judith Graham Poole in 1965. [28] Since that time technical advances have improved the yield of factor VIII in cryoprecipitate, and the preparations now generally contain approximately 100 IU factor VIII/bag. Thus, the patient with hemophilia A would require exposure to 216 donor U (bags) for treatment of a single routine hemorrhage, in comparison with the 10,00020,000 donor exposures associated with exposure to a single lot of commercial concentrate. Before the development of effective viral attenuation of commercial lyophilized factor VIII concentrates, cryoprecipitate was safer from transfusion-transmitted viral disease because of the reduced number of donor exposures. However, recombinant or viral attenuated concentrates are now preferred because cryoprecipitate is not easily viral attenuated. [20]

Desmopressin

Desmopressin (1-desamino-8-D-arginine vasopressin [DDAVP]), a synthetic vasopressin analog, increases factor VIII and vWF levels, allowing successful treatment of dental and surgical patients with mild and moderate hemophilia A and von Willebrand disease (vWD) with desmopressin alone. [29] [30] [31] Desmopressin is now considered the treatment of choice in patients with mild and moderate hemophilia A who respond to the drug. It is not efficacious in the treatment of severe hemophilia A or severe type III vWD or in patients with any form of hemophilia B.

The individual response to desmopressin is variable; [32] in hemophilia A, the range of responses has been reported to be 225-fold over baseline factor VIII levels. Thus, patients should undergo a trial dose with laboratory measurement of the factor VIII (or vWF) response before the use of desmopressin for the treatment of bleeding episodes and prophylaxis for dental and other surgical procedures. A single individual usually responds to a similar degree with repeated doses; [33] thus, the plasma level of factor VIII attained after a trial dose of desmopressin can be used to predict responses for the design of future therapy. For example, a patient with mild hemophilia A who achieves a 40% factor VIII level after desmopressin stimulation should be treated with desmopressin for routine hemarthroses, tooth extractions, and so forth, but desmopressin would not be sufficient for treatment of limb- or life-threatening hemorrhages or preparation for major surgery, situations in which a 100% factor VIII level is indicated.

Desmopressin is now available in both an intravenous form and a concentrated intranasal form (Stimate). [34] [35] Care must be taken to ensure that pharmacies do not substitute the dilute intranasal preparation formulated for the treatment of enuresis and diabetes insipidus; it is not effective for treatment of hemophilia and vWD. The recommended dose of intravenous desmopressin is 0.3 g/kg;[29] [32] the calculated dose is diluted in 2550 ml normal saline and given as an intravenous infusion over 30 minutes. The dose of intranasal desmopressin is 150 g (one puff) for patients <50 kg and 300 g (two puffs, one in each nostril) for those >50 kg. Because the maximal rise in factor VIII occurs at 3060 minutes, it is advisable to time the infusion as close to the surgical procedure as possible.

Tachyphylaxis may occur with repeated doses of desmopressin and varies from patient to patient. [36] Therefore, if repeated dosing is contemplated, factor VIII levels should be monitored and exogenous factor VIII given as needed. In general, if 24 days have elapsed between doses, a response similar to baseline can be expected.

Side effects are usually minimal and include facial flushing, headache, or mild increases in pulse rate or blood pressure that resolve when the infusion is slowed or discontinued. Rare cases of seizures associated with hyponatremia have been reported. [37] Fluids should be restricted to maintenance for 24 hours when desmopressin is administered, and serum sodium should be monitored in the patient treated with repeated doses; hyponatremia generally responds to fluid restriction. Thrombosis has occurred rarely;[38] thus, the drug should be used with caution in patients with an increased risk of thrombosis.

Antifibrinolytic Therapy

Antifibrinolytic therapy is a useful adjunctive therapy, particularly for treatment of injuries involving the oral and nasal mucous membranes. It is not effective in obtaining initial hemostasis, but it prevents clot lysis once hemostasis has been achieved by clotting factor replacement or local measures. For example, in the treatment of dental extractions, usually a single dose of clotting factor replacement before the procedure is sufficient for hemostasis, and maintenance with an antifibrinolytic agent can be substituted for maintenance clotting factor replacement. [39] [40] Tranexamic acid has recently been shown to be effective when used as a mouthwash, as well.[41] Antifibrinolytic agents have not shown any efficacy in the treatment of hemarthroses and are contraindicated in the presence of hematuria. Patients treated with prothrombin complex concentrates should not be given antifibrinolytic drugs simultaneously with an infusion (it is advisable to wait at least 46 hours) because of the increased risk of thrombotic complications.

Two antifibrinolytic agents are available in the United States, -aminocaproic acid (EACA; Amicar) and tranexamic acid (Cyclokapron). EACA is formulated in an oral tablet and an oral elixir form. The tablet is a very large 500-mg size, and with the usual adult dose of 5 g (10 capsules), even adults prefer the elixir. The oral dose of EACA is 100 mg/kg (maximum 10 g) initial dose followed by 50 mg/kg/dose (maximum 5 g) every 6 hours. The second agent, tranexamic acid, is also available in 500-mg capsules, but they are smaller and the lower recommended oral dose (25 mg/kg/dose every 68 hours) is more tolerable. Both are available in intravenous forms, but the dosing schedules are different.

Hemophilia B

The clinical manifestations of hemophilia B (factor IX deficiency) are quite similar to those of hemophilia A. Therefore,

the approach to management of hemorrhages with products containing factor IX is analogous to that using factor VIII in hemophilia A. Recovery of factor IX in plasma after an infusion is approximately half that of factor VIII (1% [U/dl] rise after 1 U/kg); therefore, twice as much product must be given to achieve a desired plasma level (Table 140-1). However, the minimal hemostatic level is lower than that of factor VIII and the biologic half-life of factor IX is longer (1824 hours). [42]

Two generally accepted dose classifications are used for hemophilia B as well (Table 140-2). Routine bleeds such as hemarthrosis and muscle hemorrhage require 2030% (U/dl) plasma factor IX levels; with major surgery and life-threatening hemorrhage, an 80% (U/dl) factor IX level should be achieved initially and levels should be maintained at >3045% (U/dl) for 57 days and then >1520% (U/dl) for another 57 days. Calculation of the dose required to achieve a desired rise in plasma factor IX is as follows:

The dose calculation for the use of recombinant factor IX (BeneFix) must be multiplied by a factor of 1.2 to compensate for the decreased in vivo recovery of the transfused product (see the section on recombinant factor IX).

Thus, for treatment of hemarthrosis in a 70-kg man with severe hemophilia B, the dose would be 30% (U/dl) × 70 kg = 2,100 IU factor IX. If recombinant factor IX is used, the dose would be 30% (U/dl) × 70 kg × 1.2 = 2,500 IU. For maintenance therapy for major bleeds or surgery, doses are smaller because of the longer biologic half-life of factor IX. For major hemorrhage, factor IX levels should be monitored.

Therapy Products

Recombinant Factor IX Concentrate

A recombinant factor IX concentrate [43] (BeneFix) has recently been licensed for the treatment of hemophilia B. In addition to the added viral safety provided by the recombinant nature of the product, the final formulation is also free from albumin or other human plasma-derived protein, adding an additional measure of safety. Thus, this product is now preferred for the treatment of hemophilia B. Transfused recombinant factor IX concentrate has a decreased in vivo recovery compared to plasma-derived products, presumably due to dissimilar post-translational processing of the protein during synthesis in cell culture. Thus, previously established dose

calculations based on plasma-derived products must be multiplied by a factor of 1.2 to compensate for the decreased recovery of the recombinant protein. However, the in vivo half-life of the product is the same as that of human plasma-derived factor IX.

Both recombinant factor IX concentrate and the highly purified coagulation concentrates have been associated with the recently recognized syndrome of anaphylaxis in the setting of inhibitor development in young children with severe hemophilia B treated with these products. [44] Therefore, previously untreated patients with severe hemophilia B should receive their initial 1020 infusions at a medical facility able to manage anaphylaxis should it occur.

Coagulation Factor IX Concentrates

Coagulation factor IX concentrates are highly purified plasma-derived products that are nonthrombogenic. [45] If a human plasma-derived factor IX concentrate is chosen over recombinant factor IX, it is strongly recommended that these highly purified products be used instead of prothrombin complex concentrates when using high or repeated doses of factor IX for major surgery or life-threatening hemorrhages, in patients with impaired ability to clear activated coagulation factors such as those with hepatocellular disease or infants, those with crush injuries or massive muscle hemarthroses, or those with a history of thrombosis. The two currently available products are virus inactivated by solvent/detergent treatment (AlphaNine) in one and ultrafiltration plus thiocyanate treatment (MonoNine) in the other. As with the use of recombinant factor IX concentrate, precautions for the management of anaphylaxis in previously untreated patients should be taken as outlined in the preceding section.

Prothrombin Complex Concentrates

Fractionation procedures developed to produce intermediate-purity factor IX concentrates use barium sulfate precipitation and column chromatography with a variety of adsorbents.[46] [47] Because the physical and chemical properties of the vitamin K-dependent clotting factors are highly similar, factors II and X, as well as varying amounts of factor VII and the physiologic anticoagulant protein C, are copurified with factor IX. Although this similarity has been used for treatment of other vitamin K-dependent coagulation factor deficiencies, the presence of these factors in an activated form is thought to be the cause of rare but life-threatening thrombotic complications, including deep venous thrombosis, pulmonary emboli, myocardial infarctions, and disseminated intravascular coagulation. [48] [49] The risk appears to be increased in patients receiving high or multiple doses of concentrate or concurrent antifibrinolytic therapy or in those with significant liver disease and impaired ability to clear activated clotting factors from the circulation. Therefore, the recommendation was made to add 100 U heparin/500 U factor IX concentrate to the vials after the product is reconstituted when using these products in high doses or when more than a single infusion is anticipated. [50] However, nonthrombogenic highly purified factor IX concentrates, either recombinant or plasma-derived, have been developed and should be used when more than a single dose of factor IX is anticipated. [51] Currently available prothrombin complex concentrates undergo dry heating at high temperatures (80°C) or vapor heating to inactivate viruses; no HIV seroconversions have been noted, and hepatitis C transmission may also have been eliminated. [23]

Plasma

Until the recent availability of pure, safe factor IX concentrates, fresh-frozen plasma was used, particularly in young children and mild and moderate hemophilia B patients in whom infrequent use and smaller doses did not result in untoward numbers of donor exposures. However, recombinant factor IX concentrate or one of the highly purified, viral-attenuated concentrates is now preferred for treatment of hemophilia B. If fresh-frozen plasma is used for the treatment of coagulation deficiencies for which there is no safe, effective concentrate, consideration should be given to the use of small pools of well-screened volunteer donors to limit the possibility of HIV or hepatitis transmission. Some blood centers are using a delayed release strategy whereby a plasma donation is not released for transfusional use until the donor has returned some months later and been found to have negative viral serology, thus reducing the chance that the initial donation was made during the window period between viral infection and the ability of screening tests to detect the infection. A viral-attenuated solvent/detergent-treated plasma product has been licensed and should add an additional increment of viral safety. [52] If parental heterozygous carriers are considered for directed donations, clotting factor levels should be evaluated before donation of plasma for affected children. Maternal plasma should be avoided because it is more likely to be implicated in the rare complication of transfusion-related acute lung injury, mediated by granulocyte antibodies. [53] Fresh-frozen plasma should be ABO type-compatible to avoid the complication of isohemagglutinin-induced hemolytic anemia. Fresh-frozen plasma should be administered immediately after thawing. [54]

Patients with intact cardiovascular systems can safely tolerate a dose of 1520 ml/kg.

Hemophilia with Inhibitors

Of patients with severe hemophilia A, 1530% develop a circulating inhibitor (antibody) to factor VIII, and thus treatment with factor VIII concentrates becomes extremely difficult or impossible. Development of an inhibitor should be suspected in a patient who does not respond to coagulation factor replacement therapy in the expected manner. Clinically, inhibitor patients can be divided into two general categories: those who develop higher titers of antibody with exposure to factor VIII (high responders) and those who maintain a low-titer inhibitor despite repeated doses of factor VIII (low responders). [55] Treatment recommendations differ for the two groups.

Low-Responder Factor VIII Inhibitors

Patients whose inhibitor titers, measured by the standard method of Kasper et al., [56] do not rise >10 Bethesda units despite exposure to factor VIII are considered low responders. Most of these individuals can continue to be treated with factor VIII concentrate, but in higher doses. For serious limb- or life-threatening bleeding, current recommendations include a bolus infusion of 100 U/kg factor VIII initially, followed by 20 U/kg/hr. Factor VIII assays should be obtained 1 hour after the bolus infusion and then at least daily so that the dose may be adjusted depending on the factor VIII level.

Joint and muscle hemorrhages and other more minor hemorrhages can usually be managed with bolus infusions of factor VIII; the exact dose should be determined in each patient based on a factor VIII recovery study after an infusion of factor VIII. Adjunctive therapy with antifibrinolytic agents is effective in the management of dental extraction and nasal and mucous membrane injury, as discussed above for noninhibitor patients.

High-Responder Factor VIII Inhibitors

Numerous therapies and combinations of therapies have been devised for treatment of the high-responder inhibitor patient; however, to date none of these is as uniformly successful as factor VIII replacement in the noninhibitor patient.

Prothrombin Complex Concentrates

Prothrombin complex concentrates are often used to treat routine joint and muscle hemorrhages in inhibitor patients. A multicenter study conducted in the late 1970s showed that the two prothrombin complex concentrates then available promoted hemostasis in 50% of bleeding episodes at a dose of 75 factor IX U/kg in comparison with a 25% placebo effect with albumin.[57] The addition of heat treatment to the manufacturing process has not altered the response rate. [58] Most physicians initially manage hemarthroses and other routine bleeds in these hemophilia patients with prothrombin complex concentrates. The dose for joint and muscle bleeds has varied from 50 to 100 U/kg; most physicians administer 75 U/kg/dose in this setting. If no response occurs after 2 or 3 infusions given 12 hours apart, it is unlikely that additional infusions will achieve hemostasis; instead, they will only increase the risk of thrombotic complications.

Activated Prothrombin Complex Concentrates

Currently two activated prothrombin complex concentrates (APCCs) are commercially available, Autoplex and FEIBA. They have been purposefully activated during fractionation, resulting in increased amounts of activated factor VII, factor X, and perhaps thrombin. These products are intended for use in patients with circulating inhibitors. Two double-blind controlled trials comparing the efficacy of APCC with standard prothrombin complex concentrates showed a small but significant advantage of FEIBA (64% efficacy) over a standard prothrombin complex concentrate (52% efficacy) in one trial [59] and no additional benefit of Autoplex over Proplex (standard prothrombin complex concentrate) in the second trial. [60] The introduction of viral attenuation of these products has had no apparent effect on their

hemostatic efficacy or safety in noncontrolled studies. [61] Although the high cost of the APCCs and their unproved efficacy over standard prothrombin complex concentrates has led most investigators to discourage their use for routine management of inhibitor patients, anecdotal reports of their utility in the management of serious bleeding episodes have led to the recommendation of their use as an initial therapy in serious hemorrhages when other available therapy would not be expected to be efficacious (e.g., patients with inhibitor titers >50 Bethesda units). The dose usually recommended is 75 U/kg. This can be repeated within 612 hours, but >3 consecutive doses and simultaneous use of antifibrinolytic therapy should be avoided, if possible. If not, the patient should be monitored for the possibility of disseminated intravascular coagulation and myocardial infarction, which has been reported with the use of APCCs as well as the standard prothrombin complex concentrates.[62]

Recombinant Factor VIIa

A recombinant factor VIIa (activated factor VII) concentrate has been developed and has been shown to achieve hemostasis in surgical and life-threatening hemorrhage in high-responder inhibitor patients who have failed conventional therapy. [63] [64] It is postulated to interact with tissue factor expressed on endothelial cells at the site of vascular injury and to activate factor X, thus bypassing the inhibited factor VIII cofactor step in factor X activation. [65] This promising product continues to undergo clinical trials and is currently available only for investigational or compassionate use.

Porcine Factor VIII Concentrates

Most investigators prefer to use a factor VIII-containing product for limb- or life-threatening bleeding if it is at all feasible to achieve a hemostatic level. Human factor VIII concentrate in high doses (see previous recommendations for low-responder inhibitors) can be tried if the inhibitor titer is <10 Bethesda units or can be lowered to <10 with plasma exchange or immunoadsorbant columns, but this is a temporary solution, because a rapid anamnestic response usually limits the ability to maintain a hemostatic level for >13 days in high-responder inhibitors. [66] A porcine factor VIII concentrate, highly purified by polyelectrolyte ion exchange chromatography, is particularly useful for treatment of hemorrhages in patients with an inhibitor titer <50 Bethesda units. [67] Occasionally, it has been efficacious in patients with higher-titer inhibitors who have antibodies that are less cross-reactive with porcine factor VIII. It is preferable to high-dose human factor VIII because of the lower risk of anamnesis.[68] In patients who do not have anamnesis, the product can be considered for routine bleeding as well. The recommended starting dose is 100150 U/kg, with measurement of plasma factor VIII levels to guide continued therapy. It is advisable to measure the antiporcine factor VIII antibody titer periodically in high-responder inhibitor patients to be prepared when a life-threatening hemorrhage occurs.

Other Therapy

In the 1970s, the first attempts were made to induce immune tolerance to factor VIII in inhibitor patients by infusions of twice-daily high doses of factor VIII (100150 U/kg) plus APCC over prolonged periods (months to years).[69] This very expensive and demanding treatment, although successful in 15

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who completed the Bonn Protocol, led others to modify the regimen by eliminating the APCC and reducing the amount and frequency of factor VIII infusions, with some success.[70] [71] Other modifications reported to have varying success rates have included the use of intravenous gamma-globulin, immunosuppressive therapy, removal of antibody by extracorporeal immunoadsorption on Staph Protein A columns, and combinations of those modalities. [72] Induction of immune tolerance in four of five patients on home-based therapy with porcine factor VIII was also recently reported. [73]

Factor IX Inhibitors

In patients with hemophilia B and factor IX inhibitors, treatment with prothrombin complex concentrates for routine hemorrhages or APCCs for more serious bleeding at the dose recommended for factor VIII inhibitor patients has been successful. However, it is not helpful for patients with the syndrome of anaphylaxis in the setting of inhibitor development because it too contains factor IX. Recombinant factor VIIa has been shown to be efficacious in the patients with factor IX inhibitors in whom it has been tried.[74] Immune tolerance induction with high-dose factor IX concentrate has been disappointing, and several cases of nephrotic syndrome have developed when factor IX was given in high doses for immune tolerance induction.[74]

von Willebrand Disease

Treatment of vWD depends on the type of vWF defect and on the patients response to desmopressin. Either quantitative or qualitative vWF defects may be present. Transfusion products appropriate for each type are summarized in Table 140-3 .

The most common quantitative defect, a functional molecule present in decreased amounts (hypoproteinemia), is termed type I vWD; [75] most vWD patients (approximately 90%) are type I, and most of these respond to administration of desmopressin with a three- to fivefold increase in plasma vWF. [29] [30] [31] [32] [36] A single dose of desmopressin is generally sufficient for minor bleeding and tooth extractions. For oral bleeding and tooth extractions, antifibrinolytic therapy is also recommended, as discussed for hemophilia. It is advisable to perform a desmopressin trial to determine the individual response before its use for major surgical procedures; in general, if the vWF activity (ristocetin cofactor) rises to >100% (U/dl), the patient can be treated with desmopressin alone. For major surgery, doses given every 1224 hours for 23 days postoperatively are usually sufficient to maintain hemostatic levels of vWF >4050 U/dl (%). vWF:ristocetin cofactor levels should be measured daily and exogenous vWF (Humate-P or vWF concentrate) administered if tachyphylaxis occurs. In patients in whom factor VIII:C levels are also low, factor VIII:C should be monitored and maintained at a hemostatic level as recommended for hemophilia A with either desmopressin or factor VIII concentrate as needed.

Patients with qualitative vWF defects (type II) [76] can be subgrouped into those with absent high-molecular-weight multimers on the basis of failure of multimerization or increased proteolysis of vWF (type IIA), or those with a loss of high-molecular-weight multimers from plasma on the basis of an increased affinity of vWF for the platelet surface due either to a vWF defect (type IIB) [77] or to a platelet glycoprotein Ib defect (platelet-type vWD). [78] Some patients with type IIA vWD have a transient response to desmopressin, and minor bleeding may be treated with desmopressin. However, for major bleeds and surgery, replacement therapy with factor VIII concentrate containing normal vWF multimers (Humate-P) (see Chap. 139) is usually required. Type IIB patients and those with platelet-type vWD may have thrombocytopenia that is exacerbated by the administration of desmopressin; therefore, desmopressin is generally considered to be contraindicated in this group, although some investigators believe that it may be useful in some patients in this group. [79] Replacement therapy with Humate-P or vWF concentrate is required for treatment of type IIB vWD; platelets may need to be given if thrombocytopenia is severe, but normal vWF should be given first. For patients with platelet-type vWD, platelet transfusion is indicated for treatment of hemorrhages; rarely, vWF replacement is also needed.

Patients with severe type III vWD lack detectable vWF; the factor VIII:C level is also low (35%) because vWF serves as a stabilizer for factor VIII in the circulation. Desmopressin is ineffective in this group because vWF stores are also absent. Therefore, vWF and factor VIII must be replaced for treatment of hemorrhages. Because vWF has a 12-hour half-life in the circulation, management of bleeding episodes with vWF replacement is similar to that described above for factor VIII replacement for hemophilia A. Humate-P is currently the only licensed factor VIII concentrate in the United States that contains normal vWF. A purified plasma-derived vWF concentrate is currently in clinical trials. A recombinant vWF has been developed, but human clinical trials have not yet begun.

Other Inherited Procoagulant Deficiencies

Factors II, VII, and X

Transfusion therapy for these very rare vitamin K-dependent procoagulant deficiencies should be approached through analysis

TABLE 140-3 -- Therapeutic Alternatives for the Treatment of von Willebrand Disease

Type of vWD	Desmopressin	vWF or FVIII Concentrate	Platelets
I	Preferred treatment if desmopressin results in therapeutic level in trial	If desmopressin is not effective or if higher levels are required	Not indicated

IIA	Not usually effective; may cause improvement for 12 hours	Preferred treatment	Not indicated
IIB	May cause further decrease in platelet count	Preferred treatment	If thrombocytopenia remains severe after vWF replacement. Not usually effective alone
Platelet-type von Willebrand disease	May cause further decrease in platelet count	May cause further decrease in platelet count	Preferred treatment
Untyped von Willebrand disease	Use with caution; follow platelet count and vWF levels	Preferred treatment in untyped patient with von Willebrand disease	Not usually required unless patient has platelet-type von Willebrand disease

of minimal hemostatic levels, as well as recovery and half-life of the transfused protein (Table 140-1). As with other clotting factor deficiencies, the use of plasma may be limited by volume constraints but is usually quite efficacious at a dose of 1020 ml/kg because of the low minimal hemostatic levels of these procoagulants. If higher plasma levels of the vitamin K-dependent clotting factors are needed, one can either use plasma exchange procedures or begin the use of prothrombin complex concentrate. The content of the various vitamin K-dependent procoagulants in each prothrombin complex concentrate is variable. [80] [81] The specific concentration of the required clotting factor in a concentrate can often be obtained from the manufacturer. Precautions regarding adverse effects are outlined above for plasma and prothrombin complex concentrates. A factor VII concentrate is in development. Vitamin K administration has no role in the treatment of these inherited coagulopathies.

Fibrinogen and Factors V, XI, and XIII

Although most patients with fibrinogen deficiencies are asymptomatic, the afibrinogenemic and some of the dysfibrinogenemic patients may occasionally have hemorrhage that requires replacement therapy. Fibrinogen can be replaced with either plasma (1020 ml/kg) or cryoprecipitate (1 donor U/5 kg) to raise the fibrinogen level to 50100 mg/dl.[82] [83] Because of its long half-life, repeated infusions need only be given every 45 days if prolonged correction of the deficiency is required.

Factor V deficiency, also called parahemophilia, is rarely associated with bleeding severe enough to require replacement therapy. When required, the only available product is plasma; preferably the product should be <1 month old. Hemostatic level is approximately 25%, and the half-life of the transfused protein is 36 hours; thus, the condition is highly amenable to treatment with plasma. [84] [85]

Factor XI deficiency, also known as hemophilia C, is variably associated with symptoms, even in those with moderate or severe deficiencies. Therefore, treatment should be reserved for bleeding episodes or for surgery in those who are known to be symptomatic. Transfusion with plasma (1020 ml/kg) results in a rise of 2550% of factor XI, which is an adequate dose for hemostasis even for severe bleeding episodes and surgery. [86] Plasma exchange may also be useful.[87] Cryoprecipitate supernatant and one of the prothrombin complex concentrates (Konyne) contain factor XI and have been used in the extraordinary circumstance in which prolonged high-dose therapy is needed.[88] A factor XI concentrate is available in Europe.

Congenital factor XIII deficiency is an extremely rare deficiency characterized by bleeding with injuries and delayed healing. The very low hemostatic level (23%) and long half-life (6 days) permit most bleeding to be treated with plasma. [89] Alternatively, cryoprecipitate may be used;[90] a factor XIII concentrate is available in Europe.[91]

The contact factors, factor XII (Hageman factor), prekallekrein (Fletcher factor), and high-molecular-weight kininogen (Fitzgerald factor), are not associated with bleeding manifestations and require no transfusion therapy.

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THERAPY FOR INHERITED ANTICOAGULANT DEFICIENCIES

Inherited deficiencies of the naturally occurring anticoagulants are associated with an increased risk of thrombosis, both arterial and venous. The most widely recognized is antithrombin III deficiency, but deficiencies of other physiologic anticoagulants, such as the vitamin K-dependent factors protein C and protein S, as well as the fibrinolytic protein plasminogen, have also been implicated in thrombophilic disorders. [92] The general approach for management of the heterozygous states is similar. Specific treatment is not instituted until the first thrombotic episode occurs because many heterozygotes never develop a thrombotic complication. At that point, after treatment of the acute thrombosis with standard therapy, lifelong anticoagulation with coumarin is usually recommended after the first or second thrombotic episode. In some cases, replacement therapy is warranted.

Antithrombin III Deficiency

Antithrombin III deficiency, a fairly rare heterozygous deficiency, has been well described as associated with venous thrombosis and pulmonary emboli. Because heparins anticoagulant activity to inactivate thrombin and factor Xa is effected through induction of a conformational change in antithrombin III, patients with antithrombin III deficiency may manifest heparin resistance when attempts are made to treat their thrombotic episodes. If significant heparin resistance is encountered, treatment with antithrombin III may be indicated. Antithrombin III may be replaced by infusion of a pasteurized antithrombin III concentrate licensed for prophylaxis of deep vein thrombosis and treatment of thrombosis in patients with inherited antithrombin III deficiency. [93] The transfused protein has a recovery of 1.4% (U/dl)/U/kg of antithrombin III administered and a biologic half-life of 3.8 days.

Protein C Deficiency

Heterozygous protein C deficiency has been variably associated with thrombotic tendencies. [94] Therefore, the incidental discovery of protein C deficiency is not an indication for lifelong anticoagulation. However, if thrombotic complications occur after treatment of the acute thrombotic event, long-term anticoagulation with coumarin is indicated. It is important that the patient be adequately heparinized during the initial administration of coumarin because protein C is a vitamin K-dependent protein with a very short half-life (46 hours); a more thrombophilic state is induced until the level of the procoagulants is also lowered and may be the cause of coumarin-induced skin necrosis. [95]

Homozygous protein C deficiency is an extremely rare disorder; affected infants have nearly all presented in the newborn period with purpura fulminans and disseminated intravascular coagulation. [96] Infants with these symptoms should have assays obtained for protein C and then be treated immediately with fresh-frozen plasma (912 ml/kg every 12 hours has been shown to control symptoms) or, if available, the vapor-heated protein C concentrate currently in clinical trials. [97] Replacement therapy should be continued until all lesions are healed. Once lesions have healed, most patients can be maintained on coumarin with occasional replacement therapy if symptoms recur.

Other Inherited Anticoagulant Deficiencies

Protein S, another vitamin K-dependent plasma anticoagulant, functions as a cofactor in protein C inactivation of factors Va and VIIIa. Heterozygous deficiency of free protein S has been described in families with an increased incidence of thrombotic episodes. [98] Currently no replacement product for protein S deficiency is available, and patients should be placed on lifelong anticoagulation after treatment of the initial thrombotic episode. Coumarin-induced skin necrosis has also been reported in association with heterozygous protein S deficiency. [99]

Other inherited deficiencies associated with hypercoagulability include dysfibrinogenemias (abnormal plasmin-binding site), plasminogen deficiencies, and tissue plasminogen activator release defects. [100] An inherited abnormality of factor V, factor V Leiden, resulting in the protein C resistance syndrome, and a mutation in the prothrombin gene leading to increased circulating levels of prothrombin have also been implicated in

the etiology of thrombosis. [101] No replacement products are currently available for treatment, and therapy depends on anticoagulation.

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Chapter 141 - Hemapheresis and Cellular Therapy

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INTRODUCTION

Bloodletting is an ancient therapy, fashionable, albeit unproved, well into the 19th century. About the time that scientific skepticism began to temper the widespread use of therapeutic phlebotomy, a new technique for blood removal, apheresis, appeared in the research laboratory. [1] The term *apheresis,* derived from a Greek verb meaning to take away or withdraw, was coined to describe removal of one component of blood with return of the remaining components to the donor. Like phlebotomy, apheresis was used first to treat patients but later became more important for collecting blood components for transfusion. In the United States, >6 million units of platelets are collected by plateletpheresis and >10 million plasmapheresis procedures are performed annually; an estimated 100,000 therapeutic apheresis procedures are performed each year in the United States for a wide variety of indications. [2]

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PRINCIPLES OF APHERESIS

The principal objective of apheresis is efficient removal of some circulating blood component, either cells (cytapheresis) or some plasma solute (plasmapheresis). For most disorders, the treatment goal is to deplete the circulating cell or substance directly responsible for the disease process. Apheresis can also mobilize cells and plasma components from tissue depots. For example, lymphocytes may be mobilized from the spleen and lymph nodes of some patients with chronic lymphocytic leukemia, and low-density lipoproteins can be removed from tissue stores in patients with familial hypercholesterolemia. Apheresis may have other, less obvious effects. Lymphocyte depletion may modify immune responsiveness in some disease states, possibly by disturbing the control mechanisms of cellular immune regulation. Plasmapheresis enhances splenic clearance of immune complexes in certain autoimmune disorders. [3] When therapeutic effect is judged by clinical improvement rather than by efficiency of solute removal, apheresis is more often a helpful adjunct than a form of first-line therapy.

Several mathematic models formulated for different clinical conditions describe the kinetics of apheresis. [4] [5] [6] Removal of most blood components follows a logarithmic curve (Fig. 141-1). This model assumes that the component removed is neither synthesized nor degraded substantially during the procedure, remains

Figure 141-1 Relation between volumes removed by apheresis and percentage of the target component remaining. The relation is valid for blood volumes during red cell exchange or for plasma volumes during plasmapheresis if the target solute remains primarily within the intravascular compartment. Solid line, continuous exchange; large dashes, discontinuous exchange with initial removal; small dashes, discontinuous exchange with initial infusion.

within the intravascular compartment, and mixes instantaneously and completely with any plasma replacement solution. When the goal of plasmapheresis is to supply a deficient substance, for example plasma factors in the treatment of thrombotic thrombocytopenic purpura, replacement follows logarithmic kinetics similar to those developed for solute removal. From Figure 141-1 it is evident that removal of 1.52 volumes will reduce an intravascular substance by about 60% and that processing larger volumes results in little additional gain.

Specific cell removal with centrifugal automated cell separators depends on the number of cells available, the volume of blood processed, the efficiency of the particular instrument, and the separation characteristics of the different cells. Most commercially available instruments remove platelets and lymphocytes extremely efficiently. Granulocytes and monocytes cannot be cleanly separated from other cells by standard centrifugal apheresis equipment (Fig. 141-2). Optimal harvesting of these cells requires special techniques such as stimulating the donor with corticosteroids or cytokines and adding sedimenting agents to enhance cell separation.

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TECHNOLOGY AND TECHNIQUES

The plasmapheresis technique that originated in the animal laboratory required manual resuspension of red cells and posed a substantial risk of microbial contamination of the components being reinfused.[1] With the introduction of sterile, disposable, interconnected plastic blood bags, plasmapheresis became relatively safe and easy. However, manual apheresis proved too inefficient and labor-intensive for collecting large component volumes and raised concerns that the separated units of red blood cells might be reinfused accidentally into the wrong donor or patient. The introduction of automated on-line blood cell separators solved these problems.[7] [8]

Automated apheresis instruments use microprocessor technology to draw and anticoagulate blood, separate components either by centrifugation or by filtration, collect the desired component, and recombine the remaining components for return to the patient. [9] The equipment contains disposable plastic software in the blood path and uses anticoagulants containing citrate or combinations of citrate and heparin that do not result in clinical anticoagulation of the patient. Most instruments function well at blood flow rates of 3080 ml/min and can operate from peripheral venous access or from a variety of multilumen central venous catheters.

Because the ideal method for treating disorders mediated by abnormal plasma components is to remove the offending substance selectively, a variety of on-line filtration and column adsorption techniques have been introduced or proposed. Ligands bound to a column matrix may be relatively nonspecific chemical sorbents, such as charcoal or heparin, or specific ligands, such as monoclonal antibodies and recombinant protein antigens. The two most successful clinical columns use staphylococcal protein A and dextran sulfate cellulose. Staphylococcal protein A has high affinity for the Fc portion of IgG1, 2, and 4 and for immune complexes containing these IgG subtypes. The dextran sulfate cellulose columns selectively remove low-density lipoproteins and have proved effective in managing patients with homozygous hypercholesterolemia (Fig. 141-3).

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THERAPEUTIC CYTAPHERESIS

Common indications for therapeutic cell removal are listed in Table 141-1 . Red cell exchange (erythrocytapheresis) is used most often to manage or prevent the acute vaso-occlusive complications of sickle cell disease. Mechanical cell separators offer the advantages of speed and ease compared to manual exchange transfusion and reduce the risks of rapid blood volume alteration and increased blood viscosity that may occur with simple transfusion. Automated procedures can be performed with all centrifugal instruments (Fig. 141-4).

Figure 141-2 Schematic distribution of cells at the collection port of a centrifugal cell separator. The number and percentage of each cell type collected can be varied by adjusting the site of collection along the interface or by changing centrifugal force, blood flow rate, or rate of cell removal.

Figure 141-3 Two-stage therapeutic plasmapheresis. Plasma is separated from cells by filtration, then passed through parallel adsorption columns to remove low-density lipoproteins from a patient with homozygous familial hypercholesterolemia.

TABLE 141-1 -- Common Indications for Therapeutic Cytapheresis

Red cell exchange
Sickle cell disease
Acute complications
Prophylaxis for recurrent stroke
Frequent severe pain crises
Hyperparasitemia (malaria, babesiosis)
Leukapheresis
Leukemia with hyperleukocytosis syndrome
Cutaneous T-cell lymphoma (photopheresis)
Peripheral blood stem cell collection
Plateletpheresis
Symptomatic thrombocytosis

Sickle cell anemia occurs in individuals who are homozygous for a single mutation in codon 6 of the -globin gene, resulting in substitution of a single amino acid. Although the defect appears simple, the pathophysiology of the vaso-occlusive crises is complex, involving hemoglobin polymerization, change in cell shape, adhesion to endothelial cells, and release of inflammatory cytokines. Clinical manifestations may vary from patient to patient. [10] The rationale behind exchange transfusion involves improving tissue oxygenation and preventing microvascular sickling by diluting the patients abnormal red cells, simultaneously correcting anemia and favorably altering whole blood viscosity and rheology. No clinical data support a single optimal level of hemoglobin A (HbA); however, as few as 30% of transfused cells markedly decrease blood viscosity; at mixtures of 50%, resistance to membrane filterability approaches normal. [11] [12] In nonemergency situations, such levels can often be achieved with a simple transfusion regimen. For simple and

Figure 141-4 Schematic of an automated red cell exchange transfusion using a two-arm procedure.

exchange transfusions, raising the level of HbA to 70% while lowering the level of HbS to 30% is generally efficacious, although even higher levels of HbA may be required to treat an ongoing crisis.

Clinical indications for exchange transfusion remain controversial. Simple transfusion has improved renal concentrating ability and splenic function in young sickle cell patients; exchange transfusion has improved exercise tolerance and reversed the periodic oscillations in cutaneous blood flow associated with this disease. [13] [14] [15] [16] Such observations have encouraged the use of exchange transfusion for acute complications of sickle cell disease such as acute chest syndrome, priapism, cerebrovascular accident, and intrahepatic cholestasis. Exchange transfusion for sickle cell patients has also been used for prophylaxis during pregnancy, before surgery, or for patients who have suffered a stroke.[17] A multicenter randomized trial of transfusion during pregnancy has shown that transfusion sufficient to reduce the incidence of painful crises did not reduce other maternal morbidity or perinatal mortality. [18] In a randomized study of sickle cell disease patients undergoing surgery, a conservative simple transfusion regimen (to increase the hemoglobin level to 10 g/dl) was as effective as an aggressive regimen (to lower the HbS level to <30%) with respect to perioperative nontransfusion-related complications. The patients in the aggressive regimen group received twice as many units of blood, had a proportionally increased red blood cell alloimmunization rate, and had more hemolytic transfusion reactions. [19] The results of this and other studies suggest that if prophylactic preoperative exchange transfusion is used, it should be limited to patients undergoing high-risk procedures in whom simple transfusion could not effectively raise the HbA level to 70%.

Transfusion prophylaxis is now clearly indicated for children at high risk for stroke. A randomized controlled study [20] demonstrated a risk reduction of 90% in the

patients who were maintained at levels of HbS <30% by simple or exchange transfusion. This result confirms earlier experience and indicates that in this group of children with sickle cell anemia, transfusion therapy should begin before the first event and continue indefinitely. Long-term erythrocytapheresis may be preferable for patients at high risk for stroke who have developed iron overload to levels associated with organ damage. [21]

Exchange transfusion, although relatively safe and convenient, carries all the complications of red cell transfusion. Patients are exposed to a large number of donors and are at a small but significant risk of contracting hepatitis and other blood-born infections. As many as 33% of all patients develop alloantibodies, and life-threatening delayed hemolytic transfusion reactions have been reported. [22] [23] In addition, an immunohematologic study of multiply transfused sickle cell patients shows that 85% of heavily transfused patients are alloimmunized to HLA and/or platelet-specific antigens. [24] Most centers avoid inducing nonhemolytic transfusion reactions and HLA alloimmunization by using leukodepleted red cells. Extended red blood cell phenotyping at diagnosis and provision of phenotypically matched blood, when practical, can reduce the risk of red cell alloimmunization and associated hemolytic transfusion reactions. [25] Despite the removal of cells during exchange, patients remain in positive iron balance, although iron accumulation is slow and chelation is rarely required to prevent transfusional hemosiderosis. [21]

Other indications for red cell exchange are rare. The procedure has been used for patients with overwhelming red cell parasitic infections, such as severe and complicated malaria and babesiosis. [26] [27] In these situations, red cell exchange decreases the concentration of circulating parasites and may help sustain life until conventional therapy and natural immunity take effect. Although the efficacy of this therapy has not been evaluated by controlled trials, prospective studies and review of published cases[26] [28] suggest the use of erythrocytapheresis for parasitemia >1015%. Automated red cell removal with volume replacement, isovolemic hemodilution, can be performed rapidly and safely in polycythemic subjects. [29] This maneuver should be reserved for polycythemic patients with an urgent clinical indication to lower the hematocrit (e.g., evolving thrombotic stroke) for which standard single-unit manual phlebotomy might be inadvisably slow.

Therapeutic leukapheresis has been used most successfully to help manage patients with acute or chronic leukemia and extremely high white blood cell (WBC) numbers, so-called hyperleukocytic leukemias. When the fractional volume of leukocytes (leukocrit) exceeds 20%, blood viscosity increases and leukocytes can interfere with pulmonary and cerebral blood flow and compete with tissue for oxygen in the microcirculation. [30] A single leukapheresis procedure generally reduces the WBC count by 2050%. Ordinarily, leukapheresis is indicated when the blast count is >100,000/mm [3] or when rapidly rising blast counts are >50,000/mm[3] (leukocrit >10%), especially when evidence of central nervous system or pulmonary symptoms appears. [31] Although repeated leukapheresis has adequately reduced the WBC count in a series of patients with chronic myeloid leukemia, the median patient survival rate was not significantly different from that of similar patients treated with conventional chemotherapy.[32] Chronic leukapheresis can provide acceptable control of the peripheral WBC count in clinical situations such as pregnancy, when cytotoxic agents may best be avoided, but cytoreduction alone does not appear to alter the course of chronic myeloid leukemia.

Cytoreduction for managing other leukemic processes has limited value. Some studies of patients with chronic lymphocytic leukemia suggested short-term clinical benefit, but long-term support of patients when the disease is refractory to chemotherapy does not appear to prolong life. [33] [34] Transient responses to leukapheresis used alone or in combination with low-dose chemotherapy have been reported in a variety of lymphoproliferative disorders. However, most patients relapse quickly and do not respond to further leukapheresis therapy. [35] [36] [37]

Lymphocyte depletion has been used therapeutically to modify patient immune responsiveness. Removal of large numbers of lymphocytes over a period of a few weeks can suppress peripheral lymphocyte counts in rheumatoid arthritis patients for up to 1 year and alter skin-test reactivity and lymphocyte mitogen responsiveness to a variety of stimulants. [38] Selected patients experience a modest but significant reduction in disease activity; however, the subset of patients who may derive substantial benefit from this therapy is difficult to identify. [39] [40] Lymphocyte removal by apheresis has also been used to treat patients with multiple sclerosis, to enhance allograft survival, and to reverse graft rejection, but evidence of clinical efficacy in these situations is sparse.

Photopheresis is an automated extracorporeal photochemotherapy treatment that involves oral administration of a light-sensitizing agent, 8-methoxypsoralen, followed by leukapheresis and ex vivo ultraviolet A irradiation of leukocytes. The treated cells are reinfused. This therapy has minimal toxicity and is highly effective in the treatment of patients with advanced cutaneous T-cell lymphoma. Additional disease states that may respond to photopheresis therapy include systemic sclerosis and other autoimmune disorders,[41] solid organ graft rejection, [42] [43] and graft-versus-host disease following bone marrow transplantation. [44] Experience with photopheresis in these settings is limited.

Therapeutic plateletpheresis is generally reserved for patients with myeloproliferative disorders and hemorrhage or thrombosis associated with an increase in circulating platelets. Many centers consider using plateletpheresis when the patients peripheral platelet count is >10^6 /mm^3 , although no consistent relation between the level of platelet elevation and the occurrence of symptoms has been found, [45] [46] and no generally accepted assay of platelet dysfunction predicts which patients are at risk. A single cytapheresis procedure can lower the platelet count by 3050%. Attempts to maintain thrombocythemic patients at normal platelet counts by cytapheresis alone have not been successful; more practical long-term chemotherapy should be instituted concurrently. Since most patients with thrombocytosis, even those with myeloproliferative disorders, do not develop symptoms, prophylactic plateletpheresis is unwarranted, regardless of the platelet count. [47]

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THERAPEUTIC PLASMAPHERESIS

Growth of therapeutic plasmapheresis during the past 50 years owes more to the development of new technology than to new insight into the pathophysiology of the diseases under treatment. Technical developments continue to outpace clinical applications. The prevailing developmental strategy has been to complement plasmapheresis with more specific procedures to remove pathologic plasma constituents. An impressive array of immunologic and physicochemical techniques, including cascade filtration, adsorption, immunoadsorption, cryoprecipitation, and photoinactivation, have been added to the plasmapheresis system for laboratory research and early clinical studies. [48]

Common clinical indications for therapeutic plasmapheresis are outlined in Table 141-2 . Committees appointed by the American Society for Apheresis, American Association of Blood Banks, and the American Academy of Neurology have published practice guidelines for using plasmapheresis in a wide variety of disease states.[49] [50] [51] Some of the least controversial indications for plasmapheresis are supported by small series of uncontrolled cases that rely on some objective clinical or laboratory measurement of patient improvement. This is the case for paraproteinimia with hyperviscosity syndrome, cold agglutinin disease, cryoglobulinemia, and poisoning with albumin-bound toxins. [52] [53] [54] [55] [56]

Evidence that both cutaneous and vascular lesions regress in familial hypercholesterolemia as low-density lipoprotein levels are controlled by plasmapheresis has encouraged the use of several related procedures in patients with homozygous disease and in poorly controlled heterozygous patients. Low-density lipoprotein apheresis is a procedure for removing apolipoprotein B-containing lipoproteins specifically from the blood by a variety of techniques, including dextran sulfate cellulose adsorption, immunoadsorption, and heparin-induced extracorporeal precipitation. Short-term safety and efficacy have been demonstrated. [57] Continuing experience with this therapy will be required to prove long-term clinical benefit in patients with refractory hypercholesterolemia and coronary artery disease.

Plasmapheresis may be used in patients with other inherited metabolic disorders, such as Refsums disease. [58] The frequency of exchange depends primarily on total body burden, rate of synthesis, and plasma concentration of the solute to be removed

TABLE 141-2 -- Common Indications for Therapeutic Plasmapheresis

Paraproteinemia with hyperviscosity syndrome
Cold agglutinin disease
Cryoglobulinemia
Drug overdose and poisoning (protein-bound toxins)
Homozygous familial hypercholesterolemia
Refsums disease
Myasthenia gravis
Post-transfusion purpura
Goodpastures syndrome
Pemphigus vulgaris
Chronic inflammatory demyelinating polyneuropathy
Guillain-Barré syndrome
Thrombotic thrombocytopenic purpura

Figure 141-5 Plasma exchange to remove plasma neutral glycolipids in a patient with Fabry disease. The plasma lipid recovery curve appears to be biphasic, reflecting initial re-equilibration from tissue stores and subsequent new synthesis of that glycolipid. Solid line, cerebrosides; dotted line, dihexosylceramides; large dashes, trihexosylceramides; small dashes, globosides.

(Fig. 141-5). Less evidence exists to support a role for repeated treatments in these disorders.

Plasmapheresis appears to have at least a temporary adjunctive role in managing some disorders characterized by circulating autoantibodies. Early success was reported in patients with Goodpastures syndrome, a disorder characterized by a specific pathogenic autoantibody directed against the renal glomerular and pulmonary alveolar basement membrane. Plasmapheresis has demonstrated similar success in a variety of disorders associated with specific autoantibodies, including myasthenia gravis, pemphigus, and Eaton-Lambert syndrome. In other autoimmune disorders, such as immune thrombocytopenic purpura and immune inhibitors to coagulation proteins, plasmapheresis may be helpful during a catastrophic event, but benefit in chronic disorders is less well established. [59] [60] [61] [62] [63] [64] [65] Controlled clinical trials of plasmapheresis have demonstrated efficacy in at least two of the polyradiculoneuropathies. [66] [67] However, intravenous immunoglobulin alone may be equally effective in the treatment of severe Guillain-Barré syndrome. [68] Although therapeutic plasma exchange has been used in a variety of other immune disorders, such as systemic lupus erythematosus and rheumatoid vasculitis, such use remains unproved and should be reserved for circumstances where a vital organ or life itself is endangered.

Controversy surrounds the practice of combining cytotoxic drug therapy with plasmapheresis to prevent rapid resynthesis of antibody, so-called antibody rebound. Although the rebound phenomenon is well established in animal models, investigational studies in healthy volunteers suggest that antibody rebound is not common. Controlled trials of plasmapheresis therapy in lupus nephritis and polymyositis have been criticized for not including a plasmapheresis-with-immunosuppression arm. Since many uncontrolled treatment protocols stipulate simultaneous use of immunosuppressive drugs and plasmapheresis, an apparent favorable outcome may result from the independent effects of the different treatments or some synergistic effect, or from neither.

Replacement Fluids for Plasma Exchange

The success of therapeutic apheresis procedures seldom depends on the composition of the replacement solution that is used. The single exception seems to be thrombotic thrombocytopenic purpura. Numerous reports support the use of fresh-frozen plasma or cryoprecipitate-poor plasma as a specific

therapeutic replacement fluid.[69] [70] The effectiveness of plasma exchange in this setting may derive from removal of antibody and/or replacement of a factor such as von Willebrand factor-cleaving protease.[71] [72] With therapeutic plasmapheresis for most other disorders, the primary function of the replacement solution is to maintain intravascular volume. Additional requirements include restoration of important plasma proteins, maintenance of colloid osmotic pressure, maintenance of electrolyte balance, and preservation of trace elements lost during a prolonged course of plasmapheresis procedures. In moderately well-nourished subjects, homeostatic mechanisms normally obviate the need for precise plasma replacement, and 5% albumin or combinations of albumin and crystalloid are usually sufficient. Other patients should receive solutions prepared specifically to meet their individual requirements. Routine supplementation with calcium, potassium, or immunoglobulins is unnecessary. Problems of decreased availability and high cost of albumin have led some centers to develop protocols for alternatives to plasma-derived volume expanders, such as hydroxyethyl starch, for full or partial volume replacement with plasma exchange.[73] These solutions are generally well tolerated. Since less than 500 ml is removed during most cell collection procedures and therapeutic cell depletions, no replacement beyond the anticoagulant and saline priming solution is required.

Complications of Therapeutic Apheresis

Automated apheresis is a minimal-risk procedure for normal healthy donors. The current generation of blood cell separators is remarkably reliable and equipped with sensitive detection and alarm systems to alert the operator to potential problems. Nevertheless, at least 59 deaths have been associated with therapeutic procedures, an estimated mortality of 3 in 10,000 procedures. However, in most reports, deaths are related to cardiac and respiratory causes in patients critically ill before apheresis, and the contributory role of the apheresis procedure is often questionable.[74] If citrate-induced hypocalcemia, vasovagal reactions, clotting of fistulas and catheters, and urticaria are included, about 10% of all therapeutic apheresis procedures have medical complications. The frequency of adverse reactions is influenced primarily by the experience of the operator and the nature of the patient population under treatment.[75]

Expected and predictable effects of therapeutic apheresis include alterations in laboratory parameters due to removal and dilution by replacement fluids. A 520% decrease in hemoglobin/hematocrit, a 2030% decrease in platelet count, and mild leukocytosis are commonly observed. Significant but transient abnormalities in coagulation tests often occur after serial plasma exchange procedures, as well as clinically insignificant decreased levels of other plasma proteins.[76]

The most common adverse effect of both donor and therapeutic apheresis is symptomatic hypocalcemia due to infusion of calcium-chelating citrate ions in the anticoagulant, and, if used as replacement fluid, anticoagulated donor plasma. This is usually manifested by mild perioral and/or acral paresthesia, requiring no intervention other than slowing the reinfusion rate. The benefit of oral calcium supplements in this setting is questionable, although the practice is widespread. Hyperventilation may exacerbate these symptoms. More severe citrate toxicity is very uncommon; signs may range from involuntary carpopedal spasm to frank tetany with spasm in other muscle groups, including life-threatening laryngospasm.[77] Controlled infusions of 10% calcium gluconate are effective in the management of these complications. Acute severe hypocalcemia leading to fatal cardiac arrhythmia has rarely been reported in apheresis patients. Because metabolism of citrate occurs predominantly in the liver and kidney, patients with conditions affecting these organs are at increased risk for severe citrate reactions. In addition, citrate toxicity has been reported due to mechanical dysfunction of the automated apheresis instrument.[78] A recent study of therapeutic plasma exchange procedures performed with and without 10% calcium gluconate infusion showed the effectiveness of continuous calcium infusion for the prevention of mild to moderate citrate toxicity;[79] however, careful monitoring is necessary to prevent inadvertent overdose if this procedure is adopted.

The most severe allergic complications occur when plasma is used as the replacement solution, and this risk increases with repeated exposure.[75] Allergic reactions to ethylene oxide, an agent used in the sterilization of plastic disposable equipment, have been reported.[80] Intravenous diphenhydramine is usually effective in managing these reactions; premedication with steroids and precautions against anaphylaxis may be necessary for sensitized individuals. Atypical (hypotension and flushing) and anaphylactic reactions have been reported in patients receiving angiotensin-converting enzyme inhibitors undergoing different apheresis procedures; these medications should be discontinued for 24 hours before apheresis.[81] Serious complications indirectly associated with therapeutic apheresis include adverse consequences of vascular access, namely hemorrhage, pneumo- or hemothorax, thrombosis, nerve damage, and infection. A retrospective review of 381 therapeutic plasma exchange procedures at one institution reported an approximately 1% incidence of severe complications, all of them related to central venous catheters.[82]

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CELLULAR THERAPY

Blood cell separators developed for hemapheresis are used increasingly for novel forms of cellular therapy. In the cell-processing laboratory, separation techniques have been developed for removing T lymphocytes, for purging tumor cells, and for selecting progenitor cells from autologous bone marrow harvests and peripheral blood stem cell collection products. [83] Hematopoietic progenitor cells can be collected from peripheral blood and cryopreserved. Such progenitors are used to reconstitute bone marrow in patients who receive ablative chemotherapy. Lymphocyte concentrates prepared from leukocyte collections are used as immunotherapy for solid tumors, leukemia, and viral complications of bone marrow transplantation. Preliminary studies promise a role for both circulating hematopoietic progenitor cells and other mononuclear cells in the therapy of immunosuppressed patients and in gene therapy.

Peripheral Blood Stem Cells

Pluripotent hematopoietic progenitor cells, most likely including primordial hematopoietic stem cells capable of reconstituting the bone marrow and the immune system, have long been known to circulate in the peripheral blood. [84] Substantial numbers of peripheral blood (progenitor) stem cells (PBSCs), as measured by colony-forming assays, can be identified in plateletpheresis collections from normal donors. Numerous recent studies have confirmed the potential for rapid and durable engraftment of PBSCs mobilized into the circulation by hematopoietic growth factors and harvested by large-volume hemapheresis procedures. [85] PBSC infusions have been used both for complete marrow replacement and for bone marrow rescue after high-dose chemotherapy. PBSC collections have several practical advantages compared to bone marrow harvests: they eliminate the risk associated with anesthesia, permit collection of hematopoietic progenitor cells despite tumor involvement of the marrow or previous pelvic irradiation, facilitate graft manipulations by obtaining greater numbers of CD34+ cells, permit repeated collection as necessary, and can be safely performed on small children and even infants. [86] [87] Numerous recent studies show that PBSC transplantation consistently results in rapid hematopoietic recovery and durable engraftment in both

autologous and allogeneic transplant settings. [88] [89] [90] [91] PBSCs are rapidly replacing bone marrow for transplantation after myeloablative therapy. Despite the 10-fold increase in the number of T lymphocytes in unmanipulated allogeneic PBSC collections, there does not appear to be an increase in the incidence or severity of acute graft-versus-host disease, although the risk of chronic graft-versus-host disease may be higher. [92] The explanation for this seeming paradox is an area of active investigation.

Ordinarily, PBSCs circulate in small numbers. The concentration of stem cells in the peripheral blood can be increased by the administration of cytokines such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF (GM-CSF), and by the administration of chemotherapy. CD34+ hematopoietic stem cell levels in the peripheral blood generally rise 2050-fold (from 13 cells/L to 40-70 cells/L) following G-CSF alone, and they may show an even greater increase (1001000-fold) when chemotherapy rebound is enhanced by administration of G-CSF. New cytokines in development, or combinations of cytokines, may further improve PBSC mobilization, resulting in reduced numbers and smaller volumes of leukapheresis procedures required to obtain adequate transplants. The appropriate mobilization strategy to use depends on the clinical situation. In the autologous transplant setting, the mobilization regimen may consist of chemotherapy with or without cytokine administration, depending on the patients diagnosis and medical condition. Patient age >50 years and the number of prior chemotherapy cycles correlate inversely with efficacy of PBSC mobilization. [93] [94]

Efforts to define optimal collection conditions have been hindered by the lack of a standardized assay. In vitro functional hematopoeitic colony assays have been used to characterize and quantitate stem/progenitor cells collected by hemapheresis. Colony assays have limited clinical utility because they require 1014 days to perform. Assays that use the CD34 antigen as a marker for primitive hematopoietic progenitor cells can be used to predict the yield of PBSCs collected by leukapheresis, and have the advantage of a relatively rapid turn-around time. [85] Despite ongoing problems with interlaboratory standardization, flow cytometric analysis of CD34 immunostained blood cells can be used effectively for real-time decision-making about the timing and adequacy of PBSC collections. [85] [94] [95] Many studies show poor correlation between rebound leukocytosis or mononuclear cell counts and PBSC product CD34+ cell and colony counts. Optimal timing to achieve an adequate single leukapheresis collection of autologous PBSCs may be more accurately predicted by preceding-day [96] or same-day measurement of peripheral blood CD34+ cell concentration. Recent and ongoing studies are evaluating a variety of approaches to achieve standardization and clinical utility of CD34+ cell quantitation, including enhanced flow cytometry and microvolume fluorimetry. [97] [98]

Although definition of a therapeutic dose remains controversial, most centers recommend a minimum of 25×10^6 CD34+ cells/kg. Within this range, recovery of neutrophil and platelet counts as early as 12 weeks post-transplant is commonly observed. Greater numbers of CD34+ cells confer a shorter time to hematopoietic recovery and concomitantly fewer blood component transfusions; however, significant improvement in transplant-related mortality or overall survival has yet to be demonstrated in this setting. Several clinical scale devices for positive selection of CD34+ hematopoietic cells are available. CD34+ selection by immune-affinity techniques (positive selection) results in removal of unwanted cells, including occult malignant cells that do not express the CD34 antigen and T lymphocytes, without compromising the engraftment potential of the purified product. [99] [100] As yet, no data confirm the clinical benefit of positive selection.

Several problems complicate the widespread use of PBSCs. For allogeneic donors, the discomfort of multiple-day cytokine administration may be a significant deterrent. Citrate toxicity is a common complication of PBSC collections (see previous section); although usually mild and transient, it may be severe and even life-threatening in some individuals. Venous access requires large-bore multilumen catheters, and these appear particularly susceptible to clotting, especially when patients receive recombinant cytokine stimulation. Hemorrhage, especially in thrombocytopenic patients, is another potentially severe complication of central venous catheter placement. Patients who have received multiple prior cycles of chemotherapy occasionally fail to respond adequately to mobilization regimens. Finally, PBSCs are stored frozen in liquid nitrogen with the cryoprotectant dimethylsulfoxide. Cryopreservation is expensive, and rapid infusion of dimethylsulfoxide causes bradycardia, hypertension, fever, nausea, and vomiting. Toxicity is readily averted, however, by appropriate premedication and careful attention to infusion rates.

Adoptive Immunotherapy

Advances in cellular immunology and cell culture technology, the availability of recombinant human cytokines, and the ability to collect large numbers of lymphocytes have resulted in the development of adoptive immunotherapy, the passive transfer of immunologically active cells. Autologous adoptive immunotherapies require the harvest and ex vivo manipulation of peripheral blood mononuclear cells for reinfusion as potent cytotoxic effector cells. The most extensive clinical studies have been performed with autologous lymphocytes, collected by hemapheresis or separated from resected tumors and expanded ex vivo 1001,000-fold. [101] [102] Peripheral blood lymphocytes, cultured in high concentrations of the lymphokine interleukin-2, generate clones of cells capable of lysing fresh tumor cells in short-term cytotoxicity assays. The effector cells, termed lymphokine-activated killer (LAK) cells, consist of a heterogeneous population of cells, primarily natural killer cells and T cells, that mediate tumor regression in animal models. They have produced objective responses in patients with advanced renal cell carcinoma, melanoma, colorectal cancer, and non-Hodgkin lymphoma. The effects appear to be dose-dependent and non-MHC-restricted and require the simultaneous administration of high-dose systemic

interleukin-2. The postulated mechanism of action involves lymphocyte trafficking to the site of tumor, followed by local expansion and cytokine release under the influence of interleukin-2. Lymphocytes separated from resected tumors, termed tumor-infiltrating lymphocytes (TIL), can be similarly expanded, to up to 10 [12] cells, and infused. TIL are reportedly 50100 times more potent than LAK cells. [103] Unlike LAK cells, TIL often recognize specific tumor antigens, and their action is MHC-restricted. Recent trials of cytokine-primed TIL in the treatment of metastatic renal cell carcinoma and resected non-small cell lung carcinoma demonstrate clinical efficacy in selected subgroups of patients. [104] [105]

The ability to identify and isolate human tumor antigens by advanced molecular genetic techniques has resulted in the formulation of novel strategies for adoptive immunotherapy of cancer.[106] Peptides derived from these tumor antigens are pulsed onto antigen-presenting cells such as dendritic cells, [107] which can then be directly infused as cancer vaccines. Alternatively, the cancer peptide-pulsed antigen-presenting cells can be used to sensitize TIL or peripheral blood lymphocytes ex vivo for reinfusion as specific antitumor effector cells. Techniques to translate these experimental strategies into clinically practicable therapies are under development.

While adoptive immunotherapy has been used primarily in the treatment of solid tumors, cellular immunotherapy has also been investigated for the treatment of acquired immunodeficiency syndrome and for relapsed chronic myeloid leukemia after allogeneic bone marrow transplantation. Initial studies in

acquired immunodeficiency syndrome involved adoptive transfer of syngeneic peripheral blood lymphocytes and bone marrow; treatment resulted in short-term immune reconstitution, but no antiviral benefit. [108] Later studies with cells expanded 1524-fold in culture resulted in increased circulating CD4 cell numbers sustained for up to 6 weeks. Current approaches include the ex vivo expansion of HIV-specific cytotoxic T lymphocytes (CTL), and modification of the T-cell receptor by gene engineering techniques to permit target cell lysis in a non-MHC-restricted fashion. [109]

Infusion of donor leukocytes in chronic myeloid leukemia patients who relapsed after bone marrow transplantation resulted in cytogenetic remission after inducing graft-versus-host disease, suggesting a role for infusional cellular graft-versus-leukemia therapy. [110] Although small numbers of cells (10^7) generally suffice, cells collected by lymphocytapheresis are often aliquoted and stored for repeated treatment. A retrospective analysis of donor leukocyte infusion for relapsed hematologic malignancies after allogeneic bone marrow transplantation shows a complete response rate of 60% in chronic myeloid leukemia patients overall, with an even higher response rate in patients with relapsed chronic-phase disease. [111] Donor lymphocyte infusions also may effect complete or partial remissions in patients with relapsed multiple myeloma after allo-bone marrow transplantation. [112] Other complications of bone marrow transplantation that may be responsive to donor leukocyte infusion include Epstein-Barr virus-associated lymphoproliferative disorders and cytomegalovirus. [113] [114] Response to donor leukocytes is often associated with the development of acute and chronic graft-versus-host disease, although the severity is usually mild to moderate and controlled by immunosuppressive therapy. Another complication of donor leukocyte infusion is transient or persistent marrow aplasia, which may be attributed to the destruction of residual host hematopoiesis by the infused mononuclear cells before recovery of normal donor hematopoiesis. [115] Strategies to minimize these toxicities without compromising the graft-versus-leukemia effect are under development.

Cellular Gene Therapy

Cellular gene therapy is a technique in which a functioning gene is inserted ex vivo into somatic cells to correct an inborn genetic error or to provide some new function to the cell. The gene-modified cells, often selected and expanded, are infused into the patient. Successful therapy requires gene identification and isolation, efficient insertion and expression, and sufficient circulation or engraftment of the corrected cells. A variety of gene insertion techniques have been employed, but current strategies focus on a variety of replication-defective viruslike vectors. [116] [117] Most of the clinical studies have involved either lymphocytes or hematopoietic progenitor cells collected by apheresis as gene targets; however, numerous other target cells (e.g., myoblasts, hepatocytes) are possible. Some strategies take advantage of a survival advantage of the gene-corrected cells in the particular patient population, while other strategies involve using gene insertion to protect the desired population from the cytotoxic effects of chemotherapy or to render cells more immunogenic. Some gene therapy protocols, such as the adoptive transfer of receptor-modified syngeneic T cells for immune restoration in human immunodeficiency virus, combine elements of adoptive immunotherapy with gene therapy techniques.[118] As yet, few patients have derived definite benefit from cellular gene therapy trials. However, a great deal of information regarding therapeutic strategy, cell biology, host immune response, and techniques for cellular biologics has been derived from these pioneering attempts.

The ability to collect large numbers of leukocytes and PBSCs and to expand and manipulate their growth and differentiation in vitro makes these cells logical candidates for cellular gene therapy. Lymphocytes collected by hemapheresis were used in the first therapeutic gene transfer protocol. [119] Two children with severe combined immunodeficiency as a result of an inherited deficiency of adenosine deaminase (ADA) received autologous T cells that had been expanded ex vivo and corrected by the insertion of a normal ADA gene. Monthly collections and reinfusions of gene-corrected cells resulted in normalization of peripheral blood T-cell counts and significantly improved immune function and clinical status in both children. ADA gene expression in T cells has persisted for 3 years after the last gene treatment infusion, suggesting that the in vivo survival of reinfused transduced T lymphocytes may be longer than previously thought. [120] In these cases, lymphocytes have functioned as a temporary biologic drug-delivery system. Leukocytes are potential vehicles for replacing defective or deficient genes in a variety of other inherited disorders and for delivery of new genes (e.g., in the immunotherapy of cancer).

Permanent correction of genetic and acquired defects might be effected by inserting the missing or defective gene into hematopoietic stem cells. A broad range of inherited disorders are candidates for gene therapy using hematopoietic progenitor cells, including those with generalized (not hematopoietic-restricted) expression. Some candidate disorders involving a defective gene not normally expressed by hematopoietic cells are the hemophilias A and B and -1-antitrypsin deficiency. [116] Hematopoietic stem cell gene insertion with retroviral vectors has been accomplished in murine and nonhuman primate models, and in pilot human clinical trials, but the numbers of gene-marked cells are small and gene expression is poor. [122] In addition to inherited disorders, some strategies under investigation involve gene therapy for hematopoietic malignancies.[124]

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Chapter 142 - Transfusion Reactions

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INTRODUCTION

A transfusion reaction can be defined as any untoward reaction that occurs as a consequence of infusion of blood or one of its components. Reactions are considered to be acute when they occur during transfusion or within several hours after the transfusion has been terminated (Table 142-1). Reactions also can be classified as delayed. Delayed hemolytic transfusion reactions, for example, usually occur days after the transfusion. Other types of delayed reactions occur long after the blood has been infusedmonths or even yearsas in the case of some transfusion-transmitted diseases. The acute fatality rate after transfusion is 11.2/100,000 patients who receive a transfusion, or about 35 acute transfusion-related deaths/year in the United States. [1] This chapter reviews various types of transfusion reactions.

TABLE 142-1 -- Differential Diagnosis of Acute Transfusion Reactions

Reaction Type	Presenting Signs and Symptoms
Acute intravascular hemolytic	Fever, chills, dyspnea, hypotension, tachycardia, flushing, vomiting, back pain, hemoglobinuria, hemoglobinemia, shock
Acute extravascular hemolytic	Fever, indirect hyperbilirubinemia, post-transfusion hematocrit increment lower than expected
Febrile reaction	Fever, chills
Allergic (mild)	Urticaria, pruritus, rash
Anaphylactic	Dyspnea, bronchospasm, hypotension, tachycardia, shock
Hypervolemic	Dyspnea, tachycardia, hypertension, headache, jugular venous distention
Septic	Fever, chills, hypotension, tachycardia, vomiting, shock

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HEMOLYTIC TRANSFUSION REACTIONS

Hemolytic transfusion reactions are caused by the immune-mediated lysis of transfused red cells. Immune-mediated hemolysis can be divided conveniently into four categories by time of reaction (acute or delayed) and by site of hemolysis (intravascular or extravascular). Thus, the four types are acute intravascular, acute extravascular, delayed intravascular, and delayed extravascular reactions (Table 142-2 (Table Not Available)).

Acute Intravascular Hemolytic Transfusion Reactions

Acute reactions occur when incompatible red cells are transfused into a patient who already possesses the corresponding antibody, and a reaction occurs within minutes. Because of naturally occurring ABO antibodies, infusion of ABO-incompatible blood is the most likely cause of a clinically significant acute intravascular hemolytic transfusion reaction.[2] Such a reaction could occur, for example, after transfusion of A red cells into an O recipient who has significant amounts of circulating anti-A. Although the titer and avidity of the antibody affect the extent of the hemolytic reaction, the clinical severity of an ABO-incompatible blood transfusion is greatly influenced by the degree of complement activation and cytokine stimulation. In an ABO-incompatible transfusion reaction, fixation of the C5b-9 complex occurs with release of C3a and C5a (anaphylatoxins 1 and 2, respectively). These low-molecular-weight peptides produce bronchospasm, mast cell degranulation, hypotension (C3a), and pulmonary dysfunction (C5a); the latter is secondary to migration of granulocytes to the pulmonary capillary bed, resulting in V/Q abnormalities.[3] Fixation of the C5b-9 complement membrane attack complex produces pores in the red cell membrane, and the resultant osmotic lysis produces hemoglobinuria and hemoglobinemia.[3] [4] Indeed, the sine qua non of an acute intravascular hemolytic transfusion reaction is the presence of both red plasma and red urine.

It is critical to distinguish, as quickly as possible, hemoglobinuria and hemoglobinemia secondary to an acute hemolytic transfusion reaction from similar signs due to other causes. Hemoglobinuria can be confused with hematuria from bladder hemorrhage, or myoglobinuria. Hemoglobinemia due to non-immune-mediated lysis can occur secondary to mechanical hemolysis from an improperly collected blood sample. The direct antiglobulin test (DAT) usually becomes positive in an immune hemolytic reaction; preparation of an antibody eluate is necessary to identify the offending antibody. An antibody elution is the removal of antibody from the red cell by various physical or chemical techniques with concentration of the antibody in the eluting solution, which is usually albumin/saline. This antibody-containing solution, the eluate, is then treated as a serum sample and used to identify the offending blood group antibody.

The time lost in making a clinical diagnosis can turn a mild treatable reaction into one that is life-threatening. Indeed, a full-blown acute intravascular hemolytic transfusion reaction is a true medical emergency. Antibody-coated red cell stroma produced during an immune hemolytic transfusion reaction induces renal vasoconstriction, resulting in acute tubular necrosis. [3] [5] Another contributing cause of ischemic renal failure involves the release of free plasma hemoglobin. It has been shown that cell-free hemoglobin binds tightly to nitric oxide. Endothelium-derived relaxing factor, a powerful vasodilator, is composed in part of nitric oxide. By binding to nitric oxide, free plasma hemoglobin blocks relaxing factor and prevents renal vasodilation and promotes renal vasoconstriction and thus renal tubular ischemia and eventual tubular necrosis.[6] The C3a and C4a generated may cause hypotension and tachycardia and may produce shock. Clinical symptoms include shortness of breath, chest pain, dizziness, and nausea. Patients often report a sense of impending doom. C3a can also cause bronchospasm, and C5a generation causes further pulmonary dysfunction. Many patients, curiously even anephric patients, often complain of lower back pain. [7] This is believed to be due to ischemic muscle pain or vasospasm, rather than to kidney pain from developing renal failure.

In addition to complement components, another class of biologic response modifiers, cytokines and interleukins (ILs), also plays a role in the clinical symptom complex associated with acute intravascular hemolytic transfusion reactions. For example, IL-1, IL-6, and tumor necrosis factor- (TNF-) have pyrogenic activity; IL-8 is a neutrophil chemotactic and activating factor. These four cytokines have been generated in in vitro models of intravascular hemolysis and IgG-mediated red cell incompatibility. [8] [9] [10] [11] The clinical variability of hemolytic transfusion reactions is likely explained by the relative balance of cytokine production in the transfusion recipient. Factors that increase the circulating levels of proinflammatory cytokines and chemokines result in more severe reactions. [12]

TABLE 142-2 -- Hemolytic Transfusion Reactions: Serologic Presentation

(Not Available)

Adapted from Blood Transfusion Therapy: An Audiovisual Program. 2nd ed. American Association of Blood Banks, Arlington, VA, 1991, with permission.

Therapy

Initial therapy consists of immediately stopping the transfusion, maintaining a patent intravenous line, supporting the cardiorespiratory system, and ensuring a brisk diuresis. Increasing renal blood flow is the best way to prevent acute oliguric renal failure. The usual therapeutic maneuver used to induce a diuresis is infusion of 0.9% NaCl or some other suitable crystalloid solution. Crystalloid should be given to maintain a urine output of 100 ml/hr for about 24 hours. Diuretics, such as furosemide, also can be used and are preferred by some to infusion of mannitol to increase intravascular volume. [13] [14] Mannitol, if chosen, must be used with caution; if acute tubular necrosis (ATN) occurs before mannitol infusion, pulmonary edema may occur due to the acute increase in intravascular volume secondary to fluid expansion. Similarly, excessive crystalloid infusion should be avoided in patients with borderline congestive heart failure. Renal damage must be minimized, however, and increasing renal blood flow helps to prevent anuric renal failure. The mechanisms responsible for the beneficial effect of increased renal blood flow likely include increased clearance of free hemoglobin, resulting in decreased binding of nitric oxide, and a return of more physiologic control of renal vasodilation. Creatinine and blood urea nitrogen (BUN) should be closely monitored; dialysis may be necessary for therapy of acute renal failure. As difficult as ATN therapy is, it is easier to treat a patient in the diuretic phase than in the oliguric phase of ATN. Support of blood pressure and respiration may require the use of vasopressors, bronchodilators, and, when necessary, intubation. Low renal levels of dopamine (12 g/kg/min) promote renal vasodilation, support renal function, and offset the likelihood of anuric renal failure. Higher levels of dopamine cause vasoconstriction and should be avoided unless the patients blood pressure requires the increased dosage. Disseminated intravascular coagulation (DIC) can occur in severe cases, and heparin may be indicated if a significant coagulopathy is evident. Heparin, however, is rarely needed in mild cases. The prothrombin time, partial thromboplastin time, and fibrinogen level should be closely monitored.

WORKUP OF AN ACUTE INTRAVASCULAR HEMOLYTIC TRANSFUSION REACTION

If an acute transfusion reaction occurs:

1. *Stop blood component infusion immediately.*
2. Maintain IV access with a suitable crystalloid or colloid solution.
3. Maintain blood pressure, heart rate.
4. Maintain an adequate airway.
5. Give a diuretic and/or institute a fluid diuresis, or both.
6. Obtain blood/urine for transfusion reaction workup.
7. Blood bank workup of suspected transfusion reaction:

    Check paperwork to ensure correct blood component was transfused to the right patient.
    Observe plasma for hemoglobinemia.
    Perform direct antiglobulin test.
    Repeat compatibility testing (cross-match).
    Repeat other serologic testing as needed (ABO, Rh).
    Analyze urine for hemoglobinuria.

If intravascular hemolytic reaction is confirmed:

1. Monitor renal status (BUN, creatinine).
2. Monitor coagulation status (prothrombin time, partial thromboplastin time, fibrinogen).
3. Monitor for signs of hemolysis (lactic dehydrogenase, bilirubin, haptoglobin).
4. If sepsis is suspected, culture as appropriate.

Laboratory Evaluation

Laboratory findings include hemoglobinuria, hemoglobinemia, and a haptoglobin level that is low to 0 mg/dl. During the hemolytic episode, the bilirubin usually increases only to 23 mg/dl if the patient has normal liver function. Elevations of bilirubin to 2030 mg/dl are not seen in otherwise normal patients, even with florid hemolysis. Very elevated bilirubin levels are seen only in patients with concurrent hepatocellular disease, such as viral hepatitis or hepatic carcinoma. Due to the lysis of red cells, levels of lactate dehydrogenase (LDH) may rise markedly. If the patient shows no signs of vasomotor instability and if hemostatic and renal function is unchanged 24 hours after the incompatible transfusion, the episode can be considered to be over, with serious sequelae unlikely.

Although ABO-incompatible blood transfusions are the most common cause of an acute intravascular hemolytic transfusion reaction, other complement-fixing antigen-antibody systems, such as the Jk^a (Kidd) or Fy^a (Duffy) blood group systems, can also produce these reactions. [15]

Acute Extravascular Hemolytic Transfusion Reaction

In an extravascular hemolytic transfusion reaction, complement either is not fixed at all or is fixed only to C3b. In either situation, because of the nature of the antigen-antibody reaction, complement activation with fixation of the C5b-9 complex does not occur. The presence of IgG on the red cell and/or fixation of complement to C3b results in an extravascular reaction because the antibody-coated cells are cleared by either the IgG receptors in the spleen or the C3b receptors in the liver. Red cell lysis does not occur in the intravascular space. Largely as a result of the lack of generation of C3a or C5a, an extravascular hemolytic transfusion reaction does not usually present as a clinical emergency. It is characterized, as are all immune hemolytic transfusion reactions, by development of a positive DAT due to recipient red cell alloantibody binding to the circulating donor red cells. Moreover, an increase in indirect bilirubin, an increase in LDH, a decline in hematocrit, and an increase in colorless urine urobilinogen occurs; hemoglobinuria and hemoglobinemia, however, are rarely present. The patient typically remains clinically stable, and renal failure, shock, and hemostatic abnormalities, such as DIC, are rarely seen unless the amount of incompatible blood infused is excessive. However, patients often have a low-grade fever, probably secondary to the generation of IL-1 or other proinflammatory cytokines. [12]

The diagnostic test of choice is, again, a DAT with an eluate. The eluate is performed to identify the antibody coating the red cells. [15] The positive DAT, again, is due to the patients antibody coating the donor red cells. This is not autoantibody, and the patients own red cells are not usually involved in the reaction.

Typically an acute extravascular hemolytic transfusion reaction requires no special therapeutic intervention. Maintaining a good urine output and monitoring renal and hemostatic function are usually sufficient. The patient characteristically recovers in a few days as the offending donor red cells are cleared from the circulation. The pathogenic alloantibody can often be identified in the patients serum. These acute transfusion reactions may occur if the patients pre-existing alloantibody was missed by the blood bank during the antibody screening process, if the unit of blood was labeled for the wrong patient, or if the unit was hung in error on the wrong patient. [2]

Delayed Hemolytic Reactions

The pathogenesis of a delayed intravascular transfusion reaction is similar to that described for an acute intravascular hemolytic reaction. However, in the delayed type, the patient develops

the antigen-antibody reaction some time (510 days) after the transfusion. The process thus occurs more slowly and is less likely to present as a clinical emergency. Hemoglobinuria and hemoglobinemia can occur but often are less profound and pronounced than with an acute intravascular reaction. This likely is due to the continual and gradual removal of antibody-coated red cells as the antibody titer rises, as well as to the more gradual generation of C3a and C5a. The same serologic diagnostic and treatment concepts used for an acute intravascular hemolytic reaction apply here. However, the need for acute intervention is much less likely. Initiating a diuresis and monitoring renal and hemostatic function may be sufficient.

Delayed extravascular hemolytic transfusion reactions occur as well. Such antigen-antibody reactions often involve the Rh system. The clinical presentation is similar to that of an acute extravascular reaction: the patient often has no acute clinical symptoms but may present with a fever, a falling hematocrit, and the development of a new DAT. Serologic evaluation of a transfusion reaction in a patient who had a positive pretransfusion antiglobulin test result, caused by autoantibody or medication, may be confusing. Such a workup requires an expert serologist to detect the new alloantibody in the patients serum and make a diagnosis of a hemolytic transfusion reaction.

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FEBRILE NONHEMOLYTIC TRANSFUSION REACTIONS

A febrile nonhemolytic transfusion reaction (FNHTR) is suspected when a temperature rise of 1°C or more occurs during or after transfusion when no other cause can be found. In addition to fever, an FNHTR is often accompanied by shaking chills. These reactions are due to cytotoxic or agglutinating antibodies in the patients plasma reacting against antigens present on transfused donor lymphocytes, granulocytes, or platelets. [16] [17] [18] [19] The antibodies often have human leukocyte antigen (HLA) specificity, although they also may be neutrophil- or platelet-specific. [20] [21] Conversely, the donor plasma also can contain antibody that can react with the corresponding cellular antigens in the recipients blood. [22] The cause of the fever relates to antibody-leukocyte or antibody-platelet interactions. The febrile reaction is mediated largely by the release of cytokines such as IL-1, IL-6, and TNF- from macrophages, monocytes, granulocytes, or lymphocytes. [23] IL-1 (endogenous pyrogen), via prostaglandin PGE_2 synthesis, probably stimulates the thermoregulatory center of the hypothalamus to produce fever. Other mediators such as macrophage inflammatory proteins (MIP-1) may also participate in the febrile response, but this reaction is not mediated through prostaglandin synthesis. [24]

The cytokines could be derived from three possible sources in the transfusion setting. The first is synthesis by recipient leukocytes in response to transfusion. The second is production by donor leukocytes after infusion into the recipient. The third mechanism is generation by donor leukocytes in vitro during storage before transfusion. [25] Red cells and units of platelet concentrates have sufficient potential sources of cytokines in the ample number (10^7 10^8) of mononuclear leukocytes capable of cytokine synthesis. Studies have shown that generation of cytokines does indeed occur in units of blood during storage and that such generation is directly proportional to the leukocyte count of the unit and the duration of storage. [26] [27] [28] Use of third-generation leukocyte reduction filters can lower the incidence of febrile reactions. Judging from reports of persistent febrile reactions despite use of these 34 $\log_{10}$ leukocyte reduction filters, such filtration needs to occur before storage and before the donor leukocytes begin to generate these proinflammatory cytokines in the blood storage bag. Heddle et al. [29] showed that febrile reactions to platelet transfusions were more likely due to proinflammatory cytokines present in the supernatant platelet-poor plasma than to the cellular elements in the platelet concentrates. Proinflammatory cytokines are not removed by bedside blood filters and enter the circulation. [30] Chemokines, however, such as IL-8, and RANTES and complement components C3a and C5a are removed, to varying degrees, by some bedside leukoreduction filters. [31] [32] Prevention of cytokine generation by prestorage leukoreduction, however, is more efficient and effective than is scavenging of the biologic response modifiers by using bedside filtration technology.

The frequency of febrile reactions has been estimated to be 0.5%/unit transfused. [33] [34] Reactions are most commonly seen in recipients who have been exposed to multiple white cell or platelet antigens, such as oncology patients or multiparous women. Oncology patients are at risk as a result of frequent transfusions, multiparous women from multiple exposures during childbirth. Both of these groups of patients can form multiple HLA, granulocyte, or platelet-specific antibodies that will react with white cells or platelets on subsequent exposure. Antigen-antibody reactions are capable of stimulating cellular activation with resultant complement fixation, cytokine generation, and activation of other biologic response modifiers. [35] [36]

Clinical Manifestations

Clinically, an FNHTR is characterized by fever and chills occurring shortly after the transfusion has begun. [37] The patient may have nausea and vomiting. Various factors, including rate of infusion of the blood and leukocyte content of the transfused blood (antigen load), may affect the severity of the clinical presentation of the febrile reaction. Febrile reactions also can occur several hours after the transfusion has ended. [17] Although an FNHTR often starts with the patients complaining of feeling uneasy or chilly, symptoms often progress from a slight tremor to true rigors. The chills or rigors are a manifestation of heat-conserving cutaneous vasoconstriction and result in muscular heat production. [38] These thermogenic responses, coupled with any effects due to release of IL-1 or other cytokines, may cause the patients temperature to rise several degrees above pretransfusion levels. Although these reactions are rarely dangerous, the severity often increases as the rate and dose of the infusion increase. Mild reactions are generally more uncomfortable and frightening than life-threatening. FNHTRs are usually self-limited, with fever persisting for no more than 810 hours. Elderly patients with a tenuous cardiovascular status and critically ill patients may develop respiratory complications, hypotension, or shock. Young adults, however, are less likely to become seriously ill even with development of a high fever. As in any transfusion reaction, the onset of fever and chills requires that the transfusion be stopped, the intravenous line kept open with normal saline or a suitable crystalloid, and appropriate samples sent to the blood bank. The patient should be reassured, as anxiety may be extreme.

Laboratory Evaluation

The workup of a febrile reaction must be undertaken promptly. Fever may also be the first sign of an acute hemolytic reaction or infusion of a unit of red cells or platelets contaminated with bacteria. The workup consists of ruling out a hemolytic reaction by reconfirming the ABO type of the patient and the donor unit, re-cross-matching to confirm patient-donor compatibility, evaluating the results of the pre and post-transfusion direct antiglobulin tests, evaluating the serum for hemolysis, and rechecking the accuracy of paperwork. [39] [40] [41] The post-transfusion DAT should yield negative findings, as an FNHTR does not involve red cell alloantibody.

As laboratory testing is being completed, the workup should extend beyond the blood bank to include bedside patient evaluation. Because fever and chills also may be caused by drugs or by diseases associated with infection or inflammation, these

factors also should be evaluated. Blood cultures of both the patient and the blood product, to rule out infusion of a contaminated unit of blood, is becoming more common at many medical centers. The difficulty lies in knowing when to order blood cultures: they are expensive and there is a 3% incidence of positive cultures due to contamination during collection (see the Bacterial Contamination section). Most routine hospital blood banks do not have the expertise or reagents needed to identify specific HLA, platelet, or granulocyte antibodies. Accordingly, the diagnosis of a febrile hemolytic transfusion reaction is usually made without isolating an identifiable antibody. The FNHTR is often a diagnosis of exclusion arrived at after ruling out a hemolytic reaction, a septic transfusion, or other possible causes of fever.

Fever usually responds to antipyretics, including aspirin, nonsteroidal anti-inflammatory agents (NSAIDs), and acetaminophen. For thrombocytopenic adult patients, acetaminophen (325650 mg PO) is the antipyretic of choice. Thrombocytopenic patients should avoid aspirin: it further compromises platelet function because of its inhibitory effect on platelet cyclo-oxygenase, an enzyme involved in the platelet release reaction. Nonthrombocytopenic patients may receive aspirin if its use is not contraindicated for other medical reasons. Many house officers routinely order diphenhydramine for treatment of febrile reactions. This therapy, however, should be reserved for pretreatment or treatment of possible allergic reactions. Unless the patient has a history of allergic reactions or shows signs of an allergic component to an FNHTR, such as flushing, hives, or pruritus, there is no benefit from the use of antihistamines for febrile reactions. In addition, antihistamines produce drowsiness in some patients.

For patients with no history of febrile reactions, routine premedication is unnecessary. Adult patients with severe reactions despite premedication may require more

intensive pharmacotherapy, including hydrocortisone sodium succinate (100 mg IV) given immediately before transfusion. Patients with severe shaking chills can be treated with meperidine intramuscularly or subcutaneously. Meperidine can stop shaking chills almost immediately. [42] Febrile reactions after granulocyte transfusions and, less frequently, after platelet transfusions can be so severe that hypotension and cardiovascular collapse can occur.

Prevention of febrile reactions when pharmacologic therapy fails relies on the use of leukocyte-depleted blood components. Several leukocyte depletion techniques are available. Centrifugation can remove approximately 70% of the leukocytes in a unit of blood; cell washing or use of frozen deglycerolized red cells can remove up to 95% of contaminating white cells. Older microaggregate blood filters can reduce leukocyte content by approximately 12 logs (9093%). [43] [44] [45] [46] [47] [48] [49] Third-generation leukocyte depletion red cell filters are also useful for preventing febrile reactions. [50] [51] [52] They can remove up to 4 logs (99.99%) of white cells, often lowering the level of white cells in a unit of blood from 10^9 to 10^5 . They also may be useful for preventing or delaying the onset of HLA alloimmunization. [53] [54] Leukocyte depletion platelet filters are also available. [55] [56] [57] [58] [59] [60] [61] Further, the results of a multi-institutional trial to reduce alloimmunization to platelets (TRAP Trial) have shown that reduction of leukocytes by filtration and UV-B irradiation of platelet concentrates were equally effective in preventing alloantibody-mediated refractoriness to platelets during chemotherapy for acute myeloid leukemia. [62]

The degree of usage of leukocyte-depleted blood components is still controversial. [63] [64] [65] Data show that a patient who has had one febrile transfusion reaction has a 1 in 8 chance of having another one. [34] Individuals with a history of recurrent severe febrile reactions should have notations made in their blood bank record to ensure future use of leukocyte-reduced components.

Although they clearly are indicated for people with multiple and recurrent febrile reactions, many physicians question whether leukodepleted blood products should be used indiscriminately for all patients. [63] [64] [65] Indeed, the immunomodulatory effects of allogeneic blood transfusions on tumor growth and recurrence and susceptibility to viral infection is poorly understood, and much research has yet to be performed. [66] [67] The cost of filtration is often cited as a reason to limit use of leukoreduction technology. Cost considerations, however, are often discounted when a significant threat, imagined or real, to the worlds blood supply is posed. Such a threat may be associated with the risk of transmission of new variant Creutzfeldt-Jakob disease. [68] There is concern that this new variant form may be transmitted by eating tissue from cows with bovine spongiform encephalopathy. In a murine model, there are some data that the agent may be present in the blood and can be associated with B cells. [69] There are already discussions as to whether all blood should be leukodepleted to address this potential risk to the worlds blood supply. [68] [70] Studies such as these [62] [68] [69] [70] may begin to influence physicians to use leukoreduction technology for more patients. Clearly, such increased use, while possibly improving the safety of the blood supply, will significantly raise the cost of medical care.

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ALLERGIC TRANSFUSION REACTIONS

Allergic transfusion reactions are believed to be most commonly due to infusion of plasma proteins. [71] The allergic manifestations produced vary. They include skin erythema with associated mild urticaria and pruritus; a confluent rash, which is intensely pruritic; extensive urticaria; severe vasomotor instability; bronchospasm; and anaphylaxis. A patient who develops hives and a mild allergic reaction during a blood transfusion does not progress to a more severe anaphylactic reaction after infusion of additional blood from the same unit. Thus, the severity of allergic transfusion reactions is not necessarily dose-related.

The exact nature of the antibodies involved in the various types of allergic reactions is unclear. The mild allergic reactions are usually IgG-mediated, but IgE may also be involved; anaphylactic reactions are most often IgG mediated. [7] Allergic transfusion reactions are quite common, occurring in approximately 1% of all transfusions. [72] [73] Most of these reactions start with pruritus, followed by the development of hives. At this point, the transfusion should be stopped and the patient given 2550 mg diphenhydramine, if there are no medical contraindications and if the patient had not already been maximally premedicated. After a short interval the transfusion can be continued, but only if the rash decreases and/or the hives disappear and the patient feels well and shows no signs of fever, chills, or vasomotor instability. For mild allergic transfusion reactions not associated with any cardiorespiratory problems, such as bronchospasm, dyspnea, hypotension, or tachycardia, it is acceptable to continue the transfusion after the reaction has subsided, as it is not likely to recur. The risk of transfusion-transmitted disease posed by infusion of another unit of blood is greater than the risk posed by continuing a transfusion that has produced only mild urticaria. This is not true, however, if the patient shows signs of systemic toxicity from the transfusion, such as hypotension, bronchospasm, or tachycardia.

The treatment of mild allergic reactions involves the use of diphenhydramine. Reactions are rarely serious and often do not recur with subsequent transfusions. The pathogenesis is thought to be recipient antibody directed against donor plasma proteins. Most blood banks, however, cannot identify the etiologic antibody; identification usually requires research laboratory techniques. Accordingly, the diagnosis of an allergic transfusion reaction is a diagnosis of exclusion. Washed red cells can be used to prevent reactions. [43] [44] Leukocyte depletion or microaggregate filters are of no value because the plasma protein passes through the filter.

Most patients who have one allergic reaction should continue to receive routine bank blood. Washed cells should not be provided until a second reaction has occurred and generally should be provided only for patients with severe or recurrent reactions. The occurrence of one or two hives or mild pruritus post-transfusion is not an indication for the use of washed cells. Anaphylactic reactions, however, can be life-threatening and may require intubation and pressor agents. The use of washed cells for patients experiencing these reactions is appropriate.

Although it is convenient to characterize a transfusion reaction as being purely febrile or allergic, in reality there is often a mix of the two symptoms, and the reaction is designated according to the predominant clinical sign.

OTHER ADVERSE EFFECTS OF TRANSFUSION

Microaggregate Debris and Adult Respiratory Distress Syndrome

Much has been published regarding the use of microaggregate filters for preventing adult respiratory distress syndrome after massive transfusion. [63] [64] [74] [75] [76] [77] [78] Microaggregate debris consists of dead platelets, granulocytes, and fibrin strands that form in blood during storage. [79] [80] [81] It has been hypothesized that infusion of blood containing microaggregate debris through standard 170-m-pore filters results in the passage of these 20- to 120-m-microaggregates into the pulmonary vasculature, producing occlusion of the pulmonary capillaries and pulmonary failure. Published studies have shown, however, that microaggregate filters are not required and do not appear to influence the onset of adult respiratory distress syndrome. [82] [83] This syndrome is most likely, and most often, attributable to concurrent hypotension and sepsis rather than to infusion of microaggregate debris, per se. Microaggregate filters may be of value during cardiopulmonary bypass surgery, when the microaggregate debris can enter the arterial circulation directly. It is likely that improvements in surgical techniques, post-trauma medical care, perfusion technology, and especially treatment of shock and sepsis have contributed more to the decreased incidence of postperfusion respiratory distress syndrome than has use of microaggregate filters. [84]

Leukocyte Reduction

There are multiple reasons emerging to leukoreduce units of blood and components. Standards of the American Association of Blood Banks require that to be considered leukoreduced for any indication, the level of leukocytes in the final component should be $<5 \times 10^{6}$. [85] This value applies to units of red blood cells and single-donor apheresis platelets. For platelets prepared from whole blood, the value is $<8.3 \times 10^{5}$ in all bags. Since the final pooled component also must have $<5 \times 10^{6}$ leukocytes, this implies that the final pool size should not exceed 6 units. Indications for leukocyte reduction range from those for which data exist (such as to decrease the incidence of HLA alloimmunization) [53] [54] [62] to other areas with fewer data (such as to decrease the incidence of tumor recurrence [86]). As more data are published, the indications for use of leukocyte reduction filters will become clearer. Although a conservative approach to their use for well-established indications would seem most prudent, changes for the indications are always being reviewed. [65] [66] [67] [68] [69] [70]

Bacterial Contamination

Bacterial contamination of stored blood can pose grave risks to the recipient. Bacteria can enter the blood bag during venipuncture as a result of inadequate skin preparation or during component preparation. [87] Some bacteria grow best at room temperature; other (psychrophilic) organisms grow optimally at refrigerated blood bank (16°C) temperatures. Gram-negative bacteria, including *Pseudomonas, Yersinia, Enterobacter,* and *Flavobacterium,* are organisms commonly associated with a contaminated unit of refrigerated blood. [88] [89] Platelet concentrates, stored at room temperature, are also known to be subject to bacterial contamination; several reports have described fatal septic transfusion reactions due to platelets containing *Salmonella* or *Staphylococcus*. [90] [91] [92] Units of blood that are contaminated need not be obviously discolored, malodorous, or clotted; by simple visual inspection it is extremely difficult to determine whether a unit is contaminated. However, Kim et al. [93] reported that visual inspection, with comparison of the blood bag segmented tubing with that of the blood bag itself, can on occasion identify units likely to be bacterially contaminated.

Individuals who receive a unit of contaminated blood may develop fever, rigors, skin flushing, abdominal cramps, myalgias, DIC, renal failure, cardiovascular collapse, and cardiac arrest. Clinically the patient presents in warm shock. These reactions may be immediate, or there may be a delay of several hours before the symptom complex becomes apparent. Reaction to infusion of a unit of contaminated blood is distinguishable from FNHTR by the formers more severe clinical presentation and is distinguishable from intravascular hemolytic transfusion reaction by the absence of the latters characteristic hemoglobinuria and hemoglobinemia. Reactions following transfusion of infected units of blood are often attributable to endotoxin produced by gram-negative bacteria. The presence of bacteria alone, however, can also cause many of these symptoms. Also, the symptom complex is often attributable, in part, to cytokines and interleukins that are generated in the contaminated, stored blood in vitro. These biologic response modifiers produce severe reactions in vivo following transfusion. [26] [27] [28] [94] [95] [96] Nitric oxide, for example, produced locally in smooth muscle by the synergistic action of TNF-, IL-1, and -interferon, probably mediates refractory hypotension and vasoplegia associated with septic shock. [95] [97]

If, during transfusion, a patient who appeared well suddenly develops rigors and shock, infusion of an infected component should be considered. If on further evaluation this diagnosis is still considered likely, the patient should be treated immediately, since delay significantly contributes to the chance of a fatal outcome. Blood pressure, pulse, respiration, and renal blood flow need to be supported. Blood infusion should be stopped the moment any transfusion reaction is suspected, and appropriate samples should be sent to the blood bank for a DAT and other studies. Cultures of the untransfused blood remaining in the blood bag should be sent as they may be diagnostic. Broad-spectrum antibiotics should be started immediately if infusion of contaminated blood is suspected and continued until the culture results are reported. These reactions are rare [98] but can be fatal. Use of recombinant IL or cytokine inhibitors is still too experimental to be useful clinically.

Circulatory Overload (Hypervolemia)

Hypervolemia is a form of transfusion reaction that should be considered in patients who, during a blood infusion, develop sudden severe headache, dyspnea, tachycardia, tachypnea, congestive heart failure, or other signs of fluid overload. Unless the patient is actively hemorrhaging, blood should never be infused rapidly, as the acute expansion of the patients intravascular volume may exceed the capacity of the cardiovascular system to compensate. [99] Rapidly transfusing a patient who is anemic but who is euvolemic and not actively bleeding produces no benefit and may cause harm. This caveat applies to transfusion of any blood component. Patients with compromised cardiopulmonary status may not tolerate acute blood volume expansion and may develop right- or left-sided heart failure. This is especially true for infants and the elderly. If symptoms occur, the

transfusion should be stopped and some form of volume reduction, either diuretics or phlebotomy, instituted. If there is a concern that the patient may not tolerate infusion of a full unit of blood or component within the 4-hour period of time allotted for infusion of blood components, the blood bank can divide the product into smaller portions, which can be transfused in aliquots. As a general guide, infusions in nonbleeding adults should occur at <23 ml/kg/hr. The rate should be lowered to 1 ml/kg/hr for patients at risk of fluid overload. Diuretics may be given to patients with compromised cardiopulmonary status before transfusion.

The initial stages of transfusion-induced hypervolemia may be difficult to distinguish from hemolytic transfusion reaction as well as from febrile or allergic reactions. The absence of hemoglobinuria and hemoglobinemia or the absence of a positive post-transfusion DAT result should serve to distinguish the reaction from one due to

immune hemolysis, and the absence of fever, chills, or urticaria from the febrile or allergic types of reactions.

Transfusion-Related Acute Lung Injury (Noncardiogenic Pulmonary Edema)

On rare occasions individuals being transfused with red cells or platelets develop pulmonary edema within 4 hours of starting a transfusion, without an elevation in cardiac pressure.[22] [100] [101] [102] [103] [104] [105] [106] Several reports of this type of transfusion-related acute lung injury (TRALI) suggest that these cases may be more common than expected. The chest radiograph reveals a pulmonary edema pattern, and there is respiratory insufficiency with a decrease in O_2 saturation, but without development of elevated left-side cardiac pressure. Copious amounts of pulmonary edema fluid are produced, as is characteristic of hypervolemia; however, the noncardiogenic reaction usually follows infusion of volumes of blood too small to produce fluid overload. The patient may also develop fever, chills, cyanosis, dyspnea, tachypnea, or hypotension. The postulated pathologic mechanism includes a reaction between donor HLA or white cell antibody and recipient leukocytes; complement activation also may occur when leukoagglutinins present in the recipient react with leukocytes contained in the infused donor blood, with aggregation of white cells.[107] [108] As a result of the HLA, Ag-Ab reaction, the activated leukocytes generate adhesive molecules on their surface (CD11/CD18). These proteins permit the leukocytes to attach to the cell membrane of the pulmonary endothelial cells and by diapedesis enter the interstitial space between the pulmonary capillaries and the alveolar epithelium. Once they are in the interstitial space, the neutrophils degranulate and release destructive enzymes, which produce a capillary leak, resulting in fluid filling the alveolar air sacs. Thus, pulmonary leukostasis with pulmonary edema occurs as a result of microvascular occlusion and capillary leakage. [109] [110] Complement-activated granulocytes also produce toxic oxygen radicals that damage pulmonary endothelial cells, resulting in an increase in pulmonary vascular permeability and further passage of fluid into alveolar spaces. TRALI has been estimated to occur once in 4,500 transfusions. [103] [105] [106] Silliman et al.[111] have postulated a two-step approach to the pathogenesis of TRALI. After an initial neutrophil activation step, neutrophil-priming lipids, which they believe play a key role in the process, further activate the granulocytes and produce the clinical syndrome. [106] [111] Rodent model systems for TRALI have also been described.[112]

Since this diagnosis usually requires that cardiac monitoring (Swan-Ganz) catheters be in place, TRALI is difficult to diagnose outside the operating room or intensive care unit. When a patient shows signs of noncardiogenic pulmonary edema, as with all other reactions, the infusion should be stopped. These HLA/neutrophil antigen-antibody reactions may be idiosyncratic and often do not recur.

TABLE 142-3 -- Diagnosis of Transfusion-Related Lung Injury (TRALI)

Onset	Within 4 hours of start of transfusion
Frequency	1:4,500 transfusions (probably underreported)
Signs/Symptoms	Decreased O_2 saturation, fever, hypotension, tachypnea, dyspnea, diffuse pulmonary infiltrates, normal cardiac pressures (requires Swan-Ganz catheter), copious amounts of pulmonary edema fluid
Pathogenesis	HLA/granulocyte-specific antibodies (usually of donor origin) reacting with recipient leukocytes. Activates recipient neutrophils; stimulates complement activation; generates CD11/18 on PMN surface, resulting in pulmonary capillary adherence and diapedesis and eventual pulmonary capillary leak syndrome. Latter is associated with generation of proteolytic enzymes and toxic O_2 metabolites, which cause endothelial cell damage. Neutrophil priming lipids may also play a role.
Diagnosis	Chest x-ray; blood gases; blood for HLA or anti-neutrophil antibodies
Differential Dx	Fluid overload; septic transfusion; anaphylaxis
Treatment	STOP TRANSFUSION; ventilatory support (administer O_2 , intubate PRN), support blood pressure, administer steroids; diuretics of *no* value

Data from Levy et al.[167]

To remove leukocyte antigens from units of red cells or platelets, leukocyte reduction filters may be useful; washed red cells are useful for that component as well. Treatment is symptomatic (Table 142-3). Multiparous women who are blood donors and blood donors who have been multiply transfused should not be used as plasma donors, as their serum may contain high titers of leukoagglutinating antibodies. [113]

Anaphylactic Reactions

When plasma containing IgA is transfused to patients with IgG anti-IgA antibodies, anaphylactic reactions can occur. Anaphylactic reactions have also been reported following transfusion of platelets administered through some types of bedside leukoreduction filters. [114] [115] [116] [117] [118] [119] The pathogenesis of this syndrome appears to be the activation of the contact pathway (prekallikrein converting to kallikrein) induced in plasma by the negatively charged surface of some leukoreduction filters. Kallikrein activation stimulates the conversion of high-molecular-weight kininogen to bradykinin. The bradykinin thus generated by the negatively charged bedside filter enters the circulation and produces a symptom complex characterized by pain, cutaneous flushing, and hypotension without fever or chills. The syndrome is often much more severe in patients already taking angiotensin-converting enzyme (ACE) inhibitors. ACE is identical to kininase II, which is responsible for degrading brady-kinin. Blockage of the kininase II degradation of bradykinin by ACE inhibitors results in a prolonged bradykinin half-life and a reaction that can be very severe. Accordingly, physicians should let the blood bank know of any patients taking ACE inhibitors who require blood transfusion. Prestorage leukoreduction avoids this problem, as the half-life of bradykinin is about 15 seconds. Reactions have been reported with platelet as well as red cell transfusions, but most commonly with platelet transfusions administered through a bedside leukoreduction filter, with a negative surface charge. While these reactions are rare, they can be quite serious.

Hypothermia

Hypothermia can occur with rapid infusion of large quantities of refrigerated (16°C) blood. [120] [121] [122] Data have shown that rapid infusion of blood (1 unit every 5 minutes) may lower the temperature of the sinoatrial node to <30°C, at which point ventricular fibrillation may occur. [123] Most transfusions need not be given this rapidly. Thus, for routine transfusion, blood does not have to be warmed. Indeed, overwarming blood can cause thermal injury and produce hemolysis, DIC, or shock. Further, a delay in infusing blood due to the extra time required for warming could impede resuscitation. Although routine warming of blood for trauma patients is not recommended, use of warming techniques for some trauma patients may reduce the incidence of coagulopathies associated with major trauma.

If blood is to be warmed, only an electric blood warmer should be used. The temperature must be monitored and kept below a level that could cause hemolysis. [85] [124] Usually this is <42°C. Heating blood under running hot tap water or heating in a microwave device is unacceptable; microwave devices produce hot spots that can cause hemolysis.[125] [126]

Electrolyte Toxicity

Citrate, a component of the preservative solution used in blood storage, functions as an anticoagulant by chelating calcium and interfering with the coagulation cascade. Rapid transfusion of citrated blood thus can be associated with a drop in ionized calcium levels. [127] [128] [129] Citrate-containing blood products, however, are routinely infused without any problem, as the citrate is rapidly metabolized to bicarbonate. In patients with normal liver function, citrate infusion is thus very unlikely to produce reactions. Mild to severe citrate toxicity can be seen, however, in individuals undergoing cytapheresis when large volumes of citrated plasma are reinfused.[130] [131] [132]

The effects of hypocalcemia range from mild circumoral paresthesias to frank tetany. However, severe citrate toxicity, even with massive transfusion, is very rare. More commonly, the reaction is mild and self-limiting and can be treated by merely slowing the rate of reinfusion. [133] If prolonged Q-T intervals or signs of tetany are seen, calcium can be administered. Infusion of calcium is itself often associated with the development of ventricular arrhythmias and cardiac arrest. [134] Calcium should not be infused routinely, even after large-volume blood transfusions. *Under no circumstances should calcium be added to a unit of blood, as it would recalcify the unit and cause clots to form in the bag.*

Hypomagnesemia, presumably due to chelation of magnesium by citrate, has also been reported. Actual clinical complications of transfusion-induced

hypomagnesemia, however, have not been well documented.[135]

Hyperkalemia due to infusion of stored blood is a rare occurrence. Although hyperkalemia is often thought to be a problem in massive transfusion, in reality development of hypokalemia is of greater concern. With storage, leakage of potassium from red cells to the extracellular fluid occurs. However, following infusion into a recipient, the red cells reverse the biochemical storage lesion, and intracellular potassium levels are restored. Further, as the citrate is metabolized to bicarbonate, the blood becomes alkalotic, contributing to hypokalemia.[136] [137] [138] In massive transfusion, this may not uncommonly result in the need for administration of potassium. Extracellular potassium increases at the rate of about 1 mEq/day during the first few weeks of storage. If this presents a concern for neonates or patients with renal failure, fresher blood can be requested. As there is increased potassium leakage from red cells following exposure to 25 Gy irradiation to prevent post-transfusion graft-versus-host disease, a maximum 28-day shelf life from the day of irradiation is imposed on this blood component. [85] Ammonia toxicity, previously a concern with stored blood, rarely presents problems today. [139]

Plasticizers

Plasticizers are chemicals used to make the rigid polyvinyl chloride plastic used in blood bags more malleable. The traditional plasticizer is di-2-ethylhexylphthalate (DEHP).[140] There has been some question as to whether DEHP is carcinogenic and whether its breakdown product, monoethylhexylphthalate, may produce problems due to production of peroxisomes, which may be destructive to vital organs. [141] [142] [143] [144] This plasticizer is being replaced with a citrate-based [145] [146] plasticizer, and new-generation blood bags are commercially available. Ironically, DEHP plasticizers may actually serve a useful function in that they appear to stabilize the red cell membrane and also may have a beneficial effect on platelet morphology. [147] [148] [149]

Graft-versus-Host Disease

Graft-versus-host disease (GVHD) occurs when immunologically competent lymphocytes are introduced into an immunoincompetent host who cannot destroy the donor lymphocytes. The immunocompetent donor lymphocytes engraft, recognize the host as foreign, and then attack host tissues. GVHD occurs after allogeneic bone marrow transplantation and less often after transfusion of nonirradiated cellular blood components, especially when the blood donor and recipient share some HLA antigens.[150] There is an increased danger from post-transfusion GVHD, in part because of the frequent failure of physicians to recognize and treat the reaction promptly. Another major factor, however, is the propensity of the donors lymphocytes to produce recipient bone marrow aplasia. In postbone marrow transplant GVHD, the bone marrow is of donor origin, and bone marrow aplasia does not occur. In post-transfusion GVHD, however, the bone marrow is of foreign (host) origin and the donors lymphocytes attack it, thus producing aplasia.

Post-transfusion GVHD is fatal in >90% of cases, primarily because of aplasia of the recipients bone marrow. Reports have shown that haploidentical directed donor units of blood may produce fatal post-transfusion GVHD even in immunocompetent recipients. [151] [152] The use of irradiated blood (>2500 cGy) is now recommended in clinical situations in which post-transfusion GVHD is considered likely, such as when patients receive blood transfusions from their relatives. Leukocyte reduction filters should not be used as prophylaxis against GVHD, as the exact number of leukocytes needed to produce

RISK GROUPS FOR TRANSFUSION-ASSOCIATED GVHD

Risk well defined

- Bone marrow transplant recipients
- Congenital immunodeficiency syndromes
- Intrauterine transfusion
- Transfusions from first-degree blood relatives
- Hodgkin disease

Risks under review

- Premature newborns
- Hematologic malignancies other than Hodgkin disease
- Solid tumors
- Organ transplant recipients

No risks defined

- Term newborns
- AIDS

From Anderson KC, Goodnough LT, Sayers M et al: Variation in blood component irradiation practice: implications for prevention of transfusion-associated graft-versus-host disease. Blood 77:2096, 1991, with permission.

the disease is not known with certainty. Case reports of fatal GVHD in patients who received leukoreduced but not irradiated blood have been published. [153] Several articles have addressed this subject.[154] [155] [156] [157] Radiation of red cells produces a membrane defect, however, which causes a slow leakage of potassium and hemoglobin.[158] Accordingly, the FDA has ruled that irradiated units of red cells shall have an outdate not to exceed 28 days from the time of irradiation. GVHD continues to be a rare but extremely serious complication of blood transfusion. [159] [160] [161]

Hemosiderosis

One milliliter of red cells contains 1 mg iron. A unit of blood with 250 ml red cells thus contains approximately 250 mg iron, and 4 units of blood 1 g iron, roughly the amount stored in the bone marrow. Males and nonmenstruating women lose only about 1 mg iron/day. Continued use of transfusion therapy in individuals with an extravascular type of hemolytic anemia, such as those with thalassemia or sickle cell anemia, in which iron is not lost from the body but is recycled, can thus result in the accumulation of excessive tissue stores of iron. Over long periods of time, the iron that is stored in parenchymal cells results in death of the cell and eventual organ failure.[162] Transfusion of reticulocyte-rich blood (neocytes) generally has not been found to be of value for decreasing the transfusion requirement in patients with chronic hemolytic anemias.[163] While young red cells have a longer half-life and should decrease the transfusion requirement, neocytes are not widely accepted as being clinically useful. Iron chelation therapy, such as that with deferoxamine, is now widely used and in conjunction with neocyte therapy actually may be able to

maintain patients with chronic hemolytic anemias in negative iron balance. [164] [165]

Air Emboli

With replacement of evacuated glass bottles by plastic blood bags, the risk of air embolism from phlebotomy or transfusion has virtually disappeared from transfusion practice. Air, however, still may be pumped into patients by the roller pumps contained in various transfusion devices, especially apheresis and intraoperative salvage machines.[166] All such devices currently manufactured, however, contain air in-line sensors. However, any operators using this equipment must be well trained and remain alert to the potential risk of air embolization at all times while the patient is being treated. Patients who receive air intravenously experience acute cardiopulmonary insufficiency. The air tends to lodge in the right ventricle, preventing blood from entering the pulmonary circulation. Acute cyanosis, pain, cough, shock, and arrhythmia may occur, and death may result unless immediate action is taken. The patient should be placed head down on the left side; this will usually displace the air bubble from the pulmonary valve.

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Chapter 143 - Transfusion-Transmitted Diseases

Jay E. Menitove

INTRODUCTION

The risk of acquiring a transfusion-transmitted infection decreased significantly during the past decade as a result of improvements in testing, donor screening, and permanent deferral of donors with positive viral test results. Current estimates indicate that 1 or 2/1,000 transfusion recipients are at risk of receiving a unit contaminated with a viral, bacterial, or parasitic agent. In contrast, an estimated 50% of recipients are at risk of significant morbidity or mortality if not transfused. [1] (Table 143-1) Transfusion medicine specialists anticipate further improvements in transfusion-transmitted disease risk reduction through nucleic acid amplification assays, implementation of viral inactivation techniques, and further improvements in donor screening. [2] [3] [4] [5] [6]

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RETROVIRAL INFECTION

Human Immunodeficiency Virus-1

The initial report of hemophilic patients with the acquired immunodeficiency syndrome (AIDS) appeared in 1982. Subsequently,

TABLE 143-1 -- Risk of Transfusion-Transmitted Infection

Infection	Risk per Unit
Human immunodeficiency virus-1	1:676,000
Human T-lymphotropic virus-I/II	1:640,000
Hepatitis A	1:1,000,000
Hepatitis B	1:63,0001:233,000
Hepatitis C	1:103,000
Hepatitis G	1:25100
Malaria	1:4,000,000
Chagas disease	(?)
Creutzfeldt-Jakob disease	No cases reported
Symptomatic Bacterial Sepsis	
Red blood cells	1:500,000 (?)
Platelets	1:2,0001:12,000 (?)

donor history questioning was revised and by 1985, antibody testing for human immunodeficiency virus (HIV) was available. The window period between HIV infection and seroconversion decreased to 22 days from 56 days between 1985 and 1996. [5] [7] [8] In 1996, blood centers implemented p24 HIV-1 antigen testing to narrow the window to 16 days. Antigen testing supplements rather than replaces HIV antibody testing because HIV-1 p24 antigen appears transiently during the acute phase of HIV infections (Fig. 143-1). Genomic amplification techniques, such as polymerase chain reaction (PCR) assays, offer the potential for further reducing the transfusion-transmitted infection risk and are under investigation currently. Preliminary studies suggest that HIV-1 PCR testing detects HIV genomic signals 11 days after infection. [7] [8]

The Centers for Disease Control and Prevention (CDC) reported 8,450 cases of AIDS in recipients of blood transfusion through June 30, 1997 including 37 adults/adolescents and 3 children who received blood from HIV-seronegative donors. [9] In addition, during the 1980s, 4,799 hemophilic and other patients with coagulation disorders acquired AIDS as a result of plasma derivative therapy. Viral inactivation processes for coagulation factor concentrates and immune globulin preparations using heat (pasteurization) or physical chemical properties (solvent/detergent treatment) dramatically reduced the risk of transfusion-transmitted HIV.

Mathematical models using estimates of the seronegative window interval, the probability that donations occur during this window, and the frequency blood donors develop new or incident infections provide the basis for calculating the residual risk of transfusion transmitted infections. [10] [11] Based on an observed rate of 7.7 confirmed positive HIV donations per

Figure 143-1 The lines depict the serologic and nucleic acid profile of an acute HIV infection. *(Reprinted from Busch,[6] with permission of Marcel Dekker, Inc.)*

100,000 collections, a predicted rate of 1/380,000 donations would transmit HIV using anti-HIV testing alone. However, HIV-seropositive donors react positively in other blood screening tests; 31% of anti-HIV-seropositive donations have anti-hepatitis B core antibodies. [12] This reduces the residual risk to 1/450,000660,000. [10] [11] HIV-1 p24 antigen testing reduces the window period an additional 27%, 16 days instead of 22 days. Preliminary estimates suggested HIV-1 p24 antigen testing would prevent 510 transfusion-associated HIV infections in the United States annually, reducing the residual risk to 1/676,000 (range: 572,000838,000). Eighteen months after HIV-1 p24 antigen testing was implemented, only two donors were HIV-1 p24 antigen positive and HIV antibody negative. [13]

Blood centers notify donors found repeatably reactive in HIV antibody screening tests despite indeterminate or negative Western blot or immunofluorescence test results. Experimental data indicate very few blood donors with HIV indeterminate supplemental tests are infected with HIV. [14] Low-risk blood donors, on occasion, have false-positive supplementary test results. [15]

Human Immunodeficiency Virus-2

Human immunodeficiency virus-2, a retrovirus linked more closely to the simian immunodeficiency virus than to HIV-1, was recognized initially in West Africa. Sixty-two cases of HIV-2 were reported in the United States as of June 30, 1995. [16] The interval between HIV-2 infection and AIDS is longer than that associated with HIV-1.

Antibody testing for HIV-1 detects 6091% of HIV-2 infected persons, whereas HIV-2 antibody test kits detect >99%. [17] Blood donor testing for both HIV-1 and HIV-2 antibodies began in 1992. HIV-1/2 combination test kits identified only two donors with HIV-2 infection during the 36 months following test introduction. [16] [18]

HIV-1 Group O and Group M, Subtype G Infection

The HIV-1 group O viruses were isolated from central and west African patients in 1994. These HIV-1 variants share 6570% homology with HIV-1 and 56% homology to HIV-2. Through 1997, two HIV-1 group O-infected persons were identified in the United States. [19] [20] Prior to the development of test kits designed specifically to identify HIV-1 group O-infected donors, donors are deferred if they were born in, lived in, traveled to and received a blood transfusion, or had risk exposures in HIV-1 group O endemic African countries.

Most HIV-1 infections are caused by a group M subtype B viruses. An AIDS case attributed to infection with an HIV-1 group M subtype G virus was reported in 1997. The serum reacted in routinely available HIV antibody test kits. Ongoing surveillance studies monitor emergence of new HIV variants and the effectiveness of routine HIV test kits for detecting these viruses. [20]

Human T-Lymphotropic Virus-I and -II

Human T-lymphotropic virus type I (HTLV-I) antibody testing for blood donations started in 1988. HTLV-I is the etiologic agent for adult T-cell leukemia/lymphoma (ATL), HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and uveitis. Patients with ATL have HTLV-I provirus integrated into CD4+ flower-like cells. [21] ATL occurs in 24% of infected persons following a latent period that extends to several decades. The illness is characterized by leukemia, generalized peripheral lymphadenopathy, hepatomegaly, impared liver function tests, splenomegaly, skin lesions, bone lesions, and hypercalcemia. [22] HAM/TSP occurs in <1% of HTLV-I infected

individuals. Patients with transfusion-associated HAM/TSP develop neurologic symptoms for a median of 3.3 years after transfusion. [22] This illness is characterized by slowly progressive chronic spastic paraparesis, lower limb weakness, urinary incontinence, impotence, sensory disturbances, low back pain, hyper-reflexia, and impaired vibration sense.

As a retrovirus closely related to HTLV-I, HTLV-II shares 60% sequence homology with HTLV-I. The two major subtypes of HTLV-I, African and Austronesian, maintain 90% homology and the two major subtypes of HTLV-II, A and B, have 94% homology. [23] HTLV-II is tropic for CD4+ lymphocytes but does not appear to cause any hematologic malignancy. Persons infected with HTLV-II have an increased risk of pneumonia, tuberculosis, fungal infections, bladder and kidney infections, urinary incontinence, and arthritis. The few cases of HTLV-II HAM reportedly involved HTLV-IIa. Because HTLV IIa predominates in the United States, HTLV-IIb cannot be considered less neuropathologic. [24] [25]

Risk factors for HTLV-I and -II infections in blood donors include low educational attainment, accidental needle sticks, previous blood transfusion, seven or more lifetime sex partners, and a sex partner from an HTLV endemic area. Injection drug use or having sex with a drug user associates significantly with HTLV-II but not with HTLV-I infection. [26]

By 1992, 0.1% of first-time blood donors had positive HTLV-I antibody results; repeat donors had lower rates. [10] The 51-day (range: 3672 days) interval between exposure and HTLV-I antibody detection creates a mathematically derived residual risk of 1/640,000 (range: 260,0002,000,000). However, only 30% of infected cellular components transmit the virus. [27] Antibody test kits for HTLV-I detect 5591% of specimens infected with HTLV-II. HTLV is not transmitted by noncellular components such as plasma and cryoprecipitate. The residual risk should decrease following implementation of test kits designed specifically to detect HTLV-II in addition to HTLV-I in 19971998.

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HEPATITIS INFECTION

Hepatitis A

Hepatitis A virus (HAV) is a nonenveloped RNA virus in the Picornaviridae family. It is transmitted primarily by the fecal-oral route and has an incubation period of 2045 days, with an average of 28 days. The virus replicates in hepatocytes followed by viremia and simultaneous shedding in the bile and feces. Subsequently, patients develop anorexia, fever, malaise, and nausea. Alanine aminotransferase (ALT) levels become abnormal and jaundice develops. Detectable anti-HAV antibodies arise contemporaneously with clinical symptoms. Unlike hepatitis B, C, D, and G, there is no carrier state. Transfusion-transmitted HAV infection occurs as a result of donation during the asymptomatic period. [28]

Numerous cases of hepatitis A transmission occurred in recipients of factor VIII and IX concentrate despite treatment with the solvent/detergent process. [29] [30] In addition to solvent/detergent resistance, HAV is relatively thermostable. It is inactivated by pasteurization and vapor heating. [31]

Hepatitis B

Hepatitis B virus (HBV) is a double-stranded, enveloped DNA virus in the Hepadnaviridae family. The viral particles contain an inner viral DNA core and an outer lipoprotein envelope that includes the hepatitis B surface antigen (HBsAg). The e antigen (HBeAg) is a soluble product that is co-translated with the core protein and serves as a marker for viral replication. HBV is transmitted through contaminated needles, sexual contact, occupational and other exposure to blood, and at birth. The average incubation period is 59 days (range: 3787 days) and may be asymptomatic in 50%. [1] [32] Five to 10% of those infected become chronic viral carriers.

Approximately 4 weeks after exposure, hepatitis B core (HBcAg) and hepatitis Be antigens (HBeAg); pre-S2, pre-S1, and S antigens (HBsAg); polymerase and transcriptase activity; and the X antigen protein appear. HBsAg persists in acute infection for up to 4 months (average 63 days). [33] ALT levels increase within a few weeks of HBs antigenemia. At approximately the same time as IgM antibodies occur against HBcAg (IgM anti-HBc), symptoms develop. Antibodies against HBsAg(HBsAb) occur several weeks later and protect against HBV infection. [32] Because anti-HBc is present during the interval between disappearance of HBsAg and appearance of anti-HBs, anti-HBc serves as a marker for preventing potentially infectious blood donations by HBsAg-negative persons who do not have sufficient quantities of HBsAb. Anti-HBc also identifies chronically infected HBV donors with undetectable HBsAg levels. [34] [35]

Transfusion-associated HBV infection occurs because not all HBV DNA carriers have detectable HBsAg and anti-HBc, and HBV variants with mutations may be silent serologically. [11] [36] Calculating HBV residual risk on the basis of HBsAg positivity in repeat donors identifies only 42% of incident HBV cases. [11] [33] [37] Using this approach and disregarding an effect for anti-HBc testing, the HBV risk is 1/63,000 units. If estimates are made on the basis that 0.03% of blood donors are positive for HBV, HBsAg test sensitivity is 99.9%, and anti-HBc test sensitivity is 99%, then the residual risk is 1/233,000 units. [1]

Hepatitis C

The hepatitis C virus (HCV), a single-stranded RNA virus of 9,500 nucleotides coding for 3,000 amino acids, is a member of the Flavivirdae family.

The 5 end of the HCV genome contains a highly conserved noncoding region that is used in PCR amplification assays. Structural elements are downstream and include the nucleocapsid or core and the two envelope regions, E1 and E2/NS1. The first nonstructural (NS) region, NS1, blends with E2. The subsequent NS2, NS3, NS4, and NS5 regions have protease, membrane binding, and polymerase functions [38] (Fig. 143-2). The initial genomic isolate, 5-1-1, is located at the 3 end of NS4. Expansion of the 5-1-1 clone produced the c-100-3 antigen used in first-generation HCV antibody detection kits. Second-generation anti-HCV assays include c22-3 core region antigens, and c33c NS3 antigens. Newer assays contain NS5 region antigens and a reconfiguration of antigens used in earlier assays without the c100-3 antigen. [38] Enhanced sensitivity appears related to increased detectability of c33c. [39]

Blood donors and patients with chronic hepatitis from the United States and western Europe are infected usually with HCV genotypes 1a, 1b, 2a, 2b, and 3a. Type 1b is prevalent in southern and eastern Europe. In Japan, Taiwan, and parts of China genotypes 1b, 2, and 2b predominate. [38] The various genotypes are associated with similar replication, transmission, and immune clearance rates. [40]

Hepatitis C virus is transmitted by injection drug use, percutaneous exposure such as tattooing, health care-related exposure, blood transfusion, and possibly sexual or household contact with HCV-infected persons. No identifiable risk appears for 50% of cases. Among blood donors, up to 0.36% are seropositive for HCV. [41] Donors 3039 years of age had the highest HCV seropositivity rate, as did those with a remote history of injecting drugs, prior blood transfusion, and possibly intranasal cocaine use. [42]

HCV Frequency in Recipients

Surrogate or indirect testing to reduce the risk of non-A, non-B post-transfusion hepatitis began in 1986 and 1987 in the United

Figure 143-2 The HCV genome encodes a single-stranded RNA virus in the Flaviviridae family. The structural organization contains 9,500 nucleotide bases that encode 3,000 amino acids. The figure denotes recombinant antigens comprising the various HCV test kits. *(From Alter.* [36] *)*

States and included ALT and anti-HBc assays. In retrospective analyses, these tests reduced the transmission of hepatitis C by 70%. [43] [44]

Introduction of first-generation HCV test kits in May 1990 reduced the risk of acquiring HCV per unit to 0.03% from 0.19%. [45] Second-generation HCV assays detect HCV 82 days (range: 54192 days) after infectivity or almost 6 weeks earlier than first-generation assays. [46] Based on studies of repeat blood donors, the residual risk following second-generation HCV testing is 9.70 (range 3.4736.11) per million donations or 1/103,000 units. [11] Newer assays decrease the window period between infectivity and detection to 70 days. [47] It is uncertain whether these new tests alter the residual risk because no cases of transfusion-transmitted HCV infection occurred at several institutions after second-generation assay implementation. [48] [49]

Approximately 90% of hemophilic patients treated with nonvirally inactivated coagulation factor concentrates are anti-HCV positive. HCV RNA remains in dry-heated (60° or 80°C) concentrates, but is not present when concentrates are heated in the lyophilized state at 80°C for 72 hours. HCV RNA was detected in some wet-heated or solvent/detergent-treated concentrates derived from anti-HCV unscreened plasma. HCV RNA is eliminated by wet-heating or solvent/detergent treatment of plasma from anti-HCV negative donors.[50]

Several lots of intravenous immune globulin preparations transmitted HCV between January 1993 and February 1994. [51] [52] [53] [54] [55] Although the plasma donors were second-generation anti-HCV negative, putative neutralizing antibodies were insufficient to inactivate contaminating HCV viruses. Currently, immune globulin preparations undergo viral inactivation procedures. In one report, immunodeficient patients receiving implicated lots of intravenous immune globulins developed significant liver failure and associated hepatitis-related mortality in contrast to idiopathic thrombocytopenic purpura patients who had clinically mild courses despite evidence of chronic infection.[55]

Natural History

Acute post-transfusion HCV infection causes no symptoms or jaundice in 7080% of cases.[38] The bilirubin level exceeds 2.5 mg/dl in only 30% and the mean peak ALT level is 708 /L. Nonetheless, the illness becomes chronic in 6080% of patients. Liver biopsies in HCV-positive blood donors who had a risk exposure 1620 years previously showed mild to moderate chronic active hepatitis and cirrhosis in 90%. Although donors with normal ALT levels had milder forms of disease, only 15% had normal liver histology.[56]

In a report from a tertiary care institution, 67% of patients had fatigue and hepatomegaly after a mean follow-up interval of 22 years. [57] Twenty percent had chronic hepatitis, 23% chronic active hepatitis, 51% cirrhosis, and 5% hepatocellular carcinoma. Other investigators found an 20% incidence of liver failure among patients surviving 15 years after developing post-transfusion hepatitis. [58] A study involving pediatric oncology patients with HCV infection showed a relatively benign clinical course after an average 14 year follow-up period.[59] The insidious nature of HCV infections and the deleterious effect of alcohol are demonstrated in a study involving patients observed an average of 18 years after transfusion. [60] Those with transfusion-related acute hepatitis had liver disease-related mortality rates of 3.3% compared to 1.12.0% in patients without clinical evidence of hepatitis. Although the difference is significant statistically, 71% of those with liver disease suffered from alcoholism.

HCV Testing Strategies

Hepatitis C virus RNA viremia appears within several days to 2 weeks after HCV infection. ALT levels increase, on average, 68 weeks postexposure. The highest HCV RNA levels occur just before clinical or laboratory evidence of hepatitis. Anti-HCV becomes detectable 1012 weeks after exposure. Antibodies against c 100-3 and 5-1-1 often disappear in patients with resolving infections, but c22-3 and c33c antibodies persist in chronic hepatitis. [38]

Rarely, seroconversion is delayed. A health care worker suffering a needle stick injury and simultaneous exposure to HIV and HCV seroconverted 9.513.5 months after exposure.[61]

In practice, the benefit of newer HCV tests containing NS5 epitopes may be more theoretical than observed. [39] [46] [48] [49] [62] [63] These assays potentially are more effective in identifying persons infected with type 2b and 3a HCV subtypes. [62] [63]

Nonspecific test reactions occur with the enzyme-linked immunosorbent assay (ELISA)-based screening tests. Supplemental tests, such as the recombinant immunoblot assay (RIBA), use HCV recombinant antigens on nitrocellulose membranes. Fifty to 85% of recipients of blood from RIBA-positive donors become infected, approximately the same rate as recipients of blood from HCV RNA-positive donors. ELISA-positive donors found negative by RIBA or PCR testing are unlikely to be infected with HCV.[64]

Currently, ALT as a surrogate or indirect test for hepatitis is not recommended in light of anti-HCV test effectiveness. [43] [44] [47] Nucleic acid amplification testing of volunteer blood donors is under investigation. During a pilot study, one HCV-seronegative, RNA-positive specimen was detected among 20,000 samples tested. [65] Nucleic acid amplification assays, potentially, could detect 650 HCV-infected donations annually in the United States.

Notification of Transfusion Recipients About HCV Risk

Approximately 7% of the estimated 4 million US citizens infected with HCV acquired their infection from blood transfusion. Most of these transmissions occurred before second-generation tests were implemented. Because many HCV infected patients are unaware of their infection and some therapeutic interventions may be effective, programs for notifying those at risk for HCV, including transfusion recipients, were initiated in 1998.

Hepatitis D

Hepatitis D virus, or delta hepatitis, is a defective RNA virus that replicates only in the presence of HBsAg. All patients are HBsAg positive, antibodies are present against HBV, HDV antigen is found in the liver, or HDV RNA is present in the serum. Approximately 1.48% of HBV donors are coinfected with HDV. [1] [66] Transmission occurs simultaneously with HBV or is superimposed on chronic carriers of HBV. Screening donors for HBsAg decreases the risks of HDV transmission.

Hepatitis E

Hepatitis E is a nonenveloped RNA virus associated with water-borne epidemics and fecal-oral spread. Viremia may be protracted, 45112 days. [67] The illness is associated with significant morbidity and mortality, particularly in developing countries.

Hepatitis G

The GB agent derives from the serum of a 34-year-old surgeon, GB, that was obtained 3 days after he developed jaundice in the 1960s. Plasma from marmosets containing the GB agent was passaged serially into tamarins (*Sanguinui labiatus*). Acute hepatitis developed 711 days later. Using a subtractive PCR methodology, characterization of two unique RNA molecules with limited amino acid identity to nonstructural HCV proteins led to identification of two flavivirus-like genomes in the GB-agent plasma, GBV-A and GBV-B.[68]

Degenerate PCR primers designed to amplify the helicase regions of GBV-A, GBV-B, and HCV aided in the identification of the helicase gene sequence of a novel virus from patients with non-A, non-B, non-C, non-D, non-E (non-A-E) hepatitis. The

Figure 143-3 (Figure Not Available) GB viruses A and C are related closely but share less homology with GBV-B and HCV. *(From Leary TP, Muerhoff AS, Simons JN et al: Sequence and genomic organization of GBV-C: a novel member of the flaviviridae associated with human non-A-E hepatitis. J Med Virol 48:60, 1996. Reprinted by permission of Wiley-Liss, Inc., a division of John Wiley & Sons, Inc. © 1996.)*

flavivirus, GBV-C, shares 59% nucleotide identity and 64% amino acid identity with GBV-A and is distinct from HCV. [69] In another laboratory, investigators identified an RNA flavivirus containing 9,392 nucleotides from the serum of a patient initially thought to have non-A, non-B viral hepatitis. Although subsequent testing showed evidence of HCV, a new virus, (HGV), related closely to GBV-A and distantly to HCV and GBV-B was identified. [70] Extensive analyses of the polyprotein sequences of GBV-C and HGV showed amino acid sequence identity at 95% and nucleotide identity at 85%. As a result, GBV-C and HGV are considered isolates of the same virus[71] [72] (Fig. 143-3 (Figure Not Available)).

Frequency of HGV Infection in Blood Donors

In studies conducted shortly after the identification of the HGV/GBV-C virus, 13 (1.7%) of 779 consecutively screened volunteer blood donors in the United States with normal ALT levels contained HGV/GBV-C RNA. Of 709 donors with elevated ALT levels, 11 (1.5%) were HGV/GBV-C RNA positive. One of the donors with elevated ALT levels was HGV/GBV-C and HCV positive and another was HGV, HBV, and HCV positive. [70]

Studies conducted subsequently in several international locations found 1.34.2% of donors HGV/GBV-C RNA positive. At one center, the prevalence increased to 7.4% among donors with anti-HCV and HCV RNA.[73]

Frequency of HGV/GBV-C Infection in Transfusion Recipients

Transmission of HGV/GBV-C occurs through transfusion. [70] [74] [75] [76] [77] [78] The incidence of HGV/GBV-C in transfusion recipients followed prospectively for acute hepatitis in the 1970s and from whom serum samples from all donors are available, reveals that only 4%, 3 of 79 patients, with non-A, non-B hepatitis have HGV/GBV-C as the only viral marker.[74] Eighty percent of the transfusion-associated hepatitis cases were caused by HCV. Ten percent of those with acute HCV hepatitis were co-infected with HGV/GBV-C. In contrast, only 0.6% of nontransfused patients followed in the control group were HGV/GBV-C RNA positive, reinforcing the concept that HGV/GBV-C is transmitted by transfusion. Approximately one-third of HGV/GBV-C-infected patients showed viral clearance within 3 years of infection.

The patients with HGV/GBV-C infection after transfusion had ALT levels less than five-fold elevated, no jaundice, and no symptoms. No correlation between HGV/GBV-C RNA levels and ALT levels was seen. The severity of hepatitis in HGV co-infected patients paralleled that seen in HCV-alone infected patients, suggesting that the extent of liver disease in co-infected patients is related to HCV infection, not HGV/GBV-C.

More than 14.2% of transfusion recipients in a French study

were HGV/GBV-C infected including 24.7% of bone marrow transplant patients.[75] In the United Kingdom, 61% of patients receiving a bone marrow transplant for acute leukemia were HGV/GBV-C positive compared to 33% receiving chemotherapy despite similar transfusion histories. No differences in HGV/GBV-C positivity was seen in patients treated before or after HCV screening was introduced. Liver function test abnormalities were seen in the same proportion in HGV/GBV-C infected and noninfected patients.[77]

Among patients undergoing cardiac surgery, the risk of transmission is 0.46% per donor unit in Tawian. The risk did not change following introduction of HCV testing.[78] Thirty-six percent of infected patients were viremic for >6 months. Patients on dialysis also have a higher incidence of HGV/GBV-C; ALT levels are not elevated.[79]

Approximately 918% of hemophilic patients are HGV/GBV-C RNA positive.[70] [80] [81] In addition, 32% of hemophilic patients in one study had anti-HGV envelope protein E_2 . Accumulating evidence suggests antibodies to the HGV/GBV-C envelope protein E_2 develop in those clearing HGV/GBV-C RNA, and thereby, may serve as a marker for identifying transiently viremic persons. [81] [82] [83] [84] Solvent/detergent treatment appears to inactivate HGV/GBV-C in coagulation factor concentrates. [80] [81]

Frequency of HGV/GBV-C Infection in Community-Acquired Non-A-E Hepatitis

Selected serum samples from patients with acute viral hepatitis enrolled in the CDC Sentinel Countries Viral Hepatitis Study were tested for HGV/GBV-C RNA. [85] HGV/GBV-C RNA was present in 9% of patients with non-A-E hepatitis, 20% of those with HCV infection, 25% of those with HAV infections, and 32% of those with HBV infection. HGV/GBV-C did not change the clinical course of acute disease in patients with hepatitis A, B, or C. Liver function test abnormalities were not seen in patients with HGV/GBV-C infection alone. Chronic hepatitis occured in 60% of patients with HCV alone and 61% of patients co-infected with HCV and HGV/GBV-C. That is, this study did not implicate HGV/GBV-C as the etiologic agent of non-A-E hepatitis. In another study involving histopathologic examination, HGV/GBV-C infection did not modify the course of HCV infection. [86]

Is HGV/GBV-C a Hepatitis Virus?

The results of studies in transfusion recipients and in patients with community-acquired HGV/GBV-C suggest that HGV/GBV-C does not cause significant liver disease.[48] [74] [77] [78] [85] [87] Occassional patients with fulminent hepatitis have HGV/GBV-C. [88] [89] It appears that HGV/GBV-C infection in patients with aplastic anemia is the result of transfusion rather than etiologic. [90] In situ hybridization studies have not been conducted to show HGV/GBV-C replication in hepatocytes. It appears the designation of HGV/GBV-C as a hepatitis virus is premature. Nonetheless, it is clear that HGV/GBV-C is tranfusion transmitted.

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HERPES VIRUS INFECTION

Cytomegalovirus Infection

Cytomegalovirus (CMV), a large, enveloped, double-stranded DNA herpes virus, resides intracellularly in leukocytes. The subpopulation of leukocytes responsible for transmitting CMV is undergoing debate currently. CMV causes acute episodes and also latent recurrences. Secondary or reinfections are less well documented. Cellular blood components transmit CMV, not plasma or cryoprecipitate. CMV infection causes clinical symptoms predominantly in immunosuppressed patients. [91] [92] Immunocompetent patients infected through transfusion rarely develop significant clinical illness.

In immunosuppressed patients, CMV infection leads to pneumonitis, hepatitis, gastroenteritis, retinitis, and other inflammatory conditions. Primary infection in pregnancy does not result in unusual toxicity for the woman. However, congenital CMV infection is associated with jaundice, hepatosplenomegaly, microencephaly, and thrombocytopenia. Fetal mortality rates approach 20%.[91] [93] Premature infants (<1,200 g birth weight) born to CMV-seronegative women are at risk for similar disorders including hemolytic anemia. Morbidity varies considerably from a few percent to 50% of seronegative infants receiving unscreened blood. CMV-seronegative marrow transplant patients appear particularly susceptible to CMV infection with 30% of those infected developing symptomatic CMV disease. [94] Seronegative renal and liver transplant recipients are at risk for transfusion-transmitted CMV infections, but the risk associated with receiving seropositive solid organs outweighs that attributed to blood transfusion. CMV-seronegative HIV-infected patients represent another group at risk for transfusion-transmitted primary CMV infection (Table 143-2).[91]

At least 50% of blood donors have antibodies against CMV, thereby indicating prior CMV infection. One study found CMV DNA in only 0.9% of normal blood donors, but 44% of CMV antibody-negative donors had evidence of CMV RNA.[95] Another study found the risk of transfusion-acquired CMV infection to be <1/698 donor exposures.[96]

Two percent of CMV-seronegative marrow transplant patients given CMV-seronegative blood develop CMV compared to 2857% following infusion of CMV unscreened blood components.[94] Serologic screening failures reflect antibody titer levels below detectable limits despite continued presence of intracellular virus, transent viremia that binds circulating antibody, or other limitations in test efficacy.

Other techniques for reducing the risk of transfusion-transmitted CMV infection include leukocyte reduction, saline washing, and use of frozen deglycerolized blood components. Currently, filters that reduce the leukocyte content to $<5 \times 10^6$ leukocytes per transfusion and selection of blood from CMV-seronegative donors are the predominate methods used to reduce the risk of transfusion-transmitted CMV.[91] [94] [97]

Comparison of Leukocyte-Reduced and CMV-Seronegative Blood Components

A prospective, randomized, controlled study compared the effectiveness of components leukocyte-reduced by filtration and CMV-seronegative blood for preventing CMV infection and disease in 502 seronegative allogeneic and autologous marrow transplant recipients. [94] Seropositive patients were not studied because of the confounding effect of CMV reactivation. Five patients developed CMV infection 21100 days after transplant, 0.8% of those receiving seronegative blood and 1.2% of those receiving filtered blood. The actuarial probability of CMV infection 100 days after transplant was 1.3% for patients receiving seronegative blood and a statistically similar 2.4% for those receiving filtered blood. In a secondary analysis that included all

TABLE 143-2 -- Indications for Use of CMV-Reduced Blood Products

Premature infants (<1,200 g) born to CMV-seronegative mothers
CMV-seronegative allogeneic and autologous progenitor cell transplant recipients
CMV-seronegative HIV-positive patients
CMV-seronegative recipients of solid organs from CMV-seronegative donors
CMV-seronegative patients who are potential progenitor cell transplant recipients
CMV-seronegative pregnant women

CMV infections occurring immediately after transplant to day 100, the CMV infection rates and CMV disease rates were 1.4% and 0% in the seroselected group and 2.4% and 2.4% in the group receiving filtered components. The difference in disease acquisition is significant statistically but may reflect CMV infection prior to blood component randomization. Most transfusion medicine experts, on the basis of this and other studies, believe appropriately performed leukocyte reduction provides results equivalent to those associated with selection of seronegative components.

CMV Reactivation and Co-infection (Second Strain Infection)

Second strain infections have been reported in recipients of solid organ transplants, but evidence linking blood transfusions as the source is not available. Because reactivation of previous infections occurs in seropositive patients, the benefit of providing reduced-risk CMV components for these patients is uncertain. In addition, the use of leukocyte-reduced components for CMV-seropositive patients to decrease the risk of reactivation is under investigation.

Epstein-Barr Virus Infection

Tranfusion transmission of Epstein-Barr virus (EBV), a herpes virus, is unlikely because 90% of the adult population has evidence of previous exposure and infection is deterred by host virus-specific cytotoxic T lymphocytes. The cytotoxic T lymphocytes lyse EBV-infected B lymphocytes when viral peptides are expressed on the lymphocyte surface.[98]

Human Herpes Virus 8 Infection

Human herpes virus 8 (HHV-8), a herpes virus linked by molecular and serologic studies to Kaposis sarcoma, has been detected in a blood donor. In vitro

experiments document transmission to virus-negative B lymphocytes and umbilical cord blood mononuclear cells. These observations suggest the virus may be transmitted by transfusion. However, Kaposis sarcoma occurs infrequently among HIV-infected transfusion recipients and hemophilic patients indicating the risk is remote.[99] [100] [101]

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PARASITIC INFECTIONS

Malaria

In the United States, malaria occurs in travelers, military personnel, and immigrants from endemic countries. Occasional cases result from mosquito transmission, blood transfusion, or organ transplantation. Approximately three transfusion-associated malaria cases occur per year in the United States. [102] [103] Malarial parasites, *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* maintain viability in red blood cells (RBCs) stored at 4°C, platelet concentrates stored at room temperature, and following RBC cryopreservation and thawing. Malaria is not transmitted by RBC-free components such as plasma and cryoprecipitate. The incubation period following transfusion ranges from 7 to 50 days (average 20 days).

The clinical presentation includes chills, fever, and splenomegaly. Fatigue, nausea, vomiting, headache, and diarrhea occur. Anemia may be severe with associated hemoglobinemia and hemoglobinuria, especially with *P. falciparum* infection. Glucose-6-phosphate dehydrogenase-deficient RBC are resistant to malaria. Persons lacking Duffy RBC antigens are refractory to *P. vivax* infection.[104] Patients with sickle cell trait have partial resistance to *P. falciparum*. Babesiosis should be considered in the differential diagnosis. Prevention of transfusion-transmitted malaria results from deferring blood donors emigrating or returning from malaria endemic regions.

Babesiosis

Babesiosis is caused by *Babesia microtia* or *B. equi* that is transmitted by the bite of an infected *Ixodes scapularies* or *I. pacificus* tick. Approximately 15 cases of transfusion-transmitted babesiosis have been reported in the United States. [1] Persons with a history of babesiosis are deferred from donating blood. [103] [105]

Toxoplasmosis

Toxoplasmosis is caused by the intracellular protozoan parasite, *Toxoplasma gondii*, whose usual host is the domestic cat. The parasite is transmitted by infected cats or cat litter and eating raw or undercooked pork, goat, lamb, beef, or wild game. Approximately 2025% of the US population is infected. [1] Transfusion-associated disease occurs in immunocompromised patients.[103]

Leishmaniasis

Visceral leishmaniasis, such as that reported among veterans of the Persian Gulf War in 19901991, follows infection with *Leishmania tropica.* No cases of transfusion-transmitted visierotropic leishmaniasis have been reported. [103]

Chagas Disease

American trypanosomiasis or Chagas disease consists of an acute phase that varies from no symptoms to fever, skin rash, conjunctivitis with palpebral edema, lymphadenopathy, or hepatosplenomegaly. The acute phase usually resolves in 48 weeks unless severe myocarditis or meningioencephalitis intervenes. The latter is associated with fatal outcomes. Following a latent stage of undetermined duration, the chronic phase occurs in up to 3040% of infected patients manifest by cardiac disease, megacolon, or achalasia. [106] [107]

The flagellate protozoan parasite, *Trypanosoma cruzi* causes Chagas disease. The disorder is widespread in Latin America. Humans become infected when bitten by *T. cruzi*-infected insects of the Reduviidae family. Once infected, low-level parasitemia persists. Vector transmission is unlikely in the United States, but 50,000100,000 infected Latin American immigrants reside in the United States. Among the four transfusion-associated cases of Chagas disease reported in the United States and Canada, *T. cruzi*-infected Latin American immigrants provided platelets in three. All four patients were immunocompromised. The organisms remain viable in whole blood stored at refrigerator temperatures for 18 days, for >8 months in citrated blood samples stored at room temperature, and following freezing and thawing. In South America, 1349% of recipients of parasitemic blood become infected. There is concern that additional transfusion-associated Chagas disease cases will occur as immigration increases to the United States from Central and South America. [106] [107] [108]

Risk factors for *T. cruzi* infection include birth or residence in endemic regions such as Central America, South America, or Southeastern Mexico; living in dwellings with palm leaf-thatched roofs or mud walls where vector insects reside; or receipt of a blood transfusion in Latin America. Studies in which blood donors are asked about these risk factors identify a cohort for additional serologic screening. Using assays for *T. cruzi* antibodies, 34% of donors who lived in poor housing or received a blood transfusion in endemic areas had *T. cruzi* antibodies.[108] One study, conducted in California, found 1/340 donors had a risk factor for Chagas disease. [109]

Recommendations for screening donors by history questions, antibody testing, or a tiered approach remain in abeyance pending further investigation of test sensitivity and specificity. Chemoprophylaxis with Gentian violet or other agents and blood filtration offer alternative approaches. [107] [110] [111] Because infection

occurs in endemic regions rather than the United States, testing donors with risk factors on one occasion offers a potential alternative to testing each donation. Of note, infection did not occur in 11 recipients of blood obtained in Los Angeles and Miami from donors seropositive for *T. cruzi*.[108] [112]

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CREUTZFELDT-JAKOB DISEASE

Creutzfeldt-Jakob disease (CJD) is a subtype of the spongioform encephalopathies. Similar neurodegenerative disorders occur in sheep (scrapie), cattle (bovine spongioform encephalopathy [BSE]), and humans (Kuru, Gerstmann-Straussler-Schneiner disease, fatal familial insomnia, and new variant CJD). The median age of onset is 6167 years and few patients with CJD are younger than 30. The incidence of classic CJD is approximately one case per million population, an estimate that has not fluctuated.[113] [114] [115]

The disease arises sporadically, has a familial association, or follows an environmental exposure. Sporadic cases have no known cause. The acquired cases are linked to iatrogenic events that include use of contaminated human pituitary-derived growth hormone, dura mater transplants, a single case report involving a corneal transplant, and contaminated depth electroencephalographic electrodes. The latency period following human growth hormone factor administration ranges from 3 years to >20 years. The average duration between dura mater graft placement and onset of CJD is 89 months (range: 16193 months). [115] [116] Accumulating evidence suggests that new variant CJD and BSE, or mad cow disease, are caused by the same agent. Transgenic or inbred mice innoculated with brain tissue from sporadic, iatrogenic, new variant CJD, or BSE developed spongioform encephalopathy. [117] [118] The familial cases have a mutation in chromosome 20.[113]

According to the most widely accepted theory, an infectious agent, a prion protein (PrP) on proteinaceous infectious particle, causes CJD. The agent has some attributes of a virus, such as strain variation, but resists inactivation by boiling and ionizing or ultraviolet irradiation. The prion is a modified form (PrP^{Sc}) of a host-encoded glycoprotein (PrP^{c}). The 35,000-dalton PrP^{c} glycoprotein is not pathogenic but is required for replication of the infectious agent. PrP^{Sc} occurs through a post-translational process involving a conformational change in PrP^{c}.[113] [115] In spontaneous or familial cases, PrP^{c} is converted to PrP^{Sc}.

Approximately 85% of CJD cases occur randomly. These sporadic CJD cases present clinically with memory loss or confusion, behavioral changes, or gait instability. Myoclonic or choreoathetoid movements associated with electroencephalographic periodicity occur during the 4-month illness. Histologic examination of the brain shows spongioform changes, gliosis, and neuronal loss. Immunologic staining reveals prion-positive amyloid deposits.

The inherited forms of CJD are autosomally transmitted and are associated with coding disorders in the prion protein gene (PRNP). Recognized mutations occur at codons 102, 105, 117, 145, 178, 180, 198, 200, 210, 217, and 232. The phenotypic expression of inherited CJD is variable and inconsistent within families. [119]

Sporadic, iatrogenic, and new variant CJD occurs predominantly in persons homozygous for methionine in PRNP codon 129. In iatrogenic CJD, heterozygosity or homozygosity for valine in codon 129 does not alter disease expression but may affect the incubation time. [120]

To date, no cases of transmission by blood or plasma derivatives have been reported. In preliminary reports, no cases of CJD occurred in 179 recipients of blood components from 14 US blood donors who later developed CJD.[121] In the United Kingdom, 21 patients who received blood transfusions and 29 who donated blood among 202 definite and possible cases of CJD were investigated. [122] Transfusion recipients did not differ from age- and sex-matched controls. Six individuals who subsequently developed CJD donated >10 units of blood; the incidence of CJD in the geographic areas in which the transfusions occurred was unchanged.

Despite these data, concern related to transmission by other tissues led to recommendations deferring blood donation by those at high risk for developing CJD: persons who received human pituitary-derived growth factor and dura mater transplants and persons whose families are at increased risk for CJD, including those with more than one genetically related family member with CJD. If a donor develops CJD subsequent to donation, in-date blood components must be retrieved and destroyed. Physicians caring for recipients of previous blood donations from at-risk donors (with exceptions of those with only one family member with CJD), are notified so they can decide whether to inform patients that they received such blood components. Plasma derivatives, albumin, and antihemophilic factor concentrates are recalled if plasma donors subsequently develop CJD. More than 37 such recalls of plasma derivatives occurred between 1993 and 1997 involving >1,500 lots and products worth several million dollars. Because CJD occurs in older individuals, exclusion of plasma from donors older than 50 or 60 years would prevent some recalls. This approach is controversial because risks of other transfusion-transmitted diseases should be lower in this group than in younger donors needed to replenish the lost donations.[114]

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SPIROCHETE INFECTIONS

Syphilis

Serologic tests for syphilis (STS) were introduced for blood screening in the United States in 1938 and were required by regulation in 1958. No cases of transfusion-transmitted syphilis have been reported recently. [123] [124]

Treponema pallidum, the spirochete causing syphilis, loses viability after 7 days storage at refrigerated temperatures. Experiments to determine viability following storage at room temperature have not been performed. The concomitant administration of antibiotics to some patients receiving transfusions probably decreases the occurrence of transfusion-associated syphilis.

A report shows STS-reactive donations are HIV seropositive 12 times more frequently than STS-seronegative donations. However, syphilis testing removes only 1.4% of HIV antibody-negative, HIV-1 p24 antigen-negative, infectious donations at an estimated cost of more than $16 million per case prevented, compared to $17,00032,000 per case prevented by HIV antibody screening and $810 million per HIV case prevented by HIV antigen testing. [124]

Lyme Borreliosis and Other Tick-Borne Illnesses

Borrelia burgdorferi, the agent responsible for Lyme disease, is transmitted to humans by *Ixodes scapularis* tick bites. Spirochetemia likely occurs postinfection and may be present in asymptomatic persons. Nonetheless, no clinical or serologic evidence of transfusion-transmitted Lyme disease was demonstrated in cardiothoracic surgery patients receiving blood collected in New England during the peak deer tick season. [105]

Donors potentially exposed to Rocky Mountain spotted fever and ehrlichosis have been deferred for 4 weeks following endemic exposure.

PARVOVIRUS INFECTION

Parvovirus B19, a nonenveloped DNA virus, causes a viral exanthum called fifth disease. Approximately 50% of adults are seropositive for parvovirus B19 antibodies; most infections occur in those 615 years of age. The virus is tropic for hematopoietic

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progenitor cells through its receptor, the RBC P antigen. The P antigen is expressed on megakaryocytes, endothelium, placenta, fetal liver, and fetal heart cells, but erythroid cells are most susceptible. Following infection, neutralizing antibodies appear. Some patients with sickle cell anemia, thalassemia, and other entities associated with enhanced RBC production develop acute aplastic or hypoplastic anemia following infection. Others at risk for aplasia following parvovirus infection include immunodeficient patients, HIV-infected patients, solid organ transplant recipients, and children with malignancies. Fetal loss may follow acute parvovirus infection during pregnancy.[125] Immune globulin infusion reverses aplasia in some patients.

Neither solvent/detergent nor heat treatment at 100°C for 30 minutes after lyophilization inactivates parvovirus. [126] [127] [128] More than 80% of hemophilic patients have evidence of previous parvovirus infection. In general, these patients do not suffer serious or long-term hematologic sequelae regardless of HIV serostatus. [129] Albumin solutions are pasteurized at 60°C for 10 hours, a process that probably does not destroy the virus. However, the ethanol fractionation process used to prepare albumin inactivates or eliminates the virus.[126]

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BACTERIAL CONTAMINATION

Bacterial contamination of RBC and platelet components occurs as a result of bacterial entry during component pooling or aliquoting, minute holes in blood containers, transient bacteremia at the time of phlebotomy, or contaminated containers.

Criteria for suspecting septic transfusion reactions include temperature elevations of 13.5°F or 12°C accompanied by rigors, tachycardia, and systolic blood pressure changes indicating significant hypertension (>30 mm Hg) or hypotension (<30 mm Hg) within 90 minutes of transfusion onset. Additional signs and symptoms include nausea, vomiting, diarrhea, chest or back pain, and cyanosis.

Septic complications caused 16% of the 182 transfusion-associated fatalities reported to the United States Food and Drug Administration between 1986 and 1991. Of the reported fatalities, 72% followed platelet transfusion and 28% followed RBC transfusions. Microorganisms contaminating platelet concentrates included coagulase-negative staphylococci, diphtherioids, streptococci, *Salmonella* species, *Escherichia coli, Serrati marcescens, Pseudomonas aeruginosa, Bacillus cereus*, and *Klebsiella* species.[130] [131] [132] Recent reports found *Aspergillius terreus* and *Yersinia enterocolitica* in contaminated platelet units.[134] [135] Under-reporting of septic platelet transfusion fatalities may understate the actual incidence 10-fold. [131] Assuming bacteria enter the container at the time of phlebotomy, the microorganisms enter log phase growth after several days in these room temperature-stored components. In vitro studies indicate contaminated platelet concentrates stored for 14 days had 2.6×10^3 10×10^4 colony-forming units per milliliter compared to 1.2×10^7 4.4×10^{13} colony-forming units per milliliter in concentrates stored for 5 days. [132] The contamination rate varies between 0.04% and 0.3% per unit for platelet concentrates prepared from whole blood and from 0% to 0.42% per single donor (apheresis) platelet concentrate.[132] [134] [135] [136] [137] [138] [139]

Yersinia enterocolitica causes the majority of septic RBC transfusion reactions. Twenty-one episodes resulting in 13 deaths were reported between 1985 and 1996. [140] During this interval, approximately 44 million patients received RBC transfusions. Because RBCs are stored at 16°C, it is not surprising that psychrophilic organisms such as *Yersinia, Pseudomonas putida*, and *P. fluorescense* predominate. A lag period of 3 weeks intervenes between organisms entering the collection container and accumulation of sufficient bacteria and endotoxin to produce clinical symptoms. The contamination rate in filtered, leukocyte-reduced blood may be lower than that observed in nonfiltered components.[130] [131] [139]

When septic reactions are suspected, the transfusion must be stopped immediately and followed by supportive care and broad-spectrum antibiotic coverage. The laboratory investigation includes container and patient blood cultures. A Gram stain of the remaining fluid in the container is most likely to be positive when bacterial counts exceed 10^6 colony-forming units per milliliter. In several reports involving platelet transfusions, the Gram stain was positive in more than two-thirds of symptomatic cases.[130]

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Chapter 144 - Autologous Blood Transfusion

Margot S. Kruskall

INTRODUCTION

Although the concept of collecting, saving, and transfusing autologous blood has existed for more than 100 years, practical applications for these techniques appeared limited until recently. With the emergence of the human immunodeficiency virus and its highly efficient transmission via blood has come an increased interest in using the patients own blood for transfusion.

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ADVANTAGES OF AUTOLOGOUS BLOOD TRANSFUSION

The substitution of autologous blood components for those collected from other (allogeneic) donors eliminates transfusion-transmitted diseases such as hepatitis and AIDS. Immunologic complications related to the transfusion of foreign cells, including hemolysis and febrile reactions to white cells, are also prevented. Other advantages are less clearly established. For example,

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erythropoiesis may be sufficiently stimulated in the repeatedly bled autologous donor to hasten recovery from postoperative anemia. [1] Intraoperatively salvaged red cells are spared the acquired membrane defects (storage lesion) and 2,3-diphosphoglycerate deficiency of refrigerated allogeneic cells. [2] Some investigators believe that allogeneic blood transfusions may contribute to immunosuppression in the recipient, [3] and therefore to an increased risk of cancer spread; [4] autologous blood should not. However, a recent randomized trial of autologous versus allogeneic blood transfusion in patients with colon cancer was unable to detect any differences in prognosis, thereby rendering doubtful the contribution of allogeneic blood transfusion to immunosuppression. [5]

An important drawback to techniques of autologous blood transfusion is their increased expense relative to the technically and administratively simpler allogeneic transfusions they replace. [6] In addition, the availability of autologous components may result in their use in situations in which transfusion might not have otherwise been considered, [7] and patients with suboptimal compensatory erythropoiesis and donation-induced anemia at the time of surgery are also more likely to receive transfusions. [8] Recent analyses have estimated very large cost-benefit ratios associated with autologous collections in advance of surgery (for example, $500,000 per quality-adjusted life-year in the setting of coronary artery bypass surgery). [9] Such costs could be reduced if autologous protocols were used only in appropriate settings and if the procedures for such collections were streamlined. [10] [11] Other potential problems, such as the risks of blood donation in patients with cardiac disorders [12] [13] or complications from reinfusion of red cells damaged during their salvage from the surgical field, [14] [15] are of debatable significance.

METHODS OF COLLECTION

Three approaches are popular and sometimes used in combination in an individual patient: autologous blood donations in advance of an anticipated blood loss during surgery, salvage of intra- and postoperatively shed blood, and intraoperative acute hemodilution.

Autologous Blood Donations

Preoperative

The typical volunteer allogeneic blood donor is allowed to give 1 unit of blood (450 ml ± 10%) no more than once every 8 weeks, in order to prevent such complications as iron deficiency. However, provided that bone marrow erythropoiesis can be stimulated and satisfactory iron supplies maintained, blood can be collected as frequently as once a week from an autologous donor.[16] Although the shelf life of refrigerated red blood cells is limited to 42 days, storage for up to 10 years is possible at 65°C or lower, using glycerol as a cryopreservative.

From a cardiovascular standpoint, phlebotomy is well tolerated by a variety of seemingly high-risk donors, including the elderly, [17] [18] children,[19] pregnant women,[20] [21] and patients with coronary artery disease. [1] [22] By contrast, anemia frequently develops during the donation interval and limits the number of autologous units that can be collected. In addition to marginal iron stores, erythropoietin levels often do not increase during the donation interval, probably because the hematocrit of most donors is not allowed to fall to below 30%. [23] Recent research suggests that this situation may be improved by the administration to autologous donors of the recombinant growth hormone erythropoietin.[24] Many variables affect the response to this drug in blood donors, including the route of administration (intravenous vs. subcutaneous), baseline iron stores, and the method of iron supplementation (oral vs. parenteral). [25] These variables are under active study. The expense of erythropoietin therapy has limited its application to situations in which autologous blood donation might otherwise be difficult or impossible, such as in the already anemic patient.[26] [27]

Patients planning to undergo an elective orthopedic surgical procedure make ideal autologous donors (Table 144-1): these patients typically require blood transfusions either during surgery or during the first few postoperative days, and most have sufficient time before the operative date to make multiple donations. [28] [29] Autologous blood has also been collected from patients undergoing elective open heart surgery and other vascular bypass grafts. [1] [18] The reduction or elimination of allogeneic blood use by these patients is testimony to the value of autologous collections in such operations (Fig. 144-1). The use of preoperatively donated autologous blood has also been reported for a variety of other surgical procedures, including radical prostatectomy, hysterectomies and other gynecologic procedures; colorectal, biliary, and gastric surgical procedures; and neurosurgical procedures. [18] [30] The need for autologous blood deserves periodic reevaluation, because its role may be affected by improvements in surgical techniques. Radical retropubic prostatectomy is one such example. Ten years ago many hospitals encouraged patients undergoing this procedure to donate autologous blood; [31] [32] [33] now, in some hands, less than 2% of patients require any blood whatsoever, and autologous blood donations can be eliminated. [34] Plastic surgical procedures also rarely require allogeneic blood transfusions, and the collection and use of autologous blood appears unnecessary.[35]

Other Uses

Autologous blood has been collected from women during pregnancy for use during childbirth, but this approach is controversial. A number of recent clinical studies of blood donations during pregnancy have found no complications for either the mother or the fetus. [20] [21] Nevertheless, the transfusion rate at delivery is quite low, less than 2.5% in many institutions,[36] and most autologous donations are unused. The use of autologous donations may best be directed toward patients with multiple gestations or placenta previa, in which populations the likelihood of transfusion may exceed 25%. [37]

Long-term (frozen) storage of autologous red cells in the absence of a planned transfusion episode is largely ineffective and expensive. Medical emergencies requiring transfusion are of an unpredictable but low incidence, and the likelihood is not very great that sufficient blood could be donated and stored and that

TABLE 144-1 -- Common Indications for Autologous Blood Techniques

Surgical/Obstetric Procedures	PreoperativeDonations	Perioperative Salvage
Intra-abdominal vascular procedures	Yes	Yes
Open heart surgery	Yes	Yes
Total hip replacement	Yes	Yes[a]
Total knee replacement	Yes	Yes[b]
Scoliosis repairs	Yes	Yes
Radical prostatectomy	Yes	Yes[c]
Placenta previa	Yes	No[d]
Multiple gestations	Yes	No[d]

Adapted from Kruskall,[116] with permission.

[a]The need for postoperative salvage has not been conclusively established.
[b]Intraoperative salvage is unnecessary when a tourniquet technique is used; postoperative salvage may be of value when a procedure is performed without cement.
[c]The risk of cancer spread after transfusion of intraoperatively salvaged blood has not been established.
[d]The safety of blood containing amniotic fluid has not been conclusively established.

Figure 144-1 Proportion of patients undergoing first-time elective coronary artery bypass surgery who required transfusion. **(Left)** Autologous donors versus other patients. **(Right)** Autologous donors

according to the number of units of autologous blood donated. White bars, no transfusion; gray bars, autologous blood transfusions only; red bars, allogeneic blood transfusions. *(From Owings et al.,[1] with permission.)*

such blood could be sent where it was needed and prepared in a timely fashion (i.e., thawed and washed free of its glycerol cryoprotectant).

Other autologous components have applications in surgery. Although autologous plasma is easily separated and stored, there is little need for this component in elective surgery.[38] However, autologous fibrin glue can be prepared using the cryoprecipitated portion of the plasma mixed immediately before use with thrombin. This tissue adhesive has been used for a variety of purposes in surgical procedures, including the control of bleeding in cardiovascular and thoracic surgery, [39] adhesion in middle ear surgery,[40] closure of the dura in neurosurgery, [41] adhesion of skin grafts,[42] and closure of gastrointestinal fistulas.[43] Fibrin glue has also been used in pancreatic and hepatic resection and trauma,[44] [45] although episodes of severe hypotension have been reported, possibly related to allergic reactions to systemically absorbed bovine thrombin.[46] Another rare complication, the development of life-threatening bleeding in association with acquired factor inhibitors, is caused by immunization to small amounts of bovine factor V in the thrombin; these antibodies recognize cross-reactive epitopes on human factor V. [47] [48] [49]

Autologous platelet-rich plasma has been prepared at the start of open heart surgery, before bypass, using apheresis equipment, for return to the patient following heparin reversal.[50] Both thrombocytopenia and an acquired platelet defect, due to activation and -granule release, occur when blood passes through the membrane oxygenator during cardiopulmonary bypass.[51] The theoretical advantages of transfusing autologous platelet-rich plasma are improved hemostasis and a reduction in transfusion requirements. Initial studies provided enthusiastic confirmation. [50] [52] [53] [54] More recent prospective, blinded studies have been unable to demonstrate a reduction in blood use after either primary or repeat open heart surgery. [55] [56] Furthermore, the harvesting of platelet-rich plasma has been followed by intraoperative heparin resistance, possibly due to the release of platelet factor 3 and other procoagulants from platelets damaged during collection. [57] Further studies of this autologous component are needed before its utility can be advocated.

Intraoperative Blood Salvage

Interest in salvaging blood shed at surgery has been stimulated by the introduction of automated instruments for autologous blood salvage over the past 30 years. The first model suctioned blood from the surgical field into a reservoir, from which it could be rapidly reinfused into the patient. [14] Unfortunately, a design flaw allowed the reinfusion pump to continue to operate even when the reservoir was empty, and several deaths from air embolism were reported.[58] This situation improved with the availability of other high-speed models with safer air detectors. Techniques for washing blood to remove excess surgical irrigant fluids and other contaminants appeared at around the same time.[59]

Systems for blood collection without further washing are usually modifications of disposable suction devices (Table 144-2). Blood is collected under low vacuum pressure into a

TABLE 144-2 -- Autologous Blood Salvage Systemsa[a]

System	Hardware	Software	Characteristics of Collected Blood				
			Hematocrit	Free Hemoglobin	Platelet Count	CoagulationFactors	Fibrin Degradation Products
Collection without washing	Rigid plastic cannister	Plastic bag	Low (25%)	Very high (200 mg/dl)	Low (100,000/mm^3)	Low (3575%)	High (300 mg/dl)
Collection followed by washing	Integral or separate blood cell processor	Disposable plastic bowl and tubing	High (60%)	Low (<50 mg/dl)	Very low (10,000/mm^3)	Absent (0%)	Absent (0%)

Data from Noon[2] and Silva et al.[117]

[a] Typical results of laboratory tests are shown. Transfusion of large volumes of salvaged blood results in similar alterations in these tests in the recipient.

plastic bag seated within a hard outer cannister. An anticoagulant such as citrate can be added. As soon as the bag is full, or within 4 hours of the start of collection (to prevent bacterial overgrowth), the contents of the bag are reinfused into the patient by gravity without any further processing, except passage through a standard blood filter.

Alternatively, the contents in the bag can be washed to remove free hemoglobin, surgical irrigant solutions, and other debris. Although this step is theoretically attractive and increasingly popular, its necessity before reinfusion of shed blood has not been established. [60] [61] Instruments that include both a reservoir for collecting salvaged blood and a centrifugal washer have been developed. Large aliquots (500 ml) can be fully washed in as little as 3 minutes. As a result of this speed, autologous blood salvage has become practical in situations in which blood loss may be extremely rapid, such as trauma or liver transplantation.

The hematocrit of unwashed blood is typically low because of dilution from irrigating surgical fluids and some degree of mechanical hemolysis. [62] Free hemoglobin levels are sometimes greater than 1,000 mg/dl in unwashed blood, and hemoglobinemia and hemoglobinuria may occur after the transfusion, but renal sequelae are surprisingly infrequent.[61] [63] Despite this evidence of red cell injury, the survival rate of [51] Cr-labeled salvaged cells is normal in most patients studied. [64] [65]

Transfusion of salvaged blood has resulted in coagulation abnormalities in animals and humans, including hypofibrinogenemia, prolonged prothrombin time and partial thromboplastin time, elevated concentrations of fibrin degradation products, and thrombocytopenia. [15] [66] Some authors originally interpreted these results as evidence of disseminated intravascular coagulation, possibly incited by phospholipids and other material released from damaged red cells and platelets. A more likely alternative is that the laboratory data reflect the characteristics of the salvaged blood itself, which, after exposure to serosal surfaces, becomes deficient in coagulation factors and platelets and, in the case of unwashed blood, has high levels of fibrin degradation products (Table 144-2).[67] In those few cases in which the progression of coagulation abnormalities over time has pointed to disseminated intravascular coagulation rather than simple hemodilution, [14] [68] the patients underlying condition (shock due to massive hemorrhage) appears to have been the cause. A relationship between postoperative coagulopathies and salvaged autologous blood remains tenuous.

Fat, fibrin, bone fragments, and microaggregates often contaminate salvaged autologous blood. However, infusion of unwashed blood has not been proved harmful in either animals or humans, possibly because most particulate material is removed by routine blood filters. [69] [70] Other contaminants, such as heparin, topical antibiotics, hemostatic agents, and biological substances such as tissue enzymes, can be removed, but not necessarily completely, by washing. [71] [72] Complete removal of bacteria also is not possible, even when the salvaged blood is washed with antibiotics. [73] Thus, collection of blood from a contaminated site (e.g., with intestinal contents) is probably contraindicated, although some investigators have argued that, if no other blood is available, such transfusions may be lifesaving. [74] [75] Tumor cells have also been found in blood salvaged during cancer operations; their malignant potential after salvage and transfusion is unknown, and many consider cancer another contraindication. [76] [77]

Approximately one-half the blood lost during surgery can be salvaged; the rest is usually irretrievably absorbed in drapes and sponges or damaged during collection.[65] The use of salvaged autologous blood has been associated with a 50% reduction in allogeneic blood use in orthopedic procedures such as spinal surgery[78] and hip replacement[79] [80] and is also effective in such vascular surgical procedures as aortic reconstruction. [81] In cardiac surgery, the largest volume of blood that can be processed for return to the patient comes from the membrane oxygenator.[82] [83] Although the blood is technically not shed, in that it is still in the extracorporeal circuit, the processing is helpful in concentrating the red cells and removing cardioplegic solutions. Autologous blood salvage has also proved useful in large volume blood loss such as that occurring in liver transplantation, in which the volume salvaged averaged 25 units in one study, [84] [85] and in trauma.[86] Blood has also been salvaged from the hemoperitoneum associated with ectopic pregnancy,[87] during radical prostatectomy, [88] and during splenectomy.[89] Autologous salvage has been a useful adjunct in the treatment of some Jehovahs Witnesses, whose literal interpretation of the Bible includes abstention from routine allogeneic blood transfusion.[90]

Both the cannister systems and red cell processors used to collect intraoperatively shed autologous blood can also be employed to collect postoperative blood drainage, such as that from the mediastinum after open heart surgery, [91] from the knee or hip after orthopedic procedures, [92] or from the peritoneal cavity after hepatic injury.[93] Because blood salvaged from a serosal cavity has little residual fibrinogen and platelets, clotting is not a problem, and the addition of anticoagulants is usually unnecessary.[94] As in intraoperatively salvaged blood, free hemoglobin levels are substantially elevated. Nonetheless, red cells in the shed blood exhibit normal survival on radioisotopic studies. [95] Shed mediastinal blood following open heart surgery contains high levels of cardiac muscle enzymes, especially creatine kinase, as well as lactate dehydrogenase from hemolyzed red cells; [96] [97] reinfusion of shed blood results in elevated levels of both enzymes and can confound the diagnosis of myocardial infarction in the postoperative period. [98] [99] Reinfusion of shed mediastinal blood reduces the need for allogeneic transfusions. [100] However, the volume of red cells salvaged may be variable. For example, although the volume of postoperative drainage is often substantial in orthopedic surgery, [101] [102] red cells may represent only a small portion; thus, one study reported a mean of only 55 ± 29 ml of red cells in drains following hip surgery. [103] Arthroplasty procedures performed without cement are associated with larger perioperative blood losses, and postoperative red cell salvage may be more effective in such cases. [104]

Hemodilution

The collection of autologous blood during surgery for later reinfusion at the end of the procedure was first suggested in open heart operations, in which it was hoped that a supply of platelets undamaged by exposure to the membrane oxygenator might reduce the incidence of coagulopathies. [105] Hemodilution itself reduces red cell loss: a patient with a hematocrit of 45% and a 2-liter blood loss during surgery loses roughly 900 ml of red cells but with a hematocrit of 20% from hemodilution loses only 400 ml of red cells. Hemodilution is probably less expensive to accomplish than preoperative autologous blood donation and may be the only option available when surgery is performed in other than elective settings. [106] Proponents claim that the induced anemia may even be beneficial to the patient, in that oxygen delivery at a hematocrit of 30% is enhanced by an increased cardiac output resulting from the decreased blood viscosity. [107]

Reductions in allogeneic blood needs have been reported after marked intraoperative hemodilution (after the hematocrit is lowered by 50%). [108] [109] More modest hemodilution (e.g., removal of 2 units of blood at the beginning of surgery) is also beneficial according to some workers, [109] [110] [111] but not others.[34] [112] [113] [114] Furthermore, one group has provided evidence that hemodilution may jeopardize patients at risk of ischemic myocardial injury. [115] More research is needed to establish the safety and ideal protocols for this form of blood conservation.

FUTURE DIRECTIONS

Even if human immunodeficiency virus and hepatitis could be completely eliminated from blood components, the risk of other transfusion-transmissible diseases (both known and undiscovered) is ample reason for the continued use of autologous blood protocols wherever feasible. Recombinant erythropoietin is likely to play an increasingly important role in facilitating autologous blood donation, as are improvements in blood salvage devices that make their use easier and less costly.

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Chapter 145 - Transfusion Medicine in Hematopoietic Stem Cell and Solid Organ Transplantation

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BONE MARROW TRANSPLANTATION

Introduction

Bone marrow transplantation (BMT) is currently used to treat a broad spectrum of malignant and nonmalignant diseases. Refinements in testing for HLA compatibility, coupled with new approaches to both prevent and treat graft-versus-host disease (GVHD), have resulted in widespread use of allogeneic BMT. [1] Only 40% of patients have HLA-matched related donors; however, allogeneic BMT using either related donors other than HLA-identical siblings or unrelated HLA-matched donors has demonstrated promising preliminary results.[2] Moreover, there is increased use of autologous BMT, with or without purging of marrow tumor cells, to treat both hematologic cancers and solid tumors.[3] Increasingly, hematopoietic transplantation is being effected not only with bone marrow but also with stem cells derived from mobilized peripheral blood (PBSC) [4] and umbilical cord blood (UCB), [5] leading to the more generic term hematopoietic stem cell transplantation (HSCT). [6] These products are being further processed by positive or negative selection, in vitro expansion, and genetic manipulation (see Chap. 142). Likewise, blood components are increasingly being manipulated by irradiation, filtration, and washing, and apheresis components are being selected to provide highly enriched sources of platelets, plasma, granulocytes, and lymphocytes. Taken together, these products are all part of the developing field of hematopoietic cellular therapy in which the blood bank plays a lead role. Whether or not the HSC processing facility falls directly under the blood bank, the transfusion medicine experience is crucial, especially with the expanding interest that the United States Food and Drug Administration (FDA) is taking in this area.

Other chapters in this edition cover in detail elements of HSCT that integrally involve the blood bank, including the mobilization and optimal collection of PBSC, and the functions of the HSC processing facility, including the in vitro manipulation of bone marrow, PBSC, and UCB products. This chapter will restrict its focus to the traditional blood bank role of providing safe and effective blood component therapy to HSCT patients: ABO incompatibility in allogeneic HSCT, alloimmunization, transfusion-associated (TA) GVHD, cytomegalovirus (CMV) infection, basic HSC processing as it relates to good manufacturing practices and federal regulation, documentation of engraftment, immune cytopenia after HSCT, and the use of hematopoietic growth factors to hasten engraftment and to lessen the toxicity of high-dose ablative treatment. Transfusion medicine expertise is essential in all these aspects of HSCT, both in current practice and in furthering research efforts. [6]

Blood Component Support

The blood component laboratory plays a critical role in HSCT by providing appropriate red blood cell (RBC), platelet, and blood component support both before and after HSCT. The criteria for transfusion of blood components to patients undergoing HSCT are not different from transfusion criteria used more generally, but the magnitude of support required is great. The unique nature of HSCT patients, however, does require specific transfusion strategies to avoid alloimmunization, CMV infection, and TA GVHD, as well as strategies to prevent the potential complications of ABO incompatibility in allogeneic transplants. Finally, as is true in other settings, the blood bank should not only determine which products are required but also review ongoing transfusion practice.

Several donor and/or patient factors may influence hematologic engraftment, immune reconstitution, and the blood product support required after HSCT. [7] In all marrow recipients, engraftment may be compromised due to disease and/or treatment-related effects on the marrow microenvironment. Reconstitution after allogeneic HSCT may be relatively enhanced since the donor marrow is healthy; however, engraftment may be adversely affected by regimens employed in the recipient to prevent or treat GVHD. Moreover, in vitro T-cell depletion of donor marrow, which can effectively abrogate GVHD, has in some patients resulted in failure to engraft and graft rejection. Autologous marrow may be intrinsically compromised due to the patients underlying disease and cytotoxic therapy received prior to marrow harvesting, to in vitro techniques used for removal of tumor cells, or to cryopreservation. In syngeneic HSCT, donor marrow is histocompatible and healthy and neither manipulated nor cryopreserved. However, the underlying disease and prior treatment of the recipient may, as is true in other types of HSCT, compromise the marrow microenvironment and thereby adversely affect engraftment.

After HSCT, there is a period of pancytopenia lasting 14 weeks when patients require multiple RBC and platelet transfusions. [6] [8] [9] [10] For example, patients with aplastic anemia undergoing allogeneic HSCT received a median of 9 (182) and 44 (6468) units of RBCs and platelets, respectively, primarily during the first 4 weeks after grafting.[9] Clinical parameters may correlate with transfusion needs. In patients with acute nonlymphocytic leukemia in first remission who underwent HSCT, for example, the method of prophylaxis against GVHD, the development of acute GVHD, the method of prophylaxis against infection, and donorrecipient ABO compatibility all influenced the magnitude of transfusion support required. [11] In particular, patients with grades 3 and 4 acute GVHD required more blood product support than those with grades 1 and 2 acute GVHD: a median of 217 units of platelets and 27.5 units of RBCs in the former group, compared to 91 units of platelets and 14 units of RBCs in the latter patients. In recent years, a major focus has been to reduce the period of pancytopenia by the use of growth factors (e.g., G-CSF, GM-CSF, and erythropoietin) and the use of peripheral blood stem cells (PBSC) grafts rather than bone marrow. The latter, in particular, may reduce the period of pancytopenia to 1014 days, with a significant decrease in platelet and RBC use. [12]

Although complications related to GVHD and infection are fewer in autologous than in allogeneic HSCT recipients, the magnitude of transfusion support nonetheless is also significant in recipients of autografts. In a study of patients with non-T-cell acute lymphoblastic leukemia who underwent either monoclonal antibody (MoAb)-purged autologous HSCT or allogeneic HSCT, autologous marrow recipients engrafted more quickly, had shorter hospital stays, and had fewer early deaths, suggesting that they may have less transfusion requirements than allogeneic HSCT recipients. [13] The blood component laboratory must therefore be an integral part of the planning for and daily management of the HSCT service to meet these demands in a timely fashion.

Importance of ABO Compatibility in Allogeneic HSCT

ABO incompatibility between marrow donor and recipient may be major, with isohemagglutinin in the recipient directed against donor RBC antigens; minor, with isohemagglutinin in the donor directed against recipient RBC antigens; or both (Table 145-1). Major ABO incompatibility carries the risk of severe hemolytic reactions at the time of HSCT, graft rejection, or delayed engraftment in the setting of high-titer hemolytic isohemagglutinin in the marrow recipient. [6] [10] Attempts to overcome major ABO incompatibility have included depletion of RBCs from the bone marrow graft prior to HSCT [14] [15] and/or removal of isohemagglutinin from the recipient by large-volume plasma exchanges or immunoadsorption.[16] In addition, some investigators have supplemented these techniques with pre-HSCT transfusions of donor type blood or purified A or B substance to adsorb recipient isohemagglutinins completely. [17] [18] Although studies suggest that major ABO-incompatible HLA-matched transplants have resulted in no increase in patient mortality, incidence of rejection, delayed engraftment, or GVHD compared with ABO-compatible controls,[19] [20] [21] some reports suggest that RBC engraftment can be delayed in this setting.[6] [10] [15] Current practice in major ABO-incompatible HLA-matched HSCT is to deplete RBCs from all marrow before HSCT, but to use methods for depletion of recipient isohemagglutinin only when it is present in high titer and may result in delayed erythropoiesis and hemolysis after HSCT. Prolonged red cell aplasia despite adequate granulocyte and platelet engraftment has been described in a number of case reports. Therapies have included plasmapheresis, high-dose steroids, and erythropoietin, with variable results to date. [22] [23]

Potential adverse outcomes of minor ABO incompatibility between donor and recipient include rapid immune hemolysis at the time of infusion of donor marrow due to passive transfer of isohemagglutinin in the marrow plasma, or delayed immune hemolysis caused by anti-RBC antibodies produced by the donor marrow. [6] [10] There is no effect of minor ABO incompatibility on graft rejection, incidence or severity of GVHD, or patient survival. [19] [20] Although exchange transfusion of the recipient before HSCT using red cells of the donors blood group has been used

TABLE 145-1 -- Examples of Transfusion Restrictions After ABO-Incompatible HSCT

ABO Type		Type of Mismatch	Transfusion Restriction	
Recipient	Donor		RBC	Plasma
A	A	None	A or O	A or AB
A	O	Minor	O	A or AB
A	AB	Major	A or O	AB
A	B	Major/Minor	O	AB

to prevent hemolysis caused by passive transfer of isohemagglutinin in the marrow product, this is rarely a clinically significant problem and can more easily be avoided by removing plasma from the marrow prior to infusion. Minor ABO incompatibility can result in adverse reactions due to the production of anti-A and/or anti-B antibodies by donor marrow lymphocytes early (13 weeks) post-transplant, particularly in patients on cyclosporine therapy or those receiving T cell-depleted allografts.[24] [25] [26] This may be particularly severe and associated with reactive hemolysis in the setting of minor ABO-incompatible HLA-matched HSCT using unrelated donors.[27] The rapidity of engraftment and early related production of isohemagglutinins is somewhat surprising, given the delay in humoral reconstitution noted after HSCT.[7] Current practice is to transfuse either group O or donor group RBCs to dilute the recipient red cells and to monitor patients closely for evidence of hemolysis; in rare cases, exchange transfusion has been required due to very rapid engraftment of donor lymphocytes, production of anti-RBC antibodies, and related hemolysis. As a general principle, ABO-incompatible transplants require separate specific restrictions in terms of the type of RBC and plasma to be infused (Table 145-1). RBCs should be compatible with both donor and recipient plasma, while plasma should be compatible with both donor and recipient RBCs. ABO antigens are widely expressed on endothelial tissues; thus, HSCT recipients may be considered ABO chimeras even after full RBC engraftment.

Alloimmunization and Platelet Refractoriness

Patients undergoing HSCT are dependent on large numbers of cellular blood product transfusions, often before and always after HSCT. Studies suggest that sensitization to HLA antigens increases with number of transfusions and related donor exposures; [28] therefore, patients eligible to undergo HSCT are at high risk to develop alloimmunization. HLA sensitization, in particular, may have adverse effects on response to platelet transfusion and on marrow engraftment. The latter is most evident in the setting of aplastic anemia: an analysis of 625 patients with aplastic anemia who received allografts from HLA-identical donors demonstrated either no or transient engraftment in 68 (11%) patients.[29] Of a variety of clinical parameters analyzed, post-HSCT treatment with cyclosporine and avoidance of pre-HSCT blood transfusions were associated with improved survival. Although graft failure associated with histocompatibility differences between donor and recipient is often attributed to rejection by host T lymphocytes, persistent host antibodies specific for donor antigen may also mediate graft failure, whether by antibody-dependent cell-mediated cytotoxicity or complement-mediated cytotoxicity. Specifically, host anti-HLA class I antibodies have been associated with graft failure and death; host anti-ABO antibodies have persisted for up to 18 months post-HSCT and resulted in erythroid hypoplasia. [30] There is therefore a need to prevent alloimmunization in patients who are candidates for later HSCT. While the use of family members as blood donors pre-HSCT has been reported not to be harmful in patients with malignancy,[31] transfusions from the potential marrow donor should be avoided due to the risk of sensitization of the patient to both HLA and non-HLA antigens in this setting.

Patients are also at high risk to become alloimmunized post-HSCT. In this setting, repeated failure to obtain satisfactory responses to platelet transfusions, as measured by the 1-hour post-transfusion corrected count increment (CCI), is a well-recognized problem. There are many patient-specific, nonimmune causes of refractoriness (Table 145-2) that are common in as many as 67%[32] of post-HSCT thrombocytopenic patients. Platelets, however, express HLA class I antigens, small amounts of ABO, and a number of platelet-specific antigens, all of which

TABLE 145-2 -- Causes of Platelet Refractoriness

Patient Factor	Clinical	Blood Bank
Alloimmunization	Fever	ABO match
Bone marrow transplant	Sepsis	Platelet storage time
Gender	Splenomegaly	Platelet crossmatch
Circulating IV Ig	Clinical bleeding	HLA match
Transfusion number	RBC use	
	DIC	
	Neutropenia	

Adapted from Friedberg et al.,[54] with permission.

may be the target of an immune response. Strategies to prevent alloimmunization and to provide suitable platelet products are a major focus of the transfusion service in treating HSCT patients. A retrospective analysis of patients with severe aplastic anemia who underwent allogeneic HSCT documented the development of refractoriness to transfusion of random donor platelets in 34% of patients; indeed, the number of platelets transfused (if 40 units) as well as lymphocytotoxic antibodies correlated with refractoriness.[33] Finally, Galel et al.[34] also documented alloimmunization in 31% of transplant patients, with similar incidence in recipients of autologous (34%) and allogeneic (27%) recipients. Half of the alloimmunization in each group was demonstrated pre-HSCT. They identified patients with Hodgkins disease and women with children to be at particular risk for developing alloimmunization. Most importantly, they concluded that HSCT does not prevent the development of alloimmunization in either autologous or allogeneic HSCT recipients. It is therefore important to test for anti-HLA antibodies whenever transplant recipients become refractory to random donor platelet transfusion, since response to random donor platelet transfusion is poor in the sensitized host, and HLA-matched, family member, or crossmatch-compatible platelets may be useful in this setting (see Chap. 137).[35] [36] [37] [38] [39]

It would appear that patients undergoing HSCT would be an appropriate group in which to employ strategies to avoid alloimmunization. These include minimizing donor exposure by ensuring the appropriateness of all transfusions, the use of single-donor platelets, [40] [41] or HLA-matched platelets,[42] and/or ultraviolet (UV) irradiation[43] [44] or leukodepletion[45] [46] [47] [48] of platelets for transfusion. Leukodepletion has received particular attention as a means of reducing alloimmunization, as filtration and apheresis technology can now economically provide blood products with <10 ^{6} residual leukocytes. Both prospective and uncontrolled studies have indicated that the risk of alloimmunization and platelet refractoriness can be reduced with the use of leukopoor products. [45] [46] [47] [48] For example, Saarinen et al.[46] showed that 0 of 26 pediatric patients receiving leukopoor blood components became refractory, compared to 11 of 21 historical control patients who received standard products. Likewise, van Marwijk Kooy et al.[45] found in a prospective randomized trial that 46% (12 of 26) of patients receiving standard platelets (mean of 35 × 10^{10} leukocytes/dose) became clinically refractory, while this occurred in only 11% (3 of 27) of patients receiving leukopoor products. De novo anti-HLA antibodies were detected in most refractory patients in both groups. Further, recipients of leukopoor products who became refractory did so later (>4 weeks) than the standard therapy group.

Most recently, the TRAP trial[49] has reported that there was no difference in the rate of alloimmunization when comparing pooled, filtered platelet concentrates, filtered single-donor apheresis platelets, and UV-B-irradiated platelet concentrates in patients with newly diagnosed acute myeloid leukemia. Although still the subject of investigation, these data may imply that leukodepletion by filtration is the most effective method for the prevention of alloimmunization. Further, the timing of filtration may be important. An animal model study[50] has suggested that prestorage filtration is more effective than poststorage filtration in preventing primary refractoriness. Indeed, in a prospective study of aplastic thrombocytopenic patients receiving prestorage leukoreduced platelets and RBC, Novotny et al. [51] found that anti-HLA antibodies developed in only 3 of 112 (2.7%) patients with negative risk factors for prior HLA sensitization. Conversely, 16 of 52 (31%) patients with a history of prior pregnancy or transfusion of nonleukoreduced blood developed antibodies. Nevertheless, most (95%) of the alloimmunized patients could be supported with prestorage leukoreduced products without the need for HLA-matched or crossmatched platelets.

Available data suggest that leukodepletion does not prevent secondary alloimmunization in recipients previously sensitized by transfusion or pregnancy, and there is as yet no effective means of reversing alloimmunization once it occurs. Taken together, prevention of alloimmunization is probably best achieved by the combination of prestorage leukodepletion and limiting donor exposure, avoiding unnecessary transfusion, and the use of single-donor apheresis platelets. Overall, the only potential adverse effect of depleting leukocytes from blood products would be removal of a purported graft-versus-leukemia effect, [52] but whether white blood cells within transfused cellular blood components mediate such an effect remains controversial. [53]

Once platelet refractoriness is documented, there are a number of strategies that may assist in obtaining therapeutic platelet concentrations in vivo. Obviously, if a nonimmune cause is apparent such as sepsis or disseminated intravascular coagulation, treatment of the underlying cause is indicated, and the use of ABO-matched, fresh (12 days old) platelets may be beneficial. [54] A recent survey showed that nonimmune causes may be present in up to 88% of patients treated for hematologic malignancy that become platelet-refractory,[32] while immune causes could be demonstrated in 25%, either by positive lymphocytotoxicity assay or in platelet crossmatch. In the latter cases, the use of HLA-matched platelets or crossmatched platelets are indicated. Even so, overall 4050% of HLA-matched or crossmatched platelets fail[39] to produce an expected increment. Due to the polymorphism of the HLA loci, it is often not possible to find complete (grade A) HLA-A and -B locus matches, leading to the use of platelets mismatched at one or more loci. Available evidence suggests that the optimal response requires either a grade A or BU match (Table 145-3), in which case up to 80% of transfusions may be successful.

TABLE 145-3 -- Degree of HLA Matching for HLA-Matched Platelets

Match Grade	Description	Examples of a Donor Phenotype for a Recipient Who Is A1,3;B8,27
A	Four antigen match	A1,3;B8,27
B	No mismatch present	
B1U	One antigen blank or unknown	A1,-;B8,27
B1X	One crossreactive group	A1,3;B8,7
B2UX	One antigen blank and one crossreactive	A1,-;B8,7
C	One mismatched antigen	A1,3;B8,35
D	Two or more antigen mismatch	A1,32;B8,35

Data from Vengelen-Tyler.[116]

Cytomegalovirus Infection

Cytomegalovirus infection and disease is a major cause of morbidity and mortality in immunocompromised patients. Especially at risk are CMV-seronegative patients who receive allogeneic BMTs, as these patients receive the most intensive conditioning regimens and are further immunocompromised by GVHD and its prophylaxis and treatment. Such patients may demonstrate a 2857% infection rate, with approximately 30% developing CMV disease, including interstitial pneumonitis and gastroenteritis, more often with fatal outcomes, despite significant advances in anti-CMV treatment protocols. [55] [56] Cellular blood components transfused from CMV-seropositive donors to seronegative recipients are a major cause of seroconversion, [56] as evidenced by early studies with prophylactic granulocyte infusions showing that lack of seroconversion was found only in seronegative recipients of granulocytes from seronegative donors. Transfusion with seronegative blood products appears to lessen the CMV infection after allogeneic HSCT when both donor and recipient are seronegative, but not when either is seropositive. [57] [58]

In some studies, treatment of patients with immunoglobulin has been efficacious in this setting and may also reduce CMV sequelae in seropositive recipients. [59] The use of both immunoglobulin and CMV-seronegative blood products appears to confer no additional benefit. CMV antigen can be detected in peripheral blood leukocytes after allogeneic transplantation, but whether antigenemia is more sensitive than rapid culture methods to focus antiviral prophylaxis in HSCT patients remains to be determined.[60] CMV infection, strongly associated with GVHD,[61] may become less frequent in all patients undergoing allogeneic HSCT due to the development of effective prophylaxis for GVHD. In addition, acyclovir can lessen the incidence of CMV infection and related morbidity, thereby improving the survival of seropositive allogeneic HSCT recipients. [62] [63] Finally, reports document successful therapy for CMV pneumonia that has developed after HSCT, with ganciclovir and either intravenous immunoglobulin or CMV immunoglobulin. [64] [65] On the basis of these and other studies, it has been the practice to reserve the exclusive use of CMV-seronegative blood components for allogeneic HSCT when both the donor and recipient are seronegative.

Cytomegalovirus infection in autologous HSCT recipients is less common, perhaps related to the rarity of GVHD in autologous HSCT recipients. Indeed, equivalent numbers of autologous and allogeneic HSCT recipients seroconvert to or excrete CMV, but clinical sequelae are less common than in allogeneic HSCT recipients. [66] Therefore, methods for CMV prophylaxis, such as immunoglobulin, seronegative blood products, or post-transplant acyclovir therapy, have not been used in autologous HSCT recipients. However, pretransplant CMV serology is also predictive of CMV infection after autologous HSCT [67] and engraftment may be delayed after autologous HSCT in patients with CMV infection. Moreover, analysis of 159 autologous HSCT recipients, all of whom received CMV-unscreened blood products, documented a probability of CMV infection in 22.5% in CMV-seronegative patients and 61.1% in CMV-seropositive recipients. [68] In this series, CMV pneumonia developed in 11 patients at a median of 100 days post-HSCT and was fatal in 9 cases. This study concluded that the risk of CMV disease is not negligible. While previously the standard of practice was not to use CMV-seronegative blood products in the setting of autologous HSCT, the recent acceptance of widely available, leukodepleted components as "CMV-safe" (see following) has led many centers to use these components in all HSCT recipients, both autologous and allogeneic.

The traditional source of CMV-negative blood components has been blood derived from seronegative donors, identified by either enzyme-linked immunoassay or latex agglutination testing. As CMV seropositivity may vary from 50% to 90% in different communities, seronegative donors are often in limited supply. While the use of CMV-seronegative blood components in seronegative recipients greatly reduces CMV transmission, a CMV infection rate of 14% is still observed, most likely due to the inadequacies of the serologic testing methods employed and the natural background rate of seroconversion. [69] In the last two decades there has been mounting evidence that latent CMV resides in the leukocytes and that their removal can render seropositive blood CMV-safe. Early trials of leukoreduced blood products in this setting were uncontrolled and hampered by the relatively crude methods available for leukoreduction. As early as 1977, an open trial in seronegative heart surgery patients showed that whole blood leukoreduced by centrifugation (58% reduction of leukocytes) resulted in 12.5% seroconversion, as compared to 67% in uncentrifuged blood recipients.[70] Since then a number of reports have shown decreased seroconversion with blood products leukoreduced by saline washing,

freeze-deglycerolization, and filtration. For example, filtration of red cells was shown to decrease transfusion-acquired CMV infection in infants, [71] and the use of filtered blood components was shown to decrease CMV seroconversion in patients undergoing treatment for acute leukemia. [72] [73] Data from Seattle suggest that provision of filtered red cells and platelet transfusions from unscreened donors to CMV-seronegative patients undergoing autologous HSCT may prevent seroconversion or infection in the recipient. [74] In addition, De Witte et al. [75] showed that leukocyte depletion of blood products by centrifugation, coupled with acyclovir prophylaxis, may also prevent primary CMV infection in CMV-seronegative allogeneic HSCT recipients. Each of these studies has supported the idea that leukoreduction significantly decreases the risk of CMV transmission.

Most recently, Bowden et al.[76] reported a randomized prospective trial of >500 HSCT patients selected to receive either seronegative products or filtration-leukoreduced products to test the hypothesis that they are equivalent at preventing CMV transmission. Although the conclusion of the studythat the rates of CMV transmission are no different for the two preparationshas been widely accepted, the study has been criticized on a number of counts that should be considered carefully. The statistical analysis excluded patients who seroconverted within 21 days of HSCT, on the basis that these patients must have been exposed to CMV prior to transplant. Five patients seroconverted in the first 21 days, and their inclusion significantly affects the results of the study (Table 145-4); some patients in the leukocyte reduction arm received seropositive unfiltered blood in error; and finally, bedside filtration was used, which has subsequently been shown to suffer from a relatively high rate of failure. [77] [78] Nevertheless, patients receiving leukoreduced blood showed a low level (2.4%) of CMV infection, which is in keeping with the historical figures of 14% CMV seroconversion with the use of CMV-seronegative components. The authors conclude with the statement: We believe that the results

TABLE 145-4 -- Incidence and Actuarial Probability of CMV Infection and Disease After BMT by Study Arm

	Analysis Period (days post-BMT)	Actuarial Probability		p Value
		Seronegative (%)	Leukoreduced (%)	
CMV infection	21100 days	1.3	2.4	1.00
CMV disease	21100 days	0	1.2	0.25
CMV infection	0100 days	1.4	2.4	0.50
CMV disease	0100 days	0	2.4	0.03

Data from Bowden RA, Slichter SJ, Sayers M et al.[76]

of this study justify abandoning the maintenance of dual inventories of seronegative and unscreened blood products. In fact, the need to perform serologic screening of blood products of CMV could be eliminated altogether. [76]

Leukoreduced components are now widely accepted as being equivalent to seronegative products for the prevention of CMV transmission. Efficient means of leukocyte removal from platelets and red cells using filtration methods have been developed, and the AABB has set standards that define leukopoor blood as containing $<5 \times 10^6$ leukocytes.[79] It is not clear what the optimal level of leukodepletion is to prevent CMV transmission; however, 5×10^6 represents a reasonable goal attainable by current technology.

The widespread availability of CMV-safe leukoreduced blood raises the question as to whether there is a role for the use of such components in CMV-infected HSCT patients. Two factors need to be considered: second-strain infections and viral reactivation. In solid organ transplants, it is well documented that CMV-positive recipients may develop CMV disease derived from the viral strain associated with the graft. [80] To date, there is no substantial evidence of second-strain infections associated with HSCT or transfusion. Of greater concern in HSCT is the possibility that transfusion may lead to reactivation of latent infections in the CMV-seropositive recipient. For example, Adler et al. [81] observed that CMV-seropositive cardiac surgery patients who receive CMV-seronegative blood are more likely to show a postsurgery increase in anti-CMV titer than those who received no blood products. Other studies have shown that CMV reactivation is higher than the background rate in transfused sick patients. The possibility that transfusion in itself may cause viral reactivation, and whether this activation can be prevented by the use of leukoreduced blood components, is the subject of a current large NIH-sponsored trial (Viral Activation Transfusion Study) among patients with human immunodeficiency virus (HIV) infection.

The AABB has published recommendations based on the above studies that suggest the use of CMV-seronegative or leukoreduced blood components for all CMV-negative HSCT recipients, both allogeneic and autologous, as well as all CMV-negative HIV-infected patients, pregnant women, and patients receiving chemotherapy likely to induce severe neutropenia. [69] It is left to individual physicians to decide whether the equivalent CMV-positive patient group should receive similar CMV-safe products, pending the outcome of the viral activation studies.

Transfusion-Associated Graft-versus-Host Disease

All patients undergoing HSCT are at risk for the development of TA-GVHD after receiving cellular blood products. The most commonly reported manifestations are skin rash, abnormal liver function tests, and severe pancytopenia. The degree of pancytopenia has generally been profound, perhaps related to the HLA disparity evident between donor and recipient in TA-GVHD. In contrast, GVHD occurs after allogeneic HSCT when marrow and donor have been chosen by virtue of histocompatibility. Transfusion-associated GVHD results in an overall 84% mortality rate after a median of 21 days (range 81,050 days). [82]

Transfusion-associated GVHD is effectively prevented by gamma irradiation of the blood product prior to transfusion. Moreover, if any transfusions are given to the marrow donor during marrow harvest, they must also be irradiated. A survey of blood component irradiation practices in the United States in 1991 found that 88% and 81.4% of allogeneic and autologous HSCT recipients, respectively, received irradiated cellular components. [83] Although it is reassuring that the overwhelming majority of patients received irradiated components, it is disturbing that not all patients received them. A recent documentation of several cases of fatal TA-GVHD in recipients of autologous HSCT[84] further suggests that irradiation of cellular components transfused to HSCT recipients is not yet uniform practice. Doses of radiation used to treat cellular blood components before transfusion have varied in the past from 1,500 to 3,500 rads; [83] however, at least one case of TA-GVHD has been documented following irradiation with 1,500 rads, [85] and recent studies suggest that irradiation to 2,000 rads is required to reduce mitogen-responsive lymphocytes by 5 to 6 logs compared to unirradiated controls.[86] [87] Based on these observations, the FDA has established guidelines regarding irradiation doses of blood products intended for clinical use. These guidelines require 2,500 rads (25 Gy) to the central part of the container, with 1,500 rads (15Gy) as the minimum dose to any other point. [88]

A potential alternative method to prevent TA-GVHD would be to deplete lymphocytes from blood products prior to transfusion. It has been demonstrated in murine systems, as well as in humans, that T lymphocytes mediate GVHD and that the incidence and severity of GVHD after allogeneic HSCT can be reduced if T cells are eliminated from the donor marrow prior to grafting by a variety of techniques. [89] [90] [91] While techniques are available for the preparation of leukopoor red cells and platelets that contain 10^6 lymphocytes, cases of GVHD after transfusion of leukopoor components have been reported. [92] [93] A canine model has been used to demonstrate that UV rather than gamma irradiation of transfused leukocytes can abrogate TA-GVHD in recipient animals. [94] However, future studies are needed to determine whether UV light can avoid TA-GVHD in transfusion recipients without adverse effects on in vitro function or in vivo recovery of UV-treated red cells or platelets. At present, irradiation remains the only widely accepted method of preventing TA-GVHD, although it is associated with RBC membrane changes that shorten their life span and potassium leakage that may pose problems in pediatric patients and those undergoing massive transfusion. [86]

Finally, immunocompetent patients may also be afflicted with TA-GVHD. Risk factors include the use of fresh blood, transfusion of recipients who share an HLA haplotype with HLA homozygous blood donors,[95] [96] and the recipients of HLA-matched platelet transfusions. Homozygosity for HLA types is more likely to occur among first-degree family members (e.g., parents, children, and siblings) and in population groups with limited HLA heterogeneity. Thus, the risk of transfusion of blood from HLA homozygous donors to unrelated HLA heterozygous patients is 1 in 874 in Japan and may be as high as 1 in 7,174 in the United States. [97] Petz et al.[98] pointed out that the incidence of TA-GVHD in the United States is substantially less than the population at risk. While underreporting may play a role, it is likely that other factors, including the use of blood that is stored for up to 6 weeks (with decreased leukocyte viability) and the rejection of HLA homozygous cells based on minor transplantation antigens, may play a role. Nevertheless, TA-GVHD is an eminently preventable disorder that is often untreatable once diagnosed. The FDA and AABB[88] now require that all transfusions from (1) any donor who is a blood relative of the recipient; (2) a donor unit intended for intrauterine transfusion; (3) to an immunoincompetent or immunodeficient recipient; or (4) a recipient of HSCT be irradiated. There is also a strong rationale for irradiation of products given to any

patient receiving intense chemotherapy, HLA-matched products, [99] [100] or granulocyte transfusions.

Prestorage Leukodepleted Products and Febrile Nonhemolytic Transfusion Reactions

Adverse consequence of residual white blood cells within platelet and RBC products include febrile nonhemolytic transfusion reactions (FNHTRs), alloimmunization, transmission of infection, GVHD, and immunomodulation.[101] Current filtration and apheresis technologies can yield leukopoor cellular components, RBCs and platelets that contain <10[6] residual leukocytes. [45] [46] [47] [48]

As noted above, it would appear that transfusion of such components can abrogate alloimmunization and avoid CMV transmission. Leukoreduced products are also beneficial in preventing FNHTR. Defined as an increase in temperature >1°C during or soon after transfusion, these reactions occur more commonly with platelet transfusions (30.8%) than with RBCs (6.8%).[102] The routine use of antipyretics may prevent most episodes of fever, but not the accompanying chills, cold, and discomfort. It was thought that these reactions were due to antibodies in the recipient to donor leukocyte antigens (including HLA), and it was known that their incidence could be reduced by removal of leukocytes prior to transfusion. [48] In a series of seminal papers, Heddle et al. [102] [103] [104] showed that FNHTRs to platelets correlate not with white cell content, but with the age of the product. Further, if the plasma is removed from platelets and infused separately, reactions occur significantly more often to the plasma than to the platelet content. These data support the concept that many FNHTRs are caused by cytokines released by leukocytes in blood components. Indeed, these and other authors have demonstrated a progressive rise in interleukin (IL) 1-, IL-6, IL-8, and tumor necrosis factor on storage of platelet and RBC products. [102] [105] [106] [107] [108] This cytokine increase is abolished by prestorage leukodepletion, which may provide an alternative explanation for the prior observations that leukoreduction decreases the incidence of FNHTR.

In summary, it would appear that transfusion of exclusively leukoreduced cellular blood components has proven efficacy in abrogating alloimmunization in patients at risk, in preventing CMV transmission, and in reducing the occurrence of FNHTR. Taken together, these findings provide a strong rationale for the exclusive use of leukoreduction products for HSCT recipients, [109] with leukoreduction preferably occurring soon after collection (prestorage) to maximize the benefit with respect to reduction of cytokine accumulation and FNHTR.

Federal Regulation and Bone Marrow Processing

As the use of BMT has increased as a treatment strategy, the blood component laboratory has become increasingly involved in HSC processing. Historically, bone marrow was not a licensed blood component, and no federal regulations concerning its collection, transportation, processing, storage, or transfusion existed. The FDA has signaled its intent to begin active regulation, and it is instructive to review the history of such regulation in blood banks: blood components are regulated both as drugs and biologicals. Good manufacturing practice regulations were defined for the drug industry in 1962 and were incorporated into law for both drugs and blood in the late 1970s. The Clinical Laboratory Improvement Act in 1967 and its amendment in 1988 specified additional regulations for blood bank laboratories. Early on, the blood industry was regulated primarily under the biologicals regulations in the Code of Federal Regulations. [110] In the 1980s the FDA began enforcing standards for drugs. Examples of such standards include the use of current good manufacturing practices, equipment validation, personnel proficiency and competency testing, and the establishment of independent quality assurance units within each establishment. [111] The blood industry responded by voluntary self-policing through the standard-setting and inspection functions of the AABB. [112] Nevertheless, many hospital blood banks are now subject to multiple regular inspections by the FDA, AABB, and Joint Commission for the Accreditation of Health Care Organizations. In 1995 the FDA proposed extending these regulations to HSCs, envisioning at least three categories: minimally manipulated products, which include bone marrow and PBSCs that are reinfused either directly after collection or after cryopreservation; manipulated products, which would include any enrichment strategy or genetic manipulation; and UCB transplants. The latter two categories would be subject to the investigational new drug (IND) process whereby the FDA controls clinical drug trials. [113] [114] The draconian nature of these proposals has been a cause of major concern in the HSCT community. More recently, the FDA has reaffirmed its intent to regulate highly manipulated HSC products, including genetically altered or in vitro expanded cells, by the IND process, and has proposed a different approach for minimally manipulated stem cell products. The latter now includes positively and negatively selected HSC from PBSC and UCB. These products would not require IND approval but would be subject to similar regulations as are in place for standard blood components, such as a requirement for establishment registration with the FDA and regular FDA inspection. [115]

In response to these developments, the community has begun to develop self-policing mechanisms. The AABB has published standards for HSC laboratories since 1993.[116] [117] Recently, the International Society for Hematotherapy and Graft Engineering and the American Society for Blood and Marrow Transplantation have established the Foundation for Accreditation of Hematopoietic Cell Therapy as an independent standard-setting and inspection entity devoted to establishing enforceable standards for the collection, storage, and manipulation of HSC grafts and the clinical practice of HSCT. [117] [118]

These developments make it clear that HSCT will be increasingly regulated and inspected. The blood bank, with many years of experience in these matters, offers a logical source of leadership in ensuring compliance with federal regulations and the provision of safe, effective HSC grafts.

Previous chapters dealt with the collection of PBSCs and the manipulation of HSC grafts to decrease GVHD and graft rejection while enhancing graft-versus-tumor effects, as well as the rapidly developing area of gene therapy. We will only briefly review the principles of processing here. Basic HSC processing may vary from performing simple cell counts and bacterial cultures prior to immediate reinfusion of allogeneic HSC to processing consisting of several steps, including concentration and washing; sedimentation with hydroxyethyl starch, Ficoll-Hypaque, or Percoll; positive or negative (purging) selection; cryopreservation and storage; and thawing and transfusion. The first goal of HSC processing is depletion of RBCs, plasma, and fat with minimal loss of progenitor cells. Marrow processing was originally done manually by simple centrifugation; at present, cell separators are used for the preferential concentration of progenitor cells with increased elimination of other hematopoietic cells. A variety of automated procedures have now been published for marrow processing using various cell separators, and engraftment has been documented after transfusion of such marrow.[119] [120] [121] [122] As is true in the preparation of other blood components, it is also critical to develop techniques to avoid bacterial contamination during the collection and processing of bone marrow. [123] [124] [125]

In allogeneic BMT, purging has been used to deplete T cells in donor marrow before grafting to prevent GVHD. [90] [126] Although the incidence of GVHD has thereby been decreased, concomitant increases in graft failure and rejection and relapse rates have lessened enthusiasm for this approach to abrogate GVHD.

HSC processing may also include depletion (purging) of tumor cells from autologous marrow or PBSC before grafting. The various methods of purging are based on physical properties, sensitivity to chemotherapy, and/or expression of cell surface antigens, [126] and may involve either negative selection of tumor cells or positive selection of stem cells. Each of these has its own set of optimal conditions, some of which have been well defined and others of which are still under investigation. One may postulate that malignancies that either arise from the marrow (e.g., leukemias, myelomas) or are histologically evident within the marrow (e.g., relapsed lymphomas, breast cancer, neuroblastoma) will require purging. Purging of tumor cells from

autologous marrow is being evaluated to allow patients with overt tumor involvement to undergo autologous BMT and to deplete subclinical residual malignant cells when tumor is not evident on cytopathologic examination. In particular, three lines of evidence suggest that there may be up to 10^8 tumor cells in marrow when histopathologic examination is normal: (1) the ability to derive tumor cell lines from marrows that appear to be pathologically normal; [127] [128] (2) the observed time to relapse and known tumor-doubling times in patients with hematologic cancers, which suggest that tumor cells were present when not pathologically evident; and (3) the use of more sensitive techniques, such as gene rearrangement or polymerase chain reaction (PCR) technology, [129] [130] which can confirm the presence of a single malignant cell within 10^6 marrow cells.

The conditions for pharmacologic purging of autologous marrow tumor cells on a cell separatorthat is, marrow RBC and drug concentrationhave been established. [131] [132] Moreover, when using cell separator technology for MoAb-based purging of autologous marrow tumor cells, a 45 $\log_{10}$ depletion can be achieved using only 50% of the MoAb and complement in 50% of the time required for manual techniques.[131] Purging of tumor cells from autologous grafts prior to BMT has permitted this approach to be used in patients with up to 20% histopathologic involvement with tumor. [133] PCR has demonstrated that immunologic purging using anti-B cell MoAbs and complement lysis could successfully remove PCR-detectable lymphoma cells from autografts in only half of the patients; [134] importantly, those patients who were reinfused with autologous marrow containing residual lymphoma cells detectable by PCR had an increased incidence of relapse. [131] Sharp et al.[135] have used a

culture technique sensitive for detecting occult lymphoma cells in marrow to analyze histologically normal marrow harvested from patients with lymphoma who were candidates for high-dose therapy and autologous BMT, and showed that detection of lymphoma cells in marrow was an adverse prognostic factor, independent of other clinical features. Finally, Gorin et al. [136] examined 263 patients with standard-risk acute myelocytic leukemia in first complete remission who underwent autografting after total body irradiation, and noted a higher leukemia-free survival rate and a lower relapse rate in recipients of mafosfamide-purged marrows than in recipients of nonpurged marrows. Most importantly, Rill et al. [137] used gene marking techniques to demonstrate directly that tumor cells contaminating a bone marrow graft can contribute to disease relapse. [137] These authors used retroviral-mediated gene transfer of neomycin resistance to mark the bone marrow cells of eight patients with neuroblastoma in clinical remission. Marked marrow was reinfused at the time of autologous BMT. Three patients relapsed within 8 months of BMT. Analysis of tissue derived from these patients demonstrated that the neomycin-resistant tumor cells were present in each case. Similar results have been reported by Deisseroth et al.[138] in chronic myelogenous leukemia (CML) patients. These experiments are not able to address the issue of whether relapse is exclusively derived from contaminating cells in the marrow graft, a finding that would argue strongly for purging of autologous grafts. An alternative strategy, indirect purging by positive selection of HSCs, has found recent favor, with at least three CD34 selection systems at or near commercialization. [139] [140] [141] These systems all use the CD34 expression of hematopoietic precursor cells for positive selection, with yields and purities in the 5090% range. In this manner, a 1010,000 fold depletion of contaminating tumor cells is achieved. Numerous studies have now demonstrated that CD34-selected cells can engraft and reconstitute the hematopoietic system; however, the clinical efficacy of such treatment in reducing tumor relapse is the subject of ongoing clinical trials. CD34-selected HSCs also provide the ideal substrate for gene therapy trials and for in vitro expansion experiments.

A major component of current good manufacturing practice in both autologous and allogeneic HSC processing is quality control of the product at each step in the procedures. These assays include the viability and sterility of the product and an assessment of the number of stem cells present, using blood stem cell assays. Although there is as yet no standardized assay for the pluripotential HSC, routinely used assays include colony forming units-granulocytes/macrophages (CFU-GM) and CD34+MNC content.[142] [143] Ultimately, rapid and complete engraftment remains the true test of successful in vitro manipulation.

Autologous and allogeneic HSC products that are not infused on the same day as collection are depleted of erythrocytes, granulocytes, and plasma; frozen in 10% dimethylsulfoxide (DMSO) in a rate-controlled freezer, set to cool at a constant 1°C per minute; and then stored until the time of infusion in either liquid or vapor phase of liquid nitrogen. [144] Alternatively, recovery of CFU-GMs from unfractionated bone marrow stored in vapor phase liquid nitrogen has been reported to be higher in 5% DMSO and 6% hydroxyethyl starch than in that from marrow stored in 10% DMSO alone.[145] Moreover, marrows frozen by simple immersion in a 80°C freezer, without controlled-rate freezing, and stored at this temperature have resulted in satisfactory engraftment after autotransplantation. Quality-control measures in both cryopreservation and thawing again must ensure both sterility and viability of stem cells.

The RBCs that are separated from donated marrow can be transfused to the marrow donor, either the patient undergoing autologous BMT or the allogeneic marrow donor, after the marrow harvest.[146] [147] Homologous transfusions can thereby be avoided.

Documentation of Engraftment

Use of HSCT in the treatment of patients with hematologic malignancies is based on the assumption that the high doses of chemotherapy eradicate the malignant clone, but also result in permanent ablation of host hematopoiesis and lymphopoiesis. Ablation of the recipients marrow eliminates cells that could cause rejection of the donor bone marrow and also provides space for engraftment of donor cells. Infused HSCs, then free of competition, can replace recipient marrow and re-establish normal hematologic and immune function.

Although the transplant preparative regimens are thought to be ablative, there have been several previous reports demonstrating recovery of recipient hematopoiesis after BMT.[148] [149] These surviving recipient cells can be either normal hematopoietic host cells or tumor cells. When neoplastic cells are identified post-HSCT, they often but not invariably result in clinical relapse. [150] When these cells are normal recipient hematopoietic cells, they can either reject the donor bone marrow cells [151] or become tolerant to donor cells and contribute to the establishment of mixed hematopoietic chimerism. [152] The phenomenon of graft rejection has been studied extensively both in experimental animal models and in clinical transplants, but the phenomenon of coexisting donor and recipient cells has only recently been recognized.

Several techniques have been used to evaluate mixed chimerism after allogeneic HSCT. Cytogenetic analysis has been used frequently to differentiate between donor and recipient cells in patients with sex-mismatched donors; when donor and recipient are sex-matched, characteristic polymorphic regions or satellites can be used to distinguish the donor or recipient origin of the dividing cells. [152] [153] Donor and recipient RBCs have been distinguished by analysis of surface antigens and enzymatic content.[154] [155] This is possible only if donor and recipient differ in a given red cell antigen, and transfusion with red cells bearing this antigen is avoided. More recently, DNA analysis using Y-chromosome probes[156] or probes for highly polymorphic regions on other chromosomes have been used to evaluate

chimerism.[157] [158] These DNA restriction fragment length polymorphisms (RFLPs) are used more often because they allow distinction and quantitation of donor recipient cells in almost all BMT patients also because they can identify the origin of nondividing cells. In an analysis at our institution, mixed chimerism after transplant was found by RFLP analysis in 53% of patients, by cytogenetic analysis in 21% of patients, and by RBC phenotyping in 44% of patients. [159]

Immune-Mediated Cytopenias

Hemolysis can be avoided in the setting of ABO incompatibility between donor and recipient of allogeneic BMT as described earlier. Autoimmune hemolytic anemia has also rarely been reported after allogeneic BMT. [160] After bone marrow grafting, severe unexplained thrombocytopenia and granulocytopenia may also complicate the postgraft recovery of the patient.[161] Antibodies to platelets and granulocytes of donor origin have been demonstrated in recipients of both allogeneic and autologous bone marrow grafts. In the case of autografts, such antibodies are by definition autoantibodies, and similar antibodies after allografting may also have an autoimmune origin. It is likely that this is the result of transient immune system imbalance, common to both allo- and autografts, in the early postgraft period. The extent to which these antibodies affect the peripheral counts probably depends on the ability of the grafted marrow to compensate for the rate of antibody-mediated cell destruction. Patients have, in many cases, responded to conventional therapy including steroids, splenectomy, and intravenous immunoglobulin.

Role of Recombinant Hematopoietic Growth Factors

The hematopoietic growth factors are glycoprotein hormones that regulate the proliferation and differentiation of hematopoietic progenitor cells and function of mature blood cells. Erythropoietin (EPO), a glycoprotein produced in response to hypoxia in the kidney, induces RBC production by stimulating the mitotic activity of erythroid progenitor cellsburst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E) and early erythroid precursor cellsin the bone marrow. Recombinant human (rHu) EPO is already of proven efficacy in the treatment of the anemia of chronic renal failure [162] and anemia in patients with acquired immune deficiency syndrome treated with zidovudine.[163] It may also be useful to treat the anemia of cancer, since endogenous EPO levels may be inappropriately low. [164] [165] [166] Specifically, levels of endogenous EPO have been reported to be inappropriately low for the degree of anemia in patients after both autologous and allogeneic BMT.[166] [167] Supplemental rHu EPO may therefore also be useful to hasten RBC engraftment and to decrease RBC transfusion requirements. [166] [167] [168] In the case of pure RBC aplasia following major mismatched allogeneic HSCT, EPO has also proven efficacious in promoting red cell engraftment. [22] [23]

Infectious complications during prolonged neutropenia remain a major cause of morbidity and mortality in HSCT. The advent of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) for the reduction of the duration and depth of postchemotherapy neutropenia has led to an explosion of their use in oncology services. The clinical experience with G-CSF and GM-CSF has been extensively reviewed, and the American Society of Clinical Oncology has published formal recommendations for their indications for use. [169] [170] [171] [172] With respect to HSCT, it is clear that G-CSF and GM-CSF can successfully shorten the period of neutropenia and reduce infectious complications in patients undergoing high-dose cytotoxic chemotherapy with autologous BMT and PBSC transplantation, although recent reports have questioned the cost-effectiveness and benefit of such therapy in treating febrile neutropenia. [173] [174] G-CSF and GM-CSF are also highly effective at mobilizing CD34+ progenitor cells into the peripheral circulation. These cells may be harvested by apheresis and have been shown to be sufficient for full hematopoietic reconstitution in both the autologous and allogeneic HSCT setting. [4] [175] G-CSF and GM-CSF may also be used to treat patients with graft failure after BMT[176] and appear not to exacerbate GVHD when used early after allogeneic BMT. [177] No formal trial has compared the use of G-CSF versus GM-CSF, but a recent survey suggests that 82% of physicians prefer the use of G-CSF to GM-CSF, based on a more favorable profile of side effects. [178] With G-CSF, this is predominantly in the form of bone pain, which occurs in about 20% of patients given G-CSF after myelotoxic chemotherapy and is responsive to simple analgesics. GM-CSF has been reported to induce lethargy, myalgia, bone pain, anorexia, weight change, skin eruptions, and flushing, all of which are generally well

tolerated by patients.

Cytokines that stimulate platelet production have been a major focus of research, as thrombocytopenia remains an important dose-limiting constraint and a major expense related to chemotherapy. A number of cytokines have been shown to be involved with megakaryocyte differentiation and platelet formation. While clinical data is available on a range of potential thrombopoietic agents, such as IL-1, IL-3, stem cell factor (SCF), and IL-6, the most promising are IL-11 and thrombopoietin (TPO) and its truncated, pegylated form called megakaryocyte growth and development factor (MGDF). [179]

IL-11 has completed phase III trials as a postchemotherapy thrombopoietic drug and a small body of clinical data is available. IL-11 is a pleotropic cytokine expressed in hematopoietic tissue, as well as in brain, spinal cord neurons, gut, and testis. In hematopoietic tissue it stimulates myeloid, erythroid, and all stages of megakaryocytic progenitors, in synergy with IL-3, IL-4, IL-7, IL-12, IL-13, SCF, flt3 ligand, and GM-CSF. [179] In vivo treatment of rodents, nonhuman primates, and humans with IL-11 leads to marked stimulation of megakaryocytopoiesis. Following successful animal studies, IL-11 has been tested clinically. In phase I studies, 16 women with advanced-stage breast cancer on high-dose chemotherapy were treated with increasing doses of IL-11 (up to 100 g/kg/d) before and after four cycles of combined chemotherapy. IL-11 was associated with a dose-dependent attenuated thrombocytopenia after chemotherapy with increased bone marrow cellularity, megakaryocyte maturation, and platelet production. Side effects including a reversible anemia, probably due to fluid retention, arthralgias, myalgias, fatigue, nausea, headache, and edema. No fever was noted, but a rise in acute phase reactants, including C-reactive protein, fibrinogen, and haptoglobin, was apparent. [180]

Tepler et al.[181] extended these studies in a group of 27 cancer patients who had required platelet transfusion during a prior round of chemotherapy. They found that while placebo-treated patients invariably (26 of 27 patients) required platelet transfusions on a subsequent round of chemotherapy, 30% (8 of 27 patients)($p = 0.23$) did not require platelet transfusions when given 50 mg/kg of IL-11 daily. No difference in the number of days of hospitalization, neutropenic fever, or RBC transfusions was noted. More recently, Isaacs et al.[182] randomized 77 women with advanced breast cancer to receive either 50 g/kg/d of IL-11 or placebo for either 10 or 17 days during two cycles of dose-intensive chemotherapy. Again, the use of IL-11 significantly reduced the need for platelet transfusions. These studies suggest that IL-11 has the potential to reduce significantly the duration and extent of postchemotherapy thrombocytopenia. This may allow greater dose intensification and will reduce the costs and risks associated with platelet transfusions in these patients.

Thrombopoietin, or as it was previously known c-MPL ligand, appears to be a specific platelet-inducing cytokine that is

also showing promise in early clinical trials. TPO supports all stages of platelet production in vitro, affecting progenitor proliferation and increasing megakaryocyte number and ploidy in synergy with other cytokines.[183] Thrombocytopenic states that are associated with decreased megakaryocyte number induce high levels of plasma TPO, and preclinical studies show that TPO, and its truncated, pegylated form MGDF, are effective at inducing high platelet counts and reducing chemotherapy-induced thrombocytopenia in nonhuman primate models. Phase I trials of MGDF, or more correctly polyethylene glycol-conjugated (PEG)-rHu MGDF have been reported. Basser et al.[184] administered PEG-rHu MGDF to patients with advanced cancer in incremental doses and found a dose-dependent increase in platelets in the 13 patients receiving drug compared to the 4 patients receiving placebo. Patients receiving 0.3 or 1.0 g/kg had an increase in blood platelets of 51584%, starting on day 6 of administration and continuing after the drug was stopped. Platelet count peaked between days 12 and 18 and remained above normal for up to 21 days. These studies were extended to 41 patients with advanced cancers receiving carboplatin (600 mg/m ^{2}) and cyclophosphamide (1,200 mg/m^2) chemotherapy plus filgrastim (G-CSF) 5 g/kg.[185] PEG-rHu MGDF given for 720 days at doses of 0.035.0 g/kg/d enhanced platelet recovery in a dose-related manner when compared to placebo. The platelet nadir occurred earlier, but there was no difference in the depth of the postchemotherapy nadir. Recovery to baseline platelet counts occurred significantly earlier in patients treated with PEG-rHu MGDF (median 17 days for 0.3 or 0.5 g/kg PEG-rHu MGDF versus 22 days for placebo; $p = 0.014$). Of special interest was the finding that circulating peripheral blood progenitor cell counts were increased in patients receiving the combination of filgrastim and PEG-rHu MGDF, compared with the group receiving filgrastim alone. Further, patients who had previously received PEG-rHu MGDF as part of the phase I trial exhibited faster recovery, suggesting that pretreatment with PEG-rHu MGDF before chemotherapy may be beneficial.

Similar results have recently been reported by Fanucci et al. [186] who treated 53 stage III or IV non-small cell lung cancer patients receiving carboplatin and paclitaxel chemotherapy without other cytokine support. PEG-rHu MGDF was given for 16 days until the platelet count reached 600,000/l. All patients also received the study medication for 10 days prior to chemotherapy. These authors found that the 38 patients receiving PEG-rHu MGDF experienced a median platelet nadir of 188,000 compared to 111,000 in the placebo (12 patient) group ($p = 0.013$). Baseline platelet levels were recovered by day 14 in the treated group, compared with day 21 in the placebo group ($p < 0.001$).

PEG-rHu MGDF therapy in the above trials was not associated with fever or symptoms or signs of inflammation or activation of an acute phase response. Of note were the findings that PEG-rHu MGDF therapy sometimes resulted in thrombocytosis of >1,000,000/l and that two patients in both the Fanucci and Basser studies had thrombotic events in the treated groups, while none occurred in the placebo groups. Overall, PEG-rHu MGDF appears to be a safe drug without major systemic side effects: however, no reported study has yet evaluated the role of PEG-rHu MGDF in preventing platelet transfusions in severely thrombocytopenic patients.

Conclusion

The provision of appropriate blood component support has in the past been critical for the development of curative treatment programs, such as aggressive combination chemotherapy in childhood leukemias. The blood component laboratory will continue to play a central and broadening role in the treatment of cancer, specifically in the harvesting, processing, and transfusion of HSCs from marrow, UCB, and peripheral blood sources to restore hematologic and immune function after high-dose ablative therapies. The use of recombinant growth factors appears to facilitate the harvesting of HSCs from peripheral blood and to hasten engraftment after transplantation. Innovative treatment strategies, including intensive myeloablative therapies, may now be used for a broad spectrum of patients with currently incurable cancers. Transfusion medicine expertise will be essential to ensure that appropriate blood components are provided before and after HSCT, to collect HSCs, and to avoid immune and infectious complications in patients treated in this fashion.

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SOLID ORGAN TRANSPLANTATION

Improved surgical technique and advances in prevention of allograft rejection have led to the tremendous growth in solid organ transplantation during the past decade. Readers are referred to the United Network for Organ Sharing (UNOS) web site (www.unos.org). During 1996, 20,000 solid organ transplants were performed in the United States, including >11,000 kidney, 4,000 liver, and 2,000 heart transplants. The use of cyclosporine and tacrolimus, either singly or in combination, has extended graft and patient survival.[187] These drugs are not cytotoxic agents and thus lack marrow toxicity. They block transcription of IL-2 by interfering with Ca++-activated transcription factors. Release of other T-cell cytokines, such as interferon-, is also downregulated. The overall effect is directed primarily at suppression of T-cell activation. Because corticosteroids prevent the release of IL-1 from accessory cells, the combination of cyclosporine or tacrolimus with prednisone has been highly successful. Azathioprine remains a commonly used adjunctive immunosuppressive. Newer agents such as rapamycin (sirolimus) and mycophenolate mofetil are under active investigation. [188] [189] [190] [191]

Transfusion-Transmitted CMV Infection

The implications of CMV infection are different in solid organ transplant recipients compared with BMT recipients. Following solid organ transplantation, the recipients immune system is not chimeric, and immunosuppression is less extensive than that required for allogeneic marrow transplants. As a result, solid organ recipients appear to have better immunity against infections such as CMV, which cause less postoperative morbidity. Nevertheless, for some patients CMV is an important pathogen and appears to accelerate rejection of most transplanted organs. Most serious CMV infections result from primary transmission from the donor allograft rather than from blood transfusion.[192] CMV-seronegative recipients of CMV-seropositive allografts are therefore at greatest risk. Lung transplant recipients are especially prone to morbidity and mortality related to CMV infection. [193] CMV infection occurs more often if the immunosuppressive regimen includes MoAb therapy such as OKT3. In a study of liver transplant recipients, symptomatic CMV disease, CMV hepatitis, invasive fungal disease, and death were all more likely to occur in patients who acquired CMV from the donor organ rather than from transfusion.[192] Thus, when either the donor or the recipient is CMV-positive, the use of CMV-negative blood components should be considered investigational. However, in the case of heart, lung, kidney, or pancreas transplants when both donor and recipient are CMV-negative, blood components with a reduced risk of transmitting CMV should be used. Transfusion with either CMV-seronegative blood or leukoreduced blood will effectively prevent CMV transmission by transfusion. CMV-negative or leukoreduced blood is not generally used for adult liver transplant cases because of the large number of products required. CMV infection syndromes in the postoperative period are usually adequately

treated by antiviral agents such as ganciclovir. [194] In the setting of renal transplantation, multicenter studies have suggested benefit from the use of CMV hyperimmune globulin. However, such globulin preparations are extremely expensive and are not considered to be a cost-effective therapy. Current interest has centered on the use of PCR for the early detection of CMV infection as a guide to the pre-emptive use of ganciclovir. [195] [196]

Pretransplant Blood Transfusions

Following the initial investigations by Opelz et al., [197] numerous studies documented that pretransplant blood transfusions promoted prolonged renal allograft survival. As a result, deliberate use of random donor transfusions before cadaver donor renal transplantation and donor-specific transfusions (DSTs) before living-related donor transplants became widespread. A proportion of deliberately transfused patients became HLA alloimmunized, however, and the resulting HLA incompatibilitydetected during pretransplant crossmatchingeliminated them as candidates for transplantation. The introduction of cyclosporine in the 1980s led to improved graft survival, and the benefit of pretransplant transfusions became more difficult to document. [198] As a result, most transplant centers abandoned deliberate pretransplant transfusions in favor of a strategy that avoided HLA alloimmunization through the use of erythropoietin and leukoreduced blood components. [199] [200] However, even in the cyclosporine era, some studies have shown benefit from pretransplant transfusions, [201] especially when the donor and the recipient share HLA haplotypes.[202] The beneficial effect of pretransplant blood transfusions appears to be long-lasting. [203] The exact role of pretransplant conditioning by transfusion for kidney or heart transplants[204] remains controversial.

Despite decades of investigation, the mechanism(s) responsible for the immunomodulatory effect of pretransplant transfusions remains uncertain. [205] Three basic mechanisms have been proposed: (1) pretransplant transfusions may select for patients who are immunologic nonresponders because responders to DSTs would make HLA antibodies directed against the donor and thus be eliminated from transplant due to an incompatible crossmatch. (2) Transfusions may exert an immunosuppressive effect if donor cells or HLA peptides locate to the recipient thymus where responding clones are deleted (so-called central tolerance). [206] (3) Transfusions may exert an immunosuppressive effect by altering T-cell reactivity outside the thymus (peripheral tolerance). Peripheral tolerance may result from the production of anti-idiotypes,[207] [208] from the development of suppressor T cells, [188] from the persistence of donor veto cells,[209] from Fas-mediated or other cell-mediated signals to apoptosis or anergy, [210] [211] from downregulation of natural killer cell function, [212] or from an alteration in recipient immunity that favors a shift from Th1-type cells to Th2-type cells.[213]

Renal Transplantation

ABO Compatibility

ABO compatibility between donor antigens and recipient antibodies is critical for successful renal transplantation. ABH antigens are richly expressed on vascular endothelial cells[214] and renal cells[215] and are excreted in the urine.[216] As a result, ABO-incompatible grafts are at high risk for rejection, and transplantation against ABO barriers is generally contraindicated. [217] [218] Because group O recipients are restricted to group O donors, several experimental protocols have investigated the use of group A_2 donors (who express group A antigens less strongly than group A_1 individuals) for group O recipients. [219] [220] Group O recipients of A_2 kidneys require greater degrees of immunosuppression, and graft survival is inferior to that obtained with group O donors. Transplantation of nonmatched but compatible grafts (e.g., group B donor into AB recipient) does not result in accelerated graft rejection. However, passenger lymphocytes that accompany the graft may continue to secrete ABO antibodies during the early postoperative period. Therefore, recipients of ABO-compatible but unmatched allografts may experience hemolysis secondary to ABO alloantibodies of graft lymphocyte origin. (See the next section on Orthotopic Liver Transplantation.)

HLA Compatibility

The importance of serologic HLA compatibility between donor antigens and recipient antibodies is well established for renal transplantation. Recipients who express warm-reactive antibodies to HLA class I antigens of the donor are at risk for hyperacute rejection characterized by complement activation, intravascular coagulation in the graft, organ ischemia, and necrosis that may occur within minutes of establishing blood flow to the graft. [188] [221] A variety of techniques are used for HLA crossmatching, and there is controversy regarding which method has the highest predictive value. Standards of the American Society for Histocompatibility and

Immunogenetics (www.swmed.edu/homepages/ASHI) require that the final crossmatch employ a technique with greater sensitivity than the basic microlymphocytotoxicity test. Such techniques may use longer incubation times, wash steps, an antiglobulin reagent, B-cell targets, dithiothreitol, or flow cytometry. [222]

Recognition of the importance of HLA-serologic compatibility led to the practice of monthly HLA antibody screening of serum from HLA-sensitized patients awaiting kidney transplants. Antibody specificities that are identified are recorded, and the corresponding antigens are avoided when allocating suitable kidney allografts. Some programs also consider compatibility results using archived serums when allocating allografts to recipients who are known to be HLA-sensitized. Analysis of recipient HLA specificities has revealed that identification of antibodies directed against common public antigens may be more clinically meaningful than antibodies to private HLA antigens. [223] [224] [225]

HLA Matching

Routine practice does not require HLA matching of donor and recipient when allocating cadaver kidneys. However, retrospective studies of graft survival have suggested that certain HLA mismatches are more likely to result in graft rejection than others, [226] and large retrospective analyses have provided evidence that greater degrees of HLA matching between the donor and recipient result in longer graft survival. [227] [228] While not all have agreed with these findings, [229] the potential benefits have prompted some organ banks to allocate cadaver organs based on the degree of HLA match rather than simply on the results of HLA compatibility testing. [230] [231] A review of >23,000 renal transplants reported to the United Network for Organ Sharing registry showed that the half-life for a subset of matched allografts was 17.3 years, in contrast to a half-life for unmatched allografts of only 7.8 years. [232] Nevertheless, matched cadaver transplants do not survive as well as matched living-related transplants. The difference may reflect not only injury to the graft during preservation but also the influence of histocompatibility mismatches not recognized by existing HLA typing techniques.

DNA-based typing methods are being used to make HLA antigen assignments to donors and recipients. HLA DR and DQ typing by DNA-based methods has become routine, and molecular typing for HLA A, B, and C antigens is being widely introduced. Molecular typing methods have revealed that the HLA region is extraordinarily complex, with far more alleles than had been recognized by serologic methods. Because as many as 15% of samples may be incorrectly typed by serologic

methods, [230] prior data on the benefit of HLA matching may have been clouded by admixture of unmatched grafts that were thought to have been matched. Reanalysis based on improved molecular typing and elimination of mismatches has revealed a beneficial effect on graft survival for HLA DR-matched kidney transplants. [233] It has also been suggested that the degree of molecular matching may indicate which patients can be treated with reduced amounts of postoperative immunosuppressives.

Orthotopic Liver Transplantation

Orthotopic liver transplantation (OLT) has become the treatment of choice for life-threatening end-stage hepatic failure. [234] Successful OLT requires a close working relationship among the surgical team, anesthesia services, and blood services because the operation sometimes requires spectacular quantities of blood support. Improved surgical technique, use of venovenous bypass for those cases in which the vena cava is interrupted, argon laser-directed electrocoagulation, and better preservative solutions for storing the donor liver ex vivo have contributed to decreased transfusion requirements during surgery. [235] [236] Nevertheless, considerable variation exists among blood use in different programs.

Causes of Extreme Bleeding

Intraoperative transfusion requirements for OLT cannot be predicted on the basis of preoperative coagulation screening. [237] Rather, severe portal hypertension, previous right upper quadrant surgery, vascular abnormalities of the portal vein, poor left ventricular function, pulmonary hypertension, poor nutritional state, and comorbid diseases are the most important predictors of massive transfusion and decreased survival. Prior transjugular intrahepatic portosystemic stent shunt does not appear to worsen surgical outcomes during OLT. [238] [239] [240]

OLT requires a difficult right upper quadrant dissection of the recipient hepatic bed that can be complicated by severe portal hypertension, friable collateral vessels, adhesions from prior procedures, or inflammation from the patients underlying disease. Removal of the liver requires isolating the blood supply of the organ and clamping and cutting the portal vein, hepatic artery, common bile duct, and either the hepatic vein or the inferior vena cava above and below the liver. The period after the recipient hepatectomy and before re-establishing blood flow to the new graft is referred to as the anhepatic phase of surgery. During this phase, venous blood draining the bowel and lower extremities may need to be diverted through an extracorporeal circuit if a portion of recipient vena cava was removed during the hepatectomy. A more recent surgical technique preserves the vena cava and results in less bleeding.

Patients who require OLT usually have multiple hemostatic abnormalities associated with acute or chronic liver failure. Decreased coagulation factors, splenomegaly with thrombocytopenia, dysfibrinogenemia, and chronic fibrinolysis with elevated fibrin split products may all be present in varying combinations. In addition, some patients develop a profound intraoperative fibrinolysis. [241] [242] Tissue plasminogen activator (tPA), presumably released from recipient blood vessels or from the vessels of the graft in response to shock, acidosis, pharmacologic pressors, ischemia, or preservation injury, circulates in high concentration. Normally cleared by the liver, tPA released during and immediately after the anhepatic phase of surgery is not cleared from the bloodstream. As a result, tPA activity can reach 10100 times normal levels, and a pathologic systemic lytic state ensues. A sudden decrease in all coagulation factors occurs, with particularly marked declines in fibrinogen and factor VIII. [243] Proteolysis of von Willebrand factor may also occur. [244] Less severe cases may not demonstrate systemic lysis but may lyse clot at sites of previous hemostasis. As the liver allograft begins to function, tPA is rapidly cleared and fibrinolysis decreases. The administration of antifibrinolytic agents such as epsilon-aminocaproic acid, [245] tranexamic acid, [246] or aprotinin [247] [248] can significantly improve hemostasis and may prevent exsanguination in selected patients.

A properly functioning graft is essential to the success of OLT. Grafts damaged during the demise of the donor, during harvesting or ex vivo preservation, or during transplantation are associated with both severe hypotension and coagulopathy. Hypotension results from a pronounced decline in total systemic vascular resistance and is often refractory to aggressive pharmacologic pressor support. The cause of the vascular collapse is not known. The ensuing shock exacerbates the derangement in hemostasis. With the introduction of better graft-preserving solutions, the incidence of severe primary graft nonfunction has decreased. [249]

Transfusion Support

A transfusion device designed to deliver large volumes of blood components in a rapid fashion is essential for OLT. These devices consist of a sterile holding reservoir, high-capacity blood filter, roller pump, in-line blood warmer, and air detection device. Blood is pumped through large-bore catheters placed in the antecubital or central veins. Intraoperative blood-recovery devices are a useful adjunct to blood support during OLT, particularly in cases requiring massive transfusion. [250] [251] Blood suctioned from the operative field is anticoagulated with citrate and collected in a sterile holding reservoir. The shed blood is then centrifuged, the supernatant is discarded, and the residual packed red cells are washed with saline. The saline-suspended packed red cells may then be pumped to the rapid transfusion device or transferred to a plastic blood-collection bag for later reinfusion.

OLT demands careful but aggressive component therapy guided by the clinical course and the results of intraoperative coagulation monitoring. [236] [252] Serial intraoperative hematocrit and coagulation monitoring using a limited number of tests with rapid turnaround time provides valuable information for rational blood component management. Some OLT centers monitor coagulation using the thromboelastogram, a device that measures whole blood coagulation by impedance to an oscillating cylinder. [253] [254] Other programs use traditional measurements such as the prothrombin time, fibrinogen, and platelet count. Either method of monitoring is valuable provided that testing is done serially, that results are reported rapidly, that changes are correlated in the operating room with the clinical picture, and that abnormalities are interpreted by someone with experience in the coagulopathy of OLT.

Red cell support for patients with multiple red cell alloantibodies can present a special challenge to blood services supporting OLT programs. [235] [255] For difficult patients with clinically significant, low-titer, high-frequency alloantibodies, preoperative plasmapheresis can be used to remove antibody. Patients with medium-titer clinically significant alloantibodies can sometimes be managed initially with antigen-negative cells and then switched to antigen-untyped cells should massive blood support be required. Antigen-negative units are then reinstituted at the end of surgery to prevent postoperative hemolysis. Patients with high-titer alloantibodies may require special planning and cooperation among blood suppliers to provide a sufficient quantity of antigen-negative blood. Patients with primary biliary cirrhosis or other causes of end-stage liver failure who also have IgA deficiency may have anti-IgA antibodies and require special preparation for transplant surgery. [256] Probably as a consequence of postoperative immunosuppression, Rh-negative individuals transfused with Rh-positive blood at the time of transplant are not likely to make

anti-D.[256] Nevertheless, Rh-negative women of childbearing age without anti-D are initially supported

in many programs with a reasonable number of D-negative units and then switched to D-positive cells should more blood be needed. In our program, Rh-negative males and females who are not of childbearing potential are treated from the outset with Rh-positive blood. Because many OLT patients are massively transfused, delayed hemolytic transfusion reactions in the postoperative period, if directed against high-frequency antigens, can result in dramatic hemolysis.

The use of fresh-frozen plasma (FFP), platelet concentrates, and cryoprecipitate is guided by the clinical course and by the results of intraoperative monitoring. [236] Although care must be given to prevent dilutional coagulopathy, it is not necessary to mix equal ratios of red cells and FFP or platelet concentrates. Reasonable goals of component therapy in the absence of fibrinolysis or other complications include an INR <2 or <2.5, a platelet count >50,000100,000/l, and a fibrinogen >100 mg/dl. Antifibrinolytic agents are indicated during or shortly after the anhepatic phase of surgery in patients who demonstrate a systemic lytic state. [257] A loading dose and continuous infusion of epsilon-aminocaproic acid, tranexamic acid, or aprotinin has been used with success. Hourly bolus doses have also been used. Tranexamic acid reduced operative blood loss during OLT when studied in a randomized, prospective, placebo-controlled trial. [246] However, in a separate randomized, placebo-controlled trial of 80 patients, the routine use of aprotinin was not shown to reduce overall transfusion requirements. [258] Antifibrinolytics should probably be applied to selected patients who show evidence of fibrinolysis. For all patients, local hemostasis provided by meticulous and advanced surgical skill remains critically important.

Complications of Massive Transfusion During OLT

Patients undergoing massive transfusion during OLT are susceptible to all the major complications of massive transfusion, including hypothermia, dilutional coagulopathy, electrolyte imbalance, pulmonary dysfunction, and transfusion-transmitted infections. Despite massive transfusion and the immunosuppression of the recipient, TA-GVHD is a rare complication in the setting of solid organ transplantation. [235] [255]

OLT patients are uniquely at risk for life-threatening hypocalcemia as a result of citrate toxicity. [260] Magnesium is also chelated by citrate, and low magnesium concentrations may accompany massive transfusion during OLT surgery. [261] Since the liver is the main organ for citrate metabolism, and since OLT patients can receive very rapid infusions of large volumes of FFP that is rich in Na_3-citrate, the quantity of citrate infused per kilogram body weight per minute can quickly exceed the rate of citrate removal.[262] Citrate toxicity is more pronounced if concomitant renal failure is present because the kidney is the major site of citrate excretion. The principal effects of citrate toxicity are cardiovascular. An initial blunted response of cardiac output to left ventricular volume loading is followed by hypotension resulting from poor cardiac output and decreased systemic vascular resistance. If misinterpreted as hypotension due to hypovolemia, a more rapid transfusion of citrate-rich blood components will worsen cardiac output. Citrate toxicity is aggravated by hypothermia and hyperkalemia. Because a widened Q-Tc interval on the electrocardiogram has low specificity and poor predictive value, intraoperative monitoring of the ionized calcium level is very important during OLT. With severe depression of the ionized calcium or magnesium, bizarre electrocardiographic disturbances and fatal arrhythmias can develop.

ABO Compatibility

Under normal circumstances, the donor liver must be ABO-compatible with the recipient. Transplantation of ABO-incompatible livers has occurred by error or under urgent circumstances and has had limited success. [263] [264] [265] ABO-incompatible liver transplants may occur more frequently in pediatric cases when a compatible donor organ is not available and recipient blood group alloantibodies are poorly developed. Data from the UNOS scientific registry found that ABO mismatch was among the strongest independent predictors of adverse outcome after emergency liver transplant. [266] In another series, survival of emergency ABO-incompatible grafts was 30% compared with 76% for emergency ABO-compatible grafts.[267] The immune response to ABO-incompatible grafts is consistent with a pattern of hyperacute rejection.[267] [268] Transfusion support for ABO-incompatible grafts is not standardized, but the use of RBCs and FFP compatible with both donor and recipient would seem desirable. ABO-nonmatched but compatible donors (e.g., group O donor for group A_1 recipient) occasionally occurs in OLT, but nonmatched compatible grafts may have decreased survival rates compared with matched grafts. Approximately 30% of patients transplanted with compatible but nonmatched livers suffer postoperative hemolysis of recipient red cells mediated by ABO antibodies produced by passenger lymphocytes of donor origin. [269] This complication has been reported after all major solid organ transplants, including liver, [269] kidney,[270] heartlung,[271] spleen,[272] and pancreas.[273] It is most common after liver transplantation, probably by virtue of the greater number of passenger lymphocytes that accompany this large organ. Hemolysis develops approximately 1 week after surgery and is generally detected by the development of a positive direct antiglobulin test (DAT) with ABO alloantibodies recovered in the eluate. Although the DAT may be only weakly reactive, rather brisk hemolysis typical of complement-fixing ABO antibodies can occur with a rapid fall in hematocrit and a dramatic rise in bilirubin. Acute renal failure from intravascular hemolysis is rare but has been reported. [274] Treatment is based on transfusion of red cells compatible with antibodies produced by both the recipient and the graft. Red cell exchange is usually not required. Care must be taken in routine postoperative compatibility testing for patients who receive unmatched transplants. A group A_2 recipient whose group O hepatic graft is producing warm-reactive IgG anti-A_1 can have an acute hemolytic transfusion reaction to a group A_1 donor unit transfused postoperatively despite a negative antibody screen, negative DAT, and a compatible immediate-spin pretransfusion crossmatch.

HLA Testing for OLT

HLA testing for OLT has not reached the level of clinical importance found in renal transplantation. Donor livers are not allocated on the basis of the degree of donor/recipient HLA matching, and an HLA-incompatible pretransplant crossmatch does not preclude the use of the graft. Nevertheless, there are reports suggesting that recipient HLA antibody directed against donor antigens may contribute to graft rejection under some circumstances. [275] [276] [277] In addition, highly sensitized recipients who are refractory to routine platelet transfusions are difficult to support during massive blood loss at the time of surgery. [259] An unexpected finding was that recipients with demonstrable HLA antibodies in a pretransplant serum sample often no longer react with donor-type antigens after the transplant, suggesting that HLA antibodies are adsorbed in vivo from the recipients plasma by the incompatible graft. [278] As a result, patients have had successful combined kidneyliver transplants using a donor kidney that was originally HLA-incompatible but became compatible hours after liver transplantation from the same donor.

Cardiac Transplantation

Cardiac transplantation has become an established therapy for life-threatening cardiac dysfunction. One-year graft survival

rates of approximately 80% have been achieved through the use of effective immunosuppressive regimens, improved recipient selection criteria, the use of heart assist devices to bridge the waiting time between diagnosis and transplant, the development of percutaneous endomyocardial biopsy for improved detection of early rejection episodes, and more effective management of postoperative infectious complications. As a result of these improvements, research has shifted from short-term problems such as acute rejection to factors influencing long-term morbidity-free survival. Factors that most influence allograft survival after heart transplantation include ABO compatibility, HLA matching, HLA compatibility, upregulation of adhesion molecules on vascular endothelium, duration of cold ischemia, and infection with CMV.

ABO and HLA Testing

Because ABO antigens are present on cardiac endothelial tissue, ABO compatibility of donor and recipient is a prerequisite for heart transplantation. As with other solid organ transplants, ABO-compatible but unmatched allografts are used. However, ABO-identical transplants may have a better overall survival than ABO-compatible but unmatched grafts.

The contribution of HLA matching to improved graft survival is difficult to assess. Because of the short allocation time, HLA typing of the donor organ is mostly retrospective. Numerous reports, however, suggest that HLA DR matching may improve graft survival. [279] [280] Opelz and Wujciak[281] found no further decline in graft survival rate as the number of HLA mismatches increased beyond three. They speculated that the higher doses of immunosuppressive drugs administered to heart transplant recipients compared with kidney transplant recipients might have accounted for their findings. With the advent of rapid HLA typing by PCR using

allele-specific primers, allocation of hearts based on HLA matching may become more feasible and widespread.

The clinical significance of HLA antibody testing in cardiac transplantation remains controversial. Some groups have found significant differences between long-term survival of patients with a panel reactive antibody level of <10% compared with >10%. [282] [283] Other groups have reported that the presence of specific antidonor HLA antibodies rather than the level of panel reactive antibody was a better predictor of rejection. [284] [285] HLA antibodies induced by pregnancy may represent a different risk factor from those induced by prior transfusion or transplantation. [286] Pregnancy-related HLA antibodies are generally monospecific to paternal antigens and tend to become nondetectable either because the antigenic stimulus is gone or because of the development of anti-idiotypes.

Other Risk Factors for Cardiac Rejection

During the last decade it has become clear that allograft rejection is associated with expression of cell adhesion molecules (CAMs). [287] Specific CAMs including ICAM-1, ICAM-2, and LFA-3 are found to be upregulated on vascular endothelial cells of rejecting heart transplants. [288] By promoting leukocyte adhesion to the endothelium, CAMs appear to participate in the development of cardiac allograft vasculopathy. [289] Expression of VCAM-1 and E-selectin may result from CMV infection. CMV infection appears to correlate with shortened allograft survival, a higher frequency of repeat-rejection episodes, [290] and the development of accelerated coronary atherosclerosis. [291] [292] As with other solid organ transplants, most serious CMV syndromes originate with infection from the allograft. Nevertheless, CMV-seronegative recipients of CMV-seronegative cardiac allografts should be transfused with either CMV-seronegative or leukoreduced blood components. Prolonging the duration of cold ischemia has a significant negative effect on the survival of heart transplants. In addition, postoperative ischemia appears to play an important role in myocardial injury and rejection. [287] Differentiation of ischemia from acute rejection is important for management of immunosuppression in the early post-transplant period. [293]

Other Solid Organ Transplants

Single lung, pancreas, small bowel, combined organ, and multivisceral transplants have each been successfully performed in patients with a variety of end-stage organ failure syndromes. Cadaver lung transplants and living-related partial lung transplants have been used in the treatment of end-stage pulmonary disease, including cystic fibrosis. A variety of techniques have been developed to prevent rejection of the bronchial anastomosis between donor and recipient. Two-year survival rates of 6080% have been reported. [294] Good success has been achieved with pancreas or combined kidneypancreas transplants for diabetes, particularly if transplantation is used before the onset of retinopathy. [295] The pancreatic graft is placed in the pelvic bed with the pancreatic duct draining into the urinary bladder. Small bowel and multivisceral transplants have been successfully performed in a limited number of individuals, but recipient and graft survival has been limited by problems of rejection, infection, and postoperative lymphoproliferative disorders. [296]

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Chapter 146 - Red Cell Substitutes

Robert M. Winslow

INTRODUCTION

Red cell substitutes are designed to replace red blood cells for transfusion. Solutions such as saline, Ringers lactate, albumin, dextran, and the starches are widely used to replace lost blood volume in surgery and trauma, but the term blood substitutes is usually used in reference to oxygen-carrying solutions such as those based on some form of hemoglobin or perfluorocarbon emulsions. These substitutes would carry out the primary function of red cells: transport of oxygen to tissues. The goal of developing a red cell substitute has been elusive: for centuries, an alternative to allogeneic blood for transfusion has been sought by scientists, the military, and industry. Early attempts included the use of milk, wine, gum, and red cell hemolysates. [1] In the modern era (since about 1965), three general types of products have been under development: modified hemoglobin solutions, perfluorocarbon emulsions, and lipid vesicle-encapsulated hemoglobin. None is yet approved for clinical use.

The current driving force for the development of red cell substitutes is the perceived danger of transfusion of allogeneic blood (Table 146-1). In fact, blood is safer now than it ever has been. However, the aggregate risks listed in this table are frightening to many patients, their families, and their doctors as well, and the demand for a safe and efficacious alternative is increasing. Beyond the risks listed, in regions of the world where the

TABLE 146-1 -- Risks of Transfusion of Allogeneic Blood in the United States

Event	Risk
HIV 1÷2	1÷676,000
HTLV I÷II	1÷641,000
Hepatitis B	1÷66,000
Hepatitis C	1÷125,000
Fever, chills	1÷100
Hemolytic transfusion reaction	1÷6,000
Fatal hemolytic transfusion reaction	1÷100,000

Data from Klein[2] and Schreiber.[3]

frequency of human immunodeficiency virus infection is high, development of these solutions would be particularly important.

Because of the risks of allogeneic blood transfusion, and because of the large markets that could be generated for red cell substitutes, considerable efforts are now being expended by industry to develop safe products. It is likely that red cell substitutes will find their way into clinical practice within the next decade.

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PRINCIPLES

Several major issues must be addressed to develop a successful red cell substitute.

1. *Oxygen transport:* Must a red cell substitute transport oxygen in the same way as red cells do? Oxygen affinity of cell-free or encapsulated hemoglobin may be very different from that of red cells. Perfluorocarbons carry oxygen physically dissolved rather than chemically bound, and therefore the dissociation curve is linear. Whether these factors are important, physiologically or clinically, is still not known.
2. *Plasma retention:* The normal red cell life span is about 120 days. However, the plasma half-life of cell-free hemoglobin may be only 12 hours and that of encapsulated or surface-modified hemoglobin and perfluorocarbon emulsions 2448 hours. Are these times clinically useful? If the effect of a red cell substitute is so short that a transfusion with allogeneic blood is only delayed, not eliminated, then the usefulness of the product becomes questionable.
3. *Efficacy:* It will be necessary to demonstrate efficacy for any red cell substitute to be used clinically. At present, it is taken as a matter of faith that a solution carrying oxygen will be more useful than one with no oxygen carrier, if it is without side effects. However, conclusive demonstrations will be necessary because no red cell substitute currently being developed is without side effects. For example, if a particular substitute increases systemic pressure and vascular resistance (as many hemoglobin-based solutions do), this property could counteract any increase in oxygen transported.
4. *Toxicity:* No candidate product developed to date is without toxicity. Cell-free hemoglobin has vasoconstrictor properties that could limit its use in shock and trauma. Lipid vesicles and perfluorocarbon emulsions stimulate macrophages to elaborate cytokines that can produce diverse effects, including fever and flulike symptoms.
5. *Commercial viability:* To be successful, a red cell substitute must be competitive with allogeneic red blood cells in effect, toxicity, and cost. The cost of providing human red cells for transfusion is now very low.[2] The technology required for the production of red cell substitutes is complex and likely to be expensive.

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CLINICAL APPLICATIONS

Products

Hemoglobin-based Products

The red cell substitute products that have gained the most attention are based on hemoglobin, an extraordinarily complex molecule consisting of four polypeptide chains, each made up of about 140 amino acids. Normally packaged in the red cell, free in solution it is fragile: it tends to oxidize, is unstable and toxic, and is excreted by the kidneys as the subunits fall apart. Over the years, the strategy for making a hemoglobin-based red cell substitute has been based on cross-linking hemoglobin to correct these problems.[1]

Hemoglobin has the desirable properties of a high capacity to bind oxygen and to release it cooperatively; this makes it attractive for use as a red cell substitute. However, hemoglobin, free in solution, has several unique properties. Its oxygen affinity is high because outside the red cell the allosteric effectors (2,3-diphosphoglycerate [2,3-DPG] and ATP, for example) are not present. Its effectiveness as an oxygen carrier is limited because it dissociates into half-molecules (dimers) (haptoglobin is rapidly saturated, and excess dimers are quickly removed from the circulation by the kidney after filtration in the glomerulus). Once filtered, a high concentration of protein in the renal tubules can cause tubular obstruction and consequent renal failure. Cell-free hemoglobin binds nitric oxide, an endothelium-derived relaxing factor that may contribute to vasoconstriction. Outside the red cell, hemoglobin exerts an oncotic pressure similar to that of albumin; thus, if large amounts are to be administered to a patient, shifts of fluid between the interstitial and intravascular spaces would be expected. Finally, iron, when released from hemoglobin, can promote the formation of toxic oxygen radicals.

Thus, to be an effective oxygen carrier in the cell-free state, hemoglobin must be chemically modified to avoid these problems. Whether all these must be solved completely for a product to be useful is a matter of intense debate. All the reactions currently considered useful in the production of hemoglobin-based red cell substitutes use chemical modification at one or more sites on the surface of the protein (Table 146-2). Differences in the reactions are determined by the dimensions and reactivity of the cross-linking reagents. Since the function of hemoglobin in binding and releasing oxygen is intricately connected to the transition between T and R conformation (see Chap. 22), it is not surprising that the P_{50} and yield are highly variable. Even small differences among structures of the reagents can yield products with quite different properties. In addition, the conditions of the reaction are very important, not only in regard to the state of ligation (i.e., oxygen saturation), but also to the presence of agents or molecules that lack or compete for certain reactive sites.

A further complication of these reactions is that many nonhemoglobin proteins, copurified with hemoglobin, contain reactive groups and may also be modified to produce new,

TABLE 146-2 -- Classes of Hemoglobin Modifications

Molecular Size	Modification	Examples
Monomers	Amino-terminal modification	Carbamylation Carboxymethylation Pyridoxylation Acetaldehyde dialpha (genetic)
	Lysine EF6(82) modification	Mono-(3,5-dibromosalicyl) fumarate
	Valine NA1(1)-lysine EF6(82) cross-link	2-Nor-2-formylpyridoxal 5-phosphate (NFPLP) $(Bis\text{-}PL)P_4$
	Lysine $G6(99)_1$ -lysine $G6(99)_2$ cross-link	Bis-(3,5-dibromosalicyl) fumarate (DBBF)
	Three-point	Trimesoyl tris(methyl phosphate)
	2,3-DPG analog	Pyridoxal 5-phosphate Bezafibrate, clofibrate
Multimers	Surface, multisite	Glutaraldehyde Polyaldehydes Ring-opened dials Diimidate esters
Conjugates	Surface, multisite	Dextran-aldehyde Polyethylene glycol Polyoxyethylene

Modified from Winslow,[1] with permission.

Figure 146-1 The reaction of DBBF with hemoglobin. *(From Winslow,[1] with permission.)*

potentially toxic contaminants. It has been difficult to produce a pure modified hemoglobin for toxicity studies when most processes start with relatively crude "stroma-free" hemoglobin.[4]

An example of a cross-linked hemoglobin currently being commercialized is a product studied intensively by the U.S. Army and a number of academic laboratories. Cross-linking of hemoglobin isolated from outdated human blood is carried out with bis-(3,5-dibromosalicyl)fumarate (DBBF) [5] (Table 146-2). The reaction results in

a four-carbon covalent link between adjacent chains at position 99 (Lys $_1$ 99-Lys$_2$ 99) (Fig. 146-1). This covalently cross-linked hemoglobin cannot break down into subunits in the circulation and therefore cannot be excreted as filtered globin chains.

The production process is complex.[6] Stroma-free hemoglobin is separated from red cell membranes and deoxygenated to achieve the proper molecular conformation for cross-linking, and the 2,3-DPG pocket is blocked reversibly with an allosteric effector. It is then cross-linked, heated to pasteurize it and also to remove unreacted hemoglobin, and passed through a series of cross-flow filters. It is finally sterilized by filtration through a 0.2-m filter.

The product has many favorable properties (Table 146-3). Oxygen dissociation curves show a P_{50} under physiologic conditions of 28 mmHg. The degree of cooperativity (the slope of the

TABLE 146-3 -- Properties of a Single Batch of Cross-Linked Hemoglobin

Property	Measurement/Value
Formulation	Ringers acetate
Volume	16.1 L
Hemoglobin concentration	9.8 g/dl
P_{50} , 37°C	29 mmHg
Hills parameter (n)	2.31
Inorganic phosphate	<1 g/ml
pH	7.4
Sterility	Pass
Pyrogen test	Negative
Endotoxin	<0.1 EU/ml

Adapted from Winslow,[7] with permission.

dissociation curve) is quite similar to that of blood (Hill coefficient 2.62 for blood, 2.31 for cross-linked hemoglobin), and the Bohr [8] and carbon dioxide[9] effects are nearly intact.

Theoretically, this product should have in vivo oxygen transport properties quite similar to those of whole blood. The intravascular persistence is markedly extended in the rat: uncross-linked hemoglobin has a half-life of about 1.2 hours and cross-linked hemoglobin one of 4.3 hours. In the rabbit, the persistence is longer, about 16 hours for cross-linked hemoglobin, for monkey about 14 hours, and for pig about 7 hours. [10] There is no doubt that cell-free hemoglobin transports oxygen: many studies in the literature have demonstrated the ability of hemoglobin solutions to resuscitate animals in lethal hemorrhagic shock. [1]

Other hemoglobin-based products are also being developed. For example, a product very similar to the cross-linked hemoglobin described above has been produced as a recombinant protein in *Escherichia coli*. [11] Starting material for hemoglobin modification can also be produced in transgenic animals. [12] Hemoglobin also can be polymerized using a number of polyfunctional reagents (Table 146-2) to yield molecules with markedly increased molecular weights. One example is human hemoglobin reacted first with pyridoxal-5 phosphate and then polymerized with glutaraldehyde. [13] This product has a reduced colloidal osmotic (oncotic) pressure and longer intravascular persistence compared to smaller molecules, but the polymerization reaction is notoriously difficult to control. [14] [15]

Another promising approach is to couple hemoglobin to polyethylene glycol (PEG) [16] or a similar molecule, such as polyoxylethylene[17] or dextran.[18] These conjugated hemoglobins may also have prolonged plasma retention times and might have reduced interactions with the reticuloendothelial system.

Liposome-Encapsulated Hemoglobin

Since hemoglobin is normally packaged inside a membrane, it seems intuitively correct that encapsulated hemoglobin would be the ultimate solution to the red cell substitute problem. In 1957, Thomas Chang reported the use of microencapsulated hemoglobin as artificial red blood cells. [19] Since that time, dramatic results have been reported in the complete exchange transfusion of laboratory animals, [20] [21] but progress toward development of an artificial red cell for human use has been slow because of problems of reticuloendothelial and other macrophage stimulation. [22] Other problems include maintaining sterility and large-scale production.

In the 40 years that have followed Changs initial descriptions of encapsulated hemoglobin, much work with lipid vesicles (liposomes) has been carried out. Liposomes have served as models for understanding natural cell membranes. They also have been used investigationally as vehicles for gene transfer, as targeted carriers, for pharmacologic agents, and even as lubricants for degenerated joint surfaces. The most extensively studied liposomes used to encapsulate hemoglobin are composed of phospholipid in combination with cholesterol and other lipids that confer flexibility and stability, such as ganglioside GM $_1$ or cholesterol. [23] When injected into animals, such liposomes are rapidly coated with IgG, albumin, and other opsonins. [24] Newer formulations include the use of surface components such as PEG or dextran, which can stabilize the liposomes in the circulation. [25]

The limitations to the development of liposome-encapsulated hemoglobin as a red cell substitute are difficulties in stabilizing the final product and the massive scale-up that would be required to produce a commercial product. The size of most liposome particles is approximately 0.21.0 m, too large to be filter-sterilized. Also, neither the liposome nor its hemoglobin contents can withstand pasteurization temperature. Other potential approaches to the solution of these problems involve

nonphospholipid liposomes[26] and those made from polymerizable phospholipids[27] and other polymers.[28]

Perfluorocarbon-based Products

A recent resurgence has occurred in the development of perfluorocarbons for two reasons. First, Fluosol-DA 20% (Fluosol, Green Cross Corporation, Osaka, Japan) was approved for marketing by the FDA for use in coronary angioplasty in 1990. Second, new perfluorocarbon emulsions now being developed by industry carry much more oxygen than previous products.[29] Still, there is a fundamental difference between perfluorocarbon- and hemoglobin-based red cell substitutes: oxygen is transported by perfluorocarbons as dissolved gas, whereas hemoglobin carries oxygen chemically bound to the protein itself (Fig. 146-2). The oxygen dissociation curve for hemoglobin is sigmoid, while oxygen dissociation from perfluorocarbons is linear. The lower curve in Figure 146-2 is for Fluosol, the upper one for an emulsion of Perflubron and egg-yolk phospholipid (Oxygent, Alliance Pharmaceutical Corporation, San Diego, CA), which contains about five times more fluorocarbon and therefore five times more dissolved oxygen.[30]

Initially, it would appear that it is better to have a sigmoid oxygen dissociation curve because (1) it is natural and (2) maximal oxygen carriage in blood is achieved at an alveolar P_{O_2} of about 100 mmHg, whereas only about 20% of the perfluorocarbon carries oxygen at the same P_{O_2} . To carry as much oxygen in this fluorocarbon as 7 g/dl hemoglobin, an arterial P_{O_2} of about 450 mmHg is needed. Therefore, it seems that the utility of fluorocarbon red cell substitutes will depend on a high inspired P_{O_2} . However, in many elective surgical procedures, arterial P_{O_2} well above 300 mmHg can be achieved.

In practice, a margin of safety could be achieved with relatively low doses of perfluorocarbon emulsions, which could have a significant impact; increased tolerance to a reduced hematocrit would further reduce the transfusion trigger. In a theoretical analysis of transfusion, mixed venous P_{O_2} is nearly constant from a hematocrit of 2545% because as oxygen is added in the form of transfused red cells, cardiac output decreases. [31] If the hematocrit is kept low but oxygen is added in the form of perfluorocarbon, a significant increase in mixed venous P_{O_2} occurs because the cardiac output remains high. Cell-free hemoglobin solutions would not offer this

advantage if their use does not result in increased cardiac output.

Figure 146-2 Oxygen capacity of Oxygent compared to blood. The Oxygent is a 60% (w/v) emulsion described by Flaim, [30] and the blood contains 15 g/dl hemoglobin.

Safety

Demonstration of the safety of red cell substitutes is a critical issue, because the risks of transfusion of allogeneic blood are well known. A substitute should be at least as safe as red cells unless a decisive therapeutic advantage can be demonstrated.

In a review of almost a century of clinical trials with red cell substitutes, reported side effects involved renal dysfunction, systemic symptoms (fever, chills, nausea, headache, flushing, vomiting, allergic reactions, tachycardia, bradycardia, hypertension, rigors, low back pain, chest pain, abdominal pain, decreased platelets, and increased partial thromboplastin time.[1] Many of these effects could be explained by the depletion of nitric oxide, in the case of hemoglobin-based products, or by stimulation of macrophages, in the case of liposomes or perfluorocarbon emulsions. Many are smooth muscle effects, and some involve macrophages and platelets. Recent clinical trials with hemoglobin-based products have not been extensively reported in the literature. However, it is clear that preclinical animal studies have not been completely successful in predicting human reactions to the products. [32]

Cell-free hemoglobin is widely distributed in the tissues after administration. Studies of the distribution of cross-linked hemoglobin in the intact animal show that significant amounts of hemoglobin are retained in the kidney, spleen, liver, adrenal gland, lung, heart, brain, and muscle well after any hemoglobin is detected in the plasma.[33] [34] [35] Thus, cell-free hemoglobin is distributed in almost every tissue of the body, and therefore we might expect toxic effects that could be quite unpredictable or unknown. Extensive histologic studies have been carried out in animals after exchange transfusion and have been summarized. [1]

Perhaps the effect of cell-free hemoglobin of most concern is its known ability to cause vasoconstriction and hypertension. This vasoconstriction can be mediated in part by the reaction of hemoglobin with nitric oxide, an endothelium-derived relaxing factor. [36] Nitric oxide is synthesized from arginine in endothelial cells (as well as in other cells) by an enzyme, nitric oxide synthase, that produces nitric oxide and citrulline. It binds to a heme group in guanylate cyclase that activates cGMP. Nitric oxide diffuses extremely rapidly out of endothelial cells into the interstitium and into smooth muscle cells, where it binds to a heme group in guanylate cyclase, activating cGMP and moving calcium from the unbound to bound state. The result is smooth muscle relaxation.

Nitric oxide diffuses into the lumen of the vessel, where it stimulates platelets and polymorphonuclear leukocytes, and into tissue, where it can stimulate macrophages. Hemoglobin binds nitric oxide very tightly, more tightly, in fact, than it binds oxygen, whether hemoglobin is in the red cell or free in solution. [37] The reaction is virtually irreversible. After synthesis of nitric oxide in endothelial cells, it diffuses into the smooth muscle cells, causing relaxation. Whether this interaction of hemoglobin with nitric oxide will limit clinical usefulness of hemoglobin-based red cell substitutes remains to be determined.

In addition to its effect as a scavenger of nitric oxide, cell-free hemoglobin may also induce vasoconstriction by disrupting normal autoregulation of vascular tonethat is, it may make oxygen so readily available to regulatory arterioles that reflexive vasoconstriction might occur, which paradoxically limits blood flow. [38] [39] This concept is suggested by the direct observation in the microcirculation [40] and might lead to new design strategies for cell-free blood substitutes.

Perfluorocarbon emulsions have the most extensive history of use in humans. Fluosol was approved by the FDA for use in coronary angioplasty and therefore has been given to many humans. In addition, similar formulations have been used on the battlefield in China and Afghanistan, although data are generally

not available. Perfluorocarbon emulsions have also been tested in humans as imaging agents.

The principal toxicity of perfluorocarbon emulsions appears to be their stimulation of macrophages. [41] [42] This can result in pulmonary hypertension and elaboration of thromboxane in swine and could lead to nonspecific symptoms such as fever, chills, and flulike symptoms in humans. [43]

Biocompatibility studies with liposome-encapsulated hemoglobin have been generally favorable, [21] but such products tend to be removed from the circulation by the phagocytic cells in the reticuloendothelial system. [44] This leads to significant enlargement of the liver and spleen. The direction of current research is to attempt to prolong the intravascular persistence to minimize this problem.

Efficacy

It seems intuitively obvious that a plasma expander that carries oxygen would be superior to one that does not, and that experimental proof of this concept would be relatively straightforward. However, the problem of efficacy can be appreciated by considering the difficulties in showing efficacy for red cell transfusion. The problem is a lack of clear end points: no single measure of oxygen transport is accurate and easily obtainable. It might be possible to show improved clinical outcome after transfusion of red cells to patients with extremely low hematocrits, but the bulk of allogeneic blood is given intraoperatively in response to blood loss, not severe anemia.

Most demonstrations of efficacy have been either by exchange transfusions with test material, or resuscitation from shock. [24] However, shock resuscitation is exceedingly complex: the most urgent requirement is for volume replacement. Clinical trials involving trauma patients are particularly difficult to design because of the problems of controls and informed consent. Future clinical trials will most likely be aimed at, for example, reduced use of allogeneic blood rather than at specific oxygen transport parameters, which may be controversial at best. For example, one trial with Fluosol during surgery showed that its use did not reduce the need for allogeneic blood transfusions in the postoperative period. [45]

Clinical Trials

Early trials with various cell-free hemoglobin solutions were reviewed and showed an array of side effects that affect every organ of the body. [1] However, most of these are mild or reversible, and only 1 death in >211 patients was reported in the early literature; this patient was terminally ill and would likely have died even without administration of hemoglobin.[46]

Several blood substitute products are in various stages of advanced clinical trials (Table 146-4). The greatest concern for hemoglobin-based products is that the known vasoactivity of the solutions could lead to hypertension or underperfusion of ischemic tissue. Perfluorocarbon emulsions are also being tested in humans, and a major concern appears to be thrombocytopenia.[47] No liposome-based product has yet been approved for use in human trials.

IMPLICATIONS/FUTURE APPLICATIONS

Potential Clinical Applications

Replacing all use of allogeneic blood with red cell substitutes is both unnecessary and naive. The red cell substitute candidates now being developed will probably be used initially in surgical hemodilution to provide a margin of safety and perhaps to reduce the need for the two or three units of blood used in most surgeries.

Many clinical applications in addition to hemodilution for the products now being developed will be targeted by industry (Table 146-5).[48] Applications for perfluorocarbon emulsions other than as red cell substitutes could be even more important than the use in trauma, surgery, and shock. For example, emulsions have been shown to increase the radiosensitivity of solid tumors, [49] to be excellent nuclear magnetic resonance and ultrasound imaging agents, [50] to be capable of removing gaseous microemboli during cardiopulmonary bypass, [51] and to measure tissue P_{O_2} .[52]

Availability in the Future

It seems unlikely that a cell-free hemoglobin as a red cell substitute with vasoactive effects will be accepted broadly by cliniciansindeed, vasoconstriction is a hallmark of the shock state. Perfluorocarbon emulsions may very well be the first red cell substitutes to reach the clinic. However, it is unlikely they will find wide application as such. More likely, they will find use as imaging agents, liquid-breathing agents, or adjuncts to radiotherapy of solid tumors. In addition, the low cost and simplicity of production of perfluorocarbon emulsions are favorable qualities for commercialization.

Liposome-encapsulated hemoglobin may very well be the ultimate solution to the red cell substitute problem. However, to be successful, an inexpensive and simple process will need to be developed, and any problems of reticuloendothelial blockade and engorgement of organs such as the liver and spleen will have to be thoroughly studied and understood.

The present commercial climate is such that few if any of these products will be used in scientific studies that can be reviewed in the peer-reviewed literature until they are approved for use by the FDA. This unfortunate situation has retarded development in the past and is likely to do so in the future. [32]

TABLE 146-4 -- Commercial Blood Substitute Products

Product (Manufacturer)	Composition	Indications
PHP (Apex Bioscience)	Pyridoxylated human hemoglobin conjugated to polyoxyethyene	Septic shock
HemAssist (Baxter Healthcare)	Human hemoglobin internally cross-linked with bis(3,5-dibromosalicyl) fumarate (DBBF)	Trauma Hemodilution
Hemopure (Biopure Corp.)	Glutaraldehyde-polymerized bovine hemoglobin	Hemodilution Sickle cell disease
PEG-Hemoglobin (Enzon)	Bovine hemoglobin conjugated topolyethylene glycol	Radiosensitization of solid tumors
PolyHeme (Northfield Laboratories)	Glutaraldehyde-polymerized human hemoglobin	Trauma Surgery
Optro (Somatogen)	Recombinant di-alpha human hemoglobin	Hemodilution Erythropoiesis
Oxygent (Alliance Pharmaceutical)	Emulsified Perflubron	Hemodilution Cardiopulmonary bypass
Oxyfluor (HemaGen/PFC)	Emulsified perfluorodichlorooctane	Cardiopulmonary bypass

TABLE 146-5 -- Potential Clinical Applications for Red Cell Substitutes

Trauma
Hemodilution (elective surgery)
Red cell incompatibility
Ischemia disease (e.g., percutaneous transluminal coronary angiography and stroke)
Extracorporeal circulation
Cell culture media
Hematopoietic stimulation
High blood-use surgery
Cardioplegia
Tumor therapy
Chronic anemia
Organ transplantation
Sickle cell anemia
Research on circulation and oxygen delivery

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Chapter 147 - Hematologic Complications of Liver Disease and Alcoholism

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INTRODUCTION

Liver disease and its major handmaiden, alcohol abuse, have profound effects on hemostasis and hematopoiesis. Liver disease, regardless of etiology, can be associated with abnormal bleeding due to anatomic derangements and impairment of hepatic synthetic and clearance functions. Abnormal lipid metabolism may cause red blood cell (RBC) structural defects. Alcohol and its metabolites have direct effects on hematopoiesis, behaving as a toxin and impairing nutritional physiology. Although a few well-described hematologic syndromes have been described, in most instances multiple and complex derangements are present simultaneously in the same patient, challenging the diagnostic and therapeutic skills of the hematologist.

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EFFECTS OF LIVER DISEASE ON HEMOSTASIS

Abnormal hemostasis is a common complication of liver disease, and determination of its underlying pathogenesis is essential for rational management. Mechanisms resulting in these defects include

1. Diminished hepatic synthesis of coagulation factors V, VII, IX, X, and XI, prothrombin, and fibrinogen (reflected in prolongation of the prothrombin time [PT] and partial thromboplastin time [PTT])
2. Dietary vitamin K deficiency due to inadequate intake or malabsorption (based on intrahepatic or extrahepatic cholestasis or intestinal malabsorption)
3. Dysfibrinogenemia
4. Enhanced fibrinolysis, due to decreased synthesis of $_2$ -plasmin inhibitor
5. Disseminated intravascular coagulation (DIC)
6. Thrombocytopenia due to hypersplenism

Abnormalities of Protein Synthesis

With the exception of factor VIII, [1] the liver is the major site of synthesis of the blood coagulation proteins and related regulatory proteins. [2] [3] [4] Parenchymal diseases of the liver, including cirrhosis, hepatitis, and infiltrative disorders, can impair synthesis leading to a deficiency of plasma proteins involved in hemostasis. [5] [6] [7] [8] Often, these abnormalities may be subclinical. Measurement of specific coagulation factors may be reduced but not attended by clinical bleeding or prolongation of the PT or PTT. In inflammatory liver disorders, the plasma levels of some coagulation proteins, such as factor VIII and fibrinogen, may increase as an acute phase reaction.[6] [9] [10] When the PT and PTT are prolonged, however, more extensive hepatic dysfunction is present and is associated with a poor prognosis. Hypofibrinogenemia, with plasma fibrinogen levels <100 mg/dl, is an especially grave sign. [11]

Acquired Defects of Vitamin K-Dependent Carboxylation

The vitamin K-dependent blood coagulation proteins, including prothrombin and factors VII, IX, and X, are synthesized in the liver. These proteins undergo a unique post-translational processing step where glutamic acid residues in the N-terminal region of these proteins are converted to -carboxyglutamic acid residues by a vitamin K-dependent carboxylase.[12] [13] [14] [15] [16] In some liver disorders, such as cirrhosis and hepatitis, an acquired deficiency of this carboxylation step may occur. Although generally mild, it may lead to the circulation of des--carboxylated proteins in the blood (Fig. 147-1).[17] Although biologically inactive and synthesized at the expense of biologically active forms, their levels are sufficiently low that they do not contribute significantly, if at all, to coagulation abnormalities associated with liver disease. They can, however, serve as useful markers of this acquired vitamin K-dependent carboxylation defect. Des--carboxy (abnormal) prothrombin, normally absent from plasma, circulates at detectable levels in >90% of patients with hepatoma (Fig. 147-2).[18] [19] [20] The tumor itself is responsible for the production of this protein because surgical removal or reduction in tumor mass with chemotherapy is associated with reduction or elimination of the abnormal prothrombin. Unlike the situation in vitamin K deficiency, levels of both the abnormal and normal prothrombin may be high simultaneously in patients with hepatoma. Therefore, this defect is not associated pathophysiologically with bleeding that may complicate management in primary hepatocellular carcinoma. Together with

Figure 147-1 Plasma levels of abnormal (des--carboxy) prothrombin in liver disease. The abnormal prothrombin level (g/ml) is displayed on the y axis on a logarithmic scale. The fully carboxylated native prothrombin is shown on a linear scale on the x axis. Severe vitamin K deficiency, pink triangle; warfarin therapy, white square; acute hepatitis, gray circle; cirrhosis, red square; normal, black circle. *(From Blanchard et al., [17] with permission.)*

Figure 147-2 Plasma levels of abnormal (des--carboxy) prothrombin in primary hepatocellular carcinoma. Comparison in patients with primary hepatocellular carcinoma, chronic active hepatitis, and metastatic carcinoma. The abnormal prothrombin level (ng/ml) is displayed on the y axis on a logarithmic scale. *(From Liebman et al., [18] with permission.)*

measurement of serum levels of -fetoprotein, serologic measurement of abnormal prothrombin allows identification of >84% of patients with hepatomas.

Vitamin K Deficiency

Nutritional deficiencies are common in alcoholism and alcoholic liver disease. However, with a daily minimal requirement of 100200 g, [21] inadequate intake does not lead to clinically significant vitamin K deficiency even with substandard diets. Because vitamin K is a fat-soluble vitamin, defects related to absorption can occur. This is especially so when impairment of bile acid metabolism occurs in primary biliary cirrhosis, [22] intrahepatic or extrahepatic cholestasis, [23] and during therapy with bile acid binders (e.g., cholestyramine).[24] In addition, because vitamin K is synthesized by intestinal bacteria, the use of oral antibiotics may result in frank deficiency. [25]

Dysfibrinogenemias

A defective functional form of fibrinogen has been described in some patients with liver disease, including cirrhosis and hepatocellular carcinoma. [26] [27] The defect in the molecule appears to be a post-translational event resulting in excess sialic acid residues following glycosylation in the hepatocyte. [26] [28] The action of thrombin on this abnormal fibrinogen appears to result in the formation of defective fibrin monomers with impaired ability to polymerize. [29] [30] Both the thrombin time (TT) and the reptilase time are usually prolonged, whereas the fibrinogen level measured immunochemically remains normal.

Increased Fibrinolysis, Fibrinogenolysis

Several types of pathophysiologic derangements can lead to factor deficiencies through increased factor consumption. Depressed synthesis of the major plasma inhibitor of plasmin, $_2$ -plasmin inhibitor, which is produced by the liver, results in enhanced fibrinolytic activity of the blood. [31] [32] This plasma protease inhibitor forms a complex with and inactivates plasmin.[33] Impaired synthesis correlates with the severity of the liver disease and pathologic fibrinolysis is manifested by decreased fibrinogen levels, increased fibrin degradation products (FDP), and accelerated euglobulin lysis. [34] [35]

Tissue-type plasminogen activator (t-PA) levels and its plasma inhibitors have been studied in patients with liver cirrhosis. Abnormal fibrinolysis, as measured by the dilute whole blood clot lysis time, correlated with a disproportionate reduction in the t-PA-inhibitor activity, even when t-PA and its inhibitor were increased. [36] Attempts to inhibit fibrinolysis through the use of antifibrinolytic agents have not been uniformly successful [37] and in the presence of possible DIC are contraindicated. [5]

DIC

A clear role for DIC in the pathogenesis of bleeding in liver disease is complicated by other causes of factor and platelet depletion, the cardinal signs of this disorder. Thus, decreased titers of clotting proteins and thrombocytopenia secondary to hypersplenism make it difficult to assess the contribution of DIC in an individual patient.[38] In some patients with cirrhosis, accelerated catabolism of fibrinogen, prothrombin, and plasminogen is improved by administration of heparin. [37] [38] [39] [40] Accelerated catabolism of fibrinogen occurs in acute liver necrosis, biliary tract obstruction, cholangitis, and liver contusion. [41] [42] [43] Entry of endotoxin into the circulation from the gastrointestinal tract in patients with portal hypertension has been suggested as another mechanism inducing DIC, as has impaired reticuloendothelial function, especially in alcohol-induced liver disease. [44]

Although the pathogenesis of DIC in cirrhosis is often puzzling, DIC is frequent in patients who have had surgical peritoneovenous shunts (LeVeen and Denver shunts) placed between ascitic fluid and the venous circulation. [45] [46] [47] [48] The cells suspended in ascitic fluid may induce coagulation. [48] [49] Collagen-like protein may induce platelet aggregation [50] or activate factor XII.[5] A concomitant drop in fibrinogen level and platelet count occurs, and occasionally, a disproportionate fall in the former has suggested a primary role for activation of fibrinolysis. [51] Clinically significant bleeding requires interruption of the shunt. Therapy with heparin has not been helpful. Although a role for antiplatelet agents, aspirin and dipyridamole, has been suggested, [50] they may add to the bleeding tendency in these patients.

Reduced levels of antithrombin III in chronic liver disease may contribute to the development of DIC [52] and reflect impaired hepatic synthesis. Correction of the antithrombin III deficiency by transfusion increases the survival of fibrinogen. [52] Low levels of heparin cofactor II have been reported in a few patients who may have undergone DIC.[53]

Platelets

Thrombocytopenia is commonly observed in patients with chronic liver disease and is ascribed to portal hypertension with its accompanying congestive splenomegaly.[54] This results in a shift in the platelets from the circulation to the enlarged spleen and may not affect platelet survival. [55] The reduction in the effective circulation is seldom in itself sufficient to induce a bleeding disorder but may contribute by additive effects to the other coagulation disturbances.

MANAGEMENT OF THE HEMOSTATIC DEFECTS IN PATIENTS WITH LIVER DISEASE

Evaluation and management of defective hemostasis in patients with liver disease should include a careful history and physical examination, appropriate laboratory testing to establish etiology, and institution of corrective therapy when required. Laboratory abnormalities in the absence of clinically significant bleeding may require only careful monitoring, whereas overt bleeding or preparation for invasive or surgical procedures requires replacement therapy.

Prolongation of the prothrombin time (PT) and partial thromboplastin time (PTT) usually indicates plasma clotting factor deficiency either as a result of impaired hepatic synthesis or secondary to vitamin K deficiency. When combined with diminished levels of factor V, factor XI, or fibrinogen, which are not vitamin K dependent, then liver dysfunction is apparent. Some of these abnormalities may be complicated by the presence of inhibitory activity in the plasma such as fibrin degradation products (FDP). In addition, a low fibrinogen level can result from increased fibrinolysis/ fibrinogenolysis, disseminated intravascular coagulation (DIC), or structurally abnormal fibrinogen. Measurement of the thrombin time (TT) shows a prolongation under all these conditions. Evidence for increased fibrinolytic activity is obtained by finding elevated FDP or the D-dimer. Distinction between plasma clotting factor deficiency and plasma inhibitory activity is obtained by determining whether the PT and PTT can be corrected in a 1:1 mix of normal plasma and patient plasma. Reduction of the platelet count commonly accompanies the coagulation factor disturbances and is attributable to hypersplenism, recent alcohol abuse, and if especially severe, to DIC.

If the PT is >3 seconds prolonged, and if the patient is not actively bleeding, a trial of vitamin K can be given when the laboratory findings are consistent with plasma clotting factor deficiency. A dose of 10 mg daily subcutaneously for 3 successive days is sufficient to correct the abnormality in those few patients in whom malabsorption of the vitamin is present.

More characteristically, hepatic dysfunction is the underlying mechanism and replacement therapy is warranted in patients actively bleeding or who are to undergo invasive diagnostic or therapeutic procedures. Fresh frozen plasma (FFP) remains the mainstay of replacement therapy. Its major drawback is the large volume that may be required in patients with unstable cardiovascular conditions. Although small volume replacement factor IX concentrates, which are rich in vitamin K-dependent factors, have occasionally been used, their administration has been associated with thromboembolic problems or DIC. The amount of FFP infused will vary with the clinical status of the patient and the frequency of administration dictated by the plasma half-life of the clotting factors. The desired initial infusion is 2530% of the plasma volume (12001500 ml in a 70-kg adult) to achieve at least partial correction of the hemostatic defect. With continued active bleeding, half the initial dose may be required as often as every 6 hours, considering that the half-life of several clotting factors varies from 5 to 8 hours. In the presence of ascites, in which the volume of distribution is increased, or in the presence of fibrinolysis or DIC, an increased dose of FFP will be required as determined by serial measurement of the PT and PTT. Red blood cell transfusions will also be required in the actively bleeding patient to maintain adequate hemoglobin levels and a normotensive state. In very advanced liver disease, hypofibrinogenemia may be severe (i.e., <100 mg/dl); cryoprecipitate can be transfused at the rate of 1 U/3 kg body weight. Each bag contains about 300 mg fibrinogen and, because one-half the transfused fibrinogen is metabolized within 3 or 4 days, a daily transfusion of 1 U/15 kg of body weight can maintain the fibrinogen concentration at adequate levels. Many of these patients may have some degree of thrombocytopenia, but when the platelet count is <20,000/mm^3 and bleeding is active, platelet transfusions should be considered. Determining the platelet count 1 hour after transfusion provides a useful guide to the adequacy of retention. In the presence of splenomegaly, this may be minimal due to platelet pooling in the spleen. In instances where a qualitative platelet dysfunction is suspected, desmopressin may be tried at a dose of 0.3 g/kg intravenously.

Establishing a diagnosis of DIC in bleeding patients with liver disease is especially challenging. Prolongation of the PT, PTT, TT, low fibrinogen levels, elevated FDP, and thrombocytopenia may have multiple pathophysiologic mechanisms. Because factor VIII coagulant activity is often elevated in liver disease and reduced in DIC, its measurement helps to distinguish these disorders. A diagnosis of DIC is more likely to be associated with precipitating factors such as sepsis, shock, or peritoneovenous shunts. The use of heparin in these situations has always been controversial, especially in the presence of active bleeding, so that reliance must be placed on replacement therapy with FFP, cryoprecipitate, and platelets. In the setting of liver diseases and DIC, a role for antithrombin III concentrates or antifibrinolytic agents has not been established.

Orthotopic Liver Transplantation

Hemostatic alterations in orthotopic liver transplantation can be divided into preoperative, anhepatic, reperfusion, and convalescent phases. [56] Superimposed on the preoperative hemostatic defects, hepatic clearance is absent during the anhepatic phase so that levels of t-PA increase while the plasminogen activator inhibitor-1 disappears, and fibrin(ogen)-related antigens increase. [57] [58] During reperfusion, there is further increase in excessive fibrinolysis, perhaps as a result of the release of tissue plasminogen activators from the vessels of the transplanted liver. [57] [59] Some evidence suggests that the administration of aprotinin throughout the operation may decrease hemorrhagic blood loss.[60] However, the role for this agent or purified human antithrombin III remains to be determined. [61] [62] [63] Within a few days of the convalescent phase, the coagulation factor levels return to normal, and the inhibitors of coagulation (antithrombin III, plasminogen, protein C, and protein S) normalize over 12 weeks.[56]

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EFFECTS OF LIVER DISEASE ON HEMATOPOIESIS

Chronic liver disease causing cirrhosis leads to portal hypertension and congestive splenomegaly. Portal hypertension can then lead to excessive blood loss from variceal and other types of upper gastrointestinal bleeding, whereas congestive splenomegaly represents a major cause of hypersplenism. When blood loss continues on a chronic basis, iron deficiency can supervene. Thrombocytopenia, leukopenia and anemia, or any combination thereof, may result from excessive trapping by the enlarged, congested spleen. In addition, the anemia of chronic disease, as seen in other disorders of inflammation, infection, or

malignancy, further complicates the hematologic presentation in these patients. Altered lipid metabolism may lead to structural changes in the RBC membrane, occasionally causing unusual hemolytic syndromes.

Hypersplenism

Splenomegaly is a common finding in patients with chronic liver disease and portal hypertension. The degree of reduction in the blood counts is a reflection of both splenic size and the capacity of the bone marrow to compensate for this reduction. Anemia results from RBC pooling in the enlarged spleen, decreased RBC survival, and increased plasma volume.[64] [65] [66] Neutropenia has been attributed both to increased pooling and decreased survival. [64] [67] Thrombocytopenia is primarily the result of increased platelet pooling. [54] Because of the moderate degree of these cytopenias and the operative risks in these patients, splenectomy is rarely indicated. Shunting procedures, both splenorenal and portocaval, have variable results on the hematologic manifestations. [68] [69] [70]

Effects of Liver Disease on Erythrocyte Membranes

Structural abnormalities causing RBC shape changes are common in liver disease. Target cells are frequently seen in the peripheral blood smear and result from increased surface area due to excess membrane cholesterol and phospholipid transferred from lipoproteins in the plasma. [71] These cells have a normal survival and resist osmotic lysis.[71]

Spur cells are a grave finding in some patients with advanced liver disease. This shape transformation can occur in normal RBCs incubated with plasma from affected patients.[72] Although membrane cholesterol is increased, total phospholipids are normal, and the cells show decreased resistance to osmotic lysis. [73] Splenic modeling of these cells contributes to their reduced survival, and hemolysis is usually moderate to severe. [74] Although splenectomy may improve the anemia, patients with this acquired disorder are rarely surgical candidates.

A unique feature of patients with chronic liver disease is the presence of thin macrocytes in the peripheral smear, unrelated to vitamin B_{12} or folic acid deficiency.[75] RBC diameter is increased, but the mean corpuscular volume (MCV) remains normal. Unlike target cells and spur cells, thin macrocytes do not become normal when transfused into normal subjects, nor are normal cells transformed into thin macrocytes when transfused into patients with liver disease. [75] [76] RBC survival does not appear to be affected.

Anemia Associated with Viral Hepatitis

Viral hepatitis may be associated with a mild anemia secondary to bone marrow suppression and shortened RBC survival. [77] [78] Because hepatic clearance of oxidizing metabolites may be impaired in patients with viral hepatitis, depression of reduced glutathione occurs. [79] This can be especially problematic for patients with glucose-6-phosphate dehydrogenase deficiency. [80] [81]

Aplastic anemia (Chap. 19) is a potential consequence of viral hepatitis; one study has estimated an incidence of 0.10.2%. [82] The average age is 18 years, with a male preponderance.[83] Hepatitis C is a more common cause of hepatitis-associated aplastic anemia. [84] Aplasia usually develops within 2 months of the hepatitis, and without bone marrow transplantation, has a high mortality rate. In a large interinstitutional study, 9 of 32 patients undergoing orthotopic liver transplantation for acute non-A, non-B hepatitis, developed aplastic anemia 17 weeks after transplant. No evidence for aplasia was found prior to transplant. No other cases of aplastic anemia were identified among 1,463 patients undergoing transplantation for all other indications, confirming the high risk for development of aplastic anemia in patients with acute non-A, non-B hepatitis after this procedure. [85]

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HEMATOLOGIC EFFECTS OF ALCOHOL ABUSE

Metabolism of Ethanol

Hepatocyte cytosol contains alcohol dehydrogenase, which constitutes the major pathway for the catalytic conversion of ethanol to acetaldehyde. [86] In this reaction, hydrogen is transferred from the substrate to the cofactor, nicotinamide adenine dinucleotide (NAD), and then to its reduced form, NADH, producing acetaldehyde. This oxidation step results in an excess of reducing capacity of the cell, and the shift in redox potential alters the lactate pyruvate ratio. [87] [88] The acetaldehyde produced is then converted in the mitochondria to acetate, which is catalyzed by acetaldehyde dehydrogenase, generating further NADH, a major factor in the toxicity of ethanol.[86] Increased lactic acid contributes to acidosis, and decreased uric acid excretion by the kidney leads to hyperuricemia. Gluconeogenesis is impaired and triglyceride accumulation favored. Impaired oxidation of fatty acids, resulting from reduced availability of NAD in the citric acid cycle, leads to the accumulation of esterified fatty acids. Further deposition of triglyceride and phospholipids occurs as a result of activation of the second pathway for ethanol metabolism, the cytochrome P-450-dependent microsomal ethanol-oxidizing system located in the endoplasmic reticulum. In the presence of oxygen and NAD phosphate as cofactor, acetaldehyde accumulates. With heavy ethanol intake, <20% of the substrate is metabolized through this pathway. [86] [89] The lipid accumulation in the hepatocyte is further enhanced by the direct toxic effect of acetaldehyde. Mitochondrial function is altered, as is the impairment of microtubule formation. This effect of acetaldehyde on protein synthesis interferes with the transport of lipoprotein from the liver, this defect may be the pathobiologic basis for the formation of the fatty liver. Engorgement of the hepatocyte by an increased protein load when accompanied by necrosis and polymorphonuclear inflammation characterizes alcoholic hepatitis. Although this manifestation is not necessarily a precursor to the development of cirrhosis, it is frequently a prelude. [86]

The hematologic sequelae of alcoholism are the consequences of those alterations secondary to portal hypertension and hypersplenism, lipid abnormalities reflected in membrane structural defects, disturbed synthesis of the coagulation proteins, and the direct toxic effects of alcohol and acetaldehyde.

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DIRECT EFFECTS OF ETHANOL ON HEMATOPOIESIS

Bone Marrow

Excessive alcohol consumption affects the bone marrow in several ways. These include vacuolization of hematopoietic precursors, megaloblastic changes in the absence of folate deficiency, sideroblastic changes, hypocellularity, and increased numbers of plasma cells. [90] [91] [92] [93] [94] [95] [96] Because a characteristic pattern of bone marrow injury is not diagnostic of alcoholism, some or all of these features may not necessarily coexist. Because the progeny of bone marrow precursors may lack certain synthetic capacities, it is likely that some of the described abnormalities of the peripheral blood cells may have been inflicted in the bone marrow. [97]

Vacuolization of proerythroblasts occurs with high frequency in heavy imbibers (12 pints of 80-proof whiskey daily). Vacuoles can be seen within 1 week of such intake and quickly disappear within a few days of abstinence. [91] They appear to result from surface invagination of the cell membrane, leading to endocytosis and subsequent vacuole formation. [98] Cytoplasmic localization in the proerythroblast is characteristic although occasional nuclear vacuoles can be seen. Promyelocytes share the injury to a lesser extent, as do other precursor cells. In vitro studies with normal bone marrow incubated in the presence of alcohol reproduce these findings. [98] Chemical identification of

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the inclusions has not been achieved. Similar morphologic changes had been described in association with chloramphenicol use and in other clinical disorders. [99]

Marrow hypocellularity has been observed in occasional patients with excessive alcohol intake and was attributed to the toxic effect of ethanol or its metabolites because of the absence of other factors. [94] [100] Further evidence of a toxic effect of alcohol on the bone marrow has come from in vitro studies of this agent on in vitro colony formation. [101] [102] [103] Depressed burst-forming unit erythroid and colony-forming unit (CFU) erythroid have been observed, [101] whereas higher ethanol concentrations impair CFU-granulocyte/macrophage and CFU-macrophage. [102] [103] The pluripotential stem cell escapes this injury, thus accounting for the common in vivo observation that these toxic effects are reversible on cessation of alcohol abuse. [101] [104]

Sideroblastic changes are commonly present in erythroid precursors of alcoholic patients. [105] [106] [107] Structural studies show accumulation of siderotic granules in the mitochondria surrounding the nucleus and give the characteristic ringed appearance on Prussian blue stains of the marrow. Generally, these morphologic findings are more readily appreciated in the more mature erythroid cells. Defective iron utilization for hemoglobin synthesis underlies this process, producing a dimorphic anemia that generally reverses within days to 2 weeks of alcohol withdrawal. [108] In most instances, this is not the sole cause of the anemia but may occur in up to one-third of alcoholic patients, usually in association with folate deficiency and acute blood loss. [105] [107] Earlier studies suggested that the defect resulted from ethanol impairment of the conversion of pyridoxine to pyridoxal phosphate, which is catalyzed by pyridoxine kinase. [108] Pyridoxal phosphate, the cofactor for aminolevulinic acid synthase, although measurably low in the serum of chronic alcoholics, does not necessarily correlate with the finding of ringed sideroblasts. [106] [109] [110] In addition, decreased kinase activity has not clearly been demonstrated to account for low pyridoxal phosphate levels, although the latter has been shown to undergo enhanced degradation in the presence of acetaldehyde. [110] [111] [112] Because a direct role for vitamin B_6 deficiency has not been clearly shown in these alcoholic patients, an inhibitory role of ethanol on heme biosynthetic pathways (Chap. 27) may be more likely. Overall heme production is reduced in the presence of ethanol, as is globin synthesis. [113] [114]

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MACROCYTOSIS

Macrocytic RBCs are a common finding in alcoholism.[115] When present in the absence of folate deficiency or reticulocytosis, a diagnosis of alcohol abuse can be considered.[116] [117] The MCV is generally in the 100110 fl range, the cells are round and not ovalocytic, and, together with the finding of an elevated, -glutamyl transpeptidase in the serum, can be of diagnostic import. In a significant number of heavy drinkers, an increased value for RBC distribution width obtained from automated cell counters has also been described,[118] but others have not substantiated the value of this determination.[116] Unlike the readily reversible cell injury described for alcohol-mediated toxicity, this form of RBC change remains for 24 months of abstinence.

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FOLATE AND B_{12} METABOLISM

When megaloblastic anemia (Chap. 28) is found in the alcoholic patient, it is almost always secondary to folate deficiency; the hematologic manifestations of macrovalocytosis, hypersegmentation of polymorphonuclear leucocytes, leukopenia, and thrombocytopenia are indistinguishable from other causes of folate deficiency. Although liver disease is common in this group of patients, the vitamin deficiency primarily arises as a result of poor dietary intake and the effect of ethanol on folate metabolism.[96] [119] [120] Other causes of nonalcoholic cirrhosis are uncommonly associated with folate deficiency, nor is it seen in well-nourished drinkers. Secondary effects on folate homeostasis may occur as a result of poor retention by the cirrhotic liver and increased urinary loss. [121] [122]

Evidence for a direct effect of ethanol on folate metabolism has come from several studies, both human and animal, in which alcohol and dietary intake were carefully controlled. In folate-deficient humans, megaloblastic changes can be seen within a few days when ethanol is added, but such changes take longer to develop when it is not.[96] Furthermore, folate supplements prevent these morphologic changes. Hematosuppression, including white blood cell and platelet counts, reverts to normal within 12 weeks of a hospital diet.[107] The explanation for these observations has not been fully clarified. Among the suggested mechanisms have been impairment of jejunal absorption,[123] block in the delivery of *N*-5-methylfolate from the liver to the circulation,[119] interruption of a putative enterohepatic circulation, [120] [124] and increased urinary excretion of folate.[121] [122] In animal models, conflicting data have emerged on the effect of ethanol on hepatic polyglutamate folate synthesis and retention. Both increased synthesis and decreased formation have been described. [119] [125] Furthermore, the clearance of methyltetrahydrofolic acid is normal in alcoholic patients, and its uptake is stimulated by ethanol in hepatocytes in vitro. [126] [127] Extraneous factors such as gastrointestinal blood loss, hypersplenism, hemolysis, and infection further confound the interpretation of folate balance in these patients.

Clinically significant vitamin B_{12} deficiency rarely occurs in the alcoholic patient. This is rather surprising because impairment of absorption could occur secondarily to the frequently seen gastritis (parietal cell injury), pancreatitis (interference with pancreatic enzyme release of B_{12} from R-binders), or ileal malabsorption (vitamin B_{12} uptake) in these patients.[128] [129] Whether as a result of liver injury or abnormal transcobalamin binding, serum levels of the vitamin are frequently elevated. Even when low serum levels have been observed, concomitant folate deficiency may have been causal because serum vitamin B_{12} levels reverted to normal within a few days of adequate folate intake.[130]

Iron Metabolism

Iron deficiency is commonly seen in alcoholic patients, usually as a result of gastrointestinal bleeding. However, because of the frequent association with folic acid deficiency, the usual laboratory parameters (MCV, serum iron, and transferrin saturation) may be normal or even increased. [91] [96] Serum ferritin levels may be unreliable because of coexisting liver disease or complicating inflammatory disorders. [131] Simple chronic anemia, with low serum iron and low serum iron-binding capacity, will be found in patients with infection or malignancy. Bone marrow iron stores are probably the most reliable means of ruling out iron deficiency in this complicated setting when folate deficiency has been ruled out.

Alcohol abuse may increase the body iron burden. This can result from excessive ingestion of iron-containing spirits (e.g., red wine) or increased iron absorption. [132] [133] There has been some confusion between idiopathic hemochromatosis and alcoholic cirrhosis with increased stainable iron in the liver. Patients with alcoholic cirrhosis can be divided into two groups: (1) patients who have mild-to-moderate increase in stainable iron, but relatively normal body iron stores, and (2) patients with gross iron deposition and increased total body iron stores of the magnitude seen in idiopathic hemochromatosis (1550 g iron stores). Based on studies of human leukocyte antigens, the first group shows no evidence of the genetic disease and the liver iron concentration is usually less than twice the upper limits of normal. [134] The second group appears to have idiopathic hemochromatosis in addition to alcoholism. [135] Additionally, in alcoholic patients the synthesis of desialated transferrin may lead to preferential hepatic uptake of iron. [136] [137] Recently, a candidate

hemochromatosis gene, HFE, has been identified.[138] Located on chromosome 6, a G A transition results in a missense mutation that changes cysteine 282 with a tyrosine. Approximately 83% of the hemochromatosis patients studied were homozygous for this mutation. Although other mutations likely occur, determination of these defects by currently available methods should aid in differentiating acquired from inherited disorders of iron overload. [139]

RBC Survival

A direct effect of alcohol on RBC survival once cells leave the bone marrow has not been demonstrated. [71] However, alcohol is the most common etiologic factor in acute and chronic liver disease that may be associated with hemolysis. A mild hemolytic state is common in cirrhosis, reducing RBC survival to 50% of normal. [140] In the Zieve syndrome, alcohol-induced fatty liver is associated with hypertrigylceridemia, hemolysis, and jaundice. [141] Hemolysis is usually transient, and its pathophysiology appears to be unrelated to the hypertriglyceridemia. [71] A similar transient hemolytic state showing RBC stomatocytosis has been described. [142] Rarer still has been the hemolytic anemia associated with severe hypophosphatemia. [143] In many of these alcoholic patients, additional features such as bleeding, alcohol withdrawal, folate depletion, and hypersplenism make interpretation of the reticulocyte count difficult.

Platelet Production and Function

Thombocytopenia is a well-recognized consequence of excessive ethanol intake. [91] [93] [96] With abstinence, the platelet count begins to rise within a few days, returns to normal by 1 week, and may reach supranormal levels (rebound thrombocytosis) within 13 weeks. Ethanol, or its metabolites, is the etiologic agent, and thrombocytopenia may be present in the absence of complicating liver disease, hypersplenism, nutritional deficiency, or DIC. The major toxic effect appears to be mediated at the bone marrow level, although there is some evidence for decreased platelet survival. [144] Megakaryocytes in the bone marrow are usually adequate in number,[145] [146] but may be reduced.[96] [103] [147] CFU-megakaryocytes grown in the presence of ethanol show diminished numbers in vitro. [102]

Not only does alcohol affect platelet production, but immoderate drinking can lead to several abnormalities of platelet function. Impaired platelet aggregation, [148] [149] [150] decreased thromboxane A_2 release, [148] and prolongation of the bleeding time, even in the absence of thrombocytopenia, have been observed. [144] [151] Effects on storage pool adenosine diphosphate and platelet cyclic adenosine monophosphate have also been described. [144] [152] The degree to which impaired platelet function contributes to abnormal bleeding in alcoholic patients is not clear. Whether the decreased risk of cardiovascular disease, but an increased risk of cerebrovascular accidents, observed in these patients can be attributed to platelet function defects is not known. [153] [154]

Granulocyte Production and Function

The effect of ethanol consumption on granulocyte production is less clear than its effect on RBCs and platelets. Neutropenia is more commonly a result of hypersplenism or folate deficiency. [155] Even though alcohol injury to bone marrow can produce vacuolization of myeloid precursors, the white blood cell count and marrow granulocyte reserve remain normal. [96] [156] In vitro incubation of bone marrow with high levels of alcohol can inhibit granulocyte colony formation. [104] In occasional patients, heavy alcohol intake in combination with severe bacterial infections has been associated with marked neutropenia and bone marrow myeloid arrest at the myelocyte stage. [157]

The effect of alcohol on granulocyte function has long been suggested by the poor delivery of neutrophils to sites of acute infection. Movement into skin abrasions is decreased and has been attributed to defective adherence. [156] [158] Although chemotaxis is reduced, phagocytosis and intracellular bacterial killing are not affected. [158] [159] [160]

Immune Function

Decreased numbers of circulating lymphocytes have been described in alcoholic subjects. [155] Active drinkers fail to develop increased antibody titer responses to immune challenge and delayed hypersensitivity responses to new antigens. [155] In animal studies, ethanol ingestion decreases natural killer cell activity and delayed hypersensitivity responses. [161] Although the mechanisms underlying impaired cellular and humoral immune functions in alcoholic subjects have not been fully clarified, epidemiologic data support the association.

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Chapter 148 - Hematologic Complications of Renal Disease

Nicholas Dainiak

INTRODUCTION

Chronic renal insufficiency is characterized by an elevation in serum urea nitrogen and creatinine concentrations with or without reduced urine output. It leads ultimately to functional disorders involving fluid and electrolyte balance as well as virtually every organ system in the body. Disturbances of the hematopoietic system range from cytopenias and hypocomplementemia to disorders of hemostasis. Abnormalities include the development of splenomegaly and hypersplenism and increased susceptibility to infection. In turn, these sequelae may worsen cytopenias and bleeding. Unfortunately, most of the hematologic and immunologic disturbances present in chronic renal

insufficiency progress or develop even after an optimal program of dialysis and related therapy has been initiated. [1]

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PATHOPHYSIOLOGY OF RENAL DYSFUNCTION

The classic evolution of renal failure to terminal uremia and azotemia was first described by Bright in 1936. [2] Although the full clinical spectrum of abnormalities in uremia is rarely seen today because of therapeutic intervention, pallor and anemia persist as hallmarks of severe renal insufficiency. Patients with a blood urea nitrogen concentration of >100 mg/dl rarely have a hematocrit value above 30%. [3] Attempts to explain this relationship have changed in concert with evolving concepts concerning the pathogenesis of uremia. [4] Although excretory failure leading to retention of nitrogenous waste products is pathogenetically more closely related to acute neurobehavioral abnormalities, failure of renal biosynthetic processes and hormonal regulation has been recognized as related to chronic organ abnormalities. Renal biosynthetic failure encompasses not only impaired secretion of metabolites (including serine, leucine, and arginine) by the kidney but also impaired production of renal hormones. [5] Among the renal hormones are erythropoietin (EPO), renin, 1,25-dihydroxycholecalciferol vitamin D_3 and prostaglandins. Defective production or release of these hormones leads to a wide spectrum of clinical sequelae, including anemia, hypertension, disturbed calcium metabolism, altered renal blood flow, and impaired sodium and water excretion. [6] [7] [8] Recently, the concept that defective hormonal feedback systems may lead to uremia has been advanced. [9] [10] Since the diseased kidney maintains sodium balance despite a loss of functioning nephrons, circulating inhibitors of sodium transport may be present. Although little is known about the chemical identity of such inhibitors (or uremic toxins), considerable evidence indicates that sodium transport is inhibited and sodium/potassium/ATPase levels are decreased. [11] [12] [13] [14] Such abnormal regulators are distinct from low molecular mass toxins, (including >70 nitrogenous compounds), and are also distinct from oligopeptides and polypeptides (i.e., middle molecules ranging in molecular mass from 300 to 2,000 daltons), which may be retained as a consequence of impaired renal excretory function. [5] In addition, endocrine toxins, including parathyroid and natriuretic hormones, may be present in increased quantities in response to reduced renal mass. As a result of the ubiquitous actions of these substances, many features of uremia may be explained by disruption of normal hormonal feedback systems. [15] [16]

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ETIOLOGY AND PATHOGENESIS OF HEMATOLOGIC AND IMMUNOLOGIC DISTURBANCES

Abnormalities in renal excretory function, renal biosynthetic processes, or hormonal regulation may be responsible for the hematologic sequelae of renal failure. Except for defective production of the hematopoietic hormone EPO, evidence for a direct relationship between a specific abnormality in renal function and a hematologic complication remains circumstantial.

Hypoproliferative Anemia

Anemia characteristically accompanies the uremic syndrome. It is virtually always present in acute forms of renal failure associated with interstitial nephritis, tubular necrosis, and glomerulonephritis, [17] and it is often the presenting symptom of chronic renal insufficiency as well. [3] Over 90% of patients receiving chronic dialysis therapy have anemia, and in 25% of these cases, the anemia is severe enough (hematocrit <25%) to require repeated blood transfusions. [18] Although the cause of renal failure correlates poorly with the degree of anemia, patients with polycystic disease are less anemic than those with other renal disorders on a per-milligram serum creatinine basis. This may in part be related to induction of EPO synthesis or release in response to local renal hypoxia that develops as a consequence of pressure exerted by space-occupying cysts. In contrast, renal failure associated with other disorders may be complicated by a greater degree of anemia than anticipated. For example, blood loss and iron deficiency invariably complicate renal failure due to Goodpasture syndrome and Henoch-Schönlein purpura. Likewise, brisk hemolysis may occur in patients with renal insufficiency associated with connective tissue disease (such as systemic lupus erythematosus), and the production of EPO by kidneys that have been damaged by radiation may be inordinately depressed. [19] [20] [21]

A low red blood cell mass in uremia is most often the result of diminished red cell production. The principal cause of this defect is a decrease in renal EPO production relative to the degree of anemia that is present. Although low, normal, and high levels of plasma EPO have been measured by radioimmunoassay and bioassay, [22] [23] [24] [25] [26] [27] EPO levels are inappropriately low when adjusted for the degree of anemia (Fig. 148-1).[28] [29] [30] Furthermore, a positive correlation is present between serum EPO level and hematocrit, and between hematocrit and creatinine clearance in patients with a relatively mild degree of renal failure (i.e., creatinine clearance of 240 ml/minute[28]). Anephric patients have the lowest EPO levels, a finding that complements the observation that erythropoiesis is severely impaired (but not abolished) in patients who have undergone bilateral nephrectomy. [28] [29] [30] [31] As

Figure 148-1 Levels of erythropoietin versus hematocrit in uremic patients with or without intact kidneys. Shaded area represents predicted values. *(From Caro et al., [6] with permission.)*

shown in Figure 148-1 , anephric individuals have extremely low but nevertheless detectable plasma EPO. The source of this extrarenal EPO is unknown but may include macrophages in the bone marrow or spleen,[31] [32] Kupffer cells in the liver,[33] [34] [35] [36] or undefined cellular components of submandibular glands. [37] [38] In addition to EPO, plasma from anephric individuals may also contain an insulin growth factor-like activity that supports the growth of mature erythroid progenitor cells.[39]

Although the presence of a circulating erythropoietic factor was postulated at the turn of the century, [40] was confirmed to exist in 1953,[41] and was demonstrated to be of renal origin in 1957,[42] remarkably little is known about its site of production. The renal cellular origin of the hormone has been ascribed to glomerular, [43] [44] [45] [46] [47] mesangial,[48] [49] cortical,[50] [51] [52] [53] tubular,[54] medullary,[55] [56] and juxtaglomerular[57] [58] cells at one time or another. Recently, the EPO gene has been used as a probe to detect EPO mRNA accumulation under hypoxic conditions.[59] [60] Results of this experimental approach suggest that tubular [61] or peritubular[62] cells produce EPO in response to hypoxia. Nevertheless, because the healthy kidney contains no detectable stores of EPO, [63] insight concerning cellular or subcellular mechanisms leading to defective EPO production in renal failure has not been forthcoming.

Even less is known about the forces that drive EPO production or release. Clinical observations in patients with patent ductus arteriosus and erythrocyte overproduction suggested that an oxygen sensor for hypoxia is located below the diaphragm. [64] [65] Although the sensing mechanism for hypoxia is believed to reside in the kidneys, virtually no information concerning its cellular localization is available. It has been suggested that the regulatory feedback mechanism for EPO production remains intact in patients with mild renal insufficiency but that the entire system operates at a reduced level. [29] [30] This may result in a suboptimal EPO response to hypoxia. Such a mechanism may be the antithesis of an abnormally high set point for EPO in production, which has been suggested to be responsible for EPO-dependent erythrocytosis.[66] Nevertheless, serum EPO levels are increased in patients with chronic renal failure who develop acute hypoxemia (Pa o_2 <65 mm Hg),[67] a finding that may be explained by extrarenal EPO production.[68]

In addition to relative EPO deficiency, other processes may contribute to hypoproliferative anemia in renal disease (Fig. 148-2). Uremic toxins that impair proliferation of erythroid progenitor cells and erythroid precursors in the bone marrow have been described. These include serum inhibitors of erythroblast maturation and heme synthesis,[69] [70] as well as inhibitors directed at progenitor cells. The latter toxins have been identified in bone marrow culture systems as spermine, [71] parathyroid hormone,[72] ribonuclease,[73] and various serum lipoproteins. However, the physiologic relevance of these observations is not established. Uremic serum has been found to inhibit colony-forming unit-granulocyte macrophage (CFU-GM) proliferation as well as megakaryocyte cell lines growth; [74] spermine has been found to inhibit CFU-GM proliferation.[75] These results are discordant with findings in vivo where both the white blood cell count and platelet count are characteristically normal in patients with renal insufficiency. Furthermore, uremic serum contains normal levels of polyamines. [76] Likewise, serum lipoproteins from healthy (nonanemic) individuals have been found to suppress hematopoietic progenitor cell proliferation. [77] Finally, the concentration of ribonuclease required for inhibition of erythroid progenitor cell growth in vitro exceeds levels found in uremic serum. [73]

Hemolytic Anemia

A diminished red cell mass may result not only from impaired red cell production but also from decreased red cell life span (Fig. 148-2). Reductions in red cell survival rates to approximately one-half normal have been measured in patients with advanced renal failure. [78] [79] This defect has been shown to be extracorpuscular and may be due to retention of uremic solutes that function as abnormal metabolites or to mechanical factors in the environment of the red cell as it travels through abnormal vasculature.[80] [81] Cross-transfusion studies indicate that uremic red cells survive normally in normal recipients, whereas the life span of normal red cells is shortened when transfused into uremic patients. [82] [83] Moreover, the red cell life span occasionally normalizes after intensification of dialysis [84] or a change to continuous ambulatory peritoneal dialysis, [85] thereby suggesting that metabolic factors are involved.

In contrast to its environment, the red cell appears to be generally normal in renal failure. Laboratory evidence for appropriately increased activities (for age) of many red cell enzymes and adenosine triphosphate (ATP) [14] [86] [87] support this concept. An appropriately elevated 2,3-diphosphoglycerate (2,3-DPG) level and depressed

hemoglobin affinity for oxygen suggest that the red cell carries and releases oxygen in a normal fashion. Although the oxygen affinity of hemoglobin is appropriately reduced in uremia, carbamylation of hemoglobin resulting from the action of isocyanic acid (the active form of cyanate formed by spontaneous dissociation of urea in plasma) at NH_2 terminal residues of - and -chains is associated with increased oxygen affinity. [88] This paradox may be explained in studies of red cells

Figure 148-2 Levels of defects in the production and life span of the red cell.

from uremic patients by a concentration effect. Relatively low urea concentrations (1060 mM) are associated with stronger binding of 2,3-DPG to hemoglobin and stabilization of the deoxyform of hemoglobin, while higher urea concentrations (120 mM) show a decrease in P50 and increased affinity of hemoglobin for oxygen. [89] Structural changes in the hemoglobin molecule (rather than carbamylation of hemoglobin) predominate at the lower urea concentrations that are typically found in uremic patients.

Nevertheless, slightly decreased transketolase and superoxide dismutase levels [90] [91] and depressed Na^+ /K^+ /ATPase[92] have been reported in red cells from uremic patients. Reduced antioxidant defense mechanisms have been found in red cells from patients with chronic renal failure. Recently, a decreased level of reduced glutathione (GSH) was measured in red cells from renal failure patients maintained on hemodialysis. [93] The defect was thought to be the result of lack of NADPH due to decreased glucose-6-phosphate dehydrogenase (G6PD) activity. These findings may be physiologically important when the red cell membrane is exposed to oxidant drugs or compounds.[94] Therefore, therapy that generates factors that stress the red cell antioxidant system may result in accelerated hemolysis (see later). In the absence of such stress, only a small minority of uremic patients have underlying anemia that is principally due to diminished red cell survival.

Erythrocytosis

In some patients with impaired renal function the red cell mass is elevated rather than depressed. Isolated erythrocytosis has been described in association with a variety of renal disorders, including hypernephroma, cystic renal disease, ureteral obstruction, and renal artery stenosis. [90] [91] [92] In addition, it may complicate the clinical course of 1020% of patients undergoing renal transplantation. [95] Erythrocytosis may be the harbinger of acute or chronic allograft rejection [96] and post-transplant renal artery stenosis. [97] [98] In many cases, it is accompanied by excessive EPO production.[96] [97] [98] [99] [100] [101] Even moderate levels of erythrocytosis can be associated with elevated serum EPO levels. [102] [103] [104] The etiology of increased EPO levels is unclear but may be related to impaired renal blood flow resulting in local hypoxia and stimulation of the renal oxygen sensor. [95] Selective catheterization of veins of native and transplanted kidneys has demonstrated elevated EPO levels in blood exiting from native (diseased) kidney or kidney remnants. [105] Whether such EPO production is autonomous or subject to feedback regulation but at an elevated EPO set point is unknown. [106] Alternatively, hepatic EPO production may be induced by administration of androgenic steroids. [107] [108] It appears that administration of exogenous EPO at the time of renal transplantation does not predispose to development of post-transplant erythrocytosis. [109]

Since erythrocytosis may occur in patients lacking native kidneys [110] and since confounding variables such as androgenic steroid administration, smoking, hepatic insufficiency, diabetes mellitus, cardiac dysfunction, and pulmonary disease may complicate renal failure, the etiology of EPO-dependent erythrocytosis in the renal transplant recipient may be multifactorial. Moreover, because uremic serum from patients with renal failure contains factors that are recognized by anti-EPO (i.e., share antigenic determinants with EPO) but that are free of biologic activity and are smaller in molecular mass than native EPO, the relationship between elevated EPO levels and erythrocytosis requires further characterization. [111]

Leukocyte Dysfunction

Renal failure is accompanied by a high rate of infection, which often leads to death. [112] [113] Mucosal barriers to infection may be defective, vascular and peritoneal access devices are frequently used, and immunosuppressive agents are sometimes administered, all of which predispose to infection, particularly by staphylococci. Multiple defects in the complex network of immunoregulation also have been described, although a clear explanation for infection has been elusive. Granulopoiesis is appropriately stimulated, provided that cytotoxic immunotherapy is not being used. Megaloblastic morphologic changes are only rarely seen and when present are usually unexplained. [114]

By contrast, granulocyte pool sizes may be prominently disturbed in uremia, particularly during hemodialysis. Mild neutrophilia is commonly seen, a finding that may be related to alterations in the distribution of granulocyte pools. [114] [115] [116] A low mobilizable marrow pool of granulocytes has been found in uremic patients after hydrocortisone injection.[117] Presumably, marrow granulocyte reserves have already been called upon, leading to diminished pool sizes that are assessed by steroid release tests. On the other hand, marrow release appears to be normal in response to complement, as assessed by the C3e-responsive pool. [118] More marked changes occur in granulocytes during hemodialysis. Severe transient neutropenia occurs within 2 hours after initiation of dialysis, a finding that initially appeared to be due to sequestration of granulocytes in the dialysis apparatus. [119] Further study of this phenomenon, however, disclosed that administration of autologous plasma that had been incubated with dialyzer cellophane resulted in complement-mediated leukostasis in pulmonary vessels. [120] [121] Precisely how complement activation alters granulocyte adhesiveness and whether this mechanism fully explains hypoxemia during hemodialysis remain unknown.

Mild functional abnormalities of granulocytes have been observed in uremia. Impaired granulocyte migration into a Rebuck skin window, [122] abnormal chemotactic activity in the presence of autologous uremic plasma or serum,[123] and uremic factors capable of inhibiting chemotaxis[124] have all been described. Some of these defects are reversible with successful renal transplantation. [125] The cause of reversible functional defects in leukocytes is poorly understood but multiple factors may be involved, including coexisting acidosis, hyperglycemia, hyperosmolarity, and protein-calorie malnutrition.

In contrast to mild disturbances of granulocytes, significant lymphopenia and lymphocyte dysfunction have been described in uremia. Absolute lymphopenia occurs in patients with renal failure with or without hemodialytic therapy. [115] [116] [117] [118] Use of high-permeability biocompatible membranes (polyacrylonitrile or ANG9) rather than low-permeability membranes may reduce plasma levels of factors that alter the number and/or function of T cells and B cells, including interleukin-1 (IL-1), IL-6, and tumor necrosis factor- (TNF-).[126] [127] Lymphopoietic hypoplasia and thymic hypoplasia have been described in neonates of women with renal insufficiency as well.[5] Whereas T cells are slightly depressed in number, B-cell lymphopenia may be prominent. [128] [129] [130] In addition, a variety of immunologic abnormalities have been found in nephrotic syndrome (Table 148-1).[131] [132] [133] [134] [135] [136] [137] [138] [139] [140] [141] [142] [143] [144] [145] [146] [147] [148] [149] [150] [151] [152] [153] [154] These include hypocomplementemia, defective opsonization, impaired immunoglobulin production, abnormal humoral function (assessed by the presence of soluble factors that inhibit lymphocyte proliferation and factors that inhibit monocyte migration), and impaired cellular immunity.

Although serum immunoglobulin A, G, and M levels have been reported to be diminished, [135] [136] some researchers have found normal values in uremic patients.[128] [137] On the other hand, striking defects in cellular immunity have been observed consistently, both in vitro (Table 148-1) and in vivo. Reduced delayed hypersensitivity reactions to common antigens such as mumps, *Trichophyton,* and *Candida* are well described.[149] [155] [156] Extended survival of skin allotransplants [157] [158] [159] and prolonged cardiac transplant survival [160] occur as well. In addition, alterations in the immune surveillance system may be related to the high incidence of malignancy known to occur in uremic patients.[161] [162]

TABLE 148-1 -- Immunologic Disturbances in Nephrotic Syndrome

Immune Response	Disturbance	Reference
Opsonization	Low factor B, D serum levels	[131] [132]
	Impaired neutrophil chemotaxis/chemiluminescence	[133]
	Impaired reticuloendothelial cell function	[134]

Immunoglobulin production	Low serum immunoglobulin levels	[135] [136]
	Diminished immunoglobulin synthesis in vitro	[138]
	Decreased immunoglobulin reactivity	[139] [140]
Humoral immunity	Serum inhibition of lymphocyte proliferation	[141] [142]
	Serum toxicity to lymphocytes	[143]
	Production of soluble inhibitors, including monocyte migration inhibitory factor, immune response suppressor	[144] [145] [146]
Cellular immunity	Impaired lymphocyte blastogenesis	[147]
	Reduced delayed hypersensitivity reactions	[148] [149]
	Suppression of antibody-dependent cell-mediated cytotoxicity	[150] [162]
	Impaired transfer of tuberculin sensitivity	[153] [154]
	Cutaneous anergy	[153] [154]

Both natural killer cell activity and antibody-dependent cell-mediated cytotoxicity are inhibited by uremic serum, an observation that may be important in host defense against malignancy.[127] [150] [162]

Abnormalities of Hemostasis

Abnormal hemostasis, with a proclivity for bruising and bleeding, is commonly seen in chronic renal disease. Although qualitative and quantitative disturbances of the platelets, clotting cascade proteins, and blood vessels are known to occur in uremia, the pathogenesis of these defects remains obscure. One potentially important factor may be retention of low-molecular-weight toxins. Guanidinosuccinic acid and phenolic acid are known to reduce platelet factor 3 availability and to inhibit adenosine diphosphate (ADP)-induced platelet aggregation. [163] [164] By contrast, prostaglandins that have a tonic vasodilating effect in chronic renal disease [165] [166] activate platelets and induce activation of the coagulation cascade, particularly within glomerular capillary loops. [167] Dialyzer membrane composition and flow design may also play a role in activating platelets of hemodialyzed patients. [168]

In addition, clotting abnormalities, particularly those involving the platelet, may develop as a consequence of fluctuations in the hematocrit value. According to one hypothesis, platelet adhesion to the subendothelium increases progressively as the hematocrit rises. [169] As red cells flow through vessels at relatively high shear rates, platelets diffuse radially, thereby increasing the chances of platelet collision with the vessel wall. [170] This process favors thrombus formation. Disproportionate increases in hematocrit value and whole blood viscosity may be further aggravated by the therapeutic use of diuretics. An inverse correlation exists between bleeding time and hematocrit, and red cell transfusion improves several clotting parameters, including platelet retention on glass beads, prothrombin consumption, serum thromboxane B_2 production, and intraplatelet cyclic AMP levels. [171] [172]

Alterations in clotting factor levels occur in uremia as well. Decreased blood levels of factors IX, XI, and XII have been reported [168] and are believed to result from increased urinary losses of these relatively low molecular weight proteins. Blood levels of other clotting factors (factors II, V, VII, VIII, X, and XIII) are often increased.[173] [174] [175] [176] Because the latter changes correlate with the degree of hypoalbuminemia,[177] it is possible that they develop secondary to increased hepatic synthesis. Nevertheless, no direct relationship has been established between clotting factor levels and the development of the thrombotic diatheses (such as renal vein thrombosis) that are known to complicate renal insufficiency.[178]

An abnormality of the von Willebrand factor (vWF) has also been identified in uremia, a finding that may bear directly on the role of platelets in primary hemostasis. Ristocetin cofactor activity is lower than normal in chronic renal failure patients, and elevated vWF antigen levels are detected. [179] These findings are supported by the observations that in uremia, vWF activity is consistently lower than its antigen level, and that hemorrhagic tendency is corrected by infusion of either cryoprecipitate[180] or the synthetic derivation of the antidiuretic hormone 1-deamino-8- D-arginine vasopressin.[181] These findings correlate with shortening of an abnormal bleeding time and reversal of the coagulopathy, suggesting that abnormalities in the factor VIII-vWF antigen level and functional activity lead to a prolonged bleeding time and clinical coagulopathy.

Disturbances of coagulation are also common in pre-eclampsia and eclampsia. [182] [183] Intravascular hemolysis and coagulation abnormalities are no more severe in women who develop acute renal failure than in those who do not.[184] It is unknown whether the deposition of thrombi and development of renal cortical necrosis occur as the primary event or as a secondary event in obstetric acute renal failure. [185]

CLINICAL AND LABORATORY PRESENTATION

Signs and symptoms of uremia, including hypertension, metabolic acidosis, fluid overload, and disturbances of the gastrointestinal, cardiovascular, and nervous systems, are usually the major clinical manifestations of renal insufficiency. In addition, pallor, fatigue, and high-output cardiac failure due to anemia and bleeding or intravascular coagulation may become major clinical problems that demand appropriate therapy.

Anemia

Normocytic, normochromic anemia with a corrected reticulocyte count of 1% (i.e., normal reticulocyte index in the face of anemia) is characteristically present. Morphologically, the red cells frequently show the characteristic even scalloping of echinocytes or burr cells. [186] The clinical significance of this morphologic change is uncertain because the presence or absence of echinocytes does not correlate with the severity of hemolysis and because even normal red cells reversibly transform into echinocytes in vitro.[187] Occasionally, asymmetrically distributed, large spicules appear on the surface of uremic red cells (acanthocytes) or grossly deformed and fragmented red cells (schistocytes) appear. In contrast to echinocyte formation, the presence of schistocytes is a manifestation of a pathologic process involving the microcirculation that has occurred in vivo. When severe, these changes are the hallmark of the hemolytic-uremic syndrome.

In the absence of a complicating nutritional deficiency or drug-related hemolytic process, the bone marrow aspirate appears normal. Mild granulocyte hyperplasia may be observed, and erythroid precursors are normal in appearance and maturation. [188] Occasionally, marrow fibrosis (i.e., osteitis fibrosa) is apparent on bone biopsy. Here, decreased marrow space is available for erythropoiesis, regardless of circulating EPO levels. Osteitis fibrosa, a complication of secondary hyperparathyroidism and renal insufficiency, may play a pathogenic role in the

APPROACH TO EVALUATION OF WORSENING ANEMIA

Hematologists are frequently asked to evaluate worsening anemia in patients with chronic renal insufficiency. One logical approach to the diagnostic workup of such patients is outlined here. Baseline laboratory information includes examination of the blood smear and calculation of the reticulocyte index (corrected for level of hematocrit). Individuals with hypoproliferative red cell production indices may have nutritional deficiency or an abnormal process involving the bone marrow (i.e., dyshematopoiesis, myelofibrosis, or an unrelated disorder such as myelophthisis). When microcytosis is present, a serum ferritin level measurement usually confirms the diagnosis of iron deficiency. If inflammation is present, a marrow aspiration to assess tissue iron stores may be required. Alternatively, microcytic anemia can also be observed in patients with aluminum toxicity. Because aluminum is ubiquitous (being particularly concentrated in glassware), aluminum measurements may be erroneous because of contamination of plasma samples by collecting and transport materia.[348] A red cell aluminum assay whose measurements are not altered by aluminum contamination of blood samples has been developed.[349] Alternatively, aluminum excretion after deferoxamine administration can be measured[348] and aluminum concentrations in the bone can be quantitated[350] in order to establish the diagnosis of aluminum toxicity.

The diagnosis of macrocytic, hypoproliferative anemia should be approached by measurement of serum and/or red cell folate and vitamin B_{12} levels. If results are normal or equivocal, a bone aspiration may be required to establish the diagnosis of marrow nutritional deficiency or another marrow disorder. Furthermore, liver function tests must be performed since macrocytic anemia may be associated with liver disease even in the absence of folate deficiency, and particularly because uremic patients are at risk for developing hepatic dysfunction. The presence of hyperproliferative anemia characterized by an elevated reticulocyte index necessitates a hemolytic workup. Careful examination of the blood smear for spherocytes and schistocytes is necessary when chemical evidence of hemolysis is present. The spectrum of microangiopathic changes on the blood smear, thrombocytopenia, and renal insufficiency should raise the possibility of hemolyticuremic syndrome. Alternatively, microangiopathic changescan be seen with underlying vasculitis, malignant hypertension, or connective tissue disorders that may be responsible for both renal disease and hemolysis. The sudden development of accelerated hemolysis accompanied by spherocytes should raise the possibilities of immune hemolysis, dialysis-associated hemolysis (i.e., that associated with copper, chloramine, formaldehyde, nitrate, or zinc toxicity) or drug-related hemolysis (either drugs with high oxidative potential or those known to induce antibody-mediated hemolysis; see Table 148-2). Excessive use of phosphate-binding antacids may cause a similar hemolytic picture.

Assessment of hyperproliferative anemia in the absence of chemical evidence for intravascular hemolysis should raise the possibilities of brisk erythropoietic response to blood loss and red cell sequestration in an enlarged spleen. If splenomegaly is present, one should measure uptake of chromium 51-tagged red cells over the spleen. Increased uptake occurs with splenic sequestration,

Assessment of hyperproliferative anemia in the absence of chemical evidence for intravascular hemolysis should raise the possibilities of brisk erythropoietic response to blood loss and red cell sequestration in an enlarged spleen. If splenomegaly is present, one should measure uptake of chromium 51-tagged red cells over the spleen. Increased uptake occurs with splenic sequestration, suggesting several possible causes and indicating splenectomy as a potential therapeutic option. Alternatively, splenomegaly may develop as a consequence of hepatic disease and portal hypertension. In the absence of splenomegaly, a careful search should be made for blood loss (via the gastrointestinal tract, into the retroperitoneum, or via dialysis). An ultrasound examination to evaluate for a retroperitoneal collection of blood and multiple stool guaiac tests to evaluate for gastrointestinal loss may yield the appropriate diagnosis.

production of anemia since the amount of marrow fibrosis correlates well with correction of anemia that follows parathyroidectomy. [189] [190] The extent of fibrosis also correlates with response to EPO treatment (see below).[191] The relationship of elevated parathyroid hormone levels to in vivo suppression of erythropoiesis [71] or to reduction in red cell survival [192] is unknown. As osteitis fibrosa progresses, the alkaline phosphatase level rises. Ultimately, myelofibrosis develops, accompanied by extramedullary hematopoiesis and hepatosplenomegaly.

Frequently, iron deficiency associated with blood loss andor folic acid deficiency complicates renal anemia (Table 148-2). [78] [79] [80] [81] [91] [193] [194] [195] [196] [197] [198] [199] [200] [201] [202] [203] [204] [205] [206] [207] [208] [209] [210] [211] [212] [213] [214] [215] [216] [217] [218] [219] [220] [221] [222] [223] [224] [225] Gastrointestinal tract blood loss (believed to be due to platelet dysfunction) or iatrogenic blood loss (via phlebotomy, dialyzer, or fistula) may lead to depletion of iron stores and erythroid hyperplasia. Blood loss is exaggerated in hemodialysis patients because of the heparinization that is required during dialysis. Blood losses may average as much as 1 Lmonth in patients undergoing long-term hemodialysis.[193] [194] [195] [196] [197] In contrast, patients dialyzed peritoneally do not develop iron deficiency without gastrointestinal blood loss. Rarely, iron deficiency may develop in association with urinary loss of transferrin. [198]

Several factors contribute to the development of clinically apparent folic acid deficiency. Since red cell survival is decreased, demand for folate is higher than normal.[78] [79] [80] [81] [199] This demand may not be met when patients are advised to restrict protein intake, although the need for supplemental folic acid is not clearly established.[200] [201] Nevertheless, folate is removed during dialysis.[200] In addition, absorption or metabolism of dietary folate may be suppressed by many of the drugs used in treating uremic patients, including diphenylhydantoin, barbiturates, cholestyramine, dietary amino acids, oral contraceptives, and inhibitors of dihydrofolate reductase (such as methotrexate and pyrimethamine).[202] Megaloblastic maturation and dyshematopoiesis are characteristically present in marrow aspirates of uremic patients with folate deficiency.

Red cells from uremic patients with renal insufficiency are hypersensitive to oxidant challenge. [94] [204] Most enzymes of the oxidant defense system have been shown to be normal or slightly below normal in activity, [205] [206] [207] [208] rendering cells hypersensitive to hydrogen peroxide-induced hemolysis. [209] Cross-incubation studies suggest that increased oxidant sensitivity is due to uremic plasma factors since normal red cells can be made hypersensitive to ascorbate-generated oxidants by incubation in uremic plasma.[210] The production of oxidants such as chloramine during dialysis leads to brisk hemolysis (Table 148-2). Other dialysis-associated alterations in uremic red cells, such as overheating, mechanical fragmentation, and dehydration/overhydration, may also lead to accelerated hemolysis. Drugs having a high oxidative potential may also induce hemolysis and worsen anemia. Furthermore, since malignant hypertension and vasculitis may complicate renal failure, uremic patients are prone to develop microangiopathic hemolysis. [219] Finally, hypophosphatemia associated with excessive use of

TABLE 148-2 -- Correctable Causes of Anemia of Renal Failure

Mechanism	Etiology	Reference
Blood loss, iron deficiency	Iatrogenic loss Dialyzer loss Shunt or fistula loss Gastrointestinal loss Urinary loss of transferrin	[193] [194] [195] [196] [197] [198]
Folate deficiency	Increased demand Restricted oral intake Dialyzer loss Drug-induced inhibition of absorption or metabolism	[78] [79] [80] [81] [199] [200] [201] [200] [202]
Accelerated hemolysis		
Dialysis-associated toxicity	Toxicity due to exposure to copper, chloramine, formaldehyde, or nitrates Overheating of erythrocytes Mechanical erythrocyte fragmentation Dehydration/overhydration of erythrocytes	[211] [212] [213] [214] [208] [215] [216] [217]
Drug-associated	High oxidative potential agents: thiol-containing drugs, phenylhydrazine-like drugs Immunohemolytic agents: -methyldopa, penicillin, quinidine, etc.	[91] [207] [218]
Microangiopathic	Malignant hypertension Vasculitis	[219]
Red cell phosphate depletion	Excessive antacid intake	[220]
Hypersplenism	Red cell sequestration/work hypertrophy Chronic hepatitis Transfusion-induced hemosiderosis Marrow fibrosis Silicone toxicity	[221] [222] [223] [221] [222] [224] [225]

phosphate-binding antacids (i.e., aluminum gels) may lead to several red cell abnormalities, including depletion of ATP stores, impaired glycolysis, reduced red cell deformability, and hemolysis.[220]

Although uremic patients often develop hypersplenism as a complication of their underlying disease or its therapy (Table 148-2), only a small percentage actively sequester and destroy red cells in the spleen. [221] The latter patients have a red cell survival rate that is one-fourth of normal, demonstrate increased uptake of radioactive chromium-labeled red cells, and have a favorable response to splenectomy. However, most patients with mild splenomegaly do not actively sequester red cells in the spleen, suggesting that they will have a poor response to splenectomy. [226]

Aluminum toxicity has been recognized in dialysis patients. High levels of aluminum develop as a consequence of long-term use of aluminum-containing antacids that facilitate phosphate binding or of aluminum-containing dialysate. The precise level at which aluminum becomes toxic is debatable and is in part determined by exclusion of other known causes of organ dysfunction. [227] [228] An early manifestation of aluminum toxicity is microcytosis. [229] [230] Later, hypochromic anemia, dialysis encephalopathy, vitamin D-resistant osteomalacia, and muscle weakness develop. [230] [231] The mechanism responsible for the development of microcytic anemia may involve suppression of enzymes involved in heme synthesis. [232] Alternatively, aluminum may interfere with iron uptake by the red cell since it is similar to iron with respect to trivalency and avid binding to transferrin. [233] [234] Aluminum-induced anemia can be reversed by use of a dialysate containing deionized water [234] or by chelation with desferrioxamine. [235] Today, aluminum has been removed from the dialysate in many dialysis units, thereby decreasing the incidence of aluminum toxicity in the United States.

Bleeding and Abnormal Coagulation Tests

Clinical bleeding in uremia manifests most often as purpura, menorrhagia, and occult or frank gastrointestinal blood loss. Bleeding occurs in up to one-half of all patients with chronic renal insufficiency. [236] Characteristically, the platelets are quantitatively normal, although dialysis-associated thrombocytopenia with clinical bleeding may occur. [168] [173] Platelet dysfunction characterized by abnormalities in bleeding time, platelet aggregation and adhesiveness, prostaglandin (PGI_2) production, and factor VIII-vWF complex activity is well documented. [163] [164] [165] [166] [167] [168] [236] [237] Dialysis has been reported to improve abnormalities in many of these tests and to ameliorate bleeding [238] by an unknown mechanism(s).

Hemolytic-Uremic Syndrome

Brisk hemolysis and thrombocytopenia may occur in the setting of acute renal failure. This clinical picture has emerged as a distinct syndrome, the hemolytic-uremic syndrome (HUS), which is relatively common in infants and children who develop sudden anemia, renal failure, central nervous system dysfunction, and gastrointestinal bleeding. HUS often follows a prodrome of infectious symptoms involving the digestive or respiratory tracts (or both). Although the inciting agent may be viruses, bacteria, drugs, or pregnancy, little is known of the pathogenetic mechanisms involved. Several theories have been postulated, including the induction of a generalized Schwartzman phenomenon by microorganisms or their toxins, prostacyclin deficiency, and genetic predisposition. Mitomycin treatment of solid tumors may be complicated by HUS, and more rarely, other chemotherapeutic agents (i.e., cisplatin and bleomycin) may also be associated with secondary HUS. [239] Recently, HUS has been reported after transplantation of bone marrow and solid organs. [240] [241] HUS may also be the presenting clinical form of infection by the human immunodeficiency virus (HIV). [242]

Shigellosis, an acute infectious inflammatory colitis, may be complicated by oliguria and a marked drop in hematocrit (10% in 24 hours) that occur as the dysentery is resolving. *S. dysenteriae* type 1 infection and *E. coli* O157:H7 produce high levels of Shiga-family toxins that appear to trigger HUS by binding to renal vascular endothelium where ribosomal activity is inhibited. [243] [244] Binding of toxin to the Cb3 plasma membrane receptor is followed by receptor-mediated endocytosis and inactivation of the host ribosomal RNA complex. In the United States, outbreaks of life-threatening *E. coli* infection have been reported after the consumption of undercooked meat, and are responsible for 250 deaths annually. [245] [246] Most cases have been reported from the northern latitude states, including Washington, Oregon, Minnesota, and Massachusetts. [247] HUS develops in 6% of patients who are infected with *E. coli* O157:H7, and appears 214 days (median, 6 days) after the onset of abdominal cramping, diarrhea, and bloody stools. [248] Outbreaks of *Shigella* infection have been reported in day-care centers and summer camps. [249] Leukemoid reactions in excess of 50,000/L, increased numbers of CD3-CD57-CD20 cells, a low CD4/CD8 ratio, profound hyponatremia, and marked hypoglycemia have been reported in Shiga toxin-associated HUS. [250] A mortality of 510% has been reported despite prompt, aggressive therapy. In up to 40% of those who survive acute HUS associated with Shiga-like toxins, progressive renal damage develops that requires long-term dialysis or renal transplantation. [251] [252]

The histopathologic hallmark of HUS is deposition of hyalin material in arterioles of the kidney. Hyalin deposits are thought to be composed of fibrin and platelets and are indistinguishable from deposits found in thrombotic thrombocytopenia purpura. The peripheral blood smear classically shows fragmented red cells and thrombocytopenia. These changes result from mechanical trauma, as cellular elements traverse renal arteriolar and glomerular vessels whose endothelial lining has been injured. The reticulocyte index is invariably elevated, and signs of overt hemolysis are evident, including indirect hyperbilirubinemia, elevated blood lactate dehydrogenase levels, and low or undetectable haptoglobin levels. Consistent with hemolysis, EPO levels are elevated even though uremia is present. [253]

The mortality from HUS in children varies from 5% to 20%, and in adults is considerably higher. The prognosis is related to the degree of renal insufficiency and to the extent of central nervous system involvement. Neurologic sequelae develop in up to 50% of cases and include altered consciousness, seizures, and bizarre posturing. [254] Therapy is supportive in nature and includes dialysis and blood component replacement. The role of heparin is controversial; other forms of therapy, such as steroids, immunosuppressive agents, and antimetabolites, have not produced clear-cut benefit. Some patients improve following plasmapheresis or exchange transfusion. [255]

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THERAPY AND PROGNOSIS

The management of anemia in acute and chronic insufficiency consists of nutritional replacement, including repletion of low iron, vitamin B_{12}, and folic acid stores, conservative blood component transfusion therapy, and an optimal program of dialysis and related therapy aimed at correcting uremia or reversing its effects. Specific therapy for anemia and bleeding complications has been employed since the mid-eighties when recombinant human erythropoietin (rhEPO) became available. The impact of rhEPO in clinical nephrology has been so great that treatment recommendations are now thought of in terms of the pre- and post-EPO eras.

Correction of Anemia in the Pre-EPO Era

Hemodialysis ameliorates symptoms of anemia, but rarely does anemia fully resolve.[256] Although the metabolic clearance (Km) of EPO decreases with the intradyalitic interval, serum EPO levels are unchanged after dialysis.[257] [258] These findings suggest that removal of uremic inhibitors of stem cells or EPO action may be clinically important. Alternatively, it is possible that amelioration of anemia is due to improved renal function. When the clinical spectrum of tachypnea, tachycardia, and other symptoms of high-output cardiac failure develop in association with tissue hypoxia disproportionate to the degree of azotemia, blood component therapy is required. Because transfusion of whole blood may place patients at risk for the development of pulmonary edema, infusion of packed red blood cells is usually recommended. When laboratory parameters for folic acid deficiency are evident, oral supplementation should be given. Routine folate administration, however, may be unnecessary.[201] It has been reported that supplementation with folic acid improves response to rhEPO, suggesting that the demand for folic acid is increased during rhEPO therapy.[259] Iron deficiency is easily correctable when documented in uremic patients. Because intestinal absorption of iron is normal in renal failure, oral supplementation of 65 mg elemental iron twice daily with meals is recommended.[203] [257] [260] Occasionally, parenteral infusion of iron may be necessary in individuals whose total rate of iron losses (Table 148-2) exceeds the daily iron absorption rate. Adequate iron replacement during EPO therapy is essential for an optimal response to rhEPO.

Androgens effectively increase the red cell mass in one-half of patients undergoing dialysis.[108] Although their efficacy in treating the anemia of primary bone marrow failure is debatable,[261] [262] their utility in treating anemia of end-stage renal disease is well established.[263] [264] [265] [266] Nevertheless, the hematocrit usually remains at <36% and rarely achieves normal levels. Symptomatic individuals with a hematocrit of <25% are good candidates for androgen therapy, although it should be kept in mind that side effects include masculinization, acne, fluid retention, and hepatotoxic effects (cholestasis, peliosis hepatitis, and hepatic adenomas). Parenteral administration of androgens may be more effective than administration by other routes.[267] Agents belonging to several classes (testosterone esters, 17-alkylated compounds, and norsteroids) are commonly used.[108] It has been suggested that androgens having an angular configuration (5-epimers) stimulate stem cell proliferation to a greater degree than those whose configuration is planar (5-epimers), which may act primarily by enhancing EPO production.[108] Nandrolone decanoate (Deca-Durabolin) and fluoxymesterone are popular choices for both nephrectomized and non-nephrectomized individuals.[268] [269] Patients are unlikely to respond to androgens if a favorable clinical outcome has not been achieved after 69 months of therapy at different dosages.[108] The cost-effectiveness of androgen therapy versus EPO therapy has been emphasized.[270] [271] A response to androgens that is equivalent to that of EPO has been observed in a subset of patients (male patients aged 50 years and older).[271] Since androgens of one class may be effective while drugs of another class may be ineffective, a method to select appropriate androgen therapy would be helpful to the clinician. An in vitro marrow culture assay has been found to predict response to androgens of a specific class.[272] It appears that both adequate numbers of erythroid progenitor cells and normal sensitivity to androgens of a specific class must be present to achieve a favorable clinical response.

Correction of Anemia in the Post-EPO Era

Perhaps in no other way has the therapy of renal anemia been more affected than by the introduction of rhEPO to clinical medicine. By analogy with other hormone deficiency states, it is predictable that a hormone will be effective when administered to individuals who either lack the capacity to generate a sufficient hormone level in response to a physiologic stimulus or produce a hormone fragment that is biologically inactive. In view of the central role played by EPO in erythropoiesis, the finding that EPO corrects the anemia of end-stage renal disease in humans[273] [274] [275] [276] [277] and small animals[278] is not surprising. Typically, rhEPO administration results in a dose-related, sequential increase in the reticulocyte count and hematocrit, accompanied by complete loss of a transfusion requirement (Fig. 148-3). Increases in red cell transferrin uptake accompany this erythropoietic effect.[275]

In the post-EPO era, clinical research on the treatment of anemia of renal failure has focused on the pharmacokinetic/pharmacodynamic properties of rhEPO. Although some dialyzer membranes have been found to adsorb EPO, calculations of Km for EPO before and after dialysis indicate that rhEPO may be safely administered at any time during the course of dialysis.[257] [258] Early studies indicated that subcutaneously administered rhEPO was not only effective but also absorbed more slowly than intravenously administered rhEPO.[279] A relatively low serum level that is sustained with a higher 48-hour serum concentration is achieved with subcutaneous administration. These serum concentrations are sufficient to stimulate erythropoiesis and maintain a target hemoglobin level of 10.5 g/dl, an effect that is equivalent to that obtained with intravenous administration of the drug.[280] [281] Most investigators have concluded that the subcutaneous route is associated with a decreased dose requirement in both the correction and maintenance phases of therapy.[282] [283] [284] [285] [286] [287] Owing to the high cost of therapy,

Figure 148-3 Effect of recombinant human erythropoietin on transfusion requirement, hematocrit, reticulocyte index, and serum iron parameters in a patient with renal insufficiency. *(From Eschbach et al.,[275] with permission.)*

recent studies have focused on decreasing the overall dose of rhEPO. Whereas the absorption rate is independent of dose, clearance is dose dependent and decreases with increasing dose.[288] Once weekly subcutaneous therapy is equivalent to three times weekly intravenous therapy.[289] Moreover, a reduction in subcutaneous dosing frequency from two or three times weekly to once weekly results in a similar target hemoglobin level in studies with follow-up of up to 45 weeks duration.[290] [291] These results are consistent with the notion that triggering of a limited number of clones of erythroid progenitors to undergo terminal differentiation is sufficient to initiate a gradual wave of accelerated erythropoiesis.[292] Finally, rhEPO can be safely administered undiluted into the dry peritoneal cavity with satisfactory results,[293] thereby avoiding local pain associated with subcutaneous injection.[294]

The efficacy of rhEPO also has been demonstrated in predialysis patients with anemia.[295] There has been no evidence that rhEPO therapy accelerates the deterioration of renal function.[295] [296] Anemia is promptly corrected, provided that iron deficiency is avoided, and health-related quality of life is improved in predialysis patients.[297] Once weekly subcutaneous injection is as efficacious as two or three times weekly injection schedules.[298] Similarly, rhEPO administration improves anemia in patients with failing renal allografts, although higher doses of drug may be needed in these patients than in patients with renal failure not associated with

allograft dysfunction.[299]

Improvement in anemia after the administration of rhEPO is accompanied by loss of fatigue, improved sexual function, enhanced brain function, and a psychological sense of well-being.[276] [278] [300] [301] A clinically minor transient increase in platelet count (from approximately 220 × 10^9 /L to 245 × 10^9 /L) has been documented.[302] [303] Because early studies suggested that the risk of thrombotic complications is increased after administration of rhEPO, [304] [305] [306] the potential effects of rhEPO in vivo on

Box 148-1. APPROACH TO INITIATION AND ADJUSTMENT OF RH EPO THERAPY

The greatest benefit to quality of life in patients with chronic renal failure is achieved when a hemoglobin level of 10.5g/dl has been achieved. [351] This represents the currently recommended target hemoglobin that should be achieved in all patients, provided that severe hypertension or venous access problems do not coexist.[352] A variety of protocols may be followed to initiate therapy with rhEPO.[353] Initial assessment of the patient should include obtaining baseline laboratory data such as a complete blood cell count, serum ferritin level, transferrin saturation, and serum aluminum level (for patients who have received oral phosphate binders or have been dialyzed using a bath containing aluminum). Because up to one-third of patients may experience hypertension following rhEPO therapy, the blood pressure must be managed, beginning with nonpharmacologic therapy (i.e., fluid control) and adding antihypertensive agents as necessary. Initial therapy with 50100 U/kg given intravenously or subcutaneously three times weekly is a reasonable approach.[354] Response to therapy should be monitored with measurements of hemoglobin and hematocrit (three times weekly), iron status (monthly), renal function tests, phosphorus level, and blood pressure (three times weekly).

When adjusting rhEPO dosage, it is important to understand that a response may take 26 weeks to appear, as it requires time for erythroid progenitor cells to undergo terminal differentiation and release into the circulation. rhEPO dosage should be increased if the hematocrit fails to increase by 56 percentage points after 68 weeks of therapy. rhEPO therapy should be withheld when the hematocrit exceeds 36% (in spite of dose), or when the blood pressure cannot be controlled by nonpharmacologic or pharmacologic methods. A reduction in weekly rhEPO dosage may be required when switching from intravenous therapy three times weekly to subcutaneous therapy once weekly. During such a change, it is reasonable to retain the same initial weekly total dose and carefully monitor for a hematologic response and potential toxicity. When changing the dosage regimen, it is important to allow for sufficient observation time (generally at least 4 weeks) in order to monitor closely for excessively rapid increases in hematocrit level (>4 percentage points in 2 weeks) and iron status at regular intervals (maintaining a minimum transferrin saturation of 20% and minimum ferritin level of 100 ng/ml).

A suboptimal response to rhEPO therapy may be due to inadequate dosing, iron deficiency, malignancy, inflammation, infection, vitamin deficiency, occult blood loss into the gastrointestinal tract, skin, or at the time of dialysis, hemolysis, aluminum toxicity, and secondary hypoparathyroidism. In addition, use of angiotensin-converting enzyme (ACE) inhibitors has been associated with resistance to rhEPO therapy.[355] [356] It is important to consider iron-deficient erythropoiesis in rhEPO-treated patients with normal or elevated serum ferritin levels since iron demand from accelerated erythropoiesis may exceed the rate of mobilization of iron stores. In this circumstance, functional iron deficiency can be diagnosed by detection of microcytosis and low transferrin saturation. [357] Therefore a careful search for one or more of these potential causes for EPO resistance should be conducted when rhEPO requirements are inordinately high.

the hemostatic and thrombolytic systems have been studied in detail. [303] [307] The results of these studies indicate that rhEPO improves the platelet/vessel wall interaction, possibly by improving platelet adhesion to the subendothelium via serotonergic mechanisms. The bleeding time is significantly shortened, while platelet aggregation in response to collagen and ristocetin is improved. Although levels of protein C and S and antithrombin III are decreased following rhEPO administration, these changes are counterbalanced by diminished activity of inhibitors of thrombolysis ($_2$ -antiplasmin, C1 esterase inhibitor, and plasminogen activator inhibitor). [303] [307]

Correction of anemia with rhEPO is associated with improvements in cardiovascular parameters, including a decrease in cardiac output and resting heart rate. [308] [309] [310] [311] Improved exercise capacity has been documented after 1 year of therapy and is accompanied by reduced left ventricular hypertrophy and exercise-induced cardiac ischemia.[310] [311] These changes in cardiovascular function may in part explain why rates of hospitalization are reduced in new hemodialysis patients receiving rhEPO versus those not receiving therapy.[312] In addition, rhEPO therapy has been associated with reversal of insulin resistance, improved glucose tolerance, and normalization of amino acid levels (valine, leucine, and isoleucine) and plasma lipid levels (triglycerides, total cholesterol, and low-density lipoprotein cholesterol). [313] [314] Although the mechanisms of these actions are unknown, improved local oxygenation of target tissues, reduced plasma lactate levels, and diminished iron toxicity to hormone-producing organs have been speculated to be important.

The side effects of EPO rarely require discontinuation of therapy. Most patients develop a mild sensation of aching in the long bones during administration of the drug. Approximately one-third of patients develop diastolic hypertension, an effect that is not explained by hypervolemia since blood pressure elevation occurs despite intensive efforts to prevent hypervolemia by maintaining patients at their dry weight. [276] It has been suggested that hemodialysis patients with a family history of hypertension are particularly susceptible to EPO-induced hypertension, whereas age, sex, etiology of renal dysfunction, and duration of hemodialysis therapy have no predisposing effect.[315] Mean 24-hour systolic and diastolic ambulatory blood pressure measurements are significantly increased, particularly in patients with borderline systolic hypertension.[316] Mechanisms of vascular wall damage in uremia are undefined. Progression of vascular injury during hemodialysis is well described.[317] [318] Although a rise in peripheral vascular resistance has been reported after EPO treatment, [319] levels of circulating vasoactive substances (including renin, adrenaline, angiotensin II, dopamine, endothelin, and atrial natriuretic peptide) remain unchanged. [320] [321] Early reports suggested that rhEPO may have a direct vasopressor effect on renal blood vessels.[322] Subsequent study using laser-Doppler flowmetry indicates that rhEPO does not alter microcirculatory blood flow. [323] Together, the results of these multiple studies show no apparent direct vasoconstrictor effect of rhEPO.

Initial concerns that correction of anemia with rhEPO might impair dialysis efficacy [300] [324] have not been verified. High-flux dialysis neither worsens azotemia nor shortens access survival, and may actually improve urea and acid-base balance. [301] Increasing the intensity of dialysis in patients with anemia who receive inadequate dialysis has been shown to improve anemia.[325] The response to rhEPO was found to correlate with a reduction in the levels of blood urea nitrogen and

serum albumin, with a poor response associated with a urea reduction rate of <65% during dialysis. [325] Together, these results suggest that correction of anemia in patients receiving rhEPO is enhanced by maintaining an intense dialysis regimen.

The correction of erythropoiesis to normal levels by EPO therapy may uncover iron deficiency and aluminum toxicity. Most patients receiving hemodialysis require 610 g of parenteral iron annually to maintain a normal serum iron level and transferrin saturation. [326] The most common cause of acquired EPO resistance is development of iron deficiency associated with increased utilization of iron as erythropoiesis is accelerated. [327] [328] Nearly one-half of patients with renal insufficiency who receive EPO therapy will need iron replacement therapy. Several screening tests have been used to identify patients with iron deficiency while receiving rhEPO therapy for anemia associated with renal failure. These include the transferrin saturation (in patients without hypoproteinemia) and the mean corpuscular volume (MCV). [329] [330] [331] [332] Administration of intravenous iron dextran improves not only the serum iron level and transferrin saturation but also the reticulocyte count and hematocrit (Fig. 148-4 (Figure Not Available)). Iron supplementation adequate for restoring a response to EPO may be given by both oral and intravenous routes of administration. [333] [334] It has been suggested that when evidence of iron deficiency has been documented (such as the development of microcytosis or a decrease in transferrin saturation), oral iron replacement therapy be initiated. [335] If oral iron therapy fails to improve the anemia, replacement of iron stores by either intravenous iron dextran therapy or blood transfusions may be considered.

Aluminum overload may diminish bone marrow response to EPO. In a study of >300 patients, an inverse correlation between serum aluminum levels and magnitude of hematologic response to EPO was documented.[336] Finally, resistance to rhEPO therapy in chronic renal failure patients on dialysis has been attributed to secondary hyperparathyroidism.[337] Those uremic patients who have a poor response to rhEPO therapy have a greater degree of fibrosis of the bone marrow (Fig. 148-5), a greater percentage of osteoplastic and eroded bone surfaces, and a higher serum level of parathyroid hormone relative to patients having a favorable response to therapy.[191] By contrast, the percentages of osteoid volume and surface, osteoid thickness, and stainable aluminum content of the bone in these individuals were similar in both the favorable and unfavorable response groups. Treatment of secondary hyperparathyroidism by parathyroidectomy or parenteral calcitriol therapy results in improvement in levels of hemoglobin and hematocrit[338] (Fig. 148-6) and regression of myelofibrosis.[339]

Correction of Bleeding

Infusions for uremic bleeding have not been confirmed by others [340] and their use is not recommended for routine management of uremic bleeding. In patients with hemorrhagic complications or those undergoing major surgical procedures, the adequacy of dialysis should be checked. [341] Acute bleeding can be treated with infusions of DDAVP at a dose of 0.3 mg/kg given intravenously or subcutaneously. [181] [341] DDAVP can also be administered intranasally at a dose of 3 mg/kg.[341] The hemostatic effect of DDAVP lasts only a few hours and may be lost if the drug is administered repeatedly. [341] Because the favorable effect of cryoprecipitate has not been uniformly observed, its use is not recommended.[340] [341] Long-lasting hemostatic competence may be achieved with infusions of conjugated estrogens[341] [342] [343] at a cumulative dose of 3 mg/kg administered over 5 days in individual doses given intravenously. [341] [342] [343]

Transfusional Hemosiderosis

Iron overload due to iron administration in the form of blood transfusion or parenteral iron supplementation frequently complicates end-stage renal failure. Serum ferritin levels of >300 mg/ml may be anticipated when iron intake exceeds daily losses over a protracted period of time. Because hemodialyzed patients with transfusional iron overload may develop widespread organ dysfunction, including glucose intolerance, impaired cardiac ventricular function, reduced pituitary reserves,

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Figure 148-4 (Figure Not Available) Effect of iron dextran (Imferon) administration on iron deficiency state (as measured by ferritin level and percentage of transferrin saturation), and hematologic response to treatment with recombinant human erythropoietin (as measured by hematocrit and reticulocyte count). *(From Nissenson et al.,[18] with permission.)*

Figure 148-5 Photomicrographs of cancellous bone tissue from iliac crest biopsies stained with toluidine blue. **(A)** Extensive marrow fibrosis (MF) in a specimen from a patient with severe hyperparathyroidism. Mineralized bone (MB) appears as solid dark areas. **(B)** Mild paraosseous marrow fibrosis in a specimen from a patient with less severe hyperparathyroidism. Note that whereas the marrow space is completely obliterated in the sample showing extensive MF, it is readily detectable in the sample showing less severe MF. *(From Rao et al.,[191] with permission.)*

Figure 148-6 Response of hemoglobin and EPO levels to therapy for hyperparathyroidism. Shown are hemoglobin levels before and after either intravenous calcitriol therapy (closed symbols) or parathyroidectomy (open symbols). PTHi, intact serum parathyroid hormone level (normal level, 1060 pg/ml); Tf, transfusion of packed red blood cells. *(Modified from Garcia-Canton et al.,[338] with permission from S. Karger AG, Basel.)*

and noncardiac myopathy,[344] care must be taken not to administer parenteral iron without careful assessment of iron stores. Because gastrointestinal absorption is diminished in iron-overloaded uremic patients, [345] oral iron may be administered safely, unless the patient is genetically predisposed to hemochromatosis. Desferrioxamine may be used to remove iron stores from dialysis patients who are iron-overloaded. [346] [347] When using chelators, it is important to calculate the interval of treatment relative to transfusional iron intake and dialyzer iron loss.

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Chapter 149 - Hematologic Aspects of Pregnancy

Thomas P. Duffy

INTRODUCTION

Pregnancy constitutes a major challenge for the hematologic and hemostatic resources of a womans body and requires a dual focus on the mother and fetus to recognize and treat any complications in this arena. As pregnancy progresses, a hypercoagulable state develops in anticipation of the hemorrhagic insult that accompanies placental separation.[1] The expectant mother is thus doubly jeopardized by an increased risk of thromboembolism and the many hemorrhagic complications of the pregnant state. In addition, therapies chosen to manage a maternal problem must be modulated according to their potential for damage to the fetus.[2] Any antecedent hematologic lesion will obviously further complicate and be complicated by a pregnancy. The hematologic problems of pregnancy are therefore the shared responsibility of the obstetrician, pediatrician, and the hematologist as their varied expertise and knowledge are integrated for the benefit of the mother and child.

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ANEMIA

The physiologic expansion of the expectant mothers blood volume consists of an increase in plasma volume (4060%), which is approximately twice the simultaneous expansion in the red blood cell (RBC) mass (2050%); this hydremia of pregnancy results in a fall in hematocrit to the 3032% range, requiring a revision downward (to 10 g%) of the lower limits of a normal hemoglobin during pregnancy[3] (Table 149-1). The expanded blood pool of the mother compensates for the increased metabolic and perfusion needs of the fetal-placental unit and serves as a reservoir to compensate for blood loss at delivery of the infant.

Anemia occurring during pregnancy is most frequently a reticulocytopenic, hyporegenerative anemia attributable to deficiencies of iron or folate, or both. To provide iron for fetal hemoglobin synthesis and to anticipate the losses due to bleeding, approximately 4 mg of absorbed iron each day or a total of 1,000 mg of additional iron is needed during the course of pregnancy. This amount is greater than the normal 500-mg iron storage pool present in most women, and an iron-deficient state with low ferritin levels frequently occurs in the mother.[4] [5] However, delivery of iron to the placenta continues, with a rerouting of the normal vector of iron transport to the heavy concentration

TABLE 149-1 -- Plasma Volume, Red Cell Volume, Total Blood Volume, and Hematocrit in Pregnancy

	Nonpregnant	Weeks of Pregnancy		
		20	30	40
Plasma volume (ml)	2,600	3,150	3,750	3,850
Red cell mass (ml)	1,400	1,450	1,550	1,650
Total blood volume (ml)	4,000	4,600	5,300	5,500
Body hematocrit (%)	35.0	32.0	29.0	30.0
Venous hematocrit (%)	39.8	36.4	33.0	34.1

From Hytten, [3] with permission.

of transferrin receptors on the placental trophoblastic membranes.[6] This major demand on the iron stores overwhelms most womens marginal storehouses of iron. Iron supplementation (300 mg ferrous sulfate daily) is administered to pregnant women in anticipation of this drain on their limited stores. [7]

Folate deficiency may also complicate pregnancy because of a similar escalation in the need for this essential cofactor in nucleic acid synthesis during pregnancy; folate lack not only poses the threat of anemia during pregnancy but also the complication of neural tube defects and cleft palate formation in the fetus. [8] The total body stores of this vitamin are small and short-lived. Nausea and vomiting during pregnancy may significantly impair its intake. Pure folate deficiency results in a macrocytic anemia or even pancytopenia, but the expected elevation of RBC mean corpuscular volume may be masked during pregnancy by the contribution of concomitant iron deficiency.[9] This combined deficiency may produce normocytic indices, although review of the peripheral smear often reveals a dimorphic population of RBCs and the hypersegmentation of polymorphonuclear neutrophils that indicate megaloblastic hematopoiesis. RBC sizing index curves may uncover the two distinct cell populations that characterize combined iron-folate deficiency. Most prenatal vitamins contain folate in addition to iron, so proper prenatal care of expectant mothers should obviate these problems.[10] [11] There is little concern that folate will mask vitamin B_{12} deficiency because women with this deficiency are usually older or infertile, or both.

A few other causes of reticulocytopenic anemia are directly related to pregnancy. Several cases of aplastic anemia complicating pregnancy have been described; the outcome is usually determined by the severity of the marrow lesion and the threat of leukopenia-associated infection. [12] [13] [14] Although some consider this a chance association, the resolution of the aplasia after delivery or abortion and the documented recurrence of aplasia in subsequent pregnancies makes a causal, perhaps immunologic, mechanism likely. The severity of the aplasia and the ability to support the patient help to determine the decision regarding continuation or termination of the pregnancy. Pure RBC aplasia has also been associated temporally with pregnancy. [15] Congenital aplastic anemia or Fanconi anemia may paradoxically not be manifest until the childbearing age. Instances of successful childbirth have occurred in such patients with meticulous hematologic support. [16]

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LEUKOCYTOSIS/LEUKEMOID REACTIONS/LEUKEMIA

An elevated white blood cell count with a differential revealing a rare myelocyte or metamyelocyte can occur in pregnancy. [17] Dohle bodies may also be present; this may lead to confusion in assessing the possibility of intercurrent infection. Because the count returns to normal after delivery, it is likely that the leukocyte elevation is secondary to the steroid or cytokine alterations that accompany pregnancy.

Differentiation of a leukemoid reaction from leukemia should not present any unique difficulties when this question arises during pregnancy. Flow analysis and chromosomal studies

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help resolve any confusion in the rare pregnant patient with leukemia. When leukemia or other hematologic malignancies complicate pregnancy, management is influenced by the stage of pregnancy at which diagnosis is made, the clinical effects of the disease, and the anticipated toxic effects of chemotherapy or radiotherapy (or both) on the mother and child. [18] Because the most active stage of organogenesis is the first trimester, recommendations regarding the continuation of pregnancy would differ greatly in this setting from those of a woman with leukemia or lymphoma at a later stage of pregnancy. There are several reports of successful, uncomplicated births after chemotherapy for leukemia and lymphoma, [19] [20] but the most common outcomes are abortion and fetal loss. Promyelocytic leukemia has been treated with retinoic acid during pregnancy without fetal complications. [21] When chronic leukemias occur during pregnancy, leukapheresis may serve as a delaying device until delivery is possible. [22]

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THROMBOCYTOPENIA

Although some of the data conflict, ample evidence shows that the platelet count does not deviate from normal limits during an uncomplicated pregnancy. [23] Thrombocytopenia (<150,000/L) in the expectant mother requires consideration of the usual causes of a lowered platelet count as well as diatheses unique to pregnancy. Most authors would restrict these concerns to women with platelet counts of <100,000/L; a platelet count of 100,000150,000/L, newly discovered during an otherwise uncomplicated pregnancy, is not thought to pose any threat to the fetus. [24] This so-called gestational thrombocytopenia is not considered an indication for fetal blood sampling or therapeutic intervention, although the cause of the thrombocytopenia should be investigated and identified if possible. [25]

Finding and eliminating the cause of thrombocytopenia have special significance because it can threaten both the mother and the fetus. Thrombocytopenia in the fetus has important implications for determining the method of delivery and defining the postnatal needs of the infant. The situation is even more complicated in patients with qualitative platelet abnormalities such as the Bernard-Soulier syndrome and Glanzmann thrombasthenia; these patients require platelet support during delivery, even if the numbers of platelets are normal, to compensate for their lack of effective platelet function. [26]

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IMMUNE THROMBOCYTOPENIC PURPURA

Immune thrombocytopenic purpura (ITP) has a pronounced incidence in young adult women. The immunologic alterations of pregnancy may create the same sensitization, and thus, it is not surprising that ITP may complicate the course of pregnancy. The same diagnostic criteria are used as for standard ITP. [27] Platelet-associated IgG is frequently detectable in ITP, but this sensitive assay is relatively nonspecific. Demonstrable serum antiplatelet antibodies are more specific for this diagnosis and are usually directed against platelet glycoproteins GPIIb/IIIa or GPIb/9. [26]

Therapy for ITP during pregnancy is basically the same as during the nonpregnant state. [29] Steroids, starting at 1 mg/kg of prednisone and titering according to the platelet response, are initiated for platelet counts <30,000/L; some clinicians reserve steroid use for thrombocytopenic patients with prolonged bleeding times (>20 minutes) or with demonstrated bleeding.[30] If the patient is refractory to steroids or if there are contraindications to steroid use, intravenous immunoglobulins constitute an additional effective therapy for ITP. [31] Because the platelet-elevating effect of the latter is relatively short-lived (3 weeks), it may be necessary to punctuate the regimen with weekly to biweekly immunoglobulin infusions. With the availability of these two modalities, it is usually possible to avoid splenectomy, an operation that carries the risk of fetal loss. If splenectomy is required, the first portion of the second trimester is probably the optimal time for this intervention.

The fetus is also a candidate for development of thrombocytopenia because of transplacental passage of antiplatelet IgG in ITP. Either fetal scalp vein sampling during labor[32] or umbilical vein sampling before labor [33] is used for diagnosis and to influence the method of delivery of the affected fetus; a fetal platelet count of >50,000/L is considered adequate for vaginal delivery. Initial enthusiasm for using the level of maternal serum antiplatelet antibodies as a means for anticipating thrombocytopenia in the fetus has waned;[34] it has been documented that the infants platelet count in a previous pregnancy is an excellent predictor of the count in subsequent pregnancies of mothers with ITP. [35] There is support for the administration of steroids to mothers with ITP in the 3 weeks before delivery for their effect on the platelet count of the mother; [36] there is controversy as to the transplacental passage of steroids and their postulated positive effects on the fetal platelet count. [37] [38] Administration of immunoglobulins to a thrombocytopenic mother does not appear to influence the platelet count of the fetus. [39]

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PRE-ECLAMPSIA AND ECLAMPSIA

The specific pathophysiologic mechanisms that cause eclampsia are not known. Abnormal platelet-endothelium interactions, with excessive platelet consumption, are thought to be major components of this hypertensive disorder.[40] [41] There is also support for a more minor contribution from disseminated intravascular coagulation (DIC) in eclampsia,[42] because low antithrombin III levels[43] and elevated D-dimers of fibrin split products[44] are observed. The platelet decrement usually outdistances any evidence for fibrinogen consumption in eclampsia. The marked imbalance in the thromboxane/prostacyclin plasma levels in eclamptic patients has been suggested as a causal factor in the development of this process. The sevenfold thromboxane excess contributes to both increased platelet consumption and vasoconstriction.[45] The role of acetylsalicylic acid in preventing the development of eclampsia remains controversial, creating questions regarding the primacy of platelets and arachidonic metabolites in causing eclampsia.[46] [47] [48] [49] [50] Plasma endothelin levels are also elevated, suggesting some causal role for this potent vasoconstrictor as well.[51]

When thrombocytopenia is discovered in a patient with classic evidence for pre-eclampsia (>30-mm Hg rise in systolic or >15-mm Hg rise in diastolic pressures from baseline, proteinuria 0.3 g/L/24 hours, and edema) or eclampsia (seizures/coma), the cause of the thrombocytopenia is easy to identify. Thrombocytopenia develops in 50% of patients with pre-eclampsia. However, thrombocytopenia may also accompany hepatic forms of toxemia, in which the more classic signs of pre-eclampsia are absent.[52] Acute fatty liver of pregnancy is one of these variants. Clinical attention is drawn to and focused on the hepatic abnormalities. Liver biopsy specimens of eclamptic patients demonstrate the same fatty changes with the fat content of the liver paralleling the degree of thrombocytopenia.[53]

The hematologic alterations in pregnancy sometimes extend to encompass a clinical entity that includes a hemolytic anemia.[54] The so-called HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) may or may not be associated with increased blood pressure, edema, or proteinuria.[55] Most patients with the HELLP syndrome present with malaise and right upper quadrant or epigastric pain, often diverting the physicians attention from the recognition of a toxemic state.

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THROMBOTIC THROMBOCYTOPENIC PURPURA

Several cases of thrombotic thrombocytopenic purpura (TTP) during pregnancy have been reported in the literature, but the

appearance of a TTP-like picture during the third trimester of pregnancy may actually be toxemia masquerading as TTP. [56] The pentad of TTP (fever, hemolytic anemia, thrombocytopenia, renal disease, and central nervous system disease) may all be present in toxemia except for fever. The management of a patient with this constellation during the last trimester of pregnancy is delivery of the infant and not the complex interventions used to treat classic TTP. When TTP appears earlier in pregnancy, steroids and plasma infusions [57] with and without plasmapheresis have been used successfully; uterine evacuation has also been used with successful reversal of the picture. [58]

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DIC

The most frequent causes of bleeding in obstetric patients are the many pathologic precipitants of DIC. These have their origin in the entrance of the products of conception into the systemic circulation.[59] Slow DIC is now infrequently a problem because its cause, fetal death in utero, is easily recognized by ultrasound and can be corrected by induction of delivery with blood component support to correct any coagulation abnormalities; delivery of the dead fetus removes the source of the DIC, which is thought to be the tissue thromboplastin released from the decomposing fetus.[60]

A rapid and frequently fatal form of DIC accompanies clostridial sepsis; this unfortunate aftermath of illegal abortions is also characterized by severe intravascular hemolysis, attributable to the direct bacterial by-product attack of lecithinase on the lipid component of the RBC membrane.[61] Fast DIC also accompanies abruptio placentae[62] and amniotic fluid embolism.[63] The major complication of the former is hemorrhage, whereas the latter is usually complicated by cardiopulmonary collapse in addition to hemorrhage. Treatment of DIC associated with abruptio placentae consists of stabilization of the clotting disorder with the necessary blood component support, followed by delivery of the infant.

The epidemic of cocaine addiction has seen an increased incidence of abruptio placentae in which cocaine is thought to play a causal role. Amniotic fluid embolism is usually fatal (85% mortality), primarily as a result of cardiovascular collapse. This life-threatening entity occurs most frequently during a difficult delivery in a multiparous woman. Hypoxemia, shock, and hemorrhage in this setting should suggest the diagnosis, and aggressive cardiopulmonary support, blood component administration, and steroids have been recommended for its management.

Fast and slow DIC represent the most dramatic occurrences of DIC during pregnancy. However, evidence for DIC also exists in patients who have hypertonic saline- and urea-induced abortions.[64] This complication is usually fleeting and disappears shortly after the fetus is delivered. No therapeutic intervention is usually necessary. Heparin therapy is not indicated in this situation or in most other forms of DIC complicating pregnancy. Successful heparin treatment of DIC associated with a single fetal death in a twin pregnancy has been reported;[65] this may have implications for management of a multiple pregnancy now that selective destruction of some of the fetuses is performed.[66]

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PREGNANCY AND THROMBOSIS

Pregnancy constitutes a hypercoagulable state with a progressive increase in the concentration of clotting factors and a decrease in the bodys fibrinolytic capacity. A specific plasminogen activator inhibitor is produced by the placenta. [67] This situation, when coupled with stasis due to obstruction of venous return by an enlarging uterus, explains the dramatically increased incidence of venous thrombosis that occurs during pregnancy and the puerperium. [68] Thrombosis of the pelvic and lower extremity vessels is common. Diagnosis and treatment are influenced by their potential for damage to the fetus (see box, Anticoagulation Therapy During Pregnancy).

ANTICOAGULATION THERAPY DURING PREGNANCY

No form of anticoagulation is without significant risk during pregnancy. [69] A firm diagnosis of thrombosis or embolism, or both, must be made before initiating any therapy. This may entail using the gold standard of venography, with shielding of the uterus, to document the thrombosis if impedance plethysmography is not available. The risk of the radiographic procedure is thought to be offset by the greater certainty that venography allows in dictating the need for anticoagulation. [70] The same is true for ventilation/perfusion scans or pulmonary angiography as a means of diagnosing pulmonary embolism in the same population.

The maternal and fetal morbity and mortality due to anticoagulation make family planning counseling essential for an individual who requires chronic anticoagulation. [71] Patients with artificial prosthetic valves require continuation of anticoagulation throughout pregnancy and must recognize that only one-third of pregnancies in which coumadin is administered during the first trimester result in normal live-born infants. [72] [73] Heparin administration is not clearly superior, although the risk of abnormalities in live-born infants is less. Heparin does not cross the placenta and is not associated with any specific embryopathy. Heparin, however, leads to bone demineralization in the mother and in about 10% of patients results in thrombocytopenia. Low-molecular-weight heparins appear to offer advantages over standard-dose heparin during pregnancy, both for prophylaxis and treatment of thrombotic disorders; bone demineralization and thrombocytopenia are not avoided by the use of this form of anticoagulation. [74] [75] [76] [77]

Because both coumadin and heparin pose serious risks during pregnancy, some clinicians use them sequentially to dampen the risk of one and capitalize on the convenience of the other. Standard heparin or low-molecular-weight heparin is used during the first trimester, with a subsequent switch to oral coumadin; close to the end of pregnancy, heparin or low-molecular-weight heparin is used and then stopped as described above at the time of delivery. Heparin or coumadin can be reintroduced postpartum because neither drug is a threat to nursing infants. Thrombolytic agents are contraindicated in pregnancy because of their risk of severe hemorrhage.

Because of the high incidence of recurrent thrombotic events with subsequent pregnancies, expectant mothers with a previous history of deep venous thrombosis or pulmonary embolism have usually been prophylactically anticoagulated. Low-molecular-weight heparin, with its once daily dosing, is an attractive option for this indication during pregnancy. [78] Anticoagulation is sometimes withheld as prophylaxis; dextran is administered at and around the time of delivery, and heparin is initiated in the postpartum period; a small series has demonstrated the safety and utility of this alternative management. [79]

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PREGNANCY AND THE HEMOGLOBINOPATHIES

The vogue for prophylactic exchange transfusions in sickle cell anemia has waned and has given way to watchful anticipation

MANAGEMENT OF SICKLE CELL DISORDER

Management of the patient with sickle cell disorder during the delivery period remains controversial. Some would exchange transfuse such individuals if general anesthesia is required for delivery; the potential for an anesthetic accident with hypoxemia is the basis for this recommendation. Epidural anesthesia lessens this risk, but hypotension secondary to venous pooling may occur even with this modality; attention to fluid expansion, use of pressure leg wraps, and unweighting uterine compression of the vena cava by positioning the patient on the left lateral side lessen this risk.

of the patients course by the hematologist and obstetrician together. [80] Many series have demonstrated a high risk of stillbirths, abortions, and fetal growth retardation as the outcome of nontransfused sickle-cell pregnancies, but the contribution of poor prenatal care in this socioeconomically deprived group may have been the critical factor in these fetal losses. With good antenatal care and recognition of any backdrop of organ dysfunction, the usual patient with hemoglobin SS disease tolerates pregnancy without major morbidity for mother or child. Transfusions are reserved for any acceleration of the sickling disorder, worsening of anemia (hemoglobin <6 g%), or obstetric complications. [81] In light of the severe hemolytic component of this disease, folic acid must be adequately supplemented to guarantee that a hyporegenerative megaloblastic crisis does not occur. Iron therapy may also be needed because these young women frequently have meager iron stores even in the setting of hemolytic anemia (see box, Management of Sickle Cell Disorder).

The same concerns apply to individuals with other interacting hemoglobins (SC, S thal, and others) that participate in the sickling phenomenon. Sickle trait should pose no threat to pregnancy except for the need to recognize and treat properly the higher incidence of kidney infections. The probable contribution of infection to precipitation of premature labor makes such infection a more significant threat during pregnancy.

Thalassemia major is not a frequent problem for the obstetrician because such patients often suffer with failure of pubertal growth and delayed sexual development. Hypogonadotropic hypogonadism due to hemosiderin deposition in the hypothalamus makes these patients anovulatory and afertile. [82] However, there have been several reports of successful pregnancies in this patient population with a high risk of fetal loss. Cardiac iron disease may be present and may lead to heart failure during pregnancy; close attention to maintaining the hemoglobin at a level of 10 g/dl is important. Thalassemia intermedia and minor are not characterized by any special problems during pregnancy; ferritin levels in these groups may be adequate to permit dispensation from the need for iron supplementation during pregnancy. [83]

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COAGULOPATHIES DURING PREGNANCY

The most common coagulopathy creating problems during pregnancy and delivery is von Willebrand disease (vWD). [84] Recommendations regarding the management of vWD must consider the spectrum of severity of the different subtypes of vWD and the relatively sparse experience in managing some of the more recently defined subtypes (see box, Therapy for von Willebrand Disease). Fortunately, in most of these patients, an increase in factor VIII coagulant and factor VIII antigen accompanying pregnancy frequently permits delivery without undue risk of bleeding. There remains the delayed risk of bleeding after delivery in these individuals because of the rapid return of coagulant levels to a nonpregnant deficient state. In such patients, the bleeding time is an important parameter to monitor and correct with desmopressin or concentrate as necessary.

Some individuals with vWD, especially those with the type III variant, have persisting very low to absent von Willebrand factor (vWF) levels throughout pregnancy and demonstrate a poor response to desmopressin infusions. Factor VIII concentrates that contain vWF should be administered to these patients if a cesarean section is performed; support should continue for 57 days after the delivery. The previous recommendation, that all such patients with severe vWD undergo cesarean section to protect the fetus, should be modified now that prenatal sampling of fetal blood allows identification of infants with the disorder.

The remainder of the inherited coagulopathies are not a major threat unless the coagulant levels are <25% of normal. [87] Fresh frozen plasma infusions or factor concentrates during and 45 days after delivery are the universal solution to these deficiencies, with monitoring of coagulant levels to gauge the adequacy of the replacement therapy. Factor XIII deficiency presents a special problem during pregnancy because of its association with spontaneous recurrent abortions and marked uterine bleeding; regular plasma infusions or factor XIII concentrates during pregnancy have sustained a normal pregnancy in the presence of factor XIII deficiency. [88]

Acquired inhibitors of coagulation factors may occur in the postpartum period with factor VIII antigen the most common target for these circulatory anticoagulants. Porcine factor VIII, factor VIII inhibitor bypass activity, and recombinant factor VII all are options to treat bleeding complications of this coagulopathy, although standard factor VIII concentrates are usually successful in neutralizing low levels of the inhibitor. The inhibitors often persist for months without any benefit from immunosuppression; fortunately, there is little risk for recurrence of the inhibitor in subsequent pregnancies. [89]

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LUPUS ANTICOAGULANT AND PREGNANCY: ANTIPHOSPHOLIPID SYNDROME

The lupus anticoagulant (LA), an immunoglobulin of the IgG or IgM subclass, prolongs phospholipid-dependent coagulation tests by interfering with the essential phospholipid skeleton of

THERAPY FOR VON WILLEBRAND DISEASE TYPE IIB

The type IIB subtype presents an additional layer of complexity because a progressive diminution in platelet count accompanies the pregnancy-associated increased synthesis of this variant form of vWF. [85] The appearance of thrombocytopenia during pregnancy is, in fact, grounds for considering this diagnosis, wherein the interaction of the abnormal vWF and platelets results in thrombocytopenia.[86] Because of this interaction, administration of desmopressin to such patients has been proscribed, although there remains some question as to the appropriateness of this admonition. Patients with type IIB vWD have had normal vaginal deliveries without excessive bleeding even in the presence of significant thrombocytopenia. Factor VIII concentrate that contains vWF can successfully prevent bleeding in this condition when needed.

the coagulation cascade. [90] A positive test result for syphilis, a so-called biologic false-positive test, and anticardiolipin antibodies (both tests having phospholipids as their target antigens) are frequent accompaniments of the LA. Individuals possessing such antibodies often develop thrombocytopenia and thromboses involving both the arterial and venous circuits; [91] the antibodies may be a factor implicated in creating the thromboses because plasma from LA patients inhibits vessel wall generation of prostacyclin, a vasodilator and powerful inhibitor of platelet aggregation. The procoagulant role of LA plasma may not be restricted to prostacyclin inhibition because other investigators have demonstrated defective thrombomodulin activation of protein C in the presence of LA plasma; [92] reduction in levels of annexin V, a potent phospholipid anticoagulant protein on vascular endothelial cells, is another possible mechanism of thrombosis and pregnancy loss in the antiphospholipid-antibody syndrome. [93] Whatever the role of the phospholipid antibody in the process, the LA is paradoxically a marker for an increased risk of thrombotic episodes, including strokes.

Nowhere is this thrombotic tendency more threatening than in the association of the LA with first-trimester abortions and fetal deaths in the second and third trimesters.[94] The lesion in many of these women is a thrombotic process involving the decidual and placental vessels; the absence of such findings in other LA patients forces the conclusion that the pathogenetic mechanisms of the antiphospholipid antibodies must still be clarified. Because the fetal loss syndrome is well identified in mothers with anticardiolipin antibodies who lack LA, the phospholipid target of the antibody appears to have the most significance, especially when the target of the antibody includes the epitope $_2$ -globulin.[95]

Any mother with systemic lupus erythematosus (SLE) or a history of fetal loss should be screened for antiphospholipid antibodies. The LA is not restricted to women with SLE, and there is no relationship of the antibody to the activity of the disease or even to anti-DNA activity. [96] The activated partial thromboplastin time or the more sensitive Russells viper venom time[97] is a good screen for the presence of the LA; a low titer-positive serologic test result for syphilis is sometimes the clue that LA is complicating a pregnancy. Because the anticardiolipin antibody is only loosely associated with LA in patients with SLE, separate testing for this antibody should also be performed because it may be an earlier and more important predictor of fetal distress [98] (see box, Therapy for Lupus Anticoagulant).

THERAPY FOR LUPUS ANTICOAGULANT

Optimal treatment of the pregnant patient with the LA remains to be determined. The use of prednisone and aspirin as a means of reversing the poor outcome of such pregnancies has not been validated when compared with placebo controls. Therapeutic intervention with standard heparin has permitted successful pregnancies; thromboprophylaxis with low-molecular-weight heparin also appears to be efficacious in managing thromboembolic disease in these patients. [99] [100] [101] Doppler ultrasound monitoring of placental blood flow has been suggested as a means of charting the pathologic effects of the antibody on the vascular bed and helping define the need for infant delivery. [102] The complex clinical scenarios in these patients and the gaps in our knowledge regarding the pathophysiology of the disease make necessary a close collaboration among obstetricians, hematologists, and pediatricians in their care.

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HYPERCOAGULABLE STATES AND PREGNANCY/MYELOPROLIFERATIVE DISORDERS

Primary or essential thrombocythemia in a pregnant woman may lead to the development of multiple placental infarcts, fetal loss, or fetal growth retardation. [103] [104] A combination of dipyridamole and aspirin has been administered with a successful outcome in such pregnancies; plateletpheresis has been performed immediately before delivery with effective lowering of the platelet counts. Interferon- has been used successfully during pregnancy in essential thrombocytosis without fetal complications.[105] Of note, essential thrombocytosis has also resolved during pregnancy with the disappearance of its threat of thrombosis. Monitoring of thrombocythemic pregnancies with Doppler ultrasound studies of placental blood flow has been recommended as a means of gauging the need for platelet reduction by pheresis earlier in the pregnancy.

Pregnancy complicated by polycythemia vera has been infrequently reported; the perinatal outcome has been poor, with significant fetal loss. [106] Attention must be paid to maintenance of a hematocrit in the 40% range with phlebotomy used to maintain that level. Iron supplementation needs careful monitoring and limitation to avoid erythrocytosis. Because of the frequent association of thrombocythemia with erythrocytosis in polycythemia vera, some clinicians recommend the use of low-dose heparin during and after delivery, although this approach is not without controversy. There is usually a rapid rebound in the hematocrit after delivery, requiring special vigilance to prevent threatening erythrocytosis at that time. Interferon- has been used successfully to control disease activity in myeloproliferative disorders during pregnancy without damage to the fetus; this agent may permit a smoother control of the blood counts, but experience with its use during pregnancy is still limited. [107]

Activated protein C resistance plays a prominent and threatening role in enhancing thrombosis during pregnancy and with oral contraceptive use. Because 5% of the white population is heterozygous for the gene and such heterozygotes are hypercoagulable, this mutation is the most common cause of a hypercoagulable state in the general population[108] and the most frequent cause of thrombophilia during pregnancy. Coinheritance of activated protein C resistance with other prothrombotic disorders such as protein C deficiency or homocysteinuria exaggerates the risk of thrombosis in the latter disorders. Identification of activated protein C resistance as a cause of thrombosis during pregnancy calls for standard anticoagulation administration and consideration of anticoagulant prophylaxis during subsequent pregnancies. Universal screening of pregnant women or oral contraceptive users is not recommended although any family or personal history of thrombosis would be an indication for such screening. [109] [110]

Deficiencies in proteins C and S and antithrombin III are all associated with an increased risk of venous thrombosis and fetal loss. Protein C deficiency dramatically increases the risk of deep venous thromboses during pregnancy; [111] [112] there is a 25% incidence of deep venous thrombosis in pregnancy and the puerperium in patients with this autosomal dominant inherited disorder. Therapy recommended for management of protein C deficiency is ambulatory full-dose subcutaneous heparin; protein C concentrates are also now available to correct this deficiency. [113] Protein S deficiency carries the same risks and implications for anticoagulation.

Antithrombin III deficiency is also a common inherited procoagulant state. Thrombotic events may occur during pregnancy or after delivery in 70% of such cases. [114] [115] Management of these patients has included heparin (20,000 U subcutaneously, twice a day) during the first 44.5 months and coumadin in the remainder of pregnancy to the 36th or 37th week; heparin is then introduced and withdrawn before delivery, at which time

a single infusion of antithrombin III (3,500 U) has been given. Low-molecular-weight heparin is an attractive alternative to this management. Heparin is again restarted in the postdelivery period until coumadin is introduced.

Approximately 50 cases of pregnancy in women with paroxysmal nocturnal hemoglobinuria have been reported; [116] these pregnancies have been complicated by venous thromboses involving the lower extremities and the hepatic and cerebral vasculatures. Anticoagulation with low-dose heparin or low-molecular-weight heparin is recommended for these patients if they are confined to bed during the pregnancy. This disease may also witness worsening of marrow aplasia during pregnancy and an increased number and severity of hemolytic crises. Transfusions with washed RBCs to maintain the hemoglobin at 10 g% is suggested, with particular attention toward preventing hemolytic crises after delivery. The fetus in such pregnancies needs monitoring for neonatal isoimmune hemolysis because of the risk of maternal sensitization after transfusions.

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Chapter 150 - Hematologic Problems in the Surgical Patient: Bleeding and Thrombosis

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INTRODUCTION

Surgery represents a dual challenge to the hemostatic system. First, bleeding must be stopped rapidly at the operative site, but thrombosis must be prevented also. Hemostatic failure can result in a variety of bleeding complications that are well known to the surgeon. They may be mild with an increased transfusion requirement or excessive oozing at the operative site, or more severe, resulting in blood loss with shock in the postoperative period, internal hematomas that compromise organ function, or occasionally death from uncontrolled hemorrhage. On the other hand, thrombotic complications occur frequently in the postoperative period. They are related to hemostatic changes resulting from surgery, including local damage to vessels, venous stasis, and alterations in the fibrinolytic and coagulation systems. The prevention and treatment of thrombosis in surgical patients requires difficult choices and administration of drugs that interfere with hemostasis in the perioperative period when the risk of bleeding is high. Surgeons are well aware of the hemostatic requirements of operative procedures and manage most cases routinely and many that present with hemostatic problems without assistance. However, consultation is often requested for complex or difficult problems, including preoperative evaluation of hemostasis in patients with known hemostatic defects, treatment of unexpected intra- or postoperative bleeding, prophylaxis of deep vein thrombosis in high-risk patients, and treatment of thrombosis in the perioperative period.

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PREOPERATIVE EVALUATION OF HEMOSTATIC RISK

Preoperative evaluation of hemostasis occurs at two levels. First is the routine evaluation needed in all patients before surgery to identify those with potential abnormalities of hemostasis. An adequate history is the most effective screening modality for adults and may be based on a questionnaire [1] (Chap. 105), whereas the need for routine laboratory testing is controversial. Those against routine testing [2] point to retrospective studies[3] [4] indicating that they rarely detect unexpected bleeding disorders and also emphasize the problems in evaluating false-positive abnormalities. The bleeding time is a poor predictor of abnormal surgical bleeding. [5] A recent retrospective study found that the bleeding time in asymptomatic individuals had only a 5% positive predictive value for postoperative bleeding, [6] and the poor correlation between surgical bleeding and the bleeding time has also been found in a prospective study of patients having cardiac surgery. [7] A number of recent prospective studies have also concluded that routine screening laboratory tests (bleeding time, prothrombin time [PT], partial thromboplastin time [PTT]) in asymptomatic patients are not predictive of peri- or postoperative bleeding. [8] [9] [10] [11] [12]

Screening programs that recommend laboratory testing point to the asymptomatic nature of some hemostatic abnormalities that may cause surgical bleeding and the occasional failure to obtain a detailed history. [1] [13] [14] A prospective study of preoperative screening in children before tonsillectomy found both history and laboratory screening to have high specificity but a low positive predictive value for perioperative bleeding. [14] Another study found that perioperative blood loss in adult cardiothoracic surgery patients could be predicted with a model, including the bleeding time, PT, and platelet count. [15] Given the variety of potential hemostatic defects, however, no simple screening system will identify all patients at increased risk of bleeding. The level of hemostatic risk for the proposed surgery (Table 150-1) forms the basis for an approach to preoperative evaluation (Table 150-2). A hemostatic history should be obtained in all patients, and in patients having low-risk surgery no laboratory tests are required. For moderate-to-high risk surgery additional screening would include a PTT, PT, and assessment of the platelet count.

In practice, a hematologist is rarely consulted for routine screening because surgeons have adopted approaches based on their training and local practice patterns. Rather, consultation is sought because of a history suggesting a bleeding disorder or an abnormal test that is found on screening. The approach of the consultant cannot be one of screening because the judgment of the referring physician that a bleeding disorder may be present indicates a greatly increased probability of finding an abnormality. If a referral is obtained as a result of an abnormal screening test result, this finding must be pursued and the abnormality fully explained. However, for all referrals the history is of central importance and must include a thorough review of any bleeding episodes, including hospital records and results of prior hemostatic testing, as well as careful attention to the family history. The physical examination should focus on evidence of bleeding and also identifying systemic disorders such as hepatic or renal disease. If the history of bleeding is negative or minimal, appropriate laboratory testing would include a PT and PTT and a biochemical profile to evaluate hepatic and renal function. A complete blood count and examination of the peripheral blood smear are useful to identify a myeloproliferative disorder or thrombocytopenia. If the history is suggestive of a hemostatic abnormality, then a full evaluation is indicated and additional specific testing is usually required because von Willebrand disease, mild factor VIII, factor IX, factor XI deficiencies, factor XIII deficiency, platelet functional defects, and fibrinolytic abnormalities are not identified by global screening tests.

TABLE 150-1 -- Risk of Bleeding with Surgical Procedures

Risk	Type of Surgery	Examples
Low	Nonvital organs involved, exposed surgical site, limited dissection	Lymph node biopsy, dental extraction
Moderate	Vital organs involved, deep or extensive dissection	Laparotomy, thoracotomy, mastectomy
High	Bleeding likely to compromise surgical result, bleeding complications frequent	Neurosurgery, ophthalmic surgery, cardiopulmonary bypass, prostatic surgery, surgery to stop bleeding

TABLE 150-2 -- Preoperative Hemostatic Evaluation

Routine Screening

Surgical Risk	Approach
Low	History only
Moderate or high	History, PTT, PT, platelet count

Consultation

History	Approach
Negative or minimal for bleeding	PT, PTT, platelet count, biochemical profile, complete blood count and differential, review of peripheral blood smear
Suggestive of bleeding disorder	Add to above as indicated: bleeding time, ristocetin cofactor, von Willebrand antigen, factor VIII, factor IX, factor XI, factor XIII assays

MANAGEMENT OF PATIENTS WITH HEMOSTATIC ABNORMALITIES

Patients with known hemostatic abnormalities are often referred in anticipation of surgery for assessment of bleeding risk and recommendations regarding perioperative management. Generally, operation in the presence of a hemostatic defect is associated with increased operative risk, and this should be adequately discussed with both patient and surgeon. Even when the diagnosis is clearly established and adequate replacement therapy available the operative risk is increased by the possibility of bleeding. Several general considerations affect evaluation of risk and planning management. First, the risk of bleeding with the specific surgery planned must be evaluated (Table 150-1). The support of hemostasis for an extensive procedure such as pneumonectomy will be much more difficult than that for a lymph node biopsy. Second, both the need for surgery and its urgency must be carefully considered. Greater risks are more warranted for correction of a life-threatening condition such as bowel perforation than for elective cosmetic surgery. Finally, the nature and severity of the hemostatic abnormality and the ability to correct it during the operative period are of fundamental importance. Consideration must also be given to the duration of replacement that will be required with appreciation of potential bleeding associated with events in the postoperative period such as removal of sutures and deep drains and the need for physical therapy, especially after orthopedic surgery.

The following sections discuss perioperative management of some of the more common acquired coagulation abnormalities; therapies for congenital factor deficiencies and von Willebrand disease are discussed elsewhere in this text.

Thrombocytopenia

Thrombocytopenia is one of the most common acquired hemostatic abnormalities in hospitalized patients, and the availability of platelet transfusion has made consideration of both emergency and elective surgery reasonable even in severely thrombocytopenic patients. The best index of the likelihood of bleeding in thrombocytopenic patients is the platelet count, with a minimal risk of bleeding if the platelet count is 80,000/l and significant likelihood of increased bleeding if the count is 20,000/l (Table 150-3). Differences in platelet function must also be considered in evaluating the risk of thrombocytopenic bleeding because large platelets usually seen in idiopathic thrombocytopenic purpura (ITP) are more effective than normal, whereas platelets in myeloproliferative disease or uremia, or after administration of drugs that affect platelet function (especially aspirin or other nonsteroidal anti-inflammatory drugs) will be less effective. Other critical considerations for surgery in thrombocytopenic patients include clinical conditions that reduce platelet recovery or life span after transfusion, including splenomegaly, fever, infection, active bleeding, and presence of auto- or alloantibodies.

In preparation for elective surgery, therapy for the disorder causing the thrombocytopenia is the most satisfactory approach. For example, steroid or intravenous -globulin therapy for ITP may increase the platelet count so that transfusion is not needed, and surgery in patients with leukemia should be scheduled, if possible, after achievement of hematologic remission. The platelet count required for safe performance of surgery is controversial. A National Institutes of Health Consensus Development Conference recommended transfusion at a platelet count <50,000/l; [16] others suggest transfusion at platelet counts <80,000/l for general surgery and <100,000/l for cardiopulmonary bypass.[17] A reasonable approach involves considering the bleeding risks associated with surgical intervention (Table 150-1) in deciding both the necessary platelet count to be achieved and the duration of required platelet support after surgery. For low-risk surgery, a single transfusion to increase the platelet count to >50,000/l followed by close observation may suffice. Transfusion to keep the platelet count >50,000/l for moderate-risk surgery and >100,000 l for high-risk surgery is appropriate. The duration of platelet support required has not been carefully studied, but for moderate- or high-risk surgery platelets may be needed for <1 week because platelets are principally

TABLE 150-3 -- Surgical Hemostasis in Common Clinical Problems

Thrombocytopenia		
Platelet count	80,000/l <80,000 and>20,000/l 20,000	Bleeding unlikely Platelet transfusion may be needed Bleeding likely, platelet transfusion indicated
Acquired platelet dysfunction		
Bleeding history or bleeding time	>15 min	DDAVP if bleeding time shortens Platelet transfusion if DDAVP ineffective
Liver disease		
Prothrombin time	3 sec over control	Bleeding unlikely
	>3 sec over control	FFP indicated, vitamin K may be helpful Prothrombin complex may be needed but has thrombotic risk
Fibrinogen	<100 mg/dl	Cryoprecipitate indicated
Thrombocytopenia, platelet dysfunction		As above, but platelet recovery poor because of short half-life
Accelerated fibrinolysis (short eublogulin clot lysis time)		Antifibrinolytic may be indicated

Abbreviations: DDAVP, desmopressin; FFP, fresh frozen plasma.

required for primary hemostasis. The platelet count should be monitored closely during the postoperative period with the expectation that platelet survival will be shortened by infection, fever, or bleeding. A special case is splenectomy for patients with ITP when the primary site of platelet destruction is being removed. Platelet transfusion in anticipation of splenectomy is not effective because of the short platelet survival, and transfusion, if necessary, after the splenic artery has been clamped, should be more effective.

Liver Disease

The hemostatic abnormality of liver disease is complex and may be due to one or more of several factors, including decreased synthesis of enzymes and inhibitors of the coagulation and fibrinolytic systems, synthesis of abnormal fibrinogen, decreased clearance of activated factors, abnormal platelet function, and thrombocytopenia.[18] [19] The severity of the hemostatic abnormality parallels the overall severity of the liver disease, which represents the greatest determinant of surgical risk. Patients with severe decompensated liver disease and markedly abnormal coagulation tests are at great risk of bleeding, and surgery should be avoided

except as a lifesaving measure. In evaluating hemostasis in patients with less severe disease, the PT provides a good measure of decreased synthesis of vitamin K-dependent coagulation factors and factor V. The PTT is less helpful because it is relatively shortened by a high factor VIII until late in liver disease. In addition, a platelet count is needed to identify thrombocytopenia (usually secondary to hypersplenism) and a bleeding time may be useful to determine whether platelet function is abnormal in the setting of a normal or near-normal platelet count. A fibrinogen assay should be performed, as should a thrombin time to test for dysfibrinogenemia. Tests for fibrinogen/fibrin degradation products, D dimer, and euglobulin clot lysis time are useful in evaluating disseminated intravascular coagulation (DIC) or accelerated fibrinolysis.

In patients with mild disease and a PT prolongation of <3 seconds, serious surgical bleeding is unlikely in the absence of other hemostatic abnormalities and prophylactic intervention is not necessary for low- or moderate-risk surgery. For high-risk surgery or greater degrees of hemostatic abnormality, transfusion of fresh frozen plasma is the most useful approach for correcting the coagulation abnormality. The volume needed is guided by the correction of the PT, and the success of this approach is limited by the volume required in patients with low factor levels. It should be noted that the PT of fresh frozen plasma is about 15 seconds, so complete correction of the patients PT cannot usually be achieved. Exchange transfusion may be of value in extreme circumstances. Administration of platelets should be considered for thrombocytopenia, although recovery will be decreased in the presence of splenomegaly. Administration of desmopressin acetate (DDAVP) is useful in correcting abnormal platelet function in some cases. [20] Prothrombin complex concentrates provide vitamin K-dependent factors in a smaller volume than plasma and have been used successfully before liver biopsy or surgery [21] [22] but cannot be generally recommended because of the occasional occurrence of severe thrombotic complications [23] [24] due to the presence of activated factors in available preparations. Vitamin K deficiency is a correctable abnormality in patients with associated bile duct obstruction or those taking broad-spectrum antibiotics, and administration of 10 mg vitamin K will usually shorten the PT if vitamin K deficiency is a contributory factor. Insertion of peritoneovenous shunts is frequently associated with development of a consumption coagulopathy [25] [26] due to entry into the circulation of procoagulants from ascitic fluid. Treatment with antithrombin III (AT III) replacement has not been successful, [27] but administration of heparin has led to decreased consumption of fibrinogen and platelets in this condition. [28] AT III replacement also failed to affect blood loss or transfusion in liver transplant recipients. [29]

Platelet Dysfunction

Patients with platelet dysfunction represent a large group in whom preoperative consultation is sought, usually because of finding a prolonged bleeding time with a normal platelet count. The etiology may be obvious in cases of renal or liver disease or myeloproliferative syndromes, but may also require evaluation for intrinsic platelet dysfunction (storage pool disease or platelet release defects) or von Willebrand disease. The most frequent cause of platelet dysfunction is administration of drugs such as aspirin. A careful drug history is therefore essential and drugs that interfere with platelet function should be discontinued before surgery and avoided in the perioperative period if possible. The bleeding time may be useful in assessing the efficacy of therapeutic modalities such as DDAVP.

Treatment of the underlying disease is the most effective approach in many acquired disorders of platelet function such as renal disease or myeloproliferative syndromes. Platelet transfusion is an effective treatment for intrinsic platelet dysfunction, but the dose required to achieve hemostasis is difficult to predict and depends in part on the severity of the underlying platelet abnormality. In these patients there is no need for prophylactic platelet transfusion for low- or moderate-risk surgery, but they do require close observation and treatment if abnormal bleeding occurs. Most hematologists would recommend prophylactic platelet transfusion for high-risk surgery if the bleeding time is prolonged to >15 minutes. Recently, DDAVP has been shown to shorten the bleeding time effectively in some patients with platelet dysfunction due to many but not all congenital or acquired abnormalities. [20] [30] [31] [32] [33] [34] [35] [36] [37] It is given in a dose of 0.3 g/kg intravenously and results in variable shortening of the bleeding time, noted within 1 hour and lasting 812 hours. Repeated doses can be given at approximately 24-hour intervals, but tachyphylaxis may restrict the effectiveness of subsequent doses. It has the advantage of decreasing transfusions, and its side effects are limited to occasional facial flushing, headache, mild tachycardia, and hypotension. The mechanism of DDAVP action is not well understood but is likely to be due at least in part to an increase in von Willebrand factor. [36] It may also increase the surface expression of the platelet von Willebrand factor receptor. [35]

Renal Disease

Patients with renal disease have platelet dysfunction disorders accompanied by increased bleeding times and abnormal in vitro platelet functional study results [38] [40] that may be related to defective platelet interaction with the vessel wall, [41] or as recently suggested, to increased nitric oxide synthesis by uremic platelets and endothelium. [42] The approach to therapy is given in Chapter 131 .

Antiplatelet, Anticoagulant, and Fibrinolytic Therapy

The most frequently encountered hemostatic abnormalities in patients before surgery are in those receiving antiplatelet, anticoagulant, or fibrinolytic drugs. Patients taking aspirin as antiplatelet therapy or for its anti-inflammatory or analgesic effects represent the largest such group. Some studies have shown a mild increase in surgical bleeding in patients taking aspirin [43] [44] [45] [46] [47] [48] although this has not been confirmed in other studies. [49] [50] [51] [52] Two reports concluded that aspirin should be stopped preoperatively only if the bleeding time on aspirin was outside the normal range. [53] [54] For elective surgery it is prudent to discontinue aspirin approximately 1 week before operation, although in the

absence of other hemostatic abnormalities, recent or current aspirin use should not represent a contraindication to surgery and requires no prophylactic therapy. The effect of aspirin may, however, seriously potentiate the bleeding tendency in the presence of other hemostatic abnormalities such as thrombocytopenia, uremia, or congenital factor deficiencies, and in such patients may contribute significantly to postoperative bleeding.

Subcutaneous heparin in low doses such as 5,000 U every 12 hours or low doses of low-molecular-weight heparin given subcutaneously daily are recommended as prophylaxis for postoperative venous thromboembolic disease in some patient groups (see later). This results in minimal prolongation of the PTT [55] [56] and only a slight increase in frequency of wound hematomas. [56] [57] [58] Higher doses of heparin given as treatment for acute thrombosis can increase in surgical bleeding, but the management of such patients is not difficult because the half-life of heparin is under 2 hours [59] (Table 150-4). Consequently, the heparin can be withheld 6 hours before anticipated surgery, and the PTT should return to control levels. [60] If more rapid neutralization is required for emergency surgery, heparin can be neutralized by protamine sulfate given intravenously in a dose of 1 mg for every 100 U heparin expected to be in the circulation. In patients receiving therapy for thrombosis, heparin should be interrupted for the minimum time consistent with obtaining good surgical hemostasis. In these patients, low-dose subcutaneous heparin or prophylactic doses of low-molecular-weight heparin can be substituted in the immediate preoperative period and continued in the early postoperative period. Therapeutic heparin can be restarted as early as 12 hours after surgery if there is no evidence of bleeding at the operative site; it should be restarted without a bolus at no more than the expected maintenance infusion rate. [60] The time at which full-dose heparin is resumed will depend on the type of surgery and the clinical course.

Low-molecular-weight heparin given subcutaneously has a longer half-life. [61] Consequently, if it has been given at therapeutic doses it should be discontinued 1224 hours before surgery.

Perioperative management of patients taking warfarin is more difficult because of the longer duration of its anticoagulant effect (Table 150-4). The risk of thrombosis or embolism during interruption of anticoagulant therapy must be balanced against the benefit of surgery, and this may be difficult in patients with recent venous thromboembolic disease, intracardiac thrombosis, or prosthetic heart valves. For elective surgery with low bleeding risk (Table 150-1), a reasonable approach is to reduce the dose preoperatively to achieve an International Normalized Ratio (INR) of <2.0 at which level surgery can be safely performed. For surgery with a moderate or high-risk for bleeding, this degree of anticoagulation is unacceptable, and warfarin should be held long enough before surgery to allow the INR to correct to <1.5, [60] and the patient switched to heparin, if continued antithrombotic therapy is needed. In most patients with steady-state INRs of 23, this will mean omission of four doses. [60] [62] Continuous intravenous heparin is convenient if there is reason for the patient to be hospitalized, or subcutaneous low-molecular-weight heparin can be administered on an outpatient basis. Anticoagulation can be reinstituted postoperatively with heparin. The patient should be started back on oral anticoagulants as soon as possible postoperatively because it generally takes 3 days for the INR to reach 2.0. [60] For emergency surgery in patients at moderate or high risk of bleeding, rapid reversal of the anticoagulant effect is required, and this can be achieved with vitamin K or fresh frozen plasma. Fresh frozen plasma contains all vitamin K-dependent coagulation factors, reliably and rapidly corrects the PT to near normal, and is the preferred approach in patients needing rapid correction. Its value is limited by the volume that must be administered, although 2 U of fresh frozen plasma usually corrects a therapeutic PT to within a level safe for surgery. Another limitation is that the half-life of factor VII is 5 hours, so that the PT should be monitored and additional fresh frozen plasma administered as needed. Parenteral administration of 510 mg of vitamin K will reverse the effect of warfarin with a delay of 612 hours, although its use is complicated by relative resistance to warfarin effect after its administration, so that reinstituting oral anticoagulation can be difficult. Administration of vitamin K intravenously in a low dose of 0.51.0 mg is also effective in reversing the anticoagulant effect and does not result in warfarin resistance. [63] Administration of both fresh frozen plasma and vitamin K is prudent in patients needing emergency surgery with high risk of bleeding. If the volume of plasma needed to correct the PT is too large, then prothrombin complex concentrates

can be substituted.

Fibrinolytic therapy results in pronounced hemostatic abnormalities including decreases in plasma plasminogen, $_2$ -plasmin inhibitor, fibrinogen, factor VIII, and factor V, and increases in fibrin(ogen) degradation products and abnormalities in platelet function. [64] [65] In combination with the capacity of fibrinolytic therapy to lyse needed hemostatic plugs, these abnormalities contribute to the bleeding complications seen with fibrinolytic therapy and make surgery hazardous. [66] Elective surgery should be postponed after fibrinolytic therapy until the hemostatic abnormalities are corrected. However, the need for emergency surgery is not uncommon, particularly with the increased use of fibrinolytic therapy for acute myocardial infarction and the occasional need for revascularization procedures soon after its administration. There are several reports [67] [68] [69] [70] [71] of successful coronary artery bypass graft surgery immediately after fibrinolytic therapy, although there was excessive bleeding and increased transfusion requirements were observed.

The two issues that must be considered in a patient receiving fibrinolytic therapy who requires emergency surgery are the presence of the plasminogen activator in the circulation and its effects on the hemostatic system. The effect of circulating plasminogen activator can be inhibited by administration of epsilon-aminocaproic acid (EACA), usually given in a loading dose of 6 g, administered either orally or intravenously, followed by a dose of 1 g/hr. This will have the unwanted effect of inhibiting

TABLE 150-4 -- Surgery in Patients Taking Anticoagulants

	Bleeding Risk	Elective Surgery	Emergency Surgery
Warfarin	Low	Adjust dose to INR 2.0	Discontinue warfarin
	Moderate	Adjust dose to INR 1.5	Discontinue warfarin; FFP to reduce INR to 1.5
	High	Substitute heparin if needed and hold heparin in perioperative period	Discontinue warfarin; FFP-vitamin K to reduce INR to <1.5
Heparin	Low or moderate	Discontinue high dose and give low-dose subcutaneous heparin or LMWH	Discontinue high-dose and give low-dose subcutaneous heparin or LMWH
	High	Discontinue 612 hr before surgery for unfractionated heparin or 1224 hr for LMWH	Discontinue; give protamine sulfate if needed

Abbreviations: FFP, fresh frozen plasma; INR, International Normalized Ratio; LMWH, low-molecular-weight heparin.

physiologic fibrinolysis and possibly increasing the chance of developing a thrombosis, a potentially serious problem in a patient with recent myocardial infarction. However, the half-life of fibrinolytic agents is short, varying from 6 minutes for tissue plasminogen activator (t-PA), to 23 minutes for streptokinase and 16 minutes for urokinase, although in the case of t-PA a low level of fibrinolytic activity can be identified for longer periods of time. [72] Because of the short half-life of fibrinolytic agents, administration of EACA should be required only if surgery is needed within 12 hours after discontinuing the fibrinolytic agent.

In cases in which surgery is delayed for longer periods of time, one must correct the hypocoagulable state resulting from fibrinolytic degradation of coagulation proteins. Cryoprecipitate, which is rich in fibrinogen, von Willebrand factor, and factor VIII, is the first approach to replacement. Ten bags can be given initially, along with 2 U fresh frozen plasma to help replete factor V. Monitoring improvement of coagulation abnormalities can be difficult in the emergency setting although the thrombin time is a useful guide to fibrinogen replacement. If operative bleeding occurs despite administration of cryoprecipitate and fresh frozen plasma, then EACA should be given, and platelet transfusion considered to correct potential platelet dysfunction. [73] [74]

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INTRAOPERATIVE AND POSTOPERATIVE BLEEDING

Excessive bleeding during or after surgery is a serious and potentially life-threatening complication that requires immediate evaluation and a rapid approach to diagnosis and institution of treatment. The first consideration is to determine whether the bleeding is related to a hemostatic defect or to a local lesion that requires surgical intervention (Table 150-5). Failure to surgically control bleeding vessels at the operative site is the most frequent cause of postoperative bleeding. This results in excessive blood loss limited to the site of surgery that may be rapid if a large vessel is involved. Therefore, very rapid bleeding confined only to the operative site and seen as excessive blood in operative drains or the dressing is usually due to a surgical problem. This is in contrast to bleeding due to a hemostatic defect, which often causes a generalized and slower oozing at the operative site and frequently involves additional sites outside the operative field. The patient should, therefore, be examined for signs of petechiae or purpura particularly at venipuncture sites or indwelling catheters, and the urine, stool, and nasogastric aspirate should be examined for blood if they are available. Laboratory tests are an essential part of the evaluation,

TABLE 150-5 -- Approach to Intra- or Postoperative Bleeding

1. Decide whether the bleeding is due to a local cause or to hemostatic failure Local: single site of bleeding and rapid rate, minimal or no abnormalities of coagulation tests. Hemostatic failure: multiple sites of bleeding, unusual bleeding pattern, abnormal coagulation tests.
2. Causes of perioperative hemostatic failure Pre-existing hemostatic abnormality, DIC, primary fibrinolysis; also consider contribution of dilutional thrombocytopenia and drug effects. Special cases: cardiopulmonary bypass, platelet dysfunction, prostatic surgery.
3. Diagnosis of hemostatic failure Review preoperative coagulation tests and operative record; interview family members; obtain PT, PTT, thrombin clotting time, platelet count, peripheral blood smear; consider reptilase time, fibrinogen, fibrinogen degradation products, or D dimer, euglobulin lysis time, bleeding time.

and screening tests are nearly always ordered before consultation is requested. In addition to the PT, PTT, and platelet count, it may be useful to obtain a bleeding time to evaluate platelet function if the platelet count is normal. A fibrinogen level, a test for fibrin(ogen) degradation products or D dimer, and euglobulin lysis time are needed to search for evidence of DIC or fibrinolysis. Many patients are receiving therapeutic or prophylactic heparin or have heparin instilled into indwelling catheters from which blood is obtained. This results in heparin-induced prolongation of clotting tests, which complicates the evaluation. This possibility can be evaluated by mixing studies to identify a coagulation inhibitor and by comparing the results of a thrombin clotting time that will be prolonged by heparin with a reptilase time, which will be normal. The peripheral blood smear should be reviewed to examine platelet size and number and to identify possible red blood cell fragmentation that may occur in DIC.

When evaluating hemostatic tests in surgical patients, one must consider changes that normally occur in response to surgery. These vary depending on the extent of tissue dissection and duration of the procedure. Consumption of coagulation factors and hemodilution from crystalloid infusion both lead to acute reductions during surgery, and this is typically followed by changes resulting from the acute phase response in the subsequent days. In moderately extensive surgery such as abdominal aneurysm repair or total hip replacement, typical changes include decreases in platelet count, factor VIII, von Willebrand antigen, antithrombin III and protein C during and immediately after surgery. [75] [76] [77] [78] [79]

Increases in fibrinogen, platelet count, factor VIII, and plasminogen activator inhibitor-1 in the first postoperative week reflect changes due to the acute phase response.[80] If hemostatic failure is thought to contribute to bleeding, the potential causes should be considered quickly (Table 150-5). A pre-existing hemostatic abnormality may have been undetected before surgery, and the family history and patients past medical history should be reviewed. Results of preoperative coagulation tests should be checked and reinterpreted, realizing that mild deficiencies of factor VIII, factor IX, and factor XI may be present without prolongation of the PTT, and that platelet functional abnormalities will not be identified without a bleeding time. Diagnosis of factor XIII deficiency requires a specific test, and inherited fibrinolytic abnormalities including deficiency of $_2$ -plasmin inhibitor[81] [82] and of plasminogen activator inhibitor-1 [83] [84] can cause postoperative bleeding and are not identified by screening coagulation tests. Other diagnostic considerations include consumption coagulopathy, possibly due to a mismatched blood transfusion, a period of profound hypotension, fat embolism, or amniotic fluid embolization.

Massive transfusion is often considered as a cause of intraoperative coagulopathy but rarely causes bleeding by itself. Transfusion of more than one blood volume (5,000 ml in a 70-kg adult) of banked blood predictably decreases the platelet count, but the microvascular bleeding occasionally seen in patients receiving massive transfusion cannot be directly related to the thrombocytopenia. [85] [86] [87] Therefore, routine platelet transfusion in the absence of unusual bleeding has not been recommended even during massive transfusion.[88] However, in the presence of other hemostatic abnormalities such as drugs affecting platelet function, the dilutional thrombocytopenia may be a significant contributing factor to the development of bleeding, particularly if it is suggestive of a hemostatic defect. In the presence of excessive operative bleeding, aggressive platelet transfusion to a platelet count 100,000/l is indicated.

Certain surgical procedures induce unique hemostatic changes. Thus, perfusion through the extracorporeal membrane oxygenator results in hemostatic changes during cardiopulmonary bypass. Orthotopic liver transplantation is associated with major hemostatic problems due to preoperative deficiencies

caused by severe liver disease and hypersplenism and additional intraoperative problems during the anhepatic phase of the procedure requiring careful replacement with blood products.[89] [90] Excessive fibrinolysis may also occur during liver transplantation and treatment with antifibrinolytic agents may be needed in appropriate cases.[91] [92] Excessive fibrinolysis related to hepatic or mesenteric ischemia can also occur during aortic aneurysm repair, which requires clamping above the celiac axis.[79] Excessive urinary bleeding after prostatectomy can be caused by local fibrinolysis related to high concentrations of urokinase; therapy with EACA can be beneficial,[93] but care must be taken to avoid treatment of patients with upper urinary tract bleeding, in whom fibrinolytic inhibition can result in ureteral obstruction.

The hemostatic problems with cardiopulmonary bypass requires special consideration (Chap. 131). The platelet count, hematocrit, and levels of coagulation and fibrinolytic factors are reduced to 50% of baseline after starting bypass and are maintained throughout the procedure, with the exception of factor V, which shows further reduction to <20%.[94] [95] [96] [97] These changes may be caused in part by exposure to artificial surfaces and also by a tissue factor dependent pathway related to surgical trauma.[98] The bypass procedure results in significant platelet dysfunction reflected by release of -granule constituents, the generation of platelet microparticles, abnormal in vitro aggregation tests, and a prolonged bleeding time that usually corrects within 1 hour postoperatively. [94] [95] [96] [97] [98] [99] There are no apparent changes in platelet surface expression of the important receptors, GPIIb-IIIa and GPIb-IX. [100]

Several hemostatic abnormalities contribute to postoperative bleeding after cardiopulmonary bypass. Inadequate neutralization of heparin by protamine sulfate results in a prolonged PTT and thrombin time with a normal reptilase time and is treated by administration of additional protamine sulfate. The most significant contributor to post-bypass hemostatic failure is abnormal platelet function that can be detected by an excessively prolonged bleeding time with a normal platelet count. This abnormality usually corrects within several hours but may require treatment with platelet transfusion if associated with abnormal bleeding. [101] The appearance of the heparin effect several hours after adequate protamine sulfate neutralization, termed heparin rebound, has been suggested as a cause of post-bypass bleeding, [102] but is rare. Excessive fibrinolysis and DIC are uncommon causes of postoperative bleeding and should be well documented prior to instituting therapy with heparin or antifibrinolytic agents.

Desmopressin has been administered to patients undergoing surgery with cardiopulmonary bypass because of the frequent development of abnormal platelet function and the ability of DDAVP to improve hemostasis in a variety of conditions with abnormal platelet function. Salzman and colleagues [103] conducted a double-blind prospective randomized trial in 70 patients undergoing cardiopulmonary bypass. Patients that received DDAVP at the conclusion of bypass had reduced operative and early postoperative blood loss compared to those assigned to placebo. Reduced blood loss was related to an increase in plasma von Willebrand factor concentration.[104] Several subsequent studies, however, gave conflicting results,[105] [106] [107] [108] and recent meta-analysis of 17 randomized, double-blind, placebo controlled trials including 1,171 patients found that DDAVP reduced overall blood loss by 9%, but had no statistically significant effect on transfusion requirements. [109]

Evidence of fibrinolytic activation with cardiopulmonary bypass has led to trials examining the effects of antifibrinolytic agents on intra- and postoperative bleeding. Several prospective trials have found significant decreases in both bleeding and transfusion requirements [110] [111] [112] [113] and many centers are now routinely using aprotinin, EACA, or tranexamic acid. Despite reasonable concerns over increasing thrombotic risk with antifibrinolytic agents, clinical trials have shown little evidence of increased thrombotic complications.

PROPHYLAXIS OF DEEP VEIN THROMBOSIS

The clinical consequences of postoperative venous thromboembolic disease include acute symptomatic deep vein thrombosis (DVT) and the later development of the postphlebitic syndrome as well as pulmonary embolism, which may be fatal. Accurate epidemiologic information regarding postoperative DVT is available based on routine screening using sensitive objective imaging methods. The risk of developing venous thrombosis varies in different surgical groups, and the need for prophylaxis is greater in those with higher risk (Table 150-6). The risk is lowest in young patients undergoing surgical procedures lasting <30 minutes with minimal immobility. Patients at moderate risk include those over age 40 having general abdominal or thoracic surgery lasting >30 minutes, and they have an expected incidence of venous thrombosis of 2030%, proximal vein thrombosis 210%, and pulmonary embolism 13%. Within low- and moderate-risk groups, additional factors that contribute to a further increase in risk include malignant disease, congestive heart failure, prior venous thromboembolic disease, obesity, and inherited hypercoagulable states. In certain high-risk orthopedic groups, the overall incidence of venous thrombosis

TABLE 150-6 -- Recommendations for Prophylaxis of Deep Vein Thrombosis

Category	Patients	Approximate Risk[a]			
		Calf DVT	DVT	PE	Recommendation
Low	Surgical patients under 40, surgery lasting < 30 min, no additional risk factors	12%	0.51%	00.1%	Ambulation, leg exercises
Moderate	Surgical patients over age 40, having abdominal or thoracic surgery lasting >30 min	2030%	210%	13%	Low-dose heparin
	Neurosurgery or other patients with high bleeding risk				Pneumatic compression
High	Hip fracture	4080%	2040%	510%	Warfarin,[b] LMWH
	Hip replacement				Warfarin,[b] adjusted-dose heparin, LMWH
	Knee replacement				LMWH, external pneumatic compression
	Open prostatectomy				Pneumatic compression
	Gynecologic malignancy				Pneumatic compression

Abbreviations: DVT, deep vein thrombosis; PE, pulmonary embolism; LMWH, low-molecular-weight heparin.

[a]Without prophylaxis.
[b]Given in low-dose regimen.

is 4080%, with proximal vein thrombosis in 2040%, pulmonary embolism in 510%, and fatal pulmonary embolism in 15% of patients receiving no prophylaxis. [114]

The natural history of postoperative DVT indicates that thrombi are dynamic and may either resolve or extend. Thrombi begin forming during or soon after surgery, initially in the calf veins. Most clots are asymptomatic, and many spontaneously regress but approximately 20% extend into veins proximal to the knee in the absence of treatment.[1] Most pulmonary emboli originate from proximal leg vein thrombosis,[116] and many are asymptomatic or may be misdiagnosed.[117]

Strategies for management are based on the frequency of thromboembolic disease in different surgical groups, expected clinical outcomes, and cost effectiveness. The simplest approach relies on treatment of clinically diagnosed disease, but is unacceptable in moderate- or high-risk groups because post-operative DVT is frequently asymptomatic, and the initial presentation of pulmonary embolism may be sudden death. A second potential strategy is monitoring with noninvasive tests such as ultrasound or plethysmography and treatment of affected patients only. This approach would, however, be both expensive and cumbersome, and it also raises difficult problems in management of false-positive monitoring studies. The most effective approach is primary prophylaxis in moderate- or high-risk patients using anticoagulants or methods to reduce venous stasis.

Anesthesia, operative pain, bed rest, and lack of leg muscle activity reduce venous blood flow, and this predisposes to thrombosis. Simple measures to counteract venous stasis that are applicable to nearly all patients include leg and foot exercises while in bed and early ambulation. The use of elastic stockings is of little documented benefit, but graduated compression stockings that exert greater pressure at the ankles and less pressure proximally may be of value in low- or moderate-risk patients.[118] [119] [120] External pneumatic compression devices provide active leg compression with inflatable boots that rhythmically compress calf or both calf and thigh sequentially. Such compression increases venous blood flow [121] [122] and also increases blood fibrinolytic activity; [123] [124] both effects may contribute to its efficacy. The absence of side effects makes pneumatic compression an attractive prophylactic modality, particularly in patients at increased bleeding risk. External pneumatic compression has been effective in patients after general abdominal surgery, [125] [126] [127] urologic,[128] neurologic,[129] and gynecologic surgery including that for malignancy[130] and knee replacement.[131] A system that mechanically extends the foot to cause plantar venous compression has also been shown to be effective in limited studies.[132]

The greatest experience with anticoagulant prophylaxis of postoperative venous thromboembolic disease has been with low doses of heparin and is based on the rationale that lower concentrations are needed to prevent than to treat venous thrombosis. Heparin administered in a dose of 5,000 U subcutaneously results in a peak plasma level of 0.050.15 U/ml approximately 2 hours after injection[133] with a slight but detectable increase in the PTT. [55] Administration of this dose every 812 hours beginning 2 hours before surgery results in a risk reduction of approximately 70% as determined by meta-analysis of over 70 controlled trials. [58] Large prospective, randomized trials [57] [134] [135] also demonstrated significant reductions in both pulmonary embolism and fatal pulmonary embolism in patients over 40 years of age undergoing major surgery who receive low-dose heparin prophylaxis compared to controls. Analyses of the published experience suggests 5070% reduction in risk from postoperative pulmonary embolism with the use of low-dose heparin. [114] Side effects of this regimen include bruising at injection sites and a 2% increase in wound hematomas.[58] Monitoring of the PTT is not needed with prophylactic low-dose heparin. The effectiveness and safety of low-dose heparin makes it appropriate prophylaxis for most moderate-risk surgical patients. However, surveys indicate that many surgeons do not use low-dose heparin prophylaxis because of the perceived infrequency of clinically significant thromboembolic complications and fear of bleeding. [136] [137] [138]

Several approaches have been used to improve on the effectiveness of low-dose heparin, including the addition of ergot, a venoconstricting agent, to decrease venous capacitance.[139] This has not met with wide acceptance because of the occasional occurrence of vasospastic side effects and the limited evidence of the superiority of the combination compared to low-dose heparin alone. Other studies have suggested that adjusting the heparin dosage based on PTT may increase its

effectiveness and extend the usefulness of heparin prophylaxis to high-risk patients. [140] [141] Postoperatively, the plasma concentration of antithrombin declines, and this observation led to consideration that heparin prophylaxis could be improved by supplementation with antithrombin. This was tested in two prospective, randomized trials following total hip [142] and knee [143] replacement, demonstrating that the combination of antithrombin and heparin was safe and more effective than a standard regimen of dextran. Also, the risk of developing DVT was related to the extent of postoperative decline in plasma antithrombin concentration. [144]

Heparin preparations with a restricted low-molecular-weight (LMW) range have relatively greater activity in promoting inactivation of factor Xa by antithrombin than inactivation of thrombin. [61] In comparison with standard, unfractionated heparin, LMW heparin has greater bioavailability after subcutaneous injection and a longer plasma half-life, allowing for once daily injection rather than two or three times daily for unfractionated heparin. Data are conflicting regarding the relative effectiveness of LMW heparin in comparison with unfractionated heparin in prevention of venous thrombosis in moderate risk surgical patients. Meta-analysis suggests approximate equivalence to unfractionated heparin after general surgery. [145]

The protection afforded by low-dose, unfractionated heparin is insufficient in some surgical patients with a particularly high risk of developing venous thrombosis, especially following certain orthopedic procedures. For example, after hip fracture, total hip replacement, or total knee replacement, the risk of venous thrombosis without prophylaxis is 4080%, with the frequent development of proximal leg vein clots and an incidence of pulmonary embolism of 510% and fatal pulmonary embolism 15% without prophylaxis. [114] [146] Several alternative prophylactic modalities have been tested in these high-risk patients. Dextran has been shown to be effective after total hip replacement or fracture [147] [148] but is not widely used because of the need for intravenous administration and the occasional precipitation of congestive heart failure from the fluid load. External pneumatic compression was effective in a limited number of studies after total knee replacement [131] [149] [150] and also in high-risk patients after prostatectomy [128] and gynecologic surgery for malignancy. [130] It is more effective than no prophylaxis after total hip replacement [151] [152] but is less effective than warfarin in preventing proximal vein thrombosis. [153] Although the usual low-dose heparin regimen has not been sufficiently effective in high-risk patients, some evidence indicates that subcutaneous unfractionated heparin given in a dose to increase the PTT to the upper normal range may be more efficacious. [140]

Oral anticoagulants have been shown to be consistently effective in preventing venous thrombosis in high-risk patients. In a classic study, Sevitt and Gallagher [154] demonstrated a reduction in both clinical and autopsy evidence of pulmonary embolism and DVT with coumarin prophylaxis compared to a control group. Although the effectiveness of warfarin has been amply confirmed in subsequent studies, concern over bleeding complications has limited its general acceptance. Recent studies have, however, demonstrated that a lower intensity of

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anticoagulation is effective without increasing the bleeding risk even in surgical patients. Low-dose warfarin regimens begun preoperatively were effective in patients undergoing joint replacement [155] or gynecologic surgery. [156] Paiement et al. [157] have also reported a low incidence of venous thrombosis and bleeding complications after hip replacement with the use of warfarin begun the night before surgery, and warfarin has also been effective when started postoperatively. [158] Francis et al. [159] reported no difference in outcomes comparing a warfarin regimen beginning 710 days preoperatively with one starting the night before surgery in patients undergoing knee replacement. With the use of warfarin in hip replacement, meta-analyses have identified rates of deep vein thrombosis of 24% [160] and 19%, [161] whereas large single studies have found rates between 14% and 23%. [153] [158] [162] [163] Warfarin has been less effective with knee replacement, with rates of DVT from 3855% in recent studies. [158] [159] [162]

Low-molecular-weight heparin is effective in high-risk patients, and meta-analyses have identified rates of postoperative DVT of between 12% and 17%, [145] [160] [161] similar to findings in recent large North American trials. [162] [164] [165] [166] [167] LMW heparin has also been compared directly with warfarin following hip replacement. In three studies, there was no significant difference in postoperative venous thrombosis rates, [152] [162] [163] whereas one showed a significantly lower rate with LMW heparin (15%) compared to warfarin (26%). [168] LMW heparin has also been compared with unfractionated heparin following hip replacement [97] [98] [99] and meta-analyses of published trials indicate that it is more effective. [145] [161] [162] Following knee replacement, LMW heparin has been shown superior to placebo, [169] graduated compression stockings, [170] or warfarin. [152] [162] [163] The optimum duration of prophylaxis after joint replacement surgery is not certain, and the period of risk may extend several weeks postoperatively. [171] [172] A recent double-blind study demonstrated a lower incidence of venous thrombosis 1 month after surgery when prophylaxis was extended postdischarge. [173]

Hip fracture is associated with a high risk of postoperative venous thrombosis similar to that with joint replacement. Low-intensity warfarin is effective in reducing risk [174] [175] [176] and LMW heparin [177] [178] or dermatan sulfate [179] achieves similar risk reduction. Patients suffering from multiple trauma are heterogeneous, but they also have a high risk of venous thrombosis. In one recent prospective study 58% of patients admitted to a regional trauma unit developed DVT [180] and a prospective randomized trial has demonstrated that LMW heparin is more effective than unfractionated heparin in preventing DVT in multiple trauma patients. [181]

Recommendations for prophylaxis in patients at low, moderate, or high risk are given in Table 150-6 . Early ambulation and leg exercises are recommended for patients in low-risk groups. If low-risk patients have additional factors predisposing to thrombosis, such as congestive heart failure, history of venous thromboembolic disease, severe obesity, or congenital hypercoagulable states, then low-dose heparin is indicated. Patients over age 40 having abdominal or thoracic surgery lasting >30 minutes represent a moderate-risk group, and the use of low-dose heparin (5,000 U twice daily) beginning 2 hours before surgery and continuing until the patient is fully ambulatory, is recommended. In patients with malignancy or additional risk factors the use of an every 8 hour regimen is preferable. External pneumatic compression is a good alternative in patients with an increased bleeding risk. Warfarin, LMW heparin, and adjusted-dose heparin are suitable in patients following hip fracture or hip replacement and LMW heparin and compression are recommended after knee replacement. Pneumatic compression or LMW heparin is appropriate in high-risk patients with surgery for gynecologic malignancy. Pneumatic compression is preferable following open prostatectomy because evidence indicates that heparin increases the risk of development of lymphoceles. [182] [183]

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THERAPY FOR THROMBOSIS

The principles of treatment for established thrombotic disease following surgery do not differ from those in other settings except there must be a careful consideration of the risk of bleeding related to the specific surgical procedure (Chaps. 124 and 125). This often results in a difficult clinical decision, balancing the risks of worsening the thrombotic disease due to suboptimal anticoagulant therapy against the risk of bleeding with more intensive anticoagulation. Consideration of the bleeding risk associated with the specific surgical procedure (Table 150-1) is important in making these decisions. For patients at highest risk, such as those who have had intracranial or ophthalmologic surgery, anticoagulant therapy may not be appropriate until 57 days after surgery, and fibrinolytic therapy should be delayed for even longer. By contrast, after minor external procedures patients at low risk for bleeding can be treated aggressively with anticoagulants 12 days after surgery with close observation of the surgical site. Patients at moderate bleeding risk represent a difficult group because of the potentially serious consequences of bleeding at the operative site and into the thoracic cavity, abdomen, or retroperitoneum. Recent trauma or surgery is a significant predictor of bleeding complications during heparin therapy[184] [185] and even prophylactic low-dose heparin increases the risk of wound hematomas. [58] Therapeutic anticoagulation with heparin in the first week following total hip replacement may result in bleeding complications in up to 50% of patients. [186] Careful control of anticoagulant intensity will reduce bleeding risks as indicated by reports of successful abdominal or thoracic surgery [187] [188] or orthopedic surgery[153] [157] without undue bleeding in patients receiving oral anticoagulation with INRs of 22.5.

Both LMW heparin and unfractionated heparin are available for parenteral treatment of thrombosis (Chap. 122). LMW heparin offers advantages of subcutaneous administration and a longer half-life. In the postoperative period, however, unfractionated intravenous heparin may be preferred for treatment of thrombosis because its anticoagulant effect disappears more rapidly after the infusion is stopped if bleeding occurs. Each case must be individually considered, but experience suggests that heparin may be given in the early postoperative period by continuous infusion to keep the PTT in the low therapeutic range with close observation for evidence of bleeding. For fibrinolytic therapy, the risks of bleeding are higher. This is related primarily to the potential for dissolution of fibrin in hemostatic plugs at the surgical site. Clinical trials of fibrinolytic therapy have shown that most bleeding complications are related to prior invasive procedures such as arterial punctures [66] [189] and fibrinolytic therapy for venous thromboembolic disease should be delayed 710 days following surgery because heparin anticoagulation is an acceptable alternative treatment. For patients with acute myocardial infarction, however, the decision is more difficult because of the greater benefits of early treatment with fibrinolytic therapy. This must be carefully considered in the risk/benefit analysis in treating patients who develop myocardial infarction in the postoperative period.

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Chapter 151 - Hematologic Manifestations of Childhood Illness

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INTRODUCTION

The hematologic response to systemic illness in children is similar to that in adults. However, a number of disorders occur more frequently in children and some are unique to the pediatric population. In addition, interpretation of the hematologic response is predicated on knowledge of the normal developmental changes that occur within the hematopoietic system throughout childhood (Table 151-1). This chapter focuses on the hematologic manifestations of common or unique systemic diseases that occur in the neonate, child, or adolescent. Illnesses that often require hematologic consultation are emphasized. Systemic diseases that produce hematologic abnormalities that

TABLE 151-1 -- Normal Hematologic Values in Childhood

Age	Red Blood Cells						White Blood Cells				Coagulation			
	Hb (g/dl)		Hct (%)		MCV (fl)		Total		Neutrophils (%)	Lymphocytes (%)	PT (sec)[a]		PTT (sec)[a]	
	Mean	(range)	Mean	(range)	Mean	(range)	Mean	(range)	Mean	Mean	Mean	(range)	Mean	(range)
Birth (term)	18.5	(14.522.5)	56	(4569)	108	(95121)	18.1	(9.030.0)	61	31	16	(1320)	55	(4565)
2 mo	11.2	(9.414.0)	35	(2842)	96	(77115)								
6 mo2 yr	12.5	(11.014.0)	37	(3341)	77	(7084)	11.3	(6.017.5)	32	61				
26 yr	12.5	(11.513.5)	37	(3440)	81	(7587)	8.5	(5.015.5)	42	50				
612 yr	13.5	(11.515.5)	40	(3545)	86	(7795)	8.1	(4.513.5)	53	39				
1218 yr							7.8	(4.513.5)	57	35				
Male	14.5	(13.016.0)	43	(3749)	88	(7898)								
Female	14.0	(12.016.0)	41	(3646)	90	(78102)								

Abbreviations: Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; PT, prothrombin time; PTT, partial thromboplastin time.

Data from Rudolph and Hoffman[350] and Nathan and Oski. [2]

[a] The normal range for the PT and PTT varies among laboratories. The time at which normal adult values are attained is 1 week for the PT and 29 months for the PTT. The platelet count is within the adult range from birth.

are similar in adults and children are discussed in other chapters. For a comprehensive review of the subject, the reader is referred to a published textbook. [1]

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INFECTIOUS DISEASE

Infection, especially viral infection, is the most common problem encountered by the pediatrician. Although most infections do not produce significant hematologic sequelae, all classes of microorganisms have been implicated in the pathogenesis of hematologic abnormalities that range from mild and clinically irrelevant to severe and life-threatening. In this section, we discuss the changes seen in red blood cells (RBCs), white blood cells (WBCs), platelets, or the coagulation system that are routinely encountered, are associated with a specific infection, or have a potentially serious clinical impact.

RBC

The anemia of chronic inflammation or infection in children is similar to that seen in adults in terms of both clinical and hematologic findings and pathogenesis. [2] However, anemia with acute infections occurs more commonly in children than in adults.

Anemia of Acute Infections

A mild to moderate anemia of uncertain etiology may occur in the setting of both acute viral infections or more serious bacterial infections. In a study of children with mild viral or bacterial infections in the outpatient setting, anemia was documented in 5% of children 412 years, 17% of children 6 months to 4 years, and 33% of infants 611 months of age.[3] In 14 of 15 young children the anemia resolved within 34 weeks. However, multiple mild infections may predispose the infant to develop a more chronic, mild anemia or low normal hemoglobin that may be due to iron deficiency, thus warranting a trial of iron. [4] In children hospitalized with moderately severe inflammatory processes, the incidence of mild anemia (hemoglobin 10.111.0 g/dl) is as high as 78%. [5] Specific acute bacterial infections associated with a high incidence of anemia (4474%) include bone and joint infection, [6] typhoid fever,[7] brucellosis,[8] and invasive*Hemophilus influenzae* infections.

The anemia associated with*H. influenzae* meningitis has been the most thoroughly studied of the anemias of acute infection. The majority of children with *H. influenzae* meningitis have mild anemia on admission with hemoglobin in the 911 g/dl range, and up to 90% become anemic during the course of the illness. [9] [10] This is in contrast to meningitis secondary to*Streptococcus pneumoniae* or*Neisseria meningitidis*, in which anemia is uncommon. The pathophysiology of the anemia of*H. influenzae* disease appears to be multifactorial. Shurin et al. [11] have shown that*H. influenzae* capsular polysaccharide, polyribosyl ribitol phosphate, binds to erythrocytes and in the presence of antibody and complement can result in intravascular and extravascular hemolysis. They further hypothesize that polyribosyl ribitol phosphate alone may induce more rapid clearance of RBC, perhaps on the basis of decreased RBC deformability. In addition, hypoferremia may limit bone marrow response to hemolysis.

Acute Hemolytic Anemia

Acute hemolysis has been observed with infections from all classes of microorganisms but is relatively uncommon. [12] [13] The anemia may be mild to severe and occurs in two clinical settings: (1) the child who presents predominantly with symptoms and signs of infection and is found to have anemia and (2) the child who presents with the manifestations of acute hemolytic anemia.

The mechanism of hemolysis in patients presenting with an infectious disorder depends on the infecting organism, but in most cases hemolysis is extravascular. [14] Reported mechanisms include:

1. Release of hemolysins (*Clostridium perfringens* sepsis)
2. Invasion of the RBC (malaria)
3. Alteration of RBC surface:
   a. Direct adherence by the organism (*Bartonella*)
   b. Alterations of antigenic phenotype by neuraminidase (influenza)
   c. Cold agglutinin (mycoplasma,*Listeria*, Epstein-Barr virus [EBV])
   d. Absorption of capsular polysaccharide (*H. influenzae*)
4. Mechanical mechanisms (microangiopathy associated with disseminated intravascular coagulation [DIC] or hemolytic-uremic syndrome)
5. Oxidation damage in individuals with congenital enzyme deficiencies (hepatitis and glucose-6-phosphate dehydrogenase [G6PD] deficiency, [15] *Campylobacter jejuni* in the neonate).[16]

Acute, infection-associated hemolytic anemia in one study lagged behind the clinical infection by 37 days. [17] Most children were shown to have adsorption of microbial antigens to the RBC surface suggesting an innocent bystander mechanism of erythrocyte sensitization, ultimately leading to hemolysis. The minority in this series had classic autoantibody-mediated hemolytic anemia.

Autoimmune hemolytic anemia in children is usually transient, is not associated with underlying systemic disease, and has a low mortality rate. [18] Children frequently have a history of concurrent or recently resolved infection, especially viral upper respiratory infection. In the typical acute, transient cases, 5968% of children have such a history, whereas 020% of those with the less common chronic course have a history of infection.

Aplastic Crisis

Temporary arrest of RBC production has been observed in children with infections, [19] but anemia is uncommon because of the long RBC life span. However, in two situations severe anemia has been linked with infection and cessation of erythropoiesis: (1) B19 parvovirus infection in patients with an underlying hemolytic anemia and (2) transient erythroblastopenia of childhood.

The B19 parvovirus has been a known pathogen in animals for years but has only recently been linked with human disease. [20] It is the etiologic agent of fifth disease (erythema infectiosum), a mild illness with a characteristic slapped cheek facial erythema and a generalized reticular rash. In normal volunteers infected with B19 parvovirus a mild, transient, and clinically irrelevant drop in the hemoglobin and reticulocyte count was observed. [22] In normal children this infection is not usually associated with hematologic abnormalities, although reports of both hematologic and nonhematologic effects are increasing. [21] [23] However, in children with sickle cell disease, spherocytosis, and other hemolytic anemias, B19 parvovirus infection can produce a severe anemia associated with peripheral reticulocytopenia and marrow erythroblastopenia, the aplastic crisis. Recovery within 12 weeks is the rule, but transfusion may be necessary. B19 parvovirus infection has also been associated

with prolonged anemia and reticulocytopenia in children with acute lymphoblastic leukemia in remission, [24] [25] children with solid tumors receiving chemotherapy, [26] immunodeficiency,[27] postrenal transplant,[28] autoimmune hemolytic anemia,[29] [30] and as the initial manifestation of human immunodeficiency virus infection. [31] Human parvovirus has also been identified as a cause of non-immune hydrops fetalis. [32]

Transient erythroblastopenia in childhood (TEC) is a syndrome characterized by temporary arrest of RBC production with moderate to severe anemia in previously normal infants and toddlers. Although no specific infectious agent has been proven to cause TEC, the frequency of a history of infection within 13 months, the seasonal clustering, and the similarity to childhood idiopathic thrombocytopenic purpura (ITP) all suggest a possible viral etiology. B19 parvovirus has not been associated with TEC.[33]

WBC

Children, as a rule, have the expected leukocyte response to infection. It should be remembered that infants and young children normally have a lymphocyte predominence (Table 151-1) and any leukocyte response to infection must be judged on the basis of age-related normal values.

The predictive value of the peripheral WBC and differential counts in suspected bacterial infections has been extensively evaluated in infants and children. [34] Todd has shown in hospitalized children that a neutrophil count >10,000/mm ^{3} or bands >500/mm^3 is associated with an 80% chance of having a bacterial infection. [35] In children undergoing evaluation for possible meningitis, Lembo et al. [36] found that a ratio of immature-to-total neutrophils of >0.12 was more strongly associated with, and more sensitive for, bacterial meningitis than were the total WBC or the total band count. Febrile children between the ages of 3 and 48 months are at increased risk for bacteremia, especially with *S. pneumoniae*. McCarthy et al.[37] demonstrated a threefold increase in risk of bacteremia in febrile (>40°C) children, <2 years old, who had a WBC count of 15,000/mm^3 . In this setting, the WBC count was a more sensitive indicator of the presence of pneumonia or bacteremia than was the absolute neutrophil or band count.

There are recognized exceptions to the anticipated leukocyte response to infection that may serve as a clue to the diagnosis. In typhoid fever and brucellosis, leukopenia and neutropenia are prominent early in the illness. Shigellosis is associated with a variable leukocyte count, but it is often normal, with a greater percentage of bands than neutrophils. Illnesses associated with lymphocytosis include pertussis (whooping cough), infectious lymphocytosis, infectious mononucleosis, and other viral infections. Neutropenia can be seen in bacterial sepsis from meningococcus, pneumococcus, staphylococcus, and other bacteriae and is associated with a poor prognosis. Black children (and adults) normally have lower WBC and neutrophil counts than whites and have less of a leukocytosis and neutrophilic response to serious infection. [38]

Neutropenia

The most common cause of neutropenia (neutrophil count <1,500/mm^3) in children is viral infection. A number of specific viruses are associated with neutropenia, including hepatitis, roseola, rubella, mumps, adenovirus, coxsackie A21, EBV, and influenza. [39] However, the most common clinical setting is the incidental discovery of neutropenia in the child with a nonspecific viral syndrome. Usually the neutropenia in this situation continues for <30 days and is rarely associated with infectious complications.[40] Neutropenia has also been associated with a number of bacterial, rickettsial, and fungal infections. [41]

Eosinophilia

The most common cause of eosinophilia worldwide is parasitic infection. In the United States, visceral larva migrans is the most common cause of exaggerated eosinophilia (WBC 30,000100,000/mm^3 with 5090% mature eosinophilis) in children. [42] Mild to moderate eosinophilia (400/mm^3) is most often seen in allergic children but is also characteristic of *Chlamydia* pneumonitis in infants.

Platelets/Coagulation

Thrombocytosis

Thrombocytosis (>500,000/mm^3) is known as an acute phase reaction to infection, but it has been infrequently identified in children in the past. [43] There is a particularly high incidence of thrombocytosis in patients with bacterial infections, especially pneumonia with empyema [44] and *H. influenzae* meningitis.[45] Inflammatory cytokines, such as interleukin (IL)-1, may play an etiologic role in the reactive thrombocytosis of infection. [46] Thrombocytosis may be more common in simple acute infections than previously recognized. Heath and Pearson documented a 13% incidence of thrombocytosis in ambulatory patients; children with an increased platelet count were more likely to have a diagnosis of infection. [47] There were no apparent sequelae to the thrombocytosis and antiplatelet therapy is not indicated. Although the most common cause of thrombocytosis in children is infection, [48] the differential diagnosis of an elevated

platelet count is extensive [49] and rarely includes underlying childhood malignancy. [50]

Thrombocytopenia

Thrombocytopenia can be seen in patients suffering from infections with all types of organisms. Common viral agents include varicella, EBV, rubella, mumps, measles (wild or vaccine strains), and cytomegalovirus (CMV). The primary mechanism of the thrombocytopenia is immune destruction, although a direct viral effect on the platelet or megakaryocyte has been demonstrated. Because childhood ITP is felt to be secondary to infection in most instances, the definitions of thrombocytopenia with infection and childhood ITP tend to merge. Children with thrombocytopenia from infection usually have a transient course, although instances of chronic thrombocytopenia from specific viral infections (e.g., varicella) have been documented. [51]

Thrombocytopenia is also associated with bacterial sepsis. The low platelet count may be an isolated finding or associated with DIC. Corrigan documented a 61% incidence of thrombocytopenia in 45 children with sepsis. [52] The degree of thrombocytopenia was mild to moderate (64% with >50,000/mm), but ranged as low as 8,000/mm^3 . There was no evidence of DIC in 39% of those with low platelet counts. Thrombocytopenia in the setting of bacterial sepsis is also probably mediated by an immune mechanism with elevated platelet-associated IgG. [53]

Petechial bleeding without thrombocytopenia can be seen in both bacterial and viral disease, especially with the meningococcus, streptococcus, and echoviruses. The explanation for the petechial rash in these infections is either vasculitis or platelet dysfunction.

DIC/Purpura Fulminans

Disseminated intravascular coagulation is uncommon after childhood infections and if present is usually accompanied by shock and at least a 50% mortality rate. The most common organisms producing DIC are bacterial, especially the gram-negative bacteria (meningococcus, *H. influenzae, Aerobacter*, and others) but also gram-positive organisms (*Staphylococcus aureus*, group B streptococcus, and *S. pneumoniae*, particularly in the asplenic host). DIC is also associated with disseminated viral (varicella, measles, rubella), rickettsial (Rocky Mountain spotted fever), fungal, mycoplasma, and parasitic infections.

Purpura fulminans is a rare syndrome, seen in extremely ill children with DIC. Purpura fulminans is characterized by the rapid progression of ecchymotic skin lesions, especially of the extremities, that may progress to gangrene and ultimately result in amputation. [54] This syndrome has been described as a postinfectious purpura with scarlet fever, upper respiratory tract infection, and varicella as the most common proceding illnesses with a latent period of 090 days following infection. [55] A similar clinical picture can be seen in children with DIC and acute bacterial sepsis, especially meningococcemia.

There is increasing evidence that DIC associated with purpura fulminans is associated with deficiency of the naturally occurring anticoagulants. Children with postvaricella purpura fulminans have been shown to have acquired protein S deficiency, antiprotein S antibody, and presence of a lupus anticoagulant. [56] Proteins C and S have been shown to be low in children with infectious purpura [57] and the severity of protein C deficiency has been associated with morbidity and mortality. [58]

The frequency of an inherited thrombophilia in children who develop purpura fulminans is unknown, although the association has been reported. [59]

Treatment of purpura fulminans consists of antibiotics for suspected bacterial infection, volume replacement for shock, and heparin. Although there is controversy regarding the routine use of heparin in DIC, its use in purpura fulminans has been associated with an improved outcome when started early in the course of the disease and continued for 23 weeks. [60] Theoretically to improve the efficacy of heparin, it is reasonable to infuse fresh frozen plasma or antithrombin III (AT III) concentrates[61] if the AT III level is low. There is anecdotal evidence that infusion of AT III concentrates without heparin or protein C concentrates may decrease morbidity and mortality.[62] [63] In addition, recombinant tissue plasminogen activator has been used in an attempt to restore organ perfusion by dissolution of diffuse microvascular thrombosis with apparent success.[64] Other treatments, such as regional sympathetic blockade, topical nitroglycerin, and local infusion of tissue plasminogen activator have been used to improve regional blood flow to the affected part. [65] [66] [67] The mortality of postinfectious purpura fulminans has declined from 90% in the past to 18%,[34] although patients with acute bacterial sepsis with purpura fulminans continue to have a high mortality rate.

Coagulation Inhibitors

Acquired inhibitors of coagulation in children with infection are uncommon, transient, and usually mild, [68] [69] but may be associated with severe bleeding.[70] They are usually detected after a viral illness, during penicillin therapy, [71] or incidentally. Both specific inhibitors of coagulation factors (especially factors VIII and IX) and lupus anticoagulants have been demonstrated. Significant bleeding is usually seen only in those children with specific factor inhibitors, although hemorrhage has also been described with lupus anticoagulants.[72] Treatment of symptomatic patients with prednisone has been associated with improvement in bleeding manifestations. Complete resolution without recurrence is the most common event.

Pancytopenia

Pancytopenia in a child should alert the clinician to disorders such as leukemia, aplastic anemia, or disseminated neuroblastoma. Infectious causes of pancytopenia are uncommon, and disseminated disease is most often present. Organisms implicated in patients with pancytopenia include *Mycobacterium tuberculosis*, atypical*Mycobacteria, Histoplasma capsulatum, Salmonella typhi, Mucor* species,*Brucella* species,*Fusobacterium necrophorum*, and*Erlichia canis*.[41] [73] Virus-associated or reactive hemophagocytic syndrome is an additional, though rare, cause of pancytopenia. [74] Children with human immunodeficiency virus (HIV) and concomitant infection with*mycobacterium avium intracellulare* or parvovirus B19 have been reported to have pancytopenia. [75] [76]

HIV Infection in Children/Adolescents

Infection with HIV is more common in adults but is now recognized as a leading cause of immunodeficiency in infants and children. [77] Acquisition of HIV in the majority of infected children (most of whom are <2 years old) is by vertical transmission from an infected mother to her infant. In children <13 years old with acquired immunodeficiency syndrome (AIDS), 80% have a parent with AIDS or AIDS-related complex (ARC), 13% have a history of blood transfusion, and 5% have hemophilia or another coagulation disorder.[78] Other adult routes of infection (homosexuality, intravenous needle use) are possible, especially in the adolescent and the sexually abused child.

The definition of AIDS in childhood differs from that for adults in two ways. [79] Multiple, serious bacterial infections and lymphocytic interstitial pneumonitis/pulmonary lymphoid hyperplasia are accepted as indicative of AIDS among children and not adults. In children <15 months old, because of the possibility of passively acquired maternal antibody, diagnosis of HIV infection is difficult and laboratory criteria are more stringent.

The Centers for Disease Control and Prevention (CDC) has published a revised classification of HIV infection in children <13 years of age based on infection status, clinical status, and immunologic status. [80]

The majority of children with AIDS present with oral thrush, chronic interstitial pneumonitis, hepatosplenomegaly, lymphadenopathy, or failure to thrive. Other clinical characteristics distinguishing pediatric HIV infection include a shorter incubation period, more pronounced hypergammaglobulinemia, more serious bacterial and CMV disease, and the rare occurrence of Kaposi sarcoma or other malignancies. However, as in adults, there is a broad spectrum of disease manifestations, with all organ systems potentially affected and progressive immune and clinical deterioration.

The hematologic manifestations of AIDS in children are similar to those in adults (Chap. 154) and depend on the state of the HIV infection and the presence of coexistent disease.[81] Anemia is by far the most common finding (>90%). Severe anemia (hematocrit <25%) in one study correlated with development of an opportunistic infection and death within 7 months. [82] Leukopenia and neutropenia are commonly seen in HIV-infected children (47% and 41%, respectively) with severe neutropenia associated with opportunistic infections. Immune neutropenia as well as circulating anticoagulants have been described. [83] [84] Lymphopenia is progressive but until late in the course less prominent in children than adults. Thrombocytopenia is present in 1330% of pediatric AIDS patients and can be associated with clinically significant and even fatal hemorrage. The mechanism of the thrombocytopenia in most cases is immune destruction with a high percentage of patients having antiplatelet antibodies or immune complexes, [85] although amegakaryocytic thrombocytopenia has been reported. [86] Variable therapeutic responses to both corticosteroids and intravenous IgG have been demonstrated; some children will have spontaneous remissions. [85] [87] The evaluation and treatment of hematologic abnormalities in HIV-infected children have been reviewed by Hilgartner. [88] Consensus guidelines for the diagnosis and overall management of children with HIV infection have also been published. [89]

Isolated thrombocytopenia as a presenting manifestation of HIV infection has been reported in a number of children, usually infants. [90] There have been no associated clinical stigmata of AIDS or ARC, and patients have been responsive to standard treatment (intravenous IgG or prednisone) often with sustained remissions. In a few patients with prolonged follow-up, no further manifestations of HIV infection were seen. Although it has been suggested that HIV testing may be indicated in all children with ITP, it seems most reasonable to check the HIV status of those with risk factors for AIDS and those outside the typical age group for ITP, especially infants. [91]

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COLLAGEN VASCULAR DISEASE/ACUTE VASCULITIS

Juvenile Rheumatoid Arthritis

Juvenile rheumatoid arthritis (JRA) includes a group of disorders with a varied clinical presentation, course, and outcome. [92] Systemic JRA, which occurs in 30% of patients, is a multisystem disease characterized by fever, rash, polyarticular (often destructive) arthritis, hepatosplenomegaly, and lymphadenopathy. These patients commonly demonstrate hematologic abnormalities that are proportional to disease activity. In the polyarticular presentation, more than four joints are involved but the systemic findings are absent. This group, which makes up 25% of patients, may also exhibit hematologic findings. Pauciarticular JRA is characterized by involvement of fewer than four joints and is rarely associated with hematologic abnormalities.

The incidence of anemia is 5060% in patients with systemic or polyarticular JRA and 10% in pauciarticular arthritis. The anemia usually correlates well with disease activity, worsening during acute flare-ups, but there is no relationship to the duration of illness. [93] The RBC may be normochromic/normocytic or microcytic/hypochromic. The reticulocyte count is usually low. [94] Iron studies often show low serum iron, increased free erythrocyte protoporphyrin, low-normal or elevated total iron-binding capacity, and normal or low serum ferritin. Serum erythropoietin levels are usually mildly elevated (but not as high as in iron deficiency). [95] The bone marrow does not show erythroid hyperplasia in response to the anemia and has diminished (but not absent) iron stores. [96] The etiology of the anemia may be anemia of chronic disease, iron deficiency anemia, or both.

Although it is difficult to differentiate the anemia of chronic disease from iron deficiency, studies in patients with systemic-onset chronic JRA suggest defective iron supply as the primary cause.[97] Transferrin receptor levels are inversely related to hemoglobin levels in this population. Serum transferrin receptor levels have previously been shown to differentiate the anemia of chronic disease from iron deficiency. [98] Oral iron has been effective in raising the hemoglobin in anemic patients with JRA[94] and intravenous iron has been effective in raising the hemoglobin level in children unresponsive to oral iron. [97] [99] Excessive production of IL-6 has been documented in patients with JRA and may provide an explanation for the abnormalities in iron metabolism. IL-6 may enhance ferritin synthesis and increase hepatic uptake of serum iron. Increased ferritin results in reticuloendothelial iron blockage and diminished iron absorption. [97]

Less common causes of anemia in JRA include erythroid aplasia,[100] suppression of erythropoiesis by circulating inhibitors, [101] hemolysis, and a macrocytic anemia probably related to increased folate clearance and low plasma and RBC folate levels. [102] [103]

In systemic JRA, leukocytosis with mean WBC counts up to 30,000/mm^3 and neutrophilia with a left shift occur in 90% of patients, especially those with active disease. Leukocytosis is less common in polyarticular arthritis and usually absent in pauciarticular disease. [104] Leukocytosis is so prevalent in systemic JRA that the presence of neutropenia should alert the clinician to question the diagnosis and ensure that other possibilities such as systemic lupus erythematosus (SLE) and acute lymphocytic leukemia (ALL) are not overlooked.[105] [106] ALL in children may present with fever, joint pain, hepatosplenomegaly, and isolated cytopenias. Because about 4% of children with ALL are misdiagnosed as having JRA, [107] it is important to perform a bone marrow aspirate to rule out leukemia in any patient thought to have systemic JRA prior to starting corticosteroid therapy.

Nonetheless, neutropenia has been reported in several patients having JRA. [108] Other causes of neutropenia are bone marrow suppression due to therapy with gold or nonsteroidal anti-inflammatory drugs [109] and, in adults, Felty syndrome, the triad of rheumatoid arthritis, splenomegaly, and neutropenia. [110] Studies of neutrophil function have demonstrated mild defects in chemotaxis and phagocytosis. [111] [112] About 50% of patients with systemic JRA have >5% eosinophils on the peripheral smear.[113] Basophilia[114] and plasmacytoid lymphocytes[115] have also been reported. Studies of cellular immunity are conflicting, demonstrating normal or increased T- and B-lymphocyte number, increased immunoglobulin synthesis and secretion, and either impaired or normal delayed hypersensitivity and lymphoproliferative response to mitogens.[116] [117] [118] [119] Monocyte dysfunction with decreased Fc receptor expression, decreased nitroblue tetrazolium activity, and decreased complement-mediated phagocytosis has been described.[120]

The platelet count is elevated in 50% of patients with systemic JRA. [104] IL-6, a cytokine that stimulates thrombopoiesis, is

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elevated in patients with active systemic JRA and increased levels of IL-6 are correlated with elevated platelet counts. [121] Thrombocytopenia may result from bone marrow suppression by gold therapy, [109] [122] the rare consumptive coagulopathy, [123] or platelet trapping in Felty syndrome. [93] Because thrombocytopenia is uncommon in JRA, an unexplained low platelet count should lead one to consider alternative diagnoses, such as SLE or ALL. [124] On the other hand, isolated thrombocytopenia may be the only presenting sign in a child who later develops JRA or another collagen vascular disease. Therefore, one should consider JRA and SLE in the differential diagnosis of ITP in the child who is older than 9 years and female. Appropriate screening tests for autoantibodies (e.g., antinuclear antibody, direct Coombs test) should be performed at diagnosis and periodically if new symptoms develop. [125] [126]

Disseminated intravascular coagulation may occur in children with systemic JRA after hepatic damage, from aspirin or gold therapy, or during disease flare-ups treated with nonsteroidal anti-inflammatory drugs when serum albumin is low. [127] These patients are often very ill and may require corticosteroid therapy as well as platelet and coagulation factor replacement to control the coagulopathy. [123] [128] The incidence of coagulation abnormalities in nonbleeding patients with systemic JRA is controversial. One study demonstrated prolonged prothrombin time (PT), partial thromboplastin time (PTT), and elevated fibrinogen, factor VIII, and fibrinopeptide A levels in up to 50% of these patients; [129] other reports have not confirmed such findings. [128] In addition, decreased fibrinolytic activity and increased plasminogen activator inhibitor are found in patients with active JRA, especially those with the systemic form. [130] Antibodies against factor VIII [131] and the lupus anticoagulant [132] are occasionally seen in children with JRA.

Kawasaki Syndrome

Kawasaki syndrome is an acute multisystem disorder characterized by abrupt onset of fever unresponsive to antibiotics; bilateral conjunctival injection, reddening of the lips, tongue, or oral mucosa, reddening, induration, or peeling of the hands or feet; polymorphous truncal rash; and cervical lymphadenopathy. This disorder occurs most commonly in children under the age of 2 years and has many features of a severe vasculitis; the most serious complication is development of coronary artery aneurysms, which occurs in 20% of children and is responsible for the 3% mortality rate associated with coronary artery thrombosis or rupture. The etiology of Kawasaki disease is unknown. The immunologic and clinical characteristics of this disorder are similar to diseases associated with superantigen production, of which toxic shock syndrome is a classic example.[133] [134]

Children with Kawasaki syndrome may have a mild normochromic, normocytic anemia with reticulocytopenia. [135] Rare patients with autoimmune hemolytic anemia

have been reported.[136] Leukocytosis is almost universal, with mean neutrophil counts of 21,000/mm^3 . Ninety-five percent of patients have neutrophilia, with a left shift persisting up to 3 weeks.[137] The finding of vacuoles and toxic granulation in neutrophils is a helpful adjunct in the diagnosis of Kawasaki disease. [138] The role of activated neutrophils and monocytes in aneurysm development is unclear because both impaired and enhanced phagocyte function are described. [139] [140] Studies of cellular immunity show normal total T-cell numbers but decreased suppressor T cells, causing relatively elevated T-helper cell levels during the first 4 weeks of disease.[141] The change of T-cell subsets plus B-lymphocyte stimulation may contribute to the exaggerated production of all major immunoglobulin classes during the first 8 weeks of the disease.[142] Circulating immune complexes and high C3 (but not C4) levels are found during weeks 13. During the acute phase increased levels of the cytokines IL-1, IL-6, IL-8, interferon-, and tumor necrosis factor are noted in the circulation and IL-1, IL-2, interferon-, and tumor necrosis factor in blood vessels and skin biopsies.[133] Impressive thrombocytosis occurs in 85% of patients by the second week, peaking during the third. [134] Platelet counts of up to 2 million are not uncommon and the mean platelet count is 700,000/mm^3 .[139] [143] However, 2% of patients may have thrombocytopenia caused by a consumptive coagulopathy. [144] Platelets demonstrate hyperaggregation on exposure to adenosine diphosphate, epinephrine, and collagen in vitro. These abnormalities may persist for as long as 9 months after diagnosis.[145] During the first month, levels of factor VIII, fibrinogen, thromboxane B_2 , and thromboglobulin are increased. AT III and fibrinolysis activity are decreased. The PT, PTT, and thrombin time are usually normal.[146]

Prevention and treatment of existing coronary aneurysms are the primary therapeutic goal. Aspirin suppresses platelet aggregation but does not affect aneurysm formation. Combining aspirin with high-dose intravenous immunoglobulin infusions reduces aneurysm formation, decreases fever, and normalizes laboratory signs of inflammation.[147] [148] Corticosteroid therapy increases aneurysm formation and should be avoided. [149] Guidelines for the treatment and long-term management of Kawasaki syndrome have been published.[150] [151]

Henoch-Schönlein Purpura

Henoch-Schönlein purpura (HSP) (anaphylactoid purpura) is a systemic vasculitis characterized by unique purpuric skin lesions, transient arthralgias or arthritis, especially affecting the knees and ankles, colicky abdominal pain, and nephritis. [152] [153] [154] Recognition of HSP is important, not so much for its hematologic abnormalities (which are rare) as for the unusual nonthrombocytopenic purpuric lesions, which are frequently confused with the hemorrhagic rash of ITP.

This vasculitis occurs most commonly in children 37 years old, often 13 weeks after an upper respiratory illness. The presenting complaint in 50% of children is a characteristic rash, which may begin as urticaria. As these eruptions fade, they are replaced by brownish red maculopapular lesions and petechiae. The petechiae coalesce, forming areas of raised or palpable purpura on the buttocks, legs, and extensor surfaces of the arms, with a symmetric distribution. The rash may fade and can recur for months, especially with increased activity. Children <3 years of age often have painful soft tissue swellings of the scalp and face (especially periorbital areas) and dorsum of hands and feet.[152] Infantile acute hemorrhagic edema is an acute vasculitis in infants <2 years, which may be a benign form of HSP. [155]

Sixty-seven percent of patients experience colicky abdominal pain, often associated with vomiting, hematemesis, or melena from submucosal hemorrhage and edema of the small bowel wall. With severe edema, bowel wall may become a leading point for intussception. [152] [156]

Renal involvement occurs in 50% of patients, especially boys and older children. Hematuria, either microscopic or gross, may occur with proteinuria during the first 3 weeks of the illness. With progressive involvement, hypertension, impaired renal function, and, finally, renal failure in 15% of children may occur with an associated mortality rate of 3%.[152] [157]

Anemia occasionally develops as a result of gastrointestinal tract blood loss or decreased RBC production caused by renal failure. The leukocyte count is normal. Despite the impressive purpura, the platelet count is normal or increased with normal platelet function. [152] [158] Coagulation factor levels are usually normal, though transient decreases in factor XIII activity[159] and vitamin K deficiency from severe vasculitis-induced intestinal malabsorption [160] have been reported. Bleeding in the gastrointestinal tract, the lungs, or rarely, the central nervous system,

is due to a necrotizing vasculitis and not a hemostatic defect. [156] [158]

Henoch-Schönlein purpura is considered an IgA-mediated inflammation of small vessels. Biopsy of skin or other involved tissue reveals a leukocytoclastic vasculitis. Immune complexes of IgA with complement, IgG, or IgM have been found circulating in the serum[161] and deposited in blood vessel walls of kidney, and in intestinal and skin lesions.[162] The mechanism of production, accumulation, and deposition of IgA immune complexes in the blood vessel is unclear. [163] It has been suggested that HSP may be a systemic form of IgA nephropathy.[164] Both disorders have identical renal biopsy findings and are characterized by mesangial proliferation, occasional focal sclerosis, and crescent formation. [165] [166]

Treatment is mainly supportive, although corticosteroids have been used to provide symptomatic relief with severe joint or abdominal complaints. [167] They do not alter skin or renal involvement. The prognosis is good for full recovery, except in the children with renal failure.

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CARDIOPULMONARY DISEASE

Congenital Heart Disease

Congenital heart disease occurs in about 1% of live births. [168] Structural heart malformation usually follows predictable patterns such that six defects account for 70% of all cardiac disorders: ventricular septal defect, atrial septal defect, tetralogy of Fallot, patent ductus arteriosus, pulmonary stenosis, or aortic stenosis. Children with cardiac abnormalities may be acyanotic or cyanotic, depending on the underlying lesion. Hematologic abnormalities occur most often in children with cyanotic congenital heart disease (CCHD).

Polycythemia, or an increased RBC mass for age, is the bone marrow response to chronic hypoxemia in patients with CCHD. The decreased arterial oxygen saturation stimulates erythropoietin production, which in turn increases erythropoiesis. The resultant increased RBC mass increases the oxygen-carrying capacity of the blood, resulting in improved tissue oxygenation. [169] With adequate compensation, erythropoietin levels fall to normal while higher RBC production is maintained.[170] A second compensatory mechanism is an increase in 2,3-diphosphoglycerate (2,3-DPG) levels in the RBC when the arterial oxygen tension is <70 mm Hg. The higher 2,3-DPG level causes a right shift of the oxyhemoglobin curve, resulting in greater oxygen release to the tissues.

Polycythemia in the cyanotic child is beneficial up to a point. Because the relationship between the hematocrit and blood viscosity is a hyperbolic curve, minor increases in the hematocrit above 70% cause marked increases in blood viscosity. [171] This higher viscosity results in impaired blood flow through the smaller veins with ultimately less tissue oxygen delivery. This impairment is magnified in severe polycythemia (hematocrit >75%) such that headache, irritability, dyspnea, and even pulmonary, renal, or central nervous system thrombi may occur. [172] To prevent these complications, the hematocrit should be maintained around 60% through the use of exchange transfusions. Small aliquots of the patients blood are slowly removed and replaced by equal volumes of plasma or 5% albumin. [187] Care should be taken to remove blood slowly as vascular collapse, cyanosis, stroke, and seizures have been reported with too rapid an exchange. [173] Apheresis (erythrocytopheresis) has also been shown to be an effective means of decreasing viscosity in polycythemic patients. [174]

Infants with CCHD are at risk of developing iron deficiency anemia. This may result from the combination of poor iron stores at birth (especially in premature infants), increased iron needed for enhanced erythropoiesis, poor iron intake due to poor feeding, and ongoing iron losses as a consequence of phlebotomy or exhange transfusion. These children may exhibit symptoms of iron deficiency (irritability, anorexia, poor weight gain) or worsening cyanosis. The hemoglobin may be normal for age but inappropriately low for the degree of hypoxemia. Low RBC indices and hypochromic microcytic RBC are better indices of iron deficiency in this setting. [175] Polycythemic children with iron deficiency anemia are at increased risk for cerebral vein thrombosis because of the poor deformability of the iron-deficient RBC, which further increases blood viscosity. [176] [177] To prevent this complication and to allow for maximal tissue oxygenation, all infants should be fed iron-rich infant formula and receive iron replacement therapy as needed. [178]

Routine screening of patients with congenital heart disease has demonstrated coagulation abnormalities in 2059% of children with acyanotic defects and 4050% of those with cyanotic heart disease[179] (Table 151-2). Only 11% of children with CCHD have any clinical evidence of bleeding preoperatively. However, children with underlying hemostatic defects have a greater frequency and severity of postoperative bleeding. [179] [180] Presurgical testing should include at least platelet count, bleeding time, PT, and PTT. Fibrinogen, fibrin split products, thrombin time, platelet function, and clot lysis time should be assessed with any history of bleeding or any abnormality revealed on the screening tests. Children with polycythemia have contracted plasma volumes; therefore, when collecting blood samples, extra care should be taken to ensure the proper 1:9 ratio of 3.8% sodium citrate to blood to prevent artificial abnormalities in coagulation tests.

The etiology of the coagulation abnormalities in CCHD is unclear. Earlier reports suggesting a role for consumptive coagulopathy have not been confirmed. [171] [181] [182] Protein C levels in 8 of 29 term infants with CCHD were significantly lower than controls with no evidence of familial deficiency. Of these, two had thrombotic complications, and four had consumptive coagulopathy. [183] Platelets have shortened survival times (even with normal counts), and normal to increased numbers of megakaryocytes in the bone marrow are reported.[184] This increased platelet destruction does not appear to be due to DIC. Both the platelet and coagulation abnormalities are directly proportional to the degree of hypoxemia and polycythemia. [182] For example, children with oxygen saturation >60% have mean platelet counts of 315,000/mm^3 , whereas those with saturation <60% have a mean of 185,000/mm^3 .[184] The mild platelet and coagulation abnormalities are usually improved or corrected after

TABLE 151-2 -- Coagulation Abnormalities in Congenital Heart Disease

	Acyanotic	Cyanotic
	(%)	(%)
Prolonged bleeding time	11	28
Prolonged PT		20
Prolonged PTT		19
Thrombocytopenia	1240	036
Abnormal platelet aggregation	14	3870
Increased fibrinolysis	12	010
Abnormal clot retraction	10	
Low fibrinogen	16	12
Increased fibrin split products		Occasionally
Decreased factors II,V,VII,VIII,IX,X,XI,XII		Occasionally
Decreased protein C		25[183]
Decreased large multimers of von Willebrand factor	100[a]	

Adapted from Lascari,[1] with permission.

[a]Twelve patients studied; true prevalence unknown.

surgical repair of the heart defect. [179] [185] With bleeding or if surgery is not possible, the coagulopathy may be improved by correction of polycythemia to a hematocrit of 60% by using slow plasma exchange transfusion.[186] [187] [188]

Cystic Fibrosis

Cystic fibrosis (CF) is a multisystem disorder of exocrine gland dysfunction characterized by chronic pulmonary disease, pancreatic exocrine insufficiency, hepatic dysfunction, abnormal reproductive organ function, and intestinal obstruction associated with abnormally high sweat electrolyte levels. [189] It is an autosomal recessive disease with an incidence of 1/2,000 live births.

Severe hemolytic anemia due to vitamin E deficiency may be the presenting manifestation of CF. Dolan initially linked deficiency of vitamin E with severe hemolytic anemia in infants who presented with pallor, edema, hypoproteinemia, and thrombocytosis. [190] This complication can be seen as early as 6 weeks of age. [191]

Many children with CF are chronically hypoxic, yet they do not have the excepted augmented erythroid response. [192] In a study of 42 children with CF, Vichinsky et al.[193] showed that none had polycythemia and 30% (especially the boys and the older children) had a normochromic, normocytic, or hypochromic microcytic anemia with reticulocytopenia. Compared to children with congenital cyanotic heart disease, there was no appropriate increase in RBC 2,3-DPG levels, no right shift of oxyhemoglobin curve, and either a low or a normal erythropoietin level. In vitro assays showed normal erythroid progenitor cell numbers and no serum inhibitor of erythropoiesis. Up to 66% of 25 children studied had abnormalities consistent with iron deficiency, and all responded to oral or parenteral iron therapy. It appears that the etiology of anemia in CF is multifactorial. A blunted erythropoietic response to hypoxia plus iron deficiency secondary to iron malabsorption or poor dietary iron intake may each be partially responsible for the development of anemia. If iron deficiency persists despite adequate oral supplementation, one should consider the possibility of ongoing blood loss or iron malabsorption that may necessitate the use of parenteral iron replacement.

Studies of neutrophil function in children with CF are conflicting. Some reports indicate impaired chemotaxis, chemiluminesence, granule release, and superoxide production,[194] [195] [196] whereas others have demonstrated factors in patient sputum that actually enhance neutrophil and monocyte responses to stimulants. [197] [198] Although these sputum factors may improve neutrophil killing ability, they may also worsen neutrophil-mediated lung damage. Evaluation of immune function has revealed impaired lymphoproliferative responses to *Pseudomonas* and other gram-negative bacterial antigens, defective opsonization, increased levels of circulating immune complexes, and decreased numbers of T-helper cells in 30% of patients. [199] The contribution of these findings to frequent pulmonary infection is still under investigation.

Children with CF usually do not experience clinically significant bleeding due to impaired hemostasis despite the risk of liver disease and vitamin K deficiency from malabsorption. Routine coagulation tests usually yield normal results, with an occasional prolonged PTT reported. A study by Corrigan et al. [200] revealed that 60% of 24 patients had a more subtle deficiency of prothrombin activity thought to be due to vitamin K deficiency. However, Cornelissen et al., [201] using direct measurement of vitamin K and PIVKA-II, found only 1 of 24 patients to be deficient. Routine vitamin K supplementation is not recommended, although it may be indicated in individuals with severe liver disease and those with demonstrated coagulation abnormalities.

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ANOREXIA NERVOSA

Anorexia nervosa is a psychiatric disorder occurring in about 1/800 adolescent girls; it is characterized by refusal to maintain a minimal normal body weight, intense fear of being fat, body image distortion, and amenorrhea. The profound weight loss is accompanied by hypothermia, hypotension, edema, lanugo, and metabolic changes, and has a mortality rate of 518%.[202]

A mild, normochromic, normocytic anemia with reticulocytopenia occurs in 30% of patients.[203] Acanthocytes or spur cells have been reported and may be due to low serum -lipoprotein levels. The causes of the anemia are most likely decreased RBC production and relative increase in plasma volume. A few patients have had slightly decreased RBC survival. Despite low serum iron and decreased marrow iron stores in 80% of patients, iron deficiency anemia is uncommon except during recovery when iron supplementation is necessary. Serum B_{12} and folate levels are usually normal.[204]

Fifty percent of patients have leukopenia, with an absolute decrease in numbers of neutrophils, lymphocytes, and monocytes.[203] [205] The neutropenia may be quite severe. An increased incidence of infection usually does not occur, although with increasing use of central venous catheters more serious bacterial infections are being reported.[206] Studies of neutrophil compartments have shown normal bone marrow reserves despite marrow hypoplasia and a normal to slightly decreased size of the marginal pool.[204] [205] Impaired neutrophil chemotaxis, intracellular killing of staphylococcus, and decreased complement levels have been demonstrated in patients with anorexia. These are associated with occasional skin abscess formation.[203] Lymphocytes may have impaired proliferative response to mitogens, poor or absent delayed hypersensitivity, decreased numbers of circulating T cells,[207] and a reduction in $CD8^+$ T cells.[208]

Patients with anorexia nervosa have no apparent bleeding diathesis. The platelet count is normal to slightly decreased [203] [209] and in vitro platelet aggregation to epinephrine, adenosine diphosphate, and collagen is exaggerated.[210] [211] Coagulation defects are uncommon except for vitamin K deficiency reported in bulimia.[212]

The hematologic changes in anorexia nervosa are directly correlated with total body fat mass depletion.[213] A bone marrow pattern on magnetic resonance imaging suggestive of gelatinous transformation of bone marrow (serous atrophy) is seen in patients with the lowest hematologic parameters. Direct examination of the bone marrow reveals hypoplasia with loss of fat stores and replacement by a gelatinous acid mucopolysaccharide ground substance.[214] Focal or extensive necrosis may be present.[215] Bone marrow histiocytes are relatively increased in number and have prominent blue-green granules.[204] With nutritional treatment, bone marrow hypoplasia reverses, the gelatinous material disappears, and the hematologic abnormalities, including neutrophil defects and low complement levels, resolve by 8 weeks.[203] [204]

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HEMATOLOGIC ASPECTS OF POISONING

Poisoning is an important problem in children. [216] It has been estimated that 6 million children, most under the age of 5 years, ingest some toxin each year. The effects of toxins on the blood are diverse, usually nonspecific, and in most situations overshadowed by the nonhematologic manifestations of the exposure. [217] However, with certain toxins, bleeding, anemia, or change in the appearance of the blood may be an important component of the clinical sequelae of an acute exposure.

The abnormalities of hemostasis after poisoning are numerous and the mechanisms vary. Bleeding may be the only

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manifestation of warfarin toxicity secondary to an overdose of the drug or ingestion of a rodenticide containing warfarin. Any hepatotoxic substance (e.g., iron or acetaminophen) may lead to decreased synthesis of clotting factors and resultant coagulopathy. Bleeding in these circumstances is delayed for at least 24 hours, although there appears to be an early coagulopathy in iron poisoning that may be due to a direct effect on clotting protein function and not hepatotoxicity. [218] DIC has been seen after ingestion of mushrooms of the*Amanita* genus[219] or a bite from the brown recluse spider (*Loxosceles reclusa*).[220] Poisonous snake bites can result in coagulation abnormalities characterized by hypofibrinogenemia with or without thrombocytopenia or a DIC-like syndrome. [217] Severe thrombocytopenia has been described with elemental mercury poisoning. [221]

Acute hemolytic anemia may be the presenting manifestation after exposure to drugs and toxins in children with G6PD deficiency or hemoglobin Zürich [222] [223] or (rarely) in normal children. [224] Severe hemolytic anemia has been seen after the bite of the brown recluse spider [220] [225] and after a wasp sting. [226]

Exposure to certain toxins may result in characteristic color change of the blood, which in turn may be reflected clinically in abnormal skin color. The child with methemoglobinemia (see following discussion) presents with a slate gray cyanosis unresponsive to 100% oxygen administration. On exposure to air, the blood retains a distinct brown color. Patients with toxic exposure to carbon monoxide or cyanide have increased levels of carboxyhemoglobin or cyanhemoglobin, respectively, resulting in a "cherry-red" color of the blood and skin, but only with high concentrations of the offending hemoglobin.

Infants (4 months of age) are at particular risk for developing methemoglobinemia because of a reduced amount (60% of normal) of cytochrome b_5 reductase present in neonatal RBC. Methemoglobinemia has been described in infants with diarrheal illness and in infants exposed to exogenous agents. [227]

Yano et al.[228] described 11 cases of transient methemoglobinemia in infants <1 month old who presented with vomiting, diarrhea, and acidosis. In prospective studies of infants <6 months of age with diarrheal disease, 64% had elevated levels of methemoglobin with a mean (± SD) of 10.5 ± 12.3%, 31% were cyanotic, most infants were small and/or failing to thrive, there was no association of methemoglobinemia and acidosis, and all recovered from their illness. [229] [230] Although most infants with endogenous methemoglobinemia can be managed with support and hydration, treatment with methylene-blue (1 mg/kg) is indicated in the symptomatic or more severely affected (methemoglobin >2030%) child.

Nursery epidemics of methemoglobinemia have been reported in normal newborns exposed to disinfectants or aniline dyes used to mark diapers. [231] Infants fed formulas made with well water containing a high concentration of nitrates have developed methemoglobinemia. EMLA cream, a eutectic mixture of the local anesthetics lidocaine and prilocaine, has been effective in decreasing pain in infants undergoing circumcision. [232] Methemoglobinemia has been reported with EMLA use in this situation, but only in overdose. [233] Other ingestions associated with methemoglobinemia include phenazopyridine (Pyridium), [234] dapsone,[235] metoclopramide,[236] and nitroethane (an artificial fingernail remover). [237] Although the list of oxidants reported to cause methemoglobinemia is long, [238] methemoglobinemia due to exogenous agents is uncommonly seen in infants and children.

Lead Poisoning

Lead poisoning in children has been a serious public health problem for decades. However, the most serious toxicities of lead (e.g., encephalopathy) commonly seen in the past are rarely encountered today, primarily because of measures instituted to decrease lead exposure (no-lead paint, no-lead gasoline) and screening programs in high-risk areas. Mean blood lead levels in the United States have declined from 15 g/dl between 1976 and 1982 to 3.6 g/dl between 1988 and 1991. [239] Nonetheless, lead toxicity remains a problem, especially in high-risk children. With increasing evidence that lower levels of lead exposure are associated with a significant decline in neurodevelopmental outcome, in 1991 the CDC lowered the intervention level of lead in the blood from 25 g/dl to 10 g/dl. [240] Reviews of the public health issues relating to lead poisoning in children have been published. [241] [242]

The primary hematologic effect of lead is interference at multiple points along the heme synthetic pathway. [243] The two most important effects are inhibition of -aminolevulinic acid (ALA) dehydratase [244] and ferrochelatase, [245] resulting in the accumulation of heme intermediates such as protoporphyrin. A shortened RBC survival accompanies lead poisoning and is probably due to decreased activity of pyrimidine 5-nucleotidase (also resulting in basophilic stippling of the RBC) and possibly inhibition of G6PD and the pentose shunt. [246]

The anemia of lead poisoning has classically been described as a hypochromic, microcytic anemia, as might be expected from the effects of lead on heme synthesis. Although anemia has been said to be a common finding in lead intoxication, in reality, anemia is uncommon unless the lead poisoning is severe or there is associated iron deficiency.

A strong association exists between lead poisoning and iron deficiency in children. Both tend to occur in the same population of predominantly lower socioeconomic status. Experimentally, iron deficiency has been shown to increase lead absorption, retention in tissues, and toxicity. [247] Iron deficiency also decreases lead excretion during chelation.[248] Lead may impede iron absorption and metabolism, thus leading to a vicious cycle of increasing lead toxicity and worsening iron deficiency. In a study of children with lead poisoning (blood lead 30 g/dl) 86% were found to have iron deficiency, and 100% of those with more severe lead poisoning (CDC risk classification III) [249] were iron deficient. [250]

There have now been a number of reports in children with lead toxicity documenting the infrequent occurrence of anemia without concomitant iron deficiency. Cohen et al.[251] found anemia in 12% and microcytosis in 21% of iron sufficient children with severe lead poisoning (CDC classes III and IV). The combination of anemia plus microcytosis, however, was found in only 1 of the 58 children. In less severely affected children (CDC classes I to III) Yip et al., [250] found a 30% incidence of anemia, but of those with either mild or no iron deficiency, only 6% were anemic. [250] Clark et al.[252] using multiple linear regression analysis, found transferrin saturation to be the most important predictor of mean corpuscular volume, hemoglobin, and zinc protoporphyin levels in children with lead poisoning. Two important points emerge from the foregoing information: (1) children with significant lead poisoning may have neither anemia nor microcytosis, and (2) children with documented lead

poisoning should be screened for underlying iron deficiency. The immediate treatment and long-term management of lead poisoning are beyond the scope of this chapter.[253]

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HEMATOLOGIC ASPECTS OF DRUGS

Although hematologic effects of poisoning due to toxins or drug overdoses are well documented, iatrogenic adverse hematologic effects of prescribed medications are also an occasional reason for referral to a hematologist. The hematologic effects

of drugs are sometimes predictable and may be exploited for therapeutic benefit; however, rare or unexpected hematologic toxicity of drugs may cause considerable morbidity or even mortality, as well as pose diagnostic and therapeutic challenges to the clinician. Few data exist regarding the overall incidence of such adverse events in the pediatric population[254] and most are in the form of case reports. Table 151-3 lists peripheral blood cytopenias that have been associated with medications given to pediatric patients.

Drug-induced hematologic cytopenias have been reported in association with a large number of medications. A variety of mechanisms may apply to such cytopenias, including bone marrow suppression,[255] [256] aplasia,[257] [258] and nonimmune-[259] [260] [261] or immune-mediated[262] peripheral destruction. Antimicrobial and anticonvulsant medications are the most frequently reported classes of drugs implicated in hematologic toxicity in the pediatric age group, and valproate is the single most common drug reported in this context. Valproic acid has usually been associated with mild to moderate thrombocytopenia, occurring in 20% of children on long-term therapy.[263] [264] Severe and multilineage blood cytopenias have also been reported. [265] [266] The occurrence of macrocytosis and the Pelger-Huët anomaly are frequent manifestations of valproate hematopoietic toxicity.[263] The mechanisms by which cytopenias occur in valproate toxicity are not fully understood and may be multifactorial. Both immune-mediated effects and myelodysplasia have been reported. [265] [266] [267] The bone marrow findings may occasionally resemble acute leukemia.[267] Thrombocytopenia induced by valproate is frequently dose related [263] [264] and may respond to dose reduction, thereby allowing continued use of an agent effective in seizure control.

Adverse hemostatic effects from prescribed medications have also been reported in association with a wide range of drugs. Drug-induced bleeding diatheses may be secondary to hepatic failure[268] [269] or from impaired hepatic synthesis of procoagulant proteins.[270] Coagulopathy related to altered vitamin K metabolism may occur in the setting of broad-spectrum antibiotic use.[271] Consumptive coagulopathy has rarely been reported in association with various drugs. [272] [273] [274] A tendency to pathologic thrombosis has been described due to oral contraceptives [275] and L-asparaginase therapy.[276] The latter most closely corresponds to depletion of AT III, the major physiologic regulator of thrombin.[276]

TABLE 151-3 -- Drug-Associated Cytopenias Reported in Pediatric Patients

	Anemia	Thrombo-cytopenia	Neutropenia	References
Acyclovir	X		X	[279]
Captopril	X		X	[255] [280] [281]
Carbamazepine	X	X	X	[282] [283]
Cephalosporins	X		X	[284] [285]
Chloramphenicol	X	X	X	[286]
Chlorpromazine	X		X	[287] [288]
Cimetidine			X	[289]
Cotrimoxazole	X	X	X	[290] [291]
Cyclosporin	X	X		[260] [292]
Ethosuximide	X	X	X	[257] [293]
Methyldopa	X			[294]
Miconazole	X			[295]
Penicillins	X		X	[296] [297]
Phenazopyridine	X			[298]
Phenytoin	X	X		[299] [300]
Procainamide			X	[301]
Rifampin	X	X		[302]
Sulfasalazine	X	X	X	[303] [304]
Valproate	X	X	X	[263] [266] [267]
Tetracycline	X			[305] [306]

The lupus anticoagulant has been associated with phenothiazines and related agents. [277] [278]

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HEMATOLOGIC ASPECTS OF METABOLIC DISEASES

A number of congenital disorders of metabolism may manifest hematologic abnormalities as part of the clinical presentation. Such global metabolic defects have diverse manifestations; especially prominent are neurologic findings, failure to thrive, and unexplained metabolic acidosis. Although the hematologic findings may be overshadowed by the systemic illness, the recognition of a characteristic pattern of signs and symptoms may lead expeditiously to the correct diagnosis. Table 151-4 is a compilation of inborn disorders of metabolism that may present in infancy or childhood with hematologic cytopenias.

SPLENOMEGALY IN CHILDREN

Splenomegaly is a problem frequently encountered by both the pediatrician and the pediatric hematologist. Although the spleen is rarely a site of primary disease, it may reflect systemic involvement with a variety of disorders. The spleen is the largest collection of lymphoid tissue in the body, with a unique association between the bloodstream and the reticuloendothelial compartment of the spleen. [343] Splenomegaly may result when there is antigenic stimulation of the lymphoid system (e.g., infection), obstruction of blood flow within or distal to the spleen (e.g., portal vein obstruction), exaggeration of one of the normal functions of the spleen due to an underlying abnormality (e.g., hemolytic anemia, splenic sequestration), or infiltration of the spleen by a foreign cell (e.g., leukemia, storage diseases).

The spleen tip is normally palpable in preterm infants; up to 30% of full-term neonates have a palpable spleen. [344] A spleen can be felt in up to 510% of normal children, but most of these are in the infant/toddler age group. As a general rule, a spleen easily palpable below the costal margin in any child over the age of 34 years must be considered abnormal until proven otherwise. That some palpable spleens may indeed be normal is attested to by the study of McIntyre and Ebaugh that 3% of healthy college freshmen have palpable spleens, of which about one-third persist. [345] Pretenders of splenomegaly include the left lobe of the liver, a left upper quadrant tumor such as Wilms tumor or neuroblastoma, the wandering spleen, [346] and the proptotic spleen (seen in children with depressed diaphragms from obstructive pulmonary disease, such as asthma or bronchiolitis).

A list of causes of splenomegaly in children is presented in Table 151-5 .[347] The most common cause of acute splenomegaly in children, especially young children, is a viral infection. Splenic enlargement in this setting is mild to moderate and usually transient. When history and physical examination suggest a viral etiology, a complete blood count with differential, platelet count, and reticulocyte count should be performed to rule out unsuspected leukemia or hemolytic anemia and to determine whether there is an atypical lymphocytosis. The child should be rechecked in 4 weeks (or sooner if symptoms persist). If splenomegaly persists beyond 46 weeks, the splenic enlargement may be considered chronic.

When evaluating the child with chronic splenomegaly, one must consider all of the possibilities noted in Table 151-5 . However, clues from the history and physical examination may suggest a specific etiology and direct a tailored approach to the diagnostic laboratory evaluation. If, on the other hand, there is no apparent cause of the enlarged spleen, a number of screening laboratory tests should be performed, including a complete blood count with differential, platelet count, and reticulocyte count; evaluation of the peripheral smear; sedimentation rate; liver function tests; antibody titers to EBV, CMV, and

TABLE 151-4 -- Hematologic Manifestations of Metabolic Disease with Onset in Infancy and Childhood

Finding	Category	Disease	Defect	Associated Findings	References
Anemia					
	Lysosomal enzyme defects	Gaucher disease, type 1	Glucocerebrosidase deficiency	Splenomegaly, thrombocytopenia, bone abnormalities, delayed puberty, lipid engorged macrophages (Gaucher cell) in bone marrow	[307]
		Niemann-Pick disease, type A	Acid sphingomyelinase deficiency	Feeding difficulties, hepatosplenomegaly developmental delay, neurodegenerative course, cherry-red macula, lipid laden macrophages (foam cell) in bone marrow	[308]
		Wolman disease	Acid lipase deficiency	Emesis, diarrhea, hepatosplenomegaly, thrombocytopenia, vacuolization of lymphocytes, lipid-laden macrophages in bone marrow, adrenal calcification	[309] [310]
	Defects of heme synthesis	Congenital erythropoietic porphyria	Uroporphyrinogen III cosynthase deficiency	Hemolysis, staining of diapers, photosensitivity, developmental delay, splenomegaly, thrombocytopenia, erythrodontia	[311]
		Erythropoietic protoporphyria	Ferrochelatase deficiency	Hemolysis, photosensitivity, neurologic findings, hepatobilliary dysfunction	[311]
	Defects of amino acid metabolism	Tyrosinemia, type 1	Fumarylacetoacetate hydrolase deficiency	Failure to thrive, emesis, hepatopathy, neurologic findings, thrombocytopenia, bleeding, cabbage-like odor, renal tubular dysfunction	[312]
	Defects of organic acid metabolism	Isovaleric acidemia	Isovaleryl-CoA dehydrogenase deficiency	Acidosis, pancytopenia, emesis, neurologic findings, odor of sweaty feet	[313]
		Methylmalonic acidemia	Methylmalonyl-CoA mutase deficiency	Acidosis, leukopenia, thrombocytopenia, neurologic findings, failure to thrive, emesis	[314]
		Mevalonic aciduria	Mevalonate kinase deficiency	Acidosis, cataracts, neurologic findings, hepatosplenomegaly, thrombocytopenia	[315] [316]
		Propionic acidemia	Propionyl CoA carboxylase deficiency	Ketotic hyperglycinemia, acidosis, neurologic findings, pancytopenia	[317]
		Pyroglutamic aciduria	Glutathione synthetase deficiency	Hemolysis, neutropenia, neurologic findings, metabolic acidosis	[318] [320]
	Membrane transport defects	Lysinuric protein intolerance		Malabsorption, protein intolerance, hyperammonemia, neurologic findings, glomerulonephritis, pulmonary hemorrhage, thrombocytopenia, leukopenia	[321]

	Glycolytic pathway defects	Triosephosphate isomerase deficiency	Triosephosphate isomerase deficiency	Hemolysis, neurologic findings, myopathy	[322]
		Phosphoglycerate kinase deficiency	Phosphoglycerate kinase deficiency	Hemolysis, neurologic findings	[323]
	Defects of vitamin metabolism	Cobalamin metabolic defects	Cobalamin transport and utilization defects	Megaloblastosis, pancytopenia, failure to thrive, neurologic findings	[324]
		Folate metabolic defects	Folate transport and utilization defects	Megaloblastosis, ringed sideroblasts, thrombocytopenia, diarrhea, failure to thrive, neurologic findings	[325] [326] [327]
	Defects in metal metabolism	Wilson disease		Hemolysis, liver dysfunction, neurologic findings, Kayser-Fleischer rings, arthropathy	[328]
	Defects of purine and pyrimidine metabolism	Hereditary oroticaciduria	Uridine-5-monophosphate synthase deficiency	Megaloblastosis, leukopenia, orotic acid crystaluria, immunodeficiency	[329] [330]
		Lesch-Nyhan syndrome	Hypoxanthine-guanine phosphoribosyl-transferase deficiency	Megaloblastosis, hyperuricemia, nephrolithiasis, neurologic findings, self-mutilation	[331]
	Defects of carbohydrate metabolism	Galactosemia	Galactose-1-phosphate uridyltransferase deficiency	Hemolysis, failure to thrive, neurologic findings, jaundice, emesis, diarrhea, hepatic dysfunction, cataracts, gram-negative sepsis	[332]
		Glycogen storage disease type 1b	Deficient hepatic transport of glucose-6-phosphate	Neutropenia, hypoglycemia, acidosis, hepatomegaly, xanthomas, inflammatory bowel disease	[333] [334]
		Hereditary fructose intolerance	Aldolase b deficiency	Poor feeding, emesis, failure to thrive, neu-rologic findings, hypo-glycemia, liver dysfunc-tion, thrombocytopenia, ringed sideroblasts, coagulopathy, hemophagocytosis, shock	[335]
	Mitochondrial disorders	DIDMOAD syndrome (Wolfram syndrome)	Mitochondrial DNA deletions	Megaloblastosis, ringed sideroblasts, neutropenia, thrombocytopenia, diabetes insipidus, diabetes mellitus, optic atrophy, deafness	[336]
		Pearsons syndrome	Mitochondrial DNA deletion	Ringed sideroblasts, vacuolization of marrow precursors, exocrine pancreatic dysfunction, metabolic acidosis, thrombocytopenia, neutropenia	[337] [338]
	Lipoprotein disorders	Abetalipoproteinemia		Hemolysis, acanthocytosis, bleeding, emesis, diarrhea, failure to thrive, neurologic findings	[339]

Neutropenia

	Lysosomal enzyme defects	Aspartylglucosaminuria	Aspartylglucosa minidase deficiency	Recurrent infections, diarrhea, hernias, lens opacities, neurologic findings, vacuolated lymphocytes	[340]
	Defects of organic acid metabolism	Barth syndrome (endocardial fibroelastosis-2)		Cardiomyopathy, endocardial fibroelastosis, skeletal myopathy, short stature, 3-methylglutaconicaciduria, abnormal mitochondria	[341]
		Isovaleric acidemia	Isovaleryl-CoA dehydrogenase deficiency	Acidosis, pancytopenia, emesis, neurologic findings, odor of "sweaty feet"	[313]
		Methylmalonic acidemia	Methylmalonyl-CoA mutase deficiency	Acidosis, anemia, leukopenia, neurologic findings, failure to thrive, emesis	[314]
		Propionic acidemia	Propionyl-CoA carboxylase deficiency	Ketotic hyperglycinemia, acidosis, neurologic findings, pancytopenia	[317]
		Pyroglutamic aciduria	Glutathione synthetase deficiency	Hemolysis, anemia, neurologic findings, metabolic acidosis	[318] [319] [320]
	Defects of vitamin metabolism	Cobalamin metabolic defects	Cobalamin transport and utilization defects	Megaloblastosis, pancytopenia, failure to thrive, neurologic findings	[324]
	Defects of carbohydrate metabolism	Glycogen storage disease type 1b	Deficient hepatic transport of glucose-6-phosphate	Anemia, hypoglycemia, acidosis, hepatomegaly, xanthomas, inflammatory bowel disease	[333] [334]
	Mitochondrial disorders	DIDMOAD syndrome (Wolfram syndrome)	Mitochondrial DNA deletions	Megaloblastosis, anemia, ringed sideroblasts, thrombocytopenia, diabetes insipidus, diabetes mellitus, optic atrophy, deafness	[336]
	Mitochondrial disorders	Pearsons syndrome	Mitochondrial DNA deletion	Sideroblastic anemia, vacuolization of marrow precursors, exocrine pancreatic dysfunction, metabolic acidosis, thrombocytopenia	[337] [338]

Thrombocytopenia

	Lysosomal enzyme defects	Gaucher disease, type 1	Glucocerebrosidase deficiency	Splenomegaly, anemia, bone abnormalities, delayed puberty, lipid-engorged macrophages (Gaucher cell) in bone marrow	[307]
		Wolman disease	Acid lipase deficiency	Emesis, diarrhea, hepatosplenomegaly, anemia, vacuolization of lymphocytes, lipid-laden macrophages in bone marrow, adrenal calcification	[309] [310]
	Defects of heme synthesis	Congenital erythropoietic porphyria	Uroporphyrinogen III cosynthase deficiency	Hemolysis, staining of diapers, photosensitivity, developmental delay, splenomegaly, anemia, erythrodontia	[311]
	Defects of amino acid metabolism	Tyrosinemia type 1	Fumarylacetoacetate hydrolase deficiency	Failure to thrive, emesis, hepatopathy, neurologic findings, anemia, bleeding, cabbage-like odor, renal tubular dysfunction	[312]
	Defects of organic acid metabolism	Isovaleric acidemia	Isovaleryl-CoA dehydrogenase deficiency	Acidosis, pancytopenia, emesis, neurologic findings, odor of sweaty feet	[313]
		Methylmalonic acidemia	Methylmalonyl-CoA mutase deficiency	Acidosis, leukopenia, anemia, neurologic findings, failure to thrive, emesis	[314]

		Mevalonic aciduria	Mevalonate kinase deficiency	Acidosis, cataracts, neurologic findings, hepatosplenomegaly, anemia	[315] [316]
		Propionic acidemia	Propionyl CoA carboxylase deficiency	Ketotic hyperglycinemia, acidosis, neurologic findings, pancytopenia	[317]
	Membrane transport defects	Lysinuric protein intolerance		Malabsorption, protein intolerance, hyperammonemia, neurologic findings, glomerulonephritis, pulmonary hemorrhage, anemia, leukopenia	[321]
	Defects of vitamin metabolism	Cobalamin metabolic defects	Cobalamin transport and utilization defects	Megaloblastosis, pancy-topenia, failure to thrive, neurologic findings	[324]
	Defects of vitamin metabolism	Folate metabolic defects	Folate transport and utilization defects	Megaloblastosis, ringed sideroblasts, anemia, diarrhea, failure to thrive, neurologic findings	[325] [326] [327]
		Holocarboxylase synthetase deficiency	Holocarboxylase synthetase deficiency	Acidosis, hyperammonemia, respiratory distress, neurologic findings, skin rash	[342]
	Defects of carbohydrate metabolism	Hereditary fructose intolerance	Aldolase B deficiency	Poor feeding, emesis, failure to thrive, neurologic findings, hypoglycemia, liver dysfunction, anemia, ringed sideroblasts, coagulopathy, hemophagocytosis, shock	[335]
	Mitochondrial disorders	DIDMOAD syndrome (Wolfram syndrome)	Mitochondrial DNA deletions	Megaloblastosis, anemia, ringed sideroblasts, neutropenia, diabetes insipidus, diabetes mellitus, optic atrophy, deafness	[336]
		Pearsons syndrome	Mitochondrial DNA deletion	Sideroblastic anemia, vacuolization of marrow precursors, exocrine pancreatic dysfunction, metabolic acidosis	[337] [338]

toxoplasmosis; antinuclear antibody; and ultrasonic evaluation of the liver, spleen, and portal system (the latter with Doppler flow technique). Further evaluation, including bone marrow examination, may be necessary if the preceding screening tests do not reveal the cause of the splenic enlargement.

Symptoms from splenic enlargement are uncommon, although massive splenomegaly may cause abdominal discomfort and early satiety. If the spleen is sufficiently large, there may be increased destruction or sequestration of one or more of the formed elements of the blood (hypersplenism).

Management of splenomegaly is usually that of the underlying disease, where such treatment exits. Splenectomy may be indicated in selected conditions, but the potential benefits from splenectomy must be weighed against the risk of postsplenectomy sepsis, a rapidly progressive bacteremia, most commonly from *S. pneumoniae*, with a mortality rate of 50%.[348] The risk of postsplenectomy sepsis depends on the age of the patient and the nature of the underlying disorder. Patients <3 years old and those with a compromised immune or reticuloendothelial system are most susceptible. When elective splenectomy is indicated,

TABLE 151-5 -- Splenomegaly in Children

Disorders of the blood
Hemolytic anemia: congenital/acquired
Thalassemia
Sickle cell disease
Leukemia
Osteopetrosis
Myelofibrosis/myeloid metaplasia/thrombocythemia
Infections: acute and chronic
Viral
Congenital (TORCH)
Mononucleosis (EBV, CMV)
Virus-associated hemophagocytic syndrome
HIV
Bacterial
Sepsis/abscess
Brucellosis
Salmonella
Tularemia
Tuberculosis
Subacute bacterial endocarditis
Syphilis
Lyme disease
Fungal
Histoplasmosis (disseminated)
Rickettsial
Rocky Mountain spotted fever
Cat scratch disease
Parasitic
Toxoplasmosis
Malaria
Leishmaniasis (Kala-azar)
Schistosomiasis
Echinococcosis
Hepatic/portal system disorder
Acute/chronic active hepatitis
Cirrhosis/hepatic fibrosis/biliary atresia
Portal or splenic venous obstruction (Banti syndrome)
Autoimmune disease
Juvenile rheumatoid arthritis
Systemic lupus erythematosus
Autoimmune lymphoproliferative syndrome (Canale-Smith syndrome)[349]

Neoplasms/cysts Lymphomas (Hodgkins and non-Hodgkins) Hemangiomas/lymphangiomas Hamartomas Congenital or acquired (post-traumatic) cysts
Storage diseases/inborn errors of metabolism Lipidoses: Gauchers disease, Niemann-Pick disease, etc. Mucopolysaccharidoses Defects in carbohydrate metabolism: galactosemia, fructose intolerance Sea blue histiocyte syndrome
Miscellaneous Histiocytoses Reactive Langerhans cell Malignant Sarcoidosis Congestive heart failure

it is advisable (1) to postpone surgery until the patient is at least 56 years of age, (2) to administer pneumococcal, meningococcal, and *H. influenza* (if not previously vaccinated) vaccines at least 12 weeks before splenectomy, (3) to consider prophylactic penicillin for at least 4 years, and (4) to manage significant febrile illnesses as possible postsplenectomy sepsis at all times.

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Chapter 152 - Hematologic Problems in Patients with Cancer and Chronic Inflammatory Disorders

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HEMATOLOGIC PROBLEMS IN PATIENTS WITH CANCER

In patients with malignancy, hematologic abnormalities are encountered with considerable frequency. [1] [2] [3] [4] [5] [6] [7] [8] These abnormalities result directly from the malignancy or as a consequence of treatment.

Red Blood Cells

Anemia

Anemia is the most common hematologic abnormality seen in association with cancer, occurring in approximately 50% of patients during the course of their illness. [1] [2] Review of the red blood cell indices, reticulocyte count, peripheral smear, iron studies, hemolytic studies, and bone marrow examination usually delineate the etiology (Table 152-1).

Anemia of Chronic Disease

Anemia of chronic disease (ACD) is the most common type seen with nonhematologic malignancies. [9] [10] [11] It can manifest early in the course of disease in association with cancer, and the severity of anemia can parallel the extent of the underlying cancer. Although bone marrow involvement may be present, it is not required to cause this type of anemia. The etiology of ACD is unclear.

Treatment has until recently been limited to treatment of the underlying disease or blood transfusions. However, regular administration of human erythropoietin (EPO) can ameliorate the ACD associated with cancer. [12] [13] [14] [15] [16] [17]

Tumor Invasion of the Marrow

Marrow invasion by solid tumors can be seen most frequently with small-cell lung cancer, breast cancer, prostate cancer, and lymphoma. Anemia occurs as a direct result of replacement of the marrow with tumor. Peripheral smears may show a leukoerythroblastic pattern; with severe involvement, pancytopenia

TABLE 152-1 -- Causes of Anemia in Cancer

Anemia of chronic disease
Tumor invasion
Pure red blood cell aplasia
Anemia due to blood loss Acute hemorrhage Iron deficiency (chronic blood loss)
Treatment-related anemia
Anemia due to nutritional causes
Hemolytic anemias Immunohemolytic anemia Microangiopathic hemolytic anemia Drug-associated hemolytic anemia Hemophagocytic syndrome

can occur. Extensive bone marrow fibrosis can accompany marrow invasion, further exacerbating the peripheral blood abnormalities. Treatment of the underlying malignancy, blood transfusions, and more recently EPO administration have been beneficial. [15]

Pure Red Blood Cell Aplasia

Although rare, pure red blood cell aplasia has been reported with neoplasms other than thymoma, including lymphoid tumors, nonsmall-cell lung cancer, breast cancer, and gastric cancer. The bone marrow typically shows a marked reduction of erythroid precursors, and plasma EPO levels are markedly increased. In approximately one-half the cases, an autoantibody directed against red blood cell progenitors can be observed. [18] [19] In T-cell chronic lymphocytic leukemia, T-lymphocyte suppression of erythropoiesis has been described, and this may be operative in patients with other types of malignancies. [20] [21] Treatment of pure red blood cell aplasia in patients with thymoma by thymectomy is successful in one-third of cases. Resection of the malignancy in patients with other types of tumors is also successful. However, most such patients need immunosuppressive therapy, including corticosteroids, high-dose immunoglobulin, antithymocyte globulin, danazol, and cytotoxic agents. Blood transfusion support is invariably required.

Anemia Due to Blood Loss

Iron deficiency is frequently the initial manifestation of patients with gastrointestinal malignancies. Renal carcinoma and bladder carcinoma may lead to blood loss through hematuria. Postgastrectomy patients may rebleed at sites of marginal ulcerations, and achlorhydria can impair iron absorption. The clinical distinction between iron deficiency and ACD may be difficult. Serum iron and transferrin levels can be low in both, but serum ferritin levels <10 mg/L are almost always due to iron deficiency. The presence of blood in the stools or urine is presumptive evidence for iron deficiency. Bone marrow iron stores are markedly reduced or absent.

Appropriate diagnostic studies to determine the site of blood loss should be the initial step. Treatment with oral iron preparations or parenteral iron dextran administration can improve the anemia. However, the hemoglobin level may not return to normal because, concomitantly, ACD may be present.

Therapy-Related Anemia

Treatment of solid tumors with cytotoxic agents usually leads to bone marrow depression, which can result in anemia. Although granulocytopenia and thrombocytopenia induced by such treatments reverse within 47 days after their onset, the anemia may be chronic and persist throughout the course of treatment secondary to depressed erythropoiesis. Macrocytic red cell indices and megaloblastic dysplasia of the bone marrow are frequently seen. [22] Blood transfusions may be needed, and EPO administration may benefit some patients and quality of life may improve. [23] [24]

Nutritional Causes of Anemia

Folic acid deficiency due to poor dietary intake can be seen as a result of anorexia associated with cancer. In addition, postgastrectomy patients with achlorhydria will develop vitamin B_{12} malabsorption, although anemia may take 35 years to develop. [25] Malnutrition per se can lead to ACD.

Hemolytic Anemias Associated with Cancer

Immunohemolytic Anemia.

Autoimmune hemolytic anemia is principally associated with lymphoid malignancies. Approximately 45% of patients with angioimmunoblastic lymphadenopathy will develop this type of hemolysis, and 2025% of patients with chronic lymphocytic leukemia will develop autoimmune hemolysis during the course of their illness. [26] [27] Additionally, patients with non-Hodgkin lymphoma can develop typical autoimmune hemolytic anemia. [28] With solid tumors, this is less frequent but is well documented with squamous carcinomas of the lung and cervix, adenocarcinomas of the breast, lung, ovaries, stomach, and colon, as well as others (Table 152-2). Most patients exhibit the warm antibody pattern with a positive direct antiglobulin test (Coombs test). However, cold agglutinins can be seen with non-Hodgkin lymphoma. When treatment directed at the underlying illness is successful, hemolysis can be ameliorated. However, more often other measures, including corticosteroids, cytotoxic agents, high-dose intravenous immunoglobulins, and splenectomy, are necessary.

Microangiopathic Hemolytic Anemia.

Mechanical intravascular destruction of red blood cells is relatively common with metastatic adenocarcinomas, especially those that secrete mucin. [29] [30] [31] Gastric cancer is the most frequently associated malignancy. The clinical laboratory findings associated with hemolysis are present, and, in addition, the peripheral smear will show schistocytes, helmet cells, and sometimes thrombocytopenia. In severe cases, nucleated red cells are seen in the peripheral blood along with hemoglobinemia, hemoglobinuria, and hemosiderinuria. The etiology appears, in part, to be due to low-grade disseminated intravascular coagulation (DIC) occurring in response to tumor products in the circulation that activate coagulation. Tumor products can also cause enhanced circulating platelet aggregates, which may also cause mechanical red blood cell destruction. [32] [33] Tumor products and platelet aggregates may cause endothelial cell damage leading to clinical manifestations of thrombotic microangiopathy. [34] Treatment is directed at the underlying tumor, but is usually incomplete because of the advanced state of the neoplasms associated with this complication.

Drug-Associated Hemolysis.

Mitomycin C has been associated with a syndrome of microangiopathic hemolytic anemia of a severe nature, with thrombocytopenia and renal insufficiency mimicking de novo hemolytic uremic syndrome. [35] [36] [37] Many of these patients have circulating immune complexes. This type of hemolytic uremic syndrome is usually refractory to treatment, with an almost invariably fatal course. However, recent reports suggest that plasma exchange, immuno-perfusion, and azothioprine may benefit some patients, especially when circulating immune complexes are etiologic. [38] A similar clinical picture has been reported with the bleomycin/ cisplatin-related Raynaud syndrome, and after exposure to

TABLE 152-2 -- Immunohemolytic Anemias Associated with Malignancy

Warm-reactive antibody type
Hodgkin disease
Non-Hodgkin lymphoma
Chronic lymphocytic leukemia
Multiple myeloma
Wäldenstrom disease
Angioimmunoblastic lymphadenopathy
Ovarian teratoma
Thymoma
Enteric adenocarcinoma
Breast cancer
Kaposi sarcoma
Seminoma
Renal cell carcinoma
Cold-reactive antibody type
As above, plus
Chronic granulocytic leukemia
Carcinoid tumor
Adrenocorticocarcinoma

Data from Doll and Weiss. [1]

daunorubicin, cytosine arabinoside, methyl-CCNU, deoxycorfomycin, and tamoxifen. [34] [39] [40]

Some chemotherapeutic agents have caused a warm antibody type of hemolytic anemia. This has been reported with cisplatin, tenoposide, melphalan, and methotrexate. The mechanism appears similar to that seen with high-dose penicillin-associated hemolytic anemia. [41] Discontinuation of the drug usually leads to resolution of this hemolysis.

In patients with glucose-6-phosphate dehydrogenase deficiency, administration of doxorubicin can cause an oxidant stress, producing a hemolytic anemia. [42] Discontinuation of this drug leads to resolution.

Hemophagocytic Syndrome.

The hemophagocytic syndrome has rarely been reported with gastric cancer, lymphoma, and acute leukemia.[43] This syndrome is characterized by pancytopenia, fever, lymphadenopathy, and splenomegaly. The bone marrow resembles histiocytosis with hemophagocytosis by benign-appearing macrophages. This syndrome is unresponsive to treatment and is usually fatal within a few weeks.

Polycythemia

True erythrocytosis has been reported with certain neoplasms, including renal cell carcinoma, hepatocellular carcinoma, and cerebellar hemagioblastoma. [44] [45] [46] This phenomenon has been noted in approximately 13% of patients with renal cell carcinoma, and in many of these cases elevated serum EPO levels are found. Although tumor cells from these patients do contain immunoreactive EPO, erythrocytosis in patients with Wilms tumor is due to pressure-induced hypoxia of the adjacent normal renal parenchyma.[47] [48] Removal of the kidney in patients with renal cell carcinoma has frequently led to reversion of the red cell mass to normal, and recurrence of the polycythemia has occurred when metastatic disease develops.

The association of secondary polycythemia and hepatocellular carcinoma may be due to the production of extrarenal EPO. [49] [50] Other mechanisms such as diminished metabolism of androgenic-like substances have been postulated. [51] [52] Regardless of the etiology, if the tumor can be completely excised, reversal of this abnormality can ensue.

White Blood Cells

Leukocytosis

Mild-to-moderate leukocytosis can be seen with malignancies such as bronchogenic carcinoma, gastric carcinoma, and renal cell carcinoma. When the tumors are widespread and necrotic, a typical neutrophilic leukemoid reaction can be seen, with white cell counts of 50,000/mm ^{3} or more.[53] Counts as high as 200,000/mm^3 have been reported. The distinction between a leukemoid reaction and leukemia is sometimes confusing, but in the leukemoid reaction, immaturity is typically absent and the leukocyte alkaline phosphate score is high. The cause of this phenomenon may be tumor-elaborated colony-stimulating factors (CSFs). [54] [55] No specific therapy is warranted for this problem.

Leukopenia and Neutropenia

Leukopenia can be seen in association with marrow invasion due to malignancy and leukoerythroblastosis may be observed. More typically, leukopenia is a result of bone marrow depression due to chemotherapy. This is usually reversible within 47 days of onset. More rarely, prolonged leukopenia may be seen after a chemotherapeutic course with antileukemic therapy. The use of growth factors, including granulocyte-CSF and granulocyte/macrophage-CSF has led to an earlier recovery for these patients.[56] The use of these growth factors has become increasingly routine in treating infected neutropenic patients. [57] Furthermore, these agents are being increasingly used with high-dose chemotherapy regimens to ameliorate or prevent severe neutropenia. [58] [59] [60]

Neutropenia is frequently associated with the T--cell lymphoproliferative syndrome. [61] In this disorder, T-suppressor cells appear to inhibit granulopoiesis directly. This syndrome has responded to corticosteroids, cyclophosphamide, and granulocyte-CSF.

Platelets

Thrombocytosis

Thrombocytosis associated with malignancy probably represents a reactive response to the presence of malignancy and is seen with frequency in patients with cancer,[62] [63] with or without bone marrow hypercellularity and increased megakaryocytes. Whether thrombocytosis per se in part causes the hypercoagulable state seen in association with cancer is unclear. No specific treatment for this abnormality is available.

Thrombocytopenia

Modest thrombocytopenia is frequent after treatment of solid tumors with cytotoxic agents.[64] Severe life-threatening thrombocytopenia can occur in such patients, especially if they have had prior radiotherapy or incomplete recovery from prior bone marrow depressive treatment. The bleeding risk may be enhanced by concurrent infection as a result of treatment-induced granulocytopenia. In addition, the risk of gastrointestinal hemorrhage may be enhanced by breaks in mucosal integrity due to chemotherapy. Recovery from most chemotherapy occurs within 1 week after onset. However, some agents such as mitomycin C or the nitrosoureas may cause prolonged thrombocytopenia. Bone marrow invasion can also lead to thrombocytopenia, and bleomycin damage of pulmonary endothelium may lead to platelet destruction.[65]

Treatment for thrombocytopenia due to chemotherapeutic agents should follow the same guidelines established for leukemic patients. Although most patients have a spontaneous recovery of their platelets after chemotherapy, in those who have any bleeding or whose platelet counts fall too low, platelet transfusions may be necessary.

Autoimmune thrombocytopenia is most commonly seen in association with lymphoid malignancies, but also can be seen with lung cancer, breast cancer, and testicular carcinoma.[66] [67] Treatment with corticosteroids, cytotoxic agents, high-dose intravenous -globulin, and splenectomy may be necessary if treatment of the underlying neoplasm does not improve this condition.

Coagulation Abnormalities

Cancer-Related Thrombosis

Patients with cancer have a greater tendency to thrombosis as a result of a number of causal mechanisms. This hypercoagulable state has been characteristic of patients with various malignancies. In addition to cancer patients being debilitated and possibly requiring prolonged bed rest, tumors can compress or invade blood vessels, leading to stasis, and the normal hemostatic mechanisms may become disrupted.[68] [69] [70] [71] Also, release of tumor products into the circulation may be thrombogenic.

It has been demonstrated that many of the procoagulants are increased in patients with cancer. Fibrinogen and factors V, VII, VIII, IX, and XI have been found to be elevated.[72] [73] When DIC is present, antithrombin III, protein C, and protein S can be consumed, leading to an increased tendency to thrombosis. [74] Neoplastic

cells themselves can initiate clotting by releasing tissue factor, which complexes with factor VIIa in the blood to activate factor X. [75] Tissue factor has been described with lung, kidney, colon, and breast cancer. The migratory thrombotic syndrome known as Trousseau syndrome may be caused by this latter mechanism.[76] [77] This clinical syndrome has sometimes been called chronic DIC when a thrombotic tendency dominates the syndrome instead of a bleeding tendency. [78]

Mucinous adenocarcinomas of the lung, gastrointestinal tract, and ovary are associated with a high risk of thrombosis. In addition, systemic trypsin from pancreatic carcinoma can activate coagulation.[79]

Other mechanisms may be active, including monocytic production of procoagulant substances.[80] [81] [82] [83] The substances expressed include tissue factor, factor X activators, and prothrombinase complex. The lupus inhibitor and anticardiolipins are seen with some malignancies. They increase the thrombotic risk, although the actual mechanism is unknown. Increased platelet aggregation is seen in patients with cancer, which may add to the risk of thrombosis. [84] [85]

Treatment of cancer-related thrombosis starts with minimizing the conditions associated with thrombosis, such as prolonged bed rest and lower extremity edema. Although successful treatment of the underlying tumor can benefit these patients, many of these tumors are resistant to therapy and therefore anticoagulation should be considered. Some can be treated with warfarin, but heparin may also be useful. Anticoagulation in cancer patients can be fraught with difficulties, including heparin resistance and a greater bleeding risk. [86] [87] The use of thrombolytic agents is discouraged because the bleeding risk in cancer patients is high. [88]

Nonbacterial Thrombotic Endocarditis

Nonbacterial thrombotic endocarditis is a paraneoplastic thrombotic syndrome associated with predominantly neurologic complications and (less frequently) systemic embolization. [89] [90] Such patients can present with embolic strokes manifested by focal or generalized neurologic findings (or both). Cardiac findings are minimal, and fever is absent. Embolism to other sites such as the spleen, kidneys, gastrointestinal tract, and coronary arteries are detected at autopsy but are rarely clinically manifest. Additional findings at autopsy include fibrin-platelet vegetations on the mitral and aortic valves. Tumors most commonly associated with this complication include nonsmall-cell lung cancer, prostate cancer, and pancreatic cancer. Anticoagulants are of no benefit in this condition.

Cancer-Related Bleeding

Disseminated intravascular coagulation is a frequent cause of cancer-related bleeding. Although it is most frequently seen with prostate cancer, it has been reported with cancers of the gastrointestinal tract, ovary, and lung, as well as melanoma. Many of these patients bleed, usually mildly; bleeding can become massive after manipulating the tumor, as in prostate cancer surgery. Infections may exacerbate a compensated DIC and lead to bleeding. The treatment of DIC associated with cancer can be challenging. Treating the underlying disease is frequently inadequate. Judicious use of heparin may benefit some patients. [91]

Certain chemotherapeutic agents have been associated with hemostatic defects. L-Asparaginase can lead to the production of a functionally impaired fibrinogen, which may cause bleeding. [92] Mithramycin can cause platelet dysfunction and increased fibrinolysis, leading to an enhanced bleeding risk. [93] Suramin, which is used for refractory prostate cancer, has been associated with clinical bleeding. [94] Actinomycin D can antagonize vitamin K, and the anthracyclines have been reported to cause primary fibrinogenolysis. These effects will reverse with discontinuation of the agents.

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HEMATOLOGIC PROBLEMS IN PATIENTS WITH CHRONIC INFLAMMATORY DISORDERS

Rheumatic Diseases

Inflammatory rheumatic disorders are commonly associated with hematologic abnormalities, especially anemia. The anemia of rheumatoid arthritis has been a classic model for studying ACD. Anemia and other hematopoietic dysfunctions also occur frequently in systemic lupus erythematosus (SLE). Other inflammatory states associated to a lesser degree with hematopoietic disorders include mixed connective tissue disease, polymyalgia rheumatica, dermatomyositis, scleroderma, and Sjögren syndrome.

Anemia of Rheumatic Disorders

Anemia of Chronic Disease

Anemia, usually ACD, is the most common hematologic abnormality found in rheumatic disorders.[95] The exact pathogenesis of ACD still remains a matter of debate. In 1966, Cartwright[10] postulated three basic abnormalities: impaired marrow response, possibly due to diminished production of EPO; impaired release of iron from the reticuloendothelial system; and a mild decrease in red cell survival. More recently, several studies have clarified the relationship of EPO to the anemia of rheumatoid arthritis.[96] [97] [98] [99] The EPO level is inversely correlated with the hemoglobin level. Although EPO levels rise in response to the anemia of rheumatoid arthritis, the rise is less than that found in patients with the same degree of anemia who have other illnesses. However, the blunted EPO effect is not the primary cause of the anemia of rheumatoid arthritis because the EPO levels are still greater than normal. [100] [101] Recent investigations have studied the failure of the bone marrow to respond to higher levels of EPO in chronic inflammatory disorders, particularly rheumatoid arthritis. Several cytokines involved in the inflammatory response are elevated in chronic inflammatory diseases.[102] [103] These include tumor necrosis factor-alpha (TNF-), interleukin (IL)-1, IL-6, interferon- and interferon-, and the pteridine neopterin, which is a marker of immune activation. IL-6, which is increased in patients with rheumatoid arthritis, produces anemia in animals and correlates with the other more common markers of inflammation such as the sedimentation rate. However, it is not clear in humans whether IL-6 is important in the pathogenesis of the anemia of rheumatoid arthritis or is merely a marker of disease activity. [104] [105] Cytokines may be involved in the impaired EPO response to anemia in rheumatic disorders. IL-1 and TNF- inhibit EPO production from a hepatoma cell line. This effect may occur at the level of EPO mRNA. [106] [107]

The pathogenesis of the disturbances of iron metabolism in rheumatoid disorders (decreased serum iron and iron-binding capacity, normal or increased tissue iron) has been extensively studied. Early studies demonstrating a block in the release of reticuloendothelial iron using [59] Fe-labeled iron have not been confirmed when smaller doses of radiolabeled iron were used.[108] [109] [110] [111] [112] These more recent studies also suggest that the disturbances of iron metabolism may be secondary to decreased erythropoiesis. The role of cytokines in the erythropoiesis of inflammatory disorders shows their involvement in the impairment of iron metabolism. [113] Recombinant TNF induces anemia and hypoferremia in animals.[114] [115] IL-1 increases ferritin production, and this additional ferritin may trap iron, making it unavailable for red cell production. [116]

Treatment options for the ACD associated with rheumatic

CONSIDERATIONS REGARDING THE USE OF EPO IN INFLAMMATORY DISORDERS

1. Pretreatment EPO level is not predictive of an optimal response.
2. Therapeutic dosage usually needs to be larger than that used in patients with renal failure.
3. It is prudent to delay therapy until the effects of treatment of the underlying disorder are evaluated.
4. Coexisting iron deficiency will blunt the response of EPO, and iron deficiency can develop during EPO treatment.
5. In most circumstances an EPO trial should be avoided in mild anemia (i.e., hematocrit >30 vol%).
6. An adequate response to EPO is an increase of the hematocrit l6 vol% in 68 weeks of therapy or relief of symptoms of anemia, or both.
7. A maintenance dose must be determined for each responder. A useful hint is to start by decreasing the dose by one-third.
8. Chronic administration of EPO is not justifiable for trivial improvement in the hematocrit or in the absence of an overall clinical improvement.

disorders have increased since the availability of EPO (see box). A recent randomized placebo-controlled double-blind study showed that EPO administered subcutaneously to active rheumatoid arthritis exerted a beneficial effect on disease activity. [117] Pharmacologic doses of EPO often ameliorate the anemia in patients with rheumatoid arthritis.[118] [119] [120] [121] EPO can also correct cytokine-induced inhibition of erythropoiesis in animals. [122] [123] EPO does not correct the anemia of chronic renal and inflammatory disorders in the presence of concomitant iron deficiency. Iron deficiency can develop during EPO treatment. [124] [125]

Iron Deficiency Anemia

Iron deficiency anemia commonly coexists with ACD in rheumatic disorders, especially in rheumatoid arthritis. If the serum ferritin level is <10 ng/L, the diagnosis of coexisting iron deficiency needs no further iron studies. When levels of ferritin are this low, gastrointestinal blood loss, particularly in patients taking anti-inflammatory drugs, is suggested.[126] When the serum ferritin level is >30 ng/L, the diagnosis of iron deficiency is more difficult and ultimately may depend on the finding of absent bone marrow iron.[127] In an attempt to avoid the need for a bone marrow study, various combinations of iron studies have been used. One recent study using a three-step algorithm measuring the serum ferritin, mean cell volume, and iron saturation, correctly classified 94% of rheumatoid arthritis patients with iron

deficiency.[128]

Hemolytic Anemia

Acquired hemolytic anemia, usually secondary to immune mechanisms, is frequently associated with rheumatic disorders, particularly SLE. The anemia may vary from mild to severe and is typically characterized by the presence of spherocytes on the peripheral blood film. Autoimmune hemolytic anemia associated with autoimmune thrombocytopenia is occasionally seen in SLE (Evan syndrome).

Therapy of autoimmune hemolytic anemia depends on the severity of the red cell destruction. Some patients have a compensated hemolytic state and need no therapy. More often, the anemia is severe enough that treatment should be instituted with corticosteroids. Large doses are usually required (prednisone 60100 mg/day orally). Beneficial responses occur in most patients, especially those with SLE. [129] Patients with severe anemia who have not responded to corticosteroids or in whom a large maintenance dose is required should be managed by splenectomy. However, poor-risk patients with SLE or other rheumatic syndromes may be treated first on immunosuppressive drugs, danazol, plasmapheresis, or high-dose intravenous -globulin. Reported results with these therapeutic modalities are contradictory.[130] [131] [132] [133] [134] [135]

Acquired Pure Red Cell Aplasia

Pure red cell aplasia is an uncommon immunologic disorder that has been reported in association with both rheumatoid arthritis and SLE. Pure red cell aplasia should not be mistaken for ACD. Distinctive features are moderate-to-severe anemia, marked reticulocytopenia, and the findings in the bone marrow of severe hypoplasia of the red cell series and normal production of the other marrow cell lines.

Spontaneous remission occurs in about 1015% of these patients. The management of this condition requires withdrawal of all medications. If there is no response to withdrawal nor any spontaneous remission occurring within a few weeks, corticosteroids, cytotoxic agents, antithymocyte globulin, danazol, cyclosporine, plasmapheresis, high-dose -globulin, and splenectomy may be considered. [136] [137] [138] [139] [140] [141] [142] [143] EPO therapy is not helpful. About two-thirds of patients in need of treatment will respond to one or more of the other modalities.

Neutropenia

With the exception of patients with SLE, neutropenia is seen infrequently in the inflammatory arthritides. In SLE, a neutropenia has been reported to occur in about one-half of patients.[144] The neutropenia in this setting is usually not severe and is not often associated with an infectious complication. Therefore, initiation of therapy is usually not indicated. Indeed, the neutropenia may worsen with cytotoxic therapy, even though the neutropenia is thought to be due to immune destruction of mature neutrophils or marrow progenitors.[145] Occasionally the neutropenia of SLE is secondary to hypersplenism.

Felty syndrome is an uncommon but important hematologic abnormality that occurs in 1% of patients with rheumatoid arthritis, developing about 15 years after the onset of the disease and usually when the arthritis is not active. [146] The characteristic triad of Felty syndrome is rheumatoid arthritis, splenomegaly, and neutropenia. The syndrome is also associated with recurrent infections, leg ulceration, rheumatoid nodules, and lymph node enlargement. The neutropenia is usually severe, ranging between 500 and 2,500/mm^3 . The mechanism of the neutropenia has been attributed to antineutrophil antibodies, increased occurrence of immune complexes, inhibition of neutrophil production mediated by T cells, altered neutrophil distribution, reduced neutrophil function, and hypersplenism. [147] [148] [149] Elevated levels of neutrophil-bound IgG and serum IgG neutrophil-binding activity are found in about one-half of these patients with Felty syndrome. [150]

Appropriate therapy for Felty syndrome is often difficult. If infections have been infrequent and mild in nature, no intervention is needed. Repeated and serious infections or chronic leg ulceration usually require treatment. In addition to the administration of appropriate antibiotics, other therapies are usually tried with mixed results. These include cytotoxic agents, corticosteroids, methotrexate, cyclosporine, penicillamine, plasmapheresis, gold salts, and splenic radiation. The role of splenectomy remains a matter of controversy. For decades, it has been known that splenectomy normalizes the neutrophil count in about one-half of patients with rheumatoid arthritis. Prior to the modern antibiotic era, mortality and morbidity were exceedingly high following splenectomy. Today, splenectomy is

still an increased risk and postoperative morbidity is frequent. Neutropenia recurs in about 20% of splenectomized patients. Splenectomy may be more effective in patients with high levels of serum granulocyte-binding IgG. [151] With the availability of leukocyte growth factors (granulocyte- or granulocyte/macrophage CSFs), the neutropenia of Felty syndrome has been ameliorated and the overall management of these patients has been improved. Although there have been complications, including reactivation of the arthritis, thrombocytopenia, vasculitis, and skin rash, sustained responses are commonly seen. [152] [153] [154] [155] [156] [157] [158] [159] [160] [161]

Certain similarities are apparent among Felty syndrome, the T- lymphoproliferative disorder, and hairy cell leukemia. Both hairy cell leukemia and the T-lymphoproliferative disorder are malignancies that have a known association with rheumatoid arthritis. [162] Patients with hairy cell leukemia may present with complaints of arthralgias or arthritis. [163] Table 152-3 summarizes some clinical and laboratory findings of these three syndromes.

Coagulation Disturbances

Thrombocytopenia

Thrombocytopenia, usually autoimmune in type, frequently occurs in the rheumatoid disorders, especially in patients with SLE. [164] It has also been reported in dermatomyoisitis, scleroderma, and mixed connective tissue disease. Thrombocytopenia is rare in rheumatoid arthritis, and its presence should alert the consultant to the possibility of a drug-related etiology or an unrelated primary bone marrow disorder. The therapy of autoimmune thrombocytopenic purpura in SLE is similar to that used in the management of idiopathic thrombocytopenic purpura, except that some evidence shows that splenectomy is less likely to be effective in patients with SLE.[165] Patients with SLE who fail corticosteroid therapy should be considered for alternative therapies other than splenectomy. These include cyclophosphamide, azothiaprine, vincristine, danazol, or high-dose intravenous -globulin. The prognostic significance of autoimmune thrombocytopenia in patients with SLE has been a matter of dispute. The platelet count is probably not a prognostic indicator in SLE. [166]

Although thrombotic thrombocytopenic purpura is associated with SLE, the relationship appears to be an example of distinct clinical entities occurring together in an immunologically compromised host, rather than a causal association. In patients with SLE who have neurologic symptoms along with thrombocytopenia, thrombotic thrombocytopenic purpura should be considered. Aggressive plasmapheresis is the treatment of choice and is usually effective. [167] [168]

Thrombocytosis

Thrombocytosis occurs in about one-half of patients with rheumatoid arthritis. The increased platelet count is an acute-phase reactant correlating positively with the sedimentation rate and the clinical activity of the disease. [169] This reactive thrombocytosis is almost never in excess of 1 million/mm^3 . Usually, the increased platelet count is 400,000700,000/mm^3 . The reactive thrombocytosis in these patients is only rarely associated with abnormal bleeding or clotting.

The Lupus Inhibitor

An elevated partial thromboplastin time is frequently found in patients with rheumatoid disorders, especially SLE. Typically, these patients have no history of bleeding nor any familial bleeding tendency. Most often, this elevation of the partial thromboplastin time is due to the presence of a lupus inhibitor. Although the reported case was of a hemorrhagic disorder,[170] this syndrome is generally not associated with bleeding but with an increase of both venous and arterial thrombotic events. In pregnancy, the lupus inhibitor is associated with increased spontaneous abortions, presumably secondary to placental infarctions. The correlation between the lupus inhibitor and an elevated anticardiolipin antibody titer is strong. Anticardiolipin antibody titers are found in about 40% of patients with SLE and 30% of patients with rheumatoid arthritis. Hughes[171] has described an antiphospholipid antibody syndrome in which these elevated antibodies are associated with thrombosis, recurrent fetal loss, and thrombocytopenia. The diagnosis of a lupus inhibitor is easily established.

Because the important clinical association is between the lupus inhibitor and thrombotic events, a program to prevent recurrent thrombosis is essential. Most patients do not have a history of thrombotic events even when their cardiolipin antibody level is quite high. Such patients should not be placed on an oral anticoagulant. However, if there is a history of thrombosis, warfarin should be administered. If thrombosis occurs in patients on warfarin, aspirin or immunosuppressive agents may be tried, although their effectiveness in this setting is not well defined. The management of pregnant patients with the lupus

TABLE 152-3 -- Differential Diagnosis of Syndromes Associated with Rheumatoid Arthritis

	Peripheral Blood	Bone Marrow	Spleen Size	Lymph Node Size	Tartaric Acid Phosphatase Activity	Renal Abnormalities	Cell Markers
Felty syndrome	Absolute neutropenia Cytopenias No characteristic cell	Normal Hypercellular Hypocellular	Splenomegaly prominent	Enlargement common	Negative	Usually absent	Normal or variable abnormalities
T- lymphoproliferative disorder	Lymphocytosis Cytopenias (neutropenia) Characteristic large granular lymphocyte	Lymphocytic infiltration	Splenomegaly present, not prominent	Enlargement rare	Rarely positive	Commonly present	Suppressor cytotoxic T cells $CD2^+$, $CD3^+$, $CD8^+$, $CD16^+$
Hairy cell leukemia	Cytopenias Characteristic hairy cell	Dry aspirate Hairy cells on biopsy	Splenomegaly present, not prominent	Enlargement rare	Positive Nearly pathognomonic	Usually absent	B-cell origin $CD11^+$, $CD19^+$, $CD20^+$, $CD22^+$

inhibitor is the subject of debate. The literature suggests that both aspirin and corticosteroids are useful. [172] [173] However, these agents may be dangerous in pregnancy.

Hematologic Malignancies

An increased incidence of lymphoproliferative disorders has been reported in patients with many of the rheumatoid disorders but most frequently with Sjögren syndrome. The incidence of eventual lymphoma in Sjögren syndrome is >40 times that expected in the general population. The incidence of leukemia and myeloma is two to eight times expected.[174] The association of hairy cell leukemia and the T- lymphoproliferative disorder with rheumatoid arthritis has been discussed previously.

Infectious Diseases

The hematologic consequences of viral, bacterial, and fungal infections are extremely common and protean in nature. A complete review of this relationship is beyond the scope of this discussion, which is restricted to hematologic manifestations of infectious diseases that might typically result in a hematologic consultation. Human immunodeficiency virus infection and hematologic disturbances are discussed elsewhere (Chap. 154).

Viral Infections

Neutropenia

Contrary to common perception, although neutropenia is frequently seen in viral infections, mild leukocytosis, both granulocytic and lymphocytic, is probably more frequent, particularly early in the course of the viral illness. [175] Certain viral illnesses are particularly apt to be associated with neutropenia. They include influenza, hepatitis, rubella, rubeola, adenovirus, coxsackie virus A21, dengue, and mumps. The specific cause(s) for neutropenia is still not well defined. [176] The neutropenia of common viral infections is almost never profound and is generally self-limiting. A hematology consultation is often requested when there is a severe neutropenia ($1,500/mm^3$) that has persisted for >1 or 2 weeks. This situation should be investigated further, including a bone marrow study to rule out a primary bone marrow disorder.

Lymphocytosis

Lymphocytosis occurs frequently in viral disorders. A childhood illness, acute infectious lymphocytosis, often occurs in outbreaks and is usually caused by the coxsackie virus.[177] [178]

Atypical lymphocytosis is characteristically seen in infectious mononucleosis as a result of an Epstein-Barr virus (EBV) infection. The primary cell target of EBV is the B lymphocyte. The response of activated suppressor CDA^+ T cells to the attack on B lymphocytes results in the characteristic large lymphocytes seen in this disorder. Atypical lymphocytosis may also be found in cytomegaloviral disease, hepatitis, mumps, varicella, rubeola, rubella, herpes simplex, herpes zoster, influenza, and roseola infantum. The appearance of infectious mononucleosis-like illness in an older adult may raise a concern about a lymphocytic leukemia, usually of the acute variety. In this uncommon setting, lymphocyte phenotyping and bone marrow evaluation will quickly resolve the concern. Some patients maintain the clinical and serologic manifestations of infectious mononucleosis for some period following the acute infection. [179] This sequela is rare but may especially be seen in families with a genetic disposition to disorders such as the X-linked lymphoproliferative syndrome. [180]

Thrombocytopenia

Mild-to-moderate thrombocytopenia is frequently seen in patients with measles, dengue, varicella, rubella, rubeola, mumps, EBV disease, and cytomegaloviral disease. Thrombocytopenia is rare in hepatitis B and in herpes simplex virus. Important bleeding events are rare in this viral-associated thrombocytopenia. Idiopathic thrombocytopenic purpura occurs in the aftermath of a viremia in children. Usually this complication is self-limited, but at times the thrombocytopenia is severe and prolonged, and intervention is necessary. In children, corticosteroid therapy is usually successful, and splenectomy is only rarely needed. DIC has occurred during the course of viral infections, especially rubella, rubeola, varicella, vaccinia, and variola. This is often a frightening complication unexpectedly accompanying a seemingly common childhood viral infection, but it is rarely fatal. [181] Hemorrhagic complications may also be alarming in other viral infections such as those caused by the arbovirus, the adenovirus, and the enterovirus. In these settings, fatal reactions have occurred and are attributed to DIC-like mechanisms. [182]

Virus-Related Bone Marrow Failure

Epstein-Barr virus and other viral infections have been reported to cause the hemophagocytic syndrome. This peculiar disorder is characterized by fever, hepatosplenomegaly, lymph node enlargement, rashes, and pancytopenia. Bone marrow study reveals hemophagocytosis and histocytic hyperplasia. It may be a self-limiting process, but death can occur.[183] A fatal outcome is frequent when this illness occurs in patients with malignancies.

Hematologic abnormalities have been associated with uncomplicated hepatitis, including leukopenia, thrombocytopenia, anemia with macrocytosis, and atypical lymphocytosis. Fatal aplastic anemia is a rare occurrence (0.22%) in patients with infectious hepatitis. [184] Conversely, patients with aplastic anemia may have an antecedent history of abnormal liver function tests, suggesting a subclinical hepatitis. Most cases of aplastic anemia, especially the fatal ones, occur within 6 months of the onset of the hepatitis and often when the illness is improving. Bone marrow transplantation after high-dose chemotherapy is effective in 7080% of these patients.[185] Transient aplastic crises due to infection with the parvovirus B19 has been described in patients with sickle cell disease and hereditary spherocytosis. In

hematologically healthy children, parvovirus B19 causes fifth disease. In adults, this virus causes arthralgias. Parvovirus B19 has been described as the causal agent for pure red cell anemia and transient aplastic anemia in immunodeficient patients. [186] The myriad of hematologic manifestations of human immunodeficiency virus positivity and the acquired immunodeficiency syndrome are discussed elsewhere (Chap. 154).

Bacterial and Fungal Infections

Neutrophilia

Neutrophilia is the hallmark of bacterial infection. Children have higher white counts in response to these infections than do adults. Neutrophil counts of 12,00015,000/mm^3 are usual in moderate and localized infections. Systemic infections are commonly associated with neutrophil counts of 15,00025,000/mm ^{3} , and in severe pyogenic infections neutrophil counts may reach 50,00075,000/mm ^{3} . Neutrophilic leukemoid reactions (>50,000/mm ^{3}) in adults can be confused with chronic myeloid leukemia. The presence of known infection and the finding of an elevated leukocyte alkaline phosphatase generally excludes chronic myeloid leukemia. Usual causes of leukemoid reactions include metastatic disease of the bone marrow, Hodgkin disease, and carcinoma of the stomach, breast, liver, and lung. When evaluating patients with neutrophilia of uncertain origin, a review of the morphologic changes in the peripheral blood film may be helpful. Infection as the etiology of the neutrophilia should be considered when findings such as toxic granulation, cytoplasmic vacuolization, Döhle bodies, and the Pelger-Huët

anomaly are found. However, these morphologic changes are more commonly seen when infection is obvious. They are also found in a diverse variety of noninfectious states, including myeloproliferative disorders, burns, pregnancy, and drug reactions. [187] Most fungal infections do not produce neutrophilia.

Neutropenia

Neutropenia is unusual in patients with bacterial infections. However, it is found with some frequency in patients who have salmonellosis, brucellosis, pertussis, rickettsial infections, disseminated tuberculosis, and disseminated histoplasmosis, as well as in the presence of overwhelming infection. Neutropenia in overwhelming infection is an indication of exhaustion of the bone marrow neutrophil reserve and is generally considered a poor prognostic sign. Usually the neutropenia associated with bacterial infections is modest and not clinically important. Severe neutropenia in patients with bacterial infection is more likely to occur in the elderly, in alcoholics and other drug abusers, and in patients who are myelosuppressed. The therapeutic value of granulocyte- and granulocyte/macrophage-CSF for the amelioration of severe neutropenia in these settings is under investigation. Most fungal infections do not produce neutropenia.

Lymphocytosis

Lymphocytosis >4,000/mm^3 is seen most often in pertussis. In this infection the lymphocytosis is usually 15,00025,000/mm ^{3} , but occasionally it may rise to 150,000/mm^3 . Other bacterial infections commonly associated with lymphocytosis are rickettsial illnesses, tuberculosis, syphilis, and burcellosis. Lymphocytosis is often seen as part of a neutrophilic leukemoid reaction. A lymphocytic leukemoid reaction has rarely been seen in patients with tuberculosis.

Lymphocytopenia

Lymphocyte counts <1,000/mm^3 are common in many acute infections, particularly when marked neutrophilia is present. Tuberculosis, brucellosis, and disseminated histoplasmosis infections frequently cause lymphocytopenia. Lymphocytopenia in elderly patients with a bacterial infection is a poor prognostic sign. [188]

Monocytosis

A low-grade monocytosis of >800/mm^3 often accompanies the recovery phase of acute infections. Of patients with disseminated tuberculosis and patients with subacute bacterial endocarditis, 1520% have a monocytosis. [189] [190] Persistent monocytosis following recovery from an infectious disorder suggests the possibility of an underlying primary marrow disorder and should be investigated.

Eosinophilia

Eosinophilia >350/mm^3 is rare in patients with bacterial or fungal diseases. Exceptions are bronchopulmonary aspergillosis, coccidiomycosis, and chlamydia pneumonitis of infancy. Fungal infections with eosinophilia appear to be related to immune responses, particularly high serum concentrations of IgE. The most common causes of infection-induced eosinophilia are parasites in the gastrointestinal tract. Occasionally eosinophilia from parasitic infections can be quite high and can mimic that degree of eosinophilia seen in the hypereosinophilic syndrome.

Thrombocytosis

Thrombocytosis (500,000700,000/mm^3) is fairly common in some chronic bacterial infections, including tuberculosis, subacute bacterial endocarditis, and osteomyelitis. However, thrombotic events accompanying this degree of platelet elevation are quite rare.

Thrombocytopenia

Any acute bacterial or fungus infection causing bacteremia or fungemia can cause thrombocytopenia. Several mechanisms are responsible for this development, but increased consumption is probably the most common. Tuberculosis, histoplasmosis, and rickettsial diseases may be associated with thrombocytopenia in the absence of bloodstream infection. The finding of thrombocytopenia in bacterial and fungal infections usually raises the specter of DIC. In the absence of this complication, severe bleeding from thrombocytopenia in acute bacterial and fungus infections is rare. However, severe infections are the most common cause of DIC, particularly in those caused by gram-negative bacilli. Fungal infections are only rarely associated with DIC. The prognosis of infection-induced DIC is relatively good because of the likelihood of control of the infection with appropriate antibiotic therapy. Platelet transfusions are valuable while the infectious etiology is being treated. Extreme forms of DIC, such as that seen with purpura fulminans, may prove fatal. [191]

Anemia

A significant anemia is uncommon in acute bacterial and fungal infections. An exception is the complication of a hemolytic anemia seen with a *Clostridium welchi*i or *Bartonella bacilliformis* infections. The anemia of chronic bacterial infection is usually the result of ACD.

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Chapter 153 - Hematologic Manifestations of Infectious Disease

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INTRODUCTION

Many catastrophic infections may affect the patient with hematologic-oncologic diseases. From the fungal lung infections of the acute leukemia patient to the *Salmonella*-associated osteomyelitis of the patient with sickle cell disease, the infectious disease consultant is of tremendous support in the overall management of hematologic diseases. Just as important is the role of the blood specialist in assisting the clinician in managing the various hematologic manifestations of infections. Some infectious diseases may cause a number of different, and occasionally opposite, blood dyscrasias. For example, tuberculosis, depending on its degree of severity, may present as pancytopenia with myelophthisis in one individual, but may mimic acute or chronic myeloid leukemia (AML, CML) in another. *Aspergillus* infection can cause disseminated intravascular coagulation (DIC) in one patient and thrombosis in another. A wide range of abnormalities and effects on primitive and adult hematopoietic cells occur with bacteria, viruses, fungi, and protozoa [1] and are discussed in this chapter. Infection with the human immunodeficiency virus (HIV) and the acquired immunodeficiency syndrome (AIDS) are discussed in Chapter 154; infectious mononucleosis is discussed in Chapter 58 .

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ANEMIA

Anemia that results from infection can be categorized according to the same pathophysiologic mechanisms that apply to anemia in general: blood loss, decreased production, and increased destruction (Table 153-1). Anemia secondary to gastrointestinal (GI) blood loss can occur in patients with hookworm infestation and bowel involvement by shigellosis or typhoid. *Helicobacter pylori* has been implicated in gastric and duodenal

TABLE 153-1 -- Pathophysiology of Anemia

Blood loss
Gastrointestinal (hookworm)
Genitourinary (schistosomiasis)
Pulmonary (tuberculosis)
Decreased production
Aplasia
Anemia of chronic disease
Anemia of acute infection
Increased destruction
Intraerythrocyte parasitization
Immunohemolytic conditions
Autoimmune
"Innocent bystander"
Polyagglutination
Enzyme deficiency (G6PD)
Pathologic changes
Hemolytic-uremic syndrome
DIC
Hypersplenism

ulcer disease and may cause upper GI tract hemorrhage,[2] while blood loss can be significant when due to hematuria associated with bacterial cystitis, or with hemoptysis due to large cavitary pulmonary disease associated with tuberculosis or aspergillosis. Blood loss anemia, which is usually acute in these disorders, will be associated with iron deficiency anemia as well if it is chronic.

Most infections can cause some degree of marrow suppression or decreased production. In an acute or self-limited illness, the effect on blood cell counts may be imperceptible. If the illness is prolonged, the blood cell count changes will be more striking. Infection-induced myelosuppression can involve all three hematopoietic lines, resulting in aplastic or hypoplastic anemia, or the red cells alone, resulting in a number of acute and chronic infections.

Aplastic Anemia

Aplastic anemia is extremely uncommon but can result directly or indirectly from an infectious agent. Hepatitis, usually hepatitis C, has been shown to carry a significant risk for aplasia.[3] [4] Approximately 0.10.2% of all patients with hepatitis develop pancytopenia, while 5% of patients with aplastic anemia have a recent history of hepatitis. The incidence is unrelated to the severity of the infection, and the prognosis is grave. The marrow suppression generally occurs during the convalescent phase, and the mechanism is not clear. Certain viruses, among them hepatitis B, are known to be trophic for hematopoietic cells. [5] The ability to transplant compatible marrow cells into affected patients suggests that the virus does not cause damage to the marrow microenvironment. [6]

Other viral illnesses such as HIV infection, infectious mononucleosis, noncytopathic lymphocytic choriomeningitis virus (LCMV), and B19 parvovirus are also associated with aplastic anemia. Although anemia associated with parvovirus usually occurs in individuals with other underlying hematologic conditions, such as hemolytic anemia, immunodeficiency syndrome, or subsequent to transplantation, cases in previously normal individuals have been reported. [7] [8] [9] [10] [11] [12] Human parvovirus 19 is highly trophic to human bone marrow and can replicate in erythroid progenitor cells. It is believed that the occurrence of red cell aplasia is related to the fact that the B19 cellular receptor, globoside, is a blood group P antigen. Experimental studies with B19 parvovirus suggest an inhibitory effect of the organism on erythropoiesis.[7] The addition of infected sera to human marrow culture systems caused significant inhibition of erythropoiesis as measured by colony-forming unit-erythroid and burst-forming unit-erythroid growth. In the specific clinical conditions described previously, patients should be screened for B19 infection, because the viral-induced aplasia resolves quickly with immunoglobulin treatment. [13] [14] The aplasia caused by LCMV may be mediated by the interferon- (IFN-) or IFN-.[15] Replacement of the marrow by the granulomas of tuberculosis and histoplasmosis and the marrow necrosis caused by overwhelming gram-negative and gram-positive infections, mucor, dengue fever, rubella, *Brucella*, or *Salmonella typhi* may result in pancytopenia and marrow failure.[16] [17] [18] In these instances, possible mechanisms for pancytopenia include (1) a direct toxic effect causing marrow necrosis, (2) myelophthisis due to replacement by granulomas, (3) histiocytic hemophagocytosis, or (4) nutritional deficiencies as seen in anorexia nervosa. Indirectly, infectious organisms can interfere with hematopoiesis by activating macrophages or lymphocytes to release inhibitory mediators.[19] Whatever the mechanism of infection-related marrow failure, the incidence is greater in patients previously compromised by poor nutrition, alcohol abuse, or previous exposure to chemotherapy.

Pancytopenia may also result from persistence of congestive splenomegaly related to malaria, infectious mononucleosis, toxoplasmosis, hepatitis, salmonellosis, tularemia, syphilis, subacute bacterial endocarditis, tuberculosis, or schistosomiasis.

Anemia of Chronic Disease

The anemia of chronic disease (Table 153-2) is probably the most common blood dyscrasia to occur as a result of infection. [20] [21] [22] [23] The degree of anemia tends to correlate with the intensity of the fever or the degree of inflammation. The anemia may be normocytic, normochromic or microcytic, hypochromic and is characterized by a decreased reticulocyte count and low serum iron and serum transferrin levels with a slight to moderate decrease in percent saturation, and by decreased marrow sideroblasts but normal to increased body iron stores. The most common infectious diseases resulting in anemia of chronic disease are bacterial or fungal endocarditis, meningitis, empyema, cavitary pulmonary disease, abscess (especially intra-abdominal), chronic peritonitis, chronic osteomyelitis, and chronic

infectious arthritis (Table 153-2). In addition, any chronic infection such as tuberculosis, leprosy, typhoid, tularemia, brucellosis, or Lyme disease can be associated with anemia of chronic disease.

One mechanism for this anemia was initially proposed by Cartwright and Lee. [20] The reticuloendothelial system becomes activated by the chronic infection, resulting in a block in normal iron transfer. The result is that iron released by senescent red cells is picked up by stimulated macrophages and blocked from release to marrow erythropoietic precursors. Thus, marrow stores of iron (in macrophages) are sufficient, but sideroblasts are reduced to absent. Another theory relates to the effect of various cytokines whose activity is enhanced by infection. [21] Interleukin-1 (IL-1) and tumor necrosis factor- (TNF-) are two such proteins. [24] Bacteria and fungi, as well as certain endotoxins and other infectious agent by-products, are enhancers of IL-1 production and release. Among other biologic properties, IL-1 activates neutrophils to release lactoferrin and inhibits liver cells from synthesizing transferrin, thus resulting in decreased serum iron. [25] The IL-1-induced hypoferremia is thought to be a homeostatic mechanism to decrease the virulence of infection. Infusing iron into experimental animals increases the severity of infection, while reducing the iron level by chelation decreases the virulence. [26] [27] A similar mechanism involves the role of TNF in anemia of chronic disease.

TABLE 153-2 -- Anemia of Chronic Disease

Laboratory findings
Normochromic normocytic or microcytic hypochromic
Decreased reticulocyte count
Decreased serum iron
Decreased serum transferrin
Slight to moderate decrease in iron saturation
Slight to moderate decrease in sideroblasts
Normal to increased iron stores
Disorders
Endocarditis (bacterial or fungal)
Meningitis
Emphysema
Cavitary pulmonary disease
Abscess
Chronic peritonitis
Chronic osteomyelitis
Chronic infectious arthritis
Agents
Tuberculosis
Leprosy
Typhoid
Tularemia
Brucellosis
Lyme disease

Anemia of Acute Infection

The anemia of acute infection has been described in children admitted for *Hemophilus influenzae* meningitis. [28] Although this infection is more commonly associated with hemolysis, the reticulocyte count has been reported to be low in some cases, implying marrow suppression. The anemia is usually mild and rebounds without hematinics when the infection is resolved. Other acute infections in children, such as osteomyelitis, arthritis, and pneumonia, can also cause a picture not too dissimilar from anemia of chronic disease.

Hemolytic Anemia

Hemolytic anemia, the rapid destruction of red cells, can occur as a result of infections and can involve many of the same mechanisms classically described for hemolysis in general, such as immune mechanisms, underlying red cell defects, or increased susceptibility to phagocytosis by macrophages in the spleen. [1] [29] Table 153-3 categorizes infection-induced hemolysis into that associated with normal red cells, that associated with an underlying red cell defect such as an enzyme deficiency, and that related to pathologic changes secondary to the infection. These anemias can be clinically quite dramatic, as well as demanding and exciting for the laboratory involved in their evaluation.

Parasitic and Bacterial Infection

Intraerythrocyte parasitization and red cell destruction occur with malaria and babesiosis as well as with *Bartonella bacilliformis* infection. In these disorders, the red cells are lysed, deformed, or removed by the spleen either directly or through immune mechanisms. Malaria-infected tissues release millions of merozoites into the circulation that adhere to erythrocytes through specific red-cell-surface antigens. [30] *Plasmodium falciparum* merozoites adhere to red cell glycophorin and then invade the membrane, creating a route of entry for the parasite. The cytoskeleton structure opens up to allow passage and then

TABLE 153-3 -- Categories of Infection-Related Hemolytic Anemias

Intraerythrocyte parasitization
Malaria
Babesiosis
Bartonellosis
Clostridia perfringens
Immunohemolytic conditions
Autoimmune states
Cold hemagglutinin disease
Mycoplasma
Epstein-Barr virus
Donath-Landsteiner antibodies (paroxysmal cold hemoglobinuria)
Secondary and congenital syphilis
Measles
Measles vaccination
"Innocent bystander"
Hemophilus influenzae
Polyagglutination
Enteric bacteria
Oxidative effect on enzyme-deficient red cells
G6PD deficiency

Pathologic changes
- Hemolytic-uremic syndrome
- Endocarditis
- Hypersplenism
- Disseminated intravascular coagulation

Figure 153-1 Interaction of *Plasmodium falciparum* with a red cell. The parasite creates a depression in the membrane, which becomes thickened at the site of invagination. *(From Aikawa and Miller,[96] with permission.)*

reforms itself after the organism has entered the cytoplasm. Some red cells are lysed immediately by the resulting parasitization, while others, containing the characteristic Wright-Giemsa appearance (Fig. 153-1) and shapes, feed on hemoglobin and produce various proteins. These malaria by-products can protrude through the membrane and cause the cell to adhere to vascular endothelium or other red cells, or both. IgG is attracted to these abnormal proteins and prepares the cell for immune destruction by the splenic reticuloendothelial system. With continued splenic hemolysis, the spleen enlarges, causing increased red cell destruction by trapping and removing partially damaged or deformed erythrocytes. The most serious hemolytic complication is blackwater fever. In rare instances of *P. falciparum* infection, an overwhelming and acute intravascular hemolysis occurs as a result of a direct autoimmune reaction;the Coombs test is positive.

Babesiosis-induced hemolysis is similar to malaria. The disease occurs along the coast of New England, is caused by *Bacillus microti*, and is transmitted from infected deer mice by the tick from the *Ixodes* species. The parasite invades the red cell directly by a complement-mediated mechanism. The symptoms range from generalized malaise to a hectic fever and hemolytic anemia. [31] Infection in splenectomized patients can lead to catastrophic intravascular hemolysis and acute renal failure. [32]

Bartonellosis (Oroya fever) is transmitted by a sandfly, not uncommon in Peru. [33] Within 23 weeks of the bite, fever, chills, and hemolysis occur. The hemolysis is quite dramatic and severe. The organism attaches to the surface of the red cell and may or may not invade the membrane (Fig. 153-2). If untreated, the patient can die from the event.

Although it is well known that bacteria produce hemolytic toxins, rarely is there a direct effect in humans. An exception occurs in *Clostridium perfringens* infection, which can cause severe hemolysis through its -toxin, a lecithinase, which reacts with red cell membrane lipoproteins to release lysolecithin, a red cell-lysing agent. [34] [35] The resultant intravascular hemolysis can be quite sudden and dramatic. Suspicion of this infection is raised after septic abortion, biliary tract disease, traumatic wound infection, or with malignancy. A patient with CML developed a hemolytic crisis secondary to clostridial sepsis. She

Figure 153-2 (A) Wright-stained blood smear from Oroya fever (*Bartonella bacilliformis*). The parasites are pleomorphic, stain bright red, are usually spherical, and can form small chains (× 1,100). *(From Bogemann and Rastetter,[100] with permission.)* **(B)** Scanning electron microscopy showing *Bartonella* burrowing into and penetrating the membranes of red cells during hemolytic septicemia. *(From Benson et al.,[101] with permission.)*

was able to survive for almost 5 hours with a hematocrit of 0% on the oxygen-carrying capacity of free hemoglobin. [36]

Many other severe bacterial infections have been associated with hemolysis. [37] Rapidly falling hematocrits and increased reticulocyte counts without evidence of bleeding have been reported with septicemia and endocarditis due to gram-negative or gram-positive organisms. Intravascular hemolysis has been cited secondary to cholera, while hemoglobinuria has been observed in miliary tuberculosis and some spirochete infections such as those caused by *Borrelia*. The mechanism for these types of hemolysis is unclear, but evidence points to a direct effect of the infectious agent or its by-product.

Immunohemolytic Conditions

A large number of infection-related hemolytic anemias can be due to an immunohemolytic condition. The immunohemolytic conditions can be divided into three categories: (1) autoimmune, (2) antigen/antibody complexes or "innocent bystander" reactions, and (3) polyagglutination. [29] An infectious agent or its by-product can stimulate antibody production with an affinity for a red-cell-surface-specific protein and cause an autoimmune hemolytic anemia (AHA). The agent need not be present. The resultant hemolysis can be either intravascular, secondary to IgM or "cold" antibodies, or extravascular, secondary to IgG antibodies, which are usually of the "warm" type and are directed against one of the Rh antigens. Most of the infection-induced AHAs are of the cold antibody type and are referred to as cold hemagglutinin disease (CHAD). Two of the commonest infections causing CHAD are *Mycoplasma pneumoniae* and Epstein-Barr virus (EBV) associated with infectious mononucleosis. [38] [39] [40] Both infectious agents can result in IgM antibodies directed against the I, i red cell antigens: *Mycoplasma* with anti-I antibodies and infectious mononucleosis with anti-i antibodies. These cold agglutinin antibodies bind to the red cell at low temperatures, fix complement, and cause hemolysis. The incidence of CHAD with *M. pneumoniae* and EBV infections is low, but is aggravated by low external temperatures. Other

infections such as mumps, rubella, cytomegalovirus (CMV), and *Legionella* have also been known to produce CHAD. [41] [42] [43]

In secondary and congenital syphilis, an AHA can result from the production of Donath-Landsteiner antibodies. Even though these proteins are of the IgG class and react against the P antigen of red cells, they do so at cold temperatures, and the resultant clinical picture has been labeled paroxysmal cold hemoglobinuria. [44] In affected patients this can be a chronic illness and may require patients to live in warm climates. Paroxysmal cold hemoglobinuria can also occur as an acute illness following varicella, infectious mononucleosis, mumps, measles, MMR (measles, mumps, rubella) vaccination, parvovirus B19 infection, and *Mycoplasma* infection. [45] [46] During the convalescent stage of these infections or when the patient is exposed to low environmental temperatures, the individual suddenly develops pallor, jaundice, hemoglobinuria, and splenomegaly.

Rarely are infections associated with AHA secondary to warm antibodies. A case of *Chlamydia* pneumonia has been described with IgG antibodies. [47]

The "innocent bystander" mechanism for AHA is caused by the complex of the antigen with the antibody, in proximity to the red cell. An infectious agent or antibiotic medication, or both, can cause antibody formation. The resultant antigen/antibody complex in the bloodstream can nonspecifically attach to the red cell and lead to hemolysis, the red cell being the "innocent bystander." *H. influenzae* type b meningitis in children can cause this type of hemolysis. [48] The antigen of the bacteria and its resultant antibody are simultaneously present in the bloodstream of children with this infection. [49] The protein complex binds in some way to red cells, fixes complement, and causes intravascular hemolysis. Drugs that have been implicated in the "innocent bystander" type of hemolysis include antibiotics such as quinine, quinidine, sulfonamide, *p*-aminosalicylic acid, and sulfanilic acid derivatives.

The third mechanism for AHA has been termed polyagglutination. [29] Antigens that are normally hidden or masked from contact with the red cell membrane, called Thomson-Friedenreich cryptoantigens or T antigens, may become exposed by infectious agent enzyme digestion and make the red cell membrane more susceptible to normal plasma antibodies. [50] For example, neuraminidase, produced by enteric bacteria, can break down red cell surface glycoproteins, unmask the T antigen, and expose the red cell to natural blood IgM antibodies, resulting in cell agglutination or polyagglutination and eventual hemolysis. [51] AHA can be detected by the sudden clinical picture, and by a positive result on an antiglobulin or Coombs test.

Hemolysis Due to Infection in G6PD-Deficient Patients

Normal red cells cope with the oxidative stresses of infections and drugs with the help of reducing enzymes. [52] Four enzymes keep the red cell membrane and hemoglobin in the reduced state. Once oxidized, the membrane becomes rigid and susceptible to extravascular hemolysis within the spleen (Fig. 153-3). Oxidized hemoglobin or methemoglobin form inclusions within the cell (Heinz bodies) that cling to the surface of the membrane and further initiate extravascular hemolysis. The Heinz body-membrane interface is subject to splenic macrophage stimulation that causes the red cell to look as if a piece had been removed, a "bite cell." Of the four enzymes involved, glucose-6-phosphate dehydrogenase (G6PD) is most frequently deficient in various populations. Several common genetic variants such as G6PD ^{A} , G6PD^{Mediterranean} , and G6PD^{Canton} are associated with decreased enzyme activity. Historically, a higher incidence of infection-related hemolysis has been noted in blacks than in whites in the United States, which led to the hypothesis that infection causes hemolysis in G6PD-deficient patients. [53] [54] Infection-activated neutrophils produce superoxide, which converts

Figure 153-3 Pathophysiology of infection-induced hemolysis in G6PD-deficient patients. *(From Beck and Tepper,[102] with permission.)*

hemoglobin to methemoglobin. In the absence of the enzyme, methemoglobin interacts with hydrogen peroxide, Heinz bodies are formed, the red cell membrane becomes rigid, and hemolysis results. [55] [56] [57]

G6PD-deficient patients may not have had any prior clinically evident hemolysis when they first come to medical attention. Affected patients have sudden hemolysis, scleral icterus, and increased reticulocytes, with or without splenomegaly. The blood film may initially show bite cells along with polychromatophilia. Heinz body preparations may be positive if performed during the acute hemolytic episode. G6PD enzyme assays can be falsely normal during the hemolytic period, since enzyme activity is highest in young red cells, in some variants high enough to give a normal result. The cyanide-ascorbate test detects the red cells ability to prevent hemoglobin oxidation by ascorbate and can be sensitive enough to detect as few as 1015% deficient cells on a slide. [58]

The most frequent infectious organisms associated with hemoglobin oxidation are listed in Table 153-4 and include *Salmonella, Escherichia coli*, streptococci, various rickettsiae, and organisms causing viral hepatitis. In addition, influenza A virus has been shown to have a direct lytic effect in vitro on

TABLE 153-4 -- Causes of Hemolysis in Infected G6PD-Deficient Patients

Infectious agents
Salmonella
Escherichia coli
-Hemolytic streptococci
Rickettsia
Hepatitis virus
Influenza A
Medications
Antimalarials
Primaquine
Paraquine
Pentaquine
Sulfonamides
Sulfones
Nitrofurans
Analgesics

G6PD-deficient cells. [59] Many antibiotics and medications used in managing infections are direct oxidants and cause hemolysis in enzyme-deficient patients during convalescence. Table 153-4 lists the more common agents implicated clinically.

Pathologic Changes

The final group of infection-induced hemolytic diseases can be categorized by tissue damage or hypertrophy. Pathologic changes in tissues created directly or indirectly by the infecting organism is the mechanism of hemolysis, as in the hemolytic-uremic syndrome, DIC, bacterial endocarditis, or hypersplenism. The hemolytic-uremic syndrome primarily affects young adults, children, and infants and follows a nonspecific infection. [60] For example, after an upper respiratory tract infection or bout of gastroenteritis, the child experiences the sudden development of hemolysis, thrombocytopenia, and renal failure. Pathologic changes noted in the renal glomeruli are consistent with a thrombotic microangiopathy. The blood film is characteristic of a microangiopathic hemolytic anemia, with fragmented cells, burr cells, helmet cells, and schistocytes. Whatever the inciting agent, endothelial injury occurs and leads to platelet aggregation, microthrombus formation, and the mechanical destruction of platelets and red cells. A direct toxic effect on the hematopoietic cells may occur as well. DIC and thrombotic thrombocytopenic purpura are in the differential diagnosis. Usually no organism can be identified, but documented relationships have been reported with *E. coli* (verotoxin-producing strains), *Shigella*, and *S. pneumoniae*. [61] The *E. coli* verotoxin is known to cause vascular injury, while *Shigella* infections may involve an endotoxin effect, and *Streptococcus*-associated disease may be mediated by the neuraminidase effect on cells through the organisms ability to unmask the T antigens (see previous discussion). [62]

Traumatic valvular hemolysis, a result of high blood flow through abnormal mitral and aortic valves, can occasionally be seen with rheumatic heart disease and in infective endocarditis due to organisms such as *Candida albicans*. [63] Hypersplenism and DIC, other causes of increased red cell destruction, are considered later in this chapter.

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WHITE BLOOD CELL DISORDERS

Leukocyte disorders vary considerably from infection to infection and from individual to individual. A bacterial infection may cause neutrophilia in one instance and neutropenia in another. In addition to the absolute changes in numbers of lymphoid and myeloid cells, morphologic, immunologic, and functional alterations can result from infection.

Leukocytosis

The leukemoid blood picture is of great alarm to the clinician. It is most frequently caused by infection (Table 153-5). [64] [65] Although the definition varies, the basis for the entity is (1) no evidence of leukemia, (2) a white blood cell (WBC) count of >50 × 10^9 /L, with or without (3) the presence of immature cells. With some infections, the added complication of anemia and thrombocytopenia can create a difficult differential diagnostic problem that is solved only by a marrow examination, a leukocyte alkaline phosphatase determination, or cell marker studies.

Infections can mimic myeloid or lymphoid leukemia, acute or chronic. CML is probably the most common diagnosis with infection. A high WBC count with few or no immature cells can be seen with infections accompanying other disorders such as malignancy, inflammatory arthritis, or glomerulonephritis. More difficult to differentiate is the exaggerated response to disseminated

TABLE 153-5 -- Differential Diagnosis of Leukemoid Reaction Conditions

CML
Infection with underlying disorders
Meningitis (*Hemophillis influenzae, Neisseria meningitidis*)
Staphylococcus sepsis
Pneumococcal endocarditis
Diphtheria
Bubonic plague
Tuberculosis
Salmonellosis
AML
Tuberculosis
Pseudoleukemia (rebound from neutropenia)
CLL
Pertussis
Varicella
Viral exanthems
ALL
Tuberculosis
Infectious mononucleosis
Acute monocytic leukemia
Tuberculosis
Myelodysplasia
HIV
Other viruses

tuberculosis, with WBC counts as high as 200 × 10^9 /L, accompanied by a moderate left shift. [66] [67] [68] Similar blood pictures have been seen with staphylococcal septicemia and pneumonia, meningitis secondary to *H. influenzae* and *Neisseria meningitidis*, pneumococcal endocarditis, salmonellosis, diphtheria, and bubonic plague. [69] In the event of an elevated WBC count without a source of infection, it may be necessary to determine the leukocyte alkaline phosphatase concentration and in some instances to obtain a marrow sample for cytogenetic analysis.

An AML picture has also been described with tuberculosis, either in its disseminated form or when the organism invades lymphoid tissue and the spleen. [67] [70] A pseudoleukemic blood picture is not uncommon as a rebound phenomenon in antibiotic-induced agranulocytosis and after marrow suppression from severe bacterial and protozoan infections. This scenario may be extremely difficult to diagnose and requires careful follow-up observations after appropriate therapy.

Chronic lymphocytic (CLL) and acute lymphocytic (ALL) leukemias are less frequently confused with infections. In the pre-antibiotic era, potentially fatal cases of pertussis were reported to have WBC counts as high as 200 × 10^9 /L. [71] Similarly, chickenpox and the viral exanthems may be associated with extremely high mature lymphocyte counts. The age of the patient, associated symptoms, the clinical absence of adenopathy, and the presence of splenomegaly will often suffice to make the diagnosis. Rarely will it be necessary to obtain a bone marrow aspirate or analyze for lymphocyte clonality. Disseminated tuberculosis has also been reported to be associated with high WBC counts, with immature lymphoid cells resembling lymphoblasts and ALL. [68] [72] The blood findings of infectious mononucleosis (see Chap. 58) often instill the greatest fear of a diagnosis of ALL in young adults. The clinical picture, the "atypically atypical" lymphocytes (i.e., a nonuniform population of lymphocytes), in addition to abnormal liver function tests, serologic findings, and a normal marrow, will exclude ALL.

Disseminated tuberculosis may also mimic AML. [93] The recovery phase of many other infections may occasionally give rise to a high WBC count with monocytosis. Myelodysplastic

blood smears and marrow samples are being seen in greater frequency with infection and may be difficult to differentiate from the true myelodysplastic syndromes. HIV infection can cause marrow findings not greatly dissimilar from those of refractory anemia or refractory anemia with excess blasts (see Chap. 71). A leukoerythroblastic picture, immature myeloid cells, and nucleated red cells in the blood can be seen in septicemia, miliary tuberculosis, and histoplasmosis. [74] Less dramatic blood changes in the lymphoid, myeloid, and monocytic cell lineages caused by infection are considered in the following sections. Overall, the best approach

to diagnosing and excluding a primary hematologic problem is careful monitoring during the recovery phase of the infection to be sure of the causal relationship.

Lymphocytosis

Absolute increases in lymphocytes are rarely seen in acute bacterial infections (Table 153-6).[23] [75] Pertussis is the notable exception. Affected infants of <6 months of age have only minimal changes, whereas in >50% of older children the WBC count can rise to 1520 × 10^9 /L during the first 2 weeks of illness and, rarely, into the leukemoid range. *Bordetella pertussis* toxin, known as pertussigen or lymphocytosis-promoting factor, has been shown to increase the lymphocyte count in animal experiments.[76] It is suspected that pertussigen blocks the normal migration of lymphocytes out of the bloodstream and into the tissues. The cells are normal B and T cells.

Rickettsial infections such as scrub typhus, tuberculosis, syphilis, and brucellosis can also cause an absolute lymphocytosis. Acute infectious lymphocytosis varies from a very mild nonspecific illness to a more acute disorder characterized by fever, diarrhea, upper respiratory tract symptoms, central nervous system involvement, and dramatic, absolute lymphocyte counts of 30100 × 10^9 /L. Although no definite etiologic agent is known, an adenovirus and coxsackievirus A with incubation periods of 1221 days have been implicated.

Lymphocytopenia

Lymphocyte counts of <1 × 10^9 /L are quite common in acute hospitalized patients, in infections, sepsis, malaria, HIV infection, and in a number of chronic infections such as tuberculosis, histoplasmosis, and brucellosis. [77] [78] The decreased lymphocyte count (often in association with neutrophilia) probably results from elevated plasma cortisol levels. The degree of lymphocytopenia seems to correlate with the severity of the infection.

Morphologic and immunologic lymphoid changes may occur with disease. Atypical lymphoid cells characterized by an abnormal nuclear/cytoplasmic ratio with resultant increased cytoplasm are not uncommon in infectious mononucleosis, infectious hepatitis, and some chronic infections. Changes in the

TABLE 153-6 -- Infections That Cause Lymphocytosis

Acute infection
Pertussis
Acute infectious lymphocytosis
Infectious mononucleosis
Infectious hepatitis
Toxoplasmosis
Cytomegalovirus
Chronic infection
Tuberculosis
Brucellosis
Syphilis
Congenital
Secondary
Rickettsia

numbers of B and T cells and in the ratio of T-cell subsets are hallmark findings in HIV infection (see Chap. 154) but have also been shown to occur in other viral infections and in bacterial and fungal disease. [79] [80] For example, in acute bacterial infections, T cells of all types can decline in number early in infection, while numbers of B cells rise. Prolonged T-cell lymphopenia in the face of active infection is more frequent in aging patients and is considered a poor prognostic finding. In leprosy and tuberculosis, T-cell subset ratios may change, and decreased cellular immunity has been associated with a decrease in T-helper cells and an increase in T-suppressor cells.[81]

Neutrophilia

Leukocytosis with neutrophilia and a left shift commonly accompanies bacterial infections. In addition, microscopy of blood smears to detect toxic granulation, Döhle bodies, and cytoplasmic vacuoles has a high sensitivity in predicting infectious disease. [82] Most bacteria produce a WBC count of 1214 × 10^9 /L, while systemic infections will have a WBC count as high as 1530 × 10^9 /L, and massive infections such as the leukemoid type are associated with counts of up to 150 × 10^9 /L.[76] Neutrophilia is most common with suppurative infections such as an abscess, empyema, or meningitis, and is usually associated with bacteria (e.g., *Staphylococcus, Streptococcus*). Over two-thirds of cases of infection-related neutrophilia are secondary to bacteria. [1] [83] The lack of an increased WBC count directs the clinician to a problem with the host, or to the type of infectious agent. Most healthy individuals respond with some degree of neutrophilia. Patients with alcohol-related problems may have a limited or no increase in WBC count, similar to the malnourished patient and a high percentage of the elderly. Infections with *Chlamydia*, fungi, *Mycoplasma*, and rickettsiae may have little effect on the WBC count. The neutrophilic response results from increased release from the marginal pool secondary to endogenous catecholamines and increased levels of IL-1. [84] Animal research has demonstrated an almost immediately accelerated egress of neutrophils from the storage pools into the blood after bacterial innoculation. [85]

Neutropenia/Agranulocytosis

Neutropenia, defined as absolute WBC counts of <1.5 × 10^9 /L, can occur paradoxically in almost any bacterial infection, but is more likely to occur in patients with compromised marrows as a result of chemotherapy, nutritional deficiency, or chronic debilitation. Certain viral, rickettsial, and protozoan infections may also be associated with low counts, whereas it would be unusual to find neutropenia in fungal infections (Table 153-7).[1] Were neutropenia to occur during the second week or recovery phase of the infection, it might be difficult to distinguish between a drug-related and an infection-related cause. Medications such as analgesics, sulfonamides, and other microbials can be implicated. Agranulocytosis is rare with infections and is more commonly caused by antibiotic therapy. In immunosuppressed patients, however, agranulocytosis (with or without red cell aplasia) and thrombocytopenia may occur with parvovirus B19 and other viral infections such as measles.[14] [86]

Bacterial infections such as typhoid, paratyphoid, and tularemia may cause an initial rise in neutrophils, and then a fall during the bacteremic phase of the disease. [87] Low counts are typical of many viral infections. Infectious hepatitis, HIV infection, yellow fever, influenza, measles, Colorado tick fever, and many others characteristically manifest with neutropenia. Well-documented instances of neutropenia have been shown to be caused by experimentally induced sandfly fever (Fig. 153-4)[88] and infectious hepatitis (Fig. 153-5). Almost 90% of patients with sandfly fever have neutropenia during the course of the disease.

The cause of infection-related neutropenia is either a direct

TABLE 153-7 -- Infections That Cause Neutropenia

Bacterial
Salmonellosis
Tularemia
Brucellosis

Viral
Measles
Chickenpox
Rubella
Influenza
Infectious hepatitis
Yellow fever
Sandfly fever
HIV
Colorado tick fever
Dengue fever
Rickettsial
Rickettsial pox
Rocky Mountain spotted fever
Protozoan
Malaria
Kala-azar
Relapsing fever
Massive
Miliary tuberculosis

Figure 153-4 Changes in white blood cell, neutrophil, and lymphocyte counts in experimentally induced sandfly (*Phlebotomus*) fever. *(From Sabin and Paul,[86] with permission.)*

Figure 153-5 Changes in white blood cell, neutrophil, and leukocyte counts in experimentally induced infectious hepatitis. *(From Havens and Marck,[103] with permission.)*

toxic effect on growth or increased utilization. With sepsis, neutrophils are mobilized, and the circulating pool decreases acutely before the accelerated marrow egress can compensate. Many individuals will overshoot, with a marrow rebound, producing a pseudoleukemic or leukemoid picture. There is some speculation that neutropenia is a result of inhibition of growth factors, a decrease in colony-stimulating factors, or lysis of WBCs through activation of the complement system. [19]

Neutrophil Morphologic Abnormalities

Some infected patients may show no change in the number of myeloid cells but, instead, either a shift to the left, with increased bands and metamyelocytes, or morphologic changes such as toxic granulations, cytoplasmic vacuolization, or the formation of Döhle bodies. Many elderly patients with infections such as community-acquired pneumonia may not have leukocytosis. Those individuals with neither fever nor a WBC response are at higher risk of death from the infection. With massive bacterial infection, organisms are rarely seen within the neutrophil cytoplasm, and nuclear changes can result, such as the Pelger-Huët anomaly. In patients with frequent infections, especially children, one should be alerted to the possibility of an inherited or acquired functional neutrophil abnormality. These are considered elsewhere (see Chap. 41) and include problems in phagocytosis, motility, chemotaxis and adherence, and bactericidal activity.

Monocytosis

Monocytosis, defined as WBC counts of >0.95 × 10^9 /L, may be seen with a subacute or chronic infection such as tuberculosis, subacute bacterial endocarditis, syphilis, and brucellosis, as well as with many rickettsial and protozoan infections such as malaria, typhus, trypanosomiasis, and kala-azar. [23] Varicella zoster virus infection can cause a striking monocytosis. Monocytosis may also develop transiently during the recovery phase of any infection. In tuberculosis, the monocytosis may reflect a high rate of turnover and new tubercle formation. The monocyte/lymphocyte ratio (M/L) may be a useful measure of the activity of the tuberculosis infection. [89] With activity, the M/L increases owing to an absolute increase in monocytes, and the ratio may exceed 1, (normal = 0.3). A ratio of >1 has been associated with active exudates and a poor overall prognosis. [90] The healing phase is characterized by an increase in lymphocyte count, a decrease in monocyte count, and a return to the normal M/L.

Cells resembling histiocytes or macrophages may accompany monocytes in chronic infections, and the monocytes may become vacuolated. A bedside diagnostic test for subacute bacterial endocarditis consisted of obtaining a blood smear from the ear lobe and demonstrating abundant macrophage-like cells. [91]

Eosinophilia

Eosinophilia, defined as a WBC count of >0.25 × 10^9 /L, is frequent in parasitic infections that invade tissues, such as trichinosis and spirochetal and echinococcal disease, but is rare in bacterial and fungal infections. [92] In schistosomiasis, filiariasis, gnathostomia, or invasion by the liver fluke *Clonorchis sinensis* or *Capillaria hepatica*, eosinophilia is frequently observed. Intestinal parasites are less likely to stimulate eosinophils if tissue invasion does not occur. In patients with bronchopulmonary aspergillosis, coccidioidomycosis, and *Chlamydia* pneumonitis of infancy, absolute eosinophil counts may range from 0.5 to 1.0 × 10^9 /L, with rare elevations to as high as 44 × 10^9 /L. A mild eosinophilia has also been seen in the early phases of scarlet fever.

Eosinopenia

Eosinopenia correlates with the severity of various infections. The absence of eosinophils in infection indicates a bad prognosis. The mechanism of eosinopenia is probably not corticosteroid related but due to other chemotactic factors such as C5a, which causes intravascular cell destruction, tissue margination, or intravascular margination.[93] Cytokine inhibition may also play a role in decreasing marrow production and release. Basophils are rarely affected by infection and in general are not part of the leukemoid blood picture. Basophilia should alert the clinician to a chronic myeloproliferative disorder.

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PLATELET AND CLOTTING DISORDERS

Thrombocytopenia

Thrombocytopenia is a frequent complication of septicemia due to any bacterium, virus, or fungus and can be life-threatening. [1] [94] [95] Of patients with bacteremia, >65% have thrombocytopenia of some degree, while 33% will have a more significant drop to levels of $<50 \times 10^9$ /L. Despite the high incidence of abnormal counts, the thrombocytopenia is rarely associated with clinical bleeding unless simultaneous evidence of DIC is present. Occasionally a severe infection with bloodstream involvement (e.g., peritonitis, pneumonia, and abscess) can cause a drop in the platelet count.

The mechanism of thrombocytopenia without DIC may be due to (1) decreased production, (2) increased platelet utilization, (3) binding of platelets to exposed and infected subendothelial tissue, (4) clumping by endotoxin, or (5) an immune phenomenon. Infection-induced immune thrombocytopenia may be IgG mediated and caused by gram-positive and gram-negative septicemias or the development of platelet aggregates and subsequent lysis by complement or infection-related immune complexes.

Chronic infection with hepatitis C may produce a significant autoimmune reaction to platelets in up to 41% of affected patients. Elevated titers of platelet-associated IgG are usually detected and presumed to be the cause of the low platelet count.

Thrombocytosis

Thrombocytosis, with counts frequently as high as 500700×10^9 /L, is seen in chronic infections such as tuberculosis, osteomyelitis, and subacute bacterial endocarditis, as well as during recovery from a variety of fungal and bacterial infections. [96] [97] Despite reactive platelet counts as high as $9001{,}000 \times 10^9$ /L, thrombosis is rare, and no anticoagulation is necessary. The mechanism of reactive thrombocytosis is poorly understood. Some patients have impaired splenic function, which raises the possibility of a hyposplenic state with resultant increased platelets, Howell-Jolly bodies, and target cells.

DIC and Vascular Purpura

Up to 65% of all cases of DIC occur secondary to infections. [98] Serious infections often lead to the development of rapid and massive activation of the coagulation system. DIC can be caused by tissue necrosis, endothelial surface damage, and blood stagnation, all potential complications of infectious diseases. In Rocky Mountain spotted fever, for example, endothelial damage is the pathogenic mechanism. Many causative agents, bacterial, viral, parasitic, and fungal, are implicated in DIC (Table 153-8). Most impressive clinically are purpura fulminans and the Friderichsen-Waterhouse syndrome, comprising the trio in meningococcemia of vascular collapse, mucosal and cutaneous hemorrhage, and bleeding into the adrenal gland. In addition to the direct effect of the infecting agent, bacterial products and toxins such as meningococcal endotoxin can cause endothelial

TABLE 153-8 -- Infections That Cause DIC

Bacteria *Neisseria meningitidis* *Staphylococcus aureus* *Streptococcus pneumoniae* *Escherichia coli* *Neisseria gonorrhoeae* *Mycobacterium tuberculosis* *Salmonella typhimurium*
Rickettsia infection
Mycoplasmosis
Histoplasmosis
Aspergillosis
Malaria

destruction. [99] These endotoxins cause a necrotizing vasculitis, eventual vascular collapse, and a picture of DIC.

Thrombotic Complications

Thrombotic complications are infrequent results of usual and isolated infections, but the coexistence of infection and an endothelial injury can lead to platelet deposition and clot development. Endothelial injury can occur at an intravenous line or catheter site in a hospitalized patient or an intravenous drug abuser. A suppurative thrombophlebitis in the veins of the arm or leg or in the jugular, portal, or pelvic veins can result. An embolic phenomenon can result, but would be more likely in clinical situations such as fungal endocarditis with aspergillosis.

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Chapter 154 - Hematologic Manifestations of HIV Infection

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INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) is caused by infection with the human immunodeficiency virus (HIV). This retrovirus produces a slow and usually progressive deterioration in the host immune system that in its most advanced stage is complicated by particular opportunistic infections, neurologic disorders, and neoplasms. As will be described, hematologic abnormalities occur in the majority of individuals during the course of this infection, reflecting alterations in the host immune system as well as complications of secondary infections, malignancies, and therapy. Evidence has also implicated a direct role for HIV infection itself on some hematopoietic precursor cells and other cells in the bone marrow. This discussion presents a general overview of the basic epidemiologic, virologic, and immunologic aspects of HIV infection, followed by a more specific discussion of its hematologic complications.

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DEFINITION AND EPIDEMIOLOGY

A diagnosis of AIDS is established when HIV infection is complicated by particular opportunistic infections, neoplasms, or neurologic disorders. The current definition of AIDS includes a number of clinical complications that are listed in Table 154-1 .[1] In addition, this surveillance case definition has been expanded to include individuals with laboratory evidence of severe immunodeficiency, as manifested by a reduction in the number of CD4+ T lymphocytes to <200/l or in the percentage of CD4+ T lymphocytes to <14.[1] It is clear, however, that AIDS is simply an advanced stage in a spectrum of clinical disorders that occur following HIV infection.

An earlier classification system defined a symptomatic state of HIV infection as AIDS-related complex (ARC), characterized most commonly by persisting generalized lymphadenopathy with or without a number of nonspecific complaints, including arthralgias, fatigue, intermittent diarrhea, or chronic sinusitis.

TABLE 154-1 -- CDC Classification of Clinical Categories for HIV Infection

Category A. Acute HIV infection, asymptomatic infection, persisting generalized lymphadenopathy
Category B. Symptomatic HIV infection (excluding conditions in Category C) complicated by conditions attributable to compromised cellular immunity (i.e., idiopathic thrombocytopenic purpura, thrush, listerosis, peripheral neuropathy)
Category C. AIDS (Any one of the following laboratory or clinical criteria)
Laboratory Criteria
CD4+ T-cell number <200 /l
CD4+ % of lymphocytes <14
Clinical criteria
Opportunistic infections
Candidiasis of bronchi, trachea, lungs, or esophagus
Coccidioidomycosis, disseminated or extrapulmonary
Cryptococcosis, extrapulmonary
Cryptosporidiosis, chronic intestinal
Cytomegalovirus (other than liver, spleen, or nodes)
Herpes simplex (chronic ulcers, bronchitis, esophagitis)
Histoplasmosis, disseminated or extrapulmonary
Isosporiasis (chronic intestinal)
Mycobacterium avium complex or *kansasii*, disseminated or extrapulmonary
Mycobacterium tuberculosis, pulmonary or extrapulmonary
Mycobacterium, other species (disseminated or extrapulmonary)
Pneumocystis carinii pneumonia
Pneumonia, recurrent
Salmonella septicemia
Toxoplasmosis of brain
Neoplasms
Cervical cancer, invasive
Kaposis sarcoma
Lymphoma (non-Hodgkins)
Central nervous system
Encephalopathy, HIV-related
Progressive multifocal leukoencephalopathy
Wasting syndrome due to HIV
Modified from Centers for Disease Control.[1]

Although in the current revised definition, many of these individuals would now be defined as having AIDS if their CD4 cell number were <200/l, the term ARC has been used frequently in early studies of the symptomatic stages of HIV infection and will be used periodically in this review.

The pandemic of AIDS and HIV infection continues to be an international health crisis with, as of 1996, approximately 30 million people infected with HIV worldwide, including 5.8 million people infected in this year alone. [2] AIDS continues to devastate many countries in the developing world, including Southeast Asia, India, Latin America, and particularly sub-Saharan Africa, where it was estimated in 1996 that 7% of the adult population was infected by HIV. [2] [3] In the U.S. there have been >600,000 cumulative cases of AIDS diagnosed since the beginning of the epidemic in 1981 and >400,000 deaths. [4] Although the annual incidence of AIDS-related deaths has recently declined in the U.S., largely due to the beneficial effects of combination antiretroviral therapy, AIDS remains a leading cause of death for persons aged 2544.[4] Changes in the distribution of cases in the U.S. have continued to note an increase in the proportion of individuals who have become infected through

heterosexual contact, particularly adolescents and black and Hispanic women in urban areas. [4]

Although HIV has been cultured from a variety of body fluids, including blood, semen, saliva, urine, and tears, [5] infection occurs almost exclusively by sexual contact (homosexual and heterosexual), by parental inoculation of infected blood or blood products, and perinatally from mother to infant. [5] [6] [7] [8] Infection has also occurred as a result of skin and/or mucous membrane contact with blood from an infected individual, although this mode of transmission appears to be exceedingly uncommon.[9] Overall, the incidence of seroconversion to HIV following occupational exposure via a needle stick has been estimated to be approximately 0.3%. [10]

Two major groups of retroviruses have been associated with AIDS. HIV-1 is the cause of the AIDS pandemic in Europe, Central Africa, Asia, and the Americas, while a closely related but molecularly distinct virus, HIV-2, has been isolated from AIDS patients originating from West Africa, where this virus has been shown to be endemic.[11] Prospective epidemiologic studies have indicated that HIV-2 may be less pathogenic in vivo than HIV-1 with respect to the development of immunologic abnormalities and disease.[12] However, in view of the long time interval between infection and the development of AIDS, which for HIV-1 may average 10 years or more, longer periods of follow-up will be needed before the true pathologic potential of HIV-2 is known. Several different isolates of HIV-related simian immunodeficiency viruses (SIVs) have been obtained from a number of nonhuman primate species in Africa and have been particularly useful models for studies of AIDS-like illnesses in nonhuman primates and for attempts to develop an HIV vaccine. [13] Molecular studies of healthy humans in west Africa have identified variants of HIV-2 that are closely related to some SIVs, suggesting a zoogenic origin, at least for HIV-2. [14] The origin of HIV-1 is still unknown, but viruses related to HIV-1 have been isolated from chimpanzees and other nonhuman primates, strongly suggesting that HIV-1 also originated from nonhuman primates. [15] [16]

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GENETIC ORGANIZATION OF HIV AND THE VIRAL LIFE CYCLE

The genetic organization of HIV-1 (Fig. 154-1) is remarkably complex compared to other retroviruses and contains 9 genes. Similar to other retroviruses, HIV-1 contains *gag*, *pol*, and *env* genes that encode viral core proteins, enzymes (reverse transcriptase, integrase, and a viral protease), and envelope glycoproteins, respectively. In addition, the viral *tat* and *rev* genes encode regulatory proteins that play critical roles in regulating the transcription and processing, respectively, of viral messenger RNA.[17] HIV-1 also contains a number of accessory genes (*nef, vif, vpr,* and *vpu*) that either mediate interactions with the host cell or affect the infectivity of the viral particle. [18] The viral Nef protein reduces expression of cellular CD4 and HLA class I molecules on the cell surface and also increases virus infectivity.[19] [20] [21] Vif appears to affect viral assembly in a way that is poorly defined and enhances viral infectivity on particular target cells. [22] Vpr is incorporated into the viral particle, facilitates the postentry targeting of viral RNA/DNA complexes to the nucleus, and induces cell-cycle arrest in infected cells. [23] [24] Finally, the viral Vpu protein induces CD4 degradation in the endoplasmic reticulum and enhances virus release from the cell surface. [18] [25]

The structure of the HIV virion and the viral life cycle at the cellular level are shown in Figures 154-2 and 154-3 . The mature virus particle contains two coding strands of RNA and the enzyme reverse transcriptase that are packaged in the viral core by the *gag* proteins, p24, p17, p9, and p7. This core is surrounded by a cell-derived lipid membrane into which trimers of the viral envelope glycoproteins gp120 and gp41 are inserted. Gp120 binds with high affinity to the CD4 molecule that is expressed on the surface of a number of cells that play essential roles in the function of the immune system. [26] In addition to the CD4+ subset of T lymphocytes, these cells include monocytes, follicular dendritic cells, and Langerhans cells, all of which have been shown to be infectable by HIV. [27] [28] [29] [30]

Figure 154-1 Genetic organization of HIV-1. The nine genes of HIV-1 are shown. Structural proteins of the virion and enzymes required for replication are encoded by Gag, Pol, and Env (darkly shaded regions); regulatory proteins (cross-hatched areas) are encoded by Tat and Rev, arising from doubly spliced messages, and Nef. The *vif*, *vpr*, and *vpu* genes are dispensable for viral replication in vitro but are essential for viral infection in vivo. Two identical long terminal repeats (LTRs) are present at the 5 and 3 end of the genome and contain sequences critical for viral transcription and activation by cellular and viral factors.

Although binding of gp120 to CD4 is required for attachment of the virion to the cell surface, it has been apparent for many years that interactions with other cellular molecules are required for HIV to traverse the plasma membrane.[31] [32] Recently members of the chemokine receptor family of proteins have been shown to associate with CD4 to form a functional receptor complex for this virus.[33] Chemokine receptors are seven membrane-spanning domain, G-protein-coupled receptors that are widely expressed on CD4-positive and -negative hematopoietic cells and that in conjunction with their physiologic ligands, the chemokines, induce chemotaxis, extravasation, and activation of a wide variety of leukocytes. [34] Binding of the viral envelope glycoprotein to CD4 is thought to induce conformational changes that facilitate a subsequent interaction of the envelope protein with a chemokine receptor, leading to a disruption of the cellular membrane and viral entry (Figure 154-3). Differences among HIVs in their ability to infect various CD4+ cell types have largely been explained by the particular type of chemokine receptor used by an HIV isolate.[33] Thus, HIVs that preferentially infect macrophages interact with the chemokine receptor CCR5, while viruses that use CXCR4 are able to infect CD4+ T-cell lines, which characteristically express high levels of this receptor. As described later, during the course

Figure 154-2 Structure of the HIV virion. Two coding strands of genomic RNA are packaged in a nucleoid core with the p7, p9, and p24 proteins and reverse transcriptase. The core is surrounded by the p17 matrix protein lining the inner surface of the envelope glycoprotein. The envelope consists of a lipid bilayer derived from the infected cell and glycoprotein spikes that consist of the outer gp120 molecule, which contains the binding site for the CD4 molecule, and gp41, which serves both to anchor the glycoprotein complex to the envelope and to mediate fusion of the viral membrane with the cell membrane during viral entry.

Figure 154-3 HIV life cycle. Binding of the virion to the cell surface is mediated by a specific interaction of the gp120 envelope glycoprotein with the cellular CD4 molecule and a member of the chemokine receptor family of proteins (usually CCR5 or CXCR4). Penetration occurs as the viral membrane fuses with the cellular membrane in a process that requires the gp41 envelope molecule. The viral capsid is uncoated and viral genomic RNA is reverse transcribed and duplicated by the viral reverse transcriptase to produce a double-stranded copy of viral DNA. The DNA duplex is translocated to the nucleus, where it circularizes and integrates into host chromosomes. Following appropriate activating signals, the provirus is transcribed by cellular RNA polymerase and transported to the cytoplasm. Proteins are translated and processed through biochemical steps that, depending on the protein, involve glycosylation (envelope), cleavage (envelope, Gag, Pol), myristoylation (p17), and phosphorylation (Rev, Nef). Packaging of genomic RNA with viral proteins occurs as envelope glycoproteins are inserted into the cell membrane, and a new virion subsequently buds.

of infection, HIV evolves in vivo to use different chemokine receptors, and the type of chemokine receptor used represents an important prognostic indicator. [35] [36] In addition, HIV has also been shown to be able to infect some CD4-negative cells, either by using alternative receptors such as the glycolipid galactosyl ceramide [37] or by interacting directly with a chemokine receptor in the absence of CD4. [38] [39] Although CD4-independent use of chemokine receptors can markedly expand the viral host range in vitro, the relevance of these findings to pathogenesis remains to be determined.

Following adsorption of the virion to the cell surface, HIV penetrates the cellular plasma membrane and is uncoated in the cytosol. Viral RNA is reverse transcribed to single- and then double-stranded DNA by reverse transcriptase and transported as a preintegration complex to the cell nucleus, where it circularizes and, through the action of the viral integrase, is inserted at random positions in host chromosomes. [40] [41] [42] Transcription of this provirus leads to the production of genomic RNA as well as viral proteins, and following an obligatory processing step in which the Gag and Pol precursor proteins are cleaved by the viral protease, the virion assembles at the inner surface of the plasma membrane, buds outward from the infected cell, and is released as an infectious particle. A number of factors have been shown to upregulate virus production markedly, including cellular activation, transacting factors of other viruses, and cytokines. [43] These cofactors, along with the balance of viral regulatory gene products Tat and Rev, could have relevance in vivo in determining the extent of virus production in an infected individual. [43] As described in a later section, combinations of antiretroviral drugs that target the viral reverse transcriptase and protease enzymes have recently been shown to have potent antiviral effects as a result of their synergistic inhibitory effects on viral replication. [44] [45]

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PATHOGENESIS OF HIV

The mechanism by which HIV infection produces immunodeficiency in vivo remains under intense investigation and likely reflects a complex interplay of viral and host factors. Central to the evolution from asymptomatic HIV infection to full-blown AIDS is a gradual increase over time in the systemic viral load that leads to a progressive deterioration of cellular immunity. [46] In spite of active cellular and humoral immune responses, HIV continues to replicate actively throughout the course of infection, and virus is readily detectable in peripheral blood and lymph nodes at all stages of infection. [47] [48] Studies have shown that this persistent production of virus is a highly dynamic process that involves ongoing infection of new target cells and a rapid turnover of both viral particles and infected cells. [49] [50] It is estimated that 10^9 10^{10} virus particles are produced every day with a half-life of <6 hours. [49] [51] Although the vast majority of virus-producing cells have a half life of 1 day [51] and are likely killed by HIV infection itself, additional populations of infected cells, including macrophages and memory CD4+ cells, turn over more slowly and appear to represent long-term reservoirs of infected cells. [51] [52] [53] The steady-state level of viral RNA in plasma represents an important prognostic indicator and in addition to the CD4 cell number is highly predictive of the development of AIDS. [54] A transition from macrophage tropic to T-cell line tropic HIVs in an infected patient is also a poor prognostic finding, reflecting a change in chemokine receptor usage from viruses that use CCR5 to ones that use CXCR4. [35] [36]

Central to the immune abnormalities in AIDS are both quantitative and qualitative [55] [56] [57] [58] abnormalities in CD4+ lymphocytes. Proposed mechanisms for the depletion of these cells are listed in Table 154-2 and include direct effects of viral infection on mature and progenitor CD4 cells, as well as the destruction by cellular or humoral mechanisms of uninfected CD4 cells that display adsorbed or processed viral antigens on their surface. [59]

TABLE 154-2 -- Possible Mechanisms for Depletion of CD4 Lymphocytes During HIV Infection

1. Direct killing of CD4 cells by HIV
2. Syncytia formation between infected and uninfected cells
3. Cytotoxic humoral or cellular immune responses to gp120 adsorbed on uninfected CD4 cells
4. Induction of programmed cell death (apoptosis) by the interaction of gp120 with CD4 molecule
5. Defective maturation of CD4+ cells in the thymus
6. Infection and killing of lymphoid stem cells or supporting cells required for stem cell proliferation and/or differentiation
7. Antilymphocyte antibodies

Several reports have noted that the attachment of gp120 to CD4 on the surface of T lymphocytes can lead to programmed cell death or apoptosis when these cells are subsequently stimulated through their antigen receptor or when CD4 is crosslinked by anti-gp120 antibodies. [60] [61] Moreover, animal models have indicated that CD4+ cell depletion may also result from defective maturation of CD4+ T cells in the thymus. [62] In addition, immune defects are present prior to a severe depletion in CD4 lymphocytes and may reflect an imbalance of particular T-lymphocyte subsets resulting from a selective effect of HIV on memory as opposed to naive T cells. [56] [63] [64] Studies have suggested that memory but not naive CD4 cells express the chemokine receptor CCR5 and therefore might be particularly susceptible to infection by CCR5-utilizing viruses that are present in the early stages of infection. [65] Alternatively, an initial dysregulation in immunity may result from a depletion of CD4 cells involved in helping cellular immunity (TH1 cells) in contrast to those involved in promoting humoral immunity (TH2 cells). [66] One recent report noted a profound decrease in HIV-specific CD4+ T-helper lymphocyte response, suggesting that the loss of this cell population could contribute to the failure of the immune response to control viral replication. [58] In addition to the role of CD4+ lymphocyte depletion and dysfunction in the pathogenesis of AIDS, it is clear that monocytes, macrophages, and dendritic cells are targets for HIV infection and probably represent a major reservoir for virus production in vivo. [27] [30] [67] [68] [69] In view of the central role of monocytes and dendritic cells in processing and presenting antigens to T and B lymphocytes, it is likely that HIV infection of these cells plays a significant role in the development of immunodeficiency. [69]

As indicated by the current surveillance definition of AIDS (Table 154-1), neurologic abnormalities in both the central and peripheral nervous systems, as well as the development of Kaposis sarcoma (KS) and B-cell lymphomas, are common clinical manifestations of AIDS. [1] In the CNS, glial and other reticuloendothelial cells and possibly endothelial cells [70] [71] have been shown to be infected productively by HIV, raising the possibility that dementia and other neurologic defects could arise from the direct effects of viral infection, inflammatory responses, or the elaboration of toxic cytokines by infected cells. [70] [71] [72] Intense efforts are in progress to identify particular chemokine receptors that might be involved with CNS replication, with one recent report implicating the chemokine receptors CCR5 as well as CCR3 as candidates. [73]

Kaposis sarcoma is a proliferative disorder of endothelial cells that occurs predominantly in homosexual and bisexual men with HIV infection. [74] [75] This disorder has also been recognized as a classic form in older Mediterranean and Ashkenazic Jewish men, an endemic form in sub-Saharan Africa, and an iatrogenic form complicating immunosuppressive therapy for allografts. [75] In AIDS-associated KS, HIV is not detectable in the proliferating endothelial cells, suggesting an indirect role for HIV in pathogenesis. In addition, the finding that among HIV-infected people KS is largely restricted to homosexual men has

strongly implicated another transmissible agent. Recently a new human herpesvirus termed HHV-8 or KS-associated herpes virus (KSHV) has been shown to be detectable in KS lesions in AIDS-associated as well as classical, endemic, and iatrogenic forms of KS, strongly implicating a role for this infectious agent in pathogenesis. [76] [77] HHV-8 has also been detected in the semen of HIV-1-infected homosexual males, suggesting that this virus can be sexually transmitted. [78] Several studies have suggested that viral and cellular proteins are produced by HIV-infected cells that can promote angiogenesis and perhaps contribute to a dysregulation of endothelial cell growth. [74] [79] [80] [81] However, the finding that HHV-8 also encodes chemokine-like proteins that are potent angiogenic factors has further implicated a direct role for this virus in endothelial cell proliferation. [82] Although the factors responsible for dysregulation of endothelial cell growth in KS are not clear, it appears likely that a combination of HIV-induced immunosuppression and direct and indirect effects of HHV-8 are involved. In addition, given the apparent role of cytokines and chemokines in the endothelial cell growth in KS lesions, it is possible that KS does not represent a true neoplasm, and that it may ultimately be treatable by novel strategies that inhibit or modulate these angiogenic factors. [83]

A dramatic rise in the incidence of B-cell lymphomas has been reported in patients with HIV infection, [84] [85] [86] and in contrast to KS this complication has been observed in all HIV-infected patient groups, including children. [85] [86] [87] [88] The malignant B cells from these lymphomas are not infected by HIV, [85] [89] and are likely derived from a clonal outgrowth of polyclonal populations of proliferating B cells present in patients with HIV infection. [85] [86] [90] [91] Possible mechanisms for the B-cell stimulation observed in these individuals include a dysregulation in CD4 helper function, [66] or the direct mitogenic effects of Epstein-Barr virus (EBV) infection [85] [92] or HIV antigens. [93] [94] A recent study has also suggested that HIV infection of microvascular endothelial cells could promote B-cell attachment and activation, possibly contributing both to the increased incidence of lymphomas and to the peculiar predisposition of these neoplasms to involve extranodal sites. [95] Approximately 3070%

of lymphomas have been found to contain EBV DNA, strongly implicating this virus in at least some of these neoplasms. [85] [87] [89] [92] [96] Hodgkins disease has also been reported with increased frequency in HIV-infected patients, [97] [98] although the reason for this association is unclear. In at least a subset of non-AIDS and HIV-infected patients with Hodgkins disease, Reed-Sternberg cells have been shown to contain integrated copies of the EBV genome [99] [100] or to express EBV RNA,[96] raising the possibility that the reactivation of EBV that occurs in patients with HIV infection may be involved in the development of AIDS-associated Hodgkins disease. Although less common than B-cell lymphomas, peripheral as well as cutaneous T-cell lymphomas have also been reported in patients with HIV infection. [101] [102] In primary effusion lymphomas (previously termed body cavity-based lymphomas) seen in AIDS, HHV-8 has been identified in the malignant cells, implicating a direct role for this herpesvirus in these disorders. [103] [104] Unlike opportunistic infections, B-cell lymphomas may occur in individuals with only mild reductions in CD4 cell numbers. An additional discussion of AIDS-associated lymphomas is presented in Chapter 75 .

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STAGES OF HIV INFECTION

A number of stages of HIV infection have been recognized, including an acute viral syndrome, an asymptomatic stage, a variety of symptomatic conditions frequently characterized by generalized lymphadenopathy, and full-blown AIDS (Table 154-1).[1] Patients with the acute viral syndrome typically present 26 weeks after exposure with fever, malaise, myalgias, maculopapular rash, diarrhea, lymphadenopathy, or aseptic meningitis. [105] Laboratory findings include lymphocytosis with atypical plasmacytoid lymphocytes, a negative heterophile, and mild thrombocytopenia. [105] Thrombocytopenia has been a frequent hematologic complication, along with leukopenia without lymphocytosis.[106] [107] Of importance in the diagnosis of acute HIV infection, individuals may be serologically negative during the viral prodrome but typically develop anti-HIV antibodies 12 months after the onset of this illness. Circulating viral p24 antigen in the absence of anti-HIV antibody may be detectable during this acute phase, [106] and individuals typically have high levels of plasma viremia, which subsequently decreases to low or undetectable levels as a consequence of the host immune response.[46] [108] [109] [110]

The majority of HIV-infected individuals in the United States are asymptomatic but exhibit serologic evidence of infection. That asymptomatic but seropositive patients are persistently infected with HIV has been clearly established by epidemiologic studies, positive viral cultures, and the presence of viral DNA and RNA in peripheral blood mononuclear cells and plasma, respectively, detected by the polymerase chain reaction. [47] [48] Studies evaluating lymph tissues of HIV-infected patients have shown that even in this asymptomatic period there is an abundance of replicating virus in these tissues, indicating that while these individuals may be clinically well, contrary to earlier views, HIV is clearly not latent during this period. [30] [48] This prolonged period of clinical latency may be complicated by persistent generalized lymphadenopathy with or without a number of nonspecific complaints, including arthralgias, fatigue, intermittent diarrhea, and chronic sinusitis. [1] In the final stage of full-blown AIDS, levels of viral RNA in plasma and lymph nodes are usually markedly elevated, and there is a disruption of lymph node architecture producing follicular involution. Prospective studies of HIV-infected patients indicate that the median duration between HIV infection and AIDS is 10 years or longer. [111] [112] As noted previously, factors that predict the impending development of full-blown AIDS include a high level of viral RNA in plasma and the presence of viruses that use the CXCR4 chemokine receptor and induce syncytia on laboratory derived T-cell lines. [35] [36] [54] The clinical course of HIV infection is summarized in Figure 154-4 . Clinical progression over time is associated with a progressive loss in CD4 lymphocytes as well as a gradual increase in a systemic viral load.

Interestingly, a subset of HIV-infected patients, termed long-term nonprogressors, who either fail to develop clinically significant immunodeficiency or do so at a much slower rate has been identified. [113] [114] [115] These individuals exhibit low levels of detectable HIV in plasma and lymph nodes. This important group of individuals is under intense investigation, and evidence has suggested that viral, [114] [116] immunologic,[58] [113] and other host factors may be contributing factors. Recently a naturally occurring mutation in the CCR5 chemokine receptor has been observed in 15% of North American and European Caucasians that results in a nonfunctional receptor. HIV-infected individuals who are heterozygous for this mutation progress to AIDS approximately 34 times more slowly than individuals who lack this allele. [117] Remarkably, individuals who are homozygous for this mutation exhibit a relative (though not absolute) resistance to HIV infection, likely indicating the importance of CCR5 in the transmission and/or the initial establishment of HIV infection. [118] [119] It is likely that additional host factors will be identified that also influence the course of HIV infection.[119] [120] [121]

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HEMATOLOGIC ABNORMALITIES IN PATIENTS WITH HIV INFECTION

As described previously, it is clear that hematologic abnormalities in infected individuals may arise from a number of causes, including (1) direct infection by HIV of bone marrow cells or

Figure 154-4 Natural history of HIV infection in an infected individual. A typical course of HIV infection is shown. During primary infection there is active viral replication with viremia and widespread dissemination of virus to lymph organs, during which time a sharp decline in the number of CD4+ lymphocytes occurs. This is followed by a prolonged period of clinical latency, characterized by a slow but progressive decline in CD4+ lymphocytes. When the CD4+ cell number falls to <200/l, the clinical course is generally complicated by opportunistic infections. A quantitative increase in viremia occurs during this time, reflecting increased viral replication, an increasing viral load, and/or redistribution of virus from damaged lymph organs.

other supporting cells required for hematopoiesis, (2) dysregulation of the host immune system leading to destruction or inhibition of hematopoietic cells, and (3) secondary complications of opportunistic infections, malignancies, and/or therapy for these disorders. Not surprisingly, the principal hematologic problems encountered in patients with HIV infection include cytopenias of red cells, neutrophils, lymphocytes, and platelets, as well as a variety of polyclonal and oligoclonal gammopathies. Several general reviews of the hematologic complications in AIDS have been published. [122] [123] [124]

Abnormalities in Hematopoietic Cells

Anemia and Red Blood Cell Abnormalities

Anemia occurs in the majority of HIV-infected patients at some point in the course of their disease. [125] [126] [127] Both the incidence and degree of anemia have been shown to correlate with the severity of the clinical syndrome. [125] [128] When HIV infection is complicated by opportunistic infections, anemia has been seen in 7095% of patients with mean hemoglobin and hematocrit levels of 9.711.7 g/dl and 36%, respectively. [125] [129] However, even in asymptomatic patients with HIV infection or those with minimal symptoms, a mild but significant reduction in hematocrit has been reported in 1520% of patients. [125] Anemia and granulocytopenia tend to occur concurrently, with one series showing that 68% of patients with hematocrits <40 were also neutropenic, with white blood cell counts $<1.4 \times 10^3$ /l while 88% of patients with neutropenia were also anemic. [125]

Anemia in patients with HIV infection who are not undergoing antiretroviral therapy with zidovudine is typically normochromic normocytic, although a mild degree of anisocytosis and poikilocytosis is common. [130] [131] [132] [133] [134] Macrocytosis occurs in the majority of patients treated with zidovudine. [135] [136] Schistocytes are prominent in the setting of thrombotic thrombocytopenic purpura, which may complicate HIV infection, as described in a later section. [137] [138] [139] [140] [141] [142]

In addition to being a complication of the HIV infection itself, anemia is a frequent side effect of antiretroviral drugs, particularly zidovudine, as well as drugs used to treat or prevent opportunistic infections, including dapsone, primaquine, trimethoprim/sulfamethoxazole, and ganciclovir.

Depressed erythropoiesis in AIDS has been suggested by a low or inappropriately normal reticulocyte count, and in HIV-infected patients the reticulocyte count cannot be used as a reliable indicator of either hemolysis or bleeding. [123] [126] [134] Similar to the anemia of chronic disease, it is likely that inflammatory cytokines play a role in suppressing erythropoiesis in patients with HIV infection. Tumor necrosis factor and interleukin-1 have been shown to suppress erythropoiesis in vitro, and both of these cytokines can be increased in HIV-infected patients. [143] In addition, the finding on bone marrow examination of normal to increased numbers of erythroid progenitor cells, [125] [129] [144] along with a variable degree of dyserythropoiesis, [125] [133] [144] [145] has indicated that ineffective erythropoiesis may be an additional contributing factor. [122] [145] [146] A syndrome resembling paroxysmal nocturnal hemoglobinuria was described in a patient presenting with intravascular hemolytic anemia, markedly dysplastic erythroid hyperplasia on bone marrow examination, and abnormal HAM and sucrose-lysis tests. [147] Megaloblastic changes in erythroid precursors are uncommonly seen [133] [148] but have been described during therapy with trimethoprim/sulfamethoxazole, dapsone, and zidovudine. [133] [135] [136] [145] [146] [149] Several reports have noted profound erythroid hypoplasia as well as pure red cell aplasia in the setting of *Mycobacterium avium* complex (MAC) infection, [129] [130] [131] [133] [150] where suppression of erythroid progenitors due to soluble factors, including tumor necrosis factor , has been implicated. [150] [151] Pure red cell aplasia has also been reported in HIV-infected patients receiving zidovudine [152] and without any associated cause. [153] As described further in a later section, in both HIV-infected adults and children, a severe depression of erythropoiesis may occur due to chronic parvovirus B19 infection, where the presence of giant pronormoblasts in bone marrow may be diagnostic. [154] [155] [156] [157] Anemia in the setting of HIV infection can have

important prognostic significance. A low hematocrit (<25) has been shown to be a negative predictor for survival in patients with MAC infection, [158] and in one recent study HIV-associated anemia from any cause that did not resolve was highly correlated with a shortened survival. [128]

Serum erythropoietin levels in patients with HIV-associated anemia may be increased [136] [159] or reduced, [160] with one series finding reduced serum levels in 22 of 29 AIDS patients. [160] Although for untreated patients there is an inverse correlation between hematocrit and serum erythropoietin levels, the erythropoietin response to anemia is inappropriately low compared to patients with iron-deficiency anemia or bleeding. [159] [161] [162] Similar findings have been described in patients with the anemia of chronic disease. [163] Some experimental evidence has suggested that HIV infection itself may directly suppress erythropoietin. [164] Interestingly, for some patients treated with zidovudine, serum erythropoietin levels may increase dramatically in response to anemia, suggesting a restoration of the erythropoietin response. [159] [162] This finding could be related to the occasional reports of polycythemia following the initiation of zidovudine therapy. [165] [166] As described following, early clinical trials evaluating the role of recombinant erythropoietin in the treatment of anemia in patients receiving zidovudine found that patients with low pretreatment serum erythropoietin levels (500 IUL) are more likely to respond to erythropoietin therapy, as shown by an improvement in hematocrit and a decreased transfusion requirement. [160] [167]

Iron stores in anemic patients with HIV infection are typically adequate or increased, and iron indices are similar to those seen in chronic disease states, with a low serum iron and transferrin and an elevated serum ferritin. [131] [148] In both adults and children, the level of serum ferritin was found to be a marker for advanced or progressive disease. [168] [169] An elevated serum ferritin level has been shown to correlate with markers of immune activation (interferon , serum neopterin levels, and 2-microglobulin). [170] Bone marrow iron is usually adequate or increased in reticuloendothelial cells, [125] [133] although in one study reduced or absent bone marrow iron was found in 16 of 32 patients with AIDS. [130] Particularly in children with HIV infection, iron malabsorption has been implicated as a contributing factor to anemia. [171]

Sideroblasts, while uncommon, have been described in 5% of AIDS patients in some series.[125] [129] [133] [145]

Reduced serum B_{12} levels are seen in 730% of patients with AIDS, [172] [173] [174] [175] [176] although this typically occurs in the absence of neutrophilic hypersegmentation, red cell macrocytosis, or megaloblastic changes on bone marrow aspirate.[173] [174] [175] [177] When present, a low plasma B_{12} level in HIV-infected patients does not appear to be due to a reduction in the B_{12} binding proteins, transcobalamin or haptocorrin.[178] Malabsorption of B_{12} with an abnormal Schillings test has been found in AIDS patients both with[173] [179] and without[172] [174] [177] chronic diarrhea syndromes, suggesting that a true negative balance of B_{12} may exist in some patients. Correction of the Schillings test by intrinsic factor in these patients has been variable. [175] [177] In one study, patients with an abnormal Schillings test were noted to have chronic inflammatory changes on colonic and duodenal biopsy, as well as evidence by in situ hybridization of HIV-infected mononuclear cells within the lamina propria.[174] These findings have suggested that HIV infection within the gut mucosa could contribute directly to B_{12} malabsorption. However, normal Schillings tests in patients with low serum B_{12} levels have also been reported.[173] [176] [177] Although patients with reduced serum B_{12} levels are more likely to be anemic, the absence of megaloblastic changes and the lack of a hematologic response to B_{12} therapy have indicated that in most patients this reduction is not clinically significant. [123] [173] [175] Nonetheless, using a reduction in serum holo-transcobalamin II as a more sensitive indicator of negative B_{12} balance, reduced levels were reported in approximately half of patients with AIDS;[180] this has raised the possibility that subclinical B_{12} deficiency may be more significant than previously recognized. Clinically, a reduced serum B_{12} level may increase the hematologic toxicity associated with zidovudine therapy.[135] Although lower B_{12} levels have been noted more frequently in patients receiving zidovudine,[176] supplemental therapy with cobalamin and folate failed to prevent or reduce the myelosuppression associated with zidovudine. [181] Alterations in folate metabolism have also been reported in AIDS patients with reduced intestinal absorption of folate, [182] as well as increased serum and red cell folate levels, [183] [184] even in the absence of reduced serum B_{12} levels.[184] The underlying mechanism and clinical significance of these findings are unknown.

Positive direct antiglobulin tests have been reported in 2043% of hospitalized AIDS patients or patients undergoing transfusion therapy [122] [132] [185] [186] [187] and in 8% of asymptomatic individuals. [188] These reactions are usually weak, involve IgG and/or complement, and are likely due, at least in part, to the deposition of immune complexes on red blood cells, similar to that seen in non-AIDS patients with hypergammaglobulinemia. [185] Nonetheless, sensitive assays have documented the presence of true anti-red cell antibodies with anti-i and anti-U specificity in 64% and 32% of AIDS patients, respectively, [186] as well as anti-PI and anti-LEB antibodies in some patients.[122] [186] Anti-i antibodies are known to occur during acute EBV infection, and it is possible that the increased frequency of this antibody in patients with HIV infection results from active EBV or possibly cytomegalovirus (CMV) infection. [186] Cold agglutinins have been reported in up to 22% of HIV-infected patients, although no association between the presence of these antibodies and anemia has been found. [189] Although uncommon, severe autoimmune hemolytic anemia has been reported, with responses observed with steroids, intravenous gammaglobulin, splenectomy, or zidovudine. [156] [190] [191] [192] [193] [194] The relative reticulocytopenia frequently noted in patients with HIV infection may mask the diagnosis of autoimmune hemolytic anemia. [192]

Leukopenia and White Blood Cell Abnormalities

Leukopenia is common in HIV-infected individuals and, similar to anemia, occurs with a frequency that generally correlates with the severity of the clinical syndrome. Between 5785% of patients with AIDS[122] [131] and 1021% of patients with ARC[122] [129] are leukopenic, while <5% of asymptomatic seropositive patients present with leukopenia.[125] [129] Leukopenia typically involves both lymphocytes and granulocytes, although monocytopenia has also been reported in 875% of patients with AIDS.[129] [133] However, as noted previously, a reduction in the absolute number of CD4+ T cells occurs as one of the earliest immunologic abnormalities of HIV infection, and the number of these cells declines progressively over time (Figure 154-4). As noted previously, a low number of CD4+ lymphocytes is one of the most important prognostic indicators for the risk of developing opportunistic infections. [195]

A number of morphologic abnormalities have been described in peripheral blood and bone marrow white blood cells of patients with HIV infection; these are discussed in greater detail in a later section. Particularly in the setting of granulocytopenia, neutrophils may show nuclear hyposegmentation with an apparent left shift, along with the presence of monolobed or Pelger-Hüet forms[133] [134] (Fig. 154-5). In addition, other dysplastic changes of neutrophils have been described, including increased size, prominent granulation, and increased peroxidase activity. [122] [133] [134] Nonspecific morphologic findings include large vacuolated monocytes and atypical lymphocytes, particularly in patients who are lymphopenic. [133] Although myelodysplastic changes are common and do not represent a preleukemic syndrome,[123] [134] [196] rare cases of acute myeloblastic leukemia evolving from a pre-existing myelodysplastic state have been reported in HIV-infected individuals. [197] [198]

Figure 154-5 Peripheral blood and bone marrow morphologic abnormalities in AIDS. **(A)** Pelger-Hüet cell and macrocytes in a patient with AIDS-related complex undergoing therapy with zidovudine. The mean corpuscular volume on this blood sample was 118 ^{3} . **(B)** Loose granuloma formation from a bone marrow biopsy in a patient with disseminated MAC infection involving bone marrow. Acid-fast stains of this specimen showed numerous organisms within the granuloma. **(C)** Grocott stain showing cryptococcal involvement of bone marrow. Organisms in this panel are darkly stained. High magnification of this specimen in **(D)** shows numerous budding yeast forms. **(E)***Histoplasma* within a macrophage seen on Wright Geimsa stain of a bone marrow aspirate. **(F)** Non-Hodgkins lymphoma involvement of marrow (diffuse non-Burkitts type), showing a hypercellular marrow completely replaced by malignant cells. **(G)** Hodgkins disease involving mar-row, showing normal cellularity on the left portion of the panel and a pleomorphic desmoplastic reactive process on the right. High-magnification view of this specimen in **(H)** shows numerous Reed-Sternberg cells. **(I)** Giant pronormoblast in parvovirus B19 infection. **(J)** Pseudogaucher cell resulting from bone marrow histiocytes engorged with MAC organisms. **(K)** Condensed lobulated nucleus of a dysplastic megakaryocyte from a patient is shown adjacent to a normal megakaryocyte. *(Reprinted with permission from Bauer et al.[270])*

Granulocytopenia is a common complication of several drugs used in the therapy for AIDS-related infections, including trimethoprim/sulfamethoxozole and pentamidine for *Pneumocystis carinii* pneumonia (PCP), pyramethamine/sulfadiazine for CNS toxoplasmosis, flucytosine for cryptococcal and other fungal infections, and ganciclovir for CMV infection.[199] Between 1928% of patients receiving trimethoprim/sulfamethoxozole for PCP develop neutropenia, [200] [201] [202] often in the setting of fever, rash, and elevated liver transaminases.[202] As described in a later section, dose-dependent neutropenia in patients receiving zidovudine has been reported in 816% of patients during long-term therapy,[135] [203] but it may also occur in an apparently idiosyncratic manner with profound bone marrow hypoplasia [204] and agranulocytosis. [205] Although more commonly seen in the setting of advanced HIV infection, granulocytopenia has also been reported to complicate the viral prodrome of acute HIV infection.[107]

Not surprisingly, granulocytopenia complicating AIDS has been associated with an increased incidence of infection with bacterial and fungal pathogens similar to those seen in non-HIV neutropenic patients. These infections have included bacteremia (*Staphylococcus* species, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*) and fungemia (*Cryptococcus* and *Candida* species) as well as pulmonary aspergillosis, pyomyositis malignant external otitis, neutropenic enterocolitis, and pseudomonal keratitis.[124] Risk factors for bacterial infections have included a granulocyte count <500/l and central venous access catheters. [206] [207]

Although the mechanism of granulocytopenia in AIDS remains under intense investigation, the role of HIV infection on bone marrow function is still controversial and has recently been reviewed.[208] Bone marrow biopsies in patients with AIDS and symptomatic HIV infection are either normocellular or hypercellular in 60100% [125] [129] [131] [133] [134] [144] [148] and are frequently associated with dysplastic changes,[129] [134] [144] suggesting that ineffective myelopoiesis may be occurring. In vitro culture of bone marrow from HIV-infected patients for granulocyte and macrophage (CFU-GM) and erythroid (BFU-E) colony-forming units (CFUs) or of CD34+ progenitor cells has been reported to be either normal[209] [210] [211] or reduced.[212] [213] [214] [215] [216] [217] [218] Infection of cultured bone marrow cells by HIV-1 has been demonstrated in vitro, and in some,[215] [219] [220] [221] but not all, studies[209] has been reported to inhibit colony formation. However, the direct evaluation of CD34+ cells or CFUs from patients with AIDS for HIV DNA by the polymerase chain reaction technique has in several, [209] [210] [214] [215] [222] [223] [224] but not all,[225] studies failed to show a significant degree of infection. A single study, however, using in situ hybridization of bone marrow from patients did report HIV genomes in a high proportion of patients, detectable in immature myeloid cells, histiocytes, endothelial cells, and nucleated red cells. [146] Nonetheless, the predominant view from most studies is that with the exception of

the megakaryocyte (discussed in a later section), few hematopoietic progenitor cells are directly infected by HIV.

A number of indirect mechanisms of HIV-mediated suppression of hematopoiesis have also been suggested. Suppressive effects and/or apoptosis induced by the viral envelope glycoprotein, gp120, on CFU formation have been observed in vitro in some, [221] [226] [227] but not all, studies.[209] In addition, earlier studies have implicated suppression of myelopoiesis by T lymphocytes, [212] anti-gp120 antibodies, [211] and a glycoprotein produced by cultured bone marrow cells of HIV-infected patients.[213] It is also likely that cytokines produced from HIV-infected cells, including transforming growth factor- [228] and tumor necrosis factor,[229] play a role in suppressing hematopoiesis. Viral gene products have also been implicated in suppressing myelopoiesis, including Tat, reported to suppress cultured bone marrow cells by inducing macrophages to produce transforming growth factor-, [228] and the *gag* gene product p24.[230] A possible autoimmune mechanism for the destruction of peripheral blood granulocytes has also been suggested by the presence of antineutrophil immunoglobulins in as high as 67% of patients with AIDS and ARC. [231] [232] [233] There is also some evidence that bone marrow stromal cells may be infectable by HIV, implying that hematopoiesis could be affected through viral-induced alterations in the bone marrow microenvironment.[234] Nonetheless, as discussed following, whatever the mechanism of AIDS-associated leukopenia, a dramatic rise in peripheral blood neutrophils is typically seen in AIDS patients following therapy with recombinant GM-CSF [235] or G-CSF,[236] clearly indicating that competent myeloid stem cells with the capacity for differentiation are present, even in patients with advanced immunodeficiency.

Thrombocytopenia

Thrombocytopenia is a frequently reported complication of HIV infection [237] [238] [239] and has been described in both adults and children. [240] [241] [242] [243] [244] Depending on the patient population analyzed, thrombocytopenia, defined as a platelet count <100,000/l, has been reported in 38% of seropositive individuals [132] [241] [245] [246] [247] and 3045% of patients with fully developed AIDS. [122] [246] In some studies an inverse correlation was found between the platelet count and the CD4+ lymphocyte number.[245] However, isolated and occasionally severe thrombocytopenia may occur even in asymptomatic individuals as the presenting manifestation of HIV infection. Thrombocytopenia has also been reported during acute HIV infection as part of the acute viral syndrome described earlier. [106] [248]

The degree of HIV-related thrombocytopenia is generally mild to moderate, with mean counts ranging from 43,000 to 57,000/l. [240] [249] However, a severe reduction to levels <10,000/l has also been described. [240] [249] [250] Roughly one-third of patients with thrombocytopenia present with a history of easy bruising, petechiae, or bleeding,[251] although in the majority, significant spontaneous clinical bleeding usually does not occur. [122] [237] Not surprisingly, life-threatening hemorrhagic complications, primarily due to CNS bleeding, may occur in HIV-infected hemophiliacs, particularly with platelet counts <50,000/l. [252] Unlike classic immune thrombocytopenic purpura (ITP), HIV-related thrombocytopenia frequently occurs with other hematologic abnormalities, usually neutropenia with or without anemia in 60% of cases.[122] [123] However, as noted previously, particularly in asymptomatic seropositive patients, it generally occurs as an isolated hematologic abnormality. [125] The presence or absence of thrombocytopenia in seropositive individuals appears not to be a prognostic indicator for the development of AIDS, [240] [242] [253] although one study of hemophiliacs noted an increased mortality in patients with thrombocytopenia independent of the CD4 lymphocyte count. [254] Interestingly, in as high as 1150% of patients, thrombocytopenia may regress spontaneously without therapy, [122] [240] [242] although these findings are not always observed.[255]

The mechanism of thrombocytopenia in HIV infection appears to involve both increased platelet destruction as well as ineffective platelet production. In one study, the survival of [111] Indium-labeled autologous platelets was found to be significantly reduced compared to normal controls in both untreated and zidovudine-treated patients with HIV-related thrombocytopenia.[256] Platelet survival was also decreased in HIV-infected patients without thrombocytopenia, although to a lesser extent. [256] Most but not all reports indicate that there is significant platelet sequestration and/or destruction in the spleen in HIV-associated thrombocytopenia. [256] [257] [258] One report indicated that platelet destruction was the predominant factor early in the course of disease, while decreased platelet production was the predominant factor later in the course of disease.[258] The high

response rate to splenectomy of HIV-associated thrombocytopenia, even in patients with advanced disease, certainly suggests that the spleen is probably a major site of platelet sequestration and/or destruction. [240] [259] [260] [261] [262] The observed decrease in platelet survival is most likely immunologically mediated. In HIV-related thrombocytopenia, there are marked increases in platelet-associated immunoglobulin and complement, and circulating immune complexes are increased 24 times that seen in classic ITP. [242] [249] [250] [258] Among HIV-infected patients, the presence of circulating immune complexes has been shown to correlate positively with the presence of thrombocytopenia.[258] An analysis of circulating as well as platelet-associated immune complexes has shown anti-idiotypic antibodies directed against antibodies to the HIV-1 envelope glycoprotein, gp120. [263] In at least some individuals, a true autoimmune process has been suggested by the detection of antiplatelet activity in IgG eluted from platelets;[231] [242] [249] [258] [264] one study suggested that at least some patients may exhibit antibodies to platelet glycoproteins IIb-IIIa [265] similar to that seen in classic ITP, although this has been somewhat controversial. [264] IgM rheumatoid factor containing IgG antiplatelet (anti-GPIIIa) antibodies has also been reported in HIV-infected patients and shown to correlate with thrombocytopenia. [266] Interestingly, two reports demonstrated that antibodies eluted from patient platelets could recognize both platelet glycoprotein IIIa and HIV envelope glycoproteins, suggesting that cross-reactive epitopes between viral envelope and platelet glycoproteins[267] [268] could potentially mediate a true autoimmune destruction of platelets in some patients.

Although megakaryocytes are typically increased in HIV-related thrombocytopenia, a kinetic analysis has shown a significant reduction in platelet production. [256] As described in a later section, megakaryocytes are characteristically dysplastic, [134] [144] [269] [270] and circulating platelets have been shown to exhibit an inappropriately low mean platelet volume, similar to that seen in myelosuppressive disorders. [271] A number of studies have strongly indicated that this dysplasia may be related to direct infection of the megakaryocyte by HIV.[258] [272] [273] [274] Megakaryocytes have been shown to express CD4 and to be able to bind HIV. [275] [276] [277] Megakaryocytic cell lines are susceptible in vitro to infection by particular isolates of HIV, [276] [278] and one report indicated that this infection could be markedly enhanced by tumor necrosis factor ,[279] similar to the effects of this cytokine on T-cell and monocytic cell lines. [280] [281] [282] [283] Finally, studies of megakaryocytes directly from HIV-infected patients have shown viral RNA (Fig. 154-6) and viral proteins, strongly suggesting that these cells are infected in vivo. [258] [284] [285] Interestingly, in one study HIV transcripts were not detected in CFUs for cultured megakaryocytes, suggesting that megakaryocytes may not be susceptible to infection until they are more differentiated or that infected megakaryocytes may be unable to proliferate or differentiate in vitro. [285] One study described an isolate of HIV-1 that was selectively cytopathic for megakaryocytic cell lines, indicating that viral factors could be involved in determining the extent to which megakaryocyte dysfunction and thrombocytopenia occur in infected patients. [286] Thus, unlike other hematopoietic precursors, there is clear evidence that HIV directly infects megakaryocytes, contributes to ineffective thrombopoiesis, and, coupled with antiviral immune responses, leads to thrombocytopenia in a subpopulation of patients. Future studies that evaluate chemokine receptor expression on megakaryocytes and the extent to which they are involved in viral infection will be of particular interest. [287]

A number of drugs frequently used in the setting of HIV infection may cause thrombocytopenia. These include trimethoprim/sulfamethoxazole, pentamidine, pyrimethamine, ganciclovir, fluconazole, -interferon, trimetrexate, eflornithine, rifabutin, and clarithromycin. [124] In addition, other secondary

Figure 154-6 Megakaryocyte infection by HIV. In situ hybridization with a ^{35}S-labeled HIV-specific probe in a bone marrow biopsy from an HIV-seropositive patient. In panels **(A)** and **(B)**, numerous grains are apparent, indicating the presence of HIV RNA within the megakaryocyte cytoplasm. In panel **(B)**, a dysplastic megakaryocyte largely denuded of cytoplasm is shown (arrow) that also is reactive with the HIV probe. *(Reprinted from Zucker-Franklin and Cao,[284] with permission.)*

causes of thrombocytopenia must also be considered in HIV-infected patients, including thrombotic thrombocytopenic purpura and bone marrow infiltration by lymphomas and opportunistic infections.

Abnormalities in Coagulation

Antiphospholipid antibodies detectable as lupus anticoagulants or anticardiolipin antibodies have been identified in 2282% of patients with HIV infection, including homosexual men, intravenous drug users, and hemophiliacs. [288] [289] [290] [291] [292] Interestingly, despite the association in non-HIV-infected patients between lupus anticoagulants, anticardiolipin antibodies, and a false-positive VDRL, the VDRL in HIV-infected patients without a history of syphilis is usually nonreactive, probably reflecting differences in specificity of the antiphospholipid antibodies in patients with and without HIV infection. [288]

Patients with a lupus anticoagulant typically present in the absence of a bleeding history with a prolonged activated partial

thromboplastin time (APTT) that does not correct after mixing with normal plasma and incubation at 37°C. A prolongation in the tissue thromboplastin time is also usually seen.[288] The Russell viper venom clotting time (RVVCT) that measures clot formation by the direct activation of factor X is the most specific indicator of a lupus anticoagulant.[122] [288] [293] In HIV-infected patients, lupus anticoagulants are typically IgMs [288] and can be partially adsorbed with phospholipids obtained from commercially available lipid extracts or platelet membranes.[122] [293] In one study of HIV-infected patients with prolonged APTTs, lupus anticoagulants were described that were not detectable by a mixing test. However, functional inhibitory activity was demonstrated by a prolonged RVVCT or by correction of the APTT with increasing concentrations of exogenous phospholipid.[294] Lupus inhibitors were initially described in 2073% of patients with AIDS [123] [288] [293] and in as high as 43% of asymptomatic seropositive patients,[295] although in another series this was found to be considerably less frequent, particularly in nonhospitalized patients, where it was not observed in 142 HIV-infected patients.[291]

Anticardiolipin antibodies (ACAs) that may or may not have activity as lupus inhibitors are detectable by enzyme-linked immunosorbent assays (ELISA) and in HIV-infected patients are usually IgG immunoglobulins.[289] [292] [295] In one study a higher frequency of ACAs in patients with AIDS compared to asymptomatic patients (84% vs. 50%) was found,[289] although another study of hemophiliacs found no difference in the frequency of ACAs among hemophiliacs with or without AIDS.[295] One study found that ACAs were detectable in 30% of hospitalized and 8% of nonhospitalized seropositive patients.[291] No correlation has been found between the presence of ACAs and the presence or absence of thrombocytopenia,[296] the CD4 cell number,[295] or the risk of progression to AIDS.[295] Despite the association of a positive ACA titer with antinuclear antibodies (ANAs) in >90% of patients with lupus, ANAs have rarely been reported in patients with AIDS,[289] although one study did note low-titer ANAs in 19 of 151 patients with AIDS or ARC.[297]

The presence of a lupus inhibitor or ACAs does not predispose to clinical bleeding, and in the absence of thrombocytopenia or an elevated prothrombin time is not, in and of itself, a contraindication to an invasive procedure.[122] [123] [288] [293] However, depending on the specificity of the antiphospholipid antibody, other coagulation defects may be present, including a reduced level of factor VII, resulting in a prolongation in the prothrombin time,[298] and abnormal platelet aggregation studies associated with an abnormal bleeding time.[288] Consequently, the decision to perform invasive procedures on an HIV-infected patient in whom a lupus anticoagulant is identified should be individualized; a careful bleeding history is required. In addition, the presence of a lupus inhibitor in hemophiliacs may complicate factor replacement therapy, where the resulting prolongation of the APTT may be misinterpreted as a factor VIII inhibitor and interfere with monitoring for factor VIII replacement therapy.[122] In contrast to patients without HIV infection, where the presence of a lupus inhibitor is associated with thrombotic events and fetal loss, a lupus inhibitor is rarely associated with thrombosis in patients with HIV infection.[288] [293] Nonetheless, HIV-infected patients who have lupus inhibitors and/or ACAs have been reported with avascular necrosis, skin necrosis, strokes, and other thrombotic events,[293] [299] [300] [301] and in some of these patients low-grade disseminated intravascular coagulation and depressed levels of protein C have also been implicated in thrombotic complications.[302]

The mechanism for the high frequency of antiphospholipid antibodies in HIV-infected patients is unclear but presumably involves the generation of phospholipids from either endogenous or exogenous sources. Destruction of immune or hematopoietic cells directly or indirectly by HIV could induce membrane damage and the liberation of phospholipids as immunogens. Alternatively, secondary infections could provide exogenous phospholipids or further contribute to the release of endogenous phospholipids from damaged tissues. The combined results of two studies noted a frequency of lupus anticoagulants or ACAs in 39 of 50 AIDS patients with PCP and in only 1 of 34 AIDS patients with other opportunistic infections, suggesting an association between *Pneumocystis carinii* and antiphospholipid antibodies.[303] Moreover, in both studies patients were described who developed these antibodies with the onset of the pneumonia and who subsequently lost the antibody with successful therapy for the infection. However, another study found no correlation between the presence of an opportunistic infection and a lupus inhibitor.[122] The high frequency of ACAs, even in asymptomatic individuals, however, indicates that other factors aside from PCP are likely involved.

Several reports have described a remarkably high frequency of acquired protein S deficiency in HIV-infected patients, with a reduction noted in 1773% of seropositive patients.[304] [305] [306] [307] [308] [309] Reduced protein S levels have also been reported in HIV-infected children.[310] This deficiency reflects a true reduction in both free and total protein S levels, with no alteration found in the level of C4b-binding protein, which binds 6070% of circulating protein S.[304] [305] Some studies have noted a correlation of reduced protein S levels with a diagnosis of AIDS and a low CD4 cell number,[306] [311] while another study found an association with an increasing duration of HIV infection and elevated levels of ACAs.[305] The mechanism of protein S deficiency in these patients is unclear and could involve decreased synthesis and/or abnormalities in endothelial cell function. Protein S is synthesized in liver and endothelial cells, and the presence of normal levels of other hepatic coagulation factors in patients studied does suggest that endothelial cell dysfunction could be involved.[305] [306] Increased levels of antigenic von Willebrand factor have been described in seropositive patients, possibly reflecting abnormal endothelial cell function.[305] [307] One study suggested that autoantibodies to protein S could also be playing a role.[309] Thromboembolic complications have been reported in HIV-infected patients with reduced protein S levels.[304] [305] [306] [312] [313] Thus, in contrast to the presence of a lupus anticoagulant, protein S deficiency may predispose to thrombosis in HIV-infected patients in certain settings.

Gammopathies

Polyclonal hypergammaglobulinemia was among the first immunologic abnormalities described in AIDS and is seen in both asymptomatic and symptomatic seropositive patients.[90] [314] [315] Elevated levels of IgG, IgA, and IgM are present in spite of depressed in vitro B-cell proliferative responses to mitogens.[90] [91] Interestingly, in 318% of HIV-infected patients, monoclonal or oligoclonal immunoglobulin bands can be demonstrated by serum protein electrophoresis or immunofixation.[316] [317] [318] [319] [320] This high frequency of monoclonal gammopathy in patients with a mean age of 35 is in marked contrast to a frequency of 0.09% for age-matched controls.[317] [321] Paraproteins can be either IgG or IgM and usually occur in the absence of a depression of other immunoglobulins and without Bence Jones proteinuria.[316] [317] In one study, an analysis of paraproteins in seven HIV-infected patients demonstrated five to represent a polyclonal response to HIV antigens, suggesting an exaggerated humoral immune response to particular viral components.[319]

Clinically, hypergammaglobulinemia may contribute to rouleaux formation of red cells on a peripheral blood smear,[129] the generation of circulating immune complexes,[322] [323] and the formation of a number of abnormal antibodies reactive with platelets,[231] [246] [249] [264] red cells,[122] [185] [186] lymphocytes,[324] [325] granulocytes,[231] [246] and phospholipids.[288] [289] [291] [293] Remarkable cases of a

hyperviscosity syndrome secondary to diffuse polyclonal hypergammaglobulinemia have also been described in HIV-infected patients presenting with visual blurring, characteristic changes on funduscopic examination, and epistaxis, with an elevated serum viscosity level.[326] [327] Hyperviscosity syndromes have also been reported in HIV-infected children during the administration of intravenous gammaglobulin.[328] Although uncommon, both multiple myeloma and isolated plasmacytomas have been described in HIV-infected patients,[146] [329] [330] including one case of myeloma in which the paraprotein was directed against the HIV p24 *gag* gene product.[331] Amyloidosis with AA-type fibrils has also been reported in a single patient with AIDS who presented with nephrotic syndrome and demonstrated characteristic green birefringence after Congo red staining of kidney, liver, spleen, and bone marrow.[332] Paradoxically, HIV-infected hemophiliacs with a history of acquired inhibitors to factor VIII frequently lose this activity during the course of their HIV infection, and may benefit from the reintroduction of factor VIII as part of their management.[333]

The mechanism of hypergammaglobulinemia in HIV-infected individuals appears to involve polyclonal activation of B cells in vivo. Although B cells obtained from patients are poorly responsive to mitogens in vitro, Lane et al.[90] demonstrated that cultured B cells from patients with HIV infection secrete immunoglobulin spontaneously, suggesting that circulating B cells in these patients were preactivated in vivo and, paradoxically, refractory to further stimulation in vitro. B cells from normal donors can be directly activated without T-cell help by EBV infection as well as CMV and HIV antigens, and one or more of these factors could be responsible for the apparent activation of B cells in HIV-infected patients. HIV gp120 has been implicated in functioning as a superantigen that can directly activate B-cell subsets that express particular immunoglobulin genes.[93] [94] [334] HIV has also been proposed to infect endothelial cells and stimulate B-cell proliferation indirectly by upregulating the costimulatory and adhesion molecules, CD40 and VCAM-1, respectively.[95] As noted previously, paraproteins in some patients may have anti-HIV activity,[319] [331] [335] indicating that one component of the hypergammaglobulinemia may be linked to the immune response to HIV antigens. Alternatively, as noted earlier, immune dysregulation may result from an imbalance of CD4 lymphocyte subsets that promote either cellular or humoral immune responses, resulting in sustained polyclonal B-cell activation.[66]

The profound stimulation of B cells present even in asymptomatic individuals with HIV infection is closely linked to the development of generalized lymphadenopathy[315] and likely plays a role in the pathogenesis of B-cell lymphomas observed in these patients.[336] [337] [338] Although the risk for developing B-cell lymphomas is greatest in patients with a severe depletion of CD4+ lymphocytes (<50/L),[339] unlike opportunistic infections, lymphomas may occur in the absence of clinically significant immunodeficiecy. It will be of great interest to determine if this sustained B-cell stimulation and the risk of lymphoma decrease following the

initiation of highly active antiretroviral therapy.

Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura (TTP) is an uncommon but well-described complication of HIV infection that has been described in both adults and children. [138] [139] [141] [142] [340] [341] [342] TTP can occur at any point in the natural history of HIV infection and has been described in patients with fully developed AIDS as well as the initial manifestation of HIV infection. [138] [139] [141] TTP has also been reported following and before a diagnosis of HIV-related ITP. [333] [344] Clinically, patients present with the typical TTP syndrome of fulminant microangiopathic hemolytic anemia and thrombocytopenia, with a variable proportion of individuals exhibiting fever, elevated creatinine, and neurologic abnormalities. [141] [345] More protracted cases of TTP have also been described. [346] Schistocytes and an elevated lactate dehydrogenase (LDH) level are typically present, although depending on the degree of bone marrow compromise by the underlying HIV infection, the reticulocyte count may be less than expected for the degree of anemia. Neurologic abnormalities have included mental status changes, seizures, transient focal neurologic defects, and coma. [138] [139] [141] Examinations of tissues from autopsy specimens, as well as lymph node and gingival biopsies, have documented characteristic histologic changes of fibrin deposition in the microvasculature. [138] [139] [347] Responses to therapy in HIV-associated TTP are similar to those seen for the classic syndrome, with 5085% of patients responding to regimens that included plasmapheresis with or without fresh-frozen plasma infusions, antiplatelet agents, corticosteroids, or vincristine. [138] [139] [140] [141] One case report of a patient with relapsing TTP suggested a response to zidovudine therapy. [348]

The mechanism for HIV-associated TTP and its relation to HIV infection itself are unclear. As with TTP in the absence of HIV infection, the pathogenesis of this disorder may involve primary endothelial cell damage [349] and/or factors that promote or inhibit platelet agglutination. [350] The hemolytic uremia syndrome has also been reported in HIV-infected patients presenting with renal failure and microangiopathic hemolytic anemia. [351] [352] Interestingly, TTP has also been reported in patients infected with a different human retrovirus, human T-cell lymphotropic virus (HTLV-I), [342] [353] which is closely associated with adult T-cell leukemia and a demyelinating neurologic disorder termed HTLV-associated myelopathy. [354] [355]

Porphyria Cutanea Tarda

Several recent reports have described acquired porphyria cutanea tarda (PCT) in HIV-seropositive patients. [356] [357] [358] [359] [360] [361] [362] Acquired PCT is a disorder of heme synthesis characterized by a reduction in uroporphyrin decarboxylase activity in liver cells with an elevation in heme precursors in plasma, primarily uroporphyrinogen. These metabolites are oxidized and excreted in the urine as uroporphyrin, causing an increased urinary uroporphyrin-to-coproporphyrin ratio (see Chap. 27). As in non-HIV-associated PCT, patients present with manifestations of delayed-type photosensitivity with increased skin fragility, vesicles, bullae, hyperpigmentation, and hypertrichosis, particularly on the face and dorsum of the hands. [356] However, in contrast to classic PCT, polycythemia and sclerodermoid changes are uncommon. [356] Hepatic dysfunction appears to be a common cofactor, since the majority of patients have abnormal liver function tests, serologic evidence of viral hepatitis, or a history of excessive alcohol consumption. [356] [357] [360] [361] [362] Therapeutic measures directed at avoiding sun exposure and removing precipitating agents (ethanol, estrogens, barbiturates) are generally successful, although additional measures including phlebotomy, antimalarials, [356] [358] and, in one case, therapy with zidovudine [358] may also be beneficial.

Diffuse Infiltrative CD8 Lymphocytosis Syndrome

A remarkable syndrome has been described in HIV-infected patients characterized clinically by bilateral parotid gland enlargement, xerostomia, and xeropthalmia. [363] [364] Biopsies of minor salivary or parotid glands have shown diffuse infiltration by benign CD8+ lymphocytes. Extraglandular infiltration by CD8+ cells is also common, with some patients presenting with lymphocytic interstitial pneumonitis, peripheral neuropathy, or lymphocytic infiltration of gastric mucosa, kidney, thymus, and

liver. [363] [365] Peripheral blood typically shows a normal total white cell count with a CD8+ T-cell lymphocytosis of approximately 50% and an elevated total CD8 count. CD4+ T cells in these patients are mildly to moderately depressed, indicating that this syndrome usually occurs in patients with minimal immune deficits. In contrast to patients with Sjögrens syndrome, serologic markers of rheumatologic disorders (rheumatoid factor, antinuclear antibodies, anti-Ro/SS-A, anti-La/SS-B) are negative, and HLA typing has shown an association with HLA DR5 or DR6. [365] [366] Complete responses in individual patients have been observed following treatment with zidovudine or chlorambucil. [363] However, numbers of treated patients are too small to permit general therapeutic recommendations. Phenotypic analysis of peripheral and infiltrating CD8+ lymphocytes, the detection of HIV antigens in salivary tissue, and the association of this syndrome with HLA DR5 suggest that this disorder results from a genetically determined and exuberant polyclonal cellular immune response to HIV itself, possibly correlated with an abnormal tissue expression of adhesion molecules. [364]

Morphology of Hematopoietic Cells in HIV Infection

Peripheral Smear

As noted previously, cytopenias of all peripheral blood cells have been observed in patients with HIV infection (Table 154-3). With the exception of thrombocytopenia, which can occur in asymptomatic individuals with relatively mild immune deficiency, anemia and leukopenia are both more frequent and severe in patients with advanced immunodeficiency. [122] [125] Peripheral red blood cells in patients with anemia are typically normochromic and normocytic and exhibit a varying degree of anisocytosis and poikilocytosis. [129] [132] [133] [134] The perturbation in red cell size and shape is reflected in an increased red cell distribution width. [123] Macrocytosis is rarely seen. [129] However, in patients receiving therapy with zidovudine, macrocytosis is present in the majority of patients, occasionally with mean corpuscular volumes as high as 120 or greater (Fig. 154-5). [135] [136] Rouleaux formation of red cells may also be seen; this likely reflects the presence of concomitant hypergammaglobulinemia. [129] [133] As noted previously, schistocytes and nucleated red cells are present in patients with HIV-associated TTP. [137] [138] [139] [141]

Peripheral blood neutrophils are typically left-shifted [129] and may exhibit a number of morphologic abnormalities, including enlarged size, hyposegmentation, and Pelger-Hüet anomalies (Fig. 154-5). [129] [134] Atypical plasmacytoid lymphocytes are occasionally seen in asymptomatic individuals but are particularly common in lymphopenic patients with AIDS [129] [133] and during acute HIV infection. [367] Large atypical monocytes have also been described with prominent vacuolization and fine nuclear chromatin. [129] [133]

Bone Marrow Histology

Bone marrow examinations in HIV-infected patients are usually performed to evaluate peripheral cytopenias or when systemic infections or malignancies are suspected. Thus, with the exception of patients with isolated thrombocytopenia, the majority of information available on bone marrow histology in patients with HIV infection is from individuals with advanced immunodeficiency. It is clear, however, that in this population, abnormalities are frequently seen in all marrow cellular elements, as well as in the marrow matrix itself (Table 154-3). With the possible exception of particular types of dysplastic changes in megakaryocytes [269] [270] (described further later), there appear to be no distinctive features of bone marrow histology in patients with HIV infection or AIDS. [122] [123] [368]

TABLE 154-3 -- Morphologic and Histologic Abnormalities of Peripheral Blood and Bone Marrow in Patients with ARC and AIDS

Peripheral Blood
Red cells
Normochromic normocytic anemia
Anisocytosis and poikilocytosis
Rouleaux
Schistocytes
Reticulocytopenia
Basophilic stippling

Macrocytosis (during zidovudine therapy)
Neutrophils
Enlarged
Left-shifted
Hyposegmented with Pelger-Hüet forms
Other
Plasmacytoid lymphocytes
Large vacuolated monocytes
Bone Marrow
Cytologic features
M/E ratio 25:1
Hypercellular, normocellular, or hypocellular
Erythroid dysplasia
Erythroid hypoplasia (MAC infection, zidovudine)
Myeloid dysplasia
Left-shifted, hyposegmented, maturation arrest at metamyelocyte stage
Megakaryocytes
Adequate to increased
Dysplastic (hyposegmented, micromegakaryocytes, denuded nuclei)
Plasmacytosis
Lymphoid aggregates/infiltrates
Histiocytes
Loose granulomas (aggregates of plasma cells, lymphocytes, histiocytes)
Erythrophagocytosis
Increased eosinophils
Iron adequate to increased (reticulo-endothelial cell distribution)
Marrow matrix
Increased reticulin
Necrosis
Serous atrophy

Adapted from reviews by Perkocha and Rodgers[123] and Zon and Goopman.[122]

Marrow cellularity is typically normocellular to hypercellular, even in the setting of peripheral cytopenias, although 525% of AIDS patients in some series have exhibited hypocellular marrows.[125] [130] [131] [144] [146] [148] In general, the peripheral blood counts do not correlate with the overall degree of marrow cellularity, and patients with granulocytopenia and anemia are more likely to have normocellular or hypercellular than hypocellular marrows. [125] The myeloid-to-erythroid ratio usually ranges from 27:1, reflecting the combined effects of myeloid hyperplasia seen even in patients with peripheral granulocytopenia, and erythroid hypoplasia. [123] [134] [144] [146] A higher degree of erythroid hypoplasia in some series likely reflects the increased use of zidovudine, which, as described in a later section, can suppress erythropoiesis.

A remarkable degree of dysplasia has been noted in the morphology of myeloid, erythroid, and platelet precursors. Myeloid maturation is in general left-shifted, [125] [144] with one series noting a relative maturation arrest at the metamyelocyte stage. [129] A number of myelodysplastic changes in the myeloid lineage have been described, including the presence of abnormal myeloblasts with high nuclear/cytoplasmic ratios and abnormal folded or cleaved nuclei, [134] as well as large myelocytes, metamyelocytes,

and bands with megaloblastic-appearing nuclei. [129] [134] [144] [145] In contrast to the megaloblastic changes of myeloid cells seen in B_{12} deficiency, granulocytes in patients with AIDS are often hyposegmented rather than hypersegmented.[129] [134]

Dyserythropoiesis, although initially reported less often than myelodysplasia, has been increasingly recognized. [145] [146] Abnormalities have included erythroblasts with lobulated, binucleated, and fragmented nuclei, as well as basophilic stippling in more mature red blood cells. [129] [134] [144] In one series, all patients with dysplastic changes were found to be anemic and exhibited marked anisocytosis and poikilocytosis on peripheral smear. [125] Frank megaloblastic changes in erythroid precursors have been reported but appear to be less common than megaloblastic features of myeloid cells. [134] However, erythroid megaloblastic changes have been described and are likely more common in patients receiving folate antagonists or zidovudine. [133] [135] [136] [145] [146] [149] Erythroid hypoplasia that may be severe has been reported in some patients with AIDS or ARC,[148] in patients receiving zidovudine,[136] [152] [204] and in the setting of systemic MAC infection. [129] [133] [150] [158] Pure red cell aplasia or severe hypoplasia secondary to persistent parvovirus B19 infection has also been described in immunocompromised patients with [154] [156] [157] [369] [370] [371] and without[369] [372] [373] [374] HIV infection. Remarkable responses have been observed with intravenous gammaglobulin therapy due to the presence of neutralizing immunoglobulin in commercial gammaglobulin preparations.[154] [157] [369] [372] [375] Chronic parvovirus B19 infection should be suspected if characteristic vacuolated giant pronormoblasts are seen on marrow aspirates or biopsies[154] [156] [157] [372] [376] (Fig. 154-5), but this may warrant consideration in any patient in whom a profound depression in erythropoiesis occurs out of proportion to other marrow elements. In general, marrow morphology is a poor predictor of parvovirus infection, and more sensitive assays are often required, including polymerase chain reaction analysis of viral DNA in plasma and immunoperoxidase stains or in situ hybridization analysis of marrow samples.[375] [377]

Megakaryocytic dysplasia has become increasingly recognized in patients with fully developed AIDS as well as in those with isolated thrombocytopenia. [146] [231] [270] Dysplastic changes that have been described have included micromegakaryocytes with denuded nuclei and megakaryocytes with hyposegmented or fragmented nuclei [134] [144] [145] [146] [269] [270] (Fig. 154-5). Ultrastructural analysis of megakaryocytes from AIDS patients with thrombocytopenia has demonstrated marked ballooning or blebbing of the peripheral zone, a finding not encountered in megakaryocytes from individuals with non-HIV ITP. [269] In addition, abnormal clustering of megakaryocytes on bone marrow biopsy sections, similar to that seen in myeloproliferative syndromes, has also been described. [134] As noted previously, dysplastic changes observed in megakaryocytes (Fig. 154-6), the detection of HIV RNA in megakaryocytes from infected patients, and the clinical response of 5068% of patients with HIV-related thrombocytopenia to zidovudine are all consistent with the view that the megakaryocyte itself is a target for HIV infection in vivo. [284] [285]

Although several of the dysplastic changes noted in HIV-infected patients resemble those seen in preleukemia syndromes, myeloid leukemia in AIDS, though reported, is very uncommon.[197] [198] [378] Thus, it appears that the significance and the etiology of dysplasia in patients with HIV infection are different than those described for patients without HIV infection who present with peripheral cytopenias and marrow dysplasia. [123] [134] As noted previously, direct infection of marrow

precursors by HIV may contribute to these defects, although this issue remains controversial. [209] [210] [211] [215] [219] [220] [221] [284] [379]

Lymphoid aggregates are common and have been reported in 1050% of patients with AIDS and ARC. [125] [130] [131] [132] [134] [144] [145] [146] [148] Lymphoid aggregates may be either small, well circumscribed, and composed of small round lymphocytes, or large, poorly defined, and mixed with histiocytes. [133] [145] Infiltrating lymphocytes have also been described that are atypical or cleaved and located in paratrabecular areas. [144] [148] However, when paratrabecular localization of lymphocytes is seen, it is important to rule out the possibility of a non-Hodgkins lymphoma, since lymphomas in AIDS patients involve bone marrow in up to 50% of cases. Bone marrow has also been described as the initial or only site of involvement for both non-Hodgkins and Hodgkins lymphomas complicating HIV infection [380] [381] (Fig. 154-5).

Plasmacytosis is also extremely common in bone marrow and has been seen in 3185% of patients. [125] [130] [145] [146] In individual patients, plasma cells may represent as high as 40% of bone marrow cells in the absence of a neoplastic disorder. [382] The morphology of plasma cells has varied from being normal to moderately dysplastic with large bi- or trinucleated cells. Russell bodies with multiple globules have also been described. [382] Although myeloma has clearly been reported in AIDS, patients may present with monoclonal spikes on serum protein electrophoresis and marrow plasmacytosis with plasma cell aggregates and atypical forms, but without a clonal proliferation of plasma cells. [383] Thus, in the absence of lytic bone lesions or other clinical evidence of a plasma cell neoplasm, a diagnosis of myeloma should be made with caution.

Additional cellular abnormalities of bone marrow include increased numbers of histiocytes, noncaseating granulomas, and the presence of loose granulomas consisting of aggregated histiocytes, lymphocytes, and plasma cells [130] [131] [134] [144] [146] [148] [382] (Fig. 154-5). Pseudogaucher cells have also been described from a patient with MAC infection in which large foamy histiocytes on Wright Geimsa stain were shown to contain numerous mycobacterial organisms by periodic acid-Schiff and Ziehl-Nielsen stains. [384] An additional case of this morphologic manifestation of MAC is shown in Figure 154-5 . Histiocytic phagocytosis of erythroid cells and occasionally granulocytes and platelets has been described, [133] [385] but this is a nonspecific finding that may also occur in a variety of viral, fungal, and bacterial infections. Severe hemophagocytic syndromes have been reported in HIV-infected patients presenting with pancytopenia. [386] [387] [388] Although peripheral blood eosinophilia is rarely seen, [129] marrow eosinophilia is common and has been reported in 961% of patients with AIDS. [125] [144] [148] [382]

In most series, bone marrow iron stores are adequate to increased [125] [131] [133] [134] [148] in reticuloendothelial cells, indicating a defect in iron utilization similar to that seen in other chronic disease states. [163] Interestingly, one study in children described decreased iron stores in 27 of 78 patients (44%). [368] Sideroblasts with or without the presence of ringed forms have been described but are uncommon. [125] [129] [131]

Abnormalities in the bone marrow matrix are frequently seen and include increased reticulin or fibrosis, [125] [129] [131] [134] [144] [146] [148] [382] and serous atrophy or gelatinous transformation. [130] [144] [146] [148] [382] The increase in marrow reticulin may be focal or diffuse and may be increased in areas of granuloma formation [131] [148] or lymphoid aggregates. [148] In the majority of patients in some series, bone marrow aspiration is difficult or not uncommonly dry, [129] [131] [133] probably as a result of one or more of these abnormalities. While atypical proliferation of endothelial cells has been noted on histologic sections of marrow, [148] KS involving bone marrow is rarely seen. [389] [390]

Bone marrow histology or culture may be particularly useful in documenting opportunistic infection and should be performed in HIV-infected patients with undiagnosed fevers or constitutional symptoms, especially those with low CD4 cell numbers. [391] [392] [393] In one series of 47 patients with AIDS in whom a bone marrow aspiration or biopsy was performed to evaluate unexplained fever, pathogens were detected in 20 patients

TABLE 154-4 -- Infections Involving the Bone Marrow in Patients with AIDS

Infection	References
Mycobacterium avium complex	[129] [130] [131] [133] [394] [396]
Mycobacterium tuberculosis	[394] [396]
Mycobacterium xenopi and *kansasii*	[396]
Histoplasma	[130] [396]
Cryptococcus	[394] [396] [397]
Toxoplasma	[131]
Cytomegalovirus	[144]
Leishmania	[398]
Pneumocystis carinii	[400] [401] [402]
Disseminated cat scratch	[399]
Parvovirus B19	[154] [156] [157] [369] [370]

(42.5%). [394] Although it has been argued in the diagnosis of mycobacterial infection that blood cultures have a sensitivity comparable to that of bone marrow cultures in diagnosing disseminated infection, [395] bone marrow examination with special histologic staining can certainly provide a more rapid method of diagnosis for both opportunistic mycobacterial and fungal infections. [396] Infectious agents reported to involve the bone marrow in patients with AIDS are listed in Table 154-4 and include MAC, [129] [130] [131] [133] [393] [394] [396] *Mycobacterium tuberculosis*, [393] [394] [396] *Mycobacterium xenopi*, and *kansasii*, [396] *Histoplasma* [130] [396] (Fig. 154-5), *Cryptococcus* [394] [396] [397] (Fig. 154-5), *Toxoplasma,* [131] CMV, [144] *Leishmania,* [398] disseminated cat-scratch disease, [399] and *Pneumocystis carinii.* [400] [401] [402] Marrow granuloma in patients with mycobacterial infection may be either loosely formed or absent despite the presence of abundant organisms on acid-fast stains [130] [394] (Fig. 154-5). As noted previously, parvovirus B19 infection should be suspected in the setting of profound erythroid hypoplasia if giant pronormoblasts are present (Fig. 154-5). [154] [156] [157] [369] [370]

Extralymphatic presentation of non-Hodgkins lymphomas occurs in up to 90% of patients with HIV infection and has been reported to involve the bone marrow in 50% of cases. [403] As noted previously, bone marrow involvement may be the presenting site of lymphoma. [337] [403] The lymphomas are typically high-grade Burkitts or non-Burkitts lymphomas (Fig. 154-5), although more well-differentiated types have also been described. [337] [403] Lymphomas should be suspected in the clinical setting of constitutional symptoms, an elevated LDH, and the presence of teardrops or nucleated red blood cells on peripheral blood smear. Hodgkins disease, also noted with increased frequency in HIV-infected patients, is, in comparison to patients without HIV infection, more likely to involve extralymphatic sites, including the bone marrow (Fig. 154-5). [337] [381]

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HEMATOLOGIC COMPLICATIONS AND EFFICACY OF ANTIRETROVIRAL THERAPY

Zidovudine (AZT)

3azido-2,3-dideoxythymidine (AZT), or zidovudine, was the first antiretroviral drug shown to improve the survival of patients with AIDS. [404] [405] This thymidine analogue contains an azido group in place of the 3 hydroxyl group of the nucleoside sugar moiety. Zidovudine is phosphorylated by cellular kinases, and following incorporation into an elongating strand of viral DNA by reverse transcriptase, AZT-triphosphate prevents subsequent 5 to 3 phosphodiester linkages from forming, resulting in premature termination of the viral DNA chain.[404] [405] [406] An increased sensitivity of reverse transcriptase to zidovudine compared to cellular DNA polymerases confers greater activity on viral rather than cellular DNA synthesis. [404] [406] [407] The antiviral effects of zidovudine are dependent on its phosphorylation by cellular kinases. [406] In addition to its activity as a chain terminator, AZT-monophosphate competitively inhibits thymidine kinase, resulting in a reduced level of intracellular thymidine-5-triphosphate (dTTP) and an inhibition of DNA synthesis. [404] [405]

Early studies demonstrated that zidovudine could prolong survival in patients with fully developed AIDS [160] and delay the progression to AIDS in asymptomatic patients with HIV infection whose CD4 cell numbers were <500/l. [408] [409] Zidovudine can also improve HIV-related dementia and other neurologic defects [410] [411] [412] and has been shown to reduce maternal-to-fetal transmission of HIV when administered to the mother before and to the fetus after birth. [413]

Hematologic toxicities associated with zidovudine are, in general, dose-related and are more likely to develop in patients with pre-existing neutropenia and anemia, low serum B_{12} levels, and CD4 cell numbers <100/l. [135] [414] However, severe pancytopenia occurring within 46 weeks of starting zidovudine has also been reported as an apparently idiosyncratic reaction and may persist several months after discontinuation of therapy. [204] Patients on chronic zidovudine therapy typically develop macrocytic red cell indices, although generally without hypersegmentation of neutrophils. [135] [415] The incidence of anemia has decreased considerably with the lower doses of zidovudine used in current protocols. [127] Unlike thrombocytopenia (discussed further later), HIV-related anemia and neutropenia usually do not respond to zidovudine therapy. As noted previously, bone marrow examinations of patients receiving zidovudine have demonstrated a number of abnormalities in the erythroid lineage, including erythroid hypoplasia, aplasia, or maturation arrest. [136] Interestingly, among patients who develop severe pancytopenia and marrow hypoplasia as an early complication of zidovudine therapy, macrocytosis is not seen. [204] Zidovudine is metabolized primarily by glucuronidation in the liver, and drugs that are also glucuronidated, particularly probenecid, [405] [416] may prolong the half-life of zidovudine and potentiate its toxicity. The effects of myelosuppressive drugs such as chemotheraputic agents will be worsened by concomitant therapy with zidovudine. [417]

The hematologic toxicity of zidovudine is likely related to inhibition of cellular DNA polymerases as well as a depletion of intracellular thymidine pools. [404] [405] [418] Studies on the effects of zidovudine on bone marrow cultures have demonstrated a time- and dose-dependent inhibition of marrow erythroid and granulocyte/macrophage precursors, with the BFU-Es exhibiting 5- to 8-fold more sensitivity to zidovudine than CFU-Es, CFU-GMs, and CFU-GEMMs. [418] As discussed further in the following sections, the concurrent administration of recombinant erythropoietin or myeloid colony-stimulating factors (GM- or G-CSF) can ameliorate the anemia and leukopenia that may complicate zidovudine therapy. [167] [236] [419] [420] [421]

Other Reverse Transcriptase Inhibitors and Protease Inhibitors

A number of other nucleoside analogs are now available to treat HIV-infected patients, including DDI, DDC, D4T, and 3TC, as well as nonnucleoside analogs such as navaripine. In general, these compounds have little hematologic toxicity and can be used in patients who cannot tolerate zidovudine. [405] In one retrospective study of patients receiving DDI, a significant improvement in hemoglobin level, granulocyte number, and platelet count was observed and found to persist for up to 1 year following therapy.[422] Macrocytosis has not been observed with either DDC or DDI. [423] [424] However, nonhematologic toxicities are

TREATMENT OF ANEMIA IN HIV INFECTION

As in any patient with a low hematocrit, the diagnostic evaluation of anemia in a patient with HIV infection requires a careful consideration of factors that contribute to decreased or ineffective red cell production, increased peripheral destruction, and blood loss. In addition to direct effects of HIV and/or chronic disease states on hematopoietic precursors, other important etiologies include marrow involvement by lymphomas and opportunistic infections (particularly MAC or fungal pathogens) and blood loss due to gastrointestinal involvement by lymphomas and CMV infection. As noted previously, pure red cell aplasia secondary to persisting parvovirus B19 infection has been well described in HIV- infected patients and typically responds to intravenous gammaglobulin therapy. [154] [156] [157] [369] [370] [377] Available evidence does not indicate that decreased iron, B_{12}, or folate levels contribute to the chronic anemia that is typically seen in HIV-infected patients, although consideration of these potentially reversible etiologies is always warranted. [123] [181] Immune hemolytic anemia, although reported, is similarly rare [190] [192] [193] [194] [404] [434] despite the frequency of positive anti-red cell immunoglobulin tests, [122] [185] [186] but when present has been reported to respond to corticosteroids, splenectomy, and, in a single case, zidovudine. [192] [193] [434] Microangiopathic hemolytic anemia secondary to TTP, although uncommon, may be fulminant but has responded to regimens that included plasmapheresis with or without antiplatelet agents and plasma infusion. [137] [138] [139] [140] [141]

Potentially reversible drug etiologies of anemia include zidovudine, trimethoprim/sulfamethoxazole, dapsone, and, in the setting of antimycobacterial therapy, isoniazid. Reversible megaloblastic panhypoplasia has been reported secondary to trimethoprim and dapsone therapy for PCP, and folinic acid has been recommended, particularly with suspected vitamin B_{12} deficiency, to ameliorate the hematopoietic toxicity of these and other folate antagonists. [149] Although patients receiving dapsone frequently exhibit manifestations of low-grade hemolysis with an increase in LDH, severe hemolysis may also occur, particularly in the setting of G6PD deficiency. [435] [436]

As noted previously, therapy with zidovudine is frequently complicated by anemia and occurs more commonly in patients with advanced immunodeficiency and low serum vitamin B_{12} levels. [135] [176] Although the anemia secondary to zidovudine therapy is generally dose-related and typically responds to dose reduction, apparently idiosyncratic reactions have been reported and may cause profound and prolonged marrow hypoplasia even after zidovudine is discontinued. [204] The long-term administration of zidovudine to some patients may require transfusion support, although, as described following, in some patients this may be ameliorated by the use of recombinant erythropoietin. [167] [419] [420]

Chronic transfusion support was initially a common practice in the treatment of AIDS patients. However, transfusion requirements have decreased in recent years due to the use of lower doses of zidovudine, the availability of alternative antiretroviral drugs, particularly protease inhibitors, and the use of erythropoietin. In general, the indications, benefits, and complications of transfusion support of patients with HIV-associated anemia are similar to those of the non-HIV-infected patient. [437] [438] There is some evidence that hepatitis C infection as a complication of transfusion therapy can have a more aggressive clinical course in the setting of HIV infection. [439] As with non-HIV-infected patients, chronic transfusion therapy may cause clinically significant iron overload complicated by hepatic fibrosis. [440] Interestingly, although transfusion-associated graft-versus-host disease has been observed in immunosuppressed patients, this complication has not been reported in patients with HIV infection, perhaps as a result of cytopathic effects of HIV on CD4 lymphocytes that are likely required to initiate or sustain the graft-versus-host reaction. [441] [442]

Several clinical trials have evaluated the effects of recombinant human erythropoietin in treating anemia in patients with HIV infection receiving zidovudine. [167] [419] [420] Erythropoietin has been shown to improve the hematocrit and decrease transfusion requirement in patients whose levels of endogenous erythropoietin levels were <500 IU/L (Fig. 154-7). No benefit was seen in patients with pretreatment erythropoietin levels >500 IU/L, who made up approximately one-third of the zidovudine-treated patients. [167] [419] A similar clinical benefit was also seen in zidovudine-treated patients who received erythropoietin in combination with recombinant granulocyte colony-stimulating factor (G-CSF). [420] Similar results have been described in HIV-infected patients not receiving zidovudine. [443] [444] Quality of life assessments have demonstrated that patients receiving erythropoietin for anemia experienced a greater energy level and an improvement in their activity level. [445] Although studies in AIDS patients did not demonstrate significant side effects from erythropoietin therapy, toxicities are well described in other patient groups and include rash, fatigue, and headache, as well as more severe cerebrovascular and cardiovascular complications, including stroke, seizure, hypertension, and myocardial infarction. Finally, an additional study demonstrated that G-CSF in the absence of erythropoietin treatment may also increase red cell production and the number of circulating BFU-Es, raising the possibility that additional therapeutic approaches using combinations of hematopoietic growth factors may be useful in the treatment of HIV-associated anemia. [446]

In patients with endogenous erythropoietin levels <500 IU/L, the recommended starting dose of erythropoietin is 100 Ukg subcutaneously or intravenously three times a week for 8 weeks. Doses are then increased by 50 Ukg every 4 weeks until a rise in hematocrit of 5% is observed or a hematocrit of 36% is achieved. The dose should not exceed 300 Ukg. [124]

Figure 154-7 Response to recombinant erythropoietin in patients on zidovudine therapy. Mean hematocrit concentrations are shown for zidovudine-treated patients with endogenous erythropoietin levels of 500 mU/ml who were treated with either recombinant erythropoietin (r-HuEPO) or placebo. r-HuEPO dose in the treated group was 100200 U/kg IV or SC three times per week. Bars represent 95% confidence intervals. No benefit was seen in patients with endogenous erythropoietin levels >500 mU/ml. *(Reprinted from Henry et al.,[167] with permission.)*

common, particularly for the nucleoside analogs, and include peripheral neuropathy and gastrointestinal toxicity, particularly acute pancreatitis. [414] [422] [423] [424] [425]

For both nucleoside analog and nonnucleoside-based reverse transcriptase inhibitors, drug-resistant strains of HIV characteristically emerge due to the selection of viral mutants with specific amino acid changes in the viral reverse transcriptase. [426] [427] These drug-resistant variants have been seen even when reverse transcriptase inhibitors are used in combination. [405] [428] [429] Recently, a number of compounds that inhibit the viral protease have become available, including saquinavir, indinavir, and ritonavir. When used in combination with one or more reverse transcriptase inhibitors, these regimens have been highly effective in reducing the levels of circulating viral RNA to low or even undetectable levels, [44] [430] a finding that is expected to have substantial clinical benefit. Although these complex drug regimens can be difficult to administer and may be poorly tolerated in some patients, in general combination antiretroviral regimens that do not include zidovudine have little hematologic toxicity. [44] [430] In protocols that incorporate interferon, leukopenia can be a significant dose-limiting toxicity. [431] [432] [433]

TREATMENT OF HEMATOLOGIC COMPLICATIONS OF HIV INFECTION

The evaluation and treatment of cytopenias in patients with HIV infection must take into account a number of possible contributing factors, including (1) the effects of drugs used to treat HIV infection itself or complicating opportunistic infections, (2) direct marrow involvement by pathogens or neoplasms (particularly lymphomas), (3) generalized suppressive effects of chronic disease states on myelo- and erythropoiesis, (4) possible nutritional deficiencies, (5) immune-mediated destruction of mature or immature hematopoietic cells, and (6) direct effects of the underlying HIV infection on bone marrow stem cells (Table 154-5). Consequently, the approach to the treatment of cytopenias in patients with HIV infection requires a careful consideration of multiple iatrogenic, infectious, neoplastic, and immune etiologies, many of which are potentially reversible.

Neutropenia

A number of drugs as well as infectious and neoplastic complications of AIDS can produce leukopenia, although reversible iatrogenic or secondary etiologies are often not found. Commonly used medications whose use is frequently complicated

TABLE 154-5 -- General Approach to the Differential Diagnosis of Cytopenias in Patients with HIV Infection

1. Hematologic toxicity of drugs disseminated intravascular coagulation
Antiretroviral therapy (i.e., zidovudine, interferon)
Antibiotics (i.e., trimethoprim/sulfamethoxazole, dapsone, pentamidine, ganciclovir, flucytosine)
2. Direct marrow involvement by infections (i.e., MAC, parvovirus B19)
3. Marrow involvement by malignancies (i.e., non-Hodgkins and Hodgkins lymphomas)
4. Autoimmune destruction of hematopoietic cells
5. Nutritional deficiencies (B_{12} , folate)
6. Generalized myelosuppressive effects of chronic disease states
7. Direct effects of HIV infection of marrow stem cells (i.e., megakaryocytes)
8. Miscellaneous (i.e., blood loss, TTP, disseminated intravascular coagulation)

by neutropenia include zidovudine, interferons, trimethoprim/sulfamethoxazole, sulfadiazine, ganciclovir, pentamidine, rifabutin, flucytosine, and chemotherapeutic agents (for lymphomas or KS).[199] [200] [202] [414] [433] However, even when drugs can be implicated as the cause of neutopenia, management decisions are frequently complicated by risks associated with discontinuing or reducing the dose of drugs required for treating infections or neoplasms. Some reports have noted an improvement of HIV-related neutropenia following the initiation of antiretroviral therapy, [422] and it is likely that further improvements will be reported with the use of combinations of antiretroviral agents (i.e., reverse transcriptase inhibitors and protease inhibitors).

The early observation that AIDS-related neutropenia could respond dramatically to therapy with GM-CSF provided a rationale for using hematopoietic growth factors in a number of clinical settings in which neutropenia is common. Groopman et al. [235] initially described responses in 16 of 16 AIDS patients treated with intravenous recombinant GM-CSF. A remarkably rapid and dose-dependent increase in peripheral blood neutrophils, monocytes, and eosinophils was observed (Fig. 154-8). Increased marrow cellularity was seen even in patients who had hypocellular marrows prior to therapy. Toxicities were mild and included fever, myalgias, headache, and flushing. Similar responses were subsequently described for patients treated with G-CSF. [236] Although responses to intravenous GM-CSF or G-CSF were transient, subsequent studies have demonstrated that long-term subcutaneous therapy with these growth factors can produce a sustained increase in granulocyte counts from weeks to months without an apparent depletion in marrow stem cell activity.[420] [421] [447] This effect has been observed in a variety of clinical settings in which neutropenia is common, including (1) patients receiving zidovudine either with [431] [432] or without[236] [420] [421] [447] interferon, (2) patients with CMV infection receiving ganciclovir, [448] (3) patients with HIV-related lymphomas receiving chemotherapy, [449] and (4) patients with HIV-associated neutropenia complicated by bacterial infections.[450] These studies demonstrated that GM-CSF or G-CSF, given at low doses, could elevate the granulocyte count and allow continuation of myelosuppressive therapy.[451] [452] In one study of patients with AIDS-associated non-Hodgkins lymphomas, GM-CSF therapy reduced the number of febrile episodes and days of hospitalization.[449] Further studies will be needed to determine the ultimate impact of hematopoietic growth factors on quality of life and survival.

Although some in vitro studies have shown that GM-CSF but not G-CSF could stimulate replication of particular HIV isolates, [283] with one exception,[421] clinical trials to date have not demonstrated laboratory evidence of a sustained enhancement of viral replication in vivo. [431] [432] [447] [449] [453] Interestingly, GM-CSF has been shown to augment markedly the antiretroviral effects of zidovudine in vitro, apparently by increasing intracellular levels of the biologically active triphosphate form. [454] [455] In addition, several reports have, depending on the assay used, described functional defects in neutrophils from patients with HIV infection, although no consensus exists as to specific defects involved. [456] [457] [458] [459] [460] In two studies, neutrophil function was observed to be enhanced following GM-CSF therapy. [460] [461] Neutrophils from patients treated with GM-CSF have also exhibited enhanced killing of HIV-infected target cells in the presence of antibodies to the HIV envelope glycoprotein. [462] Thus, myeloid growth factors may have a number of therapeutic roles both in promoting antiviral effects and in augmenting host immunity. However, the significance of these findings in terms of clinical benefit to patients remains to be demonstrated.

The usual starting dose of G-CSF for HIV-associated neutropenia is 5 g/kg/day given subcutaneously; it may be increased to 7.5 g/kg/day and then 10 g/kg/day. [124] Depending on the clinical setting, attempts may be made to reduce

TREATMENT OF THROMBOCYTOPENIA IN HIV INFECTION

As noted previously, studies on the mechanism of thrombocytopenia in HIV-infected patients have demonstrated decreased platelet survival [256] and the presence of circulating and platelet-associated immune complexes, [242] [322] [323] as well as the presence of true antiplatelet antibodies in some patients. [231] [242] [246] [249] [264] A number of medical approaches that have been successfully used to treat classic ITP have also been used in HIV-related thrombocytopenia with varying degrees of success, including corticosteroids, [240] [241] [251] intravenous gammaglobulin, [463] [464] and danazol. [251] Although responses are seen in as high as 6085% of patients receiving prednisone, usually in 4-week courses of 1 mg/kg/d followed by a rapid taper, these responses are sustained in only 10-20% of patients. [240] [241] [251] However, the long-term or repetitive use of an immunosuppressive agent in the setting of HIV infection is a major concern. Intravenous gammaglobulin may produce a rapid improvement in the platelet count, as seen in classic ITP, in approximately 90% of patients and represents the treatment of choice in the setting of a medical emergency or as a supportive measure for an invasive procedure. [463] [465] Responses typically last several days or occasionally weeks, but as with corticosteroids are usually transient. Encouraging results with anti-Rh(D) immunoglobulin [466] [467] [468] [469] and even dapsone [470] have been reported, presumably as a result of reticuloendothelial modulation from IgG-coated or hemolyzed red blood cells. Vincristine has also been reported to improve the platelet count in small numbers of AIDS patients with thrombocytopenia. [241] [471] In a more investigational approach, long-term correction of the platelet count was observed in a single AIDS patient following treatment with an anti-CD16 monoclonal antibody, reactive with the low-affinity Fc gamma receptor, again indicating that therapeutic measures to induce reticuloendothelial blockade may improve platelet survival. [472]

In contrast to the typically transient improvement following medical management of HIV-related ITP, splenectomy has been highly effective in producing a sustained increase in platelet counts in approximately 90% of patients. [240] [259] [260] [261] [262] Improvement has been seen even in patients who are refractory to steroids and gammaglobulin before splenectomy and those with severe thrombocytopenia with platelet counts <10,000/l. [260] Although in most reported series there has been no increase in the morbidity of splenectomy in HIV-infected patients, [260] the use of this procedure, while effective, clearly must be individualized. Among hemophiliacs, in whom relatively mild thrombocytopenia can lead to life-threatening hemorrhagic complications, [252] complete and last- ing responses were seen following splenectomy in four of four patients studied. [473] Most reported series have shown that splenectomy does not accelerate the clinical progression to AIDS. [240] [251] [262] As a possible alternative to splenectomy, some benefit with low dose splenic irradiation has also been reported, including patients who have failed to respond to zidovudine or gammaglobulin. [474] [475] [476]

Several reports have shown responses to zidovudine in approximately 50% of patients with HIV-related thrombocytopenia. [238] [477] [478] [479] [480] [481] [482] In one blinded placebo-controlled trial of 10 patients with platelet counts ranging from 20,000 to 100,000/l (mean 53,000/l), zidovudine therapy (250 mg q6h) resulted in a mean increase of 53,000/l in all patients during 8 weeks of treatment, while no patients receiving placebo showed improvement. [477] Relapses occurred in three of five patients on discontinuation of drug. Another prospective trial showed that the response to zidovudine was dose-dependent and durable. [482] In spite of concern that the well-documented myelosuppressive effects of zidovudine could pose risks for thrombocytopenic patients, thrombocytopenia, in contrast to neutropenia and anemia, is a rare complication of zidovudine. [135] [160] The mechanism for the beneficial effects of zidovudine on HIV-related thrombocytopenia is unclear, but in view of the evidence for HIV infection of megakaryocytes, as noted previously, it is likely that the antiviral activity of this agent acts to promote more effective thrombopoiesis. One kinetic analysis of platelet production demonstrated a significant improvement following zidovudine treatment. [256] Successful therapy of HIV-related thrombocytopenia has also been described for another antiretroviral drug, DDI, in both children [483] and adults. [484] [485] One large series of patients receiving long-term DDI therapy showed a general improvement in hemoglobin, granulocyte, and platelet counts. [422] Interestingly, in one report a beneficial effect on HIV-related thrombocytopenia was seen for zidovudine but not DDI. [486] Alpha-interferon has been reported to produce an increase in the platelet count in thrombocytopenic patients with HIV infection, [487] [488] [489] [490] [491] although it is unclear if this benefit is mediated by its antiviral, cytotoxic, or immunosuppressive effects. [237] Future studies using thrombopoietin to treat HIV-associated thrombocytopenia will be of interest, given the evidence for impaired platelet production. In view of the progress that has been made in reducing HIV replication with combinations of antiretroviral drugs, it is likely that there will be a decline in the incidence of HIV-related thrombocytopenia in the coming years.

Figure 154-8 Response to recombinant GM-CSF in an AIDS patient with neutropenia. Total leukocyte counts and differentials are shown for an AIDS patient treated with intravenous recombi-nant GM-CSF (1.0×10^4 U/kg/d) from days 117. *(Reprinted from Groopman et al., [235] with permission.)*

therapy to alternate-day treatments with a goal of maintaining the granulocyte count between 5001,000/l.

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FUTURE DIRECTIONS

The pandemic of AIDS and HIV infection will continue well into the next decade and will continue to pose major health care problems throughout the world. Many of the advances in therapy described in this review are unavailable or too expensive to be used in the populations of the world most affected. Although studies with a simian immunodeficiency virus model have shown that protective immunity can be generated by infection with an attenuated virus, [492] [493] the prospect for an effective vaccine for HIV in the near future is remote. [494] [495] [496] The treatment of HIV-infected patients with combinations of antiretroviral drugs has shown a dramatic reduction in the level of replicating virus, a result that is likely to have substantial clinical benefit. [497] [498] However, given that HIV remains detectable in long-lived populations of CD4 cells, [52] [53] the likelihood that HIV infection can be cured is small. Nonetheless, the prospects for controlling HIV infection are very encouraging. Newer drugs with fewer side effects and improved pharmacokinetic properties are clearly needed to simplify therapeutic regimens that are often too difficult or toxic for patients to tolerate. Research areas in the years ahead will also be focusing on new approaches, such as chemokine receptor antagonists to prevent viral entry as well as inhibitors of other viral proteins, such as the viral integrase. In addition, several studies have shown that hematopoietic cells can be engineered to become resistant to HIV infection and/or replication. [499] [500] [501] [502] [503] [504] These approaches may hold some promise as ways to reconstitute the immune system in patients once HIV replication has been suppressed. [504]

As has been the case with pharmacologic approaches to antimicrobial and antineoplastic therapy, improvements in controlling HIV infection have resulted from synergistic effects of effective agents that have different mechanisms of action. With a better understanding of HIV pathogenesis and the effects of this virus on the hematopoietic system, the use of antiretroviral agents and supportive modalities such as hematopoietic growth factors may be able to control or even reverse many of the problems discussed in this review. Clearly, hematologic complications of HIV infection will remain an active area for basic research and a challenge to clinicians in the years ahead.

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Chapter 155 - Bone Marrow Aspiration and Morphology

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BACKGROUND

History and Ontogeny of the Bone Marrow

After the blood itself, the bone marrow is the largest and most widely distributed organ in the body. Our bone marrow contains about 1 trillion cells and releases approximately 200 billion red blood cells, 100 billion white blood cells, and 400 billion platelets each day. [1]

For centuries, the marrow was considered a source of warmth and energy. Before its discovery as a blood-forming organ, it was believed to be the source of bone nutrition. It was not until the work of Neumann and Bizzozero that a relationship between the blood and bone marrow was established. In 1868, Neumann noted that bone marrow was an important organ for blood formation and was involved in de novo formation of red blood cells. [2] Bizzozero independently concluded that non-nucleated cells form from bone marrow nucleated cells and that white blood cells are also made in the bone marrow. [3] [4] Neumann continued to investigate bone marrow and was the first to identify leukemia as a disease of the marrow. [5] He also coined the term myelogenous leukemia. [6] Neumann was also the first individual to recognize that at birth, all bones that contain marrow contain red marrow. As the individual ages, the peripheral marrow becomes more fatty and develops into yellow marrow. [7]

Bone marrow is the last site of hematopoiesis during the prenatal period. In humans, bone marrow hematopoiesis becomes apparent at approximately 20 weeks of gestation. [8] However, some observers have found evidence for bone marrow activity as early as 1215 weeks of gestation. [9] At the time of birth, almost all hematopoiesis, under normal conditions, occurs in the bone marrow. Extramedullary hematopoiesis is sometimes observed in stressed situations, such as severe hemolytic anemia.

Anatomy of the Bone Marrow

The marrow is a richly cellular and highly vascular tissue. It consists of two components: the hematopoietic cell compartment and a highly organized stromal component that supports the proliferation of these hematopoietic cells. Whereas the hematopoietic cells are transient in the marrow, the stroma remains and serves as a backbone upon which the hematopoietic cells grow and differentiate. Within the hematopoietic areas of the bone marrow, hematopoiesis appears to be highly compartmentalized. Erythropoiesis takes place in distinct anatomic units surrounding a central macrophage, known as erythroid islands. [10] Granulopoiesis also appears to take place in somewhat less appreciable morphologic foci and is often associated with a stromal cell. [11] [12] Megakaryopoiesis occurs adjacent to the sinus endothelium with cytoplasmic processes of the megakaryocytes appearing to penetrate the endothelial wall. [13] [14] Both the hematopoietic cells and the stromal cells of the bone marrow are separated from the blood by a vascular wall that is of a large caliber and is relatively thin-walled. It is through these vessel walls that the mature cells of bone marrow migrate. This appears to occur as a transendothelial process, with the exiting cells appearing to make pores within the endothelial cells. [15]

At birth, all bone cavities are completely filled with hematopoietic cells and hence appear red, but with advancing age the hematopoietic elements are gradually replaced by adipose tissue. By adolescence, hematopoietic marrow is found only in the cavities of the centrally located bones, such as the sternum, ribs, vertebrae, clavicles, scapulae, skull, pelvis, and proximal ends of the femurs and humeri. A slow reduction in the red marrow mass then continues throughout life. This phenomenon has implications for using age to determine where bone marrow aspirations and biopsies might be performed. Thus, for the tibia and femur, approximately half of the hematopoietic cells are replaced by fatty material after the first decade of life, whereas for the vertebral column and iliac crest, half of the bone marrow remains hematopoietic for up to 60 or more years of life.

Historical Perspective of Bone Marrow Examinations

The first bone marrow biopsy was reported in 1905 by Pianese. [16] [17] It was performed in an infant with leishmaniasis. In 1909, Pianese trephined the tibia and femur, aspirating the marrow with a needle attached to a syringe. [18] In 1923, Seyfarth used a surgical trephine to obtain marrow from the ribs and the sternum. [19] Because trephination resulted in excessive bleeding and infection, it was not a generally well-accepted procedure. Airinkin, in 1927, eliminated the trephine complications by using a short lumbar needle to puncture the manubrium and sternum. [20] He obtained a marrow aspiration by placing a syringe on the needle hub. In 1945, Vandenberghe and Blitstein were the first to use the iliac crest to obtain bone marrow, [21] and Heidenreich obtained marrow from the spinous processes. [22] In 1952, Bierman used the posterior iliac crest as the site for bone marrow aspiration, claiming it to be a safe site. [23]

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PERFORMANCE OF THE BONE MARROW ASPIRATION AND BIOPSY

Criteria for Aspiration and Biopsy

The bone marrow aspiration and biopsy are now well-accepted procedures for evaluating both the cellularity of the marrow and the nature of the cells present. Before deciding whether, or what kind of, bone marrow examination is necessary, it is important to obtain a clinical history and to clarify what information is needed. Although in most instances, both aspiration and biopsy are necessary to evaluate the clinical situation fully, there are instances in which an aspiration, by itself, will give the information required. In the evaluation of a patient who is felt to have immune thrombocytopenia, a bone marrow aspiration, appropriately handled as discussed later in this chapter, allows the hematologist to determine whether the thrombocytopenia is associated with increased numbers of megakaryocytes. In pediatric practice, the hematologist is sometimes consulted to perform a bone marrow biopsy to determine whether the individual has a constitutional chromosomal abnormality. This

not infrequently occurs in the neonatal intensive care unit. For such an analysis, a bone marrow aspiration is adequate and often gives results regarding potential chromosomal abnormalities within 12 days. The only other instance in which an aspiration alone is indicated is in the follow-up of individuals with leukemia, when the only question being asked is, Are these patients still in remission? Otherwise, it is the norm to have a bone marrow biopsy accompany a bone marrow aspiration. This is especially true in assessing the cellularity of the bone marrow (for example, in patients with cytopenias) and in looking for evidence of infiltration associated with either malignancies or storage diseases.

Bone marrow cytologic examination may also be obtained by an incisional biopsy. This is usually performed by the surgeon at the time of a staging laparotomy for Hodgkin disease. Occasionally the site at which the bone marrow aspiration and biopsy are performed is determined by prior examination of the patient. For example, when looking for metastatic cancer or multiple myeloma, it is of value, if possible, to aspirate an area where there is tenderness or where there is radiologic evidence of potential infiltration.

The only contraindication to performing a bone marrow biopsy is the presence of hemophilia or other related disorders. Thrombocytopenia, no matter how severe, is not a contraindication. There are, however, complications of a bone marrow aspiration or biopsy. Fatalities, although rare, have been reported, especially with sternal aspirations and biopsies.

Rarely, a bone marrow needle may break. If this occurs, an attempt to extract the distal segment with a hemostat should be made. If it is unsuccessful, a surgeon should be consulted. Hemorrhage from bone marrow aspiration can occur at any site, especially in the thrombocytopenic individual. This can usually be prevented by applying a pressure bandage at the site. Other rare complications include pulmonary emboli following sternal aspiration and infected bone marrow aspiration sites, especially in immunocompromised patients.

Although it has been traditional to utilize the sternum for bone marrow aspiration but not for biopsy, our preferred site for both aspiration and biopsy in the adult as well as the child and infant is the posterior iliac crest and spine. In many premature infants and some full-term infants, the iliac bone has not completely ossified, and therefore an alternative bone, such as the anterior tibia, should be utilized. It is still worthwhile, because of both ease and safety, to attempt to use the iliac bone initially, even in neonates.

Aspiration of the posterior iliac bone marrow is usually quite cellular, even in the elderly. However, if an individual has had total nodal radiation therapy for Hodgkin disease, this bone may be permanently altered. In obese patients, it is necessary to use a long needle if the posterior iliac crest is aspirated.

Bone Marrow Aspiration and Biopsy

Before performing the examination, the patient should be reassured that the study is safe and that although some pain will occur, analgesic agents will be used, and pain will terminate at the end of the procedure. For the overly anxious patient, the use of a tranquilizer can be helpful. In children, especially those with procedure phobias, the use of lorazepam under carefully controlled conditions has been very beneficial. This drug produces both relaxation and an antegrade amnesia that many children prefer. A safe, conscious sedation policy should be in place prior to using drugs such as lorazepam in children.

Adults and some older or heavier children are asked to lie on one side after removing their clothing. In young or thin patients, it may be easier to perform the aspiration with the patient lying prone or with a pillow under the abdomen and pelvis. Since this procedure is performed without the patients seeing it, it is important to tell the patient what will be and is being done. The iliac crest and spine are felt with the fingers and a small mark is made in the appropriate area with the fingernail. After double gloving, the area is swabbed with an antiseptic solution. After cleansing the area, the outer pair of gloves is removed. Lidocaine or some other anesthetic agent is then injected where the mark was made. We utilize an air gun to give the initial anesthetic agent and follow it with a needle long enough to anesthetize the periosteum directly. It is worthwhile then to allow 12 minutes for the anesthetic to produce a maximum effect.

The kinds of needles used for aspiration are quite numerous. We prefer using a needle with a stylet fixed by a Luer-Lok or some other locking device. It is important to use a sharp needle, one long enough to penetrate the bone easily. In order to be sure that the needle is entering correctly, the second and third fingers on the hand not being used to insert the needle should be placed on the iliac crest or spine and the needle inserted between them. The needle and stylet are pushed into the bone with a slight rotary motion. When it is felt that the needle is firmly in place, the stylet is removed and a 10- or 20-ml plastic syringe is attached. The patient is then told that he/she may feel an unpleasant sensation, and the plunger of the syringe is pulled back vigorously with no more than 0.5 ml of bone marrow and blood aspirated. The smaller the volume, the less the contamination with peripheral blood. The sample is then given to an assistant, who prepares it as described later in this chapter. If additional samples are needed, another syringe may then be placed on the needle. When the procedure is completed, the stylet is reinserted, the needle removed, and pressure applied to the area.

If a biopsy is performed, a small incision with a #11 scalpel blade is made before insertion of the biopsy needle and stylet. A different site should be chosen for the biopsy, if it is performed after the aspiration. After insertion of the biopsy needle into the bone, the stylet is removed. The needle is then advanced between 1 and 2 cm, depending on the size of the patient. This is performed with very little rotation of the needle. After advancing the needle, the hub is rotated a few times in one direction, followed by a few times in the other direction. The needle is inserted a small additional amount to break the attachment, and the rotations are repeated. The thumb is then placed on the hub of the needle, and the needle is extracted with slight lateral movements accompanying the removal. The bone marrow biopsy is then placed on a slide, where imprints are made before processing for cytologic investigations.

Morphologic Preparation of Bone Marrow

A complete diagnostic evaluation of a bone marrow sample may involve any or all of the following techniques: morphologic examination of stained smears, cytochemistry, culture for microorganisms, histologic examination of stained sections, immunocytochemistry, cell marker analysis by flow cytometery, cell culture assays, cytogenetics, and molecular biologic studies of gene rearrangements or translocations. It is important that the sample is prepared in a fashion that allows the maximal use of as many of these techniques as is necessary to answer the clinical question.

Several morphologic preparations can be made from the bone marrow sample obtained by aspiration or biopsy. [24] Each has its advantages and limitations, as listed in Table 155-1 . The sample considered to have the best morphology and most representative distribution of cell types is the direct smear, since its preparation involves the least manipulation of the sample and no anticoagulant is present. This is the preparation that should preferably be used for a bone marrow differential count. In some cases, the marrow is too hypocellular to allow

TABLE 155-1 -- Morphologic Preparations Made from the Bone Marrow Sample

Preparation	Major Purpose	Limitations
Direct smear	Relative percentage of marrow elements; best morphology	Too few cells to evaluate in some hypocellular marrows
Particle	Marrow cellularity	Parts of smear (near particles) may be too thick for best morphology
Concentrate	Concentrates nucleated cells, especially megakaryocytes and tumor clumps	Relative proportions of cells may be distorted; morphologic changes due to EDTA
Mixed layer	Enriched for macrophages; evaluation of iron stores	Similar to particle
Biopsy imprint	Tumor cells; useful in case of a dry tap	Similar to particle

an adequate number of cells to be examined on the direct smear. Here, the bone marrow concentrate (essentially a buffy coat preparation) is useful since the leukocytes are enriched relative to red blood cells. Megakaryocytes and tumor cell clumps are also enriched, particularly at the feathered and side edges of the smear. However, because the buffy coat layer is not sharply demarcated and merges imperceptably with the red cell layer, there is some variability in the degree of enrichment of nucleated cell types of differing densities. Often, erythroid precursors are significantly enriched relative to granulocyte precursors. Furthermore, the exposure to an anticoagulant such as ethylene-diamenetetracetic acid (EDTA) required during the preparation of the buffy coat may result in artifactual nuclear clefting and cytoplasmic vacuolation, particularly of abnormal cells. [25] The bone marrow particle preparation is useful for estimation of overall cellularity, since large spicules are concentrated in this specimen. However, the morphology is often not as good as in the direct smear, since portions of the smear near the particles may be relatively thick. Macrophages are frequently enriched in the particles found in the mixed layer and are useful in evaluating iron stores. The bone marrow biopsy imprint is important to detect metastatic tumor in cases in which the aspirate is uninformative and to allow immediate morphologic examination of the marrow in case of a dry tap, which occurs in about 4% of marrow aspirations. [26] This is useful in view of the high incidence of pathologic changes (93%) in these cases. [26] There is considerable evidence also that metastatic malignancy, lymphoma, or multiple myeloma is missed more frequently on the aspirate than the biopsy. [27] [28] [29] However, the sensitivity of bone marrow aspirate for detection of metastatic tumor is dependent on the thoroughness of examination of all smear preparations. A recent study, in which the presence of tumor cells on each aspirate smear was examined separately, showed that tumor cell clumps are found in only one of eight smears in 5% of cases with positive aspirates. [30]

Technical Considerations in Smear Preparation

Preparation of good quality peripheral blood and bone marrow smears on a consistent basis is more difficult than it would appear. There is no substitute for practice in blood smear preparation under the guidance of an experienced technologist. The pusher slide used to make the smear should be narrower than the glass slide on which the drop of blood/marrow is placed, to ensure that the side edge of the smear can be examined. Important variables affecting smear quality are the size of the blood/marrow drop, angle at which the spreader slide is held, speed at which the smear is made, and hematocrit of the sample. High pusher slide angle, rapid movement of the pusher slide, and high hematocrit all tend to produce thicker smears. The ideal smear should be about two-thirds the length of the glass slide, with an even transition from thick to thin areas, terminating in a feathered edge.

Preparation of Bone Marrow Smears at the Bedside

In addition to the syringes and needles required for the actual aspiration, it is important to have the following materials ready for the preparation of the sample:

Glass slides, coverslips

Gauze

Forceps

Sample collection tube with EDTA (liquid EDTA for best morphology)

Heparinized tubes with culture medium for special studies

Pusher device (forceps with hemacytometer cover glass)

The glass slides should be placed in a convenient location before the procedure so that no time is lost in the preparation of the direct smears. About 0.5 ml of marrow is placed on a glass slide and the remainder immediately added to the EDTA anticoagulated sample collection tube and mixed well. The marrow placed on the glass slide is examined to determine whether bone marrow has been obtained, as indicated by the presence of spicules, which appear as fatty droplets, granules, or small chunks of bone. These concentrate at the feathered edge when a smear of the bone marrow is made.

Nine bone marrow direct smears are made from the pool of marrow by placing a tiny drop on a clean glass slide and making a smear in the same fashion as a blood smear. Alternatively, the pusher may be dipped directly into the pool of marrow and then the smear quickly pushed out on a fresh glass slide. This step should be done quickly, as the marrow pool is not anticoagulated. Four bone marrow direct particle coverslips are made by placing a small drop of the spicule-containing marrow pool on a coverslip or the end of a glass slide, placing another coverslip/slide on top, allowing the marrow to spread, and quickly pulling apart. It is very important to get spicules on this preparation so that the cellularity of the particles can be assessed. In addition, two direct squash particle slides are made by draining the marrow pool on a gauze (leaving behind the particles), placing a slide on top, and slowly pulling apart.

When a biopsy is performed, the bone is pushed out onto the slide. Several touch preparations (usually five) are made without damaging the bone (by lightly touching the bone on different sites on the slide). The bone is placed into 10% formalin for fixation and histologic sectioning. Several blood smears should also be prepared.

Preparation of Bone Marrow Concentrate

The EDTA tube containing marrow is mixed and pipetted into Wintrobe tubes as would be done for a buffy coat. The Wintrobe tube is spun at 1,500 g for 10 minutes. The layers of the Wintrobe tube are shown in Figure 155-1 , with approximate normal ranges for the nucleated cell containing layers (the plasma and red cell layers are variable and reflect the amount of blood aspirated with the marrow).

The fat layer is discarded. The mixed layer is placed on a slide to make a squash particle slide as described previously. A drop of plasma is placed on a parafilm watchglass. The myeloid-erythroid (M-E) layer is removed (if there is not much of an M-E layer, the top of the RBC layer is taken also) and mixed with an equal amount of plasma. Several (1015) push

Figure 155-1 Layers of the Wintrobe tube: the fatty layer is on top and is yellow or milky in color (12%); the mixed layer is second, and is not always present. It contains particles with a high content of fat cells (<1%). The plasma layer is next, followed by the myeloid-erythroid layer. This layer appears as a buffy coat and contains nucleated cells not associated with fat cells (38%). At the bottom of the tube are the red blood cells (i.e., the hematocrit of the marrow sample).

smears of this concentrated bone marrow sample are made in the usual fashion. Smears containing any particles that are seen in the concentrated sample can also be made.

It is important to retain several unstained smears from each preparation for special stains. Unstained slides are labeled before storage at room temperature on the side opposite the smear with an identifying number using a marker. When special stains are performed, the slides should be marked on the edge with a diamond point pencil to prevent sample mixup. The finished bone marrow slide folder should contain Wright-Giemsa stained smears of blood, direct marrow smear, marrow particle, marrow concentrate, and marrow biopsy imprint, as well as unstained smears of the mixed layer. Marked iron accumulation in macrophages can be seen on the unstained mixed layer smear as a golden-brown pigment.

Preparation of Bone Marrow Sample for Special Studies

At the time of bone marrow aspiration, it is important to consider whether cell marker studies, cell culture assays, cytogenetic analysis, or molecular biologic assays are likely to be required for clinical diagnosis. Fortunately, the initial sample preparation at the bedside is similar for most special studies, so that an extra sample may be obtained to be used if preliminary morphologic examination suggests the need for additional testing of the bone marrow aspirate.

The general rule is to obtain a viable, sterile, anticoagulated marrow specimen sufficient for the required assays. It is essential to determine the sample preparation and storage requirements of the individual laboratory performing each test, since assay procedures may vary. The most commonly used procedures are outlined in the following sections.

Preparation of Bone Marrow Sample for Cell Marker Studies

Bone marrow may be collected in EDTA or heparinized for cell marker studies. Bone marrow samples are stable at room temperature for 24 hours prior to staining, allowing shipment to a reference laboratory for analysis if necessary. Either EDTA or heparin is suitable for cell marker analysis, but EDTA may be preferable because of the possible interference of heparin with some molecular studies, as discussed later. EDTA has also become the standard anticoagulant for T-cell phenotyping in peripheral blood. In cases of inadequate aspirate in which phenotypic data is critical, mechanical or collagenase disaggregation of the biopsy preparation may permit cell marker analysis. [31] [32]

Long-term storage of interesting or complex samples is often desired, in order to allow later testing with additional antibodies or molecular biology techniques. The best way to accomplish this is to freeze aliquots of the marrow sample for storage in liquid nitrogen. The standard technique of freezing cells in DMSO using a controlled rate (1°C/minute) liquid nitrogen freezer, followed by rapid rewarming from liquid nitrogen storage, has been shown to preserve lymphocyte markers. [33] If a controlled-rate freezer is not available, a modification of the two-step method published by McGann and Farrant [34] [35] can be used. Ficoll/Hypaque separated cells (10^7 /ml in RPMI 1640, 10% fetal calf serum, and 5% DMSO) are held in 1-ml vials at 30°C for 5 minutes (in methanol cooled with dry ice), then quickly plunged into liquid nitrogen. Cells are thawed by rapid rewarming at 40°C and gradual addition, over 5 minutes, of five volumes of tissue culture medium with 10% fetal calf serum before centrifugation to remove the DMSO. Cell viability should be determined after thawing, as this may be variable, especially if the sample contains many myeloid cells.

Preparation of Bone Marrow Sample for Cytogenetic and Cell Culture Studies

In order to maintain cells in an optimal condition for assays requiring cell proliferation, it is advisable to collect marrow at room temperature in a sterile tube containing tissue culture medium (RPMI 1640 with 10% fetal calf serum) with anticoagulant as described previously. The shorter the storage period the better, although overnight storage in general does not significantly affect the validity of cytogenetic results. However, it is necessary to test proposed storage conditions to be sure that they will yield valid results with the specific assay being performed.

Preparation of Bone Marrow Sample for Molecular Analysis

Preparation and storage of marrow cells for molecular diagnostic tests is similar to cell marker studies, although sample requirements should be checked with the specific laboratory before obtaining the marrow sample. Heparin may contaminate DNA preparations and interfere with endonuclease digestion or PCR reactions. DNA is relatively stable, so any sample suitable for cell marker or cytogenetic analysis should yield DNA suitable for Southern blot or PCR, if enough cells are present. Even paraffin-embedded tissue can be used for PCR of genomic DNA sequences. In contrast, mRNA is very liable to digestion by ubiquitous nucleases, once cell membranes are disrupted. If RT-PCR assays are required or samples are to be stored for future RT-PCR analysis (e.g., bcr/abl, RAR/PML translocations), RNA preparations should be made from buffy coat or Ficoll/Hypaque density gradient separated mononuclear cells as soon as possible after preparation. Alternatively, RNA can be isolated from aliquots of cells frozen and stored under viable conditions (e.g., in liquid nitrogen).

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INTERPRETATION OF THE BONE MARROW ASPIRATION AND BIOPSY

Overview

Careful formulation of the clinical question being asked is the starting point for evaluation of the bone marrow aspiration and biopsy. Clarifying what information is needed ensures that appropriate samples for special studies to address all relevant diagnostic possibilities are obtained. Morphologic evaluation of the bone marrow as well as interpretation of special procedures, such as cell marker studies, is dependent on an assessment of the patients history, physical exam, and results of laboratory studies already performed.

The evaluation of bone marrow aspiration and biopsy involves at least two phases. The first is immediate examination of a Wright-Giemsa stained bone marrow aspirate preparation, typically the direct smear, to arrive at a preliminary diagnosis. This allows the physician to take appropriate clinical action or order additional special studies based on unexpected findings.

The final diagnosis should be based on the complete picture provided by clinical history, peripheral blood smear examination, laboratory tests, bone marrow biopsy and aspiration, and all special studies (cytochemical stains, cell marker studies, cytogenetics, molecular diagnostic assays, etc.) performed on the bone marrow sample. Given the complexity and variety of these diagnostic assays, the challenge for the clinical laboratory, pathologist, and hematologist is to organize these activities so that the diagnostic picture is clearly evaluated and reported with a minimum of redundant effort and inconsistent interpretations. This necessitates a close working relationship between the involved laboratories, or establishment of a hematopathology laboratory which has as its mission the coordination of all these activities to provide a complete diagnostic evaluation.

Morphologic Examination of Blood and Bone Marrow Smears

Accurate evaluation of blood or bone marrow smears is dependent on adequate smear preparation and staining. Short smears, overly thick or thin smears, or those without feathered or side edges, are inadequate and repeat smears should be made.

At medium power (20× objective), the smear should be examined for distribution of leukocytes (excess leukocytes at the feathered or side edges may bias the leukocyte differential). Platelet clumps at the feathered edge or platelet satellitism (Plate 155-1 are suggestive of EDTA-induced platelet aggregation, which may affect the platelet count. The smear should also

SOME CONSIDERATIONS IN BONE MARROW INTERPRETATION

1. Formulate clinical question prompting the bone marrow examination on the basis of clinical history, blood counts, and peripheral smear.
2. Determine need for aspirate only or aspirate plus biopsy.
3. Immediately review direct marrow aspirate smear for provisional diagnosis and to make sure appropriate special studies are ordered while sample is fresh.
4. Interpret bone marrow in light of clinical information, laboratory studies, peripheral smear examination, review of all bone marrow preparations including marrow biopsy, and evaluation of all special studies.
5. Examine bone marrow for nonhematopoietic or tumor cell infiltration (biopsy is most sensitive, but marrow concentrate may reveal tumor clumps in the side or feathered edges).
6. Examine marrow aspirate smears for intracellular organisms or abnormal storage products.
7. Estimate overall bone marrow cellularity and adequacy of each hematopoietic lineage from the biopsy and particle preparation, and confirm by examination of direct aspirate smear. Bone marrow concentrate is useful in evaluating megakaryocyte adequacy.
8. Assess adequacy of differentiation in each hematopoietic lineage.

be scanned for abnormal cells, smudge cells, nucleated red cells, red cell agglutination (Plate 155-2) or rouleaux (Plate 155-3), fibrin strands (Plate 155-4 ; these may indicate partial clotting of the sample and falsely low platelet count), parasites (plasmodia, Plate 155-5), and background staining suggestive of paraproteinemia.

Blood cell morphology is examined under oil immersion at 50× or 100×. The higher magnification is required for accurate evaluation of cytologic detail, while examination at 50× is most useful for the leukocyte differential. The most important variable affecting interpretation of blood cell morphology is selection of the appropriate part of the smear to examine. As shown in Figure 155-2 , the optimal area to examine is the transitional area between the thick part of the smear and the feathered edge, where there are only a few overlapping red cells and central pallor of normal red cells is evident. Skilled technique in preparing

Figure 155-2 Optimal area for examination of the blood smear is indicated by the box. Red cells are separate, with only a few overlapping cells per oil immersion field. Leukocytes are sufficiently spread to view their cytoplasmic contents. In the thin part of the smear, near the feathered edge, red cells are flattened and appear as large spherocytes (right), whereas in the thick part of the smear (left), red cells are rounded up and overlapping. Leukocytes also round up in this area of the smear, hindering proper identification of abnormal cells.

the smear is necessary to ensure that this transitional area is in fact represented on the smear. The most common error in blood smear interpretation results from drawing inappropriate diagnostic conclusions based on morphology of cells examined in nonoptimal portions of the blood smear. In the thin portion of the smear, red

cells are large and flattened, losing their central pallor and appearing as unusually large spherocytes. Leukocytes are flattened and often show nuclear degeneration or cellular disruption due to excessive shear forces during smear preparation. In thick areas, red cells are overlapping and stacks of erythrocytes (rouleaux) are normally present, obscuring erythroid morphology. Leukocytes are rounded and appear to have a high N/C ratio, potentially causing serious diagnostic confusion. The thick portion of the blood smear, however, may be useful for identifying microfilariae and malaria parasites. The side edges of the smear should be examined, since large cells, such as malignant cells and immature precursors, tend to accumulate in this area. These large cells are also disproportionately found in the feathered edge, but here their morphology is often distorted.

At high power, leukocyte subpopulations can be assesed either qualitatively or quantitatively by performing a 100 cell leukocyte differential (Plates 155-6 , 155-7 , 155-8 , and 155-9). Leukocytes are examined for degree and type of granulation (Plates 155-10 and 155-11) as well as abnormal nuclear structure (Plates 155-12). Red cells are systematically examined for average size, presence of polychromatophilia, inclusions and hemoglobin crystals (Plates 155-13). Some common red cell morphologic variants are shown in Plates 155-14 , 155-15 , 155-16 , 155-17 , 155-18 , 155-19 , 155-20 , 155-21 , 155-22 , 155-23 , 155-24 , 155-25 , 155-26 . Platelet number is estimated and abnormalities in platelet size (Plate 155-27) noted. A rough estimate of platelet number can be obtained by multiplying the average number of platelets per 100× oil immersion field (based on 10 fields) by 20,000 (this varies somewhat depending on the optics of the microscope).

The observer should be aware of some common artifacts that complicate interpretation of blood and bone marrow smears. These include artifactual loss of central pallor in red cells at the thin edge of the smear, rouleaux (occuring normally in the thick portion of the smear), crenated red cells (a uniform spiny shape that can occur upon prolonged storage), and a sharply demarcated hypochromic central pallor with refractile borders (Plate 155-28 , resulting from excessive ambient humidity at the time of smear preparation, or from low hematocrit). Cryoglobulin aggregates may resemble platelets (Plate 155-29). Granulocytes undergo apoptosis in samples stored for long periods prior to smear preparationthe pyknotic nucleus often resembles that of a nucleated red cell. Lymphoid cells, particularly abnormal ones, are subject to nuclear lobulation upon storage. This phenomenon is also observed in cytocentrifuged cells (for instance, in a body fluid preparation).

Adequacy of Bone Marrow Specimens

Before any specimen can be interpreted, a judgment must be made as to its adequacy for the diagnostic purpose. Biopsies that consist mostly of cortical bone, cartilage, or muscle and little or no medullary cavity are clearly inadequate for evaluation of hematopoiesis. Typically, at least a 0.5 cm core of marrow should be present, especially when looking for nonuniform abnormalities such as metastatic tumors. A recent survey found the incidence of inadequate pediatric biopsies submitted as part of a multicenter clinical trial to be about 17%. [36] Extensive crush artifact or hemorrhage may also obscure diagnostic findings in biopsy specimens that do include hematopoietic marrow. Morphologic interpretation may be adversely affected by excessively thick (>5 m) sections. It should also be recognized that even an adequate biopsy is only a sampling of the bone marrow, and interpretation could be affected by local factors, such as radiation-induced fibrosis and the nonuniform pattern of tumor metastasis.[37]

Adequacy of the bone marrow aspirate is sometimes difficult to assess, since the characteristic tissue architecture is not as readily evaluated as on the biopsy. Here the best indicator is the appearance of particles, or fragments of intact marrow, which are observed on the particle preparation. These are recognized by their characteristic mixture of fat and cellular elements, the proportion of which varies depending on marrow cellularity. Presence of marrow particles reassures the observer that the marrow space was entered and marrow was successfully aspirated. It does not assure, however, that all types of cells in the marrow space were aspirated effectively as single cells. If no particles are found, and no hematopoietic progenitors characteristic of the bone marrow (i.e., megakaryocytes, myeloid, or erythroid progenitors) are present, the possibility exists that the marrow cavity was not entered, or the marrow elements were not effectively aspirated (dry tap). The cells that do appear in the aspirate specimen in such cases likely originate from the peripheral blood. Adult aspirates containing >30% small lymphocytes and monocytes (in the absence of lymphoproliferative disease) are likely to be significantly contaminated with peripheral blood, as suggested by studies comparing proliferative activity of cells in simultaneously obtained aspirate and biopsy specimens. [38]

Assessment of Bone Marrow Cellularity

A major purpose of the bone marrow examination is to evaluate the adequacy of bone marrow development in each hematopoietic lineage. The most reliable assessment of overall hematopoietic cellularity is based on the biopsy specimen. [39] [40] A visual estimation of the percentage of marrow space occupied by hematopoietic elements plus stroma is the typical parameter used to assess cellularity (Plates 155-30 and 155-31). The reference range for marrow cellularity decreases with age (from 90% in infants to 50% in the elderly for the iliac crest), and so this value must be interpreted in light of the patients age. Erythroid cellularity can be estimated visually by looking for erythroid aggregates, which are clusters of darkly staining cells scattered throughout the marrow cavity. Adequacy of megakaryocyte numbers is also fairly readily evident at low power by the frequency of these large multilobulated cells.

Cellularity is less reliably assessed in aspirate preparations, because the tissue architecture has been disrupted and the sample is inevitably mixed to a varying degree with peripheral blood. Furthermore, cells that are abundant in the bone marrow, but tightly adherent to supportive elements in the tissue, may be grossly underrepresented in the aspirate.

In spite of these caveats, the density of cellular elements in particles appearing in the aspirate smear is usually a reasonable indicator of bone marrow cellularity (Plate 155-32). These particles are most easily observed in the particle preparation and direct smear. Somewhat less reliable, but nevertheless useful, is the overall density of hematopoietic precursors in the body of the bone marrow aspirate preparations. The most common interpretive problem is a falsely low estimate of cellularity based on the aspirate [40] resulting from dilution with peripheral blood during aspiration. A cellular aspirate specimen with good marrow particles nearly always indicates good cellularity of the biopsy as well.

The myeloid:erythroid (M/E) ratio can be derived from marrow differential counts or estimated, and is a rough indicator of significant alterations in cellularity of either myeloid or erythroid lineages. This value can be adequately interpreted, however, only in the context of overall cellularity. Otherwise, it is not clear whether a lowered M/E ratio represents erythroid hyperplasia or myeloid hypoplasia. The relative number of myeloid and erythroid cells is best evaluated on the direct smear or particle preparation, because the concentrate is often enriched in erythroid elements. Megakaryocyte number is not as easily estimated as in the biopsy. The best approach is to look

for megakaryoctes in particles (most particles should contain one or more megakaryocytes), in the direct smear (there should be at least 510 megakaryocytes in the readable portion of the slide), and in the feathered edge of the marrow concentrate (in a properly prepared concentrate, this should contain numerous megakaryocytes).

Infiltration of the Bone Marrow

One of the first questions to be addressed in examination of the bone marrow is whether the marrow cavity is infiltrated with cells other than the precursors of the major hematopoietic lineages and other accessory cells that should normally be present. This information is critical because it may provide clues to diagnoses other than those of primary hematologic disorders. If the differential diagnosis includes disorders that result in infiltration of the bone marrow, a bone marrow biopsy should be obtained if possible. The biopsy is clearly superior to the aspirate in sensitivity of detection of most types of infiltrative diseases of the bone marrow, although in many cases, the diagnostic cells will also appear on the aspirate preparation. [41]

Metastatic Tumor

Nonhematopoietic tumors are usually recognizable as such on the biopsy (Plate 155-33), although the tissue of origin may not be obvious if the tumor is anaplastic. The differential diagnosis should be pursued with special studies (e.g., immunocytochemistry) as dictated by the histologic appearance of the tumor and the patients history. Nonhematopoietic tumors are recognizable on aspirate preparations primarily by their tendency to form three-dimensional clumps (Plate 155-34). These are sometimes preferentially found in the feathered or side edges of the smears, and may be so infrequent that they are found only on the concentrate smear. A common interpretive problem is in distinguishing tumor clumps from the frequent clumps of damaged hematopoietic cells typically observed, particularly in the concentrate smear (Plate 155-35). Nucleic acid released from damaged cells (often found in manipulated samples, such as the concentrate) is very sticky, and so entraps hematopoietic cells in sizeable clumps. The best strategy is to examine cells at the periphery of clumps, where their morphology can best be evaluated. Nonhematopoietic tumor cells may also be observed as single cells with typically dense nuclear chromatin, high N/C ratio, abnormal nucleoli, and vacuolated

cytoplasm (Plate 155-36), but such isolated cells are not always present, since nonhematopoietic tumors are typically very adherent to each other and the supportive structures in the bone marrow.

Lymphoma cells infiltrating the marrow space are relatively adherent to underlying marrow structures and therefore better represented in the biopsy than the aspirate preparations. The biopsy is more sensitive in detection of lymphomatous involvement, even in small-cell lymphomas, which would be expected to be most easily aspirated.[42] In the biopsy, differentiation of malignant lymphoma (Plate 155-37) from lymphoid aggregates (Plate 155-38), common in the elderly and in autoimmune disorders,[43] is sometimes difficult. The best diagnostic indicators of malignant lymphoma are atypical cytologic features of the lymphoid cells themselves, a monomorphous appearance of the infiltrate, and location immediately adjacent to bony trabeculae (Plate 155-37). The aspirate preparations are useful in that lymphoid morphology may be more easily seen on the smears than in the biopsy sections. In contrast to solid tumors, lymphoma cells do not appear as adherent clusters in the aspirate. In some cases, special studies (e.g., immunocytochemistry, flow cytometry) may be required to distinguish a malignant lymphoma involving the bone marrow from an atypical lymphoid reactive process. Examples of immunohistochemical markers include cyclin D1 for mantle cell lymphoma and bcl-2 for follicular lymphoma. Of particular interest is the adaptation of in situ hybridization techniques for routine immunohistochemistry laboratories to allow detection of monoclonal kappa or lambda mRNA expression in bone marrow biopsy specimens.[44] This circumvents the limited sensitivity of immunohistochemical detection of surface monoclonal Ig light chain. Hairy cell lymphoma is characterized by a rather distinctive pattern of monomorphous infiltrate of cells with abundant cytoplasm, giving rise to a fried egg appearance to the tissue. The aspirate often fails to yield any of these adherent cells, and when the marrow is replaced by lymphoma, the result is a dry tap.

Multiple myeloma primarily involves the bone marrow, reflecting the normal migratory pattern of plasma cells to the bone marrow. The proportion of myeloma cells as estimated from the biopsy is often higher than it would appear from the aspirate, probably related to the adhesive properties of these malignant cells. In both biopsy and aspirate, atypical cytologic features (high N/C ratio, fine nuclear chromatin, and atypical nucleoli) should be looked for, as these indicate a more aggressive biologic behavior. Binuclearity on its own does not represent an atypical feature.

Fibrosis

Bone marrow fibrosis is a feature of some primary hematologic disorders (e.g., myelofibrosis) and may accompany infiltrative disorders of the bone marrow. A bone marrow biopsy is necessary to assess the presence and degree of fibrosis. To reliably detect early fibrosis, a reticulin stain of the biopsy specimen should be performed (Plate 155-39). The effect of fibrosis on the bone marrow aspirate is typically to reduce the number of cells obtained, resulting in a dry tap if the fibrosis is severe.

Storage Disorders

Accumulation of lipid or other storage products in bone marrow macrophages can be a diagnostic clue to storage disorders, such as Gaucher (Plate 155-40) or Niemann-Pick (Plate 155-41) disease. Macrophages are most readily found in the feathered edge of bone marrow direct smear or concentrate preparations. The detailed morphologic appearance of the cytoplasm can best be identified on the aspirate, although abnormal macrophages can be identified on both biopsy and aspirate preparations.

Infectious Diseases

A frequent indication for bone marrow aspiration is identification of infectious organisms by culture or histologic examination. The biopsy section is better suited for identification of granulomas (Plate 155-42), which can be infrequent, underlining the need for an adequate biopsy size. The presence of granulomas is an indication for staining for mycobacterial or fungal organisms, since granulomas are almost always present when mycobacterial organisms are identified by special stains. [45] [46] However, the differential diagnosis of bone marrow granulomas is extensive. [47] Intracellular organisms, such as leishmania[48] (Plate 155-43), histoplasma (Plate 155-44), and toxoplasma, [49] or viruses (cytomegalovirus) can be identified on bone marrow aspirate smears. Bone marrow examination may be particularly useful in the rapid diagnosis of infectious disorders in high-risk populations, such as AIDS patients. [46] [50] Erythrophagocytosis by bone marrow macrophages is associated with severe viral infections (Plate 155-45).

Assessment of Differentiation in the Hematopoietic Lineages

The cytologic features associated with differentiation in the major hematopoietic lineages are central to the evaluation of the

bone marrow in hematologic disorders. Bone marrow biopsies prepared according to standard procedures are fixed in formalin or mercury based fixatives (B5, Zenker) and decalcified prior to paraffin embedding and sectioning. The preservation of cytologic detail is dependent on fixation, sectioning, and staining techniques, so that consistency in these protocols is essential for reliable interpretation. Plastic embedding of undecalcified biopsies allows thin (2 m) sections to be produced, which can result in superior preservation of cytologic detail. [51] Antigen preservation in these sections for immunohistochemistry may be unreliable, but modified protocols have been described which improve the retention of antigenic epitopes. [52] [53]

Myeloid

Maturation of myeloid precursors can be assessed on both biopsy and aspirate preparations. [41] The biopsy provides a good overall assessment of maturation. Abnormally large clusters of immature myeloid precursors (abnormal localization of immature precursors) observed in biopsies of patients with myelodysplasia have been associated with a poor prognosis. [54] Fine cytologic detail is often more easily identified on the aspirate preparations. The direct smear is the best starting point, since these cells are not exposed to EDTA anticoagulant, which can induce vacuolation and subtle nuclear changes in abnormal cells. Myelocytes and later precursors are normally nearly as abundant as mature neutrophils; promyelocytes are infrequent (<10% of myeloid elements), and blasts should number <5% of nonlymphoid hematopoietic cells in the marrow. Abnormally large precursors, hypogranulation of late neutrophilic precursors, and nuclear hyposegmentation of neutrophils (pseudo-Pelger-Huet anomaly; Plate 155-46) suggest myelodysplasia if observed in a significant proportion of neutrophilic cells. It should be recognized that these features are not specific for primary hematopoietic disorders, and may particularly be seen in AIDS patients. Abnormally large precursors and hypersegmentation of neutrophils suggest folate or vitamin B_{12} deficiency.

Erythroid

Like myeloid maturation, erythroid maturation can be assessed in both aspirate and biopsy preparations, although cytologic detail is usually more easily seen on the aspirate and adequacy of numbers is more reliably assessed on the biopsy. Delay in nuclear maturation relative to cytoplasmic hemoglobinization, abnormal nuclear shape, or binuclearity are observed in myelodysplasia (Plate 155-47). These features are also associated with megaloblastic anemia, but the nuclear maturation delay is much more severe and is accompanied by a more significant increase in cell size. The marked hypercellularity and nuclear immaturity of erythroid precursors in megaloblastic anemia can suggest acute leukemia on initial evaluation of the bone marrow biopsy. Erythroid precursors containing abnormal iron deposits are observed in certain types of myelodysplastic syndromes (RARS), with the iron-laden mitochondria frequently forming a ring around the nucleus (Plate 155-48).

Megakaryocytic

The most common question involving the megakaryocytic lineage is a numeric one: is there sufficient megakaryopoiesis to support the diagnosis of peripheral platelet destruction in a case of thrombocytopenia? This is addressed by examination of either biopsy or aspirate specimen. Normal megakaryocytes must be distinguished from osteoclasts, which are large, multinucleated, and have similar-appearing cytoplasmic granulation, but whose nuclei are separate. Lymphocytes and other hematopoietic cells may sometimes be normally observed within the cytoplasm of megakaryocytes, a phenomenon known as emperipolesis. Mature megakaryocytes in conditions accompanied by increased peripheral platelet destruction often have reduced cytoplasm, suggesting exhaustion by premature release of platelets, but this is not a definitive diagnostic finding. An important feature of myelodysplastic syndromes is the appearance of abnormally small but mature megakaryocytes (as identified by cytoplasmic platelet-like purple granulation or histochemical staining for gpIIb/IIIa) [55] with 12 round unlobulated nuclei. These micromegakaryocytes (Plate 155-49), although not specific, are suggestive of myelodysplasia [56] if found frequently in the marrow, typically in the feathered edge of the concentrate

preparation. In myelodysplastic and myeloproliferative syndromes, these cells can be small enough to be confused with lymphocytes in the marrow or peripheral blood.

Lymphoid

The bone marrow is the primary site of the antigen-independent (precursor) phase of B-cell development. Therefore, variable numbers of B-cell precursors are normally found in the marrow. This number is highest in infants, in whom they may be nearly as frequent as the myeloid and erythroid elements, and decreases with age. Normal B-cell precursors (termed hematogones by some observers[57] [58]) show some immature features (moderately fine chromatin, larger cell size, fairly high N/C ratio), and may be confused with recurrent leukemia, especially after cessation of chemotherapy in children with lymphoblastic leukemia, [59] when a rebound of these normal cells is typically seen.

Macrophage

Macrophages are typically indistinct on both biopsy and aspirate preparations, with small nuclei and indistinct cytoplasmic borders. Easily found macrophages suggest an increase in their numbers, which may occur as a consequence of chemotherapy or secondary to infectious, inflammatory, or storage disorders. Phagocytosis of cellular debris by macrophages is a common event secondary to high cellular turnover, but prominent erythrophagocytosis suggests familial or virus-induced hemophagocytic syndromes.

INTERPRETIVE CONSIDERATIONS IN RELATION TO SPECIAL STUDIES

Often the hematologist or pathologist must reconcile seemingly conflicting information from bone marrow aspirate, biopsy, and multiple special studies. In some cases, the apparent conflict is explainable based on the underlying biology and the different information provided by these tests.

In interpreting cell marker studies by flow cytometry, the reported percentage of lymphocytes or abnormal cells is typically based on analysis of a population defined by light scatter that excludes myeloid precursors and most erythroid cells. Because this is a selected population, the proportions of abnormal cells thus obtained may differ from that observed in unmanipulated bone marrow aspirate smears. Certain types of lymphoma may be difficult to aspirate and may therefore be undetected in the cell marker study even though the marrow biopsy is positive. Another limitation of flow cytometry is that the abnormal cells cannot be morphologically identified on the instrument, and so if they do not possess a distinctive marker, a small subset of abnormal cells, although easily identified in the aspirate, may be difficult if not impossible to definitively distinguish from normal precursors on the flow cytometer. Immunohistochemical staining circumvents many of these problems, but not all antibodies are capable of reacting with antigenic determinants in fixed deparaffinized tissue.

Southern blot for clonal gene rearrangements or translocations is sensitive to about a 510% abnormal population. Typically, an abnormal population large enough to be identified at a molecular level by Southern blot is likely to be recognizable on morphologic examination if the cells possess distinctive morphologic features. Polymerase chain-reaction-based assays are much more sensitive than visual observation, and are designed to identify residual abnormal cells in bone marrow samples that appear morphologically to be in remission.

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Chapter 156 - Automated Analysis of Blood Cells

Daniel H. Ryan

Automated blood cell analysis and manual morphologic examination of blood cells each offer significant advantages and disadvantages. It is unlikely that either approach will totally supplant the other. Without the speed and walk-away capabilities of modern automated instruments, clinical laboratories would be unable to handle a large volume of samples efficiently. Automated instruments also provide superior accuracy and precision in quantitative blood cell measurements. However, the complexity and remarkable variation of the formed blood elements should be recognized as a formidable challenge for any automated instrument. A large number of samples screened on automated instruments still require manual examination of the blood smear for definitive diagnosis of morphologic abnormalities.

A current trend in instrument design is to incorporate as many analysis parameters as possible into one instrument platform, in order to reduce the need for manual review of problem samples and to minimize the need to run a single sample on multiple instruments. Automated blood cell counts, leukocyte differentials, smear preparation, reticulocyte counts, and even CD4 counts are being performed on an integrated set of automated instruments. [1] Increasingly, laboratories are planning to incorporate such instruments into a highly automated combined chemistry/hematology laboratory setting where samples are automatically sorted, aliquoted, and brought to the appropriate instrument for analysis by a robotic track system. [2] [3] [4] The goal is to provide cost-effective automated analysis and reporting for the majority of samples, selecting those that need manual review, and automatically preparing stained smears from those selected samples.

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COMMON TECHNIQUES USED IN BLOOD CELL ANALYSIS

Packed Cell Volume

The oldest method of quantitative measurement of blood cells is the measurement of packed volume of formed elements of the blood after centrifugation. The best example is the spun hematocrit, but instruments have also been devised to estimate leukocyte and platelet count from a blood sample centrifuged in a special device.[5]

Image Analysis

Computerized image analysis for leukocyte differential counts[6] has been largely superseded by blood counters that analyze cells in flow, offering increased throughput and versatility.

Impedance Particle Counting: The Coulter Principle

This is the basis for cell count and volume measurements on most automated clinical hematology instruments. Cells can be counted and their size estimated by measuring change in electrical resistance as cells in solution flow through a narrow aperture across which a DC current is maintained. [7] The magnitude of the voltage change is directly proportional to cell size, but is affected by the shape of the cell as it passes through the aperture, thus necessitating separate calibration for cells with different deformability parameters, such as red cells[8] and leukocytes.[9]

Light Scatter

A focused light beam striking a cell will be scattered in all directions. The amount of light scattered at a relatively low angle from the incident light path is primarily dependent on cell size. The amount of light scattered at a wide angle is primarily affected by structural complexity within the cell (granules, nuclear shape). Although there is some overlap, simultaneous measurement of light scattered at different angles allows certain leukocyte types to be distinguished. Because of overlap, an unanticipated excess of any one cell type (for instance, incompletely lysed red cells in a leukocyte analysis) may invalidate the quantitative results.

Light Absorption

Absorption of incident light by cells as a result of accumulation of a cytochemical dye for detection of peroxidase activity is used by some instruments in conjunction with light scatter measurements to identify the five major leukocyte populations. [10] This method is generally less sensitive than fluorescence labeling, but has been used for immunophenotyping with alkaline-phosphatase conjugated antibodies. [11]

Conductivity

A relatively new technique in clinical instruments is measurement of conductivity of cells to high frequency alternating current to identify granulocytes.

Fluorescence

Measurement of emission of fluorescent light is sensitive and broadly applicable to analysis of multiple cellular characteristics, such as immunophenotype (using labeled antibodies), DNA content, RNA content, and other cellular characteristics.

Blood analyzers increasingly combine analytical principles, such as light scatter and fluorescence (flow cytometers), impedance, conductivity, and light scatter (automated blood cell counters), progressively blurring distinctions between flow cytometers and automated cell counters. These instruments represent significant clinical advances, but require an increasing degree of interpretive sophistication on the part of the laboratory technologist.

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INTERPRETATION OF AUTOMATED BLOOD CELL ANALYSIS

In a typical automated blood cell counter, a portion of the aspirated sample is lysed for hemoglobin and leukocyte analysis. A separate aliquot is diluted without lysis for red cell and platelet analysis. Some parameters are calculated from the primary data, including hematocrit (= RBC count × MCV), MCHC (= hemoglobin/hematocrit), MCH (= hemoglobin/

RBC count), and RDW or PDW (= estimate of variance in the red cell or platelet volume distribution).

Hemoglobin and Hematocrit

All hemoglobins in the lysed aliquot, including oxy-, carboxy-, carbonmonoxy-, and methemoglobin are converted by potassium ferricyanide to cyanmethemoglobin, which has a broad and easily measured absorbance peak at 540 nm.[12] The hemoglobin is preferable to the hematocrit (Hct) for clinical use, since it is directly measured, and not subject to some of the infrequent artifacts that affect the Hct measurement. Lipemia may falsely elevate the hemoglobin measurement, but this artifact is usually detectable by examination of the sample and comparison of the hemoglobin and (independently calculated) Hct. Hematocrit measured as packed cell volume (spun Hct) is slightly higher than the automated Hct, due to the inclusion of trapped plasma in the packed red cell column. [13] The amount of this trapped plasma, typically about 23% of the packed red cell volume,[13] varies depending on red cell morphology and technical factors, and may be as high as 6% in microcytic anemias.[14] Although blood counters are typically calibrated to yield a hematocrit equivalent to the packed cell volume for normal blood, the spun Hct is likely to be higher than the automated Hct in patients with hypochromic or poorly deformable (i.e., sickled) red cells, due to increased plasma trapping, or in patients with high Hct (e.g., newborns). Hemoglobin can be accurately measured by recently available point of care instruments, in particular those using a photometric measurement similar to the larger automated instruments.[15] This approach may be of particular value in critical care settings. [16]

Erythrocyte Count and Indices

The MCV is directly derived from the mean red cell volume determined by impedance or light scatter, whereas the other indices are calculated as described previously. The MCV is the most clinically useful red cell index. [17] [18] Although the MCV has been widely used to direct the evaluation of anemias, its diagnostic sensitivity in both iron-deficiency anemia and megaloblastic anemia has been questioned. [19] [20] The MCH closely parallels the MCV, and provides little additional diagnostic information.[18] The MCHC is more frequently reduced in iron-deficiency anemia than other causes of microcytosis, [21] but the drop in MCHC occurs relatively late, [22] and the diagnostic utility of the parameter is poor. [23] An elevated MCHC is associated with hereditary spherocytosis, [24] but this finding is not always present. Numerous discriminant functions based on red cell indices have been proposed to differentiate iron deficiency from thalassemia trait, but cannot substitute for definitive tests in diagnosing individual patients. [21] [25]

The MCV and red cell count are subject to several artifacts. The most common of these is autoreactive red cell antibodies active at room temperature, which aggregate red cells in the instrument, lowering the red cell count, while increasing the MCV. [26] Typically, the red cell count is lowered to a greater degree than the MCV is increased (since large aggregates are not counted), causing a falsely elevated MCHC. Warming or prediluting the sample usually reverses this effect, which may occur in healthy subjects. Artifactually low MCV measurements resulting from osmotic effects have been infrequently reported in extreme hyperglycemia. [27]

Red Cell Distribution Width (RDW)

The RDW is a quantitative estimation of anisocytosis, and is typically computed as the coefficient of variation of red cell size distribution. Anemic disorders can be classified on the basis of RDW. [28] In particular, the RDW is more frequently elevated in iron-deficiency anemia than in thalassemia or anemia of chronic disease. [28] [29] However, there is significant overlap among these patient groups, even when only clear-cut presentations are considered, [21] complicating interpretation of the results in individual patients. [30] This parameter may be useful in screening high-prevalence populations or in conjunction with other more specific tests, such as serum ferritin.[31] The RDW is used in the clinical laboratory as an indicator of anisocytosis in order to select samples from the automated blood counter for morphologic red cell analysis.

Reticulocyte Count

In the standard manual reticulocyte count, new methylene blue is used to stain residual RNA and aggregate the RNA to make it easily visible. Automated reticulocyte analysis relies on detection by staining with either methylene blue [32] or fluorescent RNA-binding dyes in single cells. [33] This methodology has been adapted to flow cytometers and automated blood cell counters,[32] as well as dedicated reticulocyte counters. [33] Leukocytes and large platelets or platelet aggregates must be carefully excluded, as these cells will contain variable amounts of RNA. [34] A major advantage of automated reticulocyte counting is improved precision,[33] especially in the low-normal range, where manual methods are imprecise as a result of the small number of reticulocytes counted. [35] Reference ranges should be re-evaluated when introducing an automated reticulocyte method, as methodology-dependent variations in reticulocyte counts have been observed. [36] Recovery after bone marrow transplant may be detectable slightly earlier by the automated reticulocyte count as compared with the standard reticulocyte count, platelet, or neutrophil counts. [37] [38] Standardization of staining procedures, data analysis, [39] and quality control[40] [41] are important, since the reticulocyte RNA content is a continuous variable without clearly separated positive and negative populations (this is also a problem with manual reticulocyte counting). [42]

Reticulocyte Maturity Index

Immature or shift reticulocytes with increased RNA can be recognized by increased staining with precipitating RNA dyes [43] or as polychromatophilic cells on a blood smear.[44] Increased RNA content by flow cytometry may be an indicator of reticulocyte immaturity. Increased reticulocyte RNA content is an early sign of erythroid recovery following bone marrow transplant, [38] [45] [46] and varies in anemic states in accordance with the expected degree of erythropoietic stimulus. [47] [48]

Platelet Count

Counting of platelets in whole blood has traditionally been difficult because of their small size, wide size range, tendency to aggregate, and potential overlap with more numerous red cells or debris.[49] Current instruments deal with these problems by mathematical analysis of the platelet volume distribution to insure that it represents the log-normal distribution (skewed to the higher volumes) expected in both normal and diseased subjects. If the volume distribution does not conform to this shape, the presence of cell fragments, debris or microcytic red cells is suspected, and manual techniques must be used. Similar to the MCV and RDW, a mean

platelet volume (MPV) and platelet distribution width (PDW) is calculated from the platelet volume distribution.

Even with automated instruments, the platelet count is measured less precisely than the other components of the blood count (expected analytic variability of about 22%).[50] Variability tends to be higher in the markedly thrombocytopenic range, where there may be the greatest need to accurately measure small changes in platelet number. The lower limit of linearity

is an important limiting factor in automated instruments,[51] because of the difficulty in distinguishing platelets from cellular debris and other particles. In the markedly thrombocytopenic range, the platelet count may be flagged by the instrument as suspect, necessitating a time-consuming manual confirmation. In spite of these limitations, current automated instruments provide accurate information for clinical decision-making in thrombocytopenic patients.[52] Flow methods using specific platelet-associated markers have been shown to increase accuracy at low platelet concentrations,[53] but these are not yet adapted for routine clinical use.

The most important cause of a falsely low platelet count is inadequate anticoagulation of the specimen (indicated by small clots or fibrin strands visible on smear). Some individuals possess autoreactive antibodies that recognize platelet surface epitopes, which are exposed under divalent cation-free conditions (i.e., EDTA- or citrate-anticoagulated blood).[54] The resulting platelet clumps show up as an abnormal peak in the leukocyte volume histogram (the aggregated platelets are incompletely lysed by detergent), and can also be observed in the feathered edge of the smear. Platelet adequacy can be estimated from a heparinized sample or fingerstick smear (both of which typically show some platelet clumping resulting from incomplete inhibition of platelet activation). Falsely high platelet counts can be caused by cytoplasmic fragments of degenerated leukocytes, microcytic red cells, cryoglobulins, or debris. In all these instances, the platelet volume distribution would be expected to demonstrate a significant deviation from the expected log-normal relationship, and would be flagged as abnormal.

Platelet Analysis

MPV

The MPV is inversely correlated with platelet count,[55] and must therefore be interpreted in conjunction with the platelet count using a nomogram of reference ranges.[56] An elevated MPV has been associated with destructive thrombocytopenia,[57] and a decreased MPV with hypoproliferative thrombocytopenia.[56] Increased MPV may be a result of stimulation of megakaryopoiesis,[58] rather than simply decreased platelet age.[57] [59] MPV is increased in several clinical settings associated with cardiovascular risk,[60] and may be a risk factor for recurrent myocardial infarction[61] and restenosis after coronary angioplasty.[62] Interpretation of MPV is complicated by the continuously changing MPV observed in patients recovering from destructive thrombocytopenia[57] and the instability of MPV in blood collected in EDTA[63] (but not citrate[64]). The platelet distribution width (PDW), when interpreted in combination with the platelet count and MPV, may help to distinguish reactive from primary thrombocytosis.[65] Routine clinical use of either of these parameters awaits further definition of diagnostic utility.

Reticulated Platelets

The RNA content of platelets can be quantitated by flow cytometry using the RNA-binding dye thiazole orange.[66] Recent studies in animals suggest that reticulated platelets are in fact young platelets, analogous to reticulocytes.[67] An increased percentage, but not absolute number, of reticulated or RNA-containing platelets is observed in destructive thrombocytopenia, suggesting that young platelets are also subject to destruction.[66] [68] [69] A subset of patients with clinically defined ITP do not show increased RNA-containing platelets;[68] it is not known whether these patients represent a subgroup complicated by deficient thrombopoiesis.[70] In contrast, patients with hypoproliferative thrombocytopenia have a normal percentage, but markedly decreased absolute numbers, of RNA-containing platelets.[66] As is the case with reticulocytes, appearance of reticulated platelets precedes platelet recovery after stem cell transplantation.[71] [72] Platelets must be carefully distinguished from RNA-containing cellular fragments and other debris using light scatter parameters, platelet-specific markers,[73] or by physical separation of platelets.[74] These technical issues as well as difficulty in standardization limit widespread routine clinical application of this assay.[75]

Platelet Function

Flow cytometry analysis can detect decreased surface expression of platelet surface integrin adhesion molecules in congenital platelet function disorders.[76] Platelet activation results in conformational changes in surface gpIIb/IIIa, and exposure of granule membrane proteins, such as P-selectin (CD62P) and CD63. Binding of specific antibodies to determinants on these molecules can therefore serve as an estimate of the degree of platelet activation in a clinical sample.[77] [78] Platelet CD63 expression in early pregnancy may indicate increased risk factor of preeclampsia.[79] [80] Platelet activation is observed in acute coronary syndromes[81] and after coronary angioplasty,[82] but its prognostic value in cardiovascular disease and prothrombotic states remains to be established.

Leukocyte Count and Differential

Three major normal leukocyte populations (lymphocytes, monocytes, and neutrophils; the three-part differential), can be distinguished by electrical impedance volume measurement of leukocytes in which the cytoplasmic contents have been partially removed by detergent lysis of the cell membrane. Eosinophils and basophils are not sufficiently distinctive to be enumerated using this single-parameter approach. Analysis of the volume histograms is used to indicate the presence of increased numbers of eosinophils, basophils, reactive lymphocytes, or abnormal cells, whose volume tends to fall between the three major normal cell types. If present in sufficient quantity, nucleated red cells, clumped platelets, fibrin strands, malaria parasites, and cryoglobulins will show up on the leukocyte volume histogram. Samples are flagged by the instrument for manual review if any of these abnormalities are found.

Five normal leukocyte populations (the five-part differential), can be distinguished by several different techniques. One approach is to use a combination of light scatter and absorption to identify stained leukocytes in flow (Technicon[83]). A peroxidase stain is used to detect neutrophils and eosinophils. Eosinophils have very high peroxidase staining but relatively low forward light scatter, because their large granules scatter most of the incident light at high angles rather than low angles. Basophils are identified by resistance to lysis using a different detergent. Leukemic cells are typically large but lack peroxidase positivity (large unstained cells).

A commonly used instrument (Coulter) identifies the five major blood leukocyte types by electrical impedance, light scatter, and electrical conductivity, as shown in Figure 156-1 . Other approaches to the five-part leukocyte differential include differential resistance to lysis of eosinophils and basophils in specific detergents at different temperatures (Sysmex), or patterns of light scatter determined by measurements at four different angles, using a polarized light source (Abbott Cell-Dyn). These instruments are being successfully used in clinical laboratory settings.[1] [84]

A significant proportion of samples submitted for automated blood count and differential (about 2050% depending on patient population and instrument type) are flagged by the instrument for manual review.[85] [86] [87] The correlation of automated and manual differential counts in samples not flagged for manual review is excellent with instruments reporting either three-part[88] or five-part[89] differential. The automated differential has

Figure 156-1 Automated five-part leukocyte differential. **(A)** Simultaneously measured volume, electrical conductivity and light scatter in lysed whole blood analyzed by the Coulter STKS automated blood counter. As shown, each parameter distinguishes different groups of leukocytes, allowing numerical estimation of the five major leukocyte subgroups by comparing data from each measurement. Abnormal cells typically lie in between these normal populations. **(B)** Two-parameter histogram of discriminant function (DF) 1 (derived from scatter data) vs. volume, showing the cell populations identified. Basophils are indistinguishable from lymphocytes on the basis of scatter and volume alone. **(C)** Two-parameter histogram of DF2 (derived from conductivity data) vs. volume. Basophils have higher conductivity than lymphocytes, but are not separable from other granulocytes. **(D)** Two-parameter histogram of DF3 (conductivity, gated on scatter to eliminate cells with high light scatter) vs. volume. Basophils can now be seen as a separate population, since they have lower light scatter than other granulocytes, but higher conductivity than lymphocytes or monocytes.

an advantage in precision over the standard differential, because thousands, rather than 100, cells are counted.[90] However, in practice the substantial physiologic variation in the differential leukocyte count may complicate interpretation of small changes in percentages.[91]

The false negative rate (i.e., failure to detect significant numbers [typically 5%] of abnormal cells) in the newer instruments which report a five-part differential varies from 120%.[85] [92] [93] [94] Circulating lymphoma cells and reactive lymphocytes are among the more common causes of false negative results. [85] The false negative rate for significant red cell or platelet abnormalities is about 2.5%. [95] Reliable detection of infrequent abnormal cells is a problem for both automated counters and conventional microscopy. The best approach is to use all available clinical and laboratory information to identify patients in whom careful morphologic examination of the blood smear for a specific abnormality is of diagnostic relevance. [96] The data generated by automated blood counters can be used to direct attention to those blood films most likely to contain diagnostic information. [97]

The blood count[98] and leukocyte differential count [95] are considered by many observers to be overutilized as screening tests relative to their diagnostic utility. Given the current ordering patterns for these tests, automated differential counters offer a rapid and cost-effective means for screening blood for significant quantitative and qualitative leukocyte abnormalities, but definitive characterization of any cell populations flagged by these instruments as atypical still requires visual examination of the blood smear by a trained observer.

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CELL MARKER ANALYSIS BY FLOW CYTOMETRY

Principles of Flow Cytometry

Flow cytometers use the general principles of automated cell analysis mentioned previously, particularly light scatter, but are especially designed for sensitive and quantitative fluorescence measurements of single cells. A very brief overview of technical aspects of flow cytometry is presented here. Excellent reviews and textbooks covering this subject in detail are available. [100]

Cell Preparation and Staining

For accurate surface labeling and flow cytometric analysis, cells must be viable and available as a single cell suspension. Bone marrow and blood cells are easily kept in suspension as single cells, but solid tissue samples must be mechanically or enzymatically disaggregated, sometimes resulting in loss of the cells of interest and a false negative result. This is not an infrequent occurrence in some lymphomas, praticularly large cell lymphoma, where the cells are fragile and highly adherent.

Fluorescence Measurement

The fluorescence emitted by each cell as it passes through the light beam is optically focused and amplified. Discrimination of up to four fluorescent dyes is possible on most standard clinical flow cytometers, using optical filters placed in front of separate light detectors.

Selecting the Right Population to Analyze

The flow cytometer senses particles passing through the light beam by detecting pulses of scattered light. It cannot identify cells morphologically, and thus the operater must set decision criteria (gates) to tell the flow cytometer which are the populations of interest for display of the fluorescence data. Modern flow cytometers store all of the correlated light scatter and fluorescence data, so that if the operator has selected the wrong population, the data can be replayed on the computer, selecting a different population for analysis. The major subpopulations of hematopoietic cells can be recognized (albeit with some overlap) by analysis of light scatter and expression of common leukocyte antigen CD45 (Fig. 156-2). Because monocytes and myeloid cells may nonspecifically bind antibodies, it is critical to select the proper cell population for analysis. Leukemic populations can be distinguished from most normal cells and therefore more readily phenotyped by selecting a population with weak CD45 expression and low wide angle light scatter (cell granularity). [101] [102] By including CD45 with each marker combination, this same population can be selected for each analysis performed on the sample.

Principles of Cell Marker Analysis

Flow cytometry is broadly applicable beyond the field of hematology. [103] The most common applications in hematology are detection of differentiation or maturation-related cellular proteins using labeled monoclonal antibodies, or DNA content using DNA binding dyes. Numerous cellular proteins have been recognized using monoclonal antibodies (termed CD for cluster designation referring to a cluster of antibodies identifying the same antigen; the designations are regularly updated).[103] Some clinically useful lineage-associated markers are listed in Table 156-1 , and the pattern of expression of selected markers in the major hematopoietic lineages is shown in Figure 156-3 .

Rationale for Immunophenotyping of Hematologic Neoplasms

The clinical relevance of immunophenotyping is based on the assumptions that the phenotype of the abnormal cell is an interpretable pattern and not a random collection of markers and that the phenotype of a cell reflects important biologic characteristics (i.e., clinical aggressiveness, drug sensitivity). In view of their markedly altered genotype and clinical behavior, it is remarkable that leukemic cells usually phenotypically resemble some normal stage of differentiation. However, our understanding of the biologic role of most cell markers is extremely incomplete. Therefore, these markers are likely to be mere surrogates for the actual factors that determine response to chemotherapy and clinical aggressiveness. For this reason, diagnostic interpretation of phenotypic data must always be verified empirically by careful clinical studies. Based upon the recent expansion of knowledge linking morphologically, immunophenotypically, and cytogenetically defined disorders with molecular mechanisms (recently reviewed in detail[104]), we now have an unprecedented opportunity to determine the most clinically relevant approach to classification of hematologic neoplasms.

Pattern Recognition in Immunophenotyping of Hematologic Malignancies

A pattern of marker expression is more convincing and more likely to be clinically relevant than expression of any single marker. The diagnostic importance of recognizing characteristic combinations of surface markers in lymphoid malignancies is illustrated in Figure 156-4 . Flow cytometry analysis of neoplasms requires pattern recognition; it has been stated that flow cytometric diagnosis is more analogous to reading a bone marrow aspirate than determining a serum sodium level. [105] The recognition of populations of cells with distinctive

Figure 156-2 Selection of cells for flow cytometry analysis using light scatter. Proper selection of the cell population of interest is the first and most important step in data analysis of flow cytometry studies. Several normal cell populations are recognizable by their CD45 expression and light scattering properties, as shown in **(A)**. The boxed area includes cells with weak CD45 expression and low wide angle light scatter, which is a fraction enriched in early precursor and progenitor cells. Acute leukemia cells typically show these features **(B, C)**, making it possible to select a cell population relatively enriched in abnormal cells and depleted of normal cells on which to analyze surface marker expression. Panel **(D)** illustrates the need to look at all cell subpop-ulations to be sure malignant cells are not being excluded from the analysis. The population with high CD45 expression above the boxed area represents lymphoma cells, which are similar in their CD45 expression and light scatter properties to normal lymphocytes, but show monoclonal surface immunoglobulin expression and B cell markers on further study.

TABLE 156-1 -- Lineage Association of Diagnostically Useful Hematopoietic Markers

Marker	Lineage Association
B cell	
Immunoglobin	B[a]
CD22	B
CD20	B
CD21	B
CD19	B
T cell	
CD3/TcR	T
CD2	T, NK
CD5	T, B subset
CD4	T, Mo, eos, MK
CD7	T, myeloid progenitor
CD1	Thymocyte, Langerhans
Progenitors	
CD34	All progenitors
TdT	Lymphoid progenitors
CD10	Pre-B, activated B, PMN
Myeloid/monocyte	
MPO	My, Mo
CD33	My, Mo
CD13	My, Mo
CD14	Mo
CD15	My, Mo
CD11c	My, Mo
Megakaryocyte	
CD41a	MK, endothelium
CD61	MK, endothelium
Erythroid	
Glycophorin	Erythroid
Spectrin	Erythroid
NK/cytotoxic T	
CD56	NK, plasma cell
CD16	NK, PMN
CD57	Cytotoxic T

B, B cell; T, T cell; AMoL, acute monocytic leukemia; NK, natural killer cell; Mo, monocyte; TdT, terminal transferase; MPO, myeloperoxidase; My, myeloid cells; MK, megakaryocytes.

[a]Commonly used markers are grouped according to their major lineage association. Lineage associations of each marker are shown to the right of the CD designation. The more specific markers are at the top of the list in each lineage category.

immunophenotype and correlation of these populations with morphologically relevant cells is the key skill of flow cytometric interpretation. The major steps in cell marker analysis and interpretation by flow cytometry are summarized in Table 156-2 . A recent study has emphasized the importance of pattern recognition over numerical analysis in the diagnosis of hematologic neoplasia. [106] The importance of visual analysis of histograms to recognize abnormal expression density or coexpression of markers is often obscured by the standard practice of reporting percentage of positive cells for each marker, which may be misleading when a weakly staining population of neoplastic cells is present. [107] [108]

Lineage Specificity of Markers

Although terms such as myeloid marker are convenient and commonly used, the functional role of any surface protein is seldom restricted to cells of a given lineage. For instance, the neutral endopeptidase CD10, initially identified as a marker of immature B cells, is expressed by other cell types including neutrophils and renal tubular epithelium. Although neoplastic cells do tend to resemble normal cells in a particular lineage at a particular stage of differentiation, upon close examination neoplastic cells often display an incorrect phenotype when compared with any known normal cell. This can take the form of asynchronous (lineage-correct markers appearing in the wrong order) or mixed lineage (markers associated with different lineages appearing in one cell) differentiation. It should be recognized that aberrant marker expression may also reflect leukemic expansion of a rare normal population that is difficult to detect by standard techniques. [109] Careful study of acute leukemia cells at diagnosis with multicolor antibody panels reveals such unusual marker combinations in a majority of cases. These can be used to allow detection of small numbers of residual leukemia cells after initiation of treatment. [110]

Demonstration of Monoclonality

Monoclonal origin can be detected in immunoglobulin-expressing B-cell malignancies by light chain restriction (i.e., a population expressing only kappa or lambda light chain).[111] There is no comparable phenotypic marker for monoclonality in T cells. Light chain analysis is more sensitive in identifying B-cell lymphoma than the percent of total B cells, with a / ratio of >3:1 or <1:2 strongly suggesting the presence of a monoclonal population. [112] Quantitatively restricted expression of surface Ig by monoclonal B cells has been used to detect small monoclonal B cell populations undetectable by analysis of / ratio. [113] Nonspecific binding should be excluded by simultaneous staining with anti- and anti- antibodies or combining the anti-light chain antibody with a B-cell marker [114] (Fig. 156-5).

Cell Marker Analysis in Acute Leukemia

Classification of Acute Leukemia

In diagnosis of a hematologic malignancy, cell marker studies are most useful if a morphologically recognizable abnormal population is present. This is because of phenotypic overlap between leukemic and normal progenitors and the relative lack of leukemia-specific markers. Once the diagnostic sample is carefully analyzed, it

may then be possible to detect minimal residual disease based on expression of unusual marker combinations as mentioned previously.

Immunophenotyping allows reproducible lineage assignment of some leukemias which would otherwise be difficult to classify, [115] [116] particularly in differentiating lymphoid from immature myeloid leukemias (Table 156-1). Cytoplasmic markers (peroxidase, cCD13[117] [myeloid], cCD22[118] [B lineage], cCD3[119] [T lineage]) may be more specific indicators of lineage commitment in acute leukemia. [118] There is significant immunophenotypic overlap among the different FAB categories, [120] but important associations exist between immunophenotype and some morphologic or molecular entities, as recently reviewed. [104] In myeloid leukemia, these include CD14 expression in M4/ M5;[121] aberrant expression of CD2 (a lymphoid-associated marker) in M3 [122] and M4eo;[123] lack of HLA-DR in M3;[124] aberrant CD19 expression in t(8;21) M2[125] and monocytic leukemias;[126] CD34 expression and chromosome 5 or 7 abnormalities in M0;[127] [128] [129] and CD61 or CD41 (platelet-associated glycoproteins) expression in megakaryoblastic leukemia (but false-positive results due to platelet adherence to monoblasts must be avoided [130]). Acute lymphoblastic leukemia can be classified into B-cell precursor (CD19+, usually CD10+), pre-B (cytoplasmic Igm+), B cell/Burkitt (surface IgM+), or T cell (CD7+/CD2+) based on immunophenotype.[104] In some studies, the B-cell precursor phenotype is associated with the best overall prognosis in childhood ALL. [131] A characteristic mixed lineage phenotype (CD19+/CD10+/CD15+) in infants is associated with chromosome 11q23 translocations involving the HRX gene and an adverse prognosis.[132] A fairly characteristic

Figure 156-3 Differentiation based expression of cell markers (lineage maps). Expression of clinically useful surface markers during differentiation in the **(A)** B cell, **(B)** T cell, **(C)** myeloid,[162] [163] and **(D)** monocytic lineages. A general idea of the variation of expression intensity of a specific marker is indicated by the height of the line corresponding to each marker. CD5 is expressed on a subset of normal B cells and on B-CLL. The phenotype of activated B cells and corresponding lymphomas is complex and varied. While most normal pre-B cells are TdT, pre-B ALL is TdT+, an example of asyncronous differentiation. Asynchronous differentiation is also seen in myeloid leukemias; they often express late markers such as CD11b, CD14 and CD15 along with earlier markers CD34 and HLA-DR, so that leukemia phenotypes do not always match a normal differentiation stage.

Figure 156-4 Diagnostic importance of multiparameter analysis. **(A)** Fluorescence histogram of CD20 vs. CD5 in normal lymph node. Only a small number of CD20+/CD5+ cells are typically observed in normal tissues. **(B)** Fluorescence histogram of CD20 vs. CD5 in B-cell CLL. The characteristic co-expression of CD5 by the malignant B cells is clearly demonstrated by two-color analysis. Note that expression of both CD20 and CD5 is quantitatively lower in the malignant cells than the normal B (CD20+) or T (CD5+) cells. **(C)** Fluorescence histogram of CD8 vs. CD4 in normal blood mononuclear cells. Note virtually exclusive expression of the two markers on different subsets (T-suppresser/cytotoxic and T-helper/inducer). **(D)** Fluorescence histogram of CD8 vs. CD4 in T-cell lymphoblastic lymphoma, showing co-expression of CD4 and CD8, a common thymocyte phenotype that is typical of T-ALL and T cell lymphoblastic lymphoma. **(E)** Fluorescence histogram of CD10 vs. CD45 in remission bone marrow after chemotherapy. Although 60% of the lymphoid cells are CD10+, they show a sequence of loss of CD10 correlated with increasing CD45 expression characteristic of normal B-cell maturation, as indicated in the histogram. **(F)** Fluorescence histogram of CD10 vs. CD45 in B-cell precursor ALL. Note the high CD10 expression, CD45 negativity, and lack of evidence of progressive maturation of the cells.

TABLE 156-2 -- Practical Considerations in Hematopoietic Immunophenotyping

1. Morphologically identify cells of interest
2. Formulate the clinical question
3. Select appropriate antibody panel
4. Identify light scatter/CD45 population likely to contain cells of interest
5. Are the other populations consistent with expected normal cell types?
6. What is the overall phenotype of the cells of interest?
Level of confidence (from phenotype, morphology, surface Ig clonality) that population is abnormal
Best fit for lineage identity
Look for atypical or mixed-lineage markers (used to detect residual disease)
Assess differentiation stage and look for specific phenotypes associated with clinical and molecular leukemia/lymphoma subtypes
7. Restain with additional markers if needed based on initial data.

CD10+/CD19+/CD34 phenotype is found in a subset of pre-B ALL patients and is strongly associated with the t(1;19) translocation (E2A-PBX1). [133]

It is becoming increasingly evident that immunophenotypic patterns are most useful not as separate independent prognostic indicators, but as clues to the existence of more specific molecular abnormalities underlying malignant transformation and clinical behavior. For instance, expression of myeloid antigens in childhood ALL is not associated with an adverse prognosis in the overall patient population. [134] However, the group of patients with a true bilineage phenotype [135] does include a subset of patients with t(9;22) and 11q23 translocations, which definitely indicate a poor prognosis.

Phenotypic changes are generally not sufficiently characteristic to allow diagnosis of myelodysplastic syndromes or to differentiate the various subgroups.

Minimal Residual Disease

Highly sensitive molecular assays such as RT-PCR have recently made it possible to detect residual leukemic cells at concentrations of 10^{5} 10^{6} . These assays are applicable to patients whose leukemic cells contain a clonal genomic rearrangement amenable to PCR amplification. An alternative approach is to detect residual leukemic cells by virtue of their expression of combinations of cell markers found infrequently (<10^{3} 10^{4}) in normal bone marrow or blood.[136] Such combinations are surprisingly frequent, if a thorough investigation is made. Common aberrant phenotypes include cross-lineage marker expression (CD2, CD19 in AML), asynchronous marker expression (i.e., an early plus a late marker expressed together), over- or under-expression of a marker relative to normal cells, [137] and abnormal light scatter profile (i.e., a big CD2+ cell, or a small CD13+ cell). [138] Such unusual marker combinations are found in 87% of AML, [138] with a high proportion having more than one aberrant phenotype suitable for follow-up studies. [139] Phenotypic shifts occur in relapsed cells, but the diagnostic aberrant phenotypes are infrequently altered. [140] Although the sensitivity of PCR-based assays is inherently higher, flow cytometry offers the advantage of simple and reliable quantitation, and the ability to sort cells of interest for studies.

Recent results using immunophenotypic approaches to detection of minimal residual disease suggest that even though flow cytometry assays may be intrinsically less sensitive than molecular assays, the detection of residual cells in the range of

Figure 156-5 Detection of monoclonality by flow cytometry. Fluorescence histograms of: anti-kappa vs. anti-lambda **(A,B),** CD20 vs. anti-kappa **(C,D),** and CD20 vs. anti-lambda **(E,F)** in a reactive lymph node **(A,C,E)** or a lymph node replaced by B-cell lymphoma **(B,D,F).** Note the separate +/ (15%) and /+ (12%) populations in the benign reactive node **(A)**, each of which is identified as a true B-cell population by co-expression of CD20 **(C,E)**. Note the +/ (81%) population without a corresponding /+ (<1%) population in B-cell lymphoma, consistent with a B-cell monoclonal proliferation.

25 × 10^{3} at the time of completion of induction or intensification chemotherapy is highly predictive of clinical outcome. [138]

Cell Marker Analysis in Lymphoproliferative Disorders

B-Cell Neoplasms

B-cell chronic lymphoproliferative disorders are identified by expression of B-cell associated markers and monotypic surface immunoglobulin. Some lymphomas, particularly large cell lymphomas, may lack detectable surface immunoglobulin expression. [104] A number of clinically important lymphoproliferative disorders express a fairly characteristic immunophenotype, while others are not clearly distinguishable on the basis of cell marker studies. [141] Clinical presentation, morphology, and histopathologic appearance are critical in drawing optimal diagnostic conclusions in all instances. The most distinctive phenotypes are found in hairy cell leukemia (CD103+/CD11cbright+/CD25+), CLL (CD5+/CD23+/FMC7) and mantle cell lymphoma (CD5+/CD23/FMC7+). Monoclonal light chain expression and an expanded CD20+/CD5+ population may permit diagnosis of early CLL in patients with relative lymphocytosis, but with normal absolute lymphocyte counts. [142] The hairy cell phenotype is sufficiently distinctive to allow detection of small numbers of residual leukemic cells. [143] Quantitative expression of surface markers is a useful parameter, particularly in CLL, where CD20 and surface Ig are weakly expressed relative to normal B cells and most other lymphoproliferative disorders. CLL is distinguishable from prolymphocytic leukemia by these features and by the lack of CD5 expression in prolymphocytic leukemia. Interestingly prolymphocytic transformation of CLL usually retains a typical CLL phenotype. Follicular lymphomas are distinguishable from CLL by intensity of surface Ig expression, CD10 positivity (in most cases), and by lack of CD5 expression. Multiple myeloma (in common with normal plasma cells) lacks many of the characteristic B-cell markers (CD19, CD20, surface Ig). Diagnosis may be more easily accomplished by immunohistochemical staining for cytoplasmic immunoglobulin light chain. Interestingly, both normal [144] and neoplastic[145] plasma cells co-express several myeloid-associated markers. Diagnosis of Hodgkin lymphoma by flow cytometry is seldom practical due to the infrequency of the abnormal cells.

T-Cell Neoplasms

Immunophenotypic diagnosis of T-cell lymphoproliferative disorders is more difficult, since these cells usually express markers found normally in mature T cells. However, on careful analysis, the majority of both mature and immature T-cell lymphomas show either loss of an expected T-cell marker (typically CD7 or CD5), loss or co-expression of CD4 and CD8, or expression of CD1, suggesting aberrant or asynchronous differentiation. [146] Because the mixture of cells making up the normal T-cell population is phenotypically complex, detection of small malignant T-cell populations (i.e., 2% Sezary cells) on this basis is generally not feasible. Most instances of large granular cell (LGL) lymphoproliferative disease display a characteristic cytotoxic T-cell phenotype [147] (CD3+/CD57+/CD56/CD16±), although a minority resemble true natural killer cells (CD3/CD57±/CD56+/CD16+). Only the former type can be identified by T-cell antigen receptor gene rearrangement.

Other Flow Cytometry Applications in Hematology

Enumeration of CD34+ Progenitors

Adequacy of peripheral blood stem cell harvests can now be rapidly assessed by flow cytometric measurement of CD34+ cell concentration. [148] CD34+ cell measurement is as effective as CFU-GM assays in predicting successful engraftment,[149] but is more rapid and is easier to perform. This assay is increasingly utilized by bone marrow transplant centers, but technical aspects of the assay (for instance, gating strategy for cell selection [150] and choice of red cell lysis methods[151]) that affect the quantitative results have not yet been adequately standardized. [150] [152] [153]

Paroxysmal Nocturnal Hemoglobinuria (PNH)

The diagnosis of PNH has entered a new era with the ability to directly measure expression of phosphatidylinositolglycan

(PIG)-anchored surface molecules in different hematopoietic lineages by flow cytometry. [154] Consistent with detailed studies of complement lysis sensitivity, flow cytometry analysis reveals subpopulations of Type I, II, and III erythrocytes with progressively decreasing expression of PIG-linked proteins. [155] The flow cytometry assay is more sensitive than standard complement lysis sensitivity tests, [156] and offers the ability to analyze blood neutrophils and monocytes, which are usually more severely deficient in PIG-linked protein expression than red cells. [156] [157] Lymphocytes are typically least affected.[158] Recommended surface markers for sensitive and reliable PNH diagnosis are CD59 (all lineages), CD55 (neutrophils, monocytes, lymphoid cells), and CD14 (monocytes) (Fig. 156-6).[156]

DNA Content

Staining of fixed cells with dyes that bind stoichiometrically to DNA permits direct analysis of DNA content in individual cells.

Figure 156-6 Quantitative flow cytometry for diagnosis of PNH. Single-parameter histograms of fluorescence intensity vs. cell number in blood cells from a patient with paroxysmal nocturnal hemoglobinuria (color tracing) and a normal control (black tracing). Each peak represents a population of cells; position on the x-axis corresponds to fluorescence intensity on a log scale. Preparations of red cells, neutrophils, and mononuclear cells were stained with the indicated monoclonal antibodies and the appropriate cell population selected for analysis by light scatter gating. Fluorescence of cells stained with control irrelevant antibodies was lower than 1 unit on the log scale. **(A)** Red cells. There is only a small subpopulation in the PNH patient with decreased CD59 expression, and no reduction in CD55 expression. **(B)** Neutrophils. Note the clearly defined subpopulation showing weak expression of CD55, CD59, and CD24, consistent with type II PNH cells. The diagnosis of PNH is more evident on examination of the neutrophils than red cells, perhaps as a result of preferential destruction of the most severely affected red cells in the circulation. **(C)** Monocytes. A population of very weakly staining (type II) PNH monocytes is evident on the CD14 histogram. Note that surface expression of PIG-linked surface molecules (CD59 and CD14) is not equally affected in this lineage. This study is an example of the ability to quantitate surface molecule expression using flow cytometry.

Flow cytometry can detect a gain or loss of 520% (depending on instrument precision and percentage of aneuploid cells) in DNA content of G0/1 cells. B-precursor ALL patients with a DNA index (DNA content relative to normal cells) of >1.16 have significantly better relapse-free survival regardless of age or white count than those with DNA index of 1.16.[159] DNA content is of limited diagnostic value in chronic lymphoproliferative disorders and myeloid leukemia. Cells in the S phase of cell cycle can be recognized by DNA content between G0/1 and G2. Estimated percentage of S phase cells (using DNA content analysis) in lymphomas is correlated with morphologic risk categories, and may provide additional prognostic information. [160] Reproducible estimation of the percentage of S phase cells presents considerably more technical challenges than measurement of DNA content, because of the greater complexity in modeling the S phase to account for variability in G0/1 and G2 peaks, as well as the presence of nuclear fragments and debris.

Leukocyte Adhesion Deficiency (LAD)

A congenital defect in production of the common 2 chain of the leukocyte integrin adhesion molecule family causes defective migration of neutrophils and an increased susceptibility to recurrent infections, and is detectable by loss of surface expression of the beta-2 integrin family on leukocytes. [161]

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Chapter 157 - Laboratory Detection of Hemoglobinopathies and Thalassemias

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INTRODUCTION

Hemoglobinopathies are a result of the production of globin polypeptides with an abnormal primary structure. Sickle cell anemia is an example. In thalassemia, the primary structure of globin is usually normal, but there is an abnormal distribution of normal hemoglobins. For example, in -thalassemia, hemoglobin (Hb) A_2 and fetal hemoglobin (Hb F) are increased. The diagnosis of inherited disorders of hemoglobin requires the ability both to find abnormal hemoglobins and to detect increased or decreased amounts of normal hemoglobins. Finding these disorders in neonates and in adults is important clinically. When clinical or laboratory findings suggest common or rare hemoglobin disorders, a definitive diagnosis must be established before counseling and treatment planning are started. Population screening is directed at finding heterozygotes for the most common hemoglobin disorders. These include sickle cell disease, Hb E disease, and the - and -thalassemias. In pregnancies at risk for these disorders, antenatal diagnosis is possible. Newborn screening can detect homozygotes for hemoglobin disorders at birth, providing affected individuals the benefits of early treatment. Although tests used for definitive diagnosis and screening can be similar, each rationale requires a different approach (Tables 157-1 , 157-2 , and 157-3).

Testing for hemoglobin disorders ranges from detection of the globin proteins produced by the affected genes by isoelectric focusing (IEF), electrophoresis, high-performance liquid chromatography (HPLC), and mass spectroscopy, to gene analysis by restriction endonuclease cleavage, specific oligonucleotide hybridization, and DNA sequencing. Some methods provide a preliminary diagnosis and others a definitive diagnosis of disorders of hemoglobin structure or synthesis. These methods have been reviewed.[1] [2] [3] [4] [5] [6] [7] No single laboratory test alone is adequate for the detection of all hemoglobinopathies and thalassemias. The various techniques used currently and their pitfalls are discussed below. Blood counts and erythrocyte indices can modulate the interpretation of more definitive studies and will first be reviewed briefly.

MEASUREMENT OF HEMATOLOGIC PARAMETERS

Hematologic indices, measured by electronic cell counting, play an important role in the differential diagnosis of hemoglobinopathies

TABLE 157-1 -- Screening Protocols for Heterozygote Detection in Adults

For -thalassemia trait
Microcytosis detected by electronic cell counters.
Hb A_2 determination. If elevated, probable -thalassemia trait.
Provide counseling. If Hb A_2 normal, consider iron deficiency, -thalassemia, or rare types of -thalassemia.
For -thalassemia
Microcytosis in the absence of -thalassemia or iron deficiency [a]
For initial screening of sickle cell trait, Hb C trait, Hb E trait
Isoelectric focusing, or electrophoresis at pH 8.6 and 6.1, or high-performance liquid chromatography
If AS, AC, or AE pattern (Hb A concentration >Hbs S, C, E),[b] a carrier state is likely; provide counseling.

[a] Simple means are not generally available to detect mild forms of -thalassemia whose diagnosis still requires the detection of -globin gene deletions. Definitive diagnosis is only required for genetic counseling. Otherwise, a presumptive diagnosis by exclusion is usually sufficient.
[b] By convention, the hemoglobin fraction of the greatest concentration is listed first.

and the thalassemia syndromes, but they are nonspecific. [1] [7] The peripheral blood film also provides important clues to the nature of these disorders. In thalassemia, microcytosis is usually, but not always, present. The Hb E syndromes and, to a lesser extent, the Hb C disorders are also associated with a decreased mean corpuscular volume (MCV). A normal value for the MCV generally excludes the possibility of Hb E or -thalassemia trait. Some milder types of -thalassemia may have normal MCV. In sickling hemoglobinopathies without associated thalassemia or iron deficiency, the erythrocytes are usually normocytic. [8] [9] [10] [11] [12] [13] [14] Cell counters that measure the electrical resistance of cells (Coulter principle) may overestimate the volume of the poorly deformable sickle cell. Analyzers using the light scattering (Mie) principle are more accurate for this determination. [15] With this method, determining the distribution of red blood cell (RBC) density that may be useful for evaluating certain treatments and the clinical course of sickle cell anemia is also possible. RBC distribution width (RDW) may help differentiate thalassemia carriers from people with iron deficiency. In iron deficiency, the RDW is high; in most other conditions with microcytosis RDW is normal. Many different discriminant functions using blood counts and RBC indices have been proposed to allow easy differentiation between thalassemia and iron deficiency. None are sufficiently robust to be recommended for diagnostic purposes.

TABLE 157-2 -- Newborn Screening for Hemoglobinopathies

It is recommended that pretest education be given to prospective parents so that they can better understand the implications of disease detection or the presence of a carrier state.
Hemoglobin separation by isoelectric focusing, high-performance liquid chromatography, or cellulose acetate electrophoresis (pH 8.6).
If a normal result (FA) is obtained, notify the patients that the results appear normal. If clinically or hematologically indicated, the analysis should be repeated at 6 months.
If the study shows an FS or FSC pattern indicating possible sickle cell anemia or Hb SC disease, confirm by citrate agar gel electrophoresis (pH 6.1) and refer the patient for hematologic evaluation, family studies, parental counseling, long-term care, and prophylactic penicillin therapy. Perform confirmatory studies as outlined in the adult protocol at 6 months of age.
If the study shows an FSA, FCA (indicating possible Hb S- or Hb C- $^{+}$ thalassemia) or FE, FC pattern (indicating possible Hb E disease, Hb C disease, or Hb E or Hb C with ^{0} thalassemia, (Hb A_2 is not easily measured in the presence of Hb C or Hb E), confirm by citrate agar gel electrophoresis (pH 6.1) and refer the patient for hematologic evaluation, family studies, parent counseling, and long-term care. Perform confirmatory studies as outlined in Table 157-3 .
If the study shows Hb Barts and Hb H, suspect Hb H disease.
If the study shows an FAS, FAC, or FAE pattern, perform citrate agar gel electrophoresis (pH 6.1). If the results confirm sickle cell trait, Hb C trait, or Hb E trait, the parents should be provided counseling.
If the results indicate the presence of another variant, obtain another sample at 6 months of age and refer to reference laboratory.
If another pattern is obtained, obtain sample at 6 months of age and refer to reference laboratory.
In patients with a presumptive diagnosis of sickle cell disease, Hb E- ^{0} thalassemia, -thalassemia, or Hb H disease, perform confirmatory testing as in Table 157-3 or by DNA analysis that can provide definitive results much earlier than hemoglobin-based studies.

TABLE 157-3 -- Primary Evaluation of Suspected Hemoglobin Disorders

Initial laboratory determinations
Measurement of hemoglobin concentration and packed cell volume (PCV), red blood cell indices (mean corpuscular volume and RDW)
Hemoglobin separation by either isoelectric focusing, cellulose acetate electrophoresis (pH 8.6), or high-performance liquid chromatography, measurement of Hb F
Evaluation
Hemoglobin concentration and PCV measurement

If a normal value is obtained, proceed with other initial studies as clinically indicated.
If a decreased value is obtained, complete the initial studies and if indicated, obtain hematologic evaluation.
If an abnormally high value is obtained, measure hemoglobin-oxygen affinity and complete other studies as clinically indicated.
Measurement of the mean corpuscular volume
If a normal value is obtained, proceed with the other initial studies.
If a decreased value is obtained, quantify Hb A_2 and Hb F and determine the iron and iron-binding capacity or serum ferritin level.
If the findings indicate -thalassemia trait (elevated Hb A_2) obtain appropriate family studies and counseling.
If the findings indicate iron deficiency, complete medical evaluation and treatment.
If normal Hb A_2 and iron values are obtained, if indicated, obtain family studies and specialized laboratory determinations for the detection of -thalassemia or other abnormalities. Refer if needed for counseling or treatment.
Hemoglobin separation studies
If a normal pattern is seen and the other determinations are normal, notify the patient that all results appear normal.
If the hemoglobin separation study shows SS, SA, SC, do confirmatory testing and then refer if needed for hematologic evaluation, family studies, counseling, and long-term care.
If Hb F is elevated and Hb A reduced or absent, consider thalassemia or hereditary persistence of fetal hemoglobin and provide counseling and treatment.
If Hb H is present, provide counseling and treatment.
If an AS pattern is obtained, confirm by citrate agar gel electrophoresis (pH 6.1), and perform sickle solubility test.
If sickle cell trait is confirmed, provide counseling.
If sickle cell trait is not confirmed, refer to reference laboratory to determine the hemoglobin abnormality and provide counseling.
If an AC, CC, AE, or EE pattern is obtained, perform citrate agar gel electrophoresis (pH 6.1) to confirm presence of Hb E or Hb C. Do sickle solubility test.
If solubility test is negative, counsel or treat as needed.
If solubility test is positive, refer to reference laboratory to determine the hemoglobin abnormality and provide counseling.
If another pattern is obtained, refer to reference laboratory to determine the hemoglobin abnormality and counsel.

Modified from Honig GR, Adams JG: Human Hemoglobin Genetics. Springer-Verlag, Vienna, 1986.

HEMOGLOBIN DIAGNOSIS BASED ON PROTEIN DETECTION

The following methods all detect abnormal hemoglobin protein or abnormal quantities of normal hemoglobin. Some can be adapted to measure the concentration of hemoglobin fractions.

Commonly Used Techniques

IEF

Hemoglobin is a charged molecule. When a hemoglobin solution, or hemolysate, is applied to a supporting medium like a cast polyacrylamide gel and placed in an electrical field, it migrates to the anode or cathode from its point of application.

Some, but not all, hemoglobin variants have charge differences from normal hemoglobin and migrate differently in an electrical field. For example, sickle hemoglobin (Hb S) has two more positive charges than Hb A per hemoglobin tetramer, Hb C has four more positive charges, and Hb J has two less positive charges. Thus, Hb S migrates more slowly than Hb A toward the anode, Hb C migrates more slowly than Hb S, and Hb J migrates more rapidly than Hb A. Isoelectric focusing uses a pH gradient in a polyacrylamide gel. [16] [17] [18] The electric field also focuses the hemoglobin fractions into sharp, distinct bands at their isoelectric points. As can be seen in Figure 157-1 (bottom), hemoglobin variants that are resolved poorly by electrophoresis are clearly differentiated by this technique. Although more costly than electrophoresis, the resolving power of this technique and the ability to separate Hb C and Hb E has made it the method of choice for initial screening in many laboratories, especially for neonatal screening. However, gels must be evaluated visually and considerable skill is required to interpret isoelectric focusing gels because many extraneous minor hemoglobin bands are often present. Also, quantitation of hemoglobin fractions is difficult and only qualitative information is obtained.

Hemoglobin Electrophoresis at Alkaline pH

Hemoglobin electrophoresis has used various solid support media, but cellulose acetate membranes are now the material of choice. They are inexpensive, can be prepared quickly and easily, provide sharp resolution of hemoglobin bands, allow measurement of the relative quantities of the major hemoglobin bands by densitometry or elution and spectroscopy, and furnish a permanent record after the membranes are fixed and cleared. [19]

However, when applied to newborn screening, Hb A and Hb S are poorly resolved from Hb F, which makes up about 80% of the newborn hemolysate. It is imperative that in newborns another method, such as citrate agar gel electrophoresis, is used with cellulose acetate. Cellulose acetate electrophoresis of some common hemoglobin variants is shown in Figure 157-1 (top) and more complex patterns are diagrammatically depicted in Figure 157-2 .

Citrate Agar Gel Electrophoresis at Acid pH

To more definitively identify abnormal hemoglobins by electrophoresis, examining their mobility using more than one support medium, buffer, and pH is useful. Electrophoresis on agar gels

Figure 157-1 (Top) Cellulose acetate electrophoresis (TRIS-EDTA, borate buffer, pH 8.6) of some common hemoglobinopathies. Migration is from cathode to anode. The samples are: (1) Hb AA (adult, normal); (2) Hb AS (adult, sickle cell trait); (3) Hb SS (adult, sickle cell anemia); (4) Hb SC (adult, Hb SC disease); (5) Hb FA (newborn, normal); (6) Hb FAS (newborn, sickle cell trait); (7) Hb FS (newborn, sickle cell anemia); and (8) Hb FSC (newborn, Hb SC disease). When high concentrations of Hb A and Hb F are present, their resolution is poor. Hb A_2 is a very faint band and does not show up well in all samples. **(Middle)** Citrate agar gel electrophoresis, pH 6.1. The samples are arranged as in the top panel. Note the excellent resolution of Hb F from all other hemoglobin types. The migrations of Hb C and, to a lesser extent, Hb S depend on their concentrations. Thus, there are differences in the mobilities in samples 4 and 8. Controls of appropriate concentrations eliminate any confusion in this regard. **(Bottom)** IEF using pH 59 ampholines in polyacrylamide gel. The samples are arranged as in the top panel. Note that Hb Fis more clearly resolved from Hb A anf Hb S than on cellulose acetate. The high resolving power of this technique leads to the resolution of several minor hemoglobin bands that are not seen by other methods. These bands represent glycosylated and acetylated hemoglobins as well as various methemoglobin hybrids. *(Electrophoretograms courtesy of Robert L. Barlow.)*

Figure 157-2 Diagrammatic representation of some common electrophoretic patterns on cellulose acetate (pH 8.6) and citrate agar gel (pH 6.1). The electrophoretic mobilities of the common variants are listed above the electrophoretograms. The numbers beneath the cellulose acetate electrophoretic patterns indicate the positions of hybrid hemoglobins formed with Hb G Philadelphia (an -globin mutant). Position 1 can be occupied by $_2{}^G{}_2$ (e.g., see Hb AG) or by this hybrid and $_2{}^G{}_2{}^C$ (e.g., see Hb AC/G). Position 2 can be occupied by $_2{}^G{}_2{}^S$ (e.g., see Hb AS/G) or by this hybrid and Hb C (e.g., see Hb SC/G). Position 3 is occupied by $^G{}_2{}_2$ (e.g., see Hb FA/G). Position 4 can be occupied by Hb G (e.g., see Hb AG) or by Hb S and Hb G (e.g., see Hb AS/G).

with citrate buffers at pH 6.1 is the complementary electrophoretic method most often used. In this technique, most hemoglobins move cathodically from their point of origin and with different relative mobilities than seen with cellulose acetate electrophoresis at alkaline pH. [19] [20] Citrate agar electrophoresis can detect some variants that do not separate from Hb A, S, or C by cellulose acetate electrophoresis and should be used with cellulose acetate electrophoresis to resolve confusion in the identification of many common variants. It is especially useful for detecting hemoglobinopathies in the newborn owing to the distinct separation of Hb F from Hb A and the major common variants. The migration patterns of some common hemoglobin variants on citrate agar gels at pH 6.1 are shown in Figures 157-1 (middle) and 157-2 . Hb D and Hb G, which comigrate with Hb S on cellulose acetate, migrate with Hb A on citrate agar (boxes). Citrate agar gel electrophoresis is extremely helpful for differentiating between Hb E, Hb C, and Hb O Arab that all comigrate on alkaline electrophoresis (Fig. 157-2). Thus, citrate agar gel electrophoresis remains one of the few methods that unambiguously distinguishes Hb S and Hb C from other common variants, and it is an important adjunct to cellulose acetate electrophoresis.

HPLC

Hemoglobins can also be separated by HPLC[21] (Fig. 157-3). The advantages of this technique are:

1. The resolution of various hemoglobins, including Hb F, is excellent.
2. The procedure is usually automated by an interface to a microcomputer, which can produce a reliable interpretation of the chromatogram.
3. The various hemoglobin fractions are quantified by this method.

This technique is used as the primary screening procedure in some newborn screening programs. Its limitation is that it cannot always resolve Hb S or Hb C from other variants with the same charge. Also, the equipment is expensive to purchase and maintain and greater technical skills are required with some systems. Reversed-phase HPLC is useful for globin chain separation but is not used for primary diagnosis and is restricted to specialized laboratories. [22]

Figure 157-3 HPLC on a CS-300 cation exchange column (Brownlee Labs). The samples are the same as those in Figure 157-1 .

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INTERPRETATION OF COMMONLY USED METHODS OF HEMOGLOBIN SEPARATION

Isoelectric focusing, electrophoresis, and HPLC of hemoglobin are usually done for limited clinical indications: (1) heterozygote screening; (2) neonatal screening for disease carriers; (3) evaluation of anemia, microcytosis, erythrocytosis, syndromes suggesting a sickling disorder; or (4) quantitation of the percentage of normal and abnormal hemoglobin components to monitor chronic transfusion programs. Except for chronic transfusion monitoring and neonatal screening, there is rarely an indication for repeat studies once a diagnosis has been established because after the neonatal period, the vast majority of patients will have a stable distribution of normal and abnormal hemoglobins.

The major clinical questions to resolve when ordering these studies are:

1. Is an abnormal variant present?
2. What is (are) the abnormal variant(s)?
3. How abundant is (are) the variant(s)?
4. Is (are) the variant hemoglobin(s) relevant to the clinical picture that prompted the electrophoresis in the first place?
5. Is a normal hemoglobin present in abnormal quantities?

Most studies will be ordered to evaluate the possibility of a sickle cell syndrome, a thalassemia syndrome, or the presence of common hemoglobin variants such as Hbs C, E, D, O, and G, which often accompany these conditions.

The use of IEF or electrophoresis to detect an unstable hemoglobin as a cause of an unexplained hemolytic anemia, a hemoglobin with altered oxygen affinity as the cause of erythrocytosis or cyanosis, or the presence of methemoglobinemia must be approached with a proper appreciation of the actual diagnostic value of protein-based studies. For these rare abnormal hemoglobins, such studies are usually reliable only for confirmation and further characterization of variants causing these clinical syndromes. Functional tests to establish the presence of an abnormal hemoglobin that is likely to be relevant to these clinical syndromes should be the primary diagnostic maneuvers. A heat or isopropanol stability test and a search for Heinz bodies are obtained when evaluating the possibility of hemolysis from unstable hemoglobins. The P_{50} should be measured when a variant with high- or low-affinity hemoglobin oxygen affinity is suspected. Spectrophotometric analysis for methemoglobin best detects this oxidized form of hemoglobin. As discussed in Chapters 22 and 31 , one should also search for characteristic clinical and hematologic features of these syndromes. Many variants that are unstable or have altered oxygen affinity are silent electrophoretically so that normal electrophoresis will not rule these out. Only if an abnormal band or peak is present will IEF, electrophoresis, or HPLC be informative; the mobility of the abnormal band may also provide clues to the likely identity of the hemoglobin.

Although there are >800 known mutants of hemoglobin, the bulk of abnormal results will arise from a handful of variants like Hbs S, C, D, E, G- Philadelphia, and Lepore. This reflects the fact that sickling syndromes, thalassemias, and a few other hemoglobinopathies account for >99% of the abnormal hemoglobins encountered. Most remaining variants occur rarely and sporadically among human populations and the bulk of them are irrelevant clinically. [23]

Interpreting IEF or electrophoresis results is not always simple because several common variants have identical charge differences and comigrate (Figs. 157-1 and 157-2). For example, on cellulose acetate electrophoresis, Hb D and Hb G comigrate with Hb S, and Hbs E, C, O, and A_2 have identical mobilities. Hb C and Hb A_2 are well separated, however, on citrate agar where Hb A_2 travels with Hb A. Both Hb D and Hb E comigrate with Hb A on citrate agar electrophoresis. By using both techniques of electrophoretic separation distinguishing these

A VARIANT MIMICKING Hb S

A 24-year-old African-American woman with sickle cell disease was referred for further testing because of conflicting results of hemoglobin studies. One child was told at a health fair that she had only normal hemoglobin. But she knew from recent studies in her science class, that if her mother had sickle cell anemia she must have the sickle cell trait. An older sibling had sickle cell trait. The mother had symptoms of sickle cell disease and was anemic.

The pedigree and the results of hemoglobin electrophoresis in the reference laboratory are shown in Figure 157-4 . The mother, (I 2) is a mixed heterozygote for Hb S and the -globin variant, Hb D Los Angeles. This combination results in sickle cell disease because Hb D Los Angeles, present in 0.10.4% of African-Americans, polymerizes with Hb S. Cellulose acetate electrophoresis does not resolve Hb D from Hb S. If this is the sole method of diagnosis, the single major band of Hb S seen in the mother will be misinterpreted as homozygosity for the sickle hemoglobin gene when, in fact, it represents both Hb S and Hb D. Agar gel electrophoresis at pH 6.1 causes Hb D to migrate with Hb A, permitting its distinction from Hb S and producing in I 2 a pattern appearing like sickle cell trait. At the health fair, hemoglobin screening was done by solubility testing only. This method is incapable of detecting variants that do not contain the Hb S mutation and is an ill-advised screening test. Hence the report that this child was normal. When his blood was examined by electrophoresis, (II 2) the pattern on cellulose acetate membranes at alkaline pH, like his sister (II 1), resembled sickle cell trait. But, agar gel electrophoresis, showed only a Hb A band that actually contained both Hb A and Hb D.

Of the several Hb D variants, only Hb D Los Angeles causes severe sickle cell disease when it coexists with Hb S. Hb D should be suspected when a sickle cell trait pattern is accompanied by a negative solubility test. Hb D trait is innocuous.

Figure 157-4 Electrophoretic results and pedigree of a family with the -globin variants Hb D Los Angeles and Hb S. Figure 157-4

A VARIANT MIMICKING Hb D

An African-American couple was planning a family and sought genetic counseling for hemoglobinopathies. After performing both cellulose acetate electrophoresis and citrate agar gel electrophoresis, the mother was diagnosed with sickle cell trait and the father was diagnosed with Hb D Los Angeles trait. The pedigree is shown in Figure 157-5 and the electrophoretic results are included in Figure 157-2 . The couple was told that there was a 25% chance with each pregnancy that the child would have Hb S/D Los Angeles disease (see previous box).

After discussion, the couple decided that the risk was worth taking. When the first child was born, the electrophoretic pattern on cellulose acetate at alkaline pH was confusing because Hb A was present with multiple variant bands and was not consistent with any of the outcomes discussed by the genetic counselor. When specimens of the childs blood and the fathers blood were sent to a reference laboratory, the child was shown to have Hb S trait with Hb G Philadelphia trait, and the father was shown to be a simple heterozygote for Hb G Philadelphia. Hb G Philadelphia is an -globin chain variant that is relatively common in African-Americans. Because it is an -globin chain variant, it affects the electrophoretic mobility of Hb A_2 and Hb F as well as Hb A. Thus, it should have been picked up in the father because of the minor variant Hb A_2 band cathodic to nor al Hb A_2 (Fig. 157-2 , AG). In addition, as an -globin variant, Hb G Philadelphia usually comprises 20% of the total hemolysate whereas Hb D Los Angeles, a -globin variant usually comprises 40%. The quantitative differences between the two hemoglobins, however, is not always reliable because the percentage of Hb D Los Angeles may be reduced in the presence of -thalassemia. Conversely, Hb G Philadelphia is usually linked to a deletion of the other -globin gene in *cis*, and when in *trans* to a chromosome that also has an -globin gene deleted, can comprise up to 45% of the total hemolysate. The multiple bands in the newborn are explained in Figure 157-2 (FAS/G).

The distinction between Hb G Philadelphia and Hb D Los Angeles is not trivial. Unlike Hb D Los Angeles, Hb G Philadelphia does not participate in the polymerization of Hb S. Thus, it would be impossible for these parents to have a child with a clinically significant hemoglobinopathy.

Figure 157-5 Pedigree of a family with Hb S and Hb G Philadelphia. Figure 157-5 .

variants from Hb A is possible. IEF and HPLC used alone can differentiate some of these variants.

The fractional percent of each hemoglobin is an important factor in abnormal hemoglobin identification and in the diagnosis of certain types of thalassemia. For example, Hb A_2 rarely accounts for >6% and is never >12% of the total hemoglobin. A band in the position of Hb A_2 on cellulose acetate that comprises 2550% of the total hemoglobin is probably Hb C or Hb E. Hb Lepore comigrates with Hb S on cellulose acetate but is never >1015% of the total hemoglobin; Hb S, on the other hand, is rarely <2530% of the total hemoglobin.

For most common hemoglobinopathies, IEF or hemoglobin electrophoresis results can usually be interpreted by considering (1) the number of abnormal bands seen and their mobility compared with Hb A; (2) the amount of each hemoglobin found, expressed as percentage of the total; and (3) this information related to the clinical context of the case by considering the patients age, hematologic parameters, and RBC morphology.

In adults, three hemoglobin bands or peaks are present on IEF, cellulose acetate electrophoresis, and HPLC: Hb A ($_2$ $_2$), forming 95% of the total; Hb A_2 ($_2$ $_2$), constituting 23% of the total; and Hb F ($_2$ $_2$), barely visible if at all, and accounting for 0.51% of the total. These components should be used as a frame of reference when evaluating abnormalities; one should focus not simply on the abnormal bands but also on the deviations of the overall pattern from the normal. For example, are the Hb A_2 and Hb F bands present at the appropriate positions but in altered amounts? Are any normal bands missing from their usual positions? **S**pecific globin gene mutations will have very different impacts on this pattern. The hemoglobin profile of a patient represents the complex interaction of products of several globin genes: four loci, two loci, four loci, and two loci. The following principles should be remembered:

1. Mutations affecting the -globin locus will tend to change only *one* band and this will usually be a major band, in terms of the quantity of hemoglobin. This reflects the fact that there is only one copy of the -globin gene per haploid cell and that -globin is a constituent of only Hb A. Two -globin genes are expressed in the diploid erythroid cell, and this accounts for 95% percent or more of the total non-globin chains. Based on gene dosage and this level of output, one would expect, as a first approximation, heterozygosity for a -globin variant to yield a single new band that would account for about half the total hemoglobin. As a corollary, Hb A levels would be reduced nearly half. The actual quantity of normal and abnormal hemoglobins may deviate somewhat from 50% for complex reasons (Chap. 31).
2. In contrast, -globin variants should produce altered Hb A, A_2 , and F bands because each of these hemoglobins contains -globin. Four functioning -globin genes exist in the diploid erythroid cell. Therefore, a mutation of one globin gene might then be expected to create variants forming 25% of the total Hb A, 25% of the total Hb A_2 , and 25% of the Hb F. This approximation is reasonable in practice, but not precise, because the two -globin alleles are not equally expressed. Moreover, the frequent occurrence of -globin variants in areas of the world where -thalassemia is extremely common often results in complex linkages of structural variants to thalassemic alleles, a feature that complicates predictions of the quantity of hemoglobin types based on gene dosage alone. Consequently, -globin variants usually, but not always, amount to 25% or less of the total hemoglobin and tend to produce multiple new hemoglobin bands.
3. -Globin variants will alter a single major hemoglobin band during fetal and neonatal life but, after the first year of life, will alter only the faint Hb F band and are rarely detected.
4. Mutants of the -globin gene will alter only the Hb A_2 band

and are never clinically significant. However, an elevation of the amounts of normal Hb A_2 suggests the probable presence of -thalassemia trait.

The actual amounts of different hemoglobin types that accumulate in vivo depend on the output from individual globin genes, the stability of the globin and hemoglobin products, and on the post-translational assembly of -dimers. From a practical viewpoint, this is most relevant to sickle cell disease.

The more positively charged the -variant, the lower its affinity for -chains. This usually has little impact on the steady-state accumulation of hemoglobin tetramers because a slight excess of -chains is always available to bind non-chains. However, mild forms of -thalassemia are extraordinarily common in the same populations in which sickle cell anemia and other -chain variants are often found. Individuals heterozygous for -chain variants who also carry -thalassemia genes exhibit altered ratios of the individual hemoglobin components. For example, the positively charged ^{S} -globin chain binds -chain less well than does ^{A} -chain. Because -thalassemia limits the number of -chains, normal ^{A} and abnormal ^{S} compete for the limited -globin pool and more Hb A than Hb S forms. Therefore, a typical Hb A/Hb S ratio in individuals with sickle cell trait and -thalassemia is 70:30 compared to 60:40 in uncomplicated sickle cell trait. [24] In contrast, Hb S is *less* positively charged than Hb C. In patients inheriting both ^{S} and ^{C} genes (Hb SC disease), the Hb S/Hb C ratio is 50:50, reflecting a similar affinity of each variant for -globin chains.

Heterozygosity for both Hb S and $^+$ thalassemia causes higher amounts of Hb S than Hb A because the output of the -thalassemia locus is usually reduced to <30% of normal. Typical Hb S/Hb A ratios in Hb S-$^+$ thalassemia are 70:30 to 90:10.[25] [26] [27] Hb E is a very common variant in southeast Asians and is inefficiently expressed (Chap. 29). It rarely forms >2530% of the total hemoglobin. Because it is highly positively charged, its percentage drops dramatically to 1015% in patients who also inherit -thalassemia. Hb C and Hb O, in contrast, usually accumulate in amounts closer to those of Hb S in sickle cell trait. Individuals with Hb E will have a thalassemic phenotype and a lower percentage of the abnormal hemoglobin, which provides strong evidence for the diagnosis.

Hemoglobin Lepore is moderately common among southern Europeans. As noted in Chapter 29 , Hb Lepore arises from fusion of the and genes, eliminating the normal - and -globin loci from that chromosome and replacing them with a poorly expressed thalassemic gene. Therefore, heterozygous Hb Lepore should be suspected if a hemoglobin band migrates like Hb S but comprises only 510% of the hemolysate. Homozygotes for Hb Lepore have no Hb A. The cells of these patients are also profoundly microcytic, like those of patients with classic forms of -thalassemia.

By using these principles, interpretation of the most common types of hemoglobinopathies should be possible. To summarize:

1. -Globin variants affect multiple bands and are usually <25% of total hemoglobin in heterozygotes.
2. -Globin variants affect one band and form 50% of total hemoglobin.
3. -Globin and -globin variants affect only the minor Hb F and A_2 bands in adult life.
4. The fractional percent of the variant can be as informative as its point of migration in the hemoglobin separation system.

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ADDITIONAL PROTEIN-BASED METHODS FOR CHARACTERIZING HEMOGLOBINS

The Sickle Solubility Test

Sickle hemoglobin has unique properties that allow its chemical detection. It is insoluble and precipitates in high-molarity phosphate buffer at neutral pH when reduced with sodium dithionite. This observation forms the basis for the sickle solubility tests. [28] The sickle solubility test has no role as a primary screening test or as the sole means of detecting Hb S. It identifies only the presence of Hb S and not its quantity and so cannot distinguish among sickle cell trait, sickle cell anemia, and Hb S-$^{+}$ thalassemia. It cannot detect other nonsickling variants. The solubility test is not applicable in neonates who have a large amount of fetal hemoglobin and fails to detect low concentrations of Hb S. A reliable solubility test is a useful adjunct to the IEF, electrophoretic, or HPLC identification of Hb S in adults. This test is quite specific for Hb S and will help distinguish it from variants that may mimic it electrophoretically. This test is also useful for the identification of the rare variants with two amino acid substitutions, one of which is the sickle mutation (Hb C Harlem, C Ziguinchor, S Antilles, S Travis, S Providence, and S Oman). Solubility tests sometimes give false-positive results but, with reliable reagents, this problem is rare.

SICKLE CELL ^{0} THALASSEMIA

A 34-year-old African-American woman (I 2), early in her third pregnancy, was referred to clarify the possibility of her carrying a fetus at risk for sickle cell disease. Her first child (Fig. 157-6 , II 1) with her first husband (I 1) had sickle cell anemia and was ill. Her second child (II 2) by her present husband (I 3) was also ill. Yet, the referring laboratory told her that because her husband had no abnormal hemoglobin present on IEF, it was not possible for the child to have any type of sickle cell disease.

On IEF, I 1 and I 2 had typical findings of sickle cell trait. II 1 and II 2 had 90% and 80% Hb S, respectively. Their Hb F levels were 7% and 14% with Hb A_2 of 3% and 6%. I 3 had Hb A, 94%, Hb F, 1%, and Hb A_2 , 5%. He was not anemic but had an MCV of 70 fl. A blood film showed microcytosis, target cells, and basophillic stippling.

II 3 is not normal. He has ^{0} thalassemia trait. Therefore, each pregnancy with his current wife has a 25% chance of resulting in a fetus with Hb S-^{0} thalassemia. Hb S-^{0} thalassemia is a phenocopy of sickle cell anemia and patients can have all of the complications of this disease. Although an abnormal hemoglobin is not found in carriers of -thalassemia, microcytosis and elevated Hb A_2 suggest this diagnosis.

Figure 157-6 Pedigree of family with Hb S-^{0} thalassemia. Figure 157-6

Quantitation of Hemoglobin and Globin Chain Fractions

Measuring the fractional percentages of the hemoglobin components present can be important. Quantitation of Hb S and Hb A levels help distinguish Hb S-$^{+}$ thalassemia from sickle cell trait; the proportion of Hb S in sickle cell trait is often reduced in the presence of -thalassemia; the quantitation of Hb A_2 in adults is important because it is so often elevated in -thalassemia carriers. [29]

Several reliable procedures for hemoglobin quantitation exist and include elution from cellulose acetate strips and spectrophotometric analysis of the eluted fraction, densitometry of stained and cleared cellulose acetate strips, chromatography on minicolumns, and HPLC. [30] The elution method is highly reliable for measuring Hb A_2 and variant hemoglobin levels in adults. Because Hb F migrates between Hb A and Hb S, this method is not used for newborn samples. Densitometry is unreliable for measuring Hb A_2 and Hb F.[31] [32] The minicolumn method is reliable but has been largely supplanted by HPLC, [33] which is probably the method of choice for quantitation of the major hemoglobins in the newborn.

The quantitation of Hb F is extremely important in the diagnosis of the thalassemias, sickle cell disease, the Hb E syndromes, and the hereditary persistence of fetal hemoglobin (HPFH) syndromes in adults. Most human hemoglobins rapidly denature at alkaline pH and can then be precipitated with ammonium sulfate. Fetal hemoglobin, however, is not denatured and remains soluble under these conditions. The alkali resistance of Hb F provides a rapid and simple technique for quantifying Hb F in human blood samples.[34] Fetal hemoglobin can also be quantified by HPLC and immunologic methods.[33] [35] The analysis of the distribution of Hb F in the RBC is useful in distinguishing among various forms of -thalassemia and HPFH. [36] The ^{A} - and ^{G} -globin chains of Hb F can be separated and quantified by reversed-phase HPLC, and this is useful for differentiating among the types of HPFH. [37] Hb F-specific antibodies can measure the number of F cells and the amount of Hb F per F cell.[38] [39]

Mass Spectroscopy

Replacing the laborious methods of tryptic peptide separation and amino acid analysis by HPLC, mass spectrometric methods allow the protein-based detection of uncommon or unique globin structural variants. Using electrospray ionization mass spectrometry, differences among most abnormal globins may be detected. [40] When this method is insufficiently sensitive, other forms of mass spectrometry, like liquid secondary ion, fast atom bombardment, and tandem mass spectrometry are used to

distinguish among abnormal tryptic fragments of globin and analyze the sequence of their amino acids. [1] These analyses have limited applications and are restricted to a few specialized laboratories.

Capillary Isoelectric Focusing

In this recent modification of thin-layer IEF discussed above, hemoglobins are separated by their isoelectric points in pH gradients established in fine capillary tubes. Available commercial systems allow automated data analysis, require small sample size, are rapid and sensitive, conserve expensive reagents, and provide quantitative information.[41] These advantages argue for the more widespread application of this procedure as a primary screening technique.

Immunologic Detection

Specific antibodies can be raised to normal hemoglobin and some hemoglobin variants. [35] [42] The major applications of immunologic detection of hemoglobin are for the measurement of Hb F by radial immunodiffusion[43] and for quantifying the -globin chain of Hb Barts, a technique of value in the detection of carriers of the common type of severe thalassemia in Asians.[44] [45]

Hemoglobin Functional Studies

Some variant hemoglobins are characterized by abnormal functional properties. Variants with abnormal oxygen affinity may be recognized by abnormal oxygen dissociation curves or determination of the P_{50}. Several automatic methods of obtaining oxyhemoglobin dissociation curves have been described. [46] Most unstable hemoglobins have been found to precipitate more readily than Hb A when exposed to heat or when incubated at 37°C in a buffer containing isopropyl alcohol. [47] The Hb M variants may be identified by their spectral changes although often their detection is very difficult.

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DNA-BASED METHODS

DNA-based methods of detecting hemoglobin abnormalities have the advantage of specificity and the current disadvantage of cost and availability. Most often they are limited to reference laboratories. They are limited in that they do not provide important ancillary information that may come with the protein-based techniques, like levels of Hb F, which are prognostically useful in sickle cell anemia.

A mutation that causes a hemoglobinopathy or a thalassemia can be precisely identified in material as limited as a single cell or blastocyst, enabling preimplantation diagnosis.[48] In principle, hemoglobin disorders can be detected by analyzing fetal cells that find their way into maternal blood, [49] but, more practically, DNA-based methods lend themselves to antenatal diagnosis. Here, different methods, covered elsewhere in this volume, like single-strand conformation polymorphism, denaturing gradient gel electrophoresis, restriction endonuclease analysis of mutations, detection of mutations by allele-specific oligonucleotide probes, and mutation detection by DNA sequencing, have all been used. [4]

-Thalassemia gene identification using specific oligonucleotides is available commercially and is useful for heterozygote detection in selected populations, often as an antecedent to antenatal diagnosis. Rapid methodologic advances coupled with price decreases may soon see DNA-based techniques used in neonatal screening programs.

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LABORATORY PROTOCOLS

Laboratory methods should be based on the reason for abnormal hemoglobin detection. Table 157-1 illustrates useful protocols for heterozygote detection, Table 157-2 provides an approach to neonatal disease detection, and Table 157-3 illustrates the evaluation of suspected hemoglobin abnormalities in patients.

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Chapter 158 - Antenatal Diagnosis of Hematologic Disorders

Kim Kramer
Harvey J. Cohen

INTRODUCTION

The past three decades have taken the concept of antenatal diagnosis from a blind needle uterine insertion for the acquisition of amniotic cells to a refined ultrasound-guided transcervical swab for villus sampling. In the past, karyotyping was performed in 4 weeks; today, single gene defects can be detected in a matter of hours. What once necessitated 50 ml of amniotic fluid in the 20th week of gestation now routinely requires as little as 20 mg from chorionic villus sampling at 6 weeks of gestation.[1] Indeed, we are now able to detect single DNA base pair alterations in one blastomere from an extracorporeal embryo.

With this explosive surge in methods to diagnose molecular defects earlier, more quickly, and more effectively comes the need for earlier, safer, and more effective interventional and therapeutic techniques. The practice of antenatal diagnosis once served only for elective termination of fetuses affected by a known genetic

disorder. It is now possible to provide in utero treatments that can alter the natural history of many genetic disorders. This is of particular relevance for the hematologist, who is often involved in an entire array of clinical conditions that can be detected and treated in the antenatal period.

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METHODS OF ANTENATAL DIAGNOSIS

Prenatal diagnostic techniques differ in their indications, invasiveness, diagnostic accuracy, and risks. The choice of technique depends on the gestational age, indications for the procedure, safety considerations, turnaround time, and maternal and fetal risks. Before choosing a procedure, thorough consideration must be given to the potential use of the information obtained.

Amniocentesis

The concept of obtaining amniotic fluid cells shed from fetal skin, lung, and gastrointestinal tract for diagnostic purposes had its origin in 1930 in the setting of amniography.[2] It was not until the 1950s, however, that amniocentesis was introduced as a method for the diagnosis and management of erythroblastosis fetalis [3] and sex chromatin analysis.[4] In 1966, Steele and Breg[5] cultured amniotic fluid cells in sufficient quantity to be karyotyped, leading to the first in utero diagnosis of Down syndrome by Valenti et al. in 1968.[6] By 1970, an estimated 10,000 procedures had been performed in high-risk pregnancies, with a fetal morbidity and mortality rate of <1%.[7]

Although routine amniocentesis is often performed at 1620 weeks gestation, the clinical and laboratory experience with first-trimester procedures is encouraging. [8] After a thorough ultrasound examination of the fetus, placenta, and uterus, a 22-gauge spinal needle is inserted transabdominally or transvaginally. [8] Approximately 1 ml/week of gestation is withdrawn; successful fluid acquisition approaches 99.8% and can be used for cytogenetic and biochemical studies. [8] With enhanced cell growth, prenatal diagnosis for some disorders is possible as early as 5 days; [9] reliable karyotyping is completed by approximately 14 days. [10] The procedure boasts an extremely high diagnostic reliability, with an estimate of 1 miskaryotype per 5,000 procedures and a culture failure rate of <1%. [11] Procedure-related fetal loss rate is approximately 0.5%.[12] Complications are rare and usually minor; they include amniotic fluid leakage, vaginal cramping, fetal trauma, blood-stained amniotic fluid, and infection.[13] Concerns over the possibility of positional deformities and pulmonary hypoplasia due to removal of amniotic fluid early in gestation have been raised, although no conclusive evidence for such associations exists. [14]

Chorionic Villus Sampling

Fetal chorionic villi were successfully sampled using a transvaginal hysteroscope as early as 1968, [15] although chromosome analysis from chorionic villus sampling (CVS) in the first trimester became possible only in the early 1980s. Since that time, CVS has emerged as a means of making earlier, quicker, and more reliable prenatal diagnoses. The test, commonly performed under ultrasound guidance at 912 weeks gestation, involves the aspiration of 2030 mg of villi from the placenta via a transabdominal or (more conventionally) a transcervical approach (Fig. 158-1).[16] Chorionic villi may be analyzed directly for a rapid karyotype or cultured for DNA and enzyme analysis.[17] The polymerase chain reaction (PCR) has shortened the diagnostic time to a few hours. In general, CVS permits earlier detection of a genetic defect and provides a greater number of cells for genetic studies. These advantages need to be weighed against a slightly poorer performance with respect to fetal loss rate and diagnostic accuracy. Unlike amniocentesis, CVS does not allow the diagnosis of structural problems such as neural tube defects. Other complications with CVS exist, including fetal hemorrhagic lesions, [18] [19] post-CVS amnionitis,[20] and reported limb and

Figure 158-1 Chorionic villus sampling depicting the transabdominal and transcervical approach.

oromandibular malformations. [21] No clear proof of limb-reduction abnormalities exists, although the risk appears greatest in CVS obtained prior to 9 weeks gestation.[17] Fetomaternal hemorrhage occurs in up to 50% of women and appears to be independent of technical approach. [22] Maternal contamination is a potential pitfall that can be avoided with experience in the dissection and preparation of the samples for assay. [23] Currently, CVS remains the optimal procedure for diagnosing many metabolic disorders and single gene defects.

Fetal Blood Sampling

In utero blood sampling was introduced in 1973 via fetoscopy. A fiberoptic endoscope is passed transabdominally into the amniotic cavity, followed by the passage of a needle through the fetoscope into the umbilical vessels for blood acquisition. [24] Although it is a reliable method of obtaining pure fetal blood, procedure-related fetal deaths approach 25%.[25] Other methods of fetal blood acquisition include embryoscopy, placental aspiration, hepatic vein sampling, and cardiac puncture. Since 1983, fetal blood sampling (FBS), also called cordocentesis, has become a safe and reliable means of accessing the fetal circulation. [26] It is generally performed from 15 weeks gestation until the last trimester of pregnancy but may be performed earlier. [27] Under direct high-resolution ultrasound guidance, a spinal needle is inserted transabdominally through the uterus and into the umbilical vessel. Approximately 13 ml of blood is aspirated, and the purity of the sample is assessed to determine if there is maternal blood or amniotic fluid contamination. [28] Difficulty in obtaining the sample occurs if fetal movements are frequent and strong enough to dislodge the needle, if the placenta is placed posteriorly, if the umbilical cord is small, and in the case of oligohydramnios and maternal obesity, causing poor ultrasound visualization.[29] Indications for FBS continue to decrease as more congenital disorders are diagnosed earlier with molecular techniques by CVS and amniocentesis. The most common indications for FBS in the 1990s include the assessment and management of alloimmunization disorders, intrauterine growth retardation, nonimmune hydrops, fetal blood pH and acidbase status, neural tube defects, and congenital infections. [30] FBS is also used for rapid karyotyping, and if DNA studies are not informative. Serious complications, although

unusual, include bleeding from the umbilical puncture site, fetal bradycardia, [31] irregular uterine contractions, chorioamnionitis, cord hematoma, premature rupture of membranes,[26] and fetomaternal hemorrhage. [32] The procedure-related fetal loss rate is approximately 12%. [31] [33] Gestational age and duration of the procedure appear related to fetal loss within 48 hours of FBS. [34] Evidence suggests that the risk of FBS is increased in abnormal pregnancies, most likely due to underlying pathology.[35] The success of FBS, in part, requires a highly capable clinical laboratory aware of the most commonly requested fetal serologic, hematologic, and serum chemistry studies. FBS remains the most desirable way of accessing the fetal circulation for both the diagnosis and therapeutic management of numerous congenital hematologic disorders.

Noninvasive Prenatal Diagnosis

Every known invasive prenatal procedure carries the risk of fetal death. Retrieval of trophoblasts from the lower uterine cavity has been proposed as a minimally invasive method of obtaining fetal DNA.[36] [37] It remains to be established whether trophoblasts are present in most or only a few transcervical samplings. The

reliability of transcervical sampling has been questioned by several investigators [38] but may ultimately be improved with refinements in technique.

Several attempts have been made to detect fetal cells in the maternal circulation for the noninvasive diagnosis of selected inherited disorders. More than a century has elapsed since Schmorl, a German pathologist, identified trophoblasts in lung tissue of women dying of eclampsia. [39] In the 1950s, trophoblasts were detected in the uterine vein,[40] and Kleihauer found fetal cells in maternal blood by using a stain for fetal hemoglobin. [41] Nearly three decades have passed since Walknowska et al.[42] cultured fetal lymphocytes from maternal blood for karyotyping. Recent efforts have focused on defining the cell types to be retrieved, the quantity of fetal cells in maternal blood, fetal cell enrichment techniques, and the feasibility of clinical applications.

Target fetal cell types from maternal blood include trophoblasts, lymphocytes, granulocytes, nucleated erythrocytes, and platelets (Table 158-1). Currently, the fetal nucleated erythrocyte appears to be the most desirable target cell. The frequency of fetal erythrocytes and CD34+ hematopoietic progenitor cells in sorted subpopulations from maternal blood was recently determined by Little et al. [43]

Techniques of fetal cell separation include fluorescent flow cytometry, fluorescence-activated cell sorting, and the use of immunomagnetic beads. Improved fetal nucleated erythrocyte sorting has recently been reported using intracellular antifetal hemoglobin antibodies [44] and fetal thymidine kinase assays.[45] Genetic analysis has been accomplished by PCR amplification of fetal Y chromosome and paternal-specific sequences, and by fluorescent in situ hybridization.

The retrieval of fetal cells from maternal blood currently remains in the investigational phase because of low sensitivity and low specificity. Success has been met where a known unique paternal mutation or polymorphism exists. Fetal inheritance of a paternal beta-globin mutation, Hb Lepore-Boston, has been detected in maternal blood,[46] as has paternally inherited -thalassemia mutations.[47] Limited experience exists for the diagnosis of other genetic disorders, such as sickle cell anemia,[48] and for the determination of fetal Rh(D) status.[49] The possibility of diagnosing X-linked inherited diseases in maternal blood was recently reported by Sekizawa et al.,[50] who diagnosed Duchenne muscular dystrophy using a single fetal erythrocyte in maternal blood. Successful efforts have been reported for the diagnosis of fetal aneuploidy and fetal sex determination. [51] It remains to be determined if there is an ideal gestational age for sampling, whether ABO incompatibility limits the availability of fetal cells, and whether abnormal pregnancies affect the fetal cell yield. [52] Although techniques to improve diagnostic accuracy are needed, the future of noninvasive prenatal testing is optimistic.

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WHITE BLOOD CELL DISORDERS

Severe inherited immunodeficiency states encompass a wide spectrum of disorders involving absent or abhorrent components of the lymphoid or phagocytic system. Although rare, many of these disorders are life-threatening (Table 158-2). The issue of prenatal diagnosis arises in families that already have a severely affected child. A prerequisite for antenatal diagnosis includes extensive phenotypic or genotypic analysis of the index patient.

The relatively late development of the fetal immune system largely restricts confident phenotypic antenatal diagnosis to fetal blood analysis during the second trimester of pregnancy. The most primitive lymphoid stem cell arises in the fetal liver by 7 weeks gestation and is detectable in the developing thymus by 89 weeks. [53] Phenotypically mature T lymphocytes can be detected in the fetal circulation and peripheral lymphoid tissues by 1213 weeks. [54] Synthesis of IgM, IgG, and IgA has been demonstrated by B lymphocytes at this stage.[55] Likewise, the synthesis of fetal liver complement components C2, C3, C4, C5, and Clq inhibitor takes place in the first trimester.[53] Insufficient numbers of neutrophils in the fetal circulation before 7 weeks

TABLE 158-1 -- Target Fetal Cells in the Maternal Circulation

Cell Type	Advantage	Disadvantage
Trophoblasts	Unique morphology Uniquely fetal	Possible mosaicism, often filtered in the pulmonary circulation Difficult to analyze by fluorescent in situ hybridization [291] Specificity of antibodies unclear
Lymphocytes	May be sorted by HLA status in selected cases	Persistence in maternal circulation as long as 27 years [292] Not inherently different from maternal cells Produced late in gestation
Granulocytes	Functional assessment of single cells	Least studied Frequency of fetal cells not consistent
Nucleated red cells	Common in fetal blood, rare in adult blood Amenable to cytogenetic analysis Express unique antigens and unique fetal hemoglobin for positive identification	Increased number in preeclampsia of maternal vs. fetal origin [293]
Platelets		Lack genomic DNA

TABLE 158-2 -- Hematologic Disorders Diagnosed Antenatally

White blood cell disorders
Chronic granulomatous disease
Chédiak-Higashi syndrome
Wiskott-Aldrich syndrome
Leukocyte adhesion deficiency
X-linked agammaglobulinemia
X-linked lymphoproliferative disorder
Ataxia-telangiectasia
Severe combined immunodeficiency (SCID)
X-linked SCID
ADA-deficient SCID
Hyper-IgM immunodeficiency
Bare lymphocyte syndrome
Human immunodeficiency disease (HIV)
Platelet disorders
Alloimmune thrombocytopenia
Autoimmune thrombocytopenia
Glanzmanns thrombasthenia
Bernard-Soulier syndrome
Thrombocytopeniaabsent radii syndrome
Gray platelet syndrome
May-Hegglin syndrome
Congenital amegakaryocytic thrombocytopenia
Red blood cell disorders
Hemoglobinopathies
Sickle cell anemia
-thalassemia
-thalassemia
HbE, HbC, HbD, HbH, Hb Lepore, HbO-Arab
Alloimmunization
Rh disease
Kell incompatibility
RBC enzyme deficiency (G6PD, PK)
Congenital anemias
Fanconi anemia
Diamond-Blackfan anemia
Nonimmune hydrops fetalis

Coagulopathies
- von Willebrand disease
- Hemophilia A
- Hemophilia B
- Isolated factor deficiencies
- Hypo- or A-fibrinogenemia
- Thrombophilia
- Protein C deficiency
- Protein S deficiency
- Antithrombin deficiency

gestation restrict phenotypic identification of phagocytic disorders until the second trimester. [56] Entertaining earlier antenatal diagnosis of the congenital white blood cell disorders has only recently become possible with molecular techniques and a greater understanding of the associated genetic defects.

Chronic Granulomatous Disease

Granulocyte function in chronic granulomatous disease (CGD) has been evaluated by demonstrating the inability of neutrophils to reduce nitroblue tetrazolium (NBT) dye to blue formazan particles, as well as absent superoxide anion production and NADPH oxidase activity. [57] Prenatal diagnosis of CGD was first performed by Newberger et al.[58] in 1979; they adapted the NBT test on blood samples obtained by placental vessel puncture visualized by fetoscopy. Unsuccessful attempts were made at performing NBT reduction on cultured amniotic fluid cells. [59] No consistent differences have been observed between normal cultured amniotic cells and those from CGD patients.[60] Matthay et al.[61] adapted the luminol-enhanced chemiluminescence method on pure fetal blood samples to exclude the diagnosis in one fetus at risk. Recently, CGD has been diagnosed postnatally by a single cell granulocyte oxidative burst activity by flow cytometry. [62] Routine flow cytometric diagnosis appears reliable.[63] Analysis at the DNA level became possible as our understanding of the genetic heterogeneity of the disease improved. CGD is caused by a diversity of mutations that may affect multiple genes.[64] Using closely linked flanking markers to a part of the NADPH oxidase complex, Lindof et al. [65] performed the antenatal diagnosis of X-linked CGD on cultured amniotic fluid cells. In 1990, Nakamura et al. [66] demonstrated monoclonal antibody staining against cytochrome b558 villus macrophages in an unaffected fetus at 8 weeks gestation. More specifically, direct sequence analysis of amplified fetal DNA may demonstrate a single base pair substitution of a known genetic defect in question. [67] [68]

Chédiak-Higashi Syndrome

Antenatal assessment of Chédiak-Higashi syndrome has primarily relied on the secondary manifestations of the disorder, including the identification of characteristic abnormally large acid-phosphatase-positive lysozomes present in neutrophils, eosinophils, basophils, lymphocytes, and fibroblasts. [69] Identification of peroxidase-positive granules in neutrophils from midgestation FBS has led to the positive phenotypic identification of affected fetuses. [69] [70] Phenotypic expression has also been tested in cultured amniotic fluid cells in the feline model [71] and in human chorionic villi.[69] Recent identification and mutation analysis of the complete Chédiak-Higashi gene on chromosome lq42-43 will allow more precise genotypic diagnosis. [72]

Leukocyte Adhesion Deficiency

Leukocyte adhesion deficiency is a rare autosomal recessive disease caused by deficient leukocyte surface glycoproteins (integrins) that leads to severe and often fatal infections. Prenatal diagnosis is possible by FBS at 2022 weeks gestation by demonstrating integrin expression on fetal leukocytes. [73] Distinct mutations of the gene encoding the common subunit CD11/CD18 shared by three leukocyte surface glycoproteins (integrins) have been identified and mapped to chromosome 21, [74] [75] suggesting the potential of using PCR-amplified DNA from CVS or amniocentesis for antenatal diagnosis.

Severe Combined Immunodeficiency Syndromes

The severe combined immune deficiency (SCID) syndromes include a heterogeneous group of disorders resulting in profound impairment of both cellular and humoral immune function. The inherited defect is largely X-linked, caused by mutations in the IL-2R chain gene. [76] About 20% of SCID lesions are associated with adenosine deaminase (ADA) deficiency and purine nucleoside phosphorylase deficiency, resulting in a virtual absence or functional deficiency of T and B lymphocytes. [77] The high lethality of the disorder makes prenatal diagnosis and identification of the carrier states desirable. Prenatal diagnosis is possible by immunophenotypic, functional, and molecular analysis.[78] The value of FBS in the diagnosis of various forms of SCID has been recognized by many investigators. [79] [80] [81] [82] As reference ranges of fetal leukocyte and lymphoid subpopulations have been established, monoclonal antibodies and flow cytometry may be used to determine cell subsets in fetuses at risk and to assess IL-2R expression.[78] [83] T- and B-lymphocyte HLA-DR and $_2$ microglobulin can also be detected by immunofluorescence.[84] A profound lymphopenia and an absence of T- and B-cell markers in pure fetal blood confirm the diagnosis of SCID. [84] Where ADA deficiency is the known defect, ADA and dATP measurements in chorionic villi, fetal red cells, and cultured amniotic fluid cells may be used to perform rapid prenatal diagnosis. [85] [86] [87] The most

promising method is the genotypic analysis of SCID by identification of the IL-2R mutation [78] [88] or direct mutation analysis of the ADA gene.[89]

The bare lymphocyte syndrome is a SCID variant in which T and B lymphocytes lack expression of HLA antigens. [90] Demonstrating HLA antigen expression on fetal blood lymphocytes using immunofluorescent monoclonal antibodies and flow cytometry may exclude the diagnosis in infants at risk. [90] Recently, a restriction fragment length polymorphism (RFLP) has been identified, allowing rapid genotypic analysis for a subgroup of patients with HLA class II-deficient immunodeficiency. [91]

Wiskott-Aldrich Syndrome

The X-linked recessively inherited Wiskott-Aldrich syndrome (WAS) is characterized by eczematoid dermatitis, thrombocytopenia, and reduced cellular and humoral immunity. The absence of a defined lymphocyte population or a constant immunologic abnormality has made phenotypic antenatal diagnosis difficult. Normal mean platelet volumes and platelet counts by FBS have been used to exclude the diagnosis, [92] but these measurements are unreliable where abnormal hematologic parameters progress after birth.[93] Kenney et al.[94] found that patients with WAS have structurally abnormal lymphocytes with short blunted microvilli under electron microscopy; they used this morphologic marker to correctly identify an affected fetus. Progress in genotypic diagnosis was made by indirect DNA analysis combined with studies of X-chromosome inactivation patterns as early as 9 weeks gestation. [95] [96] [97] [98] The WAS gene is mapped to the proximal arm of the X chromosome between Xp11.22-11.3.[99] [100] Several mutations and DNA flanking markers have been found in the WAS gene that facilitate reliable diagnosis of both carriers and affected fetuses.[101]

X-Linked Agammaglobulinemia

X-linked agammaglobulinemia is a congenital antibody deficiency disease caused by defective B-lymphocyte differentiation due to a defective cytoplasmic tyrosine kinase (BTK). In families seeking genetic counseling, carrier status can be identified by assessing nonrandom X-chromosome inactivation patterns in the maternal B-lymphocyte population.[102] [103] [104] [105] [106] Because of the variability in the number of B cells in the midtrimester fetus, prenatal diagnosis using lymphocyte subpopulations is helpful in excluding the disease only if a normal number of cells are seen. [103] The BTK gene has recently been cloned and the genomic organization determined.[107] Several flanking markers surround the locus and have been used to diagnose the disorder as early as 10 weeks gestation using CVS. [104] Nonallelic genetic heterogeneity with RFLPs may complicate the diagnosis, although a method of calculating this risk has been developed. [105] [106] An ordered approach to diagnosis includes adequate tests for primary carrier status, linkage analysis using RFLPs where applicable, direct mutational analysis when possible, and confirmational immunologic studies later in gestation.

Hyper-IgM Syndrome

Patients with the hyper-IgM syndrome have a primary underlying immune disorder caused by the inability to initiate switching from one immunoglobulin subtype to another. The disease is inherited in an X-linked or autosomal recessive manner. Prenatal diagnosis by immunophenotypic analysis was limited due to the absence of

immunologic abnormalities during fetal development and the random pattern of X inactivation in lymphoid cells of obligate carriers. [108] Recently, X-linked hyper-IgM immunodeficiency was diagnosed at 12 weeks gestation using a highly polymorphic dinucleotide repeat in the CD40 ligand gene of fetal DNA isolated from CVS. [109]

X-Linked Lymphoproliferative Disorder

The X-linked lymphoproliferative disorder is characterized by a selective severe or fatal immunodeficiency to the Epstein-Barr virus. [110] The X-linked lymphoproliferative mutation has been mapped to the distal arm of the X chromosome at Xq25-q26. [111] [112] Prenatal diagnosis has been reported using multiplex PCR with highly polymorphic markers on CVS-obtained fetal DNA. [113]

Immunodeficiency with Ataxia-Telangiectasia

Ataxia-telangiectasia (AT) is an autosomal recessive disorder characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, immunodeficiency, increased incidence of malignancy, and a 23 times increased sensitivity to ionizing radiation. The underlying molecular abnormalities all result in defective DNA replication and repair. Prenatal diagnosis is possible by demonstrating spontaneous chromosome breakage and radioresistant DNA synthesis in cultured villus cells and/or amniotic fluid cells, and by analyzing the response of amniocytes to x-ray stress. [114] [115] The presence of a clastogenic factor, a low-molecular-weight protein in the serum of affected patients, has also been identified in the amniotic fluid of an affected fetus. [115] The first prenatal diagnostic testing for AT by genetic analysis was performed by Gatti et al.[116] in 1993. The entire coding sequence of the AT gene has recently been characterized, making possible mutation screening in carriers and fetuses at risk.[117] A high frequency of distinct AT gene mutations has been identified. [118]

Human Immunodeficiency Virus Infection

Until recently, no treatment could reduce the risk of maternalfetal human immunodeficiency virus (HIV) transmission. In the last 5 years much knowledge concerning the transmission of HIV in the perinatal period has been gained. Efforts have focused on effective screening programs for HIV infection in pregnant women, patient education, and interruption of the transmission cycle.

The United States Centers for Disease Control and Prevention has published guidelines urging health care providers to offer HIV counseling and antenatal HIV testing to all women.[119] Voluntary universal antenatal HIV screening programs in pregnant women appear to result in a higher percentage of women accepting testing and a subsequent increase in the detection of previously unrecognized infections. [120] Reasons for declining testing have included poor patient knowledge about vertical transmission, the assumption of no risk, being in a stable relationship, not wanting to think about HIV when pregnant, and not wanting to know if one is HIV-positive.[121] Culturally and linguistically appropriate efforts have improved womens knowledge regarding vertical transmission and treatment options. [122]

The risk of vertical transmission is reduced by the administration of maternal and neonatal zidovudine therapy [123] and the use of bottle feeding.[124] This has contributed to practical and ethical arguments concerning mandatory prenatal HIV screening for all pregnant women. Prenatal testing for fetal HIV infection has emerged as a challenging task. Fetal HIV infection probably occurs after 13 weeks gestation, and concerns have been raised about the small but serious risk of fetal exposure to maternal HIV due to fetomaternal hemorrhage with amniocentesis, FBS, or CVS.[125] Routine fetal serologic studies are of limited use, given that almost all children of HIV-infected mothers have normal immunologic findings, even at birth. [126] HIV reverse transcriptase activity and viral antigens have been demonstrated in amniotic fluid as early as 15 weeks gestation, although it is unknown if such a finding invariably means fetal infection. [127] [128] More diagnostic is the finding of HIV antigens or

genome in fetal cells obtained by FBS. Given the infrequent early in utero HIV transmission rate, midtrimester FBS is currently of limited value to women in making reproductive decisions.[129]

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RED BLOOD CELL DISORDERS

Hemoglobinopathies

The hemoglobinopathies are the most commonly inherited recessive diseases in humans, with an estimated 240 million heterozygotes worldwide and 200,000 homozygotes or compound heterozygotes born each year.[130] The global nature of these conditions, coupled with the desire for disease prevention and prediction, has propelled molecular biology research. Application to antenatal assessment has progressed at a comparable rate, leading to the International Registry for Prenatal Monitoring of Hereditary Anemias, a documentation of the thousands of individuals examined to date. [131] Initial methods of testing involved determining the relative rates of fetal , , and chain synthesis. This has gradually been replaced by DNA analysis of fetal amniocytes or chorionic villi. The combination of heterozygote screening, counseling, and prenatal testing has dramatically reduced the number of affected infants born in many countries throughout the world. [130]

Sickle Cell Anemia

Hollenberg et al.[131] first predicted that the detection of sickle cell hemoglobin in utero could identify affected fetuses. In 1972, Kan et al. [132] detected sickle cell trait in a 15-week-old fetus by incubating umbilical cord blood with radioactive leucine, followed by separation of the globin chains using carboxymethyl cellulose chromatography (CMC). Other methods for globin chain analysis include electrophoresis on polyacrylamide gels containing urea, acetic acid, Triton X-100, and hemoglobin immunofluorescence.[133] [134] High-pressure liquid chromatography (HPLC) by ion exchange correlates highly with CMC and can separate globin chains to provide an answer in 15 minutes. [135] Isoelectric focusing is an alternative method for analyzing hemoglobin tetramers instead of globin chain synthesis in pure fetal blood samples.[136] [137] Maternal blood contamination is a potential source of error in any of the above methods, skewing globin chain percentages. [138]

Kan and Dozy[139] first performed antenatal diagnosis of sickle cell disease at the DNA level by indirect molecular techniques. Polymorphism of the DNA sequence adjacent to the -globin gene was established, and the normal HbA fragment was differentiated from the HbS allele of a different length. Several altered restriction endonuclease sites have been found using the enzymes HpaI, DdeI, MstII, CvnI, and OxaNI. [140] [141] PCR allows restriction digestion and direct visualization of DNA fragments more rapidly and without the need for radioisotopes. [142] Direct assays detecting the HbS mutation in enzymatically amplified fetal DNA are possible using allele-specific oligonucleotide probes. [143] [144] The sickle cell base alteration has also been detected by the color complementation methods with allele-specific oligonucleotide primers conjugated to fluorescein, described by Chehab and Kan. [145]

Thalassemias

In contrast to the structural variant of HbS, the thalassemias are characterized by a decreased production of - or -globin chain synthesis. Screening programs and elective reproduction cessation were the initial methods of preventing the birth of an affected child. More precise methods were devised using second-trimester FBS and evaluation of globin chain synthesis. Knowing that a rise in the / ratio occurs normally throughout gestation, CMC was used to calculate the / ratio. Quantitative analysis of -chain production was used to suggest the presence of the homozygous state. Diagnostic errors were known to occur in patients having high -producing + thalassemia major and low -producing -thalassemia trait.[131] CMC was gradually replaced by HPLC, [146] isoelectric focusing,[147] and gel electrophoresis.[148] Other mutant -globin chain variants such as HbE, Hb Lepore, HbC, and Hb O-Arab have been detected by CMC using fetal blood. [131] As the molecular defects in the -globin gene producing thalassemia were characterized, restriction enzymes, gene mapping, and oligonucleotide probes specific for point mutations of interest have been more commonly used for antenatal diagnosis.[130] [149] A rapid and simple modified PCR-based method has been developed for the detection of the most common -thalassemia and HbE mutations in target populations. [150] Although rarer alleles are expected to be identified in the future, it is estimated that the 54 known alleles account for 99% of -thalassemia gene defects in the world.[149] The combination of PCR and specific restriction enzymes or probes is currently the most commonly used method of antenatal assessment and continues to revolutionize programs aimed at disease prevention.

Detecting -thalassemia by gene deletion assessment was performed in 1976 by Kan et al. [151] A quantitated reduced amount of fibroblast DNA hybridization could indicate -thalassemia trait, Hb H disease, or -thalassemia fetal hydrops syndrome. [151] [152] Hybridization techniques can detect rarer forms of thalassemia caused by gene deletions,[153] as can the lack of amplified globin gene DNA.[154] Recently, a simple monoclonal antibody test was described as having a high sensitivity and specificity in Southeast Asian populations carrying the (- ^{SEA}) deletion.[155] This test may be applied to the screening of pregnancies at risk for hydrops fetalis due to homozygous (- ^{SEA}) deletions.

Rh Disease

In the 1950s, severe hemolytic disease due to red blood cell alloimmunization accounted for >1,000 deaths per year. [156] Since the introduction of anti-D immunoglobulin prophylaxis in the 1970s, a dramatic decrease to 34 deaths per year has occurred. [157] Nevertheless, maternal Rh sensitization and fetal hemolytic disease due to other blood group antibodies remain significant problems that may lead to anasarca, fetal cardiac failure, and death. [158] Diagnostic and therapeutic advances were made in the late 1950s by Liley et al.; an indirect index of fetal erythrocyte destruction was estimated by amniotic fluid bilirubin spectrophotometric measurement.[159] Intraperitoneal infusion, introduced in 1963, was the first method used to treat seemingly severely affected fetuses. [160] Gaining access to the fetal circulation allowed more direct measurement of fetal hematocrit, reticulocyte count, and Coombs test, and has provided a more direct route for transfusion therapy. [158] Fetal Rh phenotyping is firmly established by obtaining a pure fetal blood sample. Bennett et al. [161] described the use of PCR to determine the RhD genotype of both amniotic cells and fetal blood samples in 15 fetuses, with complete correlation. Such a method of fetal RhD typing permits accurate first-trimester identification of infants at risk without invading the fetomaternal circulation, and appears accurate and reliable. [162] Similar PCR-based determinations of Rhc, Rhe, and Kell blood group typing of fetuses at risk for hemolytic disease have been described. [163] [164] [165]

Nonimmune hydrops fetalis may occur in a number of conditions, including the fetal aplastic anemia associated with parvovirus B19 infection. The gestational age with the greatest risk for fetal hydrops appears to be the second trimester. [166] Parvovirus B19 DNA has been obtained from fetal blood using PCR and parvovirus B19 specific primers, and has been used with fetal ultrasound to diagnose fetuses at risk for severe disease.

Reversal of parvovirus B19 hydrops has occurred with antenatal transfusion therapy. [167]

Enzymopathies

Inherited red blood cell enzyme defects may result in clinically undetectable altered erythrocyte survival or function, intermittent hemolytic crises, or a state of chronic

hemolytic anemia. The most common enzyme defects involve glucose-6 phosphate dehydrogenase and pyruvate kinase. The diagnosis often relies on demonstrating decreased erythrocyte enzyme activity. Normal enzymatic activities are available for >12 glycolytic enzymes in fetal erythrocytes at 1724 weeks gestation. [168] Two other disorders in the heme synthetic pathway, acute intermittent porphyria and protoporphyria, can also be detected by assays of uroporphyrinogen I synthetase and heme synthetase, respectively.[169] [170] Analysis at the molecular level will likely prove to be a more reliable means of antenatal diagnosis of these disorders in the future.

Other Congenital Anemias

Two other rare fetal congenital anemias, Fanconi anemia and Diamond-Blackfan anemia, may be diagnosed in utero. [171] [172] Fetal blood counts and the number of circulating erythroid progenitor cells can be compared with normal values for gestational age. [173] Ultrasound demonstrating abnormalities of the radii or thumbs support these diagnoses.[171] A more definitive test for Fanconi anemia is based on demonstrating hypersensitivity of chorionic villi and amniocytes to clastogen-induced chromosomal breakage.[174] The characterization of the Fanconi anemia gene for complementation group C now permits the detection of affected pregnancies by molecular identification of specific mutations. [175] [176]

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PLATELET DISORDERS

The antenatal diagnosis of platelet disorders is aimed at identifying and treating fetuses at risk for hemorrhagic complications. Early detection may identify infants likely to benefit from in utero treatment, delivery by cesarean section, or treatment in the immediate neonatal period (see Table 158-2).

Alloimmune Thrombocytopenia

Neonatal alloimmune thrombocytopenia (NAIT) is a potentially severe disease caused by fetomaternal incompatibility for platelet-specific antigens, most commonly the PlA antigen system. As many as 1 in 2,000 fetuses may be affected,[177] with a mortality rate as high as 13%.[178] Half the cases of NAIT occur in a first-born child.[179] Although the risk of intracranial hemorrhage appears to be highest during delivery, 2550% of such hemorrhages appear to occur earlier in gestation. [180] Prenatal diagnosis is generally indicated in families with a previously affected neonate, since the risk of subsequent affected pregnancies is as high as 7585%, with a tendency to increasing disease severity. [181] Parental platelet phenotyping is first performed to determine pregnancies at risk. PlA1-negative women who are HLA-B8, DR3 appear to be most at risk for having an affected infant. [182] Maternal anti-PlA1 antibody levels have not been useful in predicting the severity of fetal thrombocytopenia.[183] Rapid genotype assays for PlA haplotypes may aid in predicting fetal risk. [184] FBS at 2022 weeks gestation is the most reliable method of assessing fetal platelet phenotype and platelet count. [185] Fetuses with platelet counts severely depressed early in gestation (i.e., by 20 weeks) appear to be at greatest risk of developing intracranial hemorrhage. [186] Early therapeutic interventions with in utero transfusions of PlA1-negative platelets, together with frequent ultrasound examinations, have been shown to increase the fetal platelet count and decrease the complication rate. [183] [187] Fetal scalp vein platelet count estimations have been used at the time of delivery to determine which infants should be delivered by cesarean section. This procedure, however, is associated with a high frequency of falsely low fetal platelet counts in the setting of amniotic fluid contamination, and does not address the risk of early gestational intracranial hemorrhage.

Advances in the diagnosis of PlA1-associated NAIT using allele-specific oligonucleotide probes have been made by McFarland et al. [188] A single nucleic acid base substitution in the gene encoding glycoprotein IIIa of the PlA1 antigen differentiates it from PlA2 and accounts for the alloantigenic polymorphism in the PlA system. [189] DNA-based phenotyping has been used on fetal leukocytes and amniocytes and is applicable to CVS obtained earlier in pregnancy. [188] [190] Its greatest advantage lies in the potential for earlier, more accurate diagnoses, and it eliminates the problems and risks associated with platelet serologic typing from FBS.

Autoimmune Thrombocytopenia

Autoimmune thrombocytopenia in the neonate can occur by passive placental transfer of maternal IgG, resulting in the destruction of fetal platelets. For unclear reasons, the morbidity and mortality of fetal autoimmune thrombocytopenia is much lower than that associated with alloimmune thrombocytopenia. [191] Indeed, the nadir platelet count in affected infants often occurs after delivery. [192] Given that idiopathic thrombocytopenic purpura (ITP) is a fairly common disease in childhood and in women of childbearing years, it is important to determine which fetuses may be at greatest risk for severe thrombocytopenia and thus would benefit from a cesarean section with postnatal observation. Little correlation exists between maternal and fetal platelet counts. The observation that maternal platelet-associated IgG levels were predictive of neonatal thrombocytopenia has not been confirmed, [192] nor has the measurement of amniotic fluid or cord blood antibody levels. [193] Likewise, the platelet count of a previous baby is not a reliable predictor. [194] Fetal thrombocytopenia does appear to be more frequent when maternal thrombocytopenia is present at the time of delivery.[195] The only reliable way to establish the presence of fetal thrombocytopenia is by FBS. Given the low risk of antenatal fetal morbidity, the benefit of an invasive procedure such as FBS has not been proven. FBS prior to delivery is recommended by some investigators as a reliable means of detecting fetal thrombocytopenia to aid in perinatal planning. [196] Avoidance of fetal head trauma and delivery by cesarean section is generally reserved for pregnant patients with ITP and documented fetal platelet counts <5070,000/mm^3 .[192] [197]

Many pregnant women have platelet counts in the range of 80150,000/mm^3 , especially in the second half of gestation. [198] This benign thrombocytopenia of pregnancy, also called pseudo-ITP, poses no maternal or fetal risk and is not associated with neonatal thrombocytopenia. In the absence of an associated bleeding tendency, no specific antenatal or perinatal precautions need be taken. At times, this incidentally detected condition may be difficult to distinguish from chronic ITP. [192]

Glanzmanns Thrombasthenia

Patients with Glanzmanns thrombasthenia have a normal circulating number and appearance of platelets, but platelets fail to aggregate in response to normal agonists. Absence or deficiency of glycoprotein IIb/IIIa, the fibrinogen receptor needed for normal platelet aggregation, causes a variable hemorrhagic tendency. [199] The GP IIb/IIIa complex is immunologically detectable as early as 19 weeks gestation, allowing radiolabeled anti-GP IIb/IIIa antibodies to be used in the assessment of fetuses at risk.[185] [200] The risk of fetal death due to post-FBS hemorrhage

appears to be particularly high with this disorder, thereby limiting antenatal diagnosis to parents who have decided on terminating an affected pregnancy. [201] This risk may be removed by diagnosis at the gene level with CVS. Several mutant alleles all purported to cause Glanzmanns thrombasthenia have been described. [202] The use of gene markers, however, is limited because the Glanzmanns phenotype corresponds to different genetic mutations. [203]

Bernard-Soulier Syndrome

Bernard-Soulier syndrome is a rare autosomal recessive inherited bleeding disorder characterized by mild thrombocytopenia, giant platelets, and absent ristocetin-induced von Willebrand factor (vWF) binding. The platelet membranes of affected individuals have absent or deficient GP Ib/IX and GP V. [204] Mutant alleles in the genes encoding these proteins have been described. [205] Antenatal diagnosis is theoretically possible by FBS at 1822 weeks for membrane assessment of GP Ib/IX content.[206] Affected women who have had multiple blood and platelet transfusions have an increased risk of fetal thrombocytopenia or immune hydrops fetalis.[207]

Other Inherited Platelet Disorders

Several other inherited platelet disorders may be detected in utero. Thrombocytopenia-absent radii syndrome is initially suspected by characteristic radiographic or ultrasonographic abnormalities and may be confirmed by FBS documenting thrombocytopenia. [208] This syndrome is differentiated from amegakaryocytic thrombocytopenia, in which neonatal thrombocytopenia is followed by pancytopenia later in childhood. The latter disorder had been diagnosed in utero by detecting thrombocytopenia in one infant at risk.[209] Gray platelet syndrome, a rare platelet disorder caused by defective platelet -granules, may be assessed antenatally by demonstrating the presence or absence of -granules by electron microscopy. [201] [203] Similarly, the demonstration of a normal platelet count and volume in the absence

of abnormal spindle-shaped leukocyte inclusion bodies excluded the May-Hegglin anomaly in an infant at risk. [201]

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COAGULOPATHIES

Components of the fetal hemostatic system begin to appear at approximately 1011 weeks gestation.[210] Reference values for numerous coagulation factors have been obtained from fetal blood samples.[211] Important insights into the genetics of inherited coagulopathies have enabled prenatal diagnosis by direct defect detection or by DNA polymorphism-based gene tracking.

von Willebrand Disease

One of the most commonly inherited bleeding disorders, von Willebrand disease (vWD) is a heterogeneous autosomally inherited disease caused by a qualitative and/or quantitative deficiency of vWF. More than 20 distinct clinical and laboratory subtypes of vWD have been described. [212] Variability of levels in the heterozygous state make the phenotypic diagnosis difficult. vWF behaves as an acute-phase reactant, and levels are often markedly elevated in the neonate. [213] Prenatal diagnosis of vWD has been reported using assays detecting factor VIII-related antigen and activity in fetal blood samples. [209] [214] [215] Advances in vWD molecular biology have led to both prenatal diagnosis and rapid neonatal diagnosis of certain subtypes. Peake et al. [216] used PCR to amplify DNA from CVS at 10 weeks gestation to identify a fetus with type 3 disease. The diagnosis was confirmed at 18 weeks by demonstrating undetectable levels of VIIIc, vWF Ag, and vWFRiCo in fetal blood. Cord blood leukocyte DNA testing of this sequence has been reported in the rapid diagnosis of types 1 and 2B in the neonatal period. [217] [218] Characterization of molecular defects by single point mutations or RFLP analysis is now possible in the majority of type 2A, 2B, and 2N cases and some type 3 cases. [219]

Hemophilia

Deficiency or abnormality of human factor VIII or IX results in the inherited bleeding disorder hemophilia A or B, respectively. The diseases are X-linked recessive, with an incidence of 1 in 5,000 males. In the era prior to antenatal diagnosis, presumed carriers of severe hemophilia were advised not to conceive children. [220] With the technology offered by amniocentesis for prenatal sex determination, others practiced the termination of all male fetuses. Because measurements of fetal clotting factors may provide a method of detecting affected fetuses,[221] FBS at 1820 weeks offered hemophilia carriers the chance to give birth to a known unaffected son. [222] Since factor levels may vary widely with inflammation, stress, exercise, and pregnancy, ambiguous results at a fairly late stage of pregnancy were occasionally obtained.[223] Fetal plasma assays have since been surpassed by analysis at the DNA level. Today, CVS is the most widely used method, although analysis by amniocentesis, FBS, and preimplantation genetic studies have been used in selected cases. [224] Direct DNA analysis has identified large numbers of point mutations, deletions, and insertions.[225] Most families carry a unique mutation, enabling RFLPs to be widely used in DNA analysis for fetuses at risk. [226] RFLPs within or closely linked to the factor VIII gene can establish carrier status and enable rapid prenatal diagnosis by detecting a mutant factor VIII allele. In the United States and Italy, St14, BclI, and XbAI markers have been the most useful in diagnosing hemophilia A; St14 is informative in 90% of cases. [227] The combined use of the enzymes TaqI, XmnI, MspI, BamHI, SstI, MnlI, DdeI, and HhaI can ensure definitive diagnosis of hemophilia B in up to 90% of people of European descent. [225] Such technology is limited when mothers are not heterozygous for the mutant or marker gene and when linkage disequilibrium exists. [228]

Other Factor Deficiencies

Congenital deficiencies of prothrombin, [229] and factors V,[230] VII,[231] X,[232] XI,[233] XII,[234] and XIII,[235] and familial multiple factor deficiencies [236] have all been described. Prenatal diagnosis by conventional specific functional and antigenic assays on fetal blood samples has been performed. [237] Awareness of the diagnosis antenatally is important in providing parents with desired information and in reducing the potential morbidity associated with vaginal delivery, scalp electrodes, vacuum extractions, forceps, and long labor.[237] Factor concentrate given just prior to delivery may benefit infants most at risk.

Hypercoagulable States

Severe thromboembolic disease may occur as a result of moderate to severe deficiency of the coagulation cascade inhibitory protein S or C, or antithrombin III. Disseminated intravascular coagulation and purpura fulminans have been documented hours to days after birth in infants with homozygous or compound heterozygous protein S or C deficiency.[238] [239] Protein S and C levels in newborns are 2040% normal adult levels; [240] thus, detecting levels <1% by FBS may identify infants most at risk for purpura fulminans. More reliable methods are now possible with molecular techniques that can identify affected fetuses earlier in gestation. The gene for protein C has been localized to chromosome 2. [241] Family diagnosis in a pedigree of protein C deficiency has been performed using DNA

ETHICAL ISSUES IN ANTENATAL DIAGNOSIS

The value of advances in antenatal assessment can be assessed only by also examining the personal, cultural, legal, religious, social, political, and economic factors. Ethical arguments encountered in prenatal diagnosis largely focus on the right of reproductive choice and on maternal rights versus fetal rights. Multiple and often contradictory values and belief systems influence reproductive choice decisions. Arguably, the foremost ethical justification for prenatal diagnosis lies in its potential to prevent the suffering of a future child affected by a serious and untreatable genetic disorder by selective abortion. [279] This in turn may prevent the inevitable suffering otherwise forced on a family and society. Such a premise would require clear distinctions as to what is a serious and untreatable disorder. Potential decisions about termination are often focused on the severity of the disorder and only marginally influenced about when in gestation the decision is made.[280] In a study of womens opinions on the use of prenatal diagnosis, strong positive attitudes toward diagnostic procedures were found, particularly if treatable abnormalities were detected.[281] In some instances, the availability of prenatal diagnosis has increased the birth rate, as in the Greek Cypriot population at risk for homozygous -thalassemia. This group previously had a low birth rate or terminated pregnancies without a diagnosis because of the risk involved. [282] The >90% termination rate for fetuses having thalassemia major in Europe further emphasizes the cultural variations in the need for antenatal assessment. [283] For other diseases, however, prenatal diagnosis is not widely accepted, as is evident by its exceedingly low practice in pregnancies at risk for sickle cell anemia in the United States.[284] The unpredictability of the severity of sickle cell anemia contributes to its infrequent antenatal assessment.

The influence on reproductive genetics by political parties, civil liberty groups, and religious forces is enormous. Political intervention has influenced the access to new technologies (e.g., RU486, the gag rule prohibiting physicians in federally funded clinics from engaging in abortion counseling), the availability of private insurance and federal funds for prenatal procedures, and the availability of research resources (i.e., fetal tissue research). [285] The prospect of malpractice litigation under certain circumstances has induced physician anxiety: there are physicians in France jailed for purportedly delaying HIV testing. [286]

Many questions are raised regarding the purpose of pre- natal diagnosis. A serious and controversial issue concerns assessment for fetal sex selection, a practice reportedly rare in industrialized countries but relatively common in developing countries. [287] Prenatal testing of the fetus conceived for the purpose of benefiting another sibling is another issue reviewed by legal and ethical advisory committees. [279] In one instance, a family sought prenatal diagnosis to bear a healthy child to serve as an HLA-identical bone marrow donor for their son affected by Wiskott-Aldrich syndrome. The physicians involved concluded prenatal testing should not be used to benefit a third party.[279] Here, the concept of parental reproductive autonomy is a strong rebuttal. Prenatal diagnosis for the purpose of the prevention of late-onset genetic disorders is another issue arising with advanced antenatal technology. Our ability to detect offspring who have genes rendering them more susceptible to cancer, diabetes, heart disease, and depression further clouds the picture.

Although it is an arduous and yet to be perfected procedure, preimplantation genetic screening is now technically feasible. The genetic structure of the embryo formed by in vitro fertilization may be assessed, followed by uterine transfer of the genetically unaffected embryo. Preconceptive testing of oocytes is yet another possibility, whereby biopsy of the first polar body permits knowledge of the DNA content remaining in the ovum. [288] Although strong ethical objections to such practices are raised, preimplantation genetic screening may appeal to those opposed to selective termination but who are at risk for a genetically affected offspring.

More universal acceptance of antenatal assessment can be expected with the development of intrauterine therapies, although procedures such as in utero fetal stem cell transplantation, fetal liver cell transplantation and germ line genetic therapy have posed ethical questions in and of themselves. Selective termination is no longer the common conclusion in prenatal diagnosis. Genetic counseling has been shown to reduce anxiety and depression [289] and may serve to prepare a couple for parenting a child with special needs. [290] Physicians devoid of provider prejudice have legal and ethical duties to inform parents of tests widely accepted in the medical community as standard prenatal care. Health care providers are obligated to communicate fully in helping families make the most informed reproductive choices. This will ultimately improve perinatal care, allay parental anxiety where possible, and provide psychosocial support for those involved.

polymorphism of the gene.[242] Several RFLP sites within or near the protein C gene have been discovered, implying the potential for early diagnosis and improvement in carrier detection. Prenatal diagnosis of compound heterozygous deficiency of protein C by direct detection of the mutation sites has been reported. [243] Two protein S genes have been identified and localized near the centromere of chromosome 3. [244] [245] Several protein S gene mutations in patients with phenotypic protein S deficiency and thrombosis have been identified. [246] Further research is needed to reveal genetic alterations in affected individuals and the RFLPs that may be useful in prenatal diagnosis and carrier detection.

Deficiency of antithrombin predisposes affected individuals to repeated thrombotic events. Inherited in an autosomal dominant pattern, homozygosity has been reported in children of consanguineous families. [247] [248] Undetectable antithrombin levels obtained by FBS in the second trimester may detect severely affected infants. Recently, prenatal diagnosis of combined antithrombin and factor V deficiency by genotypic analysis from CVS at 12 weeks gestation in a fetus at risk was successfully performed.[249] The diagnosis of the heterozygote form is best deferred until later in the postnatal period. [249]

FUTURE DIRECTIONS

Considerable progress has been achieved in our understanding of the molecular basis of several congenital hematologic disorders. The future of antenatal assessment relies on the refinement of prenatal diagnostic procedures that do not endanger fetal or maternal well-being and on improving available therapeutic options. Reliable, noninvasive screening techniques applicable to all pregnant women may one day be realized.

Preimplantation genetic analysis of aspirated blastomeres has become possible and is a valuable asset to in vitro fertilization programs. The genetic indications for preimplantation diagnosis

are largely the same as those for prenatal diagnosis. Thus far, approximately 40 healthy children have been born worldwide after preimplantation diagnosis excluded genetic disease.[250] Defects must be detectable by PCR or, for X-linked diseases, by fluorescent in situ hybridization. Experience has been gained with preimplantation sex determination[251] and in the diagnosis of hematologic conditions, including -thalassemia, [252] RhD blood typing,[253] and sex-linked diseases. [254] Preimplantation diagnosis is currently hindered by the limited quantity of material available for genetic analysis and the potential for misdiagnosis by allele dropout. [255] Recently, simultaneous detection of multiple gene expression in human preimplantation embryos was described. [256] This novel RT-PCR method permits the comparison of gene expression at different stages of development and may further our understanding of gene activation and regulation.

Advances for the correction of selected hematologic disorders with in utero therapy continue to be made. The immaturity of the fetal immune system and the presence of marrow space makes the fetus an ideal transplantation host, requiring neither extensive preconditioning regimens nor HLA-identical transfusions. [257] Hematopoietic stem cell transplantation, first demonstrated in mice,[258] sheep, and nonhuman primates[259] led to several attempts in human fetuses.[257] [260] [261] The source of hematopoietic stem cells appears critical to engraftment. [260] [261] [262] Fetal liver cells, alone or with syngeneic fetal thymus and skin cells, have engrafted following umbilical vein and intraperitoneal transfusions. [260] [261] [262] Full immunologic reconstitution of human fetuses with SCID has occurred following in utero stem cell transplantation.[261] [263] In 1996, Flake et al.[264] reported the successful treatment of X-linked SCID by in utero transplantation of haploidentical paternal enriched hematopoietic progenitors that resulted in split chimerism in which T cells were of donor origin. Complete cure or significant improvements have been demonstrated in conditions associated with severe aplastic anemia, thalassemia, bare lymphocyte syndrome, and inborn errors of metabolism. [260] [261] In utero stem cell transplantation offers the advantages of increased probability of engraftment and chimerism, [265] decreased risk of acute graft-versus-host disease, [257] [266] a more sterile environment,[266] and a selective growth benefit for normal hematopoietic cells. [264] Early gestational age improves the chance for full, rapid development of transplanted stem cells. This exciting therapeutic modality has the potential to eliminate or ameliorate many inherited hematologic disorders before the onset of clinical manifestations of the disease. Permanent engraftment will likely be limited to hereditary disorders with a growth advantage of donor cells over recipient cells (i.e., immune deficiency syndromes, Fanconi anemia).[267] A concern with in utero stem cell transplantation is the possibility of transmission of viral infections to immunocompromised fetuses.

An alternative method of in utero treatment may be seen with gene transfer techniques. When incorporated into a retroviral vector, foreign genes have been successfully incorporated into murine hematopoietic stem cells, hepatocytes, skin cells, and endothelial cells. [268] [269] [270] [271] [272] Phenotypic correction of Fanconi anemia in human hematopoietic cells with a recombinant adeno-associated viral vector has been reported; the corrected cells have a selective advantage over defective hematopoietic stem cells.[273] In utero gene transfer and expression of neomycin-resistance genes in fetal sheep hematopoietic cells have been demonstrated.[274] Cord blood cells as a target for genetic modification have been increasingly studied. The in vivo proliferation of retrovirally marked cord blood cells has been evaluated in the ovine system[274] and is actively being studied in infants diagnosed prenatally with ADA deficiency. [275] Thus far, it remains to be proven that transduction in primary cells is permanent and that the viral vector is fully integrated. [276] Improvements in the gene transfer efficiency of cord blood cells may permit effective treatments for a variety of inherited hematopoietic disorders.

The future is likely to find prenatal testing increasingly used to assess fetal donor prospects. One unit of umbilical cord blood contains sufficient hematopoietic stem cells to fully reconstitute a child and in some cases an adult with a disease treatable by bone marrow transplantation. [277] [278] Predictive genetic testing and HLA typing of cultured fetal cells enable one to know early in pregnancy if a fetus is genetically normal and a possible allogeneic bone marrow donor for an affected family member. Cord blood cells may become an increasingly valuable source of progenitor cells for transplantation, immunotherapy, and gene therapy. [267]

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Chapter 159 - Electrophoretic and Immunochemical Analysis of Human Immunoglobulins

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INTRODUCTION

Human immunoglobulins occur in five classes, based on amino acid sequence differences in the constant regions of their heavy polypeptide chains. A given immunoglobulin molecule may have either type or type light chains, a distinction determined by primary structural differences in the constant regions of the molecule. The various immunoglobulin heavy chain classes and subclasses (Table 159-1), termed isotypes, are encoded by immunoglobulin genes on chromosome 14. and light chain genes are encoded on chromosomes 2 and 22, respectively. Detailed information on immunoglobulin genes, on B-cell development and activation, and on molecular structure and biologic functions of immunoglobulins is given in Chapter 36 . The availability of heavy chain-specific antisera that identify human , , , , or chains, and light chain-specific antisera that recognize human or chains, is central to two of the

TABLE 159-1 -- Selected Properties of Immunoglobulins in Human Serum

Class (Isotypes)	Heavy Chain Type	Light Chain Type	Molecular Weight	Normal Adult Serum Concentration[a]	Subclasses (%)	Normal Electrophoretic Mobility (range)	Half-life (days)	Other Comments
IgG		or	150,000	565 1,765 mg/dl[b]	IgG1 (6270)[e] IgG2 (1133) IgG3 (36) IgG4 (0.56)	From most cathodal portion of -globulin area to $_2$ -globulin region	23	Crosses placenta; neonatal levels approximate adult concentration; some IgG myelomas are euglobulins[f]
IgA		or	170,000 (monomer)	85385 mg/dl[b]	IgA1 IgA2	From fast - to $_1$ -globulin area	6	In IgA myeloma a portion of paraprotein may form higher molecular weight polymers that differ from monomer in electrophoretic mobility
IgM		or	900,000 (Pentamer)	45250 mg/dl[b]	None recognized	From fast - to $_2$ -globulin area	5	In Waldenström macroglobulinemia significant IgM monomer may be present; IgM paraproteins often are euglobulins[f]
IgD		or	185,000	0trace[c]	None recognized	Similar to IgM and IgA	2	Concentration in normal serum too low to be detected by zone electrophoresis, IEP, or IFE; rare myeloma; very rare HCD
IgE		or	200,000	10200 IU/ml[d]	None recognized	Similar to IgM and IgA	2	Concentration in normal serum too low to be detected by zone electrophoresis, IEP, or IFE; very rare myeloma; elevated levels in allergic or parasitic diseases

Abbreviations: IEP, immunoelectrophoresis; IFE, immunofixation electrophoresis; HCD, heavy chain disease.

[a] See Table 159-2 for age-adjusted changes in concentrations of IgG, IgA, and IgM.
[b] By nephelometry.
[e] Approximate percentage of total IgG (data from Specialty Laboratories, Inc., Santa Monica, CA 90404).
[f] Euglobulins are insoluble in the low ionic strength buffers commonly employed in electrophoretic procedures, including IEP and IFE. This property often causes spontaneous precipitation at or near the origin and impedes analysis (see text).
[c] Undetectable in many normal individuals.
[d] By ELISA assay. Mayo Medical Laboratories (Rochester, MN), using a radioimmunoassay, lists a normal adult range of 20367 U/ml.

principal methods for analysis of human immunoglobulins. [1] Of greatest utility in clinical diagnostic laboratories are polyclonal antisera, usually prepared in goats or rabbits. At this time, monoclonal antibodies have a more limited role because they precipitate poorly with the proteins to which they are directed, and their use therefore requires additional steps to immobilize the protein(s) of interest.

Most pathologic immunoglobulin disorders involve IgG, IgA, and IgM, and these are emphasized in this chapter. IgD and IgE occur only in trace amounts in normal human sera (Table 159-1) and cannot be detected by the standard electrophoretic methods currently in use, unless the concentration of these proteins is abnormally elevated, as in the rare IgD or IgE myelomas.

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CONCEPTS OF MONOCLONAL AND POLYCLONAL IMMUNOGLOBULINS

One of the most striking features of the immunoglobulins synthesized by normal individuals is their heterogeneity, not only with respect to class, subclass, and light chain type, but also as reflected by an enormous diversity in antibody specificity. The latter property is related to amino acid differences in the variable regions of the heavy and light chains making up the immunoglobulin molecule. Within this extremely diverse array of circulating immunoglobulins are the products of individual cells of the B-lymphocyte lineage. Each antibody with its unique primary structure arises from a unique B cell and its progeny (i.e., a clone). However, so many clones contribute to the immunoglobulin pool in normal serum that the immunoglobulin molecules produced by an individual clone cannot be detected within the collective background of immunoglobulins synthesized by all the other clones. Thus, the great heterogeneity of immunoglobulins in normal serum reflects their highly polyclonal origin.

All of the immunoglobulins synthesized by a clone of antibody-forming cells share identical amino acid sequences in both constant and variable regions and therefore are identical in class, subclass, and light chain type. Post-synthetic glycosylation of heavy chain constant regions varies among the immunoglobulin classes, but is generally similar for immunoglobulin molecules formed by a given clone. It follows from these principles that the immunoglobulin proteins arising from a single clone typically have the same net electrical charge, and therefore the same electrophoretic mobility. Such monoclonal immunoglobulin proteins migrate electrophoretically as a very sharp, dense band. This is in contrast to the broader, polydisperse migration of normal serum immunoglobulins that reflects synthesis by a vast number of clones, resulting in a polyclonal pattern. At the molecular level, this electrophoretic (charge) heterogeneity of normal immunoglobulins is thought to be determined mainly by primary sequence differences in the immunoglobulin variable regions, with some contributions from their class and subclass. Thus, antibodies with specificity for a given antigenic epitope may have a monoclonal origin and, if so, would be expected to exhibit a sharply restricted electrophoretic mobility.

However, the quantity of immunoglobulin synthesized by each clone of antibody-forming cells is normally modulated in such a way that its immunoglobulin products are not in sufficient concentration to be distinguished from immunoglobulins contributed by all the other clones.

In contrast, in myeloma, Waldenström macroglobulinemia, some B-cell lymphomas, heavy chain diseases, or primary amyloidosis, a single clone of plasma cells or immunoglobulin-synthesizing B lymphocytes emerges from the general B-cell population, expands in an unregulated fashion, and synthesizes a homogenous immunoglobulin product at a high enough rate to be detectable against the background of polyclonal immunoglobulins.

In this chapter, the historical term paraprotein is sometimes used to designate such a monoclonal immunoglobulin, even though the original concept that all monoclonal proteins represent intrinsically defective molecules, having no counterpart in the normal host, is no longer accepted. On the other hand, heavy chain disease, in which only portions of an immunoglobulin heavy chain are synthesized, and light chain disease (Bence Jones myeloma), in which qualitatively normal light chains are synthesized and secreted without heavy chains, do represent disordered synthesis beyond the abnormality of uncontrolled clonal expansion.

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METHODS FOR CLINICAL EVALUATION OF HUMAN IMMUNOGLOBULINS

Assessments of abnormalities of human immunoglobulins in serum, urine, or other body fluids utilize zone electrophoresis, immunoelectrophoresis (IEP), and immunofixation electrophoresis (IFE) for qualitative and semiquantitative analysis. Nephelometry or radial immunodiffusion are used for quantitative measurement of immunoglobulins. Other specialized procedures are employed for evaluation of anomalous proteins such as cryoglobulins.

Zone Electrophoresis

All electrophoretic methods separate proteins within a complex mixture by virtue of differences in their net electrochemical charges. Zone electrophoresis is a general term for procedures that subject proteins to an electrical field in, or on, a supporting matrix that permits subsequent fixation of the separated proteins and their detection by staining with protein-binding dyes. The supporting medium for the most familiar form of zone electrophoresis consists of strips of cellulose acetate. Agarose gels, supported on a clear plastic film sheet, are now more widely used than cellulose acetate because of their capacity to resolve serum into a greater number of discrete bands.[2] After fixation and drying, stained zone electrophoretic patterns (on cellulose acetate or dried agarose gels) can be scanned by a densitometer to produce a tracing in which each band in the stained pattern is translated into an inscribed peak whose height and width reflect the intensity and electrophoretic dispersity, respectively, of the band being scanned. When this is applied to serum separated on cellulose acetate, the traditional serum protein electrophoresis (SPEP) profile results (Fig. 159-1A). By determining the total protein concentration in the sample, each main peak (e.g., albumin, $_1$ - or $_2$ -globulins, -globulin, or -globulin) can be assigned an approximate protein concentration (by mathematical integration). Similar SPEP analyses are performed with stained protein patterns obtained by zone electrophoresis in agarose gel. Such patterns are shown in the uppermost sections of each of the immunofixation electrophoresis figures (Figs. 159-4 , 159-5 , 159-6 , and 159-7).

It must be remembered that the classical -globulin peak does not encompass the full range of immunoglobulin mobilities. Some human immunoglobulins migrate as -globulins and occasionally into the $_2$ -globulin area. By convention, an immunoglobulin population (with a negative net charge) that migrates more strongly toward the positive pole (anode) is said to have fast electrophoretic mobility, and immunoglobulin molecules found close to the cathode are said to be slow.

Figure 159-1 Zone electrophoresis on cellulose acetate with densitometric scans. **(A)** Normal human serum. **(B)** Serum with large monoclonal spike in the mid -globulin area. Note that background of normal polyclonal -globulin on either side of the spike is severely diminished. Further evaluation by IEP revealed that the monoclonal spike was IgG-.

Zone electrophoresis (SPEP) has been widely employed for many years as a first screening test for evaluation of serum immunoglobulins. This technique can establish the presence of moderate to large monoclonal spikes (Fig. 159-1B), polyclonal hypergammaglobulinemia, or generalized (pan-) hypogammaglobulinemia. By itself, this method has significant shortcomings: (1) it cannot identify the class or light chain type of a monoclonal protein; (2) it may be too insensitive to detect a low

concentration of monoclonal immunoglobulins or free light chains in serum, especially if the background immunoglobulins are normal or increased; and (3) it is incapable of detecting selective IgA or IgM deficiency. SPEP performed in agarose gel is more sensitive than SPEP in cellulose acetate in detecting small monoclonal immunoglobulin populations, although shortcomings (1) and (3) above still apply.

The use of SPEP in combination with quantitative assays of IgG, IgA, and IgM (e.g., by nephelometry or radial immunodiffusion) can greatly add to the power of this evaluation. Some clinical laboratories offer quantitation of total or molecules by rate nephelometry. This provides a / ratio (normally about 70:30). A patient with a sizable spike by SPEP might be found to have very high serum IgA and light chain values by nephelometry, not infrequently with a depression of IgG, IgM, and total molecules, making the diagnosis of an IgA myeloma very likely.

Evaluation of a paraprotein disorder should also include IEP or IFE analysis of both serum and concentrated urine because the greater sensitivity of the latter procedures would permit a more complete analysis. This includes the detection of a second, lesser paraprotein (aside from a major serum spike seen in SPEP), such as free monoclonal light chains in serum or urine (Bence Jones protein), occasional biclonal paraproteins or the presence of multimers of the main paraprotein that have slightly different electrophoretic mobility. Formation of such multimers (e.g., dimers and tetramers) occurs quite often in IgA myeloma and can result in a falsely polyclonal appearance of an abnormal peak seen in SPEP.

Urine electrophoresis on cellulose acetate or agarose gel (UPEP), usually performed after the urine has been concentrated 50- to 200-fold, can reveal (1) a restricted band representing a Bence Jones protein (BJP); (2) an intact immunoglobulin paraprotein that has entered the urine from the plasma; (3) both of the above; or rarely (4) immunoglobulin fragments, as in heavy chain disease. However, the precise identity of such urinary proteins will not be determined by this procedure, and subtle monoclonal bands, such as those due to low concentrations of BJP, may be missed. Thus, evaluation of urine for paraproteins is optimally done by IEP or IFE on 50- to 200-fold concentrated urine. (The extent to which the urine is concentrated is largely determined by the overall concentration of protein in the sample.) For the clinician, the accurate detection of BJP is more than academic, since the presence of BJP is associated with a greater risk of amyloidosis and renal disease (myeloma kidney). In some patients who eventually prove to have primary amyloidosis, a low concentration of monoclonal light chains in the urine may be a decisive clue. Thus, evaluation for a paraprotein disorder should always include appropriate studies of urine as well as serum. A 24-hour collection of urine is not necessary for this diagnostic study; the current, highly sensitive IEP or IFE analysis of concentrated urine requires only 100 mL of a random voiding. In contrast, serial quantitative assay of urinary light chains has been used in some centers for judging the efficacy of therapy. This type of assay does require a 24-hour collection.

Immunoelectrophoresis

IEP is a versatile and broadly effective procedure for the detection of pathologic proteins in serum and urine. [1] In this method, a thin (22.5 mm) layer of agar gel dissolved in 50 mM barbital buffer (pH 8.2) is formed on the surface of glass slides or on 85 × 100 mm clear, flexible plastic film sheets (Gel Bond). (Agar, rather than its purified constituent, agarose, is commonly used in IEP because its higher endosmotic [reverse] flow advantageously positions the immunoglobulins for analysis.) The patients serum or concentrated urine is placed in a small well punched into the agar, and a similar well on the same gel receives normal human serum (NHS) as a control. The slides (or film sheets) are placed in the electrophoresis apparatus, connected to the buffer wells by wicks, and then subjected to an electrical field at room temperature. After a predetermined period, or based on movement of a marker dye, the current is stopped, the agar-containing slides are removed from the

electrophoresis apparatus, and narrow troughs are cut parallel to, and on either side of, the path of protein migration (Fig. 159-2). Each trough is filled with selected polyclonal antisera to human immunoglobulins and the slides are kept in a moist environment overnight at room temperature. The antibodies diffuse from the trough into the neutral agar, where they meet their corresponding antigens (i.e., human immunoglobulins or other serum proteins) that have migrated to various points in the agar during the electrophoretic phase of the procedure. When antigen and antibody meet, a smooth curvilinear arc of precipitation is formed (Fig. 159-2). This arc of precipitation demonstrates both the presence and the electrophoretic dispersion of the protein recognized by the antiserum. The intensity and position of the arc also provide a semiquantitative estimate of the concentration of that protein present in the patients serum. Thus, IEP is a two-phase procedure: electrophoretic separation and immunoprecipitation. In common laboratory practice, the chief diagnostic interest concerns the immunoglobulins; accordingly, polyclonal anti-, anti-, anti-, anti-, and anti- antibodies are routinely employed to develop such precipitin arcs.

Figure 159-2 displays an IEP pattern of a serum with a large monoclonal IgG spike (lower patterns in Fig. 159-2A and Fig. 159-2B).

Figure 159-2 IEP analysis of a serum containing a large IgG- myeloma protein. For the initial electrophoretic separation in the agar gel, the upper small wells in each pairing received normal serum and the lower wells received the patients serum. At the conclusion of the electrophoretic separation, monospecific antisera to **(A)** human -chains (IgG-specific), **(B)** -chains, and **(C)** -chains were added to the longitudinal troughs. For ease of illustration, results with antisera of other specificities are not shown. Arrowheads point to the abnormal bowing in the IgG and -arcs that indicates the presence of an IgG- monoclonal protein.

The key diagnostic feature is the localized bulge or bowing in both the IgG arc and the arc. Note that these two arcs show bowing in precisely the same electrophoretic position, i.e., the abnormality in the IgG arc and in the arc is attributable to the same molecule. The bulge or bowing in these IgG and arcs results from an extremely high concentration of identical molecules having the same net charge migrating to one highly localized point during the electrophoretic separation; they then diffuse as a molecular cohort into the oncoming anti- or anti- antibodies from the troughs. The bowing or displacement of the IgG and arcs toward the antibody source (lower patterns in Fig. 159-2A and Fig. 159-2B) results from the shift in the equilibrium point for antigen-antibody precipitation. Antigen excess (i.e., the high concentration of monoclonal IgG) drives the region of optimal precipitation toward the antibody trough. In contrast, the IgG and precipitin arcs produced by normal serum are smooth, continuous curves without localized bowing or displacement (Fig. 159-2 , upper patterns). Note that the polydisperse precipitin arc produced by anti- antibody with this myeloma serum is significantly weaker than that obtained with normal serum (Fig. 159-2C). This indicates a depressed level of type polyclonal immunoglobulins in the serum containing the IgG spike. Such reciprocal depression of normal immunoglobulin concentrations is common in advanced myeloma.

IgA paraproteins typically have a fast - or -globulin mobility. This is also true of some IgM paraproteins, as well as the rarely encountered IgD or IgE paraproteins. However, some IgG paraproteins also migrate in this area.

The use of IEP to demonstrate type Bence Jones protein in concentrated urine is demonstrated in Figure 159-3 . It should

Figure 159-3 IEP of 50-fold concentrated urine. Each of the upper wells contained normal serum; each lower well contained the concentrated urine of the patient. Trough **(A)** received polyspecific antiserum to whole human serum; trough **(B)**, anti-; trough **(C)**, anti-. Note the clear bowing or deviation of the precipitin arc formed by anti- but the smooth, polydisperse appearance of the arc formed by anti-. This urine sample contains -type BJP.

be borne in mind that 50- to 200-fold concentrated urine from healthy individuals often displays free polyclonal light chains (both type and type) by either IEP or IFE. Some myeloma patients have both a serum paraprotein and urinary BJP. In such cases, the BJP often has a greater net negative charge (faster mobility) than the intact monoclonal immunoglobulin.

IEP (or IFE) also provides information on whether the normal immunoglobulins are depressed. In advanced myeloma, with a large monoclonal spike or with urinary BJP alone, it is common to observe depression of normal background immunoglobulin. When severe, this depression of normal polyclonal immunoglobulin may be associated with an increased clinical risk of bacterial infection. Moreover, in the absence of a detectable serum paraprotein, hypogammaglobulinemia in an older patient may be an important clue to the presence of Bence Jones myeloma, in which case IEP or IFE of urine should provide decisive evidence.

Immunofixation Electrophoresis (IFE)

Although IEP has been a major asset for the detection and isotyping of monoclonal proteins, the more recently developed technique of IFE [3] is even more sensitive in identifying monoclonal immunoglobulins or light chains in very low concentrations in serum or urine. Thus, IFE has emerged in many clinical laboratories as the dominant immunospecific electrophoretic procedure employed. Two situations in which IFE surpasses IEP warrant comment. In the presence of a normal or increased background of polyclonal immunoglobulins, IEP may fail to detect a very small monoclonal immunoglobulin, because the background immunoglobulins form unusually strong precipitin arcs within which it is difficult for a small quantity of paraprotein to produce a clear-cut deviation or bulge. Another problem occasionally encountered with IEP in this same setting is unsuccessful light chain typing of a monoclonal IgM protein, because lower molecular weight and faster diffusing IgG and IgA molecules may preemptively bind the available anti- or anti- antibodies before the more slowly diffusing, high molecular weight IgM proteins can make contact. IFE is usually successful in these situations. IFE is, however, technically more demanding and labor-intensive than IEP. IFE has the added advantage of being ready for interpretation on the same day as the electrophoretic run, and it requires less experience to interpret than IEP.

Like IEP, IFE employs a combination of zone electrophoresis and immunoprecipitation with specific antisera. IFE begins with electrophoretic separation of suitably diluted serum or urine in 1 percent agarose gel (in 50 mM barbital buffer, pH 8.6). The agarose gel is applied to the surface of the same 85 × 100 mm Gel Bond clear plastic film sheets described in the discussion of IEP. The electrophoretic separation is performed on a cooling plate connected to a recirculating, temperature-controlled water bath at 515°C (we favor 13°C). Immediately after the electrophoretic run, precut strips of cellulose acetate or lens paper, each soaked in a monospecific antiserum (e.g., anti-, anti-, anti-, anti-, or anti-), are applied to the gel surface overlying individual electrophoretic lanes. The antibodies are allowed to diffuse (for approximately 10 min.) from the strips into the gel, where they encounter and precipitate with their respective immunoglobulin antigens. The strips are removed and the agarose gel (on its plastic support) is then washed to remove all proteins that were not precipitated by a specific antibody. The final steps involve drying the gel on the plastic sheet, and staining with a protein dye, such as Coomassie blue or amido black, to reveal the bands of antibody-precipitated immunoglobulins.

Figure 159-4 , demonstrates an IFE pattern for normal serum, including both a Coomassie blue stained agarose zone electrophoresis

Figure 159-4 IFE applied to normal serum. The upper pattern (track 1) represents zone electrophoresis in which Coomassie blue dye was applied to a strip cut from the plate after electrophoresis, without addition of antiserum. The major bands (from right to left) are albumin, $_1$ -antitrypsin, haptoglobin, transferrin, C3, and -globulin (with its normal polydisperse pattern). Below the zone electrophoresis strip, each horizontal track represents concurrently assayed immunoprecipitation (IFE) after overlay with antisera as follows: track 2, anti-IgG; track 3, anti-IgA; track 4, anti-IgM; track 5, anti-; track 6, anti-. The small daggers indicate the point of application of the serum sample; this process commonly leaves a narrow stripe that must not be confused with a monoclonal band.

with no antiserum added (lane 1) and the immunofixation patterns produced by each of five specific antisera (lanes 26). All such patterns are polydisperse. Figures 159-5 and 159-6 depict sera with small monoclonal IgG or IgM bands, respectively, which were not clearly resolved by IEP. Fig. 159-7 presents the serum IFE of a patient with IgD myeloma that had been classified, at another hospital, as Bence Jones myeloma because anti-IgD had not been tested. The patient has both an intact IgD paraprotein and free type light chains. (Virtually all IgD myelomas are type .) Similarly, IFE is even more sensitive than IEP in detecting very low concentrations of monoclonal light chains in concentrated urine.

Despite its superior sensitivity for detecting monoclonal immunoglobulins at low concentrations, IFE does have some pitfalls. Optimal dilution of the serum or urine sample is essential. Too heavy a protein input can result in obscuring monoclonal bands within the darkly staining background. Overdilution may result in loss of a potentially detectable monoclonal immunoglobulin. If the patients serum concentrations of IgG, IgA, and IgM are known, that is helpful.

IFE is so sensitive that one must be concerned about overdiagnosis of monoclonal disorders and the potential to set in motion an expensive, anxiety-producing clinical workup. For example, monoclonal gammopathies of undetermined significance (MGUS), [4] which are increasingly encountered in the seventh, eighth, and ninth decades of life, are detected by IFE with far greater frequency than by more traditional SPEP or IEP. Clonally restricted light chains may also be detected in urine in the absence of other evidence for a plasma cell dyscrasia. [5] Moreover, using IFE, one, or even several, faint but reproducible electrophoretically restricted bands are sometimes found in a strongly polyclonal hypergammaglobulinemic serum. Such oligoclonal bands are often undetectable by conventional SPEP or even by repeated careful IEP. This type of oligoclonal pattern in serum has been seen in acute and chronic infections, in rheumatologic diseases, in acquired immunodeficiency syndrome or asymptomatic human immunodeficiency virus infection, in chronic liver disease, in carcinoma, or in severe drug hypersensitivity. [6] [7] [8] [9] These oligoclonal bands may be undetectable when the patients serum is reexamined several months later, and thus may represent transient, accelerated immunoglobulin synthesis by vigorously expanded but nonmalignant clone(s) of antibody-forming cells stimulated by the acute illness or active flare. In other instances, such very weak monoclonal bands persist without change over time, and should be considered a variant

Figure 159-5 IFE demonstrating a very small IgG- monoclonal protein. The upper segment is a stained zone electrophoresis of the patients serum (no antibody was added). The other tracks, in descending order, were developed as follows: track 2, anti-IgG; track 3, anti-IgA; track 4, anti-IgM; tracks 5 and 6, two different anti-- sera; track 7, anti-; track 8, anti-IgG. The arrowhead indicates the position of the monoclonal IgG- band. The small daggers mark the point of serum application.

Figure 159-6 IFE demonstrating a subtle IgM- monoclonal protein (arrowhead). Upper track: agarose zone electrophoresis. Other tracks, in descending order, were developed with anti-IgG (track 2), anti-IgA (track 3), anti-IgM (track 4), anti- (track 5), anti- (track 6), repeat anti-IgM (track 7), and repeat anti- (track 8).

form of monoclonal gammopathy of undetermined significance.

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OVERVIEW OF ELECTROPHORETIC PROCEDURES

The complete characterization of immunoglobulins often requires several different laboratory assays. A single method that will cover all situations does not exist. [1] A gradation, or hierarchy, of progressively more sensitive, and usually more expensive, methods has evolved. Some authors do not recommend the very sensitive IFE as an initial screening procedure. [10] We employ zone electrophoresis in agarose, IEP, and IFE in our laboratory. Increasingly, referring physicians, now aware of the greater sensitivity of IFE in detecting monoclonal immunoglobulins, are specifically requesting this procedure as the first-line assay. As a result, a combination of SPEP in agarose and IFE has gradually become the norm of serum or urine evaluation.

This review highlights the relative strengths and shortcomings of these diagnostic tools, thus diminishing some of the mystery in their usage. Good communication between the clinician and diagnostic laboratory personnel should solve most problems.

QUANTITATIVE ASSAY OF IMMUNOGLOBULINS

Concentration ranges for immunoglobulins in adult human serum are included in Table 159-1; age-adjusted values are shown

Figure 159-7 IgD myeloma. Zone electrophoresis of undiluted and 1/3 dilution of patient serum (tracks 1 and 2) revealed two abnormal, restricted bands (arrowheads). Concurrent IFE (tracks 310) was set up as follows: anti-IgG (lane 3), anti-IgA (lane 4), anti-IgM (lane 5), anti- (vs. undiluted serum, lane 6), anti- (vs. 1/3 serum, lane 7), anti-IgD (vs. undiluted serum, lane 8), anti-IgD (vs. 1/3 serum, lane 9), anti- (lane 10). The IFE results demonstrate that the more cathodal paraprotein (seen in lane 1) is IgD- and the more anodal band contains free- light chains (BJP).

in Table 159-2 . The technique of radial immunodiffusion [11] was the dominant assay procedure for quantitative measurement of immunoglobulins for many years. In this technique, polyclonal antiserum specific for IgG, IgA, or IgM is incorporated into melted agarose and the mixture is then allowed to gel on a glass or plastic slide, thus creating an anti-IgG slide,

TABLE 159-2 -- Age-Adjusted Serum Immunoglobulin Levels (mg/dl)[a]

	IgG	IgA	IgM
Birth-4 months	7001,480	02.2	530
46 months	3001,000	382	15109
6 months16 years	5001,550	14232	43240

[a] Adult concentrations are shown in Table 159-1 .

an anti-IgA slide, and an anti-IgM slide. Multiple wells are punched into the gels. The wells are filled with serum from each patient to be assayed, and some wells receive serial dilutions of a standard serum or other preparation containing known concentrations of IgG, IgA, and IgM. From each well, the immunoglobulins diffuse into the surrounding antibody-impregnated agarose. Because excess antigen solubilizes antigen-antibody complexes, the point of equilibrium for maximum immunoprecipitation keeps moving outward as long as there is excess immunoglobulin antigen to solubilize the inner ring of precipitate. Ultimately, at about 24 hours, the system reaches equilibrium and each well shows a halo of surrounding precipitate. The diameter of the halo, measured accurately by special optical magnifiers, is proportional to the quantity of that immunoglobulin in a patients serum. The diameter of the halo is converted to concentration by constructing a standard curve relating halo diameter to several known concentrations of the respective immunoglobulin.

Nephelometry has recently emerged as the dominant technique for immunoglobulin quantitation in large laboratories because the procedure can be automated. [11] This method is possible because an extremely fine suspension of antigen-antibody complexes results when diluted antiserum (e.g., anti-, anti-, or anti-) is added to a diluted human serum. Although invisible to the eye, these complexes produce scattering of a beam of laser light that is passed through the mixture. Such light scattering is readily detected and quantified by a photoelectric sensor. Moreover, either the amount of light scattering (endpoint nephelometry) or the speed at which it develops (rate nephelometry) is proportional to the amount of the specific protein in the serum sample. A standard curve is again constructed (by computer) from the readings given by multiple dilutions of a serum standard whose concentration of each immunoglobulin is known.

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CRYOGLOBULINS

Cryoglobulins are immunoglobulins that form insoluble aggregates at temperatures <37°C. [12] Type I cryoglobulins are of one immunoglobulin class, most commonly IgM or IgG (rarely IgA or free light chains), and are monoclonal. Many examples are associated with myeloma, Waldenströms macroglobulinemia, or lymphoma. In some instances, there is no recognizable underlying disorder (primary or essential type I cryoglobulinemia); such patients may, in time, develop an overt lymphoproliferative disease. The physical form of the type I cryoprecipitation may be amorphous, gelatinous, or crystalline.

Types II and III cryoglobulins are termed mixed in that their formation involves the interaction of two or more classes of immunoglobulin. At least one of the components is a cold-reactive anti-IgG (rheumatoid factor) that combines with the patients polyclonal IgG (or any source of normal human IgG) to form a cryoprecipitate. This cryoprecipitate is a special type of immune complex possessing a definite capacity to activate and consume complement, both in vivo and in the venipuncture tube if cooling occurs. The anti-IgG antibody appears to be the critical component mediating cryoprecipitation. The essential immunochemical difference between types II and III cryoglobulinemia is that in type II the anti-IgG component is monoclonal, most commonly IgM, but in some cases it is IgG or IgA. In type III cryoglobulinemia, the anti-IgG component is polyclonal; it may be polyclonal IgM alone or IgM in combination with IgG or IgA anti-IgG antibodies. Both type II and type III cryoglobulinemia have been associated with hepatitis C infection (i.e., detection of anti-hepatitis C antibody or viral RNA in the serum or cryoprecipitate). [13] Distinguishing between type II and type III is important because patients with type II, but apparently not type III, cryoglobulinemia are at increased risk of concurrent or subsequently developing B-cell lymphoma. Many other instances of type II cryoglobulinemia, in which an underlying disorder is not evident, are termed essential or primary, but B-cell lymphomas have developed in a substantial proportion of such patients. Patients with type III cryoglobulinemia may have a collagen-vascular disease such as systemic lupus erythematosus or a systemic viral infection, but this disorder also occurs in an essential or primary form.

Blood drawn for cryoglobulin assay is ideally transported to the laboratory in a container of warm water, permitted to clot in a 37°C bath, and subjected to centrifugation in a warmed centrifuge. This 37°C serum is placed in the refrigerator in a graduated conical tube for 7296 hours to permit complete precipitation. Many cryoglobulins, especially type I, precipitate far more rapidly. At the end of 7296 hours, the tubes are spun and the packed precipitate volume (cryocrit) is measured in the same way as a hematocrit. Normal sera show no visible precipitate under these conditions.

If the test result is positive, the supernatant serum is removed and the cryoprecipitate is washed thoroughly in cold saline and re-dissolved in a small volume of 0.15 M NaCl for analysis of its composition. For large cryoprecipitates, IEP is effective. For small cryoprecipitates and for distinction between type II and type III cryoglobulins, IFE has been highly effective.

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ACKNOWLEDGMENTS

The author is indebted to Donna R. Graham, Christine C. Waldrop, and Mary Beth Chen of the Strong Memorial Hospital Clinical Immunology Laboratory for valuable contributions in the performance of the assays shown. Thanks are also due to Judith Sterry of the Protein Chemistry Laboratory of Strong Memorial Hospital for providing the SPEP patterns on cellulose acetate. William Smith of the Medical Photo/Illustration Division, University of Rochester, provided expert photographic assistance. Finally, the author acknowledges Laurie Betts for skillful preparation of the manuscript.

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Chapter 160 - Use of Molecular Techniques in the Analysis of Hematologic Diseases

Nancy Berliner

The dramatic advances in recombinant DNA technology since the late 1970s have yielded crucial insights into the processes involved in hematopoietic cell ontogeny. Equally impressive is the effect this technology has had on the diagnostic capabilities for defining, diagnosing, and predicting the natural history of hematologic diseases. The same techniques that have allowed the elucidation of the molecular mechanisms of hematologic abnormalities have provided powerful diagnostic tools, bringing our understanding of basic science into sharply relevant clinical focus. An exhaustive review of the application of these techniques is beyond the scope of this chapter; however, a brief summary of the areas outlined in Table 160-1 provides some insight into the current and evolving uses of DNA technology in clinical hematologic practice.

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PRENATAL DIAGNOSIS

The interplay of basic science and clinical diagnostics has been well demonstrated in the analysis of hemoglobinopathies. The increasing precision with which the molecular lesions of thalassemia and sickle cell disease have been defined is paralleled by the increasing sensitivity and specificity of techniques for antenatal diagnosis of these disorders[1] (see Chap. 157). For the purposes of this discussion, the range of molecular techniques

TABLE 160-1 -- Use of Molecular Techniques for Hematologic Diagnosis

Prenatal diagnosis
Analysis of restriction fragment length polymorphisms linked to diseases of known genetic loci
Direct oligonucleotide screening to detect single base changes defined as the basis of disease
Use of PCR to amplify regions of DNA for further analysis for known or suspected molecular abnormalities in the gene of interest
Diagnosis of lymphoproliferative disease
Defining clonality of poorly defined lymphoproliferative lesions lacking classic histologic features or lymphoid surface markers
Determining extent of disease in patients with lymphoproliferative lesions
Monitoring disease activity in patients undergoing treatment for lymphoproliferative disease
Human leukocyte antigen (HLA) typing for bone marrow transplantation
Analysis of cell origins in bone marrow transplantation recipients
Documentation of engraftment after marrow transplantation
Identification of chimeric states and prediction of graft rejection
Determination of cell implicated in relapse after bone marrow transplantation
Diagnosis of follow-up of diseases associated with known cytogenetic abnormalities
Documenting aberrant translocations (e.g., Philadelphia chromosome-negative chronic myelogenous leukemia
Detection of minimal residual disease by PCR across known chromosomal breakpoints

PCR, polymerase chain reaction.

for antenatal diagnosis of sickle cell disease is described as a paradigm for these developing concepts.

With the exception of lymphocytes, as described later, the DNA content of all cells in a given individual is identical. Consequently, prenatal diagnosis by DNA analysis can be performed on DNA derived from any fetal tissue. The usual source of fetal DNA has been amniotic fluid cells. Obtaining adequate tissue for diagnosis requires 2030 ml of amniotic fluid with growth of the cells in tissue culture before DNA isolation. Amniocentesis is performed at 16 weeks gestation, and analysis may not be completed until 4 weeks later. Chorionic villus sampling has begun to supplant amniocentesis as a source of fetal tissue, because it can be performed at 812 weeks of gestation.[2] [3] With the development of more sensitive methods of DNA diagnosis, notably the polymerase chain reaction (PCR), the cellular material necessary for genetic analysis has been reduced to a very few cells; consequently, it is likely that progressively less invasive procedures for obtaining fetal cells will evolve.

Analysis of Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphisms (RFLPs) have been described in detail in Chapter 1 . Briefly, RFLPs reflect DNA mutations that alter the recognition sites for restriction endonucleases and persist as detectable polymorphisms on Southern blot analysis of genomic DNA. [4] These mutations are usually phenotypically silent, in that they usually lie within introns and do not affect the protein encoded by the gene locus in which they are found. The exception to this rule is discussed in the following paragraph.

Restriction fragment length polymorphisms can be related to diseases caused by single gene defects in several ways (Fig. 160-1). A single base change causing a disease may itself give rise to an altered restriction site. Such a chance occurrence represents the exception to the usual rule that RFLPs arise from phenotypically silent mutations in intron regions; in this instance, finding the RFLP is diagnostic of the mutant allele. It allows the direct identification of both carriers and homozygous people with the disease and is not seen in any person who does not carry the mutant allele. In such a situation, a person can be diagnosed without knowing family inheritance patterns. With respect to the mutation in sickle cell anemia, the single A-to-T change at the DNA level that causes the conversion of glutamic acid to valine at position 6 of the -chain can be detected directly using several restriction enzymes. [5] [6] [7]

In most instances, however, single-base-pair changes do not give rise to convenient restriction enzyme site alterations that allow direct detection of the mutation. Alternatively, RFLPs may arise within the gene of interest independently from the mutation that causes the disease. These reflect common mutations associated with, but not responsible for, the less frequent mutations that alter gene expression and cause disease. In these instances, the RFLP does not directly identify affected people in the general population but is diagnostic in a family known to carry a mutant gene in association with a given RFLP. The informativeness of such an RFLP depends on the origin of the

Figure 160-1 Two different types of restriction fragment length polymorphisms used in the prenatal diagnosis of sickle cell disease. **(A)** The point mutation in the globin gene that gives rise to the sickle mutation alters the recognition site for the restriction endonuclease *Mst*II, giving rise to an altered restriction fragment when DNA containing the sickle gene is digested with that enzyme. **(B)** The sickle cell gene is associated with a DNA polymorphism in the 3 flanking DNA of the globin gene. This polymorphism is clinically silent but can aid in the antenatal diagnosis of sickle cell disease. If carrier parents are heterozygous for the polymorphism in association with the sickle gene (AS in the Southern diagram), fetuses at risk can be identified by homozygous presence of the same band (SS in diagram). M, *Mst*II; H, *Hpa*I; SS, sickle cell disease; AS, sickle trait; AA, normal.

disease-related mutation as a single event within a population at a time after the RFLP arose. Thus, it requires the selection for persistence of the original disease allele in the population rather than its continued renewal by new mutations. Both sickle cell disease and the thalassemias have been shown to have arisen in selected populations in association with such polymorphisms. [8] [9] [10] In stable populations, several such polymorphisms together have been used to define a haplotype with which a given disease is associated. This has been documented by Orkin and associates [11] for several specific -thalassemia mutations.

Finally, an RFLP may not lie within the locus of the gene of interest at all, but may lie close enough to the gene that the two loci rarely, if ever, become separated by recombination. The diagnostic value of the RFLP then depends on statistical analysis of the proximity of the polymorphism to the gene of interest. Diagnosis is based on the analysis of the RFLP patterns of a given affected family to ascertain whether the distribution of alleles allows identification of the RFLP associated with the disease within that individual pedigree. Although this kind of linkage analysis is the least direct application of RFLPs to disease diagnosis, it is the most generalizable to a wide range of diseases. Because the globin gene locus has been fully sequenced and the mutant alleles extensively analyzed, most diagnoses can be made by direct diagnosis using RFLP analysis based on either altered restriction sites at the site of mutation or haplotype analysis, as described earlier. However, this more general application of RFLPs is the basis for gene mapping efforts that promise to allow diagnosis of diseases for which the mutations, and indeed the affected genes themselves, are yet to be identified.

The use of RFLPs for antenatal diagnosis was first undertaken for the diagnosis of hemoglobinopathies. The application of these techniques to the diagnosis of sickle cell disease is diagrammed in Figure 160-1 . Similar techniques have been used in parallel fashion for the prenatal diagnosis of thalassemia. The earliest studies of sickle cell disease were based on a polymorphic site for the enzyme *Hpa*I, which was located 4 kilobases (kb) downstream of the -globin gene. [3] Absence of the restriction site was seen in 87% of sickle genes in the population studied. [8] Consequently, if parents carrying the sickle gene were heterozygous for the absent restriction site, affected fetuses could be identified by homozygous absence of the same site (Fig. 160-1B). Subsequent techniques for diagnosing sickle cell disease exploited the fact that sickle cell disease, unlike thalassemia, arises from a single defined mutation, namely a single A-to-T base substitution. This allowed the identification of restriction enzyme sites that were altered by the mutation, as well as direct analysis of mutant alleles without family studies. Two enzymes that can be used to identify the sickle mutation have been identified. [5] [6] [7] One of these, *Mst*II, results in an easily distinguishable pattern diagnostic of the disease (Fig. 160-1A). This technique has also been used to diagnose other single-base mutations resulting in hemoglobins S, O-Arab, and others. [12] [13] As previously suggested, thalassemia is not readily amenable to this approach because of the heterogeneous lesions giving rise to the disease; however, certain predominant mutations within defined populations have been diagnosed in this manner. [14]

All of these studies are performed using Southern blotting techniques. [15] Genomic DNA from the fetus (and appropriate family members as needed) is digested with a diagnostic restriction enzyme, size fractionated by agarose gel electrophoresis, blotted on nitrocellulose, and hybridized to a radiolabeled probe for the -globin gene. Hybridization techniques are described in Chapter 1 .

Direct Oligonucleotide Screening for Known Point Mutations

The full sequence of the -globin gene is known, and the single base change responsible for the sickle mutation has been established. This knowledge allows more refined techniques for prenatal diagnosis of sickle cell disease, as well as for other hemoglobinopathies in which the responsible mutation is known at the level of the DNA sequence. Molecular hybridization techniques have been developed that can pinpoint single base changes in DNA sequence using short probes of 1520 nucleotides. Under stringent washing conditions, hybridization between such a short probe and the DNA in question requires that the base pairing between the two be exact. Technology for the synthesis of such oligonucleotide probes now exists. Oligonucleotide sequences that are identical to either the native or the mutant gene can be synthesized and the oligonucleotides radiolabeled and hybridized to DNA from the fetus at risk (Fig. 160-2). This DNA can be spotted directly onto nitrocellulose because the hybridization does not require restriction enzyme digestion of the DNA. The consequent rapidity of diagnosis gives this technique its primary advantage over RFLP analysis by Southern blot hybridization. The method has been used successfully for prenatal diagnosis of both sickle cell disease and thalassemias associated with known single-base changes. [16] [17] [18]

Use of PCR in Prenatal Diagnosis

The development of PCR represents one of the most important technical advances in molecular diagnostics. [19] The technique is described elsewhere (see Chap. 1) and is summarized again in Figure 160-3 . Using PCR, prenatal diagnosis is possible with as little as 1 g of DNA. Diagnosis is performed by using oligonucleotides flanking the gene to amplify the DNA of interest from minute quantities of total cellular DNA. The amplified sequences can then be analyzed by any of the techniques described. A common means of diagnosis is to hybridize the amplified DNA to oligonucleotide probes. This is currently the most rapid means of diagnosis of sickle cell disease[20] [21] and thalassemias with known mutations.[22] These techniques can also be invaluable in the rapid analysis of unknown mutations. [22] Alternatively, if a mutation alters a known restriction enzyme site, PCR-amplified DNA can be digested and analyzed by agarose gel electrophoresis.

A fluorescence amplification technique has been developed that permits direct PCR analysis of DNA for known single-base mutations. Oligonucleotide primers containing the normal and mutant sequence are synthesized, and the two primers are differentially labeled with red and green fluorescent tags. These primers are mixed with a common antisense primer, which is left unlabeled. PCR is then performed, and the reaction products are then directly analyzed in a fluorometer for differential fluorescence. [23]

Although the primary focus of this discussion has been on the prenatal diagnosis of hemoglobinopathies, similar techniques are also being used in the diagnosis of hemophilia and other congenital hematopoietic disorders as their molecular origins become better understood. [24] These techniques are not restricted to prenatal diagnosis. For example, PCR for the diagnosis of factor V Leiden has become a routine part of the evaluation of patients with suspected hypercoaguable states. [25]

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DIAGNOSIS OF LYMPHOPROLIFERATIVE DISEASE

As mentioned previously, lymphocytes are a unique exception to the rule that all somatic cells carry identical DNA. Lymphocyte ontogeny is marked by a series of DNA rearrangements and deletions by which the immunoglobulin and T-cell receptor (TCR) gene loci become functional units capable of expression. The details of gene rearrangement and the role it plays in the generation of antigen specificity and immunologic diversity are described in Chapter 15 . The discussion here is limited to a

Figure 160-2 Use of oligonucleotide probes to screen for sickle cell disease. Specific oligonucleotide probes that are homologous to the sequence of the ^{A} and the ^{S} -globin genes are synthesized. Because the oligonucleotides are only 19 nucleotides long, hybridization under appropriately stringent conditions will yield stable hybrids only if the DNA sequence is identical to the probe sequence. The single base change that gives rise to the sickle mutation is sufficient to destabilize the DNA-oligonucleotide duplex and allows the distinction of normal (AA), sickle trait (AS), and sickle cell disease (SS) DNA. After isolation, the DNA is blotted directly onto nitrocellulose without the necessity of restriction enzyme digestion and hybridized to the radiolabeled oligonucleotide probes. The blots are washed at high stringency and autoradiographed. Predicted results are as diagrammed in the lower panel.

description of the direct clinical applications of molecular probes to the investigation of the characteristics and natural history of lymphoproliferative disease.

The use of the Southern blotting technique to elucidate the clonal rearrangements of the immunoglobulin light chain genes is outlined in Figure 160-4 . Similar general principles apply to the rearrangement of the heavy chain genes and the light chain loci, as well as the TCR gene loci. In nonlymphoid cells, all the immunoglobulin and TCR genes are in the germline (unrearranged) state. Southern blot analysis of DNA derived from nonlymphoid cells using a probe for the immunoglobulin heavy chain gene reveals a single band.

The immunoglobulin genes in all B cells, and the TCR genes in all T cells, have undergone somatic rearrangement. Because there is flexibility of the precise site at which the rearrangement of these genes takes place, not all the recombinations maintain a reading frame that allows for successful translation of the mRNA into a functional protein. If a nonfunctional rearrangement takes place on one chromosome, a further rearrangement takes place on the homologous chromosome. Because of the large deletions that take place in the course of recombination, it is not possible for two rearrangements to take place on the same chromosome. Consequently, every lymphoid cell has either one or two rearranged heavy chain genes, and one or two rearranged light chain genes. If Southern blot analysis could be performed on a single cell, each lymphocyte would show either one or two rearranged bands. However, because the DNA from >10,000 cells is required to detect a single-copy gene by Southern blot analysis, these bands will be undetectable in a polyclonal proliferation of lymphocytes. The only detectable band is the germline band contributed by the unrearranged heavy chain genes in the lymphocytes that have rearranged only one of the two heavy chain loci.

In a monoclonal proliferation of B cells, however, all the cells contain an identical pattern of heavy chain rearrangement. Southern blot analysis thus reveals one or two rearranged bands, corresponding to one or two rearranged heavy chain loci.

Defining Clonality of Lymphoproliferative Lesions

The process of malignant transformation freezes a tumor cell population at a given stage of differentiation and gives rise to a proliferation of cells manifesting an identical pattern of DNA rearrangement. Southern blot analysis consequently offers a sensitive means of establishing the clonality and lymphoid origin of cells showing somatic rearrangement of their immunoglobulin or TCR gene loci. Southern blot analysis of DNA from a range of tissues, including lymph nodes, pleural fluid, and peripheral blood, has been used to establish the diagnosis of lymphoma in lesions that lack either definitive histology or surface markers. [25] It has also been used to examine the clonality of poorly defined lymphoid lesions such as T-cell lymphocytosis, [26] [27] [28] angioimmunoblastic lymphadenopathy,[29] and

Figure 160-3 Use of PCR to amplify a portion of DNA for analysis. One microgram of total genomic DNA is denatured at high temperature and annealed to oligonucleotide primers flanking the DNA of interest. By using the temperature-stable *taq* polymerase, the primers are extended to synthesize a copy of the DNA between the two primers. The temperature is then raised to denature the products, and the process is repeated for 30 cycles. The primary product of this reaction is a fragment of DNA bounded by the two oligonucleotide primers (short-short hybrids). By this means, a minute quantity of total DNA may be used to generate specific DNA in large quantity for analysis.

Figure 160-4 Southern blot analysis of light chain rearrangement to establish clonality of populations of B lymphocytes. **(A)** The Southern technique and its predicted results in different cell populations. **(B)** The molecular configuration of the -locus. **(Left)** Genomic DNA, as represented in peripheral blood granulocytes, is analyzed. Because all of these cells retain their immunoglobulin genes in the germline configuration, a single band on Southern blot analysis corresponds to the germline -locus. **(Middle)** Polyclonal lymphocytes also show only a germline band on Southern analysis. However, as shown in part B, this does not show that the cells do not have rearrangement of the -locus. Instead, it demonstrates that there are insufficient cells of any one clone to be detectable as a discrete band on Southern analysis. Because a significant fraction of the cells retain one -locus that is unrearranged, a germline band is detectable. (In normal peripheral lymphocytes, this band also partly reflects T cells, which do not rearrange their immunoglobulin gene loci.) **(Right)** The Southern pattern produced by monoclonal tumor cells bearing a light chain. All the cells of the population have an identical rearrangement of the -genes that will be apparent as a rearranged band on Southern blot analysis. The figure demonstrates the pattern of cells in which one -locus has rearranged. If both chromosomes are rearranged, there will be two rearranged bands, and the germline band will no longer be present.

lymphoproliferative lesions related to the acquired immunodeficiency syndrome. [30]

Gene rearrangement studies must be interpreted in light of the genetic events tied to lymphocyte ontogeny that they reflect. Because a chromosomal locus can rearrange only one time, only one band corresponding to the DNA should be seen on each chromosome. More than two rearranged bands is indicative of an oligoclonal population of cells. In addition, there is a strict hierarchy of recombinatorial events. The immunoglobulin heavy chain locus rearranges before the light chains, and light chains rearrange before the -chains. [31] Similarly, the -chain of the TCR is the first of the TCR loci to rearrange. [32] If a functional rearrangement does not occur in either -locus, and the -locus rearranges successfully, the -regions are often subsequently deleted altogether. Finally, in some lymphoid tumors, immunoglobulin heavy chain rearrangements are found in the company of T-cell -chain rearrangements. Such lineage infidelity has never involved light chain rearrangement and is thought to be a marker of malignant derangement of a very early lymphoid cell.

The sensitivity of gene rearrangement studies allows the detection of very small clonal populations amounting to as little as 1% of the total cellular population studied.[33] The significance of small clonal populations has been debated, and the equation of monoclonality with malignancy has been challenged. [32] For example, patients with angioimmunoblastic lymphadenopathy have been observed to have small clonal populations of B and T cells in lymph nodes and peripheral blood that are frequently transient. It seems likely that this represents temporary overgrowth of small clones of cells secondary to defective immunoregulatory control rather than malignancy.[29]

Determining Extent of Lymphoproliferative Disease

Immunoglobulin and TCR gene rearrangements have been used in the staging of patients with lymphoproliferative disease. Southern blot analysis using probes for these loci has been shown to be sensitive enough to detect clonal populations of approximately 1% of the total cell population studied. This translates to detection of the DNA represented by 10^4 cells.[32] [33] Consequently, it is a highly sensitive means of detecting peripheral blood or bone marrow involvement with lymphoma in patients with known lymphoproliferative disease in whom marrow involvement is insufficient for histologic diagnosis.

Gene rearrangement studies have also been used to validate the use of flow cytometry as a means of detecting peripheral blood involvement with B-cell lymphoma. [34] The test allows cytofluorometric identification of small populations of clonal B lymphocytes by detection of an imbalance in the normal distribution of surface immunofluorescent staining for and light chains. Peripheral blood mononuclear cells are labeled with fluorescent-tagged rabbit heteroantisera to and light chains and then analyzed in a flow cytometer. The success of the technique is based on the empiric observation that a given clone of B cells has a uniform expression of surface light chains, and that the histograms of and light chain distribution in normal polyclonal B cells are identical. The presence of a predominant clone distorts the histogram of the light chain expressed and causes the shift of one curve relative to the other. The difference between the curves may then be analyzed by computer.[35] [36] The sensitivity of the flow cytometric technique allows detection of clonal populations in the 510% range. Gene rearrangement studies have confirmed that the finding of a positive test result correlates with the finding of a circulating population of cells with the same DNA rearrangement as that found in the primary tumor.[32] Although Southern blot analysis is more sensitive than flow cytometry, it appears that the latter, simpler, technique is an extremely effective means of detecting minimal bone marrow involvement with lymphoma.

Monitoring Disease Activity in Treated Patients

The sensitivity of immunoglobulin and TCR gene rearrangement studies makes them an excellent method for detecting minimal residual involvement of the bone marrow in patients undergoing chemotherapy or bone marrow transplantation for the treatment of lymphoid malignancy. In a study of children treated with chemotherapy for acute lymphocytic leukemia, this technique allowed detection of an estimated 1 in 5001,000 leukemic cells in remission marrows. [37] [38] Subsequent studies have suggested that detection of any number of residual leukemic cells by this technique is highly predictive of relapse, although negative studies do not guarantee long-term remission.[38] [39]

Use of PCR Analysis of Immunoglobulin and TCR Loci in Lymphoproliferative Disease

Because PCR can potentially amplify the DNA of a single gene from a single cell to a detectable level, it can detect minimal involvement with cells carrying a characteristic gene rearrangement well below the limits of detectability by any other technique. However, PCR for diagnosis of lymphoproliferative disease is hampered because the sequence of rearranged immunoglobulin or TCR loci in a given lymphoma is specific to that patients tumor and theoretically requires design of patient-specific primers to amplify tumor DNA. Modifications of the PCR technique have allowed for the design of primers that can monitor minimal residual disease by PCR. Two major techniques have been used. The first is to design two consensus primers, one to conserved sequences within the flanking DNA of the V region, and the other to a similarly conserved sequence flanking the J region; these regions lie too far apart in unrearranged DNA to allow efficient PCR. Only rearranged immunoglobulin or TCR loci will allow amplification of a distinct fragment; clonal populations yield an amplified species by this technique. PCR is then used to detect a characteristic footprint of the rearranged DNA within the tumor that can then be followed as a marker for residual disease. [40] [41] [42]

A more sensitive technique has been developed by which PCR fragments generated using consensus sequences are then subcloned and sequenced, with subsequent synthesis of patient-specific oligonucleotide probes, termed *allele-specific oligonucleotides.* PCR is then performed using one consensus primer and one allele-specific oligonucleotide; this has in fact resulted in the detection of malignant cells at the theoretic detection limits of PCR, namely 1 in 10 ^{5} 10^6 cells.[43]

Use of PCR of the immunoglobulin and TCR loci for detection of residual lymphoproliferative disease has proved highly predictive of relapse in patients with acute lymphocytic leukemia.[44] [45] However, absence of detectable diagnostic fragments by either Southern blot analysis or PCR has not been shown to guarantee ongoing remission. In some patients with previously normal results, positive PCR studies were seen to develop before relapse. [46]

The negative prognostic significance of detection of residual disease by these techniques contrasts with the results found for detection of residual *bcr-abl* transcripts after bone marrow transplantation for chronic myelogenous leukemia (CML), as is discussed in greater detail later.

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HUMAN LEUKOCYTE ANTIGEN TYPING FOR BONE MARROW TRANSPLANTATION

Molecular techniques are of growing importance in the analysis of potential donors for bone marrow transplantation. Serologic human leukocyte antigen (HLA) testing identifies large groups of cross-reactive antigens. It has been found that genotypic incompatibility results in acute graft-versus-host disease, despite

serologic identity.[47] When evaluating sibling donors, serologic testing and mixed lymphocyte culture (MLC) assays are usually adequate to confirm the haplotype identity of the donor and recipient. However, with the advent of unrelated donor transplantation, it has become increasingly important to guarantee genotypic identity by molecular techniques. In unrelated donor transplantation, MLC reactivity has been shown to be a very poor predictor of graft-versus-host-disease. [48] Most laboratories have abandoned MLC testing and are routinely using PCR amplification of DNA followed by hybridization to sequence-specific oligonucleotide probes to establish specific HLA-DR antigen typing.[49] [50] [51] Similar assays of HLA class I loci are also being developed and are anticipated to replace serologic testing in the future.[52]

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ANALYSIS OF CELL ORIGINS IN BONE MARROW TRANSPLANT RECIPIENTS

Restriction fragment length polymorphism analysis is a powerful technique for analyzing the engraftment of patients from their donors after bone marrow transplantation. Southern blot analysis allows the laboratorian to distinguish virtually any two individuals by the use of any of the growing panel of defined DNA polymorphisms.[53] Peripheral blood from the patient and the donor can be analyzed before transplantation to define polymorphic sites at which they differ. Alternatively, after transplantation, DNA from a nonhematopoietic tissue from the patient (i.e., skin) can be compared with DNA from the donor.

This approach has been used to document engraftment, graft failure, and graft loss. It has also been used to document hematopoietic chimerism in a patient with severe combined immunodeficiency, in whom the T cells were shown to be of donor origin and the B cells and granulocytes were shown to be of patient origin. [54] [55] Similar studies can also be used to determine the cell implicated in the relapse of leukemia after transplantation. [56] [57]

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DIAGNOSIS AND FOLLOW-UP OF DISEASES ASSOCIATED WITH KNOWN CYTOGENETIC ABNORMALITIES

Several hematologic malignancies are associated with characteristic chromosomal translocations that mark the abnormal cell population. The Philadelphia chromosome in CML and the t(14;18) translocation in follicular lymphoma are two examples of such translocations in which the genes involved in the chromosomal breakpoint have been defined. Southern blot analysis, using probes for the genes involved in the translocation event, permits the detection of a rearranged band in DNA from cells carrying the translation. In a manner similar to that used for the detection of small populations of lymphoma cells, this method can be used to detect small residual populations of malignant cells after treatment with chemotherapy or bone marrow transplantation.

The advent of PCR has rendered this methodology much more powerful. By means of probes for the *bcr-abl* translocation, this technique has been used to demonstrate residual CML cells in patients after chemotherapy and bone marrow transplantation. [58] [59] Similarly, probes of t(14;18) have been used to detect residual follicular lymphoma cells in patients apparently in remission in terms of histologic and conventional Southern blot analysis. [60] With respect to t(14;18), detection of residual disease was highly predictive of relapse in one study. [61] However, in CML, *bcr-abl* transcripts were detectable by PCR in almost all patients within the first 6 months after bone marrow transplantation. This finding does not appear to be predictive of imminent cytogenetic or hematologic relapse and may support the hypothesis that the cure of CML depends on the graft-versus-leukemia effect, at least in some patients. [62] The long-term prognostic significance of repeated amplifiable *bcr-abl* transcripts in patients in remission remains to be determined. Similarly, in M2 acute myelogenous leukemia (AML) associated with the t(8;21) translocation, there is a high rate of detectable fusion *AML1/ETO* transcripts from the translocation seen in patients in long-term remission. [63] [64] This is not associated with a threat of imminent relapse. In one study, patients after allogeneic transplantation for AML associated with this cytogenetic abnormality did not have detectable transcripts.[64] However, because most patients with this subtype of AML do not require bone marrow transplantation, [65] it is important to note that there is no established predictive value for detectable *AML1/ETO* fusion transcripts after successful chemotherapy for this disease. With this caveat in mind, PCR promises to be an extremely important tool for following the natural history and predicting the course of these diseases.

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APPENDIX: Normal Blood Values: Selected Reference Values for Neonatal, Pediatric, and Adult Populations

Sharon M. Geaghan

TABLE 1 -- Reference Ranges for Leukocyte Counts in Children and Adults[a]

Age	Total Leukocytes		Neutrophils			Lymphocytes			Monocytes		Eosinophils	
	Mean	Range	Mean	Range	%	Mean	Range	%	Mean	%	Mean	%
Birth	18.1	9.030.0	11.0	6.026.0	61	5.5	2.011.0	31	1.1	6	0.4	2
12 hr	22.8	13.038.0	15.5	6.028.0	68	5.5	2.011.0	24	1.2	5	0.5	2
24 hr	18.9	9.434.0	11.5	5.021.0	61	5.8	2.011.5	31	1.1	6	0.5	2
1 wk	12.2	5.021.0	5.5	1.510.0	45	5.0	2.017.0	41	1.1	9	0.5	4
2 wk	11.4	5.020.0	4.5	1.09.5	40	5.5	2.017.0	48	1.0	9	0.4	3
1 mo	10.8	5.019.5	3.8	1.09.0	35	6.0	2.516.5	56	0.7	7	0.3	3
6 mo	11.9	6.017.5	3.8	1.08.5	32	7.3	4.013.5	61	0.6	5	0.3	3
1 yr	11.4	6.017.5	3.5	1.58.5	31	7.0	4.010.5	61	0.6	5	0.3	3
2 yr	10.6	6.017.0	3.5	1.58.5	33	6.3	3.09.5	59	0.5	5	0.3	3
4 yr	9.1	5.515.5	3.8	1.58.5	42	4.5	2.08.0	50	0.5	5	0.3	3
6 yr	8.5	5.014.5	4.3	1.58.0	51	3.5	1.57.0	42	0.4	5	0.2	3
8 yr	8.3	4.513.5	4.4	1.58.0	53	3.3	1.56.8	39	0.4	4	0.2	2
10 yr	8.1	4.513.5	4.4	1.88.0	54	3.1	1.56.5	38	0.4	4	0.2	2
16 yr	7.8	4.513.0	4.4	1.88.0	57	2.8	1.25.2	35	0.4	5	0.2	3
21 yr	7.4	4.511.0	4.4	1.87.7	59	2.5	1.04.8	34	0.3	4	0.2	3

From Dallman PR: Blood-forming tissues. In Rudolph AM (ed): Pediatrics. 16th ed. Appleton-Century-Crofts, New York, 1977, p. 1178, with permission.

[a]Numbers of leukocytes are in thousands per mm^3 , ranges are estimates of 95% confidence limits, and percentages refer to differential counts. Neutrophils include band cells at all ages and a small number of metamyelocytes and myelocytes in the first few days of life.

TABLE 2 -- Reference Hematologic Values in Children and Adults[a]

Age	Hemoglobin (g/dl)		Hematocrit (%)		Red Cell Count (10^{12} /L)		MCV (fl)		MCH (pg)		MCHC (g/dl)	
	Mean	2 SD	Mean	2 SD	Mean	2 SD	Mean	2 SD	Mean	2 SD	Mean	2 SD
Birth (cord blood)	16.5	13.5	51	42	4.7	3.9	108	98	34	31	33	30
13 days (capillary)	18.5	14.5	56	45	5.3	4.0	108	95	34	31	33	29
1 wk	17.5	13.5	54	42	5.1	3.9	107	88	34	28	33	28
2 wk	16.5	12.5	51	39	4.9	3.6	105	86	34	28	33	28
1 mo	14.0	10.0	43	31	4.2	3.0	104	85	34	28	33	29
2 mo	11.5	9.0	35	28	3.8	2.7	96	77	30	26	33	29
36 mo	11.5	9.5	35	29	3.8	3.1	91	74	30	25	33	30
0.52 yr	12.0	10.5	36	33	4.5	3.7	78	70	27	23	33	30
26 yr	12.5	11.5	37	34	4.6	3.9	81	75	27	24	34	31
612 yr	13.5	11.5	40	35	4.6	4.0	86	77	29	25	34	31
1218 yr												
Female	14.0	12.0	41	36	4.6	4.1	90	78	30	25	34	31
Male	14.5	13.0	43	37	4.9	4.5	88	78	30	25	34	31
1849 yr												
Female	14.0	12.0	41	36	4.6	4.0	90	80	30	26	34	31
Male	15.5	13.5	47	41	5.2	4.5	90	80	30	26	34	31

From Dallman PR: Blood-forming tissues. In Rudolph A (ed): Pediatrics. 16th ed. Appleton-Century-Crofts, New York, 1977, p. 1111, with permission.

[a]These data have been compiled from several sources. Emphasis is given to studies employing electronic counters and to the selection of populations that are likely to exclude individuals with iron deficiency. The mean ±2 SD can be expected to include 95% of the observations in a normal population.

TABLE 3 -- Reticulocyte Counts in Infants and Children by Flow Cytometry

Age	n	Percentage	Absolute Count (10^9 /L)
014 days [a]	21	5.33 + 1.99	318.44 + 50.04
		(1.61/8.28)	(238.9/404.1)
14 days1 yr [a]	32	2.03 + 0.52	89.00 + 22.94
		(1.09/3.00)	(45.8/137.8)
13 yr [a]	63	2.01 + 0.72	88.38 + 20.49
		(1.11/5.00)	(50.2/141.0)
38 yr [a]	60	2.19 + 0.66	98.83 + 27.97
		(0.95/4.03)	(45.4/163.1)
812 yr [a]	18	2.07 + 0.78	90.20 + 27.16
		(1.18/3.78)	(54.8/154.4)
12adult [a]	14	2.25 + 0.99	91.62 + 24.80
		(1.32/4.91)	(58.6/146.2)

Age	n	Percentage	Absolute Count (10^4 / l)
Term newborns [b]	150	2.44 + 1.15	7.42 + 2.50
		(1.1/5.5)	(4.9/15.0)
Preterm newborns [b]	30	2.55 + 1.01	7.18 + 2.88
		(0.9/6.5)	(4.0/17.1)
1 wk16 yr [b]	750	1.16 + 0.39	5.87 + 1.23
		(0.6/1.9)	(5.0/6.5)

[a]Data from 188 pediatric outpatients (018 years of age) for whom all hematologic parameters are within age-appropriate reference ranges. Unpublished data from Lucile Packard Childrens Hospital at Stanford. Analysis was performed on Beckton Dickenson FACSCAlibur flow cytometer. Results are expressed as mean + 1 SD (min/max).
[b]Data from Herkner KR, Bock A et al: Reticulocyte counting and maturity grading in pediatrics using the Sysmex R-1000 automated reticulocyte analyzer. Sysmex J Int 2:4, 1992. Results are expressed as mean + 1 SD (min/max).

TABLE 4 -- Estimated Blood Volumes

Age	Plasma Volume (ml/kg) (PV)	Red Cell Mass (ml/kg) (RCM)	Total Blood Volume (ml/kg)	
			From PV	From RCM
Newborn	41.3	43.1	82.1	86.1
	46.0		78.0	84.7
17 days	5154	37.9	8286	77.8
112 mo	46.1	25.5	78.1	72.8
13 yr	44.4	24.9	73.8	69.1
	47.2		81.8	
46 yr	48.5	25.5	80.0	67.5
	49.6		85.6	
79 yr	52.2	24.3	87.6	67.5
	49.0		86.1	
1012 yr	51.9	26.3	87.6	67.4
	46.2		83.2	
1315 yr	51.2		88.3	
1618 yr	50.1		90.2	
Adults	3944	2530	6888	5575

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TABLE 5 -- Red Cell Enzyme Activity in Adults and Term Infants[a]

Enzyme	Adults (n = 20)	Infants (n = 10)
Hexokinase	12.9 ± 2.1	34.0 ± 6.0
Phosphoglucose isomerase	406 ± 37	560 ± 112
Phosphofructokinase	148 ± 24.5	84.5 ± 24
Aldolase	24.5 ± 3.7	42.0 ± 10.0
Glyceraldehyde-3-phosphate dehydrogenase	885 ± 127	884 ± 245

Triosephosphate isomerase	26,323 ± 3,240	29,111 ± 4,100
Phosphoglycerate kinase	2,795 ± 144	3,926 ± 528
Phosphoglycerate mutase	751 ± 99	1,049 ± 160
Enolase	252 ± 54	517 ± 121
Pyruvate kinase	179 ± 16	256 ± 50
Lactic dehydrogenase	2,033 ± 287	2,756 ± 425
Glucose-6-phosphate dehydrogenase	215 ± 18	328 ± 40

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[a]Infant samples were obtained from newborns weighing more than 2,800 g whose gestational age was 39 weeks or greater. Blood was drawn within 24 hours of birth. All the newborns were clinically healthy. Adult samples were obtained from healthy, normal volunteers. Units per 100 ml of RBCs.

TABLE 6 -- Red Cell Glycolytic Intermediate Metabolites in Normal Adults, Term Infants, and Premature Infants[a]

Metabolite	Normal Adults (n = 10)	Term Infants (n = 10)	Premature Infants (n = 11)
Glucose-6-phosphate	24.8 ± 9.8	45.2 ± 8.7	66.8 ± 34.8
Fructose-6-phosphate	5.4 ± 1.0	9.9 ± 2.3	20.5 ± 8.9
Fructose-1,6-diphosphate	4.6 ± 1.0	3.8 ± 0.7	3.6 ± 0.8
Dihydroxyacetone phosphate	4.9 ± 3.5	11.9 ± 5.0	18.6 ± 10.7
Glyceraldehyde-3-phosphate	2.6 ± 0.7	1.9 ± 1.6	6.5 ± 3.2
3-Phosphoglycerate	61.6 ± 12.4	58.2 ± 14.4	47.5 ± 14.2
2-Phosphoglycerate	4.3 ± 1.8	4.9 ± 1.6	4.4 ± 2.5
Phosphoenolpyruvate	8.8 ± 2.6	7.6 ± 2.9	7.4 ± 3.0
Pyruvate	73.5 ± 33.1	70.4 ± 32.3	78.4 ± 4.15
2,3-Diphosphoglycerate	4,423 ± 1,907	3,609 ± 800	3,152 ± 2,133

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[a]Premature infants had birth weight below 2,200 g and gestational age of <37 weeks, and were clinically healthy at the time of investigation.

TABLE 7 -- Reference Values for Hemoglobin F and Hemoglobin A_2 in the First Two Years of Life

Age	Hb F (%)[a]		Hb A_2 (%)[b]	
	Mean	± 2 SD	Mean	± 2 SD
17 days	75	6180		
2 wk	75	6681		
1 mo	60	4667	0.8	0.41.3
2 mo	46	2961	1.3	0.41.9
3 mo	27	1556	2.2	1.03.0
4 mo	18	9.429	2.4	2.02.8
5 mo	10	2.322	2.5	2.13.1
6 mo	7	2.713	2.5	2.13.1
8 mo	5	2.312	2.7	1.93.5
10 mo	2.1	1.53.5	2.7	2.03.3
12 mo	2.0	1.35.0	2.7	2.03.3
1316 mo	0.6	0.21.0	2.6	1.63.3
1720 mo	0.6	0.21.0	2.9	2.13.5
2124 mo	0.6	0.21.0	2.8	2.13.5

[a]Schroter W, Nafz C: Diagnostic significance of hemoglobin F and A $_2$ levels in homo- and heterozygous -thalassemia during infancy. Helv Paediatr Acta 36:519, 1981, with permission.

[b]Metaxotou-Mavromati AD, Antonopoulou HK, Laskari SS et al: Developmental changes in hemoglobin F levels during the first two years of life in normal and heterozygous -thalassemia infants. Pediatrics 69:738, 1982, with permission.

TABLE 8 -- Serum, Ferritin, Iron, Total Iron-Binding Capacity, and Transferrin Saturation in Children and Adults

Age	Male Subjects		Female Subjects	
Ferritin	**ng/ml**	**g/L**	**ng/ml**	**g/L**
130 days[a]	6400	6400	6515	6515
16 mo[a]	6410	6410	6340	6340
712 mo[a]	680	680	645	645
15 yr[b,c]	624	660	624	660

69 yr [b] [c]	1055	1055	1055	1055
1014 yr [b]	2370	2370	640	640
1419 yr [b]	2370	2370	640	640

Iron[d]	**g/dl**	**mol/L**	**g/dl**	**mol/L**
130 days	32112	5.720.0	29127	5.222.7
31365 days	27109	4.819.5	25126	4.522.6
13 yr	2991	5.216.3	25101	4.518.1
46 yr	25115	4.520.6	2893	5.016.7
79 yr	2796	4.817.2	30104	5.418.6
1012 yr	28112	5.020.0	32104	5.718.6
1315 yr	26110	4.719.7	30109	5.419.5
1618 yr	27138	4.824.7	33102	5.918.3

Iron-Binding Capacity[d]	**g/dl**	**mol/L**	**g/dl**	**mol/L**
130 days	94232	16.841.5	94236	16.842.2
31182 days	116322	20.857.6	89311	15.955.7
182365 days	176384	31.568.7	138365	24.765.3
13 yr	204382	36.568.3	184377	32.967.5
46 yr	180390	32.269.8	162352	29.063.0
79 yr	183369	32.866.1	167336	29.960.1
1012 yr	173356	31.063.7	198383	35.468.6
1315 yr	193377	34.567.5	169358	30.364.1
1618 yr	174351	31.162.8	194372	34.766.6

Transferrin Saturation[b] [c] [e]		
15 yr	0.070.44	0.070.44
69 yr	0.170.42	0.170.42
1014 yr	0.110.36	0.020.40
1419 yr	0.060.33	0.060.33
Transferrin[d]	**g/L**	**g/L**
130 days	0.972.05	0.922.08
31182 days	1.063.25	1.283.09
182365 days	1.783.57	1.463.64
13 yr	1.963.65	1.493.82
46 yr	2.023.50	1.743.99
79 yr	1.493.53	1.863.68
1012 yr	1.733.80	1.853.77
1315 yr	1.713.74	1.933.91
1618 yr	1.943.48	1.814.16

[a] Soldin SJ, Morales A et al: Pediatric reference ranges on the Abbott Imx for FSH, LH, prolactin, TSH, T_4, T_3, free T_4, free T_3, T-uptake, IgE, and ferritin. Clin Biochem 28:603, 1995. Study was based on hospitalized patients. Values represent 2.5 and 97.5th percentiles.
[b] Lockitch G, Halstead AC et al: Age- and sex-specific pediatric reference intervals for zinc, copper, selenium, iron, vitamins A and E and related proteins. Clin Chem 34:1625, 1988. Study was based on healthy children; children with anemia, microcytosis, and thalassemia syndromes were excluded. Values represent the 0.025 and 0.975 fractiles.
[c] No significant differences between males and females; range derived from combined data.
[d] Soldin SJ, Brugnara C et al: Pediatric Reference Ranges. 2nd ed. AACC Press, Washington, DC, 1997, pp. 100, 101, 143.
[e] Transferrin saturation calculated from iron (mol/L)/TIBC. Note that the lower reference limits for serum iron and transferrin saturation in this study are below the limits used to define acceptable levels for nutritional health for these two analytes (see ONeal RM, Johnson OC, Schaefer AE: Guidelines for the classification and interpretation of group blood and urine data collected as part of the National Nutritional Survey. Pediatr Res 4:103, 1970).

TABLE 9 -- Reference Ranges for Serum Haptoglobin by Age

	Hemoglobin-Binding Capacity (mg/dl)	
	Mean	**Range (95%)**
Cord blood	0	0
17 days	10	041
14 wk	28	045
13 mo	59	4195
36 mo	91	64134
612 mo	115	43160
15 yr	109	51160
510 yr	107	62186
>10 yr	110	41165

From Kalil M, Badr-El-Din MK, Kassem AS: Haptoglobin level in normal infants and children. Alex Med J 13:1, 1967, with permission.

TABLE 10 -- Plasma Levels of Folic Acid and Vitamin B_{12} in Children

	Males	Females
Folic Acid	**nmol/L**	**nmol/L**
01 yr	16.350.8	14.351.5
23 yr	5.734.0	3.935.6
46 yr	1.129.4	6.131.9
79 yr	5.227.0	5.430.4
1012 yr	3.424.5	2.323.1
1318 yr	2.719.9	2.716.3
Vitamin B_{12}	**pmol/L**	**pmol/L**
01 yr	216891	1681,117
23 yr	195897	307892
46 yr	181795	2311,038
79 yr	200863	182866
1012 yr	135803	145752
1318 yr	158638	134605

From Hicks JM, Cook J et al: Vitamin B_{12} and folate: pediatric reference ranges. Arch Pathol Lab Med 117:704, 1993, with permission. Data were collected from hospitalized patients; 2.597.5th percentile values adapted from the Hoffman technique. Specimens from >100 patients in each age group were analyzed by radioimmunoassay.

TABLE 11 -- Plasma Levels of Vitamin E (-Tocopherol) in Children

Age	Males and Females	
	mol/L	**g/ml**
Prematures [a]	18	0.53.5
Full term [a]	28	1.03.5
25 mo [a]	514	2.06.0
624 mo [a]	819	3.58.0
212 yr [a]	1321	5.59.0
16 yr [b]	721	3.09.0
712 yr [b]	1021	4.09.0
1319 yr [b]	1324	6.010.1

[a]Meites S (ed): Pediatric Clinical Chemistry. 3rd ed. AACC Press, Washington, DC, 1989, pp. 295296.
[b]Lockitch G, Halstead AC et al: Age- and sex-specific pediatric reference intervals for zinc, copper, selenium, iron, vitamins A and E and related proteins. Clin Chem 34:1625, 1988. Study was based on healthy children; values represent the 0.025 and 0.975 fractiles.

TABLE 12 -- Serum Erythropoietin Levels in Term Infants During the First Year of Life

(Not Available)

From Yamashita H, Kukita J et al: Serum erythropoietin levels in term and preterm infants during the first year of life. Am J Pediatr Hematol Oncol 16:213, 1994, with permission.

TABLE 13 -- Plasma Erythropoietin Reference Ranges in Children[a]

Age (yr)	n	Male Subjects	n	Female Subjects
13	122	1.717.9	97	2.115.9
46	89	3.521.9	76	2.98.5
79	79	1.013.5	80	2.18.2
1012	98	1.014.0	90	1.19.1
1315	100	2.214.4	148	3.820.5
1618	66	1.515.2	77	2.014.2

From Krafte-Jacobs B, Williams J, Soldin SJ: Plasma erythropoietin reference ranges in children. J Pediatr 126:601, 1995, with permission.

[a]Values are expressed in mIU/ml. Data were obtained from a total of 1122 hospitalized and outpatient children ages 118 years, using a commercially available ELISA methodology. Values are 2.5th97.5th percentiles.

TABLE 14 -- Comparison of Selected Coagulation Factor Values in Newborns[a]

Age	Fibrinogen (mg/dl)	F II (U/ml)	F VIII (U/ml)	F IX (U/ml)	F XII (U/ml)	Antithrombin (U/ml)	Protein C (U/ml)
Term							
Hathaway and Bonnar (1987) and Manco-Johnson et al. (1988) [b]	240 (150)	0.52 (0.25)	1.5 (0.55)	0.35 (0.15)	0.44 (0.16)	0.56 (0.32)	0.32 (0.16)
Andrews et al. (1987, 1988) [c]	283 (177)	0.48 (0.26)	1.0 (0.50)	0.53 (0.25)	0.53 (0.20)	0.63 (0.25)	0.35 (0.17)
Corrigan (1992) [d]	246 (150)	0.45 (0.22)	1.68 (0.50)	0.40 (0.20)	0.44 (0.16)	0.52 (0.20)	0.31 (0.17)
Preterm							

Hathaway and Bonnar (1987) and Manco-Johnson et al. (1988) [b]	300 (120)	0.45 (0.26)	0.93 (0.54)	0.41 (0.20)	0.33 (0.23)	0.40 (0.25)	0.24 (0.18)
Andrews et al. (1987, 1988) [c]	243 (150)	0.45 (0.20)	1.1 (0.50)	0.35 (0.19)	0.38 (0.10)	0.38 (0.14)	0.28 (0.12)
Corrigan (1992) [d]	240 (150)	0.35 (0.21)	1.36 (0.21)	0.35 (0.10)	0.22 (0.09)	0.35 (0.10)	0.28 (0.12)

From Hathaway W, Corrigan J: Report of scientific and standardization subcommittee on neonatal hemostasis. Thromb Haemost 65:323, 1991.

[a]Data are expressed as mean and lower limits of normal (in parentheses). Preterm, 3036 weeks gestational age.
[b]Hathaway W, Bonnar J: Hemostatic Disorders of the Pregnant Woman and the Newborn Infant. Elsevier Science Publishing Co., New York, 1987; Manco-Johnson M, Marlar R et al: Severe protein C deficiency in newborn infants. J Pediatr 113:359, 1988.
[c]Andrews M, Paes B et al: Development of the human coagulation system in the full-term infant. Blood 70:165, 1987. Andrews M, Paes B et al: Development of the human coagulation system in the healthy premature infant. Blood 72:1651, 1988.
[d]Corrigan JJ Jr: Normal hemostasis in fetus and newborn: coagulation. In Polin RA, Fox WW (eds): Fetal and Neonatal Physiology. WB Saunders, Philadelphia, 1992, pp. 13681371.

TABLE 15 -- Reference Values for Coagulation Tests in Healthy Children Aged 116 Years Compared with Normal Adults[a]

Coagulation Tests	Age			
	1 5 yr	6 10 yr	11 16 yr	Adult
	Mean (Boundary)	Mean (Boundary)	Mean (Boundary)	Mean (Boundary)
PT (s)	11 (10.611.4)	11.1 (10.112.1)	11.2 (10.212.0)	12 (11.014.0)
INR	1.0 (0.961.04)	1.01 (0.911.11)	1.02 (0.931.10)	1.10 (1.01.3)
APTT (s)	30 (2436)	31 (2636)	32 (2637)	33 (2740)
Fibrinogen (g/L)	2.76 (1.704.05)	2.79 (1.574.0)	3.0 (1.544.48)	2.78 (1.564.0)
Bleeding time (min)	6 (2.510) [b]	7 (2.513) [b]	5 (38) [b]	4 (17)
II (U/ml)	0.94 (0.711.16) [b]	0.88 (0.671.07) [b]	0.83 (0.611.04) [b]	1.08 (0.701.46)
V (U/ml)	1.03 (0.791.27)	0.90 (0.631.16) [b]	0.77 (0.550.99) [b]	1.06 (0.621.50)
VII (U/ml)	0.82 (0.551.16) [b]	0.85 (0.521.20) [b]	0.83 (0.581.15) [b]	1.05 (0.671.43)
VIII (U/ml)	0.90 (0.591.42)	0.95 (0.581.32)	0.92 (0.531.31)	0.99 (0.501.49)
vWF (U/ml)	0.82 (0.601.20)	0.95 (0.441.44)	1.00 (0.461.53)	0.92 (0.501.58)
IX (U/ml)	0.73 (0.471.04) [b]	0.75 (0.630.89) [b]	0.82 (0.591.22) [b]	1.09 (0.51.63)
X (U/ml)	0.88 (0.581.16) [b]	0.75 (0.551.01) [b]	0.79 (0.501.17) [b]	1.06 (0.701.52)
XI (U/ml)	0.97 (0.561.50)	0.86 (0.521.20)	0.74 (0.500.97) [b]	0.97 (0.671.27)
XII (U/ml)	0.93 (0.641.29)	0.92 (0.601.40)	0.81 (0.341.37) [b]	1.08 (0.521.64)
PK (U/ml)	0.95 (0.651.30)	0.99 (0.661.31)	0.99 (0.531.45)	1.12 (0.621.62)
HMWK (U/ml)	0.98 (0.641.32)	0.93 (0.601.30)	0.91 (0.631.19)	0.92 (0.501.36)
XIIIa (U/ml)	1.08 (0.721.43) [b]	1.09 (0.651.51) [b]	0.99 (0.571.40)	1.05 (0.551.55)
XIIIs (U/ml)	1.13 (0.691.56) [b]	1.16 (0.771.54) [b]	1.02 (0.601.43)	0.97 (0.571.37)

From Andrew M, Vegh P et al: Maturation of the hemostatic system during childhood. Blood 80:1998, 1992, with permission.

[a]All factors except fibrinogen are expressed as units per milliliter, where pooled plasma contains 1.0 U/ml. All data are expressed as the mean, followed by the upper and lower boundary encompassing 95% of the population. Between 20 and 50 samples were assayed for each value for each age group. Some measurements were skewed due to disproportionate number of high values. The lower limit, which excludes the lower 2.5% of the population, is given. PT, prothrombin time; APTT, activated partial thromboplastin time; VII, factor VII procoagulant; vWF, von Willebrand factor; PK, prekallikrein; HMWK, high molecular weight kininogen.
[b]Values that are significantly different from values in adults.

TABLE 16 -- Reference Values for the Inhibitors of Coagulation in Fetuses, Full-term Infants, and Adults[a]

Parameter	Fetuses (Weeks Gestation)			Newborns (n = 60)	Adults (n = 40)
	19 23 (n = 20)	24 29 (n = 22)	30 38 (n = 22)		
ATII (%)	20.2 (1231) [c]	30.0 (2039)	37.1 (2455) [d]	59.4 (4280) [d]	99.8 (65130)
HCII (%)	10.3 (616)	12.9 (5.520)	21.1 (1133) [d]	52.1 (1999) [d]	101.4 (70128)
TFPI (ng/ml) [b]	21.0 (16.029.2)	20.6 (13.433.2)	20.7 (10.431.5) [d]	38.1 (22.755.8) [d]	73.0 (50.990.1)
PC Ag (%)	9.5 (614)	12.1 (816)	15.9 (830) [d]	32.5 (2147) [d]	100.8 (68125)
PC Act (%)	9.6 (713)	10.4 (813)	14.1 (818) [c]	28.2 (1442) [d]	98.8 (68129)
Total PS (%)	15.1 (1121)	17.4 (1425)	21.0 (1530) [d]	38.5 (2255) [d]	99.6 (72118)
Free PS (%)	21.7 (1332)	27.9 (1940)	27.1 (1840) [d]	49.3 (3367) [d]	98.7 (72128)
Ratio of free PS to total PS	0.82 (0.750.92)	0.83 (0.760.95)	0.79 (0.700.89) [d]	0.64 (0.590.98) [d]	0.41 (0.380.43)
C4b-BP (%)	1.8 (0.6)	6.1 (012.5)	9.3 (514)	18.6 (340) [d]	100.3 (70124)

From Reverdiau-Moalic P, Delahousse B et al: Evolution of blood coagulation activators and inhibitors in the healthy human fetus. Blood 88:900, 1996, with permission.

[a]Values are the mean, followed in parentheses by the lower and upper boundaries including 95% of the population. Abbreviations: Ag, antigen; Act, activity.
[c]$P < 0.05$.
[d]$P < .01$.
[b]Twenty samples were assayed for each group, but only 10 for 19- to 23-week-old fetuses.

TABLE 17 -- Complement Fractions C3 and C4 in Males and Females, by Age

	Age	g/L
C3		

Zillow et al. (1993) [a]	Healthy neonates	0.300.98
Lockitch et al. (1988) [b]	05 days	0.261.04
	119 yr	0.510.95
	Adult	0.450.83
C4		
Lockitch et al. (1988) [b]	05 days	0.060.37
	119 yr	0.080.44
	Adult	0.110.41

[a]Zillow G, Zillow EP et al: Complement activation in newborn infants with early onset infection. Pediatr Res 34:199, 1993. Results are 0100th percentiles. Thirty-two healthy full-term newborns, birth weights between 2,500 and 4,020 g, were tested. All mothers (vagina, rectum) and infants (ear, gastric) were culture negative.
[b]Lockitch G, Halstead AC et al: Age- and sex-specific pediatric reference intervals: study design and methods by measurement of serum proteins with the Behring LN nephelometer. Clin Chem 34:1618, 1988. Results are 0.0250.975 fractiles, with permission.

TABLE 18 -- Plasma Levels of Immunoglobulin A in Males and Females, by Age

Age	Male			Female		
	n	mg/dl	g/L	n	mg/dl	g/L
Soldin et al. (1996) [a]						
130 days	61	120	0.010.20	56	119	0.010.19
31182 days	52	756	0.070.56	60	159	0.010.59
183365 days	37	9107	0.091.07	23	1590	0.150.90
13 yr	105	18171	0.181.71	127	25141	0.251.41
46 yr	138	60231	0.602.31	135	47206	0.472.06
79 yr	111	77252	0.772.52	105	41218	0.412.18
1012 yr	104	61269	0.612.69	115	73239	0.732.39
1315 yr	104	42304	0.423.04	118	82296	0.822.96
1618 yr	112	89314	0.893.14	100	90322	0.903.22
Lockitch et al. (1988) [b]						
012 mo			0.001.00		0100	
13 yr			0.241.21		24121	
46 yr			0.332.35		33235	
79 yr			0.413.68		41368	
1011 yr			0.642.46		64246	
1213 yr			0.704.32		70432	
1415 yr			0.573.00		57300	
1619 yr			0.744.19		74419	

[a]Soldin SJ, Bailey J, Beatey J et al: Pediatric reference ranges for immunoglobulins G, A and M on the Behring nephelometer. Clin Chem 42:S308, 1996, with permission.
[b]Lockitch G, Halstead AC et al: Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. Clin Chem 34:1618, 1988. Results are 0.0250.975 fractiles, with permission.

TABLE 19 -- Immunoglobulin E in Males and Females, by Age

Age	Females (KIU/L)	Males (KIU/L)
Soldin, Lenherr et al. (1995) [a]		
012 mo	<8	<12
13 yr	<28	<90
410 yr	<137	<163
1118 yr	<398	<179
Soldin, Morales et al. (1995) [b]		
012 mo	020	224
13 yr	255	2149
410 yr	8279	4249
1115 yr	5295	7280
1618 yr	7698	5268

	IU/ml
Cord blood[c]	0.022.08
Lindberg and Arroyave (1986) [d]	
<1 yr	06.6
12 yr	020.0
23 yr	0.115.8
34 yr	029.2
45 yr	0.325.0
56 yr	0.217.6
67 yr	0.213.1

78 yr	0.346.1
89 yr	1.860.1
910 yr	3.681.0
1011 yr	8.095.0
1112 yr	1.599.7
1213 yr	3.983.5
1316 yr	3.3188.0

[a]Soldin SJ, Lenherr S, Kumar A: Pediatric reference ranges for IgE. Clin Chem 41:S92, 1995.
[b]Soldin SJ, Morales A et al: Pediatric reference ranges on the Abbott Imx for FSH, LH, prolactin, TSH, T4, T3, free T3, T-uptake, IgE, and ferritin. Clin Biochem 28:603, 1995. Study was based on hospitalized patients. Values represent 2.5th and 97.5th percentiles.
[c]Ownby DR, Johnson CC, Peterson EL: Maternal smoking does not influence cord serum IgE or IgD concentrations. J Allergy Clin Immunol 88:555, 1991. Values constitute 95% of the measured samples.
[d]Lindberg RE, Arroyave C: Levels of IgE in serum from normal children and allergic children as measured by an enzyme immunoassay. J Allergy Clin Immunol 78:614, 1986. Three hundred forty-six nonatopic children were selected for study by ELISA methodology.

TABLE 20 -- Immunoglobulin M in Males and Females, by Age

Age	Male			Female		
	n	mg/dl	g/L	n	mg/dl	g/L
Soldin et al. (1997) [a]						
130 days	61	12117	0.121.17	56	19104	0.191.04
31182 days	52	27147	0.271.47	60	9212	0.092.12
183365 days	37	41197	0.411.97	23	4216	0.042.16
13 yr	105	63240	0.632.40	127	70298	0.702.98
46 yr	138	64248	0.642.48	135	81298	0.812.98
79 yr	111	49231	0.492.31	105	62270	0.622.70
1012 yr	104	58249	0.582.49	115	81340	0.813.40
1315 yr	104	57298	0.572.98	118	69361	0.693.61
1618 yr	112	59291	0.592.91	100	86360	0.863.60
Lockitch et al. (1988) [b]						
012 mo			0.0 2.16		0216	
13 yr			0.282.18		28218	
46 yr			0.363.14		36314	
79 yr			0.473.11		47311	
1011 yr			0.462.68		46268	
1213 yr			0.523.57		52357	
1415 yr			0.232.81		23281	
1619 yr			0.353.87		35387	

[a]Soldin SJ, Bailey J, Beatey J et al: Pediatric reference ranges for immunoglobulins G, A and M on the Behring nephelometer. Clin Chem 42:S308, 1996. Plasma or serum.
[b]Lockitch G, Halstead AC et al: Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. Clin Chem 34:1618, 1988. Results are 0.0250.975 fractiles. Plasma or serum.

TABLE 21 -- Plasma Levels of Immunoglobulin G in Males and Females, by Age

Age	Male			Female		
	n	mg/dl	g/L	n	mg/dl	g/L
Soldin et al. (1997) [a]						
130 days	61	260986	2.609.86	56	2211,031	2.2110.31
31182 days	52	195643	1.956.43	60	390794	3.907.94
183365 days	37	184974	1.849.74	23	407774	4.077.74
13 yr	105	5071,305	5.0713.05	127	5501,407	5.5014.07
46 yr	138	5711,550	5.7115.50	135	6751,540	6.7515.40
79 yr	111	7001,680	7.0016.80	105	5891,717	5.8917.17
1012 yr	104	8181,885	8.1818.85	115	7051,871	7.0518.71
1315 yr	104	7091,861	7.0918.61	118	8911,907	8.9119.07
1618 yr	112	6321,979	6.3219.79	100	9532,108	9.5321.08
Lockitch et al. (1988) [b]						
012 mo			2.7316.60		2731,660	
13 yr			5.3310.78		5331,078	
46 yr			5.9317.23		5931,723	
79 yr			6.7317.34		6731,734	
1011 yr			8.2118.35		8211,835	
1213 yr			8.9318.23		8931,823	

1415 yr			8.4220.13		8422,013	
1619 yr			6.4618.64		6461,864	

[a]Soldin SJ, Bailey J, Beatey J et al: Pediatric reference ranges for immunoglobulins G, A and M on the Behring nephelometer. Clin Chem 42:S308, 1996.
[b]Lockitch G, Halstead AC et al: Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. Clin Chem 34:1618, 1988. Results are 0.0250.975 fractiles.

TABLE 22 -- Immunoglobulin G Subclasses (IgG_1 , IgG_2 , IgG_3 , IgG_4)[a]

Age	IgG_1	IgG_2	IgG_3	IgG_4
Cord blood, preterm	3.49.7	0.71.7	0.20.5	0.20.7
Cord blood, term	5.813.7	0.65.2	0.21.2	0.21.0
5 yr	5.612.7	0.44.4	0.31.0	0.10.8
6 yr	6.211.3	0.54.0	0.30.8	0.20.9
7 yr	5.410.5	0.93.5	0.31.1	0.21.1
8 yr	5.610.5	0.74.5	0.21.1	0.10.8
9 yr	3.911.4	0.74.7	0.41.2	0.21.0
10 yr	4.410.8	0.64.0	0.31.2	0.10.9
11 yr	6.410.9	0.94.3	0.30.9	0.21.0
12 yr	6.011.5	0.94.8	0.41.0	0.20.9
13 yr	6.111.5	0.97.9	0.21.1	0.10.8
Adults	4.89.5	1.16.9	0.30.8	0.21.1

From Miles J, Riches P: The determination of IgG subclass concentrations in serum by enzyme-linked immunosorbent assay: establishment of age-related reference ranges for cord blood samples, children aged 513 years and adults. Ann Clin Biochem 31:245, 1994.

[a]Values for males and females, expressed in g/L. Twenty schoolchildren from each age group were tested. Only samples with C-reactive protein within the reference range were included. ELISA methodology was used on serum.

TABLE 23 -- Serum Levels of Immunoglobulin Light Chains in Males and Females

Age	Kappa (g/L)	Lambda (g/L)	Kappa/Lambda Ratio
Newborn	8.10 (7.78.7)	4.70 (4.55.0)	1.74 (1.71.9)
Premature	4.29 (3.14.9)	2.91 (2.63.1)	1.70 (1.61.9)
1 mo	4.46 (3.64.8)	2.32 (1.93.5)	1.80 (1.62.0)
2 mo	2.58 (2.42.7)	1.88 (1.82.0)	1.66 (1.61.7)
3 mo	2.07 (1.72.5)	2.25 (2.02.3)	1.09 (1.01.3)
4 mo	2.09 (1.92.4)	2.40 (1.62.6)	0.98 (0.81.0)
5 mo	2.36 (2.03.2)	2.54 (2.42.9)	1.02 (1.01.1)
1 yr	4.46 (3.05.3)	3.91 (2.74.8)	0.99 (0.91.2)
2 yr	5.32 (3.66.4)	3.82 (2.84.0)	1.42 (1.31.5)
3 yr	4.98 (4.15.4)	3.67 (3.14.8)	1.31 (1.11.4)
4 yr	6.78 (4.88.3)	4.87 (4.05.5)	1.51 (1.21.7)
5 yr	6.58 (5.47.1)	4.01 (3.84.7)	1.55 (1.31.7)
6 yr	8.02 (6.08.5)	4.01 (3.06.0)	1.61 (1.61.7)
7 yr	5.90 (4.67.5)	4.08 (3.94.5)	1.64 (1.41.8)
8 yr	8.34 (7.39.1)	4.64 (5.16.4)	1.50 (1.41.7)
9 yr	8.26 (7.19.6)	4.95 (3.35.5)	1.69 (1.51.8)
10 yr	9.03 (8.29.6)	5.32 (4.95.7)	1.80 (1.31.9)
11 yr	7.18 (6.08.3)	4.10 (3.84.8)	1.67 (1.41.8)
12 yr	9.17 (8.29.6)	4.90 (4.16.0)	1.82 (1.72.0)
13 yr	9.59 (8.210.5)	5.86 (5.36.1)	1.64 (1.41.7)
14 yr	9.45 (8.010.8)	5.50 (4.95.8)	2.09 (1.92.2)
15 yr	10.18 (7.211.0)	4.75 (4.15.0)	2.25 (1.72.3)
16 yr	9.37 (7.011.2)	5.09 (4.65.6)	1.89 (1.52.2)

From Herkner KR, Salzer H et al: Pediatric and perinatal reference intervals for immunoglobin light chains k000a0a and lambda. Clin Chem 38:548, 1992. Median and ranges given as 10th90th percentiles.

TABLE 24 -- Reference Ranges for Pediatric Lymphocyte Subsets at Different Ages

Age		Total Lymphocytes	CD4	CD8	CD2	CD3	CD19	Helper/ Suppressor Ratio
06 mo	%	.62.72	.50.57	.08.31	.55.88	.55.82	.11.45	
(n = 10)	A	5.47.2	2.83.9	0.352.5	3.95.3	3.55.0	0.433.3	6.22
612 mo	%	.60.69	.49.55	.08.31	.55.88	.55.82	.11.45	

(n = 9)	A	5.36.7	2.63.5	0.352.5	3.84.9	3.44.6	0.433.3	6.22
1218 mo	%	.56.63	.46.51	.08.31	.55.88	.55.82	.11.45	
(n = 9)	A	4.95.9	2.32.9	0.352.5	3.54.2	3.23.9	0.433.3	6.22
1824 mo	%	.52.59	.42.48	.08.31	.55.88	.55.82	.11.45	
(n = 10)	A	4.45.5	1.92.5	0.352.5	3.13.9	2.83.5	0.433.3	6.22
2430 mo	%	.45.57	.38.46	.08.31	.55.88	.55.82	.11.45	
(n = 9)	A	3.95.2	1.52.2	0.352.5	2.63.6	2.33.3	0.433.3	6.22
3036 mo	%	.39.53	.33.44	.08.31	.55.88	.55.82	.11.45	
(n = 10)	A	3.35.1	1.22.0	0.352.5	2.23.5	1.93.1	0.433.3	6.22
3 years	%	.22.69	.27.57	.14.34	.65.84	.55.82	.09.29	
(n = 73)	A	1.65.4	0.562.7	0.331.4	1.24.1	1.03.9	0.201.3	3.24

Values are expressed as 95th percentile reference range; %, relative % A, absolute cell count × 10^9 /L. Samples analyzed by Epics Profile (Coulter) flow cytometer following whole blood lysis.

From Kotylo PK, Fineberg NS et al: Reference ranges for lymphocyte subsets in pediatric patients. Am J Clin Pathol 100:111, 1993. See also Denny T, Yogev R et al: Lymphocyte subsets in healthy children during the first 5 years of life. JAMA 267:1484, 1992. Copyright 1992, American Medical Association.

TABLE 25 -- Total Bilirubin in Premature and Full-term Infants

	Premature mg/dl	Full-term mg/dl
Cord blood [a]	<2.0	<2.0
01 days [a]	<8.0	<6.0
12 days [a]	<12.0	<8.0
25 days [a]	<16.0	<12.0
>5 days [a]	<2.0	0.21.0

	mol/L	mg/dl
Bottle-fed infants [b]	<212	<12.4
Breast-fed infants [b]	<253	<14.8

[a]Behrman RE (ed): Nelson Textbook of Pediatrics. 14th ed. WB Saunders, Philadelphia, 1992, p. 1803.
[b]Maisels MJ, Gifford K: Normal serum bilirubin levels in the newborn and the effect of breast feeding. Pediatrics 78:837, 1986. Represents 97th percentile rank for white neonates of >2,500 g birth weight.

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